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# Regulation of mouse somatostatin receptor type 2 gene expression by glucocorticoids

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**Abstract Somatostatin is a regulatory peptide with important inhibitory functions. Its effects are mediated by five receptors, sst1–sst5. Previous studies revealed that sst2 contains three alternative, tissue specific promoters. Here, molecular mechanisms for the regulation of sst2 transcription by glucocorticoids were investigated. Reporter gene assays in NG 108-15 cells and electrophoretic mobility shift assays revealed that a glucocorticoid responsive element at position −1044 on the second promoter mediates dexamethasone induction. These findings, the existence of a cAMP response element and the tissue dependent activity (brain, pituitary and gastrointestinal tissues) indicate the importance of the second sst2 gene promoter.**



*Key words:* Somatostatin receptor type 2; Transcription regulation; Promoter; Glucocorticoid; Glucocorticoid responsive element

## 1. Introduction

Somatostatin acts as a potent inhibitor of endocrine and exocrine secretions and neuronal functions and is involved in various regulatory circuits in the central nervous system and peripheral tissues. For example, somatostatin plays important roles in the pituitary as the physiological counterpart of growth hormone releasing factor and within the gastrointestinal tract. There, it inhibits the release of many intestinal peptides and pancreatic hormones and enzymes (reviewed in [1]). In addition, stable analogs of somatostatin are used clinically to treat tumors, because also growth of cancer cells is inhibited by somatostatin action [2,3]. All these effects are mediated by five different somatostatin receptors (sst1–sst5) [4–13]. In our laboratory we are focussing on the investigation of the structure and the regulation of the sst2 gene (see Fig. 1). We have previously shown that this receptor subtype is alternatively spliced at the 3′ end resulting in the generation of two isoforms (sst2A and sst2B) which differ within their C-termini and exhibit different pharmacological properties [13,14]. Furthermore, it was demonstrated that transcription of the sst2 gene is initiated from three alternative promoters which are active in a tissue and cell dependent manner [15]. Gene regulation studies revealed that these promoters are not equally responsive to extracellular stimuli, as for instance to agents that elevate the intracellular cAMP level [16]. Increasing knowledge of such regulatory mechanisms may be of more than academic interest since an adequate level of sst receptor expression in cancer tissue is a prerequisite for an effective medical treatment with somatostatin analogs.

It is well known that glucocorticoids (GC) are involved in the regulation and maintenance of physiological functions in many systems. Among other functions, GC are also involved in the fine-regulation of somatostatin actions. It has been reported that GC up- and downregulate gene expression of the somatostatin gene itself [17,18], as well as somatostatin receptor genes [19–21]. For example, it was demonstrated that the number of sst receptors in certain brain areas increased in response to GC [20]. At a cellular level it was shown that mRNA levels of different sst subtypes as well increase as decrease in pituitary $GH_4C_1$ cells in a time dependent way [21]. Glucocorticoids are known to modulate gene expression via an intracellular glucocorticoid receptor (GCR). This, when activated, may function as a transcription factor enhancing transcription or it may be directly involved in the inhibition of transcriptional activity (reviewed in [22,23]). This study reports attempts to reveal the molecular basis for the transcriptional activation of the sst2 gene by glucocorticoids.

## 2. Materials and methods

### 2.1. sst2 promoter constructs

The construction of the sst2 promoter-chloramphenicol acetyl transferase reporter gene plasmids using the reporter gene vector plasmid system pBLCAT2/pBLCAT3 [24] is described in detail elsewhere [16]. In addition to those plasmids, two reporter genes with internal deletions of the second sst2 promoter were constructed as follows: construct P2-1137 was first cut at two internal PstI sites at nt −555 and −593 and then digested with Bal31 enzyme. This digest was stopped at different time points and the remaining plasmid was religated leading to the internal deletion constructs P2-1137del-968-232 and P2-1137del-1049-114. To yield construct G4.tk.CAT an oligonucleotide (5′-GTTTTCTGATTTTTCATGTTCTAGAAACC-3′) was inserted into the blunted *Bam*HI site of pBLCAT2. All reporter plasmids were sequenced after construction.

