Interaction of Composite Protein Complex with the Fibroin Enhancer Sequence*

Toshiharu Suzuki and Yoshiaki Suzuki

From the Department of Developmental Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

To characterize proteins that bind to the upstream region of the fibroin gene, we used an electrophoretic mobility assay and a DNase I footprinting assay. A crude nuclear extract from the posterior silk glands forms a sequence-specific complex with the upstream region, −234 to −66, which includes a signal for tissue-specific transcriptional enhancement. This specific complex is composed of at least two factors of proteinous nature and the DNA. The composite proteins specifically protect an −13-base pair (−167 to −155) region in a DNase I footprinting assay. This region contains an interesting repeated sequence, AATTTAATTT. Crude nuclear extracts from the middle silk glands and the ovarian tissues, but not a HeLa whole cell extract, also give the band complexes of similar electrophoretic mobility to the one formed in the posterior silk gland extract. However, under a higher EDTA condition, these complexes are more unstable than the one constructed in the posterior silk gland extract. These results suggest a possibility that these proteinous molecules in the complex, although apparently ubiquitous in Bombyx cells, might be specifically modified in the posterior silk gland cells to play an important role in the specific expression of the fibroin gene.

Studies of regulatory mechanisms of eukaryotic type II gene expression are progressing at the molecular level. Besides RNA polymerase II, proteins factors required for faithful transcription have been identified, and molecular roles of these factors are being analyzed in detail (1–3). However, the mechanism of specific gene expression is only poorly understood. It is important to characterize the molecular event in tissue- and/or stage-specific gene expression and to elucidate molecular mechanisms of transcriptional enhancement of class II genes. Upstream sequences of the class II gene play, in general, an important role for the enhancement of expression. For example, a large number of genes are known to have an enhancer sequence, and some of them also have other regulatory sequences of its 5′-upstream region (4–8).

The fibroin gene of the silkworm (Bombyx mori) is one of the first characterized and analyzed for its upstream sequence (9, 10). The gene is expressed specifically in the posterior silk gland, and a large amount of transcripts from this gene are preferentially detected at the larval feeding stages (11). Pre-}

viously, from posterior silk gland cells, we developed a cell-free extract that can transcribe the fibroin gene faithfully and preferentially (12). Functional analyses of the upstream region of the fibroin gene in this homologous in vitro system showed that the 5′-flanking sequence upstream from the core promoter region enhances the transcription of the fibroin gene (12). In addition, it is shown that the upstream region is composed of at least two elements for transcriptional enhancement. One is the distal region, −238 to −73, which contributes to tissue-specific enhancement, and the other is the proximal region, −72 to −32, which enhances transcription by factor(s) present in a species-specific and constitutive manner (13). In spite of accumulating knowledge on interesting functions of the fibroin gene upstream sequence, factors responding to these regions are poorly understood. In the immunoglobulin gene, nuclear factors that bind to the transcriptional control element have been identified (14–16). NF-κB, which is one of these binding proteins, can be induced in pre-B lymphoid cells and binds to a defined site in the κ-immunoglobulin gene upstream element. Appearance of this protein is correlated with immunoglobulin gene expression. However, induction of NF-κB is not restricted to B lymphoid cells (17). In phorbol diester-inducible genes, nuclear factors that bind to the enhancer elements have also been identified (18). These factors can also be activated in the cells after the stimulus with phorbol diester (19). Judging from the function of the upstream region of the fibroin gene, we should also assume a participation of proteinous factors binding to the upstream region for the expression of this gene (20). We already know that natural fibroin genes purified from fibroin producer and nonproducer cells were identical in their primary sequences, and methylation modifications were not detected in the upstream regions (21, 22).

Accordingly, we suppose that differential transcription of the fibroin gene in vivo may depend on cellular factors which function through binding to the upstream elements.

In this report, we describe the identification and characterization of proteins that bind to the upstream DNA element of the fibroin gene. This report shows for the first time that composition of at least two proteins is essential to obtain binding capacity to the upstream DNA element and/or to form a stable protein-DNA complex, and this composition specifically binds to an −13-bp sequence containing a repeated sequence of AATTTAATTT. These proteins are probably associated with one of the candidates of regulatory factors on fibroin gene expression.

