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Bombyx Y-Box Protein BYB Facilitates Specific DNA Interaction of Various DNA Binding Proteins Independently of the Cold Shock Domain

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A new member of the Y-box protein family of the silkworm *Bombyx mori* (BYB) was co-purified with the fibroin gene enhancer-binding protein FMBP-1, and stimulated the binding of FMBP-1 to its cognate DNA element. However, the stimulatory effect was not specific to FMBP-1, BYB also enhancing the binding of mammalian transcription factors OTF2, SP1 and AP2 to their specific binding elements. Besides the above transcription regulatory factors, BYB facilitated the binding of basal transcription factor TBP, and enhanced transcription from the adenovirus 2 major late promoter in a reconstituted transcription system. Moreover, BYB stimulated the reactions of some restriction endonucleases under cold conditions. The C-terminal region of BYB was sufficient for these stimulatory effects, and the highly conserved cold shock domain (CSD) in the N-terminal region was dispensable. GST-pull down experiments showed that the C-terminal region could interact with DNA independently of the CSD. The above results suggest that the C-terminal region of BYB causes the active interaction of various DNA binding proteins with their targets. Such a function of the C-terminal region of BYB may partly explain the functional diversity of Y-box proteins.

Key words: cold shock domain, DNA binding protein, FMBP-1, nucleic acid chaperone, Y-box protein.

Abbreviations used: Ad2MLP, adenovirus 2 major late promoter; BYB, *Bombyx* Y-box protein; GST, glutathione S-transferase; EMSA, electrophoresis mobility shift assay; FMBP-1, fibroin-modulator-binding protein-1; TBP, TATA box-binding protein; TF, transcription factor.

A number of genes encoding members of the Y-box protein family have been cloned from various eukaryotes (*1*–*3*). The first member of the family, human YB-1, was isolated as a factor that binds to a DNA element containing an inverted CCAAT sequence, known as the Y-box (*4*). The DNA binding domain of Y-box proteins has been highly conserved throughout evolution and is named the cold shock domain (CSD). CSDs are >40% identical and >60% similar to cold shock response protein CspA in *Escherichia coli* (*5*), and >50% identical to each other in eukaryotes. Not the amino acid sequence but the structure of the C-terminal region is also conserved, and alternative acidic and basic/aromatic stretches are found in vertebrates (*3*, *6*). An acidic stretch is not obvious in invertebrates, but basic/aromatic stretches or RGG repeats are observed in the C-terminal region (*2*, *7*). The high level of conservation of these CSD proteins in from bacteria to man suggests that the Y-box proteins have essential roles in biological processes. The Y-box proteins were later found to interact with a wide variety of DNAs and RNAs with generous selectivity (*8*–*11*). Correlating to the interaction with various nucleic acids, diverse functions of the Y-box proteins have been proposed; transcriptional activation or repression, DNA repair, translational repression, RNA packaging and RNA chaperoning (*2*, *3*).

Y-box elements have been found in the promoters of many genes including vertebrate germ-cell specific genes (*12*) and cell-growth associated genes (*13*). Human multidrug resistance gene-1 (MDR-1) also has an inverted CAAT sequence in its regulatory region (*14*). Another Y-box protein binding element, the CT-rich element, is also observed in its transcriptional regulatory regions (*15*, *16*). These Y-box protein binding elements are involved in the expression of such genes in either a stimulative or suppressive manner (*17*–*21*). One of the *Xenopus* Y-box proteins, FRGY2, is contained in maternal mRNP complexes in the oocyte (*11*). The Y-box protein in *Drosophila*, Yps, is involved in the mRNA localization machinery, and necessary for the correct localization of *osker* mRNA in the oocyte (*22*, *23*). In these complexes, the Y-box proteins seem to repress the translation of mRNA until an appropriate developmental time when the function of the stored mRNA is necessary (*2*, *24*, *25*). FRGY2 has been

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shown to stimulate the transcription of oocyte-specific genes, but to repress translation of the newly synthesized mRNA (*19*). Thus, the Y-box proteins are involved in the regulation of gene expression through both transcription and translation, but their precise functions and mechanisms of action are still not well understood.

We isolated a Y-box protein orthologue from *Bombyx* named BYB. BYB was co-purified with fibroin gene transcriptional modulator binding protein FMBP-1 (*26*), and enhanced the binding of FMBP-1 to specific DNA elements. BYB also stimulated the interaction of various DNA binding proteins with their cognate targets. These activities were observed irrespective of the absence of the Y-box protein binding sequences in the target DNAs, and occurred independently of the CSD.

MATERIALS AND METHODS

Silk Gland Extracts—Larvae of a *Bombyx mori* hybrid strain (Kinshu × Showa) reared on an artificial diet or mulberry leaves were dissected on day 1 of the fifth instar stage and the posterior silk glands (PSG) were collected. Crude nuclear extracts were prepared as described (*27*) with several modifications for large-scale purification. About 300 pairs of posterior silk glands were minced in 60 ml of extraction buffer and then homogenized. The homogenate was centrifuged, and the pellet was suspended again in 100 ml of the extraction buffer and homogenized. A further 140 ml of the buffer was added to the homogenate and proteins were extracted by the addition of 24 ml of a saturated $(NH_4)_2SO_4$ solution. The mixture was centrifuged at 180,000 × *g* for 4 h and the supernatant was collected by decantation. Proteins were precipitated by the addition of powdered $(NH_4)_2SO_4$ and collected by centrifugation at 10,000 × *g* for 1 h. The precipitate was dissolved in dialysis buffer (20 mM Hepes, pH7 .9, 100 mM KCl, 12.5 mM $MgCl_2$, 17% (v/v) glycerol, 0.1 mM EDTA, pH 8.0, and 2 mM DTT) and then dialyzed against the same buffer.

Column Chromatography of BYB Protein—Posterior silk gland extracts were fractionated on a phosphocellulose column as described (*28*) with minor modifications. Extracts prepared from ~300 pairs of posterior silk glands were dialyzed against K300 buffer (20 mM Hepes, pH 7.9, 300 mM KCl, 12.5 mM EDTA, pH 8.0, 15% (v/v) glycerol, and 2 mM DTT), loaded on a phosphocellulose column (10 ml), and then fractionated. Fractions eluted with K500 buffer (20 mM Hepes, pH 7.9, 500 mM KCl, 1 mM EDTA, pH8.0, 15% (v/v) glycerol, and 2 mM DTT), which contained FMBP-1 activity, were collected and loaded directly onto a calf thymus DNA-cellulose column (0.3 ml) pre-equilibrated with the K500 buffer. The column was washed with 3 ml of N500 (500 mM NaCl instead of 500 mM KCl in K500 buffer), and 300 μl each of N550 and N600 buffer containing 10 mM hepthyl-thioglucoside and 1 μg/ml of luepeptin, and then proteins were eluted with N900 buffer. The protein composition was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 2.5% (w/v) stacking and 12% (w/v) separation gels. The proteins were then detected with a Silver Stain II kit (Daiichi Chemical Co.).

EMSAs—The electrophoresis mobility shift assay (EMSA) was performed as described (*26*). During the course of protein purification, 2 μl aliquots of eluent from the columns or the diluent and protein solutions recovered from polyacrylamide gels were used for EMSA without adjusting the salt concentration. The nucleotide sequences of the top strands of the probes used in this study were as follows: +290, aattGATGAATCTATGTAAATACTGGGCA GAC (*26*); AP2/SP1, aattAACTGGCCGCGGGCGGGTAGTTCGATCA; Ad2MLP, aattGGG GGGCTATAAAAGGGGGTGGGGGCGCG. Double-stranded oligonucleotide probes were labeled by filling in the sticky ends shown by aatt with Klenow polymerase, and single-stranded probes were labeled with T4 polynucleotide kinase. OTF2A was purchased from Roche Diagnostics, and one unit was defined as the activity necessary for shifting 50% of the probe DNA under the conditions as per the manufacturer's instructions. AP2, SP1 and TBP were purchased from Promega Co, and one unit was defined as the activity necessary for complete footprinting. All these factors were of human origin and supplied as recombinant proteins.

