Contribution of a Nuclear Factor 1 Binding Site to the Glucocorticoid Regulation of the Cytosolic Aspartate Aminotransferase Gene Promoter*

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Michèle Garlatti, Martine Aggerbeck, Jacqueline Bouguet, and Robert Barouki

From INSERM U-99, Hôpital Henri Mondor, 94010 Créteil, France

Two regions of the cAspAT gene promoter mediate the glucocorticoid regulation of this gene in the Fao hepatoma cell line. The proximal region was localized by deletion studies and stable transfections in the Fao cells to the sequence −553/−398. This region includes the glucocorticoid-responsive element (GRE) A sequence, which consists of two overlapping GREs and which can mediate the glucocorticoid regulation of a heterologous promoter. DNase I footprinting studies have shown that a site 80 base pairs upstream of the GRE A was protected by liver and brain nuclear extracts (site P9). The binding was displaced by an excess of an oligonucleotide containing a typical NF1 binding site and by NF1-specific antibodies. In electrophoretic mobility shift assay using the P9 oligonucleotide as a probe, several complexes were formed. Most complexes were common to liver and brain but were less abundant when testis extracts were used. At least one complex was specific to the liver. All complexes, with the exception of two, were competed for by the NF1 oligonucleotide. Furthermore, the sequence of the P9 site showed a 7/9-base pair homology with a cAMP site.

The regulation of gene expression by glucocorticoid hormones is mediated by an intracellular receptor which displays both DNA binding and transactivation properties (1). The receptor interacts usually as a dimer on partially palindromic sites called glucocorticoid-responsive elements (GRE). However, in some gene promoters, GREs are not sufficient to convey hormonal regulation. In these cases, other binding sites for transcription factors, usually located in the vicinity of a GRE, are also required, and constitute, with the GRE, a so-called glucocorticoid-responsive unit, or hormone response domain (2). Following initial observations on the tryptophan oxygenase gene promoter (3) and the mouse mammary tumor virus long terminal repeat promoters (4), several studies have established that glucocorticoid regulation of artificial promoters was strongly enhanced when a binding site for one of several transcription factors was present in the vicinity of a GRE (5, 6). These sites were either the CACC sequence, the nuclear factor 1 site, the octamer sequence, the Sp1 element, or a duplication of the GRE itself. The analysis of natural promoters showed that several sites are usually required for efficient regulation. In addition to the mouse mammary tumor virus and the tryptophan oxygenase promoters, the glucocorticoid-responsive units of the tyrosine aminotransferase (7) and of the phosphoenolpyruvate carboxykinase promoters (8) have been extensively analyzed. In the latter case, even two closely located GREs are not sufficient to confer an efficient regulation which requires two additional sites for accessory factors. Furthermore, the phosphoenolpyruvate carboxykinase glucocorticoid responsive unit cooperates with proximal promoter elements to mediate an efficient regulation (9). Clearly, the mechanism of gene regulation by glucocorticoids is complex, and additional studies on different gene promoters are required to allow a better understanding of this biological process.

We are studying the hormonal regulation of the cytosolic aspartate aminotransferase gene promoter. This gene is different from other models for glucocorticoid hormone action in that it is a housekeeping gene characterized by a widespread basal expression and a tissue-specific glucocorticoid regulation (10). The cAspAT gene basal promoter binds transcription factors of the C/EBP family and the NF1 family (11). The binding pattern differs according to the tissue, which presumably provides the possibility for tissue-specific regulation. Two regions of the cAspAT gene promoter contribute to the glucocorticoid regulation: a distal one (−1.983/−1.718 kilobases) which also carries the negative regulation by insulin, and a proximal one (−553/−318 base pairs) (12). The latter one contains an unusual glucocorticoid-responsive sequence consisting of two overlapping GREs that can bind two receptor dimers in a highly cooperative manner (13). Because of this unusual structure and because of the complexity of the cAspAT gene promoter, it was of interest to search for other factors that can cooperate with the glucocorticoid receptor. Here we show that proteins of the NF1 family bind 80 base pairs upstream of the GREA and that this binding is necessary for an efficient glucocorticoid activation of the cAspAT gene promoter.

