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Chimeric retinoic acid/thyroid hormone receptors implicate RAR-α1 as mediating growth inhibition by retinoic acid

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Retinoic acid (RA) affects the growth and differentiation of cells in culture, usually to decrease the growth rate. In amphibian limb regeneration RA has the remarkable ability to affect pattern formation by changing positional identity, but its initial action on the limb is to inhibit division of the blastemal progenitor cells. Newt limb blastemal cells also show this inhibition in culture. In order to investigate the role of different RA receptors (RARs) in the RA response, the hormone binding domain of the newt RARs α1 and δ1 was replaced with the corresponding region from the *Xenopus* thyroid hormone receptor-α (TR-α). In COS cells transfected with each of the chimeras, transcription was activated after exposure to thyroid hormone (T3). Their profile of activity on three different response elements was indicative of RAR specificity and not TR specificity. After transfection of cultured newt blastemal cells with a DNA particle gun, the chimeras were equally active in stimulating T3-dependent transcription of two different synthetic reporter genes. Blastemal cells were transfected with chimeras or control plasmids along with a marker plasmid expressing β-galactosidase, exposed to RA or T3 and labelled with [^{3}H]thymidine followed by autoradiography. The α1 chimera gave T3-dependent inhibition of growth, comparable to the effect exerted by RA itself, whereas the δ1 chimera and control plasmids were inactive. The results imply that RAR-α1 mediates the effects of RA on blastemal cell growth.

Key words: cell growth/retinoic acid/retinoic acid response element/thyroid hormone

Introduction

Retinoic acid (RA) affects the proliferation and differentiation of a wide variety of cell types both in culture and *in vivo*. Although certain exceptions have been proposed (Ide and Aono, 1988; Paulsen *et al.*, 1988), its general action in culture is to decrease the growth rate and often to promote differentiation of precursor cells or established cell lines (Strickland and Mahdavi, 1978; Breitman *et al.*, 1980; Kim *et al.*, 1987). Its anti-neoplastic properties have been underlined recently by the therapeutic use in provoking differentiation of human leukaemic promyelocytes carrying the t(15;17) translocation (Huang *et al.*, 1988). There has also been much interest in its remarkable ability to respecify positional identity in developing and regenerating limbs (Brockes, 1989; Stocum, 1991; Tabin, 1991; Bryant and Gardiner, 1992). Limb regeneration in urodele amphibians such as the newt or axolotl proceeds by local formation of a blastema, a growth zone of mesenchymal progenitor cells which give rise to the regenerate (Wallace, 1981). The initial effect of RA on the blastema is to inhibit division (Maden, 1983), but subsequently an RA-treated blastema gives rise to extra structures that are indicative of a unidirectional change in axial specification (Maden, 1982; Stocum and Crawford, 1987). These diverse effects of RA are thought to be mediated by nuclear receptors of the steroid/thyroid superfamily which act as ligand-dependent transcription factors.

Studies in mouse and man have led to the identification of three genes coding for the retinoic acid receptors RAR-α, RAR-β and RAR-γ (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Zelent *et al.*, 1989). Sequence comparison of the receptors has shown that whereas the DNA-binding and ligand-binding domains are well conserved, there is considerable divergence in the NH_2-terminal A region. In addition each gene encodes multiple isoforms, of which the principal ones derive from alternative promoter usage and splicing at the NH_2-terminus (Kastner *et al.*, 1990; Leroy *et al.*, 1991; Zelent *et al.*, 1991). The distribution of the RARs has been analysed in detail, particularly during mouse development, by *in situ* hybridization with probes specific for each of the three RARs but not for individual isoforms (Dolle *et al.*, 1989, 1990; Ruberte *et al.*, 1990, 1991). These studies indicate that while RAR-α appears to be ubiquitous, RAR-β and RAR-γ show marked spatial and temporal regulation during embryogenesis. Furthermore, a study of transactivation by the various isoforms in transfection assays with different reporter genes has revealed clear differences in their activity that are dependent on the promoter and cell context (Nagpal *et al.*, 1992). Nonetheless there is little information that allows distinctions to be drawn about which isoform is responsible for mediating a particular physiological response to RA. A recent study with an RA-resistant subclone of human HL-60 leukaemic cells has shown that RAR-α, -β and -γ as well as RXR-α are all able to mediate granulocytic differentiation (Robertson *et al.*, 1992). In other experiments on HL-60, a synthetic RAR-α antagonist has been shown to counteract RA effects (Apfel *et al.*, 1992). It is clearly a challenge to identify the precise contributions of each isoform in different cell types, and to evaluate the possibility of functional redundancy.

In this report, we focus on the ability of RA to decrease the growth rate of cultured limb blastemal cells from the newt. The major isoform expressed in the newt limb and blastema is δ1 (Ragsdale *et al.*, 1989, 1992), which appears to be the urodele equivalent of γ1, although these receptors have diverged extensively in the A region. In addition, we have identified an α1 isoform which has high sequence

