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A GATA-binding protein expressed predominantly in the pupal ovary of the silkworm, *Bombyx mori*

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Abstract

Preferential localization of Bkm (Banded krait minor-satellite) DNA sequences on Y/W chromosomes of higher eukaryotes, which remain highly condensed in somatic cells but undergo extensive decondensation in the germ cells during early stages of development, led to the postulation for the existence of a sex- and tissue-specific Bkm-binding protein (BBP). Accordingly, we purified and characterized a BmBBP expressed predominantly in pupal ovary of the silkworm *(Bombyx mori*). 2D-PAGE revealed BmBBP as moderately basic (pI 7.8–8, in the range expected for DNA-binding proteins) and Matrix Assisted Laser Desorption/Ionization Time of Flight exhibited a value of 37.5-kDa. BmBBP neither contains nor requires divalent metal ions for its DNA-binding activity, suggesting that it does not belong to the well-studied GATA-family of transcription factors. BmBBP is unusually strong in its DNA-binding characteristics to Bkm (GATA-repeats), which suggests its probable role in bringing about coordinated chromatin conformational changes to activate genes present in associated chromosomal domains. Fluorescence immuno-localization studies employing specific anti-BmBBP antibodies revealed its presence in the follicle cells and in the ooplasm, as well as the nucleus of different developmental stages of oocytes.

Keywords: Bombyx mori; GATA-binding protein

1. Introduction

Bkm (Banded krait minor-satellite) DNA sequences, predominantly containing GATA repeats are found in widely separated orders of eukaryotes (Singh et al., 1981; Singh and Jones, 1982). They are preferentially concentrated on the sex chromosomes (Y in mammals and W in snakes and birds) of the heterogametic sex. They are also reported to be present on the autosomal region of mouse chromosome-17, which causes hermaphroditism on deletion (Singh and Jones, 1982; Kiel-Metzger and Erickson, 1984). In snakes the W chromosome is highly condensed in all somatic tissues but is extensively decondensed in developing oocytes (Singh et al, 1979). In mouse and humans, the Y chromosome remains condensed in all somatic cells, but decondenses in germ cells during spermatogenesis. A sex- and tissue-specific Bkm-binding protein (BBP) which specifically binds to GATA repeat was isolated earlier from snake ovary (Singh et al., 1994). The BBP possesses no similarity with the known GATA family of transcription factors. Similar proteins were demonstrated to be present in human and mouse testicular tissues (Singh et al., 1994). The silkworm, *Bombyx mori* also possesses the female heterogametic sex determination system (ZZ male and ZW female) similar to that of snakes (Tanaka, 1916). Further, *B. mori* is the only known lepidopteran species in which W chromosome is shown to be strongly female determining (Tazima, 1964; Suzuki et al., 2001 Niimi et al., 2001). Based on the chromosomal sex determination analogy of snakes, it was proposed to investigate whether such a sex- and tissue-specific BBP exists in silkworms. In the present study, we purified and characterized a BBP, which is predominantly expressed in the pupal ovary of the silkworm, *B. mori*.

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2. Materials and methods

2.1. Materials

Pupae and early 5th instar larvae of the MU_{303} silkworm strain were collected from silkworm breeding centres at Bidadi, Karnataka and APSSRDI, Hindupur, Andhra Pradesh, India.

2.2. Processing the tissues and preparation of tissue extracts

The silk cocoons were cut open and pupae were sexed before they were dissected to obtain the required tissues. Ovaries were dissected each day of pupal development from day 6 to day 10 (from the day of silk secretion). The oocytes were collected as one pool at each day of development. Testes were collected from pupae at day 8 of development. Ovaries and testes were collected in insect ringer solution. Silk glands were dissected from the 5th instar larvae at day 5. The tissues were flash-frozen in liquid nitrogen and kept at −70°C until they were used for protein isolation. For preparation of extracts, 10 g of the respective tissue was suspended in and washed with 5 volumes of TNGE buffer (20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 10 mM EDTA, 1 mM PMSF and 4% glycerol). The tissue was re-suspended in 10 volumes of TNGE buffer containing 0.5% Triton X-100 and subjected to homogenization in narrow cylindrical jars covered with ice, using a Polytron-tissue homogenizer with medium-size probe at a speed setting of 4/10 for 2 min each, over a 10 min period. An interval of 1 min (for cooling the probe) was allowed between different pulses of homogenization. The homogenate was checked for nuclear breakage under the microscope and then centrifuged at 12 000 rpm in an SS-34 rotor (of Sorvall RC 5B centrifuge) for 1 h, for sedimenting the debris. The supernatant collected is referred to as the tissue extract. BBP precipitate was prepared from the tissue extract between 60–80% saturation of ammonium sulphate and is referred to as Asp-BBP.

2.3. Preparation of $(GATA)_{16}$ and Bkm2(8) DNAs

Oligonucleotides containing poly-GATA and poly-CTAT were synthesized, annealed, phosphorylated and cloned into the *Sma*I site of pUC18. The insert was released by digestion with *Eco*RI and *Hind*III, which flank the *Sma*I site. The 120 bp long insert contained 16 repeats of GATA (Singh et al., 1994). A *Bam*HI/*Pst*I insert of the *Drosophila* CS314 clone (545 bp long) containing 66 copies of GATA repeats was cloned into pUC18 (Singh et al., 1981) and designated as Bkm2 (8). The insert was released by digestion with *Eco*RI and *Hin*dIII restriction enzymes and purified by gel elution after electrophoresis on an agarose gel.

2.4. End labeling of $(GATA)_{16}$ and Bkm2 (8) DNA

End-labeling of double-stranded DNA was carried out as described in Sambrook et al., (1989). 120 bp $(GATA)_{16}$ was end-labeled by filling-in the enzyme site overhangs with Klenow (*E.coli* DNA-Polymerase I, large fragment) at 37°C for 90 min in Klenow buffer containing 10 mM of each of dGTP, dCTP and dTTP, 40 mM DTT and an appropriate amount of ^{32}P-dATP. The reaction was terminated by addition of EDTA to a final concentration of 35 mM. Labeled $GATA_{16}$ in 300 mM Na-acetate buffer, pH 5.5 was precipitated at 0°C overnight with isopropanol in the presence of sheared *E.coli* DNA as the carrier. The precipitate was washed with ethanol, dried and redissolved in sterile deionised water. The 545 bp fragment of Bkm2(8) was also end-labeled as described above.

