Purified glucocorticoid receptors bind selectively *in vitro* to a cloned DNA fragment that mediates a delayed secondary response to glucocorticoids *in vivo*

(specific protein–DNA interactions/delayed secondary glucocorticoid response element/ α_{2u} -globulin gene/late or indirect responses)

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ABSTRACT We have identified and characterized a 206-base-pair region downstream from rat α_{2u} -globulin promoter that specifically mediates a delayed secondary response to glucocorticoids. Unlike positive primary glucocorticoid response elements (GREs), this regulatory element, termed delayed sGRE, dictates an inductive process preceded by a time lag of several hours and blocked by the protein synthesis inhibitor cycloheximide. Reminiscent of GREs and negative GREs (nGREs), a delayed sGRE confers hormonal regulation upon a linked heterologous promoter from a downstream position with respect to transcription start site and, remarkably, also interacts selectively with purified glucocorticoid receptor. These results imply that receptor binding to a delayed sGRE *in vivo* may mediate certain secondary responses to glucocorticoid hormones.

Steroid hormones associate specifically with receptor proteins, form hormone–receptor complexes, and regulate the coordinated generation of a limited number of rapid primary (p) responses and a larger number of secondary (s) responses (1–3). s Responses are considered indirect responses; the hormone-induced accumulation of s-response mRNAs (4–8), but not p-response (9) mRNAs, is blocked by inhibitors of protein synthesis. At least two classes of s response are distinguishable. For most s responses, hormone-induced alterations in the level of s-response mRNAs and proteins follow a time lag of several hours (1, 4–7). Other s responses resemble p responses by displaying little or no time lag but are blocked by protein synthesis inhibitors (ref. 8 and references therein). We refer to these s responses as *delayed secondary* and *early secondary* responses, respectively.

Studies in several systems have characterized DNA sequences within or near p-response genes that bind specifically to steroid hormone receptors *in vitro* and rapidly alter transcription rates *in vivo* (3, 10). For example, fragments of mouse mammary tumor virus (MTV) DNA that selectively bind to glucocorticoid receptors *in vitro* (11, 12) are positive glucocorticoid response elements (GREs) functioning as receptor-dependent transcriptional enhancer elements *in vivo* (13, 14). In contrast, DNA sequences upstream of certain glucocorticoid repressed genes, also recognized by glucocorticoid receptors, act as negative GREs (nGREs; refs. 10, 15, 16). We will refer to DNA sequences that mediate the early and delayed s response to glucocorticoids as *early sGRE* and *delayed sGRE* (where sGRE indicates secondary GRE), respectively.

Previous investigations of rat α_{2u} -globulin (RUG) gene expression revealed that induction by glucocorticoid hor-

mones (i) is a delayed s response (4–6), (ii) acts at the transcriptional level (18), and (iii) appears to depend on sequences within RUG gene and/or flanking DNA (6). Therefore, according to our definition, segments within and/or near this gene must function as a delayed sGRE.

Although the phenomenon of delayed s response has been elegantly described in *Drosophila* (1, 19) and clearly documented in mammalian cells (4–7), the location and functional properties of delayed sGREs have not been defined. Delayed sGREs may affect transcription rates by acting as any one of the elements of transcriptional response (20). Thus, for example, they might overlap a promoter, be situated at a relatively fixed distance from a promoter, or act from a distance in a position- and orientation-independent manner. Because of the *indirect* nature of the delayed s response, delayed sGREs need not specifically bind the steroid receptor; instead, they could be controlled by putative p-response proteins (pRP) whose genes themselves are regulated directly by GREs/nGREs. Conceivably, activity of certain delayed sGREs could involve pRP and/or other factors as well as direct binding to glucocorticoid receptor (1, 3, 4, 17, 19).

Mutational analysis has led to the proposal that DNA sequences between 115 and 160 base pairs (bp) upstream of the RUG promoter, by binding to a hypothetical steroid hormone-induced protein, act as the delayed sGRE essential for RUG's s response to glucocorticoids (6). Alternatively, those mutated sequences may act in a dominant fashion to uncouple the interaction of upstream/downstream delayed sGREs with the RUG promoter (see ref. 21 and the citations therein). We have tested various RUG DNA fragments for delayed sGRE activity by methods that preserved the intactness of promoter sequences. We show that certain RUG DNA fragments, distinct from the RUG promoter, bind selectively to purified receptors *in vitro* and confer delayed s responsiveness upon a heterologous promoter and gene *in vivo*.

