Glucocorticoid and estrogen regulation of a rat T-kininogen gene

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ABSTRACT

We have examined the regulation of a rat T-kininogen gene by glucocorticoid and estrogen. Expression of the endogenous gene in a rat hepatoma cell line is increased 5-fold and 2-fold in response to dexamethasone and 17 β -estradiol-3-benzoate, respectively. Various deletion constructs of the 5' region of an isolated T-kininogen gene were fused to a chloramphenicol acetyltransferase (CAT) gene and introduced into the hepatoma cells by electroporation. Analysis of the CAT activity in cell extracts after treatment with glucocorticoid or estrogen revealed that a fragment from -167 to +52 is sufficient to confer full induction. An additional deletion in this region was unresponsive, while a larger fragment (-612 to -100) linked to a heterologous promoter did result in regulated expression. These results suggested that the sequence responsible for the hormonal response was located at -167 to -100 from the transcription start site. This 67 bp region contains a consensus for the core sequence of the glucocorticoid responsive element (GRE) and the estrogen responsive element (ERE). Interestingly these elements are located within 7 bp of each other and both sequences overlap a 16 bp palindrome that may be important in hormone receptor-DNA recognition.

INTRODUCTION

Tissue injury brought about by bacterial infections, burns, or malignant growths triggers a systemic inflammatory response in mammals. One aspect of this response is increased activity of the adrenal gland and a concomitant rise in the concentration of circulating glucocorticoid hormone (1). Furthermore, there is a change in the profile of serum proteins produced by the liver. Albumin and transferrin levels are decreased, while a range of other proteins are dramatically increased. As a group, these proteins have been referred to as acute phase inflammatory reactants (2).

In rats, a cysteine protease inhibitor is induced 8-10 fold in response to inflammation (3,4). It has been called α 1-major acute phase protein to reflect this induction (5). More recently, it is referred to as T-kininogen (T-KG) because of the high degree of sequence similarity to the low molecular weight K-kininogen (6,7). T-kininogen contains a bradykinin moiety as do the K-kininogens, however the amino acids flanking this nonapeptide are different in T-kininogen. As a result, T-kininogen will only serve as precursor of bradykinin if the protein is treated with excess trypsin (8). The

principal function of T-kininogen therefore appears to be as an inhibitor of cysteine proteases possibly released by cell lysis in an inflammatory area.

We are interested in examining the regulation of acute phase reactants and of T-kininogen in particular. It has been shown that various cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF), as well as steroid hormones can regulate the expression of a number of acute phase reactants (9,10,11,12). We have initially chosen to study the hormonal regulation of T-KG with dexamethasone and estrogen. Control of T-KG gene expression with glucocorticoid would be consistent with the observation of increased hormonal levels during an inflammatory response. In addition, Oh-ishi et al. (13) recently demonstrated higher levels of T-KG protein in the serum of female versus male rats. Nucleotide consensus sequences for binding of the glucocorticoid or estrogen receptor-steroid complexes have been identified (14,15). Moreover, recent investigations have indicated that additional sequences may be important in mediating the glucocorticoid induction. In particular, Schüle et al. (16) have demonstrated that a pentanucleotide sequence, CACCC, which is essential for β -globin gene transcription, is also required for the dexamethasone induction of tryptophan oxygenase. In order to determine the molecular mechanism for the hormonal regulation of the T-KG gene, we have established a cell culture/DNA transfection system. Recently, we isolated and characterized a rat T-kininogen gene (manuscript in preparation). Nucleotide sequences from the 5' end of this gene have been examined for the ability to support glucocorticoid or estrogen induced expression of a reporter gene in a transfected cell culture system. A region from -167 to +52 was found to confer hormonal responsiveness and contains the consensus sequences for a GRE and an ERE separated by only 7 bp.

