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Molecular Cloning of the *Bombyx mori* Ubiquitin Homologue Gene That Is Up-regulated Upon Infection

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Ubiquitin can be covalently attached to cellular proteins as a post-translational modification and is involved in metabolic stresses, such as heat shock and immune response. We have isolated and sequenced a cDNA encoding ubiquitin from the silkworm, *Bombyx mori*. The insert in the clone is 533 nucleotide long with an open reading frame of 387 nucleotides that encodes a protein of 129 amino acids with a molecular weight of 14.8 kDa. The amino acid sequence shared high homology with the ubiquitins known so far. The result of dot blot hybridization showed that the *B. mori* ubiquitin gene is up-regulated upon *E. coli* infection, suggesting that the *B. mori* ubiquitin plays an immune-related role.

Key words : Ubiquitin, *Bombyx mori*

Introduction

Ubiquitin is a small protein that is found in all eukaryotic cells (Goldstein *et al.*, 1975). Its sequence is so highly conserved that the human and moth proteins share almost 100% identity at the protein level (Bishoff and Schwartz, 1990).

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by ubiquitin system. In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein (Hershko and Ciechanover, 1998). This small protein, ubiquitin can be covalently attached to cellular proteins as a post-translational modification and is involved in a variety of cellular functions, including regulation and stress response (Glotzer *et al.*, 1991; Hochstrasser *et al.*, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992; Wilkinson, 1996), and is implicated in the immune response, development, and programmed cell death (Hershko and Ciechanover, 1998). In addition, there is the report that ubiquitin system is related to aging (Niedzwiecki and Fleming, 1993). Aberrant proteins are selectively degraded within mammalian cells at elevated rates (Hershko and Ciechanover, 1982). In the degradation of heat-denatured proteins in mammalian cells, the ubiquitin dependent degradation system is activated (Parag *et al.*, 1987). Also, the ubiquitin/proteosome pathway is responsible for the degradation of the bulk of celluar proteins during homeostasis (Rock *et al.*, 1994), as well as the increased degradation of proteins that occurs upon metabolic stresses, such as heat shock (Finley *et al.*, 1987; Niedzwiecki and Fleming, 1993). Noxious stimuli can facilitate the denaturation or damage of intracelluar proteins, which then become targets for ubiquitination and subsequent proteolysis. In yeast, loss of the ubiquitin gene is not in itself lethal, although mutant cells can no longer withstand even mild injuries (Finley *et al.*, 1987).

In order to study the relationship between the immune response and ubiquitin, we isolated and characterized the ubiquitin gene from *B. mori*. In this paper, we reported that a cDNA structure and characterization of ubiquitin homologue gene that is up-regulated by invaded microorganisms.

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Materials and Methods

Biological materials

The silkworms, *Bombyx mori*, were reared on an artificial

diet at 24-27°C and 70-90% humidity. The 5th instar *B. mori* larvae were immunized with a subcutaneous injection of *E. coli* K12 strain DH1, grown overnight in LB liquid medium at 37°C with shaking, on the 7th segment of the abdomen.

cDNA library screening

From the cDNA library of *B. mori* injected with *E. coli* (Kim *et al.*, 1996), the clone containing the ubiquitin gene was isolated with ^{32}P-labeled 501 bp ubiquitin homolog gene from *Antheraea yamamai* ESTs as a probe (Yun *et al.*, 2000). Hybridization of the plaque-blotted Hybond N$^+$ membranes (Amersham Pharmacia Biotech Co.) were performed in 5× SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 5× Denhardt's solution, and 0.5% SDS at 65°C overnight with probe. The membranes hybridized with probe were washed twice in 2× SSC, 0.5% SDS for 5 min at room temperature, washed twice with 2× SSC, 0.2% SDS for 15 min at 65°C, and washed more than twice with 0.2× SSC, 0.2% SDS for 15 min at 65°C until the background signals were reduced.

DNA sequencing and database search

The complete sequence of the cDNA encoding *B. mori* ubiquitin was determined with a dye terminator cycle sequencing method using an automatic DNA sequencer (Perkin Elmer Co., ABI 377). For sequencing, double-stranded DNA was prepared using Wizard Plus SV Minipreps DNA Purification System (Promega Co.). DNA (300-500 ng) was mixed with T3, T7 primer (3.2 pmole) and 8 μl of Terminator Reaction Mix (Perkin Elmer Co.), and was reacted by Polymerase Chain Reaction (PCR) method. Twenty-five cycles of PCR were performed at 96°C for 30 s, 50 15 s, and 60°C for 4 min. The resulting PCR products were electrophoresed on the 4.5% denatured polyacrylamide gel and analyzed by DNA Sequencing Analysis Software (Perkin Elmer Co.). After then, amino acid sequence was deduced from the cDNA sequence. The homology of the nucleotide sequences of cDNA and deduced amino acid sequences with other species were analyzed through GenBank database.

