Glucocorticoids activate somatostatin gene transcription through co-operative interaction with the cyclic AMP signalling pathway

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The somatostatin (SS) gene is transcriptionally regulated via the cyclic AMP (cAMP) response element (CRE), located in the proximal promoter (−41 to −48 bp). We have previously reported that glucocorticoids induce dose-dependent cell-specific alterations in the steady-state SS mRNA level. Here we have investigated direct transcriptional control of the SS gene by glucocorticoids. We have examined transcriptional interaction between glucocorticoids and the cAMP signalling pathway and mapped the 5′ upstream regulatory region of the SS gene involved in glucocorticoid transactivation. Transcriptional regulation was determined by analysis of chloramphenicol acetyltransferase (CAT) activity in PC12 rat pheochromocytoma cells and A126-1B2 (protein kinase A-deficient mutant PC12) cells, by acute transfection of 5′ flanking SS DNA (−750, −250 and −71 bp) ligated to the reporter (CAT) gene. Dexamethasone (DEX) induced a dose-dependent 2.2-fold stimulation of SS gene transcription in PC12 cells, but not in A126-1B2 cells. Other steroid and thyroid hormones tested, and retinoic acid, were ineffective, while cAMP and forskolin stimulated gene transcription 4–5-fold in PC12 cells but not in A126-1B2 cells. DEX exerted an additive effect on cAMP-induced gene transcription. Deletion of the promoter from −750 to −71 bp (but not from −750 to −250 bp) abolished all stimulatory effects of DEX without affecting cAMP responsiveness. Mutation of the CRE abrogated both DEX- and cAMP-dependent gene enhancement. Gel electrophoretic mobility shift assays confirmed that the −250 to −71 bp region of the SS promoter (but not the −71 to +55 bp domain) binds specifically to a glucocorticoid response element-sensitive nuclear protein(s) from PC12 cells, suggesting a putative glucocorticoid receptor interaction with SS promoter DNA. We conclude that glucocorticoids regulate SS gene transcription positively. Glucocorticoid-induced transactivation shows dependence on protein kinase A activity, and may be mediated via protein–protein interaction between the glucocorticoid receptor and the CRE binding protein. DNA sequences upstream from the CRE between −250 and −71 bp in the SS promoter appear to be the target of glucocorticoid action.

INTRODUCTION

Somatostatin (SS) synthesis and gene expression is modulated by a number of prominent agents and mediators such as cyclic AMP (cAMP), oestrogens, testosterone, interleukin-1, insulin, growth hormone and *N*-methyl-D-aspartate receptor agonists [1–11]. In mammals, synthesis of SS is directed by a single gene which contains two known regulatory elements in its proximal promoter region: a variant of the TATA box 26 bp upstream from the start of transcription, and an octameric enhancer sequence, the cAMP response element (CRE), located further upstream between nucleotides −48 and −41 [3,12,13]. In addition, a SS gene upstream enhancer element (SMS-UE) has recently been identified in the more distal promoter between nucleotides −113 and −83 which confers islet D-cell-specific gene expression [14,15]. The promoter region does not contain canonical steroid or thyroid hormone response elements. Activation of the adenylate cyclase/cAMP pathway plays an important role in stimulating both secretion and gene transcription of SS [3–6]. cAMP activates transcription of SS and other cellular genes through the CRE, which binds the nuclear protein CREB (CRE binding protein); the transcriptional efficacy of CREB is regulated through phosphorylation by the cAMP-dependent protein kinase A [4,5]. Several other transcriptional factors related to CREB and capable of interacting with the CRE have also been identified, e.g. ATF-1, CRE-BP1/ATF-2, CREM, ATF-a, and so on [14]. The SMS-UE region represents a tripartite *cis* regulatory unit which is activated by a protein complex consisting of CREB and islet-cell-specific and α-CAAT binding factor-like transcription factors, and which is in turn synergistically coupled to the CRE located in the proximal promoter [15–17]. It is not known whether agents such as gonadal steroids, interleukin-1, insulin, growth hormone and *N*-methyl-D-aspartate receptor agonists, which modulate SS mRNA levels, act transcriptionally, and if so, what the intracellular signalling mechanism is.

We have previously demonstrated that dexamethasone (DEX) induces tissue-specific time- and dose-dependent alterations in SS peptide production and mRNA accumulation in normal SS-producing tissues *in vivo* and *in vitro* [18]. In addition, DEX produced biphasic patterns of change in SS secretion and steady-state mRNA levels in a rat SS-producing islet tumour cell line ($1027B_2$), with dose-dependent stimulatory and inhibitory components [18]. This suggests a complex molecular mechanism of glucocorticoid action on the SS gene, involving regulation at various levels. In the present study we have examined the transcriptional control of the SS gene by glucocorticoid as one putative level of control. Using the chloramphenicol acetyl-

Abbreviations used: CAT, chloramphenicol acetyltransferase; cAMP, cyclic AMP; CRE, cAMP response element; CREB, CRE binding protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GR-DBD-(440–525), glucocorticoid receptor DNA binding protein (residues 440–525); GRE, glucocorticoid response element; DEX, dexamethasone; MT-CREB, metallothionin-CREB; PEPCK, phosphenolpyruvate carboxykinase; SMS-UE, somatostatin gene upstream enhancer element; SS, somatastatin.

