Flimsy Cocoon Mutant of *Bombyx mori* Larva Produces a Reduced Amount of Fibroin mRNA

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ABSTRACT. The proportions of fibroin mRNA synthesized in the posterior silk gland of the flimsy cocoon mutant (*flc*) of *Bombyx mori* at days 3, 4 and 5 of the fifth instar were 1.2%, 0.7% and 1.8% of the labeled total RNA, respectively, whereas those in heterozygous larva (+/*flc*) at the same stages were 1.6%, 3.2% and 8.5%, respectively. The total amount of fibroin mRNA that accumulated in one larva at day 5 of *flc* was less than 10 µg and that of +/*flc* was about 110 µg. The intracellular distribution of the mRNA also was measured. The proportion in the nuclear fraction was 0.5% of the total labeled RNA for *flc* and 0.8% of that for +/*flc*; in the cytoplasmic fraction the proportion was 0.2% for *flc* and 2.4% for +/*flc*. Thus, the reduced amount of fibroin mRNA in the *flc* mutant is considered to be due to the degradation of the synthesized mRNA rather than to a reduction in the synthesis of mRNA. It is believed that this degradation causes abnormal fibroin production. The primary action site of the *flc* gene is not known.

In *Bombyx mori* some mutants have unusual cocoon formation. *Nd* mutants are characterized by low or no fibroin synthesis which produces an unusual cocoon consisting mainly of sericin, or no cocoon is formed (4, 5). The accumulated amount of fibroin mRNA in these mutants was 0.3 to 0.7% of the total RNA, whereas that in the wild type was 2.0% (8). The flimsy cocoon mutant which produces a thin cocoon of reduced weight is designated *flc*. The cocoon weight of homozygous larva carrying the recessive *flc* gene was about 30% that of the heterozygous larva (+/*flc*). The reduction in cocoon weight is due to fibroin production in *flc* (3). Here we report that the reduced amount of fibroin in the *flc* mutant is caused mainly by the decreased amount of fibroin mRNA, and that a reduction in mRNA occurs in the cytoplasm.

MATERIALS AND METHODS

Materials. The strain of *B. mori* used was the b-60 stock maintained at the Institute of Silkworm Genetics, Faculty of Agriculture, Kyushu University. Larvae that are homozygous (*flc*) and heterozygous (+/*flc*) for *flc* were produced by crossing *Ze*+/*+flc* females to +/*flc* /+/*flc* males. Gunpo×Shyugyoku was used as the wild type. The eggs of this strain were purchased from Gunze Co., Ayabe City, Kyoto, Japan. All larvae were reared at 25°C on mulberry leaves.

RNA extraction and the estimation of the amount of fibroin mRNA. [³²P]RNAs were

prepared by labeling two or three larvae for about 24 h with $^{32}\text{PO}_4^{3-}$ (0.5 to 1 mCi per larva) at days 2, 3 and 4 of the fifth instar. The posterior silk glands were dissected out in cold $1\times\text{SSC}$, and the total RNA was extracted with cold phenol after pronase digestion by a method similar to that of Suzuki and Brown (7). RNase free DNase treatment was carried out at 37°C for 10 min at pH 7.6 in Tris-HCl buffer (6). The RNA in the flow through fraction from a Bio-Gel A50m column was digested with RNase T_1 . Its oligonucleotides were analyzed on a DEAE-Sephadex A25 column (8). To estimate the amount of fibroin mRNA, the purity (%) of fibroin mRNA in each sample was calculated by the following equation $p=100(b-x)/b-a$, where a, b and x are the percent of tetranucleotides of RNase T_1 digests of; a, the purest mRNA (1.7%); b, the artificial contaminant of 40s ribosomal RNA (11.6%) or heterogeneous nuclear RNA (9.5%); and x, the sample to be tested (8, 9).

Separation of nuclei and cytoplasm. Larvae were labeled for 24 h with $^{32}\text{PO}_4^{3-}$ at day 3 of the fifth instar for *flc* or on day 4 of the same instar for the wild type. The method of the separation was as described by Suzuki and Giza (9). Although 98% of the total DNA was recovered in the nuclear fraction, 70 to 80% of the total RNA was found in the cytoplasmic fraction (see Table 2). The RNA extraction from each fraction and the estimation of the amount of fibroin mRNA were carried out as described above.