2.5. Gel mobility shift assays

Gel mobility shift assays were performed according to the method reported earlier (Fried and Crothers, 1981). An appropriate amount of tissue extract (15–100 μg) in 50 mM Tris-HCl pH8.0 containing 300 mM NaCl, 50 mM DTT, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM PMSF and 10% glycerol was incubated with 1–2 μg of sheared *E. coli* DNA at room temperature for 10 min. To this, approximately 3–4 ng of end-labeled $GATA_{16}$-DNA (containing 30,000–50,000 cpm) was added and incubated for a further period of 30 min at room temperature. The sample was adjusted to 10 mM Tris-HCl, pH 7.5, 0.025% bromophenol blue, 0.025% xylene-cyanol and 6% glycerol by adding stock buffer, loaded on a 6% native polyacrylamide gel and electrophoresed using 90 mM Tris-borate buffer, pH 8.00, containing 1 mM EDTA. The $GATA_{16}$-probe was loaded in an adjacent well as control. Electrophoresis was carried out at 100 V, till bromophenol blue moved out of the gel. The gel was dried and subjected to autoradiography.

2.6. SDS-PAGE and electroblotting of proteins

SDS PAGE of the samples was carried out as described earlier (Laemmli, 1970). 10% polyacrylamide gels were run in duplicate; one was stained with Coomassie brilliant blue R-250 and the other was processed for a Southwestern assay. For high sensitivity, silver staining of SDS PAGE gels was performed as described previously (Oakley et al., 1980). SDS PAGE (slab) gels were electroblotted both for the Southwestern and Western assays. The gels were incubated with gentle shaking for renaturation in 20 mM Tris-HCl pH 7.4 containing 50 mM NaCl and 10 mM $MgCl_2$ for 3 h or preferably overnight and equilibrating with 20 mM Tris, 192 mM glycine and 20% methanol onto 0.45 μm pore size nitro-

cellulose membrane using the semi-dry graphite method described earlier (Towbin et al., 1979).

2.7. Southwestern assay

This assay was essentially performed by the methods described earlier (Bowen et al., 1980; Pathak, 1997). The protein blot was incubated in binding buffer, 10 mM Tris-HCl, pH 7 containing 50 mM NaCl and 1 mM EDTA (100 μl per cm^2 surface area of the blot) for 10 min and subjected to pre-treatment in the same buffer containing 0.2% BSA and 25–50 μg of sheared *E.coli* DNA (pre-treatment buffer) for 2 h at room temperature. At the end of this step, a DNA–protein interaction was performed in pre-treatment buffer containing 10^5 cpm of ^{32}P labeled BKm2(8) probe/ml of buffer for 90 min at room temperature. The blot was washed in binding buffer with three changes for 15 min each, dried and exposed in a Phosphor imager cassette.

2.8. Two-dimensional gel electrophoresis of proteins

This was performed by the method developed earlier (O'Farrell, 1975). Proteins were separated in the first dimension in tube gels of 1.5 mm diameter containing 5% polyacrylamide, 8 M urea, 2% NP-40, 5% 2-mercaptoethanol and 2.5% of ampholytes in the pH range of 3–10 (2% of pH 5–8 and 0.5% of pH 3–10). For the second dimension, the gels extruded out of the tubes were incubated in the SDS-PAGE sample-loading buffer for 30 min at room temperature, fused at the top of a 10% SDS-PAGE slab gel and subjected to electrophoresis. One of the I-D gels was cut into 5-mm sections and subjected to determination of the pH gradient that developed in the gel during the I-D electrophoresis.

2.9. Fluorescence immunostaining

Slides with tissue sections were re-hydrated in PBS through descending grades of methanol solutions prepared in PBS (90%, 70%, 50%, 30%). Tissue sections were permeabilized by immersing them in PTX (0.1% Triton X-100 in PBS) for 2 min. Blocking was done by incubating in PBTX (0.1% Triton X-100, 1% BSA in PBS) for 3–4 h at room temperature on a shaker. Slides were then removed and 80 μl of BBP antibody prepared in PBTX in 1:250 dilution was layered on the tissue section. The slides were covered with parafilm and incubated for 12 h at 4°C in a tray with moistened tissue. The antibody solution was washed off by rinsing twice with PBTX followed by an extended wash with PBTX for 2 h at room temperature. The slides were then incubated with 1:400 dilution of biotin-conjugated goat anti-mouse anti-IgG at room temperature for 2 h. The unbound antibody was washed off by rinsing twice and given a 1 h wash with shaking at room temperature in PTX. The PTX wash was followed by two changes in PT (PBS, 0.1%Tween 20) for 30 min each. The following steps were carried out in dim light: 80 μl of PT with 1 μl of avidin-conjugated cy-3 was layered on the slide, covered with parafilm, and incubated for 45 min at room temperature. The slides were then rinsed in PT for 30 min and mounted in 80% glycerol containing an antifade (1 mg/ml), covered with a cover slip and analysed by confocal microscopy.

2.10. Preparation of GATA affinity column

A GATA-Sepharose affinity column was prepared by coupling the 120 bp $GATA_{16}$ DNA to CNBr-activated Sepharose 4B (Pharmacia) employing the procedure outlined earlier (Kadonaga and Tjian, 1986). CNBr-activated Sepharose (1 g) was suspended in deionised water (50 ml) at 4°C for 15 min and washed several times with water followed by 1 N HCl (200 ml) on a sintered glass filter for removing preservatives and activation of the binding sites. The resin was finally washed with 100 ml of 20 mM potassium phosphate, pH 8. $GATA_{16}$-DNA in 20 mM potassium phosphate buffer, pH 8 was added to the washed resin and subjected to end-over-end mixing at room temperature overnight. The resin was centrifuged and washed with 100 ml of deionised water and unreacted sites on the Sepharose were blocked by incubating the resin with 200 mM glycine, pH 8, for 2–3 h at room temperature. This was followed by 100 ml washes with 10 mM and 1 M potassium phosphate buffers (both at pH 8) and 1 M NaCl in 100 mM potassium phosphate. The resin was finally stored in TNGE buffer (20 mM Tris-HCl, pH 8, containing 150 mM NaCl, 10 mM EDTA, 1 mM PMSF and 4% glycerol).

