The analysis of regulatory sequences required for high level induction of rat α_{2u} -globulin gene expression by dexamethasone

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ABSTRACT

The α_{2u} -globulins are the major urinary proteins in adult male rats. They are encoded by a gene family, the expression of which is under multihormonal control in the liver. Glucocorticoids are positive regulatory hormones and we have analyzed the contribution of 5'-upstream sequences to the induction by dexamethasone of two cloned members of the family transfected into mouse L-cells. The results demonstrate that sequences from -762 bp to -226 bp of clone 91 are required for the 24-fold level of induction that was observed. Addition of 5.5 kb of upstream sequence beyond -762 bp did not alter the level of induction significantly, whereas deletion of the DNA between -762 bp and -226 bp reduced inducibility to about 4-fold. Sequencing of this region revealed that an element, 5'-AGAACAgtTTCAAA-3', similar to the 15 bp consensus glucocorticoid

response element 5'-AGAACA_{nnn}TGTACC-3', is situated 513 bp upstream of the transcription start site. We infer that this element or its left half site is necessary for the dexamethasone-induced expression of clone 91 from the observation that a second gene, clone 2, that contained a base substitution at position 5 in the left half site was not inducible. It now appears that at least three distinct cis-acting regulatory regions, all of which bind to the glucocorticoid receptor *in vitro*, may contribute to the full induction of clone 91 by dexamethasone. These are: the distal upstream region identified by this study, a proximal upstream region that binds not only the receptor but also α_{2u} NF1, a constitutively expressed nuclear protein required for induction and a region within the fourth intron that contains five tandem receptor binding sites.

Journal of Molecular Endocrinology (1998) **20**, 129-139

INTRODUCTION

The regulation of gene expression by glucocorticoids has been the subject of intensive study ever since the pioneering work of Tomkins (Tomkins *et al.* 1966, Rousseau *et al.* 1973). Hormone-activated receptors bind specific DNA sequences, known as glucocorticoid response elements (GRE), which are typically present in the promoters of hormone-responsive genes (Payvar *et al.* 1983, Yamamoto 1985, Evans 1988, Beato *et al.* 1989, Godowski & Picard 1989, Mangelsdorf *et al.* 1995). Upon binding to the GRE, the receptor interacts with the transcription complex to either stimulate or suppress RNA transcription. The consensus sequence for the GRE is 5'-AGAACA_{nnn}TGTACC-3' (Truss & Beato 1993), but the half

site AGAACA as well as TGTGCC, which is related to the right half site of the 15 bp consensus, have both been shown to bind the receptor (Payvar *et al.* 1983, Scheidereit *et al.* 1983, Chan *et al.* 1991).

One of the best documented examples of glucocorticoid-regulated transcription is found in the mouse mammary tumor virus (MMTV). Inducibility is conferred by a region within 200 bp of the start of transcription (Chandler *et al.* 1983, Ponta *et al.* 1985) that includes a distal receptor binding site that is similar to the 15 bp consensus GRE and three proximal binding sites of sequence AGAACA (Payvar *et al.* 1983, Scheidereit *et al.* 1983). Mutations within or removal of any of the four binding sites substantially reduce the level of induction (Cato *et al.* 1988, Chalepakis *et al.* 1988),

implying that an interaction occurs *in vivo* between receptors bound to both the complete GRE and the half sites. Exhaustive mutagenesis of the 15 bp GRE has revealed that the receptor does not make contact with the third position of the GRE since mutations at this position in either half site were tolerated (Nordeen *et al.* 1990). However, inducibility is particularly dependent upon the remaining bases, especially within the left half element, AGAACA. For example, any change to the G at position 2 in this half site resulted in a loss of inducibility, whereas several changes at the corresponding position in the right half site were without effect (Nordeen *et al.* 1990).

We are interested in the control by glucocorticoids of the α_{2u} -globulin gene family in adult male rats and the contribution of the individual genes to hormone-induced changes in protein levels. The effects of hormones such as glucocorticoid, androgen and growth hormone *in vivo* have been well documented (Kurtz & Feigelson 1978, Roy 1979). Castration of male rats, which causes a deficiency of the sex hormones, results in decreased expression of α_{2u} -globulin. Treatment of castrated male rats with dexamethasone results in a rapid induction of α_{2u} -globulin synthesis. The level of expression falls back to the control level in 24 h. Administration of progesterone, which is known to compete for the glucocorticoid receptor, inhibits the induction of α_{2u} -globulin synthesis (Kurtz *et al.* 1978) and this suggests that dexamethasone induction is a receptor-mediated process. Since *in vitro* analysis has shown it to be somewhat delayed following the administration of dexamethasone (Addison & Kurtz 1986, Hess *et al.* 1990) and inhibited by cycloheximide and emetine (Chen & Feigelson 1979, Addison & Kurtz 1986, Hess *et al.* 1990), induction is most appropriately described as a secondary and not a primary response. In one of the earliest models describing steroid hormone action, secondary response genes were postulated to be activated by the products of primary response genes (Ashburner *et al.* 1973). Recent experiments on the action of ecdysone in *Drosophila* confirm the general features of this model but it is now clear that the class of secondary response genes is heterogeneous (for review, see Thummel 1995). Of particular interest in the context of the work to be described here are the 'early late' response genes of *Drosophila*, such as DHR3 and E78B, which are induced directly by the hormone but which also require ecdysone-induced protein synthesis for maximum induction.

