Transcriptional Regulation of the Generic Promoter III of the Rat Prolactin Receptor Gene by C/EBPβ and Sp1*

(Received for publication, May 7, 1998, and in revised form, July 10, 1998)

Zhang-Zhi Hu, Li Zhuang, Jianping Meng, and Maria L. Dufau‡

From the Section on Molecular Endocrinology, Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Three promoters are operative in the rat prolactin receptor gene as follows; promoter I (PI) and II (PII) are specific for the gonads and liver, respectively, and promoter III (PIII) is common to several tissues. To investigate the mechanisms controlling the activity of promoter III, its regulatory elements and transcription factors were characterized in gonadal and non-gonadal cells. The TATA-less PIII domain was localized to the region −437 to −179 (ATG +1) containing the 5'-flanking region and part of the non-coding first exon. Within the promoter domain, a functional CAAT-box/enhancer binding protein (C/EBP) (~386) and an Sp1 element (~386), which bind C/EBPβ and Sp1/Sp3, respectively, contribute individually to promoter activation in gonadal and non-gonadal cells. However, significant redundancy was demonstrated between these elements in non-gonadal cells. Additionally, an element within the non-coding exon 1 (~338) is also required for promoter activity. Activation of PIII by the widely expressed Sp1 and C/EBPβ factors explains its common utilization in multiple tissues. Moreover, whereas the rat and mouse PII share similar structure and function, the mouse PI lacks the functional SF-1 element and hence is inactive. These findings indicate that promoter III is of central importance in prolactin receptor gene transcription across species.

Prolactin is the most functionally diversified pituitary hormone that exerts a wide range of biological actions involving lactation, reproduction, growth and metabolism, water-salt balance, immune regulation, and maternal behavior through specific prolactin receptors (PRLR)† present on various prolactin target tissues (1–3). The functional diversity of this hormone and the wide distribution of its receptors have suggested that a complex form of regulation determines the levels of PRLR expression in individual prolactin target tissues. This has been revealed by the recent demonstration of multiple and tissue-specific promoter control of the prolactin receptor gene expression in rat gonadal and non-gonadal tissues (4, 5). PRLRs are present as long and short forms and are encoded by multiple PRLR mRNA transcripts, which are derived from alternative transcription initiation from different PRLR promoters at the 5'-end, alternative splicing of the 5'-noncoding exon (exon 2), and the coding exons (3, 4). Three PRLR gene promoters have been identified in the rat. Promoter I, specifically utilized in gonadal cells, contains a functional SF-1 consensus element that binds steroidogenic factor-1 (SF-1), a specific zinc finger DNA-binding protein also known as Ad4BP (6), which is an essential transcriptional activator for this promoter (5). Promoter II is specifically utilized in the liver (4, 7) and its activity is regulated by the liver-specific hepatic nuclear factor 4 (HNF4) through binding to an HNF4 element in the promoter region (7). By contrast, the generic promoter III (PIII) is commonly utilized in the rat gonads and liver and as a sole promoter in the rat mammary gland (4, 5). Sequence analysis of PII revealed C/EBP- and Sp1-binding elements. It is likely that this promoter is also utilized in other PRLR-expressing tissues that have not been examined. However, the mechanisms underlying regulation of the commonly utilized PRLR promoter III has not been investigated.

In the present study, we aimed to define the promoter domain of PIII and to identify the cis-elements and trans-factors that regulate its promoter activity in gonadal and non-gonadal cells. We demonstrated that C/EBPβ and Sp1 bind to the 5'-flanking region and regulate the PIII activity in homologous and heterologous cells. In addition, a proximal downstream sequence element (DSE) was essential for basal promoter activity. Finally, we have determined that the promoter III homolog in the mouse shares structural and functional similarity with the rat PIII, whereas in this species, the PI homolog exhibits minimal promoter activity due to mutation of the SF-1 element that renders this element non-functional. Taken together, these findings indicate that PIII is of central importance in the control of transcription of the prolactin receptor gene.

MATERIALS AND METHODS

Animals—24-Day-old immature female rats (Charles River, Wilmington, MA) were injected subcutaneously daily for 3 days with 1.5 mg/day 17β-estradiol (Sigma). On the 4th day, the animals were sacrificed (CO2 asphyxiation) and ovaries removed for preparation of ovarian granulosa cells. The studies were approved by the NICHD Animal Care and Use Committee (protocol 97039).

Culture of Cell Lines and Primary Rat Ovarian Granulosa Cells—A stable steroidogenic cell line, mouse Leydig tumor cells (MLTC), which expresses prolactin and luteinizing hormone receptors (kindly provided by Dr. R. V. Rebois, National Institutes of Health, Bethesda, MD), was maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum and 1x antibiotic/antimycotic mixture (Life Technologies, Inc.). The human hepatoma cell line...
Mutagenesis to the translation initiation codon (PIII (Promega, Madison, WI). The pGL2 constructs were numbered relative to the translation start sites are present. A C/EBP-like domain of PIII resides at 437-345) containing this region was examined for promoter activity, it had only minimal activity in both cell types.

