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Glucocorticoid receptor binds cooperatively to adjacent recognition sites

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In order to define the mechanism of synergistic induction mediated by multiple glucocorticoid response elements (GRE), the affinity of the glucocorticoid receptor to a single or duplicated GRE was analyzed by gel retardation, nitrocellulose filter binding and by footprinting experiments. Direct measurement of the relative affinity and indirect determination by competition showed >10-fold higher affinity of the glucocorticoid receptor to a duplicated GRE when compared to a single element. Maximal stability of the GRE–receptor complex was obtained using two closely spaced GREs positioned on the same side of the DNA helix. Increasing the distance or changing the helical position of the GREs considerably increased the off rate of the receptor. DNase I footprinting shows in addition to the protection of the GRE region, an altered pattern in the nonprotected intervening DNA indicating structural alteration of the DNA helix by the receptor bound to adjacent GREs.

Key words: glucocorticoid receptor binding/cooperativity/ eukaryotic expression system/vaccinia virus

Introduction

Steroid hormone receptors are conditional transcription activator proteins (Yamamoto, 1985; Evans, 1988; Beato, 1989). Native receptors are inactive in the absence of their cognate hormones and bind with high affinity and specificity to their target sites only after the ligand is bound to the receptor, a step which was called 'activation' or 'transformation' (Jensen *et al.*, 1968). This was confirmed by genomic footprinting studies which demonstrated that the hormone responsive element of the tyrosine amino transferase gene was occupied *in vivo* only in the presence of glucocorticoids (Becker *et al.*, 1986). However, hormone-independent interaction of glucocorticoid receptor with specific binding sites in crude cytosol has also been described (Willmann and Beato, 1986). Recent analysis has shown that hormone binding is required to transform the receptor from an inactive complex with the heat shock protein hsp90 to the active state in which the receptor is able to bind to specific DNA sequences (Joab *et al.*, 1984; Housley *et al.*, 1985; Sanchez *et al.*, 1987; Groyer *et al.*, 1987; Denis *et al.*, 1988; Pratt *et al.*, 1988). Deletion of the hormone-binding domain results in a weakly but constitutively active glucocorticoid receptor (Godowski *et al.*, 1987; Hollenberg *et al.*, 1987).

Steroid receptors specifically interact as dimers (Tsai *et al.*, 1988; Kumar and Chambon, 1988) with short partial or perfect palindromes of 15 bp length (Scheidereit and Beato, 1984; Karin *et al.*, 1984; Strähle *et al.*, 1987; Klock *et al.*, 1987; Klein-Hitpass *et al.*, 1988b). In some cases such as the long terminal repeat (LTR) of the murine mammary tumor virus (MMTV) (Scheidereit and Beato, 1984; Buetti and Kühnel, 1986) and the rat tyrosine amino transferase (TAT) gene (Jantzen *et al.*, 1987), a clustering of glucocorticoid response elements (GRE) has been observed. The importance of such a linkage of GREs for the magnitude of the hormone response has been demonstrated for these two cases. Similar observations have been made on the estrogen response element (ERE) of the chicken vitellogenin gene (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988a). In addition, duplication of a GRE has been shown to increase the rate of transcription from an adjacent promoter by at least one order of magnitude, when compared to a single element (Strähle *et al.*, 1988; Schüle *et al.*, 1988).

An appealing explanation for this functional synergism is cooperative binding of the receptor to GREs. Cooperative binding is well documented for the λ repressor (Hochschild and Ptashne, 1986; Griffith *et al.*, 1986 and the lac repressor (Besse *et al.*, 1986; Krämer *et al.*, 1986; Borowiec *et al.*, 1986; Mossing and Record, 1986). Cooperativity of binding has also been described for the interaction of the heat shock activator protein with the two adjacent heat shock response elements of the *Drosophila hsp*70 gene (Topol *et al.*, 1985) and for a transcription factor binding to two tandem repeats in the SV40 enhancer (Davidson *et al.*, 1988). Electron microscopic analysis of purified progesterone receptor binding to clustered sites of the uteroglobin gene showed formation of DNA loops which most likely resulted from receptor interaction (Theveny *et al.*, 1987).

We used the gel retardation assay and nitrocellulose filter retention to analyze the interaction of the glucocorticoid receptor to a single or a duplicated GRE. As a source of receptor, we used unfractionated liver cytosol or cytosol prepared from HeLa cells expressing high levels of receptor, as a result of infection with a recombinant vaccinia virus containing glucocorticoid receptor cDNA. The latter system enabled the use of nitrocellulose filter binding for determination of the off rate of receptor–GRE complex and analysis of binding in DNase I footprinting experiments. Our results demonstrate that the synergism between two GREs observed at the level of transcription (Strähle *et al.*, 1988) can probably be explained by cooperative binding of the receptor to adjacent GREs.