Induction occurs mainly at the transcription level (Kulkarni *et al.* 1985) and several studies have been conducted to determine the regulatory sequences

and transcription factors involved. From the results, a complex picture is beginning to emerge. On the one hand, results from clone RAP 01 have shown that a region between -642 bp and -452 bp upstream of the transcription start site showed significant binding affinity to the glucocorticoid receptor *in vitro* (Van Dijck *et al.* 1987). When a fragment from clone 207 containing sequences between -639 bp and +1395 bp was introduced into transgenic mice, its transcription in the liver responded to hormones as in the male rat liver (Soares *et al.* 1987) and the expression of a hybrid coding region driven by this promoter region was efficiently induced by dexamethasone in castrated mice (Sakar & Feigelson 1989). Taken together, these data indicate that sequences between -452 bp and -640 bp play an important role in glucocorticoid induction and they suggest that a direct interaction of the hormone-receptor complex with the upstream genomic DNA may occur.

Studies on clone 91 suggest that additional elements of the cis-acting region that controls inducibility are located within 235 bp of the transcription start site. A full length version of this clone, which included about 10 kb of upstream sequence, was inducible by dexamethasone in mouse L-cells (Kurtz 1981) and a truncated clone containing 235 bp of the 5'-upstream region still responded to glucocorticoids (Kurtz *et al.* 1982). Reporter constructs driven by the promoter-proximal 235 bp of the upstream region also responded to the hormone although the level of induction varied considerably from 2- to 4-fold for a *tk* reporter (Kurtz *et al.* 1982) to about 17-fold for a CAT reporter (Schwartz & Kurtz 1996). Hormone responsiveness is correlated with receptor binding to a restriction fragment between -252 bp and -118 bp (Van Dijck *et al.* 1987) but none of the footprints formed by the proteins in a liver extract on DNA of the proximal upstream region (Addison & Kurtz 1986, Van Dijck *et al.* 1993) involves sequences that resemble either the 15 bp GRE consensus or its two half sites. Thus the significance of this binding is difficult to interpret at present.

To identify the cis-acting elements within the proximal region that conferred inducibility, linker scanning mutagenesis of the region was undertaken from which it was concluded that sequences between -115 bp and -160 bp were essential (Addison & Kurtz 1989). A protein, α_{2u} NF1, is responsible for the two footprints in this region and, interestingly, it is expressed constitutively and is not specific to the nuclear extracts of hormone treated cells (Addison & Kurtz 1989).

A third region within the fourth intron of clone 91 also appears to be a functional component of the

FIGURE 1. Restriction maps of the two α_{2u} -globulin genes, clones 91 and 2. The heavy line indicates the transcribed region.

regulatory system. A 206 bp fragment from the intron, which binds to the glucocorticoid receptor *in vitro*, bestows a delayed hormone responsiveness upon a heterologous promoter (Hess *et al.* 1990). The binding sites within the region do not comprise full length GREs but rather all consist of a family of hexamers whose consensus 5'-TGTGCC-3' is similar to the right half site of the GRE.

The nature of the interactions amongst the various components of the regulatory system for clone 91 is unclear as is the significance of the DNA binding of the receptor to three distinct genomic regions. In this study, the relative contributions of promoter proximal and distal elements to dexamethasone-induced RNA accumulation were examined by transfecting clone 91 constructs with variously deleted upstream regions into the dexamethasone-responsive mouse L-cells. A construct, from which all DNA upstream of -226 bp had been removed, exhibited 2- to 4-fold levels of induction, but full inducibility required the presence of elements between -226 bp and -762 bp. Sequencing of this region revealed the presence of two possible GREs and we present evidence that indicates only one is a functionally active component of the regulatory system.

MATERIALS AND METHODS

DNA manipulations

Bacteria and λ phage culturing, plasmid and λ phage DNA preparation and subcloning were performed according to standard methods (Sambrook *et al.* 1989).

Genomic library screening

A commercial rat genomic library in λ DASH II (Stratagene, La Jolla, CA, USA) was screened according to the manufacturer's instructions with an end-labeled oligonucleotide KF40, extending from -8 bp to +32 bp relative to the transcription start site of clone 91. Positive plaques were isolated and subjected to a further PCR screening using the oligonucleotides KWO1 (5'-CTTTATACTCTCTCTGGGTTT-3') and KWO3 (5'-AGATGACATCGCCAAGT-TTC-3'). These two primers are located in a promoter proximal region which is highly divergent in the 13 members of the α_{2u} -globulin gene family sequenced to date (W Addison, personal communication) and their sequence is identical to that of clone 91. The plaques were isolated and suspended in 12 μ l H₂O. Four microliters of this suspension were mixed with 3 μ l 10 $\times$ PCR buffer, 4 μ l 1 mM dNTP, 2 μ l 1 μ M KWO1, 2 μ l 1 μ M KWO3, 7 μ l 30 mM magnesium chloride, 0.5 μ l Taq DNA polymerase, and 7.5 μ l H₂O. PCR was performed under the following conditions: one cycle of 5 min at 95 $^{\circ}$ C, 1 min at 58 $^{\circ}$ C and 2 min at 72 $^{\circ}$ C, followed by 30 cycles of 1 min at 94 $^{\circ}$ C, 1 min at 58 $^{\circ}$ C and 2 min at 72 $^{\circ}$ C. Five λ clones produced bands of the predicted size and the inserts were subcloned into pBluescript KS+ for the purposes of restriction analysis and sequencing. Of these five, only pL21 shared with clone 91 an *Eco*RI site in the second exon (Fig. 1). Since sequence analysis of the highly variable region between -238 bp and +63 bp showed pL21 to be identical to clone 91, we concluded that it was indeed another isolate of this clone. Although pL21 contained only a partial coding sequence, it had a