RESULTS

Dominant Mutations of PIII Resides at 437 to 179—1.3 kilobases of genomic DNA (1427 to 179) containing 5′-flanking region and partial first exon sequence (El) of the rat PRLR gene was initially examined for promoter activity in luciferase reporter gene construct in transiently transfected gonadal (MLTC) and non-gonadal (HepG2) cells. This fragment containing promoter III exerted strong promoter activity in both cell types, in contrast to P1 or PII, which showed specific activation in MLTC or HepG2, respectively (3). Subsequently a series of deletions of this genomic fragment in luciferase reporter gene constructs were characterized in MLTC and HepG2 cells (Fig. 1). Deletions from 1427 to 437 had no significant effects on the luciferase activity in both MLTC and HepG2 cells (constructs 1–6). However, deletion from 437 to 344 reduced the promoter activity to 23 and 58% of full activities in MLTC and HepG2 cells, respectively (construct 7), indicating that the 437 to 344 region is required for full promoter activation in both cell types. However, when the 5′-flanking fragment (506 to 345) containing this region was examined for promoter activity, it had only minimal activity in both cell types (15% of full activity) (construct 8). These results indicated that both the 5′-flanking region and part of the first exon downstream of the transcription start site (TSS) are also necessary for full promoter activation, and therefore the promoter domain of PIII resides at 437 to 179. This 258 bp promoter region contains neither a canonical TATA box sequence nor consensus initiator sequences, although CT-rich sequences surrounding the translation start sites are present. A C/EFP-like element was identified at 398 in the 5′-flanking region of the promoter domain, 58 bp upstream of the major transcription start site at -340 (4). Three bp downstream of this C/EFP element resides a (CTC), repeat, a variant Sp1-binding site, referred as Sp1 (386). Two additional Sp1 consensus sequences downstream of the TSS are present at -273 and -261, referred as Sp1 and Sp1, respectively. In addition, an AP2 consensus sequence 8 bp downstream of the major TSS is also present. These putative regulatory elements were further explored.

Characterization of the Mouse PRLR Genomic Clone from BAC (Bacterial Artificial Chromosome) Library—A BAC DNA clone with 120-kilo base insert containing the mouse PRLR genomic regions corresponding to both the rat promoters III and I was obtained from BAC library (Genome Systems, Inc., St. Louis, MO) by hybridization screening using rat PI and PII region probes. The BAC DNA was prepared as described (1, 2). A series of deletions of this genomic fragment in luciferase reporter gene constructs were characterized in MLTC and HepG2 cells (Fig. 1). Deletions from 1427 to 437 had no significant effects on the luciferase activity in both MLTC and HepG2 cells (constructs 1–6). However, deletion from 437 to 344 reduced the promoter activity to 23 and 58% of full activities in MLTC and HepG2 cells, respectively (construct 7), indicating that the 437 to 344 region is required for full promoter activation in both cell types. However, when the 5′-flanking fragment (506 to 345) containing this region was examined for promoter activity, it had only minimal activity in both cell types (15% of full activity) (construct 8). These results indicated that both the 5′-flanking region and part of the first exon downstream of the transcription start site (TSS) are also necessary for full promoter activation, and therefore the promoter domain of PIII resides at 437 to 179. This 258 bp promoter region contains neither a canonical TATA box sequence nor consensus initiator sequences, although CT-rich sequences surrounding the translation start sites are present. A C/EFP-like element was identified at 398 in the 5′-flanking region of the promoter domain, 58 bp upstream of the major transcription start site at -340 (4). Three bp downstream of this C/EFP element resides a (CTC), repeat, a variant Sp1-binding site, referred as Sp1 (386). Two additional Sp1 consensus sequences downstream of the TSS are present at -273 and -261, referred as Sp1 and Sp1, respectively. In addition, an AP2 consensus sequence 8 bp downstream of the major TSS is also present. These putative regulatory elements were further explored.
amined for their nuclear protein binding capability by EMSA and for their functionality in promoter activation by mutagenesis analysis (see below).

To examine whether this atypical TATA-less promoter can direct faithful transcription initiation in transfected cells, the PII constructs (−437/-179) including wild type and mutated construct of the putative functional C/EBP element (see also below) were transfected into MLTC cells, and mRNA from these cells was isolated and analyzed by 5′-RACE PCR. An extended band of −350 bp was revealed from the PCR products of either the wild type or mutant constructs by Southern hybridization using a nested probe (P 74, Fig. 2). Therefore, the transcription start site derived from the size of the 5′-RACE PCR products for transfected PII constructs in MLTC cells was consistent with the major TSS at −340 previously identified in vivo by primer extension analyses in both gonadal and non-gonadal cells (4). Since the transcription initiation is mainly dictated by the core promoter structure (e.g. TATA box, initiator, GC box) in most genes (12), but not by the enhancer element, the fact that similar results were obtained from both the wild type and C/EBP mutant constructs strengthened the notion that the core PII domain directed faithful transcription initiation in cultured cells.