Recovery of Proteins on SDS-PAGE—To examine the activity of proteins, protein bands in polyacrylamide gels were detected by negative staining with zinc (*29*), recovered, then denatured and renatured as described (*30*). Proteins were dialyzed finally against NP40 buffer (50 mM Tris, pH 7.9, 100 mM NaCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 1 mM DTT, 20% (v/v) glycerol, and 0.1% (v/v) NP40) and stored at –80°C until use.

Amino Acid Sequencing of BYB—When the concentration of a protein was necessary before SDS-PAGE, 2–5 μl of StrataClean resin (Stratagene) equilibrated with N500 buffer was added to the protein fraction, mixed, and precipitated by centrifugation. The loading dye solution was added to the resin pellet, and then the suspension of the resin and loading dye was applied directly to the SDS-PAGE gel. With this treatment, p36 protein in several milliliters of a solution derived from about 1,000 pairs of PSGs was separated in a single lane of a standard preparative gel. The gel was stained with Coomassie brilliant blue R250, and the band of p36 was excised, destained, and digested with lysile-endopeptidase. The proteolytic peptides were extracted and fractionated by HPLC. The amino acid sequences of five polypeptides (as shown in Fig. 3A) were determined with a protein sequencer (Applied Biosystems Inc. 492) at the Center for Analytical Instruments, National Institute for Basic Biology.

Cloning of BYB cDNA—DNA fragments between the sequenced peptides were amplified by PCR with degenerate primers using cDNA from PSGs at 12 h after the fourth ecdysis stage as a template, cloned into pBluescript and then sequenced. From the unique internal sequence of the cold shock domain, a 30-mer probe (GATGGCAGTCTGATGCACAAACACATCTTC) was synthesized and used for library screening.

Poly(A)$^+$ RNA was prepared from PSGs at 12 h after the fourth ecdysis stage with a Quickprep mRNA purification kit (Amersham Pharmacia Biotech). Other methods for the construction of the cDNA library were as described by Mach *et al.* (*31*). A library of 2.26×10^5 independent clones was amplified once and used. On screening of the library with the above probe, many positive

clones were obtained. We isolated seven clones and analyzed them further.

Recombinant BYB Proteins—To obtain GST-BYB fusion protein containing the entire coding region of BYB, a DNA fragment comprising from the nucleotide at position 78 to the 3′ end of the cDNA was prepared and subcloned into pGEX-4T1, and named pGEX-BYB. To prepare a protein depleted of the N-terminal region of BYB, a DNA fragment comprising from position 432 to the cDNA end was prepared and subcloned into pGEX-4T2. In this clone (pGEX-BYBdel.N), the coding region comprising amino acid positions 120 to 259 of BYB was fused to GST. To prepare a protein depleted of the C-terminal region, pGEX-BYB was digested at nucleotide position 501 and the cDNA end, and then self-circularized. In this clone (pGEX-BYBdel.C), the coding region comprising amino acid positions 1 to 141 was fused to GST. GST-BYB fusion proteins (Fig. 9) were produced in *E. coli* and purified with glutathione-Sepharose (Amersham Pharmacia Biotech Inc.), and used either directly or after purification by recovery of the bands from the SDS-PAGE gel.

Antibody Preparation—GST-BYBdel.N protein was used to immunize rabbits. After the third booster, serum was collected. The anti-human TBP antibody was purchased from Promega Co.

Western Blotting—Proteins were separated by SDS-PAGE and then transferred to a PVDF membrane. BYB was detected with an ECL Western blotting kit (Amersham Pharmacia Biotech Inc.). The membrane was processed as recommended by the manufacturer, and 1/500 diluted 1st anti-BYB antiserum and 1/1,000 diluted 2nd anti-rabbit antibody antiserum were used.

Transcription—The in vitro transcription system was reconstituted with recombinant basal transcription factors and purified RNA polymerase II as described (*32*). TFIIB (50 ng), TFIIF (120 ng), TBP (45 ng), RNA polymerase II (200 ng), and closed circular template of Ad2MLP (400 ng) were used in a total volume of 20 μl.

GST-Pull Down with the C-Terminal Region of BYB—To 10 μl of glutathione-Sepharose equilibrated with binding buffer (0.6 × concentration of the NP40 buffer), 500 ng of GST-BYBdel.N or GST protein was added. The mixture was centrifuged, and the pellet was recovered. After washing the Sepharose resin with the binding buffer, 2 ng of isotope-labeled Ad2MLP probe and 10 ng of SP1, AP2 or BSA were added, followed by incubation at room temperature for 1 h, and then washing with the binding buffer (1 ml × twice). Finally, GST or GST-fusion protein was eluted by the addition of 5 mM glutathione and 50 mM Tris pH8.

Reaction of Restriction Endonucleases—The 10 μl reaction mixture comprised 0.3 μg of λ DNA, 50 mM Tris (pH8.0), 10 mM $MgCl_2$, 100 mM NaCl, and 1 μl of BYB (2 ng of BYB and 500 ng of BSA) or BSA (500 ng) in NP40 buffer. The mixture was incubated with 0.1 (at 37°C) or 0.3 (at 4°C) units of EcoRI. At the end of the reaction, each tube was chilled on ice, and then the contents were mixed with 3 μl of DNA loading dye and stored at –20°C until analyzed on 1% (w/v) agarose gels. The reactions of various restriction endonucleases were performed under the same conditions irrespective of the optimal conditions for each endonuclease.

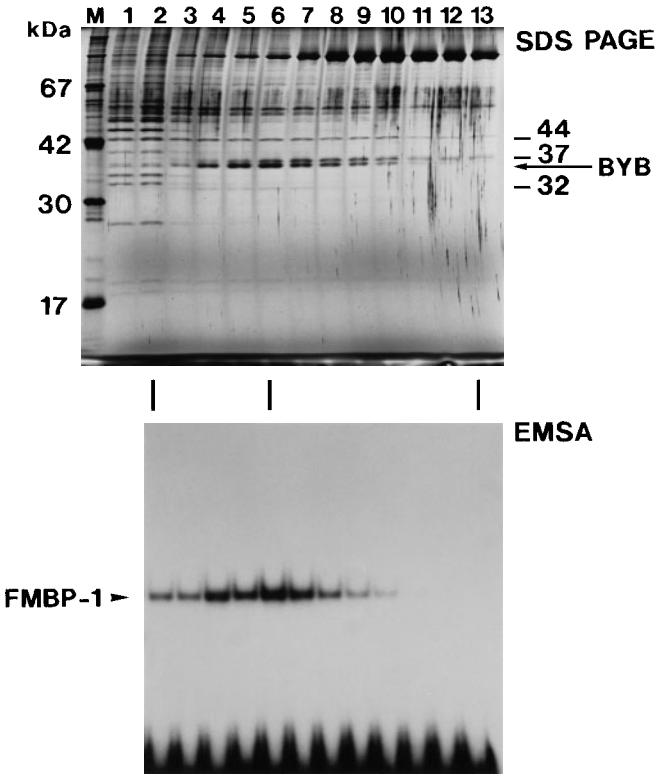

Fig. 1. **Co-elution of a p36 protein with FMBP-1 activity from a DNA-affinity column.** The 0.5 M KCl eluate from the phosphocellulose column of PSG extracts was loaded directly onto a DNA-cellulose column and fractionated. After washing the column with 0.5 M NaCl buffer, proteins were eluted with 300 μl each of the buffer containing increasing concentrations (50 mM steps from 550 mM [lane 1] to 1150 mM [lane 13]) of NaCl. For SDS-PAGE, 30 μl of each faction was concentrated and loaded on a single lane, and 2 μl was used for EMSA with the +290 probe. BYB, p36; 44, p44; 37, p37; and 32, p32 protein.