### 2.2. Cell culture, transfections and CAT assays

NG 108-15 cells were cultured at 37°C with 10% $CO_2$ in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin with additional 0.1 mM hypoxanthin, 10 μM aminopterin and 17 μM thymidine. For transfections $10^6$ cells were plated on 5 cm tissue culture dishes and allowed to grow overnight. Medium was replaced and cells were transfected with 8 μg plasmid DNA using the method described by Chen and Okayama [25]. After 16–18 h medium was renewed again and cells were allowed to grow for 2 days in the presence of 1 μM dexamethasone or vehicle (ethanol) before harvesting. Harvesting and CAT ELISA were performed according to the protocol of the CAT ELISA kit from Boehringer Mannheim.

### 2.3. Extraction of nuclear protein

The nuclear protein extraction procedure of NG 108-15 cells for

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EMSA has been described in detail in a recent publication from this laboratory [16].

*2.4. Electrophoretic mobility shift assay*

Synthetic oligonucleotides (Metabion, Martinsried) carrying putative GCR binding sites G1 to G5 were labeled with [γ-32P]ATP (Amersham, Braunschweig) according to standard methods [26]. For each band shift reaction 5000 cpm labeled probe DNA was incubated with 3 μl NG 108-15 cell nuclear extract for 60 min on ice in a 20 μl reaction mixture containing 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 1 μg poly(dIdC) DNA. Some reactions also contained various amounts of competitor DNA. For immunoshift experiments nuclear extract was preincubated with 3 μg GCR antibody ('P-20', Santa Cruz Biotechnology, Heidelberg). Samples were loaded onto 6% polyacrylamide gels and separated electrophoretically. The gels were then dried and exposed to Kodak XO-MAT AR films. Sequences of the oligonucleotides were: classical GRE: 5′-AGGATCTGTACAGGATGTTCTAGATCG-3′, G1: 5′-CACACAGACACGTGTGTGCTTACTC-3′, G2: 5′-CCCCCAGAGGAGACTGTACCTTCGCA-3′, G3: 5′-CTGCCACGGCTGACCTGTCCACCAGGT-3′, G4: 5′-GTTTTCTGATTTTTCATGTTCTAGAAACC-3′, G5: 5′-CTGACTCTGCAACCTGTGCCCTGTTCCCCG-3′.

*2.5. Statistical analysis*

For statistical evaluation student's *t*-tests were performed. Asterisks (*) indicate significant different values ($P < 0.05$).

## 3. Results

*3.1. The second sst2 promoter may be stimulated by glucocorticoids*

We have recently published the structure of the sst2 gene upstream regulatory region and sequences of the three sst2 promoters [15]. Searching for putative binding sites for the glucocorticoid receptor (GCR), five DNA stretches, termed G1–G5, were found within these sequences. Requirements were that these sequences (a) share at least a homology of 60% with the classical GRE consensus sequence and (b) contain the core sequence 5′-TGTNC-3′ at the 3′ half of the imperfect palindromic classical GRE. The T residue at the 12th position distinguishes GRE elements from similar putative estrogen receptor binding sites [22,23]. The putative sst2 GRE elements and the classical GRE sequence are shown in Table 1. To investigate a possible role of these elements in activation of transcription of the sst2 gene in response to GC transfection experiments were carried out (Fig. 2). For this purpose various chloramphenicol acetyl transferase reporter gene constructs containing parts of the three sst2 promoters were made. These constructs were tested in transiently transfected NG 108-15 cells (a neuroblastoma×glioma hybrid cell line) for their ability to respond to dexamethasone treatment. As controls reporter plasmids containing the thymidine kinase promoter of the herpes simplex virus (pBLCAT2 [24]) were used. The basal transcriptional rate of these constructs in NG 108-15 cells has been already described elsewhere [16]. Construct P1-425 which carries sequences of the first sst2 promoter (lane 1) did not confer dexamethasone responsiveness

