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Isoform-specific immunological detection of newt retinoic acid receptor δ1 in normal and regenerating limbs

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SUMMARY

Retinoic acid (RA) exerts a variety of effects on the regenerating urodele limb including positional respecification of the blastema. The major RA receptor expressed in the newt limb and blastema is the δ1 isoform and, in order to detect δ1 in this context, we have made five affinity-purified antibodies against fusion proteins and peptides from non-overlapping regions of the molecule. These antibodies have been evaluated by reaction with transfected COS-7 cells, newt limb cells in culture and newt limb tissue sections. The most informative antibodies were RP6, directed against N-terminal region A sequence, and RP8, directed against C-terminal sequence. In western blots of blastemal extracts, δ1 protein was detected as two major bands of immunoreactivity at positions consistent with the employment of two candidate methionine initiators identified by cDNA sequencing. Staining of adult limb sections with RP6 and RP8 showed reactivity in half of the nuclei in epidermal and mesenchymal tissues, a heterogeneity that was observed with adjacent nuclei in muscle fibres. In the regenerating limb, nuclei in the blastemal mesenchyme and wound epidermis were strongly reactive, although no axial variation in expression was detected.

Key words: retinoic acid, retinoic acid receptors, limb regeneration, newts

INTRODUCTION

Limb regeneration in urodele amphibians such as the newt and axolotl is a key system for evaluating the effects of retinoic acid (RA) on pattern formation (Brockes, 1989; Stocum, 1991; Bryant and Gardiner, 1992). Regeneration proceeds by local formation of a blastema, a mound of undifferentiated mesenchymal progenitor cells enclosed by a jacket of epidermis. A significant effect of RA on regeneration is to respecify the positional identity of the limb blastema (Niazi and Saxena, 1978; Maden, 1982). Blastemal cells arising at different positions on the proximodistal axis (running from shoulder to fingertips) normally regenerate only those structures distal to their level of origin (Stocum, 1984). Exposure to an appropriate concentration of RA elicits regeneration of additional, more proximal structures, thus producing a partially duplicated limb. In addition to positional respecification, the regenerating limb shows several effects of RA that are generally characteristic of tissue responses observed in other vertebrates. For example, cell division in the blastemal mesenchyme is transiently inhibited, and the wound epidermis around the blastema shows the enhanced mucopolysaccharide synthesis that is observed in mucous metaplasia (Maden, 1983). Cartilage elements, a recognised target of retinoids in development, show fissures in the vicinity of the amputation plane, as well as a loss of metachromatic staining intensity (Maden, 1983). In addition, there is an enhanced generation of mononucleate blastemal cells from the tissues of the mature limb by the process of dedifferentiation (Kim and Stocum, 1986; Stocum and Crawford, 1987; Scadding, 1989). It is a challenge to understand the basis of these diverse effects, and an important starting point is to relate them to the heterogeneity in retinoic acid receptors (RARs) (Ragsdale and Brockes, 1991).

RARs are ligand-dependent transcription factors of the steroid/thyroid hormone superfamily (Petkovich et al., 1987; Giguere et al., 1987). In mouse and man, three major forms of RAR, termed , and , have been identified (Benbrook et al., 1988; Brand et al., 1988; Zelent et al., 1989). Each form has at least two variants or isoforms as a consequence of alternative promoter usage and differential splicing at the N terminus (Kastner et al., 1990; Leroy et al., 1991; Zelent et al., 1991). There have been extensive studies of the expression and distribution of the three major forms, particularly using in situ hybridisation to sections of the mouse embryo, although the probes used in these experiments were not isoform specific (Dolle et al., 1989, 1990; Noji et al., 1989; Osumi-Yamashita et al., 1990; Ruberte et al., 1990, 1991a,b). These studies demonstrate that the receptor is ubiquitously distributed, whereas and show characteristic spatial and temporal regulations that suggest these forms have distinct functions in development. In the urodele, studies on cDNA clones from the newt limb blastema have identified two isoforms of RAR that have significant homology with mouse and

human counterparts, a RAR based on a partial cDNA clone and a third form referred to as (Ragsdale et al., 1989, 1992a,b; Giguere et al., 1989). RAR is related to mouse and human RAR but is distinct in the sequence of its N- and C-terminal regions. There are two isoforms of which 1 is the most highly expressed of all RARs in the limb and limb blastema, as analysed by northern blotting and RNase protection, and is expressed at low levels in a variety of other tissues (Ragsdale et al., 1992b). Furthermore the sequence of the N-terminal A region of 1, as determined from cDNA clones, is distinct from that of other vertebrate RARs and includes two potential methionine initiators, identified as A and B, as well as several potential CUG initiators located upstream and in frame (summarised in Fig. 1). By its pattern of distribution and exceptional sequence, the 1 isoform appears to be a strong candidate to mediate some of the more exceptional effects of RA on limb regeneration, including that of positional respecification.

