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The Effects of Retinoic Acid on the Regenerating Limbs of the Larval Korean Newt (*Hynobius leechii*)

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The effects of retinoic acid (RA) on the regenerating limbs of Korean newt (*Hynobius leechii*) larvae have been studied. Intraperitoneal injection of RA at 4 days post-amputation caused the proximalization of regenerate structures in the proximodistal (PD) axis of the limbs amputated through either the distal zeugopodium or the distal stylopodium. The mean level of proximalization (MLP) increased as a dose-dependent manner, and the MLP was also dependent on the level of amputation at a given dose of RA. At the dose of 150 μg/g body wt., MLP's in either amputation level reached close to a peak value, and an increasing number of inhibition of regeneration or duplication in the transverse plane occurred at the dose of 200 μg/g body wt. and above. The changes induced by RA treatment suggest that positional values in the cells of the regenerating limbs have been modified in the three cardinal axes including PD and transverse axes in a graded mode. Furthermore, from the comparison of data in two different amputation levels, it can be speculated that the sensitivity of cells to RA effect along the PD axis might not be linear.

KEY WORDS: ***Hynobius leechii*, Limb regeneration, Retinoic acid, Positional value, Pattern formation**

Amphibians possess remarkable power to regenerate the parts of their bodies damaged or lost by accidents or attacks from enemies (Goss, 1969). Especially, limbs of amphibians can easily be regenerated, and hence, have been used extensively in the study of regeneration as a model system of pattern formation.

Briefly, regeneration of amphibian limbs proceeds as follows. Immediately after amputation, the amputation surface is covered by actively dividing and migrating epidermal cells in the stump close to the amputation level. The epidermis covering the amputation surface is now called 'wound epidermis' and an extensive disorganization of stump tissue begins under the wound epidermis. As a result of the dissolution of stump tissues, cells in the stump lose their characteristics of differentiated state and now they are called 'dedifferentiated cells.' Those dedifferentiated cells soon start to divide and accumulate under the would epidermis, and form a pile of cells which is called a 'blastema'. As a result of active cell division, the size of a blastema increases with time and cells start to condense and produce cartilagenous matrix at the central region of a blastema. At the same time, at the distal region of a blastema, an outline of digits slowly appear and finally form the complete replica of the lost part of a limb (Stocum, 1979). The rate of limb regeneration is quite variable among species of amphibians, and urodeles usually have limb regenerative capacities entire their life whereas anurans retain the complete regenerative capacities only during their larval life (Scadding, 1977).

In the process of limb regeneration, only the parts distal to the amputation level can be regenerated. This phenomenon is referred to 'the rule of distal transformation' and interpreted to

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mean that position-related properties in the blastema cells can only be distalized to form the distal structures (Rose, 1970). These position-related properties are usually called 'positional values' which are thought to be imprinted on cells as a result of interpretation of positional information in a given field of a developing system (Wolpert, 1969, 1971). Those positional values may specify the location of cells in the three cardinal axes, proximodistal (PD), anteroposterior (AP), and dorsoventral (DV) axes, of a given system. Therefore, to understand the patterning mechanisms of a developing or regenerating system, it appears to be essential to know the nature of positional informations and positional values and how these properties are set and interpreted in the process of development or regeneration.

Recently, a group of compounds related to vitamin A, collectively called retinoids, has been shown to evoke drastic alterations in the patterns of developing or regenerating limb structures. When a small amount of retinoic acid (RA) is applied to the developing chick wing bud, an AP mirror-imaged duplicate of digits appear from the anterior side of a wing bud (Tickle *et al.*, 1982; Summerbell, 1983; Han and Kim, 1988). In amphibians, retinoids such as retinoic acid, retinol palmitate, and arotinoid induce the duplication of limb skeletons in the PD axis and often in the transverse axes when limbs were amputated and treated properly (Maden, 1982; Maden, 1983; Thoms and Stocum, 1984; Kim and Stocum, 1986a). In addition, RA can complete the AP pattern from the anterior half stump in the regenerating axolotl limbs (Kim and Stocum, 1986b). These results suggest that positional values which appear to specify the level of structures might have been changed with RA-treatment. Therefore, retinoids are generally considered to be a promising tool to find the nature of positional information and positional values and to elucidate the molecular mechanisms of pattern formation.

