The EMBO Journal vol.13 no.17 pp.4087–4095, 1994

A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1

Stefanie Heck, Michael Kullmann, Andreas Gast, Helmut Ponta, Hans J.Rahmsdorf, Peter Herrlich and Andrew C.B.Cato[1]

Kernforschungszentrum Karlsruhe, Institut für Genetik, Postfach 3640, D-76021 Karlsruhe, Germany
[1]Corresponding author

Communicated by P.Herrlich

Steroid receptors activate and repress genes. An important class of genes that they repress is controlled by the transcription factor AP-1. The activity of AP-1 is inhibited by the receptor, a mechanism exploited for the therapy of various forms of pathological hyperproliferation in humans. We show here by point mutations in the DNA binding domain and by the choice of steroid ligands that repression of AP-1 activity and transactivation functions of the glucocorticoid receptor (GR) are separable entities. While DNA binding and activation of glucocorticoid-regulated promoters require GR dimerization, we present data that suggest that repression is a function of GR monomers.
Key words: AP-1/D-loop/mineralocorticoid receptor/transrepression/transactivation/zinc finger region

Introduction

Steroid, thyroid and retinoic acid receptors are ligand binding transcription factors that not only activate but also repress the expression of specific genes. The mechanism of gene activation is far better understood than that of repression. Target genes for transactivation are selected by the receptors through the recognition of defined sequences in the control regions of these genes [see reviews by Evans (1988) and Cato *et al.* (1992a)]. Recognition is brought about by discrete sequences in the zinc finger DNA binding region of the receptor and by homo- or hetero-dimer formation of the receptors (Yu *et al.*, 1991; Bugge *et al.*, 1992; Kliewer *et al.*, 1992; Leid *et al.*, 1992; Marks *et al.*, 1992; Zhang *et al.*, 1992). The nature of the dimers formed determines the promoter element recognized (Baniahmad *et al.*, 1990; Näar *et al.*, 1991; Umesono *et al.*, 1991; Carlberg *et al.*, 1993; Mader *et al.*, 1993; Tini *et al.*, 1993). The ligands are decisive for transactivation and, particularly in the retinoic acid and thyroid receptors, determine dimerization by recruiting specific regions in the ligand binding domain (LBD) (Tsai *et al.*, 1988; Fawell *et al.*, 1990a; Yu *et al.*, 1991; Leid *et al.*, 1992; Forman *et al.*, 1992). In the case of the glucocorticoid hormone receptor (GR), and other steroid hormone receptors, transactivation occurs exclusively by homodimerization. This is mainly achieved by a region in the second zinc finger of the receptor known as the D-loop (Umesono and Evans, 1989; Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991). The resulting homodimers then bind to the palindromic glucocorticoid response element (GRE) to initiate the transcriptional activation process (Evans, 1988; Beato, 1989; Cato *et al.*, 1992a).

Repression of genes by steroids, thyroid hormone or retinoic acid affects many genes (for reviews see Miner *et al.*, 1991; Cato *et al.*, 1992b; Pfahl, 1993). One important class of target genes that are repressed by the hormones, are those under the positive control of certain members of the AP-1 transcription factor family (Fos, Jun and ATF) (reviewed by Cato *et al.*, 1992a; Ponta *et al.*, 1992; Radler-Pohl *et al.*, 1993). Each member of this family binds as a homo- or heterodimer to its preferred sequence; these preferred sequences are subtly different from each other (reviewed by Angel and Karin, 1991). As AP-1 also prevents hormone-dependent activation of GR-regulated promoters, a mutually inactive complex formed either by a direct protein–protein interaction of the receptor and AP-1 or through a third partner has been postulated (Miner *et al.*, 1991; Pfahl, 1993; Radler-Pohl *et al.*, 1993). The domains of the interacting partners have not been accurately mapped. However, deletion mutants of the GR have allowed the assignment of the N-terminal amino acid sequence between positions 272 and 400, the DNA binding domain (DBD) and the LBD as essential components for the repression (Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; Shemshedini *et al.*, 1991; Kerppola *et al.*, 1993). Interestingly, a transactivation-defective mutant (LS7, with two amino acid exchanges in the second finger of the DBD; Godowski *et al.*, 1989) represses the AP-1-dependent promoter (Yang-Yen *et al.*, 1990), a finding compatible with the idea that the transactivation function of the receptor is not required for the repression of AP-1 activity. In the case of AP-1, the so called bzip region seems part of the target required for the repression (Schüle *et al.*, 1990).

Two findings initiated the present study. (i) The GR represses AP-1-dependent promoters that carry no GRE and repression occurs in the presence of the *in vivo* occupancy of the AP-1 site, with no signs of GR binding to DNA (Jonat *et al.*, 1990; König *et al.*, 1992). This suggested that properties totally different from DNA binding and transactivation may be important for repression. (ii) Repression occurs at a lower hormone concentration than activation of a GRE promoter (Jonat *et al.*, 1990) which may indicate that repression is exerted by receptor monomers in contrast to the requirement of dimers for activation. Here we show, by creating several point mutations in the receptor and by choice of ligands, that the activation and repression functions are clearly separable. Further, mutations of amino acids in the dimerization interface (D-loop) of the GR prevent the activation

of glucocorticoid-regulated genes, but fully repress AP-1 activity. Repression thus appears to be independent of dimerization but employs a defined repressing domain.

Results

Regions of the glucocorticoid receptor required for repression

We and others have shown previously that the mineralocorticoid receptor (MR) is a poor repressor of AP-1 activity (Cato *et al.*, 1992b; Ponta *et al.*, 1992; Pearce and Yamamoto, 1993). The MR, however, transactivates GRE promoters in the presence of either its physiological ligand, aldosterone, or the synthetic glucocorticoid dexamethasone (Arriza *et al.*, 1987, 1988; Cato *et al.*, 1991). Domain-swapping experiments between MR and GR define the regions of the GR that convert the MR into a repressing molecule. Such chimeric constructs (Figure 1A) driven by the Rous sarcoma virus (RSV) promoter were transiently cotransfected with an AP-1-dependent reporter plasmid into receptor-negative CV-1 cells. AP-1 activity was stimulated by the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), and the receptors synthesized were activated by dexamethasone. The results from these experiments suggest that two GR segments are necessary for full repression of AP-1 activity. (i) The presence of the N-terminal half of the GR establishes a partial level of repression (compare GMG with MMG, or GMM with MMM in Figure 1B). (ii) The DBD participates in the repression (compare MGM with MMM, or MGG with MMG in Figure 1B). Interestingly, the DBD exerts partial repression in the complete absence of the N-terminus (compare ΔMM with ΔGM in Figure 1B). The nature of the hormone binding C-terminal domain in these chimeric constructs is apparently not important for repression, since the M and G domains are interchangeable (compare GMG with GMM, MGG with MGM, and MMG with MMM in Figure 1B). The appropriate transactivation experiments (Arriza, 1991) showed nonetheless that the non-repressing receptor chimeras (MMM, ΔMM and MMG) are expressed in the CV-1 cells (results not shown). Our results may be interpreted to indicate that several regions, particularly the N-terminus and DBD of the GR, contribute to the repressing function of the receptor. This finding is consistent with our previous results and data presented by other investigators (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; Pearce and Yamamoto, 1993).

