Identification and expression analysis of the replication factor C protein in the silkworm, *Bombyx mori*

Kohji Yamamoto*, Mohammad R. Haque and Fumiko Saruta

Department of Bioscience and Biotechnology, Kyushu University Graduate School, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

(Received November 6, 2018; Accepted January 7, 2019)

Keywords: DNA replication, over expression, phylogenetic analysis, replication factor C

INTRODUCTION

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is an agriculturally crucial animal for silk production. Approximately 820 silkworm mutants have been deposited and categorized according to the color of the pupa, skin color, patterns of the larva, and color of the eggs (http://shigen.nig.ac.jp/silkwormbase/top.jsp). The phenotypes available for *B. mori* have been used in genetic studies. The recent focus in silkworm research has been on using the silkworm as an animal model in drug discovery and development instead of mice or other animals to confirm drug efficacy, studying the function of silkworm proteins, and utilizing it as an expression host for recombinant proteins. However, the cell cycle, including initiation of DNA replication, has not yet been investigated in *B. mori*. As an initial step toward understanding this process for this species, we attempted to identify factors involved in DNA replication.

Replication factor C (RFC) fulfills essential functions in DNA replication and repair of eukaryotic cells (Mossi and Hubscher, 1998). Eukaryotes have five RFC subunits, designated RFC1 to RFC5, and all of them contribute to RFC activity and chromosome replication (Ohta et al., 2002). These subunits belong to the AAA+ protein family (Ohta et al., 2002) and feature seven well-conserved motifs, the RFC boxes II through VIII, which are crucial for the RFC complex formation (Mossi and Hubscher).

Previously, we identified a minichromosome maintenance protein in silkworms, which is one of the proteins involved in DNA replication (Yamamoto et al., 2010). In the process of identifying components important for DNA replication in this species, a cDNA encoding RFC, another DNA replication factor, was obtained. In the present study, sequence alignment and the corresponding phylogenetic tree identified the RFC cDNA as a homologue of the *B. mori* RFC subunit 2 (bmRFC2). Furthermore, the expression pattern of the RNA encoding bmRFC2 was determined by real-time PCR.

MATERIALS AND METHODS

Insect and tissue dissection

Silkworms of the *B. mori* p50T strain were reared on mulberry leaves at the Institute of Genetic Resources, Kyushu University in Fukuoka, Japan. Larvae on day three of the fifth instar were dissected. Tissues (testis, ovary, whole region of silk gland, midgut, and fat body) were excised in ice-cold 0.75% NaCl, frozen immediately in liquid N\(_2\), and stored at −80°C until use. Hemolymph was collected on ice in a tube containing solid phenylthiourea to avoid melanization, and centrifuged at 1,000 × g for 15 min at 4°C. The pellet (hemocyte) was washed with ice-cold 0.75% NaCl, frozen immediately in liquid N\(_2\), and stored at −80°C until use.

Cloning and sequencing of cDNA encoding bmRFC2

Total RNA was prepared from the larval testes; first-strand cDNA was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR) using SuperScript II reverse transcriptase (Invitrogen, www.invitrogen.com) with an oligo-dT primer. The resulting cDNA was used as a template to obtain target fragments by PCR. The used oligonucleotide primers are as follows: 5′-CGC CATATG GAAGTAGACGAACCGGAGAAG-3′ (sense) and 5′-ATA GGATCC TCACCACTCCGGGTCACCGCC-3′ (antisense) for RFC2. The underlined and double-underlined regions are NdeI and BamHI restriction enzyme sites, respectively. To subclone the PCR product into an expression vector, the primers were designed using partial sequences found in the SilkBase EST database (Mita et al., 2004). PCR was performed under the following conditions: one cycle at 94°C for 2 min, 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 2 min, and one cycle at 72°C for 10 min. The PCR products were cloned in pGEM-T Easy vectors (Promega, www.promega.com). Homology alignment was performed with CLUSTALW (version 1.83) using 10 and 0.2 as the values of the gap creation penalty and gap extension, respectively. The phylogenetic tree was

*To whom correspondence should be addressed.
Fax: +81-92-624-1011. Tel: +81-92-802-4811.
Email: yamamok@agr.kyushu-u.ac.jp
Fig. 1. Alignment of amino acid sequences of the replication factor C protein 2 (RFC2). RFC2 sequences from the following organisms were obtained from Swiss-Prot and NCBI databases: *Bombyx mori* (determined in the present study), *Homo sapiens* (no. P35250), *Mus musculus* (no. Q9WUK4), and *Rattus norvegicus* (no. Q641W4). Frames indicate the RFC boxes.

Quantitative PCR (qPCR) analysis

Knowledge of tissue distribution of bmRFC2 mRNA could help in understanding its physiological importance. To this end, cDNAs were prepared as described in the previous subsection. qPCR primer sets for bmRFC2, *B. mori* ribosomal protein 49 (Bmrp49), *B. mori* actin A3 (BmActA3), *B. mori* glyceraldehyde-3-phosphate dehydrogenase (BmGAPDH), and *B. mori* ubiquitin (BmUBC) were designed. The sequences were as follows: bmRFC2_F (forward), 5′-TACTCCAGGCTTAGTGATG-3′; bmRFC2_R (reverse), 5′-CTGTGCTGTGAAGACTACTG-3′; Bmrp49_F (forward), 5′-GATGTGTTTTATAATTC-3′; Bmrp49_R (reverse), 5′-GCATCATCAAGATTTCACAGCTC-3′; BmActA3_F (forward), 5′-CGCCAACACCCTATTGTCTTCG-3′; BmActA3_R (reverse), 5′-ATGGGGCAGAGAGCTGTGATT-3′; BmGAPDH_F (forward), 5′-TCATCGGTCTTGACTATATG-3′; BmGAPDH_R (reverse), 5′-GAGCAGATGCTTTATCTGTA-3′; BmUBC_F (forward), 5′-GTGTCCGCACTTTGTCTT-3′; and BmUBC_R (reverse), 5′-ACCTTCCCTTCGCATTCT-3′.

