Intron Loss Is Associated with Gain of Function in the Evolution of the Gloverin Family of Antibacterial Genes in Bombyx mori

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Gene duplication is a characteristic feature of eukaryotic genomes. Here we investigated the role of gene duplication in the evolution of the gloverin family of antibacterial genes (Bmglv1, Bmglv2, Bmglv3, and Bmglv4) in Bombyx mori. We observed the following two significant changes during the first duplication event: (i) loss of intronV, located in the 3′-untranslated region (UTR) of the ancestral gene Bmglv1, and (ii) 12-bp deletion in exon3. We show that loss of intronV during Bmglv1 to Bmglv2 duplication was associated with embryonic expression of Bmglv2. Gel mobility shift, chromatin immunoprecipitation, and immunodepletion assays identified chorion factor 2, a zinc finger protein, as the repressor molecule that bound to a 10-bp regulatory motif in intronV of Bmglv1 and repressed its transcription. Gloverin paralogs that lacked intronV were independent of chorion factor 2 regulation and expressed in embryo. These results suggest that change in cis-regulation because of intron loss resulted in embryonic expression of Bmglv2-4, a gain of function over Bmglv1. Studies on the significance of intron loss have focused on introns present within the coding sequences for their potential effect on the open reading frame, whereas introns present in the UTRs of the genes were not given due attention. This study emphasizes the regulatory function of the 3′-UTR intron. In addition, we also studied the genomic loss and show that “in-frame” deletion of 12 nucleotides led to loss of amino acids IHDF resulting in the generation of a pre-pro-processing site in BmGlv2. As a result, the N-terminal pro-part of BmGlv2, but not of BmGlv1, gets processed in an infection-dependent manner suggesting that pre-pro-processing is an evolved feature in Gloverins.

Insects depend on humoral (antimicrobial peptide (AMP)) synthesis and cellular responses to effectively kill the invading microbes (bacteria and fungi) as they lack adaptive immunity capable of producing antibodies (1). Typically AMPs have low molecular weight, are water-soluble, and possess broad spectrum antibacterial activity (2). Cecropins, attacins, drosocins, and diptericins have antibacterial activity against Gram-negative bacteria, whereas defensins and metchnikowin kill Gram-positive bacteria (2, 3). Specificity in the immune response against a particular class of microbes is because of specific interaction between pathogen-associated molecular patterns present on the microbes and pathogen recognition receptors of the insect (1, 4). Because of the polyclonal recognition of pathogen-associated molecular patterns, any microbial infection leads to production of the same battery of AMPs through two evolutionarily conserved pathways toll or imd (1, 4).

The Toll and IMD pathways mediate regulation of innate immune response in insects, in response to fungal and bacterial infections, respectively (1, 4). Dorsal was the first transcription factor to be identified as regulator of innate immunity in Drosophila. Ip (5) identified Dif (Dorsal related immunity factor), a second Drosophila Rel protein, in the larval fat body. Drosophila Rel proteins are found in the cytoplasm but are translocated to the nucleus upon activation of the immune pathway in a signal-dependent manner. An extracellular signal, encoded by the spatzle gene, binds to Toll, a membrane-bound receptor. Spatzle binding to Toll causes activation of a signal cascade involving Tube and Pelle, a serine/threonine kinase. This leads to phosphorylation and subsequent degradation of Cactus, an IkB homolog (1, 4). In the absence of Cactus, Dorsal and DIF are free to translocate to the nucleus, where they act as transcription factors and initiate expression of AMP genes. A third Rel factor, Relish, was isolated in a molecular screen for genes whose expression is altered after infection (6). Relish is a compound protein containing both Rel (activating) and IkB (inhibitory) domains (7). Upon infection, the Relish inhibitory domain is proteolytically cleaved by dredd, a Drosophila caspase, to release the active Rel protein (8). Genetic epistasis studies and molecular analysis of gene function show that imd, Relish components of a signaling pathway are distinct from the Toll pathway and are essential for combating Gram-negative bacterial infection (8—15).

Although the mechanism of AMP activation has remained conserved across species, the repertoire of AMPs present in
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different organisms varies (1–4) e.g. hemolin, an AMP with an immunoglobulin fold (16), has been reported only from lepidopteran insects. This prompted us to look for Lepidoptera-specific AMPs in Bombyx mori with a broader aim to understand the evolution of immune system in insects. B. mori is the only lepidopteran insect for which whole genome sequence (17, 18) and EST data base (19) are available. An analysis of B. mori genome and EST sequence revealed the presence of AMPs like cecropins, moricins, attacins, lebocin, enbocin, hemolin, and gloverins. Our analysis, based on sequence information available currently, suggests that like hemolin gloverins are also restricted to lepidopteran insects.

Gloverins are glycine-rich (16–20%) antibacterial proteins and have been reported from lepidopteran insects Hyalophora gloveri, Helicoverpa armigera, and Trichoplusia ni (20–22). They are basic, heat-stable proteins with random coil structure in solution and take α-helical structure upon interaction with lipopolysaccharide (20). We found that silkworm has four gloverin genes (Bmglv1, Bmglv2, Bmglv3, and Bmglv4). The derived genes Bmglv2–4 have evolved as a result of three gene duplication events. A significant difference was observed in the embryonic expression profile of these genes, whereas, derived genes Bmglv2–4 express in all embryonic stages but not the ancestral gene Bmglv1. This suggested that embryonic expression of derived genes was gained during duplication of Bmglv1. Embryonic regulation of AMP genes is not well studied; hence we set out to study the genetic changes that led to evolution of embryonic expression in the Lepidoptera-specific AMP gene family, gloverin. Molecular analysis suggested that embryonic expression of Bmglv1 was regulated by an intron present in the 3’ UTR of the gene. Further characterization of the regulatory role of intron V led to identification of CF2, a zinc finger transcription factor that regulates oogenesis as suppressor of Bmglv1 in the embryo. We also tested the significance of embryonic expression of daughter gene Bmglv2, by RNAi which led to reduced hatching of embryos. This indicated that Bmglv2 has a role in embryonic development. We show that this gain of function in embryonic development was linked to loss of intron V.

