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Original Article

Interdigital soft tissue separation induced by retinoic acid in mouse limbs cultured *in vitro*

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ABSTRACT The temporal pattern of separation of the soft tissue between mouse digits was examined in an organ culture model system. Mouse limbs of different gestational age were cultured *in vitro* and the pattern of separation of the digits characterized. By gestational day 12.5 (E12.5) the limbs were committed to undergo separation of the soft tissue in the interdigital space when cultured *in vitro*. Prior to E12.5 digital separation did not occur and the limb tissues were not committed to this process. The addition of 10^{-7}M retinoic acid (RA) to the media of E12 limbs was capable of inducing digit separation in the uncommitted limbs. Both the soft and hard tissue development of digits formed *in vitro* for either committed limbs or uncommitted limbs induced with RA was similar to the *in vivo* pattern.

KEY WORDS: *digit separation, retinoic acid, retinoic acid receptors, limb development*

Introduction

The development of the mammalian limb is initiated by the migration of cells from the lateral plate mesoderm and somites into locations on the ventrolateral body wall (Hopper and Hart, 1985). The migration and proliferation of cells in these locations results in the precise development of four limb buds between gestational days 8 and 9 in the mouse (Owens and Solursh, 1981; Theiler, 1989). The limb buds contain a mesenchymal core of cells that are capable of inducing the overlying ectoderm to differentiate into an apical ectodermal ridge (AER) (Saunders, 1948). The development of the AER is an essential early step in limb bud outgrowth along the proximo-distal axis (Saunders and Gasseling, 1968; Summerbell *et al.*, 1973). The cells just underlying the AER represent the progress zone and will provide the progenitors for all the mesenchymal components in the limb (Summerbell *et al.*, 1973; Bryant and Gardiner, 1992). The position and ultimate fate of the cells in the mesenchyme may be defined by their orientation with respect to three axes of development (Tabin, 1991). By the 9th day of fetal gestation, in the mouse, cells in the developing limbs have differentiated to produce regional specializations necessary for the development of specific structures (Theiler, 1989).

The distal-most aspect of the limbs undergoes a pattern of differentiation necessary for the development of the hands and digits. The hand paddle initially appears as a flattening in the dorsal-ventral dimension of the distal limb bud tissues on the 12th day of gestation in mice (Theiler, 1989; Wanek *et al.*, 1989). Within this developing structure, the mesenchymal cells become specialized to produce the cartilaginous blastema that are the precursors of the hand and digital bones (Owens and Solursh, 1981). The cartilage development results in digital rays that are connected by soft tissue in the interdigital spaces such that while digit formation is well progressed the digits do not exist as separate distinct entities. The completion of digit formation requires the separation of the soft tissue between the digital rays by the breakdown of the interdigital tissue (Saunders and Fallon, 1966). Programmed cell death and cell migration of the interdigital soft tissue occur to complete the separation of the individual digits by gestational day 15 in the mouse (Saunders and Fallon, 1966; Alles and Sulik, 1989; Wanek *et al.*, 1989; Mendelsohn *et al.*, 1992). Thus, as gestation proceeds, limb bud tissues terminally differentiate in a precise pattern of cartilage, bone and soft tissue necessary for the development of all the structures of the limb. Specific patterns of epithelial-mesenchymal interaction and induction of gene expression are necessary to achieve this coordinated developmental sequence.

Retinoic acid has been shown to be an important molecule in the development of the limb (Tickle *et al.*, 1982; Summerbell, 1983; Eichele, 1990; Tabin, 1991; Mendelsohn *et al.*, 1992). Endogenous retinoic acid may have a concentration gradient in avian limb tissue and this gradient may be correlated with specific patterns of tissue differentiation (Eichele and Thaller, 1987; Eichele, 1990). Exogenous retinoic acid has been shown to establish different concentration gradients which result in duplications and malformations in the exposed limbs (Kochhar, 1973; Tickle *et al.*, 1982;

Abbreviations used in this paper: RA, retinoic acid; RAR, retinoic acid receptor; AER, apical ectodermal ridge.

