UNIVERSITY
of
GREENWICH

Greenwich Academic Literature Archive (GALA)
– the University of Greenwich open access repository
http://gala.gre.ac.uk

Contact: gala@gre.ac.uk

Isoform-specific induction of a retinoid-responsive antigen after biolistic transfection of chimaeric retinoic acid/thyroid hormone receptors into a regenerating limb

Lauren T. Pecorino[1], Donald C. Lo[1,*] and Jeremy P. Brockes[1,2,†]

[1]Ludwig Institute for Cancer Research and [2]Department of Biochemistry and Molecular Biology, University College London, 91 Riding House Street, London W1P 8BT, UK

*Present address: Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, USA
†Author for correspondence at: Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK

SUMMARY

Retinoic acid (RA) induces secretory differentiation in the wound epidermis of a regenerating amphibian limb. We investigated the role of individual RA receptor (RAR) types in the newt wound epidermis by introducing chimaeric RA/thyroid hormone (T3) receptors ($\chi\alpha 1$ and $\chi\delta 1$) that can be activated by T3. A biolistic particle delivery system was employed to transfect cells in the wound epidermis of a regenerating limb and approximately 10% of the cells in targeted surface areas expressed marker genes. Both $\chi\alpha 1$ and $\chi\delta 1$ were comparable in their ability to stimulate transcription of a synthetic reporter construct through a RA response element after activation with T3 in situ. This activation was also comparable to that obtained by the endogenous complement of RARs in the RA-treated, transfected wound epidermis. The RA-inducible WE3 antigen, a marker for secretory differentiation, which distinguishes the wound epidermis from normal skin (Tassava, R. A., Johnson-Wint, B. and Gross, J. 1986, *J. Exp. Zool.* 239, 229-240), was used to assess the functional role of $\chi\alpha 1$ and $\chi\delta 1$. Chimaeric receptors were transfected with an alkaline phosphatase marker gene, activated with T3, and the expression of both the marker and WE3 was analyzed by double-label immunofluorescence. Newt limbs transfected with $\chi\delta 1$ showed many double-labelled cells dependent on the presence of T3, whereas contralateral limbs transfected with an alkaline phosphatase marker lacking chimaeric receptor sequences did not. Limbs transfected with $\chi\alpha 1$ did not show double-labelled cells in the presence or absence of T3, whereas in an earlier study $\chi\alpha 1$, and not $\chi\delta 1$, inhibited growth. These results indicate that specific effects of RA can be mediated by particular types of RARs and demonstrate a novel approach for studying the action of RA on its target tissues.

Key words: biolistics, wound epidermis, retinoic acid receptors, limb regeneration, mucous metaplasia

INTRODUCTION

Epithelia are a classic target for retinoid action during development and in the adult, and the effects of both deficiency and excess have been extensively documented (for reviews, see Darmon, 1991; Morriss-Kay, 1992). When keratinizing epithelia are exposed to high concentrations of retinoic acid (RA), they characteristically undergo mucous metaplasia (Fell and Mellanby, 1953). This appearance of a ciliated and secretory epithelium is also shown after treating the wound epidermis of a regenerating amphibian limb (Maden, 1983; Scadding, 1989). The wound epidermis, a specialized structure, is formed by the migration of epidermal cells over the wound surface after amputation of a limb in adult urodeles such as the newt or axolotl. The blastema, a growth zone of mesenchymal progenitor cells, forms beneath the wound epidermis and progressively reconstructs the structures of the limb distal to the amputation plane (reviewed in Wallace, 1981; Stocum, 1984). Limb regeneration is an important system for investigating the ability of RA to respecify cell differentiation, and also positional identity (Brockes, 1989; Stocum, 1991; Tabin, 1991; Bryant and Gardiner, 1992) in that a distal blastema treated with RA gives rise to structures that are proximal to its point of origin (Maden, 1982; Stocum and Crawford, 1987). The mechanism of its effect on the wound epidermis is a particular focus of the present paper.

Tassava and colleagues have isolated a monoclonal antibody that identifies the WE3 antigen, a $43\text{-}44\times 10^3$ M_r protein (Castilla and Tassava, 1992) which is an interesting marker for retinoid effects on the epidermis as well as for normal epithelial differentiation. WE3 is undetectable in normal newt skin or in the wound epidermis shortly after amputation, but it appears in the latter location during the second week postamputation, and is thus a marker distinguishing the mature wound

epidermis from skin (Tassava et al., 1986). If the regenerate is exposed to RA, WE3 is induced both in the wound epidermis, as early as one day after amputation, and in the skin (Tassava, 1992). A survey of WE3-reactive tissues in the newt shows that WE3 is expressed primarily in cells specialized for secretion and/or ion transport and it has been suggested that the wound epidermis acquires a secretory/transport function when WE3 appears (Goldhamer et al., 1989).

In order to understand the mechanism of these effects on the epidermis, it is an important step to identify the RA receptors that mediate them. At least six different members of the RAR family have been identified in the newt limb and limb blastema by cDNA cloning (Ragsdale et al., 1989, 1992a,b). This includes two isoforms of RARα, as well as two translational variants of RARδ1, the major receptor expressed in the limb. RARδ1 is also of interest because its N-terminal sequence is unique among vertebrate RARs, and it has been detected in both the wound epidermis and the blastemal mesenchyme by immunohistochemistry (Hill et al., 1993). Although it is a for-midable problem to identify the role of different RARs in the various responses to RA, one approach is the use of chimaeric RA/thyroid hormone receptors. As described in a previous study, the ligand binding domain for newt RARs α1 and δ1b has been replaced by the corresponding region of the *X. laevis* T3 receptor-α (TR-α). After transfection of these chimaeric receptors (χα1 and χδ1) into cultured cells, RA-responsive genes were activated by T3 (Schilthuis et al., 1993). In order to apply this approach in the animal, we have taken advantage of recent developments in biolistics technology (Sanford et al., 1991; Williams et al., 1991; Tang et al., 1992) to introduce plasmid DNA directly into the wound epidermis in situ. This approach to RAR function, whereby a single isoform can be activated in target tissue, should complement information coming from analysis of RAR null mice (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993). In this report, we demonstrate that, after activation with T3, χδ1 can mimic the effect of RA on WE3 expression but χα1 cannot do so. Together with the earlier study (Schilthuis et al., 1993), these results indicate that RAR isoforms mediate distinct aspects of the response to RA.

MATERIALS AND METHODS

Animals

Newts (*Notophthalmus viridescens*) were supplied by Blades Biologicals (Edenbridge, Kent, UK). Hindlimb amputations were performed as previously described (Kintner and Brockes, 1985) on animals anesthetized in 0.1% tricaine methane sulfonate (Sigma). Hormones (T3, RA, or retinol palmitate [Sigma type VII]) were added to the balanced salts solution in which the animals were maintained.