The present study was performed to evaluate the effect of RA on the regenerating limbs of as yet untested Korean newt (*Hynobius leechii*) larvae in the aspects of dose-response relationship and amputation level specific responses.

Materials and Methods

Animals & maintenance

Larval Korean newts, *Hynobius leechii*, were used in this experiment. At room temperature, larvae hatched in about 10 days from naturally spawned eggs. Larvae were kept in 20% Holtfreter solution and fed freshly hatched brine shrimp every day until 3 weeks after hatching. Thereafter, animals were reared individually in waxed paper cups and fed finely chopped beef liver. Around 6-7 weeks after hatching, animals had fully grown forelimbs and weighed 0.2-0.3 grams.

Amputation levels

The forelimbs of each animal were amputated bilaterally through either the distal zeugopodium (just above the wrist) or distal stylopodium (just above the elbow). Any limb cartilage protruding after amputation was trimmed to give a flat amputation surface.

Preparation and administration of retinoic acid

Retinoic acid (all trans type XX: Sigma) was dissolved in dimethylsulfoxide (DMSO) to make a stock solution of 50 $\mu g/\mu l$ (0.166 M). The stock solution was made up under subdued light to minimize photooxidation, and used fresh. Each animal was weighed on a top loading balance. A microliter syringe (Hamilton, USA) was used to inject each animal intraperitoneally with the desired dose of retinoic acid per gram of body weight. Control animals were injected with an amount of DMSO equivalent to the amount used to deliver the retinoic acid. All animals were injected at 4 days post-amputation, since the previous study have shown that regenerating axolotl limbs are most sensitive to the duplicating effects of RA at this stage of regeneration (Thoms and Stocum, 1984).

Staining and analysis of limb skeletons

Limbs were allowed to regenerate for 6-7 weeks after RA injection, then were fixed in Gregg's fixative and depigmented in hydorgen perioxide. Depigmented limbs were stained in Vic-

Table 1. Scoring system for the duplication of skeletons resulting from RA-treatments in regenerating newt limbs amputated through the distal zeugopodium.

Degree of duplication(DD)	Characteristics
0	normal regeneration
0.5	partial duplication of zeugopodium
1.0	complete duplication of zeugopodium
1.5	partial duplication of stylopodium
2.0	complete duplication of stylopodium
2.5	partial duplication of girdle

Table 2. Scoring system for the duplication of skeletons resulting from RA-treatments in regenerating newt limbs amputated through the distal stylopodium.

Degree of duplication(DD)	Characteristics
0	normal regeneration
0.5	partial duplication of stylopodium
1.0	complete duplication of stylopodium
1.5	partial duplication of girdle

toria blue B and cleared in methyl salicylate (Bryant and Iten, 1974).

Each regenerate was scored, as outlined in Tables 1 and 2, for the number of stump segments duplicated, with partially duplicated segments being scored as half segments. Since only partial girdles were ever duplicated, a girdle duplication was always counted as a half segment. The mean number of segments duplicated at each dose of RA was then calculated and termed the *mean level of proximalization* (MLP). The maximum possible MLP for a regenerated derived from the distal zeugopodium is 2.5; for a regenerate derived from the stylopodium, it is 1.5.