RESULTS

Co-Elution of BYB with FMBP-1 from a DNA-Cellulose Column—After DNA-cellulose column chromatography of PSG extracts, we analyzed the DNA binding activity of FMBP-1 in each fraction by EMSA and the protein composition by SDS PAGE. As shown in Fig. 1, FMBP-1 was eluted in fractions with between 550 and 950 mM NaCl. For these fractions, several major and minor protein bands were detectable, but only two proteins corresponded to the FMBP-1 activity. The major one was about 36 kDa (p36), and the minor one about 32 kDa (p32). These proteins were recovered from the gel in parallel with proteins of 37 kDa (p37) and 44 kDa (p44), renatured and then examined for FMBP-1 activity. As shown in Fig. 2, protein p32 at least bound the +290 probe (see "MATERIALS AND METHODS") and migrated the same as the FMBP-1 in the crude extracts and DNA-cellulose fractions, while proteins p36, p37 and p44 did not bind the probe (Fig. 2A and not shown). A few batches of p36 produced a minor complex with the probe on EMSA, but the mobility of the complex was much slower than that of the FMBP-1 complex. This seems likely to be a complex with the bottom strand of the probe (see Figs. 6A, 7A and B, 9B, and also Nishita and Takiya, in preparation). The

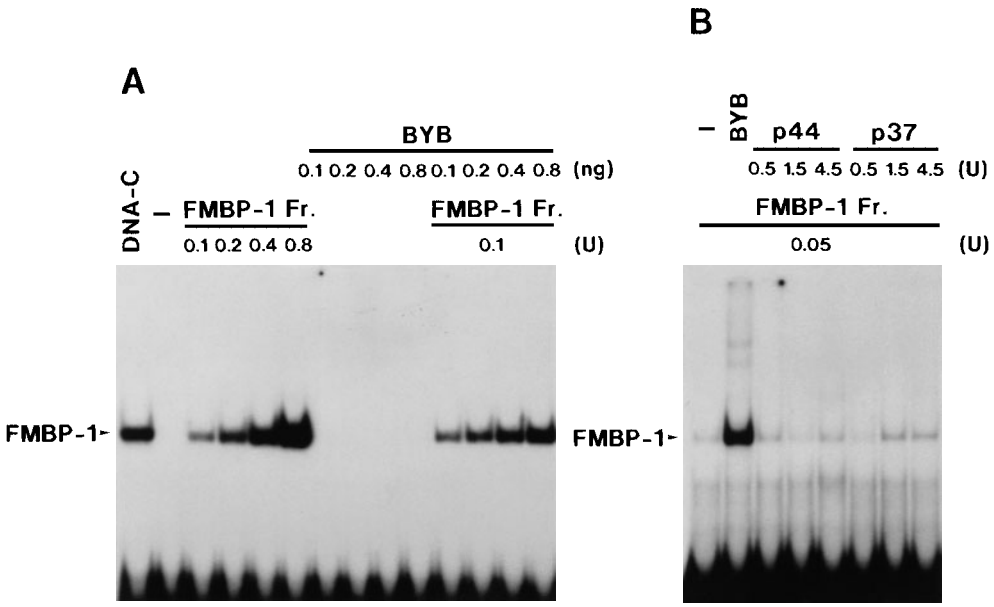

Fig. 2. **Enhancement of the binding of FMBP-1 to its cognate element by the p36 protein.** Each protein recovered from the SDS-PAGE gel after DNA-cellulose column chromatography was dissolved in 200 µl of denaturation buffer and renatured in NP40 buffer, and then used directly or after dilution. The amount of p36 protein (BYB) was roughly estimated by comparison with known proteins electrophoresed in the same gel. The concentration of p36 protein used was about 2 ng/µl. The amounts of other proteins were defined by the volume of the protein solution recovered from the preparative gel run in parallel. One unit indicates 1 µl of the original protein solution. (A) Proteins recovered from the gel at around 32 kDa (FMBP-1 Fr.) and 36 kDa (BYB) were examined for FMBP-1 activity separately or together by EMSA with the +290 probe. The fraction recovered from the gel at around 32 kDa still contained proteins other than FMBP-1, and was therefore named FMBP-1 Fr. DNA-C is the fraction obtained on the DNA-cellulose column chromatography used as a control for FMBP-1. (B) Effects of the p37 and p44 proteins on the DNA binding of FMBP-1. In the BYB lane, 0.4 ng of p36 protein was added.

effects of these proteins on the DNA binding of FMBP-1 were examined by addition together with p32 to the EMSA reactions. Clearly p36 stimulated and/or stabilized the DNA binding of FMBP-1 (Fig. 2A), but no significant effect was observed with p37 or p44 (Fig. 2B).

To establish the molecular identity of p36, we purified the protein from PSGs by column chromatography and SDS-PAGE, and determined the partial amino acid sequences of five proteolytic polypeptides (Fig. 3A). Homology searches in various databases indicated that three of them correspond to the CSD of the Y-box protein family. cDNA clones for p36 were isolated from a library of PSG and the longest one was sequenced. The cDNA was 1,396 bp long, encoding an ORF of 259 amino acids

A

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1   MADTEKAPQP QPQQQLEQQP QAQQAKTVKQ KQVIAEKVSG TVKWFNVKSG  50
51  YGFINRNDTK EDVFVHQTAI ARNNPRKAVR SVGDGEAVEF AVVAGEKGFE 100
101 AAGVTGPGGE PVKGSPYAAD KRRGYHRQYF PRQGGGRGGE GAPRRGGLGR 150
151 RGPPPNQGGA QGDEGQEGGI VPSQRSFFRR NFRGGRRGGG PGPMNRGGFR 200
201 RVRPRNFQAG QGGQNQAQRQ NGQDGDASAT TSNQQQGAKP KSNKPVGTTI 250
251 ETTTNESQA
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Fig. 3. **Amino acid sequence deduced from the cDNA for p36.** (A) The predetermined amino acid sequences of the p36 protein are indicated by arrows and the CSD is boxed. Gln-rich regions are shown by dots, basic/aromatic regions in the C-terminal half are underlined, and RGG repeats are double-underlined. (B) Comparison of the CSD amino acid sequences. Amino acids identical to those of BYB are shown by dots.