To identify those forms of the 1 protein expressed in the limb and its blastema, as well as to localise receptor expression in tissue sections, we have prepared a panel of antibodies to different regions of the molecule. Particularly in comparison with studies employing in situ hybridisation, there has been little work with antibodies to the RARs. Two studies have employed panels of antibodies directed at different regions of the mouse 1 receptor (Rochette-Egly et al., 1991), and the 1 receptor (Gaub et al., 1992). These reagents have been valuable in allowing western analysis of the forms expressed in mouse embryos and F9 embryonic carcinoma cells, and in providing evidence that 1 and 1 can be phosphorylated. To date there have been no reports of using these or other antibodies to localise RARs in tissue sections, although cells expressing transfected RAR expression constructs have been stained in culture and show nuclear reactivity (Gaub et al., 1989). The antibodies to the newt 1 receptor have enabled us both to identify different forms of the protein, and to localise expression in sections of normal and regenerating limbs.

MATERIALS AND METHODS

Animals

Newts (*Notophthalmus viridescens*) were obtained from Blades Biologicals, Edenbridge, Kent. Procedures for animal care, anaesthesia, limb amputation surgery, regeneration blastema staging and RA treatment have been described (Iten and Bryant, 1973; Kintner and Brockes, 1985; Savard et al., 1988). Tissues designated for protein extraction were stored after collection in liquid nitrogen.

Cell culture and transfection

Simian COS-7 cells were grown in 10 cm dishes in DMEM with 10% foetal calf serum and transfected by the calcium phosphate method using standard procedures. Newt limb cells, derived from explanted limb mesenchyme (TH4B) or limb blastema (B1H1), were grown on collagen coated plastic Petri dishes in medium composed of 60% Minimum Essential Medium with Earle's salts (Flow), 27% distilled water, 10% foetal calf serum, supplemented with insulin, glutamine and antibiotics as described previously (Ferretti and Brockes, 1988). Cells were maintained at 25°C in a humidified atmosphere of 2% CO_2.

Preparation of affinity-purified antibodies to fusion proteins and peptides

Fusion protein expression vectors were constructed by ligating restriction fragments of RAR cDNA into the appropriate site of a pATH *trpE* vector (Yansura, 1990; Yansura and Henner, 1990), and confirmed by DNA sequencing. For fusion RP6, an *Eco*RI-*Hin*dIII fragment of clone NR0 (Ragsdale et al., 1989) was inserted into pATH 1; it encodes 55 amino acids of 1 A region. RP16 was constructed with an *Eco*RI-*Pst*I fragment that encodes 142 amino acids from region E of RAR . RP8 was constructed with a *Pst*I-*Hin*dIII fragment that incorporates the entire RAR sequence C-terminal to that of RP16, that is, 69 amino acids from region E and 31 amino acids from region F. The fusion protein constructs were introduced into *E. coli* BB4 by transformation and cultures were induced with indole acrylic acid according to standard procedures (Yansura, 1990; Yansura and Henner, 1990). After preparation of inclusion bodies, the fusion proteins were purified by preparative SDS-polyacrylamide gel electrophoresis. For immunisation, gel slices were pulverised in a liquid nitrogen cooled mortar and then emulsified with Freund's adjuvant. Rabbits were immunised with 0.5-1 mg of purified fusion protein on at least three occasions. Sera were analysed by a standard ELISA procedure employing antigen-coated microwells.

For construction of affinity columns, the fusion proteins were electroeluted from the gel slices, and 1-5 mg of purified protein was coupled to Affigel beads according to the maker's instructions (Biorad). Rabbit antisera were fractionated with $(NH_4)_2SO_4$ and specific antibodies purified by adsorption to affinity columns followed by elution with 0.2 M glycine buffer (pH 2.2). Affinity purification was monitored with the ELISA procedure and peak fractions were pooled, concentrated and stored in aliquots at −70°C.

Peptides DH2 and DH1 cover sixty percent of the sequence between 1a and 1b initiation sites (Fig. 1). The peptides were synthesized with the F-MOC method and their amino acid sequences confirmed by amino acid analysis (DH2: MKFSDTASCRDGGE; DH1: RPEEEGKGAGGRSKLR). Purified peptides were conjugated to keyhole limpet haemocyanin (KLH) according to published methods (Gullick, 1988), and 7-8 mg of KLH-coupled peptide was emulsified with adjuvant and injected into each rabbit. Peptides were coupled directly to Affigel for affinity purification. Affinity-purified DH1 and DH2 antibodies were combined at appropriate dilutions and used as a mixture.

Preparation of extracts and western blotting

Newt limb blastemas, typically 70-100 per preparation, were extracted with ten strokes in a Dounce homogeniser with 0.4 ml hypotonic lysis buffer (0.02 M Tris-HCl pH 8.0, 0.02 M KCl, 1 mM EDTA, 1 mM PMSF, 1% protease inhibitor mixture). The homogenate was adjusted with 0.1 ml 3 M KCl, extracted with a further ten strokes, and left on ice for 30 minutes. The lysate was centrifuged at 10^5 ***g*** for 30 minutes at 2°C in a Beckman TL-100 bench top ultracentrifuge. The supernatant was analysed as the soluble fraction, while the pellet was washed twice with lysis buffer, disrupted in 0.1 ml lysis buffer and solubilised for SDS gel electrophoresis in sample buffer containing 0.025 M dithiothreitol. In later preparations, NaCl was substituted for KCl in the extraction buffer without detectably affecting the results. Extracts of transfected COS-7 cells were prepared by washing the cells in PBS, scraping them from the dish, and treating the cell pellet as described above.