Plasmids

The reporter plasmid RARE-tk-CAT (Smith et al., 1991), a gift from P. Chambon, consists of one copy of the RA-responsive element from the *RAR-β2* gene upstream of the thymidine kinase (tk) promoter which directs expression of the chloramphenicol acetyltransferase (CAT) gene. The human placental alkaline phosphatase gene (obtained from C. Cepko) was cloned under the control of the SV40 promoter in pSG5 (Stratagene) to give pSDAP. pCMV-LUC-ori was constructed by inserting a fragment of the luciferase gene into the *Hin*dIII-*Bam*HI site of pcDNA1 (Invitrogen) and N-CMV-LacF was

constructed by inserting the CMV promoter into a *Xba*I site upstream of *LacZ* containing a nuclear localization signal in pUC18 (both obtained from S. A. Johnston). Plasmids χα1 and χδ1 have been described by Schilthuis et al. (1993). In brief, they were constructed by ligation of the A-D regions of the newt RARα1 or RARδ1 cDNAs respectively to the E/F regions of the *X.laevis* T3 receptor-α at an *Eco*RI site. The chimaeric receptor was inserted between an SV40 early promoter with SV40 small T intron sequences and a poly(A) site. The plasmid also contained an alkaline phosphatase cassette under the control of the SV40 promoter.

Biolistic transfection

Limbs of anesthetized newts were transfected in situ using a biolistic particle delivery system (PDS-1000/He; Bio-Rad). Pressurized helium (2200 psi) was used to propel DNA-coated gold particles (1.6 μm) into the wound epidermis under minimum vacuum (5 inches of mercury). A target distance of 14 mm was defined by the addition of a spacer and limb collar. Microprojectiles were coated with DNA by calcium chloride (CaCl₂)/spermidine precipitation. DNA (total 20 μg) was added to 50 μl of a 60 mg/ml stock of gold particles in water. An equal volume of 2.5 M CaCl₂ and one fifth volume of 1 M spermidine were added immediately over low vortex. The microprojectiles were pelleted and rinsed with 70% and 100% ethanol before resuspending in 50 μl of 100% ethanol. The ethanol suspension (8 μl) was applied to ethanol-rinsed Kapton macrocarriers in a moisture-free box and allowed to dry before loading into the PDS 1000/He. After transfection, animals were placed into balanced salt solution (with or without hormones).

Enzyme assays

Blastemas were removed from anesthetized newts and homogenized in 0.25 ml of Promega Cell Culture Lysis Reagent. The homogenate was centrifuged at 4°C for 10 minutes at 15,000 g, and the supernatant was removed and centrifuged once again before assaying. Luciferase and CAT assays were performed as previously described (Brockes, 1992) with minor modifications. CAT assay mixtures (0.15 ml) contained 50 μl of blastemal extract and were performed in duplicate. Incubation was carried out overnight at 37°C and incorporation of ¹⁴C-chloramphenicol was determined by a double back-extraction procedure (Seed and Sheen, 1988). Luciferase assays were performed with 0.25 ml of freshly prepared reaction mixture (Promega) and analyzed on a scintillation counter.

Alkaline phosphatase was analyzed by 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) staining of sections which had been heated to 65°C for 20 minutes to inactivate endogenous phosphatases, and β-galactosidase was analyzed by 5-bromo-3-chloro-3-indolyl β-D-galactoside (X-gal) staining using standard methods on limbs fixed in 0.5% glutaraldehyde.

Antibodies

Monoclonal antibody (mAb) WE3 was provided by R. Tassava (Tassava et al., 1986). Rabbit immunoglobulins to human placental alkaline phosphatase, rhodamine-conjugated rabbit anti-mouse immunoglobulins, fluorescein-conjugated rabbit anti-mouse immunoglobulins, rhodamine-conjugated swine anti-rabbit immunoglobulins and fluorescein-conjugated swine anti-rabbit immunoglobulins were purchased from Dako.

Histochemical staining and immunocytochemistry

Limbs were removed 4 days after biolistic transfection, fixed in methanol at −20°C for 1 hour and rinsed with 10% goat serum in PBS for at least 4 hours. Tissue was frozen in Cryo-M-Bed (Bright Instrument Company) over liquid nitogen. 30 μm sections were prepared using a base sledge microtome (Leitz 1400), dried on gelatin-coated slides, and rehydrated with 10% goat serum in TN buffer (0.02 M Tris, pH 8, 0.15 M NaCl) for 10 minutes. Primary antibodies (mAb WE3 and/or rabbit immunoglobulins to human alkaline phosphatase)

were diluted 1:100 in 10% goat serum/TN and reacted with sections for 1 hour. Sections were washed in 10% goat serum/TN for 10 minutes. Secondary antibody (either fluorescein- or rhodamine-labeled swine anti-rabbit) was reacted with sections for 1 hour. Sections were blocked with 10% rabbit serum/TN for 10 minutes and if double staining, another secondary antibody (fluorescein or rhodamine labeled rabbit anti-mouse) diluted 1:50 in 10% rabbit serum/TN was reacted with the sections for 1 hour. The sections were rinsed in 10% goat serum/TN, postfixed in acid-alcohol, rinsed in 0.2 M Tris, followed by distilled water and mounted in 1% Dabco (1,4-diazabicyclo-[2,2,2,] octane; Merck) in 90% glycerol, 10% 0.1 M phosphate buffer, pH 8.6.

Representative sections were photographed with a Zeiss (Axiophot) microscope equipped with Plan-Neofluar objectives using 400 DX (Kodak) film. Fluorescein was viewed through a 450-490 excitation filter (chromatic beam splitter FT510; barrier filter BP515-565) and rhodamine was viewed through a 546 excitation filter (chromatic beam splitter FT580; barrier filter LP590).

RESULTS

Biolistic transfection of wound epidermis in situ

The biolistic particle delivery system (PDS) has recently been successful for the transfection of cultured newt blastemal cells (Schilthuis et al., 1993). These cells have proved difficult to transfect by a variety of other procedures. Biolistics employs high pressure helium to propel DNA-coated microprojectiles into target cells (reviewed by Pecorino and Lo, 1992). We have made minor modifications to the PDS-1000/He apparatus (Bio-Rad) which allow transfection of regenerating limbs of live newts (Fig. 1). These revisions were modelled on the hand-held device (Sanford et al., 1991; Williams et al., 1991) which is currently not commercially available. The two components, an additional spacer and a limb collar containing a 5 mm diameter opening, allow the limb to be positioned at a small distance from the stopping screen, and shield the rest of the animal from the path of microprojectiles (Fig. 1). After biolistic transfection, gold microprojectiles can be seen in the wound epidermis.

