The EMBO Journal vol.11 no.3 pp.1015-1023, 1992

A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor

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Communicated by M.Beato

Inhibition of gene transcription is brought about by several mechanisms. The least understood mechanism is probably silencing, the analogue to transcriptional enhancing. We provide evidence that the silencing function of the oncogene product v-ERBA or the cellular counterpart, the thyroid hormone receptor (TR, c-erbA) is located in the C-terminal part and is transferable to a heterologous DNA binding domain. Deletion analyses suggest an important role for a basic and hydrophilic amino acid stretch on both ends of the domain. In addition we show that the related retinoic acid receptor (RAR) also contains a functional silencing domain similar in size and amino acid sequence. However, the activity of this domain can be neutralized by an additional domain in the C-terminus which functions cell specifically.

Key words: retinoic acid receptor/silencer/thyroid hormone receptor/v-erbA

Introduction

Thyroid hormone (T3) and retinoic acid (RA) are required for development, growth and differentiation (for review see Schwartz, 1983; Ragsdale and Brockes, 1991). The hormonal response is mediated by binding to specific nuclear receptors (Bigler and Eisenman, 1988), which are members of the steroid/thyroid hormone superfamily (Evans, 1988; Green and Chambon, 1988). The receptors function as ligand dependent transcription factors and can mediate the cellular response to hormonal signals by direct control of gene expression. Binding of the hormone leads to regulation of specific genes in target cells. The thyroid hormone receptor (TR, c-erbA) binds sequence specifically to DNA in a hormone independent manner (Damm *et al.*, 1989). The binding site (thyroid hormone response element, TRE) shows a very flexible arrangement including palindromic, inverted palindromic or direct repeats (Baniahmad *et al.*, 1990; Näär *et al.*, 1991; Umesono *et al.*, 1991). In addition to TR and the oncogene product v-ERBA, the retinoic acid receptor is also able to bind the same DNA element (Umesono *et al.*, 1988). The T3 receptor is a potent activator after adding hormone. Without hormone, however, the receptor acts as a transcriptional repressor (Damm *et al.*, 1989; Graupner *et al.*, 1989; Sap *et al.*, 1989; Baniahmad *et al.*, 1990), which is a novel feature compared with the other well described steroid receptors like the oestrogen, progesterone and glucocorticoid receptors. In contrast to the normal, cellular TR the viral oncogene product v-ERBA fails to bind hormone due to mutations in the C-terminal ligand binding domain (Sap *et al.*, 1986; Munoz *et al.*, 1988) and represents a hormone independent repressor of transcription (Damm *et al.*, 1989; Graupner *et al.*, 1989). The v-erbA oncogene is derived from the avian erythroblastosis virus (AEV) which induces erythroleukaemia and fibrosarcomas in chickens and transforms erythroid cells and fibroblasts in culture (Graf and Beug, 1983). The integrity of the DNA binding domain (DBD) is critical for its biological activity (Privalsky *et al.*, 1988). Furthermore, it has been shown that the v-erbA oncogene product specifically supresses expression of a limited group of erythroid specific genes (Zenke *et al.*, 1988). Transcriptional repression may involve several mechanisms such as the formation of inactive heterodimers and competition at the DNA binding level (Brent *et al.*, 1989; Glass *et al.*, 1989; Hudson *et al.*, 1990). Another mechanism for negative regulation involves the concept of direct inhibition of gene transcription or silencing, in analogy to activator proteins (for review see Levine and Manley 1989; Renkawitz, 1990). The ligand-free thyroid hormone receptor or v-ERBA has been shown to repress transcription by silencing, i.e. the DNA-bound receptor inhibits transcription independently of position and orientation, and repression is effective on a complete or on a minimal promoter (Baniahmad *et al.*, 1990). Here we wanted to know which parts of the receptors are involved in such a mechanism. To exclude competition on the authentic TR binding site and to avoid any interference of receptor mutations effecting dimerization and nuclear localization, we used a heterologous DNA binding domain. Fusion proteins could be analysed for transcriptional repression and to determine the necessary amino acid sequence of the receptors for silencing. Here we confirm that repression involves direct inhibition or silencing. Deletion analysis reveals the presence of a transferable silencing domain not only in the v-ERBA protein and in the TR, but also in the retinoic acid receptor (RAR). The silencing activity is located in the C-terminal parts of these proteins and is cell type specifically neutralized by a separate domain in the wild-type RA receptor.

Results

Binding site dependent repression and cell specificity of TR, v-ERBA and RAR

To show binding site dependent repression on the HSV tk-promoter by the oncogene product v-ERBA, we transfected the reporter plasmid containing one copy of the thyroid hormone response element found in one of the chicken lysozyme silencer elements (TRE*lys*) combined with the tk-CAT fusion gene (TRE*lys* tkCAT; Baniahmad *et al.*, 1990). The reporter plasmid was cotransfected with the

expression plasmid coding for the v-ERBA oncogene protein (Figure 1). As a control reporter plasmid we used a mutated TRE binding site (TRE*mut* tkCAT). Cotransfections into L cells (Figure 2) showed reduction of the tk-promoter activity using TRE*lys*, independent of the presence of thyroid hormone (T3). We observed a weaker, but similar effect in CV1 cells. Comparable results were obtained using TRE*pal* (Glass *et al.*, 1988; data not shown) which represents another binding site for the erbA family with a palindromic sequence inverted relative to TRE*lys*. To compare the activities of different receptors binding to a TRE and proving receptor dependent repression we cotransfected TRE*lys* tkCAT together with expression vectors coding for v-ERBA, the rat thyroid hormone receptor α (TR or c-erbA), the human retinoic acid receptor α (RAR) and the control vector Δgal (Figure 1), which does not encode any polypeptide. As shown in Table I the repression was dependent on the cDNAs inserted in the expression vectors and was seen not only for the v-erbA oncogene, but also with the cellular counterpart c-erbA or TR and also with the RAR in L cells. The inductions were calculated by dividing the obtained values from induced and uninduced transfected cells. Upon addition of hormone, induction was seen only for the corresponding receptors (TR or RAR). The induced levels were above the promoter activity. Using CV1 cells TR and v-ERBA behaved similarly, whereas RAR showed no repressing activity, but acts rather as a weak activator in the absence of hormone. Similar results were seen using the complete palindromic TRE (TRE*pal*; Glass *et al.*, 1988; data not shown).

