Identification and purification of a *Bombyx mori* homologue of FTZ-F1

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ABSTRACT

Extracts from embryos and from posterior and middle silk glands of the silkworm, *Bombyx mori* contain a sequence specific DNA binding factor termed BmFTZ-F1. The factor binds to the recognition site of FTZ-F1, a positive regulator of the *fushi tarazu* gene in *Drosophila melanogaster*. BmFTZ-F1 and FTZ-F1 share the same methylation interference patterns, the same chromatographic behaviors and similar protease digestion profiles. Anti-FTZ-F1 cross reacts with BmFTZ-F1. These results indicate that BmFTZ-F1 is a *B. mori* homologue of FTZ-F1. The mobility of the factor-DNA complex formed in the silk gland extract changes depending on the developmental stages. Purification of BmFTZF1 to an almost homogeneous state reveals that the factor is a 73 kd protein.

INTRODUCTION

Recent studies have shown that sequence specific DNA binding proteins play important roles in the determination of differentiation during the development of *Drosophila melanogaster* and other organisms (1-9). The *fushi tarazu* (*ftz*) gene of *Drosophila* is expressed in 7 stripes with double segment periodicity at the blastoderm stage of embryogenesis and is transiently expressed again in a segmentally repeated subset of neuronal precursor cells in the developing nervous system at 5-12 hrs of embryogenesis (10-12). Homozygous *ftz*− mutant embryos possess about half the normal number of body segments (13). A mutant of *Drosophila* which expresses the *ftz* gene in the blastoderm stage but not in the central nervous system does not develop some neurons (12), indicating that the *ftz* gene plays an important role in the determination of those cells.

Previous studies identified a sequence specific DNA binding protein, FTZ-F1, which has 4 binding sites around the *ftz* gene (14). Expression of *ftz-lacZ* fusion genes carrying mutations at the FTZ-F1 binding site within the *cis* regulatory element for the expression of the striped pattern was reduced in transformed embryos, especially in stripes 1,2,3 and 6 (14). These results strongly suggest that FTZ-F1 is a positive regulator of the *ftz* gene and FTZ-F1 plays an important role in the development of *Drosophila* through the regulation of the *ftz* gene. FTZ-F1 binding activity is present in blastoderm embryos when the *ftz* gene is expressed. After coordinate disappearance of this activity and *ftz* gene repression, FTZ-F1 activity reappears in late embryos, larvae and adult flies. In later developmental stages, FTZ-F1 shows different mobility with FTZ-F1 in blastoderm embryos on the gel mobility shift analysis, although DNA binding property and digestion patterns of protease indicate that these factors have common structure including DNA binding domain. In addition, the amount of the factor increases dramatically during late embryogenesis without concomitant increase in the *ftz* gene expression (14). These results suggest that factors binding to the FTZ-F1 site play a role in the regulation of other genes. However, it is not easy to analyze this presumptive function due to low abundance and difficulties of purification of this factor from *Drosophila*.

The silkworm, *B. mori*, is larger than *Drosophila* and useful for the biochemical studies. Specially, the middle and posterior silk glands have been used as experimental material in studies of highly specified and temporally regulated genes (15-18). Genes which are specifically expressed in those tissues have been analyzed using transcriptionally active extracts prepared from silk glands (19,20) and factors which regulate these genes have been identified and characterized (19-23).

In this study, we describe a factor of *B. mori* which recognizes the same binding site as FTZ-F1. This factor, BmFTZ-F1, is developmentally regulated in the silkworm, exhibits similar mode of contact to the binding site of FTZ-F1 and has similar biochemical characteristics to FTZ-F1. From these results, we conclude that BmFTZ-F1 is a factor corresponding to *Drosophila* FTZ-F1. The factor has been purified to near homogeneity from the crude whole cell extract of the posterior silk glands of the silkworm.

MATERIALS AND METHODS

Materials

*B. mori* stains, C-108, P-22 and their hybrid, were used in this experiment. Agarose NA, Heparin-Sepharose CL6B, CNBr-activated Sepharose 4B and poly(dl-dC)-poly(dl-dC) were obtained from Pharmacia LKB Biotechnology. NA45 membrane was obtained from Schleicher & Schuell. Bovine serum albumin was purchased from Miles Co. Endoproteinase Glu-C was obtained from Boehringer Mannheim Biochemicals. Anti-β-galactosidase antibody and reagents for the western blotting analyses were purchased from Promega Co. The aquapore RP300 column was...
washed with 20 ml of the buffer A containing 0.1 M NaCl. The material was eluted with 3.2 ml of buffer A containing 1 M NaCl. First 0.8 ml of 1 M salt fraction (1.0M-1) was subjected to reverse phase HPLC on an Aquaprep RP 300 column (2.1 x 30mm) and proteins were eluted with a linear gradient of 10 to 70% acetonitril in 0.1% trifluoroacetic acid. Peak fractions were pooled and dried using a speed vac concentrater.