2.11. Immunization of mice

Mice were immunized employing the procedure described in Sambrook et al., (1989). Silver stained acrylamide gel bands containing 300–400 ng of BBP were homogenized thoroughly in two volumes of PBS and emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously. Three boosters followed primary injection at 10-day intervals, wherein the antigen was prepared in Freund's incomplete adjuvant. Upon completion of boosters, 0.5 ml blood was drawn from each animal by a retro-orbital puncture procedure, allowed for clotting, centrifuged and serum separated. Anti-serum was stored at 4°C and used in further studies.

2.12. Mass spectrometry

Matrix Assisted Laser Desorption/Ionisation-Time Of Flight (MALDI-TOF) study was conducted employing a 'Kratos PC-Kompact MALDI 4 V1.1.2' mass spec-

trometer. The protein sample was thoroughly mixed with an equal volume of Sinapinic acid (3, 5-Dimethoxy-4-hydroxycinnamic acid) matrix, deposited on the stainless steel target, allowed to air dry and used in the study.

3. Results

A gel mobility shift assay employing an end labeled $GATA_{16}$-probe with different tissue extracts of *B. mori* was carried out to establish the presence and relative abundance of BBP. Results shown in Fig. 1A reveal that BBP is present predominantly in pupal ovary (lanes 3 and 4) as compared to pupal testes (lanes 5 and 6). Lane 2 shows a positive control with snake ovary extract. The silk gland and other tissues also showed basal levels of BBP signal (data not shown), which perhaps arose due to non-specific interactions of the probe with other tissue proteins (see later). When we conducted similar studies with the pupal ovaries at different days of development, we found that BBP level reached its maximum between day 7 and day 8 (Fig. 1B, lanes 2 and 3). It is difficult to pinpoint the exact developmental stage of the ovary at which the expression of BBP is at its peak since the ovarioles from which the extracts were prepared contain oocytes at different developmental stages. Gel mobility shift experiments with Bkm2(8) instead of $GATA_{16}$, revealed identical results (data not shown).

The mobility shift assay (MSA) using a $GATA_{16}$ probe and tissue extracts of posterior (Fig. 2A), anterior and middle silk glands (Fig. 2B) of day 5 of 5th instar larvae and testes (Fig. 2C) and ovaries (Fig. 2D and 2E) of day 8 of pupal development, was carried out in the presence of an increasing concentration of sheared *E.coli* competitor DNA. The intensity of signal showed a drastic reduction with increasing concentration of competitor DNA and it disappeared completely at the highest competitor DNA concentration in all the tissues (Fig. 2A–C) except in the pupal ovary (Fig. 2D), where it remained more or less unchanged. These results strongly suggest that the faint signals observed in tissues other than pupal ovary are likely due to non-specific interaction of other proteins with the probe. In order to further establish the sequence specificity of the BBP, we used increasing concentrations of unlabeled $GATA_{16}$ as competitor DNA and repeated the MSA. The results (Fig. 2E) clearly show a drastic decrease in the intensity of signal with increasing concentration of the competitor DNA (lane 3). The signal is completely abolished with a 25-fold excess of cold $GATA_{16}$ competitor DNA (lane 4).

The MSA has a limitation in revealing the identity or characteristics of the molecules involved in the interaction. It only allows detection of the presence or absence of molecules specifically interacting with the probe and their relative abundance under the given assay conditions. Further, with increase in the size of the probe, the MSA takes a longer time for electrophoresis and probe–protein complexes tend to dissociate, resulting in increased mobility of the dissociated probe from the complex. Thus the assay could yield multiple mobility-shifted signals. In view of this, in further studies we performed Southwestern (instead of MSA) assays of BBP with end labeled Bkm2(8)-DNA as the probe, as described in methods.

Fractionation of pupal ovary extract with ammonium sulphate precipitation revealed that all the BBP activity, assessed either by MSA (employing ^{32}P-GATA 16

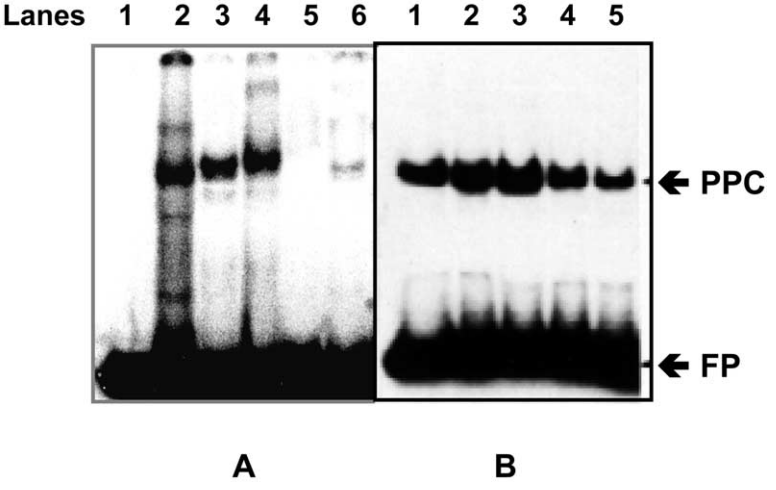

Fig. 1. Gel mobility shift assay of tissue extracts. (A) Lanes 3 and 4 are 15 μg and 30 μg extracts of pupal ovary and lanes 5 and 6 are 15 and 30 μg extracts of pupal testes. Lane 2 is a positive control containing 15 μg of snake ovary extract. Lane 1 contains only ^{32}P labeled probe. (B) Lanes 1 to 5 show retardation with 50 μg of pupal ovary extract from 6, 7, 8, 9 and 10th day of pupal development, respectively. Reactions were performed in 20 μl with 3–4 ng ((40 000 cpm) of ^{32}P end-labeled $GATA_{16}$-probe and 500 ng of sheared *E.coli* DNA as non-specific competitor. Free probe (FP) and probe–protein complex (PPC) are indicated. B represents tissue extract derived from pooled oocytes from the respective day of development, which consisted of a set of oocytes at different developmental stages.