Identification and characterization of the silencing domain

To localize the transcriptional silencing activity of the v-erbA oncogene and its cellular counterpart TR, fusions were made between the coding sequences of these receptors and the GAL4 DNA binding domain (GAL4-DBD; amino acids 1−147). The GAL4-DBD, which in addition to specific DNA binding activity contains signals for dimerization (Carey *et al.*, 1989) and nuclear translocation (Silver *et al.*, 1988 and see below), shows no or only weak *trans*-activation function (see below). Therefore, receptor deletions and mutations can be analysed for transcriptional regulation even when dimerization and nuclear translocation domains are deleted. The expression plasmids coding for the GAL4-DBD fused to the C-terminal parts of either TR or v-ERBA (Figure 3A) were transfected into L cells together with a reporter plasmid containing the GAL4 binding site (17mer, Carey *et al.*, 1990) instead of a TRE in front of the tk-promoter−CAT fusion (Figure 1). As shown in Table II and Figure 3B the GAL4-DBD had no effect compared with the control vector ΔGAL. The gal−v-erbA fusion product exerted a very strong silencing activity on the promoter independent of hormone. The GAL−TR fusion also exhibited a very strong silencer activity in the absence of hormone, but was highly inducible after adding T3 (Figure 3B). Similar results were obtained using CV1 cells (data not shown). A concentration series of the gal−v-erbA expression plasmid inhibited promoter activity significantly even at very low amounts (0.025 pmol, 6-fold reduction; data not shown). This finding and the very strong induction seen with T3 (Table II and Figure 3B) argue against squelching (sequestering of intermediary factors by

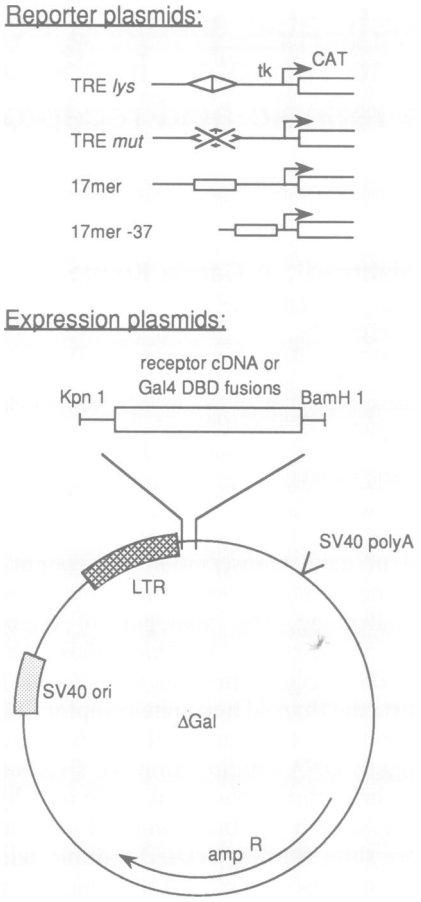

Fig. 1. Plasmids used in cell transfections. For the reporter plasmids only the reporter specific inserts are depicted. The structure of expression plasmids and the control plasmid ΔGal are shown in the lower part of the figure.

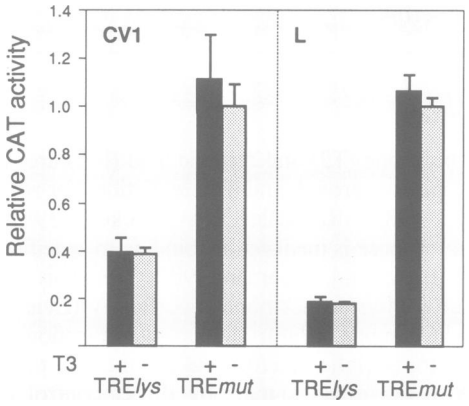

Fig. 2. Inhibition of promoter activity by the oncogene product v-ERBA. CAT values obtained with the expression plasmid v-erbA transfected into CV1 and L cells. Reporter plasmids containing a thyroid hormone responsive element (TRE*lys*) or a mutated binding site (TRE*mut*) transfected with thyroid hormone T_3 (black bars) or without hormone (dotted bars).

overexpression of transcription factors; Ptashne, 1988) being the mechanism for repression in this case. Previously we have postulated that a true silencer functions analogously to an enhancer, i.e. a silencer inhibits transcription independently of position and orientation, and independently of the complexity of the promoter. That is what we have found for v-ERBA and TR, the DNA-bound receptor inhibits

Table I. Cell type-specific induction and repression of indicated expression plasmids on TRElys tkCAT