Since the different regions of the GR may cooperate with each other, it is perhaps premature to derive definitive conclusions as to the relative magnitude of the contribution to repression of each domain. The data, however, provide encouragement for exploring the repressing mechanism of the GR by point mutations.

Individual amino acids in the DBD discriminate between transactivation and transrepression

The DBD of the GR shares the highest degree of sequence homology with other members of the steroid hormone receptor superfamily (reviewed by Beato, 1989). As this region contributes to transrepression (Figure 1B), a comparison of the amino acids within the DBDs between good and poor repressors may identify amino acid sequences important for transrepression. The relatively small number of differences between the GR and MR DBDs (Figure 2A) made systematic *in vitro* mutagenesis feasible. The positions with differences between the GR and MR were mutated singly or in various combinations (Figure 2B).

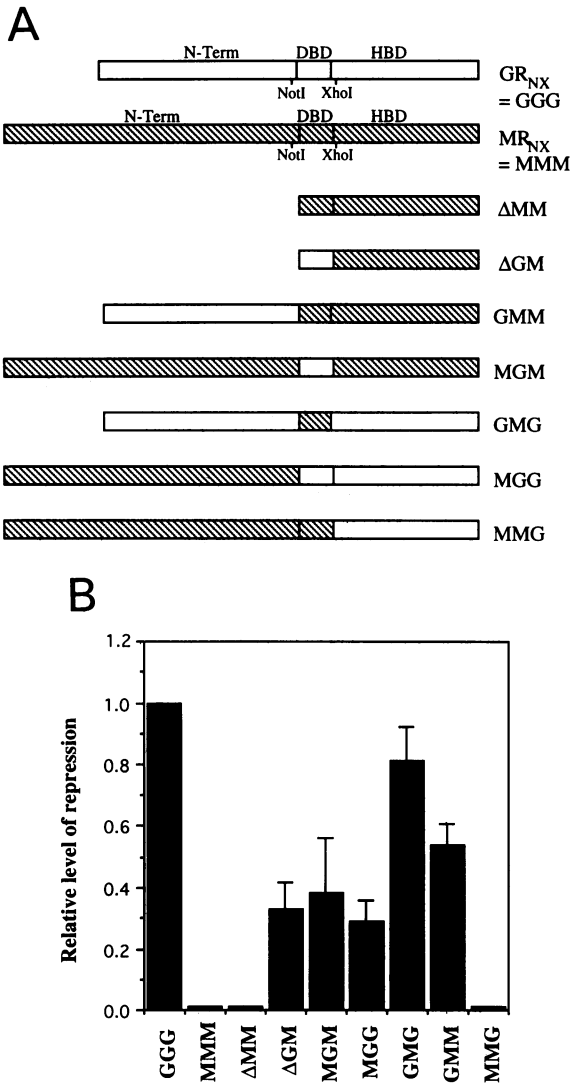

Fig. 1. Transrepression by chimeric GR−MR expression vectors. (**A**) Schematic diagram of the GR−MR constructs showing the *Not*I and *Xho*I restriction sites flanking the DNA binding domains used for exchanging the receptor domains. These receptor chimeras have been described by Arriza (1991). (**B**) Transrepression by the GR and MR as well as the chimeras was carried out in transient transfection experiments in CV-1 cells. These cells were cotransfected with 5 μg of an indicator plasmid (5 × TRE TATA CAT), 5 μg of expression vectors containing the human GR, MR or chimeric receptor cDNAs and 2 μg of the control RSV−β-galactosidase construct. The transfected cells were treated with the phorbol ester TPA (80 ng/ml) in the presence or absence of 10^{-7} M dexamethasone. Two days after transfection the cells were harvested and disrupted, and 100 μg cellular protein or 3 U of β-galactosidase was used for CAT assay. The level of repression was calculated from the level of CAT activity induced by TPA in the presence of hormone over the value in the absence of hormone. The difference between the level of repression by the empty expression vector and that of the wild-type receptor was assigned the nominal value of 1. The repression by the chimeric receptors was expressed relative to this nominal value. Presented as bar diagrams are the averages of the relative level of repression with standard deviations from at least seven independent experiments.

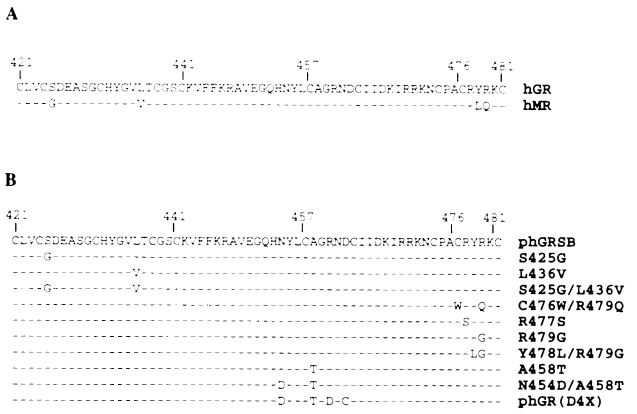

Fig. 2. Exchange of specific amino acids in the DBD of the human GR. (**A**) A diagram of the amino acids in the DBD of the human GR indicating the sequences that differ in this region from those of the human MR. The dashed line represents identical sequences and the amino acid sequences at the position of divergence are indicated. (**B**) A list of the receptor expression vectors used for transactivation and transrepression studies indicating how they diverge from the wild-type receptor phGRSB. This GR expression vector has single *Sac*I and *Bam*HI linkers flanking the DBD. This facilitates the creation of mutants by exchanging the wild-type with the mutated fragment through digestion with the restriction enzymes *Sac*I and *Bam*HI. In all transfection studies, the wild-type construct, phGRSB, did not differ from the authentic wild-type GR which lacks the restriction enzyme sites.

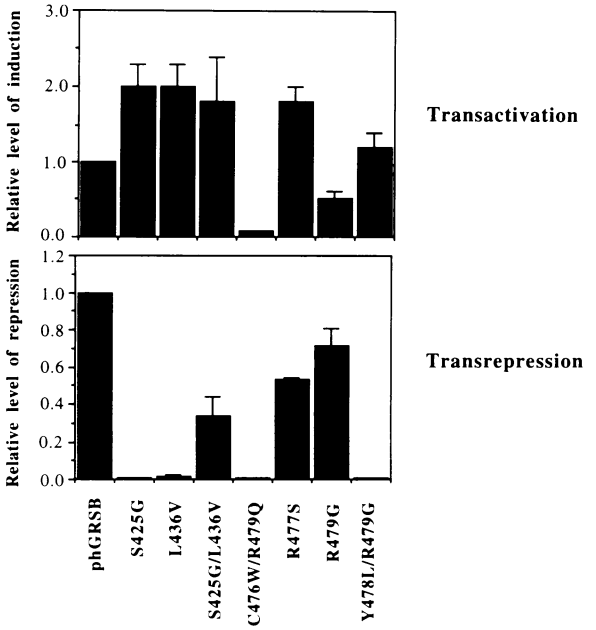

Fig. 3. Amino acid exchanges in the DNA binding domain of the human GR distinguish between transactivation and transrepression. Transactivation experiments were carried out in CV1 cells with 9 μg of the MMTV CAT construct pHCwt (Cato *et al.*, 1988), 1 μg of receptor expression vector and 2 μg of the control β-galactosidase vector. The results are presented as the level of induction of expression of the pHCwt construct by the mutant receptors relative to that of the wild-type construct. The induction factor for the wild-type construct was given the nominal value of 1. The bar diagram represents the relative induction level and the standard deviation of at least five different experiments. Transrepression experiments were performed by cotransfecting the same constructs into CV1 cells with the indicator plasmid 5 × TRE TATA CAT. The assay and presentation of the results are as described in Figure 1B. The bar diagrams represent the averages and standard deviation from at least five independent determinations.