EXPERIMENTAL PROCEDURES

Materials—Commercial silkworm strains (Kin-Shu × Sho-Wa or Shun-Rei × Sho-Getsu from the Kanebo Silk Co., Kasugai City, Japan); homologous in vitro system for fibroin gene expression (12, 13). The abbreviations used are: bp, base pair; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; NEM, N-ethylmaleimide.
Japan) of *B. mori* were reared at 27 °C on an artificial diet from Kyodo Shiryo Co. (Yokohama, Japan). DNase I and trypsin were purchased from Boehringer Mannheim, and soybean trypsin inhibitor was from Sigma. *Escherichia coli* alkaline phosphatase, T4 polynucleotide kinase, and restriction endonucleases except for *XmnI* were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). The large fragment of *E. coli* DNA polymerase I (Klenow enzyme) and *XmnI* were from New England Biolabs. Poly(dI-dC)-poly(dI-dC) was from Pharmacia LKB Biotechnology Inc. Phosphocellulose (P-11) was from Whatman. [α-32P]dATP (~3000 Ci/mmol) and [γ-32P]ATP (>500,000 Ci/mmol) were purchased from Amersham Corp.

**Phasmid DNA**—pFb5'Upl was constructed by the insertion of an EcoRI-TaqI segment (−234 to −66) of pFbΔ-238R DNA (23) to the HindIII site of pBR322 through the HindIII linkers. pFbΔ-238R DNA was constructed by the insertion of an EcoRI*-*TaqI DNA fragment (−234 to −66) into the HindIII site of pFb5*Δ*-52 through the HindIII linker (23). pFb5*Δ*-238 DNA contained the DNA segment (−238 to +585) of the fibroin gene and the larger EcoRI-BamHI DNA fragment of pBR322 (23), and pFb5*Δ*-31 DNA contained the DNA segment (−31 to +585) of the fibroin gene and the larger HindIII-BamHI DNA fragment of pBR322 (28).

**Extracts**—Nuclear extracts from posterior and middle silk glands were prepared according to the method described previously (12) except for an additional 2500-rpm centrifugation for 10 min before the Dounce homogenization step. With this additional operation, cytosol proteins like fibroin or sercin were removed, and the crude nuclear fraction was recovered. An ovarian tissue extract was also prepared by the same method as previously described (13). A whole cell extract from HeLa cells was prepared according to the method of Manley et al. (24). Protein concentration was determined using the assay of Lowry et al. (25) with bovine serum albumin standards.

**Fractionation of Nuclear Extract from Posterior Silk Glands**—A nuclear extract was fractionated as described previously by Roeder and co-workers (26). Approximately 200 mg of nuclear extract (15 ml) from about 500 larvae were loaded onto a 62-ml bed volume column (2 × 20 cm) of P-11 equilibrated with a buffer containing 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 2 mM dithiothreitol, 17% (v/v) glycerol containing 0.1 M KCl. The flow-through fraction was collected and referred to as fraction A. Adsorbed proteins were stepwise eluted with the same buffer containing 0.3 M KCl (fraction B), 0.5 M KCl (fraction C), and 1 M KCl (fraction D). Typically, the recovery of protein was 60–65% in fraction A, 20% in B, 5–10% in C, and <5% in D. Fractions were dialyzed against the same buffer containing 0.1 M KCl and 12.5 mM MgCl2 for 12 h and stored at −80 °C.

**Probes for Electrophoretic Mobility Assay and Footprinting**—An upstream element, −234 to −66, of the fibroin gene was prepared by digesting pFb5'*Up1* DNA with HindIII and purifying the 182-bp fragment with linker portions by 5% polyacrylamide gel electrophoresis. The linker portions of the DNA fragment were labeled with Klenow enzyme and [α-32P]dATP according to a standard method (27). Specific radioactivity was 0.8–4.0 × 10^6 cpm/µg.

Another upstream element, −238 to −38, was prepared as follows. pFb5*Δ*-238 DNA (23) was linearized by digestion with EcoRI. The coding strand of the resulting linear DNA was 5' end-labeled with T4 polynucleotide kinase and [γ-32P]ATP, or the noncoding strand was also 3' end-labeled with Klenow enzyme and [α-32P]dATP. End-labeled DNA was then digested with *XmnI*, and the resulting 201-bp fragment was purified by 5% polyacrylamide gel electrophoresis. Specific radioactivity of the probe was 1–5 × 10^6 cpm/µg.