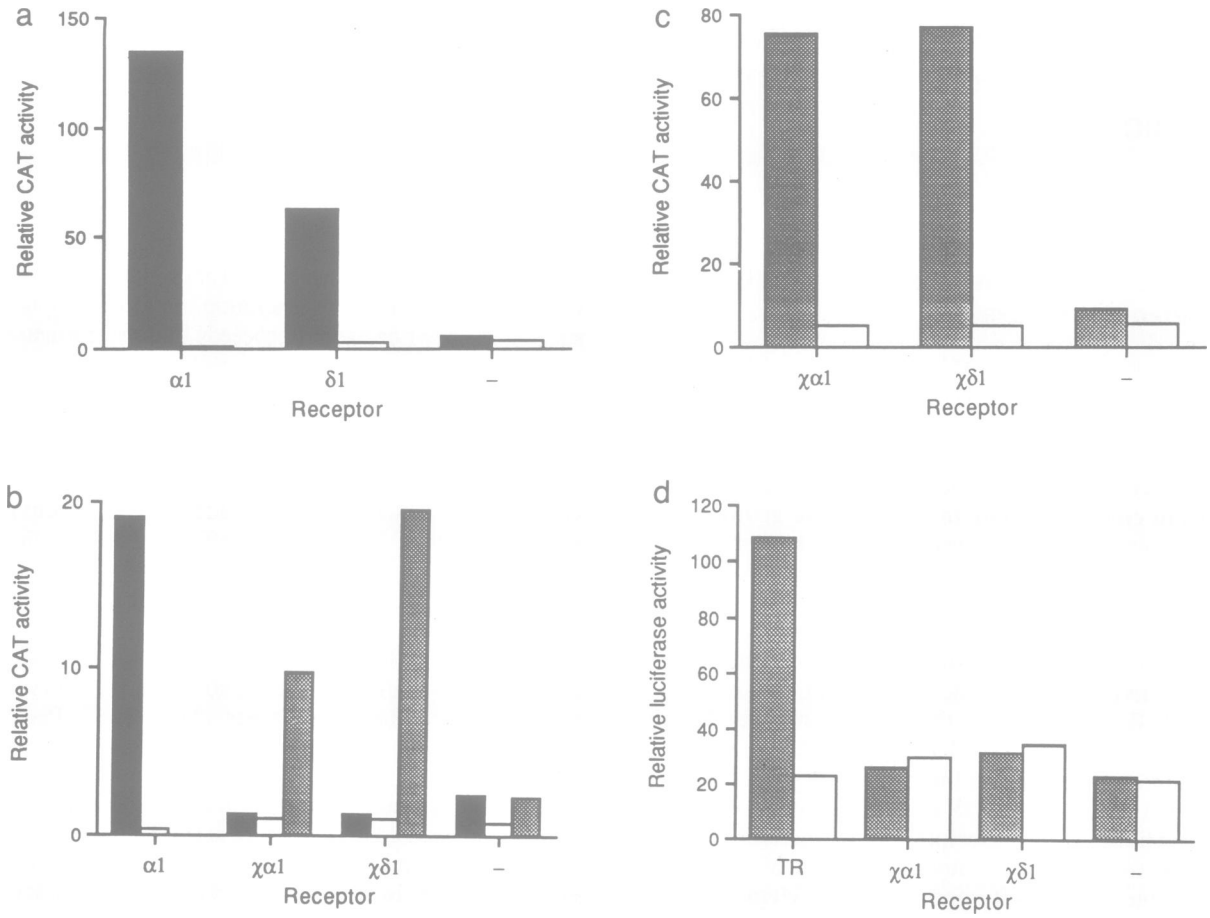

Fig. 1. (**a**) Activity of newt RARs in COS cells. COS cells were transfected with 5 μg expression plasmid for RARs, or vector control, 5 μg $(TRE3)_3$-tk-CAT and 5 μg EFβ-gal as a standard for transfection efficiency. The relative CAT activity is expressed in thousands of units after normalizing for β-galactosidase activity. Filled bars; cells treated with RA (10^{-7} M). Empty bars; untreated cells. The data shown in this and the subsequent figures are representative of that obtained in 3–5 experiments. (**b**) Activity of chimeric receptors on the palindromic TRE in COS cells. Cells were transfected with 5 μg expression construct for the chimera, or RAR-α1, or vector alone, 5 μg $(TRE3)_3$-tk-CAT and 5 μg EFβ-gal as above. After incubation in 10^{-7} M RA (filled bars), 5×10^{-7} M T3 (hatched bars) or no hormone (empty bars), the cells were extracted and the normalized CAT activity was determined. Note that the chimeras activate with T3 but not RA, while RAR-α1 activates with RA. (**c**) Activity of chimeric receptors on an RARE in COS cells. Cells were transfected with 5 μg receptor construct and 5 μg EFβ-gal as above, along with 5 μg RARE-tk-CAT. The normalized CAT activity was determined after incubation with T3 (hatched bars) or no hormone (empty bars). (**d**) Absence of activity of chimeric receptors on the direct repeat TRE. COS cells were transfected with 5 μg chimera, or TR-α expression vector, or vector alone, 5 μg EFβ-gal, 5 μg pMOMLV-tk-luc and 5 μg $(TRE3)_3$-tk-CAT. The normalized luciferase activity was determined after incubation with T3 (hatched bars) or no hormone (empty bars). As control, the CAT activity was measured to confirm that each chimera could activate another reporter in the same experiment (data not shown). The chimeras do not activate through the direct repeat TRE, but the TR-α control does so.

identity to its mammalian counterpart (Ragsdale *et al.*, 1989). We have replaced the RA-binding domain of both newt isoforms with the corresponding region of the *Xenopus* thyroid hormone (T3) receptor-α (TR-α). The chimeras were comparably activated by T3 to stimulate expression of RA reporter genes but only the chimera of RAR-α1 inhibited growth in a T3-dependent fashion. This ability to mimic the quantitative effect of RA implicates the α1 receptor as a natural mediator of this response.

Results

Construction of chimeric retinoid/thyroid receptors

Chimeric receptors were constructed by replacing the E and F regions of the newt α1 and δ1 RARs with the corresponding region of the *Xenopus* TR-α. An *Eco*RI site is present in the δ1 sequence in the E region at a position 11 amino acids from the D/E boundary (Ragsdale *et al.*, 1989). This site lies upstream of the Ti and dimerization domains of the E region (Laudet *et al.*, 1992). Equivalent *Eco*RI sites were introduced into the RAR-α1 and TR-α genes by mutation, and the chimeric receptors, χα1 and χδ1, were assembled and introduced into an expression vector (see Figure 6). The construction did not introduce or change any residues at the junction, but resulted in direct apposition of the two sequences.

