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### Research Article

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# Regulation of the Rat Liver Sodium-dependent Bile Acid Cotransporter Gene by Prolactin

## Mediation of Transcriptional Activation by Stat5

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### Abstract

The intracellular mechanism(s) underlying the upregulation of the hepatic Na<sup>+</sup>/taurocholate cotransporting polypeptide (ntcp) by prolactin (PRL) are unknown. In this report, we demonstrate a time-dependent increase in nuclear translocation of phosphorylated liver Stat5 (a member of the Signal Transducers and Activators of Transcription family) that correlated with suckling-induced increases in serum PRL levels. In electrophoretic mobility gel shift assays, nuclear Stat5 exhibited specific DNA-binding ability towards IFN- $\gamma$ -activated sequence (GAS)-like elements (GLEs; 5'-TTC/A-PyNPu-G/TAA-3') located in the -937 to -904 bp region of the *ntcp* promoter. Transient cotransfections in HepG2 cells revealed that PRL inducibility (2.5–3-fold) required coexpression of the long form of the PRL receptor (PRLR<sub>L</sub>) and Stat5. Deletion analysis mapped the PRL-inducible region to -1237 to -758 bp of the *ntcp* promoter. Linking this 0.5-kb region to a heterologous thymidine kinase (tk) promoter, or linking multimerized *ntcp* GLEs either upstream of the *ntcp* minimal promoter (-158 to +47 bp) or the heterologous promoter conferred dose-dependent PRL responsiveness. The short form of the PRL receptor failed to transactivate *ntcp* GLEs. These results indicate that PRL acts via the PRLR<sub>L</sub> to facilitate Stat5 binding to *ntcp*-GLEs and to transcriptionally regulate *ntcp*. (*J. Clin. Invest.* 1997. 99:2906–2914.) Key words: hormone • taurocholate • GAS-like elements • intracellular signaling • *ntcp*

### Introduction

A critical function of the mammalian liver is the formation and maintenance of bile flow. The major driving force for bile secretion is provided by the vectorial active transport of osmotically active agents (primarily bile salts) in a concentrative manner into the confined space of the canaliculus, followed by the passive movement of water and counter ions until osmotic equilibrium is reached (1). In the rat liver, the Na<sup>+</sup>-dependent cotransport of bile salts, such as taurocholate, across the baso-

lateral membrane accounts for ~75–80% of total bile salt uptake, and is the first step in transcellular movement across the hepatocyte. The cDNA encoding the rat hepatic Na<sup>+</sup>/taurocholate cotransporting polypeptide (*ntcp*)<sup>1</sup> has been cloned (2), and its expression in COS-7 cells has been shown to confer transport characteristics similar to those observed in vivo (3).

We have previously reported that the  $V_{\max}$  for Na<sup>+</sup>/taurocholate cotransport is increased in hepatocytes from postpartum rats compared to nonpregnant and pregnant rats (4). This increase is blocked by inhibition of secretion of the anterior pituitary hormone PRL, and is mimicked by infusion of oPRL in ovariectomized rats (4). Furthermore, the increased Na<sup>+</sup>/taurocholate cotransporter activity is also observed in highly purified basolateral membrane vesicles from postpartum rats, and these changes correlate with a similar increase of steady-state *ntcp* mRNA and immunodetectable *ntcp* protein (5–8). Despite these advances, the intracellular mechanism(s) leading to this apparent increase in *ntcp* transcription are largely unknown. However, the female rat liver is known to contain a significant number of PRL receptors (9), suggesting the role of a receptor-mediated event. The two isoforms of the hepatic PRL receptor, long (PRLR<sub>L</sub>) and short (PRLR<sub>S</sub>) forms, are members of the cytokine/hematopoietin/growth factor receptor superfamily (10). Although both forms of the receptor bind with equal affinity to PRL, only the PRLR<sub>L</sub> stimulates milk protein gene transcription, since the PRLR<sub>S</sub> lacks the signaling carboxy-terminal intracellular domain. It has been suggested that the PRLR<sub>S</sub> serves as a transporter or a binding protein to convey PRL from one cellular compartment to another (11).

Recently, the signal transduction mechanism of PRL has been studied using in vitro cell culture systems and mammary gland explants. Upon interaction of PRL with PRLR<sub>L</sub>, there is sequential receptor dimerization leading to the activation of an associated cytoplasmic tyrosine kinase, Jak-2 (a member of the Janus kinase family), which then phosphorylates a single tyrosine residue at position 580 in the PRLR<sub>L</sub> (12–17). The phosphorylated tyrosine in PRLR<sub>L</sub> recruits a latent transcription factor, Stat5 (a member of the Signal Transducers and Activators of Transcription family of transcription factors). Stat5 is in turn activated by Jak-2 via phosphorylation on a single tyrosine residue located at position 694 (18, 19). The phosphorylated Stat5 proteins form dimers that are released into the cytoplasm and translocated to the nucleus, where they bind to specific DNA sequences and activate transcription of PRL-

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1. Abbreviations used in this paper: GH, growth hormone; GLE, IFN- $\gamma$ -activated sequence (GAS)-like elements; HSVtk, herpes simplex virus thymidine kinase promoter; *ntcp*, Na<sup>+</sup>/taurocholate cotransporting polypeptide; oPRL, ovine prolactin; PRLR<sub>L</sub>, long form of PRL receptor; PRLR<sub>S</sub>, short form of PRL receptor; Stat, signal transducers and activators of transcription.

responsive genes, such as the casein gene (20, and references therein).

The recent cloning of *ntcp* and characterization of the 5' flanking region has facilitated investigations of molecular mechanisms involving its hormonal regulation. It appears that a TATA element, a binding element for a yet-unknown liver-enriched factor, and an HNF1-binding site, which are all located within -158 bp upstream of the transcription start site, are minimal requirements for the basal transcription of *ntcp* (21). In addition, several DNA-binding sequences (*cis* elements) are located in the immediate 1.3-kb 5' flanking region, with which sequence-specific regulatory proteins (*trans*-acting factors) could interact and potentially regulate the transcription of *ntcp*. Two such putative binding sites, corresponding to Stat5 consensus binding motifs TTC/A PyNPu G/TAA, were identified by sequence homology searches (21, 22). These palindromic sequences were initially identified in the mammary gland and termed prolactin-responsive elements or IFN- $\gamma$ -activated sequence (GAS)-like elements (GLEs). These sequences are highly conserved in the promoters of the  $\beta$ -casein genes of various species and other genes, such as the serine protease inhibitor-3 (SPI-3) and insulin-1 gene (23, 24). Additionally, several reports have elegantly demonstrated the presence of Stat5 protein in the rat liver nuclei late in an acute phase response (25) or after treatment with growth hormone (GH; 26, 27). Taken together, these observations led to our working hypothesis that in the postpartum period, high levels of circulating serum PRL regulate *ntcp* transcription via a tyrosine phosphorylation signaling pathway that involves the hepatic PRL receptor(s) and Stat5. The present studies were therefore designed to address whether (a) PRL activates Stat5 in the postpartum rat liver; (b) Stat5 exhibits specific binding to the putative GLEs in the *ntcp* promoter; and (c) Stat5 can *trans*-activate the *ntcp* promoter.

