The Effect of 20-Hydroxyecdysone on the Synthesis of Fibroin mRNA in the Cultured Posterior Silk Gland of *Bombyx mori*¹

Hideaki MAEKAWA, Naoko SAISHU-TAKADA and Naoko MIYAJIMA

Department of Technology, National Institute of Health, Tokyo 141, Japan

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The activity of total RNA synthesis including fibroin mRNA in the cultured posterior silk gland of *Bombyx mori* was dependent on the developmental stage of larva from which the silk gland was dissected out. The majority of the RNA synthesized in the posterior silk gland cultured for 24 hr was ribosomal RNA and transfer RNA at all investigated stages. The synthesis of fibroin mRNA primarily decrease by the application of the molting hormone, 20-hydroxyecdysone, whereas the hormone stimulated the synthesis of both ribosomal and transfer RNAs.

INTRODUCTION

The ecdysteroid titers of the larval hemolymph in the silkworm, *Bombyx mori*, were determined precisely by means of radioimmunoassay (KIGUCHI and AGUI, 1981). According to their results the levels of ecdysteroids at 7 hr before the fourth apolysis and at the fourth ecdysis are 40 ng/ml and 30 ng/ml of hemolymph, respectively. The ecdysteroid concentration at 15 hr after the apolysis (300 ng/ml) is the highest level during the molting stage. The synthesis of fibroin mRNA in the posterior silk gland of *B. mori* is entirely suppressed during the fourth molting stage (MAEKAWA and SUZUKI, 1980) when hemolymph contains a high level of ecdysteroid. Therefore, the present experiments have been undertaken in the organ culture system of the silk glands to investigate whether the ecdysteroid inhibits specifically the synthesis of fibroin mRNA.

MATERIALS AND METHODS

Animals. The *B. mori* eggs, a hybrid of Chinese and Japanese strains (Gunpo × Shugyoku) were purchased from Gunze Co., Motomiya-cho, Fukushima, Japan. The larvae were reared at 25°C on an artificial diet purchased from Kyodo-Shiryo Co., Yokohama, Japan.

Staging and organ culture. The staging during the fourth molting stage was carried out according to the spiracle index which indicates the shapes of newly forming spiracle (MAEKAWA and SUZUKI, 1980; KIGUCHI and AGUI, 1981). The stage of the used larva was expressed by the time (hr) before or after the fourth apolysis at which the epidermis

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detaches from the overlying larval cuticle. The silk glands were dissected out in the cold $1 \times \text{SSC}$ (0.15 M NaCl and 0.015 M sodium citrate) from the larvae and washed with cold $1 \times \text{SSC}$. A pair of silk glands was divided into single gland, six to ten of which were pooled and cultured at 25°C with 1 ml GRACE's medium without hemolymph (GIBCO). In some experiments ecdysteroid (20-hydroxyecdysone, Rohto Chemicals Co.) was supplemented to the medium at a concentration of 3 µg/ml which is compatible with the physiological condition (CHERBAS et al., 1980).

RNA labeling and saturation hybridization. The silk glands were cultured in the medium with 0.1 to 0.5 mCi/ml of ^3H -uridine for a given time and RNA was extracted from the posterior parts of the cultured silk glands by a method of SUZUKI and BROWN (1972). The proportions of fibroin mRNA synthesis in total RNA synthesis were measured by the technique of saturation hybridization (MAEKAWA and SUZUKI, 1980). This method is suitable to measure the amount of mRNA synthesized in a given time when there exists a great amount of the preexisting cold mRNA. The cloned fibroin gene plasmid (pFb10) (OHSHIMA and SUZUKI, 1977) was used to detect the labeled fibroin mRNA from the total labeled RNA. The ratio of the maximum of fibroin mRNA to pFb10 DNA was 1/11 in this hybridization condition. The prepared RNA was saturated to the filters immobilized pFb10 DNA amount of 2–4 µg/filter by the hybridization for 16 hr at 50°C in 0.6 M NaCl, 0.1 mM EDTA, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris-HCl (pH 7.5) and 50% formamide (for details, see MAEKAWA and SUZUKI, 1980). RNA synthesis under several incubation conditions were compared between each gland of a pair of the silk glands.

RESULTS

Incorporation of ^3H -uridine into the cultured posterior silk gland RNA

The incorporation of the labeled uridine into total RNA of the cultured glands increased with the culture period. However, the radioactivities incorporated into RNA of the glands cultured for 15 hr after dissected out at 7 hr after the apolysis, were 4.58 and 3.26×10^5 cpm per gland in the different two groups of the cultured glands (Table 1). Although there were differences among the groups of cultured glands in the counts incorporated per one gland, we could compare that between each of the homologous pair of the glands. The RNA synthesis was more active in the gland dissected out at 7 hr before the apolysis in which the ratio of the radioactivities incorporated for 26 hr and 8 hr culture was 3.62, than that at 0 hr after the apolysis in which the ratio of that for 24 hr and 5.5 hr culture was 1.30 (Table 1). It is coincident with the data on total RNA synthesis that decreased around the early stage of fourth molting (MAEKAWA, 1979; MAEKAWA and SUZUKI, 1980). The majority of ^3H -uridine incorporated into total RNA in a long labeling period (24 hr) was distributed in rRNA and tRNA which were shown in the sucrose density gradient centrifugation (Fig. 1). This profile agreed with that of RNA labeled *in vivo* (MAEKAWA, unpublished data). Therefore, it was considered that this organ culture system was under the normal condition for the RNA synthesis.