Activity of the chimeric receptors in COS cells

Figure 1a shows the activities of the parent newt RARs after transfection of expression constructs into COS cells along with a synthetic reporter gene carrying three copies of the palindromic T3 response element (TRE) of the mouse growth hormone promoter. This response element is activated by both RARs and TRs (Umesono *et al.*, 1988). A strong RA-dependent stimulation of reporter activity was observed for the newt receptors, as previously reported (Ragsdale *et al.*, 1989). The chimeric receptors were analysed in the same system along with an α1 control. As shown in Figure 1b, the chimeras were not activated by RA, in contrast to the α1 control, whereas they were strongly activated by

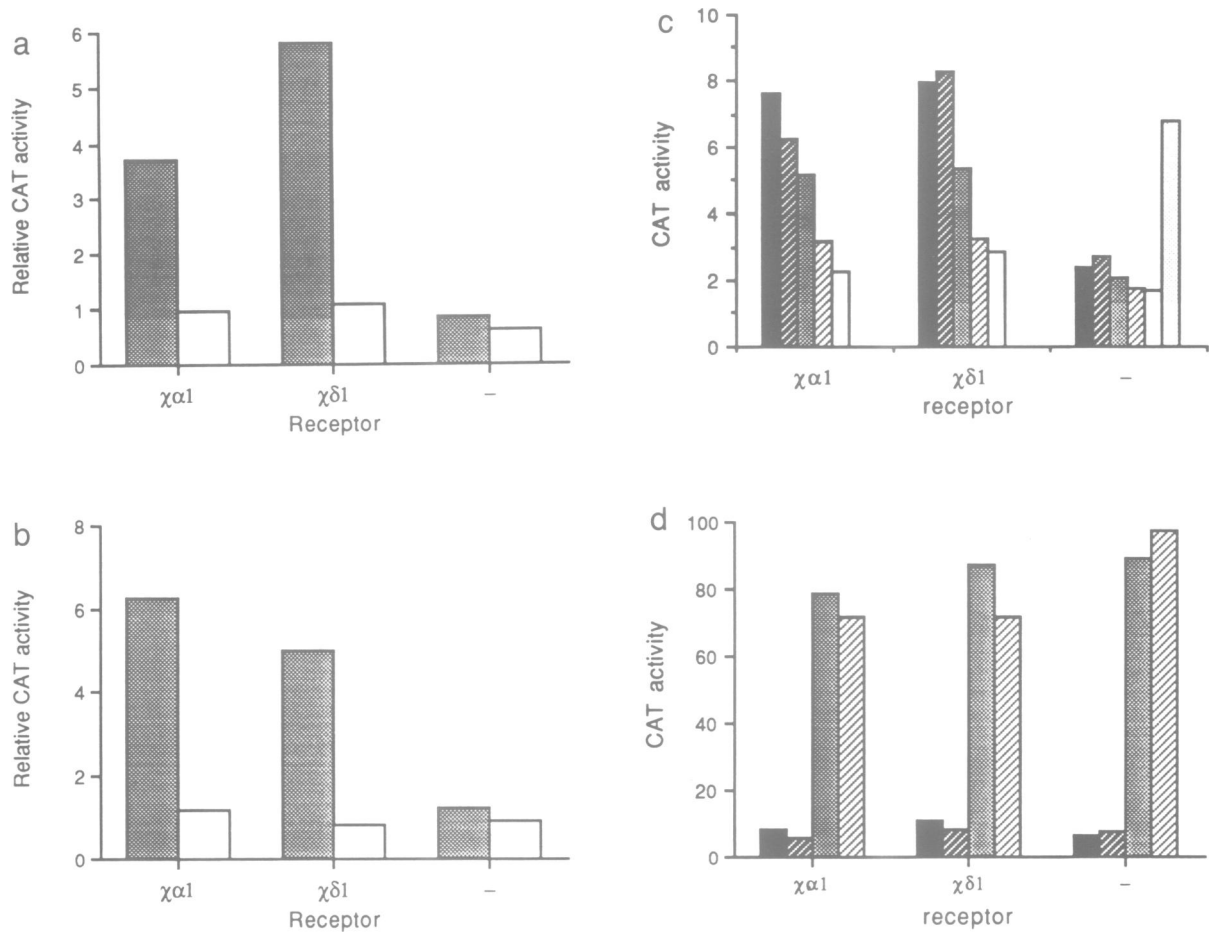

Fig. 2. (**a**) Activity of chimeric receptors on the palindromic TRE in cultured newt limb blastemal cells. Newt B1H1 cells were transfected using a particle gun with 2 μg chimeric receptor expression construct, or vector, 2 μg $(TRE3)_3$-tk-CAT and 2 μg EFβ-gal. The normalized CAT activity is shown for cells incubated in 5×10^{-7} M T3 (hatched bars) or without hormone (empty bars). (**b**) Activity of chimeric receptors on an RARE in newt limb cells. Newt cells were transfected with 2 μg receptor expression construct, or vector, 2 μg RARE-tk-CAT and 2 μg EFβ-gal. The normalized CAT activity is shown for cells incubated in T3 (hatched bars) or without hormone (empty bars). (**c**) Dose response for T3-induced stimulation of RARE by chimeras in newt cells. Newt cells were transfected as in (b) and CAT activity was determined after incubation in 0 (□), 10^{-9} (▨), 10^{-8} (▩), 10^{-7} (▨) or 10^{-6} (■) M T3. Also shown is the level of activation of the same reporter mediated by the endogenous RARs on treatment of the cells with 10^{-7} M RA (▩). (**d**) The chimeric receptors do not squelch transcription activated by an unrelated activator. Newt cells were transfected with 1 μg RGR expression construct, 1 μg GRE-tk-CAT reporter, 2 μg EFβ-gal and 2 μg chimera expression construct or vector. The normalized CAT activity is shown for cells incubated in 10^{-7} M RA (▩), 5×10^{-7} M T3 (▨), 10^{-7} M RA and 5×10^{-7} M T3 together (▨), or with neither RA nor T3 (■). In the presence of RA, reporter expression is activated by the RGR hybrid receptor; this activation is not significantly affected by either chimera.