FIGURE 2. Restriction maps of constructs containing the clone 91 α_{2u} -globulin gene and different lengths of 5'-upstream sequence. The vector used for all constructs was derived from pBR322 (Addison & Kurtz 1986). The bold line indicates the transcription unit. The 3'-downstream sequences are about 400 bp and the length of 5'-upstream sequence in each construct is indicated on the right.

much longer 5'-upstream region than the original isolate of clone 91 and therefore was fused to it to yield an extended upstream region (see below).

DNA sequencing

DNA in the proximal 5'-upstream region was sequenced with the T7 Sequencing Kit from Pharmacia Biotech (Baie d'Ufré, Québec, Canada) following the manufacturer's instructions. The primers used in sequencing were: T3 promoter primer, T7 promoter primer, KWO1, KWO3 and KWO2 (5'-CCTTCTCATATGGGCCTTCCA-3').

Plasmid construction

Plasmid 91R91 (Fig. 2) contains 762 bp of the 5'-upstream region, all of the coding region and 400 bp of the 3'-downstream region of the α_{2u} -globulin gene designated as clone 91 within a pBR322-derived vector (Addison & Kurtz 1986). This plasmid was used to construct three derivatives, 91RX2, 91RX3 and 91RX4 (Fig. 2), by replacing the 5'-upstream fragment beyond the

XbaI site in 91R91 (Fig. 2) with different 5'-upstream fragments from pL21 (see above). Plasmid 91 Da (Fig. 2) was constructed by truncating the 5'-upstream region in 91R91 immediately proximal to the *XbaI* site.

Transfection of mouse L-cells

Stable transfectants of mouse L tk⁻ aprt⁻ cells (Wigler *et al.* 1979) were obtained by a modification of the method of Kingston (1987). One day before transfection, confluent cells from one 100 mm culture plate were split 1:10 in Dulbecco's modified minimal medium (DMEM) supplemented with 10% calf serum. Two hours prior to transfection, the cells were fed with 9 ml of the same medium before being treated with 1 ml of a calcium phosphate precipitate (formed in HEPES, Kingston *et al.* 1990) containing 15 μ g plasmid DNA, 1 μ g neomycin resistant plasmid, pKOneo and 15 μ g L-cell carrier DNA. After 16 h incubation, the transfected cells were washed twice with PBS and then cultured for 48 h in DMEM supplemented with 10% calf serum and then in the same medium containing 400 μ g/ml geneticin for 2 weeks. After selection,

FIGURE 3. Detection of α_{2u} -globulin RNA by the S1 protection assay. Two independent cell lines, Da-1 and Da-2, obtained from transfection with 91 Da (see Fig. 2) and three independent cell lines obtained from transfection with 91R91: R9, R3 and R11, were treated with dexamethasone (+) or without dexamethasone (-) for 9 days as described in Materials and Methods. The S1 analysis was carried out with 25 μ g total RNA from these cell lines, as well as RNA from L-cells (L), liver RNA from an adult female rat (F), and an adult male rat (M). The protected 32 base fragment was cut out to quantitate α_{2u} -globulin RNA induction.

single colonies were isolated and cell lines were established in which the presence of α_{2u} -globulin DNA and transcribed RNA was confirmed by Southern blotting and S1 protection respectively.

Hormonal induction of α_{2u} -globulin RNA

Cell lines expressing α_{2u} -globulin RNA were maintained until confluence on DMEM+10% calf serum and then split 1:8 the day before induction. Two plates were used in each sample treatment. In the control group, cells were fed on DMEM+10% calf serum, and in the experimental group the medium was supplemented with 50 mM dexamethasone. The respective media were replaced every 2 days and the cells were harvested on the 9th day for RNA isolation. The duration of hormone treatment was chosen because a determination of the kinetics of RNA accumulation showed that following a 1 day lag, levels increased until about 9 days, after which a plateau was reached (Addison & Kurtz 1986).

RNA isolation and S1 protection assay

Total RNA was isolated from the cells in two 100 mm tissue culture plates following lysis in 4 M guanidinium and centrifugation through a CsCl step gradient (Kingston *et al.* 1991). The RNA (25 μ g) was annealed with the oligonucleotide KF40 labeled

with [γ - 32 P]ATP using T4 polynucleotide kinase. The oligonucleotide extends from -8 to +32 with respect to the start of transcription and the 32 base protected fragment was fractionated on a 15% acrylamide/urea denaturing gel, cut out and the radioactivity quantitated in a scintillation counter using the Cherenkov mode.