Results

The glucocorticoid receptor binds much more strongly to a duplicated GRE than to a single GRE

Cytosol was prepared from rat livers, or from HeLa cells which had been infected with a vaccinia recombinant vector

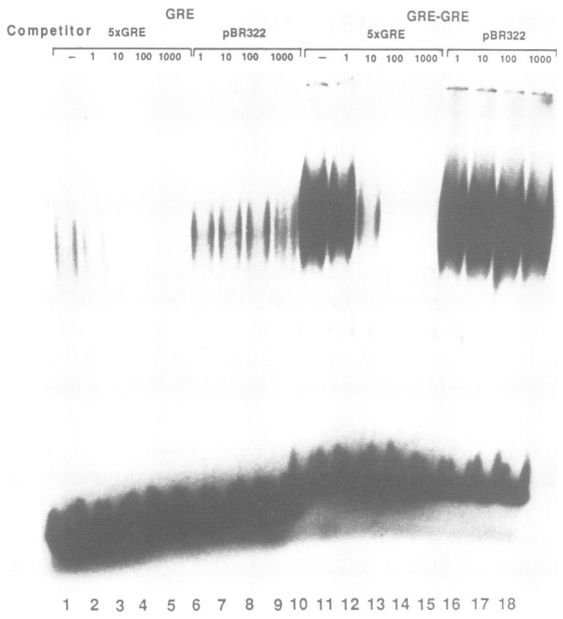

Fig. 1. Glucocorticoid receptor binding is much stronger to a duplicated GRE than to a single GRE. 10 fmol of a fragment containing one GRE (lanes 1–9) or two GREs (lanes 10–18) which were labeled to the same specific activity, were incubated with 3 μl of HeLa cell cytosol prepared from cells infected with recombinant vaccinia virus in the absence or presence of increasing concentrations of competitor DNA. The competitors used were a restriction fragment containing five GREs (lanes 2–5 and lanes 11–14) or pBR322 DNA digested with *Alu*I (lanes 6–9 and lanes 15–18). Amounts of competitor DNA are given as ng DNA.

carrying the rat glucocorticoid receptor cDNA (Miesfeld *et al.*, 1984), under control of the late promoter of the 11K protein gene (Stunnenberg *et al.*, 1988). Aliquots of the cytosol preparations containing 10^{-7} M dexamethasone were incubated at 25°C with end-labeled restriction fragments carrying either one or two GREs. The GRE sequence was identical to the 15 bp imperfect palindrome of GRE II of the rat liver tyrosine amino transferase (TAT) gene (Jantzen *et al.*, 1987) and flanked by 25 bp of vector sequence. When duplicated, the GREs were separated by a 6 bp *Spe*I restriction site, so the center to center distance was 21 bp (GRE21GRE, Figure 5A; Strähle *et al.*, 1988). Therefore, the two GREs faced the same side of the DNA helix. Binding was analyzed by electrophoresis at room temperature on a 3% non-denaturing polyacrylamide gel (Fried and Crothers, 1981; Garner and Revzin, 1981).

In the experiment shown in Figure 1, end-labeled fragments containing one GRE (lanes 1–9) or two GREs (lanes 10–18) were incubated with cytosol prepared from HeLa cells infected with recombinant vaccinia virus. The amount of radioactivity in the retarded fragment carrying a GRE dimer (lane 10) was 15-fold higher than in the corresponding fragment containing a single GRE (lane 1). A small but reproducible size difference between the retarded complexes formed with either the single or the duplicated GRE fragment was detected. The diffuse appearance did not allow the detection of an additional band migrating at the position of a single bound GRE, when a fragment carrying two GREs was used. However, kinetic experiments as shown in Figure 5 allowed the conclusion that, on the duplicated GRE, occupancy of both binding sites is prevalent. A fragment carrying five copies of the GRE II of the TAT gene was a strong competitor for receptor and hence reduced

