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A transcriptional repressor obtained by alternative translation of a trinucleotide repeat

Rainer B. Lanz[1], Stefan Wieland[1,2], Martin Hug[1] and Sandro Rusconi[1,3,*]

[1]Institut für Molekularbiologie II der Universität Zürich, UZI Winterthurerstrasse 190, 8057 Zurich, Switzerland, [2]Medizinische Universitatsklinik, Hugstetterstrasse 55, D-79106 Freiburg, Germany and [3]Biochemisches Institut, Universität Fribourg, Pérolles, CH-1700 Fribourg, Switzerland

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ABSTRACT

Triplet nucleotide repeats are ubiquitous and rapidly evolving sequences in eucaryotic genomes. They are sporadically found in coding regions of transcription regulators where they become translated in different homopolymeric aminoacid (HPAA) stretches, depending on the local frame. Poly(CAG) yields three different HPAAs (poly Gln, Ser or Ala). Current sequence databases indicate a clear bias in the size and frequency of these HPAAs according to the rule: (Gln)n>(Ser)n>>(Ala)n. Aiming to understand the reasons of this bias, we changed the translational reading frame of the highly polymorphic CAG-repeat that normally encodes poly-Gln in the N-terminal portion of the rat glucocorticoid receptor (GR). The GR mutant in which the CAG repeat is translated to poly-Ala (called GR[Ala]) is incapable of transactivation, but maintains competence for hormone binding, nuclear translocation and specific DNA binding. We show that GR desactivation is obtained only when a very precise threshold length of the repeat is reached. GR[Ala] displays a strong negative dominance when tested for transcriptional activation *in vivo* and may become useful for selective competition of receptor dependent activities in tissue culture cells and transgenic animals. We discuss the implications of our findings for the understanding of the evolutionary behaviour of trinucleotide repeats in coding sequences.

INTRODUCTION

Tri-nucleotides of the sequence 5′-CAR-3′ [R represents G or A, also known as OPA (1) repeats] have been found in the coding as well as in the non-coding regions of many mRNAs. Deletion studies of these elements have not yet been informative with regard to their function, although several human diseases could be linked to repeat length expansions [reviewed by (2)]. The most recurrent OPA motif is the CAG trinucleotide that can encode either poly-Gln, -Ser or -Ala, depending on the reading frame. These particular homopolymeric amino acid (HPAA) strings are not equally represented in nature, where their frequency as well as size distribution follows the order: poly-Gln > poly-Ser >> poly-Ala (S. R., unpublished). Furthermore, one can observe that both frequency and size of HPAAs diminish proportionally to the evolutionary degree of the organisms. Thus, it appears that HPAAs are generally eliminated during evolution and that the poly-Ala motifs are subjected to an additional, stronger negative selection. We have shown elsewhere that homopolymeric glutamine stretches can serve as weak transcription activation domains (3) and in this work we have been aiming at understanding the evolutionary bias applying to the alternative translations of the CAG repeats. For these experiments we used a very well studied transcription factor, the glucocorticoid receptor (GR) that naturally tolerates the presence of a CAG repeat in the poly-Gln frame.

The GR [Fig. 1, reviewed in (4)] is a transcription factor consisting of several modules: a DNA binding domain (DBD), a hormone binding domain (HBD) and several transactivation domains. The major transactivation function of the GR has been localized within the N-terminal half, approximately between residues 110 and 320 (5,6). Weaker transactivation functions have been described around the DNA binding region [called *tau2* (7) or *enh1* (8)], and the region TAF-2 that is overlapping with the hormone binding domain (9). The presence of multiple activation domains has so far prevented the simple construction of mutants that are unable to activate transcription while retaining all the other GR functions. We report here the generation of a *trans*-dominant negative [TDN, see (10) for comprehensive definitions] mutant obtained by changing the translational reading frame of a tri-nucleotide repeat present in the rat GR cDNA.

RESULTS

Construction and expression of GR HPAA mutants

The rat GR cDNA cloned from hepatoma cell line J.2.17.2 (11) contains a sizeable CAG repeat coding for 22 Gln residues (interrupted by one Arg) towards its amino-terminus (aa 75–96, see Fig. 1A). The length of this repeat varies among different rat strains (12), is shorter in the mouse GR and is absent in the human GR. We can tentatively conclude that the presence of poly-Gln is rather irrelevant for essential GR functions. However, we

*To whom correspondence should be addressed at: Biochemisches Institut, Universität Fribourg, Pérolles, CH-1700 Fribourg, Switzerland