Other methods
SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (25). The western blot analysis was carried out according to the protocol of Promega Co. Protein concentrations were measured using the Bio Rad protein assay.

Figure 1. Sequence specific DNA binding activity in B. mori extracts. (A) Specific binding of factor in the extract prepared from B. mori embryos to a 33bp synthetic FTZ-F1 binding site I fragment. Standard gel mobility shift analysis was carried out using the extract from embryos (lanes 1). Twenty five (lane 2) or 100 (lane 3) fold molar excess cold FTZ-F1 binding site I fragment or 200 (lane 4) or 1000 (lane 5) fold mass excess cold lambda/HaeIII DNA was added in the standard gel mobility shift analysis. F represents the position of free probe DNA. (B) Developmental profiles of the factor. The standard gel mobility shift analysis was performed using whole cell extracts from the 6day old embryos (lane 2), from the posterior silk gland at the 4th instar larvae (lane 3), from the posterior silk gland at the 4th molting stage larvae (lane 4), from the posterior silk gland at the 5th day of the 5th instar larvae (lane 7). The mobility of complex formed by Drosophila FTZ-F1 is shown in lane 1 as a control. F represents the position of free DNA. (C) Detection of minor difference in the mobility of the factor-DNA complex. Standard gel shift assay was performed using extracts from the posterior silk gland at the 2nd day (lane 2) or the 5th day (lanes 1 and 3) of the 5th instar larvae. F represents the position of free probe DNA.
RESULTS
Detection of a B. mori homologue of FTZ-F1 in the embryonic and silk gland extracts

Whole cell extract of B. mori embryo were analyzed by gel mobility shift assay using 32P-labeled FTZ-F1 binding site I fragment as a probe. As shown in Fig. 1A lane 1, the embryonic extract gave 3 shifted bands. These mobility shifts were competed by the addition of small amounts of unlabeled probe DNA (Fig. 1A, lanes 2 and 3), but not competed by non-specific DNA fragments (Fig. 1A lanes 4 and 5). These results indicate that a factor which recognizes the FTZ-F1 binding site is present in B. mori. Fig. 1B shows developmental profiles of the factor. Mobilities of factor-DNA complexes formed in the extracts from embryo (main band), the posterior silk gland at the 4th instar larvae and the middle and posterior silk gland at the 2nd day of the 5th instar larvae were the same. However, the extract from the posterior silk gland at the 4th molting stage gave a faster migrating band. Furthermore, the extract from the posterior silk gland at the 5th day of the 5th instar produced a band with a slightly slower mobility. This minor difference was confirmed by side by side comparison of the mobilities of the factor-DNA complex formed in the extracts from the posterior silk gland at the 2nd and 5th day of the 5th instar larvae (Fig. 1C). None of these bands have the same mobilities as the FTZ-F1-DNA complex (Fig. 1B, lane 1). Each mobility shift observed in these extracts was competed with unlabeled FTZ-F1 binding site I fragment but not with non-specific DNA fragments (data not shown).

Methylation interference experiment

Methylation interference experiment gives information about contact points on DNA by a DNA binding factor. As shown in Fig. 2A, methylation in four guanine residues within the FTZ-F1 binding site I fragment strongly interferes the complex formation in both Drosophila embryo extract and B. mori extract from the posterior silk gland at the 2nd day of the 5th instar larvae. These guanine residues are marked by closed triangles in Fig. 2B. This agreement in the methylation interference patterns using FTZ-F1 and BmFTZ-F1 demonstrates that not only the position of the binding site but also the DNA contact are the same. From these results, we conclude that this B. mori factor corresponds to FTZ-F1 in Drosophila and we named it BmFTZ-F1. The same methylation interference profiles were obtained using the extracts from embryo and the posterior and middle silk gland at any stage of development tested thus far, indicating that band shifts observed in Fig. 1 were produced by factors which recognize the same sequence in a similar manner (data not shown). Minor bands which were present in extract of embryo or the posterior silk gland at the 4th molting stage larvae have not been examined for methylation interference.