Upon transfection of plasmids encoding β-galactosidase or human alkaline phosphatase, expression of marker enzyme in the wound epidermis can be detected by several methods, three of which are shown in Fig. 2. When transfected skin or wound epidermis was analyzed by X-gal staining in whole mounts, approximately 10% of cells in targeted areas strongly expressed β-galactosidase (Fig. 2A). Detection of alkaline phosphatase activity in tissue sections by histochemical staining showed that positive cells were most abundant in the superficial layer of epidermis (Fig. 2B), although some were also observed in the basal layer (not shown). No positive cells were detected in the mesenchyme under the present conditions of transfection. Alkaline phosphatase was also detected by indirect immunofluorescence with a primary rabbit antibody (Fig. 2C) which gave clearer resolution of transfected cells.

Retinoid inducibility of WE3 in transfected epidermis

It was necessary to confirm the RA responsiveness of the WE3 antigen in transfected limbs. Hindlimbs at 3 days postamputation were bombarded in situ with a plasmid encoding alkaline phosphatase (pSDAP) to mark transfected cells. Animals were

maintained either in water, or supplemented with retinol palmitate (300 mg/liter) for 6 days before harvesting. The pattern of reactivity of a monoclonal antibody against antigen WE3 (mAb WE3; Tassava et al., 1986) was similar to that described by Tassava (1992). Sections of limbs maintained in water showed a few reactive cells in the basal layers of the wound epidermis. Gland cells and occasional characteristic round cells of the skin epidermis were also WE3 positive (Fig. 3A). Retinol palmitate (300 mg/liter) markedly enhanced the temporal appearance of the WE3 antigen throughout the wound epidermis and also caused normal skin epidermis to become reactive (Fig. 3B). Strong cytoskeleton-associated reactivity was observed in many cells. Comparable results were obtained with RA (10^{-7} M) in place of retinol palmitate in agreement with Tassava (1992). In view of subsequent experiments, it was important to determine if transfected cells remained responsive to retinoids. Sections of retinol palmitate-treated limbs were analyzed by double labelling for WE3 and alkaline phosphatase reactivity (Fig. 3C). A subset of WE3-positive cells
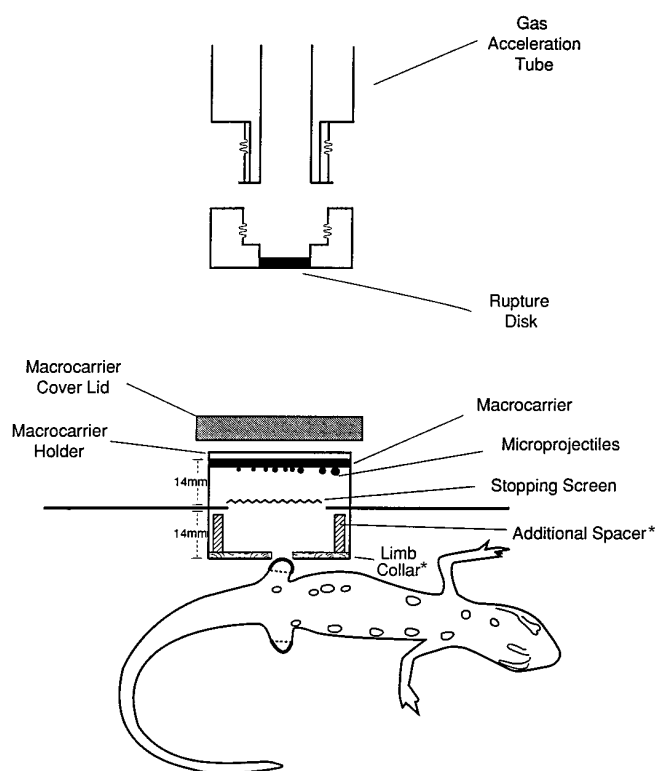

Fig. 1. Biolistic transfection of the regenerating newt limb. Schematic representation of the method for transfecting a regenerating limb in situ using biolistics. Components of the Biolistics Particle Delivery System (PDS 1000; Bio-Rad) are labelled, along with minor additions marked by asterisks which define a 14 mm target distance. The rupture disk breaks under pressure, releasing a helium shock wave that propels the macrocarrier disc and plasmid-coated microprojectiles. The disc is arrested by a stopping screen and the microprojectiles are launched towards the target. An anesthetized newt is supported against the limb collar in a padded sling affixed to the bottom of the launch assembly, thus allowing the regenerating limb to be positioned as depicted.

were also clearly positive for alkaline phosphatase demonstrating that WE3 remains inducible in transfected wound epidermis. We next tested the activity of the chimaeric receptors in this context.

Chimaeric receptors activate transcription in vivo

We investigated the ability of $\chi\alpha1$ and $\chi\delta1$ to activate a synthetic reporter gene in situ. As shown in Fig. 4, both amputated hindlimbs were cotransfected with the RARE-tk-CAT reporter, containing an RA responsive element upstream of the thymidine kinase promoter directing expression of the chloramphenicol acetlytransferase (CAT) gene, and with a luciferase reporter (pCMV-LUC) that served to normalize the level of CAT and to correct for differences in transfection efficiency. The right side also received a plasmid expressing a chimaeric receptor (either $\chi\alpha1$ or $\chi\delta1$), while the left side received vector sequences as a control. The animals were maintained either in water or with 10^{-7} M T3 to activate the chimaeric receptors.

The results are expressed as the ratio of normalized CAT activity of the right limb to that of the left limb. As can be seen in Fig. 5A, $\chi\delta1$ showed T3-dependent stimulation of reporter activity in situ. Comparable activation was shown by $\chi\alpha1$ (Fig. 5B). In order to compare directly activation by $\chi\alpha1$ and $\chi\delta1$ in the presence of T3, the chimaeric receptors were transfected into opposite hindlimbs of the same animal. T3-dependent activation of the reporter by $\chi\alpha1$ and $\chi\delta1$ was essentially the same (ratio: 1.3 ± 0.1 [mean $\pm$ standard error]; Fig. 5B) for a group of five animals. Note that activation of the reporter in response to T3 by $\chi\alpha1$ (3.14 ± 0.4) and by $\chi\delta1$ (4.3 ± 0.5) was similar to activation in response to RA by the endogenous complement of RARs (3.6 ± 0.7) (Fig. 5C). In view of this evidence for T3-dependent activation of a synthetic reporter, we investigated if the chimaeric receptors could induce expression of the WE3 antigen in the wound epidermis.

