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Mechanisms of Transcriptional Activation of the Promoter of the Rainbow Trout Prolactin Gene by GHF1/Pit1 and Glucocorticoid

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The transcription factor GHF1/Pit1, required for the expression of the prolactin (PRL) and other pituitary-specific genes, is highly conserved from fish to mammals but the mechanisms by which it activates transcription are poorly understood. The activity of the promoter (−627/+15 region) of the rainbow trout *PRL* (t*PRL*) gene fused to the luciferase reporter gene was studied using GHF1-expressing rat pituitary GC cells. Nuclear extracts of GC cells produced five GHF1-specific footprints in the t*PRL* promoter, with the position of the two most proximal ones being highly conserved in trout and mammalian GHF1-regulated genes. Deletional and mutational analyses of the t*PRL* promoter showed that the most proximal GHF1 site alone is sufficient to confer sub-maximal GHF1-dependent transcriptional activity and that a glucocorticoid response element-like motif mediates dexamethasone stimulation. It is suggested that GHF1 molecules bound to different sites of the t*PRL* promoter cannot interact simultaneously with the transcriptional apparatus. Moreover, GHF1 and the ligand-bound glucocorticoid receptor tethered to their cognate elements in the promoter could cooperate to enhance transcription by interacting simultaneously with different members of the basal transcriptional complex.

The pituitary-restricted POU domain transcription factor GHF1/Pit1 is required for the expression of the growth hormone (*GH*), prolactin (*PRL*) and thyrotropinβ (*TSH*β) genes in somatotropes, lactotropes and thyrotropes, respectively (1,2). Further, GHF1 is necessary for the phenotypic specification of the above cell types and is involved in the regulation of its own gene (1,2). The expression of the GHF1 target genes is also modulated by systemic regulators, such as cAMP, steroids and thyroid hormone (1,2), but the mechanisms by which GHF1, either alone or in concert with other activators, mediates transcriptional activation are still poorly understood. Because the structure (3) and functions (4-6) of GHF1 are highly conserved from fish to mammals, it is likely that the basic mechanisms of activation of its target genes have been retained during evolution. On the other hand, since PRL performs an impressive array of different functions in vertebrates, from osmoregulation in teleost fish to lactogenesis in mammals (7,8), the transcriptional regulation of its gene may have required not only the conservation of its GHF1-dependence, but also the acquisition of a variety of modulating mechanisms to adapt PRL expression to the contingent needs of each species. Thus, the comparative study of *PRL* gene expression may provide useful clues about how GHF1 directs transcription and cooperates with accessory factors to regulate the expression of its target genes.

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The nucleotide sequence reported in this paper has been submitted to the EMBL nucleotide sequence database (Accession No. X95907)

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In the present work, we have cloned a 5′-flanking region of the rainbow trout (*Oncorhynchus mykiss*) *PRL* (t*PRL*) gene and examined the mechanims regulating its transcriptional activity. Footprinting analysis demonstrated that the t*PRL* promoter bears five GHF1-binding sites, the position of the two proximal ones being conserved in fish and mammalian GHF1-regulated genes. Transfection studies, performed in rat pituitary GC cells, indicated that the most proximal GHF1 site plays an essential role in transcriptional activation and that a sequence homologous to the mammalian glucocorticoid response element (GRE) mediates dexamethasone (DEX) stimulation.

MATERIALS AND METHODS

Cloning and sequencing of trout PRL gene promoter. The −627/+15 5′-flanking region of the trout *PRL* promoter (627tPRL) was amplified from trout genomic DNA by PCR using the following pair of primers derived from the sequence of the chinook salmon (*O. tschawytscha*) (9): P-627 (5′-CCAGTGTTTAGAACGCTACGCCACTCG-3′) and P+15 (5′-CCTCTCCTGCTTTTTGGCTGTCGTTCC-3′). Cloned fragments were sequenced with the DyeDeoxy terminator cycle sequencing kit in a ABI 373A automatic sequencer.

Plasmids and mutagenesis. The p0-Luc plasmid was prepared from the promoterless plasmid pBL-CAT6 by replacement of the chloramphenicol acetyl transferase (*CAT*) gene with the luciferase coding region from pXP2 (6). The pCMV-βgal plasmid (10) expresses the *E. coli* β-galactosidase reporter gene under the control of the cytomegalovirus promoter/enhancer region. pCMV-tGHF1, pCMV-rGHF1 and pCMV-CAT were prepared substituting the β-galactosidase coding region of pCMV-βgal with the trout GHF1, rat GHF1 and CAT coding sequences, respectively (6).

