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Induction of Pupation by DNA Broken with Steroid Hormones in Silkworms

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The initiation of pupation by larval DNA broken with ecdysterone or cortisone was investigated by using *Bombyx mori*. A single strand scission (nick) was produced by treating mildly the DNA with the steroid hormone + Cu²⁺ and the nicked DNA was then injected into the posterior part of the ligated prepupa. It was observed that the pupation-inducing action of DNA treated with ecdysterone is stronger than that of DNA treated with cortisone. The pupation by the nicked DNA was, however, not so complete as that by ecdysterone itself. In 3 hr after injection, the degree of nicking produced in DNA of prepupae injected the broken DNA was remarkably low comparing with that produced in DNA of those injected ecdysterone itself. After 24 hr, the nicking degree became much higher and double strand break occurred in both the cases. These observations suggest that the pupation in ligated prepupae injected the nicked DNA is initiated by the synthesis of necessary mRNA.

INTRODUCTION

Based on our experience of virus induction (Yamafuji, 1964), we have assumed that the initiative process of cytodifferentiation may be appropriate DNA breakages (Yamafuji et al., 1971d). Later, it was actually demonstrated that the pupation of silkworm, *Bombyx mori*, can be induced by DNA breakers, such as steroid hormones, catecholamines and hexose oximes (Yamafuji *et al.*, 1971e). We have now proved that the pupation is initiated by single strand scission in DNA produced by ecdysterone or cortisone.

METHODS AND MATERIALS

Rearing of silkworm and induction of pupation

A stock, *Taiheix Choan*, of domestic silkworm was reared with fresh mulberry leaves at 25°C. Matured larvae at the beginning of cocoon-spinning were ligated between the third and fourth abdominal segment, and after about 20 hr, 0.02ml of inducers were injected into the posterior part. On the third or fourth day after the injection, the pupation of the hinder abdomen was estimated and recorded. In the control, 0.02 ml of 10 mM NaCl were injected.

To prepare the inducer DNA, 40 µg/ml DNA isolated from matured larvae were mixed with 1 ml of 1 µM ecdysterone (gift of Takeda Co. in Osaka) + 10 mM CuSO₄ or 1 ml of 10 µM cortisone (product of Sigma Co. in St. Louis) + 10

mM CuSO_4 and incubated at 37°C for 2 hr. The mixture was incubated in water without reagents and dialyzed in the same way. The treated DNA was injected in 10 mM NaCl solution.

Preparation of DNA

Digestive organ-free tissues of matured larvae or posterior parts of ligated prepupae were homogenized in a citric acid solution (pH 3) containing bentonite (2 mg/ml) and DNA was quickly prepared according to the method described previously (Yamafuji *et al.*, 1966). Preparation of DNA from mouse muscle and baker's yeast was also performed in the same way. Bentonite was added to prevent deoxyribonuclease action (Lundblad and Johanson, 1968).

Examination of double helix break and single strand scission in native DNA was carried out with the process reported before (Yamafuji *et al.*, 1971c).

RESULTS

Induction of pupation by DNA broken with steroid hormones

In view of the observations on the alteration of chromatin by steroid hormones (Yamafuji *et al.*, 1971e), it seemed reasonable to suppose that in the first stage of pupation the single strand scission of DNA molecules in the prepupal chromosome may be brought about by ecdysone. Although the production of the scission by ecdysterone or cortisone plus cupric ions *in vitro* has already been corroborated by using mouse liver DNA (Yamafuji *et al.*, 1971e), this was now confirmed with larval DNA (Fig. 1). The scission of silkworm DNA by ecdysterone was also enhanced by 10 mM Cu^{2+} which itself had no capability to break DNA. Double strand break, however, did not occur under the same conditions. In supplementary experiments it was substantiated that the sedimentation patterns of silkworm DNA after treating with cortisone

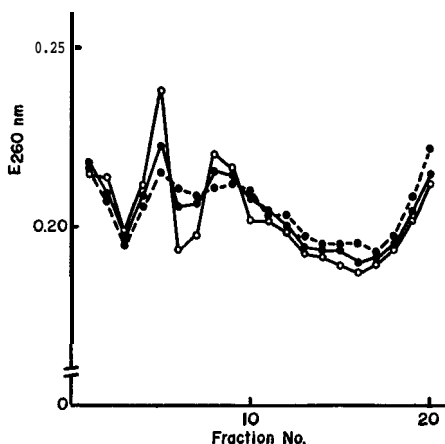


Fig. 1. Sedimentation pattern of inducer DNA prepared from matured larvae. Centrifuged at pH 12.6. o-o control, ●—● treated with ecdysterone, ●·····● treated with ecdysterone + Cu^{2+} .

