Distance-dependent Interactions between Basal, Cyclic AMP, and Thyroid Hormone Response Elements in the Rat Growth Hormone Promoter*

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Developmental stage- and tissue-specific expression of the rat growth hormone (rGH) gene is conferred by DNA sequences within 237 base pairs of the transcription start site. Although binding of a number of transcription factors including Pit-1, Sp1, GHF3, and thyroid hormone receptor (T3R) stimulates rGH expression, several studies have suggested that interactions between these factors are important in determining cell specificity and responsiveness to extracellular signals. We have directly tested this hypothesis by creating a set of nested insertion mutations at two positions in the rGH promoter. Sequences were inserted at either position -148, separating GHF-3 and T3R binding sites from the downstream Pit-1 and Sp1 binding sites, or at -51, separating the above elements from the TATA box. All insertions were made in the context of the rGH gene -237/+85' flanking DNA, linked to a chloramphenicol acetyltransferase reporter gene and tested for activity by transient transfection in GC pituitary tumor cells. Insertions at both -148 and -51 caused sharp distance-dependent reductions in serum-stimulated expression such that insertions of 23 base pairs at -51 or 44 base pairs at -148 were sufficient to isolate the effects of sequences upstream of the insertion point. Insertions at -148 reduced T3 responsiveness severalfold but had little or no effect on stimulation by forskolin, whereas insertions at -51 reduced both T3 and forskolin responsiveness. Our results are consistent with the hypothesis that expression and regulation of the rGH gene is dependent on short-range protein-protein interactions, which are more critically dependent on spacing than the relative orientation of the transcription factor binding sites.

Studies employing both cell cultures (1-6) and transgenic mice (7, 8) have shown that pituitary specificity of growth hormone expression is mediated by sequences within 237 bp upstream of the transcription start site. Within this region, binding of the pituitary transcription factor Pit-1 directs cell specificity (2, 5, 6, 9-12) and responsiveness to cAMP and phorbol esters (13-16), while a number of other more ubiquitous transcription factors including Sp1 (17), GHF-3 (18, 19), and thyroid hormone receptor (T3R) (14, 20-22) contribute to basal activity and responsiveness to thyroid hormone (T3). Although the interrelationships between these factors are not well understood, the more distal of two Pit-1 binding sites overlaps a single Sp1 site in both rat (17) and human (23) GH promoters in such a way as to prevent the simultaneous binding of these two factors. Pit-1 binding also appears to be required for thyroid hormone responsiveness of both rat (18) and human (24) GH promoters, and synergistic interactions between Pit-1 and T3R in directing rGH promoter activity have recently been reported (25). However, little is known about the spatial requirements for these interactions. Synthetic thyroid hormone response elements (TREs) have been shown to function up to 700 bp upstream of the rGH promoter (26), although the extent of T3 induction appears to be reduced when the TRE is moved from its native position.

To examine the functional relationships between rGH regulatory elements, we carried out an insertional mutagenesis of the rGH 5' flanking DNA, introducing sequences of various lengths either between the TATA box and the Pit-1 binding sites (-51 insertions) or between the Pit-1 binding sites and upstream sequences, which contain the principal TREs and GHF-3 binding site (-148 insertions). Our results show that spacing between the TATA box and more distal elements is critical for activity. Generally, insertions at -51 had a more deleterious effect on activity than insertions at -148. However, insertion of either 23 bp at -51 or 44 bp at -148 effectively abolished the effects of sequences upstream of the insertion point on serum-stimulated activity. Transfections employing cells grown in stripped serum showed that insertions at -148 reduced T3 responsiveness severalfold, but had little or no effect on stimulation by forskolin, whereas insertions at -51 reduced both T3 and forskolin responsiveness, although the effect on forskolin responsiveness appeared to be slightly greater. Taken together, these results suggest a model for rGH promoter function in which spacing between the component elements is critical for activity.

**MATERIALS AND METHODS**

**Cell Culture**—GC cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, fetal calf serum.

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**The abbreviations used are: bp, base pair(s); rGH, rat growth hormone; CAT, chloramphenicol acetyltransferase; TRE, thyroid hormone response element; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.**
fetal calf serum (FCS) (Cytosystems) (23). To test the effects of thyroid hormone (T₃) and forskolin, cells were grown for 24 h before transfection in DMEM supplemented with 10% charcoal/resin-stripped FCS (27).