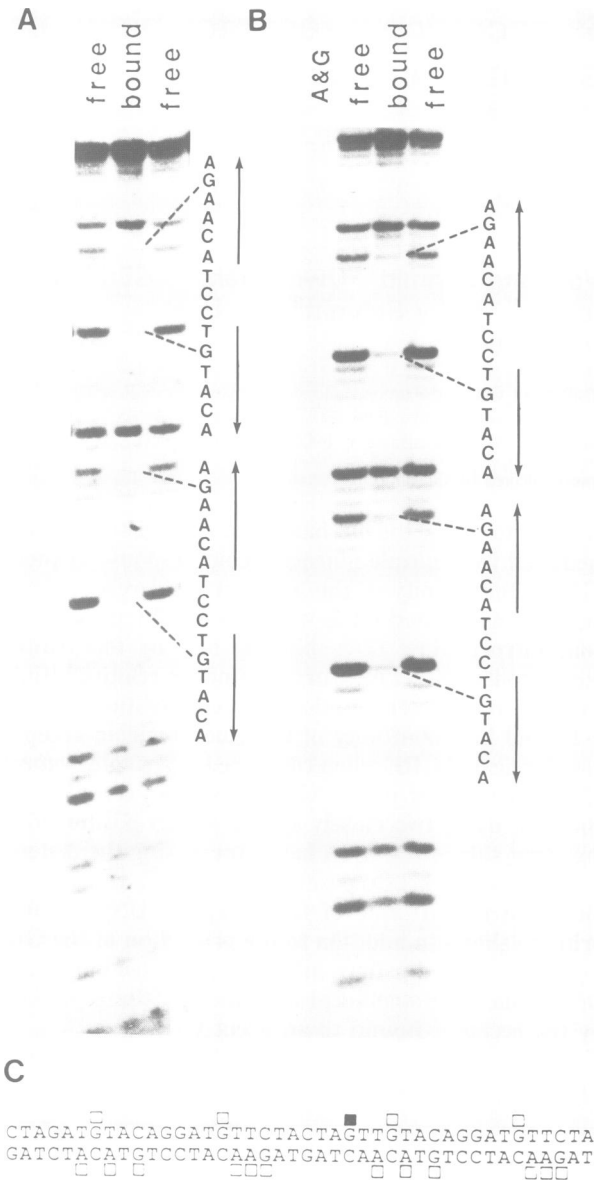

Fig. 2. Methylation interference analysis of GRE bound proteins show only contacts specific for glucocorticoid receptor binding. 40 μl of cytosol prepared from HeLa cells infected with recombinant virus or rat liver were incubated with 100 fmol of a partially methylated end-labeled DNA fragment containing two GREs. Retarded fragments were isolated, cleaved by piperidine treatment and analyzed on sequencing gel. **A)** Recombinant vaccinia virus infected HeLa cell, DMS interference pattern of the upper strand. **B)** Rat liver cytosol, DMS interference pattern of the upper strand. **C)** Summary of protections and enhancements on both strands. Purine contact sites are indicated by open squares, increased cleavage by filled squares.

band-shift complexes containing either one or two GREs (compare lanes 2–5 with lanes 11–14). In contrast, no competition was seen by increasing concentrations of pBR322 DNA (lanes 6–9 and lanes 15–18). No equivalent band-shifts were observed in cytosol prepared from cells infected with wild type virus (data not shown). Incubation with liver cytosol resulted in much weaker shifts migrating at identical position (data not shown).

The specificity of competition for the GRE-containing fragments as well as the absence of equivalent band-shifts in cytosol prepared from cells infected with wild type virus (data not shown) argued that binding of the glucocorticoid receptor was responsible for the retarded complexes

