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Metal binding 'finger' structures in the glucocorticoid receptor defined by site-directed mutagenesis

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The glucocorticoid receptor and the other members of the steroid receptor super-family share a highly conserved, cysteine-rich region which coincides with the DNA binding/transactivating domain. It has been postulated that this region is folded into two 'zinc finger' structures, similar to those originally reported for the transcription factor TFIIIA. The first potential finger domain contains four conserved cysteines and one conserved histidine, while the second contains five conserved cysteines. Using site-directed mutagenesis, we have analysed the consequences of altering the proposed finger-like structures. Our results show that most of the mutations affecting the conserved cysteines result in a total loss of glucocorticoid receptor function. In one important exception, however, a conserved cysteine (Cys500) is dispensable for glucocorticoid receptor activity and therefore cannot be involved in complexing a metal ion to form a finger structure. Moreover, the replacement of either Cys476 or Cys482 by His residues maintains partial *in vivo* activity of the glucocorticoid receptor, while their exchange for an alanine or serine residue, respectively, eliminates receptor function. These results support, at a genetic level, the involvement of cysteines of the glucocorticoid receptor DNA binding domain in metal ion complexation and define the candidate residues involved in such coordination.

Key words: glucocorticoid receptor/site-directed mutagenesis/transcription factor/'zinc fingers'

Introduction

The rat glucocorticoid receptor (rGR) is a member of a super-family of transcription factors. Transcriptional activation of gene expression is mediated by the interaction of the glucocorticoid hormone–receptor complexes with specific DNA target sequences called glucocorticoid responsive elements (GRE) (for a review see Yamamoto, 1985). Deletion analysis and sequence comparisons between the steroid receptors have identified at least two functional domains of the rGR: (i) a hormone-binding domain situated in the C-terminal part of the receptor—when this is deleted, the rGR acts constitutively (Godowski *et al.*, 1987); and (ii) a DNA-binding/transactivating domain of 86 amino acids in the centre of the receptor sequence (Hollenberg *et al.*, 1987; Rusconi and Yamamoto, 1987). The latter, highly conserved among the steroid receptor super-family, is rich in cysteines and contains two sequence motifs that resemble the DNA-binding 'zinc finger' domains first identified in the transcription factor TFIIIA (Brown *et al.*, 1985; Miller *et al.*, 1985). However, instead of the characteristic Cys/His repeating pattern of TFIIIA (Cys-X_2-Cys-X_{12-13}-His-X_{3-4}- His) (for a review see Klug and Rhodes, 1987; Evans and Hollenberg, 1988), two clusters, containing a variable number of cysteines and histidines, are found in the steroid receptor family.

These two cysteine clusters, which are encoded by two exons (Huckaby *et al.*, 1987; S.Rusconi, unpublished data), could each be folded into a zinc finger-like structure (Giguère *et al.*, 1986). Further support for this possibility comes from the finding that zinc is important for DNA binding of the estrogen receptor (Sabbah *et al.*, 1987). The first cluster of the rGR contains five cysteines (at positions 440, 443, 450, 457 and 460, of which Cys450 is not conserved among steroid receptors) and one conserved histidine (His451). The second cluster contains five conserved cysteines (at positions 476, 482, 492, 495 and 500) and one non-conserved histidine (His472) (Giguère *et al.*, 1987, 1988; McDonnell *et al.*, 1987; Petkovich *et al.*, 1987). Depending on the cysteine or histidine residues considered, several alternative finger structures are formally possible (Danielsen *et al.*, 1986; Klug and Rhodes, 1987; Miesfeld *et al.*, 1987; Evans and Hollenberg, 1988). The aim of the present work was to test the candidate residues for involvement in a metal-binding finger structure by site-directed mutagenesis.

We have substituted the Cys and His residues likely to interact with metal ions, in order to determine whether any or all of them are indispensable for rGR function. Although most of the mutations affecting the conserved cysteines turned out to be deleterious for GR activity, at least two conserved residues, His451 and Cys500, are dispensable under our assay conditions and cannot be involved in metal complexation. Furthermore, we have identified a permissive replacement of two cysteines by histidines (but not by alanine or serine), thus providing genetic support for the role of a metal ion in GR function.