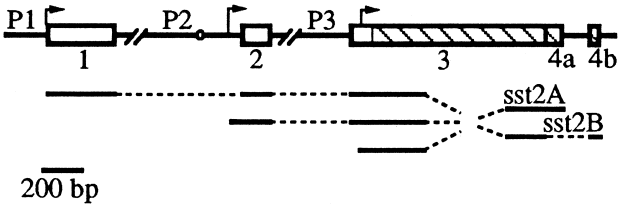


Fig. 1. Structure of the mouse somatostatin receptor type 2 gene. The schematic drawing of the genomic organization (top) shows intronic sequences as lines and exons as boxes. The protein coding sequence is hatched. The first two introns are longer than 25 kb. The gene consists of four exons, two of which are located within the untranslated upstream region of the gene. The fourth exon is divided in two parts (4a and 4b), which are alternatively spliced. The sst2 gene contains three promoters (P1–P3); the transcription initiation sites are indicated as arrows. The second promoter contains a cyclic AMP response element (CRE) shown as a small box. Below: Six different mRNA transcripts are possible for sst2. Transcripts may derive from either of the three promoters and each transcript may contain exon 4a or 4b. The latter alternative leads to the generation of two receptor protein variants (sst2A and sst2B) with different carboxy-terminal tails.

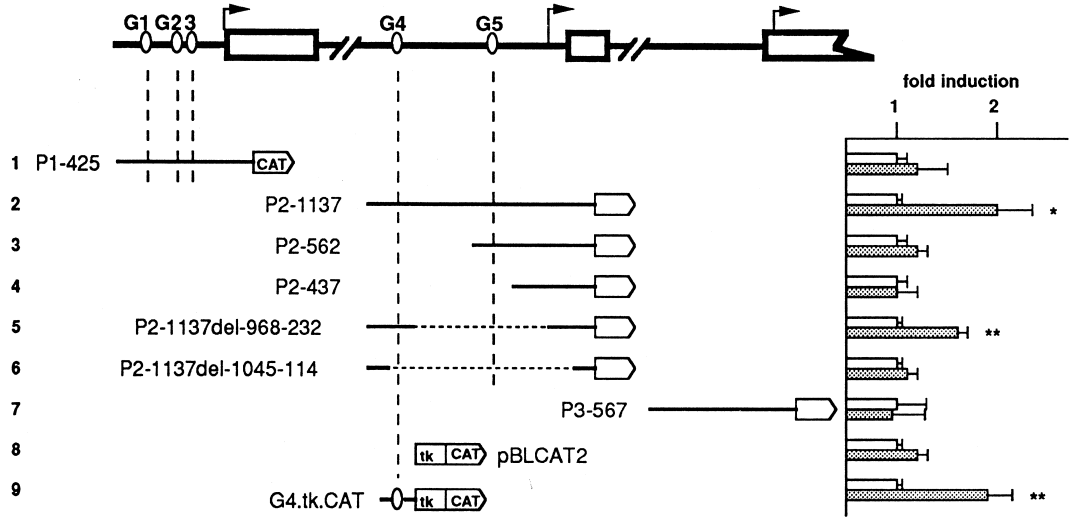


Fig. 2. Transient expression of reporter gene constructs in NG 108-15 cells. On top, a schematic drawing shows the three sst2 promoters with the relative location of the five putative GC responsive elements G1 to G5 (open ovals). Exons are shown as boxes and transcriptional start sites as arrows. Below, the results of the transfection experiments are shown. Left, the reporter gene plasmids with sst2 promoter sequences are indicated relative to their position within the gene. Right, CAT activities for the respective constructs are expressed as fold induction for dexamethasone (1 μM) treated transfected cells (grey bars) compared to vehicle treated transfected cells (white bars).