**Generation of rGH:CAT Hybrid Genes and Insertion Mutants**—Sequences containing the rGH 5′-flanking DNA −237/+7, −148/+5, and −61/+4 were inserted into pUC18 (23) to generate −237 rGH:CAT, −148 rGH:CAT, and −51 rGH:CAT. All insertion constructs were generated in the context of −237 rGH:CAT. Insertions at −148 were created by first inserting a 44-bp polylinker fragment at an FnuDII site at −148 and then using this construction to delete various shorter insertions. Insertions at −51 were generated by subcloning rGH sequences −237/−51 between the BamHI and Smal sites of pUC18 and ligating these to −51 rGH:CAT with different lengths of polylinker. Site-directed mutagenesis was carried out as described previously (17).

Transfections and CAT Assays—GC cells were transfected by electroporation as described (23) using a Bio-Rad gene pulser apparatus. Ten µg of each construct were transfected into 3 × 10⁵ cells along with 5 µg of either pRSVβGal (28) or pRSV-CAT (17) to standardize for transfection efficiency, depending on whether cells were assayed for CAT activity or mRNA. Conditions for electroporation were 960 microfarads, 300 V, 0.4 cm electrode gap in a total volume of 0.4 ml in Dulbecco’s phosphate-buffered saline. Transfected cells were plated into 4 × 10² dishes for CAT analysis or 1 × 175 cm² tissue culture flasks for analysis of RNA. Cultures were maintained post-transfection in DMEM supplemented with 10% FCS or, where the effects of case of TS and forskolin were to be tested, DMEM supplemented with either 10% stripped FCS and 10 nM T₃ (in ethanol), 10 µM forskolin (in dimethyl sulfoxide), both, or 10% stripped FCS and an equivalent volume of solvent(s) as control.

For CAT analysis, cells were harvested by scraping 20 h after transfection. CAT and β-galactosidase assays were as described previously (23). CAT activities for each dish were normalized for differences in transfection efficiency by dividing by the β-galactosidase activity determined for the corresponding dish. As CAT activities between transfections differed up to 5-fold, CAT activities were normalized to the native −237 rGH:CAT construct.

**RNA Preparation and Analysis**—Total cellular RNA was prepared by lysis of the cells in situ with 4 M guanidinium isothiocyanate, followed by purification through a CsCl step gradient (30). Transcription initiation sites were mapped using a ribonuclease protection assay as described previously (17).

**RESULTS**

To alter the spacing and alignment between promoter elements in the rGH gene, DNA sequences of varying length, derived from the pUC18 polylinker, were inserted at either position −51. Insertions of 11-1 bp or the two insertions which represent integral turns of DNA helix, alter the distance between sequences either side of an insertion point but not their alignment and are referred to as “even” insertions. Nonintegral turns of DNA helix alter both the distance and alignment of sequences either side of the insertion point and are referred to as “odd” insertions. Insertions at −148 and −51 on Serum-stimulated Activity of the rGH Promoter—To determine the effect of insertions on rGH promoter activity, transfections were performed on cells grown in the presence of 10% fetal bovine serum. The results of these experiments are summarized in Fig. 2. Activities were normalized to the −237 rGH:CAT construct, which yielded 23,289 ± 5,476 cpm in six separate transfections. Generally, insertions at −148 (Fig. 2A) had a less deleterious effect than insertions at −51 (Fig. 2B). Insertion of 15 bp at −148 decreased activity approximately 40%, while longer insertions progressively reduced activity such that insertion of 44 bp abolished any apparent effect of sequences −237/−148. At the −51 position, insertion of as little as 5 bp caused a 2-3-fold reduction in CAT expression, while insertion of 23 bp was sufficient to reduce CAT expression to the level of the −51 rGH promoter construct. For both −148 and −51 insertions, activity was decreased in proportion to the length of insertion. However, there was no apparent turn dependence between even (10 and 20 bp) and odd (5, 15, and 35 bp) turn insertions, suggesting that spacing of promoter elements is more critical for activity than their relative orientations.