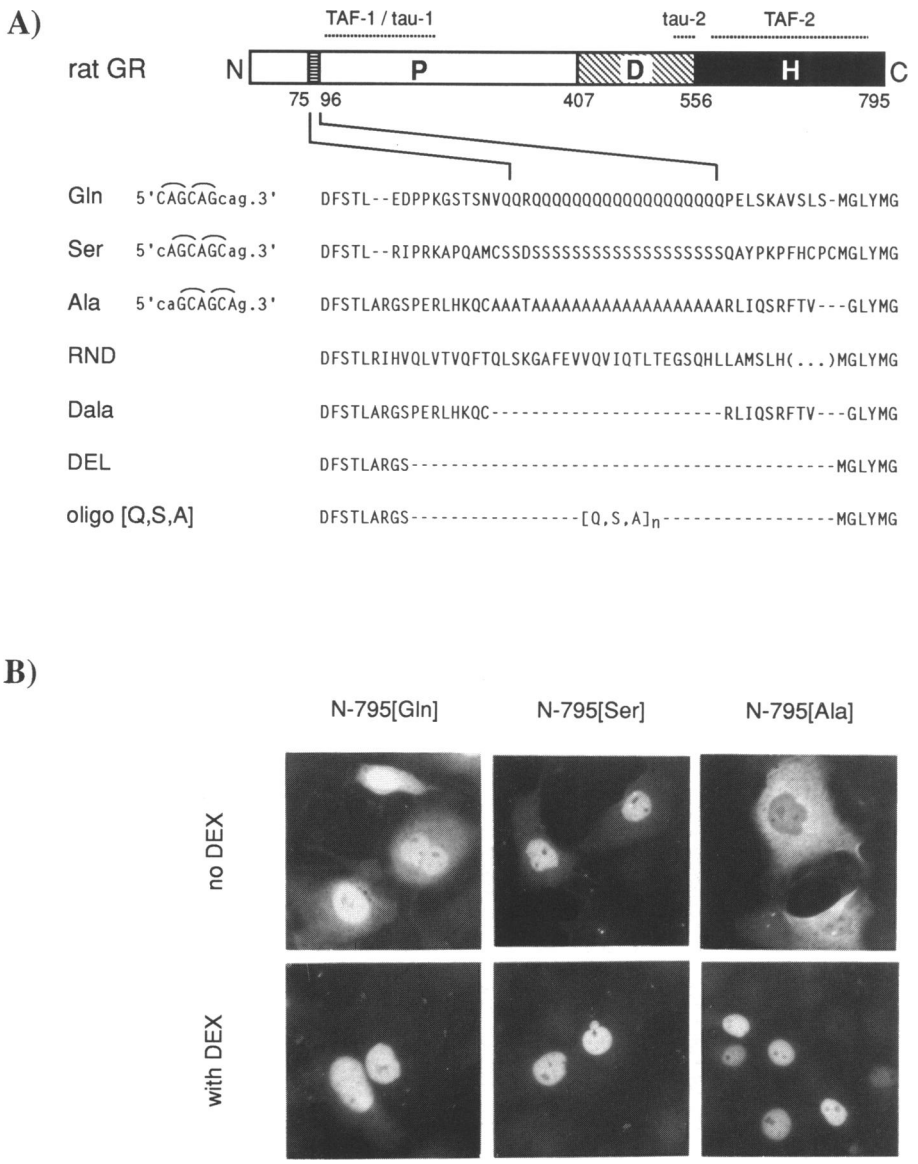


Figure 1. Construction and expression of GR HPAA mutants. (**A**) Top: Schematic representation of domain structure of the rat glucocorticoid receptor (GR). The potentiator (P), DNA-binding (D) and hormone binding (H) domain and the localization of the mutations in the N-terminal part are indicated. The position and extent of transactivation domains [TAF (9) or tau (6)] are shown on the top with dotted lines. The full length receptor is referred to as N-795, other truncated forms and chimeras are presented in Figure 2A. Relevant amino acid sequences of the wild type (poly-[Gln]), and of the mutants poly-[Ser], [Ala], [RND], [DEL], [Dala] and oligo-[Q,S,A] (top to bottom) are given. In our constructions some amino acids flanking the OPA repeat have also been changed (see top four constructs). Oligo[Q,S,A] constructs were based on the GR[DEL] parental clone. The residues immediately adjacent to the Gln, Ser or Ala repeat in the Oligo-[Q,S,A] constructs were different in each case (see Methods). (**B**) Immunofluorescence of CV-1 cells expressing GR bearing HPAA-mutations. Transiently expressing CV-1 cells were detected by indirect immunofluorescence with the monoclonal antibody BuGR1 (see Methods). After induction with 5×10^{-7} M Dexamethasone, all transiently expressed GRs show nuclear localization (bottom row). In absence of ligand, the mutant GR N-795[Ala] is found predominantly in the cytosol, whereas the wild type and the poly-Ser containing form show strong nuclear—besides cytoplasmic staining (top row). The preferential nuclear localization in absence of hormone persisted under different conditions such as lower serum or in absence of phenol-red (data not shown).