Fibroin mRNA synthesis in the cultured posterior silk gland

When the silk glands dissected out at 7 hr before apolysis were cultured for 8 hr, the proportion of fibroin mRNA synthesis to total labeled RNA was 0.9%. The value

Table 1. Incorporation of ^3H -uridine into the cultured posterior silk gland RNA and saturation hybridization of the RNA with fibroin gene DNA

Stage of silk gland and culture period	Number of gland	^3H -Uridine incorporated into total gland RNA ($\times 10^5$ cpm)	Proportion of fibroin mRNA to total labeled RNA (%)
7 hr before the apolysis			
8 hr	8	2.90	0.92
26 hr	8	10.5	2.21
Just after the apolysis			
5.5 hr	8	2.78	1.00
24 hr	8	3.61	0.36
7 hr after the apolysis			
2 hr*	6	1.58	0.074
15 hr*	6	4.58	ND
15 hr**	8	3.26	ND
24 hr**	8	4.86	ND
24 hr***	8	6.80	0.078
39 hr***	8	5.04	0.028
23 hr after the apolysis			
18 hr	8	10.05	0.015
24 hr	8	3.25	0.019

The silk glands dissected out were cultured in 1 ml of GRACE's medium containing 0.2 mCi/ml of ^3H -uridine at the stage of 7 hr before and just after the apolysis or 0.3 mCi/ml of that at the stage of 7 hr and 23 hr after the apolysis. RNA was extracted with cold phenol after pronase digestion from the posterior silk glands after the culture. Incorporation into total RNA is shown as cpm/one posterior silk gland. RNA was sized to 4–5S with formamide and hybridized by the method described in MATERIALS AND METHODS. A pair of silk glands divided each other was used to culture for different periods at each stage. At 7 hr after apolysis stage, each of a pair used is marked with the same number of '*.' ND shows 'not determined.'

increased to 2% after incubation for 26 hr (Table 1). The silk glands from the larvae at the apolysis synthesized fibroin mRNA as much as 1% of total labeled RNA when incubated *in vitro* for 5.5 hr. However, prolonged incubation of the glands (24 hr) reduced the proportion of fibroin mRNA synthesis (0.36% of the total labeled RNA) (Table 1). On the other hand, the proportions of the mRNA synthesis were low (0.02–0.08%) in the silk gland dissected out at 7 hr or 23 hr after the apolysis (Table 1).

The effect of 20-hydroxyecdysone on the synthesis of fibroin mRNA

The activities of RNA synthesis were compared in the silk glands cultured with or without 20-hydroxyecdysone (Table 2). The incorporations of ^3H -uridine into total RNA of the posterior silk glands were stimulated by the application of 20-hydroxyecdysone, regardless of the developmental stage of the silk glands. The major part of incorporated ^3H -uridine was distributed in rRNA (70%) and tRNA (25%) which was unaffected by the presence of 20-hydroxyecdysone in the culture medium (Fig. 2). When the silk glands dissected out at 10 hr before and just before the fourth apolysis were cultured for 22 hr in the presence of 20-hydroxyecdysone, the proportion of the fibroin mRNA synthesis to total labeled RNA decreased to approximately 1/10 and 1/3 of that observed in the control culture without ecdysteroid, respectively, even after considered the increase of total RNA synthesis by hormone (Table 2). 20-Hydroxy-

Fig. 1. Sucrose density gradient centrifugation of RNA extracted from the cultured posterior silk gland. The silk glands were dissected out from larvae at 7 hr before the apolysis (a) and just (b), 7 hr (c), or 23 hr (d) after the apolysis, and cultured for 24 hr or 26 hr in GRACE's medium containing ^3H -uridine (0.2 or 0.3 mCi/ml) as shown in Table 1. A part of RNA extracted from the glands was layered on a 15–30% sucrose gradient (10 ml). After the centrifugation for 16 hr at 25,000 rpm with a SW40Ti rotor, 0.3 ml fractions were collected and the radioactivity (●) and absorbance at 260 nm (○) of each fraction were determined.

Table 2. Effect of 20-hydroxyecdysone on fibroin mRNA synthesis in the cultured posterior silk gland

Stage of silk gland	Application of 20-hydroxyecdysone	Ratio of ^3H -uridine incorporated into total RNA (20-hydroxyecdysone +/–)	Proportion of RNAs to total labeled RNA (%)	
			rRNA	Fibroin mRNA
10 hr before the apolysis	+	1.3	73	0.13
0 hr after the apolysis	–		66	1.6
7 hr after the apolysis	+	2.7	71	0.026
	–		64	0.2
23 hr after the apolysis	+	1.4	66	0.011
	–		66	0.017

The silk glands from larvae of a given stage were dissected out and cultured in 1 ml of GRACE's medium including 0.2 to 0.5 mCi/ml of ^3H -uridine with or without 3 $\mu\text{g}/\text{ml}$ 20-hydroxyecdysone for 22 hr. The proportions of fibroin mRNA were determined as mentioned in Table 1. The percent of rRNA to total labeled RNA was estimated after sucrose density gradient centrifugation (see Fig. 2).