## Methods

**Reagents and antibodies.** All reagents were of molecular biology or cell culture grade. Restriction enzymes and polynucleotide kinase were obtained from GIBCO BRL (Gaithersburg, MD). Ovine PRL (oPRL, NIDDK-oPRL-19; AFP-9221-A) was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program, National Institute for Child Health and Human Development, and the US Department of Agriculture. Antibodies used in this study were Stat5 mAb (raised against ovine Stat5 amino acids 451-469; Transduction Laboratories, Lexington, KY), polyclonal rabbit anti-mouse Stat5b (raised against amino acids 711-727 of the carboxy terminus of mouse Stat5b), phosphotyrosine (PY-20) antibody agarose conjugate (Santa Cruz Biotech Inc., Santa Cruz, CA), phosphotyrosine (4G10) mAb (Upstate Biotechnologies Inc., Lake Placid, NY), and anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates (Amersham, Arlington Heights, IL).

**Animals.** Pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) timed at the first day that sperm were detected (day 0) were housed in cages with Sani-chips bedding (PJ Murphy Forest Products, Montville, NJ). The rats had free access to Agway Prolab-3000 rat chow (Charlotte, NC) and water, and were maintained on a 14-h, automatically timed light and dark cycles (lights on at 6:00 a.m., off at 8:00 p.m.) in a HEPA-filtered laminar flow unit for at least 7 d before use. Litter sizes were culled to eight pups. Pups, 4-5 d post partum, were removed from the dams overnight to decrease maternal serum PRL levels. The pups were returned to the dams and allowed to suckle for 0, 15, 30, or 60 minutes before killing of the dam by de-

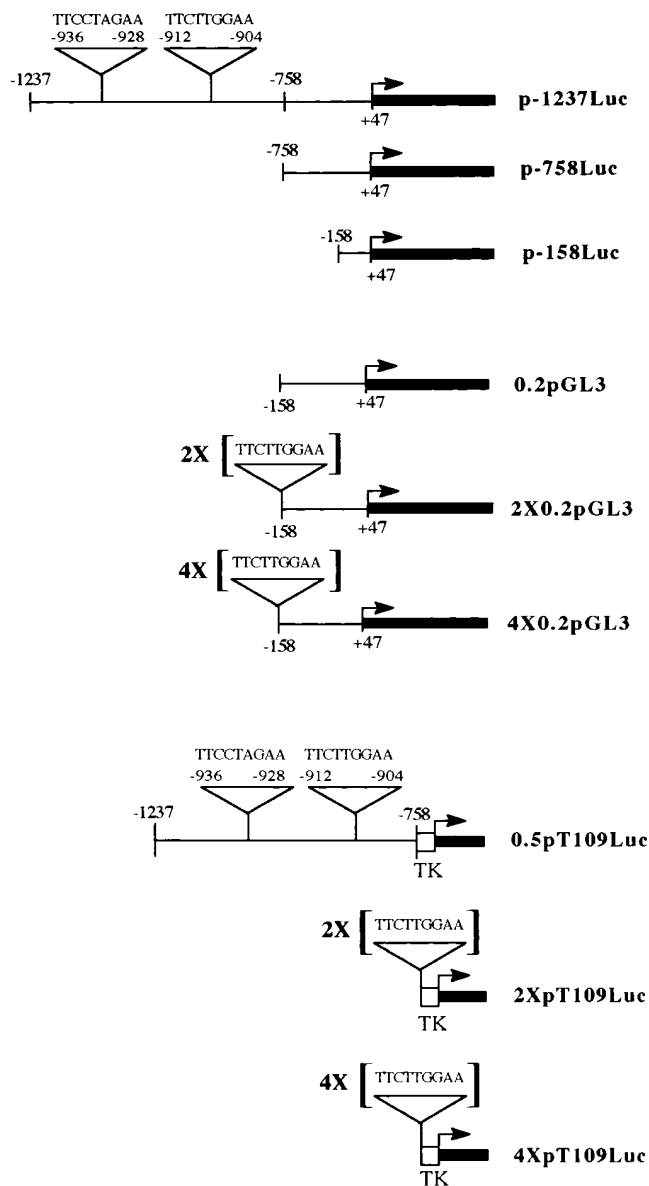
capitation and removal of the maternal liver for preparation of nuclear extracts. Three to four rats were used in each group.

**Serum PRL assays.** Blood was collected after decapitation, and serum was isolated by centrifugation for measurement of PRL levels by a standard double-antibody RIA as described previously (28).

**Nuclear extract preparation.** Rat liver nuclear extracts were prepared by the method of Standke et al. (29) with the inclusion of phosphatase inhibitors 10 mM sodium fluoride (NaF) and 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) along with the protease inhibitors antipain, pepstatin, and chymotrypsin inhibitor (2  $\mu\text{g}/\text{ml}$  each); leupeptin and aprotinin (5  $\mu\text{g}/\text{ml}$  each); trypsin inhibitor (10  $\mu\text{g}/\text{ml}$ ); and 0.1 mM PMSF in the homogenization buffer. After extraction in high salt buffer (10 mM Hepes [pH 7.6], 400 mM KCl, 1 mM EDTA, 1 mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, 1 mM DTT, and 0.1 mM PMSF), the extracts were dialyzed (for 90 min) in 250 ml of buffer containing 10 mM Hepes (pH 7.6), 40 mM KCl, 0.5 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, 1 mM DTT, and 0.1 mM PMSF. The precipitate formed during dialysis was removed by centrifugation at 10,000  $g$  for 5 min, after which the extracts were aliquoted, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Protein concentrations were measured by the method of Lowry (30) using BSA as standard.