Introns, noncoding sequences interrupting protein-coding genes, are the hallmark of eukaryotic gene organization (23). However, the role of intron in AMP gene regulation was previously not known. This is the first study demonstrating the regulatory role of an intron in an AMP gene and association between intron loss and gain of function. This study also emphasizes evolution of functional divergence in AMP gene paralogs.

**EXPERIMENTAL PROCEDURES**

**Animals—**B. mori strains Pure Mysore and Nistari were collected from the sericulture station at Hindupur, Andhra Pradesh, India. Escherichia coli (K12 strain), cultured in antibiotic free LB media, was used for infecting silkworm larvae.

**Gel Shift Assay (EMSA)—**Embryonic nuclear extracts were prepared by homogenizing embryos (40–72 h AEL) in extraction buffer (20 mM Hepes, pH 7.9, 5 mM MgCl$_2$, 0.1 mM EGTA, 12.5% sucrose, 25% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture) using a Dounce homogenizer, followed by centrifugation at 3300 × g for 20 min at 4 °C. The precipitated nuclei were suspended in 1 ml of the extraction buffer. Embryonic extracts were also prepared from w$^{1118}$ and Df(2L)Y$^{27}$ flies (these flies lack the cf2 locus). For EMSA, 100 ng of double-stranded cf2 oligonucleotide (AGTAAATATATATTTAAA) was labeled with 3 μl of [γ-32P]ATP (4 × 10$^6$ cpm) and 1 μl of polynucleotide kinase (10 units/μl) in 1 μl of PNK buffer (New England Biolabs) for 1 h at 37 °C. The labeled DNA was purified on a G-50 column. The binding reaction was performed for 30 min at room temperature by mixing 1 ng of purified [32P]-labeled double strand synthetic oligonucleotide probe (4000 cpm/μl), 10 μg of nuclear extracts, 300 ng of poly(dI-dC), and 5 mM Zn$^{2+}$ in the presence of a protease inhibitor mixture (Sigma). Cold competition was performed by preincubating the extracts with a 40-fold excess of unlabeled oligonucleotide at room temperature for 15 min. Anti-CF2 monoclonal antibody was added to the binding reaction for 30 min to perform supershift experiments. The binding reaction was analyzed by electrophoresis on native 6% polyacrylamide gels.

**In Vitro Transcription and Translation—**In vitro transcription was done essentially as mentioned in Suzuki et al. (24, 25). For in vitro translation, different expression constructs were incubated with embryonic extracts prepared from Drosophila or silkworm embryos. Embryos were collected, dechorionated by bleaching, washed 3–5 times with 0.1% Triton X-100, and transferred to hypotonic buffer at 4 °C. Embryos were further washed in 3–5 volumes of cold hypotonic buffer (10 mM Hepes-KOH, pH 7.4, 15 mM KCl, 1.5 mM Mg(OAc)$_2$, 2 mM DTT) on ice. Next, embryos were Dounce-homogenized, and homogenate was centrifuged for 15 min at 15,000 rpm at 4 °C. The supernatant was transferred to a fresh microcentrifuge tube and centrifuged again under the same conditions to remove any residual debris. Extracts were centrifuged through Sephadex G-25 Superfine columns prepared in buffer A (30 mM Hepes-KOH, pH 7.4, 100 mM KAc, 2 mM Mg(OAc)$_2$, 2 mM DTT, protease inhibitor mixture). The column was transferred to a fresh collection tube to which a volume of cold buffer A equal to the extract volume was added, and the column was centrifuged for 3 min at 200 × g at 4 °C. 100 μl of eluate was applied to a P6 (Bio-Rad) desalting column to remove salt and other contaminants, and the desalted eluate was used for in vitro translation as mentioned in Gebauer et al. (26). Entire protocol was first standardized with CantonS Drosophila embryonic extracts (data not shown), and then the standardized protocol was used for experiments with B. mori embryonic extracts. B. mori embryos were collected 40 and 56 h AEL and pooled.

**Immunodepletion of Embryonic CF2—**Immunodepletion was done largely following the protocol as described previously (27) with the following modifications. Immunodepletion of CF2 was performed in embryonic extracts in a final volume of 500 μl of buffer A. CF2-depleted supernatant was collected and used immediately for in vitro translation.

**Western Blotting—**In vitro translation reaction product was separated on a 10% minigel SDS-polyacrylamide gel (Bio-Rad). Protein samples separated by SDS-PAGE were electrophoretically transferred using a Trans-blot cell (Bio-Rad), at 200 mA overnight at 4 °C to Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences). The blots were stained
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for total protein by Ponceau S (Sigma) and blocked in 10% non-fat dry milk in 0.5% Tween 20, 0.05% SDS in PBS (Blocking buffer). The blots were incubated for 6 h at room temperature in primary antibodies and then washed four times (10 min each) in Tween 20 + PBS followed by a 2-h incubation at room temperature with the secondary antibody (Sigma). The blots were then washed three times for 30 min in Tween 20 + PBS and rinsed once in PBS. The protein bands were detected using horseradish peroxidase-enhanced chemiluminescence (Amer sham Biosciences). Anti-CF2 (mouse) and anti-GST (rabbit) antibodies were used in 1:10,000 and 1:1000 dilutions, respectively, for probing.