C/EBPβ Specifically Binds to the C/EBP Element in the 5′-Proximal Region of Promoter III in MLTC and HepG2 Cells—To examine transcription factor-binding sites in the PII domain, EMSA was employed using double-stranded DNA probes of various lengths. We first analyzed the putative C/EBP-like binding sequence TTGTGCAACAC (−398) (C/EBP consensus sequence RTTGGCGYAAY where Py indicates) within the 258-bp promoter domain (Fig. 1). When a short probe (probe III −416/−388) containing this site was used in EMSA, a prominent protein complex was revealed in the nuclear extracts from MLTC cells (Fig. 3A, lanes 1 and 9). This complex was completely inhibited by preincubation of the nuclear extract with a 250-fold molar excess of unlabeled DNA (lanes 2 and 10) but not with unlabeled mutated C/EBP sequence (C/EBPβ m2, lanes 3 and 11). Similarly, this complex was inhibited by preincubation with unlabeled C/EBP consensus oligomer (CS, lane 4) but not by its mutant (CSx, lanes 5). These results indicated that this C/EBP-like sequence was specifically bound by a protein complex related to C/EBP family of proteins present in MLTC nuclear extract. To examine the nature of this protein complex, a panel of C/EBP antibodies including one that exhibits cross-reactivity to all subtypes (pan-antibody), and those specific for C/EBP subtypes α, β, δ, and ε were used for gel supershift assay. This protein complex was clearly supershifted by C/EBP pan-antibody (lanes 6, 7, and 12) as well as by anti-β subtype antibody (lane 14) but not by antibodies to α, δ, and ε subtypes (lanes 13, 15, and 16) or normal rabbit serum (lanes 8 and 17). These results demonstrated that the C/EBP-like element in the PII domain binds specifically to C/EBPβ present in MLTC nuclear extracts.

Furthermore, the specific C/EBPβ binding complex, supershifted by C/EBPβ antibody, was also shown using a longer DNA probe (probe I, −437/-345). This probe also revealed additional specific DNA-protein complexes C1 and C2 not related to C/EBPβ (Fig. 3A, lanes 18 and 26). When three mutants of the C/EBP-like element (m1, m2, and m3) were used as unlabeled DNA competitors in EMSA, no competition for the C/EBPβ complex was observed (lanes 20–22). The most conserved mutation of C/EBP-like element, m2, was subsequently used for functional studies of this promoter (see below). The other specific protein complexes (C1 and C2) revealed by probe I were competed by unlabeled probe sequence I (lane 19) and II (lane 29) but not by the sequences of −437 to −416 or −388 to −345 (not shown), indicating the presence of distinct protein-binding sites within the region of −416 to −373 (II). These two complexes, which were subsequently identified as members of the Sp1 family of proteins, bind to a variant Sp1 element located 3′ to the C/EBP element at −386 (Fig. 3C) (contents in Fig. 3A concerning this Sp1 element, lanes 24–34, are described in detail below).

The nuclear extract of HepG2 cells was similarly examined.
C/EBP derived mRNA transcripts using specific oligomer 5'-CTGTCTCTGAGAAGAAG-3'. Above the expressed PRLR-PIII/LUC gene constructs in MLTC cells.

from wild type (WT) CAGGTAAAG-3'. DNA complex by dissociation of the putative protein-protein association (not shown). Because of the weak and more retarded C/EBP binding detected in HepG2 nuclear extract by the short probe III, the C/EBP binding was masked by the strong and similarly migrated Sp1/Sp3 binding detected by probe II in HepG2 cells (not shown). Therefore, a C/EBP element-mutated probe II (Im) with intact Sp1 site was used in EMSA of Sp1 binding in HepG2 cells (see Fig. 4A and description below). Although C/EBP binding was not detected in rat granulosa cells using either the short or the long probe by the conventional EMSA method, this binding site was shown to be functional in the study of C/EBP site mutagenesis and of C/EBP cDNA cotransfection in granulosa cells (see below) (Fig. 7).