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transferase (CAT) transcription assay, we have compared the effects of glucocorticoids with those of other steroid and thyroid hormones, and investigated transcriptional interactions between glucocorticoids and the cAMP signalling pathway. We demonstrate that glucocorticoids regulate SS gene transcription positively through a co-operative interaction with the cAMP signalling pathway, and that DNA sequences upstream from the CRE in the SS gene are necessary for this effect.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), Phenol Red-free DMEM, heat-inactivated fetal bovine serum (FBS) and horse serum, L-glutamine and other tissue culture reagents were purchased from Gibco. 8-(4-Chlorophenylthio)-cAMP, phorbol 12,13-dibutyrate, forskolin, DEX, β-oestradiol, testosterone, 3,3',5-tri-iodo-L-thyronine (T_3) and retinoic acid were from Sigma Chemical Co., St. Louis, MO, U.S.A. Restriction endonucleases and other nucleic acid modifying enzymes were from BRL, Bethesda, MD, U.S.A. Oligonucleotides of CRE and glucocorticoid response element (GRE) consensus sequences were from Promega. Mermaid and GeneClean oligo DNA purification kits were from BIO101. D-*threo*-[*dichloroacetyl*-1-^{14}C]Chloramphenicol, [1,2,4,6,7-^{3}H]dexamethasone, [α-^{32}P]dCTP and [γ-^{32}P]dATP were purchased from Amersham, Arlington Heights, IL, U.S.A. Purified bacterially expressed glucocorticoid receptor DNA-binding domain [GR-DBD-(440–525)] was from Dr. L. P. Freeman (Sloan–Kettering Institute, New York, NY, U.S.A.) [19]. All other biochemicals were of the highest quality available commercially.

Cell culture and transfections

PC12 rat pheochromocytoma tumour cells [20] and A126-1B2 mutant PC12 cells deficient in cAMP-dependent protein kinase A [21] were obtained from Dr. J. A. Wagner, Dana Farber Cancer Institute, Boston, MA, U.S.A. Both cell lines were cultured in DMEM with 10% FBS, 5% horse serum and 27.8 mM glucose. In all experiments involving culture of cells with steroid hormones, transfected cells were incubated in medium that was Phenol Red-free, supplemented with FBS and horse serum rendered hormone-free by treatment with dextran-coated charcoal.

For transient transfection experiments, 2.5×10^6 cells were plated in 10 cm-diam. plastic Petri dishes. After 48 h of culture, when they had reached ~ 70% confluency, they were transfected by calcium phosphate precipitation followed by glycerol shock [22]. A 20–40 μg portion of plasmid DNA was mixed in 125 mM $CaCl_2$ and 25 mM Hepes-buffered saline, pH 7.05, at room temperature for 30 min and subsequently incubated with the cells for 4–6 h. The medium was then discarded and cells were exposed to 15% glycerol for 3 min, washed with PBS and reincubated with 10 ml of fresh culture medium. Test agents were added 24 h after transfection and the cells were harvested by scraping 48 h later.

CAT assay

Cell were washed twice with PBS, harvested in 1 ml of 40 mM Tris (pH 7.4), 150 mM NaCl and 1 mM EDTA, and collected by low-speed centrifugation. Extracts were prepared in 150 μl of 250 mM Tris (pH 7.8) by three cycles of freezing and thawing in a solid CO_2/ethanol bath and were cleared by centrifugation at 10000 *g* at 4 °C for 10 min. Aliquots of the supernatant were assayed for protein concentration by the method of Bradford, with BSA as standard [23]. CAT assays were performed at 37 °C in a total volume of 150 μl of 250 mM Tris (pH 7.8), 0.53 mM acetyl-CoA (Pharmacia) and 0.83 μCi/ml [^{14}C]chloramphenicol (final concentrations) [22]. Under these conditions, the assay was linear for up to 8 h and with up to 300 μg of extract. Routinely, 100 μg of cell lysate protein was incubated for 3.5 h at 37 °C. The reaction mixture was then extracted by ethyl acetate and subjected to t.l.c. followed by autoradiography. CAT activity was quantified by liquid scintillation counting of excised bands containing either the acetylated or non-acetylated forms of [^{14}C]chloramphenicol, and was expressed as a percentage of [^{14}C]chloramphenicol converted to the acetylated forms. All experiments were repeated at least three times.

The plasmid pCH110 (Pharmacia) was routinely co-transfected with various CAT constructs and used as an internal control for monitoring transfecting efficiency [24]. It expresses constitutively the *lacZ* (β-galactosidase) gene driven by the Simian virus 40 early promoter. Treatment with test agents did not produce any significant variation in β-galactosidase activity in any experiment.

Plasmid constructions

The metallothionein-CREB (MT-CREB) expression vector has been described previously [25]. CREB expression was induced by exposure of MT-CREB-transfected cells to $ZnSO_4$ (90 μM). The chimaeric gene constructs pSS-750CAT and pSS-71CAT (containing SS gene sequences from positions −750 to +55 bp and −71 to +55 bp respectively ligated to the bacterial reporter gene encoding CAT) [3] were obtained from M. R. Montminy (Salk Institute, La Jolla, CA, U.S.A.). pSS-250CAT was generated by inserting a *Sal*I fragment (−250 to +55 bp) of the −750 SS promoter into a pUC19-derived pCAT-Basic vector (Promega). A CRE substitution mutation in the −250 bp promoter (Δ-CRE pSS-250CAT) was generated by replacing the octameric CRE (TGACGTCA) with a non-related sequence of equal size (GCGTAGTC) by a two-step PCR procedure for gene splicing by overlap extension [26]. Oligonucleotide primers (38-mer) were designed with the mutant sequences flanked by 15 nucleotides of wild-type sequence at either side. A −250 to +55 bp SS promoter fragment with the CRE substitution mutation was amplified by using as template the SS −250 to +55 bp construct in the Bluescript plasmid (Stratagene). The mutant fragment was inserted into pCAT-Basic. All constructs and insert orientations were confirmed by restriction mapping and/or sequence analysis using the method of Sanger et al. [27].