RT-PCR, in Vitro Transcription, and RNAi—Total RNA was isolated using TRIzol reagent (Invitrogen), dissolved in 50 µl of RNase-free water, and quantified in a spectrophotometer. 1 µg of RNA was used in 20 µl of RT reaction by using SuperScriptII (Invitrogen) and oligo(dT) or gene-specific primers. For synthesizing dsRNA Bmglv1 and Bmglv2, PCR products were cloned between BamHI and KpnI sites of pB-SK+ vector, and in vitro transcription was done with linearized template using T3 and T7 RNA polymerase, separately. Single strand RNA was purified after DNase treatment to remove template plasmid and quantified in a spectrophotometer. An equal amount of sense and antisense RNA was used for annealing in annealing buffer by heating at 95 °C for 5 min followed by slow cooling to room temperature. Respective dsRNA was injected on the 1st day of 5th instar larvae. Male and female moths eclosed from dsRNA injected larvae were allowed to mate (15 experiments each for Bmglv1-dsRNA and Bmglv2-dsRNA), and egg hatching was calculated. An equivalent volume of nontarget baculoviral ie1-dsRNA was injected in control larvae. Different concentrations of dsRNA were used for standardization of RNAi, and the data presented here are from larvae injected with 10 µg of dsRNA. A list of primers used for cloning of dsRNA target site has been provided in the supplemental material.

Chromatin Immunoprecipitation—The protocol followed for ChIP was essentially as mentioned on line with the following modifications. Instead of Staphylococcus aureus cells, protein A beads and silkworm embryo were used. Fluorescence real time PCR was done with double-stranded DNA dye SYBR green (PerkinElmer Life Sciences) on an ABI PRISM 7700 system (PerkinElmer Life Sciences) to quantify the enrichment of the cf2-binding element upon ChIP. PCR specificity was confirmed by the molecular size of the PCR product and ΔCt analysis. Reactions were done in triplicate and compared with input DNA also (in triplicate) and nontemplate control (in duplicate).

Plasmid Constructions—Full-length Bmglv1 cDNA was cloned between BamHI and KpnI sites in pFB-His-GST expression vector to generate expression plasmid pFB-His-GST-Glv1. This plasmid was later used for transforming DH10Bac bacterial cells to obtain recombinant pFB-His-GST-Glv1 bacmid that was subsequently transfected into Sf9 cells to get the recombinant virus expressing His-GST-Glv1 fusion protein. Maximum expression of the protein was observed between 70 and 96 h postinfection. His-GST-Gloverin1 fusion protein was purified using a glutathione column. To generate expression vectors with different promoters to be used in the in vitro translation reaction, Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedrin promoter of pFB-His-GST basic vector was replaced with B. mori cytoplasmic actin (BmA3-Actin) promoter (for control plasmid) or Bmglv1 or Bmglv2 promoters. For cloning the intronV of Bmglv1, genomic PCR with exon5- and exon6-specific primers (see supplemental material for primer sequences), which amplified exon5-intronV-exon6, was done, and subsequently this PCR product was cloned downstream togst only orgst + glv1 fusion ORF (Bmglv1::GST-glv1-intronV plasmid). Control plasmid lacking intronV was also generated by RT-PCR using same set of primers and cloned downstream togst or other ORFs (Bmglv1::GST-glv1-DintronV plasmid). To study the role of CF2-binding motif of intronV, a deletion construct lacking cf2 motif was generated by Msel digestion that deleted 53 nucleotides just before intronV-exon6 junction. Msel-digested product was blunt-ended, ligated, and then cloned downstream togst to generate Bmglv1::GST-glv1-IntronVΔcf2 plasmid. For immunodepletion, a plasmid expressing GST-Bmglv1 fusion protein (Bmglv1::Gst-Bmglv1(ORF)-Invcf2) was generated by cloning Bmglv1 ORF between gst and the Ex5-InV-Ex6 cassette in the plasmid Bmglv1::GstEx5-InV-Ex6.

RNase Protection Assay—Exon5-intronV-Exon6 and Exon5-intronVc2-Exon6 fragments were PCR-amplified from the respective plasmid (described in preceding paragraph) constructs using 3′-UTR forward and reverse primers (see supplemental Methods) and cloned in pCR2.1 vector (Invitrogen). The radiolabeled antisense strand was synthesized using T7 RNA polymerase (New England Biolabs) in an in vitro transcription reaction. 5 µg of RNA sample was hybridized with in vitro transcribed antisense RNA probe (3 × 106 cpm) at 45 °C overnight. RNA samples were dissolved in 25 µl of 75% formamide, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5. RNase A (100 µg/ml) was added to the reaction mix along with 200 µl of 200 mM NaCl, 5 mM EDTA and incubation was done for 1 h at 37 °C. After the RNase incubation, proteinase K (250 µg/ml) digestion was done followed by phenol/chloroform extraction and ethanol precipitation and finally subjected to denaturing (8 M urea) PAGE.

Antibacterial and Prepro-processing Assay—Antibacterial activity of BmGlv1 and BmGlv2 was determined by incubating ~105–106 cells/ml of E. coli with 5 µm of either BmGlv1 or BmGlv2 in 1× PBS, pH 7.1; and 1, 2, 4, and 6 h postincubation, the optical density of the respective cultures was taken, and a graph was plotted. Each experiment was repeated a minimum of three times. Antibacterial activity of BmGlv1 and BmGlv2 was also quantified by counting the number of surviving bacteria after 6 h of incubation in the presence of BmGlv1 or BmGlv2 (colony-forming units/ml) on antibiotic-free LB agar plates. In a control experiment, bacteria were incubated with an equivalent amount of PBS.

Bacterial challenge activates different proteases that in turn cleave the N-terminal prepro part and release mature AMPs. Hence, fat body extracts from E. coli–challenged 5th instar larvae were prepared 3, 6, 9, and 12 hours post infection and pooled. Later, purified GST-Glv1 and GST-Glv2 proteins were incubated with the pooled fat body extract for 2 h, and then the complete reaction mixture was separated on SDS-polyacrylamide gel followed by Western blotting with anti-GST antibody.
Release of GST-specific band was an indication of processing of the AMP.