Comparison by endoproteinase Glu-C

FTZ-F1 and BmFTZ-F1 were compared with a digestion pattern by proteases. As shown in Fig. 3, when factor-DNA complexes
Figure 4. Purification of BmFTZ-F1. (A) Heparin-Sepharose column chromatography. Protein elution profile is shown. The right upper panel illustrates the BmFTZ-F1 activities which were measured by the standard gel mobility shift analysis. Fractions between two arrows (fractions 13–17) were pooled for further purification.

(B) The BmFTZ-F1 activities which were measured by gel shift assay (upper panel) and silver stained SDS-polyacrylamide gel (lower panel) of fractions of different purification steps. Each panel shows the corresponding amount of activities and protein profiles of 1 µl sample of crude extract, 1 µl sample in 60 ml of Heparin-Sepharose column flow through fraction, 8 µl sample in 5 ml of 0.4M salt-washed fractions 1–2 and 8 µl sample in 0.8 ml of 1M salt-eluted fractions 1–5. (C) Silver stained SDS-polyacrylamide gel of each fraction of reverse phase column chromatography. Ori means 5 µl sample of 1.0M-1 fraction (Figure 4B) which were loaded on the reverse phase column. Elution profile is shown in the lower panel. Arrows indicate the corresponding fractions for SDS-polyacrylamide gel electrophoresis.
produced by FTZ-F1 and BmFTZ-F1 were digested with Endoproteinase Glu-C, mobilities of both complexes increased to a similar degree. But further digestion with 10 times stronger conditions did not give major effect to the mobilities and intensity of bands in both factors. These results indicates that both factors form stable factor-DNA complex against Endoproteinase Glu-C.

**Purification of BmFTZ-F1**

To study the properties of BmFTZ-F1, we purified the factor. Four hundred ml of the whole cell extract which was prepared from 2,000 pairs of the posterior silk glands at the 2nd day of the fifth inster larvae were loaded on a Heparin-Sepharose CL4B column and eluted with a linear NaCl gradient in Buffer A. Each fractions were assayed by gel shift analysis using $^{32}$P-labeled FTZ-F1 binding site I fragment as a probe. As shown in Fig. 4A, BmFTZ-F1 were eluted at about 0.6 M NaCl (Fraction 12 - 17), while most of the protein were eluted in low salt. The next step was chromatography on the FTZ-F1 binding site DNA-Sepharose column. The lower part of Fig. 4B shows the protein profiles of the fractions of each purification step by SDS-polyacrylamide gel electrophoresis and the upper part of Fig. 4B shows the amounts of BmFTZ-F1 activities which were measured by the gel shift analysis using the same amount of aliquot loaded on SDS-polyacrylamide gel electrophoresis. From the binding site affinity column, half of activity was eluted with 0.4 M salt and the other half of the activity was eluted with 1 M salt. The protein profiles of SDS-polyacrylamide gel electrophoresis show that each step of purification enriched for the binding activity and that the elution profile of 73 kd protein corresponds to the amount of BmFTZ-F1 activity in each fraction. The amount of BmFTZ-F1 in the complex formed by the fraction 1.0M-1 in the upper part of Fig. 4B was calculated from the amount of DNA present in the shifted band (200 fmoles) and the molecular mass of the protein (73kd) assuming that the molar ratio of protein to DNA in the complex is one. The predicted amount (15ng) is in agreement with the amount of polypeptide estimated from the intensity of 73 kd band in the lower part of Fig. 4B. Thus, we conclude that BmFTZ-F1 is a 73 kd protein, although FTZ-F1 is 95 kd (14). In this experiment, BmFTZ-F1 was purified 12,000 fold and the yield was about 4 µg protein (Table 1). This 1.0M-1 fraction was loaded on a C-8 reverse phase column. Although the activity could not be measured, the 73 kd protein was further purified to near homogeneity as shown in Fig.4C lane 5.

**Immunological comparison**

We have examined whether anti-FTZ-F1 serum reacts with BmFTZ-F1 or not. Reverse phase column purified BmFTZ-F1 and FTZ-F1 were subjected to SDS-polyacrylamide gel electrophoresis and the gel was stained with silver (Fig.5 lanes 1 and 2). Though this preparation of FTZF1 was partially degraded and hence 5 major bands were seen in the gel, the intact polypeptide is 95kd. Ten times more amount of samples were electrophoresed on the same gel and analyzed with western blotting method using anti-FTZ-F1 (Fig.5 lanes 3 and 4). BmFTZ-F1 did react with anti-FTZ-F1 weakly (lane 4). Neither preimmune serum (lanes 5 and 6) nor anti-β-galactosidase antibody (lanes 7 and 8) react with FTZ-F1 and BmFTZ-F1. Of course anti-FTZ-F1 did not cross react with contaminating proteins in partially purified BmFTZ-F1 fraction (data not shown). The reactivity of BmFTZ-F1 with anti-FTZ-F1 was about 10 times less than that of FTZ-F1.