5×10^{-7} M T3. A comparable stimulation by T3 was observed when the chimeras were assayed after cells were co-transfected with a reporter carrying the response element of the RAR-β2 promoter which is stimulated by RARs but not TRs (Figure 1c) (de The *et al.*, 1990; Sucov *et al.*, 1990). In contrast, the chimeras were not active on a reporter carrying the thyroid response element from the Moloney murine leukaemia virus (MoMLV) LTR, which is known to be specifically activated by TRs (Figure 1d) (Vivanco Ruiz *et al.*, 1991). As expected, this reporter was stimulated after co-transfection with an expression construct for the *Xenopus* TR-α (Figure 1d). Thus the activity of the chimeras in COS cells indicates that they retain the specificity for response elements that is characteristic of the RARs.

Activity of the chimeras in newt limb blastemal cells

The cultured newt cells used in these experiments were originally derived from a hind limb blastema (Ferretti and Brockes, 1988). They express blastemal mesenchyme markers such as reactivity with the monoclonal antibody 22/18 (Kintner and Brockes, 1985; Ferretti and Brockes, 1988) and antibodies to the K8/K18 cytokeratins (Ferretti *et al.*, 1989). The transfection efficiency for these cells is very low with conventional procedures, but using a DNA particle gun (Klein *et al.*, 1987; Yang *et al.*, 1990; Tang *et al.*, 1992), we routinely achieved frequencies up to ~10%, and this method was used for all of the experiments reported here. After transfection with reporters carrying the palindromic TRE (Figure 2a), or the retinoic acid response element (RARE) of the β2 promoter (Figure 2b), the chimeras showed similar T3-dependent stimulation of reporter activity. The concentration dependence of stimulation on the β2-RARE reporter in the range 10^{-6} to 10^{-9} M T3, a range covering that used in subsequent experiments (see below), is shown in Figure 2c. Also shown in this figure is that the fold-stimulation and magnitude of activation by the chimeras were comparable to those seen when the cells were stimulated with 10^{-7} M RA. This level of RA-dependent activation was even seen in cells expressing a chimeric receptor, demonstrating that the chimeras are not

expressed at a level that is sufficient to act as a dominant negative to the endogenous RARs (data not shown; Barettino *et al.*, 1993; Damm *et al.*, 1993).

In a separate experiment, the chimeras were shown not to squelch transcriptional activation by an unrelated activator. Expression from a reporter plasmid containing a glucocorticoid response element (GRE) upstream of the thymidine kinase (tk) promoter driving the chloramphenicol acetyltransferase (CAT) gene is activated in an RA dependent manner by an activator containing the DNA binding domain of the human glucocorticoid receptor and the RA binding domain of the human RAR-α. In newt cells, neither the α1 nor δ1 chimera, in the presence or absence of T3, affected transcriptional activation in this system (Figure 2d).

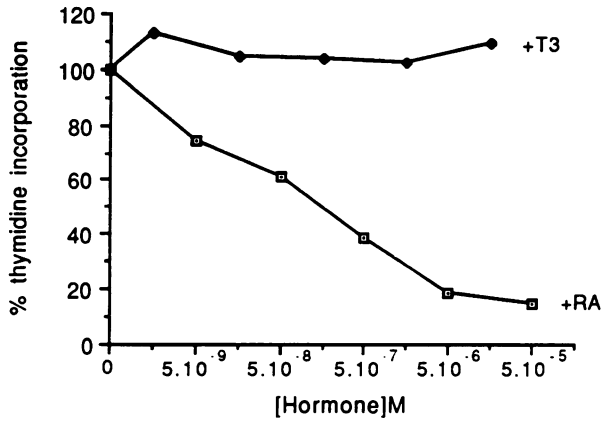

Fig. 3. Effect of T3 and RA on DNA synthesis in cultured newt cells. Newt cells were incubated with various concentrations of T3 (closed circles) or RA (open squares) for 72 h, followed by [^{3}H]thymidine for 14 h. The ^{3}H radioactivity incorporated into DNA was determined as described in Materials and methods, and expressed relative to the incorporation in cells incubated without hormone.

These observations indicate first that the chimeric receptors activate transcription in newt cells to an extent that is quantitatively similar to that produced by the endogenous RARs. Second, such activation is not limited by the availibility of other components, such as the RXRs. If this were the case the chimeras would have acted as dominant negatives to the endogenous RARs (Barettino *et al.*, 1993), yet they do not. Third, the chimeras do not interfere with transcription mediated by an unrelated activator, and hence do not exhibit squelching activity.

These findings encouraged us to analyse the functional consequence of chimera activation in newt cells.

Effect of chimera activation on growth of cultured blastemal cells

When cultured newt limb blastemal cells were exposed to various concentrations of RA for 3 days, there was a dose-dependent decrease in the growth rate as measured by incorporation of [^{3}H]thymidine (Figure 3), or by counting cell number (data not shown). If T3 was applied in the same range of concentrations to parallel cultures, no inhibitory effect was observed (Figure 3). No obvious change in cell differentiation was observed with either hormone. After transfection of newt limb cells with plasmid DNA using the DNA particle gun, transfectants continued to express plasmid