**rGH Promoter Activity Is Not Affected by Mutations Spanning the −51 Insertion Point**—To determine whether the loss of activity associated with insertion of sequence at the −51 position might be due to disruption of a factor binding site, a scanning mutagenesis, introducing blocks of four nonconservative transversions from −53 to −38 (Fig. 1), was carried out. The effects of these mutations were assessed in an RNase protection assay using an RSV-CAT construct as a cotransfected control. The effects of these experiments (Fig. 3) demonstrate that base substitutions in this region have either no effect on activity (−45/−42) or increase activity less than 2-fold (−53/−50 and −41/−38). This would suggest that the decreased activities caused by insertions at the −51 position are not due to disruption of the site at which the insertions were made. Moreover, mutations downstream of the insertion point had no appreciable effect on activity. Gel mobility shift assays carried out using restriction fragments spanning this region showed that the mutations had no effect on factor binding either in the vicinity of the insertion point (GHF-6)² and at the −148 position were also prepared so as to retain a common sequence at the 3′ end. Although the 15-bp insertion differed at the 3′ side, this entire sequence was contained within the 35- and 44-bp insertions.

Inspection of the inserted sequences and the new sequences created at their junctions with rGH sequences revealed no significant identities to binding sites for any known transcription factors. In addition, analysis of these sequences for their potential flexibility and curvature showed no strong predisposition toward the formation of structures that might influence promoter activity (31, 32).

Restriction fragments spanning the rGH sequences −237/−7 from either the native gene or each insertion mutant were linked to a CAT reporter gene (23). Additional CAT constructs containing the rGH native sequences −148+/7 or −51/+7 were also prepared. All constructs were transfected by electroporation into the rat pituitary tumor cell line, GC, together with a hybrid RSV-β-galactosidase gene cotransfected to normalize for variations in transfection efficiency. In these and previous studies (23), we found the activity of the RSV-β-gal control to be stimulated less than 2-fold by either T₃ or forskolin.

**Effects of Insertions at −148 and −51 on Serum-stimulated Activity of the rGH Promoter**—To determine the effect of insertions on rGH promoter activity, transfections were performed on cells grown in the presence of 10% fetal bovine serum. The results of these experiments are summarized in Fig. 2. Activities were normalized to the −237 rGH:CAT construct, which yielded 23,289 ± 5,476 cpm in six separate transfections. Generally, insertions at −148 (Fig. 2A) had a less deleterious effect than insertions at −51 (Fig. 2B). Insertion of 15 bp at −148 decreased activity approximately 40%, while longer insertions progressively reduced activity such that insertion of 44 bp abolished any apparent effect of sequences −237/−148. At the −51 position, insertion of as little as 5 bp caused a 2-3-fold reduction in CAT expression, while insertion of 23 bp was sufficient to reduce CAT expression to the level of the −51 rGH promoter construct. For both −148 and −51 insertions, activity was decreased in proportion to the length of insertion. However, there was no apparent turn dependence between even (10 and 20 bp) and odd (5, 15, and 35 bp) turn insertions, suggesting that spacing of promoter elements is more critical for activity than their relative orientations.

²F. Schaufele, unpublished observations.
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The activity of the native rGH promoter is largely hormone-dependent, and this has been shown using transfection assays. When hormone-depleted media were used, the activity of the rGH promoter was significantly lower than in the presence of hormones. The relative activities of insertions, both at position -148 or -51, were reduced up to 3-fold. However, this was still significantly greater than the activity of -51 rGH. CAT. The weak turn dependence observed in the basal activities of insertion mutants was also evident in the stimulated activities. This resulted in similar induction for each construct even though the induced activity was significantly reduced by longer insertions. For example, the hormone-induced activities of all -51 insertion constructs differed significantly from the native control, only some of the differences in inductions were significant. More...

Effects of Insertions at -148 and -51 on T3 and Forskolin Stimulation of the rGH Promoter—In addition to the pituitary-specific expression conferred by Pit-1/GHF-1, rGH promoter activity is stimulated by both T3 and cAMP (14, 18). To determine whether T3- and cAMP-responsive elements were affected by spacing and to examine their contribution to changes observed in serum-stimulated activity, transfections were carried out on cells grown in hormone-depleted media.