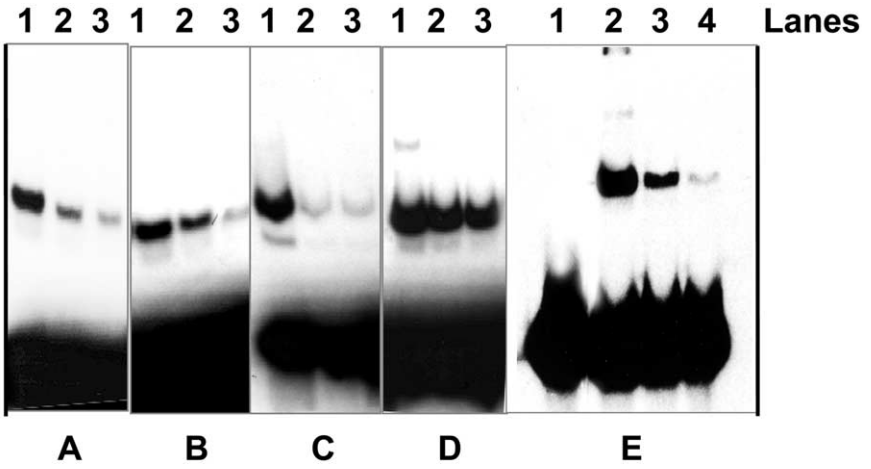

Fig. 2. Gel mobility shift assay showing specificity of BBP interaction. Tissue extracts from: (A) posterior silk glands; (B) anterior and middle silk glands (day 5 of 5th instar larvae); (C) pupal testes; and (D and E) pupal ovaries (day 8 of pupal development). Lanes 1 to 3 of A, B, C and D represent 0.5, 1 and 5 μg of sheared *E.coli* DNA as non-specific competitor; lanes 2 to 4 of E represent 0, 15- and 25-fold excess of unlabelled $GATA_{16}$- as specific competitor and lane 1 contains only the end-labeled $GATA_{16}$ probe. The reactions were carried out in 20 μl consisting of 50 μg of tissue extract, and 3–4 ng of ^{32}P end-labeled $GATA_{16}$ probe.

probe) or by Southwestern [using ^{32}P-Bkm2(8) probe] assay could be precipitated between 60–80% saturation of the salt. We refer to this fraction as Asp-BBP, which consistently contained a unique 37-kDa BBP band. For understanding the characteristics of BBP, we analysed Asp-BBP fraction by SDS-PAGE and 2-D gel electrophoresis. The results of this study are shown in Fig. 3. Gels were subjected to both Coomassie blue staining (Fig. 3A) and Southwestern assay (Fig. 3B). In both A and B portions 'a' shows separation by SDS PAGE, while 'b' shows the 2-D gel. Portions 'a' and 'b' represent parts of the same gel, which were run together in this study. It can be seen that Asp-BBP gave a homogeneous 37 kDa Southwestern band (B 'a') in SDS PAGE, while the 2-D gel gave the corresponding 37 kDa Southwestern spot (B 'b'). Neither a corresponding band nor a spot at 37 kDa were observed in the two gel types (see A 'a' and A 'b'). A circle marks the region where

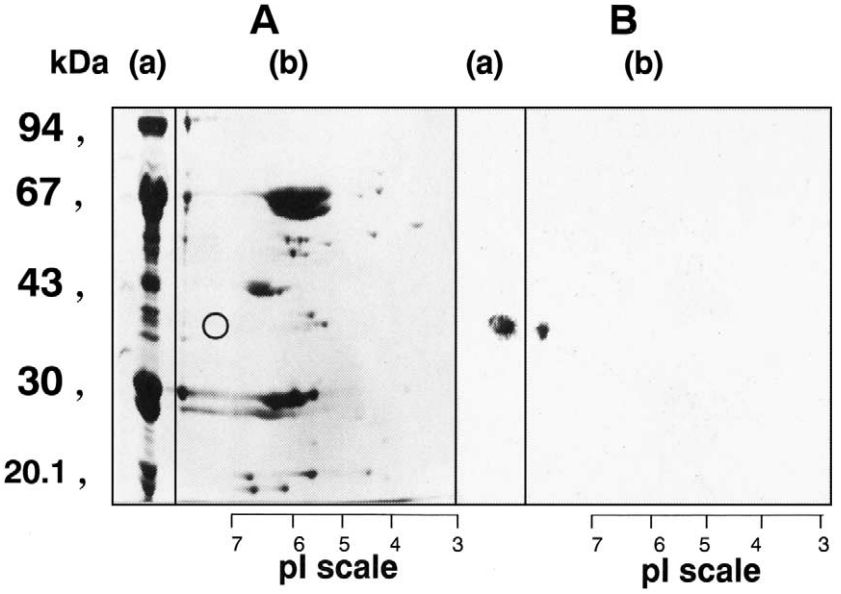

Fig. 3. SDS and 2-D PAGE profiles of Asp-BBP fraction. (A) Gels stained with Coomassie blue; (B) the same gels subjected to Southwestern assay. 100 μg of Asp-BBP fraction was resolved by iso-electric focusing, the gel fused on top of the SDS-PAGE slab-gel and run in the 2nd dimension. In A and B, portions 'a' and 'b' represent SDS-PAGE and 2-D PAGE, respectively. The proteins from SDS-PAGE (Aa) and 2-D PAGE (Ab) gels were electroblotted on to PVDF membrane and renatured by incubation with renaturation buffer and the blots were probed with end labeled Bkm2(8) probe employing adequate concentration of sheared *E. coli* DNA as the competitor. The circle denotes the expected BBP spot in the Coomassie stained 2-D gel.

the BBP spot is expected to appear in the Coomassie blue stained 2-D gel (Fig. 3). This is mostly due to the fact that BBP is present in the tissue at an extremely low level in comparison to other oocyte proteins, as expected for regulatory proteins and transcription factors. It is interesting to note that the pI of BBP was about 7.8–8 similar to that of DNA-binding proteins.

