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Comparison of the effects of vitamin A on limb development and regeneration in *Xenopus laevis* tadpoles

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SUMMARY

The purpose of these experiments was to compare the effects of vitamin A on developing and regenerating limbs in *Xenopus laevis* tadpoles. Each tadpole had one hindlimb amputated to induce regeneration while the contralateral developing limb was left intact. Tadpoles at stages 50 through 54 were treated by immersion in retinol palmitate at doses ranging from 0·3 to 75 i.u. ml^{-1}, for periods ranging from 1 to 14 days. Developing limbs usually became hypomorphic as a result of the treatment, with results varying with stage and treatment from slight phalange derangements to total disruption of pattern, or complete inhibition of limb development. Regenerating limbs gave a variety of responses including hypomorphic regeneration, proximodistal or anteroposterior duplication of skeletal elements, or complete suppression of regeneration. The response to retinol palmitate of developing limbs was clearly different from regenerating limbs. Hypotheses which might explain the results were discussed and a hypothesis which proposed a dual action of vitamin A affecting both the cell cycle and the mechanism of pattern regulation was proposed.

INTRODUCTION

Interest in the effects of vitamin A on limb regeneration have been high since the initial observation of Niazi & Saxena (1978) that vitamin A induced regenerative growth in excess of that normally seen, and subsequently the demonstration by Maden (1982) that the vitamin A effect was specifically to alter the proximodistal (PD) positional coding of cells of the regenerating limb. Vitamin A treatment caused the limb to begin regeneration with parts of the limb which were proximal to the actual level of amputation (Maden, 1983*a*). Subsequently, it was discovered that vitamin A also affected pattern regulation in chick embryos by mimicking the effect of the zone of polarizing activity and causing anteroposterior (AP) duplications after local application treatment procedures (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983).

Teratogenic effects of vitamin A in developing limbs have been studied for some time in mammals. The overall effect of vitamin A administered during limb

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development was to cause a variety of skeletal reductions (Kochhar, 1977). The duplications observed in regenerating limbs as a result of vitamin A treatment have never been observed in developing limbs with the exception of the chick studies noted above. Indeed, if duplications had been observed in developing limbs, they would undoubtably have been studied in detail years ago. This raises the question, then, of whether regenerating limbs and developing limbs are basically different in their response to vitamin A.

Since the studies on developing limbs have been carried out predominantly in mammals, while studies on regenerating limbs have been done in amphibians, one must ask whether this represents a taxonomic difference in developmental mechanisms between the two groups. Recent studies comparing developing and regenerating limbs treated with vitamin A have suggested that this is not the case. In a series of experiments on axolotl larvae, *Ambystoma mexicanum*, Scadding & Maden (1986) have shown that the effect of retinol palmitate on developing limbs is to cause skeletal reductions varying in severity with dose from slightly hypomorphic to severely hypomorphic. Observations on contralateral regenerating limbs of the same animal indicated that retinol palmitate effects are more variable and include hypomorphic limbs, proximodistally duplicated limbs, as well as total inhibition of regeneration. This suggests that there may be a fundamental difference between developing and regenerating limbs in the way vitamin A affects pattern regulation. Similarly, in the anuran *Bufo melanosticus*, developing limbs exposed to one particular treatment regime of retinol palmitate had skeletal deficiencies, while contralateral amputated limbs produced completely duplicated limb regenerates (Niazi & Ratnasamy, 1984). It is clear that vitamin A can have different effects on developing and regenerating limbs in the same animal at the same time.

Previous studies have indicated that methods of administration and dose levels can influence the effects of vitamin A on both developing and regenerating limbs, not only in a quantitative way, but also in a qualitative way. For example, in the chick embryo, sublethal systemic doses of vitamin A had no effect on limb development while local administration of retinoids in a paper carrier caused AP duplications (Summerbell & Harvey, 1983). Similarly, varying dose levels gave qualitatively different results in the regenerating axolotl limb. Low dose levels induced hypomorphic regeneration, moderate dose levels induced PD duplications, and high dose levels caused complete inhibition of limb regeneration (Scadding & Maden, 1986). In both chick and axolotl, results vary with different developmental stages as well.

Consequently, in order to be certain that apparent differences in the effect of vitamin A on developing and regenerating limbs are consistent and are not simply due to trivial differences in dose sensitivity or stage, it is necessary to carry out a systematic study of different developmental stages, different dose levels, and different treatment times. No anurans have been studied in detail in order to determine if there are consistent differences between developing and regenerating limbs. Hence, the purpose of the present investigation was to examine

systematically the differences between regenerating and developing limbs in an anuran, *Xenopus laevis*, at different developmental stages, different dose levels, and different treatment times, as part of an overall attempt to understand the mode of action of vitamin A more clearly. We also particularly wished to determine if there were any conditions under which duplications could be induced in developing limbs. Thus, this study is basically an extension of that carried out previously in axolotls (Scadding & Maden, 1986).