**Immunoprecipitation and Western blots.** For immunoprecipitation, 50  $\mu\text{g}$  of nuclear protein was incubated with 5  $\mu\text{g}$  of PY-20 mAb agarose conjugate or 3  $\mu\text{g}$  of 4G10 mAb in a total volume of 100  $\mu\text{l}$  of immunoprecipitation buffer (1% Triton X, 150 mM NaCl, 10 mM Hepes [pH 7.4], 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.2 mM  $\text{Na}_3\text{VO}_4$ , 0.5% NP-40, and 1  $\mu\text{g}/\text{ml}$  of pepstatin, aprotinin, and leupeptin), and was gently mixed in an end-over rotor at  $4^\circ\text{C}$  overnight. 25  $\mu\text{l}$  of a 50% suspension of Sepharose 6MB beads (Pharmacia Biotech, Uppsala, Sweden) was added to the samples that were immunoprecipitated with the 4G10 antibody and mixed for an additional hour. The immunoprecipitated proteins bound to agarose conjugates or beads were pelleted and washed three times with immunoprecipitation buffer, suspended in 30  $\mu\text{l}$  of  $2\times$  SDS sample buffer (3% SDS, 10%  $\beta$ -mercaptoethanol, 2% glycerol, 200 mM Tris/HCl [pH 6.8]), and boiled for 10 min. For Western blots, 20  $\mu\text{g}$  of nuclear protein or immunoprecipitated proteins were resolved on an 8.5% SDS-PAGE, and the proteins were transferred to nitrocellulose membrane. Transfer was assessed by incubating with 0.1% Ponceau, and standards were marked. The membrane was washed with distilled water to remove the excess stain, and was blocked overnight at  $4^\circ\text{C}$  in wash buffer (100 mM NaCl, 1% Tween 20, 10 mM Tris/HCl, pH 7.5) containing 3% BSA (Fraction V; Sigma, St. Louis, MO) and then incubated for 1 h with primary antibodies (1:3,000 dilution of either monoclonal Stat5, monoclonal phosphotyrosine 4G10 antibody, or polyclonal anti-rabbit Stat5b) in wash buffer containing 3% BSA. After six washes (5 min each), the membranes were incubated with anti-mouse IgG horseradish peroxidase conjugate (1:3,000) or anti-rabbit IgG horseradish peroxidase conjugate (1:5,000) for 1 h. After another six washes, the proteins were visualized using the enhanced chemiluminescence detection system (Amersham). Blots were stripped for 30 min in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM  $\beta$ -mercaptoethanol, and were blocked for 2 h before re-probing.

**Plasmid constructions.** The vector pSVoAL5' was kindly provided by Dr. Suresh Subramani (University of California, San Diego, CA). pRSV- $\beta$ -galactosidase and the cytomegalovirus-driven mammalian expression vector pL3T7CMV were generously provided by Dr. Daniel Noonan and Kenneth W. Henry II (University of Kentucky). The cDNAs for the long and short form of the rat prolactin receptors (clones R2 and F3, respectively) were gifts from Dr. Paul Kelly (INSERM, Paris, France). Ovine Stat5 cDNA (pXM-MGF) was a generous gift from Dr. Bernd Groner (Institute for Experimental Cancer Research, Freiburg, Germany). Plasmid pL3-PRLR<sub>L</sub> was constructed by subcloning the 2.3-kb EcoRI-PstI fragment of clone R2 to pL3T7CMV. pL3-PRLR<sub>S</sub> was similarly constructed by subcloning the 1.6-kb EcoRI-EcoRI fragment of clone F3 to pL3T7CMV. Fig. 1 is a schematic representation of all luciferase reporter plasmids used in



**Figure 1.** Structures of the plasmid constructs of the rat *ntcp* promoter used in transfections. The two GLEs are located between  $-936$  and  $-904$  bp in the rat *ntcp* promoter sequence. The  $-158$  to  $+47$  region constitutes the minimal rat *ntcp* promoter, and synthetic oligo nucleotides containing two or four GLEs (shown in parentheses) were fused to either the minimal promoter in the plasmid pGL3 or the heterologous thymidine kinase minimal promoter in pT109Luc to obtain the 2X and 4X constructs.

the study. The construction of p-1237Luc, p-758Luc, and p-158Luc were described previously (21). 0.2pGL3 was constructed by ligating the 200-bp HindIII fragment obtained by digesting p-158Luc at the HindIII site of pGL3 basic vector (Promega, Madison, WI). 0.5pT109Luc was constructed by inserting the 500-bp HindIII ( $-1237$  to  $-758$ ) fragment of *ntcp* upstream of the herpes simplex virus thymidine kinase minimal promoter (HSVtk) in pT109Luc (American Type Culture Collection, Rockville, MD). To create the plasmids 2XpT109Luc and 4XpT109Luc, complementary oligos corresponding to the *ntcp* GLE located at  $-912$  to  $-904$  bp ( $5'$ -agctagttgtcattcttg-gaaaaataact- $3'$ , sense;  $5'$ -agctagttattttccaagaatgacaact- $3'$ , antisense) containing a modified HindIII site at the  $5'$  ends were concatenated

to obtain two and four tandem GLEs that were then ligated to the HindIII-digested pT109Luc plasmid. 2X0.2pGL3 and 4X0.2pGL3 were created by ligating the KpnI-BamHI digest of 2XpT109Luc and 4XpT109Luc upstream of the *ntcp* minimal promoter ( $-158$  to  $+47$ ) in p0.2GL3. The orientation and integrity of all plasmids were verified by sequencing (31) using specific primers by Sequenase kit (U.S. Biochemical, Cleveland OH) or the Thermosequenase cycle sequencing kit (Amersham).

**Electrophoretic mobility shift assays.** The following double-stranded oligonucleotides were used (sense strand sequence; Stat-binding sites are in capitalized bold): A 29 mer corresponding to region  $-922$  to  $-892$  of the *ntcp* promoter ( $5'$ -gaagtgtca**TTCCTGGAA**aaataacaat- $3'$ ), a 21 mer containing the bovine  $\beta$ -casein Stat5 consensus binding motif ( $5'$ -agat**TCTAGGAA**ttcaatcc- $3'$ ), mutant Stat5 ( $5'$ -agat**TAGTTTAA**ttcaatcc- $3'$ , sense strand sequence identical to consensus Stat5 except for the ctagg  $\rightarrow$  agttt substitution in the Stat5-binding motif) and glucocorticoid response element oligonucleotide ( $5'$ -tcg-actgtacaggatgttctagtagtact- $3'$ ). The probes (50,000 cpm/ng) were  $^{32}$ P-radiolabeled with [ $\gamma^{32}$ P]ATP, using polynucleotide kinase at the  $5'$  OH blunt ends. The end-labeled, double-stranded oligos were gel purified from free ATP and single-stranded oligos on a 12% polyacrylamide gel, excised and eluted in  $1\times$  NET buffer (0.1 M NaCl, 1 mM EDTA, and 1 mM Tris-HCl [pH 7.6]). Electrophoretic mobility shift assays were conducted essentially as described (32). Nuclear extracts (5  $\mu$ g protein) were incubated for 20 min at room temperature with 10 fmol of purified probe in a 20- $\mu$ l reaction containing 5 mM Tris-HCl [pH 7.9], 15 mM Hepes-KOH [pH 7.9], 0.08 M KCl, 3.5 mM MgCl<sub>2</sub>, 5 mM EDTA, 5 mM DTT, 10% glycerol, 0.1% Tween 20, and 0.133 mg/ml poly (dI-dC):poly (dI-dC). Free probe and protein-bound probe were separated on a 4% polyacrylamide gel containing 2.5% glycerol and  $0.25\times$  TBE (25 mM Tris-HCl, 25 mM boric acid, and 0.25 mM EDTA [pH 8]). The gel was dried and exposed to autoradiography at  $-80^{\circ}\text{C}$ . In competition assays, 100 and 500 fmol (10- and 50-fold molar excess) of the specific unlabeled oligo was added to the binding reaction. In supershift studies, nuclear extracts were preincubated at  $4^{\circ}\text{C}$  for 15 min, followed by 30 min at room temperature with 1  $\mu$ l (1 mg/ml) of Stat5 polyclonal antibody before the addition of labeled oligo. In certain cases, the free probe was allowed to run off the gel to better visualize the DNA-protein complexes.