The 627tPRL fragment was cloned by blunt-end ligation into the *Bam*HI site of p0-Luc yielding the p627tPRL-Luc expression vector. The various deletions of p627tPRL-Luc were prepared taking advantage of restriction sites in the promoter sequence: p168tPRL-Luc (*Nco*I site), p110tPRL-Luc (*Bcl*I site), p58tPRL-Luc (*Nsi*I site), p627Δ1tPRL-Luc (an internal deletion between the *Nco*I to the *Bcl*I sites) and p627Δ2tPRL-Luc (an internal deletion between the *Bcl*I to the *Nsi*I sites). Site directed mutagenesis of the AGTACA sequence to GCAGTG was performed by PCR with the following pair of primers: PMut (5′-CCATGCCATGGGGTATTGATG**GCAGTG**TACCTAAGG-3′) and the antisense primer PLucAS (5′-AGCGGTTCCATCCTCTAGAG-3′), using p627tPRL-Luc as a template. To prepare p627MutPRL-Luc, the amplification product was digested with *Nco*I and *Xho*I restriction enzymes and cloned into the same sites of the p627tPRL-Luc plasmid.

Cell culture and transfections. GC, HeLa and EPC (*epithelioma papulosum cyprini*) cells were grown and transfected as described previously (6). Briefly, the cells were cultivated in Ham's F12 nutrient mixture (GC) or DMEM (Hela and EPC), supplemented with 10% fetal calf serum (FCS); transfections were performed by electroporation (GC: 300 V, 1500 μF; EPC: 250 V, 1550 μF) or calcium phosphate precipitation (HeLa). For DEX induction, GC cells were cultivated 24 h in F12 medium supplemented with 10% charcoal-stripped FCS and transfected, 4 h later they were treated with DEX (10^{-7} M final concentration). Twenty four h after transfection, the cells were washed and collected in PBS, and resuspended in 200 μl of 20 mM Tricine (pH 7.8), 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100 and 15% glycerol. The suspension was centrifuged 1 min and the supernatant solution was processed for βgal (for response normalization) and luciferase activities as described elsewhere (6).

DNase I footprinting. Nuclear extracts were prepared from GC cells by the procedure of Dignam *et al.* (11). The labelled fragments (8 ng, 2×10^4 cpm) were incubated with the indicated amounts of nuclear extracts and then partially digested with DNase I according to Galas and Schmitz (12). In competition experiments, we used a double-stranded oligonucleotide bearing a GHF1-binding site matching the −181/−151 region of the rainbow trout *GH* gene promoter (13).

RESULTS

The trout PRL promoter is efficiently transactivated by rat GHF1 in mammalian cells. Transfection of increasing amounts of a plasmid (p627tPRL-Luc) bearing the −627/+15 region of the trout *PRL* gene (Fig. 1), fused to the luciferase gene, resulted in a marked and dose-dependent expression of the reporter in GHF1-expressing rat pituitary GC cells (Fig. 2A). Cotransfection of this plasmid with expression vectors encoding either rat GHF1 (CMV-rGHF1) or trout GHF1 (CMV-tGHF1) also produced an efficient expression of the luciferase gene in both mammalian (HeLa) and fish (EPC) non-pituitary cell lines (Fig. 2B). This shows that the mechanisms regulating the GHF1-dependent transcription of the *PRL* gene are highly