Sp1-binding Sites in the PIII Region—As indicated above, a variant Sp1-binding sequence (Sp1.), CTCCTCTCCTCTTCATAGCCTTATGCAG-3'. Below the expressed PRLR-PIII/LUC gene constructs in MLTC cells. A, lane 2) that were more retarded than the complex in MLTC cells (M, lane 1). These two complexes were competed by unlabeled probe sequence (lane 3) or oligonucleotide with consensus C/EBP sequence (lane 5) but not competed by sequences with mutated C/EBP sites (lanes 4 and 6). Furthermore, these complexes could be specifically supershifted by the antibody against C/EBP subtype (lane 7), indicating that C/EBP in HepG2 nuclear extract also bound to the C/EBP-like element. Since these bands were more retarded than the single band observed in MLTC cell, it is suggested that C/EBP may associate with additional protein(s) when binding to the PIII domain in HepG2 cells. However, such proteins are not related to association of Sp1 or Sp3 with C/EBP-DNA complex since preincubation with Sp1/Sp3 antiserum did not cause supershift of the complexes or increase the mobility of C/EBP-DNA complex by dissociation of the putative protein-protein association (not shown). Because of the weak and more retarded C/EBPβ binding detected in HepG2 nuclear extract by the short probe III, the C/EBPβ binding was masked by the strong and similarly migrated Sp1/Sp3 binding detected by probe II in HepG2 cells (not shown). Therefore, a C/EBP element-mutated probe II (Im) with intact Sp1 site was used in EMSA of Sp1 binding in HepG2 cells (see Fig. 4A and description below). Although C/EBP binding was not detected in rat granulosa cells using either the short or the long probe by the conventional EMSA method, this binding site was shown to be functional in the study of C/EBP site mutagenesis and of C/EBP cDNA cotransfection in granulosa cells (see below) (Fig. 7).

EMSA utilizing probe III (−416/−388) or using probe II (−416/−373) (Fig. 4A, lane 3), indicating that C1 and C2 were specifically associated with this CTC repeat. A specific Sp1 antibody partially supershifted C1 (Fig. 3A, lanes 24 and 33, and Fig. 4A, lane 4 versus lane 3) but not C2. In contrast, C2 was completely supershifted by a specific Sp3 antibody (Fig. 4A, lane 5). When both Sp1 and Sp3 antibodies were used, C1 and C2 complexes were both completely supershifted (lanes 6). These results indicated that C1 complexes contain both Sp1 and Sp3 proteins, whereas the C2 band corresponded to binding of Sp3 protein to the Sp1 element. Therefore, the CTC repeat (Sp1) in the PIII domain can bind to both Sp1 and Sp3 proteins present in MLTC nuclear extracts.

Similar protein binding to Sp1 element was observed in HepG2 cells (using probe Im that is C/EBP-mutated and Sp1, intact) as well as in rat ovarian granulosa cells (using probe II and I). As shown in Fig. 4A, both C1 and C2 complexes were competed by preincubation with unlabeled wild type Sp1 element sequences (lanes 10 and 17) but not with (CTC)3-mutated sequences (lanes 11 and 18). Supershift assay using antibodies against Sp1 and Sp3 revealed that C1 complex corresponded to both Sp1 and Sp3, whereas C2 corresponded to Sp3, in both HepG2 (lanes 12–14) and rat granulosa cells (lanes 19–21). Similarly, C1 and C2 complexes were revealed using the long probe I (−437/−345) in nuclear extracts of rat granulosa cells (Fig. 4A, lanes 23–27), MLTC (Fig. 3A, lanes 18–24), as well as in HepG2 cells (not shown). However, Sp1 and Sp3 binding in C1 complex are more discernible in EMSA using the long probe I (Fig. 4A, lane 23) than using the shorter probe II (lanes 1, 9, and 16), likely due to difference in the size of the two probes. The C1 complexes observed with probe I correspond to Sp1 and Sp3, lower and upper band, respectively.