although it contains three out of the five putative GRE sequences of the gene (G1, G2, G3). The longest construct with sequences of the second promoter, which contains the remaining two putative GREs, G4 and G5, showed a significant 2.0-fold stimulation after dexamethasone treatment of transfected cells (construct P2-1137, lane 2). Deletions of promoter 2 sequences up to nt −562 (construct P2-562, lane 3) and −437 (construct P2-437, lane 4) both abolished the stimulatory effect. Since construct P2-562 lacks G4 but still contains G5, it is suggested that G4 may mediate the dexamethasone effect. To test this hypothesis we constructed two additional reporter plasmids with internal deletions which contain G4 or do not. The G4 containing construct showed a significantly 1.6-fold dexamethasone stimulated reporter gene expression (construct P2-1137del-968-232, lane 5), whereas the deletion construct which lacks G4 showed no stimulation (construct P2-1137del-1045-114, lane 6). The sequences of the third sst2 promoter tested in this study were not responsive to dexamethasone (construct P3-567, lane 7). To test if the G4 sequences alone are sufficient to mediate dexamethasone induced reporter gene expression a 29-mer oligonucleotide containing G4 and adjacent sequences was cloned in the pBLCAT2 plasmid upstream of the tk promoter (construct G4.tk.CAT, lane 9). Transfection experiments showed a significant 1.9-fold stimulation with this reporter plasmid compared with vehicle treated controls.

Table 1
Putative glucocorticoid response elements on the sst2 gene promoters

| Putative element | sst2 promoter | Position | Sequence |
|---|---|---|---|
| G1 | 1 | −378 | 5′-AGACACGTGTGTGCT-3′ |
| G2 | 1 | −303 | 5′-AGAGCAGACTGTACC-3′ |
| G3 | 1 | −262 | 5′-CGGCTGACCTGTCCA-3′ |
| G4 | 2 | −1044 | 5′-GATTTTTCATGTTCT-3′ |
| G5 | 2 | −488 | 5′-TCTGCAACCTGTGCC-3′ |
| GRE consensus | | | 5′-GGTACANNNTGTYCT-3′ |

The sequences of the five elements G1–G5 which are similar to the classical glucocorticoid response element (GRE) are shown along with their location with respect to the transcription initiation site of the respective promoter.

### 3.2. Glucocorticoid receptor binds to a GRE within the second sst2 promoter

The five putative GRE elements of the sst2 gene were then subjected to electrophoretic mobility shift assays to investigate binding of nuclear proteins (Fig. 3). Incubation of a radioactively labeled classical GRE oligonucleotide with NG 108-15 nuclear extract (Fig. 3A, lane 1) resulted in a typical pattern of several shifted bands: a double band on top of the gel (A), two faster migrating bands (B and C) and additionally two unspecific (see below) bands (U). In the following the specificity of the shifted complexes was studied using various non-labeled competitor oligonucleotides. Using classical GRE