Dot blot analysis

Total cellular RNAs of *E. coli*-injected *B. mori* were isolated at 12 hours post infection. Total cellular RNAs (2 μg/well) from naive or *E. coli*-injected *B. mori* were danatured by glyoxalation (McMaster and Carmichael, 1977), transferred onto a nylon membrane (Amersham Pharmacia Biotech Co.), and hybridized at 42°C in the presence of 50% formamide. The probe used to detect ubiquitin transcripts was a 533 bp *B. mori* ubiquitin isolated by library screening.

Nucleotide sequence accession number

The sequence data obtained from this study have been deposited with the EMBL/GenBank/DDBJ libraries under the accession number AF308163.

Results and Discussion

We randomly selected cDNA clones from the cDNA library of *A. yamamai* and constructed expressed sequence tags (ESTs) profile (Yun *et al.*, 2000). Among ESTs, we focused our attention on clone AY783 that had high similarity with ubiquitin genes from other species. The ubiquitin gene is related to stress response, such as heat shock and gamma irradiation (Glotzer *et al.*, 1991; Hochstrasser *et al.*, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992; Wilkinson, 1996; Delic *et al.*, 1993), and is implicated in the immune response, development and programmed cell death (Hershko and Ciechanover, 1998).

To investigate the relationship between ubiquitin and immune response, we screened the cDNA library constructed with *B. mori* injected with *E. coli* (Kim *et al.*, 1996) and containing self-defense related gene, antibacterial protein genes, nuecin (Yun *et al.*, 1997), enbocin (Kim *et al.*, 1998), transferrin (Yun *et al.*, 1999) and so on. The *B. mori* ubiquitin gene was isolated using ^{32}P-labeled 501 bp ubiquitin homolog gene from *A. yamamai* ESTs as probe. As a result of screening, positive clone was selected and sequenced. As shown in the complete nucleotide sequence (GenBank accession number; AF308163) in Fig. 1, there is a 5'-untranslated region of 46 nucleotides followed by an initiating ATG codon. The TAA termination codon occurs at nucleotide 434, thus translation of the sequence from nucleotides 47 to 433 would produce a protein of 129 amino acids with a calculated molecular mass of 14,796. The AATAAA consensus polyadenylation signal is present at

```
                                                        →
1   TTTTTTTCTTTTTCTTTCCAAGCACGTTTTTCATAGGAGCAACACAATGCAAATATTCGTCAAAACCCTT
                                                      M  Q  I  F  V  K  T  L
71  ACGGGGAAGACCATTACATTGGAGGTGGAAGCTTCCGACACTATCGAAAATGTCAAAGCTAAAATCCAAG
     T  G  K  T  I  T  L  E  V  E  A  S  D  T  I  E  N  V  K  A  K  I  Q
141 ACAAGGAAGGTATTCCTCCAGACCAACAACGTCTCATCTTTGCCGGGAAACAATTAGAAGATGGCCGCAC
    D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T
211 TCTTTCAGACTATAACATCCAGAAAGAATCCACACTTCACCTGGTTTTGAGACTTAGAGGAGGTACAATT
     L  S  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  T  I
281 GAACCTTCCCTTCGCATTCTCGCCATGAAGTATAACTGTGAGAAAATGATTTGCCGTAAATGCTATGCCC
      E  P  S  L  R  I  L  A  M  K  Y  N  C  E  K  M  I  C  R  K  C  Y  A
351 GTCTTCATCCTCGTGCTACCAACTGTCGCAAGACAAAGTGCGGACACACTAACAATTTGAGACCCAAAAA
     R  L  H  P  R  A  T  N  C  R  K  T  K  C  G  H  T  N  N  L  R  P  K
                 ***
421 GAAGATCAAGGATTAAATAATAACCAAATGGGAAATTTATTGTAACAATACTATTCTCGTAACATGCAAA
    K  K  I  K  D
491 AGAAACTTAAAATAAAAGTTTTCCTCATAAAAAAAAAAAAAAA
```