Gel shift assay

Nuclear extracts were prepared from PC12 cells as described [28] and dialysed overnight at 4 °C against 20 mM Hepes (pH 7.9 at 4 °C), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethanesulphonyl fluoride and 0.2 mM dithiothreitol. SS promoter fragments −250 to −71 bp (*Sal*I/*Bgl*II), (−71 to +55 (*Bgl*II/*Sal*I) and −250 to +55 (*Sal*I fragment) were digested from pSS-250CAT, isolated by using either Mermaid or GeneClean and end-labelled with [α-^{32}P]dCTP through a Klenow fill-in reaction. Synthetic double-stranded CRE or GRE consensus oligonucleotides were end-labelled with [γ-^{32}P]dATP by the T4 polynucleotide kinase reaction.

DNA binding reactions were carried out in the presence of 5 μg of sonicated fish sperm DNA, 0.5 mg/ml BSA and specific competitors as indicated [28,29]. Nuclear extracts (5–10 μg of protein) were incubated for 30 min at room temperature with 50000 c.p.m. (10–20 fmol) of radiolabelled probe in a total

volume of 20 μl containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 6 mM dithiothreitol, 40 mM NaCl, 0.5 mg/ml BSA, 10% glycerol and 0.002% Triton X-100. Competition experiments were carried out by incubation first with 9 pmol of unlabelled CRE or GRE oligonucleotides for 20 min at room temperature without probe, followed by 20 min with probe at room temperature. The reaction mixtures were loaded on to 5% non-denaturing polyacrylamide gels and resolved by electrophoresis at 120 V for 3 h under running conditions of high ionic strength (50 mM Tris, 380 mM glycine, 2.4 mM EDTA, pH 8.5). The gels were subsequently dried and autoradiographed at −70 °C with an intensifying screen. Results were confirmed under conditions of low ionic strength (6.7 mM Tris/HCl, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9).

RESULTS

Glucocorticoids regulate SS gene transcription positively

The presence of glucocorticoid receptors was confirmed in PC12 and A126-1B2 cells by direct analysis of cytosolic [^{3}H]DEX binding sites using established methods [30]. To determine whether glucocorticoids can influence SS gene transcription, the effect of DEX on pSS-750CAT expression was investigated initially in PC12 cells. DEX induced a significant dose-dependent increase in CAT activity from 10 nm to 0.1 μM, with a maximal 2.2-fold induction at 1 μM (Figure 1). To test the specificity of DEX action, the ability of other steroid and thyroid hormones to alter SS gene transcription was determined (Table 1). In contrast to DEX, oestradiol, testerone, tri-iodothyronine and retinoic acid at doses between 1 nM to 1 μM had no effect on pSS-750CAT expression in PC12 cells. Likewise, phorbol esters were without effect, whereas cAMP and forskolin induced a 4–5-fold stimulation of SS-CAT expression (Table 1).

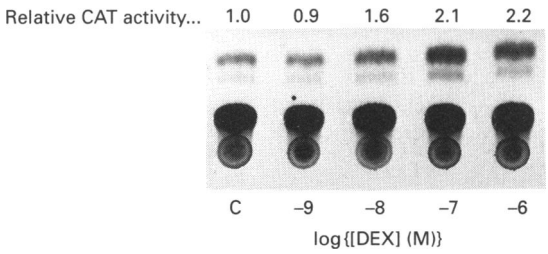

Figure 1 Effect of DEX on pSS-750CAT expression in PC12 cells

Following transient transfection, PC12 cells were incubated with DEX for 48 h and assayed for CAT. CAT activity is expressed relative to untreated controls (C). DEX induced dose-dependent stimulation of SS-CAT expression, with a maximum 2.2-fold effect at 1 μM. The Figure depicts an autoradiogram of a representative assay used to determine CAT activity in transfected PC12 cell extracts.

Table 1 Effects of DEX, cAMP, forskolin and other agents on pSS-750CAT expression in transiently transfected target cells

Results are means ± S.E.M. ($n = 5$). *$P < 0.05$ versus control; †$P < 0.05$ versus cAMP or forskolin alone.

Agents	Cell type	CAT activity (% of control)
DEX (1 μM)	PC12	212 ± 2*
cAMP (1.5 mM)	PC12	457 ± 48*
Forskolin (10 μM)	PC12	489 ± 51*
β-Oestradiol (1 μM)	PC12	108 ± 11
Testosterone (1 μM)	PC12	106 ± 13
Tri-iodothyronine (1 μM)	PC12	116 ± 16
Retinoic acid (1 μM)	PC12	101 ± 8
Phorbol 12,13-dibutyrate (1 μM)	PC12	95 ± 15
DEX + cAMP	PC12	776 ± 81†
DEX + forskolin	PC12	748 ± 70†
DEX (1 μM)	A126-1B2	110 ± 12
cAMP (1.5 mM)	A126-1B2	120 ± 21
Forskolin (10 μM)	A126-1B2	105 ± 18