In addition, two other consensus Sp1-binding sites adjacent to each other were identified 3' of the TSS within the first exon region (Sp1., TGGGCTTGGG, −273, and Sp1., CCCCCGCCGT, −261). EMSA of nuclear extracts from MLTC, HepG2, and rat granulosa cells using DNA probes (−285/−258) containing both Sp1, and Sp1, elements also showed two specific protein complexes, an upper major band and a lower minor band, bound to this probe in all three cells (Fig. 4B, lanes 1, 8, 15, and 22). These bands were competed by unlabeled oligonucleotides of...
wild type (lanes 2, 9, and 16) as well as of single mutated sequence of either Sp1b or Sp1c (lanes 3, 4, 10, 11, 17, and 18). However, competition of the protein complex was greatly diminished by double mutation of Sp1b and Sp1c in the unlabeled sequence (lanes 5, 12, and 19). Supershift assay using an Sp1 antibody indicated that Sp1 protein was part of the complex that bound to Sp1b or Sp1c (lanes 6, 13, 20, and 23). Supershift assay using Sp3 antibody indicated that Sp3 was also part of the specific complexes (Fig. 4B, lanes 24 and 25, arrows), similar to those observed for Sp1a. These results indicated that Sp1b and Sp1c are alternative binding sites for the same Sp1/Sp3 protein complexes. Similar findings for Sp1/Sp3 binding to
FIG. 4. Determination of three Sp1-binding sites in the PIII domain in nuclear extracts of MLTC, HepG2, and rat granulosa cells by EMSA. A, EMSA of Sp1 family protein binding to Sp1a site in nuclear extracts of MLTC, HepG2, and rat granulosa (rGC) cells. Double-stranded DNA probe II (lanes 1–8 and 16–22) or probe II with C/EBP mutation (IIm1, lanes 9–15) and probe I (lanes 23–27) were used in EMSA. Probes were incubated with nuclear extracts in the absence (lanes 1, 9, 16, and 23) or presence of excess molar of DNA competitors of unlabeled probe sequence (lanes 2, 10, 17, and 24) or Sp1a-mutated sequence (lanes 3, 11, and 18), in the presence of NRS (lanes 8, 15, and 22) or specific antibodies against Sp1 and Sp3 (lanes 4–6, 12–14, 19–21, and 25–27), or against C/EBPβ (lane 7). C1 and C2 DNA-protein complexes are indicated by arrows, corresponding Sp1/Sp3 or Sp3, respectively. C/EBPβ detected in MLTC using probe II is also indicated. B, EMSA of Sp1 protein binding to Sp1b and Sp1c sites in nuclear extracts of MLTC, HepG2, and rat granulosa (rGC) cells. Double-stranded oligomer probe Sp1b,c (5'-CGGGTCTTCTGGAATGGGCTTTCCATACCTTCCTGC-3') was used in EMSA. The mutated Sp1b,c sequence is 5'-CGGGTCTTCTGGAATGGGCTTTCCATACCTTCCTGC-3'. Probes were incubated with nuclear extracts of MLTC, HepG2, and rat granulosa (rGC) in the absence (lanes 1, 8, 15, and 22) or the presence of DNA competitors of unlabeled probe sequence (lanes 2, 9, and 16) or Sp1 single or double mutated sequences (lanes 3–5, 10–12 and 17–19) or in the presence of normal rabbit serum (NRS) (lanes 7, 14, and 21), specific antibodies against Sp1 (lanes 6, 13, 20, and 23) or Sp3 (lane 24), or both (lane 25). Specific Sp1/Sp3 DNA complexes are indicated by arrows.
the Sp1<sub>b,c</sub> in MLTC and HepG2 cells were observed (not shown).

**Transcriptional Activation of PIII by C/EBP and Sp1a Elements in MLTC and HepG2 Cells**—To assess the functional roles of the nuclear protein-binding sites within PIII region, luciferase reporter genes directed by promoter domains of wild type or various mutations of the protein-binding sites or putative cis-elements were constructed and transiently expressed in MLTC or HepG2 cells (Fig. 5). In MLTC cells, single mutation of C/EBP element in the promoter domain decreased the promoter activity by nearly 50% (Fig. 5, construct 2). However, the same mutation only slightly reduced the luciferase activity (to ~82% of the wild type) in HepG2 cells. Single mutation of Sp1a (construct 3) caused marked reduction in the promoter activity to 33% of the wild type in MLTC and smaller but significant reduction to 69% of the wild type in HepG2 cells (p < 0.01). Double mutation of C/EBP and Sp1a elements caused further reduction in promoter activity to 22% of the wild type in MLTC cells (construct 4). This reduction was comparable to the decrease in activity observed (to 23%) in MLTC transfected with a deletion construct that excluded the region (~437 to ~344) containing both C/EBP and Sp1a elements (Fig. 1, construct 7). These results indicated that in MLTC cells, both C/EBP and Sp1a elements are important for PIII activity, the latter being dominant. In HepG2 cells, single mutation of either C/EBP or Sp1a elements had much less impact on the activity of promoter III (82 and 69%, respectively), whereas double mutation of both elements caused a reduction in promoter activity to 38% of the wild type (construct 4). These results suggested that C/EBP and Sp1a elements both contributed to the basal promoter activity, but there was some degree of redundancy in their function in HepG2 cells.