Protein samples (10-50 μg) were separated by electrophoresis in 10% SDS-polyacrylamide gels by standard methods followed

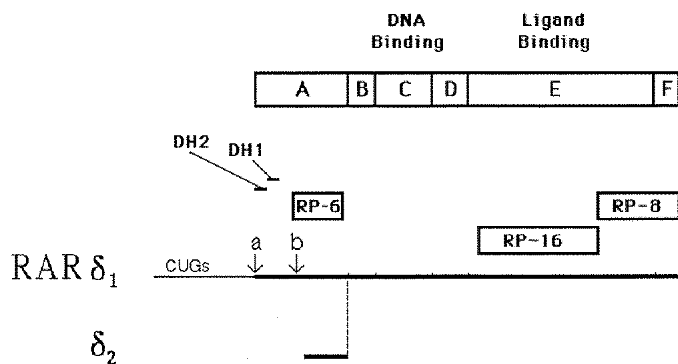

Fig. 1. Immunogens for anti-RAR antibodies aligned with RAR 1 sequence and the canonical structure of hormone nuclear receptors. The long open reading frame of RAR 1 with its potential initiation sites for translation at 5 CUG codons and methionines **a** (for 1a) and **b** (for 1b) is shown (Ragsdale et al., 1992b). Numbered from the N terminus of 1a, the extents of the various immunogens are: DH2, 2-15; DH1, 16-31; RP6, 48-102; RP16, 266-407; RP8, 406-505. The complete nucleotide sequence of RAR 1a is available from the EMBL Nucleotide Sequence Database under accession number X69944.

by semidry transfer to nitrocellulose sheets on a horizontal graphite bed apparatus. After the sheets were blocked with 5% goat serum in PBS, they were reacted with primary affinity-purified antibodies, usually at a dilution of ×100-500, followed by a secondary antibody of alkaline phosphatase coupled goat anti-rabbit IgG Fc (Promega) diluted $\times 10^4$ in PBS with 0.05% Tween 20. After incubation and washing, the filters were developed with the nitroblue tetrazolium-BCIP substrate according to the Promega instructions. Electrophoresis samples routinely included [^{14}C]methylated proteins (Amersham) as internal molecular weight markers; the markers were detected by subsequent autoradiography of the stained western blots.

Reaction of antibodies with tissue sections and cultured cells

Cryostat sections of 10-15 μm thickness were cut from unfixed newt limb embedded in 7.5% gelatin-15% sucrose in phosphate-buffered saline (PBS). Sections were dried down on gelatin-coated slides, fixed by immersion in acid alcohol (5% glacial acetic acid, 95% ethanol) at −20°C for 5 minutes and washed with PBS, followed by PBS with 5% goat serum for one hour at room temperature. Affinity-purified antibodies were diluted in PBS/10% goat serum, generally at ×25, and reacted with sections overnight at 4°C. The control antibody was hyperimmune serum to *trpE* protein diluted to yield a comparable concentration of rabbit immunoglobulin to that of the affinity-purified preparations. Sections were washed in PBS and reacted with 0.3% H_2O_2 in methanol for 30 minutes to block endogenous peroxidase activity. After further washes the bound antibody was detected using the Vectorstain ABC kit according to the makers' instructions with diaminobenzidine as substrate. In some experiments, nuclei were counterstained with 1% cresyl violet. To stain cartilage nuclei, sections were reacted with 0.5% Hoechst 33258 dye and viewed with UV epifluorescence optics. In an initial investigation into alternative fixation procedures, we found that staining with antibodies to RAR was abolished by intracardiac perfusion with 2% paraformaldehyde.

Newt limb cells in culture were washed with PBS, fixed in cold acid alcohol and processed for immunocytochemistry as above. Due to high background staining in untransfected COS-7 cells after acid alcohol treatment, we employed 100% acetone at room temperature for 5 minutes as a fixative for these cells.

RESULTS

Derivation of antibodies

To detect expression of 1 RAR protein in newt tissues a panel of five antibodies was prepared against non-overlapping regions of the molecule (Fig. 1). The antigens were derived either from bacterially expressed fusion proteins (RP6, RP8 and RP16) or from chemically synthesized peptides (DH1 and DH2). The peptide sequences were located in the A region between the two potential methionine initiators for 1a and 1b (Fig. 1), and are thus potentially specific for 1 isoforms initiating at the first 1 AUG or further upstream. RP6 sequence encompassed 1 A region sequence surrounding the 1b initiator and extending to the A/B boundary, whereas RP8 covered the C-terminal third of the E region and extended to the end of region F. RP16 was derived from most of the remainder of the E region. Antibodies to this region did not react specifically with the 1 receptor in extracts of newt tissue, and hence only its reactivity with transfected cells is considered in this account. All antibodies were raised in rabbits, and antibody activities were monitored by solid-phase ELISA assays. The experiments described here were performed with affinity-purified antibodies derived by adsorption to and elution from antigen columns (see Materials and Methods).