MATERIALS AND METHODS

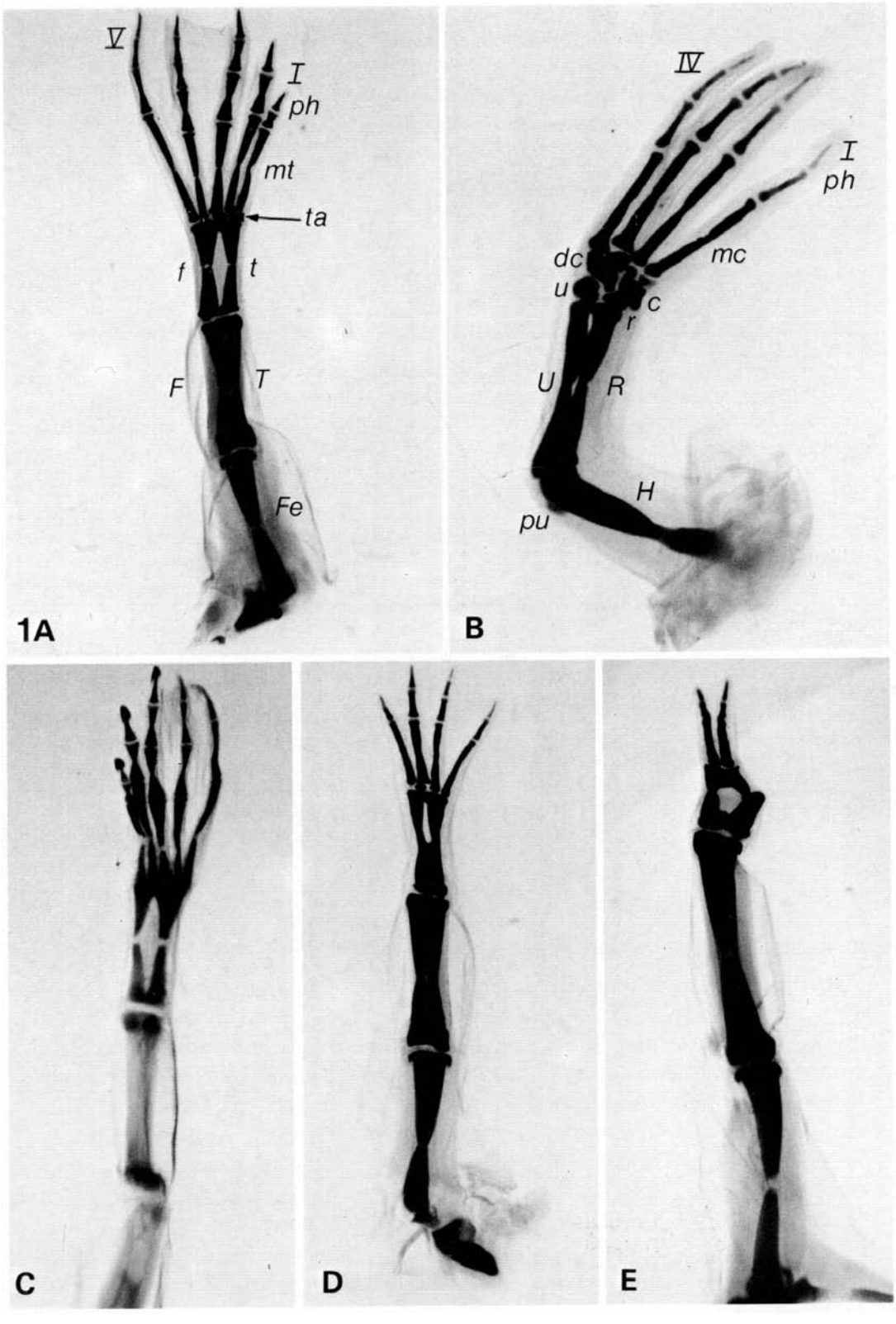
About 350 *Xenopus laevis* tadpoles were used in these experiments, having been raised in the laboratory from the induced breeding of adult frogs. Tadpoles were fed daily on a suspension of dried baby food and nettle powder. During the experiment, the tadpoles were maintained in groups of seven in 1 litre of constantly aerated tap water in plastic bins. To set up each experimental group, seven tadpoles were selected which had all reached the same stage of development (i.e. either stage 50, 51, 52, 53, or 54) as described by Nieuwkoop & Faber (1967). The tadpoles were then anaesthetized in $0{\cdot}2\,g\,l^{-1}$ tricaine methane sulphonate neutralized with sodium bicarbonate (Robinson & Scadding, 1983) and the right hindlimb amputated. At stages 50, 51, and 52 about one half of the length of the hindlimb bud was amputated, while at stages 53 and 54 the amputation plane was just proximal to the foot plate. Immediately after recovery from the anaesthetic, the tadpoles were placed in a suspension of retinol palmitate (Sigma Type VII – water dispersable) in tap water, at 0·3, 1, 3, 15, or $75\,i.u.\,ml^{-1}$. The tadpoles were maintained in this suspension for 1, 3, 7, or 14 days with changes every three or four days. At the end of the treatment period, the tadpoles were transferred to tap water and maintained therein for the balance of the experiment. Control tadpoles were amputated the same way as the experimental ones, but no retinol palmitate treatment was given.

The tadpoles were reanaesthetized and fixed after limb development or regeneration was complete at stage 59 or later. The entire tadpole was fixed in neutral buffered formalin, stained with Victoria Blue B (for cartilage), and cleared in methyl salicylate using a method similar to that of Bryant & Iten (1974). The blue-stained skeletal structures including the pelvic or pectoral girdles could then be examined and analysed. Limbs were removed from the body when necessary for photography.

The experimental design gave rise to a three-dimensional treatment matrix of five retinol palmitate concentrations, four treatment times, and five tadpole developmental stages. This gives a possible total of 100 treatment groups of which 44 were included in this experiment. All 100 possible groups were not set up since some of the lowest doses gave results which were indistinguishable from the untreated controls and some of the higher doses were lethal.

RESULTS

It is necessary to describe the normal limb skeleton of *Xenopus laevis* tadpoles as a basis for comparison and evaluation of the differences noted in regenerating and retinol-palmitate-treated limbs. The untreated intact left hindlimbs from control groups (Fig. 1A) consisted of a total of 28 skeletal elements. The leg included the femur, tibia–fibula, and tibiale (= astragalus) and fibulare (= calcaneum). The foot consisted of five very small tarsals (three lie between the proximal ends of metatarsals I to III and the tibiale, and two lie ventral to the proximal end of metatarsal I), five metatarsals, and 14 phalanges arranged on digits I through V in the formula 2–2–3–4–3. Only one case out of 36 exhibited a significant deviation from this pattern in intact control hindlimbs. Similarly, the untreated intact forelimb (Fig. 1B) consisted of a total of 26 skeletal elements. The arm included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the humerus, radio–ulna, and patella ulnaris, while the wrist and hand consisted of nine carpals, four metacarpals, and ten phalanges arranged on digits I through IV in the formula 2–2–3–3. In 52 intact untreated forelimbs examined, no significant deviations from this pattern were noted. Some of the carpals and tarsals were very small, they were not in a single plane, occasionally showed partial fusion, and often stained only lightly with Victoria Blue B, making it difficult to be confident of the precise number present in some cases. The pattern described above could be seen in Victoria Blue stained preparations by stage 58 in the hindlimbs and stage 59 in the forelimbs. Consequently, tadpoles were usually fixed at stage 59 or later for examination, since by this time the limb skeleton of the tadpoles was complete.

Untreated amputated and regenerating right hindlimbs produced regenerates essentially similar to those described previously by Dent (1962). Amputation of the hindlimb bud at stage 50, when the limb bud was about as long as it was broad, resulted in completely normal regenerates, identical in morphology to intact limbs. Amputation at stage 51, when the limb bud was about twice as long as it was broad, resulted in regenerates which were usually completely normal, although occasionally the foot was deficient in one or two phalanges. Amputation at stage 52, when the limb bud was flattening out and the first indication of the ankle constriction appeared, resulted in limb regenerates which were consistently missing one or two phalanges from digits IV or V (Fig. 1C). Amputation at stage 53, when the foot plate was developing, resulted in regenerates which were always slightly hypomorphic, usually missing one complete digit as well as several phalanges (Fig. 1D). Amputation at stage 54, when digital indentations first

Fig. 1. (A) Normal intact left hindlimb from a stage-59 *Xenopus* tadpole. The skeleton consists of a femur (*Fe*), tibia (*T*), and fibula (*F*) here fusing into the single tibia–fibula, tibiale (*t*), fibulare (*f*), five tarsals (*ta*) of which three are visible here (the other two lie beneath distal tarsal I and the proximal end of metatarsal I), five metatarsals (*mt*), and 14 phalanges (*ph*) arranged on digits I to V (I–V) in the formula 2–2–3–4–3. Magnification ×9. (B) Normal intact left forelimb from a stage-59 *Xenopus* tadpole. The arm skeleton consists of a humerus (*H*), radius (*R*), and ulna (*U*), here fusing into the single radio–ulna, and a small patella ulnaris (*pu*) at the elbow. The wrist comprises nine carpals of which seven are clearly visible here. The four larger ones are the ulnare (*u*), radiale (*r*), centrale (*c*), and distal carpal IV (*dc*). Three smaller distal carpals are also visible. Two other carpals, the radiale externum lying dorsal to the radiale, and the praepollex just medial to distal carpal I are not visible in this photograph (the praepollex is visible in Fig. 2B). The hand consists of four metacarpals (*mc*) and ten phalanges (*ph*) arranged on digits I through IV (I–IV) in the formula 2–2–3–3. Magnification ×24. (C) This right hindlimb was amputated at stage 52 and fixed at stage 65. It was scored as being within the normal range, since it only varies from normal by having a phalange formula of 2–2–3–3–2, i.e. two phalanges are missing, and the lateral metatarsals are slightly bent. Since this specimen was fixed at a later stage, ossification of the skeleton is advanced, hence there is less cartilage present resulting in fainter Victoria Blue B staining. The claws are visible on digits I, II, and III. Magnification ×8. (D) The right hindlimb was amputated at stage 53 and fixed at stage 60. It was slightly hypomorphic having four rather small digits with the phalange formula 0–2–2–3–2. Magnification ×11. (E) This right hindlimb was amputated at stage 54. The regenerated foot was extremely hypomorphic (EH–P^3) with only two small digits, each with two phalanges, and marked shortening of the tibiale and fibulare. Magnification ×9.

