A Weak TATA Box Is a Prerequisite for Glucocorticoid-dependent Repression of the Osteocalcin Gene*

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The TATA box element is not only important for establishing basal levels of transcription, but it can also be used to modulate cell type or stage specific gene activity. In the case of the human osteocalcin gene, which is transcriptionally repressed by glucocorticoids, a specific binding element for the glucocorticoid receptor (GR) overlaps a noncanonical TATA box. In the present study, the relevance and function of the TATA element in glucocorticoid-mediated repression of the human osteocalcin gene was characterized. Mutating this noncanonical TATA box into a consensus TATA box within the context of the osteocalcin promoter greatly decreased hormone-dependent transcriptional repression by GR. TATA-binding protein (TBP) bound this mutated element much more strongly suggesting a physiologically relevant role for the weak osteocalcin TATA element in the regulation of this bone specific gene. The optimization of the putative transcription factor IIB recognition site did not affect the level of GR-mediated repression. Our results support a model wherein competitive DNA binding of GR and TBP for their overlapping sites explains conditional repression of the osteocalcin gene by glucocorticoids.

Transcription of RNA polymerase II-dependent genes requires, in addition to the enzyme itself, a number of general factors that form a specific multiprotein complex near the transcription start site by interacting with basal promoter elements. The most well studied core promoter element is the TATA box, which is typically located 25–30 base pairs upstream of the transcription start site of many eukaryotic genes (1). The central step in formation of the preinitiation complex can form as TFIID cannot bind.

The central step in formation of the preinitiation complex is dependent upon this initial interaction at the TATA box. Thus, when the sequence specific factor is bound, no preinitiation complex assembly, the TATA box can also have an important function with respect to cell type or stage specific modulation of gene activity. In several reported cases, minor deviations from the consensus TATA box DNA sequence (TATA(A/T)A(A/T)A) are thought to be critical for proper regulation. For example, in the case of the rabbit uteroglobin gene, two factors, one cell type specific and the other ubiquitously expressed, have been proposed to facilitate the interaction of TBP with the weak TATA box, TACAAA, by binding the TACA site (7). Another example is the unusual inverted TTATA sequence that is involved in the negative regulation of the bone sialoprotein gene (8).

Several examples of repression of gene activity by competitive binding at the TATA box have also been postulated. In these cases, the overlap or close location of binding sites for other sequence specific transcription factors and the TATA box suggest that repression of gene expression is the result of a competition between TFIIID and a sequence specific factor (9, 10). Thus, when the sequence specific factor is bound, no preinitiation complex can form as TFIIID cannot bind.

Several cases of competitive binding with other transcription activators have been reported for gene regulation by nuclear hormone receptors (NRs), a family of ligand-dependent transcription factors that includes the receptors for steroid hormones. Upon ligand binding, NRs interact with genomic response elements and directly alter the transcription levels of linked genes. Modulation of gene activity by NRs through steric hindrance of TFIIID at the TATA box has been proposed for thyroid hormone-dependent repression of the thyroid-stimulating hormone α-gene and for the bone sialoprotein, which is negatively regulated by vitamin D (9, 11). In the latter case, a vitamin D receptor element overlaps the TATA box. A further example of gene repression by glucocorticoids involving the promoter region around −30 is reported for the type 1 vasoactive intestinal polypeptide receptor gene (12).

A DNA binding site recognized by the glucocorticoid receptor (GR) that partly overlaps the TATA box within the promoter region of the human osteocalcin gene has been identified spanning nucleotides −35/−14 relative to the transcription start site (Fig. 1) (13). The strategic and central position of the GRE makes it likely that the negative glucocorticoid effect at this gene is mediated by inhibition of formation of a functional preinitiation complex by displacement of TFIIID from the TATA box.

In the present work we have analyzed the importance of the number of associated factors (3). The affinity of the TBP/TATA box interaction has been proposed to contribute to promoter strength in vivo and in vitro, and subsequent assembly of the other general transcription factors into a functional preinitiation complex is dependent upon this initial interaction at the TATA box (1, 4–6).

In addition to its role as a nucleation point for preinitiation complex assembly, the TATA box can also have an important function with respect to cell type or stage specific modulation of gene activity. In several reported cases, minor deviations from the consensus TATA box DNA sequence (TATA(A/T)A(A/T)A) are thought to be critical for proper regulation. For example, in the case of the rabbit uteroglobin gene, two factors, one cell type specific and the other ubiquitously expressed, have been proposed to facilitate the interaction of TBP with the weak TATA box, TACAAA, by binding the TACA site (7). Another example is the unusual inverted TTATA sequence that is involved in the negative regulation of the bone sialoprotein gene (8).

