Mediators for Activation of fushi tarazu Gene Transcription by BmFTZ-F1

BmFTZ-F1による*fushi tarazu*遺伝子転写活性化に必要なメデイエーター

Feng-Qian Li

李 豊倩

Doctor of Philosophy

博士（理学）

Department of Genetics,
School of Life and Science,
The Graduate University for Advanced Studies

総合研究大学院大学生命科学研究科遺伝学専攻

1993

CONTENTS

Summary

Transcriptional activation by many eukaryotic sequence-specific regulators appears to be mediated through transcription factors which do not directly bind to DNA. BmFTZ-F1 is a silkworm counterpart of FTZ-F1, a sequence-specific activator of the fushi tarazu gene in *Drosophila*. I have isolated 18 kirodaltons (kd) and 22 kd polypeptides termed MBF1 and MBF2, respectively, that form a heterodimer and mediate activiation of *in vitro* transcription from the fushi tarazu gene promoter by BmFTZ-F1. Neither MBF1 nor MBF2 binds directly to DNA. MBF1 interacts with BmFTZ-F1 and stabilizes the BmFTZ-F1•DNA complex. MBF1 also makes a direct contact with TATA-binding protein (TBP). Both MBF1 and MBF2 are necessary to form a complex between BmFTZ-F1 and TBP. I propose a model in which MBF1 and MBF2 form a bridge between BmFTZ-F1 and TBP, and mediate transactivation by stabilizing the protein•DNA interactions.

Introduction

Promoters of eukaryotic protein-coding genes consist of two functionally different sets of DNA elements. Core promoter constitutes the recognition site of general transcription machinery and supports a basal level of transcription (Sawadogo and Sentenac, 1990). The other element including recognition sites for sequence-specific DNA-binding proteins regulates the utilization of core promoter (Mitchell and Tjian, 1989; Johnson and McKnight, 1989; Lewin, 1990). Protein-protein interactions are thought to play an important role in bringing the two promoter elements together to achieve transactivation (Lewin, 1990; Ptashne and Gann, 1990). In some cases, direct interactions between the regulatory protein and general transcription factor have been proposed (Sawadogo and Roeder, 1985; Horikoshi et al., 1988 a, 1988 b; Liberman and Berk, 1991). However, the cloning of TATA-binding protein (TBP) and its functional analyses revealed that TBP and other defined general transcription factors can not mediate regulatory protein-dependent transactivation *in vitro* (Hoffman et al., 1990; Pugh and Tjian, 1990; Dynlacht et al., 1991). It has been proposed that the TBP is associated with coactivator or mediator proteins that are required for transactivation (reviewed in Gill and Tjian, 1992). For example, some TBP associated factors (TAFs) which bind tightly to TBP restore the response to the regulatory proteins such as SP1 and CTF

(Tanese et al., 1991; Hoey et al., 1993). An activity termed USA mediates transactivation by USF and SP1 via physical interactions with TBP (Meisterernst et al., 1991). Viral proteins VP16 and E1A also make direct contact with TBP and mediates transactivation by some regulatory proteins (Stringer et al., 1990; Lin and Green, 1991; Lee et al., 1991; Horikoshi et al., 1991; Schöler et al., 1991). Although these studies established direct contact of coactivators or mediators with TBP, the mechanism by which this new class of transcription factors mediates transactivation remained unknown (Roeder, 1991; Gill and Tjian, 1992).

FTZ-F1 has been identified as a sequence specific DNA-binding protein in *Drosophila* (Ueda et al., 1990). It binds to a 9 base pairs sequence in the upstream regulatory region of the fushi tarazu (*ftz*) gene. Binding site-dependent expression of the *ftz-lacZ* fusion genes in transformed embryos showed that FTZ-F1 is a positive regulatory factor of the *ftz* gene. FTZ-F1 is present in blastoderm embryos when the *ftz* gene is expressed in a pattern of seven stripes. After coordinate disappearance of FTZ-F1 and *ftz* gene expression, the factor reappears in late embryos, larvae and pupae without concomitant expression of the *ftz* gene. The target genes of FTZ-F1 in later developmental stages are unknown. A posterior silk gland extract from the silkworm *Bombyx mori* contains a factor termed BmFTZ-F1 which recognizes the same DNA sequence as FTZ-F1 and has many biochemical characters similar to FTZ-F1 (Ueda and Hirose, 1990). Molecular cloning of cDNAs for FTZ-F1 and

BmFTZ-F1 revealed that they are members of the steroid hormone receptor superfamily and share homologies in the DNA-binding and the putative ligand-binding domains (Lavorgna et al., 1991; G.-C. Sun, Hirose and Ueda, in press). Expression of BmFTZ-F1 is intermittent, being high during larval molting, and both the larval-pupa and the pupa-adult ecdysis suggesting that the factor plays a role in molting and metamorphosis of the silkworm *Bombyx mori* (G.-C. Sun, Hirose and Ueda., in press).