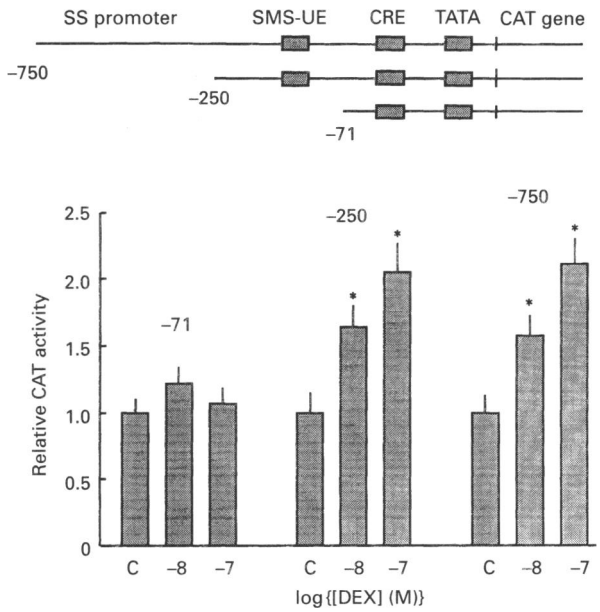

Figure 2 Effect of progressive 5′ deletion of the SS promoter fragment on DEX-induced transactivation in PC12 cells

Three promoter fragments of −750, −250 and −71 bp were used and are schematically depicted in the upper panel. Both the −750 and −250 nucleotide promoter constructs exhibited full responsiveness to DEX stimulation. Deletion to −71 bp abrogated DEX-induced stimulation of SS gene transcription. C, control. *$P < 0.05$ versus control.

The −250 to −71 bp portion of the SS promoter region is required for DEX-induced tranactivation

In order to map the promoter region responsible for glucocorticoid activation, progressive deletions of the 5′ flanking SS DNA were performed. Both the −750 and −250 bp promoter constructs exhibited full responsiveness to DEX stimulation (Figure 2). Deletion of the 5′ SS DNA to −71 bp, which retains the CRE and TATA elements but removes the SMS-UE, abrogated DEX-induced stimulation of SS gene transcription. These results further validate the specificity of the DEX effect and suggest that the CRE alone is insufficient for DEX-induced transactivation, which requires additional elements located further upstream from the CRE between −250 and −71 bp in the promoter region.

DEX potentiates cAMP-stimulated SS gene transcription

In view of the identification of DNA elements responsive to glucocorticoid stimulation upstream from the CRE in the SS gene, the potential interaction between DEX- and cAMP-induced gene transcription was explored in PC12 cells transfected with pSS-750CAT. cAMP (1.5 mM) and forskolin (10 μM) both

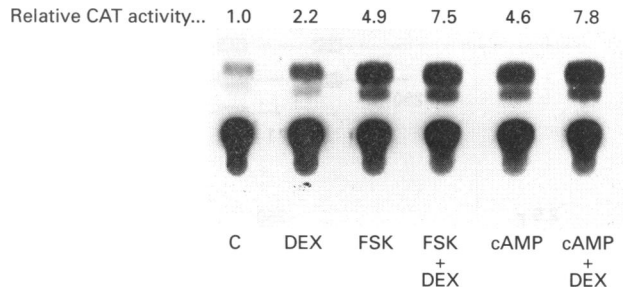

Figure 3 Additive effects of DEX and cAMP stimulation of SS gene transcription

cAMP (1.5 mM) or forskolin (FSK; 10 μM) both induced 4–5-fold stimulation of pSS-750CAT expression in PC12 cells. The combination of DEX (1 μM) with cAMP or FSK potentiated CAT activity 7–8-fold. C, control.

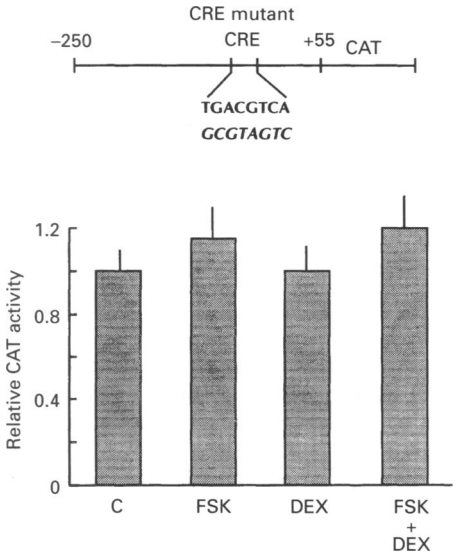

Figure 4 Effect of mutation of the CRE element on SS-CAT expression in PC12 cells

Mutation of the CRE element in the −250 promoter abolished the effects of both forskolin (FSK, 10 μM) and DEX (1 μM), and of combined forskolin and DEX.

induced a 4–5-fold stimulation of SS promoter activity (Figure 3; Table 1). The combination of DEX with cAMP or forskolin potentiated CAT activity 7–8-fold, suggesting an additive effect of DEX- and cAMP-induced transcription. The potentiating effect of DEX was also observed at a low concentration of cAMP (0.1 mM), which stimulated SS gene transcription from 3–5-fold. The pSS-250CAT construct exhibited cAMP and forskolin responsiveness as well as the additive effect with DEX, identical to that with the longer −750 bp promoter. On the other hand, the pSS-71CAT construct, which was insensitive to DEX (Figure 2), maintained full cAMP responsiveness (results not shown).