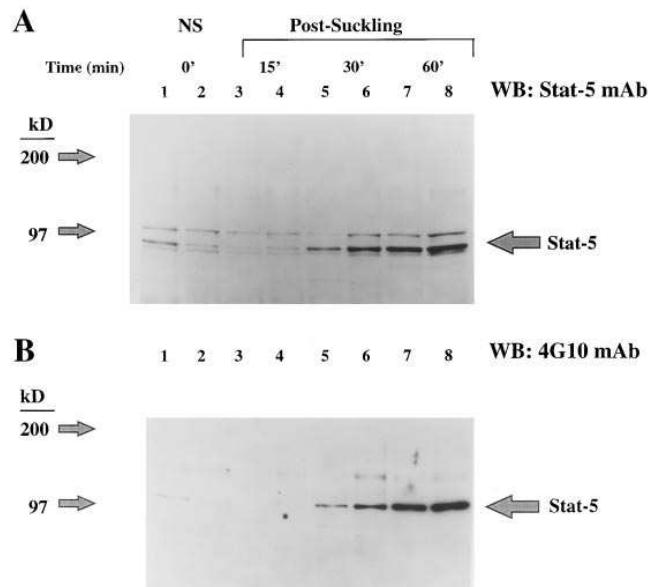
**Transient transfections.** HepG2 cells were maintained in DMEM/F12-Ham's (50:50) medium supplemented with 10% FBS (GIBCO BRL), 55  $\mu$ g/ml gentamicin (Sigma), 3.58 mM glutamine, and 1  $\mu$ g/ml insulin (GIBCO BRL). 1 d before transfection, cells were plated in phenol red-free DMEM containing 10% charcoal stripped serum (Hyclone Laboratories, Logan, UT) at 70% confluency. Transfections were done using recombinant plasmid DNA that was purified twice through cesium chloride gradient. Briefly, cells were transfected with 5  $\mu$ g of luciferase constructs, 5  $\mu$ g of pXM-MGF, and 1  $\mu$ g of pL3-PRLR<sub>L</sub> or pL3-PRLR<sub>S</sub> and pUC19 as carrier to a total of 20  $\mu$ g/10-cm plate using the calcium phosphate/DNA coprecipitation method (33). 5  $\mu$ g of pRSV- $\beta$ -galactosidase was included in each transfection to monitor transfection efficiency. Medium was removed 6–8 h after transfection, and the cells were washed twice with PBS and replated (30,000 cell/well) in a 96-well plate. Treatment was either in the absence or with seven incremental concentrations of oPRL (0.01, 0.05, 0.1, 0.5, 1, 5, and 10  $\mu$ g/ml). After 36–40 h, the cells were washed with PBS and lysed in 50  $\mu$ l of lysis buffer (25 mM Tris-phosphate [pH 7.8], 15% glycerol, 2% Chaps, 1% lecithin, 1% BSA, 4 mM EGTA, 8 mM MgCl<sub>2</sub>, 1 mM DTT and 0.4 mM PMSF). Cell extract (20  $\mu$ l) was combined with 100  $\mu$ l K-ATP/MgCl<sub>2</sub> buffer (100 mM K-phosphate, 3.65 mM ATP, 1 M MgCl<sub>2</sub>, and 50 mM Tris-HCl [pH 7.8]) and assayed for luciferase activity using a luminometer (Dynatech Laboratories, Chantilly, VA). The remaining cell extract was mixed with 200  $\mu$ l of *o*-nitrophenyl  $\beta$ -galactopyranoside (ONPG) substrate solution (100 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.8], 6.64 mM ONPG) and analyzed for  $\beta$ -galactosidase activity at 415 nm on an ELISA plate reader (Bio Rad Laboratories). Normalized luciferase response was then calculated as relative light units divided by  $\beta$ -galactosidase activity ( $\text{OD}_{415\text{ nm}}$  per

minute). Each data point generated represents a mean of triplicate measurements of a single transfection experiment. The oPRL-dependent fold induction (reported relative to normalized luciferase activity obtained in the absence of oPRL) is reported as mean  $\pm$  SD for at least three independent transfections.