Probes were preserved in a solution containing 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM EDTA at −20 °C. Probes were used entirely within 7 days for DNA binding assays and within 3 days for footprinting assays.

**Competitor DNA Fragments**—Competitor DNA fragments for electrophoretic mobility assay were prepared as follows. Competitor 1, a fragment from −238 to +187, was prepared by digesting pFb5* Δ*-238 DNA with EcoRI and *SmaI*, and the resulting 426-bp fragment was purified by 5% polyacrylamide gel electrophoresis. Competitor 2, from −31 to +187, was prepared by digesting pFb5*Δ*-31 DNA (28) with HindIII and *SmaI*, and the resulting 219-bp fragment was purified as described above. Competitors 3–5 were the products from labeled *Sau3A1* digestion of an upstream element, −234 to −66 (competitor 6), as shown in Fig. 1a. These purified DNA fragments were further purified by passing over Elutip-d columns (Schleicher & Schuell) and solubilized in a solution of 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM EDTA, and then concentrations were measured by absorbance at 260 nm.

**FIG. 1. Factors binding to upstream region of fibroin gene**. a, schematic representation is shown of probes 1 and 2 and competitor DNAs 1–6. * Sau3A1* sites as described in the text. b, electrophoretic mobility assays were performed with probes 1 (lanes 1–8) and 2 (lanes 9–16). 32P-Labeled probes 1 (~0.3 ng, 10,000 cpm) and 2 (~0.1 ng, 8,000 cpm) were incubated with a nuclear extract (5.8 µg) of the posterior silk gland cells or without extract (lanes 1 and 9) in the absence (lanes 2 and 10) or presence (lanes 3–8 and 11–16) of two different competitor DNAs. Binding reactions (10 µl) containing 15 mM EDTA are described in "Experimental Procedures." Competitor 1 DNA was added to lanes 3–5 at 3.3, 13-, and 33-fold excess and to lanes 11–13 at 2-, 10-, and 20-fold molar excess to input probes 1 and 2, respectively. A faint residual band in lane 13 was completely knocked out when a higher concentration of competitor 1 DNA was used (data not shown). Competitor 2 DNA was added to lanes 6–8 at 7-, 27-, and 67-fold and to lanes 14–16 at 20-, 80-, and 200-fold molar excess to input probes, respectively. c, electrophoretic mobility assays with probe 2 (~0.1 ng, 10,000 cpm) were performed with a nuclear extract (6.6 µg) of posterior silk gland cells (lanes 2–7) or without extract (lane 1). Binding reactions (10 µl) containing 5 mM EDTA were incubated with competitor DNAs 3–6 of 100-fold molar excess to input probe 2 (lanes 3–7) or without competitor (lane 2) as described under "Experimental Procedures." Lane 3 contains competitor 3 (~234 to −206), lane 4 competitor 4 (~204 to −171), lane 5 competitor 5 (~170 to −60), lane 6 competitor 6 (~234 to −66), and lane 7 a 346-
Electrophoretic Mobility Assay—Protein-DNA complexes were resolved on polyacrylamide gels (29) with several modifications. Usually binding reactions were carried out in a 10-μl volume containing 12 mM HEPES (pH 7.9), 60 mM KCl, 7.5 mM MgCl₂, 1.2 mM dithiothreitol, 10% (v/v) glycerol, 2 μg of poly(dI-dC)-poly(dI-dC), 0.1-0.4 ng of radiolabeled DNA probe, the indicated concentration of EDTA, and a protein sample. Reaction mixtures were incubated for 30 min at 27 °C and layered immediately onto a 4% polyacrylamide gel (acylamidobisacrylamide weight ratio of 80:1). Slab gels (0.1 × 12 cm) were pre-electrophoresed for 2 h at 160 V in a buffer containing 50 mM Tris-HCl (pH 8.3), 900 mM glycine, and 2 mM EDTA. The samples were electrophoresed at 180 V (~25-30 mA) at room temperature until bromphenol blue had run onto the bottom of the gel. The gels were placed on Whatman No. 3MM paper, dried under suction, and autoradiographed at -80 °C with Kodak X-Omat or Du Pont-New England Nuclear Cronex x-ray films.