**Phylogenetic Analysis of Bombyx Gloverins**—Best fit model was tested using Model Test as implemented in HyPhy. GTR/H11001 model was selected by both Hierarchical Model Testing and Akilike Information Criteria (AKI score /H1100513034.9) and /H92512.00475. Using these parameters, a neighbor joining tree was constructed.

**RESULTS**

Identification of Gloverin Family of AMP Genes in B. mori—We have identified four gloverin genes (named as Bmglv1, Bmglv2, Bmglv3, and Bmglv4) in the silkworm B. mori. The gene structures of silkworm gloverins revealed Bmglv1 (3.99 kb) as the largest gloverin (Fig. 1A). Gene structure of Bmglv4 could not be predicted reliably because of the presence of repetitive elements in the scaffold corresponding to exon1. Exons 2–4, which constitute ORFs, are highly conserved in all four gloverins. Phylogenetic analysis based on ORFs of the four gloverins suggested Bmglv1 as the ancestral gloverin of B. mori. AttacinB and AttacinD of Drosophila were used as outgroup. Bm, B. mori; Dm, D. melanogaster; Ms, Manduca sexta; Tn, Trichoplusia ni. C, chromosomal location of four Bombyx gloverins as determined by physical mapping. Arrows (in black) indicate the sequence of duplication, and 1–3 indicate first, second, and third duplication events, respectively.

**FIGURE 1. Location, organization, and evolution of Bombyx gloverins.** A, Bmglv1 has six exons whereas other paralogs have five. During the first duplication event loss of intronV led to fusion of exons 5 and 6 resulting in smaller Bmglv2 gene. Intron loss does not change ORF. Start codon (green arrow) lies in the 2nd exon, whereas stop codon (red star) lies in the 5th exon in all paralogs. B, phylogenetic analysis suggests Bmglv1 as the ancestral gloverin of B. mori. AttacinB and AttacinD of Drosophila were used as outgroup. Bm, B. mori; Dm, D. melanogaster; Ms, Manduca sexta; Tn, Trichoplusia ni. C, chromosomal location of four Bombyx gloverins as determined by physical mapping. Arrows (in black) indicate the sequence of duplication, and 1–3 indicate first, second, and third duplication events, respectively.
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A

| Bmglv1 | Bmglv2 | Bmglv3 | Bmglv4 | hemolin | attacin | Beta-actin |
|--------|--------|--------|--------|---------|---------|------------|
| 40     | 56     | 72     | 96     | hat     |         |            |


B

| Larval | Adult |
|--------|-------|
| hem     | att   | Bmglv1 | T | O | T | O | m |
| att     | T     | Bmglv1 | T | O | T | O | O |

C

![Expression dynamics of gloverin and other AMP genes of B. mori](image)

D

![Expression dynamics of gloverin and other AMP genes of B. mori](image)

FIGURE 2. Expression dynamics of gloverin and other AMP genes of B. mori. A, all AMPs express in embryonic stages except for Bmglv1. (Numbers at the top indicate hours AEL; hat indicates hatched larvae.) B, Bmglv1 (arrowhead), which expresses in larval gonads, is down-regulated in adult gonads. Contrary to Bmglv1 down-regulation, hemolin is up-regulated in adult gonads, and its expression was not seen in larval gonads. Attacin expression did not change appreciably. (m, λ HindIII marker; T, testes; O, ovary.) C, knockdown of Bmglv2 by RNAi leads to ~30% reduction in embryo hatching ($p = 0.001$), whereas knockdown of Bmglv1 has no effect on hatching. Data shown here are the average of five independent experiments. D, RT-PCR was done with embryonic RNA (40 h AEL) to show inhibition of Bmglv2 upon RNAi (lane 3). Lane 4 shows enhanced expression of Bmglv2 (with respect to lane 2) where larvae were injected with ie-1 dsRNA followed by E. coli infection thus confirming that ie-1 dsRNA injection does not target Bmglv2 transcript. (Lane 1, without RT control; lane 2, non-target ie-1 dsRNA control; lane 3, Bmglv2 dsRNA; lane 4, ie-1 dsRNA + Bacterial injection.). Beta actin, loading control. Numbers above the figure represent respective lanes.

erosion in intronic regions of the genes (Bmglv1 (3.9 kb) → Bmglv3 (2.9 kb)). Among the conserved introns (introns I–IV), a significant deletion in intron length is seen for intronII, during Bmglv2 to Bmglv3 duplication; however, significance of this deletion remains to be elucidated (Fig. 1B).

Sizes of different gloverins may also differ because of size variations in their UTRs. Fifth exon contributed to 3′-UTR in all the gloverins except Bmglv1, which has an additional 6th exon as well as part of 3′-UTR. Bmglv3 has the longest 3′-UTR (318 bp) and shows five unique insertions not shared by other gloverins (supplemental Fig. 1 and supplemental Table 1). Apart from this, 3′-UTRs of four gloverins also share low homology at the sequence level. Lack of conservation in A/T-rich 3′-UTRs of gloverins possibly indicates lack of conserved regulatory function.

We also confirmed the physical location of the four gloverins. Although Bmglv1, Bmglv3, and Bmglv4 were physically mapped to chromosome 28, Bmglv2 was mapped to chromosome 17 (Fig. 1C). Bmglv1 and Bmglv4 are present as tandemly duplicated genes on positive strand at 38.6 → 45.0 centimorgans, and Bmglv2 and Bmglv3 are present on the complementary strand at 0 centimorgans → 16.4 centimorgans on chromosome 17 and at 22.8 → 37.3 centimorgans on chromosome 28, respectively (Fig. 1C).