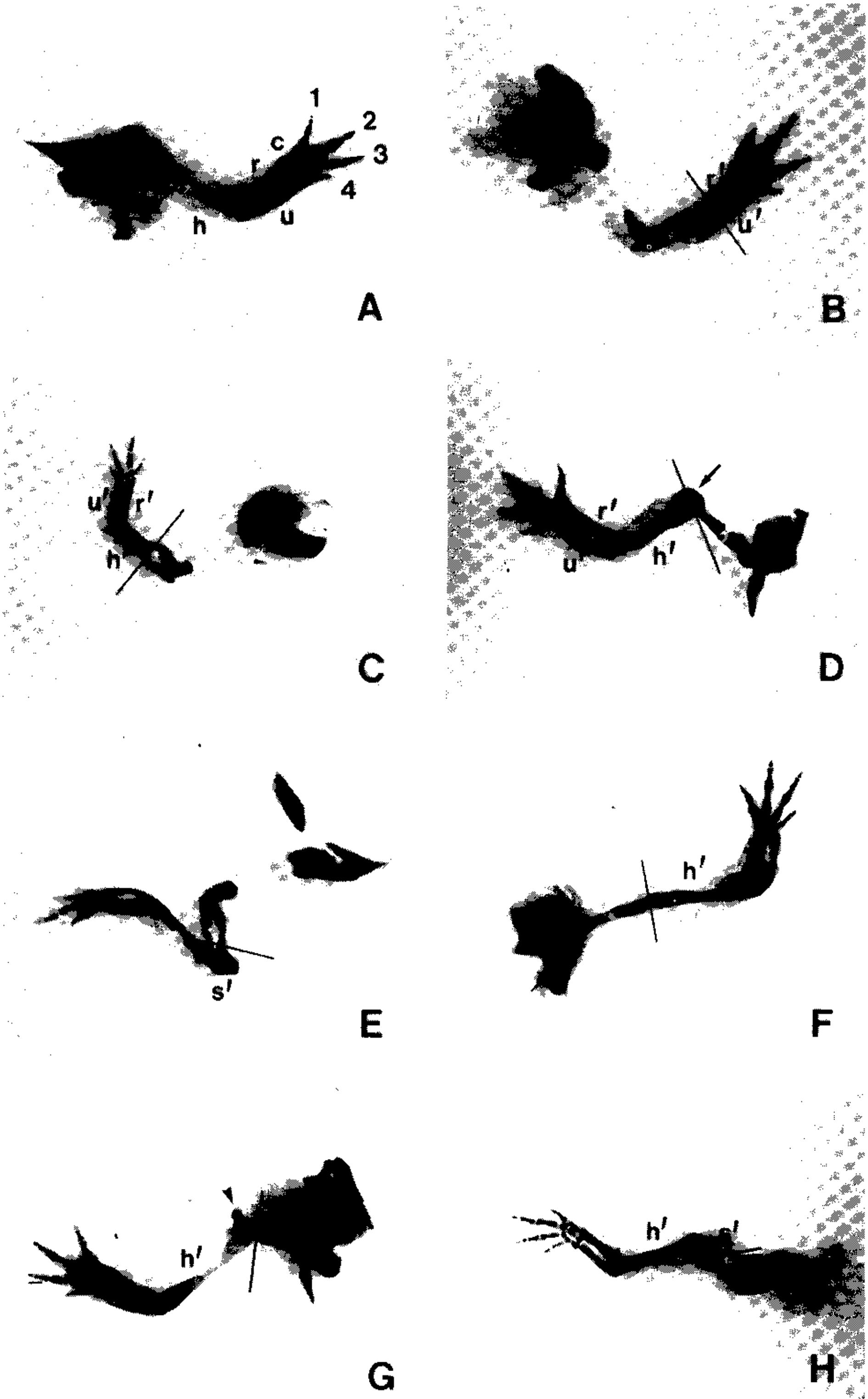
Results

Morphology of PD-dupulicated regenerates.

Regeneration from the distal zeugopodium. Retinoic acid treatment induced the duplication of stump skeletal elements in the regenerates derived from the distal zeugopodium (Fig. 1 A-E). The types of regenerates were variable from partial duplication of radius and ulna to partial duplication of girdle. When the radius and ulna were partially duplicated, the overall zeugopodial segment was longer than the normal regenerate due to the extension of duplicated skeletons to its stump. However, when the stylopodial skeletal element (humerus) duplicated, its articulation with the stump skeleton was not straight instead produced a twisted overall limb morphology.

Regeneration from the distal stylopodium. Retinoic acid treatment induced the duplication of stump skeletal elements ranging from partial humerus to partial girdle (Fig. 1 F-J). In extreme cases, the regeneration was completely inhibited or only hypomorphic regeneration occured. When the humerus was partially duplicated it usually fused with the stump humerus giving rise to abnormally long humerus in the regenerate. However, when a complete humerus or girdle was duplicated its connection with the stump skeletons was not straight and made an angle of up to 90°.