DNAase I Footprinting—DNAase I footprinting experiments were performed according to Singh et al. (15) with several modifications. Protein samples consisting of fractions A (23.6 μg) and B (6 μg) from P-11 column chromatography were incubated with 4.5 ng of DNA probe 1 (−238 to −38) in 2.5 mM EDTA in a 10-μl volume as described above. At 20 min, 1 μl of DNAase I solutions (4 μg/ml in the presence of or 1 μg/ml in the absence of nuclear protein) containing 5 mM CaCl₂ and 10 mM MgCl₂ was added to the mixtures. After 2 min, 0.75 μl of 500 mM EDTA (pH 8.0) was added to stop the DNAase I reaction (final concentration, 32 mM EDTA). Samples were loaded immediately onto polyacrylamide gel and electrophoresed as described above.

RESULTS

Specific Factors Bind to Upstream Element of Fibroin Gene—To detect factors that bind to the enhancer element (for the characterization of the element, see Ref. 30) of the fibroin gene, we have employed an electrophoretic mobility assay. Complexes representing specific protein-DNA interaction were characterized by competition of excess DNA. Two DNA fragments from −238 to −38 and −234 to −66 were used as probes (probes 1 and 2; see Fig. 1a) since the upstream element of the fibroin gene can be divided into two functional regions (13). One is the proximal region from −72 to −32 associated with the transcriptional enhancement of a constitutive and tissue-specific type, and the other is the distal region from −238 to −73 which is essential for transcriptional enhancement in a tissue-specific manner. As shown in Fig. 1b, two band complexes specific for upstream region are detected with both probes 1 and 2. One is a major product (arrow 1), and the other is a minor one (arrow 2). Binding products with a poor sequence specificity (shown with asterisks), which are not subject to competition by both competitors 1 and 2, are also detected. Of these complexes, our main effort has been concentrated on the major product. The major band complex (arrow 1) is competed specifically by a DNA fragment containing the upstream region. When competitor 1 DNA was added, the radioactivity of the major complex was completely knocked out at a competitor:probe molar ratio of ~30:1 (Fig. 1b, lanes 3-5 and 11-13, and see also Fig. 2), whereas competitor 2 was a very weak competitor even when present in 100-200-fold molar excess (Fig. 1b, lanes 6-8 and 14-16). The extent of competition by competitor 1 was quantitated and is shown in Fig. 2. The mobilities of the major complexes are approximately the same for both probes 1 and 2. These data therefore suggest that the factors constructing the major band complex recognize a region, −234 to −66, common to both probes. Since this region includes the tissue-specific enhancer sequence, in the following experiments, we have mainly used probe 2 to characterize the nature of the major band complex.

Use of a smaller competitor 6 (~234 to −66) instead of long competitor 1 (~234 to +187) revealed a complete competition

FIG. 2. Competition of major band complexes formed in nuclear extracts from Bombyx tissues. Electrophoretic mobility assays with probe 2 (~0.1 ng, 20,000 cpm) were performed with competitor 1 DNA. The binding reaction (10 μl) containing 1 mM EDTA and the nuclear extract of posterior silk gland (●, 5.8 μg), middle silk gland (●, 6 μg), or ovarian tissue (●, 1.7 μg) was incubated, and electrophoresis was carried out as described under "Experimental Procedures." The major band complexes were cut out from gels, and their radioactivities were measured in a liquid scintillation counter. Radioactivity of the major band complex that contained no competitor was defined as 100%. Assays were done in duplicate.

FIG. 3. Effect of EDTA on band complexes formed in Bombyx tissue extracts. Electrophoretic mobility assays were performed with 1 mM EDTA (lanes 1-5) or 30 mM EDTA (lanes 6-10) under an assay condition including 7.5 mM MgCl₂. Probe 2 (~0.1 ng, 20,000 cpm) was incubated with nuclear extracts of the posterior silk gland (lanes 2 and 7, 5.8 μg), the middle silk gland (lanes 3 and 8, 6.0 μg), and the ovarian tissue (lanes 4 and 9, 1.7 μg), with a whole cell extract of HeLa cells (lanes 5 and 10, 2.8 μg) or without extract (lanes 1 and 6). Lane M is a size marker, 32P end-labeled pBR322 Hinfl digests. Arrows 1 and 2 indicate the band complexes specific for sequence, and the asterisk indicates product with poor sequence specificity.