RESULTS

Full induction of clone 91 by dexamethasone requires 5' sequences upstream of -226 bp

To identify the sequences in the 5'-upstream region of clone 91 that are responsible for the induction of α_{2u} -globulin by glucocorticoids, additional 5'-upstream sequence of this clone was obtained by screening a genomic library in λ DASH II with clone 91 specific primers as described in Materials and Methods. A restriction map of clone 91 and clone 2, a second isolate which is discussed below, is shown in Fig. 1. Plasmids containing 226 bp to 6.3 kb of 5'-upstream sequences from clone 91 were constructed (Fig. 2), and used to transfect mouse L-cells as described in Materials and Methods. Cloned cell lines from transfectants of each construct were isolated and the presence of the α_{2u} -globulin gene was confirmed by Southern blotting (data not shown). The effect of dexamethasone on the level of α_{2u} -globulin RNA in several of

FIGURE 4. Effects of 5'-upstream sequence on the induction of α_{2u} -globulin RNA by dexamethasone. The average levels of induction are shown and for each construct the number of lines analyzed were respectively: 91 Da (226 bp), three lines; 91R91 (762 bp), five lines; 91RX3 (2.7 kb), five lines; 91RX2 (3.2 kb), five lines; 91RX4 (6.3 kb), six lines.

the positive cell lines was then examined using the S1 protection assay.

In the S1 protection assay, α_{2u} -globulin RNA was quantitated by the radioactivity present in a 32 base fragment, the protected part of the oligonucleotide KF40 that extends from -8 to +32 with respect to the start of transcription. The analysis of a subset of the transfected lines carrying two of the constructs (91 Da and 91R91) is shown in Fig. 3. The 40 base fragment that appears on the autoradiograph is not due to insufficient digestion with S1 nuclease. It is the protected product of an unknown RNA crossing the transcription initiation site of α_{2u} -globulin genes and has been detected previously (Addison & Kurtz 1986). This unknown transcript is present in the RNA of the transfected cell lines prior to the administration of hormone, and in the RNA of male but not female livers. It may correspond to a transcript that is 77 bp longer than the principal transcript that produces the 32 base fragment (Winderickx *et al.* 1987). Unlike the major transcript though, the accumulation of this unknown RNA is unaffected by either hormone treatment or by 5'-upstream sequence deletions. However, the presence of the 40 base fragment in S1 analyses of the transfected L-cells is a useful indicator that the introduced α_{2u} -globulin gene is integrated into a transcriptionally active and not a heterochromatic region of the genome. Some of the

FIGURE 5. The 5'-upstream sequence of clone 91. The sequence shown is that of the non-transcribed strand and the nucleotides are numbered from the transcription initiation site. The two possible GREs discussed in the text are underlined with arrows. The consensus GRE is AGAACAnnnTGTACC (Truss & Beato 1993).

cell lines transfected with plasmids 91RX2, 91RX3 and 91RX4 (Fig. 2) failed to express the longer transcript of the introduced gene and were not analyzed further.

The inducibility of α_{2u} -globulin RNA by dexamethasone, as measured by the amount of RNA accumulated in cell lines transfected with the different constructs (Fig. 2), is summarized in Fig. 4. Due to varying position effects and to differences in the copy number of α_{2u} -globulin genes at each of the different insertion sites, the level of induction differed among cell lines transfected with the same plasmid. For this reason, at least three integration sites were monitored for each construct. The data show that levels of α_{2u} -globulin RNA in cell lines transfected with constructs containing at least 762 bp of 5'-upstream sequence increase 16- to 24-fold following the addition of dexamethasone. In contrast, cell lines transfected with 91 Da, which contains 226 bp of 5'-upstream sequence exhibit a 4-fold accumulation of α_{2u} -globulin RNA. Since the α_{2u} -globulin gene in these cell lines was integrated into a transcriptionally active region, as indicated by the strong signal of the unknown RNA, it can be concluded that the loss of 5'-upstream sequences between -226 bp and -762 bp was responsible for this dramatic reduction in RNA inducibility.

Sequencing of the 5'-upstream region revealed that two sites similar to the consensus sequence of

FIGURE 6. Analysis of clone 2 α_{2u} -globulin RNA by the S1 protection assay. Five independent cell lines were transfected with constructs containing 2.7 kb 5'-upstream sequence (1, 2 and 3) or 762 bp of 5'-upstream sequence (4 and 5), the coding region and 400 bp of the 3'-downstream region of α_{2u} -globulin gene. Cells were treated with dexamethasone (+), or without dexamethasone (-) for 9 days as described in Materials and Methods. The analysis was carried out with 25 μ g total RNA from these cell lines. The protected 32 base fragment was cut out to quantitate RNA specific to the induction of α_{2u} -globulin.

the glucocorticoid receptor binding site were present (Fig. 5). The distal site at -640 bp contains a right half site that is identical to that of the consensus GRE, separated from the left half site by 2 bp, instead of the usual 3 bp spacer. The proximal site at -513 bp contains a perfect AGAACA left half site, a 3 bp spacer and a degenerate right half site. The sequence of clone 91 between -642 bp and -452 bp is identical to the same region in clone RAP 01, which has been demonstrated capable of binding glucocorticoid receptor *in vitro* (Van Dijk *et al.* 1987). From this, we conclude that DNA from this region of clone 91 is also capable of binding the glucocorticoid receptor and we infer that the decreased response of α_{2u} -globulin genes to dexamethasone in cell lines transfected with 91 Da, compared with the response in 91R91 transfected cells, is due to the loss of glucocorticoid receptor binding site(s) in this region.