EXPERIMENTAL PROCEDURES

Cell Culture

The rat hepatoma clone Fao is derived from the H4IEC3 line of the Reuber H35 hepatoma (14). Cells were maintained as described (15) and were treated, as indicated, with the various hormones and drugs for 24 h. The human hepatoma cell line HepG2 (16) was cultured under the same conditions as described by Pavé-Preux et al. (15).
Transfection Experiments
Stable Transfections—One day prior to the transfection, Fao cells (1.5 × 10^6 cells/10-cm dish) were seeded into the usual culture medium containing fetal calf serum (18). Ten milliliters of fresh medium with serum were added to the cells 2–3 h before the transfection. The CAT plasmid (10 μg) and the pSV2neo plasmid (2 μg) were introduced into the cells by the calcium phosphate co-precipitation technique followed by a glycerol shock (12). Ten ml of fresh medium with serum were added after the glycerol shock. Two days later, the cells were split 1:5 and allowed to grow for 24 h prior to the addition of the neomycin analog G418 (Life Technologies, Inc.: 250–500 μg/ml, depending on the batch). The medium was changed every 3 days. Two to three weeks later, the surviving cells were harvested and pooled for CAT assay. When needed, the cells were treated for 24 h with the relevant hormones.

Transient Transfection—HeLa2 cells were transfected as described by Garlatti et al. (11) with some modifications. Five micrograms of the reporter plasmid were co-transfected with varying amounts of a glucocorticoid receptor expression vector and with 1 μg of pSV-luc (Promega) to correct the variability of transfection efficiency. Luciferase was assayed using a kit from Promega according to the manufacturer’s instructions. Data were expressed as the ratio of CAT activity over luciferase activity. Dexamethasone (0.1 μM) was added 24 h later when required. After a 24-h treatment, cells were homogenized for CAT assay.

Chloramphenicol Acetyltransferase Assay
CAT activity was determined using the two-phase assay developed by Neumann et al. (19). Briefly, cell homogenates were prepared in 280 μl of Tris-HCl, 250 mM, pH 7.8, EDTA, 5 mM, as described previously. Cell extracts (80 μl) were treated at 65°C for 5 min, in order to inactivate the endogenous acylases, then centrifuged at 12,000 rpm for 15 min. For each reaction, 20 μl of the cell extract supernatant were added to 40 μl of a buffer solution in order to have the following final concentrations: 250 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM chloramphenicol, 30 μM acetyl-CoA to which 0.5 μCi of 3H-labeled acetyl-CoA was added (DuPont NEN, NET-290 L). The reaction was carried out for 30 min at 37°C. The solution was then transferred to a minivial and layered with 4 ml of Econofluor (DuPont NEN, NEF-969). After mixing vigorously, the two phases were allowed to separate for at least 15 min, and radioactivity was counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not the unreacted acetyl-CoA, is allowed to diffuse into the Econofluor phase. In these experiments, blanks were obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid. Proteins were assayed according to Bradford (20).

Preparation of Nuclear Extracts
Male Wistar rats, weighing 200–250 g, were used. Extracts from rat organs (liver, testis, and brain) were prepared as described by Gorski et al. (21).

DNase I Footprinting
Two probes from the cAspAT promoter (nucleotides −682 to −226) were end-labeled using the Klenow fragment of DNA polymerase I. The standard reaction was performed according to Vaulont et al. (22) with some modifications. The binding reaction was performed in a final volume of 25 μl, containing 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 2 mM dithiothreitol, 4 mM spermidine, 15% glycerol, 100 μM MgCl₂, 2 mM bovine serum albumin, 10 mM HEPES, pH 8. Two hundred fifty ng of poly(dI-dC) (Pharmacia) were used as carrier.

The clear nuclear proteins, 15–80 μg, were preincubated 15 min on ice with the competitor oligonucleotides. Then, about 1 ng of labeled probe (20,000 cpm) was added, and the incubation was continued for 15 min on ice. After adjusting the concentration of CaCl₂ to 2.5 mM and incubating for 1 min at 20°C, DNase I was added, and the digestion was carried out at 20°C for 1 min. Subsequent handling of the DNA was performed as described (22).

FIG. 1. Role of GRE A in the regulation of the cAspAT gene promoter. Plasmids p−553, −26 CAT and p−398, −26 CAT were stably transfected in the Fao cells as described under “Experimental Procedures.” G418-resistant clones were grown and pooled. CAT activity was assayed in cell homogenates 24 h after the addition of 0.1 μM dexamethasone, or 0.5 mM 8-Br-cAMP or both and compared to CAT activity of control cells. Only the fold induction by dexamethasone is shown here in cells that are treated or not with cAMP. Two different plasmid preparations have been used. The values have been obtained from two independent experiments.