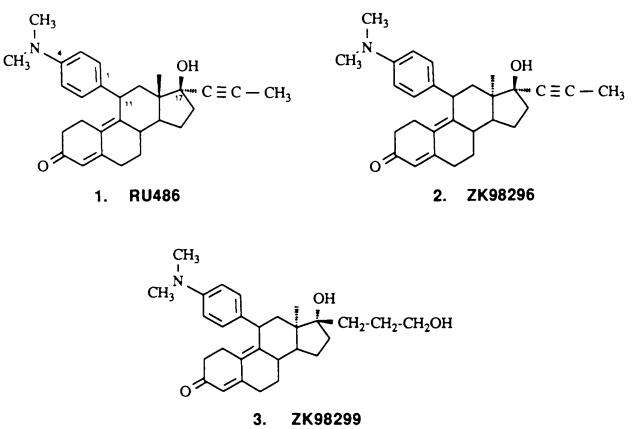

Fig. 4. Structure of the steroid analogs RU486, ZK98296 and ZK98299. Presented are the structures of the steroid analogs RU486 [11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(1-propynyl)-estra-4,9-dien-3-one], ZK98296 [11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(1-propynyl)-13α-methyl-4,9-gonadiene-3-one] and ZK98299 [11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α-4,9-gonadiene-3-one].

The sites of mutation are indicated by single letter code (e.g. in S425G, a glycine has been substituted for serine at position 425). During site-directed mutagenesis, several mutants were generated by *Taq* polymerase error. Some of these turned out to be interesting controls: C476W/R479Q and R477S in Figure 2B.

Since both GR and MR act as bona fide transcription factors, the GR mutants with MR-like changes in the DBD did transactivate (Figure 3) and bound to GRE when tested in Cos-7 cells (results not shown). Note that some mutants even transactivated better than the wild-type phGRSB construct (Figure 3). Mutants S425G, L436V and Y478L/R479G however, could not repress AP-1 activity (Figure 3). These results clearly prove that sequences in the DBD indeed contribute to transrepression and, further, that transrepression and transactivation require different structural features of the DBD. A set of mutations at the base of the second (more C-terminal) zinc finger yielded interesting results in comparison with the double mutant Y478L/R479G. The single exchange R479G had no pronounced effect on repression or transactivation but the double mutant Y478L/R479G lacked the ability to repress (Figure 3). This suggests that position 478 is relevant for repression. The single exchange R477S affected neither transrepression nor transactivation. Mutant C476W/R479Q, which converts one of the coordinating cysteine residues of the second zinc finger into a tryptophan (Figure 2B), neither bound DNA (results not shown) nor transactivated, nor did it repress AP-1 activity (Figure 3). At the first (more N-terminal) zinc finger a double mutant of S425G and L436V showed 40% transrepressing activity, although the single mutants S425G and L436V themselves did not repress at all. In this context we note that the chimera GMG, which has four MR-like amino acid changes in the GR DBD (Figure 1B), has remarkable repressing ability. This might indicate that the effect of the mutations is dictated not so much by the individual

positions of the amino acids exchanged but rather by their contribution to the overall structure of the receptor, which is influenced by several parts of the molecule.

Steroid analogs that inhibit transactivation still promote transrepression

Steroid analogs have been designed to interfere with the GR response at different levels and with varying degrees of efficacy (for review see Teutsch *et al.*, 1991). RU486, ZK98299 and ZK98296 (Figure 4) are inhibitors of glucocorticoid-dependent gene expression (Webster *et al.*, 1988; H.Wehle and A.C.B.Cato, unpublished). While in transactivation experiments RU486 on its own weakly enhanced the CAT activity of a chimeric mouse mammary tumor virus (MMTV)−CAT construct in CV1 cells transfected with the GR expression vector, ZK98299 and ZK98296 were unable to transactivate (Figure 5). The low agonistic activity of RU486 observed in this experiment, is consistent with our previous reports and those of other investigators (Webster *et al.*, 1988; Härtig *et al.*, 1993).

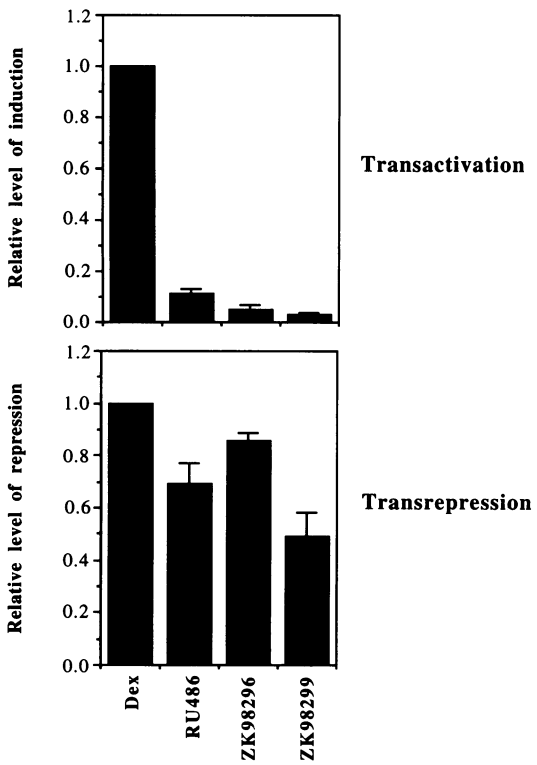

Fig. 5. Transactivating and transrepressing functions of the human GR mediated by dexamethasone and the steroid analogs RU486, ZK98296 and ZK98299. Transient transfection of the MMTV LTR construct pHCwt with the wild-type receptor expression vector GGG in CV1 cells. Eight micrograms of the MMTV LTR construct pHCwt (Cato *et al.*, 1988) and 2 μg of the glucocorticoid expression vector were cotransfected and the cells were treated with 10^{-7} M of the indicated steroids. Two days after transfection, the cells were harvested and CAT assays performed. The bar diagram represents the mean and standard deviation of four independent experiments showing the level of activation of CAT activity by the steroid analogs RU486, ZK98296 and ZK98299 relative to that induced by dexamethasone. For the determination of the repression of AP-1 activity, CV1 cells were cotransfected with the wild-type GR and the indicator plasmid 5 × TRE TATA CAT as indicated in Materials and methods. The transfected cells were treated with 80 ng/ml TPA in the absence and presence of 10^{-7} M of dexamethasone or the steroid analogs RU486, ZK98296 and ZK98299 for 36 h and CAT activity was determined (as described in Figure 1). The results are presented as the level of repression of the steroid analogs with reference to the repression by dexamethasone. The repression by dexamethasone was assigned the nominal value of 1. The bar diagram represents the average and standard deviation of at least six independent experiments.