In the absence of hormones, basal activities from constructs containing native sequences and insertion or deletion mutations were low and differed less than 2-fold (Figs. 4 and 5). It is noteworthy, however, that at both the -148 and -51 sites, even turn insertions reproducibly had a greater effect on basal activities than odd turn insertions. Although this effect was small, it affected the apparent hormone inductions, especially at the -51 sites (see below). These results indicate that the activity of the rGH promoter is largely hormone-dependent and suggest that the effects of insertions observed in the presence of whole serum may be due to the disruption of interactions involving hormone-responsive elements. Individually, both T3- and forskolin-stimulated expression of the wild-type and insertion constructs and their effects when applied together were additive (Figs. 4 and 5). Maximally stimulated activities of the native rGH promoter (Fig. 4, 40,914 ± 457 cpm, n = 2; and Fig. 5, 27,325 ± 7,161 cpm, n = 4) were similar to the serum-stimulated activities observed in Fig. 2, suggesting that addition of T3 plus forskolin mimics the effect of serum. The relative activities of -148 and -51 insertion mutants, after treatment with T3, forskolin, or both, were reduced up to 5-fold. With insertions of increasing length at -148, T3 induction was reduced 2-3-fold, whereas forskolin induction was relatively unaffected. Deletion of sequences to -148 virtually abolished induction by T3 but had no significant effect on forskolin responsiveness. As induction by T3 and forskolin were of a similar magnitude, when the two agents were added together, stimulation by forskolin diminished the effect of insertions on T3 induction. Conversely, insertions at -51 reduced induction by both T3 and forskolin, although the effects on induction by T3 alone were smaller. Although insertions reduced forskolin induction to the 3-5-fold range, this was still significantly greater than -51 rGH-CAT (2-fold), suggesting that the CREs are contained upstream of -51. Moreover, the effects of forskolin were more evident when applied in combination with T3. Under these conditions, the effect of T3 plus forskolin was significantly greater than the effect of T3 alone for native and insertion constructs but not -51 rGH-CAT. The weak turn dependence observed in the basal activities of insertion mutants was also evident in the stimulated activities. This resulted in similar inductions for each construct even though the induced activity was significantly reduced by longer insertions. For example, the hormone-induced activities of all -51 insertion constructs differed significantly from the native control, only some of the differences in inductions were significant. More...

**FIG. 1.** Factor binding sites in the rGH promoter and the location and sequences of insertions. Coordinates of binding sites for factors described in the text are numbered backwards from the transcription start site. Sequences were inserted either at position -148 or -51. The sequence of the 15-bp insertion at -148, which is contained within the 35- and 44-bp insertions, is underlined. Sequences duplicated at the 5' and 3' ends of the -51 insertions are overlined. Although the restriction enzyme TaqI cleaves at position -48, duplication of part of this site in each of the insertions resulted in a shift of the apparent insertion point to -51. The location of a T3R binding site immediately adjacent to the -51 insertion point is shown as the residues making contact with receptor (*).
Figure 2. Effects of insertions on serum-stimulated, transient expression following transfection into GC cells. Insertion and deletion mutations described in Fig. 1 and under "Materials and Methods" were transiently transfected by electroporation into GC cells grown in DMEM containing 10% FCS. Following transfection under serum-free conditions, cells were replated in serum-containing media, harvested, and lysed for assay after 24 h. A, activities of constructions containing insertions at -148 (length of insertion shown for each construct) and -148 rGH-CAT. B, activities of constructions containing insertions at -51 (length of insertion shown for each construct) and -51 rGH-CAT. Each construction was tested in three to six separate experiments.

Overall, differences in activities between the 5-bp insertion and longer insertions differed significantly for a number of treatments, whereas none of the differences in inductions between different length insertions were significant. Ribonuclease protection experiments revealed that correctly initiated transcripts from the various constructions accumulated to levels corresponding to CAT activities in response to hormone treatments (data not shown).