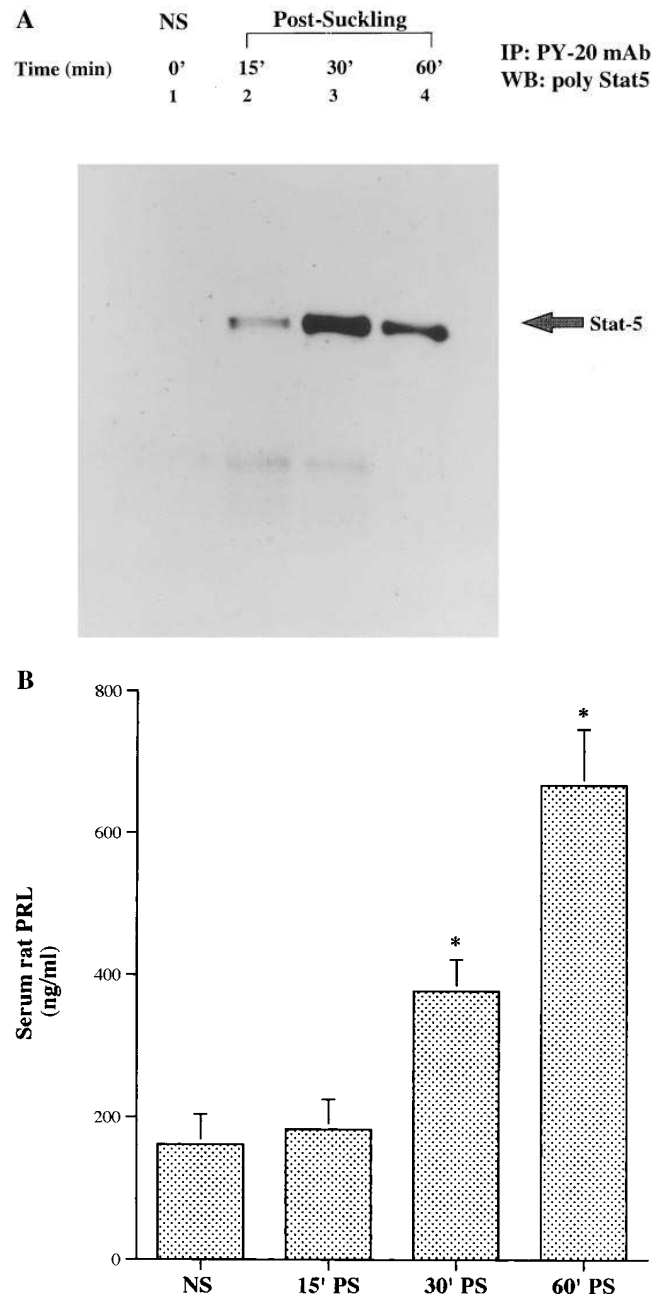
## Results

**Increased nuclear translocation of a phosphorylated Stat5-like protein in postpartum suckling rat liver.** In the postpartum rat, suckling stimulates secretion of PRL by the anterior pituitary, whereas withdrawal of suckling pups results in decline of serum PRL levels. The effects of rat PRL on hepatic phosphorylated Stat5 levels *in vivo* were investigated by quantifying nuclear Stat5 levels in the maternal livers in response to withdrawal and subsequent reintroduction of suckling.

Hepatic nuclear extracts from nonsuckling or those obtained from postsuckling rats were subjected to immunoblot analysis. The blot was probed with an anti-Stat5 mAb, and a protein of  $\sim 95$  kD that is immunogenically similar to Stat5 was quantitatively increased in a time-dependent manner in the post-suckling samples (Fig. 2 A). A parallel blot that was sequentially treated under identical conditions but probed with the secondary antibody (anti-mouse IgG horseradish peroxidase conjugate) alone revealed nonspecific bands (data not shown). Phosphorylation status determined in the same samples using the antiphosphotyrosine mAb (4G10) indicated that the  $\sim 95$ -kD protein was tyrosine phosphorylated (Fig. 2 B). To further confirm that the  $\sim 95$ -kD Stat5-like protein was tyrosine phosphorylated, nuclear extracts were immunoprecipitated with the monoclonal antiphosphotyrosine antibody PY-20 and revealed by Western blotting (Fig. 3 A) using the poly-

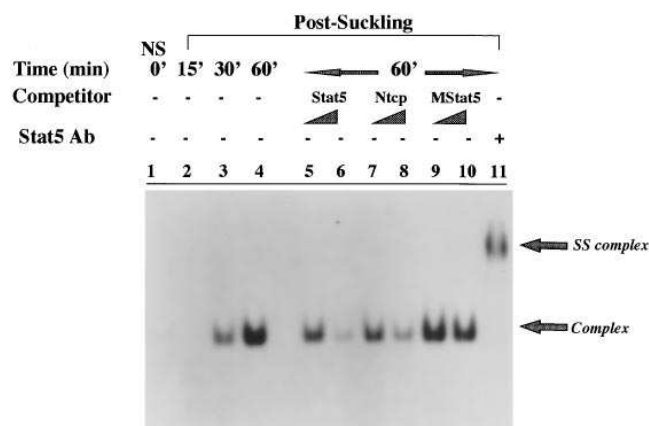


**Figure 2.** Western blotting of hepatic nuclear proteins from nonsuckling and postsuckling rats. (A) Nuclear extracts (20  $\mu$ g) from nonsuckling (NS, lanes 1 and 2) or postsuckling 15 min (lanes 3 and 4), 30 min (lanes 5 and 6), and 60 min (lanes 7 and 8), rats were probed with anti-Stat5 mAb. (B) The same blot was stripped and probed with the monoclonal antiphosphotyrosine antibody, 4G10. Each lane represents nuclear extracts from an individual animal. Similar results were obtained with three to four animals in each group.



**Figure 3.** (A) Immunoprecipitation of tyrosine phosphorylated Stat5 protein. Representative nuclear extracts (50  $\mu$ g) from nonsuckling (NS, lane 1) or postsuckling 15 min (lane 2), 30 min (lane 3) and 60 min (lane 4) rats were immunoprecipitated with the antiphosphotyrosine antibody PY-20 and probed with polyclonal rabbit anti-Stat5 as described in Methods. (B) Serum PRL levels. PRL levels from nonsuckling (NS) and 15, 30, and 60 min postsuckling (PS) rats were analyzed by a double-antibody RIA (28). Data are expressed as mean  $\pm$  SEM for  $n = 3-4$  rats. \* $P < 0.05$ .

clonal Stat5 antibody, respectively. As before, the  $\sim 95$ -kD band was predominantly detected in the nuclear extracts in the 30- and 60-min samples. Similar results were also obtained when the antiphosphotyrosine 4G10 was used for immunoprecipitation of tyrosine-phosphorylated proteins and the monoclonal Stat5 antibody was used for detection of Stat5 (data not

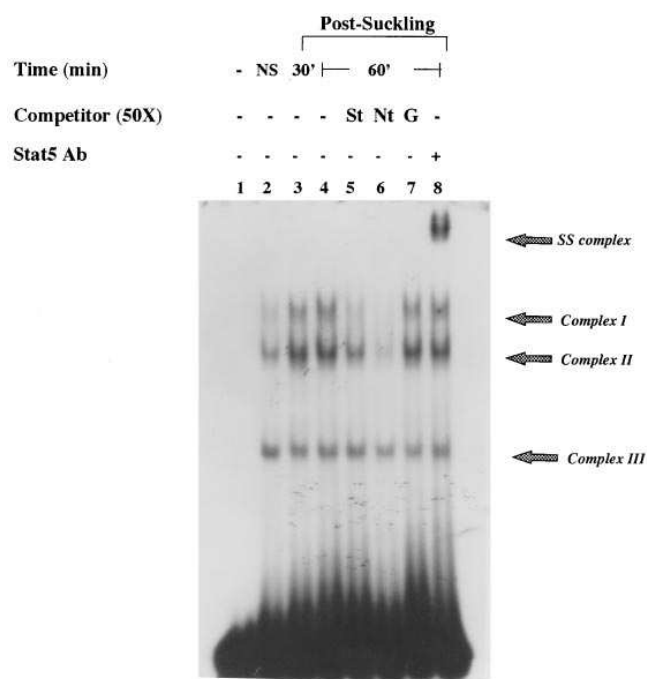


**Figure 4.** Electrophoretic mobility shift assays. Representative nuclear extracts (5  $\mu$ g) from nonsuckling (NS, lane 1) or postsuckling (15, 30, and 60 min, lanes 2–4) rats were used in band shift assays with  $^{32}$ P-labeled Stat5 consensus oligonucleotide (5'-agatTTCTAGGAA-ttcaatcc-3') as a probe. In competition assays (lanes 5–10), nuclear extracts from 60-min postsuckling rats were used. 10- and 50-fold molar excess of unlabeled Stat5 consensus (Stat5, lanes 5 and 6), *ntcp* (Ntcp, lanes 7 and 8) and mutant Stat5 (MStat5, lanes 9 and 10) oligos were used in competition assays. Polyclonal rabbit anti-Stat5 (1  $\mu$ g) was used for the supershift assay (lane 11). The same results were reproduced with nuclear extracts from independent animals (three to four in each group).

shown). The appearance of the hepatic Stat5-like phosphorylated protein in postsuckling rats correlated with increased serum PRL levels (Fig. 3 B), further suggesting that during lactation, the level of nuclear phosphorylated Stat5 is closely controlled in vivo through signals of circulating PRL which is in turn induced by the suckling stimulus from the pups.