Since results from promoter deletion analyses (Fig. 1, also see above) indicated that elements 3' of TSS within the non-coding exon 1 were required for full promoter activity, we next examined the participation of the 3'-proximal region of the promoter domain (~337 to ~179) in promoter activity. As shown in Fig. 4B, the two Sp1-binding sites present within this region (Sp1<sub>a</sub> and Sp1<sub>b</sub>) bound to Sp1/Sp3 proteins present in MLTC and HepG2 cells. Since the two Sp1 sites showed alternative binding to the same proteins (Fig. 4B), double mutation of Sp1<sub>a</sub> and Sp1<sub>b</sub> was employed in subsequent experiments. In addition, a sequence element resembling the consensus AP2-binding site (CGGGCTGGCCA, ~330), located 8 bp downstream of the major TSS (~340) and referred as DSE (downstream sequence element), was also examined for its requirement on PIII activity. However, no specific binding of AP2 protein to this AP2 consensus sequence was demonstrable by EMSA in nuclear extracts of both MLTC and HepG2 cells (data not shown). It was clearly shown that in both MLTC or HepG2 cells, double mutation of Sp1<sub>a</sub> and Sp1<sub>b</sub> had no effect on the promoter activity (Fig. 5, construct 6). However, mutation of the DSE caused reduction in promoter activities to 51–57% of the wild type in both cell types (construct 5). When both DSE and Sp1<sub>b,c</sub> were mutated, marked reduction in promoter activities to ~20 and ~14% of the wild type (construct 7) was observed in MLTC and HepG2 cells, respectively. These results were comparable to those observed for constructs with deletion of the region ~344 to ~179 (Fig. 1, construct 8). This finding indicated that Sp1<sub>a</sub> and Sp1<sub>b</sub> were functionally redundant in the presence of DSE and that Sp1<sub>b,c</sub> could in part substitute for DSE. Thus, these findings revealed that downstream sequences were required for full promoter activity of PIII. Moreover, it was demonstrated that when all four sites, the 5'-proximal C/EBP and Sp1<sub>a</sub> elements and 3'-proximal sequences DSE and Sp1<sub>b,c</sub> were mutated, the promoter activity was reduced to 5 and 7% of the wild type in MLTC and HepG2 cells, respectively (construct 8). These results demonstrated that full promoter activity of PIII depends on C/EBP, Sp1<sub>a</sub>, and the DSE with Sp1<sub>b,c</sub> being functionally redundant.

**Activation of PIII by Sp1 Protein through Sp1<sub>a</sub> Element in SL2 Cells**—To examine whether PIII can be activated by Sp1 protein through Sp1<sub>a</sub> element in SL2 Drosophila cells. Cotransfection of SL2 cells with Sp1 cDNA yielded 2–3-fold induction of wild type promoter activity or C/EBP-mutated PIII domain (both containing intact Sp1<sub>a</sub> site) and 5-fold that of the Sp1<sub>a</sub> mutant (Fig. 6). This mutant displaying a basal activity lower than wild type in the absence of Sp1 cotransfection was not activated by cotransfection of Sp1<sub>a</sub> cDNA, reinforcing the notion that Sp1 protein binds specifically to the Sp1<sub>a</sub> element and activates PIII. This finding also indicated that activation of this promoter by Sp1 in SL2 cells is independent of the C/EBP element.

**C/EBP and Sp1<sub>a</sub> Elements Are Transcriptional Activation Domains of PIII in Rat Granulosa Cells**—To examine the transcriptional activation and hormonal regulation of PIII in normal ovarian cells, primary cultures of rat granulosa cells were utilized for transient expression of PIII constructs of wild type
rat as indicated in MLTC cells by luciferase activities of the rat construct −437/−179 and its mouse homologous construct (Fig. 8C). However, the mouse counterpart of the rat gonadal-specific promoter I has only 87.5% similarity with the sequence within the rat promoter domain (Fig. 8B). Although this putative mouse promoter is generally similar to the rat PI, it contains a mutated SF-1 element (CCAGGTCA to CCACAGTCA) as determined by sequencing the genomic clone from the BAC library or independently by sequencing the PCR product of mouse liver genomic DNA (not shown). This difference in the SF-1-binding site abolished the function of this element (DNA binding and activation) as shown in mutational studies for the rat PI (5) and reduced the promoter activity of the mouse PI homolog to <10% of the rat PI (Fig. 8C). The conserved CCAAT box sequence in the mouse PI may contribute minimally to its activity, as previously shown for the rat PI (5). Since SF-1 is essential for PI activity in rat gonadal cells, lack of the functional SF-1 element in the mouse abrogates the function of this promoter (PI) and hence PRLR transcription from this promoter. It appears that PIII substitutes for PI-directed transcription of the PRLR in the mouse ovary. These findings indicate that PIII is both structurally and functionally conserved between the rat and mouse genes and are therefore of major importance in the control of PRLR gene transcription.

**DISCUSSION**

Our study has provided insights on the regulation of promoter III of the PRLR gene, a commonly utilized promoter of the prolactin receptor gene in a wide variety of tissues. The PIII activity is governed by activation of two functionally independent sites, a C/EBPβ element that binds to C/EBPβ and an Sp1 element (Sp1x) that binds to Sp1 and Sp3, members of the same family of nuclear proteins. A DSE is required for basal functions of this promoter, and the two downstream adjacent Sp1 elements (Sp1x, Sp3) may substitute for DSE function.

