CHAPTER VI

INVOLVEMENT OF cAMP IN TAIL REGENERATION IN THE SCINCID LIZARD, MABUYA CARINATA AS EVALUATED BY THE ACTIVITY LEVELS OF cAMP PHOSPHODIESTERASE : A SYSTEMIC AND LOCAL ANALYSIS

Since the discovery of cyclic 3',5'-adenosine monophosphate (cAMP) by Sutherland and his colleagues (Rall et al., 1957), this cyclic nucleotide has gained wide recognition as an essential link in the molecular chain of events that constitute specific cellular responses to various stimuli that impinge upon the cells in vivo. According to the 'second messenger' hypothesis, cAMP is supposed to act as the second messenger which is derived from ATP intracellularly by a membrane bound enzyme adenylate cyclase in response to the first messenger (hormone and/or any other agent) combining with its specific surface receptors. cAMP so produced, acting either directly or indirectly and by triggering a cascade of events then commits the cells to certain specific actions such as division, differentiation, macromolecule synthesis, metabolic alterations etc. Evidences are now available for the involvement of cyclic nucleotides in cell division (Thomas et al., 1973; Berridge, 1975; Carlone and Foret, 1979), cell differentiation (Friedman, 1976; Miller, 1977; Taban and Cathieni, 1978; Kosher and Savage, 1980), macromolecule synthesis (Sharma and Talwar, 1970; MacManus et al., 1972, 1973; Babich and Foret, 1973; Dokas, 1973; Foret and Babich, 1973; Short et al., 1975) and metabolic alterations (Rall et al., 1957; Drummond et al., 1969; Beriz et al., 1977; Ishibashi and Catton, 1978).

As a process which includes a conglomerate of all the above events, regeneration qualifies as an ideal system for evaluating functionally correlatable changes in cAMP level. Todate in fact only two attempts have been made in this context to understand the involvement of cyclic nucleotides in regeneration (Jabaily et al., 1975; Liversage et al.,1977). Both these studies (the first one on cAMP and the second one on cGMP) have been confined to newt limb regeneration. Extension of such a study to reptilian tail regeneration has not yet been attempted. Moreover, a quantitative evaluation of cAMP Phosphodiesterase (EC 3.1.4.17) (PDE) activity levels, the only known hydrolytic enzyme of cAMP (Butcher and Sutherland, 1962), though done in many biological systems has not, however, received any attention with respect to vertebrate reparative regeneration. It is known that the activity levels of phosphodiesterase can give an indirect estimate of the prevailing concentration of cAMP in any tissue (Butcher and Sutherland, 1962). Due to this fact, and owing to our practical limitations in the direct assay of cAMP, quantitative evaluation of alterations in PDE activity was thought worthwhile in the regenerate during different phases of tail regeneration in the Scincid lizard, Mabuya carinata. Since we are currently focussing our attention on involvement of systemic factors too in regeneration, liver and skeletal muscle PDE levels were also assayed along with. Apart from giving insight into the systemic responses in operation, the present study, it is hoped, might also enable in understanding the possible mechanisms of regulation of PDE activity.

MATERIALS AND METHODS

Healthy Mabuyas of both sexes weighing around 20-24 g, obtained from Hyderabad, India were maintained on insect diet at Baroda. Autotomy of the tail was done by pinching it off from 1.5-2.0 cm distal to vent. The animals with regenerating tail were sacrificed under mild anaesthesia at fixed intervals of 3, 5, 7, 10, 12, 15, 25, 40 and 60 days post-autotomy along with normal animals with intact tails. The visceral organs, liver and skeletal muscle as well as the tail (regenerating or normal, as the case may be) were quickly removed and the tissues weighed and homogenized in icecold redistilled water. A 2% homogenate was prepared for liver and skeletal muscle whereas a 4% homogenate was found satisfactory in the case of the tail tissue. In the crude homogenate the activity of cyclic AMP phosphodiesterase (PDE) was estimated according to the method described by Butcher and Sutherland (1962). The inorganic phosphate thus released due to enzymatic action was estimated by the method of Fiske and Subbarow (1925).

The amount of protein was estimated in the same homogenate by the method described by Lowry et al. (1951).

For each day and each tissue specified a total of five to seven determinations were made. The mean and standard deviation were obtained and students' 't' test was used to determine statistical significance.