Results

Experimental strategy

Site-directed mutagenesis was performed using synthetic double-stranded mutant oligonucleotides. To facilitate the exchange of wild-type sequences by mutant oligonucleotides, a mutagenesis 'cassette' harbouring 16 restriction sites within the rGR coding sequence was chemically synthesized (Figure 1b). This cassette, spanning amino acids 446–541 of the rGR, comprises part of the first Cys cluster and extends beyond the second Cys cluster (Figure 1b). The synthetic cDNA region was incorporated into the expression vectors pSTC3-556 and pSTC407-556 (Figure 1d) which encode the fragments 3-556 and 407-556 of the rGR respectively. Similarly truncated receptors, i.e. lacking the hormone-binding domain, have been reported to constitutively activate

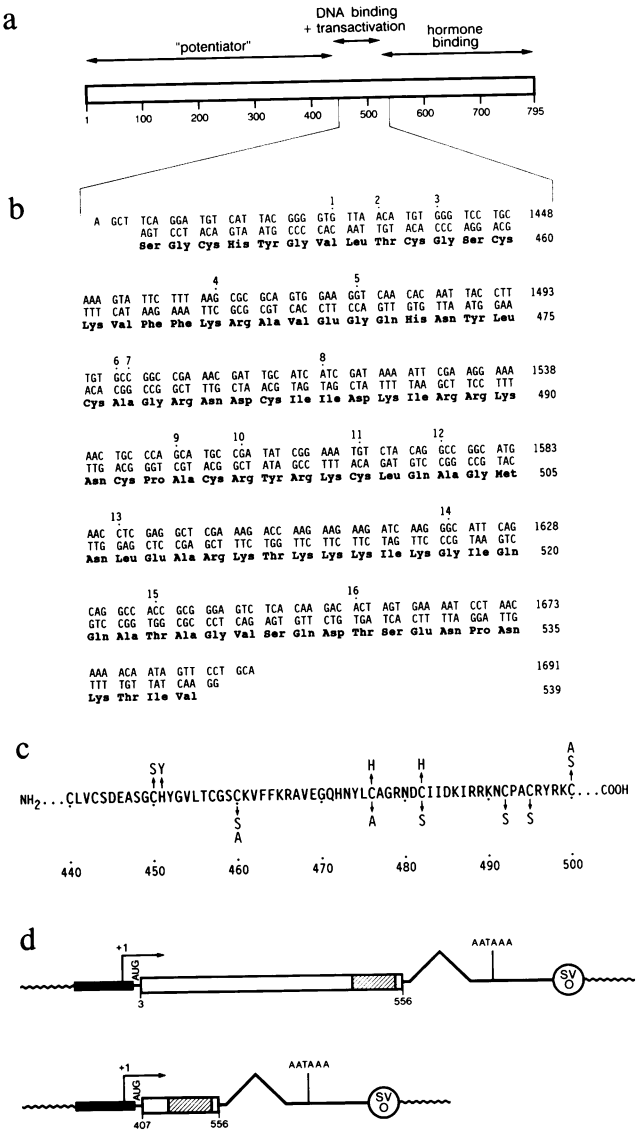

Fig. 1. (**a**) Structure of the rGR. The hormone-binding, DNA binding/transactivation and 'potentiator' domain (Giguère *et al.*, 1986; Miesfeld *et al.*, 1987) are indicated by arrows at the top of the figure. Numbers refer to the amino acid position. (**b**) Construction of a GR mutagenesis cassette. The cassette comprises 16 restriction sites, here numbered 1–16, of which two are wild-type (3 and 9) and the other 14 have been created while preserving the wild-type amino acid sequence (using a map of potential sites kindly provided by Dr Peter Lind, KabiGen, Stockholm). In this cassette, individual regions of the rGR sequence could be exchanged directly within the expression vector. Key to the sites: 1, *Hpa*I/*Hinc*II; 2, *Afl*III; 3, *Eco*0109; 4, *Bss*HII; 5, *Hin*dII; 6, *Nae*I; 7, *Eag*I; 8, *Cla*I; 9, *Sph*I; 10, *Eco*RV; 11, *Acc*I; 12, *Nae*I; 13, *Xho*I; 14, *Bsm*I; 15, *Sac*II; 16, *Spe*I. The numbers are placed at the first base of the enzyme recognition sequence. (**c**) Mutation scheme. The mutations presented in this paper are indicated with arrows. Substitutions having a null phenotype are below the sequence while those having a permissive phenotype are above. (**d**) Structure of pSTC3-556 and pSTC407-556, two eukaryotic expression vectors for the rGR (as described elsewhere in detail by S.Rusconi *et al.*, in preparation). Symbols: thick bar, cytomegalovirus (CMV) promoter/enhancer (−522/+72) (Boshart *et al.*, 1985); thin line, herpes simplex virus thymidine kinase (TK) leader, including the initiation codon AUG and three further amino acids (+55/+104) (Rusconi and Yamamoto, 1987); open box, rGR cDNA insert encoding amino acids 3–556 or 407–556 (Miesfeld *et al.*, 1986); shaded area, region corresponding to the mutagenesis cassette; thick line, rabbit β-globin splice/polyadenylation signal (Pääbo *et al.*, 1983); open circle, SV40 origin of replication (a fragment of 200 bp between the *Hin*dIII site, position 5154, and the *Sph*I site, position 120) (Tooze, 1980); wavy line, plasmid DNA.