The steroid analogs were next used to determine whether the AP-1-repressing properties of the GR were influenced. The steroid analogs at low concentrations (see Discussion) as well as dexamethasone inhibited the AP-1 response of a minimal promoter containing the collagenase AP-1 binding site (Figure 5). These data are the mirror image of those obtained with the point mutants, in that lack of transactivation does not affect the transrepressing properties of the GR. All three steroid analogs bound to the GR as demonstrated by their ability to compete for the binding of [^{3}H]dexamethasone to GR in rat liver cytosol (not shown). At equimolar concentrations, RU486 competed 3- to 4-fold better than dexamethasone. Both ZK98296 and ZK98299 competed 1.5-fold less well than dexamethasone. These receptor binding activities of RU486 and ZK98299 are in agreement with those reported for rat thymus extracts (Teutsch *et al.*, 1991). Further, the steroid analogs, in indirect immunofluorescence experiments with rat hepatoma cells, translocated the GR from the cytoplasm to the nucleus (M.Kurz and A.C.B.Cato, unpublished). In the absence of steroids, the GR is mainly located in the cytoplasm.

A plausible interpretation for the inability of the steroid analogs to mediate transactivation while promoting nuclear translocation of the GR, is that either they prevent the correct transactivating function of the receptor or they abolish binding of the receptor to GREs. To measure DNA binding, we performed electrophoretic mobility shift assays. Extracts from cells transfected with an expression clone coding for the wild-type GR, formed a GRE−protein complex if the cells had been treated with dexamethasone (Figure 6, lane 2; see arrow marked GR). Extracts from the transfected cells treated with the steroid analog RU486 showed very weak binding of the GR to DNA as compared with the control binding in the absence of hormone or upon transfection of the empty expression vector (Figure 6, compare lane 4 with lanes 1 and 3). In the presence of the steroid analogs ZK98299 and ZK98296, little or no DNA binding activity of the GR was detected (Figure 6, lanes 5 and 6). The GR levels were not altered in immunoblots after treating the transfected cells with steroid analogs of the type we have used in this study (results not shown). Thus reduced receptor levels do not explain the absence of DNA binding in the *in vitro* DNA−receptor binding studies nor could increased levels explain the effective repression of AP-1 activity.

The results from the electrophoretic mobility shift assay are, however, consistent with *in vivo* DNA footprinting experiments we carried out on the glucocorticoid regulatory region of the rat tyrosine aminotransferase gene. These experiments showed that, while dexamethasone induced binding of the GR to the regulatory elements *in vivo*, the steroid analogs were unable to do so (results unpublished). The lack of DNA binding activity of the GR loaded with the steroid analogs, explains the reduced or absent transactivation activity. That the steroid analogs still mediated transrepression demonstrates that DNA binding activity is not required for transrepression.

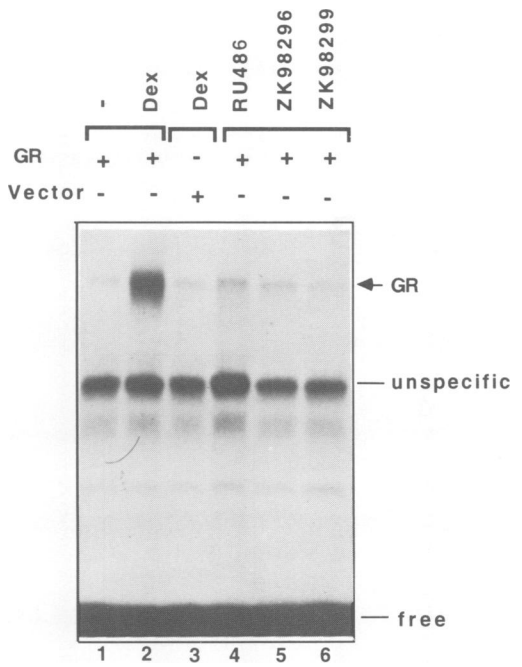

Fig. 6. DNA binding activity of the GR loaded with dexamethasone, RU486, ZK98296 and ZK98299. Wild-type GR expression vector GGG (15 μg) was transfected into Cos-7 cells by electroporation and the transfected cells were treated for 16 h with the glucocorticoid dexamethasone or with the steroid analogs RU486, ZK98296 and ZK98299 (10^{-7} M). Whole cell extracts were prepared and used in gel retardation experiments with a labeled GRE (2 fmol; 15 000 c.p.m.). Unbound labeled oligonucleotide, oligonucleotide non-specifically bound by other proteins and the specific receptor–GRE complex (GR) are indicated.

Repression is independent of GR dimerization

A prerequisite for DNA binding of the GR is its homodimerization. Since repression of AP-1 activity appears to be independent of DNA binding, we examined whether it is also independent of dimer formation. Independence of repression from receptor dimerization has previously been suggested by the lower steroid hormone concentration required for repression as compared with transactivation (Jonat *et al.*, 1990). We now interfered with dimerization by the introduction of mutations into the D-loop domain of the GR. Two types of mutations were generated: an exchange of three out of the five positions in the D-loop [Figure 2B, phGR(D4X)] (Dahlman-Wright *et al.*, 1991) and a single exchange of alanine to threonine (Figure 2B, A458T and N454D/A458T). The rationale for the single exchange (A458T) is derived from a previous observation with the androgen receptor, where this mutation at the same D-loop position severely inhibited dimerization (Kaspar *et al.*, 1993). During oligonucleotide-directed mutagenesis by PCR, a *Taq* polymerase error produced an additional amino acid exchange, N454D, outside of the D-loop, which had interesting effects (see below).

In DNA binding and transactivation studies all three D-loop mutants [A458T, N454D/A458T and phGR(D4X)] behaved identically. This is exemplified by the results of the A458T mutant in Figure 7. In DNA binding experiments, they did not bind to DNA as was reported for the D-loop mutants of the androgen receptor (Kaspar *et al.*, 1993) (Figure 7A, lanes 3 and 6). Our results on the lack of DNA binding activity with the D-loop mutants differ from reports of Dahlman-Wright *et al.* (1991) who used the DBD of the GR with amino acid exchanges in the D-loop. These authors showed that the D-loop mutant did bind to DNA, but in contrast to a wild-type DBD, the mutant did not bind cooperatively. The differences in results could be due to the fact that we used the entire GR whereas Dahlman-Wright *et al.* (1991) used only the DBD. Furthermore, in contrast to other D-loop mutants whose dimerization defects can be corrected by the use of antibodies that recognize N-terminal sequences (Fawell *et al.*, 1990b; Kaspar *et al.*, 1993), DNA binding activity was not restored to any of our GR mutants by the use of two different anti-GR antibodies (Figure 7A, lanes 7 and 8, and results not shown). These antibodies, however, recognize the N-terminal sequences in the receptor, and cause a supershift of the wild-type receptor (Figure 7A, lanes 4 and 5). According to immunoblot experiments D-loop mutants (as exemplified by the mutant A458T receptor) were expressed at even higher level than wild-type receptor (Figure 7B, compare lanes 1 and 2).

In transfection experiments, the D-loop mutants in the presence of dexamethasone did not transactivate a promoter with a single GRE such as a GRE tk CAT construct. Also, a promoter with multiple GREs, such as MMTV CAT, was only poorly transactivated (representative result with A458T in Figure 7C), consistent with results obtained with other dimerization-defective receptors (Kaspar *et al.*, 1993).