Clone 2 is not inducible by dexamethasone

Clone 2 was obtained by screening the rat genomic library in λ DASH II as described in Materials and Methods. The restriction analysis showed that clone 2 was slightly different from clone 91 (Fig. 1). Two constructs were made which contained 2.7 kb and 762 bp of 5'-upstream sequence respectively, the

coding region and about 400 bp of the 3'-downstream region of clone 2. Several independent cell lines transfected with these constructs were examined for the accumulation of α_{2u} -globulin RNA under the same dexamethasone induction conditions described above for the clone 91 transfectants. None of these cell lines showed significant accumulation of α_{2u} -globulin RNA when treated with dexamethasone (Fig. 6). Sequencing of the 5'-upstream region of clone 2 revealed that from -719 bp to +52 bp, clone 2 differed from clone 91 at 45 bases. A comparison of the two clones in the region which contains the putative GREs is shown in Fig. 7. One of the base substitutions is at the AGAACA half site of the putative GRE at -513 bp. The sequence at the other possible GRE (-640 bp) is identical to that in clone 91.

Comparing the DNA sequence of clone 2 with that of clones RAO 01 and RAP 01 in the vicinity of the two possible glucocorticoid receptor binding sites reveals that clone 2 is identical to the uninducible clone RAO 01 (Fig. 7), although differences elsewhere suggest these clones represent different members of the gene family. The inducible clones 91 and RAP 01, which likewise appear to be different members of the family, are identical at the two putative GREs. Significantly, whereas RAP 01 exhibited binding of the glucocorticoid

FIGURE 7. Comparison of the 5'-upstream sequences in clones 91 and 2 with those in RAP 01 and RAO 01 (Van Dijk *et al.* 1993). Sequences are numbered according to the transcription initiation site and the two sequences with similarity to a GRE are indicated with arrows.

receptor *in vitro*, RAO 01 did not (Van Dijk *et al.* 1987).

DISCUSSION

In general, steroid hormones regulate the expression of a given gene by altering the rate of transcription. The hormone-activated receptors bind to the response element(s) in the vicinity of the gene (Mangelsdorf *et al.* 1995), and interact either directly (Tsai & O'Malley 1994) or via other bridging factor(s) (Halachmi *et al.* 1994, Cavailles *et al.* 1995) with the transcription initiation complex to either stimulate or suppress the transcription of the target gene. The glucocorticoid receptor appears to bind to clone 91 in three separate regions: a 206 bp fragment lying within the fourth intron (Hess *et al.* 1990), a proximal upstream region between -252 bp and -118 bp and a distal upstream region between -642 bp and -452 bp (Van Dijk *et al.* 1987). The intronic region contains five footprints of the glucocorticoid receptor and mediates intermediate levels of

reporter gene induction (Hess *et al.* 1990). The footprints are all found over hexanucleotides similar to the right half site of the GRE and a 29 bp subfragment comprising just one of the footprints conferred modest inducibility upon a linked reported gene (Chan *et al.* 1991). Although the induction had the characteristics of a secondary response, namely sensitivity to cycloheximide and delayed onset, it has been pointed out that α_{2u} -globulin transcription is uncharacteristically sensitive to cycloheximide and may not reflect the inhibition of protein synthesis *per se* (Addison & Kurtz 1986). Since the hormone responsiveness of reporter genes linked to the intronic region is not seen in transfectants of either the mouse L-cells used in this study or in a49 rat hepatoma cells (Schwartz & Kurtz 1996), the contribution of the intronic region to the *in vivo* response in the liver is uncertain. We observed a 4-fold increase of α_{2u} -globulin RNA following dexamethasone treatment in cell lines transfected with 91 Da, which contains sequences downstream of -226 bp (including the fourth intron binding sites). This suggests that the intronic region does not contribute significantly to the total induction response we observe, since the -235 bp fragment by itself conferred about twice this level of inducibility upon a linked reporter transfected into L-cells (Schwartz & Kurtz 1996).

Our data show that putative GREs at -640 bp and -513 bp are included within the 5'-upstream region of clone 91 between -762 bp and -226 bp that is necessary for the high level of induction in mouse L-cells. Addition of 5'-upstream sequences beyond -762 bp did not alter the level of induction significantly, whereas deletion of the DNA between -762 bp and -226 bp (plasmid 91 Da) dramatically reduced inducibility (Fig. 2). Further evidence that an intact GRE at -513 bp is necessary for full induction derives from the observation that clone 2 is non-responsive to dexamethasone (Fig. 6). Its -513 bp GRE differs from that found in the inducible clone 91 at one position in the left half site where the C of the consensus AGAACA has been replaced by an A (Fig. 7). The left half site of a GRE binds receptor monomers preferentially over the right half site (Tsai *et al.* 1988) and is more susceptible to mutations than is the right half site (Nordeen *et al.* 1990). In fact the C to A transversion that distinguishes clones 91 and 2 caused reduced induction by dexamethasone when it was introduced into the MMTV GRE driving a CAT reporter gene (Nordeen *et al.* 1990).

