

Two silencing sub-domains of v-erbA synergize with each other, but not with RXR

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ABSTRACT

The thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) induce gene expression in the presence of specific ligand and repress transcription in the absence of hormone. This repression is mediated by an active silencing mechanism rather than by interference with DNA binding activators. V-erbA, a variant form of TR which is unable to bind hormone, represents a constitutive repressor. Here we show, using fusion proteins with the GAL4 DNA binding domain, that the minimal silencing domain of v-erbA extends from amino acids 389 to 632 and that internal deletions within this domain retain at least some repression function. Co-transfection experiments of different deletion mutants indicate that the silencing domain is composed of at least two sub-domains which are non-functional when tested individually. When combined in a heterodimeric complex, they synergize such that silencing activity is regained. In contrast to the retinoic acid receptor the retinoid X receptor does not contain a silencing domain. In addition it is unable to cooperate with the repression function of TR or v-erbA in a heterodimer.

INTRODUCTION

The members of the thyroid hormone receptor (TR) and of the retinoic acid receptor (RAR) family act on vertebrate development and homeostasis by binding to specific DNA sequences (1–3), thereby regulating transcription of target genes (4,5).

TR and RAR are ligand inducible transcription factors which repress transcription in the absence of hormone and induce expression in the presence of specific ligand (6). Depending on the promoter context or type of response element ligand dependent repression has been described as well (7–10). Here we are analyzing the repression in the absence of ligand which is mediated by an active silencing mechanism even acting on a minimal promoter composed of only a TATA box (11). Variant forms of these factors which are unable to bind hormone represent constitutive repressors (6,12). Recently the analysis of thyroid hormone resistance syndrome patients illustrated the importance of this repression function. A dominant negative TR mutant is causing a severe impairment of physical and mental development,

which is not seen upon TR gene deletion (13). Similarly, a major role for silencing is demonstrated by the chicken oncogene product v-erbA. It does not bind T3 due to mutations in its ligand binding domain (14), it functions as a constitutive silencer protein (6,11,12,15) and it interferes with normal erythropoiesis (16–20). A natural mutant of v-erbA (Pro 399 to Arg) lacks the oncogenic and the silencing activity (21,22).

Deletion analysis of v-erbA assigned the silencing function to about 300 C-terminal amino acids (a.a.) of the protein (23). Corresponding domains of TR and RAR were able to mediate both, repression and ligand dependent induction. In contrast to other repression mechanisms (7–9,24–27), nothing is known about the structural requirement for silencing domains, a prerequisite for the mechanistic understanding of this effect (24).

The finding that both TR and RAR as well as other members of the gene family form heterodimers with RXRs adds even more complexity to the transcription regulation by these factors (28–34). The natural ligand for RXR was shown to be 9-cis retinoic acid (9-cis-RA), a stereoisomer of all-trans-retinoic acid, the ligand for RAR (35,36). Heterodimerization of TR with RXR strongly enhances both, specific DNA binding measured *in vitro* (28,30,37,38) and transcriptional induction in transient transfection experiments (31,32,34,39) or *in vitro* (40). It was not known whether RXR exhibits or contributes to the repression function in the ligand free heterodimer with TR or RAR.

Here we show that the v-erbA silencing domain is composed of at least two regions, both of which are non functional on their own. Repression activity is restored when both regions are present in one protein, but interestingly also when present on two different proteins which are able to form heterodimers.

We find that a region of the RXR domain, corresponding to the minimal silencing domain of v-erbA, does not mediate transcriptional repression, although it readily displays hormonal induction. In addition it does not contribute to TR or v-erbA silencing when present in a heterodimer.

RESULTS

V-erbA contains a large silencing domain

Based on previous results (23) we wanted to define more precisely regions of v-erbA required for transcriptional repression. Therefore we fused the C-terminal part of v-erbA to the

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heterologous DNA binding domain (DBD) of the yeast activator GAL4. Amino acids 1–147 of GAL4 are sufficient for mediating several functions such as nuclear translocation, dimerization and binding to the specific upstream activator sequence (UAS), whereas the major trans-activation functions are deleted. Transcriptional regulation mediated by GAL4-DBD or by the chimeric proteins was measured using a reporter plasmid carrying the bacterial chloramphenicol acetyl transferase (CAT) gene. CAT expression was under the control of a GAL4 DNA binding site upstream of the thymidine kinase (tk) promoter. This approach allows to study the function of v-erbA domains independently of endogenous members of the thyroid hormone receptor family which bind to the T3 response elements, but not to an UAS. Expression and DNA binding ability was tested by