RESULTS AND DISCUSSION

Synthesis of fibroin mRNA at the fifth instar. In the *flc* mutant the activity producing fibroin decreases after day 3 of the fifth instar, as shown by a low incorporation of ^{14}C glycine in the posterior silk gland and by reduced amounts of gelatinous silk protein stored in the lumen (1, 2). To determine the molecular basis of this reduction in fibroin production in *flc*, we have analyzed the RNA in the *flc* larva.

The amount of bulk RNA in the posterior silk gland of *flc* during the fifth instar was 80% that of $+/\text{flc}$ (Table 1); that is consistent with the observation by Adachi and Chikushi (1). Next we determined the amount of fibroin mRNA that was synthesized and accumulated in the posterior silk gland of $+/\text{flc}$ or *flc* at days 3, 4 and 5. As shown by the brackets in Fig. 1a and c and in Fig. 2a and c, fibroin mRNA

TABLE 1. THE SYNTHESIS AND ACCUMULATION OF RNAs IN A PAIR OF POSTERIOR SILK GLANDS OF *Bombyx mori*, FLIMSY COCOON MUTANT

Genotype	Stages	Bulk RNA (μg)	Ribosomal RNA		Fibroin mRNA	
			Weight (μg)	^{32}P -cts/min (% of total labeled RNA)	Weight (% of bulk RNA)	^{32}P -cts/min (% of total labeled RNA)
$+/\text{flc}$	Fifth instar					
	day 3	1900	1600	38	—	1.6
	day 4	2600	2200	81	2.2	3.2
	day 5	3400	2800	62	3.3	8.5
<i>flc/flc</i>	Fifth instar					
	day 3	1500	1300	84	—	1.2
	day 4*	2000	—	71	—	0.7
	day 5	2400	2000	54	<0.4	1.8

* Obtained from data from the nuclear isolation experiment.

— Not determined.

Fig. 1. Bio-Gel fractionation and RNase T_1 fingerprints. [^{32}P]RNA labeled for 24 h at day 2 of the fifth instar was applied to a Bio-Gel A50m column (2.6×58 cm). The fractions shown in brackets were pooled, then digested by RNase T_1 and applied to a DEAE-Sephadex A25 column (1×10 cm). Oligonucleotides were eluted with a linear gradient of 0.1 to 0.45 M NaCl in the presence of 7 M urea. Bio-Gel fractionation, a, $+flec$; c, *flec*. Fingerprints, b, $+flec$; d, *flec* —○—, cts/min; — A_{254} .

was eluted in the flow through fraction from the Bio-Gel column (6). To estimate the amount of mRNA in the RNA samples, we pooled the RNA in the flow through fraction and digested it with RNase T_1 ; and this digest was applied to a DEAE-Sephadex column (Fig. 1b and d; Fig. 2b and d). The purity of the mRNA in the flow through fraction was estimated to be about 70% in both $+flec$ and *flec* at the day 3 stage (Fig. 1b and d). There was no significant difference between the RNAs of $+flec$ and *flec* larvae at the day 3 stage in the column profiles from the Bio-Gel and DEAE-Sephadex fractionations (Fig. 1). The proportion of mRNA synthesized was 1.6% and 1.2% of the labeled RNA of $+flec$ and *flec*, respectively (Table 1).

However, at days 4 and 5 there was a clear distinction in the column chromatography profiles of $+flec$ and *flec* (Fig. 2). The purity of fibroin mRNA in the flow

Fig. 2. Bio-Gel fractionation and RNase T₁ fingerprints. The procedure was the same as that described in Fig. 1 except that [³²P]RNA was labeled for 24 h at day 4 of the fifth instar. Bio-Gel fractionation, a, +/*flec*; c, *flec*. Fingerprints, b, +/*flec*; d, *flec* —○—, cts/min; —, A₂₅₄.

through fraction from the Bio-Gel column was 42% in day 5 larva of *flec* and 88% in +/*flec*. The proportions of mRNA in +/*flec* at days 4 and 5 were 3.2% and 8.5% of total labeled RNA, respectively, and 2.2% and 3.3% of the bulk RNA that accumulated (Table 1). These proportions are similar to those for the wild type at comparable stages (8). The mRNA proportion in the *flec* larva was estimated as 0.7% and 1.8% of the labeled total RNA at days 4 and 5, respectively.

The amount of fibroin mRNA accumulated in one *flec* larva was less than 10 µg, as calculated from the value of less than 0.4% of bulk RNA at day 5 (Table 1). This was less than 1/10 the amount for +/*flec* in which about 110 µg of mRNA accumulated. We thus inferred that such a reduced amount of fibroin mRNA cannot support normal fibroin production.