Gel Retardation Assays
Probes were oligonucleotides labeled using the Klenow fragment of DNA polymerase I. Protein-DNA binding was performed under the same conditions as those described for the footprinting experiments except that 1 μg of poly(dI-dC) was used as carrier and that 1.5–7 μg of nuclear extracts were added to the probe. After 15 min on ice, the samples were directly loaded onto a 6% polyacrylamide gel in 0.5 × TBE. The gel (0.2 × 16 cm) was pre-electrophoresed at 100 V for 1 h in the cold room (4°C). Electrophoresis was performed at 260 V for 90 min in 0.5 × TBE buffer. For supershift experiments, IgG were purified from either preimmune serum or anti-NF1 immune serum, using a HiTrap protein A column according to the manufacturer’s instructions (Pharmacia). The preimmune or specific anti-NF1 IgG (2 μg) were preincubated with rat liver nuclear extracts (2 μg) for 90 min at room temperature before addition of the probe.

Sequence of the Oligonucleotides Used in this Study
NF₁ (from adenovirus 2): TATTTCGATTTGAGACATATGATA-TGCA; P₁ (from −549 to −511 of the cAspAT gene promoter): GTCGGG-GACCTTGCTGCTGACCCCGCGCTGCGCTTAACGCAG; P₂ mut: GTCGGG- GACCTTCCGTGT2ACCGCCCGCTGCGCTTAACGCAG.

RESULTS
In a first series of experiments, we have mapped the proximal region mediating the activation of the cAspAT gene promoter by glucocorticoids. Deletion fragments of the cAspAT gene promoter were subcloned upstream of the CAT gene and the resulting plasmids were stably transfected into the Fao cells. We have previously shown that glucocorticoids stimulated promoter activity approximately 3-fold in cells transfected with the construct (−682, −26) (12). Similar experiments were performed with plasmids carrying promoter fragments that were further deleted at the 5’ end. The results depicted in Fig. 1 show that the fragment −553/−398 is required for glucocorticoid activation of the promoter. The glucocorticoid effect is even more striking in the presence of cAMP, which is known to potentiate the induction of cAspAT by glucocorticoids (12). This fragment includes a glucocorticoid-responsive sequence (called GRE A) that we have previously shown to be functional when inserted into a heterologous promoter (13). The data suggest that the half-palindromic GRE present at −26 is not sufficient to confer glucocorticoid regulation. We then asked whether other putative transcription factor binding sites in the vicinity of the GRE A could contribute to the glucocorticoid induction of the promoter activity.

The DNase I footprinting assay was carried out to identify the binding sites involved in the glucocorticoid regulation. A DNA fragment spanning the region −682/−226 was labeled at either strand, and allowed to bind to liver nuclear proteins. In the
experiment depicted in Fig. 2, four sites were protected from DNase I digestion. These sites were called P6, P7, P8, and P9. Two more proximal sites P5 and P4 are outside the portion of the gel that is shown here. Site P4 had been described in detail during the study of the promoter fragment carrying the basal activity (11). The closest site to the GRE A is P8 which is included in the promoter fragment 2553/2398. Thus, we have focused this study on the P8 site.

As shown in Table I, the DNA sequence corresponding to the P8 site displays an identity of 7/9 bases with the consensus NF1 binding sequence (23). Furthermore the P8 sequence is partially palindromic, and the distance between the two half-sites is identical to that of a typical NF1-binding site; addition of an excess of the NF1 oligonucleotides prevented the protection observed at the P8 site (Fig. 2).

A similar experiment was conducted using a fragment labeled on the noncoding strand at the distal end (Fig. 3). In this case, the P8 and P9 sites can be clearly detected when liver nuclear extracts were used. Addition of a P8 oligonucleotide (which covers the protected region) prevented protection at the P8 site, but not at the P9 site. A similar result was obtained with an NF1 oligonucleotide confirming the data of Fig. 2. Other oligonucleotides including Sp1 or CACC sites were not able to compete efficiently. These data suggest that a NF1-like protein binds at the P8 site.

When brain nuclear extracts were used instead of liver extracts, similar results were obtained for the P8 site, with a similar pattern of competition. However, there was no protection at the P9 site suggesting that binding at this site may be tissue-specific. In fact, when testis extracts were used, the P9 site was also protected, while the P8 site was only partially protected (not shown).