B

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Bombyx YB        KQVIAEKVSG TVKWFNVKSG YGFINRNDTK EDVFVHQTAI ARNNPRKAVR SVGDGEAVEF AVVAGEKGFE AAGVTGPGGE PVKGSPYAAD
Human YB1        .K...T..L. .......RN. .......... .......... KK.....YL. ......T... D..E....A. ..N......V ..Q..K....
Chicken YB1      .K...T..L. .......RN. .......... .......... KK.....YL. ......T... D..E....A. ..N......V ..Q..K....
Xenopus FRGY1    .K...T..L. .......RN. .......... .......... KK.....YL. ......T... D..E....A. ..N....E.V ..Q..K....
Drosophila yps   .E...T..T. .......... .......... .......S.. .-...K.... ......V... D..I....N. ..N....S.. ..R..QF...
Aplysia APY1     .KI..SQ... .......... ......D... .......... VK.....YL. ......K... D..E....N. ..N....E.S N.Q..K....
C.elegans F33A8  .P.K.T..K. ........N. ......T..N ..I....... IN..PN.YL. .L..N.E.M. DI.E.S..L. ..S....D.G ..Q..K....
D.japonica Y1    ..LLHTGIT. K.......R. ...VC...NQ ..I.I..S.. VKS..DHR-K ...E..EIL. DI.K.A..N. ..N.SAID.K C....E..LR
E.coli CspA        MSG.MT. I.....ADK. F...TPD.GS K.....FS.. Q-.DG--Y-K .LDE.QK.S. TIES.A..PA .GN..SL
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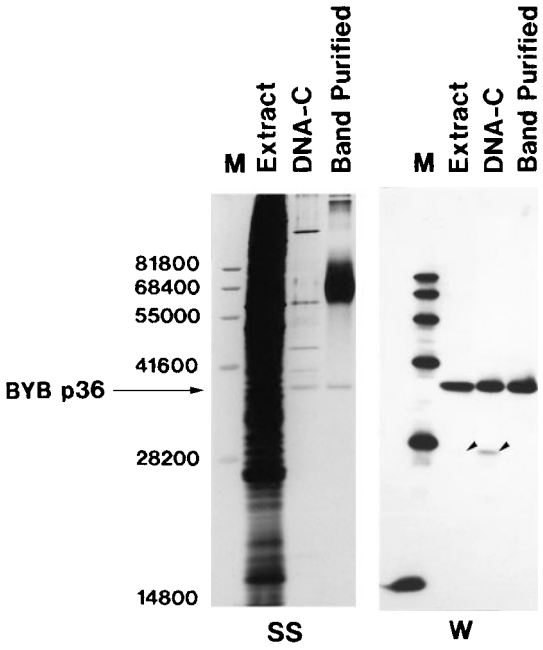

Fig. 4. **Western blotting of the BYB protein.** The combination of a crude PSG extract (Extract, 8 μg protein), the DNA-cellulose fraction (DNA-C, 30 μl), and the p36 protein purified from the gel (Band Purified, 4 ng) was electrophoresed in duplicate. After the electrophoresis, one of the gels was stained with a silver stain kit (SS), and the other one was processed for Western blotting (W). The arrow indicates the position where the p36 protein appeared and arrowheads the minor band around 28 kDa. M: marker proteins.

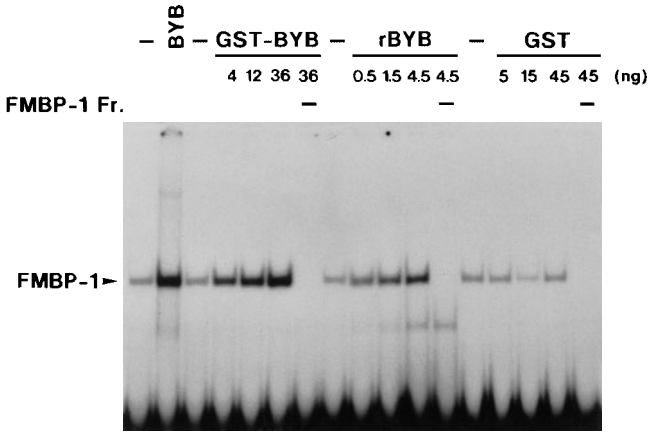

Fig. 5. **Enhancement of the binding of FMBP-1 by the recombinant BYB protein.** The recombinant BYB protein fused to GST (GST-BYB) or released from GST (rBYB) by proteolysis was added together with FMBP-1 (0.05 U) to the EMSA mixture with the +290 probe. In the BYB lane, 0.4 ng of p36 protein was added.

(Fig. 3A), and contained a poly(A) additional signal and adenine stretch at the 3′ end. All the amino acid sequences determined in advance were discovered in the ORF. The deduced protein was composed of three domains; an N-terminal region comprising amino acid positions 1 to 30, a CSD comprising positions 31 to 120, and a hydrophilic C-terminal region comprising positions 121 to 259. The CSD is 83% and 81% identical to the domain of *Drosophila* YB protein Yps and human YB-1, respectively, and 41% identical to a cold shock protein of bacteria, CspA (Fig. 3B). Because of the homology of the CSD and the similarity of the whole structure to other members of the Y-box protein family, the p36 protein was designated as *Bombyx* Y-box protein (BYB).

The Majority of BYB in Silk Glands Is 36 kDa—The molecular weight of BYB deduced from its cDNA was about 28 kDa. We prepared BYB-specific rabbit antiserum against the recombinant protein that lacked the N-terminal 119 amino acids (see Fig. 9A), and used it for Western blotting experiments. As expected, the antiserum specifically detected a 36 kDa protein in the DNA-cellulose fractions and crude PSG extracts as well as purified p36 (Fig. 4). Along with the 36 kDa band, a band around 28 kDa was detected for the DNA-cellulose fractions and PSG extracts, but the majority of the BYB was of the 36 kDa form in the PSG.

Using a probe prepared from the cDNA clone, Northern hybridization was performed with total RNA of PSG. Through the fourth intermolt, molt and fifth intermolt stages, a material of about 1.5 kb long was detected, and no obvious alterations in size and amount were observed (data not shown). Furthermore, we isolated 7 BYB clones from a cDNA library, and performed physical mapping using restriction enzymes and nucleotide sequencing of the cDNA ends. The results of these experiments showed that the clones originating from a unique mRNA described in this paper were independent (not shown).

Stimulation of the DNA Binding of FMBP-1 by Recombinant BYB Protein—To determine whether BYB encoded by the cDNA can stimulate the DNA binding of FMBP-1 the same as p36, recombinant BYB protein was produced in *E. coli*, purified and added to the EMSA reactions. The recombinant proteins both fused to GST and after separation from GST by proteolysis enhanced the binding of FMBP-1 to the +290 probe, but GST alone did not have any effect on the DNA binding (Fig. 5). Thus, cloned BYB showed the same ability to stimulate the DNA binding of FMBP-1 as purified p36.

Effects of Single-Stranded Oligonucleotides and Poly (dI-dC) on BYB—Some members of the Y-box protein family bind single-stranded and/or abasic DNAs (*8*, *9*) It is impossible to avoid contamination of a DNA by single-stranded oligonucleotides annealed to the probe in EMSAs, and the reaction mixtures usually contain a carrier DNA such as poly(dI-dC). If these DNAs had inhibitory effects on the specific binding of FMBP-1, BYB might titrate out the inhibitory effects and stimulate the FMBP-1 binding indirectly.

First we examined whether FMBP-1 binds single-stranded oligonucleotides for the +290 probe using kinase labeled at either the top or bottom strand as a probe. FMBP-1 bound neither strand of the +290 probe, while BYB preferentially bound the lower strand (Fig. 6A). Next we examined whether single-stranded oligonucleotides disturb the FMBP-1 binding to the double-stranded probe. When a 10-fold amount of a single-stranded oligonucleotide was added to the double-stranded probe, the FMBP-1 binding was not affected (Fig. 6B). Only when an extraordinary large amount (100-fold) of the single-stranded oligonucleotide was added, was the binding of FMBP-1 inhibited (~50%).