became apparent, resulted in regenerates which had four digits at best, and often had defects of the leg bones as well (Fig. 1E). Since absence of a few phalanges was characteristic of many untreated control regenerates, a "normal" limb was arbitrarily defined as one which exhibited no skeletal deletions or irregularities greater than the absence of three phalanges. Limbs with four or more phalanges missing were described as hypomorphic. Thus, stage 52 was the latest stage at which normal limb regeneration was observed. All hindlimb regenerates from amputation at stages 53 or 54 were at least slightly hypomorphic.

The results of retinol palmitate treatment on about 800 *Xenopus* tadpole limbs are summarized in Table 1. The observations from both forelimbs were combined since no left *versus* right differences were noted. Each experimental group consisted of seven (occasionally eight) tadpoles. Where there are fewer than seven hindlimbs (or fourteen forelimbs) in a group it indicates that some tadpoles in that group died during the course of the experiment and/or that some tadpoles had not reached stage 59 (and thus could not be adequately evaluated) by 3 months after amputation when the experiment was terminated. The number of forelimbs scored was often less than the number of hindlimbs since forelimb development trails hindlimb development and some tadpoles were fixed at stage 57 or 58 when hindlimb structure could be evaluated but not forelimb structure. The coding system used to describe the results is included in the legend to Table 1. Defects in the hands/feet were described as slightly, moderately, or extremely hypomorphic, and long bone defects, usually a shortening (phocomelia) or bending of the bone, were identified by coding the specific bones affected. Duplications, both proximo-distal (PD) and anteroposterior (AP) were scored by coding the specific duplications observed.

The small number of cases in some of the higher dose groups indicates that the doses used extended into the lethal range. In six additional experimental groups not recorded in Table 1, all of the tadpoles died. These were: stage 50, 3 i.u. ml^{-1}, 3 days; stage 51, 1 i.u. ml^{-1}, 3 days; stage 51, 1 i.u. ml^{-1}, 7 days; stage 52, 1 i.u. ml^{-1}, 3 days; stage 52, 3 i.u. ml^{-1}, 14 days; and stage 52, 75 i.u. ml^{-1}, 1 day. The fact that these deaths were not tightly correlated with dose and that one control group (also not in Table 1) also suffered high mortality, suggests that retinol palmitate was not the sole cause of death. It seems likely that the retinol palmitate weakens the tadpoles and reduces their resistance to bacterial infections or other disease.

Note that developing limbs may be affected by retinol palmitate while contralateral hindlimbs regenerate normally (e.g. stage 50, 0·3 i.u. ml^{-1}, 14 days). Similarly, regenerating limbs may be affected by retinol palmitate while contralateral intact hindlimbs develop normally (e.g. stage 53, 0·3 i.u. ml^{-1}, 14 days).

At stage 54, it is difficult to be certain what effects retinol palmitate has on regenerating hindlimbs because normal regeneration never occurs. Even untreated control regenerates were uniformly quite defective (Table 1). Hence, it is impossible to be certain which of the defects observed are due to the treatment and which would have occurred anyway.

Table 1. *Effects of retinol palmitate on* Xenopus laevis *limb development and regeneration*

Stage	Dose i.u. ml^{-1}	Time days	Forelimbs	Hindlimbs	
				Left developing	Right regenerating
50	0	0	8–N	7–N	7–N
50	0·3	3	14–N	8–N	7–N
50	0·3	7	8–N	4–N, 1–P^1, 1–P^2, 1–P^{23}	6–N, 1–P^1
50	0·3	14	5–N, 1–SH–P^{12}	2–N, 1–P^1, 3–P^{123}	6–N
51	0	0	12–N	6–N, 1–P^2	5–N, 1–MH–P^3
51	0·3	3	14–N	7–N	5–N, 1–A, 1–D7
51	0·3	7	7–N, 5–P^2, 2–SH–P^2	4–N, 1–SH, 1–SH–P^2, 1–P^2	5–N, 1–SH, 1–P^2
51	0·3	14	2–SH, 2–EH–P^2, 8–EH–P^{12}, 2–P^2	1–N, 1–SH, 2–MH, 1–SH–P^3, 1–EH–P^2, 1–P^2	2–A, 4–D3, 1–D4
51	3·0	3	3–N, 1–SH–P^2, 1–MH–P^{12}, 1–EH–P^1, 4–EH–P^2, 1–EH–P^{12}, 1–P^{12}	1–SH, 1–SH–P^{23}, 1–MH–P^{123}, 2–MH–P^2, 1–EH–P^2	3–N, 1–A, 2–EH–P^3
51	3·0	7	4–EH–P^{12}	1–SH–P^{123}, 1–EH–P^{23}	2–A
51	15	1	4–N, 2–EH–P^2	1–N, 1–EH–P^{23}, 1–P^{123}	1–N, 2–EH–P^3
51	15	3	2–EH–P^2	1–MH–P^{123}	1–N
51	75	1	4–N, 2–MH–P^{12}, 2–P^2	1–N, 1–MH–P^{123}, 2–P^2	2–N, 1–A, 1–EH–P^3
52	0	0	10–N	7–N	7–N
52	0·3	3	12–N	7–N	5–N, 2–SH
52	0·3	7	14–N	7–N	5–N, 1–SH, 1–EH–P^3
52	0·3	14	2–N, 4–SH, 4–MH	1–N, 4–SH, 2–MH	3–D3, 1–D3–P^{23}, 1–D4, 1–D4–SH, 1–D4–D6
52	1·0	1	2–N, 2–EH	1–N, 1–EH, 1–EH–P^2	1–N, 1–SH, 1–P^2
52	1·0	7	2–A, 4–EH–P^{12}	2–A, 1–EH	2–D3, 1–D3–EH
52	3·0	1	2–SH–P^2, 2–EH	3–MH	2–N, 1–EH–P^3
52	3·0	3	10–EH, 2–EH–P^2, 2–EH–P^{12}	1–A, 1–MH, 5–EH	7–N
52	3·0	7	4–EH–P^2, 2–EH–P^{12}	2–EH, 1–EH–P^2	1–N, 1–A, 1–MH–P^3
52	15	1	2–EH–P^2	1–EH	1–N
52	15	3	2–A, 2–EH–P^2	1–EH–P^{12}, 1–EH–P^{23}	1–A, 1–EH–P^3
53	0	0	8–N	7–N	6–SH, 1–MH
53	0·3	3	12–N	7–N	2–N, 4–SH, 1–EH–P^3
53	0·3	7	14–N	7–N	1–N, 1–A, 4–SH, 1–MH–P^3
53	0·3	14	8–N, 4–SH, 2–MH	6–N, 1–SH	2–A, 1–EH–P^3, 1–EH–P^{23}, 1–D3, 1–D4–MH, 1–D5–MH
53	1·0	1	2–N	1–N	1–N
53	1·0	3	4–SH, 5–MH, 1–EH	1–N, 5–SH	3–N, 1–SH, 1–D1–SH, 1–D6
53	1·0	7	4–EH	1–SH, 1–MH, 1–EH, 1–EH–P^3	1–A, 1–MH–P^3, 1–D3
53	1·0	14	2–EH	1–EH	1–D3–D7
53	3·0	1	3–EH, 3–EH–P^2	1–MH, 2–EH	3–N
53	3·0	3	3–MH, 8–EH, 1–EH–P^2	1–SH, 4–MH, 2–EH	2–N, 2–SH, 1–EH–P^3, 1–EH–P^{23}, 1–D6
53	3·0	7	2–EH	2–EH	1–N, 1–A
53	15	1	2–EH	1–EH	1–SH

