Identification of Novel Double-stranded RNA Produced in Midgut Epithelial Tissue of the Silkworm, *Bombyx mori*, during Infection by a Cypovirus 1

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INTRODUCTION

The genus Cypovirus (CPV) in the family Reoviridae infects the midgut epithelial cells in a wide range of insects (Francki et al., 1991). CPV virions carrying the genomic segments of double-stranded RNAs (dsRNAs) are included in polyhedra, crystallized forms of viral polyhedrin protein (Belloncik, 1989). Distinctive electrophoretic profiles of several CPV genomic dsRNAs have revealed that the *Bombyx mori* cypovirus 1 (BmCPV-1) genome is composed of 10 dsRNA segments (Payne and Mertens, 1983; Francki et al., 1991).

Segments 1, 3 and 4 of the BmCPV-1 genome have been characterized as genes encoding the putative capsid proteins Vp1, Vp2 and Vp3, respectively (Hagiwara et al., 2002), and sequences of genomic segments 5, 6, 7, 8 and 9 have already been analyzed (Hagiwara et al., 2001; Hagiwara and Matsumoto, 2000; Hagiwara et al., 1998a, b). It has been shown that segment 10 encodes the polyhedrin protein (Arella et al., 1988; Mori et al., 1989). In addition, a non-genomic small dsRNA, which was named the small polyhedrin gene segment (SP), has been found in the viral particles. SP has been shown to consist of an approximately 321-base pair sequence for the 5’ terminal 121 and 3’ terminal 200 bp of segment 10 lacking a 610-bp intervening portion, suggesting that the SP segment is a terminally conserved mutant generated from the segment 10 by an internal deletion event (Arella et al., 1988). From these findings, all eleven dsRNA segments could be extracted from and detected in the purified polyhedra or viral particles.

Recently, we found another dsRNA in a fraction of dsRNAs binding CF-11 resin from *B. mori* midgut tissues infected with BmCPV-1. The newly identified segmental dsRNA (designated S12) estimated to be approx. 0.65 kbp long by routine agarose gel electrophoresis, has never been detected not only outside but also inside BmCPV-1 polyhedra. Since the biological significance of the S12 dsRNA has yet to be elucidated, in the present study, we purified the S12 segment directly from BmCPV-1-infected midgut epithelia and performed a molecular analysis of cDNA for the novel S12 dsRNA.

MATERIALS AND METHODS

*Bombyx mori* larvae and *B. mori* cypovirus 1 (BmCPV-1)

A hybrid silkworm Kinshu-Showa was reared on an artificial diet under germ-free conditions during the larval stage. Fifth instar larvae at one day of age were injected with 25 μl of Tris-KCl buffer pH 7.4 (TK buffer) containing isolated free particles of the BmCPV-1 H strain (Hayashi and Bird, 1970) from $1.2 \times 10^7$ of polyhedra. BmCPV-1-infected midgut tissues were collected for the preparation of dsRNAs 5 days after the injection.

Purification of the BmCPV-1 dsRNA

(i) Extraction of dsRNA from the isolated BmCPV-1 polyhedra and virion

Polyhedra from the homogenates of BmCPV-1-infected midguts were isolated by repeatedly collecting insoluble precipitates in TK buffer containing 0.1% SDS as described by Mori and Kawase (1983). BmCPV-1 virions were released from the purified polyhedra after an incubation in an alkaline solution (0.05 M Na$_2$CO$_3$-0.05 M NaCl) for 1 h at room temperature, and collected by 50% sucrose density centrifugation at 105,000 × g for 3.5 h as described (Hayashi and Bird, 1970). The dsRNAs were extracted from the isolated polyhedra and virions by the acid phenol-quinidine thiocyanate-chloroform method (Sambrook and Russell, 2001). Finally, the purified dsRNAs were dissolved in DEPC water and stored at −80°C.

(ii) Affinity purification of dsRNAs from BmCPV-1-infected midguts using CF-11 cellulose resin

DsRNAs were purified from BmCPV-1-infected midguts by using the modified method of Dodds and Bar-Joseph (1983). Frozen midgut epithelia (5 g) which had been stored in liquid nitrogen was homogenized with 10 ml of STE buffer (0.2 M NaCl, 0.1 M Tris-HCl, and 2 mM EDTA, pH 7.0) containing 1% (W/V) SDS, 0.1 ml of 2-mercaptoethanol, 10 ml of 80% phenol, 10 ml of chlo-
boiled for 10 min and then transcribed to random primed method. DEPC-water containing 2 μg of S12 dsRNA was isolated from the band by the hot phenol extraction method. The 0.57-kbp fragment was amplified by 25 cycles from the uninfected midgut. As positive control experiments, the 0.57-kbp fragment was amplified by 25 cycles of the same PCR in a mixture containing randomly-primed cDNAs for the dsRNAs (100 ng) purified from infected midguts by the CF-11 affinity method as well as from polyhedra and virion particles.