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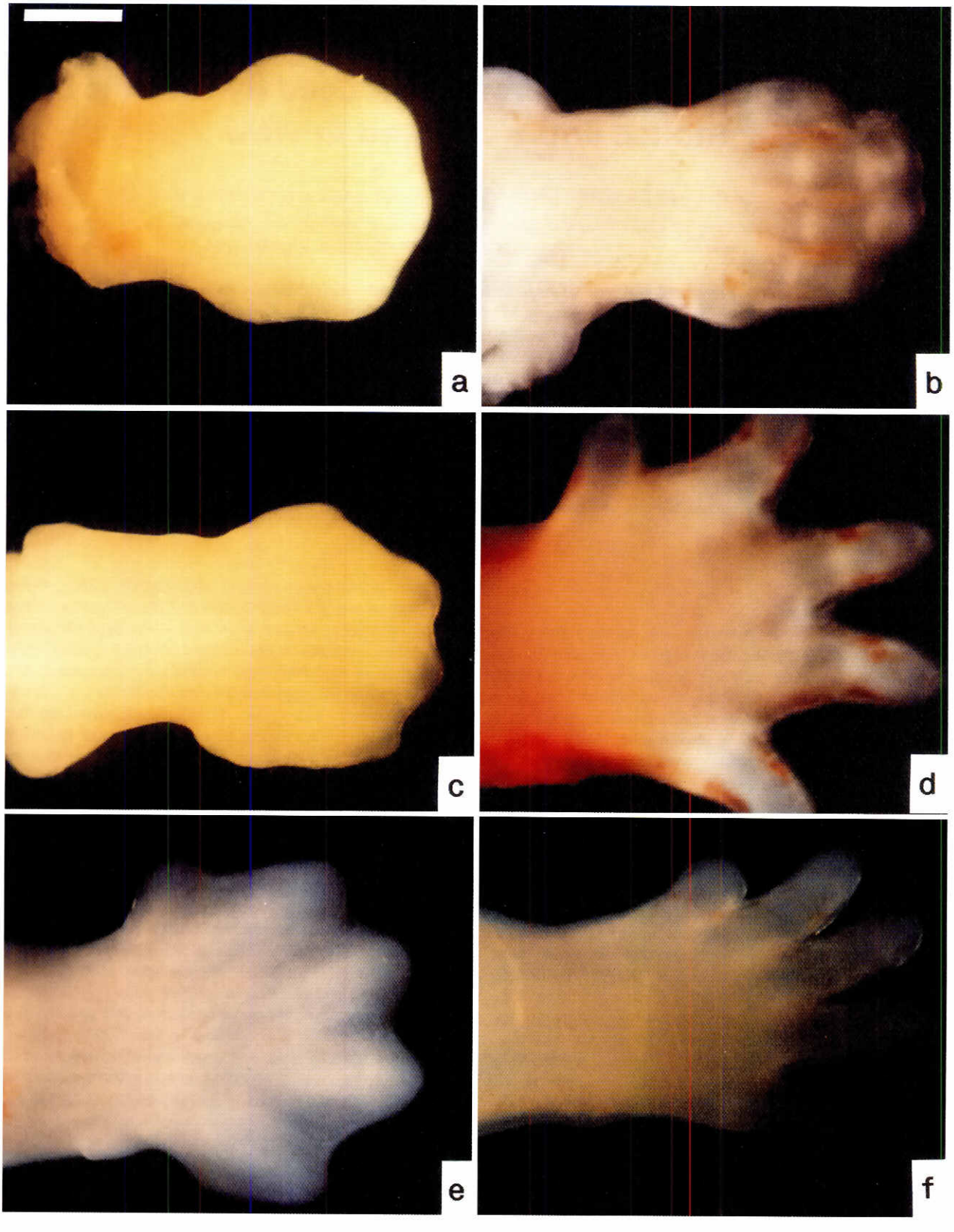

Fig. 1. Separation of the interdigital tissue in organ cultured mouse limbs of different gestational ages. *Mouse limbs were removed from fetuses of known gestational age (a-E12, c-E12.5, e-E13) and placed in organ culture as described in Materials and Methods. The limbs were allowed to develop in vitro for 3 days (b-E12+3, d-E12.5+3, e-E13+3) and the separation of the digits characterized. Bar, 500 μ.*

TABLE 1

MOUSE LIMB DEPENDENCE ON RETINOIC ACID FOR DIGIT SEPARATION *IN VITRO*

Developmental stage	Digit separation without RA	Digit separation with 10^{-7} M RA	Statistical analysis p value
E11	0/30 (0%)	0/30 (0%)	no difference
E12	3/24 (12%)	21/24 (88%)	≤ 0.004
E13	21/21 (100%)	21/21 (100%)	no difference

Limbs from fetal mice at gestational ages E11, E12 and E13 were maintained in organ culture as previously described in medium either with or without 10^{-7} M RA. Separation of the digits was evaluated in each limb after 3 days *in vitro*. The data is presented as the number of limbs with digit separation compared to the total number of limbs in each experimental group.

Summerbell, 1983; Kwasigroch *et al.*, 1984; Lammer *et al.*, 1985; Desbiens *et al.*, 1990). In mammalian development retinoic acid receptors have been localized in the interdigital tissue prior to digit separation (Dolle *et al.*, 1989, 1990; Tabin, 1991; Mendelsohn *et al.*, 1992). RARb was localized to the interdigital mesenchyme and RARg to the precartilaginous tissues and ectodermal tissues. Since the binding of retinoic acid to its receptors has been shown to induce specific patterns of gene expression (de The *et al.*, 1990; Mendelsohn *et al.*, 1991), the distribution of those receptors in the developing limb and hand may play an important role in the completion of the developmental sequence.

The goal of the present research study was to develop an *in vitro* hand and digit development model system in which separation of the interdigital soft tissue could be observed and controlled without altering the developmental sequence of the other associated tissues. The *in vitro* culture system described used a chemically defined medium to assess the role of all-trans retinoic acid in the separation of the interdigital soft tissue. This model permitted analyses of both the morphologic events associated with digit separation and the expression of specific genes that may be associated with this complex developmental event.

Results

Interdigital soft tissue separation in vitro from limbs of different gestational age

Limbs from fetuses of gestational ages E11, E12, E12.5 and E13 were cultured in BGJb culture medium for 3 days (Fig. 1, E11 data not shown). Interdigital soft tissue separation was examined in each case and correlated with the gestational age of the limb at the time of culture initiation (Table 1). The digits of E11 and E12 (Fig. 1a and b) limbs did not undergo an interdigital soft tissue breakdown in organ culture, while the digits of E12.5 and E13 limbs all became clearly and completely separated (Fig. 1c-f). The relationship between gestational age and capability for digital separation *in vitro* provided both the model system to examine the inductive influence of retinoic acid and the controls for tissue differentiation.

Comparison of digit separation in vitro and in vivo

The morphology of digits formed *in vitro* was compared to digits that had developed *in vivo*. E13 limbs were organ cultured for 5 days and the digit separation compared to tissues derived from fetuses of gestational age E14, E15 and E16 (Fig. 2). Both the patterns of soft tissue (Fig. 2a,c,e) and hard tissue (Fig. 2b,d,f) development were compared. The length of the digits, the interdigital clefting, and the appearance of the cartilage in the digits were compared. E13+5 limbs (Fig. 2e and f) had fully separated digits and contained well formed cartilage and joint spaces; however, both the soft tissue and cartilage were not as highly differentiated as was observed in the E16 *in vivo* limbs (Fig. 2c and d). The cultured limbs did not develop the claws observed in the digits *in vivo*.