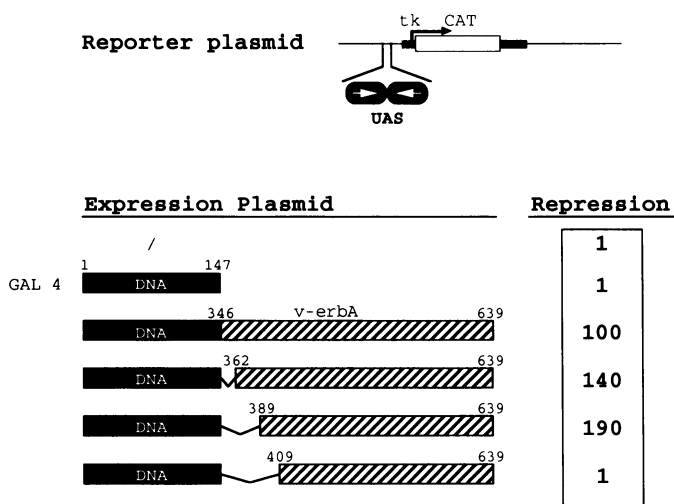


Figure 1. Amino-terminal deletion mapping of the v-erbA silencing domain. Expression vectors coding for the indicated fusion proteins were transfected into Ltk⁻ cells together with the indicator plasmid UAS-tkCAT (top line). Fold repression was calculated from CAT activities obtained in triplicate transfections relative to the CAT activity seen after co-transfection of a non-coding expression vector and the reporter plasmid (23 ± 2% CAT conversion). Experiments were repeated at least twice.

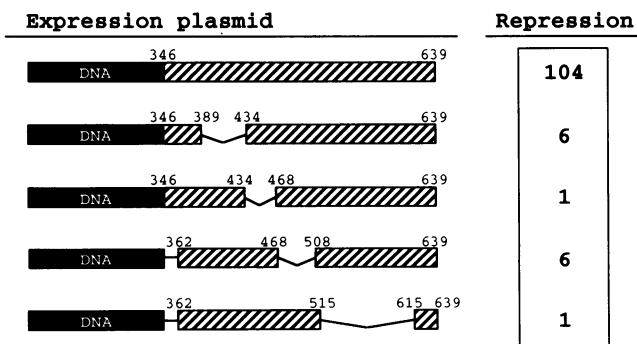


Figure 2. Internal deletions of the v-erbA silencing domain. Expression vectors coding for the indicated fusion proteins were transfected into Ltk⁻ cells together with the indicator plasmid UAS-tkCAT (see legend to Fig. 1). Expression of UAS-tkCAT together with a non-coding expression vector was 40 ± 3% CAT conversion.