MATERIALS AND METHODS

Materials

[^{14}C]Chloramphenicol (54mCi/mmol) and [α - ^{32}P]dCTP (3000Ci/mmol) were purchased from Amersham Corp. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim Biochemicals, or Promega Biotec. Chlorophenol red- β -D-galactopyranoside was a product of Boehringer Mannheim Biochemicals. Chloramphenicol acetyltransferase enzyme standard, acetyl coenzyme A, dexamethasone, 17 β -estradiol 3-benzoate, and tissue culture medium were purchased from Sigma Company. All other reagents were of the highest purity available commercially.

Cell Culture and Cell Transformation

The rat hepatoma cell line, FAZA, was grown in DME/F-12 (1:1) medium supplemented with 10% fetal bovine serum, at 37°C in 5% CO₂. Penicillin and

streptomycin antibiotics were maintained in the media. The cells were passaged by trypsinization in the presence of EDTA, followed by replating at a dilution of 1:4 to 1:5. When the cells had reached 70-80% confluence, the medium was changed and fresh medium containing 1 μ M dexamethasone (from a 1 mM stock in ethanol) or 20 nM 17 β -estradiol 3-benzoate (from a 1 μ M stock in ethanol) was added. The cells were harvested after 36-48 hours and RNA was prepared for Northern RNA blot analyses.

DNA transfection of the FAZA cells was carried out by electroporation. The cells were allowed to grow to 70-80% confluence before harvesting by trypsinization in the presence of EDTA. The cells were washed twice with electroporation buffer (272 mM sucrose, 7 mM NaPO₄, pH 7.4, 1 mM MgCl₂) and then resuspended in the same buffer at a concentration of 0.5-1 x 10⁷ cells/ml. The cells were incubated with the plasmid DNA for 10 min on ice before electroporation. The transformation was carried out with a BioRad Gene Pulser power supply. The instrument parameters giving the best results for this cell line in terms of cell viability and DNA uptake were 25 μ FD and 350 v. After electroporation, the cells were allowed to recover for 10 min at room temperature before plating in 5 ml medium in the presence or absence of 1 μ M dexamethasone or 20 nM estrogen. Normally, 800 μ l of the cell suspension was cotransfected with 20 μ g of test plasmid DNA and 10 μ g of a plasmid carrying the β -galactosidase gene, pCH110 (Pharmacia), which served as an internal control to monitor for transfection efficiency.

Assays of the Transfected Cell Extracts

Assays for CAT activity were performed as described by Gorman, et al. (17) with the following modifications: the concentration of acetyl coenzyme A was increased to 1.6 mM and the reactions were allowed to incubate for 16 hours. This was necessary due to the inefficiency of the T-KG promoter element. Additionally, the cell extracts were treated at 65°C for 10 min to inactivate deacetylase activity prior to incubation in the CAT assay.

Detection of β -galactosidase activity was performed according to a standard protocol obtained from Pharmacia (Analecets 14). Briefly, 5 μ l of the transfected cell extract was mixed with 150 μ l Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM Mg₂SO₄, 50 mM β -mercaptoethanol, pH 7.0). The reaction was started by the addition of 40 μ l of 4 mg/ml chlorophenol red β -D-galactopyranoside, and incubated at room temperature overnight. After adding 500 μ l 1M Na₂CO₃, the absorbance was measured at A₅₇₄.

Preparation and Analysis of RNA

RNA was isolated from control cell cultures and from those treated with hormone according to the method of Chomczynski and Sacchi (18). Northern RNA blot analyses followed the protocol of the Schleicher and Schuell Company for the use of Nytran nylon

membranes. The hybridization probe was an *Rsa* I fragment from the T-KG cDNA corresponding to amino acids #57-218 (6). Random oligonucleotide priming was used to label the probe with [$\alpha^{32}\text{P}$]dCTP (19).