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sequence of osteocalcin TATA box on glucocorticoid-mediated transcription repression of osteocalcin gene activity. We demonstrate that mutating the osteocalcin TATA box into a consensus TATA element greatly diminished repression by hormone-activated GR. The mutation does not influence the specific GR binding suggesting that the osteocalcin TATA box has an active function in the regulatory process. Finally, we present preliminary evidence that suggests that displacement of another polymerase II general transcription factor, TFIIA, is not important for regulation of the osteocalcin gene by mutating promoter positions reported to be responsible for efficient TFIIA function.

EXPERIMENTAL PROCEDURES

Mutations—Mutations were introduced into pOS-344Luc (14) within the promoter region of the human osteocalcin gene by standard protocols (15) using the following oligonucleotides: wild type, 5'-CTCAGCT-GGCACGCCCGCCAGGCCTCTCAGAATTTTATACCCTCTGGGC-38; TATAAAA, 5'-CTCAGCTGCCAGGCCTCAGCTTATACCCTCTGGGC-38; TACAAAA, 5'-CTCAGCTGCGCCAGGCCGGCCAGGCCTCAGCTTATACCCTCTGGGC-38; TACAAAA, 5'-CTCAGCTGCGCCAGGCCGGCCAGGCCTCAGCTTATACCCTCTGGGC-38; TACAAAA, 5'-CTCAGCTGCGCCAGGCCGGCCAGGCCTCAGCTTATACCCTCTGGGC-38.

An Mse I site was introduced by mutating the A at -3 to T to simplify screening. This mutation was shown to have no effect on reporter expression levels (data not shown).

Cell Culture—COS-7 cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified medium buffered with bicarbonate and supplemented with 5% fetal calf serum, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml).

Transient Transfection and Luciferase Assays—Cells were seeded in 6-cm plates 24 h before a transfection experiment and transfected at a confluence of 40–50% using the calcium phosphate coprecipitation technique. The precipitate contained 5 μg of supercoiled luciferase reporter plasmid DNA and varying amounts of (0–3 μg) of different expression plasmids. The overall amount of DNA was kept constant by the addition of parent expression vector. After 12–14 h of exposure to the calcium phosphate precipitate, medium was changed and the cells were treated for 24 h with 20 nM dexamethasone. Transfected cells were subsequently harvested for the luciferase assay by scraping the cells in 1 ml of phosphate-buffered saline, centrifuging for 10 min in a microcentrifuge and resuspending in 50 μl of lysis buffer (25 mm Tris-acetate, pH 7.8, 1.5 mm EDTA, 10% glycerol, and 1% Triton X-100). All experiments were performed three times in triplicate. Luciferase activity was monitored according to GenGlow luciferase assay kit (Bio Orbit) using an anthos lucy 1 luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The results are expressed as light units measured.

Protein Expression—Human glucocorticoid receptor protein was expressed in Sf9 insect cells using a baculovirus expression system as described previously (16, 17). The human TBP was produced in a Sf9 insect cells using a baculovirus expression system as described previously (16, 17).

RESULTS

One mechanism by which basal levels of transcription are established is through the binding of TFIIA to the TATA box. However, in some cases it appears that the TATA box may also be involved in the regulation of gene expression by an occlusion mechanism analogous to that found in a number of prokaryotic systems. For example, in the case of the human osteocalcin gene, the TATA box overlaps a binding site for a ligand-activated transcriptional regulator, the GR (13). Induction of this NR with glucocorticoids leads to a repression of osteocalcin gene activity to 40% of basal levels (19). Since we have defined the function of the glucocorticoid responsive element in previous studies (14), we were interested in studying the role of the sequence of the noncanonical osteocalcin TATA box in glucocorticoid-dependent repression of osteocalcin levels.