Fig. 2. Sucrose density gradient centrifugation of RNA extracted from the cultured glands. The silk glands were dissected out from larvae at 10 hr before the apolysis and cultured in GRACE's medium containing ^3H -uridine (0.2 mCi/ml) with (a) or without (b) 20-hydroxyecdysone ($3 \mu\text{g/ml}$) for 22 hr as shown in Table 2. After the centrifugation and fractionation as same condition as shown in Fig. 1, radioactivity ($\bullet$) and absorbance at 260 nm ($\circ$) of each fraction were determined.

ecdysone added to the culture medium did not affect on the fibroin mRNA synthesis of the silk glands from the larvae at 7 hr after apolysis, at which fibroin mRNA is not synthesized *in vivo* (MAEKAWA and SUZUKI, 1980).

DISCUSSION

Titer of ecdysteroides in the hemolymph of silkworm, *B. mori*, increases at the molting stages and decreases at the intermolt stages (KIGUCHI and AGUI, 1981; CALVEZ et al., 1976). In the present work, experiments have been performed to elucidate the hormonal effect on the synthesis of fibroin mRNA in the posterior silk gland. The activities of the fibroin mRNA synthesis in the cultured silk gland (Table 1) were well coincident with that in silk glands *in vivo* at any developmental stages of the silk glands, in which it has been known that fibroin mRNA is actively synthesized *in vivo* (12.6 molecules of fibroin mRNA/gene/min) at 4 hr before the fourth apolysis but the synthesis is sup-

pressed (0.084–0.023 molecule/gene/min) in a period of 7 hr after the fourth apolysis through 5 hr after the fourth ecdysis (MAEKAWA and SUZUKI, 1980).

The proportion of the fibroin mRNA to the total labeled RNA decreased by the 20-hydroxyecdysone application (Table 2). The decrease is not due to the increase of incorporation into the total RNA but the specific inhibition of the fibroin mRNA synthesis. When the silk gland from the larva immediately after apolysis was cultured *in vitro*, relative proportion of the synthesis of fibroin mRNA declined as the duration of culture period (Table 1). This could be resulted from the inhibition of the mRNA synthesis triggered by the ecdysteroid secreted in the larva because the cumulative total RNA synthesis at the 24 hr culture increased 1.3 times of that at the 5.5 hr culture (Table 1). The present result is one of few observations on the inhibitory effect of 20-hydroxyecdysone (SIEW and GILBERT, 1971; COHEN and GILBERT, 1974) on the specific mRNA synthesis (MUSKAVITCH and HOGNESS, 1980), whereas evidences concerning ecdysteroids as inducer of RNA synthesis have accumulated (KARLSON and SEKERIS, 1966; KARLSON et al., 1975; SCHELLER and KARLSON, 1977; LEPESANT et al., 1978; KRAMINSKY et al., 1980; JOWETT and POSTLETHWAIT, 1981; IRELAND and BERGER, 1982; SHIRK et al., 1983). The present results suggest that turn-off of the fibroin gene transcription at the molting stage (MAEKAWA and SUZUKI, 1980) could be a consequence of ecdysteroids action. However, pulse labeling experiments should be carried out in order to distinguish strictly a control at the level of transcription *per se* from that at the level after transcription. The effect of 20-hydroxyecdysone on the fibroin mRNA synthesis at the other developmental stages still remains to be investigated because the hormonal effects depend on the developmental stages of the animals.

It has been reported that 20-hydroxyecdysone activates the RNA polymerase I (NATORI, 1976; NISHIURA and FRISTROM, 1975) and mainly induces rRNA synthesis (SCHELLER and KARLSON, 1977). In the cultured posterior silk gland, the synthesis of rRNA and tRNA was also stimulated by 20-hydroxyecdysone (Table 2 and Fig. 2). However, it has been observed that the rRNA synthesis decreases in the silk gland *in vivo* during the early molting stage (MAEKAWA and SUZUKI, 1980). The decrease could be due to the existence of juvenile hormone, since, when the larva is allatectomized after the apolysis stage (the condition of juvenile hormone free), the reactivation of rRNA synthesis during the late molting stage occurs half a day earlier than would be expected in the normal larva (MAEKAWA, 1979). Evidences have been also presented in the other insects that the stimulation of RNA synthesis by ecdysteroids is inhibited by juvenile hormone (CHIHARA et al., 1972; OBERLANDER and TOWBLIN, 1972; CHIHARA and FRISTROM, 1973).

From the present results it is conceivable that the synthesis of fibroin mRNA *in vivo* is suppressed by 20-hydroxyecdysone regardless of the existence of juvenile hormone, although one must await the result of crucial experiments using juvenile hormone in this organ culture system.

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