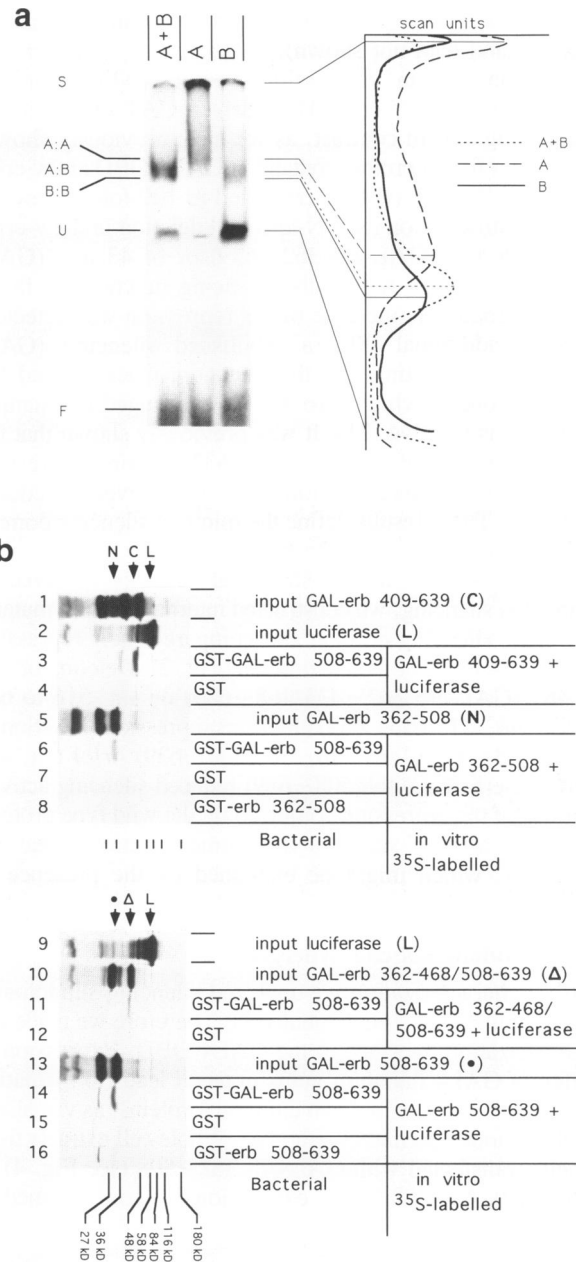


Figure 3. (a) Formation of heterodimers after cotransfection of different expression vectors. Fusion proteins GAL-erb 362–468/508–639 or GAL-erb 362–508 were expressed in COSI cells either alone (lanes A or B, respectively) or together in one experiment (lane A+B). Cell extracts were used in a gel retardation experiment using an UAS probe and the autoradiogram was scanned in order to determine migration differences of the protein–DNA complexes. The lanes were analysed using the Bio-image software and the density profile is displayed. Each band is connected to the corresponding peak in the profile. A:A, B:B and A:B indicate the positions of the A homodimer, the B homodimer and the A+B heterodimer, respectively. S indicates the slot, U an unspecific band and F the free probe. (b) GAL-erb fusion proteins interact *in vitro*: GST-Fusion proteins GST-GAL-erb 508–639, GST-erb 508–639 and GST-erb 362–508 and GST alone were expressed in bacteria, bound to Glutathione–Sepharose beads and incubated with the indicated *in vitro* translated, ³⁵S labelled proteins. The beads were washed, specifically bound proteins were analysed on SDS–PAGE and visualised by Fluorography. Lanes 4, 7, 8, 9, 10, 13 (input) show the *in vitro* labelled proteins utilised, luciferase was added in the interaction reaction as specificity control. The arrows indicate the positions of the correctly translated proteins: GAL-erb 362–508 (N), luciferase (L), GAL-erb 409–639 (C), GAL-erb 362–468/508–639 (Δ) and GAL-erb 508–639 (•).

gel retardation experiments and found to be similar for all the mutants (23 and data not shown).

Transfection of an expression vector coding for the GAL4-DBD had no effect on UAS driven CAT expression in Ltk⁻ cells (Fig. 1). In contrast, as we have previously shown, expression of a fusion protein containing GAL4-DBD and v-erbA amino acids 346–639 (GAL-verbA) led to 100-fold repression of the basal transcription rate. N-terminal deletion in the v-erbA domain of 16 a.a. (GAL-erb 362–639) or of 43 a.a. (GAL-erb 389–639) did not reduce the silencing function, in fact a slight but reproducible increase of the repression was detected. Deletion of additional 20 a.a. abolished silencing (GAL-erb 409–639). Interestingly, in this mutant prolines 397 and 399 were deleted, one of which (Pro 399) is exchanged in a natural mutant which is inactive (21). It was previously shown that the C-terminal deletion of 7 a.a. (346–632) retains repression function, whereas further deletion (346–616) severely reduces silencing (23). These results define the minimal silencing domain of v-erbA spanning a.a. 389 to 632.

In order to further define structural features involved in transcriptional silencing, we constructed internal deletion mutants in the v-erbA silencing domain and compared their repression activity relative to the complete domain (Fig. 2). Deletion of a.a. 435 to 467 (GAL-erb 362–434/468–639) or a.a. 516 to 614 (GAL-erb 346–515/615–639) abolishes repression. Deletion of a.a. 390 to 433 (GAL-erb 346–389/434–639) or of a.a. 469 to 507 (GAL-erb 362–468/508–639) reduced silencing activity to about 6% of the repression conferred by the wild type protein. This residual activity suggests that some functional features remain intact, which might be explained by the presence of subdomains.

Silencing subdomains can synergize

For further characterization we analyzed silencing subdomains for possible functional complementation. Therefore we made use of the dimerization interface of the GAL4-DBD. Expression of two different GAL4 fusion proteins in a cell leads to formation of heterodimers between the two chimeric proteins, as visualized in gel retardation experiments (Fig. 3a). Whole cell extracts from COS1 cells transfected with GAL-erb 362–508 (see Fig. 4) or GAL-erb 362–468/508–639 expression vectors formed a

corresponding DNA–protein complex with an UAS probe (lane A or B). Co-expression of both chimeric proteins leads to the formation of an intermediary complex consisting of a heterodimer (Fig. 3a, lane A+B).

To further prove the formation of heterodimers via the GAL4-DBD, an independent *in vitro* assay was performed. Fusion proteins with the Glutathione-S-transferase were bacterially expressed, bound to Glutathione–Sepharose and used to precipitate specifically interacting *in vitro* translated, ³⁵S-labelled proteins (input Fig. 3b, lanes 4, 8, 10, 13). In each experiment *in vitro* translated, ³⁵S-labelled luciferase (L) (input Fig. 3b, lanes 7 and 9) was added as a specificity control. When GST-GAL-erb 508–639 was coupled to the beads, interaction could be detected with labelled GAL-erb 508–639 (lane 14), GAL-erb 362–468/508–639 (lane 11), GAL-erb 409–639 (lane 6) and GAL-erb 362–508 (lane 3). When only GST was used no radioactive band was visible (lanes 2, 5, 12, 15). In addition, luciferase was not retained in any of these experiments, indicating that the protein–protein interactions are specific. The interaction was mediated by the dimerisation function of the GAL4-DBD, as the fusion proteins GST-erb 508–639 or GST-erb 362–508 did not interact with GAL-erb 508–639 (lane 16) or GAL-erb 362–508 (lane 1), respectively.