Effects of retinoic acid on digit separation

The gestational age dependency of digit separation provided a model for assessing the effects of exogenous agents on the separation of digits in limb tissues that were not committed *in vivo*. The effects of retinoic acid on digit separation were assessed in a dose response study. Retinoic acid was supplemented in the medium of E12 limbs at doses of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M and the limbs maintained for up to 5 days *in vitro* (Table 2, Figs. 3 and 4). Retinoic acid at dosages of 10^{-9} and 10^{-8} M had a minimal effect on the development of the limb; since the soft and hard tissues were similar to limbs cultured without RA supplementation (Fig. 1a and b, Fig. 4c and d). The addition of 10^{-6} M RA resulted in toxicity to the developing limbs, which did not continue to develop *in vitro* in the pattern observed for control limb cultures (Fig. 4a). The addition of 10^{-7} M RA to the medium induced the separation of the digits (Figs. 3 and 4b). The digit separation was compared to the degree of separation achieved during development of the hand *in vivo* and to the digit formation observed in cultures of limbs derived from fetuses of older gestational age (Figs. 1 and 4). E12 limbs cultured for 3 days with RA supplementation *in vitro* had digital separation comparable to digits of fetuses of gestational age E15 and to the digits that developed from E13 limbs maintained *in vitro* (Fig. 5). Control E12 limbs cultured in medium lacking retinoic acid did not undergo digit formation (Fig. 5). Culture of either E11 or E13 limbs in a similar dose range of RA had no effect on additional digit development. Therefore the limbs maintained *in vitro* could be induced to undergo separation of the interdigital soft tissue by a defined concentration of retinoic acid supplemented in their medium at a precise window of development.

In situ hybridization results

Whole-mount *in situ* hybridization of the cultured mouse limbs was used to localize the distribution of the retinoic acid receptors b and g. In limbs that were committed to undergo the separation of the interdigital soft tissue (E13) RARb and RARg had distinct distributions (Fig. 6). RARb was localized in the interdigital soft tissue regions (Fig. 6a) while RARg had a perichondral distribution (Fig. 6b). The distribution of the RARb and RARg mRNA was examined in E12 mouse limbs that were not committed to digital separation developmentally but could be induced to separate the digits by the addition of 10^{-7} M exogenous retinoic acid (Fig. 7). The RARb mRNA was diffusely distributed at a low level in the E12 limbs at the onset of organ culture with a slight localization to the interdigital soft tissue (Fig. 7a). With longer periods of organ culture the RARb mRNA was localized to the interdigital soft tissue (Fig. 7c and e). However, without the addition of RA the digits did not separate and the distribution of the RARb mRNA remained the same. E12 limbs supplemented with RA also had RARb mRNA localized in the interdigital soft tissue and with prolonged culture the mRNA was distributed in a more proximal direction as the interdigital soft tissue separated (Fig. 7b,d,f). The distribution of the RARg mRNA was similar in both the E12 limbs cultured with and without supplemental RA (data not shown).

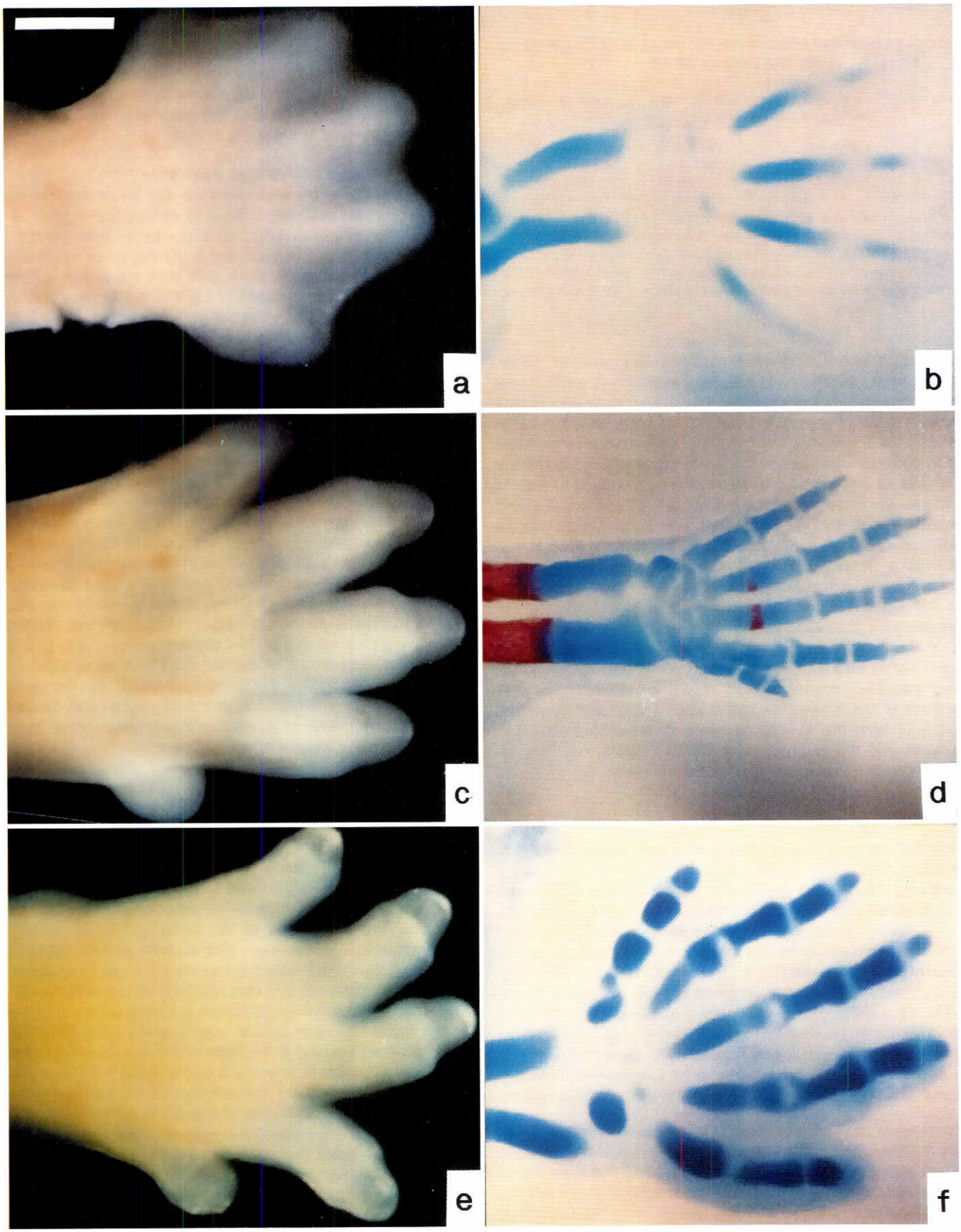
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TABLE 2