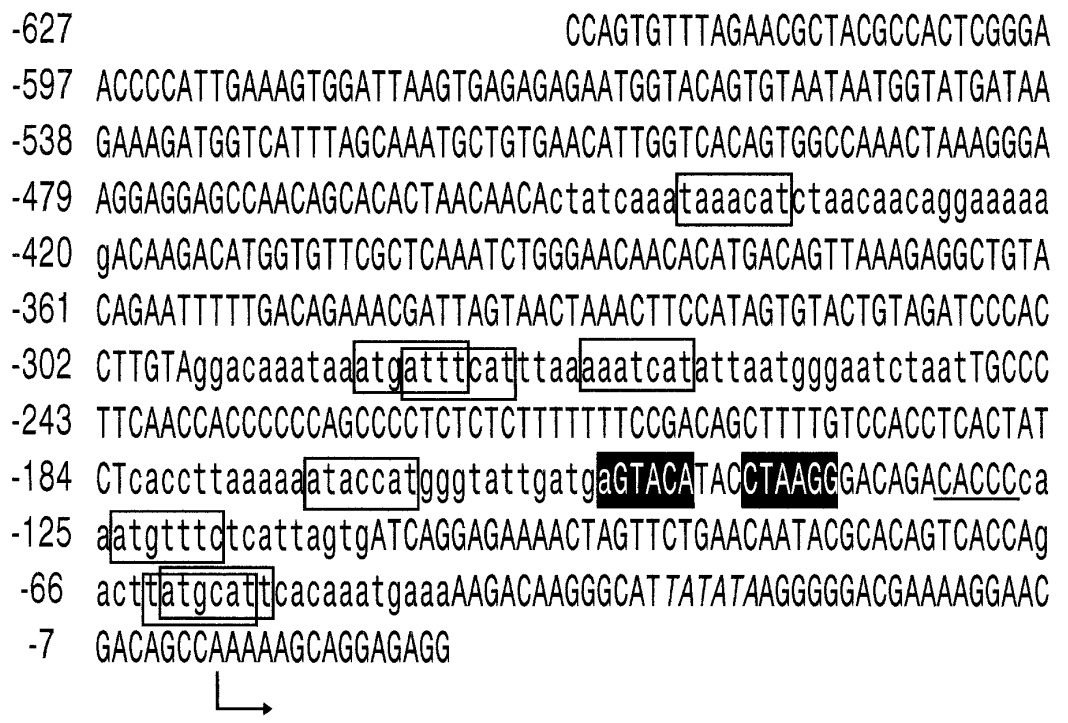


FIG. 1. Sequence of the trout *PRL* promoter. The −627/+15 region of the t*PRL* gene was amplified by PCR taking advantage of the chinook salmon sequence (9) (see Materials and Methods). The regions protected by DNase I digestion in Fig. 3 (F1 (−44/−67), F2 (−106/−127), F3 (−152/−182), F4 (−249/−296), and F5 (−419/−451)) are in lower case characters. Within the footprints, $(A/T)_3$NCAT motifs are boxed. The GRE-like element (−153/−139) is highlighted, the associated CACCC-box is underlined, and the TATA-box is in italics. The transcription start site is indicated by an arrow.

conserved in vertebrates and that GC cells represent a suitable model to study the transcriptional activity of the t*PRL* promoter.

The trout PRL promoter contains five GHF1-binding sites the position of the two proximal ones being conserved in vertebrates. The above experiments implied that rat GHF1 binds efficiently to *cis*-elements in the t*PRL* promoter. To identify precisely these elements, we performed a DNase I protection experiment using nuclear extracts of GC cells that contain high amounts of rat GHF1. As shown in Fig. 3A, these extracts produced five footprints, designated F1 to F5, that were competed for by an oligonucleotide bearing a GHF1-binding site, demonstrating that they were generated by the binding of GHF1 to DNA. The footprints contain one (F2, F3 and F5), two (F1) or three (F4) sequences whose general consensus $(A/T)_3$NCAT (Fig. 3B) fits other mammalian and fish GHF1 binding motifs (1,2,5). GHF1 binds generally as a dimer to its recognition elements, one molecule binding to the canonical motif and the second one to an adjacent A/T rich motif (1,14). The fact that A/T rich sequences flank the $(A/T)_3$NCAT motifs in the protected regions and, in addition, that the size of the footprints extends far beyond the consensus (Figs. 1 and 3A), suggest that GHF1 binds as a dimer to its recognition sites on the t*PRL* promoter.

Comparison of the proximal promoters of trout and mammalian GHF1 target genes (6, 15-17) evidences highly conserved patterns in the position of the GHF1 recognition sequences (Fig. 4). In particular, the position of the most proximal GHF1 site is strictly conserved in the promoters of trout *PRL* and *GH* genes, and rat *PRL* and *GHF1* genes. The promoters of the