Discussion

Spatial requirements for interactions between promoter and enhancer elements have been most extensively characterized for the SV40 regulatory region (33). The latter study showed that spacing and alignment was far more critical for promoter than enhancer elements. Thus, insertions between promoter and enhancer sequences were found to reduce activity 2-5-fold in a turn-dependent manner such that the insertion of whole turns of DNA helix had a less deleterious effect than insertion of nonintegral turns. Insertions between the TATA box and sequences upstream reduced TATA-dependent transcripts 4-10-fold, while TATA-independent transcripts increased up to 4-fold, both in a relatively turn-independent manner. Studies on the SV40 enhancer (34-37) have also demonstrated interactions between a number of functionally redundant modules, which may be interchanged or separated up to 50 bp. Each module may be further subdivided into a number of smaller segments (38) that most likely correspond to binding sites for individual transcription factors (39). However, unlike the enhancer modules they form, the arrangement of these sites is highly sensitive to spacing.

The results of the present study show critical distance-dependent relationships between sequence elements in the rGH promoter. Insertions at -148, which separated the GHF-3 site and TRE from elements downstream, reduced activity and T3 responsiveness but had little effect on forskolin responsiveness, while insertions at -51, which separated the TATA box from elements upstream, reduced activity and both T3 and forskolin responsiveness. These observations are consistent with the localization of a predominant TRE to the region -190/-166 (20, 22) and cAMP responsive elements to the region -104/+11 (14). Our data further indicate that cAMP response elements are contained upstream of -51. Taken together, these observations localize a CRE to the region -104/-51, which contains the proximal Pit-1 site. A number of recent studies indicate that cAMP responsiveness may be mediated by Pit-1 (16, 40, 41).

Although insertions at -51 attenuated forskolin responsiveness, this effect was most apparent when forskolin was added in combination with T3. However, unlike the strong synergism reported by Copp and Samuels (14), under the conditions of the present study, the effects of forskolin and T3 were at best only slightly more than additive. This is most likely due to differences in the time course of transfection and hormone treatment. In the present study, hormones were applied for 24 h post-transfection, whereas Copp and Samuels (14) treated the cells for 48 h. We have found that treatment...
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FIG. 4. Effects of -148 insertions on basal, T2-, and forskolin-stimulated transient expression following transfection into GC cells. Cells were grown in DMEM containing 10% charcoal/resin stripped-FCS for 48 h prior to transfection and replated post-transfection in fresh media containing vehicle (-), T3 (T3) (10 nM), forskolin (F) (1 μM), or T3 plus forskolin (T3 + F), as indicated. Constructions are labeled as shown in Fig. 2A. A, activity normalized for the -237 rGH.CAT construct stimulated with T3 + F (mean ± S.E., for two separate transfections); B, fold inductions on activities shown in A relative to no treatment control (-). Differences in fold inductions (relative to the native rGH sequence) were evaluated by a Scheffe F-test. Asterisks indicate differences significant at p < 0.05.

For 24 h results in additive effects, whereas treatment for 48 h results in strong apparent synergism that may be due, at least in part, to induction of Pit-1 by cAMP (42).

Of particular importance is the possibility that the insertions created in the rGH promoter might disrupt the binding site(s) for some previously unidentified factor(s). Since at the -148 position only longer insertions appeared to have any significant effect, it is unlikely that this position occurs within the binding site for any important factor(s). However, the sharp reductions in activity observed with all insertions at -51 suggest that this site may bind some important factor(s). Although the -51 site is adjacent to a known T3R binding site, oligonucleotides containing sequences immediately upstream of the insertion point have been shown to bind receptor (22). This region also appears to bind another factor (GHF-6) (23). However, mutations in this region had no apparent effect on GHF-6 binding. These same mutations had either no effect on rGH promoter activity or increased activity slightly. Taken together, these data suggest that the effect of insertions at the -51 position is not due to the disruption of some important factor binding site but rather to the perturbation of a critical spacing of sites either side of the insertion point.