Duplication at the transverse plane. Supernumerary limbs regenerated in the transverse plane in groups of high dose retinoic acid treatment (Fig. 1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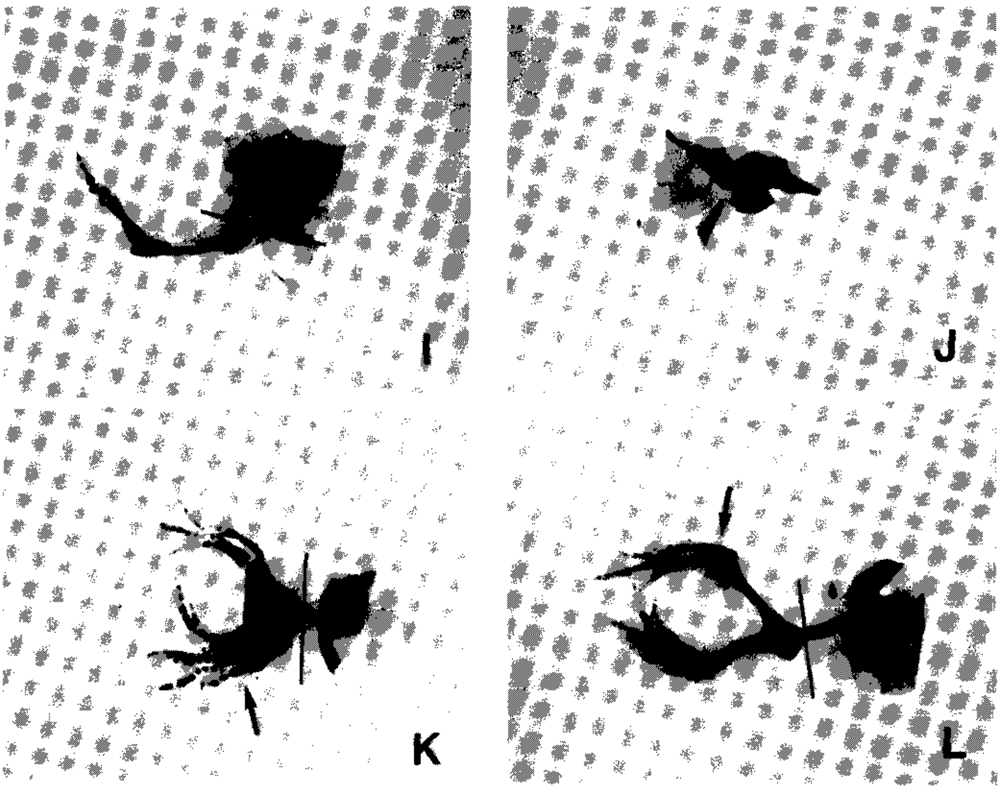

Fig. 1. Regenerates formed after amputation of forelimbs through either distal zeugopodium (A-E) or distal stylopodium (F-L) and injection of retinoic acid at 4 days post-amputation. The lines indicate the junctions between stumpts and regenerates. Magnifications, 8 ×. A. Normal regenerate. h: humerus, r:radius, u:ulna, c:carpals, 1-4 anterior to posterior sequence of digits. B. Partial duplication of radius (r') and ulna (u'). C. Partial duplication of humerus (h') and complete duplication of radius (r') and ulna (u'). D. Complete duplication of humerus (h'), radius (r') and ulna (u'). Note the extreme regression of stump zeugopodial elements, radius and ulna (arrow). E. Duplication of a complete limb including scapula (s'). F. Partial duplication of humerus (h'). Due to the direct connection of duplicated humerus with the stump humerus, overall length of the stylopodial segment is abnormally long. G. Complete duplication of humerus (h'). The protruding process (arrow head) of the duplicated humerus indicating the entire stylopodial element has been duplicated. H. Duplication of scapula (s') in addition to humerus (h'). I. Hypomorphic regeneration. Even though three segmental elements (stylopodial, zeugopodial, autopodial elements) are present, ulna and most of the carpals and fingers are missing. J. Inhibited regeneration. Only a part of the stump skeletons remains. Also note the extreme regression of the stump skeleton. K, L. Regeneration of supernumerary limbs which also show duplications in the proximodistal axis. Note extra limbs (arrow) with complicated digital patterns. Actually those limbs are composed of two limbs which are fused at the right angle.

K, L). More often, the supernumerary structures were complex and many skeletal elements were shared along the PD axis especially at the autopodial level.