Reactivity with transfected COS cells and cultured newt cells

In an initial evaluation of antibody reactivity, COS-7 cells were transfected with a construct placing the expression of RAR 1b under control of the SV40 promoter (Ragsdale et al., 1989). Transfected cultures were harvested, disrupted and fractionated into soluble and particulate fractions. Western blotting with RP8 demonstrated strong reactivity with a major band of M_r=56K (1b formula weight=51K) present in soluble and pellet fractions of transfected cells (Fig. 2, lanes 3 and 4) but not of mock-transfected cells (lanes 1 and 2) analysed in parallel, or from transfectants expressing newt RAR- 1 (data not shown). RP16 also reacted with a band of identical M_r (lanes 7 and 8) that was absent from mock-transfectants (lanes 5 and 6). In contrast, RP6 did not react detectably with these fractions except for a faint band at approximately M_r=40K that was also present in control samples (lanes 9-12). Affinity-purified antibodies to the *TrpE* core protein of the fusion constructions showed no reactivity under these conditions. In transfection experiments employing expression constructs for RAR 1a and 1-5 , which direct translation initiation upstream of the 1b initiation site, neither RP6 nor DH1/DH2 antibodies reacted with the expressed receptors (not shown). Thus all three antibodies to the A region of the 1 receptor did not react with antigen on western blots of COS-7 cell transfectants.

When transfected COS-7 cells were fixed in acetone and reacted with RP6, there was strong nuclear and light cytoplasmic staining of some cells, and this staining was absent

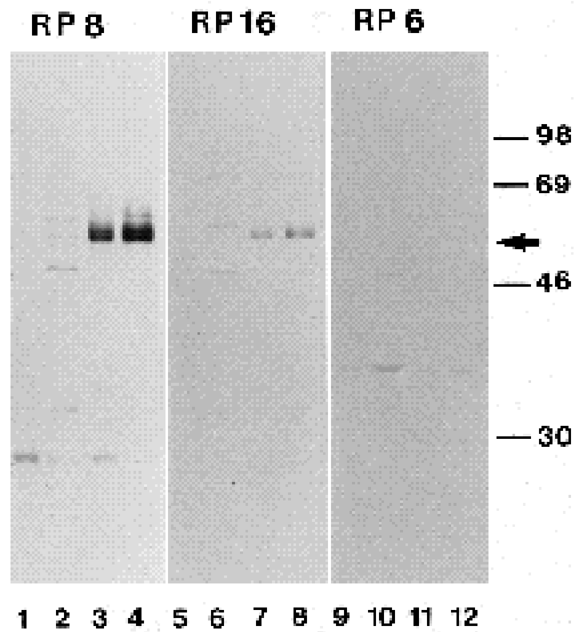

Fig. 2. Reaction of fusion protein antibodies with RAR 1b expressed in COS-7 cells. COS-7 cells were transfected with pNvRAR- 1b (lanes 3,4,7,8,11,12) or with a control plasmid, pSV2CAT (lanes 1,2,5,6,9,10). Soluble (odd-numbered lanes) and pellet (even-numbered lanes) extract fractions from cells on duplicate dishes were analysed by SDS-gel electrophoresis (10 μg protein/lane) followed by western blotting to nitrocellulose membranes. Blots were reacted with RP8 (lanes 1-4), RP16 (lanes 5-8) and RP6 (lanes 9-12). RP8 and RP16 detect a specific product of M_r=56K (arrow). Positions of relative molecular weight standards are shown at the side.

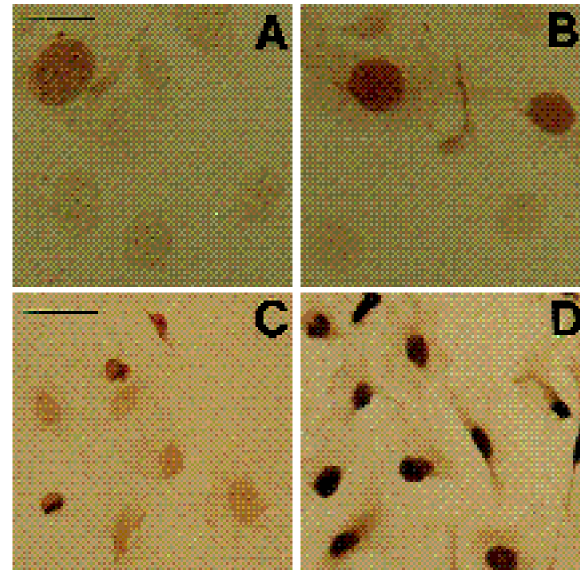

Fig. 3. Immunocytochemical detection of RAR in transfected COS-7 cells (A,B) and cultured newt limb blastemal cells (C,D). COS-7 cells were transfected with pNvRAR- 1b, fixed and stained with RP6 (A) and RP8 (B). Note positive and negative, presumed untransfected, nuclei. Scale bar: 25 μm. Newt limb B1H1 cells were fixed and stained with DH1/DH2 (C) and RP6 (D). Nuclear staining is observed in all cells. Scale bar: 100 μm.

from untransfected populations (Fig. 3A). Specific but more intense nuclear staining was elicited with RP8 (Fig. 3B). The antipeptide antibodies DH1/DH2 and control antibodies did not give detectable reactivity with fixed, transfected COS-7 cells (not shown). A somewhat different profile of reactivity was observed with cultured newt limb cells, which are known to express mRNA for the 1 receptor (Ferretti and Brockes, 1988; Ragsdale et al., 1992b). RP6 gave intense nuclear staining of all cells (Fig. 3D), and there was also significant reactivity with DH1/DH2 (Fig. 3C) and RP8 (not shown). Control antibodies did not show significant nuclear reactivity with the newt cells but did elicit cytoplasmic staining when used at low dilutions.