In order to measure the effect of heterodimers on UAS-driven transcription, we compared the reporter gene activity after co-expression of two fusion proteins to the reference transcription obtained with a single protein. To avoid differences in the transfection and expression conditions, we added equal amounts of GAL4-DBD expression plasmid in the reference transfection as well. This allows a direct comparison of the effects due to GAL4-DBD/GAL-fusion protein heterodimers (in the reference transfection) to those mediated by GAL-fusion A/GAL-fusion B heterodimers (in the co-expression experiment).

Reference expression of GAL-erb 362–508 or GAL-erb 409–639 is deficient in silencing. Co-expression of both fusion proteins leads to a 2.6-fold repression of CAT transcription (Fig. 4, line 1). Heterodimers between GAL-erb 362–508 and GAL-erb 508–639 showed a clear 6-fold synergistic repression as opposed to the reference transfections (line 2). Further N-terminal deletion of the silencing domain in Gal-erb 557–639 and Gal-erb 617–639 resulted in loss of the complementation

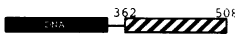

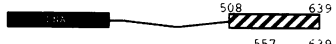
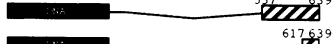

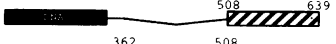
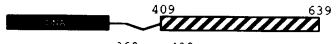
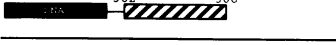

Expressionplasmid A	A	A+B	B	Expressionplasmid B	
	0.7	2.6	1.0		1
		5.9	0.8		2
		1.8	2.2		3
		1.0	0.9		4
	1.2	1.4	1.2		5
	0.7	0.4	0.9		6

Figure 4. Synergism between v-erbA subdomains in heterodimers. Expression vectors coding for the indicated fusion proteins were co-transfected with the UAS-tkCAT reporter plasmid into Ltk⁻ cells either together with a GAL4-DBD expression vector (columns A or B) for reference expression or together with a vector for a second fusion protein (column A+B) for synergistic silencing. Numbers indicate fold repression relative to transfection of a GAL4-DBD expression vector with the UAS-tkCAT reporter (28 ± 5% CAT conversion).

activity (lines 3 and 4). These data show that the silencing domain of v-erbA is composed of at least two regions which are non-functional when tested individually, but which can complement each other even when placed on two different proteins capable to form heterodimers.

In order to ensure that the complementation effect is not due to a higher amount of GAL-verbA fusion proteins in comparison to the reference transfection, we tested combinations of heterodimeric partners containing identical putative silencing regions. Neither of these combinations led to significant repression (Fig. 4, lines 5+6). In addition, increasing the amount of transfected GAL-erb 362-508 in the absence of a second expression vector did not result in silencing (data not shown).

Furthermore, we were interested to know whether a deletion mutant mediating residual silencing activity could synergize with other subdomains. Therefore we tested GAL-erb 362-468/508-639. The repression function of this fusion protein is dramatically increased when co-expressed with different heterodimeric partners (Fig. 5). Both, the N-terminal v-erbA subregion (GAL-erb 362-508) or the C-terminal region (GAL-erb 409-639) fused to the GAL4-DBD synergize efficiently in silencing activity with GAL-erb 362-468/508-639 (Fig. 5). One of these combinations (Fig. 5, line 1) yields near to wild type activity. Another internal deletion mutant with a severe defect in silencing, GAL-erb 362-434/468-639, was unable to complement GAL-erb 362-508 (Fig. 5, line 3).

We conclude that the silencing domain of v-erbA is composed of two subdomains (I+II), both of which are non functional when tested individually, but which can synergize to restore activity even when placed on two different heterodimeric partners. Subdomain I includes a.a. 389 to 508. Position 389 is determined

from Figure 1, and position 508 from plasmid A in Figure 4, line 1. Subdomain II contains a.a. 508 to 632. Position 508 is suggested by plasmid B in Figure 4, lines 1 and 2 and position 632 from previous deletions tested in homodimeric GAL fusions (23).