$\chi\delta1$, but not $\chi\alpha1$, mediates T3-dependent induction of WE3

In order to investigate the ability of the chimaeric receptors to induce WE3, right hindlimbs were transfected at 3 days postamputation with a chimaeric receptor expression plasmid that also encoded alkaline phosphatase as a marker, whereas the left hindlimb received a control plasmid lacking the receptor sequences but expressing alkaline phosphatase. Animals were maintained for 4 days either in water or T3, prior to harvesting. Newt hindlimbs were sectioned and analyzed by double-labelled immunofluorescence as before. Right or left hindlimbs that were maintained in water displayed single staining specificities. Cells that were positive for alkaline

phosphatase were negative for WE3 and vice versa (Fig. 6A). Note that at this time (7 days postamputation) a low level of reactivity to WE3 is detectable only in a few of the cells of the epidermal cap and at first it appears granular and diffuse (Tassava et al., 1986). In contrast, more than 50% of
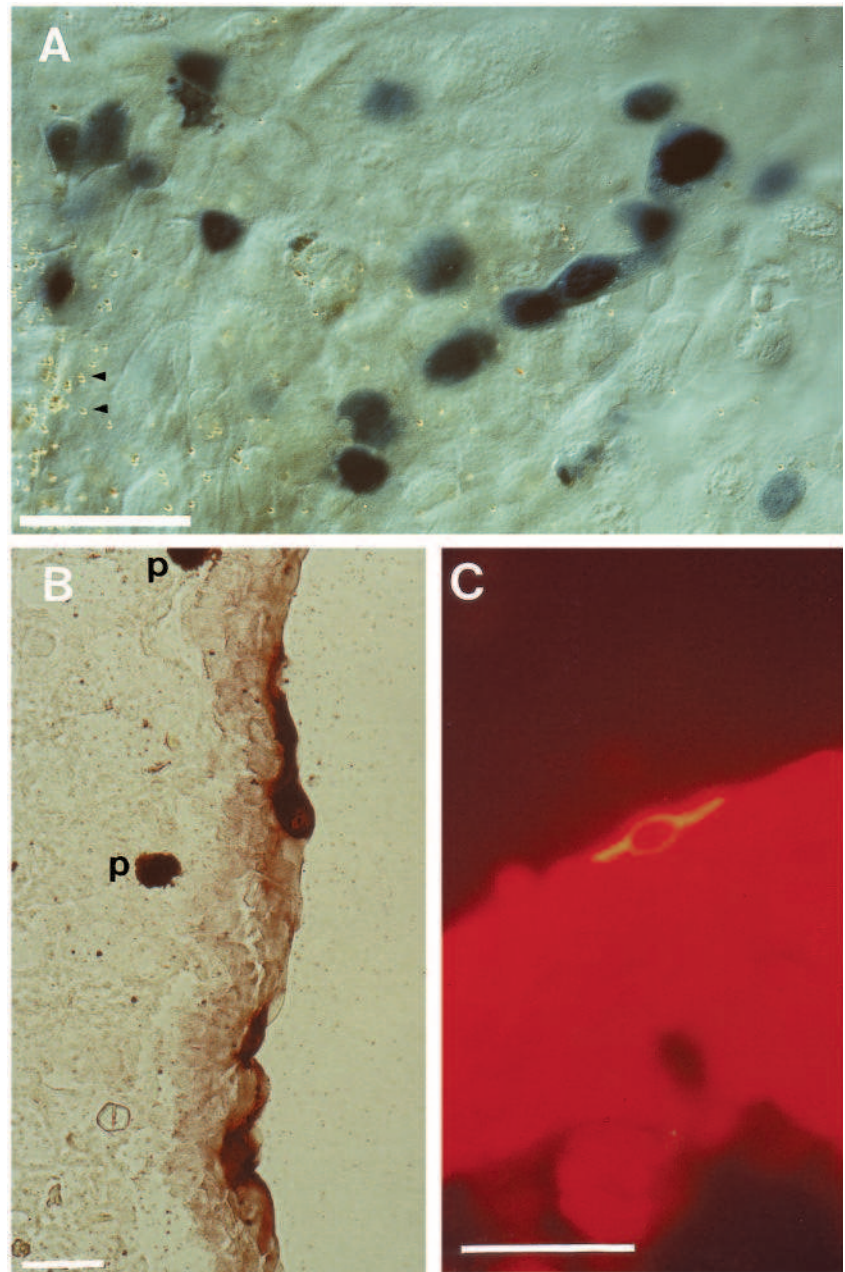