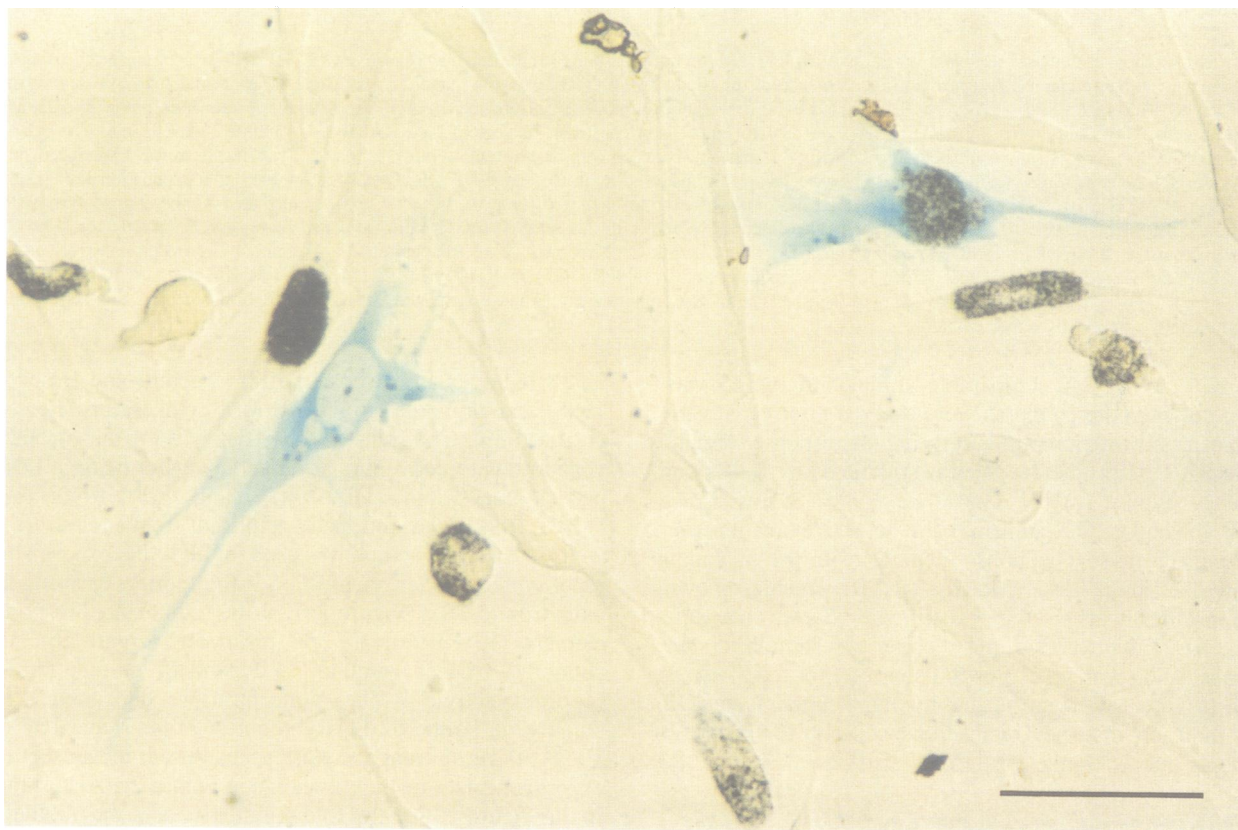

Fig. 4. Autoradiography of transfected newt cells. Cells were transfected with receptors and EFβ-gal as described in Figure 5, and incubated in T3 followed by [^{3}H]thymidine. After X-gal staining, the cells were coated with emulsion for autoradiography. One transfected (blue) cell has incorporated label and one has not. The background untransfected (white) cells show both positive and negative nuclei. The scale bar indicates 100 μm.

markers for at least 3 weeks in culture and began to incorporate [^{3}H]thymidine 7 days after transfection. It is therefore possible to determine the effect on the growth rate of activating chimeric receptors with T3.

Newt cells were transfected with chimeric or control constructions, exposed to varying concentrations of RA or T3, and incubated with [^{3}H]thymidine prior to autoradiography. In order to identify the transfected cells, all transfection mixtures contained plasmid DNA that expressed β-galactosidase. Co-transfection occurred in at least 85% of the recipients (see Materials and methods). The β-galactosidase was detected by X-gal staining, thus allowing the growth rate to be assessed by the proportion of transfected cells with silver grains over their nuclei, as illustrated in Figure 4.

The results from several experiments showed that when cells transfected with χα1 were activated with varying concentrations of T3, the decrease in growth rate was reproducibly similar to that observed when the same cell populations were treated with RA (Figure 5a). In contrast, when cells were transfected with χδ1 there was no effect

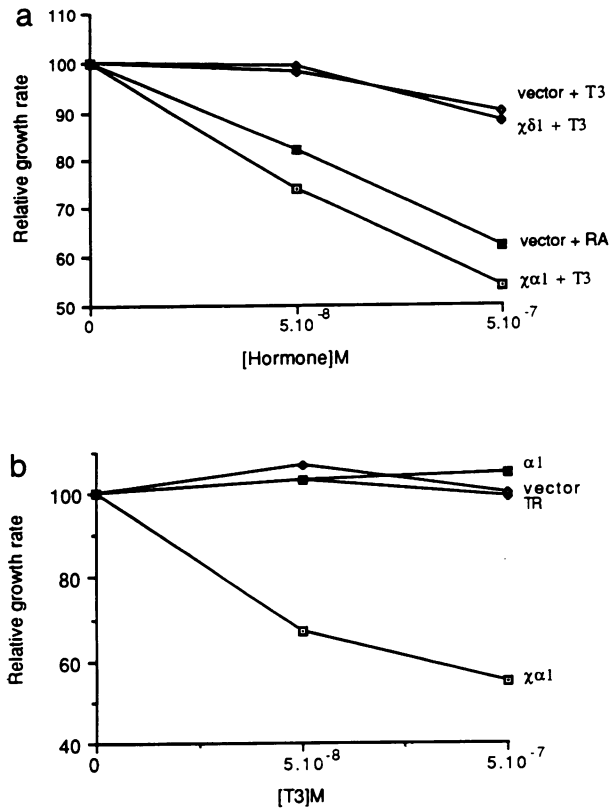

Fig. 5. Effect of chimera activation on newt limb cell growth. Cells were transfected with 2 μg chimera expression construct, or normal RAR-α1, or vector, and 2 μg EFβ-gal. After incubation with various concentrations of T3 or RA, the cultures were analysed as described in Materials and methods. The graphs show the relative growth rate of the transfected cells versus the concentration of hormone. In each case the graph shows results from a single experiment that is representative of data from at least three experiments. (**a**) Effect of T3 on growth of cells expressing χα1 or χδ1 as compared with the effect of RA or T3 on cells transfected with vector. Only χα1 is able to give a T3-dependent inhibition of cell growth comparable to that given by RA. (**b**) Effect of T3 on the growth of cells expressing vector, χα1, or the 'parental' receptors RAR-α1 and TR-α. Only χα1 gives the T3-dependent inhibition of growth. The total number of transfected cells with labelled nuclei counted for each category in all experiments was as follows; vector + T3, 1935; vector + RA, 807; χα1, 1736; χδ1, 542; α1, 2151; TR, 844.

on the growth rate in the equivalent range of T3 concentrations. It should be noted, as stated above, that the activity of the chimeras was comparable when assayed by transactivation of reporter genes. Cells transfected with either RAR-α1 or TR-α (the parent molecules of χα1) showed no T3-dependent inhibition of growth, while parallel transfections with χα1 gave the expected T3-dependent effect (Figure 5b).