considered this to be an excellent situation to examine the tolerance towards the presence of different types and lengths of HPAAs in a well-studied transcription factor. For this purpose we constructed the mutants described in Figure 1, bearing at the corresponding position a poly-[Ser] motif, a poly-[Ala] motif, a 'random' sequence [RND], a deletion of residues 71–117 [DEL] or progressively longer HPAAS (oligo[Q,S,A]). Since some amino acids flanking the OPA repeat have also been mutated during construction, we included a specific control mutant that retains the altered flanking amino acids but lacks the Ala repeat (Dala in Fig. 1A). These mutants were compared to the wild type GR by immunostaining *in situ* (Fig. 1B) for their stability and cellular localization in presence/absence of agonist. As in the case of the wild type, all mutants display an exclusively nuclear location when transiently expressed in CV-1 cells in presence of the hormone (bottom panels in Fig. 1B). In the absence of ligand, the *in situ* staining of N-795[Ser], N-795[DEL] and N-795[RND] mutant were indistinguishable from the uninduced wild type GR N-795[Gln] (for simplicity only data for mutant N-795[Ser] are shown). In contrast, the N-795[Ala] mutant shows a more

pronounced cytoplasmic or a perinuclear preference in absence of ligand (right top panel in Fig. 1B). The reasons for this distinct behavior may correlate with the other anomalous properties of GR[Ala] as described below.

The GR[Ala] mutant is unable to activate a natural responsive promoter and behaves as a *trans*-dominant negative GR

To evaluate the *trans*-activation potential, we have co-transfected effector plasmids encoding the mutant GRs along with a reporter vector in which the rabbit β-globin gene is under control of the MMTV promoter [MTV-OVEC, Fig. 2A, reviewed by (4)]. Wild type or mutant GRs were tested either as full length GR (GR N-795) or as the carboxy-truncated derivative GR N-556 that is permanently located in the nucleus and functions in absence of hormone (13). The levels of reporter mRNA were measured by quantitative S1-nuclease protection (14) and the relevant results are shown in Figure 2B and C. Small amounts of effector plasmid bearing either the wild type GR, GR N-795[DEL] or GR N-795[Dala] strongly stimulate the reporter gene in the presence of dexamethasone (Fig. 2B, compare lanes 4 with 12 and 13), suggesting that the presence or absence of the Gln repeat *per se* has little effect on the activity of the rat GR molecule. The activity of the receptor is moderately reduced when the Gln repeat is substituted with a Ser repeat and seems impaired when the segment is substituted with a 'random' sequence (lanes 8 and 9 for poly Ser and 10 and 11 for RND in Fig. 2B), whereas the mutant GR[Ala] is completely inactive (lanes 6 and 7). From immunofluorescence data we know that the lack of activity of the GR[Ala] mutant is not due to instability or incapacity for nuclear translocation (Fig. 1B) and subsequent experiments have shown that the loss of function by the poly-Ala mutation is not due to loss of hormone binding or DNA binding (see below). Only in the case of the GR[RND], the lower transactivation can partly be explained by instability of this recombinant (Table 1, column b).

Table 1. Properties of GR HPAA mutants

GR constructs		Functions						
Nr	construct[a]	Stability[b]	Binding[c]		Localization[d]		*Trans*-function[e]	
			HOR	DNA	HOR$^-$	HOR$^+$	ACT	REP
1	N-795[Gln]	10	+++		N>C	N	+++	*
2	N-556[Gln]	10	–		N		+++	*
3	N-795[DEL]	10	+++		N>C	N	+++	*
4	N-556[DEL]	10	–		N		+++	*
5	N-795[Dala]	10	+++		N>C	N	+++	*
6	N-795[RND]	1	+++		N>C	N	+	no
7	N-795[Ser]	3	+++		N>C	N	++	no
8	N-556[Ser]	3	–		N		++	no
9	N-795[Ala]	3	+++		C>N	N	–	yes
10	N-556[Ala]	3	–		N		–	yes
11	407–795	10	+++		N>C	N	+	no
12	407–556	10	+++	+++	N		+	no
13	N-154[Gln]407–556	10	+++	+++	N		+	no
14	N-154[Ala]407–556	10	+++	+++	N		–	yes
15	N-556[Gln].Ser482	10	–		N		–	no
16	N-556[Ala].Ser482	10	–		N		–	no
17	GR[Gln].AR	10			N(C	N	+++	*
18	GR[Ala].AR	10			N<C	N	+++	yes

[a]The general structure of the constructs is depicted in Figure 2A and details on sequence of the repeat are given in Figure 1A. The constructs bearing a ligand binding domain were activated with the corresponding agonist (Dexamethasone for GR and R1881 for AR). The Ser482 mutants are unable to bind DNA since the second Zn finger structure is destroyed (15).

[b]Stability was assessed by Western analysis (not shown in this work) and by *in situ* immunostaining experiments (see examples in Fig. 1B). For wild type GR the arbitrary value has been set to 10.

[c]Data from direct hormone (HOR) binding and DNA (DNA) are not shown in this work. The former were obtained by filter binding (11,30) of receptor overexpressed in cell culture, the latter were obtained either with electro-mobility shifts using nuclear extracts or with promoter interference assays (see Methods). Three '+' indicate activity similar to wild type.