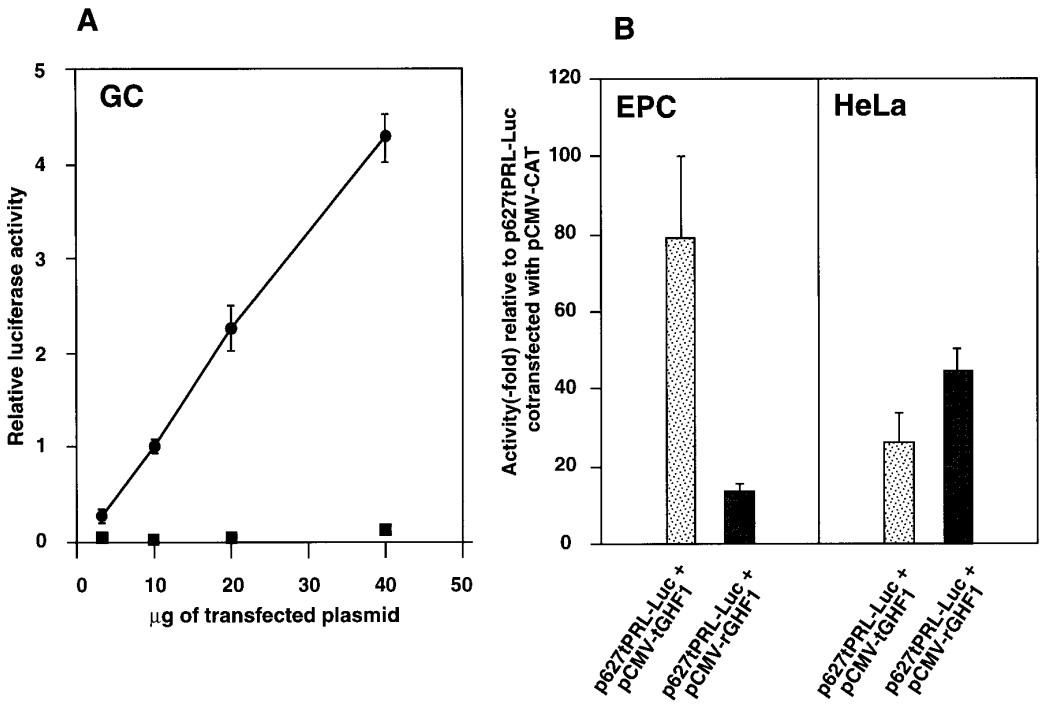
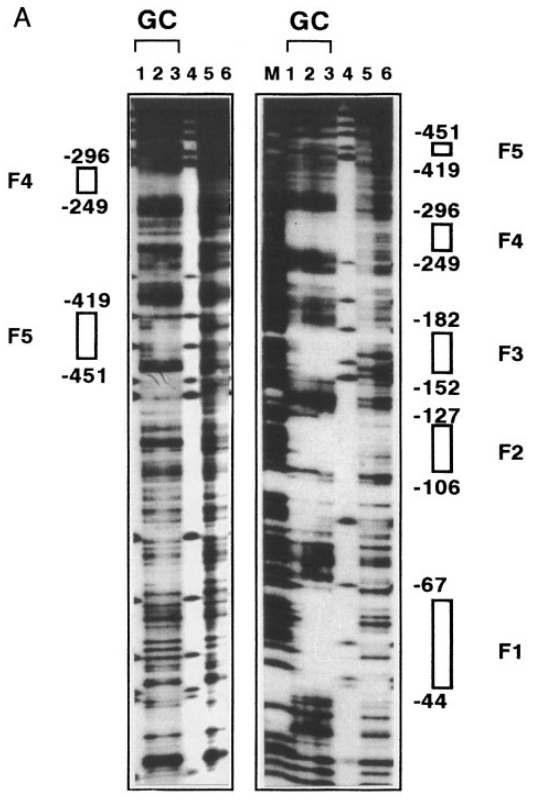


FIG. 2. Activation of the trout *PRL* promoter in pituitary and non-pituitary cells. (A) Rat pituitar GC cells (4×10^6) were transfected with 3.3, 10, 20 and 40 μg of p627tPRL-Luc (●) or the promoterless plasmid p0-Luc (■). Data are presented as fold induction relative to the value obtained with 10 μg of p627tPRL-Luc. (B) Carp EPC cells (4×10^6) and human HeLa cells (0.5×10^6) were cotransfected with 6 μg each of p627tPRL-Luc and expression plasmid (pCMV-rGHF1 producing rat GHF1; pCMV-tGHF1 producing trout GHF1; or pCMV-CAT as a negative control). In all transfections, CMV-βgal (3 μg) was included to normalize the data to transfection efficiency. After 48 h of incubation, luciferase and βgal activities were measured. Results are the mean ± SEM of two experiments, each one run in duplicate.

mammalian GHF1-regulated genes have been classified in two categories (18). In class I promoters (*GH* and *TSHβ* genes), the most proximal GHF1 site is located in the −99/−66 region, while in class II promoters (*PRL* and *GHF1* genes), it is situated in the −70/−38 region (Fig. 4). On the basis of this classification, the promoters of the trout *PRL* and *GH* genes belong to class II promoters. It is also to be observed that the second most proximal GHF1 site occupies a constant position in both class I and class II trout and mammalian promoters.

In the trout PRL promoter GHF1 activation relies mostly on the F1 site and an AGTACAtac-CTAAGG motif mediates glucocorticoid responsiveness. To investigate the contribution of the various GHF1 sites to the transcriptional activation of the t*PRL* promoter, we prepared a series of deletion mutants of p627tPRL-Luc and tested their activity in GC cells. The results (Fig. 5) indicated that the F1 site is necessary to confer an efficient activity to the promoter, and that a maximal response is obtained with the additional presence of the region encompassing the F2 site. It should be noted that in this experiment, as well as in the following ones, transfections were performed with 10 μg of reporter plasmids, a condition in which the amount of GHF1 in GC cells was apparently not limiting (see Fig. 2A). It is thus unlikely that differences in the response of the various plasmids were determined by a limited GHF1 availability.