bp HindIII-BamHI fragment from pBR322 DNA as competitors. b, and c, lane M is a size marker, pBR322 HindII digests end-labeled with 32P. Arrows 1 and 2 indicate the band complex specific for sequence, and single and double asterisks (in b) indicate products with poor sequence specificity.
of both the major and minor bands (Fig. 1c, lane 6). To delimit further the binding regions on the DNA probe for the band complex, we have also used the three smaller DNA fragments as competitors (Fig. 1c). A Sau3A1 digestion of competitor 6 (−234 to −66) results in producing three fragments covering −234 to −205 (competitor 3), −204 to −171 (competitor 4), and −170 to −66 (competitor 5). Only competitor 5, but not competitor 3 or 4, had the ability to compete both band complexes (Fig. 1c, lanes 3–5). The degree of competition of the major band complex by competitor 5 (−170 to −66) was lower than by competitor 6 (−234 to −66), but the minor band was completely knocked out by competitor 5 (Fig. 1c, compare lanes 5 and 6). An unrelated 346-bp DNA fragment from pBR322 had no effect as a competitor (Fig. 1c, lanes 7). These data also suggest that the factors constructing the major band complex recognize mainly −170 to −66 on the DNA sequence. In fact, when smaller subfragments (−234 to −202 (probe 2a), −204 to −168 (probe 2b), and −170 to −66 (probe 2c)) from Sau3A1 digestion of probe 2 were used as probes on an electrophoretic mobility assay, only probe 2b, but not probe 2a or 2b, had the ability to form the major band complex with sequence specificity (data not shown).

Similar Binding Factors Also Exist in Other Tissues of B. mori—Previously, we have shown that the distal upstream element, −238 to −73, functions in a tissue-specific manner (13). It is thus possible that factors contributing to transcriptional enhancement via the distal region may be present specifically in the posterior silk gland cells or may be activated only in these cells. To determine whether the factors that bind to the distal element are detected in other tissues, an electrophoretic mobility assay with probe 2 and nuclear extracts from other tissues of B. mori was carried out (Fig. 3).

Complexes having a similar mobility with the major band complex constructed in the posterior silk gland extract were also detected in middle silk gland and ovarian tissue extracts (arrow 1 in Fig. 3, lanes 2–4). Whereas the major band complex formed in the posterior silk gland extract was resistant to a high concentration of EDTA (Fig. 3, lane 7), the major complexes that were generated in the middle silk gland and ovarian tissue extracts reduced remarkably under the increasing EDTA concentration (Fig. 3, lanes 8 and 9). In a HeLa cell extract, a complex having a similar mobility with arrow 1 cannot be detected under conditions of both limited and increased EDTA concentrations (Fig. 3, lanes 5 and 10). Reduction of the major band under various EDTA concentrations is quantitated and shown in Fig. 4. The molar ratios of EDTA to MgCl2 necessary to reduce the radioactivity of the major band by 50% are 9 in the posterior silk gland, but 1.4 in the middle silk gland extract and 2.3 in the ovarian tissue extract (Fig. 4). It is clear that the major band complex generated in the posterior silk gland extract is more resistant to EDTA than the similar complexes generated in the two other tissue extracts. However, under a condition of limited EDTA concentration (Fig. 2), competition kinetics obtained by the use of competitor 1 was approximately the same between the three tissue extracts. In addition, the radioactivi-
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Fig. 6. Effects of trypsin and heat treatment of fractions A and B on major band complex formation. a, electrophoretic mobility assays were performed with the combinations of trypsinized or native fractions A and B. Fractions A (59 μg) and B (15 μg) from phosphocellulose column chromatography in a 15.6-μl volume were incubated at 30 °C for 20 min with or without 140 ng (fraction A) and 35 ng (fraction B) of trypsin. After incubation, 88 μg (fraction A) and 22 μg (fraction B) of soybean trypsin inhibitor (4.4-μl volume) were added, and 3-μl aliquots were assayed with probe 2 (0.1 ng, 20,000 cpm) in 5 mM EDTA. Lane 1, reaction with no protein; lane 2, with fraction A alone treated without trypsin; lane 3, with fraction A alone treated with trypsin; lane 4, with fraction B alone treated without trypsin; lane 5, with fraction B alone treated with trypsin; lane 6, with combined fractions of lanes 2 and 4; lane 7, with combined fractions of lanes 3 and 4; lane 8, with combined fractions of lanes 3 and 5; lane 9, with combined fractions of lanes 3 and 5; lane 10, with combined fractions of nontreated fractions A (8.8 μg) and B (2.2 μg). Lanes 11 and 12 are intact fraction A alone (8.8 μg) and intact fraction B alone (2.2 μg), respectively. b, electrophoretic mobility assays were performed with the combinations of heat-treated fractions A and B. Twelve μl of fractions A (2.95 mg/ml) and B (0.75 mg/ml) were heated for 5 min in a water bath at 4 or 90 °C, and 3-μl aliquots of the resulting supernatants were assayed with probe 2 (~0.1 ng, 20,000 cpm) in 5 mM EDTA. Lane 1, reaction with no protein; lane 2, with fraction A treated at 4 °C; lane 3, with fraction A treated at 90 °C; lane 4, with fraction B treated at 4 °C; lane 5, with fraction B treated at 90 °C; lane 6, with combined fractions of lanes 2 and 4; lane 7, with combined fractions of lanes 2 and 5; lane 8, with combined fractions of lanes 3 and 4; lane 9, with combined fractions of lanes 3 and 5. a and b, lane M is a size marker. Arrow 1 shows the major band complex.