RESULTS

Relative Selectivities and Efficiencies of Receptor Binding.

Titration experiments. Previous studies have suggested that the RUG/flanking DNA fragment ≈ -3000 to $+5500$ (portion shown in Fig. 1A), when introduced into the genome of mouse L cells, is responsive to glucocorticoids (26). Therefore, we examined various RUG DNA fragments spanning

Abbreviations: GRE, positive primary glucocorticoid response element; nGRE, negative GRE; sGRE, secondary GRE; RUG, rat α_{2u} -globulin; MTV, mammary tumor virus; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; Dex, dexamethasone; p and s responses, primary and secondary responses; pRP, p-response proteins.

FIG. 1. A region downstream of RUG promoter interacts selectively with purified glucocorticoid receptor and contains multiple receptor footprint sites. (A) Restriction endonuclease map of RUG genomic DNA, gene 91 (22). Solid line, 5' and 3' flanking sequences; open box, exons and introns; dotted arrow, apparent start site and direction of transcription (6); X, *Xba* I; S, *Sal* I; Y, *Sty* I; V, *Pvu* II; P, *Ssp* I; R, *Eco*RI. (B) Selectivity of receptor binding to RUG-SR [32 P]DNA: Titration and competition. Lanes 1 and 2, samples not bound to filters and containing 60% and 30% of input radioactivity, respectively. Lanes 3–7, filter-bound incubations in the absence (lanes 3) or presence (ref. 23; lanes 4–7) of receptor. (a) Mixture of labeled RUG DNA fragment SR and labeled pBR322 RR, *Nde* I (D)/R, and S/D fragments in the presence of 30, 60, and 120 ng of receptor (lanes 4–6). (b) As in a, 120 ng of receptor incubation with 5, 12.5, 25, and 50 ng of labeled DNA (lanes 4–7). (c) Labeled R digest of pA2 (25 ng; ref. 24) consisting of MTV (upper band) and pML DNA (lower band) fragments (lanes 1–2) in the presence of 50, 25, and 15 ng of receptor (lanes 4–6). (d) Twelve and one-half nanograms of end-labeled mixture of RUG-SR (upper band), pML-RR and pML-SS DNA (comigrate as lower band), and various fold mass excesses (x) of unlabeled *Xho* I fragments from tubulin (pA14; ref. 25) DNA incubated with 35 ng of receptor (lanes 4–7). The pA14 contents were as follows: lanes 1–4, 1.5x; lane 5, 5x; lane 6, 20x; lane 7, 100x. (e) End-labeled DNA fragments as in d first incubated in the absence (lane 3) or the presence (lanes 4–7) of receptor, subsequently adjusted to contain calf thymus DNA in excess of labeled DNA. Incubations were continued for 30 min at 4°C. Mass excesses of calf thymus DNA in reactions were as follows: lanes 1–4, 1.5x; lane 5, 1000x; lane 6, 2000x; lane 7, 3000x. (C) Localization of DNase I footprints of glucocorticoid receptor bound to the upper-strand DNA sequences between +1800 and +2150. G, probe DNA subjected to guanosine-specific cleavage; 1, no receptor; 2–4, 30, 40, and 60 ng of receptor; 1–4, 4 pmol of DNA. Protected sequences of >10 bp are bracketed; dots, protected regions of <6 bp; arrows, certain prominent regions of enhanced cleavage. Identical footprints were seen with four preparations of receptor in seven independent experiments.

this region for their ability to interact selectively with glucocorticoid receptor. Many such regions were observed, pointing to a complex arrangement of receptor binding sites. In this report our focus is the RUG DNA fragment SR ($\approx +1800$ to $\approx +5500$) containing a segment of RUG gene and the 3' flanking sequences (Fig. 1A).