DEX transactivation shows dependence on protein kinase A activity

To further characterize the components of the cAMP signalling pathway involved in DEX-induced transactivation of the SS gene, A126-1B2 mutant PC12 cells lacking protein kinase A activity

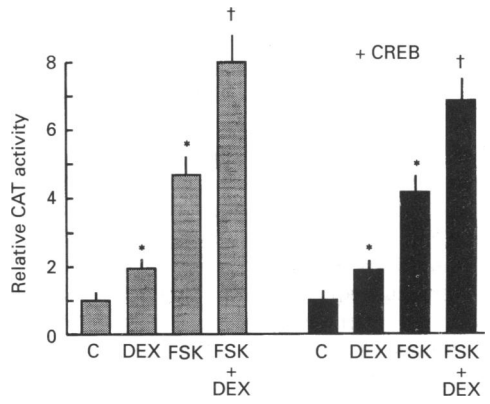

Figure 5 Effect of CREB overexpression on the co-operative interaction between DEX and forskolin in activating pSS-750CAT expression in PC12 cells

Cells were transfected with pSS-750CAT (28 μg) with or without MT-CREB (12 μg), whose expression was induced by the addition of zinc sulphate (90 μM) to the culture medium. Transfected or co-transfected cells were incubated with DEX (1 μM), forskolin (FSK, 10 μM), or the two agents in combination for 48 h. C, control. CREB overexpression did not influence DEX or forskolin effects on SS-CAT activity. Results are mean ± S.E.M. ($n = 5$). *$P < 0.05$ versus control; †$P < 0.05$ versus DEX or FSK alone.

were used (Table 1). As expected, A126-1B2 cells transfected with pSS-750CAT did not respond to either cAMP or forskolin. These cells also did not show DEX-induced SS gene transcription, confirming a requirement for protein kinase A activity for the DEX effect.

Mutation of the CRE abolishes the DEX effect on SS gene transcription

To test the effect of mutation of the CRE on DEX-induced SS gene transcription, PC12 cells were transfected with Δ-CRE pSS-250CAT. Replacement of the CRE by a non-related sequence reduced basal CAT activity (% conversion of chloramphenicol into acetylated forms) from 1.42±0.28% to 0.52±0.03% ($P < 0.01$). As expected, the mutation abolished the stimulatory effect of forskolin but additionally blocked DEX-induced stimulation, as well as the combined potentiation of CAT activity by forskolin and DEX (Figure 4). Mutation of the CRE was further validated by gel electrophoretic mobility shift assays. SS promoter fragments −250 to +55 bp cognate (containing the CRE) or CRE mutant (Δ-CRE) pSS-250CAT were radiolabelled and incubated with PC12 nuclear extract. Three retarded DNA–protein complexes were observed with the natural promoter; labelling of one of these was significantly inhibited in the presence of a 500-fold molar excess of unlabelled CRE consensus oligonucleotide (results not shown). This band was not observed with the CRE mutant promoter.

In a reciprocal experiment, the effect of overexpressed CREB on DEX-induced SS gene transcription was investigated. Co-transfection of PC12 cells with MT-CREB and pSS-750CAT had no effect on control CAT expression and did not enhance DEX-, forskolin- or combined forskolin and DEX-induced stimulation of CAT activity in these cells (Figure 5).

Gel shift assays indicate that the −250 to −71 bp SS promoter is a glucocorticoid receptor binding region

In order to obtain evidence for a potential DNA–protein interaction between the SS promoter and the glucocorticoid

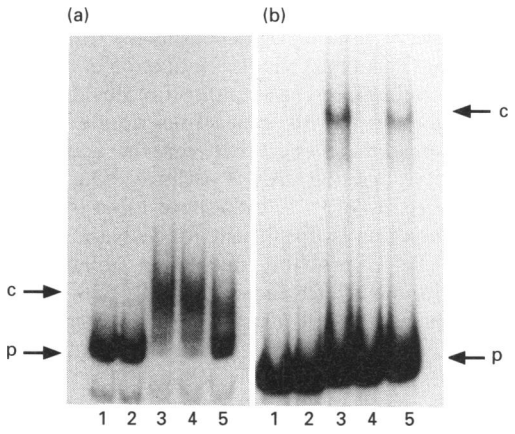

Figure 6 Gel electrophoretic mobility shift assays with ^{32}P-labelled SS promoter fragments and nuclear extract from PC12 cells

(**a**) *Sal*I/*Bgl*II fragment (−250 to −71 bp) of SS DNA as probe. Lane 1, probe alone (50000 c.p.m.); 2, with GR-DBD-(440–525) (1 ng); 3, with 7 μg of PC12 nuclear extract; 4, with PC12 extract and 9 pmol of CRE oligonucleotide; 5, with PC12 extract and 9 pmol of GRE oligonucleotide. Note the retarded DNA–protein complex (c) in lane 3, the labelling of which is competitively inhibited by preincubation with GRE (lane 5) but not CRE (lane 4). (**b**) Identical conditions to those described in (**a**), except for the radiolabelled probe (−71 to +55 bp SS DNA). A higher-molecular-mass retarded DNA–protein complex (c) is observed (lane 3), the labelling of which is specifically inhibited by unlabelled CRE (lane 4) but not GRE (lane 5). p, free probe; c, DNA–protein complex.