DIGIT SEPARATION AT DIFFERENT RETINOIC ACID DOSES

Retinoic acid concentration	Digit separation in cultured limbs
10^{-6} M RA	11/30 (31%)
10^{-7} M RA	26/30 (87%)
10^{-8} M RA	12/30 (40%)
10^{-9} M RA	9/30 (30%)
No RA	4/30 (13%)

Limbs from fetal mice at gestational age E12 were removed and maintained in organ culture as previously described. The limbs were cultured in medium supplemented with different concentrations of retinoic acid for 3 days. The limbs were examined for digit separation microscopically. Data is presented as the number of limbs that had digit separation compared to the total number of limbs in each experimental group.

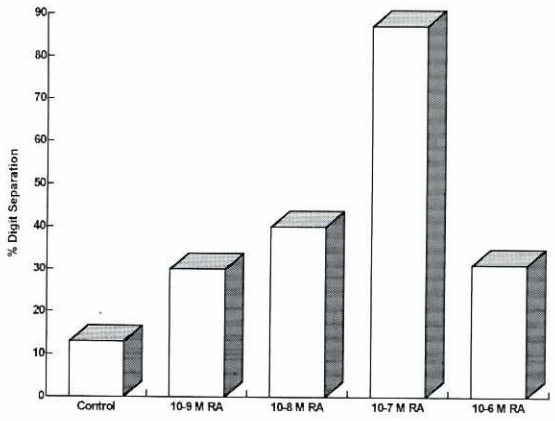


Fig. 3. Induction of digit separation in E12 limbs by different doses of retinoic acid. *Fetal mouse forelimbs were removed on gestational day 12 and placed in organ culture in media containing different concentrations of retinoic acid. The limbs were maintained in organ culture for 3 days. The separation of the digits was characterized after 3 days in culture and correlated with the concentration of retinoic acid in the culture medium.*

Discussion

Digit development requires a complex set of developmental interactions to accomplish the individual digit-specific form and function. Compounds such as retinoic acid have been shown to have a teratogenic effect on digit development following administration of excessive doses (Kochhar, 1973; Kwasigroch *et al.*, 1984; Lammer *et al.*, 1985; Mendelsohn *et al.*, 1991). Digit duplication, failure of digits to develop and absence of digital separation have all been observed. In the present study we examined the morphogenetic effect of retinoic acid on the separation of the interdigital soft tissue of limbs maintained in an organ culture model to observe the final sequence of events in the formation of individual, separated digits. Rather than exposing developing limbs *in vitro* to teratogenic doses of RA this study examined the role of RA in the completion of a normal process, the separation of the interdigital soft tissue. The addition of 10^{-7} M RA to the culture medium was capable of inducing the separation of the digits in limbs that had not yet achieved that potential during development *in utero*. Thus the model system and results presented provide a means for examining one component of digit formation, interdigital soft tissue separation, in a controlled organ culture environment suitable for analysis of the mechanism of this process.

The present studies have shown that the separation of the digits was dependent on both developmental stage-related cell commitment and the effects of retinoic acid. As early as 12 days and 12 hours of mouse fetal gestation, the cells in the interdigital spaces became committed to a pathway of cell breakdown and digit separation. Prior to that developmental stage the digits would not separate *in vitro* without an exogenous inductive stimulus. The addition of 10^{-7}M retinoic acid to the culture medium of E12 mouse limbs was sufficient to induce the interdigital cells to undergo a pattern of differentiation that permitted digit separation. Therefore the completion of the formation of individual, separated digits required either the differentiation of a specific group of interdigital cells through developmental interactions *in vivo* or through the morphogenetic effects of RA *in vitro*.

The effects of retinoic acid are mediated by a specific group of receptors (Dolle *et al.*, 1989, 1990; Tabin 1991; Mendelsohn *et al.*, 1992). The retinoic acid receptors (RARa, RARb, RARg) have been identified in the tissues of the developing limb and have a specific spatial pattern of expression (Petkovich *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989). RARa has a diffuse pattern of distribution in the tissues but both RARb and RARg have precise localizations that are related to the development of the hand. RARb has been specifically localized to the interdigital mesenchyme in the hand while RARg was limited to the cartilage-forming cells and the epidermis (Dolle *et al.*, 1989; Mendelsohn *et al.*, 1991, 1992; Lyons *et al.*, 1992). This pattern of RAR expression was found during late limb development *in vivo* (E12, E13), a stage of gestation associated with the commitment of interdigital mesenchymal cells to the separation of the digits (Alles and Sulik, 1989). Thus, *in vivo* RAR localization was temporally and spatially correlated with the breakdown of the interdigital tissues and separation of the digits.