Construction of Plasmids

Plasmids containing the T-KG promoter and various lengths of 5' flanking region fused to a CAT gene were constructed in the pIBI30/31 vectors (International Biotechnologies, Inc.) (see Fig. 2). A 1.69 kb region from a 5' Hind III site to an Alu I site 66 bp from the start of translation was cloned in front of a Hind III-BamH I fragment containing the CAT gene. This construct is called pTKGCAT. A deletion eliminating 100 bp of 5' end was prepared by cleaving first with Bgl II, then performing a partial digestion with Hind III. The desired partial product was isolated and religated in the presence of Bgl II linkers. This construct is referred to as pTKG Δ Bgl (1.59 kb). Subsequent deletion plasmids were prepared from this construct by double digests cleaving at a unique restriction enzyme site within the 5' flanking DNA and at the newly created Bgl II site. The large vector fragment was isolated and religated to produce the plasmids pTKG Δ Tth (Tth-I III, 1.19 kb), pTKG Δ Mst (Mst II, 0.66 kb), and pTKG Δ Kpn (Kpn I, 0.15 kb). A final construct was prepared by reintroducing a 70 bp *Rsa* I fragment upstream of the Kpn deletion. This vector is termed pTKGRsa70 and reconstructs the normal configuration of the T-KG gene with 167 bp flanking and 52 bp 5' untranslated sequences. Control plasmids used in each transfection were pRSVCAT and pTKG0; the latter is a derivative of pTKGCAT in which 150 bp spanning the promoter of the T-KG gene was deleted.

A plasmid containing the adenovirus promoter fused to a CAT gene was utilized to test the effect of the T-KG 5' region in a heterologous system. The adenovirus major late promoter (-40 to +170) was cloned into pIBI30 directly upstream of the Hind III-Bam HI fragment of the CAT gene. A 512 bp Mst II-Kpn I fragment was isolated from the T-KG gene and introduced 5' of the promoter element by blunt-end ligation. The resulting plasmid was termed pMLP Δ Mst.

RESULTS

Inflammation is a complex physiological response, and results in the release of numerous hormones. Glucocorticoids, in particular, are known to increase in the serum and they have been shown to modulate the expression of several acute phase proteins (10). In order to determine if T-kininogen was included in this group, the rat FAZA hepatoma cell line was treated with dexamethasone. Total cellular RNA was isolated from the cells after 36-48 hrs for a Northern blot analysis. Additionally, estrogen has been implicated as a regulator of T-KG from sex difference studies (13). Therefore, this steroid also was used to treat the hepatoma cells for analysis of the effect on the

endogenous T-KG gene. An RNA sample from cells treated with this hormone was included in the Northern analysis. As a hybridization probe, a 484 bp *Rsa* I fragment containing exons 2 through 5 was prepared from a T-KG cDNA (6). The resulting autoradiograph is shown in Figure 1. After treatment with either 1 μ M dexamethasone or 20 nM 17 β -estradiol-3-benzoate, the T-KG mRNA (1.8 kb in length) increased in abundance compared to the control samples. Quantitation by densitometric scanning indicated a 5-fold induction by glucocorticoid and a 2-fold induction by estrogen. As a control, hybridization of the same Northern blot with a probe for β -actin mRNA demonstrated an equivalent signal in all lanes (data not shown).

It is known that the increase in T-KG mRNA abundance that occurs during an *in vivo* inflammatory response is the result of increased transcription (20). However, this induction is the net result of the activities of numerous cytokines and hormones. The Northern blot analysis indicated that the FAZA cells would serve as a model system to determine the effect steroid hormones have on T-KG expression. Plasmids were therefore constructed with sequences from the T-KG gene fused to a reporter gene for transfection into this cell line. Specifically, a 1.69 kb 5' region of the T-KG gene, which included the transcription start site as defined by S1 nuclease analysis and primer extension data (21,22), was positioned upstream of a CAT gene in a plasmid vector. Figure 2 illustrates the relative position of this fragment within the T-KG genomic clone and indicates the location of several putative glucocorticoid and estrogen regulatory elements (GREs and EREs) defined by identity to a 5 bp core consensus sequence (panel A). Panel B diagrams the starting plasmid for the transfection studies, pTKGCAT, and panel C of Figure 2 describes the 5' deletion plasmids constructed to determine the minimum sequence elements necessary for hormone induced expression of CAT.