In transrepression experiments all three mutants were able to transrepress as efficiently as or even better than the wild-type GR construct (Figure 7D). The additional mutation outside the D-loop (N454D) even improved the ability of the receptor to repress AP-1 activity (Figure 7D, compare A458T with N454D/A458T). Additional mutations in the D-loop also improved the repressing ability of the receptor. Together the data suggest that dimerization of the GR does not seem to be required for transrepression of AP-1 activity. Further, the D-loop mutants confirm that the transactivating and DNA binding functions of the GR are not needed for the repression of AP-1 activity.

Discussion

As the most decisive message of this paper we propose that the DNA binding and transactivating functions of the GR are not required for repression of AP-1 activity and that the transrepressing function is mediated by a structure insensitive to mutations that destroy the major dimerization function of the receptor. Supporting evidence for these statements comes from several new point mutations in the GR and from loading the receptor with steroid analogs which results in separation of the transactivating and repressing functions. These data in conjunction with deletion and domain-swapping experiments from our and other laboratories (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; Pearce and Yamamoto, 1993; and experiments shown here) suggest a complex interaction between GR and AP-1. The interaction involves several segments of the GR, presumably jointly establishing the fully competent three-dimensional structure of the repression interface. The ligands are also conformation-determining factors (Allan *et al.*, 1992) in that hormone

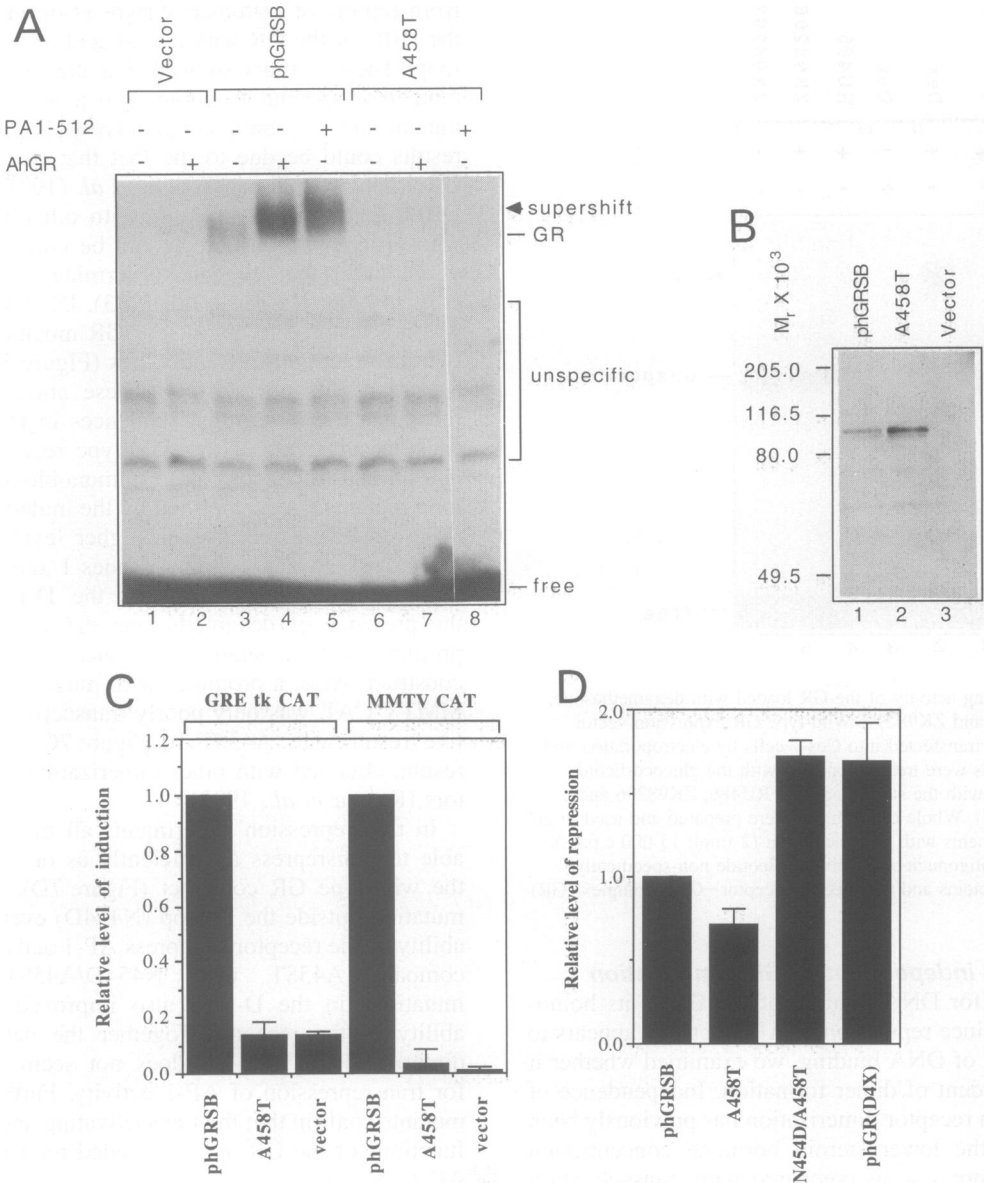

Fig. 7. A mutation in the D-loop of the GR inhibits DNA binding and reduces transactivation but does not affect transrepression. (**A**) Fifteen micrograms of the wild-type and mutant A458T receptor were transfected into Cos-7 cells by electroporation and treated with 10^{-7} M dexamethasone. Five micrograms of whole cell extract of the transfected cells were used in a mobility shift experiment with a labeled oligonucleotide (2 fmol, 15 000 c.p.m.). Where indicated, the anti-GR antibodies AhGR or PA1-512 were incubated with 1 μg poly(dI-dC) and 5 μg cellular extract for 10 min on ice before addition of the labeled oligonucleotide. (**B**) Immunoblot with 9 μg of whole cell extracts of Cos-7 cells transfected with the empty expression vector or expression vector containing the wild-type and mutant A458T receptors. After transfer of the protein onto a nylon filter, the GR was probed with the anti-GR antibody PA1-512. This antigen–antibody complex was detected by the ECL detection system. (**C**) Transactivation experiments were carried out in CV1 cells with 9 μg of the MMTV CAT construct pHCwt (Cato *et al.*, 1988) or a GRE cloned in front of the thymidine kinase promoter driving the expression of the CAT gene (GRE tk CAT) and 1 μg receptor expression vectors or empty expression vector. The receptor expression vectors used were the wild-type GR expression vector or the A458T mutant. The cells were treated with or without 10^{-7} M dexamethasone after transfection and harvested 40 h later for CAT assay. The bar diagram shows the mean and standard deviation of the level of induction of CAT activity from four experiments. The level of induced expression by the wild-type receptor was assigned the nominal value of 1.0 and the levels induced by the A458T mutant and empty expression vector were expressed relative to this value. (**D**) Transrepression experiments were performed by transfecting 5 μg of the construct 5 × TRE TATA CAT and 5 μg of the three different mutant receptor expression vectors indicated as well as the empty and wild-type receptor expression vector. The transfected cells were treated with 80 ng/ml TPA with or without 10^{-7} M dexamethasone. The results are expressed as described in Figure 1B and presented as a bar diagram of the average level of repression and the standard deviation of four different experiments.

binding is needed for repression (Jonat *et al.*, 1990); the choice of the ligand also appears to be decisive: GR loaded with dexamethasone induces transactivation and repression while GR loaded with aldosterone does not repress AP-1 but activates GRE-dependent genes (unpublished). Steroid analogs, on the other hand, induce a GR conformation unable to bind DNA and to transactivate, but competent for repressing AP-1 activity.