The receptor–DNA interactions were monitored by nitrocellulose filter binding assay (12). After filter binding, the bound fragments were eluted and analyzed on agarose gels. To estimate the recovery of DNA, reaction mixtures containing fractional amounts of input DNA were incubated in the absence of receptor and, without filter binding, analyzed on the same agarose gel. Control experiments showed that under these conditions, DNA fragments known to interact specifically with receptors were selectively retained on the filters. Thus, as previously demonstrated (12, 24), of the mixture of MTV and pML vector DNA fragments, only the MTV DNA is retained by the filters selectively, efficiently, and in a receptor-dosage-dependent manner (Fig. 1B, c). At highest receptor input, typically 50–75% of input radioactivity is bound and recovered. In contrast, in the absence of added protein, only a small amount of DNA (0.1–0.5% of input radioactivity) was retained by the filters. When the RUG-SR DNA fragment was tested in the same experiment, added purified glucocorticoid receptor prompted the preferential retention of the RUG-SR fragment relative to three

pBR322 fragments (Fig. 1B, a). Approximately twice as much receptor was required for retention of $\approx 50\%$ of RUG-SR than for retention of equivalent amounts of MTV-RR. This suggests that although the relative efficiencies of selective receptor binding to MTV-RR and RUG-SR are distinguishable, they are within the same order of magnitude. The same experiment examined the selectivity and efficiency of receptor–RUG DNA interaction by labeled DNA titration performed at a fixed concentration of receptor (Fig. 1B, b). The results demonstrate that selectivity of receptor binding to RUG-SR fragment is maintained even under conditions of excess receptor.

Competition experiments. To further monitor the selectivity of receptor interaction with RUG sequences, two types of DNA competition experiments were performed. In the first, an end-labeled mixture of RUG-SR, pML-RR, and pML-SS (Fig. 1B, d) was mixed with increasing amounts of an unlabeled *Xho* I fragment of tubulin genomic DNA that resides in the recombinant plasmid pA14 (25) and incubated with a fixed amount of receptor. Note that the labeled *Eco*RI (R) and *Sal*I (S) fragments of pML are of identical size (lower band) and control for possible difference in relative affinity of receptor for R and S staggered ends. The pML vector and tubulin genomic DNA act as negative-control sequences of bacterial and rat origin, respectively. Under the conditions of 1.5-fold mass excess of competitor DNA, there is a relatively

low degree of selectivity in the interaction of receptor with RUG DNA. As little as 5-fold mass excess of the competitor DNA substantially improved the apparent selectivity of receptor–RUG DNA interactions without reducing their efficiency. In contrast, the reduction in efficiency of receptor for interaction with RUG DNA requires substantially greater amount of competitor DNA—for example, the sample shown in lane 7 of Fig. 1*B*, *d*, contains ≈ 100 -fold mass excess of competitor DNA.

In the same experiment, a fixed amount of receptor and end-labeled DNA fragments was incubated to equilibrium; aliquots were removed and incubated in the presence of increasing amounts of denatured calf thymus DNA (Fig. 1*B*, *e*). As in the previous experiment, addition of 5-fold mass excess of competitor DNA abolished nonspecific binding, whereas, after addition of 3000-fold mass excess of competitor, the RUG–SR fragment could still be recovered. Notably, it was previously demonstrated that interaction of receptor with MTV DNA was ≈ 1000 -fold more efficient than with pBR322 DNA (24). Taken together, it appears that the relative efficiency of interaction of the purified rat glucocorticoid receptors with RUG or MTV DNA is several orders of magnitude greater than with DNA sequences that originate from bacterial plasmids, calf thymus, or from a rat tubulin gene and its flanking segments.

Footprinting of a Specific Receptor Binding Region. Additional nitrocellulose filter binding experiments suggested that sequences between $\approx +1800$ and $\approx +2900$ account, to a major extent, for the specificity of receptor–RUG DNA interaction (not shown). This facilitated the examination of the selectivity of receptor–RUG DNA interactions by DNase I footprinting (12). As one strategy, the top strand proximal to S (Fig. 1*A*) was labeled with ^{32}P . These fragments were exposed to purified receptor and subjected to mild digestion with DNase I, and the products were displayed on sequencing gels (Fig. 1*C*). Seven footprints were detected between $\approx +1800$ and $+2150$ bp downstream of the transcription start site. Receptor displayed similar but distinguishable efficiencies at different binding sites—for example, footprint 1 is 2- to 4-fold stronger than footprint 4. Additional footprinting experiments, examining other segments of RUG DNA (e.g., $+2150$ to $\approx +3900$), revealed additional binding regions; only one region bound the receptor as efficiently as the region shown in Fig. 1*C*. In general, the range of apparent affinities is similar to that observed on MTV DNA with the same receptor preparations (not shown; ref. 12).