Consistent with other genes regulated by events directly involving the TATA box, the osteocalcin gene contains a noncanonical TATA element (Fig. 1B) (in this case, TATAAAC, in which the A at position 7 is replaced by a C). Previous results suggest that TBP binds this sequence poorly and that it directs decreased basal levels of transcription (6, 20, 21). To determine if the weakness of the osteocalcin TATA box is important for glucocorticoid-mediated transcription repression, we mutated this element to a canonical TATA box, TATAAAA, and tested the effect of this mutation in cellular transfection experiments using an osteocalcin promoter construct driving the firefly luciferase gene as a reporter system. For these studies, the construct was transfected into COS7 cells where the endogenous GR is expressed at very low levels and is virtually undetectable by ligand binding or immunochromatographic assays (22). As shown in Fig. 2, mutation of the TATA box to TATAAAA increased basal osteocalcin promoter activity 1.7-fold. In addition, experiments, the cotransfected and ligand-activated GR efficiently repressed the WT osteocalcin promoter to a level between 30 and 40% in agreement with previously published results (Fig. 3A). Strikingly, equivalent amounts of GR failed to repress the osteocalcin promoter containing the consensus TATA box upon hormone induction (Fig. 3B). Thus, the nonconsensus osteocalcin TATA sequence is crucial for...
Function of a Noncanonical TATA Box

Fig. 2. A C-A change within the osteocalcin TATA box increases the basal transcription rate. COS 7 cells were transfected with an osteocalcin reporter plasmid (pOS-344Luc) either containing the WT TATA box (TATAAAC) or a canonical TATA box (TATAAAA). The figure shows the results of three experiments. The relative luciferase activity is the mean ± S.D. of three independent experiments, each performed in triplicate.

Fig. 3. Glucocorticoid-dependent repression is reduced by a consensus TATA box. COS 7 cells were either transfected with a luciferase reporter gene driven by nucleotides −344/+34 of the human osteocalcin promoter (TATAAAA) or a construct containing a canonical TATA box (TATAAAA) (B) together with an expression vector for GR. The cells were incubated with □ or without □ 20 nM dexamethasone (Dex). Luciferase activity was assayed in cells from 6-cm plates and related to the activity in cells transfected with GR in the absence of dexamethasone. The figure shows the mean ± S.D. of three experiments, each carried out with three independent triplicate analyses.

Fig. 4. GR binding to WT and mutant osteocalcin TATA box containing fragments. A, GR was expressed in a baculovirus system, and cell extracts were prepared. Aliquots of the soluble fraction were incubated with 32P-labeled oligonucleotides corresponding to nucleotides −41/−9 of the osteocalcin promoter containing the nGRE (WT) or mutants of this fragment (TATAAAA, TFIIB, and GREmut) (see Fig. 1C). B, GR binding to an optimized binding site, described for optimal GR function, enhanced specific complex formation (compare TATAAAC and TACAAAA). Extracts of cells infected with the parent virus were used as control (C). The mobility of the specific GR-DNA complex is indicated (GR).

proper osteocalcin regulation.

Although the mutated residue of the TATA box should not compromise GR binding as it is located in the 3-base pair spacer region, we performed in vitro binding studies using either a WT or the TATAAAA mutated form of a DNA fragment containing the human osteocalcin promoter-spanning nucleotides −41/−9 (Fig. 1C). In gel mobility shift assays using virally expressed GR, we showed that the receptor protein binds both fragments similarly (Fig. 4A, lanes 3 and 4). Mutation of the nGRE sequence (Fig. 1C; GREmut) eliminates the GR-specific complex, whereas nonspecific complexes were not affected (Fig. 4A, lane 6, B, lane 5). Furthermore, this band was supershifted to a slower migrating species following incubation with antibodies against GR (data not shown). A mutation within the first putative GRE halfsite (TACAAAA), creating a binding site described for optimal GR function, enhanced GR-DNA interaction (compare Fig. 4B, lanes 3 and 4).

Since we are proposing that a prerequisite for effective repression of the osteocalcin gene by glucocorticoids is a noncanonical TATA element that influences the TBP/TATA box interaction, we directly determined if TBP could distinguish between the WT and the A mutant by using virally expressed TBP in gel mobility shift assays. As shown in Fig. 5, TBP induced a specific protein-DNA complex on a DNA fragment containing the WT osteocalcin promoter fragment −41/−9. However, under the same experimental conditions, TBP bound a fragment containing a consensus TATA box within the context of the osteocalcin promoter with apparently higher affinity (Fig. 5, lane 3). The specificity of this interaction was established by using a similar element containing a G in the second position of the TATA element (TGTAAAC; Fig. 5, lanes 4 and 8). This mutation completely abolished the TBP-DNA interaction as described previously (23). The use of a synthetic DNA fragment containing an alternative mutation within the strong consensus TATA box element (TACAAAAA) resulted in a TBP-induced complex formation comparable in intensity to the weak WT osteocalcin TATA box (Fig. 5, lanes 6 and 7). When we used our osteocalcin reporter construct containing TGTAAAC, TACAAAA, or TACAAAAA as TATA element, the reporter gene activity was decreased to background levels (data not shown). This result suggests that the binding affinity of TBP to the osteocalcin TATA box is one of the major determinants for GR action at the osteocalcin promoter.