GRE-containing promoters (Godowski *et al.*, 1987; Miesfeld *et al.*, 1987).

Testing of the mutants *in vivo* was done by cotransfection with one of two glucocorticoid-regulated reporter genes, MMTV-Tag or P4-OVEC. In MMTV-Tag, the expression of SV40 T-antigen (Tag) is controlled by the GRE of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) (Chandler *et al.*, 1983; Ponta *et al.*, 1985) and is measured by the transactivation-dependent replication assay after cotransfection into CV-1 cells as described in Materials and methods. P4-OVEC (Figure 2b) contains four tandem copies of a synthetic 15-bp palindromic GRE (Jantzen *et al.*, 1987) controlling expression of the rabbit β-globin test gene, which can be measured by quantitative S1-nuclease assay (Westin *et al.*, 1987). Transcriptional activation of this reporter gene was analysed in transient assays by cotransfection into HeLa cells together with vectors expressing the wild-type or mutated rGR.

Expression from both reporter plasmids was stimulated several hundred-fold upon addition of pSTC3-556 (Table I; Figure 2a, lane 4). No detectable stimulation was observed in parallel experiments with control reporter genes that lack the GRE enhancer region (data not shown). Several recent reports have outlined the contribution of other sequence elements flanking the GRE to receptor-mediated transcriptional activation (Kühnel *et al.*, 1986; Miksicek *et al.*, 1987; Richard-Foy and Hager, 1987; Cato *et al.*, 1988; Schüle *et al.*, 1988). However, it is to be noted that in P4-OVEC, the palindromic GRE repeats were inserted immediately upstream of the rabbit β-globin gene TATA box and our results show that the GR does, in principle, not require accessory factors such as NF-1 to efficiently stimulate transcription.

Using truncated fragments of the rGR, poor levels of stimulation are generally obtained when tested in the conventional types of assays (e.g. CAT or RNA assays) (Miesfeld *et al.*, 1987; our unpublished results). In particular the short rGR fragment 407-556 barely stimulates transcription when compared with wild-type rGR or the large rGR fragment 3-556. Therefore, the latter fragment was used in the S1-analysis experiments with P4-OVEC. In the more sensitive transactivation-dependent replication assay, however, even the rGR fragment 407-556 stimulates Tag expression to substantial levels (Table I), probably due to the autocatalytic nature of this type of assay (S.Rusconi *et al.*, in preparation). For this reason, all the mutations once introduced in the cassette vectors were first tested in this assay. The activities of the most interesting mutations were then confirmed in the 3-556 context by S1-nuclease RNA determination of P4-OVEC expression.

Indispensable and dispensable cysteines for GR activity

In a first series of mutations most of the Cys residues were replaced by serine or alanine. These amino acids are roughly similar in size to cysteine but would allow neither formation of disulphide bridges nor efficient binding of metal ions such as Zn^{2+}, in a putative finger structure. We found that each of the mutations Cys460 → Ser or Ala, Cys476 → Ala, Cys482 → Ser, Cys492 → Ser and Cys495 → Ser, inserted within the segment 3-556 of the rGR, completely eliminated biological activity in the extremely sensitive transactivation-dependent replication assay (Table I). Moreover, the same