A Fragment of RUG DNA Confers Delayed Secondary Glucocorticoid Responsiveness upon a Linked Heterologous Promoter and Gene. Previous studies have suggested that multiple GREs, residing near (13) as well as within transcribed regions (27) of MTV, are responsible for mediation of primary response to glucocorticoid hormones (3). To localize and functionally characterize the delayed sGRE of the RUG gene and to investigate the biological significance of selective receptor–RUG DNA interactions, a number of recombinants were constructed with the plasmid pOTCO (Fig. 2*A*). Nine RUG DNA restriction fragments, spanning -3000 to $+5500$, were inserted at position -109 and/or $+1689$ of pOTCO. To reliably and reproducibly monitor the delayed s response, constructs were stably transfected (29–31) into various cell lines of liver and nonliver origin. In at least two cell lines, RUG DNA fragments that selectively bind to receptors *in vitro* also specifically mediated the response to glucocorticoids *in vivo*. Initial experiments suggested that a region of RUG DNA including nucleotides $\approx +1800$ to $+3900$ is most active. Analysis of 12 subfragments of this region identified the 206-bp SV fragment as the smallest fragment whose activity most resembles that of the intact $+1800$ to $+3900$ parent fragment. In contrast, RUG DNA sequences that do not bind to receptor efficiently *in vitro* (e.g., $\approx +300$ to $+700$) and actin DNA fail

FIG. 2. Chloramphenicol acetyltransferase (CAT) activity is rendered hormone-inducible in mouse mammary cell line Mm5MT bearing stably integrated copies of a TK–CAT–RUG fusion construct. (A) Upper, as in Fig. 1*A*. At least five recognition sites for purified glucocorticoid–receptor complexes (two circles) have been detected within the 206-bp SV subfragment (Fig. 1*C*). Lower, restriction maps of pOTCO, pOTCU206.1, and pGTCS sequences stably integrated in DNA of cell lines MOTCO.P, MOTCU206.1.C6, and MGTCs.C12, respectively. C, *Cla*I; G, *Bgl*II; B, *Bam*HI; other symbols as in Fig. 1. Plasmid pOTCO is an enhancerless herpes simplex virus thymidine kinase (TK) promoter–CAT fusion plasmid (28); pOTCU206.1, insertion of RUG 206-bp SV in pOTCO downstream of SV40 poly(A) addition segment (An) at $+1689$ in forward orientation; pGTCS, insertion of GRE-containing MTV and murine sarcoma virus (MSV) DNA fragments upstream of TK promoter and at $+1689$ of pOTCO, respectively. Copy number and structural integrity of the recombinant elements were determined (ref. 13; not shown). In each cell line, at least one integration event has left an intact copy of TK–CAT/insert fusion. (B) Autoradiograms showing CAT assays representative of untransfected mouse mammary Mm5MT cells (M, set d), transfected population (P), and clones (C) grown for 24 hr in the absence (–) or presence (+) of $0.5 \mu\text{M}$ dexamethasone (Dex); cultured monolayers were maintained in logarithmic growth phase in Dulbecco's modified Eagle's medium lacking phenol red and supplemented with 5% defined fetal bovine serum. $\diamond$, [^{14}C]Chloramphenicol; $\triangleright$, acetylated chloramphenicol. Similar results were obtained in independent experiments (two to four for MGTCs, two to five for MOTCU206.1 and two or three for MOTCO).

to mediate hormonal response *in vivo*. In this report, our focus is the mouse mammary cell line MOTCU206.1 bearing stably integrated copies of pOTCU206.1 in its genome (Fig. 2*A*). To serve as negative and positive p-response controls, respectively (Fig. 2*A*), the same parental mammary cells were transfected with pOTCO and pGTCS, resulting in cells MOTCO and MGTCs. Pooled extracts from 50–100 transfected colonies as well as extracts from several clonal isolates were analyzed for CAT activity (28, 33).