Bmglv1 Is Not Expressed in Embryo

Unlike Other AMP Genes—Induction of expression of AMP genes in larval and adult tissues, mainly fat body and mid-gut, upon immune challenge is an established fact (1). However, we observed basal expression of Bmglv2–4 in embryonic stages (Fig. 2A). Other than Bmglv2–4, attacin and hemolin genes are also expressed in all embryonic stages. Significantly Bmglv1 does not express in embryos (Fig. 2A). A more interesting developmental regulation of AMP expression was observed in gonads as we found that Bmglv1 is expressed in larval but not in adult gonads, whereas hemolin and other gloverins expressed in adult but not in larval gonads (Fig. 2B). Clearly, down-regulation of Bmglv1 starts in adult gonads, and complete repression is achieved in embryonic stages, and upon hatching its expression is restored. It also suggested that embryonic expression may be a general feature of AMPs. The study raised two questions as follows. (i) What is the significance of embryonic expression of AMPs in general? (ii) How is suppression of Bmglv1 achieved in embryonic stages?

4 K. Mita, personal communication.
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Significance of Embryonic Expression of Bmglv2—Embryos are naturally protected against microbial infection because of the presence of the impregnable chorion layer that provides a strong physical barrier. Hence, embryos are not prone to infection nor has any natural infection been reported in insect embryos. If the threat of microbial infection is low/absent in embryos, then to what purpose does expression of AMPs in embryonic stages serve? We hypothesized that AMPs may have some role other than killing microbes. To test this hypothesis, we knocked down the embryonically expressing gloverin paralog Bmglv2 and compared the effect of its knockdown with that of Bmglv1 knockdown. Bmglv1 and Bmglv2 dsRNA were administered to 4th and 5th instar larvae and later injected into adult moths as well. Knockdown effect was analyzed in the next generation embryo. Although RNAi of Bmglv2 led to reduced hatching, knockdown of Bmglv1, which does not express in embryo, had no such effect (Fig. 2, C and D). These results suggest a role for Bmglv2 in embryonic development that indicates a gain of function in Bmglv2 with respect to Bmglv1. Thus our results point out that embryonic expression of AMPs (Fig. 2A) is an essential and developmentally regulated process.

Bmglv1 Promoter Is Functional in Embryo—Developmental regulation of AMP genes is not well studied. Because Bmglv2 and Bmglv1 genes were quite distinct in their embryonic expression pattern, we examined the differences between the two genes to gain insight into their embryonic regulation (Fig. 2A). Promoters are the most important cis-elements that regulate spatio-temporal expression of downstream genes. One possibility was that during the duplication process, certain regulatory motifs in the promoter of Bmglv2 might have been deleted/gained leading to its expression in embryo and was further investigated. Comparative analysis of gloverin promoters revealed binding sites of all essential transcription factors, which regulate AMP genes, viz. Rel and GATA (data not shown), and we did not find any striking difference between the two promoters. Hence, we tested whether the Bmglv1 promoter was functional in embryonic stages or not. Bmglv1:gst plasmid, where gst is driven by the Bmglv1 promoter, was incubated with embryonic extract for coupled in vitro transcription and translation experiments. Synthesis of GST from Bmglv1:gst plasmid was indicative of the fact that the Bmglv1 promoter was capable of expressing in embryonic stages similar to that of Bmglv2 (Fig. 3A, lanes 2 and 3). BmA3-Actin promoter (cytoplasmic actin promoter of B. mori) construct was used as reaction control (Fig. 3A, lane 4). Because both the promoters were functional during embryonic stages, we looked for other differences between the two genes that can account for different embryonic expression profile of the two genes. It is evident that the gene structure of gloverin paralogs has largely remained unchanged with the exception of intronV, which is present only in Bmglv1 (Fig. 1B). As loss of intronV and gain of embryonic expression...
Intron V of Bmglv1 Acts Like a Repressor—Introns are non-coding parts of the gene, but they are essential segments of the genome as many of the introns are known to have regulatory functions such as enhancers and suppressors. To ascertain the embryo-specific regulatory role, if any, of intron V of Bmglv1, we constructed gest reporter plasmids under the control of the native Bmglv1 promoter. Intron V is located downstream to the ORF in the 3′-UTR region of the Bmglv1 gene; hence, to retain this natural organization, intron V along with flanking regions of exons 5 and 6 was cloned downstream to the reporter gest ORF (Fig. 3B). In the control plasmid intron V was not cloned. Both plasmids, driven by Bmglv1 promoters, were incubated with Bombyx embryonic extracts for coupled in vitro transcription and translation of the reporter gene. GST synthesis took place in the reaction where control plasmid was used, and no GST was detected in the reaction performed with the plasmid that harbored intron V (Fig. 3C), thus suggesting that intron V had inhibitory action on GST synthesis. Next we set out to dissect the mechanism of embryonic suppression of Bmglv1 gene by intron V.

Identification of Repressor Element in Intron V of Bmglv1—Intron V of Bmglv1 is 279 bp long, and to characterize the motif regulating embryonic expression of upstream ORF, EMSA was done with different fragments of intron V generated by restriction digestion (data not shown). The fragment corresponding to the last 40 bp of the intron, which has a putative CF2 binding site, showed specific shift in EMSA. Although there are two additional CF2-binding motifs in the intron, only the one present near the intron 5′-exon 6 boundary was found to be functional (supplemental Fig. 2). Later oligonucleotide (2× AGTA-AATATATATAT) corresponding to the “functional CF2-binding motif” was used as probe for gel shift. The CF2 complex could be retarded with nuclear extracts from adult ovary, testes, and embryonic extracts but not from tissues of larval origin (Fig. 3D). These results are consistent with the observation that Bmglv1 expression is not seen in the tissues where CF2 is expressed (Fig. 2A and Fig. 3D). Supershift with Drosophila CF2 antibody confirmed that the retarded complex contained CF2 protein (Fig. 3E). No gel shift was seen when embryonic extract from Df(2L) y27 flies, which lack cf2 locus, was used (Fig. 3E, lane 8). This further confirmed the specific interaction of CF2 to the intronic element. To test the interaction of CF2 to intron V under in vivo conditions, ChIP assay was performed (Fig. 3, F and G). The enrichment of intron V was seen with embryonic but not with fat-body extract (Fig. 3F). These results point out the presence of a CF2-mediated active regulatory element in the intron V of Bmglv1 and also the apparent correlation between Bmglv1 repression and expression of CF2.