Fig. 2. Detection of plasmid expression in limbs transfected by biolistics. (A) Whole-mount X-gal staining of skin one day after transfection with a β-galactosidase marker construct (N-CMV-LacF). Expression is observed in approximately 10% of cells. Arrows mark gold, DNA-coated microprojectiles. (B) A representative longitudinal section (30 μm) of a limb transfected with an alkaline phosphatase marker construct (pSDAP) and stained by BCIP. Note that the uniform appearance of pigment granules (p) in the mesenchyme can be distinguished from the cytoplasmic staining of BCIP-positive cells in the epidermis. (C) A longitudinal section in which human alkaline phosphatase was detected by indirect immunofluorescence using a rhodamine-conjugated secondary antibody to rabbit immunoglobulins. Scale bars = 50 μm.
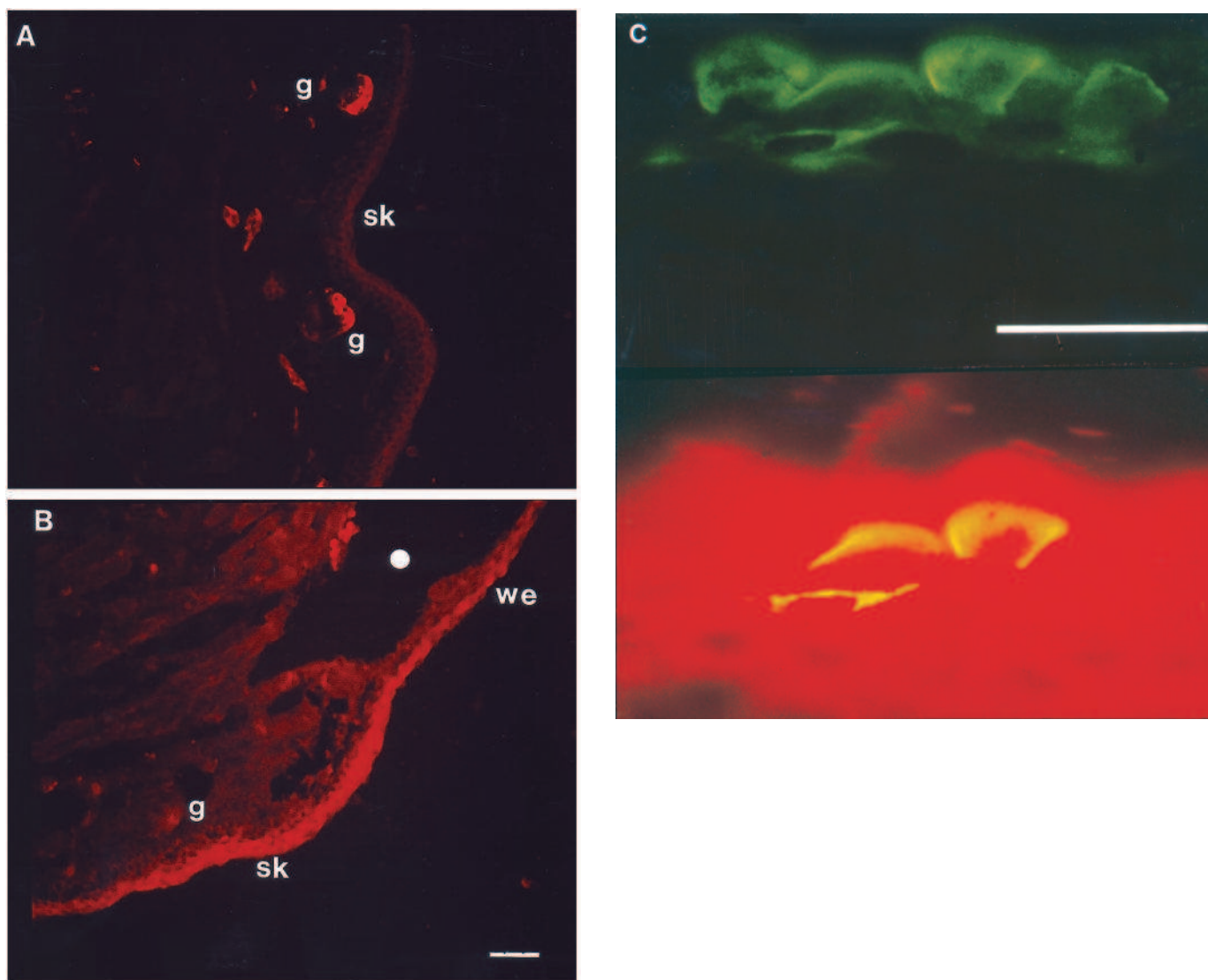
Fig. 3. mAb WE3 reactivity is induced by RA in transfected limbs. Fluorescence micrographs of longitudinal sections of limbs transfected with an alkaline phosphatase marker plasmid (pSDAP; 3 days postamputation) and harvested 6 days after transfection. (A) Representative section from a limb of a newt maintained in water. The skin (sk) is not reactive to mAb WE3. Glands (g) are strongly positive. (B) Section from a limb of a newt treated for 6 days with retinol palmitate (at 300 mg/liter). Both the wound epidermis (we) and skin (sk) are strongly positive. The white dot denotes an artifactual space between the wound epidermis and underlying structures caused by the loss of loosely adhering blastemal cells after sectioning. Scale bar = 100 μm. (C) A section of a limb treated with retinol palmitate for 6 days and analyzed by indirect immunofluorescence using double-labelling for alkaline phosphatase (rhodamine, bottom) and WE3 (fluorescein, top). Note that three transfected cells are also expressing WE3. Scale bar = 50 μm.

rhodamine-labelled alkaline phosphatase-positive cells in T3-treated animals transfected with χδ1 were also clearly fluorescein-labelled WE3 positive (Fig. 6B). The secondary antibody conjugates were reversed in several experiments in order to confirm the specificity of the double-labelling procedure and the results obtained were identical (Fig. 6D). The observation that χδ1 enhanced WE3 reactivity in response to T3 was also made by staining consecutive sections alternatively with antibody to alkaline phosphatase or mAb WE3, but double staining was preferred for unambiguous cellular identification. The staining of contralateral (left) limbs of T3-treated newts that received the control plasmid was indistinguishable from limbs maintained in water, in that double labelling was not

observed (Fig. 6C). In contrast to χδ1, when the χα1 receptor was analyzed less than 5% of transfected cells from T3-treated limbs were observed to express any detectable WE3 reactivity (Fig. 6E). These results indicate that RAR isoforms δ1 and α1 are functionally distinct in respect of this property.

DISCUSSION

Biolistic transfection
In this report, biolistic transfection has proved a powerful approach for gene transfer during limb regeneration in the adult urodele, a possibility suggested by earlier work on transfection
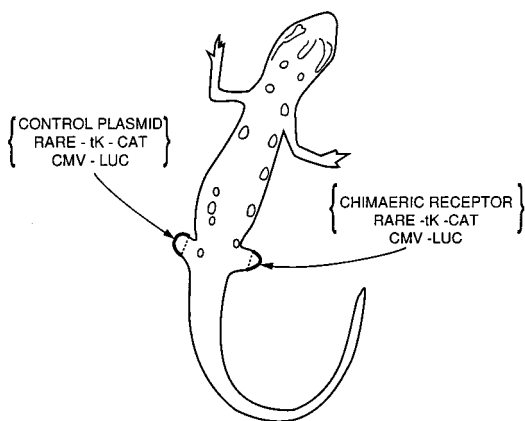
Fig. 4. Experimental design for measuring transcriptional activation by the chimaeric receptors. A reporter construct RARE-tk-CAT was cotransfected into the wound epidermis (thick line) with a normalizing plasmid, pCMV-LUC-ori, and either a plasmid encoding a χ receptor (right limb) or a control plasmid containing vector sequences alone (pUC 18; left limb) two weeks postamputation. Animals were maintained in either water alone or 10^{-7} M T3, and limbs were harvested the following day. CAT activity was normalized with respect to luciferase activity and the ratio of normalized CAT activity of the right limb to the left limb was calculated for each animal. The ratios for animals treated with T3 are expressed relative to those for animals maintained in water in parallel experiments.

of skin and liver in adult mice (Williams et al., 1991). Approximately 10% of cells in bombarded surface areas expressed transfected marker genes 24 hours after transfection, a value comparable to that obtained for the mouse tissues. Note that the level of expression decreased several days after transfection due to the loss of the cells in the superficial layer of the wound epidermis. The large urodele cells expressed levels of protein that were readily detectable either by single cell immunochemical or histochemical staining, or by enzyme assay of tissue extracts. It was particularly valuable to transfect cells in contralateral limbs with different plasmids since this allowed comparisons to be made within an individual animal, a strategy that has proved helpful in other studies on limb regeneration (Brockes, 1992).