As expected, CAT activity is not detectable in the untransfected cells (Fig. 2*B*, *d*, M) and is minimally affected by glucocorticoids in MOTCO cells (Fig. 2*B*, *c*; 1.1- to 2.0-fold). In MGTCs cells, the transfected population is induced 7.4-fold and clonal isolates are induced 10- and 11-fold (Fig. 2*B*, *a*). Notably, 11-fold regulated expression was also observed in the MOTCU206.1 cells (Fig. 2*B*, *b*) as well as in individual clones (C4, 14-fold; C6, 18-fold). We next monitored the kinetics of induction of CAT activity after various periods of exposure to Dex. As before, CAT activity in MOTCO cells is minimally affected by hormonal treatment (Fig. 3*C*; Fig. 3*D*, *C*). In MGTCs cells, however, the relative CAT activity

FIG. 3. RUG DNA dictates delayed glucocorticoid hormone responsiveness upon linked CAT gene. Autoradiograms MGTC5.C12 (A), MOTCU206.1.C6 (B), and MOTCO.P (C) show CAT assays representative of stable transfectants grown in the absence or presence of added Dex for indicated periods of time. The time scale for A and B is on top of A and that for C is on top of C. Duplicate time points in C represent CAT activity of two separate extracts from parallel experiments. (D) Fold induction of relative CAT activity (% of chloramphenicol converted to acetylated form per 50 μ g of extracted protein in 180 min) as a function of time after Dex treatment. Data were obtained by averaging two independent experiments; measurements are in the linear response range for CAT activities, for autoradiographic exposures, and of the densitometer.

increases continuously throughout the induction period, beginning at 2 hr (Fig. 3A; Fig. 3D, A), with near maximal expression achieved 12–18 hr after addition of hormone. In striking contrast to MGTC5 cells, there is an initial time lag of 6–10 hr in MOTCU206.1 cells (Fig. 3B; Fig. 3D, B), followed by a rapid induction paralleling that of MGTC5. Induction in the transfected population plus three additional clonal isolates is also delayed by 6–10 hr (not shown), suggesting that duration of time lag is minimally affected by genomic integration site. These data demonstrate that the characterized individual clones are representative of the transfected population. In addition, subsegments of 206-bp SV fragment, containing either receptor footprints 1–3 or 4 and 5 (Fig. 1C), after a delay of $\approx$ 10 hr, rendered CAT activity 7- to 9-fold and 4- to 6-fold inducible by glucocorticoids, respectively. Thus, our findings suggest that the SV fragment of RUG gene contains at least two components that dictate delayed glucocorticoid responsiveness upon the linked CAT gene.

To assess whether the induction of CAT activity might result from modulation of the linked upstream TK promoter, accurately initiated TK-CAT mRNA was monitored by RNase mapping (34). Relative transcript levels were calculated by normalization to internal control mRNAs expressed from the endogenous ERP72 (35) gene, which is not glucocorticoid regulated (Fig. 4). In MGTC5 and in MOTCU206.1 (Fig. 4, lanes 7, 8, 11, and 12), Dex increased the production of correctly initiated TK-CAT mRNA; this is induced to a greater extent than the relatively smaller amount of induction of incorrectly initiated (read-through; RT) transcripts. We assume the latter transcripts to have arisen from DNA sequences within our constructs (32) as well as from sequences flanking the genomic integration sites. The same transcripts are not induced in MOTCO cells (Fig. 4, lanes 3–6) and are not detected in untransfected cells (Fig. 4, lanes 1 and 2).

Examination of sensitivity of transcript production to an inhibitor of protein synthesis revealed that the induction of TK-CAT mRNA in MGTC5 is not blocked by cycloheximide (Fig. 4, lanes 9 and 10). In fact, cycloheximide significantly increased the basal and the induced relative transcript levels. In contrast, induction of TK-CAT RNA in MOTCU206.1 is blocked by cycloheximide (Fig. 4, lanes 13 and 14).