[d]Cellular localization was determined by *in situ* immunofluorescence (see examples in Fig. 1B) in absence (HOR$^-$) or in presence (HOR$^+$) of agonist. Symbols: N, nucleus; C, cytoplasm.

[e]The ability to *trans*-activate (ACT) or to *trans*-repress (REP) is displayed qualitatively. Specific examples are shown in Figure 2. The chimeric GR[Ala].AR is able to *trans*-repress equally well the wild type AR or GR and does it only in presence of R1881. Symbols: '+++' , *trans*-activation indistinguishable from wild type; '++', 10–30% ; '+', 3–10% ; '–', less than 0.5% ; *, not determinable; 'no' and 'yes', unable respectively able to *trans*-repress. Empty fields indicate experiments that have not been done or are not relevant for the points addressed in this work.

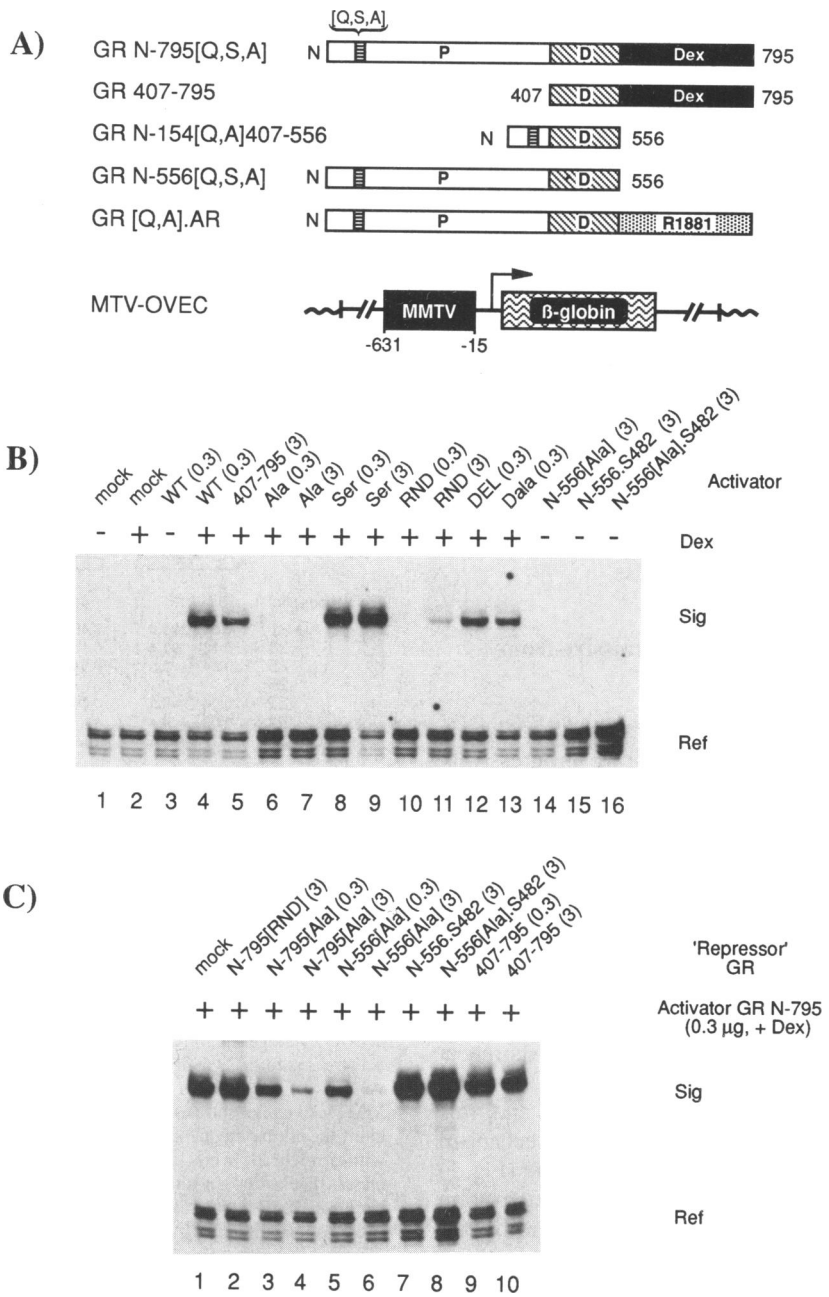


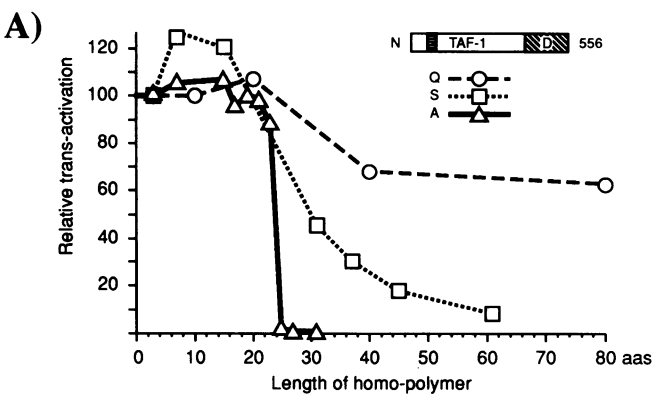
Figure 2. *Trans*-activation and *trans*-repression properties of GR HPAA mutants. (**A**) Structure of the GR segments bearing HPAA mutations and the reporter gene. The GR segments were cloned into the eucaryotic expression vector pSTC (15,26). Nomenclature: GR N-795, full-length rat GR cDNA; GR 407–795, a weak hormone-dependent GR mutant missing the TAF1; GR N-154[Q,A]407–795, the very amino portion of GR linked to a 'minimal' GR fragment that shows weak *trans*-activation (7,8); GR N-556, carboxy-truncated form that can work constitutively (7,8,13); GR[Q,A].AR(HBD), the GR amino terminus including DNA binding domain linked to the ligand binding domain of the androgen receptor (AR, a gift of A. Brinkmann). The symbols 'Dex' (Dexamethasone, Sigma) and 'R1881' (Amersham) indicate the binding domains for respective agonists. MTV-OVEC is the reporter plasmid containing the MMTV promoter/enhancer [reviewed by (28)] linked to a modified rabbit β-globin reporter fragment (14). (**B**) Effect of HPAA mutants on *trans*-activation. The transfection cocktail included 10 µg reporter MTV-OVEC and the indicated amounts of effector plasmid, other components and conditions for RNA analysis are described in Methods. If the level of transcription in lane 4 is defined as 100% (0.3 µg wild type GR), then the relative transcription levels are, from left to right: <1%*, 1%*, 2%, 100%, 46%*, <1%, 1%*, 66%, 204%*, 1%, 8%*, 41%, 45%, <1%*. <1%*, <1%*, (asterisks indicate that 3 µg effector plasmid was co-transfected). Symbols: names and bracketed numbers at top of panel, type and amount (in µg) of effector plasmid; Sig, signal from correctly initiated transcription; Ref, signal derived from co-transfected reference (-globin gene (14). Immunoblots of extracts from expressing cells indicate that some mutants differ in stability (see also Table 1). (**C**) *Trans*-repression properties of the GR[Ala] mutations. Transfection conditions as in (B). Lane 1 (activity with 0.3 µg GR N-795 plus 3 µg inert effector plasmid CMV-CAT) is defined as 100%. Both GR N-795[Ala] (lanes 3 and 4: 58% and 11%*) or GR N-556[Ala] (lanes 5 and 6: 38% and 3%*) are able to repress the action of wt GR. This is not observed in control lanes (lane 2, 126%* and lanes 7–10: 78%*, 75%*, 99%, 97%*). Other symbols as in B.