The plasmids containing the CAT gene driven by the T-KG promoter with varying lengths of 5' flanking region were transfected into the rat hepatoma FAZA cells by electroporation. The cells were then plated in medium in the presence and absence of 1 μ M dexamethasone or 20 nM 17 β -estradiol-3-benzoate for 36-48 hours. Following this incubation, cell extracts were prepared and assayed for CAT activity. The results of these assays are shown in Figure 3. Although sequences exhibiting high similarity to the consensus hormone responsive elements occur throughout the 5' region of T-KG (Figure 2A), most of those regions can be eliminated with only a slight effect on glucocorticoid or estrogen induction of CAT activity. This is evidenced by plasmids pTKG Δ Bgl, pTKG Δ Tth, and pTKG Δ Mst. When all but 100 bp was deleted, however, only a low basal level of transcription is observed. The inability of this final construct, pTKG Δ Kpn, to respond to glucocorticoid occurred despite the fact that two consensus GREs were present in this 100 bp 5' fragment. No estrogen consensus sequences are evident in this

Figure 1. Induction of T-KG mRNA in rat FAZA cells. Rat hepatoma cells were cultured for 36-48 hrs in the presence or absence of $1\mu\text{M}$ dexamethasone or 20nM estrogen. Total cellular RNA was isolated and examined in a Northern RNA blot analysis. Lane A represents control RNA, lane B is RNA isolated from cells treated with estrogen, and lane C is total RNA from dexamethasone-treated cells. The $[^{32}\text{P}]$ -labeled hybridization probe was an Rsa I fragment of the T-KG cDNA spanning exons 2 through 5. Ten μg of total cellular RNA was loaded per lane; the exposure time was 20 hrs at -70°C with an intensifier screen. The arrows indicate the positions of ribosomal RNA.

fragment, therefore the basal transcription is all that would be expected with this hormonal treatment. While it is possible that the lack of hormonal response and the low level of CAT activity with the smallest T-KG deletion (pTKGAKpn) is due to a disruption in the promoter region, this does not seem likely. The CAT activity of this construct is detectable above the background promoter-less control (pTKGO) and is nearly equivalent to the uninduced levels seen with longer constructs such as pTKGCAT and pTKGABgl. Rather, the low basal level is probably a reflection of the inefficiency of the T-KG promoter, which lacks strong consensus sequences for CCAAT and TATAA elements.

As further evidence that the hormone responsive elements were localized within the region -612 to -100 (the Mst II site to the Kpn I restriction enzyme site), this fragment was tested in a CAT plasmid with a heterologous promoter. A plasmid was constructed containing this 5' region of the T-KG gene immediately upstream of the adenovirus major late promoter (pMLPAMst). Figure 4 illustrates the results obtained from a CAT assay of cell extracts derived from transfection of FAZA cells with this construct, in the absence and presence of hormone. Both dexamethasone and estrogen produced inductions of the transfected gene similar to that seen with the constructs containing the T-KG gene promoter. In addition, use of the control plasmid pRSVCAT in the presence or absence of either steroid hormone resulted in no induction indicating the effects seen with the T-KG and MLP constructs are specific.

Figure 2. Construction of plasmids with T-KG and CAT sequences.

Panel A. 5' Genomic organization of T-KG. A phage clone containing the 5' end of the T-KG gene is diagrammed. Exons 1 and 2 are indicated as solid boxes. The expanded drawing illustrates the largest fragment of the T-KG gene used in these studies. An arrow marks the transcription start site as determined by others (21,22). The positions of consensus GRE and ERE sequences are indicated by short lines. Relevant restriction enzyme sites for mapping the genomic clone or for use in deletion analysis are abbreviated as follows: R - Eco RI, H - Hin DIII, A - Alu I, Bgl - Bgl II, Tth - Tth-1 III, Mst - Mst II, Rsa - Rsa I, Kpn - Kpn I.

Panel B. Plasmid construct pTKGCAT. The diagram illustrates the relative positions of the T-KG genomic sequences (open box) and CAT gene (solid box) in the pIBI30 cloning vector (thin line).