$\pm \text{Cu}^{2+}$ were similar to those obtained in the case of ecdysterone. It was further observed in a control test that no appreciable breakages occurred in DNA molecules which had been incubated in water without reagents.

It was desirable to prove that the single strand scission can actually be produced by the action of ecdysterone *in vivo*. For this, 0.02 ml of 50 μM ecdysterone (β -ecdysone) in 10 mM NaCl were injected into each ligated prepupa, and after 3 hr, DNA was prepared from the posterior part. Gradient centrifugation analysis disclosed that the scission could distinctly be caused in the prepupal body (Fig. 2). At the same time it was witnessed that the double

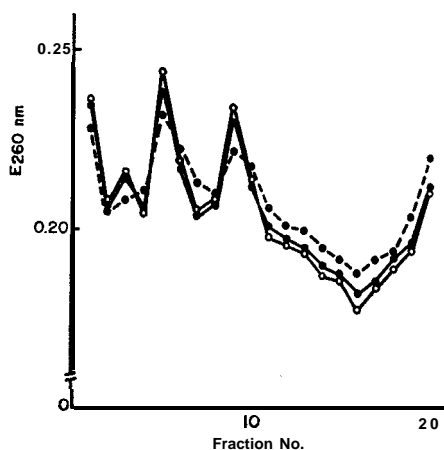


Fig. 2. Sedimentation pattern of DNA from prepupae injected with inducer DNA or ecdysterone. DNA was prepared in 3 hr after injection. Centrifuged at pH 12.6. o-o DNA from control prepupae, ●—● DNA from prepupae injected with inducer DNA prepared using ecdysterone + Cu^{2+} , ● DNA from prepupae injected with ecdysterone.

strand break did not take place in the tissues under these conditions. In this series of experiments, the control prepupae were injected with 10 mM NaCl.

On the basis of these findings, it was surmised that the pupation may be initiated by the injection of DNA appropriately broken with the steroid hormones. DNA treated with ecdysterone or cortisone + Cu^{2+} as described in the section of "Methods and Materials" was now injected into the posterior part of ligated prepupae. The experiments were carried out 40 times, including 20 preliminary ones. Since each group consisted of 10 to 20 individuals, the inducing effect was expressed in % of pupated worms, taking the average from 20 principal tests (Table 1). For comparison, 0.02 ml of 50 μM ecdysterone were injected into the posterior part of different prepupal groups: the principal injection tests were repeated 10 times and the inducing effect was calculated as mentioned above.

Table 1 shows that the pupation-inducing capacity of DNA treated with ecdysterone + Cu^{2+} is much higher than that of the one treated with cortisone

Table 1. Induction of pupation by DNA broken with steroid hormones and by ecdysterone.

Inducer	Amount of DNA or ecdy-sterone injected into each prepupa in μg	Time necessary for pupation in day	Inducing effect in %
DNA broken with ecdysterone + Cu^{2+}	0.02	3	65
DNA incubated in water	0.02	3	12
Control	—	3	0
DNA broken with cortisone + Cu^{2+}	0.08	3	42
DNA incubated in water	0.08	3	15
Control	—	3	3
Ecdysterone	0.5	4	61
Control	—	4	0

+ Cu^{2+} . This corresponds to our preceding observation that the ability of ecdysterone to produce the single strand scission is much higher than that of other steroid hormones (Yamafuji *et al.*, 1971e). The pupation in the prepupal group injected with DNA incubated in water without reagents may be due to the scission of naked DNA by a minute quantity of steroid hormones, catecholamines or sugar oximes which existed eventually in the hinder abdomen (cf. Yamafuji *et al.*, 1971e). The deviation from the average inducing effect was within 20 % in all principal tests. In the supplemental experiments with DNA from baker's yeast or mouse muscle, it was verified that no pupation is induced by the injection of DNA broken with ecdysterone or cortisone + Cu^{2+} under the same conditions.