Activities of chimaeric receptors

The distinct spatiotemporal expression patterns of RAR isoforms (Dolle et al., 1989, 1990; Ruberte et al., 1990, 1991), the synergism between RAR domains and the differential activation of various RA-responsive promoters (Leid et al., 1992; Nagpal et al., 1992, 1993) has led to the hypothesis that each RAR isoform may control particular effects of RA. The regenerating newt limb expresses at least six different RAR isoforms (Ragsdale et al., 1989, 1992a,b; Hill et al., 1993). The power of this approach using chimaeric receptors is that after transfection it is possible to activate a single RAR isoform with T3. In earlier studies on cultured newt blastemal cells, activation of $\chi\alpha1$, but not $\chi\delta1$, reproduced the effect of growth inhibition by RA (Schilthuis et al., 1993). Several distinguishing features of RAR$\delta1$ characterize it as a strong candidate for mediating the special effects of RA on limb regeneration. First,
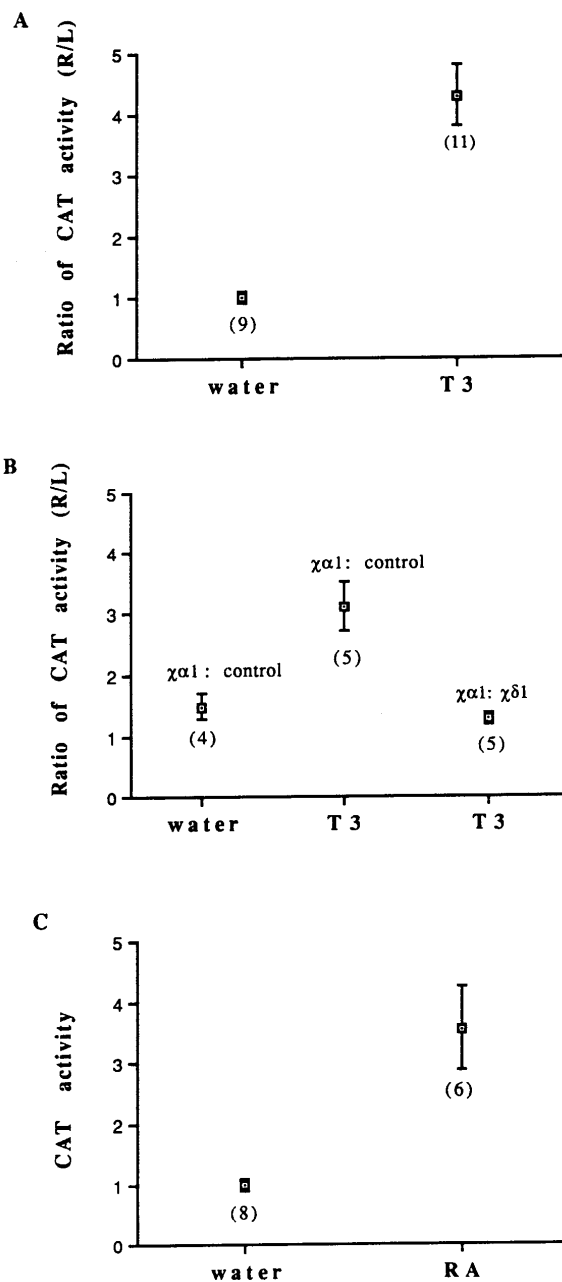

Fig. 5. Transcriptional activation of a synthetic reporter by $\chi\delta1$, $\chi\alpha1$, and endogenous RARs is quantitatively comparable. (A,B) Graphs showing the ratio of normalized CAT activity of the right limb to the left limb in animals maintained in either water or T3 (10^{-7} M) for 24 hours. In addition to RARE-tk-CAT and pCMV-LUC-ori, the right limbs were transfected with $\chi\delta1$ (graph A) or $\chi\alpha1$ (graph B) and the left limbs with control plasmid (pUC 18) as illustrated in Fig. 4. Limbs were transfected with 1.3 μg of each plasmid. Graph B also shows a direct comparison of $\chi\alpha1$ to $\chi\delta1$ (See text). (C) Graph showing normalized reporter CAT activity for treatment with either water or RA (10^{-7} M). Limbs were cotransfected with 2 μg of the RARE-tk-CAT reporter plasmid and 2 μg of the CMV-LUC normalization plasmid only. No χ plasmid was included and hence the endogenous RARs were responsible for activation of the synthetic reporter gene. (A-C) The number of newts in each group is shown in parentheses below the mean ± standard error.
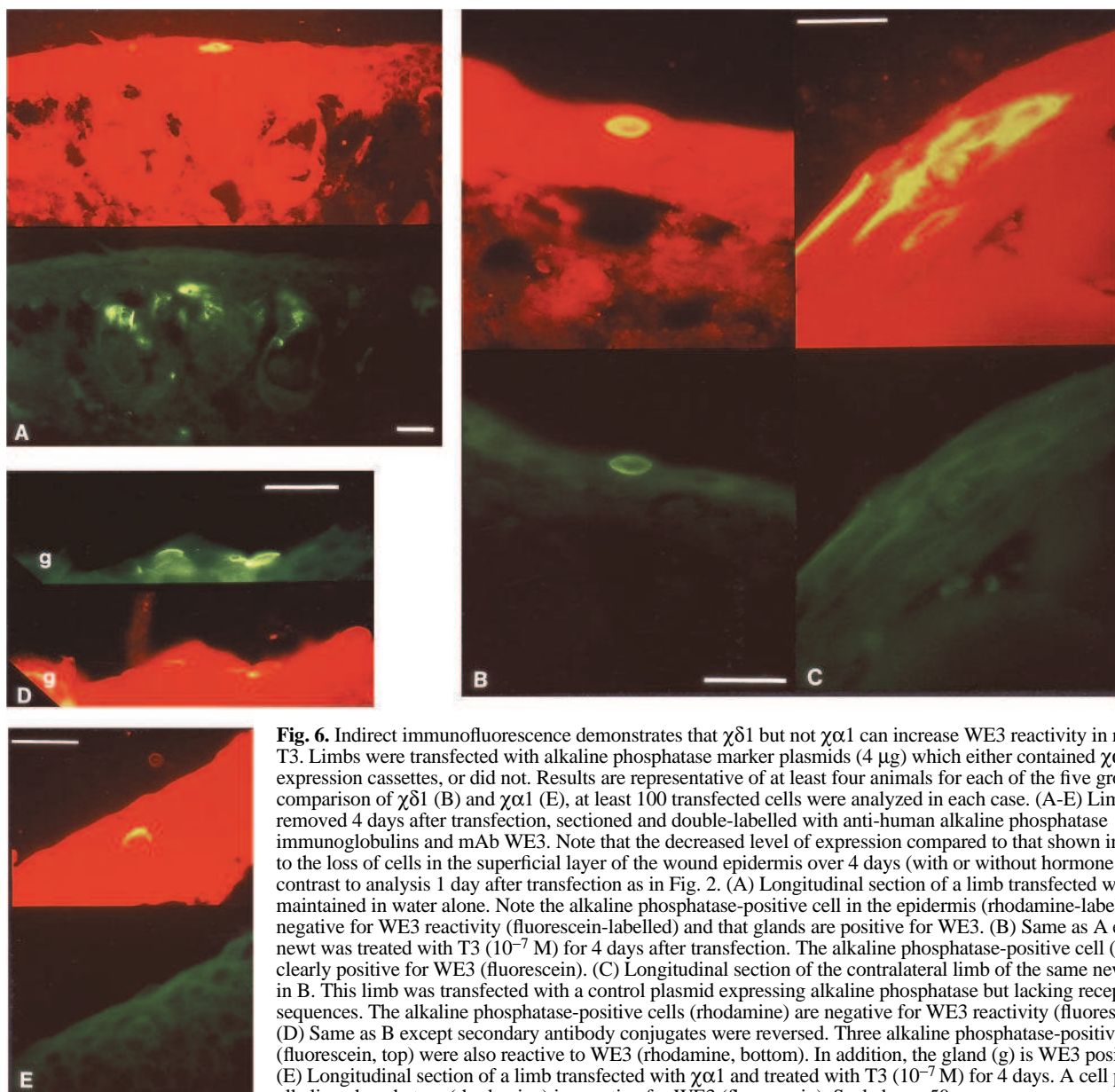
Fig. 6. Indirect immunofluorescence demonstrates that χδ1 but not χα1 can increase WE3 reactivity in response to T3. Limbs were transfected with alkaline phosphatase marker plasmids (4 μg) which either contained χα1 or χδ1 expression cassettes, or did not. Results are representative of at least four animals for each of the five groups. For the comparison of χδ1 (B) and χα1 (E), at least 100 transfected cells were analyzed in each case. (A-E) Limbs were removed 4 days after transfection, sectioned and double-labelled with anti-human alkaline phosphatase immunoglobulins and mAb WE3. Note that the decreased level of expression compared to that shown in Fig. 2 is due to the loss of cells in the superficial layer of the wound epidermis over 4 days (with or without hormone treatment) in contrast to analysis 1 day after transfection as in Fig. 2. (A) Longitudinal section of a limb transfected with χδ1 and maintained in water alone. Note the alkaline phosphatase-positive cell in the epidermis (rhodamine-labelled) is negative for WE3 reactivity (fluorescein-labelled) and that glands are positive for WE3. (B) Same as A except that the newt was treated with T3 (10^{-7} M) for 4 days after transfection. The alkaline phosphatase-positive cell (rhodamine) is clearly positive for WE3 (fluorescein). (C) Longitudinal section of the contralateral limb of the same newt represented in B. This limb was transfected with a control plasmid expressing alkaline phosphatase but lacking receptor sequences. The alkaline phosphatase-positive cells (rhodamine) are negative for WE3 reactivity (fluorescein). (D) Same as B except secondary antibody conjugates were reversed. Three alkaline phosphatase-positive cells (fluorescein, top) were also reactive to WE3 (rhodamine, bottom). In addition, the gland (g) is WE3 positive. (E) Longitudinal section of a limb transfected with χα1 and treated with T3 (10^{-7} M) for 4 days. A cell positive for alkaline phosphatase (rhodamine) is negative for WE3 (fluorescein). Scale bar = 50 μm.