Discussion

An advantage of the present approach is that it allows activation of one receptor isoform at a time in cells that express several different RARs or RXRs. The choice of the T3 binding domain to replace the RA binding domain was made for two reasons: T3 has no marked effect on limb regeneration (Hay, 1956) and, as the TR is a close relative of RARs in the nuclear receptor superfamily (Laudet *et al.*, 1992), it seemed possible that the T3 binding domain would function to activate transcription in the context of the RAR. Nonetheless it was important to determine if the hybrid receptors behave as RARs in respect of their transactivation properties. In the present case, each of the chimeras retained the specificity for response elements that is characteristic of the RAR as opposed to the TR. In addition the degree of transactivation was comparable to that observed with the parental RARs. It should be noted that we have not explored the effect of varying the precise contribution from the RAR and TR-α, and it might be important that the present constructions retain 11 amino acids from the E region of the RARs. Although the two hybrids described here behaved in an orderly fashion, we have found other cases where replacing the RA binding domain resulted in nonfunctional receptors (data not shown). In any event, the availability of functional chimeras allowed us to investigate the basis of an important effect of RA, that on cell growth in culture.

When the cultured blastemal cells were transfected using a particle gun, the cells continued to express transfected markers for at least 3 weeks. Transfected cells started to incorporate tritiated thymidine 1 week after transfection, and we have verified that the quantitative effects of RA on incorporation were comparable for untransfected cells and cells 7−10 days after transfection. This has allowed us to investigate chimera activity in the context of a 'transient' transfection assay. The most important result was that activation of the χα1 receptor with varying concentrations of T3 produced a decrease in the growth rate which reproducibly paralleled that obtained with RA, whereas control transfections with plasmid vector, RAR-α1 or TR-α had no effect. This strongly supports the view that this effect of RA is mediated by RARs, and more specifically is in agreement with properties attributed to RAR-α, though not to a specific isoform, by use of a putative α-selective antagonist (Apfel *et al.*, 1992). Since RXRs are not activated by T3 (Mangelsdorf *et al.*, 1990), it also demonstrates that ligand activation of RXRs is not necessary for this effect.

In contrast to χα1, the χδ1 construction gave no significant decrease in growth rate. When analysed for transactivation activity in newt cells, the two chimeras were the same, and it is likely, therefore, that the failure of χδ1 to affect the growth rate reflects a genuine difference in receptor function between RAR-δ1 and RAR-α1. We do not believe that the inhibition of growth by χα1 is the result of a non-specific

effect caused by over-expression of the receptor in the cell, and it should be noted that any such explanation would have to account for the fact that the effect is hormone dependent and $\alpha 1$ specific. Nevertheless, it could be argued that $\chi\alpha 1$ produces an indirect reduction in growth through squelching. Two observations indicate that this is unlikely to be correct: first, expression of $\chi\alpha 1$ or $\chi\delta 1$ in B1H1 cells does not reduce the expression from a reporter gene activated by an unrelated activator (Figure 2d); second, activation of a reporter by the endogenous RARs is not affected by simultaneous expression of either chimeric receptor in the cell, indicating that the chimeras are not expressed at levels sufficient to act as dominant negative inhibitors, for example by titration of RXRs or by competion with the RARs for binding to response elements (Barettino *et al.*, 1993; Damm *et al.*, 1993). Since $\chi\alpha 1$ and $\chi\delta 1$ activate transcription to the same extent over a range of hormone concentrations, and this level is comparable to that seen after activation by the endogenous RARs, these observations suggest that the effect of the $\alpha 1$ chimera on cell growth reflects a genuine activity of this molecule and not some non-specific inhibitory mechanism unrelated to activities of RAR-$\alpha 1$.

We have suggested elsewhere that RAR-$\delta 1$ might mediate functions specific to limb regeneration (Hill *et al.*, 1993; Ragsdale *et al.*, 1992). It cannot be ruled out that the difference observed between $\chi\alpha 1$ and $\chi\delta 1$ is a feature of the chimeras that is not a direct reflection of the parent RARs. Our results are equivocal on the issue of whether the growth inhibitory effects depend directly on transactivation, or are mediated via the inhibitory interaction with AP1 (Nicholson *et al.*, 1990; Schule *et al.*, 1991; Yang-Yen *et al.*, 1991).

This study demonstrates the power of the chimera approach for analysing the contribution of individual RAR isoforms to the response to RA. Recent work in this laboratory has demonstrated that $\chi\delta 1$ and $\chi\alpha 1$ can be shot into the wound epidermis of a regenerating limb and activated *in vivo* with T3 (L.Pecorino *et al.*, unpublished). It may therefore be possible to analyse the receptor basis of positional respecification with this method.