The retinoic acid X receptor (RXR) does not mediate transcriptional silencing

Other members of the nuclear receptor family closely related to v-erbA, like TR and RAR, show silencing activity in the absence of their specific ligands (6,23). The carboxyterminal domains of TR and RAR are 90 and 30% homologous to v-erbA, respectively. Since the corresponding domain of the retinoid X receptor (RXR) is 27% homologous to the silencing domain of RAR and since RXR is a regular component of the TR or RAR transcription complex, we wanted to know whether RXR confers a silencing function as well. Since the negative regulation was more pronounced using GAL4-DBD fusion proteins, we joined the C-terminal part (a.a. 235-462) of RXR to this heterologous DNA binding domain. The transcriptional effect on an UAS-driven tk-CAT gene of this chimeric protein in the presence or absence of ligand was tested and compared to the previously described regulation mediated by GAL-TR or GAL-verbA (23). In order to avoid interference with endogeneous factors, we used CVI cells devoid of most of the nuclear receptors. Expression of the GAL4-DBD shows no effect, expression of GAL-TR leads to clear repression in the absence of hormone and clear induction in the presence of hormone (Fig. 6) and GAL-verbA displays a constitutive silencing function, as expected. In comparison to L-cells the CVI cells show less silencing activity. GAL-RXR mediates induction upon addition of retinoic acid, but no, or a

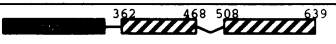
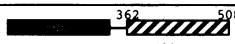
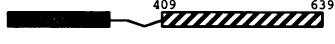
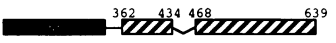
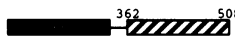
Expressionplasmid A	A	A+B	B	Expressionplasmid B	
	6.5	85.5	2.5		1
		29.1	0.9		2
	1.3	2.4	1.7		3

Figure 5. Synergism of v-erbA subdomains with internal deletion mutants. Expression vectors coding for the indicated fusion proteins were transfected into Ltk⁻ cells either together with a GAL4-DBD expression vector (columns A and B) for reference expression on a UAS-tkCAT reporter plasmid or together with a vector for another fusion protein (column A+B) for synergistic silencing. Expression of UAS-tkCAT together with a GAL4-DBD expression vector was 46 ± 2% CAT conversion.

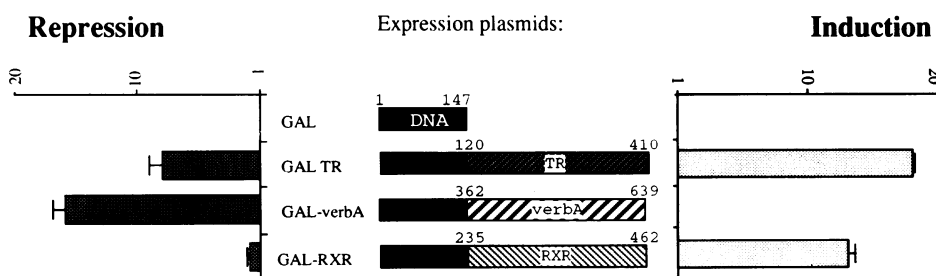


Figure 6. GAL-RXR mediates hormonal induction, but not silencing in the absence of ligand. Expression vectors coding for the indicated fusion proteins were transfected into CVI cells together with the indicator plasmid UAS-tkCAT. Fold induction of CAT-activity in the presence of hormone and fold repression in the absence of hormone are calculated relative to expression of GAL4-DBD.

very low, negative effect in the absence of ligand. For RXR induction we used all-trans RA (41), which was shown to be converted to 9-cis-RA by stereoisomerization in CVI cells (36).

These results suggest that, although the C-terminus of the RXR is functional in hormone dependent gene induction, it is unable to mediate a significant repression.

GAL-RXR does not contribute to the silencing function in a heterodimer with GAL-TR or GAL-*verbA*

RXR was shown to form heterodimeric complexes with several members of the thyroid hormone receptor family. Although, as we have shown above, it does not contain a repression function on its own, we wanted to know whether RXR would be able to help its heterodimeric partner in its silencing activity. In order to exclude effects due to differential DNA binding affinity or differential dimerization efficiency between homo- or heterodimeric complexes, we used again the GAL4 fusion proteins for this experiment. Therefore, DNA binding and dimerization is directed by the GAL4-DBD and is identical for all the homo- and heterodimers. Expression of either GAL-*verbA* or of GAL-TR along with GAL4-DBD lead to a clear repression independent of the addition of RA (Fig. 7). Replacement of GAL4-DBD with the fusion GAL-RXR shows no effect on the silencing mediated by GAL-*verbA* or GAL-TR in the absence of RA (Fig. 7). Similarly, co-expression of a large excess (up to 100-fold relative to GAL-TR or GAL-*verbA*) of GAL-RXR did not influence repression in the absence of ligands (data not shown). These results were not due to CAT assay limitations, since extended assays with large amounts of protein extract strongly increased basal level activity without showing any GAL-RXR effect on repression. In the presence of specific ligand the induction expected from GAL-RXR reduces the silencing in each case. The weaker silencing activity of GAL-TR was completely relieved.