Of the three analogs, RU486 has ambiguous properties. It is an antagonist, as it inhibits dexamethasone-dependent transactivation by the GR, but at the same time it also has partial agonist activity on transactivation (Webster

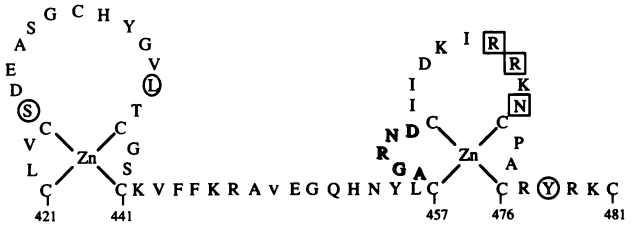

Fig. 8. A schematic diagram of the zinc finger structure of the DBD of the human GR showing the different functions associated with the various amino acid residues in this domain. The shadowed amino acids (residues 458–462) are required for dimerization (Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991). The boxed amino acids contribute to transactivation (Schena *et al.*, 1989) and the circled amino acids are those residues which, when exchanged, as shown in this study, destroy the ability of the receptor to repress AP-1 activity.

et al., 1988; Härtig *et al.*, 1993). In transrepression, most analogs show promiscuous activity. At low concentrations (10^{-8}–10^{-7} M) the analogs induce repressing capability of the GR and of the progesterone receptor (the latter not shown). At higher concentration (10^{-6}–10^{-5} M), the repression is partly or totally lost, perhaps suggesting that high concentrations push the analogs into a second binding site. At high doses, the analogs antagonize dexamethasone-dependent repression of AP-1 activity (Jonat *et al.*, 1990). Our interpretation conforms with the finding that, in addition to sharing common contact sites at the receptor with steroid agonists, RU486 may also interact with separate amino acid residues in the hormone binding domain of receptors such as the progesterone receptor (Vegeto *et al.*, 1992). With respect to a clear dissociation of the transactivating and transrepressing functions of the receptor, the analogs ZK98296 and ZK98299 were most useful in that they did not trigger any detectable transactivation by the GR but they did induce the appropriate receptor conformation for repression. Our experiments make the search for even better steroid (and by analogy, retinoic acid) analogs that exclusively repress AP-1, appear promising in view of their potential use in therapy against pathological hyperproliferation.

Point mutations in the D-loop that produce a dimerization-defective receptor (Umesono and Evans, 1989; Dahlman-Wright *et al.*, 1991) do not bind DNA or transactivate, but fully transrepress. This indicates that the repression domain is present and active in dimerization-defective receptors. Other members of the large family of steroid hormone receptors, such as thyroid and retinoic acid receptors depend, in addition to the D-loop, strongly on a dimerization interface in the hormone binding region (Glass *et al.*, 1989; Yu *et al.*, 1991; Forman *et al.*, 1992; Leid *et al.*, 1992) and it may be more difficult to produce dimerization-negative mutants. Should it be possible to produce such mutants, we would predict that they will also be functional in AP-1 repression.

Several previously reported GR point mutants affect activating and repressing functions alike (Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990). There is only one exception: the double mutation LS7 (Godowski *et al.*, 1989) destroys transactivation without affecting DNA binding or transrepression by the GR (Yang-Yen *et al.*, 1990). The D-loop mutants described in this paper permit interpretations well beyond the LS7 mutant in that they cannot bind DNA and are defective in dimerization. Irrelevance of DNA binding for repression is consistent with the outcome of the experiments with the steroid analogs (described above), with the unaltered AP-1 genomic footprint (König *et al.*, 1992) and with the DBD swap experiments that alter sequence-specific DNA binding of the resulting GR chimeras but not their ability to repress (Schüle *et al.*, 1990).

None of the amino acids we have exchanged and shown to be important for repression are required for GRE-specific DNA binding, nor are they involved in phosphate contacts at specific or non-specific sites (Luisi *et al.*, 1991). From studies of the DNA-bound structure and the solution structure, the DBD (Härd *et al.*, 1990; Luisi *et al.*, 1991) amino acid residues that we have exchanged other than those in the D-loop may well be accessible for interaction with other proteins, either AP-1 or an intermediary factor. Alternatively they may affect positions that form the contact points. A diagrammatic representation of the positions occupied by these amino acids (Figure 8; circled amino acids) shows that they are separable from other previously identified positions that are needed for transactivation [residues 469, 470 and 472 (Schena *et al.*, 1989; boxed residues)] or dimerization [residues 458–462 (shadowed residues; Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991)]. We assume that it is the way in which the DBD is folded and how the exchanged amino acids affect this folding that determines which positions contribute to repression. A further indication that the folding (conformation) of the steroid receptor is decisive in its transrepression function, is provided by studies with the steroid analogs discussed above.

In addition to the protein–protein interaction hypothesis, other mechanisms have been proposed for GR-dependent repression of promoters: GR binding to so-called negative GREs either as single elements or overlapping with AP-1 binding sites (Sakai *et al.*, 1988; Diamond *et al.*, 1990; Zhang *et al.*, 1991; Drouin *et al.*, 1993). With the described structural property of the GR necessary for repression, it should be possible to distinguish whether and to what extent different repression mechanisms exist. For example, it should be possible to determine whether or not the D-loop mutant receptors or the steroid analogs have similar effects on these other promoters.

At a composite promoter where a GR binding site is next to an AP-1 site it was first discovered that the composition of the AP-1 dimer determined the direction of GR action: with Jun homodimers, synergism is observed instead of repression while Fos–Jun heterodimers are repressed (Diamond *et al.*, 1990; Miner and Yamamoto, 1992). Interestingly, such a synergism has recently been observed with the simple AP-1 promoter used here (P.Angel, unpublished). This opens up the possibility that the AP-1 member determines the direction of the response to glucocorticoid in AP-1–GR interactions. Thus the interference by GR with the action of AP-1 appears to represent a mechanism by which the GR regulates the expression of a large number of genes whose expression falls under the control of AP-1.

Materials and methods

Cell culture and transfection

Simian CV1 and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and

5% CO_2. Transient transfection assays for transrepression were carried out using the calcium phosphate coprecipitation procedure with 5 μg 5 × TRE TATA CAT construct (Jonat *et al.*, 1990) and 5 μg empty expression vector or expression vectors containing the wild-type or mutant GR cDNA. In the transactivation studies, unless otherwise stated, 9 μg of the MMTV CAT plasmid pHCwt (Cato *et al.*, 1988) and 1 μg of the wild-type GR or empty expression vector were cotransfected into CV1 cells. In some of the transfection experiments 2 μg of a plasmid consisting of the RSV promoter driving the expression of the β-galactosidase gene was cotransfected as an internal control. The results obtained with the internal control did not differ from those obtained without. Immediately after transfection and after a 2 min shock with 15% glycerol in phosphate buffered saline, the cells were treated with inducers: TPA at 80 ng/ml and dexamethasone at 10^{-7} M. The transfected cells were harvested after 36 h and the CAT activity was determined with 80–100 μg protein or 3 U of β-galactosidase. To generate enough receptor for gel mobility shift assay, 15 μg of the empty expression vector or wild-type and mutant GR expression vectors were transfected using electroporation into receptor-negative Cos-7 cells as described by Klocker *et al.* (1992). Cos-7 cells were used in these experiments as they supported the most efficient expression of our GR constructs yielding high levels of receptor protein.