In contrast to its mammalian counterparts, whose activity is stimulated by cAMP (19,20)



B

5'							3'	
-443	T	A	A	A	C	A T	-437	F5
-292	A	A	A	T	C	A T	-286	F4
-283	A	T	T	T	C	A T	-277	F4
-272	A	A	A	T	C	A T	-266	F4
-171	A	T	A	C	C	A T	-165	F3
-118	G	A	A	A	C	A T	-124	F2
-63	T	A	T	G	C	A T	-57	F1
-56	A	A	T	G	C	A T	-62	F1
	A/T	A/T	A/T	N	C	A T	consensus	

FIG. 3. DNase I footprinting analysis performed with nuclear extracts of GC cells. (A) The t*PRL* promoter labelled at either its 3′ (left) or its 5′ (right) end was incubated with 40 μg (lanes 1 and 2), 20 μg (lanes 3) or in the absence (lanes 5 and 6) of nuclear extracts of GC cells. The DNase I digestion in lanes 1 was performed in the presence of a 250-fold excess of an unlabelled double-stranded oligonucleotide corresponding to a GHF1-binding site of the t*GH* promoter (13). DNase I digestions were performed with the following concentrations (U/ml) of enzyme: 1000 (lanes 1), 500 (lanes 2 and 3), 10 (lanes 5), and 5 (lanes 6). Lanes 4: molecular size marker. M: Maxam-Gilbert G+A sequencing ladder. (B) Sequences within the footprints have been aligned to derive a consensus for GHF1 binding to the t*PRL* promoter.

and 17β-estradiol (2) and inhibited by glucocorticoid (21,22), we found that the activity of the t*PRL* promoter was markedly enhanced by the synthetic glucocorticoid DEX, but was unaffected by cAMP and 17β-estradiol (not shown). To map the sequence(s) mediating glucocorticoid-dependent regulation, we first tested the DEX responsiveness of p627tPRL-Luc and two deletion mutants, p168tPRL-Luc and p110tPRL-Luc (Fig. 6). As shown in Fig. 6A, DEX

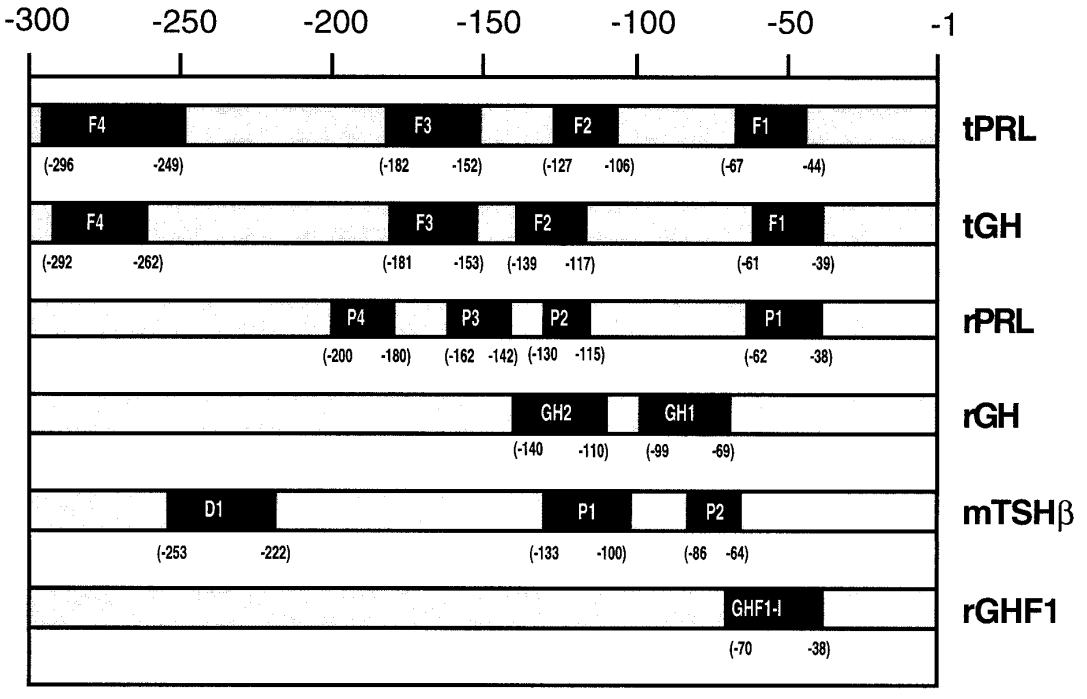


FIG. 4. The position of GHF1-binding sites is conserved in the promoters of fish and mammalian target genes. The 5′-flanking regions of trout *PRL* (tPRL) (this study), trout *GH* (tGH) (6), rat *PRL* (rPRL) (15), rat *GH* (rGH) (15), mouse *TSHβ* (mTSH*β*) (16) and rat *GHF1* (rGHF1) (17) genes have been aligned to compare the position of their GHF1 recognition sites (black boxes).