Cell-type differences in activation of PIII by C/EBPβ and Sp1 were observed. In MLTC cells, single mutation of either binding site reduces the promoter activity by ~50% or more, with the role of Sp1 being more dominant than that of C/EBPβ, indicating that both factors are required to attain full promoter
A comparison of genomic regions of rat PIII with its mouse counterpart. The box species, whereas the boxed AF064034) are compared, with sequence similarity of 96.5% between the two. Drastically between the rat and mouse upstream from it appears that C/EBP the promoter activity of promoter III of the PRLR gene. There-

**FIG. 8. Sequence comparison of the promoter region of PIII and PI between the rat and mouse and their activities in MLTC cells.** A, comparison of genomic regions of rat PIII with its mouse counterpart. The upper drawing represents the region including the exon E1 

passing the two main TSS (−340 and −351) are TCAGAGA and TGACCTT, respectively, and these represent mismatches by two nucleotides (underlined) of the consensus Inr sequence YYANuYY (17). Although it is not clear whether these sequences serve as variant Inr in PIII, the construct (−506/−345) lacking the downstream sequences including one of the two Inr-like sequences displayed minimal promoter activity. The downstream sequence element (DSE) (CGGCTGGCGA) is likely part of the core promoter elements required for efficient transcription initiation from this promoter, since its mutation reduced the promoter activity by −50% in both MLTC and HepG2 cells. However, it appears that the DSE in PIII is distinct from DPE (downstream promoter element) by sequence as well as by its location. DPE was identified in a subset of TATA-less gene promoters with a distinct 7-nucleotide sequence (A/G)G(A/T)CGTG, located at +28 to +34 downstream of the transcription initiation sites (18). DPE can be bound by TFIID but not by TATA-binding protein and is defined as part of the core promoter components (18). Although the DSE in PIII does not conform to DPE, it is an integral part of the promoter domain and may participate in or facilitate the assembly of the basal transcription machinery.

Whereas the upstream Sp1, is of major importance in PIII promoter activity, the two downstream Sp1, and Sp1, which both bind to Sp1 protein, appeared to be non-operative or redundant in the presence of an intact DSE element, in that...
mutation of the two downstream Sp1 sites did not alter the promoter function in transient reporter gene assays. However, in the presence of altered DSE, mutation of both Sp1<sub>b</sub> and Sp1<sub>c</sub>-binding sites caused further reduction in promoter activity, suggesting that Sp1<sub>b</sub> sites could partly compensate for the function of DSE when DSE was mutated. Both DSE and Sp1<sub>b,c</sub> elements are GC-rich sequences, and when they were all mutated, the promoter activity was greatly compromised. Therefore, although the Sp1<sub>b,c</sub> sites are normally redundant, they are potentially able to compensate or partially restore the promoter activity.

Unlike MLTC and HepG2 cells, we were not able to detect discernible C/EBPβ-binding complexes in the nuclear extract of rat ovarian granulosa cells using either the short probe containing only C/EBP site or the long probe containing both C/EBP and Sp1 sites. However, functional analyses of the mutated C/EBP element and the transactivation of the promoter domain by cotransfection of the C/EBPβ cDNA into this cell (Fig. 7) indicated that C/EBP element in the PII domain is a functional C/EBPβ-binding site in vivo, through an action that is independent of the Sp1 element.

In the C/EBPβ knock-out mice, ovarian follicles undergo normal growth but fail to luteinize after ovulation, suggesting that the ovary is deficient in expression of those genes that are participating in the process of luteinization and are regulated by C/EBPβ (19). It is well known that prolactin is required for the maintenance of luteinization as well as luteolysis in rodents (20). Expression of prolactin receptor is increased during follicular growth and during luteinization (21). C/EBPβ expression is increased after the luteinizing hormone surge when ovulation occurs (22). Therefore, up-regulation of both C/EBPβ and the prolactin receptor in granulosa cells is critical for luteinization. We now provide evidence that C/EBPβ is a transcriptional activator for this promoter, since overexpression of C/EBPβ in granulosa cells significantly increases the PII activity. Because PII activity is also controlled by Sp1/Sp3, the C/EBPβ knock-out mice would be expected to maintain a reduced level of PRLR, well below normal basal and hCG-stimulated levels. This marked reduction in transcription of the PRLR gene could in part explain the luteal deficiency observed in these mice.