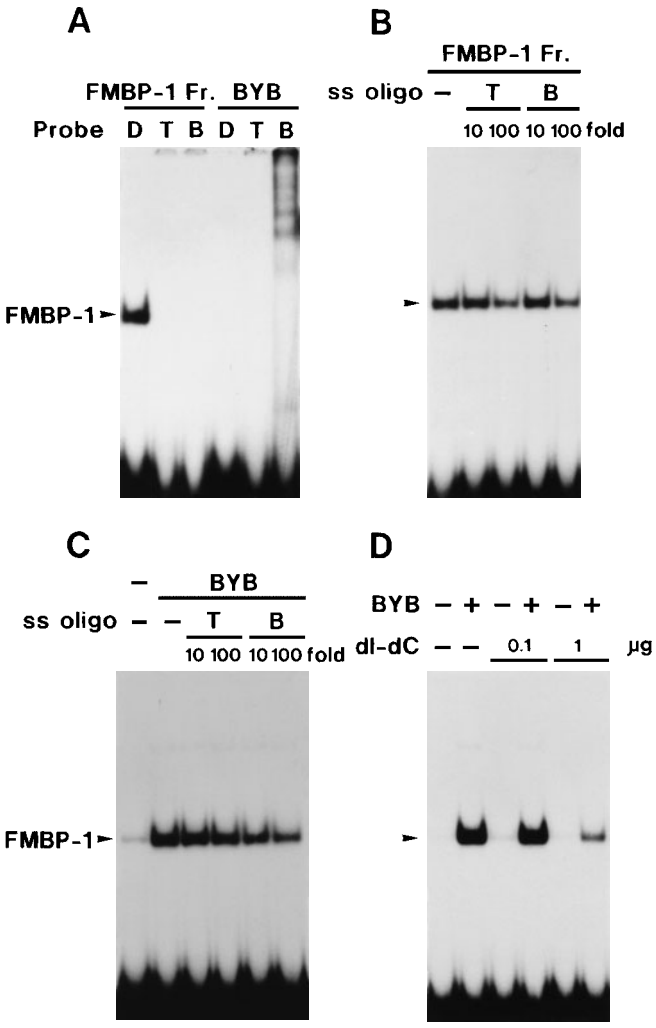

Fig. 6. **Inhibition of the stimulatory effects of BYB by single-stranded oligonucleotides and poly(dI-dC).** EMSAs were performed with double-stranded or single-stranded oligonucleotide probes in the presence or absence of various competitors. (A) The top strand, T, or bottom strand, B, of the +290 probe (0.5 ng), or the double-stranded, D, probe (0.5 ng) was incubated with 0.5 U of FMBP-1 (FMBP-1 Fr.) or 1 ng of p36 protein (BYB). (B) The +290 probe and FMBP-1 (0.5 U) were incubated with 5 ng (10-fold) or 50 ng (100-fold) of the unlabeled top or bottom strand of the +290 probe. (C) The +290 probe was incubated with FMBP-1 (0.05 U), and then enhancement of the FMBP-1 binding by p36 protein (BYB) was challenged using 5 or 50 ng of the top or bottom strand of the +290 probe. (D) FMBP-1 (0.05 U) was incubated with the +290 probe and p36 protein (+) with decreasing concentrations (1, 0.1 and 0 μg) of the poly(dI-dC) carrier.

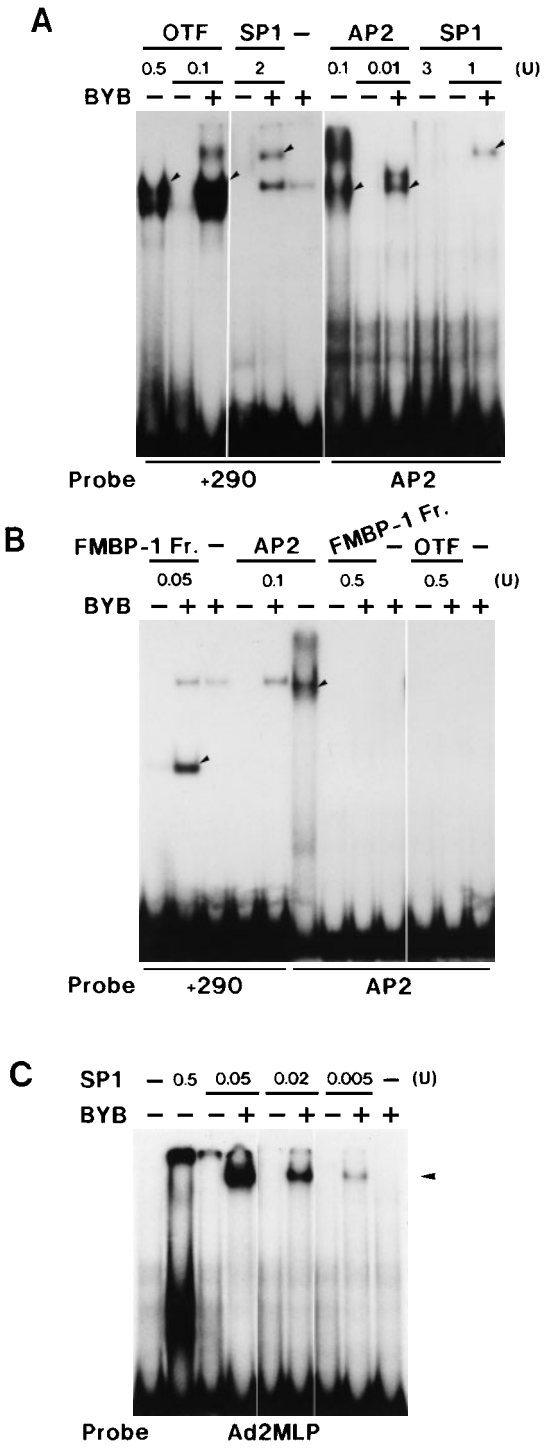

Fig. 7. **Stimulation of the specific DNA binding of various transcription regulation factors by BYB in EMSA.** (A) OTF2A, SP1 and AP2 were incubated with the +290 or AP2/SP1 probe containing their binding sequences with (+) or without (–) p36 protein (1 ng). (B) FMBP-1, AP2 and OTF2A were incubated with the +290 or AP2/SP1 probe with (+) or without (–) p36 protein. (C) Decreasing amounts of SP1 were incubated with the Ad2MLP probe. The same amount of p36 protein (1 ng) was added in the lanes denoted by (+).

The stimulatory effect of BYB itself was inhibited by the single-stranded oligonucleotides (Fig. 6C). When the top or bottom strand was added to the EMSA reaction mixtures to detect enhancement of the DNA binding of FMBP-1, the enhancement by BYB was disturbed, especially with the bottom strand. Similar effects were observed when poly(dI-dC) was used in the EMSA (Fig. 6D). Here, 1 μg of poly(dI-dC) was generally used as a carrier even when the purified protein was used. However, decreasing the amount of poly(dI-dC) to 100 ng or zero increased the stimulatory effects of BYB. Accordingly, the inhibitory effects of the single-stranded oligonucleotides or carrier DNA were not titrated out by BYB, but BYB was rather titrated out by the bottom strand and poly(dI-dC).

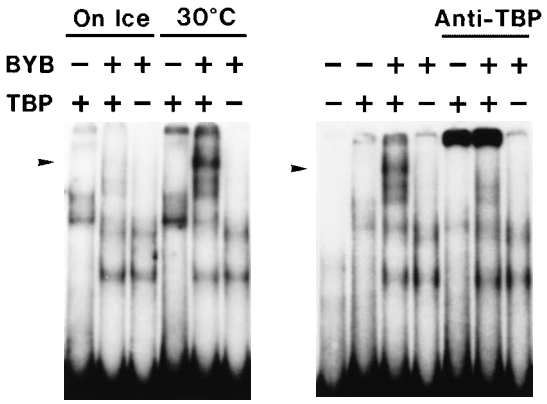

Fig. 8. **Stimulation of the DNA binding of TBP by BYB.** The effects of BYB on the DNA binding of TBP were analyzed by EMSA in the presence of poly(dG-dC) (100 ng) as a carrier instead of poly(dI-dC). Left panel: TBP (0.005U) was incubated with the Ad2MLP probe either on ice or at 30°C with (+) or without (–) p36 protein (1 ng). Right panel: After incubation of TBP with the probe in the presence or absence of p36 protein at 30°C, an anti-TBP antibody was added to the EMSA reaction mixtures. The arrowheads indicate the TBP-probe complex.