stimulated markedly p627tPRL-Luc and p168tPRL-Luc but not p110PRL-Luc, suggesting that the −168/−110 region was critical for glucocorticoid activation. This was confirmed by the fact that p627Δ1tPRL-Luc, that lacks this region, was also unresponsive to DEX (Fig. 6B). The −168/−110 region contains an AGTACAtacCTAAGG motif, located at nt −153/−139 (see Fig. 1), that bears partial homology with the mammalian GRE (23). Mutation of the AGTACA half site of this motif into GCAGTG (yielding the p627MutPRL-Luc plasmid) severely impaired the response to DEX (Fig. 6B), showing its importance in mediating glucocorticoid stimulation.

It should be observed that in the experiments of Fig. 6, in which the transfected cells were cultivated with charcoal-stripped serum to remove steroids, the activity of the various plasmids did not differ substantially in the absence of DEX. Conversely, in the experiments of Fig. 5, performed using unstripped serum, p110PRL-Luc and p627tPRLΔ1-Luc were significantly less active than p168tPRL-Luc and p627tPRL-Luc. This can be explained by the presence in the unstripped serum of glucocorticoids that stimulated the activity of the plasmids bearing the −168/−110 region of the promoter. Thus, we conclude that the F1 site alone is necessary and sufficient to mediate most of the GHF1-dependent transcriptional activity of the t*PRL* promoter, and that glucocorticoid responsiveness relies on a GRE-like motif situated between the F2 and F3 sites.

DISCUSSION

Our results demonstrate that the promoter of the trout *PRL* gene contains five GHF1-binding sites, the conserved position of the two most proximal ones indicating strong evolutionary

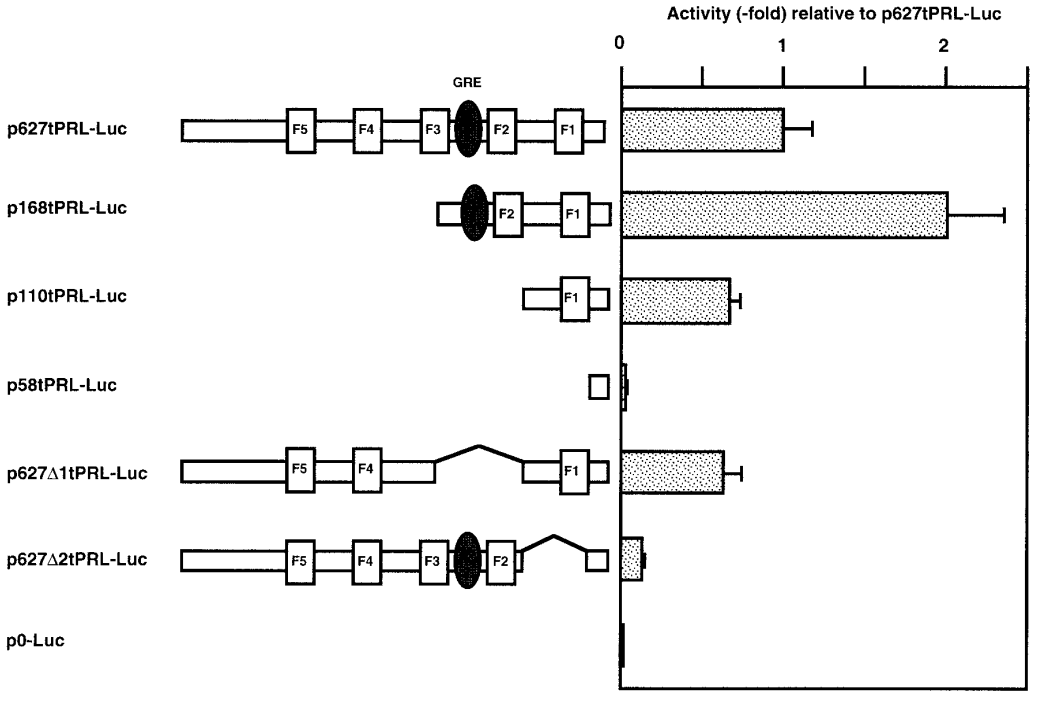


FIG. 5. Effects of deletional mutagenesis on the activity of trout *PRL* promoter in GC cells. GC cells (4×10^6) were transfected by electroporation with 10 μg of the indicated plasmid and 3 μg of pCMV-βgal. After 48 h of incubation in Ham's F12 medium supplemented with 10% unstripped FCS, luciferase and βgal activities were measured. Results are the mean $\pm$ SEM of two experiments, with each one run in duplicate. The presence and position of GHF1-binding sites (F1 to F5) and the GRE-like motif in each promoter fragment are schematically depicted on the left.