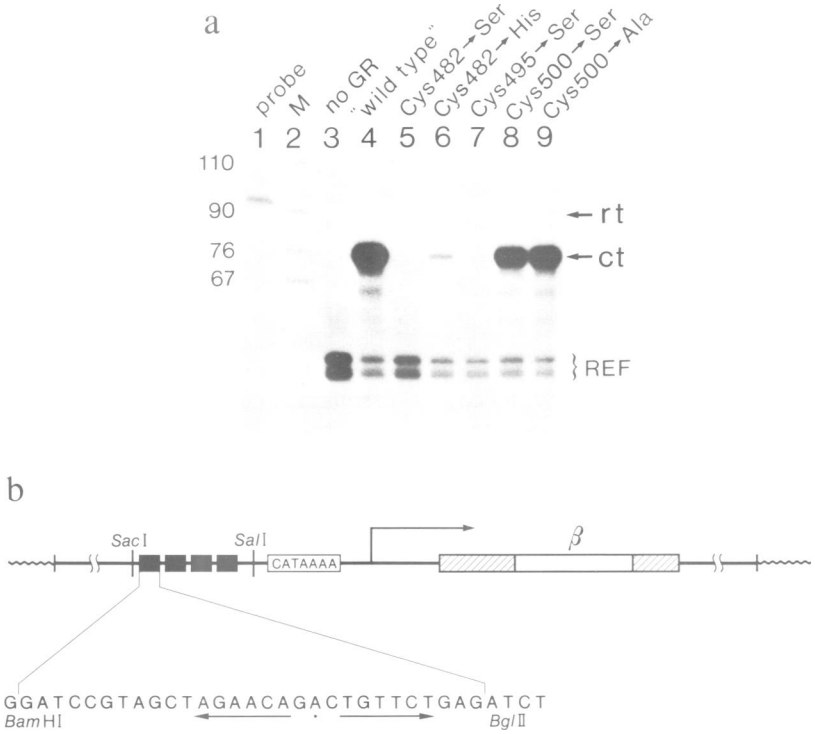

Fig. 2. (**a**) S1-nuclease mapping of transient β-globin RNA expression. HeLa cells were co-transfected with 10 μg of P4-OVEC reporter plasmid, 1 μg of OVEC-REF and 3 μg of the indicated wild-type or mutant rGR 3-556 expression vectors. Symbols: M, *Hpa*II-digested pBR322 marker DNA; ct, correctly initiated β-globin transcripts; REF, reference gene transcripts; rt, readthrough transcripts. (**b**) Schematic structure of the GR responding reporter gene P4-OVEC. Four *Bam*HI/*Bgl*II tandem synthetic repeats bearing a 15-bp palindromic glucocorticoid responsive element (GRE) sequence were inserted into the *Sac*I/*Sal*I sites of OVEC (Westin *et al.*, 1987), immediately upstream of the rabbit β-globin gene TATA box. The direction of transcription of the β-globin gene is marked (arrow).

mutant plasmids were unable to stimulate transcription to detectable levels from P4-OVEC (Figure 2a, lanes 5 and 7 and data not shown). These results demonstrate that these conserved cysteines are essential for GR function.

Unexpectedly, the replacement of Cys500 by serine or alanine had hardly any consequences for gene activation. These mutants stimulated Tag expression to levels comparable to those of wild-type rGR 407-556 (Table I) or 3-556 (Table I and Figure 2, lanes 8 and 9). From this result, we conclude that Cys500, although conserved throughout the receptor super-family, is dispensable for the transactivator functions as tested in our assay. To exclude the possibility that Cys500 was essential for the function of the complete receptor, we tested mutant Cys500 → Ala in the 3-795 segment of the rGR, including the carboxy terminus with the hormone-binding domain. This construct also showed stimulation comparable to wild-type GR in the transactivation-dependent replication assay, upon addition of dexamethasone (data not shown). Two other exchanges, namely the replacement of the highly conserved residue His451 by tyrosine and the less conserved Cys450 by serine, also maintain GR function to near or full wild-type levels respectively (Table I).

Table I. Stimulation of SV40 Tag expression by GR mutants[a]

rGR plasmid co-transfected			Stimulation factors for SV40 Tag expression
Mutation	GR fragment (amino acids)	μg	
None	407–556	0.1	79 ± 28
Cys450→Ser	407–556	0.1	73[b]
His451→Tyr	407–556	0.1	57 ± 12
Cys482→His	407–556	1	1.9 ± 0.7
Cys482→Ser	407–556	1	1.0 ± 0.1
Cys500→Ser	407–556	0.1	40 ± 6
Cys500→Ala	407–556	0.1	77 ± 33
None	3–556	0.1	203 ± 39
Cys460→Ser	3–556	1	1[b]
Cys460→Ala	3–556	1	1[b]
Cys476→Ala	3–556	1	1.0 ± 0.3
Cys476→His	3–556	1	13 ± 5.8
Cys482→Ser	3–556	1	0.9 ± 0.3
Cys482→His	3–556	1	109 ± 16
Cys492→Ser	3–556	1	0.8 ± 0.3
Cys495→Ser	3–556	1	0.8 ± 0.4
Cys500→Ser	3–556	0.1	156 ± 24

[a]values indicated were obtained in three to seven independent transactivation-dependent replication assays in the conditions described in Materials and methods.
[b]Average value of two experiments.