Cell type	Expression plasmid	CAT conv. without hormone absolute[1]	relative[2]	+T3[2]	+RA[2]	Induction by T3	Induction by RA	Repression
Ltk	ΔGal	17.95 ±0.46	1.0 ±0.03	1.54 ±0.02	1.75 ±0.28	1.5	1.8	1.0
	v-erbA	1.86 ±0.02	0.10 ±0.00	0.11 ±0.01	n.d.	1.1	–	9.7
	rTRα	3.36 ±0.03	0.19 ±0.00	2.85 ±0.12	n.d.	15.0	–	5.3
	hRARα	4.27 ±0.24	0.24 ±0.01	n.d.	1.99 ±0.15	–	8.3	4.2
CV1	ΔGal	1.79 ±0.53	1.00 ±0.30	1.04 ±0.32	1.47 ±0.08	1.0	1.5	1.0
	v-erbA	0.43 ±0.02	0.24 ±0.01	0.27 ±0.04	n.d.	1.1	–	4.2
	rTRα	0.59 ±0.05	0.33 ±0.03	6.51 ±0.62	n.d.	19.7	–	3.0
	hRARα	2.69 ±0.00	1.50 ±0.00	n.d.	3.25 ±0.49	–	2.2	0.7

[1]Absolute CAT conversion rates are expressed in pmol·mg^{-1}·min^{-1}. Average and variations are determined from independent duplicate experiments.
[2]Relative CAT assay activities normalized on the activity seen with the expression vector Δgal.

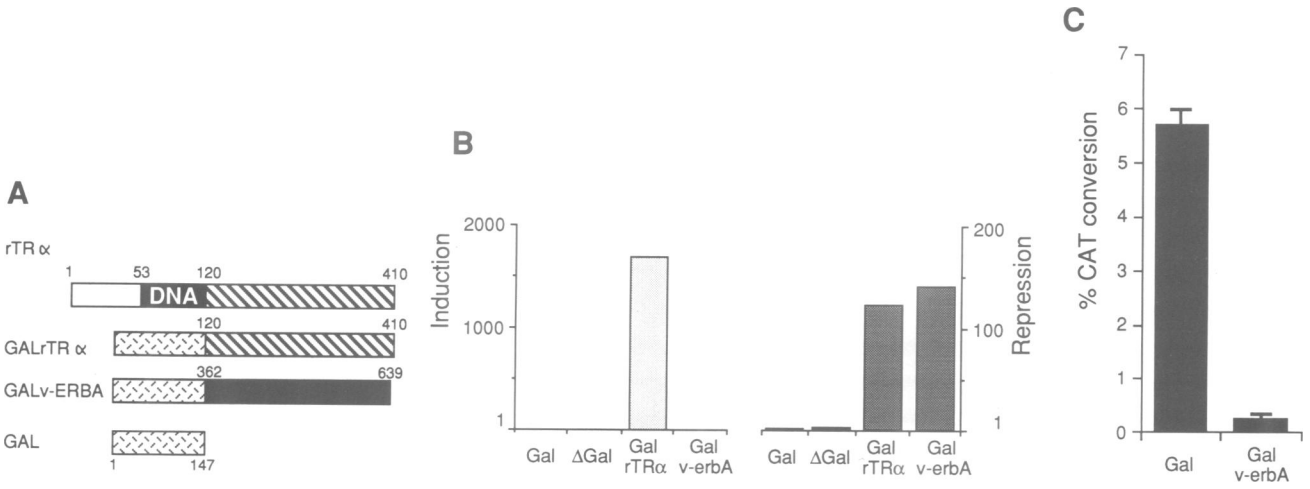

Fig. 3. Induction and repression by GAL−rTRα and GAL−v-ERBA fusions. (**A**) Diagram of the linear structure of the GAL DNA binding domain (GAL) fused to the C-terminal halves of rTRα or v-ERBA in comparison with wild-type rTRα. (**B**) DNA cotransfection experiments of the indicated plasmids with the 17mer−tkCAT reporter plasmid in L cells. Induction and repression values were calculated from CAT activities shown in Table II. (**C**) Repression in front of the TATA-box was tested by transfection of the reporter 17mer−37tkCAT and either of the expression plasmids GAL or GAL−v-ERBA in L cells.

even a minimal promoter, consisting of a TATA-box only (Baniahmad *et al.*, 1990). In order to determine, whether the Gal−v-erbA fusion product functions similarly we tested the reporter construct 17mer−37tkCAT (Figure 1). Figure 3C shows clearly, that the fusion protein functions as expected, the minimal promoter is efficiently repressed. In summary, these fusion proteins behaved like the wild-type receptors, but with much stronger activities, which can be explained by higher protein stability and/or higher DNA affinity.

In order to localize more precisely the borders of the silencing domain, we constructed a series of gal−v-erbA deletion mutants. As shown in Figure 4A, a 47 amino acid deletion in the N-terminal part of the C-terminal domain (Gal Δ409) completely destroys the silencing function. The activity of the C-terminal deletion series drops gradually within 50 amino acids. These data suggest that at least both ends of the silencing domain (amino acids 362−632) are important for function. To test whether these ends are sufficient for repression we introduced internal deletions. The construct 362−468/508−639 shows reduced activity, but is still able to confer repression, whereas construct 362−434/468−639 has lost activity. This may suggest that a functional part (part I, see Figure 6) within the silencing domain extends from amino acids 362 to 468 and that a second functional part (part II, see Figure 6) resides at the very C-terminal end as characterized by the mutant 346−582. In addition there might be a third (central) part, the function of which is destroyed in the internal deletion 362−468/508−639. If these parts function by similar mechanisms one might consider these parts as functional modules, which can be exchanged. To verify this idea, we

fused both ends of the silencing domain (amino acids 362–508 and 409–639). This fusion protein regained partial silencing activity, whereas each part, when tested individually, is inactive. For further characterization of intra-protein interactions, we duplicated a domain possessing weak silencing activity (362–616 with a 16-fold repression) and fused it to GAL4-DBD. This fusion protein demonstrated a strong silencing activity of 140-fold, which indicates that cooperative interactions occur between functional modules. Similar results were obtained using CV1 and primary chicken embryo fibroblast (CHEF) cells (data not shown). As a control, nuclear translocation was confirmed by cell immune staining after DNA transfection of active and non-active fusion proteins (data not shown). In addition, to verify DNA binding capability every fusion protein was analysed in gel retardation experiments (Figure 4B) using extracts from COS1 cells transfected with the indicated expression vectors. All the fusion proteins have the capability to bind specifically to the GAL4 binding site used as a probe.