constraints. Our transfection studies in GC cells show that the F1 site is sufficient to confer sub-maximal GHF1-dependent expression to the promoter and that a GRE-like motif mediates glucocorticoid responsiveness.

The strict conservation of the position of the most proximal GHF1 site in class II promoters correlates well with the recent demonstration that optimal activation of the rat *PRL* promoter requires a unique positioning of its proximal GHF1 binding site (18). In the t*PRL* promoter, the crucial role played by the F1 site is highlighted in experiments with stripped serum in which p110tPRL-Luc, bearing only this site, was almost as active as the plasmids bearing multiple GHF1-binding sites. Because in these experiments GHF1 availability was not limiting, it appears that the F1 site alone can mediate most of the GHF1-dependent promoter activity, suggesting a lack of synergism between the various GHF1 sites. A possible explanation of this finding is that the available contacts between GHF1 and its target factor in the transcriptional apparatus are fully engaged by a single GHF1 molecule, or its dimer, bound to a correctly positioned DNA element. This would imply that GHF1 molecules bound to different recognition sites in the promoter cannot interact simultaneously with the transcriptional machinery, although they might increase the probability of contact formation. In this context, the position of the F1 site of the t*PRL* promoter would be the most favourable to mediate GHF1 interactions with the basal transcription complex.

The sequence mediating DEX inducibility of the t*PRL* promoter represents a variant of the canonical (AGAACAnnnTGTAGC) GRE (23). This may reflect a diversity in the fish GRE,