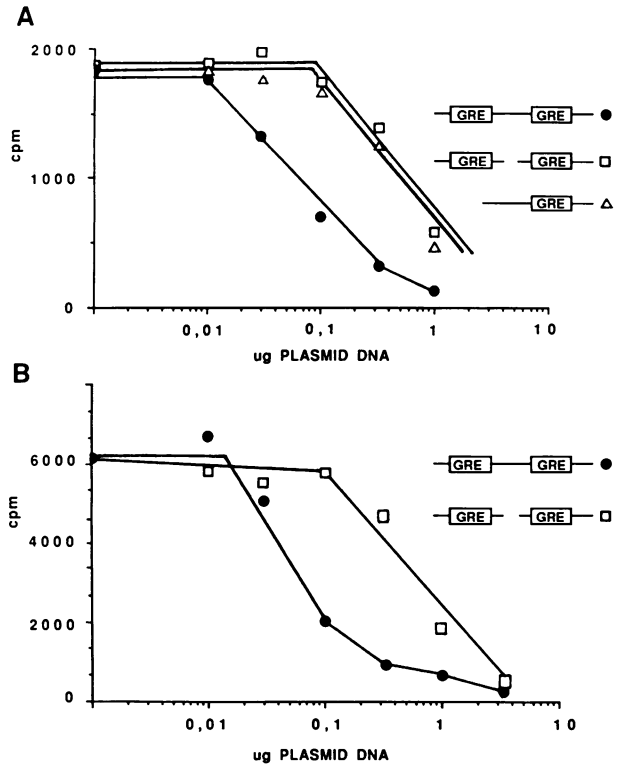

Fig. 3. Linked GREs are much stronger competitors than unlinked GREs. 50 fmol of end-labeled fragment containing two GREs (GRE21GRE, see Figure 5A) were incubated with 40 μl of cytosol at 25°C in the absence or presence of increasing concentrations of competitor DNA. The competitor DNA was *Eco*RI digested plasmid DNA containing a duplicated GRE (GRE21GRE, filled dots), GRE21GRE cut with *Eco*RI and *Spe*I (open squares), and *Eco*RI digested plasmid containing a single GRE (open triangles). Competitor DNA was made equimolar in amount of GRE and vector sequences by addition of vector cleaved with *Eco*RI. **A)** Competition experiment using rat liver cytosol. **B)** Competition experiment using cytosol prepared from HeLa cells infected with recombinant vaccinia virus.

observed. For a further confirmation of the sequences responsible for the binding, dimethyl sulfate (DMS) interference experiments were performed. A labeled fragment containing a duplicated GRE was partially methylated by exposure to DMS prior to incubation with the receptor containing cytosol. Bound and free DNA was separated, as in the other band-shift experiments, by gel electrophoresis. Then the DNA was cleaved at the methylated purines by treatment with piperidine. The resulting ladder of fragments was analyzed on a sequencing gel. Figure 2 shows that interference of binding was detectable only at positions known to be contacted by the glucocorticoid receptor (Scheidereit and Beato, 1984; Becker *et al.*, 1986; Tsai *et al.*, 1988), providing further evidence that the retardation of the labeled fragment is due to binding of the glucocorticoid receptor. There was no difference in the interference pattern between receptor prepared from cells infected with vaccinia recombinant virus (Figure 2A) or from rat liver (Figure 2B). Figure 2C summarizes the results of the analysis obtained from both strands.

Two adjacent GREs compete for receptor binding much more strongly than a single GRE

Comparison of the amount of shifted fragments containing either one or two GREs (Figure 1) indicated ~15-fold stronger binding of the receptor to fragments containing a

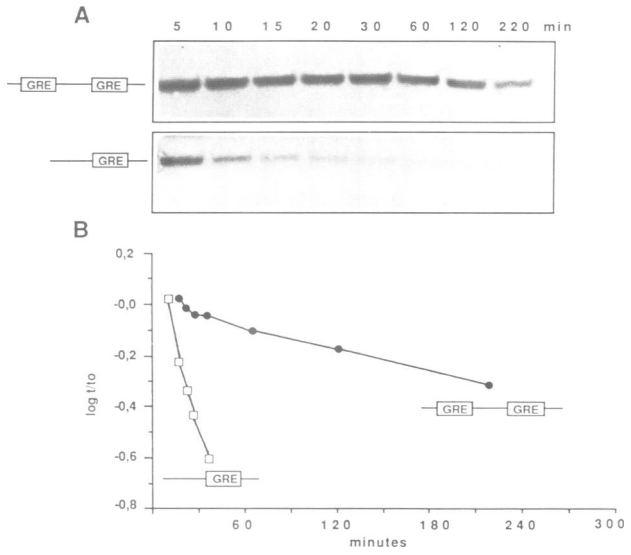

Fig. 4. Determination of the half life of the glucocorticoid receptor–GRE complex. 100 fmol of an end-labeled fragment containing a single GRE plus 50 fmol of an end-labeled fragment containing a duplicated GRE (GRE21GRE, see Figure 5A) were incubated at 25°C with 40 μl cytosol prepared from HeLa cells infected with recombinant vaccinia virus. After 30 min of incubation, the assay was challenged by the addition of 50 μg of plasmid GRE21GRE. After the time intervals indicated, aliquots were passed through nitrocellulose filters and filter-bound DNA analyzed by PAGE and subsequent autoradiography. **A)** Duplicated GRE (GRE21GRE, upper autoradiogram, 1.5 h exposure) and single GRE (lower autoradiogram, 25 h exposure). **B)** Semilogarithmic plot of the relative amount of receptor–DNA complex versus time after start of competition.

duplicated GRE than to fragments containing a single GRE. To obtain additional and independent evidence for cooperative binding, the relative binding strength was measured by comparing the amount of retarded receptor–DNA complex after competition with a single or a duplicated GRE. For that purpose, a labeled fragment containing two GREs was incubated with liver cytosol and increasing amounts of competitor DNA. Two types of competitor DNA were used: plasmid DNA containing two GREs (separated by an *Spe*I site, see Figure 5A, GRE21GRE) was either cut with *Eco*RI alone, leaving the GREs linked, or additionally with *Spe*I, separating the GREs. Furthermore , to exclude an effect on receptor binding of the eccentric position of the GRE resulting from restriction with *Spe*I, a construct containing a single GRE in the centre of the resulting *Eco*RI fragment was also used as competitor DNA. All competitor preparations were made equimolar with respect to both GRE and vector sequences. The results of the competition experiment are depicted in Figure 3A. Competition by linked GREs was >10-fold stronger than competition by equimolar concentrations of a fragment containing a single GRE. The position of the GRE within the competitor fragment had no effect on binding. Similar results were obtained when receptor expressed from recombinant vaccinia virus was used (Figure 3B).