RESULTS

In animals with original intact tail the levels of PDE was comparatively high in the tail followed by the skeletal muscle and liver in that order. Immediately after autotomy (3rd day) the levels of this enzyme dropped significantly in the case of tail and muscle and non-significantly in liver. Since then there was a gradual linear increase in the activity of PDE upto 10th day post-autotomy in both tail and muscle with the levels, nevertheless, remaining subnormal. On day 12, which corresponds to early differentiation period, the PDE activity had reached slightly above normal levels in all the three tissues. Thereafter, the enzyme activity declined continuously through 15th and 25th days ultimately registering

Table 1. Quantitative levels of cAMP Phosphodiesterase in the regenerate, liver and skeletal muscle during tail regeneration in _M. carinata_.

(Values are expressed as µg phosphorus liberated/ mg protein/min.)

Periods of tail regeneration in days	Tail	Liver	Muscle
N	2.96 ±0.49	1.14 ±0.28	1.71 ±0.31
3	1.28$\overset{*}{@}$ ±0.24	1.11 ±0.09	0.63$\overset{@}{@}$ ±0.11
5	1.45$\overset{*}{@}$ ±0.21	1.57@ ±0.31	0.89@ ±0.19
7	1.62$\overset{*}{@}$ ±0.21	1.31 ±0.18	1.25 ±0.24
10	2.11@ ±0.24	1.44 ±0.24	1.52 ±0.28
12	3.14 ±0.34	1.72@ ±0.19	2.07 ±0.24
15	2.74 ±0.24	1.66@ ±0.28	0.87$\overset{*}{@}$ ±0.16
25	2.31@ ±0.25	1.44 ±0.18	0.78$\overset{*}{@}$ ±0.13
40	0.98$\overset{*}{@}$ ±0.18	0.83 ±0.03	0.41$\overset{*}{@}$ ±0.04
60	2.94 ±0.29	0.81 ±0.11	1.01$\overset{*}{@}$ ±0.18

@ $P < 0.0025$; $\overset{@}{@}$ $P < 0.001$; $\overset{*}{@}$ $P < 0.0005$.

N - Normal (Pre-autotomy state)