**Phosphorylated Stat5-like factor in nuclear extracts binds to GAS-like element from the *ntcp* promoter.** To determine if the Stat5-like protein identified in postsuckling liver nuclear extracts exhibits DNA-binding ability, an oligo (21 mer) from the bovine  $\beta$ -casein promoter containing the Stat5 consensus binding sequence was initially used in gel shift assays (Fig. 4). A slower migrating gel shift complex was observed only in 30- and 60-min postsuckling samples. In competition assays, 10- and 50-fold excess of the unlabeled bovine  $\beta$ -casein consensus Stat5 oligo or a 29 mer corresponding to –922/–892 of the *ntcp* promoter encompassing the GLE binding motif competitively decreased specific binding. In contrast, both 10- and 50-fold excesses of unlabeled, mutated Stat5 oligo were unable to compete for specific binding, indicating strict requirements of the nuclear protein for the Stat5 cognate binding site. In addition, the DNA–protein complex was supershifted with polyclonal Stat5 antibody, indicating that the nuclear protein that is detected in a time-dependent manner after suckling is phosphorylated Stat5.

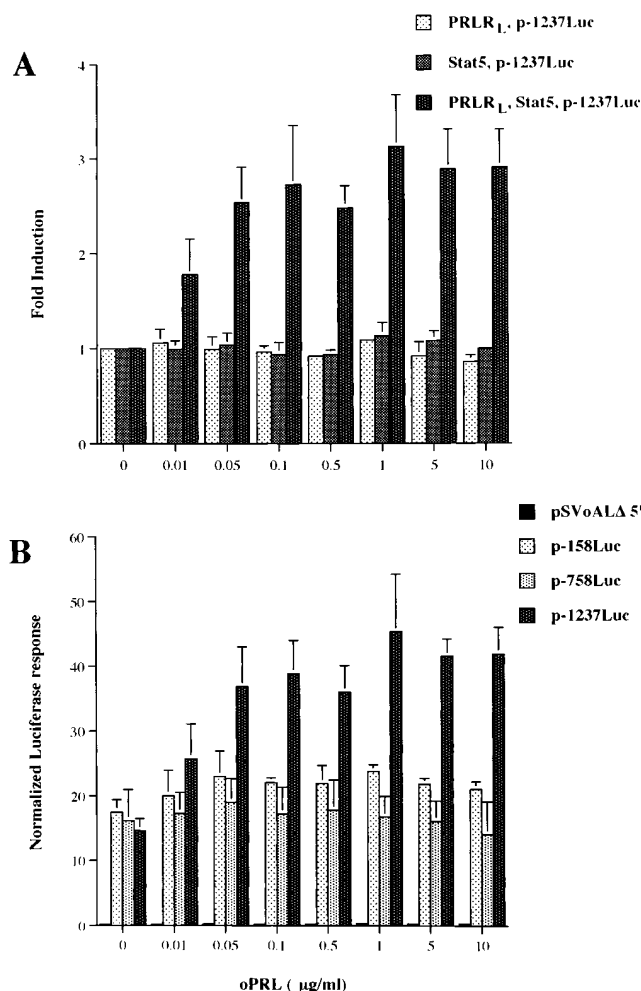
Two GLEs located at positions –936 to –928 bp (TTC CTA GAA) and –912 to –904 bp (TTC TTG GAA) were identified in the *ntcp* promoter by a sequence homology search. Both the GLEs match the consensus GAS-binding site (TTC/A C/TNG/A G/TAA). Furthermore, the nucleotide sequence of the GLE located at –912 to –904 is identical to the GAS-binding site found in the promoter of the rat  $\beta$ -casein gene at all nine nucleotide positions (20, 21). Therefore, a 29



**Figure 5.** Electrophoretic mobility shift assays. Band shift assays were performed using  $^{32}$ P-labeled Ntcp oligo (5'-gaagttgtcaTTCTTG-GAAaataacaat-3') as a probe. The labeled oligo was incubated without nuclear extract (lane 1), or with representative nuclear extracts (5  $\mu$ g) from nonsuckling (NS, lane 2) or postsuckling (30 and 60 min, lanes 3 and 4) rats. In competition assays (lanes 5–7), nuclear extract from 60 min postsuckling rats were used. 50-fold molar excess of unlabeled Stat5 consensus (St, lane 5), Ntcp (Nt, lane 6), and glucocorticoid response element (G, lane 7) oligos were used in competition assays. Polyclonal rabbit anti-Stat5 (1  $\mu$ g) was used for the supershift assay (lane 8). The same results were reproduced with nuclear extracts from independent animals (three to four in each group).

mer containing the *ntcp* GLE located –912 to –904 within its core was next evaluated for its ability to bind to nuclear proteins. As shown in Fig. 5, three DNA–protein complexes were observed. The slowest migrating complex (I) was competitively inhibited by 50 $\times$  molar excess of unlabeled Ntcp oligo and the consensus Stat5 oligo, but not by a nonspecific glucocorticoid response element oligo. Furthermore, this complex was partially supershifted with polyclonal Stat5 antibody, confirming the presence of Stat5 nuclear protein. The identity of the intermediate migrating complex (II) is unknown, although it appears to result from a specific interaction, as binding was decreased by molar excesses of both the competing Ntcp and consensus Stat5 oligos. The fastest migrating complex (III) was unchanged among the various groups, and probably results from a nonspecific DNA–protein interaction. Band shifts with an oligo encompassing the GLE in –936 to –928 of the *ntcp* promoter showed a similar pattern of DNA–protein complex formation as that of the –912 to –904 oligo (data not shown). These data indicate that nuclear Stat5 exhibits specific DNA-binding ability towards the GLEs in the *ntcp* promoter.