Western blotting of newt limb blastemal extracts

Western blotting of extracts of newt limb blastema with RP8 reproducibly demonstrated two major bands at M_r=56K and 61K (Fig. 4, lane 1). The lower band aligned with 1b reactivity present in extracts of transfected COS-7 cells (lane 2), and the position of the upper band was consistent with the relative conceptual size of the 1a receptor. In order to evaluate the reactivity of these species with RP6, limb blastemal extract was electrophoresed on a single wide gel track, which was blotted to nitrocellulose paper, and then bisected. One half was reacted with RP8 (lane 3); the other, with RP6 (lane 4). This analysis indicated that the 56K band and associated minor bands reacted with RP6. Moreover, the 61K band, though less intensely stained (lane 4), was consistently denser than in control antibody preparations. A minor band at 70K that reacted with both antibodies was also visible in some preparations (lanes 3 and 4). Thus an enhanced reactivity with RP6 for newt receptors recovered from the blastema, as compared with receptors produced by COS-7 cell transfections, was observed after both western blotting (Fig. 4) and immunocytochemistry (Fig. 3).

Soluble and particulate fractions were prepared from proximal and distal blastemas and analysed after blotting with RP8 and RP6 to determine if there was any axial variation in levels of receptor expression. RP8 labelled, as before, two major bands, which were confined to the particulate fraction (Fig. 4, lanes 6 and 8), and a minor band in the soluble fraction (lanes 5 and 7). RP6 again showed strong reaction with the lower and weak but detectable reaction with the upper of the two bands of M_r=56K and 61K (lanes 10 and 12). There were also reactivities in the soluble fractions (lanes 9 and 11) at M_r=68K and 80K which were not observed with control antibodies and whose identity is unknown. No significant proximal-distal difference in 1 expression was apparent (Fig. 4, lanes 5-12). In summary, these results are consistent with the predominant expression of blastemal 1a and 1b in a particulate, presumably chromatin bound form but also suggest the possibility of other forms that are soluble or extractable under the high salt concentrations employed.

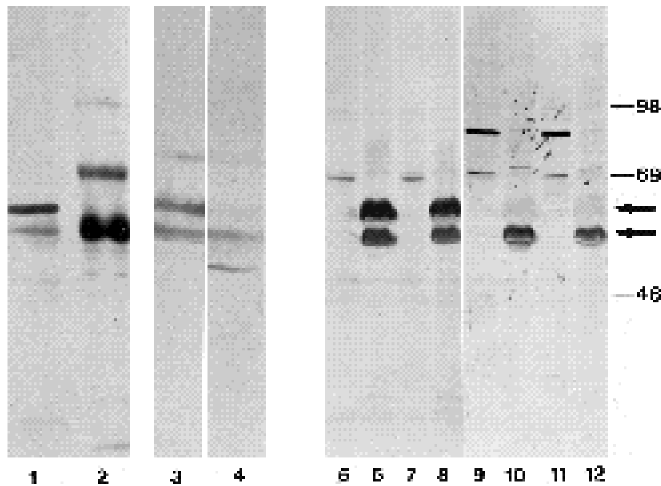

Fig. 4. Two major bands of RAR 1 immunoreactivity are detected in extracts of newt limb blastema. Lanes 1,2: Pellet fraction of newt limb blastema extract analysed by SDS gel electrophoresis (lane 1) in parallel with an extract of COS-7 cells transfected with pNvRAR- 1b (lane 2) prior to western blotting and reaction with RP8. Two bands of M_r=56K and 61K are detected in the blastemal extract and the lower one aligns with 1b protein expressed in COS-7 cells. Lanes 3,4: Single gel track loaded with limb blastema pellet fraction, blotted to nitrocellulose, bisected and reacted separately with RP8 (lane 3) and RP6 (lane 4). Note the strong reactivity of RP6 with the 56K band and weaker reactivity with the 61K band. A band at 70K is also reactive. Lanes 5-12: Soluble and particulate fractions were prepared from proximal and distal blastemas, western blotted (50 μg/lane) and reacted with RP8 (lanes 5-8) and RP6 (lanes 9-12). Lanes 5,9: soluble, distal; 6,10: pellet, distal; 7,11: soluble, proximal; 8,12: pellet, proximal. No axial difference in immunoreactivity is evident. Positions of the 56K and 61K bands (arrows, right side) and the relative molecular weight markers are noted.

Immunostaining of newt forelimb and its regeneration blastema

Sections of normal newt forelimb prepared for RP6 and RP8 immunohistochemistry showed specific reactivity with cell nuclei in epidermal and mesenchymal tissues, while no significant reactivity was observed with control rabbit antibodies. A notable and unusual feature of the reactivities of both RP6 and RP8 with all tissues of the normal limb was that approximately half of the nuclei were strongly positive whereas the remainder were negative. This is illustrated in Fig. 5 after counterstaining of nuclei with cresyl violet (Fig. 5A,B) or Hoechst dye (Fig. 5C). In the epidermis (Fig. 5A), there was a tendency for positive nuclei to predominate in more superficial layers. Heterogeneous staining was also noted for cells of the mucous glands and of the dermis, although the frequency of positive dermal nuclei was somewhat lower than that observed elsewhere in the mesenchyme. A striking finding with muscle nuclei was that this heterogeneity extended to adjacent nuclei within single muscle fibres (Fig. 5B). In cartilage, most of the peripheral nuclei in a transverse section were positive, but strong reactivity was readily detectable in the interior as well (Fig. 5C). The identification of positive and negative nuclei was not a property of a particular dilution of antibody, but was noted throughout the range of detectable reactivities. The degree of staining did not vary detectably along the proximal-distal axis of the normal limb. In approximately one-third of the limbs examined there was, however, a tendency for the epithelial cells along the dorsal, pigmented surface of the limb to be more intensely stained than their ventral counterparts.