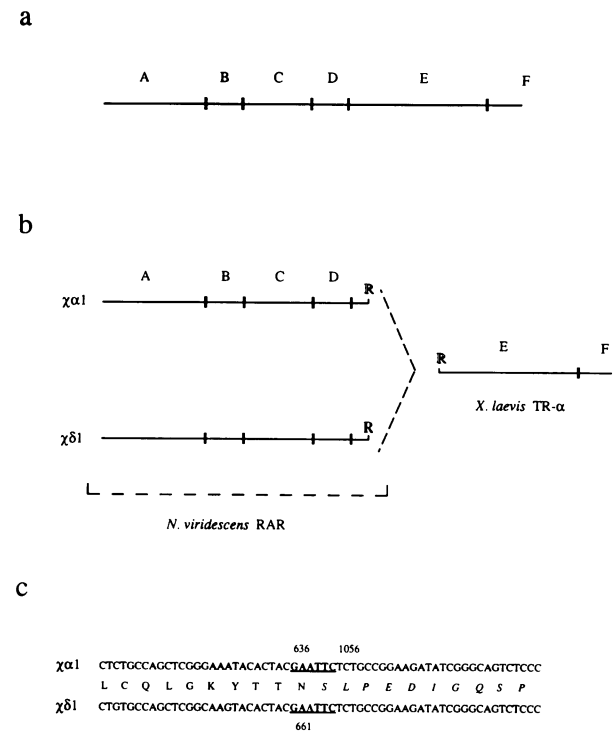

Fig. 6. (**a**) Schematic map of a consensus nuclear receptor. The C region contains the DNA binding domain, and the E region contains the ligand binding domain. (**b**) Construction of chimeric retinoid/thyroid cDNAs. The 5′ regions of cDNAs coding for the newt RARs $\alpha 1$ and $\delta 1$ were ligated to the 3′ region of a cDNA encoding the *X.laevis* TR-α (Brooks *et al.*, 1989). R (solid) indicates the endogenous *Eco*RI site in the RAR-$\delta 1$ that was used for the ligation, and the R (open) indicates the position where a matching *Eco*RI site was generated in the TR-α and RAR-$\alpha 1$ cDNAs. (**c**) Nucleotide and derived amino acid sequences at the site of fusion between the RAR and TR-α cDNAs. The nucleotide sequence of the $\chi\alpha 1$ (top) and $\chi\delta 1$ (bottom) construct is given at the site of ligation. The derived amino acid sequence (identical for both receptors in this region) is shown for the two DNA sequences. The sequence begins with the leucine that is the third amino acid of the E region in RAR-$\alpha 1$ and RAR-$\delta 1$. The *Eco*RI site is printed in bold and the TR derived amino acid sequence is printed in italics. The numbers refer to the nucleotide numbering found in the EMBL databank for the RAR and TR-α cDNAs.

Materials and methods

Cells and culture

COS-7 cells were maintained in supplemented Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After transfection cells were incubated in medium with 10% charcoal-stripped fetal calf serum. Newt (*Notophthalmus viridescens*) blastemal cells (B1H1) and limb cells (TH4B) were maintained in culture at 25°C as described previously (Ferretti and Brockes, 1988; Brockes, 1992) in supplemented 63% minimal Eagle's medium with 10% fetal bovine serum. Cells were grown in flasks or culture dishes coated with gelatin.

Plasmids

The plasmid RARE-tk-CAT (Smith *et al.*, 1991) carrying a single copy of the RAR-$\beta 2$ promoter RA-responsive element 5′ to the tk promoter driving expression of the CAT gene, and the plasmid $(TRE3)_3$-tk-CAT carrying palindromic TRE elements upstream of the tk promoter driving expression of the CAT gene (de Verneuil and Metzger, 1990) were obtained from P.Chambon. The plasmid pMOMLV-tk-Luc with the direct repeat thyroid response element from the MoMLV LTR upstream of the tk promoter driving expression of the luciferase gene (Vivanco Ruiz *et al.*, 1991) was obtained from H.Stunnenberg. The plasmid pG29G tk CAT carrying two GREs upstream of the tk promoter driving expression of the CAT gene (Schule *et al.*, 1988) and the plasmid pRGR which directs expression of a hybrid activator protein containing the DNA binding domain of the human glucocorticoid receptor and the RA binding domain from the human RAR-α, were kindly given by Dr D.Mangelsdorf and Dr R.Evans. EFβ-gal, in which expression of the *lacZ* gene is driven by the *Xenopus* EF1α promoter, was obtained from P.Krieg (Krieg *et al.*, 1989). pNvRAR-$\alpha 1$ and pNvRAR-$\delta 1$ direct expression of newt RAR-$\alpha 1$ and the 51K RAR referred to as $\delta 1b$ in Ragsdale *et al.* (1992), each under the control of the SV40 promoter in the eukaryotic expression vector pTL1, as described previously (Ragsdale *et al.*, 1989). The human placental alkaline phosphatase gene (obtained from C.Cepko) was cloned under the control of the SV40 promoter in pSG5 (Stratagene). A cassette including the SV40 promoter and polylinker from pTL1 was introduced into this modified pSG5 plasmid to give pCAP.

The plasmids encoding the chimeric receptors were constructed by fusing the A−D regions of the newt RAR cDNAs (NvRARs) to the E/F regions of the *X.laevis* thyroid hormone receptor (XITR; Brooks *et al.*, 1989). The *Eco*RI site downstream of the start of the E region of NvRAR-$\delta 1$ (Ragsdale *et al.*, 1989) was used as the point of fusion between the two partial cDNAs (see Figure 6). A matching *Eco*RI site was introduced into the cDNA encoding NvRAR-$\alpha 1$ (Ragsdale *et al.*, 1989) at the corresponding position in its E region. The restriction site was constructed by PCR, using a mutant oligonucleotide with the sequence CCGAACTGGAATTCGTAGTGTA-TTTCC. Positions that diverge from the wild type sequence are underlined. A similar strategy was used to introduce an equivalent *Eco*RI site into the E region of the TR sequence, thus allowing an *Eco*RI−*Bgl*II fragment encoding the E and F regions of the TR to be cloned into the Bluescript KS+ vector. This plasmid was used as a cassette into which the *Eco*RI fragments encoding the A−D regions of the α and δ RARs were cloned to create the coding sequences for the chimeric receptors. Sequence analysis of the chimeras revealed that while the contribution from the RARs was

as predicted, ending at the natural *Eco*RI site in the case of δ, and at the engineered site in α, the position of the junction in the TR sequence was not in the predicted site, but 24 bp further upstream. In terms of the design of the chimeras, however, this was satisfactory as it fused almost the entire E and F regions of the TR to the coding sequence for the A–D regions of the RARs, with no amino acid changes at the junction (Figure 6c). After assembly in the Bluescript vector, the entire coding sequence of each chimera was placed under the control of the SV40 early promoter in pCAP. This contains intron sequences from the SV40 small T gene and provides a poly(A) site.