Effects of RA with different dose and at different amputation level.

The most conspicuous results obtained from the RA treatment was the duplication of proximal skeletal structures from the distal amputation level. The degree of duplication increased as a dose-dependent manner, However, at high dose, either the inhibition of regeneration or the duplication in the transverse plane occured in a few cases, and the number of cases also increased with the increase of dose (Table 3).

Table 3. Dose-dependent duplication of stump skeletons in limb regenerates of Korean newt(*Hynobius leechii*) larvae.

Dose*	Amputation Level	Total Cases	Inhibition of Regeneration	Transverse Plane Duplication	PD Axis Duplication Degree of Duplication							Mean Level of Proximalization (MLP)
					Total cases	0	0.5	1.0	1.5	2.0	2,5	
0	Z	35	0	0	35	35	0	0	0	0	0	0
	S	10	0	0	10	10	0	0	0	0	0	0
50	Z	44	0	0	44	10	18	5	9	2	0	0.72
	S	33	0	0	33	18	11	4	0	0	0	0.29
100	Z	45	1(2)	0	44	7	5	6	23	2	1	1.13
	S	34	0	1(3)	34	6	6	21	1	0	0	0.75
150	Z	46	0	0	46	0	5	1	25	13	2	1.57
	S	40	12(30)	0	28	1	2	16	9	0	0	1.09
200	Z	41	1(2)	1(2)	40	0	4	4	12	16	4	1.65
	S	34	6(18)	1(3)	28	0	2	18	8	0	0	1.11
250	Z	45	4(9)	4(9)	41	1	5	4	6	15	10	1.72
	S	38	9(24)	7(18)	29	0	4	4	21	0	0	1.29
300	Z	49	12(24)	3(6)	37	0	2	0	9	8	18	2.04
	S	31	12(39)	4(13)	19	0	1	10	8	0	0	1.18

* μg of RA/g body wt. ( ): percentage
Z: Distal zeugopodium S: Distal stylopodium
(a) : Limbs showing transverse plane duplication also showed duplications in the PD axis.

At the dose of 150 μg RA/g body wt., the MLP reached to 1.57 from the distal zeugopodial level, while from the distal stylopodial level the MLP was 1.09. The values from both levels showed only slight changes at highter doses. A few cases of inhibition of regeneration occured at 200 μg RA level and the tendency was particularly noticeable in the distal zeugopodial amputation level at 250 μg RA level or higher, and also in the distal stylopodial amputation level at 150 μg or higher doses. The increase of dose also caused the duplication of skeletal structures in the transverse plane especially at the dose of 250 μg RA and above. With the same dose, the duplications in the transverse plane were more easily invoked at the distal stylopodial level of amputation than at the distal zeugopodial level of amputation.

Discussion

In the present study, RA treatment on regenerating limbs of Korean newt (*Hynobius leechii*) larvae invoked the duplications of stump skeletal structures as previously shown in other newt or salamander species (Maden, 1982; Thoms and Stocum, 1984; Kim and Stocum, 1986a). In brief, the results were: (1) RA induced duplication in both proximodistal and transverse axes; (2) the mean level of proximalization (MLP) increased with dose-dependent manners; (3) with same dose of RA, MLP was higher at the distal amputation level than at the proximal amputation level; (4) at the doses beyond one producing maximum duplication in the PD axis, regeneration was often inhibited and the incidence of duplications in the transverse plane increased concomitantly.

Morphologically, the connection between the stump skeleton and the duplicated structure was somewhat peculiar such that the duplicated structure tended to arise from the stump with an angle and from the anterior part of the stump amputation surface. Kim and Stocum (1986a) observed the same phenomenon in the RA-treated axolotl limbs during regeneration. Such phenomenon was especially obvious when the maximum duplication was obtained with RA treatment. It might be possible that the duplicates were actually arose from the anterior part of the stump amputation plane. This speculation can be supported with the observation that RA cause completion of AP pattern only from the anterior half and not from the posteroir half of limbs (Kim and Stocum, 1986b). Another speculation might be that RA-invoked changes of the cell surface in the duplicated structures were too drastic to make a smooth connection with the stump so that the two groups of cells each comprising the duplicated or the stump skeletons behaved to minimize the contact between them. The above plausibility is in a good line with the histological observation that the blastema cells tended to form an isolated ball of cells from the stump cells (Kim and Stocum, 1986c). The experimental results that the behavior of cells of different origin in regenerating axolotl limbs reflects their cellular adhesivity also support the second alternative (Nardi and Stocum, 1983). Therefore, it might be worth while to examine the cellular contribution of the stump tissue upon RA-treatment using cell markers.