Immunohistochemical analysis of staged limb blastemas demonstrated broad 1 receptor expression throughout the process of regeneration. In young blastemas, approximately fifty per cent of the mesenchymal nuclei in the proximal zone of de-differentiation were positive, whereas seventy to eighty per cent of the blastemal nuclei beneath the wound epidermis were immunoreactive. The wound epidermis itself showed a pattern of positive and negative nuclei similar to that described for the normal epidermis. In older blastemas (late bud stage) stained with RP6 (Fig. 6A) and RP8 (Fig. 6B), mesenchymal nuclei showed a gradation of staining intensity with approximately 55 per cent being scored as positive with RP6 histochemistry, in contrast to the results with cultured blastemal cells (Fig. 3D). There was no detectable variation of staining in cells along the proximodistal or anteroposterior axes within a blastema. The intensity of staining with the antipeptide antibodies DH1/DH2 (Fig. 6C) was significantly weaker than with RP6 or RP8 but nuclei in the wound epidermis were clearly immunoreactive (Fig. 6C, insert), as were occasional nuclei in the mesenchyme. Control rabbit antibodies gave no staining (Fig. 6D).

Staining patterns of proximal and distal forelimb blastemas at the late bud stage were specifically compared in four animals that received proximal amputations at the level of mid-humerus on one side and distal amputations (mid-radius\ulna) on the other (Brown and Brockes, 1991). No reproducible intra-animal differences were noted on sections stained with either RP6 or RP8 (data not shown). A similar analysis was performed to investigate any effect on receptor immunostaining in blastemas treated with RA. Four animals with distal forelimb blastemas were injected with RA in DMSO in a dose sufficient to respecify position, or with DMSO alone, as described previously (Savard et al., 1988), and were killed 5-6 days later. No modifications of RAR expression were noted in RA-treated blastemas relative to their DMSO controls.

DISCUSSION

The RARs are low level nuclear proteins belonging to a large family of transcription factors with extensive homologies, and are possibly subject to a variety of covalent modifications and non-covalent associations that may affect antibody reactivity. Our results underline that, to detect even a relatively abundant member of the family with confidence, it is desirable to have a panel of affinity-purified antibodies directed against non-overlapping regions of the molecule. One of the reagents, RP16, directed against a large section of the ligand-binding domain, reacted with 1 protein in transfected COS cells but did not react specifically with 1 in extracts of newt tissue and had to be set