Taken together, we conclude that the C-terminal regions of both TR and v-ERBA are sufficient for transcriptional repression and that this activity represents a functional domain which is transferable to a heterologous DNA binding domain. In addition, we present evidence that at least two to three functional modules reside in this domain which are required for silencing function in all cell types tested. The central region of the silencer domain contributes to silencing as well, since duplication of a part of the silencing domain can substitute for the function of the C-terminal part.

Table II. The C-termini of c-erbA and v-erbA are sufficient for repression and induction (c-erbA) by T3. Transfers carried out in L-cells

Expression plasmid	CAT conv. without hormone		+T3[2]	Induction	Repression
	absolute[1]	relative[2]			
Gal	26.18 ±0.76	1.00 ±0.03	1.15 ±0.06	1.2	1.0
ΔGal	19.01 ±0.58	0.73 ±0.02	0.51 ±0.10	0.7	1.4
Gal rTRα	0.21 ±0.01	0.01 ±0.00	13.40 ±0.04	1340	125
Gal v-erbA	0.19 ±0.01	0.01 ±0.00	0.01 ±0.00	1.0	138

[1]Absolute CAT conversion rates are expressed in pmol·mg^{-1}·min^{-1}. Average and variations are determined from independent duplicate experiments.
[2]Relative CAT assay activities normalized on the activity seen with the DNA binding domain of GAL4.

Cell specific inhibition of repression mediated by the retinoic acid receptor

Since the RAR demonstrated repression activity in L cells but not in CV1 cells (Table I), we addressed the question

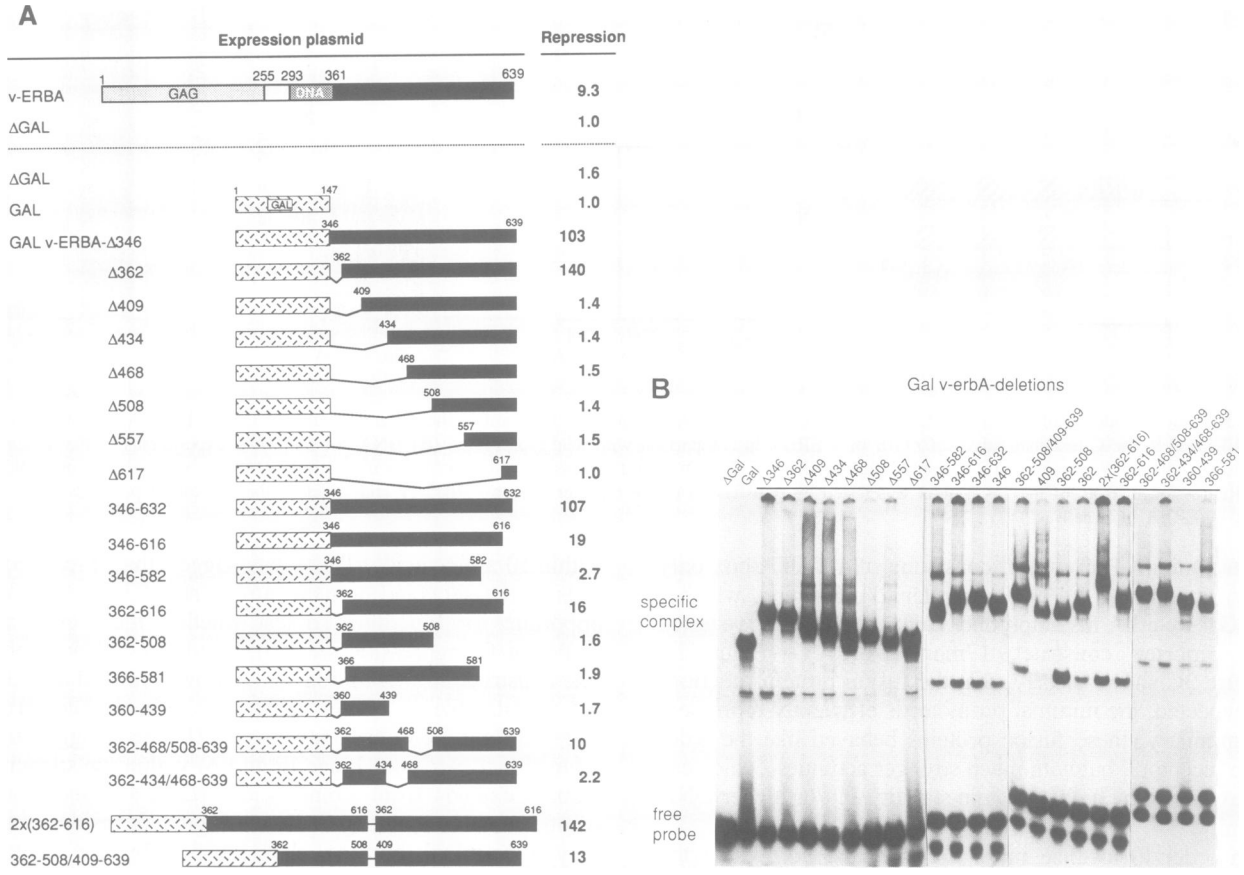

Fig. 4. Deletion mapping of the v-ERBA silencing domain. (**A**) Expression plasmids coding for the indicated fusion proteins were cotransfected into L cells together with the 17mer–tkCAT reporter plasmid. Repression values of CAT activities were calculated and are indicated. (**B**) Bandshift experiments using extracts from COS cells transfected with indicated expression plasmids. A synthetic oligonucleotide bearing the Gal4 binding site (17mer) was used as probe.