Plasmid construction

The 5 × TRE TATA CAT construct has been described by Jonat *et al.* (1990), pHCwt by Cato *et al.* (1988) and the GRE tk CAT construct by Kaspar *et al.* (1993) as the ARE tk CAT construct. The RSV−β-galactosidase construct contains the RSV promoter driving the expression of the β-galactosidase gene and was generously supplied by W.Ankenbauer, Heidelberg. Mutations in the GR DBD were constructed starting with the wild-type receptor phGRSB that contains *Sac*I and *Bam*HI sites flanking the DBD of the receptor at amino acids 403 and 532. This hGR derivative is parent to all point mutants and is indistinguishable from the wild-type receptor in DNA binding, transactivation and transrepression. The mutants were constructed by overlap extension with the PCR method of Ho *et al.* (1989). The primers used were 5′-AGGTGCCAAGGATCTGG-3′ and 5′-GCCCATTTCACTGCTGC-3′. The oligonucleotides used for the mutagenesis were as follows. A458T: 5′-CACAATTACCTATGTACTGGAAGGAATGAT-3′ and 5′-ATCATTCCTTCCAGTACATAGGTAATTGTG-3′. S425G: 5′-CTCTGCCTGGTGTGCGGTGATGAAGCTT-3′ and 5′-AAGCTTCATCACCGCACACCAGGCAGAG-3. L436V: 5′-TCATTATGGAGTCGTAACTTGTGGAAG-3′ and 5′-CTTCCACAAGTTACGACTCCATAATGA-3′. Y478L/R479G was constructed using R479G as template (Hollenberg and Evans, 1988) and the mutation introduced was 5′-CCAGCATGCCGCCTTGGAAAATGTCTTCAG-3′ and 5′-CTGAAGACATTTTCCAAGGCGGCATGCTGG-3′.

The constructs R477S, C476W/R479Q and N454D/A458T were obtained accidentally through mistakes introduced by the *Taq* polymerase during the PCR amplification.

The plasmid phGR(D4X) was constructed using N454D/A458T as template and the oligonucleotides 5′-CTATGTACTGGAGATAATTGTTGCATCATCGAT-3′ and 5′-ATCGATGATGCAACAATTATCTCCAGTACATAG-3′. Mutant R479G has already been described (Hollenberg and Evans, 1988).

Preparation of whole cell extract and determination of receptor–DNA interaction

Whole cell extract was prepared from the transfected Cos-7 cells essentially as described by Eul *et al.* (1989) except that the buffer used contained the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2.5 Kallikrein inhibitor units of aprotinin per ml, 0.6 μg of pepstatin per ml, 1 μg of leupeptin per ml, and 1 μg of antipain per ml. Gel mobility shift assay was conducted as described by Barberis *et al.* (1987). The oligonucleotide used for the mobility shift assay was obtained by annealing the following single-stranded oligonucleotides 5′-AGCTTAGAACACAGTGTTCTCTAGAG-3′ and 5′-GATCCTCTAGAGAACACTGTGTTCTA-3′. The resulting double-stranded oligonucleotide was phosphorylated using T4 polynucleotide kinase (Pharmacia) in the presence of [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham).

Immunoblot

Whole cell extracts prepared from Cos-7 cells transfected with receptor expression vectors were resolved by 8% SDS−PAGE. The proteins were transferred to a nitrocellulose filter and probed with an anti-GR antibody, PA1-512, obtained from Affinity Bioreagents (Neshanic Station, NJ). This antibody was raised in rabbits immunized with a synthetic peptide corresponding to amino acids 245–259 of the human GR. The antibody AhGR was also raised in rabbits immunized with a synthetic peptide corresponding to amino acids 150–175 of the human GR. The antigen−antibody complex was detected by the ECL detection system (Amersham, Braunschweig, Germany) according to the manufacturer's instructions.

Acknowledgements

The work presented here profited from discussion with Günther Schütz, Miguel Beato and Ron Evans. We thank R.Evans for making available to us the chimeric GR/MR constructs used in this work. We are also grateful to him for supplying us with the mutant R479G and the wild-type GR expression vector phGRSB which we used for the mutagenesis. We thank Schering, Berlin for supplying us with the steroid analogs ZK98296 and ZK98299 and Roussel Uclaf, Romainville, France for the gift of RU486. We thank Birgit Besenbeck and Anne Denninger for their excellent technical assistance. Financial support from the European Community (Concerted action BMH1-CT92-1037), Fonds der Chemischen Industrie, Schering and Deutsche Forschungsgemeinschaft (Ca 130/1-1) is gratefully acknowledged.

References

Allan,G.F., Tsai,S.Y., Tsai,M.-J. and O'Malley,B.W. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11750–11754.

Angel,P. and Karin,M. (1991) *Biochim. Biophys. Acta*, **1072**, 129–157.

Arriza,J.L. (1991) In Bonvalet,J.P., Farmann,N., Lombés,M. and Rafestin-Oblin,M.-E. (eds), *Aldosterone: Fundamental Aspects*. John Libbey Eurotext Ltd, Paris, pp. 13–21.

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M., Hendelin,B.L., Housmann,D.E. and Evans,R.M. (1987) *Science*, **237**, 268–275.

Arriza,J.L., Simerly,R.B., Swanson,L.W. and Evans,R.M. (1988) *Neuron*, **1**, 887–900.

Baniahmad,A., Steiner,C., Kohne,A.C. and Renkawitz,R. (1990) *Cell*, **61**, 505–514.

Barberis,A., Superti-Furga,G. and Busslinger,M. (1987) *Cell*, **50**, 347–359.

Beato,M. (1989) *Cell*, **56**, 335–344.

Bugge,T.H., Pohl,J., Lonnoy,O. and Stunnenberg,H.G. (1992) *EMBO J.*, **11**, 1409–1418.

Carlberg,C., Bendik,I., Wyss,A., Meier,E., Sturzenbecker,L.J., Grippo,J.F. and Hunziker,W. (1993) *Nature*, **361**, 657–660.

Cato,A.C.B., Skroch,P., Weinmann,J., Butkeraitis,P. and Ponta,H. (1988) *EMBO J.*, **7**, 1403–1410.

Cato,A.C.B., Mink,S. and Härtig,E. (1991) In Bonvalet,J.-P., Farman,N., Lombés,M. and Rafestin-Oblin,M.-E. (eds), *Aldosterone: Fundamental Aspects*. John Libbey Eurotext Ltd, Paris, pp. 23–32.

Cato,A.C.B., Ponta,H. and Herrlich,P. (1992a) *Progr. Nucleic Acid Res. Mol. Biol.*, **43**, 1–36.

Cato,A.C.B., König,H., Ponta,H. and Herrlich,P. (1992b) *J. Steroid Biochem. Mol. Biol.*, **43**, 63–68.

Dahlman-Wright,K., Wright,A., Gustafsson,J.-Å. and Carlstedt-Duke,J. (1991) *J. Biol. Chem.*, **266**, 3107–3112.

Diamond,M.I., Miner,J.N., Yoshinaga,S.K. and Yamamoto,K.R. (1990) *Science*, **249**, 1266–1272.

Drouin,J., Sun,Y.L., Chamberland,M., Gauthier,Y., De Léan,A., Nemer,M and Schmidt,T.J. (1993) *EMBO J.*, **12**, 145–156.