receptor, gel electrophoretic mobility shift assays were performed. Incubation of labelled −250 to −71 bp SS DNA with a nuclear extract from PC12 cells revealed a retarded band (Figure 6a, lane 3). The specificity of the labelled complex was tested by competition experiments with synthetic oligonucleotides. Addition of a 500-fold molar excess of unlabelled GRE consensus oligonucleotide to the binding reaction specifically inhibited complex formation (Figure 6a, lane 5), whereas the same concentration of unlabelled CRE consensus oligonucleotide was without effect (lane 4). To further validate the specificity of the SS DNA–protein interaction, parallel gel electrophoretic mobility shift assays were conducted with the −71 to +55 bp SS promoter DNA (Figure 6b). A promoter DNA complex between −71 to +55 bp SS DNA and PC12 nuclear extract was detected as a high-molecular-mass band (Figure 6b, lane 3) the labelling of which was inhibited by excess unlabelled CRE consensus oligonucleotide (lane 4) but not by GRE oligonucleotide (lane 5). In contrast to the binding observed between SS promoter DNA and the PC12 nuclear extract, purified GR-DBD-(440–525) failed to bind to either SS promoter fragments (Figures 6a and 6b, lane 2). The receptor protein was capable of retarding the mobility of labelled synthetic consensus GRE but not CRE (results not shown).

DISCUSSION

The present studies provide the first evidence of direct transcriptional enhancement of the SS gene by glucocorticoids. The effect of glucocorticoids was hormone-specific and could not be demonstrated with other steroid and thyroid hormones, or with retinoic acid. The action of glucocorticoids was dependent on a functional interaction with CRE/CREB and related binding proteins [14], and led to an additive response between the two regulatory elements.

Transcriptional control by steroid and thyroid hormones is mediated by a family of nuclear receptors that are activated by ligands and bind to *cis* regulatory elements in specific target genes [31,32]. Previous studies have provided increasing evidence for functional interactions between the nuclear receptors and other transcription factors not involving a natural promoter [33–38]. This is particularly the case with glucocorticoid-inducible genes, which frequently show GREs contiguous with DNA regulatory sequences for other transcription factors in the promoter region. These include binding sites for SP1, NF1, CACCC and CCAAT box binding proteins [33,34], CREB [35,38] and AP-1 factors [36,37], or a second GRE [34]. An interaction between the glucocorticoid receptor and these accessory factors can lead to either enhancement or repression of gene transcription. Synergism between the glucocorticoid receptor and other transcription factors was first reported for NF1, SP1 and CACCC binding proteins in genes encoding tyrosine aminotransferase and rat tryptophan oxygenase [33,34]. Functional cooperativity in these instances was shown to be critically dependent on the spacing between the regulatory elements but not their orientation, and is mediated by protein–protein interaction rather than co-operative DNA binding [33,34]. These studies additionally revealed that a single copy consensus GRE sequence isolated at a distance from the transcription start site was insufficient for gene induction, but the sequence became active when a second regulatory element including a second GRE was positioned nearby [34]. This suggests a basic model of glucocorticoid-induced transcriptional activation requiring multiple GREs or a combination of a GRE with other factor-binding sites for constitution of a hormone-inducible enhancer. More recently, the glucocorticoid receptor has been shown to repress gene expression by functional interference with several different transcriptional activators [35–38]. For instance, repression of the human chorionic gonadotrophin α-subunit and pro-opiomelanocortin genes appears to be mediated by competitive binding of the glucocorticoid receptor to DNA regulatory sequences termed negative GREs, thereby displacing transcriptional activators such as CREB in the case of the α-subunit gene [35] and the CAAT box binding factor in the case of the pro-opiomelanocortin gene [38]. For other genes such as those for osteocalcin, proliferin and collagenase, glucocorticoids repress an AP-1 site by a different mechanism involving a direct protein–protein interaction between c-*jun* and the glucocorticoid receptor leading to a mutual inhibition of their DNA binding activities [36,37].

Dependence of glucocorticoid receptor-mediated enhancement of SS gene transcription on CRE/CREB activation was shown here by two lines of evidence. First, the protein kinase-A deficient mutant cell line A126-1B2, which is incapable of inducing cAMP-mediated gene transcription due to an inability to phosphorylate and thereby transactivate CREB, was also unresponsive to glucocorticoid induction. Secondly, replacement of the canonical CRE in the SS gene upstream region with an inert sequence abolished both cAMP and glucocorticoid responsiveness. Failure of DEX to induce the −71 bp SS promoter suggests that the glucocorticoid receptor does not interact directly with the CRE/CREB complex. Since the −250 bp promoter showed full glucocorticoid responsiveness, our findings imply a glucocorticoid-sensitive region in the −250 to −71 sequence of the SS promoter. Gel electrophoretic mobility shift assays confirmed that the −250 to −71 bp region of the SS promoter (but not the −71 to +55 bp domain) binds specifically to a GRE-sensitive nuclear protein, suggesting a putative glucocorticoid receptor interaction with SS promoter DNA. Failure of the purified glucocorticoid receptor DNA binding domain protein to bind to SS DNA suggests the requirement of other components of the receptor protein and/or associated binding proteins for

responsiveness. For example, retinoid X receptor β is required for both DNA binding and transactivation of the retinoic acid receptor by its cognate response element [39]. The interaction of glucocorticoid receptor with the promoter of the glycoprotein hormone α gene is dependent on cell-specific cofactors [40]. Analysis of the SS promoter failed to reveal a classical GRE, but did disclose sequences of two possible variants (aGGCTTnnnTtTTCT at -167 bp and aaGATTnnnTGgTCT at -219 bp) which resemble atypical GREs. Further studies with promoter deletion mutants combined with DNAase I footprinting are in progress to map precisely the regulatory elements of the SS gene that interact directly with the glucocorticoid receptor.