Permissive cysteine/histidine replacement

From the properties of metal-complexing proteins, one might have expected that some cysteines could be exchanged with histidines. However, the simultaneous replacement of two cysteines by histidines in the DNA binding domain of the estrogen receptor was found to eliminate activity *in vivo* (Green and Chambon, 1987). Unfortunately, this finding is not conclusive, since this particular double substitution might well be deleterious for the finger structure (Green and Chambon, 1987). In a comparison of recently published histidine-containing putative fingers (Evans and Hollenberg, 1988, and references therein), we observed that the His residues are consistently separated by at least three amino acids in the primary sequence. This spacing might be necess-

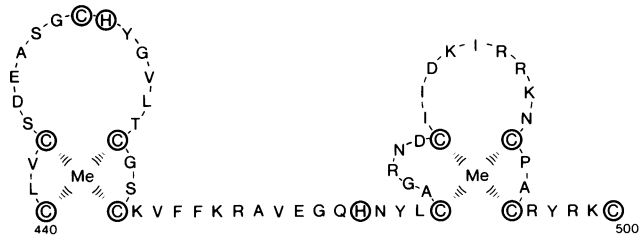

Fig. 3. Putative GR structure deduced, for the second finger, from the results presented in this paper. For numbering of the amino acids see Figure 1c.

ary to accommodate the more bulky His residues. Although this hypothesis remains to be validated, it could explain the loss of activity in the published Cys/His replacement in the estrogen receptor, in which the selected two Cys residues are separated by two amino acids only.

Following this line, we focused our attention on the Cys476 and Cys482 of the second cluster of the rGR and decided to substitute these Cys residues one by one, rather than simultaneously. This strategy proved successful. Indeed, the mutation Cys482 → His, when placed in the large rGR fragment (3-556), stimulates Tag expression 109-fold (54% of the wild-type stimulation, Table I). This result was confirmed by the clear stimulation of P4-OVEC transcription by the same mutant (Figure 2a, lane 6). This mutation Cys482 → His is, however, only partly permissive since we barely detect stimulation of Tag expression when testing the mutation in the small rGR fragment (407-556, Table I). Though the Cys → His replacement at position 476 is an even stronger down mutation, we can still observe 13-fold Tag stimulation with this mutant in the 3-556 context, i.e. 6% of wild-type values (Table I). Considering that the transactivation-dependent replication assay is more sensitive than conventional types of assays, it is not unexpected that transcription of P4-OVEC by mutant Cys476 → His in the S1-assay is still below detection level. As a negative control, insertion of alanine or serine at the same positions 476 or 482, completely abolishes biological activity (Table I; Figure 2a, lane 5 and data not shown). The maintenance of partial activity after exchanging Cys476 or Cys482 for histidine (but not by serine or alanine) supports, at a genetic level, the requirement of a metal for structural coordination in the GR.

Discussion

The DNA binding/transactivator domain of the GR contains several cysteine residues that, in analogy to the transcription factor TFIIIA, have the potential to form two metal binding fingers (Klug and Rhodes, 1987; Evans and Hollenberg, 1988). These would, however, involve four cysteines (instead of two cysteines and two histidines) which would be tetrahedrally coordinated with a metal ion (Diakun *et al.*, 1986). The GR DNA binding domain contains more than the eight absolutely conserved residues required for such an arrangement. To determine the individual role of each of the multiple Cys and His residues, we have substituted them by other amino acids using site-directed mutagenesis. While some of the mutations did not affect the receptor function, others proved deleterious. It must be mentioned that, in the type of assay reported here, the cause of the loss of function is not identified. The mutations could affect specific DNA binding, nuclear translocation, ability to activate transcription or structural stability (for instance by impeding the metal-coordinated folding).

It was not unexpected to find that the exchange of the non-conserved residue Cys450 by a serine maintains GR function. In contrast, the substitution of most of the highly conserved cysteines results in a complete loss of function (e.g. Cys460, 476, 482, 492 and 495). Surprisingly, replacement of the conserved Cys500 by serine or alanine had little consequence for gene activation. The possibility that the serine could itself coordinate a metal ion through its hydroxyl group was ruled out because of the full permissivity of a parallel replacement by alanine, a residue unable to undergo any coordination. We also tested whether Cys500 was not critical within a truncated GR but perhaps was important within the context of the complete GR, such as for interaction with cysteines outside the DNA binding region. However, this possibility was eliminated because a full-length rGR (3-795) bearing the mutation Cys → Ala500 is as active as its wild-type counterpart. The mutation His451 → Tyr also retains considerable biological activity despite the strict conservation of His451 in the steroid receptor super-family.