of whether the RAR also contains such a silencing domain and whether we can localize the cell type specificity within the receptor. Figure 5A shows the GAL−RAR fusion protein encoded by an expression vector. Cotransfection with the reporter plasmid 17mer−tkCAT into L cells revealed strong silencing activity in the absence of hormone. Addition of hormone led to high values of induction (Figure 5B). In order to localize repression function within the C-terminal part of the RAR we constructed the C-terminal deletion mutant (GAL−RAR143−403) containing amino acids homologous to the TR silencing domain. In transfection experiments this mutant was revealed as a constitutive strong repressor and not inducible by addition of hormone (Figure 5B). A further C-terminal deletion of 42 amino acids destroyed the silencing activity. Similar results were obtained using CHEF cells in transfection experiments (Table III and Figure 5C). The size and position of the RAR silencing domain are homologous to the v-erbA silencing domain. In contrast to both L cells and CHEF cells the entire C-terminal region of RAR fused to GAL4-DBD exhibited different activities in CV1 cells. It was a weak activator in the absence of hormone but still retained hormonal inducibility (~60-fold) (Table III and Figure 5C). These results confirm our observations with the wild-type receptor (see above). To analyse whether the absence of silencing is due to a missing adaptor protein in CV1 cells and/or due to different RAR specific activators which block or mask the silencing effects, we transfected the C-terminal deletion mutant (GAL−RAR143−403) which contains a complete region homologous to the silencing domain of v-erbA (see Figure 6) and which showed the strongest repression in L cells. This mutant acts as a transcriptional repressor in all cell types tested, even in CV1 cells. This result leads to the conclusion that all factors needed for transcriptional repression are