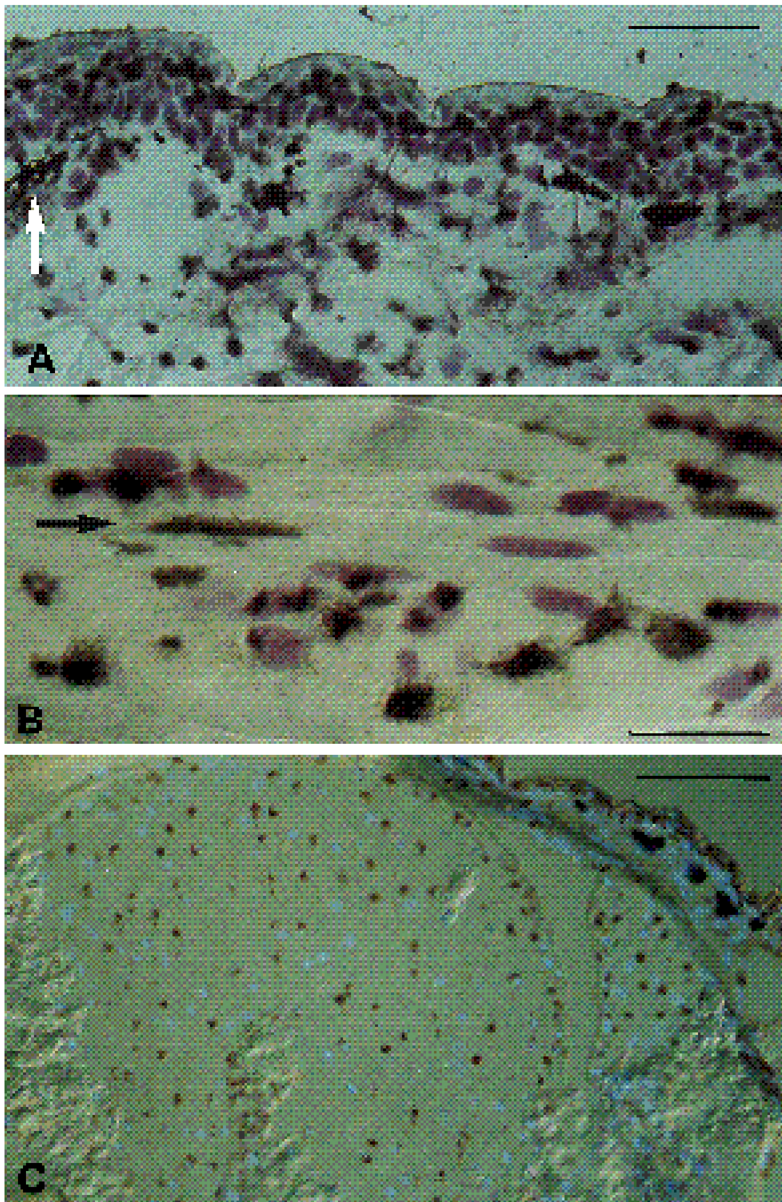

Fig. 5. Heterogeneity of RAR 1 expression in epidermal and mesenchymal nuclei of newt forelimb. Sections of (A) skin and underlying connective tissue, (B) skeletal muscle and (C) cartilage were immunostained with RP6. Nuclei were counterstained with cresyl violet (A, B) or Hoechst dye (C). Populations of positive and negative nuclei are not only intermixed (A,C) but can adjoin one another within single muscle fibres (arrow in B). Arrow in A indicates a chromatophore in the dermis. Scale bars mark 100 μm in A, 50 μm in B, 200 μm in C.

aside. The reactivities of the other three antibodies are summarised in Table 1. The EF region antibody RP8 reacted with 1 protein in all circumstances, but the region A antibodies proved markedly sensitive to context. Thus RP6 was unreactive in western blots of extracts of transfected COS cells but did recognize 1 bands in extracts of newt blastema. This could reflect some modification of the molecule in monkey cells that masks one or more determinants, although it should be noted that there is reactivity of RP6 with transfected cells fixed with acid-alcohol (Table 1). The other A region antibodies, DH1/DH2, reacted more weakly than did RP6 and did not detect transfected COS cell or newt cell material on western blots. These antibodies did, however, react with nuclei in cultured newt cells and on sections of limb tissue.

The conjunction of reactivities of RP6 and RP8 on western blots of blastemal extracts provides evidence for the expression of 1a and 1b receptors, although it is unfortunate that DH1/DH2 did not retain reactivity in this context. An alternative though less likely explanation is that one of the 56 K (putative 1b) and 61K (putative 1a) bands is a specific modification of the other; such modifications were not, however, observed after transfection of 1a or 1b constructs in COS-7 cells. Analysis of proximal and distal blastemas did not reveal any axial difference in the level or proportion of these two forms. The weak reaction of RP6 with the 1a band provides further evidence for the sensitivity of the A region determinants. The presence of minor bands in the region of M_r=70K is consistent with the expression of forms initiating upstream of the 1a AUG, a possibility that is suggested by previous western analysis of 1-5 transfectants in COS-7 cells (Ragsdale et al., 1992b). It will be important to test this point further by mutating or deleting the putative CUG initiators in 1-5 constructs. Additional antibodies will also be required to study these forms and to answer questions posed by the