Breakage of DNA in prepupae by injecting DNA broken with steroid hormones

From the observation stated above, it was inferred that the broken DNA injected may also cause breaks of DNA molecules in prepupal tissues. This could be substantiated in the experiment illustrated in Fig. 2. The degree of single strand scission produced by DNA broken with ecdysterone + Cu^{2+} , however, was remarkably low in comparison with that produced by direct injection of ecdysterone, whose pupation-inducing effect was almost the same as that of the broken DNA (Table 1). In addition, we proved recently that the synthesis of mRNA *in vitro* can be enhanced by a suitable production of single strand scission (nick) in template DNA (Yamafuji *et al.*, 1972, Omura *et al.*, 1973). These facts suggest that the initiation of pupation in the prepupal body injected with the broken DNA should be the mRNA synthesis by the introduced DNA. The specific mRNA synthesized through the function of injected silkworm DNA nicked by steroid hormones could cause the formation of catecholamines necessary for sclerotization. Since these catecholamines are also capable of breaking DNA (Yamafuji *et al.*, 1970b; Murakami and Yamafuji, 1970), the scission of low degree observed in 3 hr after injecting the broken DNA is to be attributed

to the action of dopa or dopamine. As the initiation of pupation in the prepupa injected with ecdysterone is the scission of chromosomal DNA, it is expected that the appearance of pupated individuals in such a group is somewhat delayed comparing with the group received the injection of broken DNA. This was confirmed in the experiment in Table 1. In the control, it was corroborated that the injection of broken DNA did not bring about any double strand break of cellular DNA within 3 hr, and that the steroid hormones used for the preparation of injecting DNA solutions had been completely eliminated by the dialysis performed as mentioned in the section of "Methods and Materials."

It is assumed that the catecholamine formation caused by the broken DNA injected increases with the development of pupating process and that the increase intensifies the DNA breakage in prepupal cells. Therefore, we prepared DNA from the hinder abdomen of ligated prepupae in 24 hr after the injection and fractionated it by gradient centrifugation (Fig. 3 A). It was observed that the single strand scission produced by DNA broken with ecdysterone + Cu^{2+} has become markedly stronger during subsequent 21 hr. Figure 3 also indicates that a slight nick was formed in the prepupal group injected with DNA incubated in water without reagents. It was further found that the double strand break occurs only in the case injected with the DNA nicked by ecdysterone + Cu^{2+} (Fig. 3B).

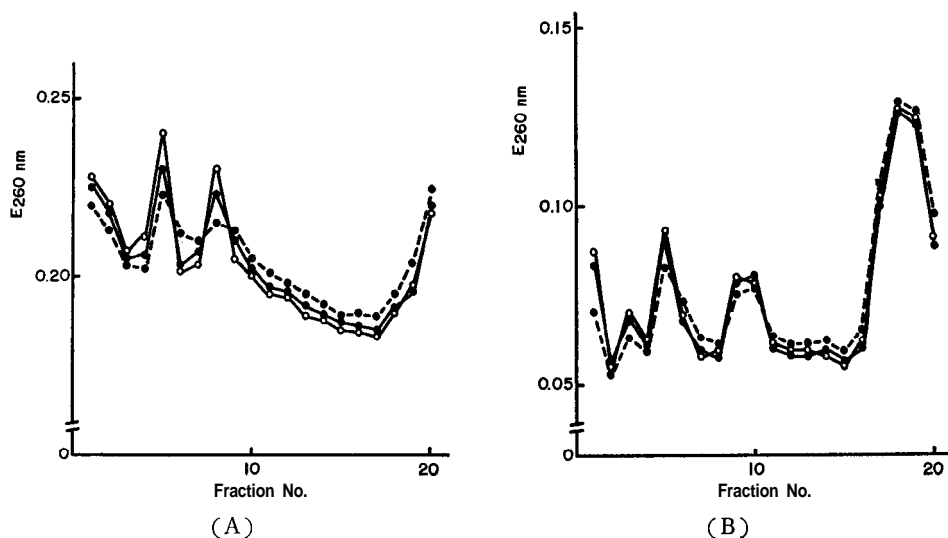


Fig. 3. Sedimentation pattern of DNA from prepupae injected with inducer DNA. DNA was prepared in 24 hr after injection. Centrifuged at pH 12.6 (A) or pH 7 (B). o-o DNA from control prepupae, ●—● DNA from prepupae injected with inducer DNA prepared after incubating in water, ●.....● DNA from prepupae injected with inducer DNA prepared using ecdysterone + Cu^{2+} .