An electrophoretic mobility shift assay was used to further characterize the binding at the P8 site. Fig. 4 shows the data obtained with liver, brain, and testis extracts using as a probe either the P8 oligonucleotide (Fig. 4A) or an oligonucleotide containing a typical NF1 site (Fig. 4B) (origin of replication of the adenovirus). Both probes gave very similar patterns of retarded complexes. A strong binding was observed with liver and brain extracts, but not with testis extracts, in agreement with the footprinting experiments. Several complexes were

| NF1 | TGGC nnnnn GCCAA |
|-----|------------------|
| P8 | TGGC TGTCA GCCCG |
| P8 mut | TGCG TGTCA GCCCG |

Table I: Comparison of the NF1, the wild type, and the mutant P8 sequences

Asterisks refer to the modified nucleotides in the P8 mut sequence.
formed in each case, an expected observation since there are several NF1 or NF1-like proteins in nuclear extracts. Complexes a, b, and c are the most abundant and display the slowest migration in the gel. These complexes were displaced by an excess of P8 or NF1 oligonucleotides. However, the NF1 oligonucleotide was more efficient suggesting that it could bind NF1 or NF1-like proteins with a higher affinity than P8. Note that one of the half-sites in the P8 sequence is not optimal for NF1 binding. Complexes e and f appear to be specific for the P8 probe (Fig. 4, A and B). As opposed to other complexes, they are poorly competed for by the NF1 oligonucleotide. The nature of these complexes is not known. However, it is unlikely that they play a significant role as they can only be seen in EMSA, not in DNase I protection experiments (absence of a footprint on the P8 site in the presence of the NF1 oligonucleotide). Furthermore, these complexes are clearly detected only when a high amount of nuclear extracts (7 μg) is added to the probe; this suggests that the abundance of the corresponding proteins is poor or that their affinity for the site is low. One interesting observation is that, although most complexes formed by liver and brain nuclear extracts are similar in migration and abundance, complex c appears to be more abundant in binding reactions with the liver extracts.

These complexes were further analyzed using a supershift assay with polyclonal antibodies directed against the C-terminal half of the NF1 protein (Fig. 4C). The anti-NF1 IgG, but not preimmune IgG, displaced complexes b, c, and d, and to a lesser extent complex a. The other complexes were barely detected in these experiments. The results confirm that proteins of the NF1 family of transcription factors are the major components of the complexes formed using the P8 probe.

In order to assess the role of the P8 site in the glucocorticoid induction of the cAspAT gene promoter, a mutation was introduced in the putative NF1 site. An oligonucleotide containing the mutated sequence (called P8 mut) was unable to compete for the complexes formed by the P8 probe and liver nuclear proteins (Fig. 5A). This suggests that the mutation has inactivated the NF1 site. This was further confirmed by gel shift assays using the P8 mut oligonucleotide as a probe (Fig. 5B). Under these conditions, there was a dramatic decrease in the intensity of the shifted bands, suggesting that the mutation did inactivate the NF1 site, and did not generate a novel site with high affinity to liver nuclear proteins.

The P8 mut mutation was then introduced into the sequence −553/−26 and the promoter activity of this mutated fragment was compared to that of the wild-type fragment (Table II). Plasmids containing the wild-type and mutated fragments cloned upstream of the CAT gene were co-transfected into the HepG2 cells with an expression vector coding for the glucocorticoid receptor. The experiment depicted in Table II shows that the mutated fragment displays a higher basal activity than the wild-type fragment. Interestingly, the 3-fold glucocorticoid activation of the wild-type promoter was significantly decreased when the P8 site was mutated. In order to assess the specificity of this phenomenon, we evaluated the effect of C/EBPβ, a well known activator of the cAspAT gene promoter (11). A C/EBPβ expression vector was co-transfected with the wild-type or with the mutated cAspAT gene promoter. Both promoters were activated to a similar extent under these conditions (data not shown). Thus, the mutation of the P8 site specifically reduces the effect of the activated glucocorticoid receptor. This suggests that proteins binding to this site contribute to the glucocorticoid regulation.

**DISCUSSION**

In this study, we have further characterized the complex regulation of the cAspAT gene promoter by glucocorticoids. This regulation is tissue-specific and is modulated both negatively and positively by insulin and cAMP, respectively (12). Two promoter regions are required for the glucocorticoid effect. We have focused this study on the proximal region which includes a functional site called GRE A. The GRE A displays a
unique structure composed of two overlapping imperfect GREs; it binds two dimers of the glucocorticoid receptor in a highly cooperative manner (13).