Although it is commonly assumed that amino acids conserved in evolution are functionally important, there are other precedents in which highly conserved residues could be mutated without loss of function (Valenzuela *et al.*, 1985). Most likely, the conserved but dispensable residues in the rGR are required for some function(s) not tested here, such as interaction with other enhancer/promoter configurations, or accessory transcription factors.

The identification of the residues non-essential for rGR function has direct consequences for finger-folding since it eliminates structure that were considered by others (Danielsen *et al.*, 1986; Miesfeld *et al.*, 1986; Klug and Rhodes, 1987; Evans and Hollenberg, 1988). The finding that neither Cys450 nor His451 are required for GR function, while Cys460 is essential, suggests that the four cysteines 440, 443, 457 and 460 of the first cysteine cluster can be folded into a finger-like structure (although Cys440, 443 and 457 were not tested directly) (Figure 3). Still in analogy with the other finger proteins typified by TFIIIA, the second cysteine cluster of the rGR would now be expected to form a loop between the doublets Cys476/482 and Cys492/495 (Figure 3). Indeed, these four residues are absolutely required for a functional receptor. His472 was not tested but is unlikely to be involved in any finger structure since it is not conserved within the steroid receptor super-family (McDonnell *et al.*, 1987). In the second GR finger domain proposed in Figure 3, it is apparent that the number of residues in the central loop would be smaller than in typical fingers (nine residues instead of the 12−13 in TFIIIA) but these could still form a so-called 'stubby' finger (Klug and Rhodes, 1987).

If the function of the essential Cys residues in the DNA binding domain of the GR is to form metal ion complexes, then the Cys and His residues in this region should be interchangeable to some extent. Here, we have shown that both Cys476 and Cys482 of the rGR were at least partially permissive for a Cys to His replacement. In the particular case of the replacement Cys476 → His, the activity of the mutant GR is considerably weaker than the wild-type but nevertheless significant: when either Cys476 or Cys482 are replaced by Ala or Ser respectively, a total loss of activity is observed. In recent experiments, in which two cysteines in the estrogen

receptor were replaced by two histidines (Green and Chambon, 1987), the failure to find receptor activity may have been due to additive effects of the double mutation or to some steric hindrance between the more bulky His residues. In the work presented here, the successful Cys → His replacement provides direct *in vivo* support for the role of metal ions in the GR function. The genetic data presented above do not allow a discussion of the recent structural models proposed for finger-like structures (Brown and Argos, 1986; Berg, 1988) but our mutagenesis cassette represents an easily accessible tool for a systematic analysis of such predictions. Final proof for the finger structure and the identification of the metal ion involved awaits biophysical studies of the GR. Based on our data, it may be useful to create a mutant GR which retains activity and contains a minimum of cysteine residues. The chance of obtaining a correct folding of the protein produced in *Escherichia coli* or by *in vitro* translation, may be increased in such variants, thus leading to better substrates for structural and biochemical studies.

Materials and methods

Materials

*Dpn*I enzyme was from Anglian Biotechnology and the Sequenase DNA Sequencing Kit was from United States Biochemical Corporation. The Gene Assembler (Pharmacia) was used for oligonucleotide synthesis.

Cell culture and DNA transformation

CV-1 cells and HeLa cells were grown in DMEM (GIBCO) supplemented with 2.5% fetal calf serum (GIBCO), 2.5% newborn calf serum (Amimed), 100 U/ml penicillin and 100 U/ml streptomycin. The cells were transfected by calcium phosphate co-precipitation (Graham and van der Eb, 1973; de Villiers and Schaffner, 1983).

Plasmids

Eukaryotic expression vectors for the rGR. pSTC3-556 (S.Rusconi *et al.*, in preparation), is described in Figure 1d and consists of a constitutively active fragment of the rGR, in which the C-terminal hormone binding domain is deleted (Godowski *et al.*, 1987). The small deletion of the three N-terminal amino acids does not affect the activity of the protein (our unpublished results).

pSTC407-556 codes also for a constitutively active receptor derivative which lacks both the hormone binding domain and the N-terminal domain (Miesfeld *et al.*, 1987); it extends from amino acid 407 to 556.

Reporter plasmids. MMTV-Tag was constructed by replacing the chloramphenicol acetyl transferase (CAT) gene of MMTV-CAT (Cato *et al.*, 1986) by the SV40 early region from a *Bgl*II-linked *Stu*I site to a *Bam*HI site, positions 5274 and 2516 respectively (Tooze, 1980).