**DISCUSSION**

In this study, we identified a sequence specific DNA binding factor, BmFTZ-F1, in the extracts of the silkworm, *B. mori*. The factor corresponds to *Drosophila* FTZ-F1 which binds to the *fz* gene promoter and regulates its transcription (14). We found following similarities between the biochemical properties of these
two factors. (1) They recognize the same sequence as shown by gel mobility shift analysis. (2) The mode of interaction to the binding sequence is the same or similar, because the same four guanine residues shows methylation interference. (3) The elution conditions of these factors from Heparin-Sepharose, binding site DNA affinity, C-8 reverse phase, Q-Sepharose and S-Sepharose columns were the same between them (a part of data was not shown). (4) The affinity of BmFTZ-F1 to the FTZ-F1 binding site I fragment is about 10^5 fold higher than that to non-specific DNA as revealed by the analysis shown in Fig. 1A. This value is the same as FTZ-F1 (14). (5) Cross reactivity of the anti-FTZ-F1 against BmFTZ-F1 indicates the presence of certain structural homology between these proteins. (6) Peptide sequencing of the purified and trypsinized BmFTZ-F1 showed that at least two peptides, 5 a.a. length and 7 a.a. length, share the same sequences between BmFTZ-F1 and FTZ-F1 (unpublished result). The presence of corresponding factor both in B. mori and Drosophila suggests that FTZ-F1 is an important factor conserved through the evolution of insects.

Despite these similarities, the mobilities of the factor-DNA complex on polyacrylamide gel were different between these factors. This is most likely due to the difference in molecular weight of these factors (95kd for FTZ-F1 (14) and 73kd for Bmftzf1). Cross reactivity of the anti-FTZ-F1 against BmFTZ-F1 was about ten times less than that of FTZ-F1. These results indicate that at least a part of those proteins is different. We don’t know whether it is caused by difference in the primary structure and/or protein modification such as phosphorylation or addition of sugars. The band of purified BmFTZ-F1 on SDS-polyacrylamide gel electrophoresis is fuzzy (Fig. 4C lane 5 and Fig. 5), suggesting that this protein is subjected to modification.

FTZ-F1 is regulated qualitatively and quantitatively during the development of Drosophila (14). Ueda et al. have shown that one of the target genes of FTZ-F1 is the ftz gene and that the expression pattern of the ftz gene is well correlated with the activity of FTZ-F1 (14). BmFTZ-F1 also shows the qualitative change during the development of the silkworm. It has been shown that the fibroin gene and the fibroin L-chain gene in the posterior silk gland are expressed at the 4th and 5th instar larvae but not at the 4th molting stage and that their expression is regulated at the level of transcription (15-18). Expression pattern of many other genes also changes through these stages. Synthesis of rRNA decreases after the 4th apolysis, but soon increases. The rate of expression becomes the highest at the 2nd day of the 5th instar and then drop again precipitously, although fibroin gene is not suppressed even at late 5th instar (15,16). Expression of heterogenous RNAs, which does not include the fibroin mRNA, are also suppressed at the late 5th instar (15). Observed difference in the mobilities of BmFTZ-F1-DNA complexes formed in the silk gland extracts prepared from the various stages of larvae suggest that BmFTZ-F1 might be involved in the regulation of a part of these genes. Preliminary analysis of cloned FTZ-F1 gene showed that FTZ-F1 has homology with hormone receptors of vertebrate and Drosophila in both binding domain and ligand binding domain (G.Lavorgna, H.U., J.Clos and C.Wu unpublished result). This result suggest that BmFTZ-F1 might be a hormone receptor which regulates its target genes. However, we could not find any candidates of the binding site for BmFTZ-F1 in the B. mori genome so far as searched from the GenBank gene bank. We also tried to clone a B. mori homologue of the ftz gene from a genomic library, but we could not find a clone which strongly hybridizes with DNA of the Drosophila ftz gene.

In this study, BmFTZ-F1 has been purified to a nearly homogeneous state. The purified factor may be useful for the analysis of protein modification which might play an important role for the regulation of the target genes. We could also get partially purified factor easily using only two steps of purification without denaturation. The preparation will be useful for the screening of the target genes of this factor by trapping the factor-DNA complex on a nitrocellulose filter. It will be also useful for the functional analysis of this factor in an in vitro transcription system. Such experiments are difficult to perform by using FTZ-F1 because only a small amount of the factor is available in a pure form (14).

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REFERENCES