We noted above that the sequence of the putative GRE at -513 bp in clone 2 is identical to that in

clone RAO 01, which did not bind to glucocorticoid receptor *in vitro* (Van Dijck *et al.* 1987). Furthermore, RAO 01 does not exhibit the footprint seen over the -513 bp sequence in the inducible clone RAP 01 (Van Dijck *et al.* 1993), which shares with our inducible clone 91 the AGAACA half site. It is not known if this footprint, which is caused by a protein found in a male rat liver nuclear extract, can be attributed to the glucocorticoid receptor but the receptor does bind to the region between -642 bp and -452 bp in clone RAP 01 (Van Dijck *et al.* 1987). Thus the loss of inducibility of clone 2 is correlated with both an alteration in the proximal GRE and the loss of receptor binding *in vitro*. Since the sequence of the possible GRE at -640 bp is identical amongst the four clones, RAP 01, RAO 01, 91 and 2, two of which are inducible and two of which are unresponsive, we infer that the 5'-distal GRE is not an active binding site for the receptor, whereas the site at -513 bp is active.

We have postulated that the altered glucocorticoid receptor binding site at -513 bp in clone 2 is responsible for the observed loss of inducibility. But it is probable that other base substitutions also contribute to this loss. Four of the base changes that distinguish clone 2 from 91 are located within footprint 2 of the α_{2u} NF1 nuclear factor that binds to the -115 bp to -160 bp region (Addison & Kurtz 1989). Although footprint 2 is important for glucocorticoid-induced transcription, induction levels of 5- to 7-fold persist when it is mutagenized within a construct bearing the proximal 762 bp of the 5'-region (Addison & Kurtz 1989). By contrast, loss of α_{2u} NF1 footprint 1 eliminates induction completely (Addison & Kurtz 1989), but since its DNA sequence is identical in clones 2 and 91, we attribute the total loss of inducibility of clone 2 (Fig. 6) to the alterations in footprint 2 coupled with the change within the GRE at -513 bp.

The glucocorticoid induction of clone 91 has features of both a primary and a secondary response, and in this respect resembles the 'early late' genes of *Drosophila* (Thummel 1995). These are directly induced by the steroid ecdysone, but require protein synthesis for full expression. Likewise, the requirement of protein synthesis for full α_{2u} -globulin induction suggests that co-activators induced by the hormone are involved. While these co-activators may be DNA binding proteins, it is tempting to speculate that they interact with glucocorticoid receptor molecules bound to the upstream and/or intronic GREs.

Footprinting studies of the promoter proximal region have failed to identify any that are caused by dexamethasone-induced proteins (Addison & Kurtz 1989, Van Dijck *et al.* 1993). However, the

constitutively expressed nuclear protein, α_{2u} NF1, which is required for induction, binds to two sites in the -115 bp to -160 bp region (Addison & Kurtz 1989). It may interact with the basal transcription complex of the gene and may participate in protein-protein interactions with glucocorticoid receptor molecules. Although the region between -226 bp and +1 bp is devoid of binding sites for dexamethasone-inducible transcription factors (Addison & Kurtz 1989), it does confer secondary responsiveness upon a linked reporter gene (Schwartz & Kurtz 1996). Perhaps an as yet unidentified protein, induced by dexamethasone, binds to the constitutively synthesized α_{2u} NF1 molecules to potentiate their interaction with the receptor and the basal transcription complex. This would explain why no new footprints appear in the proximal region following induction. In this model, the role of the upstream GRE would be to upregulate the modest level of induction (4-fold) that we observe for the proximal region alone.

It is possible that the 5'-upstream sequence between -762 bp and -226 bp to which glucocorticoid receptor binding occurs may contain the binding sites for co-activators that are involved in the dexamethasone induction. A more detailed analysis of this 5'-upstream region is required to characterize the nature of any such factors; however, an interesting feature of the 5'-upstream sequence of clones 91 and 2 is the presence of three Stat (signal transducer and activator of transcription) binding sites. These occur at -508 bp and -393 bp (TTCN3GAA) and at -212 bp (TTCN4GAA). The first two sites are the binding sites for Stat1, Stat3, Stat5 and Stat6 and the third is a binding site for Stat6 (Ivashkiv 1995). Stat proteins are expressed widely among different tissues, and are major players in the cytokine signal transduction pathway which regulates many aspects of cell growth, differentiation and activation (Ihle 1996). It has been reported that the Stat5 proteins can interact with the glucocorticoid receptor and modulate its effects on the transcription from the MMTV-LTR promoter (Stocklin *et al.* 1996). Recently it has been shown that targeted disruption of the STAT5b gene in transgenic mice causes a reduction in the elevated level of the major urinary proteins (MUP) normally seen in males to the level seen in wild-type females (Udy *et al.* 1997). The MUPs are encoded by a multigene family and are thought to be the mouse homologs of the rat α_{2u} -globulin genes to which they are 66% identical (Clark *et al.* 1984). Whether or not Stat proteins affect the glucocorticoid-regulated transcription of the α_{2u} -globulin gene is currently under investigation.