Major Band Complex Is Possibly Composed of Two Proteins and Upstream DNA Element—As a first step of purification of factors constructing the major band complex, we have tried phosphocellulose column fractionation of a nuclear extract from posterior silk glands as described under "Experimental Procedures." To detect the major band complex, probe 2 was incubated with four fractions (A–D) from phosphocellulose column chromatography, and products were resolved by electrophoretic mobility assay. Whereas the major band complex was detected reproducibly in the unfractonated nuclear extract (arrow 1 in Fig. 5a, lane N), the complex was not detected in fractionated samples (Fig. 5a, A–D). However, band complexes that showed the same mobilities as the minor ones (arrow 2 and asterisks) were detected in fractions A (arrow 2 and asterisk), B (arrow 2 and double asterisks), C (arrow 2), and D (arrow 2). To test whether factors constructing the major band complex are separated into fractions by column chromatography, reconstruction of the major band complex has been tested by combining these phosphocellulose fractions. Four (AB, ABC, ABD, and ABCD) out of all eleven combinations revealed an ability to form the major band complex, but the other seven (AC, AD, BC, BD, CD, ACD, and BCD) failed to form the complex. Minimum combination for the complex formation is, therefore, A plus B (see Fig. 5b, II). As shown in Fig. 5b, the mode of competition by competitor 1 was the same for both the reconstructed (II) and the native major band (I) complexes. To reduce both major band complexes in I and II by 50%, competitor 1 with 8–10 molar excess was necessary. Competitor 2 DNA that does not encompass the upstream region failed to compete for binding also in the reconstructed system (data not shown). These results are consistent with the observations shown in Fig. 2.

To characterize whether the factors constructing major band complex are of proteinous nature or not, fractions A and B were preincubated with trypsin (Fig. 6a) or were heated at 90 °C for 5 min (Fig. 6b), and then an electrophoretic mobility assay was carried out using probe 2 under the various combinations. Fig. 6a shows that the trypsinized respective fractions have lost the ability to construct the major band complex with nontreated counterparts (lanes 7 and 8). A broad band shown in lanes 4, 8, and 12 (Fig. 6a), which electrophoresed a little faster than the major band complex, was the product derived from nontreated fraction B (see also Fig. 5a, B). Soybean trypsin inhibitor alone (Fig. 6a, lane 6) had no effect on the construction of the major band complex. These data indicate that the factors in fractions A and B are trypsin-sensitive. Trypsin sensitivity of the major band complex was also evident in the unfractonated extract from posterior silk gland (data not shown). Fig. 6b also shows that heated fraction B lost the ability (lane 7), but heated fraction A (lane 8) maintained the ability, to construct the major band complex with the nontreated counterparts. These data indicate that the factor in fraction A is heat-stable, but the factor in fraction B is heat-labile. The factor in fraction B, when heated at
50 °C for 5 min, lost about 70% of the ability to reconstruct the major band complex with the fraction A and lost it completely when heated at 70 °C for 5 min (data not shown). Thus, we conclude that the factor A is heat-stable and the factor B is heat-labile, and at least the essential domains constructing the major band complex in both fractions A and B contain protein moiety. It is also demonstrated that at least two proteins from fractions A and B are essential to form the major band complex. A high density band constructed in fraction A (electrophoresing above the 1632-bp size marker in Fig. 5a) was a product with poor sequence specificity (data not shown), less sensitive to trypsin (see Fig. 6a, lanes 3 and 9), and heat-labile (Fig. 6b, lanes 3, 8, and 9). Factor(s) constructing the high density band in fraction A are probably not related to the major band complex.