In cultured mouse limbs the distribution of the RARb and RARg mRNA was not identical at the initiation of culture *in vitro* in the limbs committed to interdigital soft tissue separation (E12.5 and later) and limbs not committed *in vivo* to digital separation (E12). In limbs committed to digital separation (E13), the RARb mRNA was highly concentrated in a group of cells in the interdigital soft tissue while in limbs not committed to digit separation (E12) the RARb mRNA was both less highly expressed and more diffusely distributed. This distribution pattern was as expected from the pattern of RARb

Fig. 2. Comparison of the pattern of digit separation *in vitro* with the pattern observed during *in utero* development. *Fetal mouse forelimbs were removed on gestational day 13* **(a and b)** *and placed in organ culture for 5 days* **(e and f)**. *Forelimbs were removed on gestational day 16 for comparison* **(c and d)**. *The separation of the digits was characterized and compared for the organ cultured E13 limbs and the in vivo E16 limbs by both direct microscopy (a, c and e) and Alcian blue staining (b,d and f). Bar, 500 μ in a,b,e and f; 1 mm in c and d.*

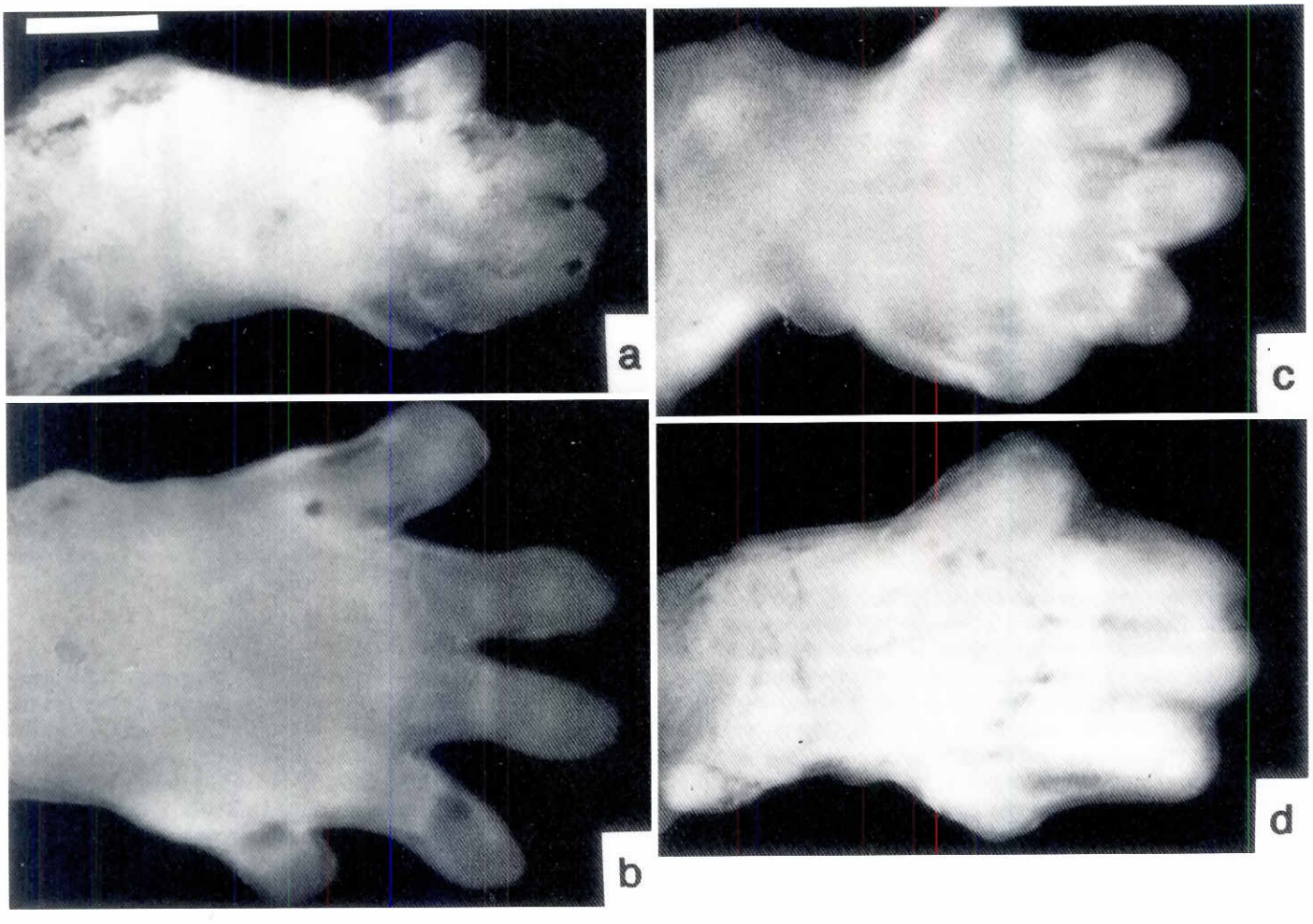
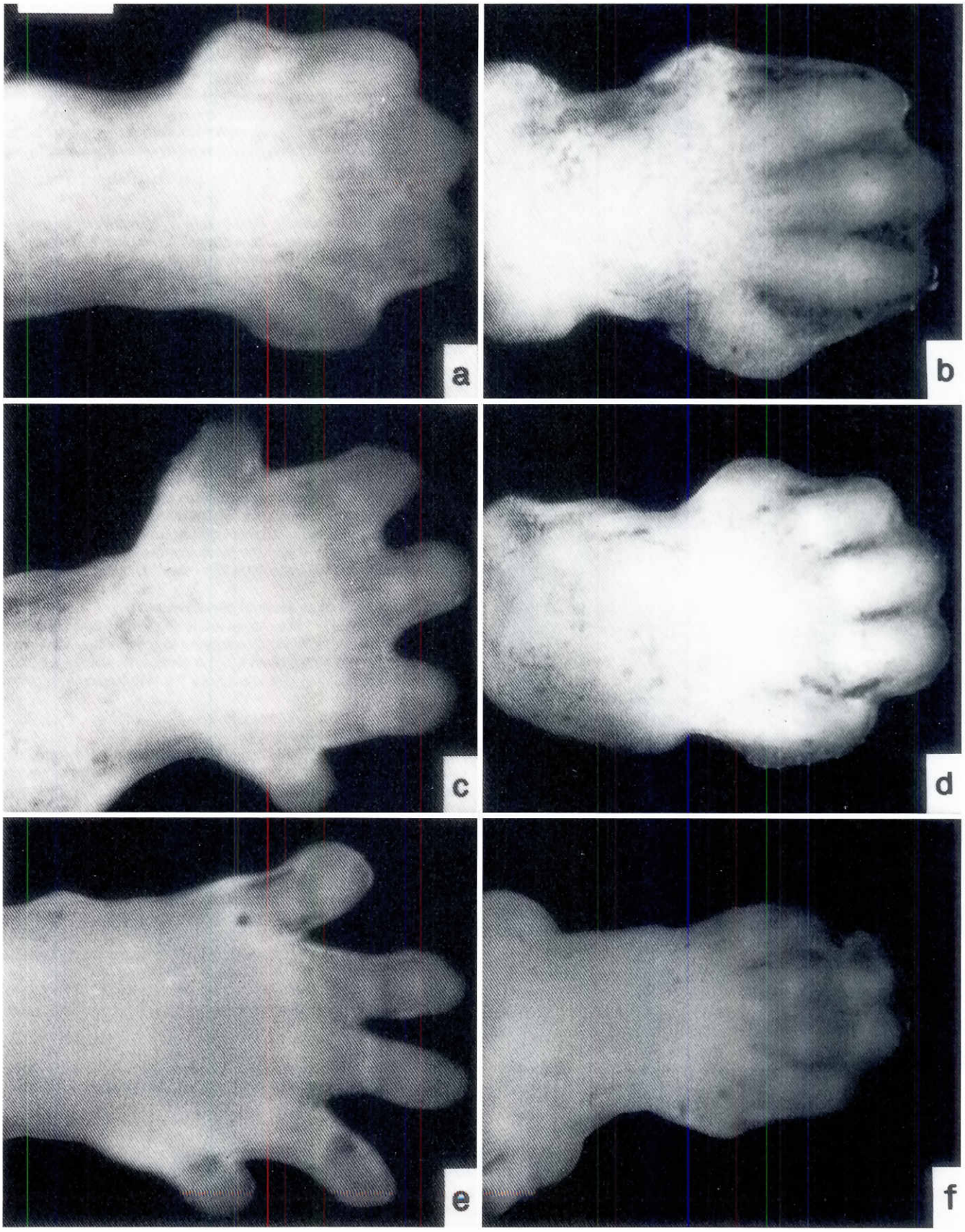