Eul,J., Meyer,M.E., Tora,L., Bocquel,M.T., Quirin-Stricker,C., Chambon, P. and Gronemeyer,H. (1989) *EMBO J.*, **8**, 83–90.

Evans,R.M. (1988) *Science*, **240**, 889–895.

Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990a) *Cell*, **60**, 953–962.

Fawell,S.E., White,R., Hoare,S., Sydenham,M., Page,M. and Parker,M.G. (1990b) *Proc. Natl Acad. Sci. USA*, **87**, 6883–6887.

Forman,B.M., Yang,C.-R., Av,M., Carsanova,J., Ghysdall,J. and Samuels,H.H. (1989) *Mol. Endocrinol.*, **3**, 1610–1626.

Glass,C.K., Lipkin,S.M., Devary,O.V. and Rosenfeld,M.G. (1989) *Cell*, **59**, 697–708.

Godowski,P.J., Sakai,D.D. and Yamamoto,K.R. (1989) In Gralla,J. (ed.), *DNA–Protein Interactions in Transcription*. UCLA Symposium in Molecular and Cellular Biology, Vol. 95. Alan R.Liss, New York, pp. 197–210.

Härd,T., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedt-Duke,J., Yamamoto,K.R., Gustafsson,J.-Å. and Kaptein,R. (1990) *Science*, **249**, 157–160.
Härtig,E., Nierlich,B., Mink,S., Nebl,G. and Cato,A.C.B. (1993) *J. Virol.*, **67**, 813–821.
Ho,S.N., Hunt,H.D., Horton,R.M., Pullen,J.K. and Pease,L.R. (1989) *Gene*, **77**, 51–59.
Hollenberg,S.M. and Evans,R.M. (1988) *Cell*, **55**, 899–906.
Jonat,C., Rahmsdorf,H.J., Park,K.-K., Cato,A.C.B., Gebel,S., Ponta,H. and Herrlich,P. (1990) *Cell*, **62**, 1189–1204.
Kaspar,F., Klocker,H., Denninger,A. and Cato,A.C.B. (1993) *Mol. Cell. Biol.*, **13**, 7850–7858.
Kerppola,T.K., Luk,D. and Curran,T. (1993) *Mol. Cell. Biol.*, **13**, 3782–3791.
Kliewer,S.A., Umesono,K., Noonan,D.J., Heyman,R.A. and Evans,R.M. (1992) *Nature*, **358**, 771–774.
Klocker,H., Kaspar,F., Eberle,J., Überreiter,S., Radmayr,C. and Bartsch,G. (1992) *Am. J. Hum. Genet.*, **50**, 1318–1327.
König,H., Ponta,H., Rahmsdorf,H.J. and Herrlich,P. (1992) *EMBO J.*, **11**, 2241–2246.
Leid,M., Kastner,P., Lyons,R., Nakshatri,H., Saunders,M., Zacharewski, T., Chen,J.-Y., Staub,A., Garnier,J.-M., Mader,S.C. and Chambon,P. (1992) *Cell*, **68**, 377–395.
Lucibello,F.C., Slater,E.P., Jooss,K.U., Beato,M. and Müller,R. (1990) *EMBO J.*, **9**, 2827–2834.
Luisi,B.F., Xu,W.X., Otwinowski,Z., Freedman,L.P., Yamamoto,K.R. and Sigler,P.B. (1991) *Nature*, **352**, 497–505.
Mader,S., Jia-Yang,C., Chen,Z., White,J., Chambon,P. and Gronemeyer, H. (1993) *EMBO J.*, **12**, 5029–5041.
Marks,M.S., Hallenbeck,P.L., Nagata,T., Segars,J.H., Appella,E., Nikodem,V.M. and Ozato,K. (1992) *EMBO J.*, **11**, 1419–1435.
Miner,J.N., Diamond,M.I. and Yamamoto,K.R. (1991) *Cell Growth Differ.*, **2**, 525–530.
Miner,J.N. and Yamamoto,K.R. (1992) *Genes Dev.*, **6**, 2491.
Näar,A.M., Boutin,J.M., Lipkin,S.M., Yu,V.C., Holloway,J.M., Glass,C.K. and Rosenfeld,M.G. (1991) *Cell*, **65**, 1267–1279.
Pearce,D. and Yamamoto,K.R. (1993) *Science*, **259**, 1161–1165.
Pfahl,M. (1993) *Endocrine Rev.*, **14**, 651–658.
Ponta,H., Cato,A.C.B. and Herrlich,P. (1992) *Biochim. Biophys. Acta*, **1129**, 255–261.
Radler-Pohl,A. *et al.* (1993) *Annals N.Y. Acad. Sci.*, **684**, 127–148.
Sakai,D.D., Helms,S., Karlstedt-Duke,J., Gustafsson,J.-Å., Rottman,F.M. and Yamamoto,K.R. (1988) *Genes Dev.* **2**, 1144–1154.
Schena,M., Freedman,L.P. and Yamamoto,K.R. (1989) *Genes Dev.*, **3**, 1590–1601.
Schüle,R., Rangarajan,P., Kliewer,S., Ransone,L.J., Bolado,J., Yang,N., Verma,I.M. and Evans,R.M. (1990) *Cell*, **62**, 1217–1226.
Shemshedini,L., Knauthe,R., Sassone-Corsi,P., Pornon,A. and Gronemeyer,H. (1991) *EMBO J.*, **10**, 3839–3849.
Teutsch,G., Gaillard-Moguilewsky,M., Lemoine,G., Nicque,F. and Philibert,D. (1991) *Biochem. Soc. Trans. (London)*, **19**, 901–908.
Tini,M., Otulakowski,G., Breitmann,M.L., Tsui,L.-C. and Giguère,V. (1993) *Genes Dev.*, **7**, 295–307.
Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.-Å., Tsai,M.-J. and O'Malley,B.W. (1988) *Cell*, **55**, 361–369.
Umesono,K. and Evans,R.M. (1989) *Cell*, **57**, 1139–1146.
Umesono,K., Murakami,K.K., Thompson,C.C. and Evans,R.M. (1991) *Cell*, **65**, 1255–1266.
Vegeto,E., Allan,G.F., Schrader,W.T., Tsai,M.-J., McDonnel,D.P. and O'Malley,B.W. (1992) *Cell*, **69**, 703–713.
Webster,N.J.G., Green,S., Jin,J.R. and Chambon,P. (1988) *Cell*, **54**, 199–207.
Yang-Yen,H.-F., Chambard,J.-C., Sun,Y.-L., Smeal,T., Schmidt,T.J., Drouin,J. and Karin,M. (1990) *Cell*, **62**, 1205–1215.
Yu,V.C., Delsert,C., Andersen,B., Holloway,J.M., Devary,O.V., Näar,A.M., Kim,S.Y., Boutin,J.-M., Glas,C.K. and Rosenfeld,M.G. (1991) *Cell*, **67**, 1251–1266.
Zhang,X.-k., Dong,J.-M. and Chiu,J.-F. (1991) *J. Biol. Chem.*, **266**, 8248–8254.
Zhang,X.-k., Hoffmann,B., Tran,P.B.-V., Graupner,G. and Pfahl,M. (1992) *Nature*, **355**, 441–446.

Received on May 24, 1994; revised on June 20, 1994