RESULTS AND DISCUSSION

We compared the electrophoretic profiles of dsRNAs isolated from the polyhedra with those purified by the CF-11 affinity method from the B. mori midguts 5 days after BmCPV-1 infection in order to investigate the presence of novel virus-related dsRNAs in the cytoplasm. Arbitrary dsRNAs specifically bind to CF-11 in the STE buffer containing 15% ethanol (see the MATERIALS AND METHODS), and pure dsRNAs could be eluted from the resin using the ethanol-free STE-buffer. As shown in Fig. 1(A), the dsRNA fraction prepared by the CF-11 chromatography clearly contained an approximately 0.65-kbp dsRNA band, while a faint 0.65-kbp dsRNA band was detectable in the dsRNA fraction isolated by the acid phenol-guanidinium thiocyanate-chloroform method, as well as eleven other well-known dsRNAs (10 genomic dsRNAs and the non-genomic 321-bp SP known to be derived from S10) as noted in the INTRODUCTION. To our knowledge, no information has been published regarding the identification of the 0.65-kbp dsRNA from BmCPV-1-infected B. mori midguts and polyhedra.

It must be clearly shown that the newly identified dsRNA designated S12 as a substrate which BmCPV-1's RNA-dependent RNA polymerase amplifies, does not exist in the pool of total RNA sequences in the uninfected midguts, in order to conclude that S12 is in verity one of
Fig. 1. Electrophoresis (A) and Northern blot analysis (B) of the double-stranded RNAs in the BmCPV-1 H strain. (A) Three micrograms of dsRNA extracted from the BmCPV-1 polyhedra by the acid phenol-guanidinium thiocyanate-chloroform method (lane P) or dsRNA extracted from the BmCPV-1-infected midguts by the CF-11 affinity method (lane C) was electrophoresed in a 1.2% agarose gel and then stained with ethidium bromide. An arrow indicates the S12 band whose molecular size was calculated based on reported dsRNAs. (B) The separated dsRNAs were transferred onto a nylon filter by the alkali-blotting method. The DNA fragment encoding the entire sequence of S12 cDNA was labeled with fluorescein and hybridized to the blot as described in MATERIALS AND METHODS. The hybridization-positive signal was developed using a Genelmage™ kit (Amersham Pharmacia).

Fig. 2. Polymerase chain reaction analysis of the S12 sequence. Twenty-five cycles of PCR under the conditions outlined in MATERIALS AND METHODS were performed for the amplification of an approx. 0.57-kbp S12 fragment (nucleotides 13-585 of the upper sequence of S12 as shown in Fig.3) indicated by the arrow from the single-stranded cDNA for dsRNAs of the isolated polyhedra (lane 1) and virion particles (lane 3), and dsRNAs purified from infected midguts by the CF-11 affinity method (lane 5), and then the amplified DNA was specifically detected by Southern blotting using the labeled 0.57-kbp S12 fragment as a probe. Negative control experiments with the same PCR steps were also performed for the amplification of the same fragment from the intact dsRNAs of polyhedra (lane 2) and virion particles (lane 4), and the dsRNAs purified from infected midguts by the CF-11 affinity method (lane 6), without reverse transcriptase treatment. Thirty-five cycles of the same PCR were performed for amplification of the same fragment from the total RNA pool with (lane 7) and without reverse transcriptase treatment (lane 8), and for the genomic DNA, extracted from uninfected B. mori midguts (lane 9).
the BmCPV-1 dsRNA components. To this end, we investigated whether the S12 partial sequence could be specifically amplified from genomic DNA and cDNA synthesized using total RNA extracted from the uninfected midgut of fifth instar larvae at five days of age. The amplification of the S12 partial sequence (0.57 kbp) from the template cDNAs for dsRNAs from the polyhedra and virions, and dsRNAs purified from infected midguts by the CF-11 affinity method was confirmed by the 25-cycle PCR using the specific primers (Fig. 2). The amplified DNA bands were not detected in the PCR mixtures containing these series of dsRNA samples without reverse transcriptase treatment as templates, indicating that no DNA encoding the S12 sequence had not contaminated these dsRNA samples. Also these data indicated that the S12 dsRNA was occluded in the virion particles. However, 35 cycles of PCR under the same conditions as for the templates, such as cDNAs for the pool of total RNA sequences and genomic DNA from the uninfected midguts, did not amplify the 0.57-kbp fragment up to a detectable level (Fig. 2). Amplification also failed using the total RNA sample without reverse transcriptase treatment. In addition, no hybridization-positive DNA band in the uninfected midgut genomic DNA digested by restriction enzymes could be detected by Southern blotting using the entire S12 sequence as a probe under highly sensitive conditions suitable for the detection of a single copy gene (data not shown). From these experiments, it is shown that S12 is a