Stimulation of Specific DNA Binding of Mammalian Transcription Regulation Factors by BYB—The specificity of the stimulation of DNA binding by BYB was investigated using mammalian transcription factors OTF2, SP1 and AP2. There is an octamer-like sequence, ATGTAAAT, for OTF2 binding and a GC box-like sequence, GGGCAG, for SP1 binding in the +290 probe, while an AP2 binding site, GCCGCGGGC, and a GC box, GGGCGG, overlap in the AP2/SP1 probe (see "MATERIALS AND METHODS"). When the +290 probe was used in the EMSA, binding of OTF2 and SP1 as well as FMBP-1 was enhanced by BYB (Fig. 7A, +290), but the binding of AP2 was not detected even with the addition of BYB (Fig. 7B, +290). On the other hand, the binding of AP2 and SP1 to the AP2/SP1 probe was enhanced by BYB (Fig. 7A, AP2), but the binding of FMBP-1 and OTF2 to the same probe was not detected regardless of the presence or absence of BYB (Fig. 7B, AP2). We further examined the binding of SP1 using another probe, Ad2MLP. When BYB was added to the EMSA reaction mixtures, the binding of SP1 was enhanced and complexes correlating to the amount of SP1 were observed (Fig. 7C). Therefore, the stimulatory effects of BYB were not factor-specific, but BYB enhanced the specific binding of various transcription regulation factors to their cognate elements.

Stimulation of Transcription by BYB in a Reconstituted System—We further analyzed the effects of BYB on another type of transcription factor, TBP. TBP is a general factor necessary for transcription by all three RNA polymerases in eukaryotes. Recombinant human TBP and the Ad2MLP probe were incubated on ice or at 30°C for 30 min in the presence of poly(dG-dC) carrier, and then complex formation was analyzed by EMSA (Fig. 8). When TBP alone was incubated with the Ad2MLP probe, two bands were observed, but these bands were detectable on incubation even on ice in the presence of poly(dI-dC) (data not shown). On the addition of BYB to the EMSA reaction mixture, a new complex appeared at 30°C. When an anti-TBP antibody was added, this complex was hyper-shifted to the top of the gel. Though the complex reacting with the antibody was detected on incubation of TBP alone with the probe, the amount was less than for the incubation of TBP with BYB (see the gel top). Accordingly, BYB enhanced the binding of the basal transcription factor TBP to the Ad2MLP probe the same as other transcription regulation factors.

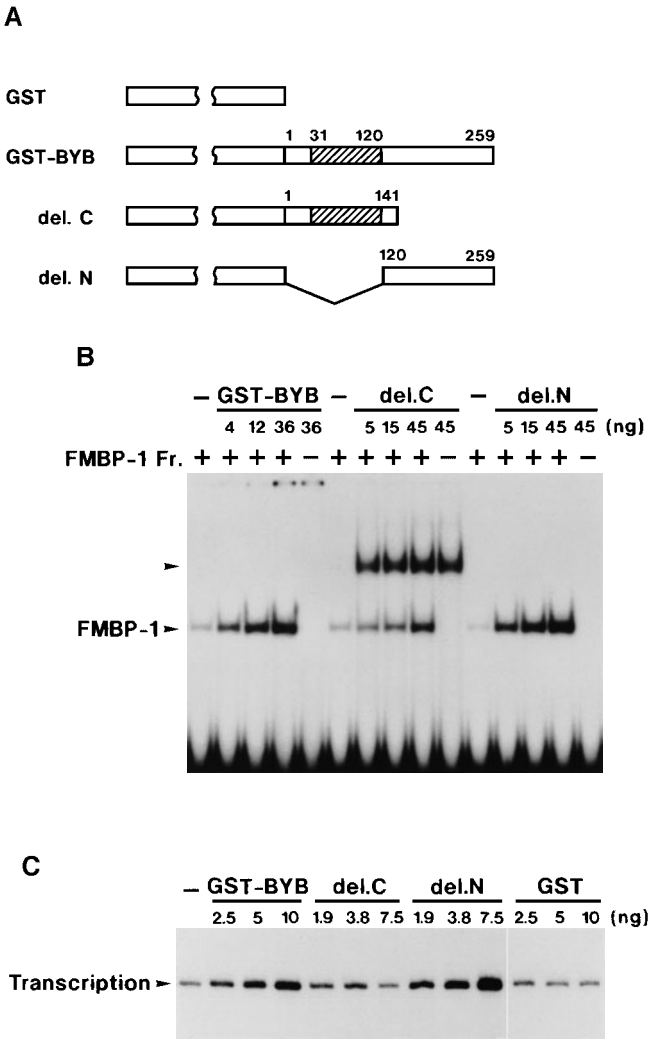

Fig. 9. **Transcriptional stimulation by the C-terminal region of BYB.** (A) Schema of the recombinant proteins. GST and GST-BYB fusion proteins are denoted by rectangles and the regions of the BYB protein are indicated by amino acid positions. The shaded box is the CSD. (B) Effects of the deletion proteins of BYB on the DNA binding of FMBP-1. GST fusion proteins of BYB (GST-BYB), GST-BYBdel.C (del.C) and GST-BYBdel.N (del.N), were purified by affinity chromatography and then added to the EMSA reaction mixtures. FMBP-1 (0.05 U) was incubated with the +290 probe with (+) or without (–) each fusion protein. The upper arrowhead indicates the complex of the N-terminal half of BYB and the bottom strand of the +290 probe. (C) Effects of BYB on transcription from the Ad2MLP. A BYB fusion protein or GST as a control was added to the reconstituted transcription system, and then transcripts were separated on a polyacrylamide gel. The relative activities in each reaction were as follows: No BYB (–) 78; GST-BYB (2.5 ng) 138, (5 ng) 197, (10 ng) 290; del.C (1.9 ng) 115, (3.8 ng) 136, (7.5 ng) 79; del.N (1.9 ng) 177, (3.8 ng) 270, (7.5 ng) 512; GST (2.5 ng) 74, (5 ng) 65, (10 ng) 63.