The stability of the receptor complex formed with a duplicated GRE is much higher than stability of the complex formed with a single GRE

The stability of the GRE–receptor complex was determined by nitrocellulose filter retention (Riggs *et al.*, 1970). For this purpose, end-labeled fragments containing either one,

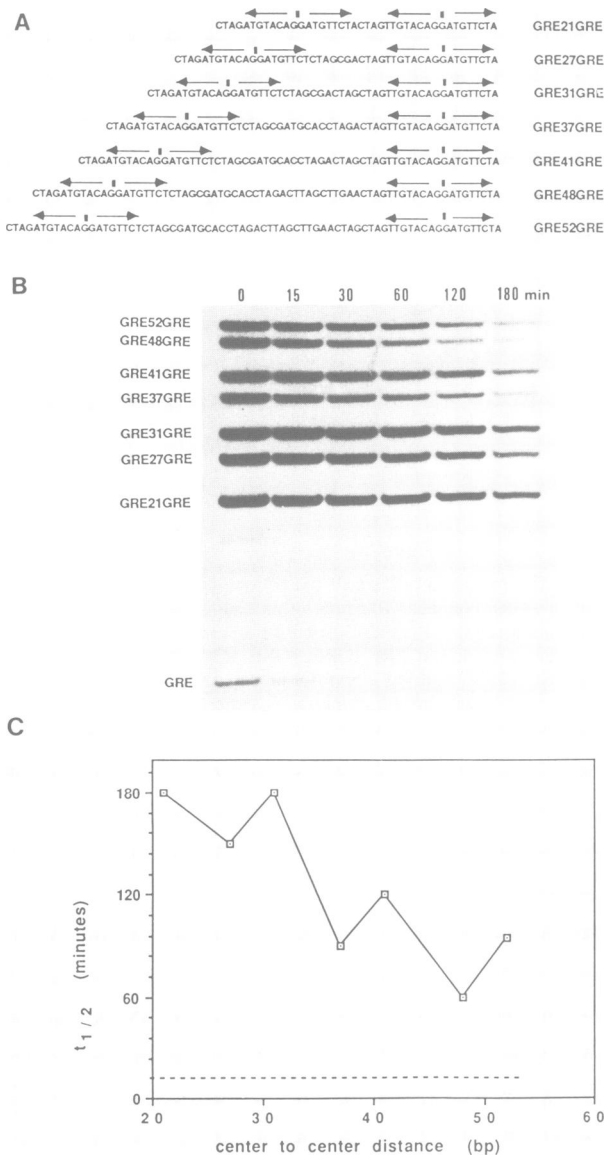

Fig. 5. Half-life of the glucocorticoid receptor–GRE complex is dependent on distance and helical position of the two GREs. 50 fmol end-labeled fragments of each of the duplicated GREs and 500 fmol of the single GRE were used for the nitrocellulose retention assay described in Figure 4A. **A)** Sequence of the distance variants. **B)** Autoradiographic analysis of the DNA retained on nitrocellulose filters after increasing time of competition. **C)** Dependence of $t_{1/2}$ on variation of center to center distance between adjacent GREs. $t_{1/2}$ of the single GRE is given by the broken line.

two or no GREs were incubated at 25°C with receptor expressed from recombinant vaccinia virus. After a binding period of 30 min, the GRE–receptor complex was challenged by a 1000-fold molar excess of plasmid DNA containing two linked GREs, thus sequestering free receptor onto this large excess of unlabeled competitor DNA. After increasing time intervals, receptor–DNA complexes were isolated by adsorption onto nitrocellulose filters and DNA was eluted from the filter by treatment with SDS. The amount of labeled DNA was determined after PAGE by autoradiography (Figure 4A) and liquid scintillation counting (Figure 4B). Binding to the single GRE was weak (note the different exposure time used) and rapidly disappeared, reaching background values after 30 min of competition. In contrast, retention of the complex formed between the