There is only one other known example of functional co-operativity between the glucocorticoid receptor and the cAMP signalling pathway, illustrated by the gene for phosphoenolpyruvate carboxykinase (PEPCK) [41–43]. The proximal region of the PEPCK gene promoter (-100 to $+1$ bp) contains the TATA and CRE elements in a virtually identical configuration to that found in the SS gene. The glucocorticoid response 'element' in this gene is a complex unit (hence termed the glucocorticoid response unit, or GRU) consisting of a tandem array (5′ to 3′) of a retinoic acid response element, an insulin-responsive sequence and two glucocorticoid receptor binding sites; the entire complex spans about 110 bp (from -455 to -363). The two glucocorticoid receptor binding sites are not homologous with the consensus GRE sequence, which they match in only 7/12 and 6/12 positions respectively, and they function independently, each accounting for half of the full response [41]. Glucocorticoids and cAMP individually induce PEPCK gene transcription, and in combination produce enhancement. This is achieved through a protein–protein interaction between the glucocorticoid receptor and the CREB occurring over a distance of 360 bp. Retinoic acid acts on the PEPCK gene synergistically with DEX, and insulin inhibits the responses of DEX and cAMP both individually and in combination. In the absence of the accessory factors, the glucocorticoid receptor binding sites alone are inert. In the case of the SS gene promoter, we found evidence of functional co-operativity between glucocorticoids and the CRE/CREB locus; however, this resulted in an additive rather than a synergistic response. Interestingly, in addition to transcriptional co-operativity between the glucocorticoid receptor and the CRE/CREB unit, SS gene expression is also capable of inhibition by insulin [9]. A survey of the SS gene promoter for these accessory factor binding sites shows two contiguous putative retinoic acid response element (-373 to -366 bp) and insulin-responsive sequence (-330 to -321 bp) motifs considerably further upstream from the potential glucocorticoid receptor responsive site. Despite these differences, there are sufficient similarities to suggest that the PEPCK gene could serve as a useful model for further studies to determine the precise nature of the transcriptional interaction between the glucocorticoid receptor, the CRE, and perhaps the retinoic acid receptor and insulin-sensitive transcription factors in the SS gene.

Since glucocorticoids act on numerous target genes, an interaction between glucocorticoids and the cAMP pathway could also occur at points more proximal to the gene through induction of one or more components of the cAMP signalling pathway. For instance, glucocorticoids have been reported to activate adenylate cyclase in GH_3 cells [44] and could conceivably enhance cAMP-dependent gene transcription via this mechanism. Such an action, however, cannot explain the ability of glucocorticoids to potentiate the effects of high concentrations of exogenous cAMP or forskolin found in the present study. Moreover, the results of the -71 bp construct argue against this possibility. It is not known whether glucocorticoids regulate the expression of protein kinase A, although glucocorticoids stimulate CREB expression in rat C6 glioma cells [45]. Induction of either of these two molecules could represent an indirect mechanism for glucocorticoid stimulation of SS gene transcription, but cannot account for the additive effect between the activated glucocorticoid receptor and the cAMP pathway. Our results with CREB co-transfection of PC12 cells suggest that overexpression of this molecule alone is insufficient for activation of SS gene transcription. This is probably due to an abundance of endogenous CREB and related factors in these cells [14,17,46], as well as the requirement not just for CREB molecules, but for phosphorylated CREB, for transcriptional activation [5,46].

The concentration of DEX at which significant induction of SS-CAT activity occurred (10 nM) falls within the physiological range, especially if allowance is made for differences in the responses of transfected and endogenous SS genes. Since cAMP is the predominant regulator of SS gene transcription, and since glucocorticoids and cAMP influence many of the same physiological processes, a co-operative relationship between the two pathways is clearly functionally relevant. Transcriptional activity of the SS gene, however, can explain only part of glucocorticoid action on steady-state SS mRNA levels that we found in rat tissues, i.e. the stimulatory component [18]. The additional, more potent, effect of glucocorticoids resulting in inhibition of SS mRNA accumulation must therefore be mediated through a post-transcriptional mechanism [18,47]. Studies of the effects of glucocorticoids on SS mRNA stability are necessary to investigate this question further.

In conclusion, we have demonstrated positive transcriptional control of the SS gene by glucocorticoids. Glucocorticoid-induced transactivation shows dependence on protein kinase A activity, and may be mediated via interactions between the glucocorticoid receptor and CREB. DNA sequences upstream from the CRE between -250 and -71 bp in the SS promoter appear necessary for the glucocorticoid effect.

We thank Dr. M. R. Montminy for the pSS-750CAT and pSS-71CAT vectors, and Dr. D. Drucker for providing MT-CREB. GR-DBD-(440–525) was obtained courtesy of Dr. L. P. Freedman. We are grateful to Drs. H. H. Zingg, J. Drouin and S. Richard for advice. We thank Mrs. A. Warszynska and Mrs G. Kent for technical assistance, Drs. A. Galanopoulou, C. B. Srikant and M. Greenwood for helpful suggestions, and Mrs. M. Correia for secretarial support. This work was supported by grants from the Canadian Medical Research Council (MT-6196). J.-L. L. received studentship support from the Fonds Pour La Formation De Chercheurs et L'Aide a La Recherche. D. N. P. is a Fellow of the Canadian Diabetes Association.