Panel C. Structure of the T-KG/CAT deletion plasmids. The open boxes represent the T-KG 5' sequences while the solid box indicates the CAT gene. The thin line represents the pIBI30 vector sequences. The deletions were made from the 5' Hin DIII site to the endonuclease restriction sites indicated. pTKGRsa70 was prepared by reinserting a 70 bp Rsa I fragment into the ΔKpn construction. The endpoints of the T-KG regions are given relative to the transcription start site.

Figure 3. 5' Regions of the T-KG gene drive induced CAT expression in response to dexamethasone and estrogen. The T-KG/CAT plasmids described in figure 2 were introduced into FAZA cells by electroporation. These transformed cells were then incubated in the presence or absence of 1 μ M dexamethasone (Panel A) or 20nM estrogen (Panel B) for 36-48 hrs. Cell extracts were prepared and assayed for CAT activity. The products of this enzymatic reaction (chloramphenicol and its acetylated forms) were separated by thin layer chromatography and subjected to autoradiography for 2 days. The negative control is pTKG0, a derivative of pTKGCAT with the promoter region deleted. The plasmid pRSVCAT was used as a positive transfection control. Standard CAT enzyme (0.1 U) was included as a control for the enzyme assay.

The sequence defined by the Δ Mst construction, which is hormone responsive, contains four putative GREs and 5 possible EREs (Figure 6). In order to identify the sequences responsible for the hormonal induction of T-KG mRNA, another plasmid was prepared by reinserting an Rsa I fragment composed of 67 bp of the adjacent 5' sequence into the pTKGΔKpn vector. This region includes both a single GRE and ERE in the context of a 16 bp palindromic sequence. The construct was introduced into the FAZA cells and after treatment with glucocorticoid or estrogen, a CAT assay was performed

Figure 4. Effect of dexamethasone or estrogen treatment on constructs with a heterologous or an unregulated promoter. A CAT plasmid containing a 512 bp Mst II-Kpn I fragment of the T-KG gene located upstream of the adenovirus major late promoter was transfected into FAZA cells. The cells were treated with dexamethasone or 17 β -estradiol-3-benzoate. Cell extracts were prepared and the CAT activity was assayed as described. The control plasmid pRSVCAT was transfected separately into the FAZA cell line. Those transfectants were subjected to the same hormonal treatment to ensure there was not a nonspecific effect on CAT mRNA in this cell line.

on the cellular extracts as shown in Figure 5. With either steroid hormone, the addition of this 67 bp fragment containing a single GRE and ERE fully restored inducibility of the CAT gene.

Finally, an effort was made to quantitate the induction observed with the CAT activities. The areas corresponding to both unreacted and acetylated forms of chloramphenicol were cut out of the thin layer chromatography plates from several experiments and counted. The values were corrected for transfection efficiency by normalization with β -galactosidase activities obtained from cotransfection with the pCH110 plasmid for each cell extract. The resulting data is presented in Table 1 as percent conversion of the substrate to an acetylated product. The induction in CAT activity observed for the intact 1.69 kb 5' region is approximately 6-fold with dexamethasone treatment and 2-fold with 17 β -estradiol-3-benzoate. Transfection of the cells with the pTKGRsa70 construction containing the functional GRE and ERE results in the same level of induction of CAT activity with each hormone. These values correlate well with the induction observed in the endogenous T-KG expression. Thus, this small region of 219 bp of T-KG gene 5' sequence is sufficient to support the full hormonal induction of a reporter gene.

The nucleotide sequence of the 5' region of the T-KG gene included in the Δ Mst deletion is summarized in Figure 6. The sequence encompasses the Δ Kpn and Rsa70

Figure 5. Identification of a small fragment conferring hormone responsiveness. The Δ Kpn plasmid, which supported only basal transcription of the CAT gene, was modified by the addition of an adjacent 70 bp Rsa I fragment containing a single consensus sequence for each hormone responsive element. This new plasmid, the parental Δ Kpn, and the Δ Mst construct, which is fully responsive, were transfected into the FAZA cells. After treatment with dexamethasone (Panel A) or estrogen (Panel B), cell extracts were prepared and assayed for CAT activity as described. The control plasmids outlined in figure 3 were also used in this experiment.