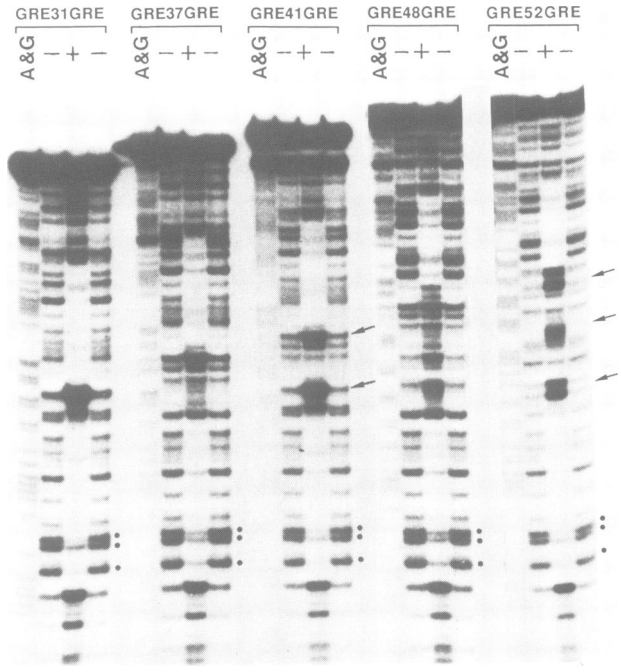

Fig. 6. DNase I footprinting shows protection of the linked GREs and enhancements in the intervening DNA. The upper strand of the distance variants indicated was asymmetrically end-labeled and used for DNase I footprinting. Cytosol prepared from HeLa cells infected with either wild type (−) or recombinant vaccinia virus (+) was used for footprinting. The location of the GRE consensus sequence is given for GRE31GRE by brackets. For further details, see text.

duplicated GRE and receptor was initially ~30-fold higher and only moderately reduced, even after 220 min of competition. Vector DNA was not detectably retained on the nitrocellulose filter (not shown). For a quantitative determination, the numerical values obtained by liquid scintillation counting of the band excised or by scanning of the autoradiograms were plotted as log(bound t/bound t_0) versus t, and half-life ($t_{1/2}$) was determined (Figure 4B). $t_{1/2}$ of the single GRE–receptor complex was 12–15 min and the $t_{1/2}$ of the duplicated GRE–receptor complex was 200 min, thus exceeding the value of the single GRE by more than one order of magnitude. This is a minimal estimate assuming that there is no inactivation of receptor during the time of the assay. Inspection of the slope of the duplicated GRE shows that it is dominated by the slowly dissociating component. There might be a more rapid decrease at early time points indicative of the presence of a small percentage of fragments which have only one GRE occupied and which therefore have a similar off rate to the single GRE–receptor complex. But it is evident that occupancy of both GREs on the same fragment is by far prevailing.

Binding of the receptor to a duplicated GRE is dependent on distance and helical position and leads to an altered conformation of the DNA between the adjacent GREs

If the cooperativity is due to direct protein–protein contacts, one might expect that binding to a duplicated GRE is influenced by distance and helical alignment of the two GREs. We therefore tested the effect of increasing distance between two GREs on the stability of GRE–receptor complex. The increments of distance were about half a helical turn; the sequences of the constructs used are given

in Figure 5A. End-labeled fragments were incubated as in the previous experiment, and the half-life was determined as in Figure 4. The autoradiogram is shown in Figure 5B. For further evaluation, $t_{1/2}$ of the different GRE–receptor complexes was determined and plotted versus the center to center distance between the adjacent GREs (Figure 5C). As evident, stability of the duplicated GRE–receptor complex decreases with increasing distance between the two GREs. When the two GREs are positioned on opposite sides of the helix the rate of dissociation is significantly increased further. For comparison, the $t_{1/2}$ of the single GRE determined in the same experiment is also given (Figure 5C, broken line).

The influence of distance and helical position on the stability of the duplicated GRE–receptor complex could reflect energy required for stretching of the receptor proteins and/or distortion of the DNA located between the GREs. To look for possible conformational changes, interaction of receptor with variably spaced GREs was analyzed by DNase I footprinting (Figure 6). The GREs were strongly protected, though protection was somewhat weaker for the GRE48-GRE, consistent with the lower stability of receptor binding. The region protected exceeds the region of the GRE consensus sequence (indicated by brackets for GRE31GRE) by 5 bp on either side. In addition to the protection of the GREs, there was increased cleavage of the intervening DNA. When the GREs were close together on the same side of the helix (GRE31GRE), there was a strongly increased cleavage of the DNA between the two GREs. If distance between the GREs was increased by one (GRE41GRE) or two helical turns (GRE52GRE) these enhanced cleavage sites (indicated by arrows) occurred at 11 bp intervals. Other cleavage sites which were also detectable on naked DNA were not affected. No regular pattern was observed when the two GREs were placed on opposite sides of the helix (GRE37GRE and GRE48GRE). In this situation, enhanced cleavage was observed at most of the cleavage sites. There was a systematic pattern of protections outside the GREs far away from the intervening DNA (indicated by dots). This pattern differed from constructs in which the two GREs are on opposite sides of the helix (GRE37GRE and GRE48GRE), as opposed to those in which the GREs face the same side (GRE31GRE, GRE41GRE, and GRE52GRE).