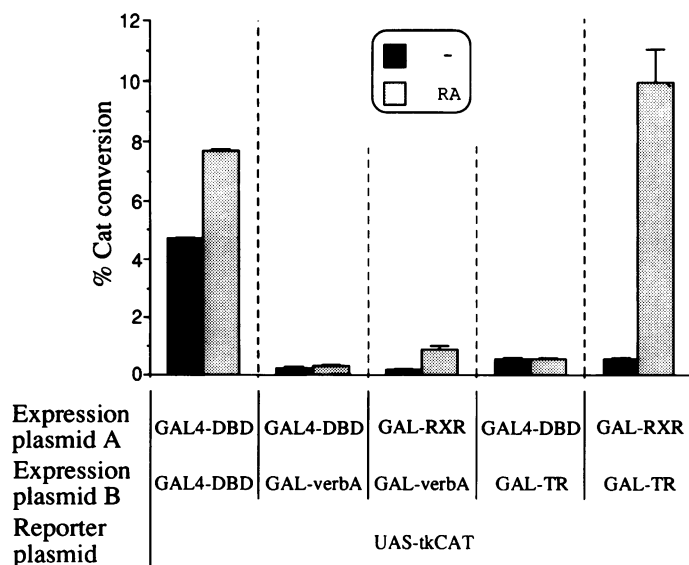


Figure 7. GAL-RXR does not synergize with *verbA* or TR in silencing. Equal amounts of expression plasmids coding for the indicated fusion proteins were transfected into CVI cells together with the indicator plasmid UAS-tkCAT. Dark boxes indicate the CAT-expression obtained in the absence of hormone, light shaded boxes show the CAT-conversion after addition of RA.

DISCUSSION

The results in this study describe the functional silencing domain of *v-erbA* and of the TR/RXR hetero-complex. We utilized the advantage of fusion proteins consisting of C-terminal parts of *v-erbA* joined to the DBD of the heterologous GAL4 yeast transcription factor. Expression of the different chimeric proteins is roughly similar as assessed by gel retardation analysis. Furthermore the GAL4-DBD ensures correct nuclear translocation, dimerization and DNA binding to the specific UAS sequence.

Functional analysis of N-terminal deletion mutants in the *v-erbA* negative domain revealed the minimal requirement of a.a. 389 to 632 for silencing function. Mutants GAL-*erb* 362–639 and GAL-*erb* 389–639 led to a weak, but reproducible increase in repression as compared to the complete silencing domain. This may suggest the presence of a trans-activation domain around position 362. Further deletion to a.a. 409 abolished the ability of the fusion protein to repress transcription. Interestingly this deletion eliminates Pro399, which had been shown previously to be involved in repression, as its mutation to Arg abolished biological oncogenic and silencing function (21,22). In this context the internal deletion mutant GAL-*erb* 346–389/434–639, which has Pro 399 deleted, is important. It is strongly impaired in silencing function, but, in contrast to deletion mutant GAL-*erb* 409–639, it still retains some negative activity. Possibly a functionally important interruption of α -helical structure in the protein is brought about by the two very close Pro 397 and 399 in the wild type protein. It could be partially restored in mutant GAL-*erb* 346–389/434–639 by the replacement with a linker sequence, which we predict by Chou–Fasman and Robson–Garnier analysis to be completely unstructured and thus flexible. Similar secondary structure predictions may allow the following assumptions. Region 360 to 420 is predicted to adopt an α -helical structure interrupted by the two prolines 397 and 399 in the wild type *v-erbA*. The functional mutant GAL-*erb* 389–639 retains this structure with a shortened N-terminal helix. Similarly in the hRAR α the prolines 170 and 172 are predicted to separate two helices, the N-terminal one of about 10 amino acids. These observations suggest that the silencing subdomain I might be defined by a structural feature consisting of two helices separated by a kink.

The fact that two of the internal deletion mutants (GAL-*erb* 346–389/434–639 and GAL-*erb* 362–468/508–639) retain some of the repressing function suggests that the silencing domain is composed of at least two functional domains. A final proof is given by the co-transfection experiments, generating heterodimers containing the putative subdomains on different molecules, presumably in a spatial arrangement different from that in the original *v-erbA* protein. Several results argue for the formation of functional heterodimers from non functional components. *In vitro* interaction assays showed that all tested GAL4-fusion proteins specifically interact, most importantly GST-GAL-*erb* 508–639 and labelled GAL-*erb* 409–639 which do not restore silencing function when cotransfected (see Fig. 4, lane 5). The dimerization interface is contributed by the common GAL4-DBD, as its deletion abolished the interaction (Fig. 3b, lanes 1 and 16). Gel retardation experiments using extracts from COS-1 cells transfected with two different expression vectors revealed the presence of heterodimeric complexes. Simply increasing the amount of transfected expression plasmid coding for a single non-functional mutant does

not lead to repression. Heterodimers formed between GAL-erb 362/508 and GAL-erb 508–639, which contain perfectly complementary sequences of the silencing domain, showed a strong synergistic silencing effect. Similarly the repression function of GAL-erb 362–468/508–639, a weakly functional mutant can be restored to nearly wild type by co-expression of several deletion mutants.