Sensitivity of the major band complex to chemical modification by N-ethylmaleimide (NEM) was also tested. As shown in Fig. 7, treatment of the nuclear extract from posterior silk gland cell with NEM eliminated the radioactivity of the major band complex, although it was not clear which factor in fraction A or B was sensitive to NEM. Inactivation by NEM may be consistent with the reduction of sulfhydryl groups of the factors. Therefore, sulfhydryl groups of the proteinous factors may be essential for protein-DNA and/or protein-protein interactions.

Footprinting Analysis of Major Band Complex—To delineate at a higher resolution the binding domain(s) of the DNA constructing the major band complex, we have carried out DNase I footprinting analysis. The 5' or 3' end-labeled probe 1 and the mixture of fractions A and B from phosphocellulose were incubated as described under "Experimental Procedures." After incubation, protein-DNA complexes were briefly digested with DNase I, and the digests were then subjected to electrophoresis. Under the above conditions, electrophoretic mobility of the band complex was not affected by DNase I (data not shown). The radioactivities in the major band complex were eluted from the gel and analyzed on a sequencing gel for protected areas (Fig. 8). Chemical degradation products of the probe were run in parallel as sequence markers. Protected regions are indicated by brackets in Fig. 8. The major band complex (Fig. 8a, lane B) appeared to protect mainly an 13-nucleotide region from -167 to -155 on both DNA strands and an additional region from -180 to -177 on the coding strand. These results indicate that the core segment is the 13-nucleotide region of -167 to -155 including a repeated 5'-AATTTAATTT-3' sequence (Fig. 8c). A sequence containing 5'-AATTT-3' is also located on a region from -117 to -113; an area (-120 to -110) around this region seems to be protected weakly (Fig. 8a, bracket 1). From these results, we conclude that composite proteins bind to a DNA element of -180 to -155 including a core region from -167 to -155 and to another element containing AATT from -120 to -110.

**DISCUSSION**

The regulated transcription of the fibroin gene in the posterior silk gland is probably mediated by a qualitative and/or quantitative change of nuclear factors. To detect possible candidates of these factors in nuclear extract of the posterior silk gland, we have employed an electrophoretic mobility assay. When a DNA fragment that contains both the distal and proximal transcription enhancing signals (probe 1) or a fragment that contains only the distal element (probe 2) was tested, two specific band complexes, a major and a minor, were detected (Fig. 1b, arrows 1 and 2). These specific complexes represent the products which are formed by interaction of the distal element with proteinous factors in the nuclear extract. In fact, the major band complex is found to be sensitive to trypsin, NEM, and heat treatment. So, we have concluded that some proteinous factors in the posterior silk gland cells are able to bind to the distal enhancer element (-234 to -66) with sequence specificity. These enhancer-binding factors may be related to the regulatory proteins for selective expression of the fibroin gene.

However, both the major and minor band complexes did not appear to be tissue-specific products because similar complexes were also detected in the middle silk gland and ovarian tissue extracts. Therefore, proteins that are required to constitute the two specific complexes might be common in the silkworm tissues. The fibroin gene is not expressed in the middle silk gland and ovarian tissue in vivo. However, transcriptional enhancement dependent on the distal enhancer is also detected, to a lesser extent, by in vitro transcription assay in the nuclear extract from the middle silk gland, but is negligible in the ovarian tissue extract (13). These observations could probably be explained by the following assumption. Factors that interact with the fibroin gene enhancer might be present in most cell types, but they are activated by specific modification in the silk gland tissues, especially in the posterior region, but not appropriately in other cell types. There is a supporting observation of this assumption; the major band complex formed in the posterior silk gland extract is highly resistant to EDTA when compared with the complexes formed in the middle silk gland and ovarian tissue extracts. Thus, we conclude that the proteins constructing the major band complex in posterior silk gland cells are different in a substantial way, from those in other cells of *Bombyx.* In fact, we have obtained a preliminary result4 that the resistance to EDTA of the major band complex from posterior silk gland extract is dependent on the factor(s) which is specific for posterior silk gland, but not for middle silk gland and ovarian tissue extracts. Such additional factor(s) constructing the major band complex may result in producing a specificity in posterior silk gland in vivo.