Binding of CF2 to Intron V of Bmglv1 Represses the Native Promoter—The physical interaction between CF2 and intron V suggested that CF2 might be important for intron-mediated suppression of the Bmglv1 gene. To establish that CF2 was required for intron V-mediated repression of Bmglv1, we performed in vitro translation of Bmglv1::Gst-Bmglv1(ORF)−ΔinV and Bmglv1::Gst-Bmglv1(ORF)−ΔinV plasmids, with whole-some and CF2-depleted embryonic extracts (CF2 protein was immunodepleted using CF2 antibody). Western blot of in vitro translation product of Bmglv1::Gst-Bmglv1(ORF)−ΔinV plasmid did not detect GST-BmGlv1 fusion protein in the reaction where complete embryonic extract was used (Fig. 4A, lane 2), but the fusion protein was detected in the reaction where CF2-depleted embryonic extract was used (Fig. 4A, lane 1). Constitutively expressing piggyBac-based BmA3-Actin::GFP plasmid (28) was used as reaction control (Fig. 4A). Synthesis of GST-BmGlv1 fusion protein in the CF2-depleted extract suggested that native Bmglv1 promoter was active in the embryo and also that the presence of CF2 led to Bmglv1 suppression (Fig. 4A).

Furthermore, to prove that CF2-mediated repression of Bmglv1 required binding of CF2 to the intron V, we repeated the immunodepletion experiment as mentioned in Fig. 4A, using Bmglv1::Gst-Bmglv1(ORF)−ΔinV plasmid that lacks the functional CF2-binding motif in the intron V (intron V−Δcf2). It is evident that in vitro translation was not affected by the presence or absence of CF2 in the embryonic extract if the CF2-binding motif was deleted (Fig. 4B, lanes 1 and 2). Taken together, data shown in Fig. 4 A and B, suggest that silencing of the Bmglv1 promoter required physical interaction between CF2 and intron V of Bmglv1. This result was further confirmed when the CF2 protein was differentially depleted by using different
amounts of CF2 antibody. Synthesis of GST-BmGlv1 protein was found to be dependent on the extent of CF2 depletion in the embryonic extract (Fig. 4C). These results establish that CF2 recruitment to intronV is essential for Bmglv1 suppression and also suggest that CF2-mediated intronic regulation is dominant over native BmGlv1 promoter.

**CF2 Blocks Transcription of Bmglv1**—We have shown that CF2 binding to intronV was required for suppression of GST-BmGlv1 fusion protein synthesis (Fig. 4, A–C). Lack of protein synthesis could be due to either suppression of transcription or of translation. If intronV acted like a cis-regulatory element, then transcription will be blocked (cis-regulation), and if the regulation was at the RNA level, then transcript will be formed but not the translation product. First, we tested whether CF2 binding to intronV suppressed transcription of Bmglv1. To elucidate the mechanism of suppression by CF2, if intronV acted like a cis-regulatory element, we performed *in vitro* transcription experiment with Bmglv1::Gst-InVcf2 and Bmglv1::Gst-InVΔcf2 plasmids. *In vitro* transcription was done with CF2-depleted and control extracts. RNA synthesis was checked by RNase protection assay (Fig. 4D). Because of deletion of the cf2 motif, transcript formed from Bmglv1::Gst-Bmglv1(ORF)-InVΔcf2 template is shorter by 53 nucleotides compared with transcript synthesized from Bmglv1::Gst-Bmglv1(ORF)-InV template (Fig. 4D, upper panel). When Bmglv1::Gst-InVcf2 plasmid was used as template in the reaction where complete embryonic extract was used, no RNA could be protected indicating absence of transcript in this reaction (Fig. 4D, lower panel, lane 3). Protection of *gst*-Bmglv1-inV-specific transcript in lane 2 indicates transcription of Bmglv1::Gst-Bmglv1(ORF)-InV template with CF2-depleted extract (Fig. 4D, lower panel). Synthesis of RNA was not affected by the presence or absence of CF2 protein when the Bmglv1::Gst-inVΔcf2 template, which lacks CF2-binding motif, was used (Fig. 4D, lower panel, lower band). CF2 bound to the intronV represses transcription from the Bmglv1 promoter, thus confirming that intronV acts like a cis-regulatory repressor element and not like a translational repressor.

This also explains that because of transcriptional repressor action of intronV, mediated by CF2, Bmglv1 transcript is not expressed in tissues where CF2 is expressed. On the other hand Bmglv2–4 genes are independent of CF2 regulation as they lack intronV. As loss of intronV was responsible for paradigm shift in embryonic regulation of Gloverin paralogs, we therefore believe that this intron loss was a critical event in the evolution of the Gloverin family of genes in *B. mori*.

**The Genomic Deletion**—First duplication was also characterized by a genomic deletion in an exon of Bmglv1. Deletions in exons have a direct effect on the nature and function of the duplicated gene as most often it leads to a frameshift in ORF that results in change in protein sequence or truncation of the original protein.