ACKNOWLEDGEMENTS

The authors are indebted to Dr Bill Addison for encouraging one of us (K S W) to pursue this problem and for providing laboratory space. We thank D Hanahan for supplying the plasmid pKOneo and Warren Gallin and Sandra O'Keefe for critical reviews of the manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to Bill Addison and to R B H. Note that the 5-upstream sequences of clones 2 and 91 have been submitted to GenBank under accession numbers AF 013498 and AF 013499 respectively.

REFERENCES

- Addison WR & Kurtz DT 1986 Nucleotide sequences required for the regulation of a rat α_{2u} -globulin gene by glucocorticoids. *Molecular and Cellular Biology* **6** 2334–2346.
- Addison WR & Kurtz DT 1989 Identification of nuclear proteins that bind to the glucocorticoid regulatory region of a rat α_{2u} -globulin gene. *Journal of Biological Chemistry* **264** 21891–21895.
- Ashburner M, Chihara C, Meltzer P & Richards G 1973 Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symposium on Quantitative Biology* **38** 655–662.
- Beato M, Chalepakis G, Schauer M & Slater EP 1989 DNA regulatory elements for steroid hormones. *Journal of Steroid Biochemistry* **32** 737–748.
- Cato ACB, Skroch P, Weinmann J, Butkeraitis P & Ponta H 1988 DNA sequences outside the receptor-binding sites differentially modulate the responsiveness of the mouse mammary tumor virus promoter to various steroid hormones. *EMBO Journal* **7** 1403–1410.
- Cavaillès V, Dauvies S, L'Horsset F, Lopez G, Hoarc S, Kushner PJ & Parker MG 1995 Nuclear factor RIP140 modulates transcription activation by the estrogen receptor. *EMBO Journal* **14** 3741–3751.
- Chalepakis G, Arnemann J, Slater E, Bruller H-J, Gross B & Beato M 1988 Differential gene activation by glucocorticoids and progestins through the hormone regulatory element of mouse mammary tumor virus. *Cell* **53** 371–382.
- Chan GC-K, Hess P, Meenakshi T, Carlstedt-Duke J, Gustafsson J-A & Payvar F 1991 Delayed secondary glucocorticoid response elements. *Journal of Biological Chemistry* **266** 22634–22644.
- Chandler VL, Maler B & Yamamoto KR 1983 DNA sequences bound specifically by glucocorticoid receptor *in vitro* render a heterologous promoter hormone responsive *in vivo*. *Cell* **33** 489–499.
- Chen CC & Feigelson P 1979 Cycloheximide inhibition of hormonal induction of α_{2u} -globulin mRNA. *Proceedings of the National Academy of Sciences of the USA* **76** 2669–2673.
- Clark JA, Clissold PM, Shawi RA, Beattie P & Bishop J 1984 Structure of the mouse major urinary protein genes: different splicing configurations in the 3'-noncoding region. *EMBO Journal* **3** 1045–1052.
- Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889–895.
- Godowski PJ & Picard D 1989 Steroid receptors: how to be both a receptor and a transcription factor. *Biochemical Pharmacology* **38** 3135–3143.
- Halachmi S, Marden E, Martin G, MacKay I, Abbondanza C & Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264** 1455–1458.
- Hess P, Meenakshi T, Chan GC, Carlstedt-Duke J, Gustafsson J-A & Payvar F 1990 Purified glucocorticoid receptors bind selectively *in vitro* to a cloned DNA fragment that mediates a delayed secondary response to glucocorticoids *in vivo*. *Proceedings of the National Academy of Sciences of the USA* **87** 2564–2568.
- Ihle JN 1996 STATs: signal transducers and activators of transcription. *Cell* **84** 331–334.
- Ivashkiv LB 1995 Cytokines and STATs: how can signals achieve specificity? *Immunity* **3** 1–4.
- Kingston RE 1987 Stable transfer of genes into mammalian cells. In *Current Protocols in Molecular Biology*, pp 9.5.1–9.5.6. Eds FM Ausubel, R Brent, RE Kingston, DD Moore, JA Smith, JG Seidman & K Struhl. New York: John Wiley & Sons.
- Kingston RE, Chen CA & Okayama H 1990 Transfection of DNA into eukaryotic cells. In *Current Protocols in Molecular Biology*, pp 9.1.1–9.1.9. Eds FM Ausubel, R Brent, RE Kingston, DD Moore, JA Smith, JG Seidman & K Struhl. New York: John Wiley & Sons.
- Kingston RE, Chomczynski P & Sacchi N 1991 Guanidinium methods for total RNA preparation. In *Current Protocols in Molecular Biology*, pp 4.2.1–4.2.8. Eds FM Ausubel, R Brent, RE Kingston, DD Moore, JA Smith, JG Seidman & K Struhl. New York: John Wiley & Sons.
- Kulkarni AB, Gubits RM & Feigelson P 1985 Developmental and hormonal regulation of α_{2u} -globulin gene transcription. *Proceedings of the National Academy of Sciences of the USA* **82** 2579–2582.
- Kurtz DT 1981 Hormone inducibility of rat α_{2u} -globulin genes in transfected mouse cells. *Nature* **291** 629–631.
- Kurtz DT & Feigelson P 1978 Multihormonal control of the messenger RNA for the hepatic protein α_{2u} -globulin. In *Biochemical Actions of Hormones*, vol 5, pp 433–455. New York: Academic Press.
- Kurtz DT, Chan KM & Feigelson P 1978 Glucocorticoid induction of hepatic α_{2u} -globulin synthesis and messenger RNA level in castrated male rats *in vivo*. *Journal of Biological Chemistry* **253** 7886–7890.
- Kurtz DT, McCullough L, Bishop DK & Manos MM 1982 DNA sequences required for hormonal induction of rat α_{2u} -globulin gene. *Cold Spring Harbor Symposium on Quantitative Biology* **47** 985–988.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & Evans RM 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.
- Nordeen SK, Suh BJ, Kuhnel B & Hutchinson III CA 1990 Structural determinants of a glucocorticoid receptor recognition element. *Molecular Endocrinology* **4** 1866–1873.
- Payvar F, DeFranco D, Firestone GL, Edgar B, Wrangé O, Okret S, Gustafsson J-A & Yamamoto KR 1983 Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. *Cell* **35** 381–392.
- Ponta H, Kennedy N, Skroch P, Hynes NE & Groner B 1985 Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. *Proceedings of the National Academy of Sciences of the USA* **82** 1020–1024.