Fig. 4. Induction of digit separation in E12 limbs by different doses of retinoic acid. *Fetal mouse forelimbs were removed on gestational day 12 and placed in organ culture in medium containing either* **(a)** 10^{-6} *M,* **(b)** 10^{-7} *M,* **(c)** 10^{-8} *M,* **(d)** 10^{-9} *M RA. The limbs were maintained in organ culture for 3 days. The separation of the digits was characterized by light microscopy. Bar, 500 μ.*

expression *in vivo*. Other studies have reported that the RARb gene expression in the interdigital spaces was maximal at the E12.5 developmental stage, an *in vivo* developmental point associated with a commitment to digit separation (Dolle *et al.*, 1989, 1990; Mendelsohn *et al.*, 1991, 1992). The E12 limbs, however, were capable *in vitro* of progressing to a stage of development with a pattern of RARb mRNA distribution identical to that observed at the E12.5 stage of development. Thus E12 limbs were committed to progress to a later stage of RARb temporal and spatial expression but were not capable of completing the process of digital separation. The addition of RA to the culture medium allowed the E12 limbs to complete the morphogenetic process of digital separation.

The present study has defined a developmental window associated with the commitment of the cells and tissues in the distal limb to complete the process of digit formation by separation of the interdigital soft tissue. The temporal and spatial expression of the RARb gene and the requirement of exogenous retinoic acid to complete the process *in vitro* may be related to the mechanism of the entire process. Initially the interdigital cells would be induced to a differentiated state associated with the onset of RARb gene expression in a precise group of cells in the interdigital mesenchyme. Thereafter these cells would be primed to respond to the exposure to retinoic acid such that the transcription of another group of genes would initiate the cascade of events required to accomplish soft tissue separation. The present results have demonstrated that the model system developed provides the means for examining the mechanism for digit separation by analyzing differences between cells and tissues that span the defined developmental window.