qPCR was performed on a StepOne real-time PCR system (ThermoFisher Scientific, Waltham, MA, USA) with TaqMan® RNaseP Fast 48-well Instrument Plate (ThermoFisher Scientific) as per the manufacturer’s instructions. The PCR program includes denaturation at 95°C for 10 s, followed by 40 cycles each of denaturation at 95°C for 5 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. All samples were analyzed in triplicate, and bmRFC2 levels were normalized against corresponding Bmrp49, BmActA3, BmGAPDH, and BmUBC levels and expressed as the bmRFC2: Bmrp49, bmRFC2: BmActA3, bmRFC2: BmGAPDH and bmRFC2: BmUBC ratios.

RESULTS AND DISCUSSION

Cloning and sequencing of cDNA encoding bmRFC2

The cDNA encoding the putative RFC2 was isolated by RT-PCR using total RNA from the silkworm testis. The nucleotide sequence of RFC2 was determined and deposited in GenBank under accession no. AB177621. This sequence contained an open reading frame of 1,023 bp, encoding 340 amino acid residues (Fig. 1), with a theoretical molecular mass and pI of 37,373 and 6.96, respectively. The deduced amino acid sequence of this putative RFC2 showed 64-66% identity with that of RFC2s from *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*. The amino acid sequence of the *B. mori* RFC revealed 12%, 23-24%, 35-36%, and 36-37% homologies to mammalian RFC1s, RFC3s, RFC4s, and RFC5s, respectively. Consensus regions, so-called RFC boxes I to VIII, were previously identified (Cullmann et al., 1995). Boxes II to VIII were discovered to have been conserved in the bmRFC2 amino acid sequence (Fig. 1). Box I is present in the large RFC subunit, similar to RFC1 (Luckow et al., 1994). The phylogenetic tree (Fig. 2) showed that the cloned RFC was included in the RFC2 group. Based on the results of the sequence alignment (Fig. 1) and phylogenetic tree (Fig. 2), this RFC appeared to be an RFC2 homologue.

Localization of the bmRFC2 transcript

qPCR analyses were performed to examine the localization of the bmRFC2 transcript. To reduce experimental errors, several housekeeping genes such as Bmrp49 (Fig. 3A), BmActA3 (Fig. 3B), BmGAPDH (Fig. 3C), and BmUBC prepared using the GENETYX-MAC software (ver. 14.0.12; GENETYX Corporation, Tokyo, Japan).
Fig. 2. Phylogenetic analysis of the replication factor C protein 2 (RFC2) amino acid sequences. The phylogenetic tree was generated with a neighbor-joining plot software. Accession numbers for each RFC2 were obtained from DDBJ and Swiss-Prot databases. The scale bar indicates branch length. The arrowhead indicates the position of bmRFC2.

(Fig. 3D) were used as internal controls. The genes used were detected in various tissues of B. mori (Peng et al. 2012). Figure 3A shows that the bmRFC2 transcript in hemocytes was expressed with a 1.8-fold higher rate than in testis, whereas it was reduced to 24% and 10% in the ovary and silk gland, respectively. Similar results are shown in Figs. 3B-D, which reveal that bmRFC2 transcripts were abundant in hemocytes, testes, midgut, and fat bodies of larval silkworms. Small amounts of the mRNA were also detected in the ovaries and silk glands. Since expression pattern of bmRFC2 transcripts were similar with several internal controls, the current result is reliable.

The tissues tested were obtained from larvae on day three of the fifth instar. Fifth-instar silkworm larvae grow rapidly, and internal tissues proliferate and differentiate

Fig. 3. Localization of the replication factor C protein 2 (RFC2) transcripts. The amount of transcript in various tissues was analyzed using qPCR, as described in the Materials and Methods section, and normalized against Bmrp49 (A), BmActA3 (B), BmGAPDH (C), and BmUBC (D). Error bars denote the standard deviations obtained from three experiments.
during this stage. Surprisingly, mRNA was not abundant in the ovary, which is one of the proliferating tissues. The question of why there was such a small amount of bmRFC2 mRNA in the ovaries and silk glands remains unanswered. Comprehensive studies on the developmental changes in mRNAs, proteins, and protein activity in various tissues are required to further understand the physiological roles of these proteins.

DNA replication in eukaryotic cells involves several enzymes and proteins that collaborate (Maga et al., 2000, Mossi et al., 2000). The RFC family plays a role in DNA replication initiation, chromosome condensation, and recombination (Kim and MacNeill, 2003). We found that RFC Boxes I to VIII are in the amino acid sequence of bmRFC2 (Fig. 1). A large amount of protein is required to examine the activity of bmRFC2 and its function in DNA replication of silkworms. We have obtained the recombinant bmRFC2 overproduced in bacteria and purified it to homogeneity using the His-tag purification system (data not shown). We are in the process of developing bacterial production and purification schemes for the other RFCs, including RFC1, RFC3, RFC4, and RFC5. The highly purified proteins would make it possible to assess their physiological role in the ovary, and to gain insight into their functions. Development of a protocol for the preparation of a large amount of the \textit{B. mori} RFC complex will contribute to detailed structural and molecular biological studies on bmRFCs.

**ACKNOWLEDGMENTS**

The current work was supported by JSPS KAKENHI (Grant Numbers 17K19272 and 16KK0172).

**REFERENCES**