The finding that overlapping GREs could constitute a functional structure has raised several questions that remain unanswered. In fact, much is known about units composed of adjacent GREs which are found in several gene promoters that are regulated by glucocorticoids. In the case of tyrosine aminotransferase, the functional regulatory unit is composed of two GREs which act synergistically (24), but also comprises other binding sites for transcription factors such as HNF3 and c/EBP (25). The glucocorticoid-responsive unit of the phosphoenolpyruvate carboxykinase gene is also composed of two imperfect GREs and of two binding sites for accessory factors (8). Conversely, in some experiments using artificial promoters, two adjacent GREs coupled to a minimal promoter were found to be sufficient to confer a potent regulation by glucocorticoids (5). Thus, depending on the promoter structure, the multimerization of GREs in an adjacent manner may, or may not, be sufficient to constitute a potent regulatory unit. Examination of the structure of several promoters suggests that additional transcription factors are often required.

Since the arrangement of the GREs as overlapping elements in the cAspAT GRE A site is different from that of other sites, it was of interest to ask whether this site was part of a larger regulatory unit. We have shown here that this is indeed the case since a NF1 binding site is critical for glucocorticoid regulation of the cAspAT gene promoter. The fact that the accessory factor in this case is NF1 is interesting because this factor is implicated in one of the best studied examples of gene regulation by steroid hormones, namely the mouse mammary tumor virus long terminal repeat promoter. In the latter case, the integrity of the NF1 site is required for efficient glucocorticoid regulation of the promoter (26, 27). Lately, it has been suggested that the chromatin structure is critical to understand the mechanisms by which the glucocorticoid receptor activates transcription (28–30). Indeed, one function of the GR is to bind to, and either disrupt or at least alter, the nucleosome structure at the mouse mammary tumor virus promoter, thus allowing NF1 to bind to DNA and activate transcription (31). In initial studies (4), only the binding of NF1 was detected in vivo after hormonal treatment, but other studies have detected the binding of both GR and NF1 (32). In the case of the cAspAT gene promoter, the inactivation of the NF1 site results not only in a decrease in the glucocorticoid effect but also in an increase in the basal activity of the promoter. Thus, the NF1 site exerts a negative effect on promoter activity in the absence of hormone. It is possible that one function of the activated glucocorticoid receptor would be to relieve this negative effect. However, we have no direct evidence that the effect of the NF1 site on promoter activity and its effect on the glucocorticoid regulation are linked.

Nuclear factor 1 has been shown to bind to several promoters and to collaborate with tissue-specific and regulated transcription factors. One interesting example is that of the eH1 site of the albumin enhancer (33). In this case, an NF1 site and a HNF3β site are in close apposition, but NF1 can inhibit transcriptional activation by HNF3β (8). In most cases, NF1 is a potent transcriptional activator (23, 34, 35). Thus, depending on the structure of the regulatory unit, NF1 can have either positive or negative effects. The mechanism by which NF1 exerts its negative effect on the cAspAT gene promoter, and actually allows glucocorticoid stimulation, is yet to be elucidated. It is noteworthy that the NF1 site is not in the immediate vicinity of the GRE A but is 80 base pairs upstream. However, this distance is optimal to bring proteins binding at those sites close together if a nucleosome is positioned on this region of the promoter.

Could NF1 contribute to the tissue specificity of the glucocorticoid regulation of the cAspAT gene? Clearly NF1 binding activity is ubiquitous. However, in addition to the initially discovered NF1, several NF1-like proteins bind to the same site (36–38). Some of these proteins are liver-specific as evidenced in EMSA by the presence of DNA-protein complexes specifically in this tissue (39). One possible model is that only the liver-specific NF1-like proteins could collaborate with the glucocorticoid receptor in the context of the cAspAT gene promoter.

The presence of different forms of NF1-like proteins and mRNAs has been shown in several studies. In one study, a NF1 site was shown to be critical to the activity of the adipocyte-
specific enhancer of the P2 gene (35). In a different example, the NF1 site of the collagen gene was shown to be implicated in the transforming growth factor β regulation of this gene as well as in its modulation by acetaldehyde (40). All these studies as well as ours demonstrate the contribution of NF1 or NF1-like proteins to various regulatory regions in gene promoters and enhancers.

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