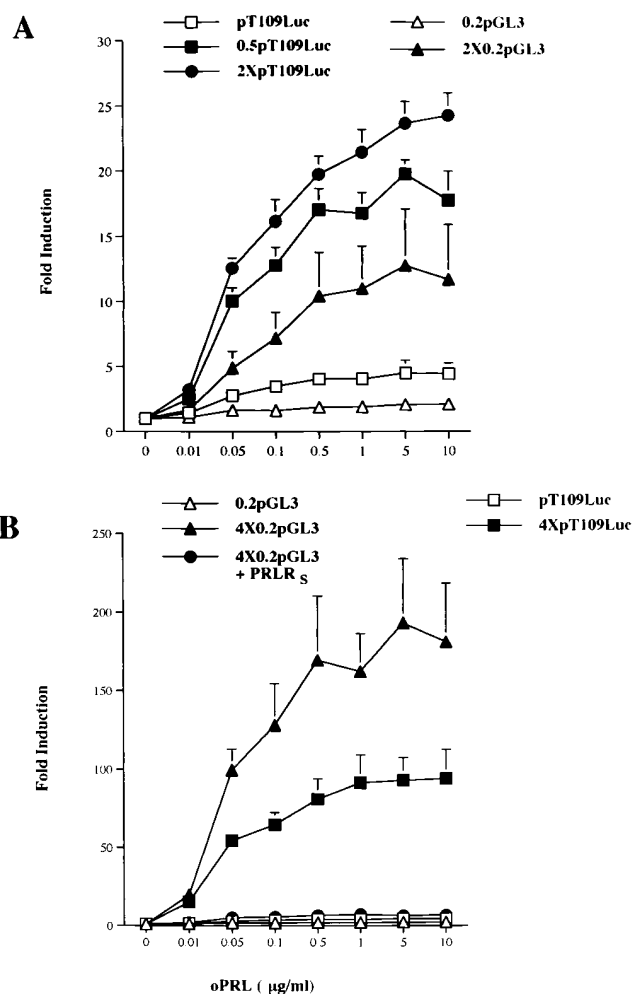
**PRL-dependent transcriptional activation of the *ntcp* promoter in HepG2 cells.** To determine if the *ntcp* promoter is PRL responsive, HepG2 cells were transiently transfected with the plasmid p-1237Luc or vector pSVoAL $\Delta$ 5', and were



**Figure 6.** (A) PRLR<sub>L</sub> and Stat5 mediate PRL inducibility of the *ntcp* promoter. HepG2 cells were transfected as described in Methods with the p-1237Luc construct either alone or cotransfected with the expression vectors for the PRLR<sub>L</sub> and/or Stat5. Luciferase activity and  $\beta$ -galactosidase activities were measured in cellular extracts 36–40 after treatments with oPRL. (B) oPRL dose–response curve of *ntcp* promoter deletion constructs. HepG2 cells were cotransfected with the appropriate promoter–luciferase plasmid constructs, pRSV- $\beta$ -galactosidase, and expression plasmids for PRLR<sub>L</sub> and Stat5, and were treated with the indicated concentration of oPRL. All data are expressed as mean  $\pm$  SD of three independent transfections.

treated with varying doses of oPRL. Consistent with earlier reports (21), negligible luciferase activity was observed with pSVoALA5'. Significant levels of luciferase activity were observed in cells transfected with the plasmid p-1237Luc, but no PRL inducibility was observed, consistent with earlier reports that HepG2 cells lack either functional PRLR<sub>L</sub>, Stat5, or both (29).

Expression vectors containing cDNAs for the PRLR<sub>L</sub> and Stat5 were cotransfected with the plasmid p-1237Luc or pSVoALA5'. As shown in Fig. 6A, addition of oPRL increased luciferase activity in a dose-dependent manner (2.5- to 3-fold) only when both the PRLR<sub>L</sub> and Stat5 cDNAs were cotransfected with p-1237Luc. In comparison, transfection of PRLR<sub>L</sub> and Stat5 cDNAs had no effect on the luciferase activity of pSVoALA5'. Exclusion of either the PRLR<sub>L</sub> or Stat5 expres-



**Figure 7.** (A) GLEs confer PRL responsiveness to *ntcp* minimal promoter. HepG2 cells were cotransfected with the appropriate promoter–luciferase plasmid constructs, pRSV- $\beta$ -galactosidase, and expression plasmids for PRLR<sub>L</sub> or PRLR<sub>S</sub> and Stat5. oPRL dose–response curves were generated as described in Methods. (B) *ntcp* GLEs confer PRL responsiveness to heterologous thymidine kinase promoter. HepG2 cells were cotransfected with the appropriate promoter–luciferase plasmid constructs, pRSV- $\beta$ -galactosidase, and expression plasmids for PRLR<sub>L</sub> or PRLR<sub>S</sub> and Stat5. oPRL dose–response curves were generated as described in Methods. Data are expressed as mean  $\pm$  SD of three to four independent transfections.

sion vectors abrogated the PRL induction, indicating that the oPRL response is mediated by the PRLR<sub>L</sub> via activation of Stat5.

In cotransfection experiments using deletion constructs of the *ntcp* promoter, the plasmids p-758Luc and p-158Luc, which exhibited significant levels of basal transcription, failed to respond to oPRL treatment when compared to the plasmid p-1237Luc (Fig. 6B). These results support the notion that although the regulatory elements necessary for basal *ntcp* promoter activity are included in the fragment –158 to +47, elements located in the region between –1237 and –758 bp, possibly the two GLEs, are required for PRL responsiveness of the *ntcp* promoter.

To further delineate the role of these GLEs in mediating PRL responsiveness, two and four multimerized GLEs were

inserted upstream of the *ntcp* minimal promoter in p0.2GL3 to create the plasmids 2Xp0.2GL3 and 4Xp0.2GL3. As shown in Fig. 7, A and B, the 2X and 4X GLEs were able to confer PRL-dependent increases in luciferase activity to the *ntcp* minimal promoter of ~ 15- and 150-fold higher than 0.2pGL3, respectively. These data indicate that the two GLEs are both necessary and sufficient for PRL inducibility. Additionally, the PRLR<sub>S</sub> failed to transactivate *ntcp* GLEs in the 4X0.2pGL3 construct, presumably because it is unable to activate Stat5 (Fig. 7 B).

In additional experiments, the ability of the -1237 to -758 bp region to modulate heterologous promoter activity was examined. Linking of this 0.5-kb PRL-responsive region upstream of the HSVtk promoter in the plasmid pT109Luc (Fig. 7 A) produced a dose-dependent increase in luciferase activity in response to oPRL stimulation. Furthermore, multimerized GLEs placed upstream of the HSVtk promoter (2XpT109Luc and 4XpT109Luc constructs) were also able to confer PRL responsiveness with the 2X construct showing a similar inducibility compared to the 0.5-kb construct (~ 20-fold higher than pT109Luc). As observed with the 4X0.2pGL3 construct, the inclusion of two additional GLEs (4XpT109Luc) had a synergistic effect on luciferase activity (~ 100-fold higher activity than pT109Luc). These results indicate that *ntcp* GLEs are capable of transactivating a heterologous promoter and functioning as enhancer elements that modulate the activity of the minimal *ntcp* promoter.