constructions as indicated by the restriction endonuclease sites. The promoter element of T-KG is unusual in that there are no consensus CCAAT or TATAA boxes or SP1 binding sites. Rather, an A-rich region has been suggested as the TATAA equivalent from S1 nuclease protection and primer extension studies (21). The sequences which correspond to the core consensus sequence for a glucocorticoid regulatory element (CTTGT) and an estrogen regulatory element (GGTCA) are indicated. Two matches for the GRE are found in the pTKGΔKpn construction, yet they are inactive in supporting dexamethasone induction. The GRE and ERE present in the active 5' fragment are indicated. Additionally, a CACCC sequence is present forty base pairs upstream of the GRE. This pentanucleotide sequence has been implicated in other systems as acting cooperatively with a GRE to yield full induction in response to glucocorticoids (16).

Table 1. Quantitation of CAT activity from T-KG/CAT plasmids

	CAT ACTIVITY - % CONVERSION ^a					
	Dexamethasone		Fold Induction	Estrogen		Fold Induction
	-	+		-	+	
pTKGCAT	0.57 ± 0.20	3.92 ± 1.77	6.9	1.06 ± 0.41	2.54 ± 0.12	2.4
pTKGΔMst	0.50 ± 0.13	2.76 ± 0.25	5.5	0.85 ± 0.31	1.20 ± 0.46	1.4
pTKGΔKpn	0.24 ± 0.11	0.19 ± 0.01	0.8	0.21 ± 0.03	0.19 ± 0.04	0.9
pTKGRsa70	0.56 ± 0.07	4.10 ± 1.41	7.3	0.66 ± 0.34	2.11 ± 0.44	3.2
pRSVCAT	75.9 ± 2.9	81.1 ± 6.4	1.1	75.9 ± 2.9	73.2 ± 5.8	1.0
pTKGO	0.09 ± 0.02	N/D ^b		0.11 ± 0.02	N/D ^b	

^aEach percentage conversion value has been normalized for transfection efficiency using data obtained for β-galactosidase activity derived from the co-transfected plasmid, pCH110, and represents the mean of at least two experiments. Within single experiments, the standard error was never more than 20%.

^bN/D, not determined.

DISCUSSION

We have been studying the regulated expression of one of the acute phase genes in rats, T-kininogen. A number of acute phase reactants exhibit induced expression in response to the cytokines and hormones produced during inflammation. Increased levels of the protein product of T-KG have also been observed in female versus male rats. This investigation was therefore aimed at characterizing the effect of glucocorticoid and estrogen on T-KG expression.

In a rat hepatoma cell line (FAZA), the mRNA for T-KG is increased 5-fold after treatment with glucocorticoid and 2-fold after estrogen treatment. Using 5' regions of the T-KG gene fused to a CAT gene and introduced into the FAZA cells by electroporation, we demonstrated that both hormonal responses could be reproduced with this exogenous gene construct. Through deletion analysis, we have identified a small fragment from the 5' end of the T-KG gene which is capable of supporting the hormonal induction of a reporter gene. This 219 bp region extends from -167 to +52 and contains consensus sequences for a GRE and ERE. In addition, these elements fall within a region capable of conferring a similar level of hormonal responsiveness on a heterologous promoter. Examination of the nucleotide sequence of the entire 1.69 kb 5' flanking region revealed multiple GRE and ERE consensus sequences. The deletion constructs indicated, however, that most of these could be eliminated while still maintaining hormonal responsiveness. For each hormone, however, when one considers the expanded consensus sequences for the steroid receptor binding sites, the elements

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CCTGAGGCTAGTGAGGCGAGCACTTCATTAGGTGAGAACCTGCTGGTTCGCTCCTTCTCTCA
-547
Mst II