Comparative analysis of silkworm Gloverins revealed an in-frame deletion of 12 bp in the exon3 of Bmglv1 (Fig. 5A). These nucleotides code for amino acids Ile, His, Asp, and Phe. ClustalW alignment of all known Gloverins suggests that the presence of IHDF is a unique feature of BmGlv1 as this sequence motif is not present in other reported Gloverin orthologs or paralogs (Fig. 5B). RHPRDVTD motif, which has the signal-processing motif, is conserved in all Gloverins except for BmGlv1, which has an insertion of amino acids IHDF between Asp and Val. This insertion might have potentially split/abrogate the processing site (Fig. 5B). Hence, we set out to study the functional consequences of the presence/absence of IHDF residues close to the prepro-processing site in BmGlv1.

**BmGlv1 Is Not Processed upon Immune Challenge**—AMPs in general are synthesized with an N-terminal prepro region, which keeps them in an inactive state (1–3). These prepro regions usually contain a signal sequence, which probably helps in their secretion. All the reported Gloverins are known to have a precursor region that has a cleavage site between arginine and aspartate in the sequence RHPRDVTD (Fig. 5B). However, the presence of amino acids IHDF between aspartate and valine of the cleavage recognition sequence has changed the sequence motif next to the cleavage site in BmGlv1. To elucidate the functional consequences of the presence of IHDF, recombinant AcNPV expressing Bmglv1 and Bmglv2 genes containing His6-GST tag at the N terminus was expressed in Sf9 cells. Purified GST-BmGlv1 and GST-BmGlv2 proteins were incubated with fat body extracts prepared from bacteria challenged and unchallenged larvae.

The fat body extracts prepared from bacteria-challenged larvae are rich in proteases that were either absent or inactive in the extracts prepared from unchallenged larvae. These proteases process AMPs by cleaving the N-terminal prepro part. We designed an assay to test the processing of GST-BmGlv1 and GST-BmGlv2 proteins. GST-specific band, being upstream to AMP, will only be released if the prepro part of the fused AMP is processed. No GST-specific band was released from either of the proteins when incubated with fat body extracts prepared from unchallenged larvae (Fig. 5C, lanes 1 and 4). However, the GST band was released from BmGlv2 but not BmGlv1 upon incubation with fat body extracts prepared from challenged larvae. Thus, release of the GST band in lane 3 indicates N-terminal processing of BmGlv2 and not of BmGlv1 (Fig. 5C). These results demonstrate that insertion of IHDF has abrogated the processing site in BmGlv1. However, lack of N-terminal processing had no effect on antibacterial activity of BmGlv1 as confirmed by zone inhibition and bacterial clearance assay. In fact, BmGlv1 has stronger antibacterial activity than BmGlv2 (0.05 mM) and cleared bacteria faster than BmGlv2 (0.1 mM) (Fig. 5, D and E) implying that lack of processing is not critical for antibacterial activity of BmGlv1.

**DISCUSSION**

In the study reported here, we have explored the effect of genome dynamics on the evolution of the Gloverin family of AMP genes. Our analysis suggests that Bmglv1 is the ancestral Gloverin, and other silkworm Gloverins evolved in due course of time as a result of three gene duplication events. One notable feature of the first gene duplication was two gain of function phenotypes associated with two deletion events. The first was in-frame genomic deletion of 12 bp that led to gain of prepro cleavage site, and the second was loss of an intron that changed the expression pattern of the duplicated gene Bmglv2.
Evolution of Prepro Domain in Gloverin Proteins—It is known that precursor AMPs are produced with an N-terminal prepro part containing signal sequence, which is important for their activation (2, 3). Processing of the N-terminal prepro part was considered as a property inherent to all AMPs. Because the processing site sequence has remained conserved even in orthologous gloverins (Fig. 1B), we reason that deletion of four amino acids was not a random event but the result of an evolutionary pressure to acquire the processing ability. In view of these results it is tempting to propose that processing of AMPs

![Prepro-processing of BmGlv1. A, exon3 of ancestral Bmglv1 has unique 12 nucleotides coding for amino acids IHDF (shown in red), but the same is deleted in exon3 of Bmglv2. B, multiple alignment of gloverins, as known now, reveals that IHDF motif, next to prepro cleavage site (downward arrow), is unique to BmGlv1. Otherwise BmGlv1 and BmGlv2 proteins are 92% similar. C, to check prepro-processing GST-BmGlv1 and GST-BmGlv2 both were incubated with fat body extract prepared from unchallenged (lanes 1 and 4) and E. coli challenged (lanes 2 and 3). Release of GST band in lane 2 indicates processing of GST-BmGlv2 into GST and BmGlv2 upon immune challenge, whereas absence of GST band in lane 2 indicates lack of processing of BmGlv1. No processing of either of the proteins is seen with extract prepared from unchallenged fat body. D, lack of prepro-processing does not affect antibacterial activity of BmGlv1 as seen in bacterial clearance assay. Shown here is the result of one representative experiment of the three such experiments done under identical conditions. E, bar diagram shows number of bacteria surviving (number of colony-forming units × 10^6/ml) after 6 h of treatment with equivalent concentrations of BmGlv1, BmGlv2, or PBS (control). Post-treatment the bacterial culture was pelleted down, washed once with PBS, and then dissolved in 200 μl of sterile plain LB broth. 100 μl of the soup was plated on antibiotic-free LB-Agar plates and incubated overnight after which the number of colonies were counted. Five replicates of each experiment were done, and p value was calculated.

Evolution of Prepro Domain in Gloverin Proteins—It is known that precursor AMPs are produced with an N-terminal prepro part containing signal sequence, which is important for their activation (2, 3). Processing of the N-terminal prepro part was considered as a property inherent to all AMPs. Because the
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is an evolved character in gloverins of B. mori. It will be interesting to investigate whether N-terminal processing in other AMPs has also evolved in a similar manner. We believe that it may be true for other AMPs as well and would most probably require study of humoral immunity in primitive insects. However, based on our data, we hypothesize that Bmglv1 may be the relic of an ancestral and more primitive immune system.