Fig. 5. Temporal pattern of digit separation of E12 fetal mouse forelimbs cultured *in vitro* in the presence of 10^{-7} M retinoic acid. *Fetal mouse forelimbs were removed on gestational day 12 and placed in organ culture in medium containing* 10^{-7} *M RA* **(a, c and e)** *or in medium without RA supplementation* **(b, d, and f)**. *The limbs were examined after 1 day in vitro (a and b), 2 days in vitro (c and d) and 3 days in vitro (e and f). The separation of the digits was compared between the limbs cultured with and without retinoic acid. Bar, 500 μ.*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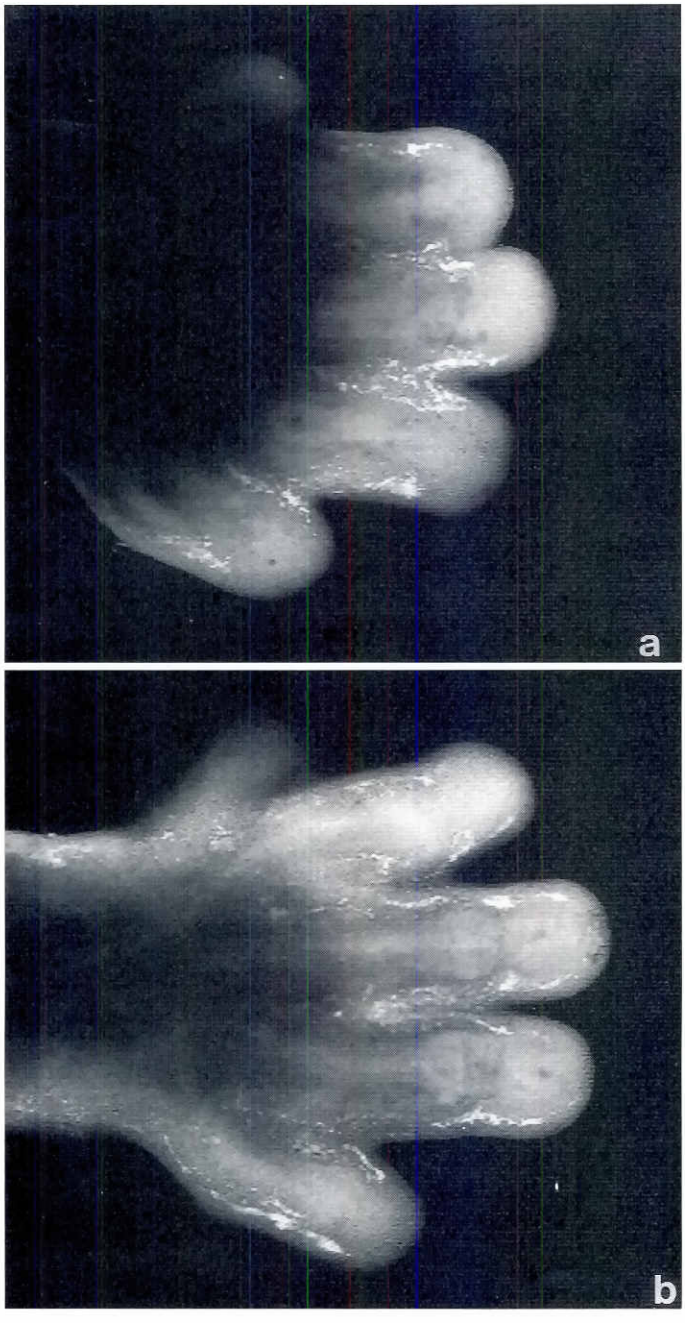

Fig. 6. Whole-mount *in situ* hybridization of RARb and RARg in E13 mouse limbs. *Fetal mouse forelimbs were removed on gestational day 13 and placed in organ culture as previously described. After 1 day of organ culture, the limbs were removed and fixed and the distribution of RARb* **(a)** *and RARg* **(b)** *mRNA were determined by whole-mount in situ hybridization with digoxigenin-labeled gene-specific cRNA probes. The spatial localization of the specific mRNA was identified by laser confocal microscopy.*

Materials and Methods

Animals

Timed-pregnant Swiss Webster mice were sacrificed on either gestational days 11, 12, 12.5 13 (E11, E12, E12.5, E13) for use of fetal tissues in organ culture and on gestational days 14, 15 and 16 (E14, E15, E16) for comparison of *in vivo* and *in vitro* limb development. The forelimbs were carefully dissected under sterile conditions from the fetal mice and either immediately placed in organ culture or placed in the appropriate fixative for a subsequent analysis.

Culture method

The dissected fetal limbs were placed on a metal grid at the air-fluid interface in Grobstein organ culture dishes (Falcon) with BGJb medium. The medium was supplemented with 0.2 mg/ml ascorbic acid and 50 U/ml penicillin/streptomycin. The limbs were maintained at 37°C in a 5% CO_2 incubator. The medium was changed every 2 days. The limbs could be maintained *in vitro* for 7 days. However, for the purposes of this study, limbs were cultured for no longer than 5 days. The cultured limbs were sampled after 1,2,3,4 and 5 days *in vitro*. The cultured limbs were examined using a dissecting microscope and photographed. Thereafter the tissues were appropriately fixed for further analysis. Experimental trials with retinoic acid involved medium supplementation with either 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M retinoic acid. The retinoic acid was present in the medium of the treated limbs throughout the entire course of culture.

Analysis

The development of the limbs *in vitro* was monitored by light microscopy, and whole-mount Alcian blue staining. The development of digit separation *in vitro* was assessed by the extent of clefting between web spaces 2, 3 and 4. If the cleft extended beyond the distal interphalangeal joint, completion of the separation of the interdigital soft tissue was interpreted.

Whole-mount analysis of skeletal development

The development of the cartilage matrix of the developing bones of the limbs was assessed by Alcian blue staining to observe the initial stages of bone formation. The limbs were placed in Alcian blue solution for 24 h and subsequently hydrated in graded alcohols. The limb tissues were cleared in 0.1% KOH.