TGGGCTAATAGAACTGAGCAGAGAGGCTGGCATGGCCAGAGGCCAGACAGGATGCCACAGGTTT
-482

GTCAGATGTCCTCAGTCTCCTGGGTGGAGCTCCAGCCCTTGGCTGAATGTCCTGCACTTGTCTGA
-417

TTTCAGGAACGCACCAGAGCTGACAAAACCTGTGTCTGTCTGTATCTGTACACCTGACGTG
-352

CCATGAGCAGTGATGTGTGGGAAGAGGTCTTGTACATTGCTCTCATTTGTTTACGTATGCAGACA
-287

CCTTTGTATCTGGGCCAGGAGTAGTAAGATGATCTGGGAAATGTTGTTTAGCATTACTG
-222

GTTTCTGCTGCAATTCCTGGGGAGAGTTGGGCAATGTTTGGGAATGGCGTGTACTCCACA
-157
Rsa I

TTCTTTCAAAGGCAACATCAAGTCACCAACCTTGTGTGCAATGCCAGCTGGGTACTGCT
-92
Kpn I
Rsa I

TGGACGTGGAGCAGAGTCTCTGTTTGTCTGAGAAGAGGGCAACCAAGGGTGACTCAGGAAT
-27

CTAAAAGGGACCAGAGTGGCGAGACAGAGAGCATAGGCAGGAGAAGTCAGAGACTGGAGGCTG
+1
+38

CTAAAAGCAGCAGCAT
A1u I

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Figure 6. Nucleotide sequence of the 5' region of T-KG. The sequence included in the Δ Mst construct is presented. The transcription start site is marked by an arrow. Putative GREs and EREs, based on similarity to the core consensus sequences, are boxed. The elements present in the fragment conferring responsiveness to dexamethasone and estrogen are double boxed. The palindromic sequence in which these elements appear is indicated by tail-to-tail arrows. The pentanucleotide CACCC sequences underlined have also been implicated in mediating glucocorticoid induction.

contained in the smallest fragment which is functional in the CAT assays exhibit the highest degree of nucleotide identity with their respective sequences. For the GRE, this represents a 12/16 match to the GRE consensus sequence (C/T)CTTGTNN(A/C)AC(A/T)TGG (14); the ERE consensus, AGGTCANNNTGACCT (15), matches 12/15 bases with the T-KG sequence. Presently, we have not tested the other 9 putative EREs and the 7 remaining GREs for responsiveness to their respective hormones. It is possible that some of these could also confer a measure of hormone responsiveness in the T-KG gene. Our data, however, indicates that the region we have identified is sufficient for the full induction observed with glucocorticoid and estrogen.

A very interesting aspect of these putative elements in the 219 bp fragment is the

context in which they appear. Both hormonal response elements occur within 7 bp of each other. This arrangement of close, nonoverlapping steroid hormone receptor binding sites has been seen in other genes induced by either glucocorticoid and progesterone (23,24) or, less commonly, by glucocorticoid and estrogen (25). In addition to the close proximity of the GRE and ERE, the intervening sequence is also of interest since it constitutes a 16 bp palindrome. The consensus sequences for these hormone responsive elements are themselves palindromes and while mutational analysis has demonstrated this feature is not absolutely required (26), sequences of this type have been implicated as possible protein binding domains due to the symmetry in the DNA structure. Thus, although mismatches destroy the consensus palindromes in each regulatory element, the GRE and ERE in the T-KG gene are incorporated into a unique palindromic sequence.

Another notable sequence feature is present in the region immediately surrounding the GRE consensus element in the rat T-KG gene. Approximately 40 bp upstream of the GRE there is a CACCC sequence. Recently, other workers identified this sequence as an integral part of the glucocorticoid responsive element for the tryptophan oxygenase gene, and demonstrated binding of a nuclear factor not related to the hormone receptor complex (16). They note that the sequence also occurs upstream of a number of other active GREs (e.g human growth factor, rat tyrosine aminotransferase, MMTV). It is possible, therefore, that there is a requirement for binding of an additional factor with the receptor-hormone complex in order to elicit full dexamethasone induction of some genes. Further investigations are necessary to determine the importance of this region in T-KG gene expression.

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