Typical Sp1 DNA-binding sequences are GC box (GGGCGG-GGCC) and GT motif (GGGTGGGCC) present in most TATA-less and GC-rich promoters. A variant Sp1-binding site CTC repeat (CTCTCTCCT) was identified in TATA-less promoter regions of a number of other growth-related genes including Wilm’s tumor-1 gene (WT-1) (23), platelet-derived growth factor-A chain (24), insulin-like growth factor-I, and epidermal growth factor receptors (25, 26), c-myc, c-myb, and vav (27–29). There are at least three close members of Sp family proteins, Sp1, Sp3, and Sp4, which share similar structural features (30, 31). The three proteins contain highly conserved DNA binding domains that all recognize the GC box and GT motif. Sp3 and Sp4 have also been suggested in transcriptional regulation. In Drosophila SL2 cells, Sp3 could suppress the Sp1-mediated transcriptional activation due to the competition with Sp1 for their common binding sites in some genes (32, 33). In other cases, Sp3 enhances the promoter activity (34–36) with equal potency (35) and sometimes is even a more effective activator than Sp1 (36). Therefore, there is differential regulation of different genes by Sp1 or Sp3. Although the activation of PII in SL2 cells by Sp1 (2–3-fold) was not as dramatic as in some other genes, the fact that Sp3 is not present in these cells may also be reflective of the degree of activation observed in this study. In mammalian cells (CV-1, HeLa, and Ishikawa cells), it was also reported that the reporter promoters (uroglobin and SV40) containing GC box or GT motif were activated by Sp1 and Sp4 but not by Sp3 (37). In the current study, we demonstrated that like Sp1, Sp3 could also bind to CTC repeats in the PII. Although it is clear that Sp1<sub>4</sub> is a positive cis-element in this promoter, the individual contribution by Sp1 and Sp3 to this promoter activation is not certain. It is believed that Sp1 is an important activator to promoter III in gonadal and hepatic cells as in numerous other Sp1-activated genes (38, 39). Since Sp3 is also a ubiquitous factor and can bind to Sp1<sub>4</sub> element, the regulatory roles of Sp3 in the PII await further study.

The activity of the PII domain (~437–179) was induced significantly by hCG/cAMP in cultured rat primary granulosa cells. However, neither C/EBP- nor Sp1-binding sites were found to specifically mediate this response to hCG/cAMP, albeit they were important for basal promoter activity. Although both C/EBPβ and Sp1 have been shown to participate in cAMP induction of transcription in some genes (40–44) through phosphorylation by protein kinase A (40, 41, 43), this appears not to be the case for this promoter domain. However, there is no consensus cAMP response element or functional AP2 site present within the promoter domain that could mediate second messenger action, thus other elements may be involved in cAMP-mediated induction of promoter activity.

The mouse PII is highly similar in structure and function to the rat promoter III as expected, suggesting that this promoter is well conserved. However, unexpectedly the homolog of the rat gonadal promoter I was critically altered in the mouse. The SF-1 element, which is essential for the rat PI activity, is a non-functional sequence in the mouse. This is reflected in the vestigial promoter activity observed in reporter assays and very low expression of the endogenous mouse counterpart of rat E<sub>1</sub> (non-coding exon 1) (4) in both MLTC and mouse ovary as compared with the E<sub>1</sub>. Hence the essential promoter for the PRLR in the mouse ovary is promoter III and the expression of the mouse ovarian PRLR is primarily regulated by C/EBPβ and Sp1, whereas promoter I is inactive due to critical base pair substitution in the SF-1 element. It is therefore expected that PRLR transcription in the ovary is reduced in C/EBPβ knock-out mice and may contribute to the deficiency in luteinization in such animals (19).

Because C/EBPβ and Sp1 are the major transcriptional activators to promoter III of the rat PRLR gene, the wide expression of these two transcription factors may explain the common utilization of this promoter in multiple tissues and in different species. This is in contrast to PI and PII, which were specifically activated by SF-1 (5) and HNF-4 (7) in the gonads and the liver, respectively.

In summary, we have identified in promoter III multiple nuclear protein-binding sites including C/EBPβ and Sp1/Sp3 and a functional DSE. C/EBPβ and Sp1<sub>b</sub> are the two major transcription factors regulating the PII activity, and DSE is also required for the basal promoter activity. Promoter III is highly active and is well conserved in the rat and mouse; therefore, it is of central importance in the regulation of the PRLR gene expression in various tissues of different species.

REFERENCES

1. Nicoll, C. S. (1974) in Handbook of Physiology (Knobil, E., and Saywer, W. H., eds) Vol. 4, Part 2, pp. 253–292, American Physiology Society, Washington, D. C.
2. Doppler, W. (1994) Rev. Physiol. Biochem. Pharmacol. 124, 93–130
3. Hu, Z. Z., Zhuang, L., and Dufau, M. L. (1998) Trends Endocrinol. Metab. 9, 94–102
4. Hu, Z. Z., Zhuang, L., and Dufau, M. L. (1996) J. Biol. Chem. 271, 10242–10246
5. Hu, Z. Z., Zhuang, L., Guan, X., Meng, J., and Dufau, M. L. (1997) J. Biol. Chem. 272, 14263–14271
6. Luo, X., Reeds, Y., and Parker, K. L. (1994) Cell 77, 481–490
7. Moldrup, A., Ormandy, C., Nagano, M., Murthy, K., Banville, D., Tronche, F., and Kelly, P. A. (1996) Mol. Endocrinol. 10, 661–671
8. Courey, A. J., and Tijan, R. (1988) Cell 55, 887–898
9. Lewis, J. G., Lin, K. Y., Kothavale, A., Flanagan, W. M., Matteucci, M. D.,