Discussion

We previously reported that multimerization of a GRE generates a very potent glucocorticoid-inducible element which is equally active from a far upstream position or immediately upstream of the TATA box (Strähle *et al.*, 1988). This synergism was highly suggestive of cooperative binding of the receptor to adjacent recognition sequences, analogous to the procaryotic araC protein (Martin *et al.*, 1986; Hamilton *et al.*, 1988; Lee and Schleif, 1989), the lac repressor (Besse *et al.*, 1986; Mossing and Record, 1986) and the λ repressor (Hochschild and Ptashne, 1986; Griffith *et al.*, 1986).

To estimate the affinity of the glucocorticoid receptor to single and duplicated GREs in cellular extracts, we used the gel retardation assay (Fried and Crothers, 1981; Garner and Revzin, 1981) and nitrocellulose filter binding analysis (Riggs *et al.*, 1970). For the following reasons we believe that the interactions we see represent binding of the glucocorticoid receptor to its recognition site.

(i) In HeLa cells, whose receptor concentration is too low to result in a band-shift under the conditions used, a shift is seen only after transfection with a recombinant vaccinia virus expressing a glucocorticoid receptor cDNA. No band-shift is seen with extracts made after infection with wild-type virus. This vaccinia expression system leads to very high receptor levels and low unspecific binding, making it very suitable for *in vitro* assays. Similar shifts are also observed using liver cytosol, but the amount of retarded fragment is much lower.

(ii) The band-shift can be inhibited by competing with DNA carrying a GRE insert but not by equivalent amounts of unrelated DNA.

(iii) Methylation interference studies show interaction only at positions known to be important for glucocorticoid receptor binding (Scheidereit and Beato, 1984; Becker *et al.*, 1986; Tsai *et al.*, 1988).

On the basis of these observations we believe that the diffuse band represents a receptor-dependent mobility shift. Our conclusion that the glucocorticoid receptor interacts cooperatively with adjacent GREs rests on the following observations.

(i) A fragment containing two GREs is retarded much more strongly than a fragment containing only a single GRE. The amount of shifted fragment is at least one order of magnitude higher with a fragment containing two GREs.

(ii) Indirect determination of affinity by competition experiments consistently shows >10-fold higher affinity of glucocorticoid receptor to a duplicated GRE than to the single GRE.

(iii) Determination of the stability of the GRE–glucocorticoid receptor complex by nitrocellulose filter retention shows a $t_{1/2}$ of 180–200 min for the glucocorticoid receptor complex with a duplicated GRE. This is >10-fold higher than the $t_{1/2}$ of the complex formed with a single GRE which has a $t_{1/2}$ of 12–15 min. These results imply an increased affinity of the receptor to the duplicated GRE of similar magnitude under equilibrium conditions. This is based on the assumption that the actual amount of GRE–receptor complex is dependent primarily on the off rate of the GRE–receptor complex. This assumption is consistent with the data obtained by gel retardation which also reflect equilibrium conditions.

(iv) Stability of the duplicated GRE–receptor complex is dependent on the intervening distance and the relative helical position of the two GREs.

(v) DNase I footprinting experiments show a distortion of the DNA between the two receptor binding sites when distance is increased.

The lower stability observed when the center to center distance between the two GREs exceeds three helical turns can be explained by the energy required for the structural alteration of the intervening DNA. Remarkably, stability of these complexes is still considerably higher than stability of the complex formed between receptor and a single GRE. Cooperative binding, which was not influenced by helical position has been observed for the GAL4 protein (Giniger and Ptashne, 1988) but no quantitative determinations have been made. Positional effects are also known from other systems. The requirement of stereospecific alignments of the enhancer and the 21 bp repeat region for initiation from the SV40 promoter has been described (Takahashi *et al.*, 1986). An effect of helical position has been observed for the

cooperative binding of λ repressor (Hochschild and Ptashne, 1986), and for the formation of the arabinose repression loop (Lee and Schleif, 1989).