Turn effects have been demonstrated for insertions between the two Pit-1 binding sites in the rGH promoter (18) and between the two most proximal Pit-1 binding sites in the rPRL promoter (43), where odd turn insertions appeared to be more deleterious than even turn insertions. In the present study, a weak turn effect resulted in slight increases in activity among the odd turn insertions at both the -148 or -51 sites. This could be due either to creating a more favorable alignment between positive control elements or to disruption of an optimal alignment of negative elements. However, the major effect of insertions at either site in the rGH promoter was distance- rather than turn-dependent. Insertions at -51 resulted in marked reductions in activity similar to those observed in the SV40 control region when insertions were made between the TATA box and promoter and enhancer elements further upstream (33). Although insertions at this position in the SV40 control region exhibited some turn dependence, the

FIG. 5. Effects of -51 insertions on basal, T2-, and forskolin-stimulated transient expression following transfection into GC cells. Transfection conditions, treatments, relative activities, and fold inductions (mean ± S.E., for four separate transfections) were as for Fig. 4. Constructions are labeled as shown in Fig. 2B. In A, all differences between the activities of insertions and the native control were significant at p < 0.05 for each treatment. Asterisks indicate significant differences between the activities of specific constructs and the 5-bp insertion construct for each treatment. In B, asterisks indicate significant differences (p < 0.05) between the activities of insertion constructs and the native control for each treatment.
major effect was distance-dependent with insertions of 5, 10, and 15 bp reducing activity to approximately 15, 20, and 5% of the wild type, respectively. Turn effects have been described for insertions between the glucocorticoid response element and CACCC box of the tryptophan oxygenase promoter (44). Our failure to observe turn-dependent effects on activity and hormone responsiveness may be due to the distance of the GFR-3 site and TRE from the insertion point (>35 bp). This could permit sufficient twisting and bending in the DNA to allow contacts between transcription factors or transcription factor complexes, which would be placed on opposite sides of the DNA helix by odd insertions and thus experience greater difficulty in interacting than configurations where shorter sequences separated their binding sites. Alternatively, turn effects could be masked by the effects of other transcription factors whose suboptimal alignment might be improved by insertions. Even if such a phenomenon were to obscure turn dependence, it could not account for the predominant distance effects we observed.

Distance-dependent interactions between steroid hormone receptors and transcription complexes assembled at the TATA box (29) have also been demonstrated in a study that showed that a glucocorticoid response element could stimulate transcription from a minimal TATA box promoter when placed close but not when placed several hundred base pairs upstream (45). However, in the more distant position, duplication of the glucocorticoid response element or addition of a binding site for one of several common transcription factors yielded a transcriptionally active unit (45). A similar observation was made for thyroid hormone receptor activation of rGH expression (26). Separating sequences containing the wild-type TRE or an up-mutant based on this TRE from a truncated 137-bp wild-type rGH promoter resulted in only modest levels of T3 responsiveness, unless present as two or three tandem copies. These findings are consistent with our observation that separating the rGH TRE by as little as 15 bp reduced T3 responsiveness 2-fold. Our findings of distance dependence of T3 responsiveness support the concept that T3 responsiveness of the rGH promoter involves interactions between T3R and some factor downstream of the TRE, possibly Pit-1 as has previously been suggested (18, 25).

The findings of the present study suggest that expression and regulation of the rGH gene is dependent on relatively short-range protein-protein interactions, which are more critically dependent on spacing than alignment of the transcription factor binding sites. This would imply that the exact distance between binding sites plays an important role in the assembly of transcription factor complexes. DNA bending by Pit-1 (46) would tend to curve the DNA between the T3R binding site and the TATA box, bringing these sites closer together. In addition, we have recently shown that a T3R-assembly factor complex binding to the upstream TRE induces a sharp 75° bend in the rGH 5’-flanking DNA centered at position −181 (47). This could result in the T3R-assembly factor complex being drawn closer to Pit-1 bound downstream and transcription factor complexes assembled at the TATA box. As DNA bending has also been described for TFIID binding at the TATA box (48), the entire array of factors bound to the rGH promoter may be drawn closely together in such a way that the spacing between factor binding sites, especially between the Pit-1 sites and the TATA box, is critical for their optimal interactions.

REFERENCES

4. Creb, M., Bertuccioli, C., Takada, R., Wan

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