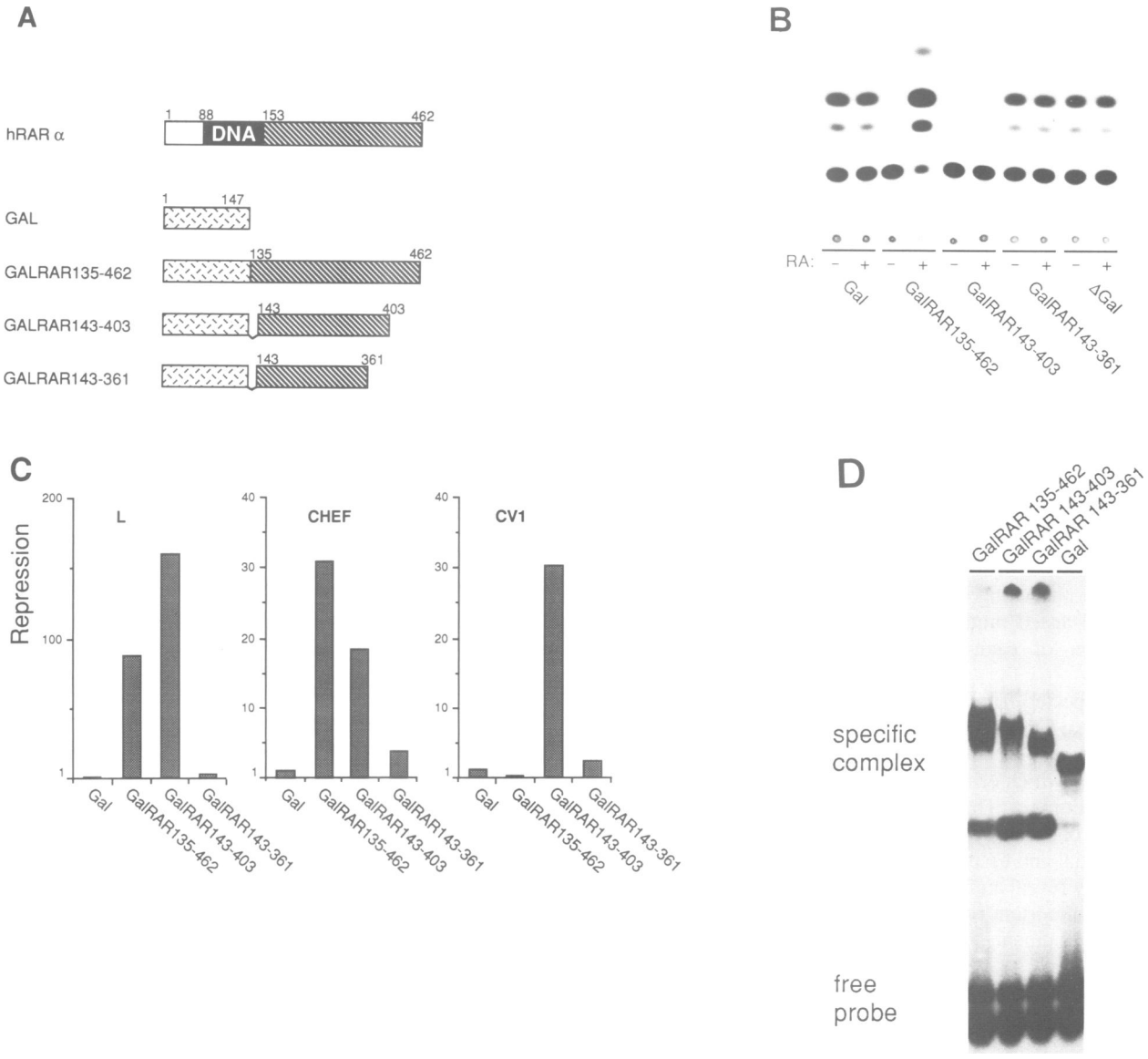

Fig. 5. Silencing domain of the hRARα. (**A**) Diagram of the linear structure of hRARα and of GAL−RAR fusions. (**B**) CAT assay obtained with cotransfected expression plasmids (as indicated) and the reporter plasmid 17mer−tkCAT in L cells with (+) or without (−) addition of retinoic acid (RA). (**C**) Cell specific repression activities calculated from CAT activities shown in Table III obtained from transfections in L, CHEF and CV1 cells. (**D**) Bandshift experiment with a ^{32}P-labelled 17mer oligonucleotide and extracts from COS cells after expression of the indicated GAL−RAR fusions.

Table III. Cell type specific repression of Gal RAR expression plasmids on TRElys tkCAT

Cell type	Expression plasmid	CAT conv. without hormone		+RA[2]	Induction	Repression
		absolute[1]	relative[2]			
L	Gal	25.42 ±0.77	1.00 ±0.03	1.06 ±0.03	1.1	1.0
	Gal RAR 135–462	0.30 ±0.02	0.01 ±0.00	22.62 ±0.89	2200	85
	Gal RAR 143–403	0.16 ±0.01	0.01 ±0.00	0.01 ±0.00	1.0	160
	Gal RAR 143–361	11.96 ±0.15	0.44 ±0.01	0.56 ±0.04	1.2	2.1
CHEF	Gal	81.49 ±4.36	1.00 ±0.05	1.14 ±0.27	1.1	1.0
	Gal RAR 135–462	2.63 ±0.02	0.03 ±0.00	13.02 ±0.14	430	31
	Gal RAR 143–403	4.44 ±1.49	0.05 ±0.02	0.02 ±0.01	0.4	18
	Gal RAR 143–361	21.56 ±2.30	0.26 ±0.03	0.38 ±0.02	1.5	3.7
CV1	Gal	7.52 ±0.02	1.00 ±0.00	1.34 ±0.02	1.3	1.0
	Gal RAR 135–462	23.14 ±0.94	3.08 ±0.13	190.30 ±0.04	62	0.3
	Gal RAR 143–403	0.25 ±0.01	0.03 ±0.00	0.06 ±0.03	2.0	30
	Gal RAR 143–361	3.22 ±0.09	0.43 ±0.01	0.60 ±0.10	1.4	2.3

[1]Absolute CAT conversion rates are expressed in $pmol \cdot mg^{-1} \cdot min^{-1}$. Standard deviations are determined from independent triplicate experiments.
[2]Relative CAT assay activities normalized on the activity seen with the DNA binding domain of GAL4.

present in all cell types tested, even in CV1 cells and that the additional 59 amino acids in the C-terminus of RAR inhibit that ability. All GAL–receptor fusion proteins were analysed for DNA binding using whole extracts from transfected COS1 cells in band shift experiments (Figure 5D) with a synthetic oligonucleotide containing the GAL4 binding site. All tested mutants demonstrated DNA binding. The migration of each GAL–receptor fusion protein was specific.

As expected from the sequence similarity, we found that not only v-ERBA and TR are similar proteins, but that the RAR as well, contains a repression function in its C-terminus, which can be transferred to a heterologous DNA binding domain. This activity can be specifically masked in at least one cell type by the very C-terminus suggesting differential and tissue specific protein–protein interactions. This may reveal an additional regulatory potential and might play an important role in the regulation of tissue specificity and development.

Discussion

Characterization of the silencing domain

The results presented here provide evidence for the existence of a silencing domain in the oncogene product v-ERBA, the rat thyroid hormone receptor α (TR; c-erbA), and the human retinoic acid receptor α (RAR). These domains are located in the C-termini and function independently of the N-terminus, of the receptor DNA binding domain (DBD) and of the cells tested. This silencing domain is modular and repression occurs in combination with a heterologous DNA binding domain such as GAL4 (amino acids: 1–147) containing a functional DNA binding region, dimerization (Carey *et al.*, 1989) and nuclear translocation signals (Silver *et al.*, 1988). Deletion analysis demonstrated that 270 amino acids of the C-terminal part of v-ERBA or 260 amino acids of the RAR are sufficient to provide strong silencing activity for the promoter. The GAL4–receptor fusions are much stronger compared with wild-type receptor activities. This effect might reflect a higher protein stability or a higher affinity to the GAL4 binding site (upstream activator sequence UAS; 17mer). In addition we show that the domain contains at least two to three important parts. Part I is located near the DNA binding domain (DBD) of the receptor and part II is positioned close to the C-terminus. In addition, sequences in the central part (between parts I and II) contribute to repression as well. More than one part must be present to provide repression in all cell types tested. These parts (modules) may be similar in function, since loss of activity by deletion of part II can be restored by duplication of the remaining silencing domain (gal–erbA362–616/362–616).