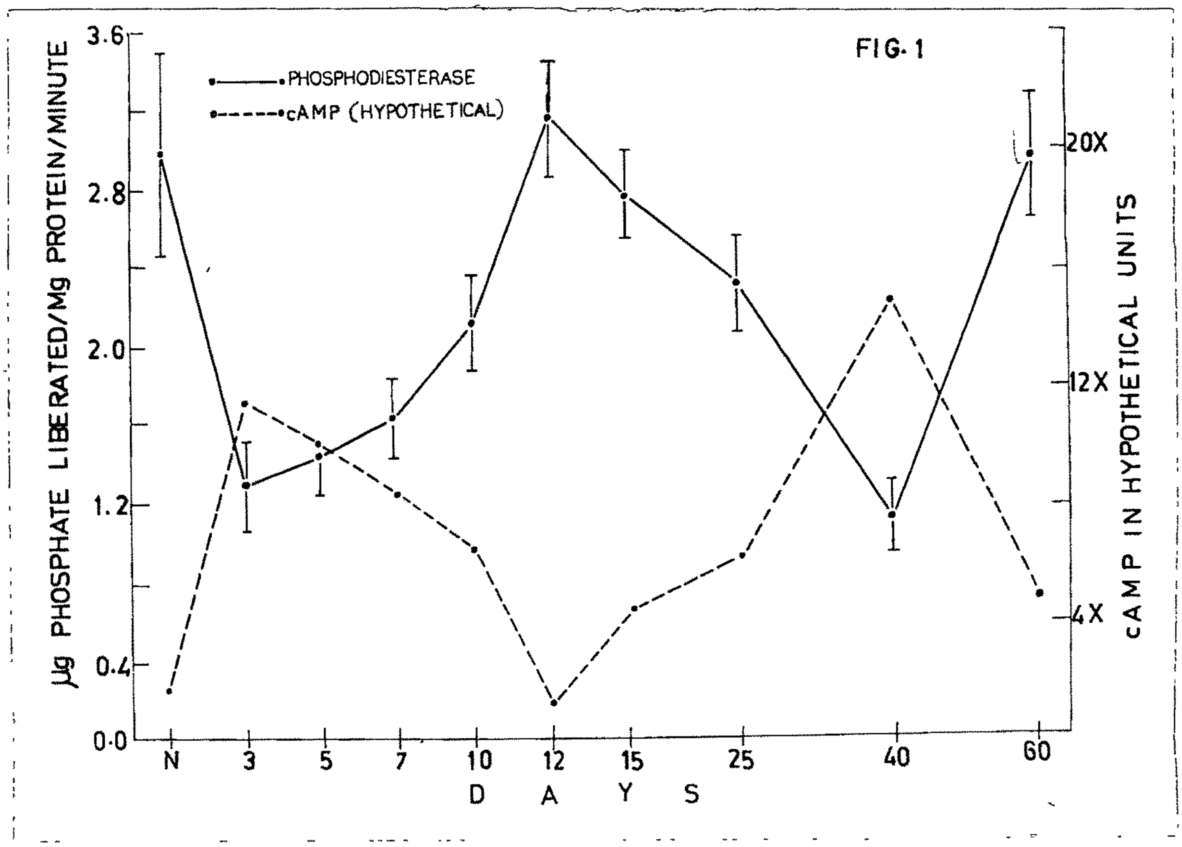

Fig. 1. Changes in cAMP phosphodiesterase activity and cAMP content (in hypothetical units) in the regenerate during tail regeneration in *M. carinata*.

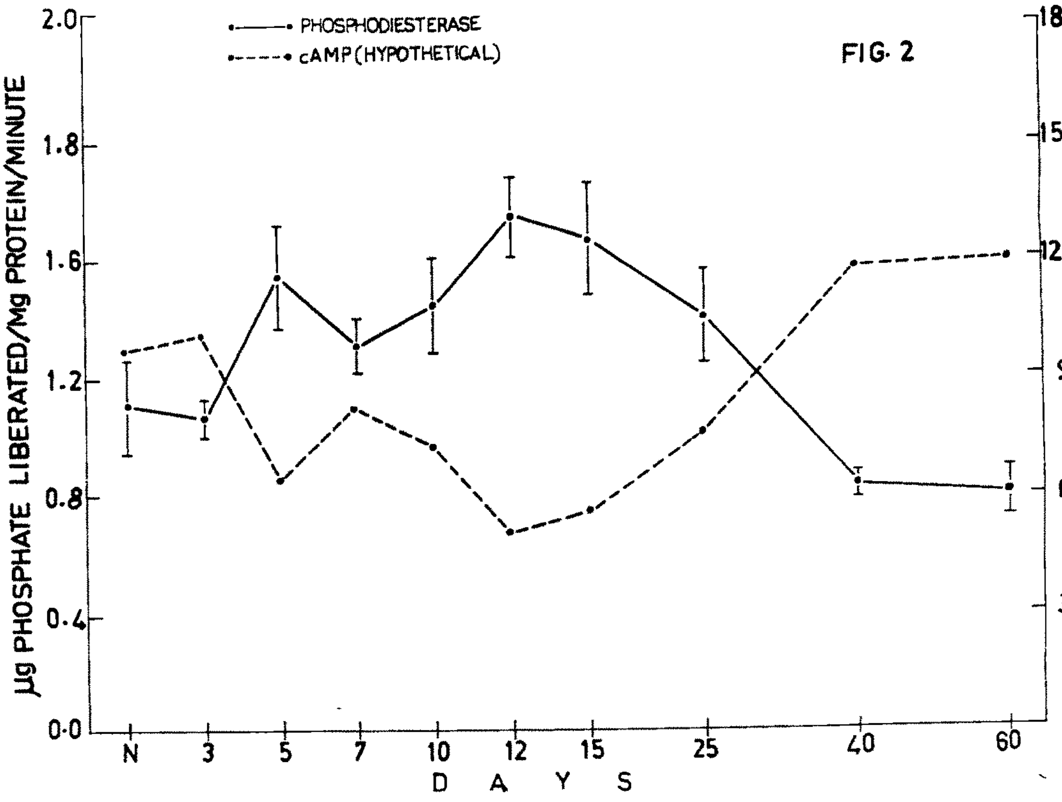

Fig. 2. Changes in cAMP phosphodiesterase activity and cAMP content (in hypothetical units) in liver during tail regeneration im M. carinata.