Fig. 1. The nucleotide and predicted amino acid sequences of the *B. mori* ubiquitin gene (GenBank accession No. AF308163). The right-hand arrow, asterisks and underlined nucleotide sequence indicate the translation start, stop codon and the putative polyadenylation signal, respectively.

```
BmUB:   1  MQIFVKTLTGKTITLEVEASDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN 60
DmUB:   1                    P                                         60
HsUB:   1                    P                                         60
ScUB:   1                    S    D   S                                60
OsUB:   1                    S    D                                A   60
CeUB:   1                                                              60

                                          *    *  *          *    *
BmUB:  61  IQKESTLHLVLRLRGGTIEPSLRILAMKYNCEKMICRKCYARLHPRATNCRKTKCGHTNN 120
DmUB:  61                  I         Q    D                    K        120
HsUB:  61                  I      Q  Q    D               V    K        120
ScUB:  61                  I     KA  S    D SV        P        R      Q 120
OsUB:  61                  I     QA  R   QD               V    K    S Q 120
CeUB:  61                  I      Q  Q    D Q         P   S    K    SSE 120

BmUB: 121  LRPKKKIKD 129
DmUB: 121        L - 128 (95%)
HsUB: 121        V - 128 (94%)
ScUB: 121        L - 128 (89%)
OsUB: 121          N 129 (89%)
CeUB: 121    I   L - 128 (90%)
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Fig. 2. Alignment of the amino acid sequences of ubiquitin family with those of the *B. mori* ubiquitin (BmUB). DmUB, *Drosophila melanogaster* ubiquitin; HsUB, *Homo sapiens* ubiquitin; ScUB, *Saccharomyces cerevisiae* ubiquitin; OsUB, *Oryza sativa* ubiquitin; CeUB, *Caenorhabditis elegans* ubiquitin. Identical residues are indicated by the blank and the asterisks indicate conserved cystein residues.

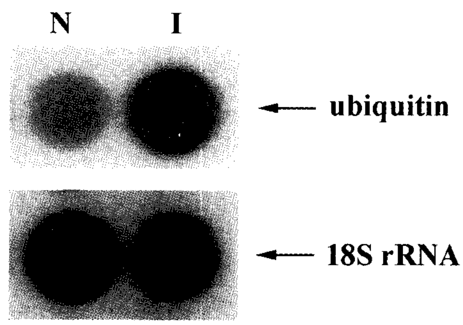

Fig. 3. Dot blot analysis of the ubiquitin transcripts from *E. coli*-induced *B. mori*. Total cellular RNAs from the naive and *E. coli*-injected *B. mori* were isolated at 12 hours post infection. The probe used to verify the involvement of immune response with ubiquitin was a 533 bp *B. mori* ubiquitin cDNA isolated by library screening. N, naive *B. mori*; I, *E. coli*-induced *B. mori*.

65 nucleotides downstream from the stop codon (TAA). The poly(A^+) tail is at 13 nucleotides downstream from the recognition sequence, which is in agreement with the fact that the signals are most often present at 11-30 nucleotides upstream from the poly(A^+) tail (Fitzgerald and Shenk, 1981).

The deduced amino acid sequences of the *B. mori* ubiquitin cDNA were aligned with insect *(Drosophila melanogaster)*, mammal *(Homo sapiens)*, yeast *(Saccharomyces cerevisiae)*, plant *(Oryza sativa)* and nematode *(Caenorhabditis elegans)* ubiquitin as shown in Fig. 2. Amino acid identity was 95%, 94%, 89%, 89% and 90% with those of *D. melanogaster, H. sapiens, S. cerevisiae, O. sativa* and *C. elegans,* respectively. The *B. mori* ubiquitin gene shared high similarity with other insect, plant, mammal and nematode. These results agree with the fact that ubiquitin sequence is so highly conserved that the human and moth proteins share almost 100% identity at the protein level (Bishoff and Schwartz, 1990). In addition, this result suggests that the ubiquitin gene is conserved in all eukaryotic cells (Goldstein *et al.*, 1975) in revolutionary respect. Another feature is that the six ubiquitins are conserved with cystein residues at 5 positions. But, in only *O. sativa,* there is glutamine instead of first cystein. In this result, we supposed that the *B. mori* ubiquitin is similar to other ubiquitin in aspect of tertiary structure and function.

On the other hand, we carried out the RNA dot blot analysis to verify whether the *B. mori* ubiquitin was related to immune response using 533 bp *B. mori* ubiquitin isolated by library screening as a probe (Fig. 3). As shown in Fig. 3, when *B. mori* was treated with 12 hours post infection with *E. coli*, there was a dramatic increase in the level of the *B. mori* ubiquitin transcripts relative to that seen in the naive. In human, ubiquitin pathway is involved in lymphocyte gamma-irradiation-induced apoptosis (Delic *et al.*, 1993). And, in insect, there is the related report that the ubiquitin/proteosome pathway plays an important role in the death of the intersegmental muscles of the moth *Manduca sexta* (Grimm *et al.*, 1996). Also, the ubiquitin/proteosome is the major proteolytic system of eukaryotic cells for the selective protein degradation. As mentioned above, the roles of ubiquitin genes are unknown, although ubiquitin genes are induced by stress, heat shock (Fornace *et al.*, 1989; Myer and Schwartz, 1996), gamma irradiation (Delic *et al.*, 1993) and so on. Also, it is not clear by which ubiquitin mechanism is induced by injection with *E. coli*, but the dramatic increase in the level of the *B. mori* ubiquitin transcripts after infection suggests that ubiquitin may be involved immune mechanism. Therefore, we suggest that when *B. mori* is invaded or injected with outer microorganism, ubiquitin might play an immune-related role as previous report (Hershko and Ciechanover, 1998).

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