To check the unlikely possibility that the reduction of fibroin mRNA took place during the RNA extraction procedure we carried out the following experiment. Labeled fibroin mRNA prepared from the wild type was coextracted with the total RNA of the posterior silk gland of the day 5 *flec* in which the amount of fibroin mRNA is reduced. The labeled fibroin mRNA was recovered mostly in the flow through

Fig. 3. Bio-Gel fractionation of re-extracted fibroin mRNA. [32 P]-fibroin mRNA was prepared from the wild type. The mRNA was homogenized with the posterior silk gland of day 5 of the fifth instar of *flc*, then the total RNA was coextracted. The RNA mixture was applied to a Bio-Gel column (2.6×58 cm). —○—, cts/min of [32 P]-fibroin mRNA; —, A_{254} .

fraction of the Bio-Gel column (Fig. 3). This is evidence that the reduction does occur *in vivo*, but not during the RNA extraction procedure.

The lack of fibroin mRNA in the cytoplasm of flc. The localization of fibroin mRNA in the nuclei and cytoplasm was analyzed by nuclear isolation (9). A successful separation of the nuclei and cytoplasm was made as shown in Table 2. The [32 P]RNA extracted from each fraction was applied to a Bio-Gel column, then after the flow through fraction had been digested with RNase T₁, the digest was applied to a DEAE-Sephadex column (data not shown). In the wild type preparation 80% of the bulk RNA was found in the cytoplasmic fraction, and 75% of the fibroin mRNA also was recovered there. In contrast, in *flc* only 30% of the fibroin mRNA was recovered in the cytoplasmic fraction, but 70% of the bulk RNA was found there (Table 2). The purity of the mRNA was 40% and 70% for *flc*, and 57% and 80% for the wild type in the nuclear and cytoplasmic fractions, respectively. There was little difference in the localization of the bulk RNA and the purity of the mRNA in each fraction between the wild type and *flc*.

We believe that $+/flc$ is indistinguishable from the wild type because *flc* is a recessive gene (3). Thus we estimated that the proportion of mRNA in the nuclear fraction of $+/flc$ at day 4, was 0.80% ($3.2\% \times 25/100$; see Tables 1 and 2) of the total labeled

TABLE 2. DISTRIBUTION OF NUCLEIC ACIDS IN THE CYTOPLASMIC AND NUCLEAR FRACTIONS OF POSTERIOR SILK GLANDS OF *Bombyx mori*, THE WILD TYPE AND THE FLIMSY COCOON MUTANT*

		DNA (%) (μg)		RNA (%) (μg)		[32 P]-Bulk RNA (%)	[32 P]-Ribosomal RNA (%)	[32 P]-Fibroin mRNA (%)
Nuclear fraction	wild	98	193	19	770	21	22	25
	<i>flc</i>	99	147	29	529	32	35	66
Cytoplasmic fraction	wild	2	3	81	3400	79	78	75
	<i>flc</i>	1	1	71	1270	68	65	33

* The contents of DNA and RNA were measured by the Diphenylamine and Orcinol reactions, respectively. Values of the [32 P]RNAs were estimated from Bio-Gel fractionation and the RNase T₁ fingerprint analysis of the RNA samples extracted from each fraction.

RNA. For *flc* it was about 0.46% ($0.7\% \times 66/100$) in the nuclear fraction. This 0.46% value corresponds to 58% of the fibroin mRNA found in the nuclear fraction of *+/flc*. Since the synthetic amount of fibroin mRNA in *flc* larva at day 3 was 75% that of *+/flc* (see Table 1), the proportion of mRNA normally synthesized in the nuclear fraction at day 4 of *flc* was estimated as 0.60% ($0.80\% \times 75/100$). Thus 77% of the estimated value was found in the nuclear fraction of *flc* at day 4. Therefore the reduced accumulation of fibroin mRNA in the *flc* mutant must be due to the degradation of synthesized mRNA rather than to a reduction in the synthesis of mRNA.

Earlier we suggested that secretion of fibroin to the lumen in the *flc* mutant was inhibited because we observed the accumulation of numerous secretory granules in the apical region (2). Because we don't know whether the reduced amount of fibroin mRNA is caused by the inhibition of the fibroin secretion, we cannot say that the *flc* gene regulates the secretion of fibroin and/or the amount of fibroin mRNA after day 4 of the fifth instar. However, we could conclude that the total amount of fibroin mRNA that accumulated in the *flc* mutant by day 5 was too little to produce a normal amount of fibroin.

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