P4-OVEC (described in Figure 2b) was constructed by inserting four copies of a 15-bp palindromic glucocorticoid responsive element (GRE) (Jantzen *et al.*, 1987) into the *Sac*I/*Sal*I sites of OVEC (Westin *et al.*, 1987), immediately upstream of the rabbit β-globin gene TATA box. All enzymatic manipulations were according to standard procedures (Maniatis *et al.*, 1982).

Mutagenesis

The mutagenesis cassette was constructed by assembling synthetic, overlapping oligonucleotides which were exchanged with the corresponding region of the rGR cDNA using the pre-existing restriction sites *Hin*dIII, *Sph*I and *Pst*I. Site-directed mutagenesis was performed directly in the rGR expression vectors pSTC407-556 and pSTC3-556 by exchange of short wild-type GR sequences with corresponding mutant double-stranded oligonucleotides between the newly created restriction sites. Each mutant receptor cDNA was verified by dideoxy chain termination sequencing (Sanger *et al.*, 1977) and at least two independent clones for each construction were tested in this study.

Transactivation-dependent replication assay (TDR assay)

The previously described 'expression selection' protocol (Tognoni *et al.*, 1985) was modified as follows. Different expression vectors encoding wild-type or mutant GR were co-transfected in CV-1 cells, along with (i) the reporter plasmid, MMTV-Tag, (ii) an excess of a small replication-competent plasmid ('parasite': SV40 origin of replication linked to an Amp^r pSP64 derivative pSTC3-237, encoding a non-functional fragment of the rGR), and (iii) as an internal standard, a non-replicating dam$^-$ plasmid (pUC18 amplified in *E.coli* GM119). In this system, the activity of the different GR mutants regulates the level of Tag expression, thus determining the rate of replication of the 'parasite' and the loss of dam$^+$ methylation (S.Rusconi *et al.*, in preparation). The extent of replication is simply assayed by quantitative transformation of competent *E.coli* after *Dpn*I digestion of the low mol. wt DNA extracted from CV-1 cells (Hirt, 1967). The transformed bacteria are plated on X-Gal/Amp plates. The number of white Amp^r colonies is proportional to the amount of *Dpn*I-resistant material, i.e. eukaryotically replicated plasmids. By comparison with the internal dam$^-$ standard plasmid (*Dpn*I resistant, which gives blue colonies on the same plate), this number is corrected for differences in the efficiency of transfection in CV-1 cells and for yield of lysate and efficiency of bacterial transformation.

Method

Wild-type or mutant GR plasmids (0.1 − 1 μg) were co-transfected in CV-1 cells (25 − 35% confluent) with the reporter gene MMTV-Tag (2 μg) along with 'parasite' (3 μg, see above for a description) and standard dam$^-$ plasmid (1 μg) in 5-cm dishes. The cells were washed twice 12 h later with Tris-buffered saline (TBS) and incubated in fresh medium. The low mol. wt DNA was extracted 48 h after transfection and subjected to *Dpn*I digestion. The *Dpn*I-resistant material was analysed by quantitative transformation of *E.coli* and plating on Amp/X-Gal plates. The ratio of white to blue colonies represents the level of Tag expression corrected for variation in the experimental parameters (see above). This value is divided by the numbers obtained in the negative controls (i.e. a parallel co-transfection in which the GR plasmid was replaced by an equivalent amount of an expression plasmid encoding non-functional receptor) to obtain a stimulation factor (Table I). The amount of co-transfected GR plasmid was calibrated to obtain maximal activity (with our standard transfection conditions, ~0.1 μg for wild-type or fully permissive mutants and 1 μg for the Cys476 → His and Cys482 → His mutants as well as for all non-permissive mutants). Details about the TDR assay will be given elsewhere (S.Rusconi *et al.*, in preparation).

RNA analysis

Transiently expressed β-globin RNA was isolated from HeLa cells co-transfected with P4-OVEC, OVEC-REF and wild-type or mutant rGR expression vectors and assayed as described in Westin *et al.* (1987). For quantities of the different plasmids see legend of Figure 2.