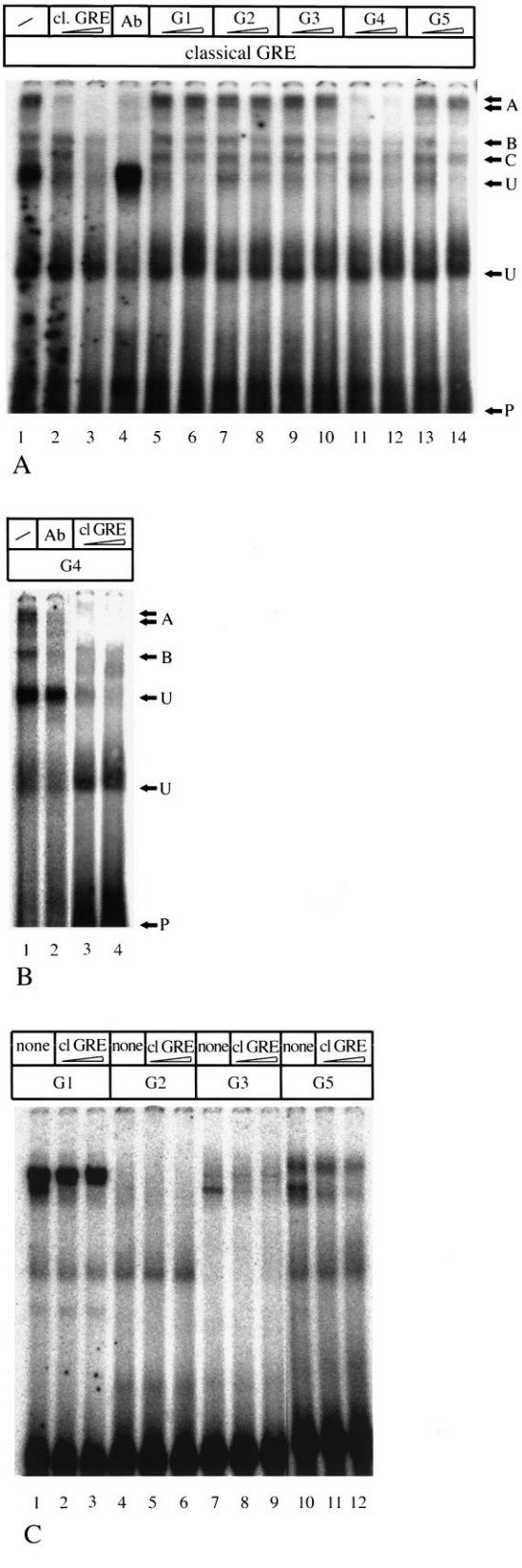


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Fig. 3. Electrophoretic mobility shift assays of the putative sst2 GRE motifs. The tables above the autoradiograms give the composition of the reactions: the lower panel designates the probe that was used in the experiment and the upper panel shows which competitor DNA or antibody was used. Abbreviations are: cl. GRE, oligonucleotide with consensus sequence of a classical GRE; Ab, antibody against GCR protein; G1 to G5, oligonucleotides with sequences of the putative sst2 elements. Competitor DNA was used in 5- and 25-fold molar excess as indicated schematically. Nuclear extract was isolated from NG 108-15 cells. The arrows mark the position of specific (A, B, C) and unspecific (U) complexes and the free probes (P).

competitor the intensity of specific bands decreased (lanes 2 and 3). Similar, immunoprecipitation of GCR protein prior to the bandshift reaction by preincubation of nuclear extract with a specific antibody resulted in a drastic decrease in intensity of the specifically shifted complexes (lane 4). When G1–G5 oligonucleotides were used to compete for the classical GRE probe (lanes 5 to 14) only G4 oligonucleotides were as effective as the classical GRE competitor (lanes 11 and 12, compare lanes 2 and 3). Fig. 3B shows the bandshift analysis of the G4 oligonucleotide. Incubation of G4 with nuclear extracts resulted essentially in the same shift pattern as the classical GRE with the exception that band C was only very weak (lane 1). Bands A and B were also recognized by the glucocorticoid receptor antibody, indicating that G4 binds the GCR (lane 2). This is supported by the fact that classical GRE oligonucleotides efficiently competed for binding to G4 (lanes 3 and 4). When using G1–G3 and G5 oligonucleotides as probes (Fig. 3C) either shift patterns that were different from the classical GRE pattern appeared for G1 (lanes 1 to 3), G3 (lanes 7 to 9) and G5 (lanes 10 to 12) or no binding at all was observed as for G2 (lanes 4 to 6). Furthermore, the shifted bands of G1, G3 and G5 could only negligible be competed by the classical GRE sequences, indicating that other proteins than GCR bind to these sequences. Bands U are regarded as unspecific since they are either competed by all oligonucleotides in higher concentrations (the upper one) or there is no apparent competition at all (the lower one).

## 4. Discussion

The somatostatin receptor 2 gene is regulated by glucocorticoids in vivo, however, the molecular mechanisms underlying this process are still largely unknown. Thus, in an early study it was shown that adrenalectomy in rats causes a decrease of somatostatin receptors in certain brain areas, which could be normalized again by GC replacement [20]. In another study the effect of the synthetic GC dexamethasone on sst2 mRNA levels in the pituitary cell line $GH_4C_1$ was presented earlier by Xu and colleagues [21]. Using mRNA hybridization techniques the authors showed that dexamethasone first, measured after 2 h, leads to an about 1.6-fold increase in sst2 mRNA levels, which was followed by a decrease in mRNA levels within 24 h. Measured at 48 h sst2 mRNA levels showed a tendency to increase again. Nuclear run-on assays and experiments in which the half life of sst2 mRNA was measured lead the authors to the conclusion that the effects of dexamethasone are mainly due to changes in the transcription rate of the sst2 gene and not to changes in mRNA stability.