Regulation of Bmglv1 Gene by 3' ‐ UTR Intron—Another significant finding of this study is the functional characterization of a regulatory element in intron of an AMP gene. The 5' ‐ and 3'-untranslated regions (UTRs) that bracket CDSSs are fundamental structural and regulatory regions of eukaryotic genes (29–32). UTRs are known to contain large numbers of introns (33), yet there is a lack of hypotheses specifically addressing the evolution of introns within UTRs. A study by Pesole et al. (33) suggests that intron density is higher in 5'-UTRs than 3'-UTRs. The observation that fewer 3'-UTRs carry introns is surprising as 3'-UTRs are generally longer than 5'-UTRs and would thus be expected to have higher intron density. The reason for the lack of introns in 3'-UTRs could be intron loss, which is most often restricted to 3' introns (34, 35). The most common mechanism of intron loss is the gene conversion of original gene by reverse transcription of spliced RNA (36–41).

Introns have been shown to affect the expression of different genes at different levels like mRNA export, stability, and translation efficiency (42–44). But the role of introns in the regulation of AMP genes is not reported. Here we have reported a CF2-dependent intronic regulation of an AMP gene. In Drosophila the CF2 protein exists in two isoforms CF2I and CF2II. The 113-amino acid-long zinc finger domain of CF2 consists of five to seven contiguous zinc fingers of the C2H2 type (45–48). The zinc finger motif of CF2 resembles zinc finger domains of the developmentally regulated Drosophila transcription factors Kruppel and hunchback. CF2 is basically a transcriptional activator and possesses transcription activation domain consisting of 17 glutamines interspersed with 7 acidic residues (49). However, this study demonstrates that CF2 can act as a repressor as well. This also suggests that action of CF2 as activator or repressor is context-dependent. This is the first study where CF2 has been shown to bind to an intron element and repress the transcription of the native gene. CF2 bound to intronV may lead to looping back of the DNA, which in turn can silence the transcription (Fig. 6). Involvement of factors other than CF2 in intronV-mediated, promoter silencing cannot be ruled out.

Intron Loss in 3'-UTR of the Gene Is Positively Selected—Fifth intron of Bmglv1 splits the 3'-UTR, implying that loss of intronV would not have affected the BmGlv1 protein; hence this intron loss could have been inconsequential from the evolutionary point of view. We show that loss of intronV did not alter the gene product, but it changed developmental regulation of Bmglv2 resulting in acquisition of a new function in embryonic development. However, this gain of function, in the derived gene Bmglv2, in embryonic development was because of its ability to express in the embryonic stages. We have shown that the embryonic regulation of promoters of both the ancestral and the derived gene is the same; still the ancestral Bmglv1 does not express in the embryo because of repression by intronV in embryonic stages (Fig. 3, A and B, and Fig. 4C). As intronV regulation is dominant over the Bmglv1 promoter, the only way to achieve embryonic expression was by losing intronV. Thus, Bmglv1 would have experienced strong pressure to lose intronV, which was eventually lost during the first duplication process. In other words loss of intronV of Bmglv1 was positively selected to achieve embryonic expression, and it was not lost randomly for being a 3'-UTR intron. Thus, our study suggests that loss of 3'-UTR introns may be associated with distinct phenotypes, and hence these introns could have experienced positive selection.

AMPs Have a Role in Embryonic Development—The significance of embryonic expression of AMPs is not clear. There are very few reports on the role of AMPs in embryonic development. Recently, hemolin expression has been shown to be important for embryonic diapause and development (50, 51). Embryonic diapause is a special physiological state that is developmentally controlled and is observed in many insects. A diapausing embryo, where most of the physiological processes are suppressed, is considered to be under stress, and it is suggested that expression of hemolin in such embryos could be part of a broad stress response (50, 51). We speculate that other AMPs,
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like gloverin paralogs, which express in the embryonic stages, might be part of the same broad stress response. However, embryonic expression of AMPs has also been reported from Drosophila, which does not undergo diapause and hence diapause may not be the only function to be affected by AMPs (52). Embryonic expression of cecropinA in Drosophila embryo was detected in tissues like yolk and embryonic epidermis but not in embryonic fat body, mid gut, or hemocytes. This is in stark contrast to CecropinaA expression in larval and adult stages where it is expressed in fat body and mid gut (1). Furthermore, GATA factor Serpent is needed for expression of AMPs in embryonic yolk but not in embryonic epidermis (52) suggesting that expression of AMPs in different tissues of the embryo is regulated by different transcription factors.

Here we have shown negative regulation of ancestral gloverin by embryonic protein CF2, which is expressed in yolk and controls oogenesis (53). This study adds one more dimension to the embryonic regulation of AMPs, and more precisely it reveals the evolution of embryonic regulation in an AMP gene family.

During the course of evolution, there have been episodes of extensive intron loss and gain because of selective forces that affect the rate of intron dynamics (54, 55). For evolutionarily conserved genes, intron insertion supposedly had adaptive effect like increasing stability of RNA, whereas intron loss was found to be deleterious (43, 44, 56). This apparent functional importance of introns could be due in part because of their effects on gene regulation. Our study demonstrates the role of intron as cis-regulators in gene evolution and thus adds another dimension to genome plasticity. The fact that intron loss achieved embryonic expression for gloverin paralogs, a property which appears to be common feature of all AMP genes except for Bmg1v1, suggests that this intron loss might have experienced positive selection. In an earlier study positive selection of intron loss in Drosophila has been shown (57). To the best of our knowledge this is the first report where positive selection for intron loss in an AMP gene has been functionally validated. In summary, our study suggests that intron loss or gain may not be a passive/random feature of genome dynamics but a result of selection pressure.

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