As the time necessary for pupation in the prepupal group injected with ecdysterone was longer by one day than that in the group injected with DNA broken by ecdysterone + Cu^{2+} (cf. Table 1), the abdomen DNA of the former

prepupae was then prepared in 48 hr after the injection. The fractionation analysis revealed (Fig. 4 A) that the single strand scission took place particularly in the highest molecular portion and that the curve in the other portions was similar to that in the DNA molecule prepared in 24 hr after injecting the nicked DNA (cf. Fig. 3 A). It was also witnessed (Fig. 4B) that the double strand break occurred more intensively than in the prepupa injected with the nicked DNA (cf. Fig. 3 B). Previously we observed that high molecular compo-

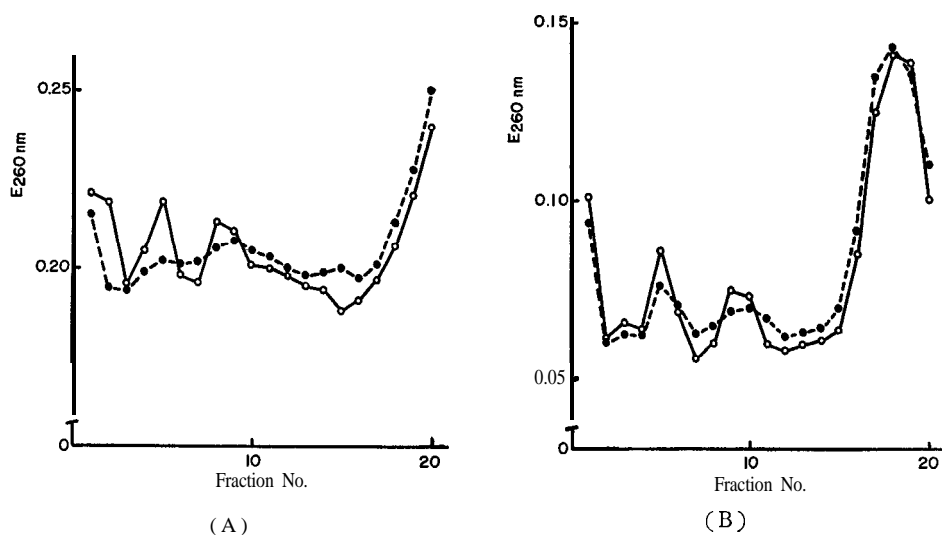


Fig. 4. Sedimentation pattern of DNA from prepupae injected with ecdysterone. DNA was prepared in 48 hr after injection. Centrifuged at pH 12.6 (A) or pH 7 (B). ○—○ DNA from control prepupae, ●—● DNA from prepupae injected with ecdysterone.

nents of larval DNA disintegrate considerably during the pupation (Yamafuji and Hashinaga, 1967). It is supposed that ecdysone, dopa and dopamine participate in this disintegration.

DISCUSSION

The first indication of chromosomal DNA breaks was obtained by our discovery of biochemical virogenesis with silkworms in 1943 which had been accomplished on the basis of a working hypothesis of endogenous virus production (Yamafuji and Shirozu, 1944). In pursuing the virogenic mechanism, we have demonstrated that the inducers can be formed metabolically (e.g. Yamafuji cycle; cf. Yamafuji, 1964) and that the larval DNA is broken by the metabolites to give viral particles (Yamafuji and Hashinaga, 1966, 1967). Although the conversion of cellular DNA into viral one was also corroborated with bacteria (Northrop, 1965, 1968, 1971; Okamoto *et al.*, 1968), we have extended our virus researches to examine whether biochemical DNA breaks can be applied

to explore various processes of cellular differentiation and anomalization (Yamafuji, 1969). In this way, we have been able to find the DNA breaking action of catecholamines (Yamafuji *et al.*, 1970b), ascorbic acid and its oxidation products (Yamafuji *et al.*, 1971c), hydroxylamine and nitrous acid (Yamafuji *et al.*, 1971d), oximes (Murakami and Yamafuji, 1970), xylans (Yamafuji *et al.*, 1970a), viral protein (Yamafuji *et al.*, 1971a) and steroid hormones (Yamafuji *et al.*, 1971b).

The experiments with these biological substances suggested that a DNA-breaker can differently act on the cell according to the degree of breakage. Our experiences have further led us to investigate whether properly nicked DNAs are capable of initiating cytodifferentiations. As it has already been proven that a puff formation in *Diptera* is accomplished by an abundant RNA production (e.g. Karlson, 1963), we have supposed that the introduction of a small amount of DNA nicked by ecdysone into ligated prepupae of *Bombyx mori* may be sufficient to cause the initiative process, namely the synthesis of mRNA for tyrosine-oxidizing enzyme in this case. Although the introduced DNA would be decomposed after a while, the supposition could be confirmed in the present study. Presumably the product in each step of pupation, such as dopa and dopamine, may successively produce special nick at different position of chromosomal DNA strands which leads to the synthesis of mRNA necessary for the formation of the next product.

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