Table 1. *continued*

Stage	Dose i.u. ml^{-1}	Time days	Forelimbs	Hindlimbs	
				Left developing	Right regenerating
54	0	0	14–N	8–N	2–A, 2–SH, 1–SH–P^3, 1–EH–P^3, 1–P^3
54	0·3	1	14–N	7–N	1–A, 4–SH, 1–MH, 1–MH–P^3
54	0·3	3	14–N	7–N	1–N, 5–SH, 2–MH
54	0·3	7	14–N	7–N	3–A, 1–MH, 1–MH–P^{23}, 2–MH–P^3
54	0·3	14	14–N	6–N, 1–SH	7–A
54	1·0	3	6–SH	1–N, 5–SH	1–SH, 4–SH–P^3, 1–MH–P^3
54	1·0	7	8–EH, 2–EH–P^2	3–MH, 1–EH, 1–MH–P^2	3–A, 1–EH–P^3, 1–D2–D6

Coding and definitions used to describe deletions are as follows:
N – Normal, missing three phalanges or less,
A – Absent, limb failed to develop or regenerate at all;
for hand/foot defects:
SH – Slightly Hypomorphic, missing 4 or more phalanges or at least one metacarpal/metatarsal absent or defective,
MH – Moderately Hypomorphic, forelimb with two digits or hindlimb with two or three digits (good quality if only two),
EH – Extremely Hypomorphic, forelimb with one digit or less, hindlimb with 2 (poor quality) digits or less;
for arm/leg defects P$^{\text{superscript}}$ was used to score shortened or significantly defective long bones (Phocomelia):
P^1 – Femur or humerus defective,
P^2 – Radius–ulna or tibia–fibula defective,
P^3 – Tibiale or fibulare defective.
Duplications in regenerating limbs were scored as follows:
D1 – extra carpals/tarsals or serial metatarsals,
D2 – extra part radius–ulna or tibia–fibula or tibiale–fibulare,
D3 – extra complete radius–ulna or tibia–fibula or tibiale–fibulare,
D4 – extra part humerus or femur,
D5 – extra complete humerus or femur,
D6 – AP duplication of extra digit or two,
D7 – AP duplication of extra complete foot.
In each entry, the first number is the number of cases observed of the deletion or duplication coded.

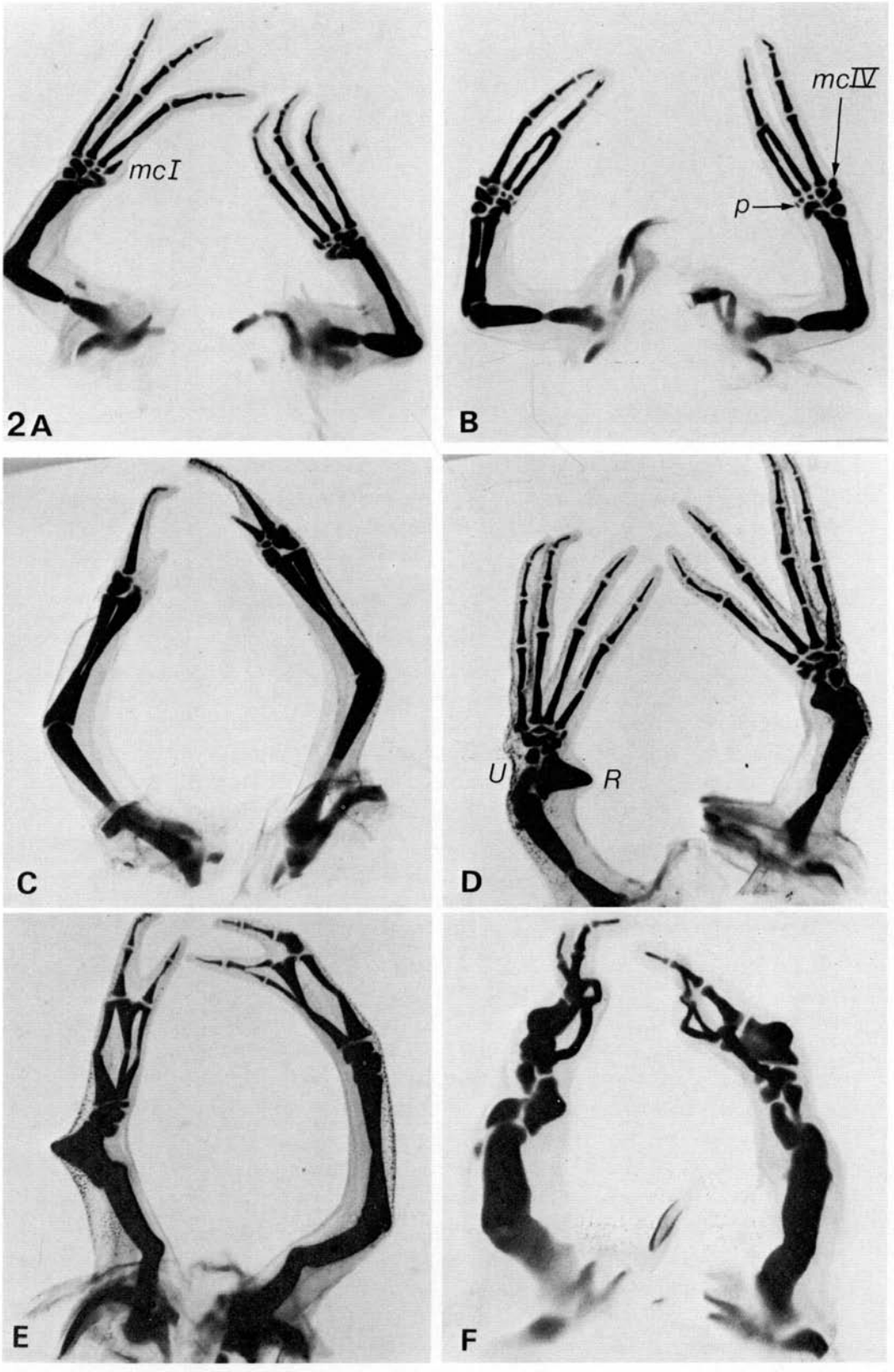
Table 2 summarizes the type and location of retinol palmitate effects on developing and regenerating limbs, irrespective of the dose employed. A number of observations can be made. Retinol palmitate had very little effect on developing limbs, especially forelimbs at stage 50. Presumably, until the limb bud is well established and begins to grow out and differentiate, retinol palmitate can have little effect. At stages 50 and 51, arm/leg defects were more common in developing limbs than hand/foot defects. However, at stage 52 and later, this was reversed and hand/foot defects predominated. Presumably, retinol palmitate affected whichever part of the limb was differentiating at the time of treatment. At stages 51 and 52, arm/leg defects were more common in developing forelimbs than in