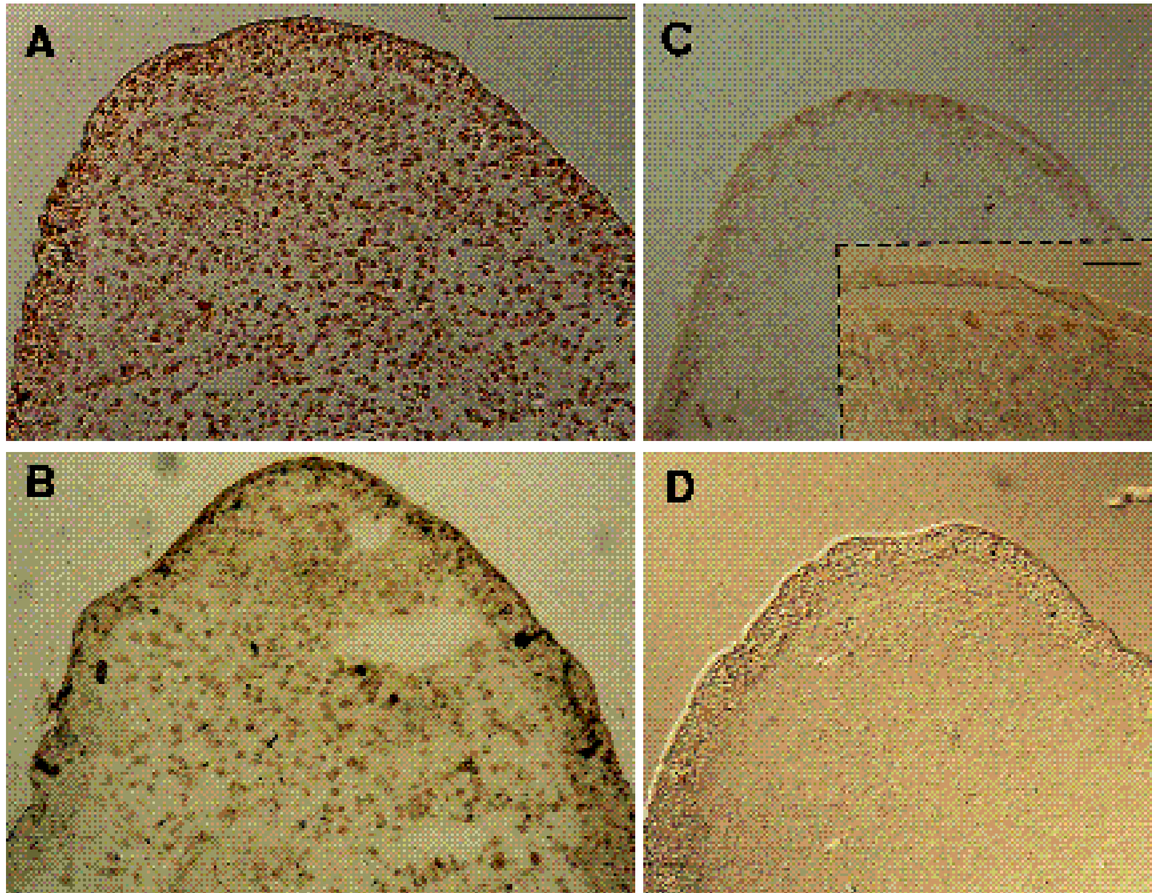

Fig. 6. Reactivities of RAR antibodies with the regeneration blastema. Sections of late bud (18 days post-amputation) blastemas were stained with (A) RP6, (B) RP8, (C) DH1/DH2 and (D) anti-*trpE* antibodies. The relative intensities shown for the different antibodies are representative of their reactivities. Scale bar for A-D: 250 μm. Insert in C illustrates at higher power DH1/DH2 staining of wound epidermal nuclei (scale bar: 45 μm).

higher molecular weight reactivities recognized in the soluble fraction.

Antibodies RP6 and RP8 gave strong staining of epidermal and mesenchymal nuclei in sections of the adult newt limb. The results were similar in all aspects of the immunohistochemistry, although RP6 reacted tissue tended to stain more intensely. RP8 was able to react with the 2 isoform in transfected cells (Ragsdale et al., 1992b), but this isoform is expressed at lower levels in the limb than 1 according to northern and RNase protection analysis, and it was not apparent in blastemal extracts by western blotting. Our analysis indicates that RP6/RP8 nuclear reactivity in sections of newt limb corresponds to the detection of 1 isoforms, and a striking aspect of this reactivity is that only half of the nuclei are positive. Previous studies employing in situ hybridisation of radioactive probes have not had the cellular resolution to observe such heterogeneity. It appears to be unrelated to the cell cycle, as it is observed in keratinocytes, cartilage, muscle fibres and connective tissue. Heterogeneous immunostaining with antibodies to other hormone nuclear receptors has been noted previously (Perrot-Applanat et al., 1985; Antakly and Eisen, 1984; Strait et al., 1991; Barsony et al., 1990), particularly in studies of the oestrogen receptor in normal and malignant breast tissue (Peterson et al., 1987; Shimada et al., 1985; Pertschuk et al., 1985; Press and Greene, 1984; King and Greene, 1984). It will be interesting to determine if RAR 2 and other members of the RAR family also show such heterogeneity. The origin and consequences of this phenomenon are unclear at present, but it may yet be significant in understanding the effects of retinoids on target tissues. The 1 receptor is expressed in all mesenchymal cell types that can contribute to the blastema, and it could mediate the effects of RA in extending the zone of cellular de-differentiation in regenerating limbs (Kim and Stocum, 1986; Stocum and

Table 1. Summary of antibody reactivities

		DH1/2	RP6	RP8
Transfected COS cells	-WB	−	−	+++
	-IHC	−	++	+++
Cultured newt cells	-IHC	++	+++	++
Blastemal tissue	-WB	−	++	+++
	-IHC	+	+++	++

Summary of the antibody reactivities presented in the Results section as determined by western blotting (WB) and immunocytochemistry (IHC). The symbol − indicates no detectable reactivity, while + to +++ indicates approximate relative reactivities to a particular source of antigen.

Crawford, 1987; Scadding, 1989). This possibility suggests the hypothesis that cells with positive nuclei would be selectively recruited into the blastema.

The immunohistochemistry for 1 protein in the blastemal mesenchyme showed a gradation of staining intensities, but there was no relation to any axis or cell group. There was no detectable difference between proximal and distal blastemas both by western analysis and immunostaining, and no difference was observed in an earlier study of message expression with RNase protection (Ragsdale et al., 1992b). It nonetheless remains a possibility that 1 participates in the ability of RA to respecify the positional identity of a distal blastema. The mammalian RAR isoform with the highest identity to 1 is 1, although the A and F regions of these receptors differ markedly (Ragsdale et al., 1989). RAR 1 transcripts, when analysed in mouse limb development by in situ hybridisation, were found to be initially uniform in the distal mesenchyme but subsequently restricted to areas of cartilage formation and were not expressed in developing muscle (Dolle et al., 1989). In addition, 1 is strongly expressed in epidermal layers of the skin. RAR 1 mRNA (Ragsdale et al., 1992b) and protein are also expressed in the newt skin and mesenchyme, but the protein is not subsequently restricted to cartilage, being found in muscle and connective tissue as well. This may reflect differences in the detection of protein and transcripts, or a difference in the expression of 1 and 1. An intriguing possibility is that the widespread expression of RAR 1 in the adult urodele limb reflects its ability to form a regeneration blastema after amputation.

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