Fig. 3. Nucleotide sequence of both strands of S12 dsRNA. Numbers indicate nucleotide positions.
BmCPV-1 virion-associated dsRNA.

To examine whether S12 is derived from well-known genomic dsRNAs by enzymatic degradation, Northern blot hybridization of dsRNAs from purified polyhedra was performed using the labeled-S12 full-length cDNA as a probe (Fig. 1(B)). The cDNA probe was found to hybridize to S12 in both dsRNA fractions from infected midguts and purified polyhedra, but not to the ten genomic dsRNAs, indicating that the S12 dsRNA segment is not derived from the genomic dsRNAs.

To analyze the nucleotide sequence, cDNA for the purified S12 segment was synthesized. A long cDNA encoding the inner central part was obtained and then cDNAs encoding both ends of S12 were cloned by the 5' RACE method. We conducted three rounds of 5' RACE for both ends and obtained three clones for each strand confirmed to have common 5' ends. It was shown that the sequence encoded by the cloned cDNAs consisted of 647 nucleotides from the 5' to 3' end (Fig. 3), showing that the length of the determined nucleotide sequence corresponded well to the molecular size of S12 estimated by agarose-gel electrophoresis (Fig. 1). The nucleotide sequence was deposited into DDBJ, EMBL and GenBank under the accession number, AB183384. Both ends of the nucleotide sequence lack a highly conserved common sequence, 5'-AGTAA for 5' termini of plus strands, and GTTAGCC-3' for 3' termini of plus strands (Payne and Mertens, 1983). The absence of the common sequence at both ends could be a result of the forced termination of the reverse transcription due to the structural complexity of both terminal regions. Alternatively, the S12 segment may not contain the consensus sequences of both ends which may be essential as an initiation signal for amplification of the ten genomic dsRNAs by BmCPV-1's RNA-dependent RNA polymerase. If the latter explanation is true, there would be an exceptional recognition of the RNA polymerase for the initiation of S12 synthesis, however, there is still not enough information on the action of the BmCPV-1's RNA polymerase to make that conclusion.

No significant homology was detected with the FASTA program between the S12 nucleotide sequence and other characterized sequences in the current data base. Also no sequences significantly homologous with known amino acid sequences were found in the S12 fragment, although we investigated the similarity of all combinations of conceivable open reading frames from ATGs to stop codons in S12.

Although there have been a number of achievements in the biochemical study of BmCPV-1 to date, an unexpected finding is that the S12 dsRNA segment is occluded in isolated polyhedra and virions, and amplified in the cytoplasm during infection. It is conceivable that the classical methods used for the purification and analysis of dsRNAs, which have been sufficient to elucidate the dsRNA composition of the virion, might have been unable to detect S12, due to its negligible quantity in the polyhedra. Since the BmCPV-1 virion was shown to contain very few copies of the S12 segment which was vigorously amplified in the midgut cytoplasm, it was also indicated that S12 might have a distinguishing characteristic hindering itself from being occluded in the progeny virion. Furthermore, in the cytoplasm during infection, the copy number of S12 segments was clearly shown to rise to the level of those in the ten genomes originally abundant in virion particles (Fig. 1), suggesting that there is a more effective system to multiply S12 than the other segments. Although we failed to find sequences homologous with other segments (Fig. 3), it would be likely that the signal sequence required for its replication, which is not similar to those of the ten genomes, is retained in S12. Besides, S12 seemed not to contain a signal for the effective packaging of itself into virion particles.

The observations showed that S12 was highly amplified in the cytoplasm during the replication of BmCPV-1 although it was not easily occluded in the progeny virion, implied that the multiplication of S12 could be involved in the morphology of the virus. Further study with a cell culture system controlling BmCPV-1 infection and S12 amplification segment will provide an understanding of the essential roles of S12 as a virion-associated dsRNA.

REFERENCES


Hagiwara, K., Kobayashi, J., Tomita, M., and Yoshimura, T.