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References

Berg,J.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 99 − 102.
Boshart,M., Weber,F., Jahn,G., Dorsch-Häsler,K., Fleckenstein,B. and Schaffner,W. (1985) *Cell*, **41**, 521 − 530.
Brown,R.S. and Argos,P. (1986) *Nature*, **324**, 215.
Brown,R.S., Sander,C. and Argos,P. (1985) *FEBS Lett.*, **186**, 271 − 274.
Cato,A.C.B., Miksicek,R., Schütz,G., Arnemann,J. and Beato,M. (1986) *EMBO J.*, **5**, 2237 − 2240.
Cato,A.C.B., Skroch,P., Weinman,J., Butkeraitis,P. and Ponta,H. (1988) *EMBO J.*, **7**, 1403 − 1410.
Chandler,V.L., Maler,B.A. and Yamamoto,K.R. (1983) *Cell*, **33**, 489 − 499.
Danielsen,M., Northrop,J.P. and Ringold,G. (1986) *EMBO J.*, **5**, 2513 − 2522.
de Villiers,J. and Schaffner,W. (1983) In Flavell,R.A. (ed.), *Techniques in the Life Sciences*, B5, *Nucleic Acid Biochemistry*. Elsevier, Ireland.
Diakun,G.P., Fairall,L. and Klug,A. (1986) *Nature*, **324**, 698 − 699.
Evans,R.M. and Hollenberg,S.M. (1988) *Cell*, **52**, 1 − 3.
Giguère,U., Hollenberg,S.M., Rosenfeld,M.G. and Evans,R.M. (1986) *Cell*, **46**, 645 − 652.

Giguère,U., Ong,E.S., Segui,P. and Evans,R.M. (1987) *Nature*, **330**, 624–629.
Giguère,U., Yang,N., Segui,P. and Evans,R.M. (1988) *Nature*, **331**, 91–94.
Godowski,P.J., Rusconi,S., Miesfeld,R. and Yamamoto,K.R. (1987) *Nature*, **324**, 365–368.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Green,S. and Chambon,P. (1987) *Nature*, **325**, 75–78.
Hirt,B. (1967) *J. Mol. Biol.*, **26**, 365–369.
Hollenberg,S.M., Giguère,U., Segui,P. and Evans,R.M. (1987) *Cell*, **49**, 39–46.
Huckaby,C.S., Conneely,O.M., Beattie,W.G., Dobson,A.D.W., Tsai,M.J. and O'Malley,B.W. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8380–8384.
Jantzen,H.-M., Strähle,U., Gloss,B., Stewart,F., Schmid,W., Boshart,M., Miksicek,R. and Schütz,G. (1987) *Cell*, **49**, 29–38.
Klug,A. and Rhodes,D. (1987) *Trends Biochem. Sci.*, **12**, 464–469.
Kühnel,B., Buetti,E. and Diggelman,H. (1986) *J. Mol. Biol.*, **190**, 367–378.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McDonnell,D.P., Mangelsdorf,D.J., Pike,J.W., Haussler,M.R. and O'Malley,B.W. (1987) *Science*, **235**, 1214–1217.
Miesfeld,R., Rusconi,S., Godowski,P.J., Maler,B.A., Okret,S., Wilström, A., Gustafsson,J.A. and Yamamoto,K.R. (1986) *Cell*, **46**, 389–399.
Miesfeld,R., Godowski,P.J., Maler,B.A. and Yamamoto,K.R. (1987) *Science*, **236**, 423–426.
Miksicek,R., Borgmeyer,U. and Nowock,J. (1987) *EMBO J.*, **6**, 1355–1360.
Miller,J., MacLachlan,A.D. and Klug,A. (1985) *EMBO J.*, **4**, 1609–1614.
Pääbo,S., Weber,F., Kämpe,O., Schaffner,W. and Peterson,P.A. (1983) *Cell*, **35**, 445–453.
Petkovitch,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature*, **330**, 444–450.
Ponta,H., Kennedy,N., Skroch,P., Hynes,N.E. and Groner,B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1020–1024.
Rusconi,S. and Yamamoto,K. (1987) *EMBO J.*, **6**, 1309–1315.
Richard-Foy,H. and Hager,G. (1987) *EMBO J.*, **6**, 2321–2328.
Sabbah,M., Redeuilh,G., Secco,C. and Baulieu,E.-E. (1987) *J. Biol. Chem.*, **262**, 8631–8635.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schüle,R., Muller,M., Otsuka-Murakami,H. and Renkawitz,R. (1988) *Nature*, **332**, 87–90.
Tognoni,A., Cattaneo,R., Serfling,E. and Schaffner,W. (1985) *Nucleic Acids Res.*, **13**, 7457–7472.
Tooze,J. (ed.) (1980) *DNA Tumor Viruses, Molecular Biology of Tumor Viruses*, 2nd edn, pt 2, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Valenzuela,D., Weber,H. and Weissmann,C. (1985) *Nature*, **313**, 698–700.
Westin,G., Gerster,T., Müller,M., Schaffner,G. and Schaffner,W. (1987) *Nucleic Acids Res.*, **15**, 6787–6798.
Yamamoto,K.R. (1985) *Annu. Rev. Genet.*, **19**, 209–252.

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