Table 2. *Summary of type and location of retinol palmitate effects*

	Stage 50			Stage 51			Stage 52			Stage 53			Stage 54		
	Forelimbs	Developing left hindlimbs	Regenerating right hindlimbs	Forelimbs	Developing left hindlimbs	Regenerating right hindlimbs	Forelimbs	Developing left hindlimbs	Regenerating right hindlimbs	Forelimbs	Developing left hindlimbs	Regenerating right hindlimbs	Forelimbs	Developing left hindlimbs	Regenerating right hindlimbs
Normal limbs	27	14	19	32	14	17	30	16	22	36	22	13	56	28	1
Arm leg defects only	–	7	1	10	5	1	–	–	1	–	–	–	–	–	–
Both arm/leg and hand/foot defects	1	–	–	30	13	5	20	4	4	4	1	7	2	1	10
Hand/foot defects only	–	–	–	2	5	1	22	20	4	40	23	12	14	10	14
Absent	–	–	–	–	–	7	4	3	2	–	–	5	–	–	14
Duplication	–	–	–	–	–	6	–	–	10	–	–	8	–	–	1
Total number of affected limbs	1	7	1	42	23	20	46	27	21	44	24	32	16	11	39

hindlimbs, presumably reflecting the fact that the forelimbs lagged behind the hindlimbs in development. In regenerating hindlimbs, retinol palmitate had little or no effect at stage 50, presumably because the treatment was over before differentiation of the regenerating limb bud occurred. At stage 51 and 52, regenerating hindlimbs responded to retinol palmitate with deletions and duplications in about equal frequencies. However, by stage 53, deletions were the predominant response, and by stage 54 duplications were rare (only 1 case out of 39). Reduction in frequency of duplications was correlated with loss of regenerative ability as the tadpole aged. At stage 53, tadpoles were losing the ability to regenerate normally after amputation, and by stage 54, normal regeneration was rare. Thus, the capacity to produce duplications seemed to be correlated with the capacity to regenerate normally.

Fig. 2 illustrates the effect of retinol palmitate on developing forelimbs. When treatment was given at stage 53, defects tended to be restricted to the hand area (Fig. 2A,B,C). Increased concentration or increased time of treatment tended to increase the severity and frequency of the effects. When treatment was given at stage 51, defects of the arm, or both arm and hand were most common (Fig. 2D,E,F). In all of these cases there is a remarkable similarity in the response of the left and right limbs to a given treatment. Even though within an experimental group there might be extreme variations in response to retinol palmitate, within each specific tadpole the response of the forelimbs was remarkably similar.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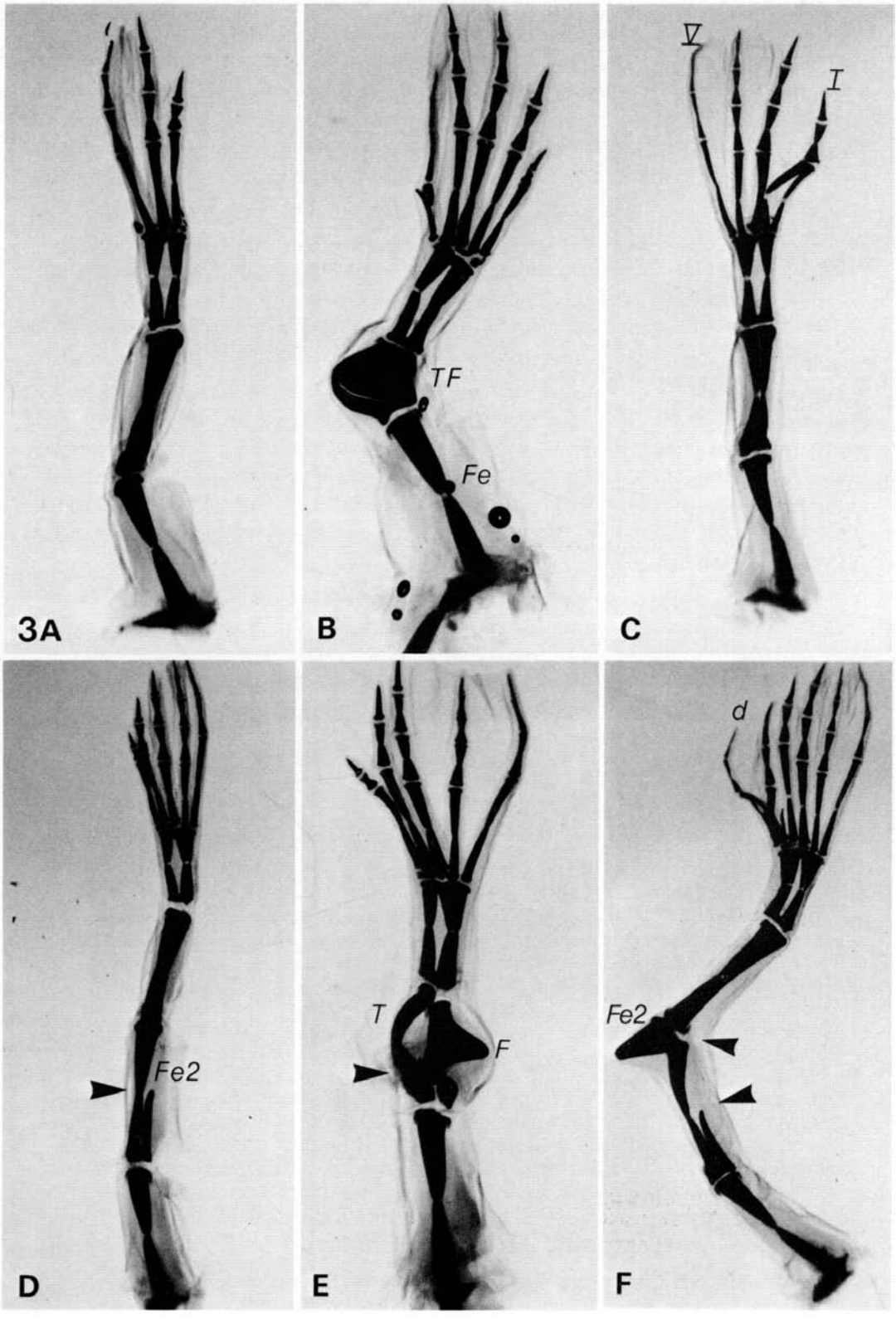
Figs 3 and 4 illustrate the effect of retinol palmitate on intact developing left hindlimbs and on regenerating right hindlimbs. In all cases, the effects of retinol palmitate on developing hindlimbs was to induce a deletion or defect in the skeletal pattern. Deletions varied from loss of a digit or two (Fig. 3A,C), to complete absence of the limb. Defects may occur in only one skeletal element (Fig. 3B) or may affect the entire leg (Fig. 4A). As for forelimbs, increased concentration of retinol palmitate and increased time of treatment tended to increase the severity and frequency of the effects. However, there were great variations within groups (Table 1), suggesting a great variability in the response of individual tadpoles. A specific treatment may have caused major defects in some tadpoles while others were unaffected.