The basis of our model for the glucocorticoid-dependent down-regulation of the osteocalcin gene is competitive binding between the basal transcription factor TFIID and a conditionally active transcription regulator, the glucocorticoid receptor, for overlapping binding sites. In this model, the relative affinity of each factor for its respective binding site is crucial for the proper regulation of the osteocalcin gene with neither factor...
binding too strongly. To more accurately assess the relative binding strengths of TBP and GR for the osteocalcin GRE/TATA box, we compared the binding affinities of TBP and GR to the osteocalcin GRE/TATA box to their respective consensus binding sites contained within the osteocalcin promoter background. In agreement with our proposed model, both TBP and GR bound the osteocalcin GRE/TATA box more weakly than they bound their consensus binding sites. As shown in Fig. 6, TBP binds the osteocalcin TATA box 5-fold less strongly than to the consensus TATA box, TATAAAA, over a wide range of protein concentrations (Fig. 6A), and GR binds the osteocalcin GRE 5-fold less strongly than to the consensus GRE, GGTACA, over a wide range of protein concentrations (Fig. 6B).

If mutually exclusive binding of the two transcription factors is a prerequisite for the glucocorticoid-mediated transcription repression effect, then an increase in GR concentration should be sufficient to perturb the enhanced binding of TFIID to the consensus TATA box within the osteocalcin gene. We therefore tested whether increased amounts of GR expression vector would be capable of repressing the mutated osteocalcin reporter construct that contains the consensus TATA box in the above described cellular transfection system. The use of an excess of GR expression vector resulted in a concentration-dependent decrease of osteocalcin reporter gene activity comparable to the level of repression at the WT osteocalcin promoter construct. The use of 5–10-fold more expression vector resulted in a similar decrease in reporter gene activity (Fig. 7). These results are indicative of equilibrium binding of GR and its competitive factor TFIID in the proposed mechanism and, furthermore, that the reduced affinity of TFIID for its DNA recognition site is critical for negative gene regulation of the human osteocalcin gene by glucocorticoids.

During the formation of the preinitiation complex, several other basal factors stabilize the TFIID-DNA complex. Among them, TFIIB is essential for the formation of the polymerase II initiation complex as it interacts with the TBP-DNA complex and recruits the polymerase. TFIIB requires at least 7 base pairs of DNA on either side of the TATA box to form a stable TFIIB-TBP-DNA complex (24–26). Lagrange et al.\(^2\) have presented evidence for the importance of two G bases at positions −3 and −6 upstream of the TATA box (positions −36 and −33 in the human osteocalcin gene) (Fig. 8A). In the case of the osteocalcin promoter, only the proximal G is conserved, which motivated us to study a possible stabilization effect of TFIIB on TFIID binding. We therefore introduced an additional mutation targeting the nonconserved A at position −6 with respect to the TATA box in the osteocalcin promoter fragment driving the luciferase reporter gene (−36 with regard to the human osteocalcin gene). In cellular transfection assays, cotransfection of GR and the mutated osteocalcin reporter construct containing the two described G residues resulted in repression of reporter gene activity upon hormone addition. Comparable to the WT promoter, the reporter gene activity was repressed to a level of 40% (Fig. 8B). Even if this base change did not directly target the putative GRE, we showed that this mutation did not disturb the GR/DNA interaction using the above described DNA binding assay. As shown in Fig. 4A, lane 5, no change in GR/DNA binding strength was detected when compared with the WT fragment. This observation strengthened the notion that only TBP binding to the osteocalcin TATA box

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\(2\) T. Lagrange, A. Kapanidis, H. Tang, D. Reinberg, and R. H. Ebright, personal communication.
has a central and dominant function in glucocorticoid mediated repression of osteocalcin expression level.