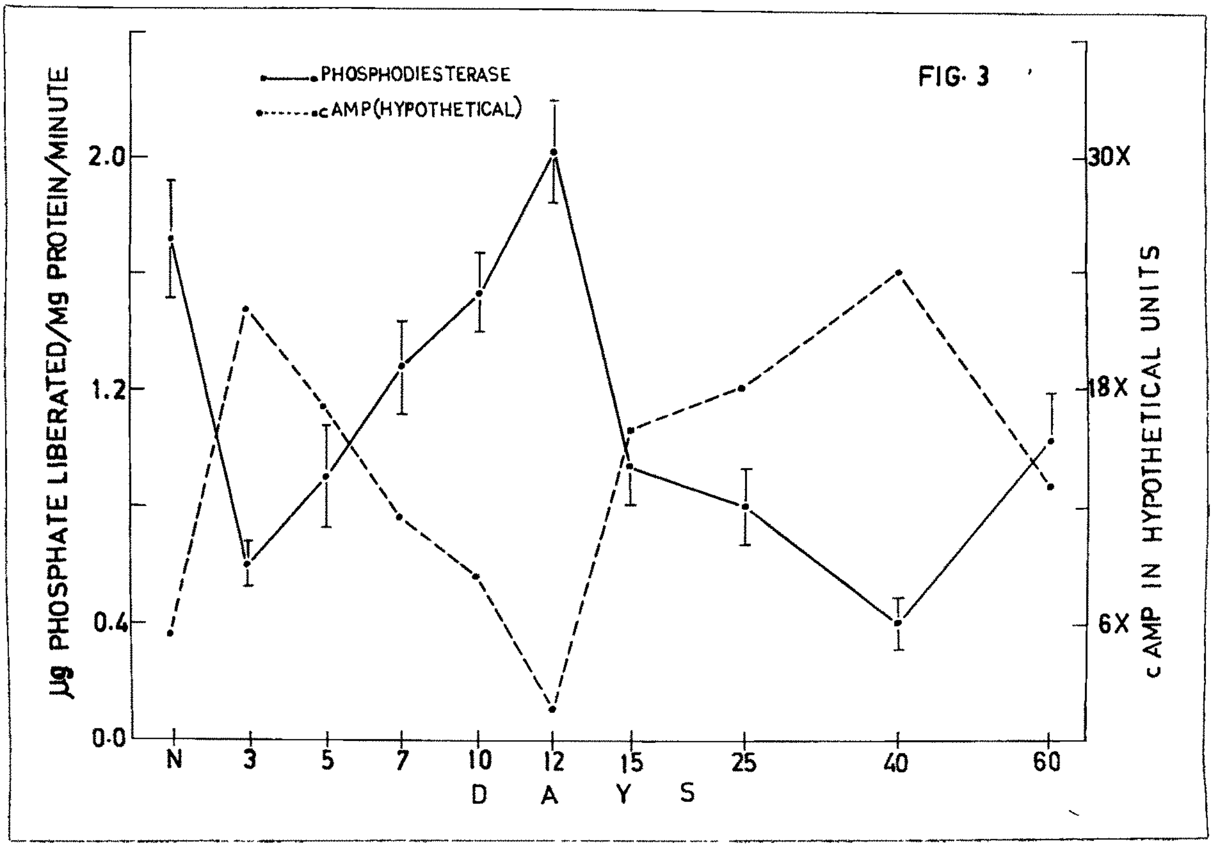

Fig. 3. Changes in cAMP phosphodiesterase activity and cAMP content (in hypothetical units) in muscle during tail regeneration in *M. carinata*.

the lowest levels of activity in all the three tissues on the 40th day by which time the regenerate is in a period of transition from differentiation to growth. Subsequently by 60th day post-autotomy, the enzyme level had reverted back to the normal in the regenerate and near normal levels in the case of muscle and liver. These changes in the activity levels of PDE in regenerate, liver and skeletal muscle are depicted in table 1, and Figs. 1-3. For sake of convenience and clarity of discussion, hypothetical levels of cAMP arrived at by keeping the reciprocal relation with its hydrolytic enzyme (PDE) have also been graphically represented in Figs. 1-3.