In general, regulation of gene expression by GC involves various mechanisms which may lead to either activation or inhibition of target gene transcription [22,23]. Inhibition of transcription includes mechanisms such as (a) direct protein-protein interactions between GCR and stimulatory transcription factors, (b) competitive binding of GCR and stimulatory factors to regulatory DNA elements and (c) interaction of GCR with negative regulatory DNA elements within gene promoters. Stimulation of gene expression by GC is known to be mediated via cis-acting promoter elements termed glucocorticoid response elements (GREs). These are conserved binding sites for the hormone activated GCR, which when bound acts as a factor enhancing transcription [27]. A complex regulation process as it is found for the sst2 gene and GC, which consists of several phases with increasing as well as decreasing mRNA levels, may result from combined inhibitory and stimulatory mechanisms. In this report we present molecular mechanisms which can explain the GC induced increase in sst2 mRNA levels. In particular, promoter sequences were identified, which might serve as functional GRE sequences. Out of five potential GRE sequences, which are found on the three sst2 promoters (G1–G5), one, namely G4, was shown to constitute a functional GRE. This element is located on the second promoter at position −1044 with respect to its transcription initiation site. It is also active in front of the heterologous viral herpes simplex thymidine kinase promoter, where a 29-mer oligonucleotide containing G4 and adjacent sequences was sufficient to confer GC stimulation in transiently transfected NG 108-15 cells. Similar to the increase in sst2 mRNA in $GH_4C_1$ cells discussed above an induction rate of 1.6- to 2-fold was observed in our transfection experiments with the sst2 promoter reporter constructs. Gel retardation experiments and immunoshift experiments revealed almost identical nuclear protein binding patterns for G4 and a classical GRE. Taken together, the results presented here convincingly indicate that the sst2 gene contains a functional GRE on its second promoter. The other two sst2 promoters did not respond to GC and do not contain GRE sequences. The putative GREs G1–G3 and G5 displayed completely different nuclear protein binding properties in the gel retardation experiments compared to the classical GRE and G4.

Astonishingly, incubation of transfected cells with dexamethasone did lead in no case to an inhibition of reporter gene expression. If mainly transcriptional changes should be responsible also for the decrease of sst2 mRNA, as suggested by Xu and colleagues [21], it must be assumed that other promoter sequences are responsible. These may either be located further upstream of the sequences isolated so far, or on an additional promoter.

The existence of a functional GRE on the second sst2 promoter emphasizes its importance for the transcriptional regulation of the gene. Thus, the second promoter also contains a functional cyclic AMP responsive element, whereas the first and the third do not [16]. Furthermore, the second promoter is active in those tissues in which somatostatin has key functions (brain, pituitary and gastrointestinal tissues) [15]. The use of alternative promoters is being recognized as a mechanism creating flexibility and diversity in gene expression in a growing number of genes (for a review see [28]). Alternative transcripts may lead to protein variants of the same gene, as it is the case for the sst2 gene within the carboxy-terminal region [13]. Furthermore, alternative transcripts of one gene may be active in different tissues [29], or responsive in different ways to extracellular and hormonal stimuli [30]. These two possibilities are also realized in the sst2 gene. This has the consequence that a regulatory stimulus is only effective in these cells and tissues that use the promoter equipped with the regulatory elements. Interestingly, it was found by Rodriguez et al. [20] that a GC regulation of somatostatin receptors occurred only in certain brain areas although they are expressed in all of the areas which were studied. Alternative promoter usage and thus alternative responsiveness may be an explanation for this. Therefore, both knowledge of tissue and cell specific promoter usage and localization of regulatory elements is

needed to understand the complexities of sst2 gene expression. More important than just understanding sst2 gene regulation may be the possibility to use stimulatory regulators in order to increase the number of sst2 receptors on certain tumor cells. This could have an additive effect to that of stable somatostatin analogs, which already are used clinically to treat certain tumors.

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