Since GR[Ala] is unable to activate but seems to respond normally to the presence of hormone (see Fig. 1B), we expected that this mutant could act as a *trans*-dominant negative (TDN) competitor for the wild-type receptor. To test this, we expressed a given amount of wild type GR and competed its action by co-expression of the [Ala] mutant and other controls. As shown in Figure 2C, only the GR[Ala] is able to compete with the wild-type GR for the GR-responsive MMTV-LTR promoter (see lanes 4 and 6 for competition in plasmid excess of N-795[Ala] or N-556[Ala]). The double mutant GR N-556[Ala]/S482, whose second Zn finger structure is destroyed (15), is unable to act as a competitive inhibitor (lane 8). This indicates that intact DNA binding function or at least integrity of the Zn-fingers is required for the TDN behaviour. We can demonstrate that the dominant negative properties of the GR[Ala] mutants are not due solely to de-activation of the major activation function [TAF1 (9)], because the truncation mutant GR 407–795 (see structure in Fig. 2A) that completely lacks TAF-1 does not act as a dominant repressor (lane 9 in Fig. 2B). Finally, the fact that the carboxy-truncated N-556[Ala] (missing the hormone binding domain, HBD) maintains the TDN properties demonstrates that the ligand binding domain is not essential for this process.

A narrow size-threshold distinguishes permissive from non-permissive oligo-Ala stretches

To better characterize the loss of function exerted by the poly-Ala stretch, we constructed GR molecules bearing progressively longer HPAA stretches and compared them to the starting construct GR[DEL] for transactivation (Fig. 3A). Extension of glutamine residues has only a minor consequence on the GR activity, whereas a long oligo-[Ser] stretch results in a progressively reduced activity (circles versus squares in Fig. 3). For oligo-[Ala] we noticed an abrupt decrease from nearly full to less than 5% transactivation when the length of the repeat increases from 23 to 25 alanines in the construct (triangles in Fig. 3). This result is very remarkable since the sole difference between GR[Ala23] and GR[Ala25] is the prolongation of the Ala-repeat by two residues. A chain of 23–25 hydrophobic residues corresponds to an average trans-membrane helix and this observation may be very important to understand the mechanism of de-activation of the GR. The original GR[Ala] mutant (Fig. 1) counts only 23 Ala residues and we believe that GR de-activation occurs in this mutant due to the immediately adjacent hydrophobic residues ($QC(A)_nRLI$), whereas in the synthetic Oligo-Ala mutants the flanking aminoacids are not hydrophobic ($RP(A)_nGRP$). This may explain why the length of the synthetic Ala-stretch needs to be slightly longer to exert the negative effect.