The glucocorticoid receptor and the estrogen receptor each form dimers which bind to their respective palindromic recognition sequences (Tsai *et al.*, 1988; Kumar and Chambon, 1988). Electron microscope analysis of progesterone receptor binding to sites on the uteroglobin gene and on the MMTV LTR has shown that loops are formed between binding sites separated by <100 bp. The size of the receptor oligomers was not precisely determined, but the average size of the proteins present on a binding site forming a loop was about twice that of the proteins bound to a single binding site (Theveny *et al.*, 1987). This suggests that a receptor tetramer is interacting with two adjacent GRE/PREs. Homotetramers lead to formation of a DNA loop between adjacent binding sites, as shown by electron microscopy, at the λ promoter (Griffith *et al.*, 1986), the lac promoter (Krämer *et al.*, 1986), and, possibly, on the arabinose promoter (Hendrickson and Schleif, 1985; Martin *et al.*, 1986; Hamilton and Lee, 1988).

Cooperative binding leads to high occupancy at low concentration of the binding protein, and to a nonlinear concentration dependence. In the critical range, relatively mild changes in the concentration of the binding protein, or—in the case of ligand-controlled binding—of ligand concentration cause strong changes of occupancy of the binding sites (Ptashne, 1986). Therefore genes with multiple binding sites for proteins capable of cooperative interaction can show a strong response at comparably low protein concentrations or at low ligand concentrations. This may explain different responses to hormones by genes carrying either a single hormone receptor binding site or multiple binding sites.

Materials and methods

Preparation of recombinant virus

The cDNA encoding the human glucocorticoid receptor (a 2.8 kb *Bam*HI–*Xba*I fragment, Miesfeld *et al.*, 1984) was cloned into the pATA-18 recombination vector consisting of a mutated 11 K vaccinia *tk*–locus (Stunnenberg *et al.*, 1988). Recombinant virus expressing hGR was prepared essentially according to the procedure of Kieny *et al.*, 1984. Vaccinia virus (strain WR) and the recombinant virus were grown in RK-13 monolayers or in HeLa S3 suspension cultures that were maintained in Eagle's medium containing 10% fetal calf serum (RK-13 and Human 143) or 5% newborn calf serum (HeLa).

Preparation of extracts

Livers from adrenalectomized male rats were homogenized in 3 vols of buffer containing 20 mM Hepes pH 7.9, 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DDT, 10% glycerol. HeLa suspension cultures were infected for 20–30 h with wild type or recombinant vaccinia virus and harvested by centrifugation. After washing with cold PBS, cells were suspended in 3 vols of buffer containing 20 mM Hepes pH 7.9, 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DDT and broken up by four cycles of freeze–thawing. Glycerol was added to a final concentration of 10%. Cytosol was prepared by ultracentrifugation, made 10^{-7} M of dexamethasone and stored as 50 μl aliquots in liquid nitrogen.

Gel retardation, nitrocellulose retention and DNase I footprinting analyses

The plasmids used containing one or two GREs cloned into the *Xba*I site of pBLCAT 2 (Luckow and Schütz, 1987) were described previously (Strähle *et al.*, 1988). GRE containing fragments were isolated after digestion with *Hin*dIII/*Bam*HI or *Hin*dIII/*Eco*RI. The fragments were labeled with Klenow polymerase using the *Bam*HI or *Eco*RI site, if not indicated otherwise.

For gel retardation analysis (Fried and Crothers, 1981; Garner and Revzin, 1981), aliquots of cytosol were incubated with end-labeled DNA fragments in 80 mM KCl and 2 mM $MgCl_2$. The assays were performed at 25°C for 30 min and analyzed by a 3% non-denaturing polyacrylamide gel. Before loading, Triton X-100 was added to 0.5% final concentration in order to reduce aggregation. After autoradiography, retarded bands were excised and radioactivity determined by liquid scintillation counting.

For nitrocellulose retention studies (Riggs *et al.*, 1970), receptor/DNA complex formation was allowed as described for gel retardation analysis. After 30 min of incubation, the assay was diluted with a 20-fold volume of prewarmed buffer, composed as above, but containing 50 μg (~1000-fold excess) of unlabeled plasmid containing a duplicated GRE (GRE21GRE, see Figure 5A). After the times indicated, aliquots were passed through 13 mm filter discs (Schleicher and Schüll, BA 85, 0.45 μm pore size). After washing of the filters, DNA retained was eluted in a solution containing 10 mM Tris pH 7.5, 1 mM EDTA and 1% sodium desoxysulfate, precipitated by addition of salt and ethanol and analyzed on a 8% non-denaturing polyacrylamide gel. After autoradiography, the amount of radioactivity in the bands was determined by liquid scintillation counting of the excised bands or by scanning of the autoradiograms.

For DNase I footprinting, a 1/10 volume of a buffer containing 100 mM $MgCl_2$, 1 mg/ml calf thymus DNA and 4 units of DNase I (RQ1 DNase, Promega) was added to the binding reaction. Digestion was allowed for 2 min at 25°C. DNA was purified by phenolization and analyzed on a 6% denaturing gel.

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