For achieving BBP purification in a single step, first we employed a $GATA_{16}$-Sepharose column for affinity purification. For reasons that were not clear, BBP could never be bound to the $GATA_{16}$ column. We suspected that BBP might be present in the form of a complex with other protein/non-protein molecules in Asp-BBP. In an attempt to further fractionate, we chromatographed Asp-BBP on a Superose 6 FPLC gel-filtration column and the protein peaks were analysed by the Southwestern assay. The profile of the FPLC elutions and Southwestern assay of the peaks are shown in Fig. 4P and Q, respectively. Almost all the BBP eluted in fractions 1 and 2 in the molecular weight region of approximately 2000–440 kDa. These results suggested that BBP exists as a high molecular weight complex in Asp-BBP and that it dissociates to a 37-kDa protein upon SDS PAGE.

The Bkm2(8) probe used in our Southwestern studies contains GATA and other interspersed repeats such as GACA (Epplen et al., 1982; Singh et al., 1984). $GATA_{16}$-Sepharose employed for isolation of BBP from the FPLC column yielded unique and homogeneous 37-kDa BBP both by SDS-PAGE and Southwestern assay, clearly demonstrating that MSA (employing $GATA_{16}$ probe) and Southwestern assay [employing Bkm2(8) probe] detected the same factor (see later). Several members of the GATA-family transcription factors which require zinc for their DNA (GATA)-binding activity are reported in the literature (Simon, 1995; Weiss and Orkin, 1995). Therefore, we proceeded to determine whether metal ion chelation affects the GATA-binding activity of BBP. Asp-BBP fraction was incubated at room temperature for 2 h with increasing concentrations of EDTA (Evans and Hollenberg, 1988), and the samples were subjected to SDS-PAGE followed by Southwestern assay. A high level of DTT was also employed to facilitate effective metal-ion chelation by EDTA. As shown in Fig. 5, Asp-BBP was incubated in 80 mM DTT in the presence of 20, 40, 60, 80 and 100 mM EDTA (lanes 2–6, respectively). It can be seen that metal ion chelation by EDTA did not cause any change in the intensity of the Southwestern signal of BBP. Also, Asp-BBP incubated in the absence of EDTA and DTT (lane 1) and Asp-BBP incubated in 100 mM DTT alone (lane 7), yielded identical Southwestern signal, suggesting that DTT by itself has no role to play in the dissociation of DNA–BBP complexes. However, efficient dissociation of DNA–BBP complexes was observed upon incubation of Asp-BBP fraction with higher levels of DTT for much longer periods than used along with EDTA (data not shown). Upon incubation of Asp-BBP with DTT and EDTA, a new doublet band of BBP with molecular mass of ~20 kDa appeared, in addition to 37-kDa protein. Since 2-D PAGE of Asp-BBP revealed only a 37-kDa protein (Fig. 3), the ~20-kDa BBP bands are likely to have arisen from degradation of free BBP dissociated from Asp-BBP during the incubation. Further, when we incubated Asp-BBP in 100 mM DTT alone at 4°C overnight, the (20-kDa doublet BBP bands did not appear (Fig. 5, lane 7).

Since increased concentration of DTT did not result in degradation Asp-BBP, we set out to purify BBP by

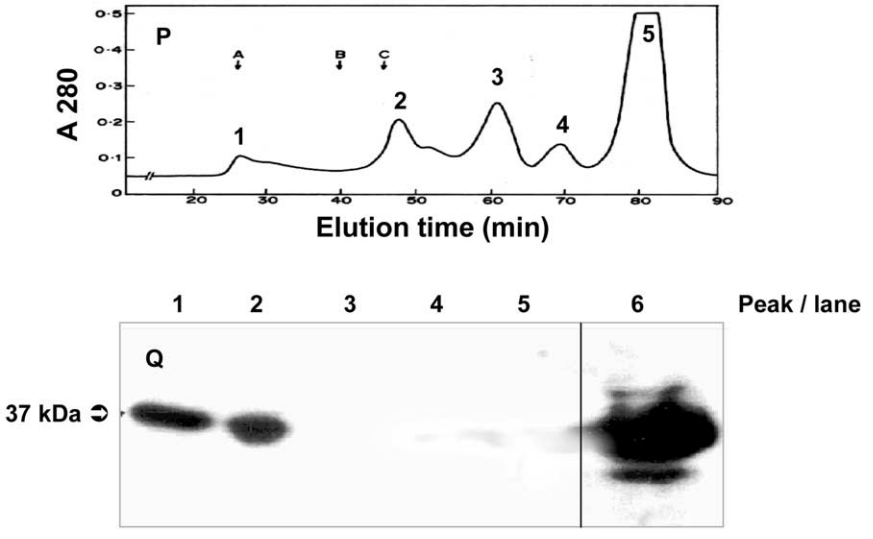

Fig. 4. Size fractionation and Southwestern assay of Asp-BBP. P. 1 mg of Asp-BBP in TNGE buffer containing 0.5% Triton X-100 was run on a Superose 6 gel filtration FPLC column. The eluted peaks are numbered. A, B and C denote the elution regions of blue dextran (2000 kDa), thyroglobulin (669 kDa), and ferritin (440 kDa), respectively, when fractionated in independent runs; Q. Lanes 1–5 are Southwestern assay of FPLC fractions using an end-labeled Bkm2(8)-probe; lane 6 is 50 μg of Asp-BBP as control.