Since the negative effect of oligo-Ala sequences at the natural poly-CAG site could be due to simple sterical disturbance of immediately adjacent sequences, we monitored the effect of various HPAAs placed at different positions along the primary sequence of the GR as shown in Figure 3. To this purpose we inserted HPAAs of different length at position 405 (upstream of the DBD), position 538 (between DBD and HBD) and position 793 (as carboxy-appendix). The mutant GRs were compared for their efficiency in stimulating transcription from the model promoter MMTV. The results were tabulated as signal/reference ratio (plain digits) and constructs encoding an Ala21 repeat (first line of tabulated results) served as 100% permissive standards (boldface digits in Fig. 3B). We observed that oligo-Ala is capable



B)

	I		II		III		IV	
position:	75		405		538		793	
Ala 21	6.63	**100.0**	9.48	**100.0**	3.02	**100.0**	7.09	**100.0**
23	6.12	**92.4**	6.10	**64.3**	4.81	**159.1**	6.10	**88.2**
25	0.24	**3.6**	0.54	**5.7**	0.50	**16.6**	5.61	**81.9**
26	-	-	-	-	-	-	3.17	**45.6**
27	0.04	**0.5**	0.04	**0.4**	0.03	**0.9**	0.43	**6.3**
31	0.02	**0.4**	-	-	-	-	0.01	**0.3**
Gln 26	4.21	**63.6**	4.71	**49.6**	9.23	**305.2**	6.26	**91.4**

Figure 3. Influence of progressive increase of HPAA repeat length and its relative position on transactivation by the GR. (**A**) Influence of repeat length. CAG repeats of both different length and translation frames have been cloned into GR N-556[DEL] and assayed for transactivation on P4-OVEC, a reporter plasmid with four idealized palindromic GR binding sites (15,29). Transfection was performed in HeLa cells, RNA analysis conditions are described in Methods. Y-axis: percentage of activation obtained compared to GR N-556[DEL]; X-axis: length of amino acid stretch encoded by the CAG repeat. Symbols: circles and dashed line, oligo-[Gln]; squares and dotted line, oligo-[Ser]; triangles and solid line, oligo-[Ala]. Note the maintenance of activity by longer oligo-[Gln] and the sudden drop when the oligo-[Ala] stretch is increased from 23 to 25 residues. Identical results were obtained with intact versions of the mutants (context N-795) assayed on the natural promoter MMTV (data not shown). (**B**) Effects of repeat insertion at different positions along the primary sequence. Top, the HPAAs (horizontally hatched box marked with arrowhead) were inserted at different positions in the four construct classes. In class I the position is the same as in the natural GR (pos. 75), whereas in classes II, III and IV the HPAAs were inserted respectively at positions 405 (just upstream of the DNA binding domain), 538 (between DBD and HBD) and 793 (carboxy appendix). Transfections were performed in CV-1 cells, 10 μg of the reporter MMTV-OVEC and 1 μg of the expression vectors encoding mutant GRs were included in the transfection cocktail, other components in transfection and RNA analysis are described in Methods. The table indicates the type of HPAA (leftmost column) and for each construct class the standardized transcription (plain characters, Signal:Reference) as well as the transcription level relative to the construct bearing a stretch of 21 Ala residues (boldface characters, percent values). The results are a compilation of several independent experiments. Empty fields indicate omitted or non-relevant analyses.

of de-activating the GR at all the tested position, although small differences in the permissivity-transition length are evident. In particular, the Ala25 repeat progressively loses its de-activation potential when moved toward more carboxy positions. This repeat reduces the activity of construct IV only down to 81% (Fig. 3B, fourth line). Similarly, the Ala27 repeat allows still more than 6% activity when placed at most carboxy position (Fig. 3B,

construct IV, fifth line). Full de-activation of construct IV was achieved only with a 31 residues repeat (sixth line). All the constructs that displayed less than 1% activity were tested for *trans*-dominance and were all found to be able to compete negatively with wild type GR. The position independence renders unlikely the idea that the negative effect of Poly-Ala in the original mutant is due to direct influence on the adjacent TAF-1 segment.

DISCUSSION

We have observed that changes of the HPAA stretch can significantly affect the transactivation function(s) of the rat GR. As shown in Table 1, neither the deletion of the repeat nor the conversion to Poly-Ser does significantly alter the activity of the GR (lines 3–5), while random amino acids result in moderate down mutations due to protein de-stabilization (lines 6 and 7 and 8). Changing to poly-Ala yields mutants that are unable to transactivate, while being relatively stable and capable of trans-inhibition (lines 9 and 10). None of our mutations of the OPA region seems to affect the other fundamental functions of the GR (columns c–e).