Sequence comparisons

Amino acid sequence comparisons of the silencing domains from the v-erbA oncogene product and of the human RARα

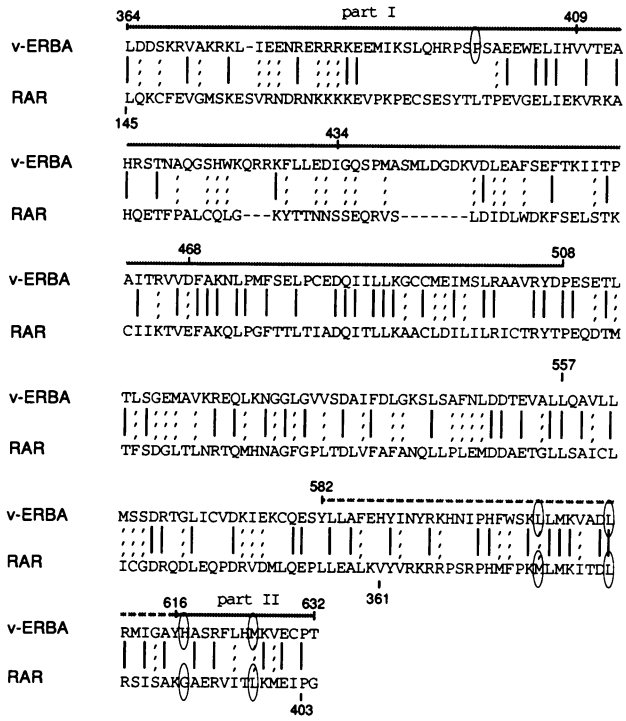

Fig. 6. Sequence comparison of the silencing domain located in the C-terminal region of the v-erbA oncogene and of hRARα. Identical amino acids are aligned and indicated with dashes, similar amino acids are pointed out by dots. The numbers represent the position of the amino acids within the wild-type receptors, part I and part II are explained in the text. Circled amino acids highlight position 399 of v-ERBA (see Discussion) and the predicted zipper structure (Forman *et al.*, 1989; Glass *et al.*, 1989; Fawell *et al.*, 1990).

revealed 32% identity and 59% similarity (Figure 6). There is no continuous stretch of identical amino acids, but rather a distribution of homologous regions spread over the entire functional domain. Both ends of the silencing domain, corresponding to parts I and II, have a hydrophilic character, whereas the central part contains more hydrophobic residues. No similarities are evident with the alanine-rich region of the Krueppel protein, which was found to be necessary for its transcriptional repression activity (Licht *et al.*, 1990) or to the Engrailed homeodomain protein (Jaynes and O'Farrel, 1991) which contains an active repression function. Sequence comparisons of part I between v-ERBA and the retinoic acid receptor implicate a stretch of basic amino acids that may be important for silencing, assuming that both receptors act via the same mechanism. This sequence has some homologies to the recently identified nuclear localization signal of nucleoplasmin (Robbins *et al.*, 1991). Deletion of this sequence does not affect nuclear localization of the fusion proteins, since the GAL4-DBD (amino acids 1−147) already contains a nuclear localization signal (Silver *et al.*, 1988) and the GAL−v-erbA fusion protein (amino acids 409−639 with the basic amino acid cluster deleted) still shows nuclear localization. We suggest that this region includes additional functions which are important for silencing. This is supported by the finding that one point mutation in the v-erbA gene, changing the proline to an arginine at position 399 abolishes the activity of v-ERBA (Damm *et al.*, 1987). This mutation is located in part I but does not alter the basic residues (Figure 6).

One C-terminal deletion mutant of v-ERBA (amino acids 362−616) missing 23 amino acids in part II of the silencing domain partially lost silencing activity. This result is consistent with the results from Forrest *et al.* (1990) who generated a 24 amino acid deletion mutant of v-erbA in the avian erythroblastosis virus (AEV). This virus retained only partial biological activity in erythroblast transformation. Part II contains the predicted α-helical structure, which may function as a zipper, allowing dimerization with identical or heterologous molecules (Forman *et al.*, 1989; Glass *et al.*, 1989; Fawell *et al.*, 1990). The dimerization domain had no effect on hormone binding but was important for the receptor to bind DNA as a dimer. However, using the GAL4-DBD (amino acids 1−147), comprising its own dimerization activity (Carey *et al.*, 1989), we clearly found binding of a dimer for the GAL4-DBD and for all GAL4−receptor fusion proteins, independent of the presence of the above protein sequence. We therefore propose that part II includes additional functional properties for silencing, possibly being mediated by other factors interacting with this domain.

Mechanism

One possible mechanism of transcriptional repression was shown by Hudson *et al.* (1990) who reported that inhibition is mediated by competitive binding of nuclear activators to a thyroid response element (TRE) located in the growth factor receptor gene. Our previous results (Baniahmad *et al.*, 1990) indicated that the presence of a TRE produced a decrease in promoter activity, which was dependent on the expression of TR in the absence of ligand. In addition, we showed that a minimal promoter consisting of a TATA-box only is sufficient as a target for repression. Using here a heterologous binding site (UAS), which has no activating properties when placed in front of the promoter or in front of just a TATA-box, we can exclude from our experiments any kind of competitions with other nuclear DNA binding factors. Therefore, it is very likely that the silencing activity of the GAL−receptor fusions must have a specific target. This silencing might occur by destabilizing the transcription initiation complex or it might have an influence on the C-terminal domain (CTD) of the polymerase II containing the heptameric repeats as shown for the yeast negative regulator SIN1 (Peterson *et al.*, 1991).