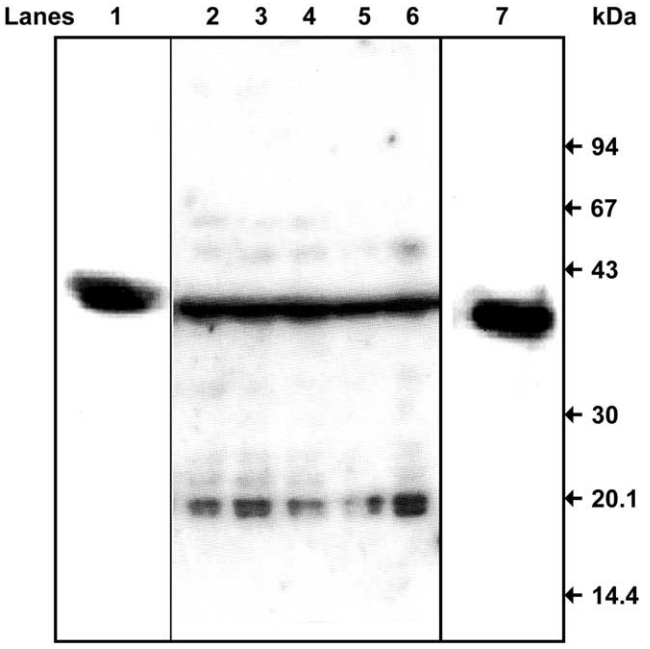

Fig. 5. Effect of incubation of Asp-BBP with EDTA as a function of its concentration: Lanes 2–6 are 200 μg of Asp-BBP incubated at 25°C for 2h with 20, 40, 60, 80 and 100 mM EDTA and 80 mM DTT, respectively; lane 1, Asp-BBP incubated at 25°C for 2 h without EDTA and DTT and, lane 7 is Asp-BBP incubated with 100 mM DTT alone at 4°C overnight. Samples were run on SDS PAGE and analyzed by Southwestern assay.

incubating the Asp-BBP at high levels of DTT for longer periods, employing $GATA_{16}$ affinity chromatography. Thus, the Asp-BBP was incubated at 4°C overnight in 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl (loading buffer) and 100 mM DTT; the sample was diluted to 10 mM DTT with the loading buffer and applied to a $GATA_{16}$-Sepharose affinity column. The column was washed with 20 bed volumes of the loading buffer and the bound BBP was eluted with a linear gradient of 50–800 mM NaCl. The eluted fractions were dot-blotted and analyzed by Southwestern assay to identify the fractions containing BBP, which were then pooled. The elution profile revealed a homogeneous peak of BBP eluting at 320 mM NaCl from $GATA_{16}$-Sepharose affinity column (data not shown). The Asp-BBP and eluted BBP fractions were analyzed by SDS PAGE followed by staining with Coomassie blue (lane c) or silver stain (lane e) and were also assayed by Southwestern assay as shown in Fig. 6A. Asp-BBP exhibited a homogeneous Southwestern BBP signal both before and after incubation with DTT (Fig. 6 A, lanes a and b). Western blot analysis of BBP employing polyclonal anti-BBP antibody, generated against pooled silver stained acrylamide gel bands of BBP, showed a specific signal corresponding to BBP (Fig. 6 A, lane f). The yield of BBP estimated by visual approximation of the silver stained BBP band (in comparison with that of other single band proteins of known quantity) is extremely low and was about 0.0002% of Asp-BBP as estimated by the Bradford method (Bradford, 1976), while the yield of Asp-BBP was about 0.1% of the tissue. Thus 50 g of silkworm oocytes could yield a maximum of 100 ng of BBP. Further, purified BBP was unstable and generated degraded bands even when stored at −20°C in different buffer conditions (data not shown). Affinity purified BBP was subjected to MALDI-TOF analysis for confirming its homogeneity and precise molecular weight. A Sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) matrix was employed for the purpose, as it appeared to be the ideal matrix for this protein. As shown in Fig. 6B, BBP revealed a molecular mass of 37 477 Da which is in agreement with the molecular weight of BmBBP observed earlier by SDS PAGE.

For gaining insight into the probable function of BmBBP, we conducted localization studies of BmBBP in pupal ovary. Paraffin tissue sections of the pupal ovary were prepared and subjected to fluorescence immuno-staining using anti-BmBBP antibody as described in Materials and methods. The studies revealed that BmBBP is expressed in the follicle cells and in the ooplasm, as well as the nucleus of different developmental stages of oocytes (Fig. 7 A3-A6).

4. Discussion

We have isolated a Bkm (GATA)-binding protein from the pupal ovary of silkworm, *B. mori*, which we have named as BmBBP. It is a low molecular weight protein (37.5 kDa), with a moderately basic iso-electric pH (7.8–8), which is expected for DNA-binding proteins. The protein appeared predominantly in the pupal ovary, while detectable levels of similar activities were observed in other tissues − silk glands and pupal testes, which could be non-specific that might arise due to the presence of some other proteins that bind to DNA. Alternatively, presence of rare copies of BBP mRNA in the terminally differentiating silk glands and pupal testes may give rise to detectable BBP and thus exhibiting MSA cannot be ruled out. We demonstrated that the MSA signals of pupal ovary BBP are specific in competition experiments, both with increase in non-specific DNA, as well as with unlabelled $GATA_{16}$. Further, BBP levels increased with age of the ovary and reached a peak by day 7 to day 8 of pupal development. Since spermatogenesis takes place during the larval stages (3rd, 4th and 5th instars) and is essentially complete by the time of pupation, it is possible that BBP was not detected in pupal testis. Due to limitations in the availability of required quantity of larval testes, we were unable to perform experiments involving this tissue. Since we speculate that BmBBP is involved in chromatin remodelling during gene expression, we believe that it is unlikely to express in testicular tissue. Our speculation