DISCUSSION

Figs. 1-3 reveal an apparent parallelism in the activity levels of (cAMP) PDE between the tail regenerate and skeletal muscle during various periods of tail regeneration. Parallel changes of lesser prominence are also depicted by the hepatic tissue especially from 7th day post-autotomy onwards. Based on the expected reciprocal relationship of PDE with cAMP, the present study indicated (irrespective of increased production of intracellular cAMP or not) two phases of increased cAMP content in the regenerate. The first phase corresponds to wound healing-preblastemic periods and the second phase corresponds to differentiation-growth periods of tail regeneration. Though increased levels of cAMP could be visualized during the first week of tail regeneration, Fig. 1 reveals the maximal content on the 3rd day. Evidently after a sharp increase in cAMP content within the first 3 days, there is a gradual reduction towards normal level during 5th, 7th and 10th days post-autotomy. The only two earlier studies on changes in the content of cAMP and cGMP during newt limb regeneration by Jabaily et al. (1975) and Liversage et al., (1977), respectively, though not successful in resolving convincingly the involvement of either of the nucleotides in the initial phases of regeneration have, nevertheless, shown an increased level of both the cyclic nucleotides in the early periods of limb regeneration. The periods selected by the above two groups of workers were unfortunately not time bound ones but were arbitrarily based on morphogenic phases of regeneration. Interestingly a significant increase in cGMP has been shown by Liversage et al. (1977) during dedifferentiation-blastema periods. Our present study indicates a high level of cAMP during the first 3 days, a period just prior to dedifferentiation and blastema formation. Bridging the chasm between these two observations are the reports wherein cAMP has been linked with a number of preparative and prerequisitive events associated with cell proliferation such as phosphorylation of histones (Greengard and Kuo, 1970; Langan, 1970; Bradbury et al., 1974), induction of ornithine decarboxylase and synthesis of polyamines (Theoharides and Cannelakis, 1975; Russel and Stambrook, 1975) and generating precursors for, and regulating DNA synthesis (MacManus et al., 1972, 1973; Foret, 1973; Foret and Babich, 1973; Short et al., 1975). It is apparent from Fig. 1 that there is a gradual decrement in cAMP content during the dedifferentiation and blastema periods, (periods of increasing exponential cell proliferation) which is interestingly paralleled by increasing content of cGMP (Liversage et al., 1977). Decreasing cAMP level and increasing cGMP level have been considered as the trigger for DNA synthesis and mitotic programme involved in cell proliferation (see reviews of Keirns et al., 1977; Miller, 1977; Schonhofer and Peters, 1977). cGMP has been considered to commit cells into proliferation by bringing about changes in transcription of ribosomal genes, RNA polymerases, stimulation of phosphorylation of acidic nuclear proteins and synthesis of phosphorybosyl pyrophosphate synthetase (Keirns et al., 1977). Our preliminary but inconclusive studies currently on also indicate a transient increase in cGMP level during the 5th and 10th days of tail regeneration. In the light of the reports and observations reviewed above, it may be tentatively surmised that both cAMP and cGMP are involved in a precisely synchronized sequential fashion in the initial molecular mechanisms which lead to extensive cell proliferation and formation of blastema. The fall in the cAMP content and increase in the cGMP content on the 5th, 7th and 10th days, might again indicate the attainment of an optimal cGMP/cAMP ratio as has been inferred in the framework of 'Yin-Yang hypothesis' of Goldberg et al. (1975). Apparently both the cyclic u nucleotides are involved in the initiation of regeneration and in this light the observations of Jabaily et al. (1975) and Liversage et al. (1977) of increased cAMP and cGMP contents during early periods of newt limb regeneration gain validity. The report of Sheppard (1972) of attainment of certain levels of intracellular cAMP as a prerequisite for mitosis also appears significant in this context. Appropriate to mention here is our unpublished observations of an increasing level of Ca^{+2} during the first week post-autotomy. Similarly Hilfeker and Higgins (1981) have also highlighted the involvement of both cAMP and Ca^{+2} in an inverse relation on initiation of nucleic acid synthesis and cell division. A tentative hypothesis that could be fashioned out of these observations is that under the initial stress of autotomy there is an increased cAMP content mediated by inhibition of PDE which leads to the building up of an increased intracellular store of free Ca^{+2} content which in its turn stimulates cGMP formation, and reduces cAMP content by activating PDE. Literature pertaining to these events have been extensively reviewed by Berridge and Rapp (1977). Besides, the fall in cAMP content corresponding to the increase in cGMP content on 5th, 7th and 10th days post-autotomy also augur well with the purported roles of these two cyclic nucleotides as inhibitor and stimulator respectively of cellular events such as lysosomal activity, phagocytosis, cell movements etc. (Ignaro, 1977; Keirns *et al*., 1977), which are all of crucial significance in aspects associated with reparative regeneration such as wound healing, dedifferentiation and blastema formation.