Cell specificity of the RAR

We present evidence that the RAR also contains a strong silencing domain in its C-terminal part. Interestingly we found differential activity with the human RARα. In the absence of hormone, it was a repressor in L cells, whereas weak activation was seen in CV1 cells. These results were confirmed by fusing the entire C-terminal part of RAR to the GAL4-DBD. This is in accordance with the observation that the RAR interacts with multiple cell type specific factors (Glass *et al.*, 1990). Deletion mutants showed that even with a strong functional silencing domain in CV1 cells, the entire C-terminus nevertheless acts as an activator. The simplest explanation would be that the C-terminus contains at least two binding sites for nuclear factors including positive and negative factors. The resulting activity would be dependent on the concentration of each of these factors in a particular cell type. Deletion of a binding site for a positive factor on the receptor would allow a better interaction with a negative factor. This might also explain the loss of transactivation

by the thyroid hormone receptor from a nine amino acid deletion present in the oncogene protein v-erbA and by analysis of chimeric receptors (Zenke *et al.*, 1990). The hormone plays an important role in stabilizing a certain conformational change allowing the receptor to interact with activating nuclear factors at much higher affinities. Recently, it has been shown that the ecdysterone receptor functions as both a transcriptional activator and repressor (Dobens *et al.*, 1991). It remains to be shown how many different members of the steroid/thyroid superfamily, including orphan receptors, contain silencing activities and how this negative regulation plays a role in development.

Materials and methods

Plasmids

The reporter plasmids contain a thyroid hormone response element (TRE*lys*, Baniahmad *et al.* 1990; or TRE*pal*, Glass *et al.*, 1988) or a Gal4 binding site (17mer, Carey *et al.*, 1989) inserted in the filled-in *SalI* site of the vector ptkCATΔH/N (Baniahmad *et al.*, 1990) or a 17mer was inserted at position −37 upstream of the TATA box (Baniahmad *et al.*, 1990).

The expression vector pABgal was constructed by replacing the coding sequences for v-erbA from the plasmid pRSverbA (Damm *et al.*, 1989) with the coding sequences of the Gal4 DNA binding domain (amino acids 1−147; *Asp*718−*Bam*HI) from pRSgal1−147 (Baniahmad *et al.*, 1991) and fusing a synthetic polylinker sequence (5'-GGGTGTCGACCAGCT-GCAGCGCTGGATCCTAAGTAGCTGAAGCTT; while the opposite strand contains a *Bam*HI compatible end) into the *Sma*I/*Bam*HI sites coding for 15 unrelated amino acids following the Gal4 DNA binding domain. The control expression vector pABΔgal was made by deleting the GAL4 coding sequence from pABgal with *SalI*/*Bgl*II and religation. The various pABgal-verbA expression vectors have been generated by insertion of the corresponding DNA fragments from pRSverbA coding for the indicated amino acids into the polylinker of pABgal. pABgal-TR was constructed by insertion of the filled-in *Nco*I−*Bam*HI fragment of pRSvrbeA12B (Thompson *et al.*, 1987) coding for the amino acids 122 to 410 of the rat thyroid hormone receptor α into the filled-in *SalI*/*Bam*HI sites. Inserting of the filled-in *Bst*EII−*Bam*HI, *Sca*I−*Sma*I or *Sca*I−*Acc*I fragments of pRShRARα coding for the human retinoic acid receptor α (Giguere *et al.*, 1988) into the polylinker of pABgal generated the constructs: pABgal-RAR135, pABgalRAR143−403 or pABgalRAR143−361, respectively. The correct reading frames of the gal−receptor fusions were verified by sequencing.

Cell culture and cell transfections

Primary chicken embryo fibroblasts (CHEF) (Solomon, 1976) were grown in DME-medium (Biochrom) supplemented with 5% fetal calf serum, 2% chicken serum, 100 U/ml penicillin and 100 μg/ml streptomycin. CVI, COSI and Ltk cells were grown as described above but without chicken serum.

DNA transfer into CVI, COSI and CHEF cells is described in Baniahmad *et al.* (1990). Ltk cells were transfected according to Choi and Engel (1988). 10^6 cells were incubated with DNA−DEAE−dextran solution (1 pmol reporter and 0.5 pmol receptor plasmids) in suspension for 30 min. After adding directly 7 ml medium, cells were seeded out on a 6 cm dish and grown for 36−40 h before harvesting. Transfection experiments with the reporter 17mer−37tkCAT were done according to Kawai and Nishizawa (1984) modified by Denner *et al.* (1990). For hormonal induction experiments, the serum was depleted of thyroid hormone and retinoic acid according to Samuels *et al.* (1979) or by charcoal-stripping. 10^{-7} M 3,5,3'-trijodthyronine or 10^{-6} M retinoic acid were used. CAT assays were done as described by Gorman *et al.* (1982) with minor modifications (Baniahmad *et al.*, 1987).

Bandshift assays

Whole cell extracts were prepared from COS cells transfected with the various expression vectors (for each 6 pmol were used) and analysed in bandshift assays as described in Baniahmad *et al.* (1991).

Cell staining

The staining of L cells for the presence and localization of the Gal4 DNA binding domain was carried out as described by Ernsberger *et al.* (1989).

Acknowledgements

We wish to thank R.M.Evans and S.M.Hollenberg for providing the wild-type receptor expression vectors and M.Ptashne and J.Ma for the GAL1−147 cDNA and for the antibody against the GAL4-DBD. We thank I.Leitner for synthezising oligonucleotides, K.Schulz and B.Wittbrodt for technical assistance and M.Short for critically reading the manuscript. Supported by a grant from the Bundesministerium für Forschung und Technologie and by the Fonds der Chemischen Industrie.

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Received on September 18, 1991; revised on December 12, 1991