NF-κB, a protein that interacts only with the κ-enhancer of the immunoglobulin gene, is not restricted in B lymphoid cells (17). NF-κB is also induced in T lymphoid and HeLa cells by lipopolysaccharide or phorbol myristate acetate stimulus, and the induction of NF-κB does not require de novo

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protein synthesis (17). According to these observations, Sen and Baltimore (17) suggested that factors regulating specific gene transcription in specific cells may be activated by post-translational modification of precursor factors present more widely. In the phorbol diester-inducible expression of genes, it is also generally accepted that change in patterns of transcription may be mediated through the post-translational alterations of pre-existing transcription factors (18, 19). Proteins that constructed the major band complex on the fibroin gene enhancer may also be regulated by ubiquitous factors for selective transcription and/or enhancement in a similar manner.

The major band complex is composed of at least two proteins. One is heat-stable, and the other is heat-labile. When a nuclear extract of posterior silk gland was fractionated by phosphocellulose column chromatography, individual fractions had no ability to form the complex by itself. When 0.1 (fraction A) and 0.3 (fraction B) M KCl fractions were combined, the major band complex was reconstructed. The reconstructed and native major band complexes were indistinguishable from each other by a criterion of competition mode (Fig. 5b). It is clear that factor A from fraction A and factor B from fraction B are separated by a phosphocellulose column equilibrated with a buffer devoid of Mg²⁺. It is important to emphasize that Mg²⁺ is essential to form the major band complex (Figs. 3 and 4). Probably, a cation like Mg²⁺ is essential for protein-protein interaction between factors A and B, and the resulting associated proteins may acquire an ability to bind to the enhancer region. Only the associated protein complex is essential to form with specificity the major band complex with the enhancer sequence of the fibroin gene. However, there remains a possibility that one factor is important to stabilize weak binding of another factor. In fact, a broad band electrophoresing a little faster than the major band complex (Fig. 5a, lanes 3 and 4) is competed specifically by a DNA fragment containing the upstream region (data not shown). Factor A may bind to the unstable factor B-DNA complex and may stabilize the binding of factor B to DNA through an interaction between factors.

The major band complex is also sensitive to NEM, which reduces the sulfhydryl groups. There are many functional interactions depending on sulfhydryl groups in biological systems. For example, an NEM-sensitive factor is required for accurate transcription (31), and the sulfhydryl-modifying reagent methyl methanethiosulfonate inhibits the DNA binding activity of glucocorticoid receptor (32). In our case, NEM may inhibit an interaction between factors A and B in the major complex, but there is also another possibility that exposed sulfhydryl groups of A-B complex may play a direct important role on binding to enhancer.

Associated proteins in the major band complex appeared to bind mainly to the −167 to −155 region on both the coding and non-coding strands. This area accommodates an interesting repeated sequence of 5′-AATTTAATT-3′. Previously,
we showed that a deletion of the upstream region from −238 to −167 caused only a small decrease, but the deletion of the region from −238 to −115 gave a large decrease of template activity in assays with both linear (23) and supercoiled (20) templates. Probably, the repeated sequence of 5′-AATT-TAATTT-3′ included in this deleted region plays an important role in template activity of the fibroin gene. A DNA fragment accommodating a region from −170 to −66 had the ability to compete the major band complex. However, the competition at 100-fold molar excess was not complete, whereas a competitor covering a wide range (−234 to −66) was able to compete for the major band complex completely at the same molar excess of DNA (Fig. 1C). Further upstream sequence beyond −171 may also play some role together with the core region from −167 to −155. In fact, a protected region indicated by bracket 3 (Fig. 8a) was at around −180 to −177 in the footprinting analysis. It is now necessary to purify factors A and B and to characterize further the functions of upstream segments interacting with the A-B complex.

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