- Rousseau GG, Baxter JD, Higgins ST & Tomkins GM 1973 Steroid-induced nuclear binding of glucocorticoid receptors in intact hepatoma cells. *Journal of Molecular Biology* **79** 539–554.
- Roy AK 1979 Hormonal regulation of α_{2u} -globulin in rat liver. In *Biochemical Actions of Hormones*, vol 6, pp 481–517. New York: Academic Press.
- Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sarkar P & Feigelson P 1989 Developmentally regulated male specific transfactor(s) enable *in vitro* transcription of a cloned α_{2u} -globulin gene. *Molecular Endocrinology* **3** 342–348.
- Scheidereit C, Geisse S, Westphal HM & Beato M 1983 The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. *Nature* **304** 749–752.
- Schwartz DA & Kurtz DT 1996 Sequence requirements for secondary glucocorticoid inducibility of rat α_{2u} -globulin genes. *Molecular and Cellular Endocrinology* **120** 153–159.
- Soares VC, Gubits RM, Feigelson P & Costantini F 1987 Tissue-specific and hormonally regulated expression of a rat of α_{2u} -globulin gene in transgenic mice. *Molecular and Cellular Biology* **7** 3749–3758.
- Stocklin E, Wissler M, Gouilleux F & Groner B 1996 Functional interactions between Stat5 and the glucocorticoid receptor. *Nature* **383** 726–728.
- Thummel CS 1995 From embryogenesis to metamorphosis: the regulation and function of Drosophila nuclear receptor superfamily members. *Cell* **83** 871–877.
- Tomkins GM, Thompson EB, Hayashi S, Gelehrter T, Granner D & Peterkofsky B 1966 Tyrosine transaminase induction in mammalian cells in tissue culture. *Cold Spring Harbor Symposium on Quantitative Biology* **31** 349–360.
- Truss M & Beato M 1993 Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrine Reviews* **14** 459–479.
- Tsai MJ & O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Review of Biochemistry* **63** 451–489.
- Tsai SY, Carlstedt-Duke J, Weigel NL, Dahlman K, Gustafsson J-A, Tsai MJ & O'Malley BW 1988 Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. *Cell* **55** 361–369.
- Udy GB, Towers RP, Snell RG, Wilkins RJ, Park S-H, Ram PA, Waxman DJ & Davey HW 1997 Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proceedings of the National Academy of Sciences of the USA* **94** 7239–7244.
- Van Dijk P, Lemaigre FP, Winderickx J, Rousseau GG & Verhoeven G 1987 Glucocorticoid receptor binding to defined regions of α_{2u} -globulin gene. *Biochemical and Biophysical Research Communications* **149** 1099–1105.
- Van Dijk P, Vos PD, Winderickx J & Verhoeven G 1993 Multiple binding sites for nuclear factors in the 5'-upstream region of two α_{2u} -globulin genes: implications for hormone-regulated and tissue-specific control. *Journal of Steroid Biochemistry and Molecular Biology* **45** 353–366.
- Wigler M, Pellicer S, Silverstein S, Axel R, Urlaub G & Chasin L 1979 DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proceedings of the National Academy of Sciences of the USA* **76** 1373–1376.
- Winderickx J, van Dijk P, Dirckx L, Volckaert G, Rombauts W, Heyns W & Verhoeven G 1987 Comparison of the 5' upstream putative regulatory sequences of three members of the α_{2u} -globulin gene family. *European Journal of Biochemistry* **165** 521–529.
- Yamamoto KR 1985 Steroid receptor regulated transcription of specific genes and gene networks. *Annual Review of Genetics* **19** 209–252.

REVISED MANUSCRIPT RECEIVED 25 July 1997