An analysis of the changes in PDE activity in liver and skeletal muscle during the first fortnight of tail regeneration (Table 1 and Figs. 2,3) reveals the maintainence of normal to above normal levels in the former, and changes parallel to those of the regenerate in the latter. These observations indicate the operation of a common regulatory factor which induces parallel changes in regenerate and skeletal muscle while in the case of hepatic tissue the enzyme remains somehow insensitive to these factors. This may be relevant in the context of a report of the inhibitory influence of extensive accumulation of cAMP on cell functions (Braun and Shiozawa, 1973) which may be applicable with reference to hepatic tissue as the hepatic tissue in *M. carinata* is involved in many biochemical and metabolic alterations during its tail regeneration. This is well supported by the report of protracted involvement of glucagon during first fortnight of tail regeneration (Ramachandran *et al*., 1980 a) and corresponding continuous depletion of hepatic glycogen content (Shah *et al*., 1977 b and Chapter II).

However, the parallel changes in the skeletal muscle and the regenerate though indicating identical sensitivity of PDE of the two tissues to a common regulatory substance should nevertheless be considered as parallel changes of differential adaptive significance. While the change in cAMP content in the regenerate can be correlated as a priori for cell proliferation, that in the skeletal muscle appears to be linkable with the observed (Chapter II) depletion of muscle glycogen store. Since the skeletal muscle phosphorylase is insensitive to glucagon, the observed depletion in muscle glycogen content should have to be triggered by the adrenaline mediated phosphorylase activation. Obviously adrenaline cannot be purported to be secreted continuously for a period as long as a fortnight and must be actually secreted only for a short period immediately subsequent to the stress of tail autotomy. In the absence of a persistant potent level of adrenaline, the maintainance of a prolonged elevated cAMP level becomes necessary for keeping phosphorylase in an active form and hence continuous glycogenolysis in muscle as an adaptive systemic response for tail regeneration. Though gradually increasing, but subnormal levels of skeletal muscle PDE activity noted during first 10 days of tail regeneration appear to be of effective adaptive significance in maintaining an elevated cAMP level in this setup.

Once again, keeping the reciprocal relationship between

PDE and cAMP, the present investigation reveals an increasing trend of cAMP content during the progressive phases of regeneration, starting from about 15th day onwards till about the 40th day post-autotomy. Increasing content of cAMP during the progressive phases marked by increasing degree of proximo-distal differentiation of the tail regenerate portends an active involvement of this cyclic nucleotide in directing the undifferentiated mesenchymal cells into committed channels of differentiation and ultimately promoting growth of the fully regenerated structure as denoted by the maximal level round about the 40th day post-autotomy. Pertinently some of the recent findings do indicate the positive influence of cAMP in cytodifferentiation and morphogenesis (Kosher and Sawage, 1980), induction of differentiation (Kram _et_ _al_., 1973; Friedman, 1976), as well as the establishment and maintainence of the differentiated state of cells (Robison, 1973; Miller, 1977). Again the increase in the cAMP content occurring during this period in the regenerate could also be correlated with the increasing phosphorylase activity (Chapter II) noted to occur at this period. With the attainment of fully regenerated condition the regenerated tail can be supposed to have acquired the original structural and functional attributes as denoted by the attainment of the normal level of PDE activity by 60th day.

Whereas the increase in cAMP in the tail regenerate during the second half of regeneration can be correlated with differentiative activities, similar changes in the skeletal muscle during the corresponding period though seemingly enigmatic may, however, have to be viewed in the light of the still persisting supranormal levels active skeletal muscle phosphorylase (Chapter II). Finally, the statistically significant parallel changes in the activity levels of PDE of skeletal muscle and tail (Table 1) and statistically non-significant and of low magnitude but similar pattern of changes of hepatic PDE too, indicate the possible operation of a common regulatory factor controlling both the local as well as systemic levels of the enzyme during the various periods of tail regeneration in *Mabuya carinata*.

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