The results obtained using the internal deletion mutants and the heterodimeric complexes point to region 434–468 as being absolutely required for function. It has to be present in at least one of the heterodimeric partners. Deletion of only these amino acids in GAL-erb 346–434/468–639 leads to a non-functional protein (Fig. 2, line 3), which cannot be complemented by a fusion protein (GAL-erb 362–508, Fig. 5, line 3) shown to synergize in other cases (Fig. 4, lines 1 and 2).

Recently, synergism in silencing between two non-functional subdomains of the hTR β C-terminal region was described using GAL4 fusion proteins and a reporter gene containing four UAS binding sites (42). In our system, using a single UAS, co-expression of the corresponding v-erbA fusion proteins GAL-erb 362–439 and GAL-erb 409–639 led to a weak, but reproducible synergism as well.

Synergism between transcriptional activating domains is a general phenomenon. Steroid receptors contain two or more trans-activation domains cooperating for full activity (43–45). In case of the human estrogen receptor two activation functions AF1 and AF2 were shown to synergize when present on different DNA binding entities (45). Recently the transcription factor TEF1 was shown to require at least two out of three subdomains for activation function, each of which is inactive when tested individually in a GAL4 fusion protein (46). Synergism within a heterodimer was described for the hormone dependent activation by RXR/RAR complexes (29,30,31,33,39). It was shown that heterodimers formed of one partner containing the N-terminal AF1 domain and the other partner containing the C-terminal AF2 domain displayed synergistic activation function (47). This synergism was also seen if one of the partners lacks a corresponding binding site in the regulated promoter. Specific synergistic silencing was described for v-erbA or TR with the protein Nep1 binding close to a thyroid hormone responsive element in the chicken lysozyme silencer –2.4 kb (11,48). Here we describe a synergism between two silencer subdomains.

The mechanism for this synergism is still unclear. Direct interaction of hTR α 1 (49) and the C-terminus of hTR β (42) with TFIIB has been demonstrated. This interaction is relieved in the presence of T3 (42), suggesting that this interaction may be involved in the silencing activity. The region of hTR β interacting with TFIIB corresponds roughly to the synergising subdomain A.A. 508–639 of v-erbA. Whether the complementary domain 389–508 interacts with some other component of the transcription pre-initiation complex remains to be shown.

V-erbA and ligand-free TR and RAR are able to mediate silencing. RAR and TR have been shown to function in a heterodimeric complex with RXR. In addition RXR is closely related to TR and RAR. Therefore we wanted to know whether RXR exhibits a silencing function of its own and/or whether RXR synergizes in repression with one or both of the silencing domains of v-erbA in a hetero-complex. The C-terminal fragment of RXR, corresponding to the minimal v-erbA silencing domain, is able to mediate the RA inducibility in the chimeric protein GAL-RXR, but does not show any silencing activity. Usually we observe that GAL4 fusion proteins show higher silencing effects on a UAS

then the natural proteins on their specific binding site (23). We therefore conclude that the hRXR α does not contain a transferable silencing function. In addition, we could show that GAL-RXR in heterodimers with GAL-TR or with GAL-verbA, respectively, does not influence their repression activity in the absence of any ligand. Only addition of RA leads to an increase of CAT transcription, due to the ligand-induced GAL-RXR.

MATERIALS AND METHODS

Plasmids

The reporter plasmid used in this study contains a GAL4 binding site (17mer)(50) inserted in front of the tkCAT gene (23).

The expression vectors pABGAL, pAB-GAL, pGAL-erbA Δ 346, pGAL-erb 362–639, pGAL-erb 409–639, pGAL-erb 508–639, pGAL-erb 557–639, pGAL-erb 617–639, pGAL-erb 362–508, pGAL-erb 360–439, pGAL-erb 362–468/508–639, pGAL-erb 362–434/468–639, and pGAL-TR were described elsewhere (23). Plasmid pGAL-erb 389–639 was obtained by cutting pGAL-verbA with *Sma*I and *Bcl*II, filling in using the Klenow fragment of DNA-polymerase I and religating, thereby deleting a fragment coding for a.a. 346 to 388. The internal deletion mutant pGAL-erb 346–389/434–639 was constructed by cutting pGAL-verbA with *Eco*RV and *Bcl*II, filling in using Klenow enzyme and religating, thereby deleting a fragment coding for a.a. 390 to 433. Plasmid pGAL-erb 346–515/615–639 was obtained by digesting pGAL-verbA with *Esp*I and *Fsp*I followed by religation, thereby deleting a fragment coding for a.a. 516 to 614. pSG-hRXR containing the coding sequence for the human RXR α was kindly provided by H.Stunnenberg (33). The RXR coding region was first cut out using *Eco*RI, filled in using Klenow enzyme and cloned into the *Eco*47III site of pABGAL. Then a *Hinc*II-*Hind*III fragment coding for a.a. 235 to 462 was obtained and inserted into the *Sma*I-*Hind*III cut pABGAL to obtain plasmid pGAL-RXR 235–462.