At high doses of RA treatment, an appreciable number of duplications in the transverse plane has been observed. The number of cases was much higher in *Hynobius leechii* than in other urodele species (Thoms and Stocum, 1984; Kim and Stocum, 1986a). RA-induced pattern duplications in the transverse plane have been observed in the regenerating limbs of anuran species (Niazi and Saxena, 1978; Maden, 1983) and in the developing wingbuds of chick embryos (Tickle *et al.*, 1982; Summerbell, 1983; Han and Kim, 1988). At present, it is not clear whether above observed results relfect only the phylogenetic differences inherent among species or age-dependent phenomenon in the course of growth from a larva to an adult.

The mean level of proximalization of duplicated skeletons was dependent on the dose of RA treatment, *i. e.*, with increasing dose, more proximal structure has been duplicated. Such phenomenon might be due to the graded changes in the positional memory of dedifferentiated cells of the blastemas as a result of RA treatment (Stocum, 1988). Currently, RA effect is thought to be mediated by the cellular retinoic acid binding protein (CRABP) and the retinoic acid receptors (RAR's) in the nucleus (McCormick *et al.*, 1988; Maden, 1988; Ragsdale *et al.*, 1989). In this process, CRABP seems to regulate the concentration of free RA in the cytoplasm and the translocated free RA in the nucleus appears to bind RAR to form RAR-RA complex and this complex recognize specific binding site of a set of genes involved in the limb regeneration. Ultimately, the dose-response effect of pattern duplication invoked with RA treatment might reflect the changes of gene expression with the increase of ligand-receptor complex (Eichele, 1989).

Our data showed that the degree of duplication by RA-treatment was also dependent upon the level of amputation. When the level of amputation was distal zeugopodium, zeugopodial elements were duplicated easily with low dose of RA whereas stylopodial element duplication needed high dose of RA. In the case of distal stylopodial level of amputation, high dose of RA treatment was required to accomplish duplication of the same segment (stylopodium). These observations suggest that position related properties along the PD axis in the newt limb might not be linear or sensitivity of cells in the zeugopodium and in the stylopodium might be different. Certainly, solutions on the above questions could be obtained through the study of limb regeneration at subcellular levels.

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한국산의 도롱뇽(*Hynobius leechii*) 유생의 다리재생에 미치는 Retinoic Acid의 효과

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한국산 도롱뇽(*Hynobius leechii*) 유생의 다리재생에 미치는 retinoic acid(RA)의 효과를 알아보았다. 도롱뇽 유생의 다리를 distal zeugopodium 또는 distal stylopodium 부위에서 절단하고 절단 후 4일째에 복강내 주사방법으로 RA를 처리한 결과 근원위 축(proximodistal axis)상에서 재생된 골격이 복제되어 근위부화한 형태를 나타내었다. RA처리에 따른 골격 재생의 평균 근위부화정도(mean level of proximalization)는 처리량의 증가와 더불어 증가하였으며 또한 절단부위를 달리하였을 경우에도 영향을 받았다. 근원위 축상에서 평균 근위부화정도의 최대치에 근접한 값은 처리량을 150 μg/g body wt.로 했을 때 얻어졌으며 이 처리량을 넘어서는 경우에는 재생의 저해 혹은 수배부로부터의 골격복제 현상이 나타났으며 그 빈도 역시 처리량의 증가와 함께 상승하였다. 이러한 실험결과는 RA처리에 의하여 유발되리라고 보여지는 positional value의 변화가 근원위 축을 비롯한 3개의 주요 축상에서 점진적으로 일어남을 시사하며, zeugopodium의 복제가 stylopodium의 복제보다 쉽게 일어난다는 사실은 근원위축상에서 세포들의 RA에 대한 민감성이 동일하지 않거나 혹은 positional value의 배열양상이 직선적이 아님을 시사하고 있다.