REFERENCES

1 Reichlin, S. (1983) N. Engl. J. Med. **309**, 1495–1501
1a Reichlin, S. (1983) N. Engl. J. Med. **309**, 1556–1563
2 Patel, Y. C. (1992) Somatostatin. In: Weil, C., Muller, E. E. and Thorner, M. O. (ed) Basic Clin. Aspects Neurosci. **4**, 1–16
3 Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G. and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6682–6686
4 Montminy, M. R. and Bilezikjian, L. M. (1987) Nature (London) **328**, 175–178
5 Gonzalez, G. A. and Montminy, M. R. (1989) Cell **59**, 675–680
6 Patel, Y. C., Papachristou, D. N., Zingg, H. H. and Farkas, E. M. (1991) Endocrinology (Baltimore) **128**, 1754–1762
7 Wernar, H., Koch, Y., Baldino, F. and Gozes, J. (1988) J. Biol. Chem. **263**, 7666–7671
8 Scarborough, D. E., Lee, S. L., Dinarello, C. A. and Reichlin, S. (1989) Endocrinology (Baltimore) **124**, 549–551
9 Patel, Y. C., Sri Prakash, M. L., Papachristou, D. N., Farkas, E. M. and Pham, K. (1988) Diabetes **37** (Suppl. 1), 101A (abstr.)
10 Rogers, K. V., Vician, L., Steiner, R. A. and Clifton, D. K. (1988) Endocrinology (Baltimore) **122**, 586–591
11 Patel, S. C., Papachristou, D. N. and Patel, Y. C. (1991) J. Neurochem. **56**, 1286–1291

12 Andrisani, O. M. and Dixon, J. E. (1990) Annu. Rev. Physiol. **52**, 793–806
13 Goodman, R. H. (1990) Annu. Rev. Neurosci. **13**, 111–127
14 Meyer, T. E. and Habener, J. F. (1993) Endo. Rev. **14**, 269–290
15 Vallejo, M., Miller, C. P. and Habener, J. F. (1992) J. Biol. Chem. **267**, 12868–12875
16 Vallejo, M., Penchuk, L. and Habener, J. F. (1992) J. Biol. Chem. **267**, 12876–12884
17 Leonard, J., Serup, P., Gonzalez, G., Edlund, T. and Montminy, M. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6247–6251
18 Papachristou, D. N., Liu, J.-L. and Patel, Y. C. (1993) Endocrinology (Baltimore) **134**, 2259–2266
19 Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. J. and Yamamoto, K. R. (1988) Nature (London) **334**, 543–546
20 Greene, L. A. and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2424–2428
21 Van Buskirk, R., Corcoran, T. and Wagner, J. A. (1985) Mol. Cell. Biol. **5**, 1984–1992
22 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051
23 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25 Drucker, D. J., Campos, R., Reynolds, R., Stobie, K. and Brubaker, P. L. (1991) Endocrinology (Baltimore) **128**, 394–400
26 Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA
27 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
28 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.) (1987) Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York
29 Drouin, J., Sun, Y. L., Chamberland, M., Gauthier, Y., De Lean, A., Nemer, A. and Schmidt, T. J. (1993) EMBO J. **12**, 145–156
30 Schneider, W. and Shyamala, G. (1985) Endocrinology (Baltimore) **116**, 2656–2662
31 Beato, M. (1989) Cell **56**, 336–344
32 Carson-Jurica, M. A., Schrader, W. T. and O'Malley, B. W. (1990) Endocr. Rev. **11**, 201–220
33 Schule, R., Muller, M., Otsuka-Murakami, H. and Renkawitz, R. (1988) Nature (London) **332**, 87–90
34 Strahle, U., Schmid, W. and Schutz, G. (1988) EMBO J. **7**, 3389–3395
35 Akerblom, J. E., Slater, E. P., Beato, M., Baxter, J. D. and Mellon, P. L. (1988) Science **241**, 360–363
36 Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M. and Evans, R. M. (1990) Cell **62**, 1217–1226
37 Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeat, T., Schmidt, T. J., Drouin, J. and Karin, M. (1990) Cell **62**, 1205–1215
38 Drouin, J., Charron, J., Gagner, J. P, Jeannotte, L., Nemer, M., Plante, R. K. and Wrange, O. (1987) J. Cell. Biochem. **36**, 293–304
39 Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. and Rosenfeld, M. G. (1991) Cell **67**, 1251–1266
40 Krishna, V., Chatterjee, K., Madison, L. D., Mayo, S. and Jameson, J. L. (1991) Mol. Endocrinol. **5**, 100–110
41 Granner, D. K., O'Brien, R., Imai, E., Forest, C., Mitchell, J. and Lucas, P. (1991) Recent Prog. Horm. Res. **47**, 319–348
42 Lucas, P. C. and Granner, D. K. (1992) Annu. Rev. Biochem. **61**, 1131–1173
43 Imai, E., Miner, J. N., Mitchell, J. A., Yamamoto, K. R. and Granner, D. K. (1993) J. Biol. Chem. **268**, 5363–5366
44 Chang, F.-H. and Bourne, H. R. (1987) Endocrinology (Baltimore) **121**, 1711–1715
45 Jungmann, R. A., Wang, X. S, Milkowski, D. M. and Short, M. L. (1992) Nucleic Acids Res. **20**, 825–829
46 Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S. and Montminy, M. R. (1992) Cell **70**, 105–113
47 Cote, G. J., Palmer, W. N., Leonhart, K. and Gagel, R. F. (1986) J. Biol. Chem. **261**, 12930–12936

Received 13 December 1993/21 February 1994; accepted 4 March 1994