Whole-mount in situ hybridization

The distribution of the RARb and RARg mRNA was examined in intact cultured limb tissues. The tissues were fixed in 4% paraformaldehyde, washed and bleached in 6% H_2O_2. The tissue was digested with proteinase K (10 mg/ml, 15 min, room temperature) and post-fixed in 0.2% glutaraldehyde in 4% paraformaldehyde with 0.1% Tween-20 for 20 min. The tissues were prehybridized (50% formamide, 5X SSC pH 5.0, 50 U/ml yeast RNA, 1% SDS, 50 µg/ml heparin) for 1 h at 70°C. The prehybridization solution was replaced by hybridization solution containing digoxigenin-labeled cRNA probes specific for either RARb or RARg (probes supplied by Prof. P. Chambon; Krust *et al.*, 1989). The hybridization was allowed to proceed at 70°C overnight with constant agitation. Following hybridization, the tissues were extensively washed at high stringency. To detect the hybridization of the labeled cRNA probes, a rhodamine conjugated anti-digoxigenin Fab fragment was incubated with the tissues. The distribution of the mRNA was detected by laser confocal microscopy.

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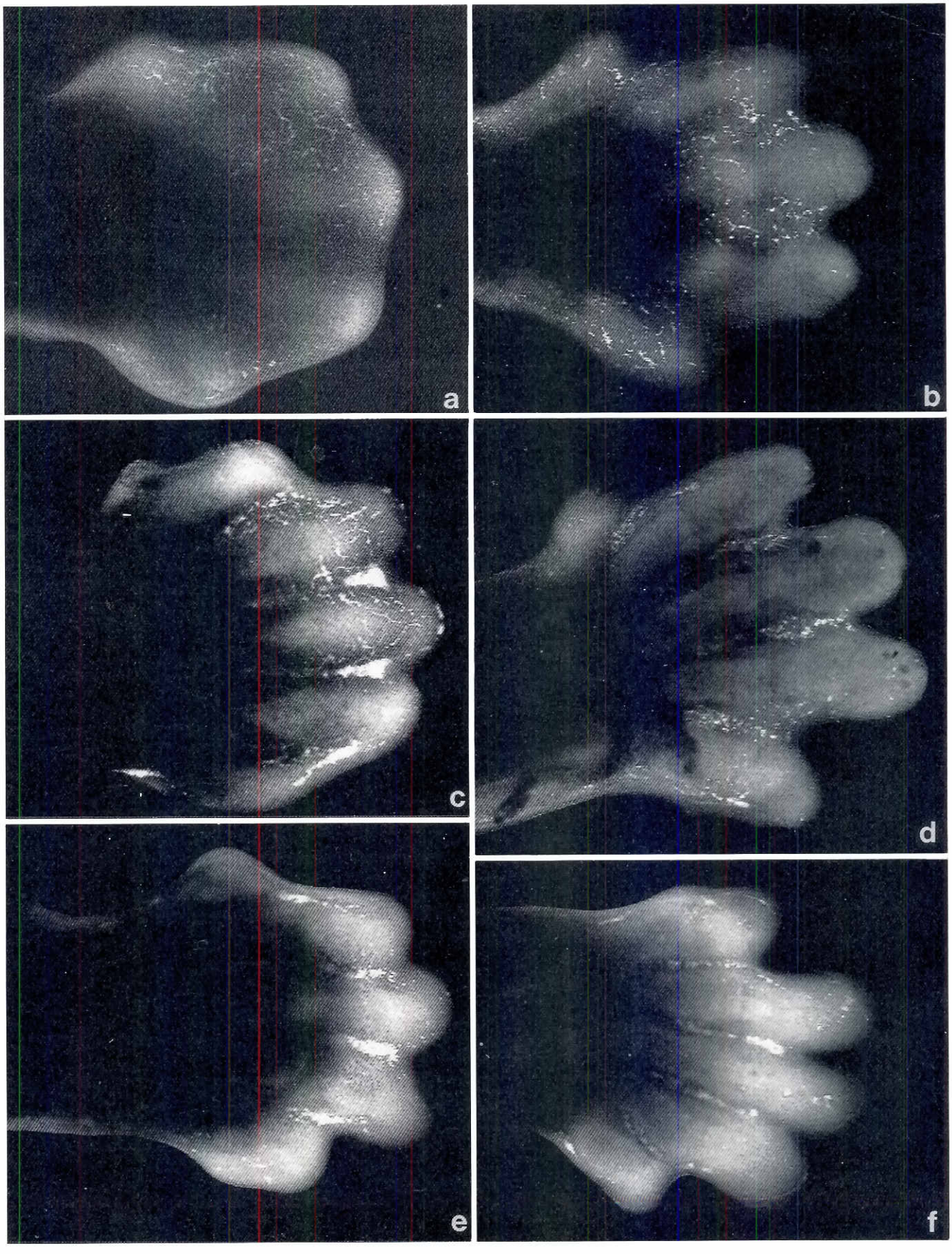
Fig. 7. Whole-mount *in situ* hybridization of RARb in E12 mouse limbs cultured *in vitro* with and without retinoic acid. *Fetal mouse forelimbs were removed on gestational day 12 and placed in organ culture in medium containing 10^{-7} M RA* **(b, d and f)** *or in medium without RA supplementation* **(a, c and e)**. *The limbs were maintained in organ culture for specified periods of time (a: 0 days; b and c: 1 day; d: 2 days; e and f: 3 days) and then fixed and processed for whole-mount in situ hybridization with a digoxigenin-labeled cRNA probe specific for RARb. The spatial localization of the RARb mRNA was determined by laser confocal microscopy.*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