The effects of retinol palmitate on regenerating hindlimbs were variable. Deletions of skeletal structures occurred in many cases (Fig. 4C) giving results similar to those illustrated for developing limbs (Figs 3A,B,C, 4A). In many cases, the deletions can be quite extensive (Fig. 4C) and frequently regeneration was completely inhibited (cf. Tables 1 & 2). Many regenerating hindlimbs produced duplications similar to those previously reported in axolotls or other anurans (Maden, 1983*a,b*). A total of 25 duplications was observed of which 19 were PD duplications only (Figs 3D,E, 4D) and three were AP duplications only (Fig. 4B,E), while three cases exhibited both PD and AP duplications simultaneously (Fig. 3F). Duplications subsequent to amputation at stages 51, 52, and 53 were common, but were not observed after amputation at stage 50, and rarely after amputation at stage 54 (Table 2). In axolotls, duplicated regenerating limbs were usually normal in morphology except for the duplication (Scadding & Maden, 1985). Similarly, in *Xenopus* PD duplicated limbs could be quite normal apart from the duplication (Fig. 3D). However, in *Xenopus* this was not always the

Fig. 2. Each photograph shows a dorsal view of both left and right forelimbs of *Xenopus* tadpole treated with retinol palmitate. (A) Forelimbs treated at stage 53 (0·3 i.u. ml^{-1}, 14 days). Both are slightly hypomorphic (SH) showing loss of digit I which is represented only by a vestige of metacarpal I (*mcI*). Magnification × 14. (B) Forelimbs treated at stage 53 (0·3 i.u. ml^{-1}, 14 days). Both are moderately hypomorphic (MH) with only two digits. Digit IV is missing and is represented by a carpal-like cartilage which is likely to be a vestige of metacarpal IV (*mcIV*). In both limbs, metacarpals I and II are partially fused and underlie a single set of phalanges. The praepollex (*p*) is visible in these specimens. Magnification × 17. (C) Forelimbs treated at stage 53 (1·0 i.u. ml^{-1}, 7 days). Both are extremely hypomorphic (EH) and hand is reduced to single digit consisting of a single metacarpal. Magnification × 12. (D) Forelimbs treated at stage 51 (0·3 i.u. ml^{-1}, 14 days). Hands are entirely normal, but arms exhibit phocomelia (P^2) in that the radius (*R*) is reduced to a triangular cartilage and the ulna (*U*) is reduced to a carpal-sized nodule of cartilage. Magnification × 15. (E) Forelimbs treated at stage 51 (3 i.u. ml^{-1}, 3 days). Hands are extremely hypomorphic and arm is phocomelic (left – EH–P^{12}; right EH–P^1). Note the bend in the humerus in each arm. An increase of this bend, such that the ends of the long bone are brought towards one another, results in a triangular cartilage as seen in radius–ulna of the left limb. This pair of limbs is unusual in that left radius–ulna is defective while right radius–ulna is normal. Magnification × 13. (F) Forelimbs treated at stage 51 (0·3 i.u. ml^{-1}, 14 days). Both limbs are extremely hypomorphic and phocomelic (EH–P^{12}). Pattern of limb skeleton is quite chaotic. Magnification × 22.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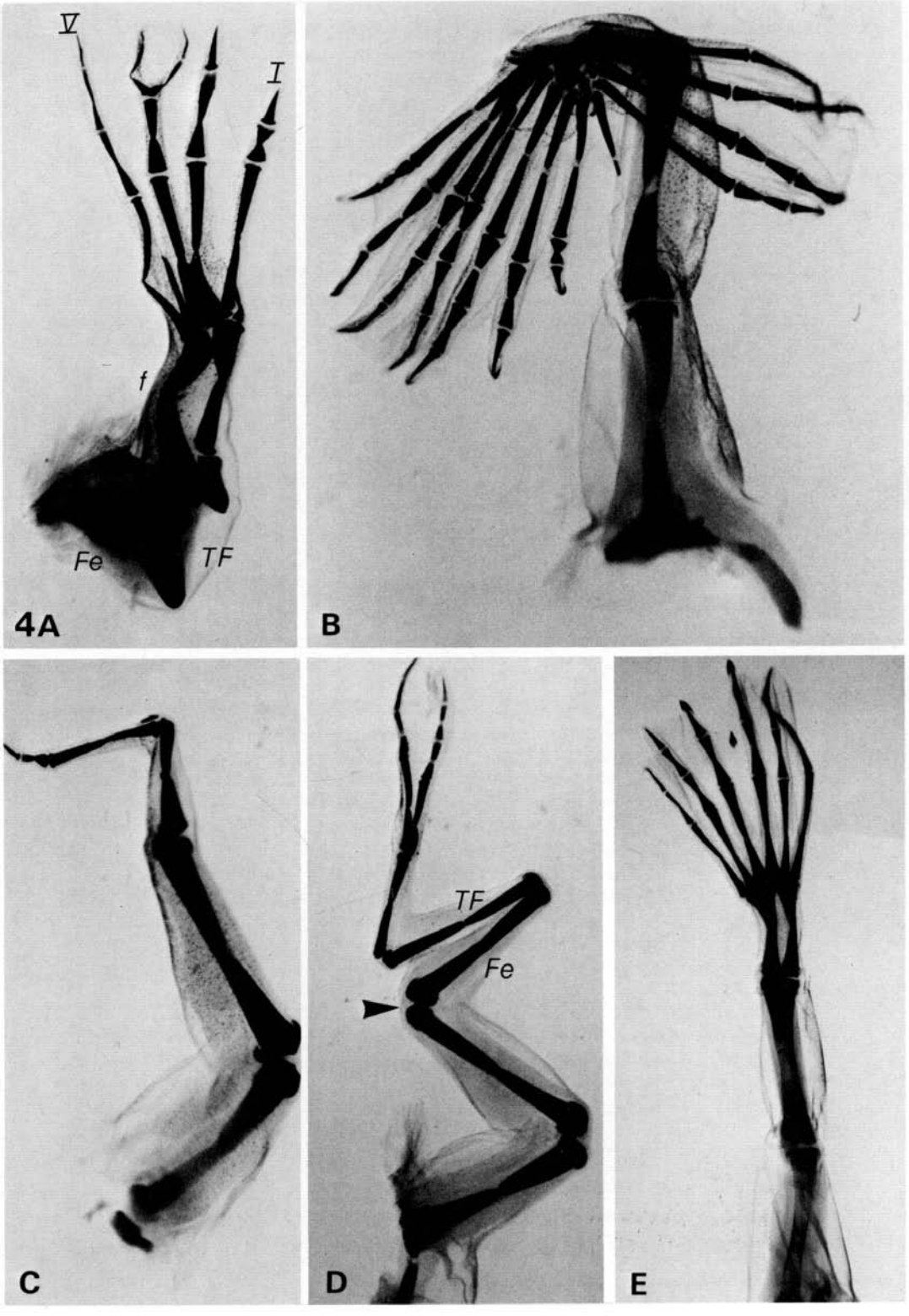
case, since duplicated limbs could also exhibit hypomorphic feet or carry skeletal defects (beyond those involved in the duplication). The commonest defect observed in about one third of the cases was for the skeletal element closest to the amputation plane to be reduced or defective (Fig. 3F). Since at stages 50 to 52 the limbs were amputated before any of the skeletal elements were visible, it was impossible to identify with certainty the amputation level in the regenerate. Consequently, it was not certain if the defective element was the most distal part of the stump, or the most proximal part of the regenerate (cf. legend for Fig. 3F). In addition, in about one quarter of the duplications either the leg bones of the duplicated regenerate were defective (Fig. 3E) or the foot was hypomorphic (Fig. 4D) or both. Overall, the response of regenerating limbs to retinol palmitate was quite variable and the type of response observed was not correlated with concentration. However, duplications occurred much more frequently at longer treatment times. With one-day treatments, there were no duplications. Of the 25 duplications observed, four were seen at 3 days, five at 7 days, and sixteen at 14 days of treatment. The optimal conditions for inducing duplications are 0·3 i.u. ml^{-1} for 14 days. These conditions induced duplications in four out of seven cases at stage 51, seven out of seven cases at stage 52, and three out of seven cases at stage 53 (Table 1). Similarly, the optimal stage for inducing duplications in regenerating limbs was stage 52, when ten out of twenty-one limbs responding to the treatment gave duplications (Table 2).