Transfection of tissue culture cells

Transfection of COS cells was performed essentially as described by Darrow *et al.* (1980). DNA (5–20 μg) was added to 430 μl H_2O and 63 μl of 2 M $CaCl_2$ and the mixture was added dropwise to 500 μl of transfection buffer (136 mM NaCl, 5 mM KCl, 11.2 mM glucose, 208 mM HEPES, 1.4 mM Na_2HPO_4; pH 7.1). After 20 min at room temperature, the mixture was added to cells and incubated for 6 h at 37°C, after which the medium was changed, hormone added as appropriate and the cells incubated for 2 days prior to assay.

Newt blastemal cells (B1H1) were transfected with a DNA particle gun (Dupont PDS-1000H biolistics machine) which employs pressurized helium to propel DNA-coated gold beads into cells. To prepare sufficient beads for two samples, 20 μl of a 60 mg/ml stock of 1–3 μm diameter beads was added to 8 μl plasmid DNA (up to 8 μg), 28 μl 2.5 M $CaCl_2$ and 6 μl 1 M spermidine. The beads were pelleted and then washed in 50 μl 70% ethanol followed by 50 μl 100% ethanol before resuspension in 20 μl of 100% ethanol. 10 μl of the suspension was loaded on to an ethanol washed Kapton disc (Bio-Rad) in a moisture free container and allowed to dry. Cells were grown to confluence in 6 cm plastic culture dishes, the medium was removed, and the dishes placed 10 cm below the nozzle of the helium gun, before being shot at a rupture pressure of 450 p.s.i. under vacuum. Approximately 50% of the cells were killed, but 1–20% of the survivors subsequently expressed the transfected DNA. After transfection, new medium was added to the cells which were incubated for 12–24 h before replating as appropriate.

Enzyme assays

Cell extracts were prepared from 6 cm diameter plates in 250 μl of either PMN buffer (0.01 M sodium phosphate, 0.1 M NaCl, 1 mM $MgCl_2$; pH 7.2) containing 0.1% NP40, or Promega cell lysis reagent (25 mM Tris–phosphate, pH 7.8; 2 mM DTT; 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; 10% glycerol; 1% Triton X-100). Assays of β-galactosidase (0.10 ml) contained 10–90 μl of extract with PMN buffer and chlorophenol red–β-galactopyranoside (0.4 mg/ml) as substrate. The mixture was incubated at 37°C for 2–20 min and analysed for absorbance at 570 nm on an automatic plate reader. Extracts from untransfected or mock-transfected cells were used as background that was subtracted from all sample readings.

CAT assays were performed in a volume of 0.15 ml containing 10–50 μl of extract, 0.13 M Tris–Cl pH 8, 0.5 mM butyryl CoA and 30 μM [^{14}C]chloramphenicol (57 Ci/mol). Reactions were performed in duplicate along with a blank assay on extracts of untransfected cells. After incubation for 2–7 h at 37°C incorporation of ^{14}C was determined by the method of Seed and Sheen using the double back-extraction procedure (Seed and Sheen, 1988). Luciferase assays were performed using 0.25 ml of rehydrated reaction mixture from the Promega assay kit according to the manufacturer's instructions, in conjunction with analysis by scintillation counting. Enzyme activity was linear with respect to the square root of the c.p.m. CAT and luciferase activities were normalized to β-galactosidase activity in all experiments.

[^{3}H]Thymidine incorporation by newt cells

Newt cells were plated in a gelatin-coated 96 well plate at an initial density of 500 cells/well. The next day, hormone (RA or T3) was added as required and cells were incubated for another 3 days. [^{3}H]Thymidine ([methyl-1',2'-^{3}H]thymidine, 124 Ci/mmol, Amersham) was added to the medium at 1 μCi/ml and the cells were grown for another 24 h prior to collection on filter discs in an automatic cell harvester. The discs were washed, dried and analysed by liquid scintillation counting.

Inhibition of cell growth

After transfection of ~2 × 10^5 B1H1 cells with the EFβ-gal marker plasmid and the appropriate receptor-encoding plasmid, or vector, cells were allowed to recover overnight. They were trypsinized and one-seventh of the surviving cells (~15 000) was replated into each of three 3.5 cm plates. A further aliquot was plated and stained the next day for β-galactosidase activity (using X-gal) to ensure that the transfection frequency was adequate. The plates were incubated for 2–3 days before hormone (RA or T3) was added at the required concentrations, and incubated for a further 7 days with one change of medium and hormone. On day 10 after replating, [^{3}H]thymidine was added (1 μCi/ml) and the plates were incubated for 7 h. The cells were rinsed in buffer A [phosphate buffered saline (PBS); 1 mM $MgSO_4$] and fixed in 0.05% glutaraldehyde. They were stained for β-gal activity with X-gal, rinsed again in buffer A, dehydrated with ethanol and air dried. Each well was coated with a thin layer of photographic emulsion (Ilford K5), exposed for 2–3 days, developed for 8 min in Phenisol (Ilford) and fixed with Hypam (Ilford) for at least 1 h.

In each well, the proportion of blue cells with labelled nuclei was determined. In the experiments described, the number of blue cells per well varied between 400 and 1500, 10–20% of which had labelled nuclei in the absence of hormone. The percent labelled cells in wells containing hormone was normalized to the value obtained in the absence of hormone (100% growth rate in Figure 5). Histochemical analysis of cells transfected with a mixture of two plasmids (EFβ-gal and the pCAP vector expressing alkaline phosphatase) by the DNA particle gun showed that co-transfection occurred in at least 85% of cases.

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