The expression plasmids GST-erb 362–508 and GST-erb 508–639 were constructed by insertion of the *Sma*I/*Hind*III fragment of pGAL-erb 362–508 or pGAL-erb 508–639, respectively, into the vector pGEX KG (51) digested with *Sma*I/*Hind*III.

The expression plasmid GST-GAL-erb 508–639 was obtained by insertion of the *Bgl*III/*Bam*HI fragment of pGAL-erb 508–639, which was filled in using Klenow, into the vector pGEX 2TK (Pharmacia), digested with *Eco*RI and filled in.

Vectors for *in vitro* translation were obtained by insertion of the *Kpn*I/*Bam*HI fragments of pGAL-erb 409–639, pGAL-erb 508–639 and pGAL-erb 362–468/508–639 into the vector Bluescript SK+, digested with *Kpn*I/*Bam*HI, and insertion of the *Kpn*I/*Hind*III fragment of pGAL-erb 362–508 into Bluescript SK+, digested with *Kpn*I/*Hind*III.

Cell culture and transfections

Ltk⁻ cells, CV1 and COS1 cells were grown in DME-medium (Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin.

DNA transfer into CV1 cells was performed using the calcium phosphate precipitation method. Ltk⁻ cells were transfected as described (52). 2×10^6 cells were suspended in DNA-DEAE-dextran solution (1 pmol reporter and 0.5 pmol expression plasmids) and incubated for 30 min. After adding directly 7 ml medium, cells were seeded on a 6 cm dish and grown for 36–48

h before harvesting. Transfections were done in triplicate and performed in at least two independent experiments. Transfections into COSI cells were done by a similar DEAE-dextran suspension method using 6 pmole of DNA on 10^7 cells. After 1 h incubation in the DNA solution, a DMSO shock was performed for 3 min, the cells were taken up in 30 ml PBS and 10 ml medium, spun down, seeded on a 15 cm dish and grown for 48 h before harvesting.

For hormonal induction experiments, the serum was depleted of thyroid hormone and retinoic acid by extensive charcoal stripping. The cells were kept for at least 24 h in depleted medium before transfection, after transfection 10^{-7} M 3,5,3'-trijodthyronine or 10^{-6} M retinoic acid was added. CAT-assays were performed as described (53).

DNA-protein binding assays

Whole cell extracts were prepared from COSI cells transfected with various expression vectors according to (54). Gel retardation experiments were performed using 20000 d.p.m. of kinase-labelled 17-mer DNA probe, 2–4 μ g of whole cell extract in an incubation mix containing 1–4 μ g of p(dI.dC), 200 mM KCl, 2 mM DTT, 14% glycerol, 5 mM $MgCl_2$, 20 mM Hepes pH 7.7. The DNA-protein complexes formed were analysed on a 5% polyacrylamide gel in $0.5 \times TBE$.

Protein-protein interactions *in vitro*

Protein-protein interactions were assayed mainly as described (42).

GST-fusion-proteins were expressed in *E. coli* BL21 cells (55). Cells were harvested by centrifugation and resuspended in NENT buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0 and 0.5% NP-40). Cells were lysed by three freeze-thaw cycles and the cellular debris was removed by centrifugation. Glutathione-Sepharose 4B beads were washed with NENT and 10 μ l of beads were incubated with 100 μ l of lysate containing the GST-fusion protein for 30 min at room temperature. Subsequently, the supernatant was removed and the beads were incubated with 10% milk powder in NENT for 15 min at room temperature. The beads were washed twice with 1 ml NENT and once with 1 ml transcription washing buffer (20 mM Hepes pH 7.9, 60 mM NaCl, 1 mM DTT, 6 mM $MgCl_2$, 8% glycerine and 0.1 mM EDTA). *In vitro* translated and radiolabeled protein were obtained using a TNT-kit (Promega). 5 μ l of crude lysate were incubated with the beads in 100 μ l transcription washing buffer for 1 h at room temperature. Finally the beads were washed (5×1 ml of NENT) and proteins were solubilized in SDS-loading buffer and analyzed on SDS-PAGE. Gels were amplified with fluorigraphic reagent (Amersham) and bands were visualised by autoradiography.

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