the N-terminal sequence departs markedly from all known vertebrate RARs and is predicted to contain a transcriptional activation domain which is unique to known RARs (Ragsdale et al., 1992b). Not only has expression of δ1 been detected in the wound epidermis and blastemal mesenchyme both by RNase protection assays and by staining sections with specific antibodies, but it is selectively high in normal and regenerating limbs and tails compared to other tissues (Ragsdale et al., 1992b; Hill et al., 1993).

In the present study, the two chimaeric receptors were equally effective at T3-dependent activation of a synthetic RA reporter CAT gene after transfection into the wound epidermis, and furthermore were able to activate such a reporter as effectively as the endogenous complement of RARs in the presence of RA. Nonetheless, expression of the retinoid responsive WE3 antigen was activated by χδ1, but not χα1, in the presence of

T3. In comparing activity of the two chimaeric receptors, we acknowledge that the transactivation assay for CAT activity is somewhat different to that for expression of the endogenous target WE3. In addition, a common reservation concerning the use of chimaeric receptors is interference with function. In conjunction with the earlier study (Schilthuis et al., 1993), we find that each chimaeric receptor (RARδ1 and RARα1) mediates a specific response to RA, thus making this possibility rather unlikely. Recently, it has been demonstrated that null mutant mice for RARγ are completely resistant to several specific RA-induced malformations (i.e. lumbosacral truncations), whereas RARα1 null mutant mice are not (Lohnes et al., 1993). These observations, along with those of the present study, suggest that RARδ, and its closest relative RARγ, are important for mediating certain effects of exogenously added RA. Interestingly, most effects on normal development in RARγ null

mutants are shown only by a small proportion of homozygotes and, in particular, although RARγ is expressed in the limb bud during morphogenesis, limb malformations are not observed (Lohnes et al., 1993). The authors attribute these features to functional redundancy between RAR isoforms. It should be noted that other urodele RAR isoforms are expressed in the limb and it will be important to investigate their properties using the present approach in order to assess the possibility of redundancy in this system.

The WE3 antigen

WE3 is expressed by many cell types with a secretory or transport phenotype (Goldhamer et al., 1989). In view of the fact that retinoids are essential for many of the cell types reactive to mAb WE3, and that WE3 is responsive to RA, it has been suggested that its appearance in the wound epidermis and other cell types identifies cells that are responsive to RA, and indeed that endogenous RA may control the appearance of WE3. The latter point is one that requires more direct evidence, but it is worthwhile to point out that a secretory role for the wound epidermis has been discussed (Singer and Salpeter, 1961), and that exogenous RA treatment enhances WE3 reactivity as well as causing mucous metaplasia. It would be interesting to compare the distribution of WE3 in normal regeneration with that of RARδ1, and to look at other cell types that express the WE3 antigen to determine which RAR isoforms might mediate expression of WE3 in non-regenerative tissues.

The present approach to understanding RAR function in the wound epidermis opens up the possibility of applying it to the cells of the mesenchymal blastema whose positional identity can be respecified by RA treatment. Studies of an extended list of markers specific to the newt blastema and wound epidermis will help to elucidate how the specialized urodele tissues differ from limb tissue in other vertebrates that cannot regenerate their limbs.

REFERENCES

Brockes, J. P. (1989). Retinoids, homeobox genes, and limb morphogenesis. *Neuron* **2**, 1285-1294.