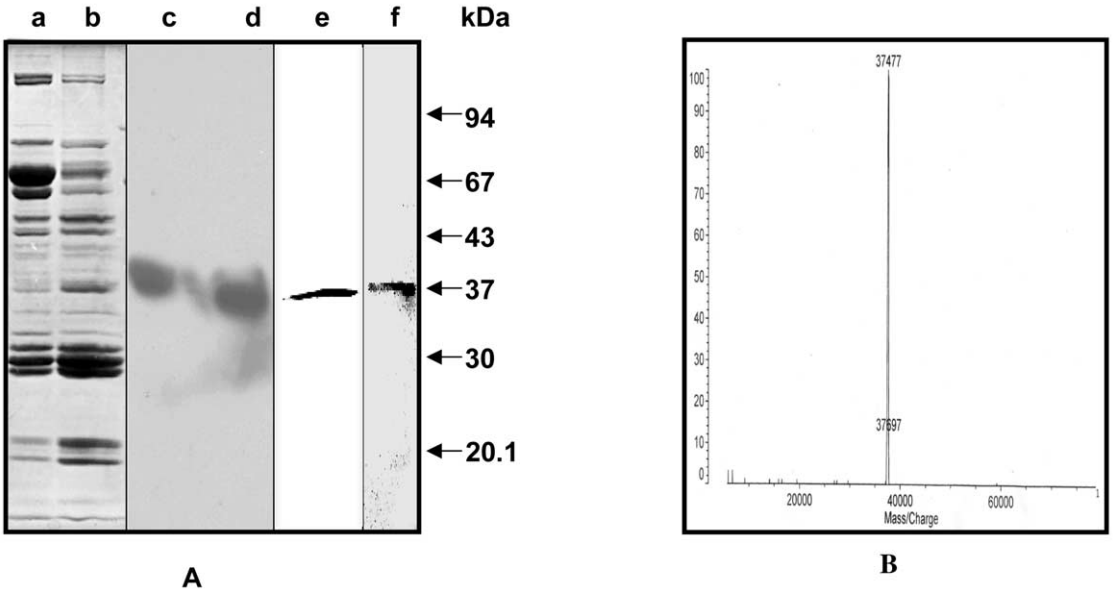

Fig. 6. (A) SDS-PAGE, Southwestern, and Western analysis of Asp-BBP and purified BBP. Lanes a and b are 100 μg of Asp-BBP stained with Coomassie blue before and after overnight incubation with 100 mM DTT at 4°C; lanes c and d are Southwestern analysis of lanes a and b; lane e is 1 μg of purified BBP stained with silver nitrate and lane f is Western analysis of lane e using anti-BBP antibody. (B) MALDI-TOF of purified BBP by employing Sinapinic acid matrix showing a homogeneous protein peak of 37.477 kDa.

emerges mainly from the earlier work in snake and mouse where BBP activity was reported to be at its peak level by the onset of spermatogenesis and oocyte development, corresponding to the decondensation of W and Y chromosomes, respectively (Singh et al., 1994). We are of the opinion that BmBBP belongs to the same class of BBPs, and may be involved in bringing about decondensation of the chromosome domain(s) containing the gene(s), the product of which may be needed at a specific stage of oocyte development. Blocks of GATA repeats have been reported to be present in at least one lepidopteran, *Ephestia kuhniella* (Traut et al., 1992); however, the hypervariable sites in this moth are present on autosomes as well (Rohwedel et al., 1993). which raises question regarding the role of BBP postulated for *B. mori* in this species. It may be interesting to investigate whether the protein corresponding to BmBBP is expressed in this species in tissue specific manner and is involved in chromatin modulation.

Earlier, several GATA-binding proteins belonging to the GATA-family of transcription factors, which specifically interact with the GATA-motif present in gene regulatory elements, have been reported from vertebrates as lineage–specific transcriptional regulatory proteins (Martin and Orkin, 1990; Romeo et al., 1990; Ho et al., 1991). Sequence analysis of these proteins has revealed strong conservation of the zinc-finger DNA-binding domain between these vertebrate transcription factors (Martin and Orkin, 1990; Romeo et al., 1990; Yamamoto et al., 1990). In *Drosophila melanogester,* a GATA (dGATAc) zinc finger protein that displays a distinct expression pattern between different embryonic tissues at different stages of development was reported (Lin et al., 1995). Another Drosophila-specific protein isoform (dGATAb), also belonging to the GATA family of transcription factors and expressed in the follicle cells, was reported earlier (Lossky and Wensink, 1995). GATA-4, another member of the same family of transcription factors present in the gonads of mouse, which is specifically expressed in sertoli cells in testes and granulosa in the ovary, has been suspected to be involved in the early gonadal development and sexual dimorphism (Viger et al., 1998). In *B. m*ori, a BmGATA ß gonadal gene (expressed in the testes as well as ovary) that expresses three alternatively spliced mRNA isoforms leading to different products involved in regulation of the expression of a class of chorion genes has also been reported (Drevet et al., 1995). We have demonstrated that BmBBP neither requires divalent metal ions, nor is affected by the presence of metal ion chelator, for its GATA-binding activity. Thus BmBBP does not appear to belong to the GATA-family of transcription factors. We further demonstrated that BmBBP reported here is a stage- and tissue-specific protein and is thus novel and distinct from other GATA-binding factors reported so far. It remains to be demonstrated whether the ovary-specific BmBBP reported here brings about chromatin modulation of the heterogametic sex to facilitate sex-specific gene expression as observed earlier in mouse and snake.

The W chromosome possesses the gene(s) responsible for female sex determination in the silkworm (Tazima, 1964; Suzuki et al., 2001; Niimi et al., 2001). These gene(s) need to be expressed or modulated during early