Fig. 3. This figure shows the effect of retinol palmitate on developing left hindlimbs of *Xenopus laevis* tadpoles (A,B,C) compared to its effect on the contralateral regenerating right hindlimbs in the same three tadpoles (D,E,F respectively). The tadpole limbs shown in D & E were amputated at stage 51 and that shown in F was amputated at stage 52. All were treated with 0·3 i.u. ml^{-1} retinol palmitate for 14 days. (A) Retinol palmitate treatment resulted in moderately hypomorphic foot as a result of the loss of two digits during development. Coded as MH. Magnification × 9. (B) Although the foot is almost normal, the tibia–fibula (*TF*) is quite defective. It appears that the tibia–fibula has bent around on itself and then fused in this position, shortening the leg and bringing the foot close to the femur (*Fe*). Coded as P^2. Magnification × 11. (C) While the leg is normal, the metatarsals are defective. Metatarsals I and V are very thin, and metatarsals I, II, and III have partially fused. Coded as SH. Magnification × 9. (D) The limb appears to have been amputated midway through the tibia–fibula (arrowhead) and the regenerate shows AP duplication beginning with part of a femur (*Fe2*). Coded as D4. Magnification × 8. (E) The limb appears to have been amputated through the proximal tibia–fibula (arrowhead). Although it is not clear from this photograph, the regenerated tibia (*T*) and fibula (*F*) are quite separate from the stump tibia–fibula, hence indicating a duplication of these elements. Coded as D3. Note that the skeletal elements closest to the amputation plane are defective, while the rest of the regenerate is normal. Magnification × 10. (F) The amputation level here is uncertain and could lie anywhere between the arrowheads, i.e. somewhere between the middle and distal end of the tibia–fibula. Consequently, it is not certain if the skeleton between the arrowheads is a defective stump tibia–fibula or a defective regenerated femur or both. The skeletal element protruding laterally seems likely to be a regenerated distal head of a femur (*Fe2*). This specimen also shows two additional digits (*d*) adjacent to a very reduced digit I. Coded as D4–D6. Magnification × 8.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DISCUSSION

This investigation analysed the response of developing and regenerating *Xenopus laevis* tadpole limbs to retinol palmitate administered at stages 50 through 54 at several combinations of concentration and treatment time. The response of developing limbs was quite uniform. In all cases, when there was an observable response to the retinoid treatment, that response was a dose-related deletion of skeletal elements and a reduction of the limb, varying from slightly hypomorphic limbs to cases of total inhibition of limb development. On the other hand, regenerating limbs gave a wide variety of responses including PD duplications, AP duplications, hypomorphic or phocomelic limbs, and complete inhibition of regeneration. This drastic difference in response to retinoids between developing and regenerating limbs noted here in an anuran and previously in the axolotl (Scadding & Maden, 1986) appears to be a consistent characteristic of amphibian limbs. This suggests that there might be significant differences in the underlying pattern regulation mechanisms. On the other hand, Muneoka & Bryant (1982) showed that in axolotls regeneration blastemas and developing limb buds were interchangeable and concluded that the patterning mechanism in developing and regenerating limbs was the same. It is apparent that developing and regenerating limbs are similar in some ways and different in others. Clearly, in their response to vitamin A they are different.

The shortened and bent leg bones observed in *Xenopus* were very similar to those reported in mice (Kochhar, 1977). Kochhar attributed the defects in mammals mainly to the inhibitory action of vitamin A on chondrogenesis and the cell cycle, although a possible inhibition of cell migration was also considered. Our observations on developing *Xenopus* limbs could be explained through similar mechanisms. However, it seems unlikely that the duplications seen in regenerating limbs could be due to the inhibition of these cellular processes.

Fig. 4. (A) Intact developing left hindlimb treated at stage 51 (3 i.u. ml^{-1}, 3 days). At this higher dose level, effects of retinol palmitate are more severe. Femur (*Fe*) and tibia–fibula (*TF*) are reduced to small triangular cartilages. Fibulare (*f*) is bent. Metatarsal IV is extremely reduced and lacks phalanges; metatarsal V is thin and bent; metatarsal II and III have fused proximally. Phalanges on digit III have bifurcated. Coded as MH–P^{123}. Magnification × 10. (B) Regenerated right hindlimb treated at stage 51 (0·3 i.u. ml^{-1}, 3 days). There are thirteen digits arranged as a primary foot and two accessory feet. The digital sequence from dorsal to ventral (right to left in the photograph) is V–IV–III–II–I–I–II–III–IV–V(very small and not visible in photograph)–IV–III–II. This is the most extensive AP duplication seen in this investigation. Coded as D7. Magnification × 10. (C) Regenerating right hindlimb contralateral to that in A above. It is extremely hypomorphic (EH) with only one digit, but without any duplication of skeletal elements. Magnification × 10. (D) A regenerating right hindlimb treated at stage 53 (0·3 i.u. ml^{-1}, 14 days). This limb shows complete duplication of the entire limb distal to the amputation level (arrowhead). Regenerate included femur (*Fe*), tibia–fibula (*TF*), and an extremely hypomorphic foot with only two digits. Coded as D5–EH. Magnification × 10. (E) Regenerated right hindlimb treated at stage 53 (1·0 i.u. ml^{-1}, 3 days). Limb exhibited AP duplication. The six digits were arranged in the sequence V–IV–III–III–IV–V indicating a partial mirror-image duplication. Coded D6. Magnification × 6.