A recent report described amino acid substitutions toward the very carboxy terminus of the GR (16) that, analogously to our GR[Ala] mutant, apparently turned off the transactivation potential of the GR while leaving the other functions intact. However, the mechanism of silencing must be different, since in our hands the same carboxy-terminal point mutants do not exhibit the expected TDN phenotype (17,18).

Negative regulation by the [Ala] motif could be due to cytosolic trapping of wt receptor by the GR[Ala]. We believe that this is unlikely, since the powerful inhibitor GR N-556[Ala] has a constitutively nuclear location (Table 1). These notions are confirmed by results obtained with chimeric constructs in which the hormone binding domain has been replaced by the counterpart from the androgen acceptor (AR, see Fig. 2A for structure). For this chimeric construct, the manifestation of the TDN phenotype requires addition of the androgen-analogue R1881 (Table 1, row 17). This shows that the GR[Ala].AR needs to be activated by the ligand in order to exert its dominant negative function. Two major points are clarified with this result: (a) it is unlikely that *trans*-repression is due to trapping of wild-type GR in the cytoplasm, since the repressor needs to increase its nuclear affinity to become active; (b) since the HBD of androgen receptor is different that the HBD of the GR, it is less likely that *trans*-repression is caused by hetero-dimerization.

It is formally possible the [Ala] motif binds a cellular repressor. However, titration experiments with over-expressed GR N-556[Ala]/S482 (which cannot bind DNA) did not rescue the repressive function of GR[Ala] (data not shown). Hence, if such a repressor exists, it should be very abundant since it could not be titrated. Poly-Ala stretches of similar length were linked to either chimeric GAL4 factors (M. Höfferer and M. H., unpublished) or to other transcription factors such as Oct 2B (S. W. and S. R., unpublished). In all cases we observed negative effects, although to a variable extent, and we noticed that most of the chimeric constructs were not able to act as repressors (S. W, M. H. and S. R, unpublished). These findings suggest that a poly-Ala stretch does not de-activate transcription factors in an autonomous fashion. Furthermore, we observed that the magnitude of the de-activation may depend on the target GREs acting at promoter or enhancer position (M.H. *et al.*, in preparation). These facts together suggest that the effect of poly-Ala is both factor- and promoter (enhancer)-specific.

To summarize, several mechanisms can be invoked to explain the loss of GR function exerted by the [Ala]-repeat. 1) General destabilization of the receptor; 2) Local disturbance of the TAF-1 domain; 3) Imposition of structural constraints that impair flexibility required for transactivation; 4) Binding of a transcriptional repressor; 5) Generation of a general transcription repressive domain; 6) Cytoplasmic trapping of the wild type GR by heterodimerization in solution; 7) Disturbance of post-translational modifications required for transactivation. Our results render the first six possibilities very unlikely. The last possibility has attracted our attention and we are currently examining whether some post-translational modification (such as Ser/Thr phosphorylation) is selectively impaired in the GR[Ala] mutants. In particular, we believe that the rather hydrophobic Ala-stretch may cause a temporary association of the nascent GR with intracellular membranes, where the normal post-translational modifications occur at a different extent. This idea is in accordance with the observed anomalous compartmentalization of the unliganded GR[Ala] (see Fig. 1B). In recent experiments we have observed that the global phosphorylation not grossly impaired in the GR[Ala] (M. H., unpublished). This does not exclude that some specific phosphorylation may be altered or that undesired modifications such as O-glycosylations that do not normally occur in the GR (19) may be favoured during that temporary change of compartmentalization. It will be interesting to observe the effect of poly-alanine stretches linked to further natural transcription factors besides Oct2. In particular, we want to verify whether the GR[Ala] mutant is also impaired in its ability to interfere with the action of AP1 on composite response elements [see (20–22) and references therein]. Recently, an oligopeptide consisting of repeated $(Lys)_4$-Ala residues has been shown to behave as a strong repressor *in vivo* and *in vitro* (23). We are currently comparing this motif with the poly-Ala segment for their behaviour when linked to different chimeric constructs. The results show that the two motifs have a distinguishable effect on transcription (M. H., in preparation). We have recently obtained transgenic animals that harbour and should express dominant negative GR constructs in selected tissues (S. Brenz Verca and Th. Rülicke, unpublished). It will be interesting to see to which extent the GR-dependent response will be impaired in tissues expressing the GR[Ala] and the physiological consequences of the competitive inhibition.

There is accumulating evidence that Ala-rich domains may be involved in transcriptional modulation. The transcription factor Tst-1 (a member of the POU family) contains an Ala-rich amino-domain that may work in a tissue-specific repressive fashion (24). Licht and co-workers (25) have proposed that Ala-rich sequences of the *Drosophila* transcription factor Krüppel may be involved in repression. It is possible that some of these Ala-rich domains have evolved from ancestral (GCN)n repeats that have been progressively immobilized by insertion of foreign codons together with wobbling of Ala codons. Therefore, we believe that the negative effects observed in our constructs may have been selected during evolution to generate some primitive transcription repressive functions.