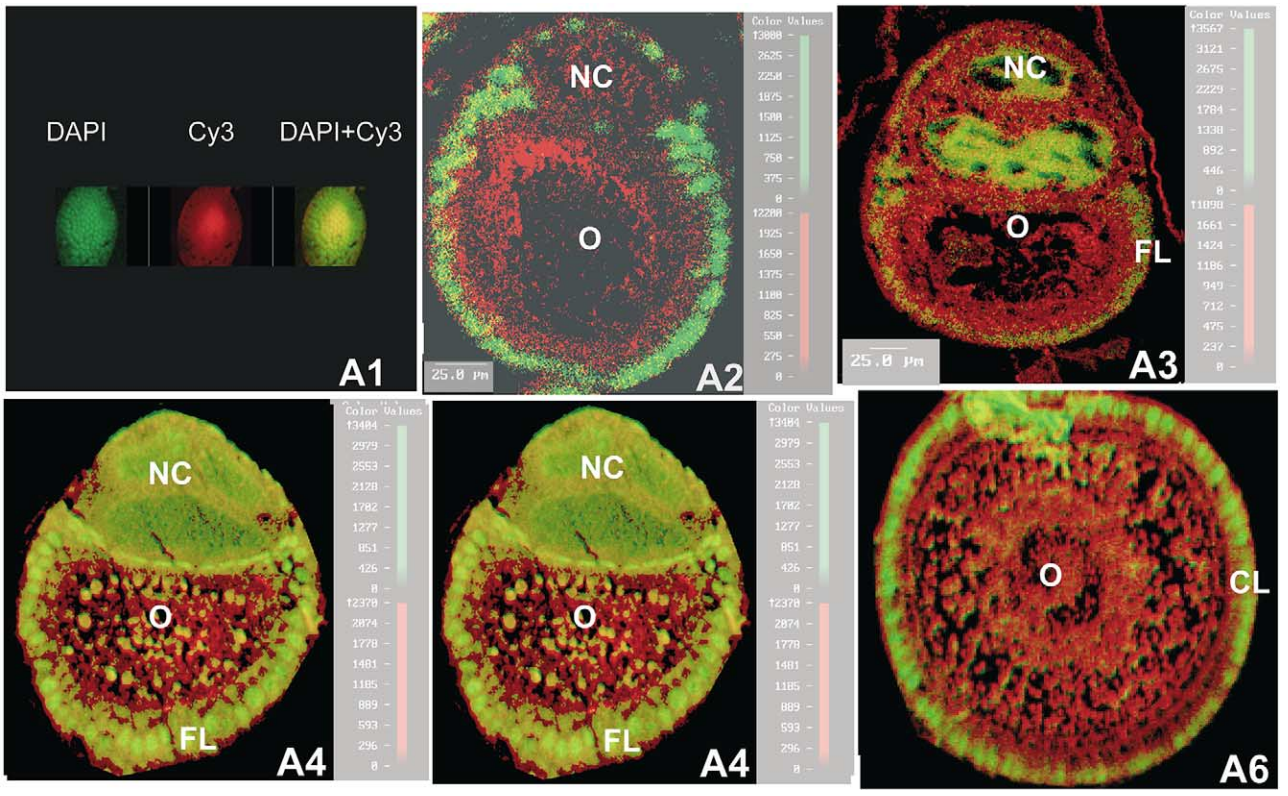

Fig. 7. Immuno-localization of BmBBP in the pupal ovary of *B. mori*, employing confocal microscopy. A1 shows avidin conjugated Cy3 which stains bright red with proteins, DAPI which normally gives green colour with nucleic acids, although we have applied pseudo-colour green and intermixing of Cy3 and DAPI produces yellow, after incubation of oocytes with BmBBP antibody. A2 shows a control experiment for immuno-localization of BmBBP in pupal ovary employing mouse pre-immune serum (1:200 dilution). A3 to A5 are paraffin sections of oocytes which are at different developmental stages (A3, Cup-shaped oocyte with abundant accumulation of yolk and nurse cells at initial stages of degeneration; A4, Follicle cells form a layer and nurse cells decrease in mass; A5, Oocyte attaining oval shape with further degeneration of nurse cells; and A6, Mature oval oocyte with degenerated nurse cells and chorion layer secreted by the follicle cells). NC, nurse cells; O, oocyte; FL, follicle layer; CL, chorion layer.

stages of development when germinal cells are differentiated. Hence the specialized mechanisms for their modulation involving GATA sequences and BBP may exist. Although GATA repeats are predominantly concentrated on the W chromosome, they are present even on the autosomes and are involved in regulation of several other cellular functions. The mechanism of gene regulation used by BmBBP is probably different from the other GATA binding proteins on the autosomes. A case in point here is that BmBBP does not require Zinc unlike other GATA binding proteins which exercise zinc finger based transcription control. Transcription factors associate with their target regulatory sequences both as monomers and dimers (Pabo and Sailor, 1992; Wolffe, 1994; Atchley and Fitch, 1997; Fernandez-Silva et al., 1997). The large excess of reduced thiols required for dissociation of BmBBP from the complex suggests a high affinity interaction between BmBBP and GATA-repeats, which in turn implies that thiol-involved interactions (disulfides) perhaps stabilize protein–protein (and thereby probably protein–DNA) interactions. These observations also suggest that BmBBP might undergo oligomerization upon binding to its target DNA sequences. Such a high affinity of DNA-protein interaction may be necessary for bringing about coordinated decondensation of the chromatin potentially present in the specifically de-condensed sex determining chromosomes in the silkworm oocytes.

Immunofluorescence localization studies have revealed the presence of BmBBP over a large portion of the oocyte nucleus all through the oocyte development and also in the follicle nuclei at the early stage of oocyte development when the oocyte assumes cup-shaped structure. These results are in agreement with the age-dependent increase of Bkm-binding activity, in both developing oocytes of snake and developing and mature mouse testes (Singh et al., 1994). Earlier, immunolocalization of the snake oocyte BBP was reported to be on the nuclear periphery in a position that is occupied by the W chromosome (Pathak, 1997). The presence of BmBBP over a large portion of the oocyte nuclei raises the question whether BmBBP plays a global nuclear role, or it is involved in a function associated with the W chromosome alone. It is difficult to answer this with

our limited results. It is important to recognize that GATA repeat units are interspersed throughout the silkworm genome and are not preferentially associated with the W chromosome (Nagaraju et al., 1995). BmBBP might play a role in bringing about coordinated decondensation of chromosomal domains containing genes, products of which may be needed in large quantity at a specific time during oocyte development. BmBBP therefore may play a role in coordinately activating genes that are located on different chromosomes. Assessment of the minimum number of GATA repeats required for the association of BBP might throw light on the physiological function of BBP proteins.

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