## Discussion

The present studies establish for the first time the mechanism(s) by which the hormone PRL upregulates hepatic bile salt transporter function postpartum; namely by activation of the latent transcription factor Stat5 via the PRLR<sub>L</sub>. These studies demonstrate striking similarities in the PRL signaling pathways in the rat mammary gland and liver that lead to tissue-specific gene expression of two target genes, namely the  $\beta$ -casein in the mammary gland and *ntcp* in the liver. As with the mammary gland, PRL activates Stat5 via PRLR<sub>L</sub>, presumably by a Jak2-dependent phosphorylation pathway. Interaction of SH2 domains of dimerizing Stat5 partners then confers the ability to bind to the GLEs in the *ntcp* promoter and transcriptionally activate the *ntcp* after translocation of Stat5 multimers to the nucleus. These data, together with earlier reports (4–8), therefore support the hypothesis that during the postpartum period, the stimulatory effects of PRL on Na<sup>+</sup>/taurocholate cotransport across the basolateral membrane of the hepatocyte appears to be primarily caused by increased transcription of *ntcp*.

Using a postpartum rat model, we were able to demonstrate a time-dependent nuclear accumulation of Stat5 that correlated with serum PRL levels. These data suggest that the level of phosphorylated Stat5 in vivo is controlled through signals of circulating PRL that are induced by suckling of the pups. Waxman et al., have reported that the administration of GH, but not PRL, in vivo results in nuclear translocation of Stat5 in the liver (26, 27). The lack of effect of PRL probably results from the use of hypophysectomized animals by these researchers, a model in which the hepatic lactogenic receptors are rapidly downregulated (34, 35). However, preliminary reports have suggested that *ntcp* is expressed in a sexually dimorphic pattern that may be attributed to the pulsatile nature of

GH secretion in male rats (36). Whether GH, which has been reported to activate multiple Stat proteins, including Stat5, regulates *ntcp* transcription by a similar mechanism as PRL is currently being investigated by this laboratory.

Gel mobility shifts and oligonucleotide competition assays showed that Stat5 in hepatic nuclear extracts from lactating animals was able to bind with similar specificities to the GAS in the bovine  $\beta$ -casein promoter (Fig. 4) as well as to the GLE located at position -912 to -904 of the *ntcp* promoter (Fig. 5). Some of the additional protein complexes formed with the Ntcp oligos, when compared to those formed by the bovine  $\beta$ -casein oligo, result in part from differences in the sizes of the two oligos and/or neighboring nucleotide sequences around the Stat5-binding site. This is supported by the observation that the core DNA-binding sequences between the GAS sites in the bovine  $\beta$ -casein (TTCTAGGAA) and *ntcp* (TTCTTG-GAA) only differ at a single position. However, the fact that a single protein complex formed with the bovine  $\beta$ -casein oligo supershifts with specific anti-Stat5 antibody indicates that Stat5 homodimers participate in DNA-protein complex formation. At this point we cannot determine whether the complex is composed of Stat5a, Stat5b homo or hetero dimers since the anti-Stat5 polyclonal antibody used was raised against a peptide corresponding to the carboxy terminus of mouse Stat5b, which differs from the corresponding sequence of Stat5a by only three amino acids. However, this antibody is reported not to cross-react with other members of the Stat family, thereby ruling out the participation of heterodimers of Stat5 and other PRL-activated Stat proteins (namely Stat1 and Stat3) in the DNA-protein complex formation (37).

We studied the mechanisms involved in PRL responsiveness of the *ntcp* promoter activity in transiently transfected HepG2 cells. A dose-dependent, PRL-induced increase in *ntcp* promoter activity was observed only when expression vectors for Stat5 and PRLR<sub>L</sub> cDNA were cotransfected with *ntcp* promoter-luciferase reporter constructs. This supports the notion that PRL-dependent transactivation is a receptor-mediated event that requires the Stat5 protein. Interestingly, of the two forms of the PRL receptor present in the female rat liver, the PRLR<sub>S</sub> is the predominant form. To date, however, the functional role of the short form of the receptor has not been defined clearly (12, 38). The present studies indicate that the hepatic PRLR<sub>L</sub>, not the PRLR<sub>S</sub>, plays a critical role in modulating bile salt transport across the basolateral domain of the hepatocyte. The PRLR<sub>S</sub> originates from alternate splicing of the PRLR mRNA and is distinguished from the PRLR<sub>L</sub> by a truncated carboxy-terminal cytoplasmic domain (39, 40). However, the juxtamembrane cytoplasmic subdomains named Box 1 (proline rich) and Box 2 (acidic region), which are conserved structural features of class I cytokine receptors, are present in both forms of the receptors (41–43). The PRLR<sub>S</sub> is thus able to maintain PRL-induced Jak-2 association via the Box 1 subdomain (44). However, truncated PRLR variants that lack the conserved cytoplasmic tyrosine residues are still capable of recruiting Stat1, Stat3, and Stat5 to the activated PRLR complex via Jak-2 (37). All together, these observations suggest that in addition to Jak-2 activation, amino acids in the COOH terminus of the PRLR<sub>L</sub> that are absent in PRLR<sub>S</sub> are also involved in Stat recruitment.

By deletion analysis, the PRL-responsive region of the *ntcp* promoter was mapped to the two GLEs located within -937 to -904 upstream of the transcription start site. Subsequently,



we demonstrated that the GLEs confer PRL responsiveness to the *ntcp* minimal promoter, and are also able to modulate the activity of a heterologous thymidine kinase promoter. These data provide strong evidence that the two GLEs in the *ntcp* promoter function in PRL-induced transcriptional regulation of this gene after the binding of Stat5 dimers. The synergistic effect on reporter gene activity observed with the multiple GLE constructs is probably caused by cooperative binding of Stat proteins to several adjacent GAS, which is facilitated by the amino-terminal domain, as has been recently reported (44). Whether or not Stat5 plays a principal role in *ntcp* regulation in the male or the nonlactating female rat liver awaits further studies. These experiments also raise an important question as to whether *ntcp* transcription can be modulated by other cytokines, such as erythropoietin, GM-CSF, and members of the IL family that activate Stat5 via their respective receptors (45).

In summary, the rat liver is an important target organ for PRL. This hormone has been shown to have diverse biological effects on cell growth, steroidogenesis, maternal behavior, osmoregulation, and immune response in various species (11, 46, 47). The present studies establish an additional physiological role for PRL in the regulation of Na<sup>+</sup>-dependent bile salt transporter function during the postpartum period. PRL may thus play an important role in maternal nutritional status by increasing uptake and biliary secretion of bile salts (4, 28, 48), which are essential for the absorption of fats and fat-soluble vitamins.

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