In the present context, our results are consistent with the notion that the 206-bp SV fragment, originating from the

FIG. 4. RUG DNA confers glucocorticoid hormone regulation upon a linked heterologous promoter. Glucocorticoid-induced increase in transcript production is blocked by cycloheximide. Untransfected cells (M), transfected population (P), and transfected clones (C) were treated (+) or without (-) added 0.5 mM Dex and 0.3 μ g of cycloheximide (Ch) per ml for 7 days. Metabolic labeling with [35 S]methionine (not shown) indicated that such treatment resulted in 60–73% inhibition of protein synthesis. Total cellular RNA was isolated (36) and 5' ends were analyzed by RNase protection (34). Autoradiographs representative of RNase protection assay are shown. The positions of the 302-nucleotide probe fragments protected by accurately initiated transcripts (TK-CAT; 203 nucleotides), read-through transcripts (RT; 279 nucleotides), and internal control (ERP72) are indicated and diagrammed below. "Probes," a mixture of TK-CAT and ERP72 probes lacking RNA and not subjected to RNase digestion. The table reports the densitometric quantitation of TK-CAT transcripts (37); intensity of the protected bands is linearly related to amount of input RNA in the 10- to 100- μ g range. All values are normalized to the internal control and presented as relative transcript level (RTL). Quantitation of ERP72 was based on autoradiographic exposure times that differ from that presented. Thus, cross-comparison of RTLs may not be visually confirmable by the above autoradiogram. +/-, Fold induction; nd, not detected. Similar results were obtained in two additional independent experiments.

fourth intron of the RUG gene, containing multiple *in vitro* receptor footprint sites, acts as a delayed sGRE *in vivo*. This delayed sGRE is distinct from the RUG promoter, acts on a heterologous promoter, acts from long range (only one position and orientation was tested here), and increases transcript production from preexisting initiation sites. These results raise the possibility that delayed sGRE interactions with receptor are intimately involved in mediation of delayed s response *in vivo*.

DISCUSSION

Delayed sGREs appear to have evolved to coordinate and temporally amplify a limited number of rapid primary responses mediated by GREs and nGREs. The anticipated diversity of s responses is likely to be reflected in the functional differences among s-hormone-response elements. Thus, delayed sGREs may mediate hormonal response by transcriptional and/or posttranscriptional mechanisms. In fact, previous work demonstrated that exonic sequences of growth hormone gene act as delayed sGRE to stabilize growth hormone mRNA (7).

DNA sequences that mediate p and s responses can coreside near and within a transcriptional unit (7). Previous studies have not clarified whether the mediation of the glucocorticoid response by RUG gene/flanking segments containing promoter mutations (6), or by a DNA fragment containing the RUG promoter (38), is a p response, an early s response, or a delayed s response.

In this work, we employed purified glucocorticoid receptors, cloned DNA fragments from a delayed s-responsive gene, and stable transfection assays to localize and functionally characterize a delayed sGRE. Our findings indicate that a RUG intronic DNA fragment that interacts selectively with purified glucocorticoid receptor *in vitro* acts from long range to confer delayed secondary glucocorticoid responsiveness upon a heterologous promoter when stably introduced into cultured cells. Given that RUG DNA fragments confer delayed s responsiveness on production of correctly initiated transcripts from downstream of CAT gene or upstream of TK promoter (this report and unpublished), it is tempting to assume that they act, primarily, by affecting transcriptional events. However, this needs to be directly tested.

We envision three disparate mechanisms whereby receptor-sGRE interactions can be involved in delayed enhancement of transcription: receptor may act (i) as a "positive regulator"—productive binding of receptor to sGRE is influenced by pRP and/or other factors; (ii) as a "block" or a "negative regulator"—pRP/other gene products function, in part, by reversing the repressive receptor-sGRE interactions; (iii) both as a negative and a positive regulator—repressive engagement of receptors at delayed sGRE is somehow altered to have inductive consequence.

Clearly, more direct assays are needed to establish any functional importance of receptor binding sites, contained within the delayed sGRE, in the mediation of delayed s response. Thus, it will be crucial to determine whether nucleotide contacts required for receptor footprint formation are also essential for delayed sGRE activity. In any case, additional mutational analysis should help to define in detail the delayed sGRE nucleotides necessary for delayed response, cycloheximide sensitivity, and possibly for specific binding to regulatory factors.

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