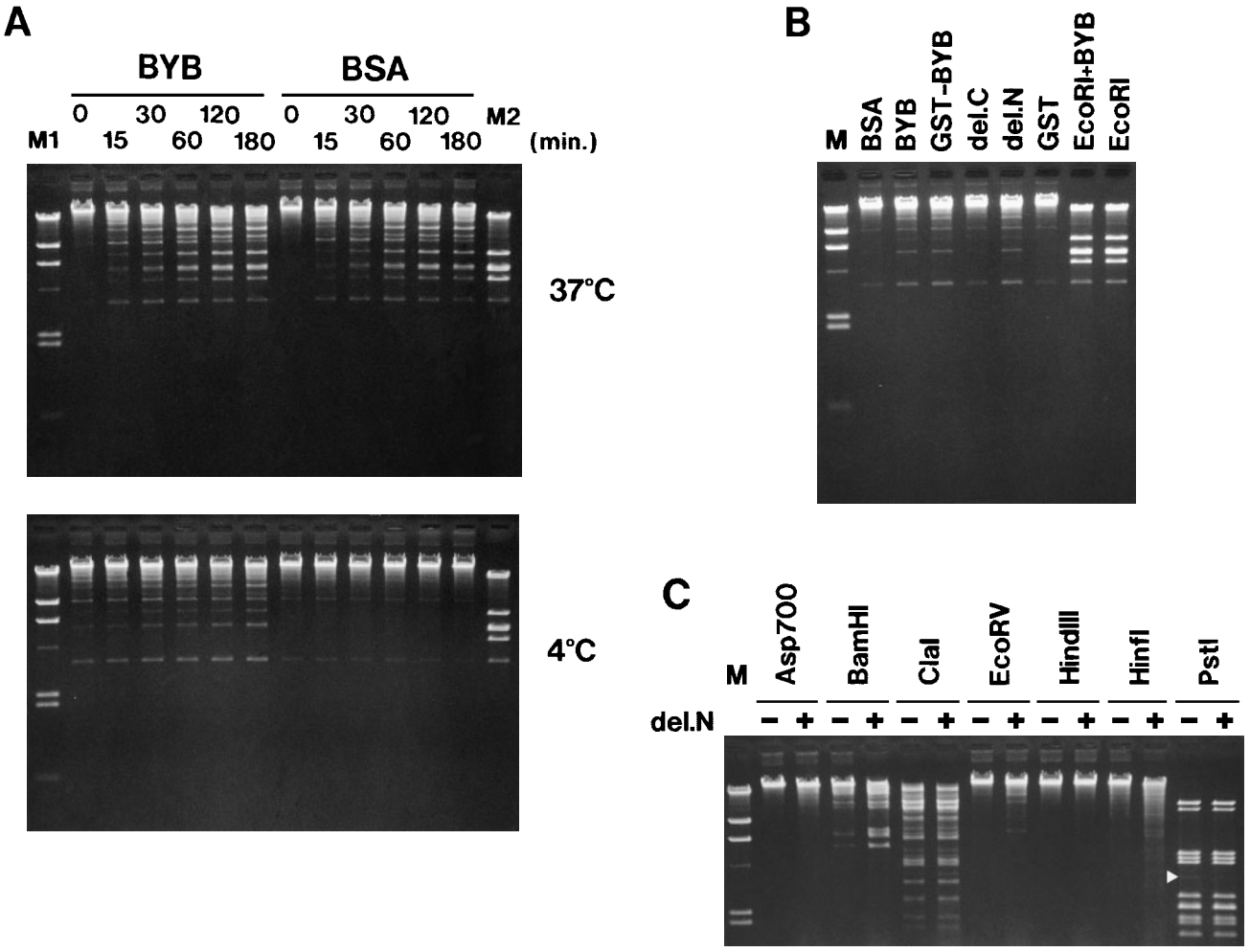

Fig. 10. **Stimulation of restriction endonuclease reactions by BYB.** (A) λ DNA was restricted by *Eco*RI at 37°C or 4°C with (BYB) or without (BSA) p36 protein and then analyzed by electrophoresis on 1% agarose gels. Each reaction was performed for the times indicated at the top of the panels. M1, complete digest of λ DNA obtained with *Hin*dIII: M2, complete digest of λ DNA obtained with *Eco*RI. (B) λ DNA was incubated with 0.3 U of *Eco*RI together with BSA, p36 (BYB, 2 ng), recombinant protein GST-BYB (4 ng), GST-BYBdel.C (3 ng), GST-BYBdel.N (3 ng), or GST (2ng) at 4°C for 3 h. The reactions denoted by *Eco*RI+BYB and *Eco*RI were performed at 37°C. (C) λ DNA was incubated with Asp700 (0.33 U), *Bam*HI (0.5 U), *Cla*I (0.33 U), E*co*RV (0.25 U), *Hin*dIII (0.33 U), *Hin*fI (0.33 U), or PstI (0.5 U) at 4°C for 3h with (+) or without (–) 100 ng of GST-BYBdel.N.

We roughly divided the BYB protein into two regions, an N-terminal half containing the CSD and a C-terminal half, produced as fusion proteins with GST (Fig. 9A), and examined which region stimulated the DNA binding of FMBP-1 (Fig. 9B). The C-terminal half of BYB, which lacked a CSD, stimulated the DNA binding of FMBP-1 the same as the whole BYB, while the N-terminal half containing the CSD stimulated the binding weakly. The C-terminal half stimulated the binding of FMBP-1 about 10 times more than the N-terminal half. Thus, the CSD conserved in various organisms is dispensable for the enhancement of the specific binding of FMBP-1 to DNA, though the N-terminal half of BYB bound the bottom strand of the +290 probe strongly (Fig. 9, and Nishita and Takiya, unpublished).

Using these deletion proteins, effects of BYB on transcription were examined in a reconstituted transcription system (Fig. 9C). The BYB fused to GST or GST alone was added to a transcription reaction system reconstituted with TFIIB, TBP, TFIIF and RNA polymerase II, and then the efficiencies of transcription from the Ad2MLP were compared. The whole protein fused to GST stimulated the transcription about 3-fold, but GST itself did not. When the deletion proteins of BYB were added, stimulation (about 6-fold) of the transcription was observed only with the C-terminal half that lacked a CSD completely, corresponding to the ability to stimulate the DNA binding.

The C-Terminal Half of BYB Interacts with DNA Independently of the CSD—The CSD of the Y box protein family is necessary for sequence-specific DNA binding, but the C-terminal region is also reported to interact with DNA and RNA (*33–35*). Here, except for the bottom strand of the +290 probe, we did not detect binding of BYB to the probes in the EMSA. However, the C-terminal region clearly stimulated the DNA binding of various transcription factors and enhanced transcription from the Ad2MLP template. We investigated whether the tail domain of BYB can interact with DNA using the GST-pull down method. The tail domain of BYB was bound to

Table 1. **Interaction of the C-terminal region of BYB with the probe DNA**.

Bound proteins	GST-BYBdel.N			GST					
Factors	SP1	AP2	BSA	SP1	AP2	BSA	SP1	AP2	BSA
Fractions	Radioactivity (cpm)						Ratio (GST-BYBdel.N/GST)		
Input	141,973	131,200	135,027	132,400	136,627	130,477	1.07	0.96	1.03
Bound	3,228	2,796	2,973	1,417	1,760	1,354	2.28	1.59	2.20
Eluate	2,355	2,172	2,294	218	374	326	10.80	5.81	7.04
Sepharose	664	485	552	1,165	1,341	1,005	0.57	0.36	0.55

The radioactivity at each step was monitored by Cherenkov counting., and the values shown are the averages of three independent experiments.

glutathione-Sepharose beads through the fused GST first, and then the AP2/SP1 probe was mixed with the beads with or without transcription factor SP1 or AP2. After washing of the beads, the bound probe was eluted with GST fusion protein using glutathione, and then the radioactivities of bound and eluted probes were compared (Table 1).

About two times the amount of probe bound the Sepharose beads via the tail domain in comparison with the control beads. Moreover, the probe bound to the tail domain beads could be eluted with glutathione, but the probe bound to the GST beads was mostly not eluted, indicating nonspecific binding of the probe to the GST beads. Accordingly, 6 to 11 times more probe bound specifically to the BYB beads than the control beads. The results were almost the same irrespective of the presence or absence of the probe-specific transcription factors SP1 and AP2. These results demonstrated that the tail domain of BYB interacted directly with the DNA.