Our initial choice of doses of retinol palmitate (15 and 75 i.u. ml^{-1}) were based on experience in other amphibians (Maden, 1983*a*,*b*; Niazi & Ratnasamy, 1984) and with postmetamorphic *Xenopus* (Scadding, 1983). However, it soon became apparent that these concentrations were lethal to *Xenopus* tadpoles. We found empirically that concentrations of 0·3 to 1·0 i.u. ml^{-1} were optimal for inducing teratogenic defects and duplication in *Xenopus* limbs. This is two orders of magnitude less than the optimal dose for axolotls. This great difference may be due to the fact that the retinoids administered in aquarium water are not dissolved but are suspended. *Xenopus* is a filter feeder and its filter apparatus may be very efficient at removing the suspended retinoids from the water. Other amphibians may only obtain the suspended retinoids through ingestion while feeding or by skin absorption. Similarly, the different efficacies of retinol acetate, retinol palmitate, retinol, and retinoic acid previously reported (Maden, 1983*a*) may be due to differential uptake rather than to differential cellular activities.

Loss of the capacity to produce duplications in *Xenopus* was correlated with the general loss of regenerative ability. Up to stage 52, regeneration is approximately normal and even at stage 53 is only slightly hypomorphic. At these stages, duplications were frequently induced in regenerating limbs by retinol palmitate. However, at stage 54, regenerative ability significantly declined, normal regeneration was rare, and a typical regenerate at this stage only had two or three digits. Also, at stage 54, duplications were rare (only one case was observed) subsequent to retinol palmitate treatment. It appears that the capacity to control the pattern of the regenerate is lost at stage 54, and consequently any ability of retinoids to alter pattern regulation and induce duplications would also be lost simultaneously. At stage 54, the main effect of retinol palmitate is to inhibit the reduced limb regeneration which does occur. Similarly, in postmetamorphic *Xenopus* where the 'regenerate' consists of a single cartilaginous spike, the effect of vitamin A is solely to inhibit this regenerative outgrowth (Scadding, 1983). Recently, Niazi, Pescitelli & Stocum (1985) reported that in urodeles, whether one obtained duplications or deletions as a result of vitamin A treatment was a function of the stage of regeneration at which the treatment was administered. Our present results in *Xenopus* are consistent with this observation.

It appears likely that there are two separate effects of vitamin A on regenerating limbs: an inhibition of cell proliferation and chondrogenesis which affects both developing and regenerating limbs, and a specific effect on pattern regulation which is seen in regenerating but not in developing limbs, leading to duplications. A hypothetical dose–response curve is given in Fig. 5. The morphogenetic effects on regenerating pattern are arbitrarily represented as a straight line running from the origin to the upper right. The lower part of this line may represent deletions due to resetting the positional code of the blastema cells to a level distal to the actual amputation plane (A). The mid part of the line may represent duplications due to resetting the positional code to levels proximal to the actual amputation plane (B). The upper right part of the line may represent other morphogenetic effects of retinoids such as AP duplications (C). This hypothesis is based in part on

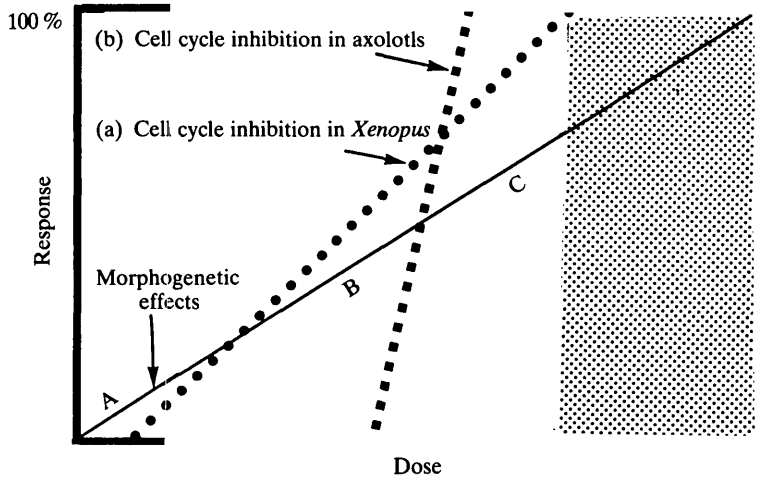

Fig. 5. Hypothetical dose–response relationships. The solid diagonal line represents morphogenetic effects of vitamin A and the dotted lines represent cell cycle inhibition in: (a) *Xenopus* (circles), and (b) axolotls (squares). See text for explanation.

the dose-related sequence of vitamin A effects as previously reported for the axolotl (Scadding & Maden, 1986).

However, simultaneously there is a second and independent inhibition of the cell cycle represented by the dotted line (circles). Thus, in the middle of the morphogenetic response range, inhibition of the cell cycle may seriously interfere with the regeneration of the duplicated limb, resulting in hypomorphic or defective duplicated limbs. But presumably, at some dose level, inhibition of the cell cycle is complete and no regeneration whatever can occur. This leaves a region of high dose levels (grey area) in which there are theoretically possible morphogenetic effects which cannot be expressed due to complete inhibition of the cell cycle. It is possible that AP duplications could lie largely in this region, and hence are infrequent in *Xenopus*.

Axolotls produce hypomorphic duplicated limbs at a much lower frequency than *Xenopus* (Scadding & Maden, 1986; Niazi *et al.* 1985). This could be due to a reduced susceptibility of the cells to the cell-cycle-inhibiting action of retinoids, such that it requires a much higher dose to have any observable effect, but then with a more vertical dose–response curve represented in Fig. 5 by the line of squares. A downward displacement of the point at which 100 % inhibition is reached might explain why some effects such as AP duplications are observed in *Xenopus* (and also *Rana*, Maden, 1983*b*) but not in axolotls. In axolotls, those high-dose morphogenetic effects may be masked by total inhibition of the cell cycle.

One possible explanation for the differences between regenerating and developing limbs may be that the wounding associated with amputation is important for the induction of duplications. This hypothesis receives support from observations on chick embryos in which duplications are only observed when vitamin A is

administered by implantation on paper (Summerbell, 1983) or on ion exchange beads (Eichele, Tickle & Alberts, 1984; Tickle, Lee & Eichele, 1985); both are methods which cause a significant wound in the limb bud. However, if vitamin A is administered systemically, it does not cause duplications (Summerbell & Harvey, 1983). It is conceivable that wounding or amputation results in the release of mitogenic factors which then counteract the cell-cycle-inhibiting effects of retinoids, allowing the expression of morphogenetic phenomena otherwise masked by cell-cycle inhibition, i.e. the dose–response curve for cell-cycle inhibition in Fig. 5 is shifted to the right. It might also be possible that limb amputation switches on the synthesis of proteins which can bind retinoids and that this retinoid binding then can influence morphogenesis, allowing a range of morphogenetic phenomena not observable in the developing limb. These hypotheses are currently being tested.

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