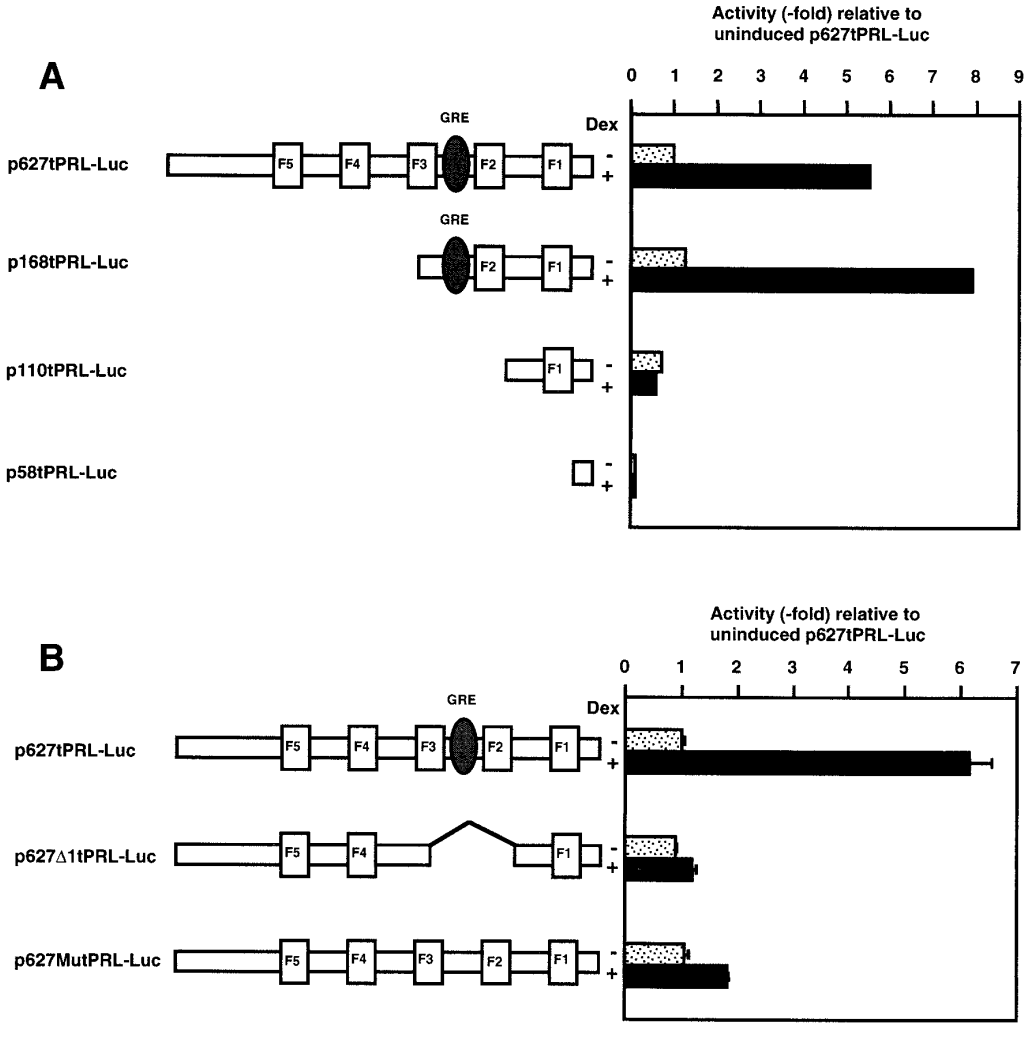


FIG. 6. Mapping of the sequence mediating dexamethasone stimulation of the trout *PRL* promoter. GC cells (4×10^6), cultivated for 24 h in Ham's F12 medium supplemented with 10% charcoal-stripped FCS, were transfected by electroporation with 10 μg of the indicated plasmids and 3 μg of pCMV-βgal, and divided in two dishes. After 4 h, DEX (10^{-7} M final concentration) was added to one of the dishes. Luciferase values were determined in uninduced (−) and induced (+) cells 24 h after transfection and normalized to βgal activity. The presence and position of GHF1-binding sites (F1 to F5) and the GRE-like motif in each promoter fragment are schematically depicted on the left. (A) The −168/−110 region of the promoter is required for DEX stimulation. Data are the mean of determinations from two independent transfections which differed by less than 10%. (B) DEX stimulation is impaired by deletion of the −168/−110 region of the promoter and by site directed mutagenesis of the AGTACA half-site of the GRE-like motif into GCAGTG. Data are the mean ± SEM of determinations from three independent transfections.

since the recent cloning of a trout cDNA encoding a glucocorticoid receptor (GR) shows a high homology with its mammalian counterpart but a slight difference in the DNA binding domain (24). However, atypical GREs mediating glucocorticoid inducibility have also been identified in several mammalian genes (25-27). Like other members of the steroid receptor superfamily, the GR is thought to interact with TFIIB (28), while GHF1 could promote transcription by favouring the recruitment of TAF_{II}s to the TFIID complex (29). Hence, by interacting simultaneously with different members of the transcriptional machinery, GHF1 and

the ligand-bound GR, tethered to their cognate recognition sites on the t*PRL* promoter, could enhance the formation of stable pre-initiation complexes triggering a high level of transcription. This is reminiscent of the mechanism by which two *Drosophila* activators, Bicoid and Hunchback, interacting simultaneously with different TAF_{II}s of the TFIID complex, bring about synergistic transcriptional activation of the *hunchback* gene promoter in a cell-free transcription system (30). Interestingly, the GRE-like sequence of the t*PRL* promoter is flanked by a CACCC-box (see Fig. 2), whose binding factor has been shown to interact and synergize transcriptionally with the ligand-bound GR in the mammalian tryptophan oxygenase and tyrosine aminotransferase genes (31-33).

The second most proximal GHF1 site should also play an important role given its highly conserved position in both class I and class II promoters. In this regard, it is noteworthy that this site is flanked by functional recognition elements for systemic activators in many GHF1-regulated genes. In fact, it is situated in proximity of a GRE in the promoters of the t*PRL* (this study) and rat *GH* (26) genes, and of a cAMP response element (CRE) in the promoters of the rat *PRL* (20), human *GH* (34) and trout *GH* (6) genes. Thus, this site may be important in the enhancement of transcription when other factors such as CREB (CRE binding protein) and nuclear receptors come into play because GHF1 bends DNA (35) and thus may favour the interaction of other activators bound to nearby sequences with the transcriptional machinery. Alternatively, GHF1 bound to F2 and possibly to its other binding sites, might also act as an architectural component leading to the formation of a nucleoprotein complex stabilized by protein:protein interactions between transacting factors tethered to adjacent recognition sequences, as shown for other activators (36).

In conclusion, the high conservation of the mechanisms involved in the transactivation of GHF1-regulated genes makes feasible the use of a heterologous model, namely rat GC cells, to study the activity of the t*PRL* promoter. This approach has provided interesting hints as to the mechanisms by which GHF1 regulates transcription and cooperates with glucocorticoids to modulate the expression of its target genes. Moreover, this investigation shows that, in different vertebrate species, the transcriptional activation of the *PRL* gene has mantained its GHF1-dependence but is variously modulated by systemic regulators such as cAMP, estrogens and glucocorticoids.

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