BYB Stimulates Reactions by Restriction Endonucleases—To determine whether BYB affects the DNA binding of proteins other than transcription factors, we chose restriction endonucleases. λ DNA was incubated with an insufficient amount of restriction endonuclease *Eco*RI, and then the time course of the reaction in the presence of BYB or BSA was compared. The difference between BYB and BSA was very slight upon incubation at 37°C, but clear at 4°C (Fig. 10A). In the absence of BYB, *Eco*RI scarcely digested the DNA at 4°C, but the restriction reaction proceeded gradually in the presence of BYB. The C-terminal region was sufficient for this stimulatory effect on the restriction reaction, and incubation of λ DNA with BYB did not affect the restriction pattern obtained with *Eco*RI (Fig. 10B). Next we examined the effects of BYB on other restriction enzymes. As shown in Fig. 10C, *Pst*I and *Cla*I restricted DNA efficiently even at 4°C, and *Pst*I digested the DNA almost completely. Detection of the stimulation effect of BYB on these enzymes was difficult, but a partially digested fragment (indicated by a triangle) disappeared upon the addition of BYB. *Hin*dIII did not restrict the DNA at all under the conditions used here regardless of the presence or absence of BYB, however, other enzymes restricted DNA partially and were stimulated by BYB (Fig. 10C).

DISCUSSION

p36 is a Bombyx Y-Box Protein—We have isolated a cDNA corresponding to the 36 kDa protein (p36) purified from PSGs of the silkworm *Bombyx mori*, and deduced its amino acid sequence. In structure, p36 is similar to the Y-box protein family in animals. It has a short N-terminal region, a highly conserved CSD and a C-terminal region containing basic/aromatic amino acid stretches. Accordingly we designated p36 as *Bombyx* Y-box protein, BYB.

Using probes designed from the amino acid sequence of p36 we isolated the cDNA for BYB, but the molecular weight of BYB calculated from the ORF is about 28 kDa. Only a 1.5 kb long RNA corresponding to the cDNA was detectable on Northern blotting, and all cDNA clones obtained in this study are expected to have originated from the same RNA. There is no evidence of alternative splicing or the existence of family proteins of BYB in the PSG of the silkworm. Western blotting with an antibody raised against the recombinant BYB showed that a 28 kDa protein reacting with the antibody exists in PSGs, but the majority of BYB is 36 kDa. These results suggest that most BYB is modified in PSGs. We can not determine the type of modification at present, but it may be worth mentioning that the difference in molecular weight between these proteins is similar to the molecular weight of ubiquitin. It is reported that protein ligation with ubiquitin or ubiquitin-like proteins is involved in the regulation of transcription and various physiological responses (*36*), though the 28 kDa recombinant BYB was sufficient for the stimulatory effects observed in this study.

BYB was eluted in the same fractions as FMBP-1 from the DNA-cellulose column following phosphocellulose column chromatography. Further fractionation of FMBP-1 by DEAE-cellulose or FMBP-1 specific DNA affinity column chromatography separated BYB from FMBP-1 (not shown). The mobility of the complex of FMBP-1 with the +290 probe in the EMSA was not affected by the addition of BYB. BYB would dissociate easily under the EMSA conditions, or BYB might dissociate from the complex after facilitating its formation. However, we can not exclude the possibility that BYB associates with the FMBP-1 complex on the specific binding elements *in vivo* and thereby plays a role in the regulation of the fibroin gene transcription directly. There is a Pro- and Gln-rich stretch in the N-terminal region and a Gln-rich stretch in the C-terminal region of BYB. A Pro- or Gln-rich region is often observed in the activation domains of transcription regulation factors, such as SP1 and AP2 (*37*,*38*). The Pro- and Gln-rich regions of BYB might have the potential to participate in the transcriptional activation of silk protein genes or generally.

Neither the +290, AP2/SP1 nor Ad2MLP probe used in this study contained a Y-box-like CAAT sequence, and BYB did not bind these double-stranded probes. Only to the bottom strand of the +290 probe, did BYB bind at a detectable level in the EMSA. Y-box proteins are able to

bind CT-rich strands of DNA (*9*, *15*, *16*), and the bottom strand of the +290 probe is CT-rich. Thus, BYB showed similar selectivity for DNA binding as other members of the Y-box family. The selective DNA binding activity of BYB was borne by the N-terminal half, mostly the CSD, and seemed to be usually suppressed by the C-terminal half (Fig. 9B and Nishita and Takiya, unpublished). The physiological meaning of this regulation of the DNA binding activity of the CSD by the tail domain is unknown.

Though the C-terminal half of Y-box proteins is relatively divergent at the amino acid level, the structure is similar, especially in vertebrates. Alternative acidic and basic amino acid stretches are repeated (*3*, *6*). Such an alternative structure is rare in invertebrate Y-box proteins, but basic stretches and/or RGG repeats are commonly observed (*2*). In the C-terminal region of BYB there are five RGGs, and three basic/aromatic stretches. These structures are reported to be responsible for nonspecific interaction of Y-box proteins with various nucleic acids (*33–35*). Consistent with previous observations, the C-terminal region of BYB had the ability to interact with the probe DNA.

Stimulation of Specific DNA Binding, Transcription and Restriction Reaction by BYB—We found that BYB stimulates and/or stabilizes the specific interaction of various DNA binding proteins with their targets. The binding of BYB to the DNA detectable on EMSA and also the Y-box sequence or CT-rich sequence in the DNA were not required for these stimulatory effects. Correlating with the dispensability of specific Y-box protein binding sequences in the DNA, the stimulation was attained with the C-terminal half of BYB lacking a CSD completely. The C-terminal half of BYB was sufficient to enhance not only the DNA binding of transcripiton regulation factors, but also transcription and restriction reactions. The transcription system used here was reconstituted with the basal factors TFIIB, TBP and TFIIF, and RNA polymerase II. Another basal factor, TFIIA, could replace BYB for stimulating the transcription in the reconstituted system (not shown). TFIIA is reported to interact with TBP, and to facilitate the binding of TBP and TFIID to the TATA box (*39*, *40*). Therefore, BYB seems to enhance the transcription through stimulation of the binding of TBP to the TATA box.

Surprisingly, BYB stimulated the reaction of some restriction endonucleases under cold conditions. In the presence of BYB, digestion of λ DNA by *Eco*RI proceeded even at 4°C, though the efficiency was very low compared with the reaction at the optimal temperature. We presumed that the stimulation of restriction reactions was achieved through stimulation of the active interaction of endonucleases with their target sequences. The active interaction of restriction endonucleases with the restriction sites is probably dependent on temperature, and BYB will facilitate the interaction even under the cold conditions.

Skabkin *et al*. (*41*) reported recently that a member of the Y-box protein family promotes both the melting and annealing of nucleic acid strands. We speculate that BYB causes a structural change in the DNA, and facilitates the active interaction of various proteins with their cognate elements. In this case, BYB and the Y-box proteins will act as a kind of nucleic acid chaperone and regulate various reactions involving protein-nucleic acid interaction. Supporting evidence was obtained in the EMSA with SP1 and AP2. When these transcription factors were incubated with specific probes, smearing and/or entrapment on the gel top were observed under the conditions used, but the addition of BYB solved these problems and the complexes gave clear bands (Fig. 7).

The nucleotide sequence data reported are available in the DDBJ/EMBL/GeneBank databases under accession number AB098537. We wish to thank Dr. Kayukawa for the assistance in the transcription experiments. We also thank Drs. Kondo and Yajima of the Central Laboratories for Key Technology, Kirin Brewery Co. for the valuable discussions. We also thank Drs. Bando and Sahara of the Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Hokkaido Univ. for the advice and for supplying the silkworms. We would also like to thank Mr. Saito and Mr. Yamada of the Field Science Center for Northern Biosphere, Hokkaido Univ. for rearing the silkworms. We also thank Ms. Makino of the Center for Analytical Instruments of NIBB for the amino acid sequencing. This research was supported partly by a Grant-in-Aid for Science Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology of Japan, and was carried out partly under the NIBB Cooperative Research Program.

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