Brockes, J. P. (1992). Introduction of a retinoid reporter gene into the urodele limb blastema. *Proc. Natl. Acad. Sci. USA* **89**, 11386-11390.

Bryant, S. V. and Gardiner, D. M. (1992). Retinoic acid, local cell-cell interactions and pattern formation in vertebrate limbs. *Dev. Biol.* **152**, 1-25.

Castilla, M. and Tassava, R. A. (1992). Extraction of the WE3 antigen and comparison of reactivities of mAbs WE3 and WE4 in Adult newt regenerate epithelium and body tissues. In *Monographs in Developmental Biology* (ed. H. W. Sauer), pp. 116-130. Basel: Karger.

Darmon, M. (1991). Retinoic acid in skin and epithelia. *Seminars in Dev. Biol.* **2**, 219-228.

Dolle, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J. and Chambon, P. (1989). Differential expression of genes encoding α, β and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature* **342**, 702-705.

Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G. and Chambon, P. (1990). Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* **110**, 1133-1151.

Fell, H. B. and Mellanby, E. (1953). Metaplasia produced in cultures of chick ectoderm by high vitamin A. *J. Physiol.* **119**, 470-488.

Goldhamer, D. J., Tomlinson, B. L. and Tassava, R. A. (1989). A Developmentally Regulated Wound Epithelial Antigen of the Newt Limb Regenerate is Also Present in a Variety of Secretory/Transport Cell Types. *Dev. Biol.* **135**, 392-404.

Hill, D. S., Ragsdale, C. W. and Brockes, J. P. (1993). Isoform-specific immunological detection of newt retinoic acid receptor δ1 in normal and regenerating limbs. *Development* **117**, 937-945.

Kintner, C. R. and Brockes, J. P. (1985). Monoclonal antibodies to the cells of a regenerating limb. *J. Embryol. Exp. Morph.* **89**, 37-55.

Leid, M., Kastner, P. and Chambon, P. (1992). Multiplicity generates diversity in the retinoic acid signalling pathways. *TIBS* **17**, 427-433.

Li, E., Sucov, H. M., Lee, K.-F., Evans, R. M. and Jaenisch, R. (1993). Normal development and growth of mice carrying a targeted disruption of the α1 retinoic acid receptor gene. *Proc Natl. Acad. Sci. USA* **90**, 1590-1594.

Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMur, M. and Chambon, P. (1993). Function of retinoic acid receptor γ in the mouse. *Cell* **73**, 643-658.

Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M., LeMeur, M. and Chambon, P. (1993). High postnatal lethality and testis degeneration in retinoic acid receptor α mutant mice. *Proc. Natl. Acad. Sci. USA* **90**, 7225-7229.

Maden, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature* **295**, 672-675.

Maden, M. (1983). The effect of vitamin A on the regenerating axolotl limb. *J. Embryol. Exp. Morph.* **77**, 273-295.

Morriss-Kay, G. (1992). *Retinoids in Normal Development and Teratogenesis.* pp. 137-212. Oxford: Oxford University.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H. and Chambon, P. (1992). Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell* **70**, 1007-1020.

Nagpal, S., Friant, S., Nakshatri, H. and Chambon, P. (1993). RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization *in vivo*. *EMBO J.* **12**, 2349-2360.

Pecorino, L. T. and Lo, D. C. (1992). Having a blast with gene transfer. *Current Biol.* **2**, 30-32.

Ragsdale, C. W., Petkovich, M., Gates, P. B., Chambon, P. and Brockes, J. P. (1989). Identification of a novel retinoic acid receptor in regenerative tissues of the newt. *Nature* **341**, 654-657.

Ragsdale, C. W., Gates, P. B. and Brockes, J. P. (1992a). A second isoform of the alpha retinoic acid receptor identified in the newt. *Nucl. Acids Res.* **20**, 5851.

Ragsdale, C. W., Gates, P. B., Hill, D. S. and Brockes, J. P. (1992b). Delta retinoic acid receptor isoform δ1 is distinguished by its N-terminal sequence and abundance in the limb regeneration blastema. *Mech. Dev.* **40**, 99-112.

Ruberte, E., Dolle, P., Krust, A., Zelent, A., Morriss-Kay, G. and Chambon, P. (1990). Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development* **108**, 213-222.

Ruberte, E., Dolle, P., Chambon, P. and Morriss-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111**, 45-60.

Sanford, J. C., DeVit, M. J., Russell, J. A., Smith, F. D., Harpending, P. R., Roy, M. K. and Johnston, S. A. (1991). An Improved helium-driven biolistic device. *Technique* **3**, 3-16.

Scadding, S. R. (1989). Histological effects of vitamin A on limb regeneration in the larval axolotl, *Ambystoma mexicanum. Can. J. Zool.* **68**, 159-167.

Schilthuis, J. G., Gann, A. A. F. and Brockes, J. P. (1993). Chimaeric retinoic acid/thyroid hormone receptors implicate RAR-α1 as mediating growth inhibition by retinoic acid. *EMBO J.* **12**, 3459-3466.

Seed, B. and Sheen, J. Y. (1988). A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene* **67**, 271-277.

Singer, M. and Salpeter, M. M. (1961). Regeneration in vertebrates: The role of the wound epithelium. In *Growth in Living Systems*, pp. 277-311. New York: Basic Books.

Smith, W. C., Nakshatri, H., Leroy, P., Rees, J. and Chambon, P. (1991). A retinoic acid response element is present in the mouse cellular retinol binding protein I (mCRBPI) promoter. *EMBO J.* **10,** 2223-2230.

Stocum, D. L. (1984). The urodele limb regeneration blastema. Determination and organization of the morphogenetic field. *Differentiation* **27,** 13-28.

Stocum, D. L. and Crawford, K. (1987). Use of retinoids to analyse the cellular basis of positional memory in regenerating amphibian limbs. *Biochem. Cell Biol.* **65,** 750-761.

Stocum, D. L. (1991). Limb regeneration: a call to arms (and legs). *Cell* **67,** 5-8.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell* **66,** 199-217.

Tang, D., DeVit, M. and Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* **356,** 152-154.

Tassava, R. A., Johnson-Wint, B. and Gross, J. (1986). Regenerate epithelium and skin glands of the adult newt react to the same monoclonal antibody. *J. Exp. Zool.* **239,** 229-240.

Tassava, R. A. (1992). Retinoic acid enhances monoclonal antibody WE3 reactivity in the regenerate epithelium of the adult newt. *J. Morph.* **213,** 159-169.

Wallace, H. (1981). *Vertebrate Limb Regeneration.* Chichester: J. Wiley.

Williams, R. S., Johnston, S. A., Riedy, M., DeVit, M. J., McElligott, S. G. and Sanford, J. C. (1991). Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci. USA* **88,** 2726-2730.