**DISCUSSION**

The affinity of sequence specific transcription factors for their binding sites is a major determinant for their action. Consensus binding sites have been defined for most of the known DNA binding transcription factors with these sites yielding maximal effects on gene transcription. Additionally, many cases have been described where nonconsensus binding sites are important for the transmission of a specific regulatory effect. This is also true for the basal transcriptional element, the TATA box, where several sites have been described in which proper regulation requires sequences divergent from that of a consensus TATA box, TATAAAA (23). For example, the rabbit uteroglobin gene depends upon a noncanonical TATA box for transmission of cell specific effects (7) and in the bone sialoprotein promoter, a noncanonical TATA box, TT-TATA, may be involved in vitamin D-dependent gene repression (8). Thus, at times a nonconsensus DNA sequence may be a prerequisite for proper gene regulation.

Competitive binding of transcription factors to a common binding site has been proposed in many cases as a functional regulatory mechanism for gene transcription. Signal transduction by steroid hormones through NRs provides several well documented examples of this mode of gene regulation. For example, in the case of the c-fos gene, the GR competes for binding with the serum response factor for a common binding site, and in the case of the bovine prolactin gene, competition takes place between GR and a cell specific positive acting DNA binding factor (11, 27). Both of these examples are quite complex in that the fundamental mechanism of repression in which displacement of an activator with another activator (GR) results in repression remains unknown. However, in the example studied here, the mechanism by which GR binding represses transcription is much simpler to understand in that GR binding displaces the basal factor TFIIID from the TATA box, thus disrupting the preinitiation complex that is required for transcription to occur.

For competitive binding to be an effective mode of transcriptional regulation, the two factors must be able to displace one another. Thus, in theory, neither factor should bind its site too tightly (although regulatory schemes involving ligand-dependent transcriptional regulators, such as NRs, should be able to bypass this requirement for that particular factor). This is particularly true with schemes that involve TFIIID as one of the members as it binds a consensus TATA box with an off-rate in excess of 2 h making displacement more difficult (28).

Consistent with this hypothesis, we show here that in the case of the human osteocalcin gene, a weaker nonconsensus TATA box is absolutely essential for glucocorticoid-mediated repression. We found that by altering the WT human osteocalcin TATA box, TATAAAC, to a consensus TATA box sequence, TATAAAA, the negative transcriptional effect of glucocorticoids on the osteocalcin transcription rate was decreased without affecting GR binding. Additionally, as predicted from previously published work (6, 20), we show that the TBP binds the consensus TATA box much more strongly than the WT osteocalcin TATA box and that the WT nonconsensus TATA box supports lower levels of transcription. Thus, when taken together, these results are consistent with glucocorticoid-mediated repression occurring via a competitive binding mechanism between TFIIID and GR at the human osteocalcin promoter.

The findings we obtained with the different TATA box variants may represent two different prerequisites for gene regulation by an occlusion mechanism, the affinity of a transcription factor for its binding site and the availability of a site
because of particular DNA architecture. Failure to repress at the WT TATAAAA sequence may occur because of one or both of these criteria. For example, GR may simply have more difficulty displacing TFII D from TATAAAA as TFII D binds this site too tightly. Alternatively, GR may have difficulty displacing TFII D from TATAAAA because the architecture of the DNA in these two TBP-TATA box complexes is predicted to be different. Several reports have suggested that TBP binding to the TATA box element induces a bend within the target site and that the degree and magnitude of this bend is directly correlated to the strength of TBP binding (29–31). The bend angle for a consensus TATA box has been described as 93 °C as compared with a TATAAAC element which bends the DNA 34 °C (20). The moderate distortion of the target DNA element observed with TATAAAC may increase the chance for a competitive factor to bind to an overlapping binding site. At this time there is no way to distinguish between these two models as no TATA box mutation has been reported that separates binding affinity from architecture. In fact, it may be that GR-mediated displacement of TFII D is hampered by both steric hinderance of the structure of the DNA as well as the off-rate of TFII D.

The observation that an optimized TFII B recognition site obtained from studies with promoters containing a standard strong TATA element did not affect GR-mediated repression of the osteocalcin gene, supports our notion that TFII D binding has a central role in our defined model system. We conclude that the reduced binding affinity and/or DNA bend angle of the TFII D-TATA box complex at the osteocalcin promoter is a major precondition for GR-mediated repression at that promoter. This supports a model involving competitive DNA binding of GR and TFII D as an explanation for conditional repression of the osteocalcin gene by glucocorticoids.

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