Genes bearing expanding trinucleotide repeat sequences that are translated into glutamine stretches have been shown to be implicated in severe diseases [reviewed by (2)]. Although a

potential structure for poly-Gln stretches has been recently proposed (31), the mechanisms by which the trinucleotide expansions result in disease outbreak are still matter of speculation. Our work extends now the knowledge in this field by showing that not only the length but also the translational frame of the repeats can influence the activity of the linked factor. A specific pathology caused by the local change of frame of a CAG repeat is unlikely to exist. However, the observation that a threshold length can decide between a strong activator and a repressor (Fig. 3A) makes us believe that some genetic defects arising by expansion of existing Ala-coding trinucleotide repeats may be discovered in the future. In any case, our studies may help understanding the evolutionary pressure that modulates the spreading of these repeats in various reading frames in coding regions of the mammalian genome and some of their most primitive biological consequences.

MATERIALS AND METHODS

Constructions of HPAA GR mutants and chimeras

A *Bam*HI–*Xba*I linker was introduced at position 71/72 in the GR-cDNA (GR[Gln], top sequence in Fig. 1A). The frame-shift mutations were created by blunt ending the protruding ends of the *Xba*I site (with Klenow polymerase to generate +1 and with mung been nuclease to generate a –1 shift). The *Nco*I restriction site downstream of the CAG repeat (aa 117) was used to restore the correct frame by compensatory repair. To create the random insertion in GR[RND], a *Nco*I–*Bam*HI-fragment of the rabbit β-globin gene providing 61 amino acids was inserted in antisense orientation. The GR[DEL] mutant was constructed by a *Bam*HI (linker)–*Nco*I collapse, whereas for GR[Dala] we used synthetic oligonucleotides containing the sequences flanking the GR[Ala] mutant. The Oligo-[Q,S,A] mutants clones were generated by using the *Bam*HI–*Nco*I starting oligonucleotide (5′-GATC-CCTCGAGGAAGACCAgcagCAGCAgcagGGAGACCGGT-ACCCCATGG-3′) containing a *Bbs*I and a *Bsa*I site (underlined) flanking four CAG-triplets. Constructs containing increasing amounts of glutamines were generated by repetitive subcloning, in which we exploited the compatible protruding ends (low case) released by the enzymes *Bsa*I and *Bbs*I that cut outside their recognition sequence. This strategy allows the rapid amplification of these (or any other) repeats. Translational frame-shift of the CAG repeat was generated by filling or digesting the cohesive ends of the *Xho*I site (CTCGAG) and complementary repair of the *Kpn*I/Asp718 site (GGTACC). The amplified repeats were re-cloned into the GR{DEL} construct (Fig. 1A) through *Bam*HI–*Nco*I sites. The flanking residues of the Oligo [Q,A,S] were as follows: LEED(Q)nGDRYP; LRRGRP(A)nGRPP; RKT(S)nRETGTYP. Maintenance of the reading frame was verified by sequencing and *in vitro* transcription/translation. Chimeric GR/AR clones were constructed by linking the first 523 aa of GR with the last 550 aa of the human AR (17).

Transfection and RNA analysis

For *in situ* immunostaining analysis (Fig. 1B), CV-1 cells were grown in DMEM supplemented with 5% fetal calf serum and penicillin/streptomycin. Ten μg effector plasmid carrying the mutated rGR-cDNA and a SV40 origin of replication was transfected together with 1 μg of an expression vector encoding SV40 T-antigen (26) using calcium phosphate co-precipitation. Cells were incubated for 24 h after removal of the precipitate. Two hours before fixation 5×10^{-7} M dexamethasone (DEX) was added in samples shown in bottom panels. The cells were fixed with –20°C acetone/methanol (3:7), dried and treated with diluted monoclonal anti-GR antibody BuGR2 (27). The complexes were visualized with FITC-labeled swine-anti-mouse antibody (Calbiochem). For the data of all other figures the transfection was performed as above with either CV1 or HeLa cells as specified. The transfection cocktail contained the indicated amount of *trans*-activator expression plasmids, the reporter plasmid (10 μg), a mock expression plasmid (CMV-CAT, used in negative controls and to compensate for different amounts of CMV bearing plasmid) and the reference plasmid [3 μg,(14)]. Where indicated, 5×10^{-7} M dexamethasone (DEX) was present in the growth medium. Transiently expressed RNA was isolated after 48 h incubation (post-transfection) and subjected to S1 nuclease analysis (14). Quantification of the signals was performed by laser densitometric scanning of different film exposures. Relative transcription is defined as the ratio {signal:reference} and selected samples were defined as 100% (see legends).

DNA binding assays

DNA binding was measured either by gel retardation assay [conditions as in (14)] or by a promoter interference assay (Döbbeling *et al.*, in preparation) in which a GRE has been placed between the TATA box and the initiation site of a reporter beta globin gene.

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