Thought a silkworm homologue of the *ftz* gene has not deen identified, the marked similarity between FTZ-F1 and BmFTZ-F1 supports the notion that they share a common mechanism for transcriptional activation. The silkworm has an advantage in getting large quantities of material for biochemical fractionation studies. This led me to start the functional analysis of BmFTZ-F1. To elucidate the mechanism of transactivation by BmFTZ-F1, I used *in vitro* transcription systems from posterior silk gland cells and human HeLa cells. Because the HeLa system does not contain a FTZ-F1 like activity, it serves as a recipient in complementation assay for active components derived from the posterior silk gland extract. The results of these analyses suggest that two proteins, MBF (*M*ediator of *B*m*F*TZ-F1)1 and MBF2, form a bridge between BmFTZ-F1 and TBP, stabilize the BmFTZ-F1•DNA and TBP•promoter interactions, and mediate transactivation by BmFTZ-F1. This is the first isolation of mediators capable of modulating tansactivator function directly.

Experimental Procedures

Preparation of silkworm extracts

All operations were done at 0-4 °C unless otherwise specified. Posterior silk glands were removed from the larvae at appropriate stage at room temperature, washed twice with phosphate-buffered saline, then washed twice with extraction buffer [20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.9 / 5 mM $MgCl_2$, 0.1 mM EGTA, 12.5 % sucrose, 25 % glycerol and 0.5 mM dithiothretiol (DTT)], frozen quickly on dry ice or in liquid nitrogen and stored at -80 °C. The frozen Posterior silk glands from the 4000 larvae at the 2nd day of the 5th instar were thawed on ice, minced with scissors in 5 ml of the extraction buffer containing protease inhibitors [0.5 mM phenylmethane-sulfonyl fluoride (PMSF) 5 μg/ml leupeptine, 2 μg/ml pepstatin and 0.75 μg/ml apropptinin] in a 50 ml beaker and transfered to two 50 ml Falcon centrifuge tubes using 80 ml of the extraction buffer containing protease inhibitors. After centrifugation at 3,000 X g for 5 min., the pellet was resuspended in 50 ml of the extraction buffer containing protease inhibitors. The suspension was homogenized in a 15 ml Kontes homogenizer with the 'A pestle' by 30 strokes and then transfered to a 200 ml beaker. An equal volume of 0.7 M KCl was added to the homogenate with continuous gentle stirring. The mixture

was stirred for an additional 30 min. The sample was centrifuged at 4,500 X g for 10 min. and the suspernatant was centrifuged at 100,000 X g for 90 min. The resulting suspernatant was taken and dialyzed against 1 liter of dialysis buffer (20 mM HEPES, pH 7.9 / 50 mM KCl, 0.25 mM EDTA, 0.5 mM DTT and 20 % glycerol) with once change of the buffer. After removing any precipitate which forms during dialysis by centrifuging at 36,000 X g for 20 min., the supernatant was quickly frozen in liquid nitrogen.

Purification of MBF1 and MBF2

The frozen posterior silk gland extract derived from 4000 larvae at the 2nd day of the 5th instar were thawed on ice then centrifuged at 36,000 X g for 20 min. The supernatant was loaded on a 50 ml column (2.4 X 20 cm) of Heparin-Sepharose CL-4B (Pharmacia). The column was washed with 100 ml of buffer A (10 mM HEPES, pH 7.9 / 0.1 mM EDTA / 0.5 mM DTT / 10 % glycerol) containing 0.1 M KCl and the mediator activity was eluted with a 300 ml linear gradient from 0.3 to 1.3 M KCl in buffer A. Fractions containing the mediator activity (fraction numbers 12-15) were pooled (300 mg protein) and directly applied to a 50 ml column of hydroxyapatite which had been equilibrated with 20 mM potassium phosphate buffer, pH 6.8 / 10 % glycerol / 1 mM DTT / 0.5 mM PMSF. The column was washed with 100 ml of the same buffer. The flow-through fractions containing the activity were pooled

(8 mg protein), dialyzed against 20 mM Tris-HCl, pH 7.9 / 50 mM KCl / 1 mM EDTA / 10% glycerol / 1 mM DTT / 0.5 % myo-inositol / 0.5 mM PMSF, and then applied to a 1 ml column of Mono Q HR (Pharmacia) which had been equilibrated with the same buffer. The column was washed with 10 ml of the same buffer. The flow-through fractions containing the activity were pooled (0.5 mg protein) and dialyzed against buffer H (20 mM Hepes-NaOH, pH 7.9 / 1 mM EDTA / 10 % glycerol / 1 mM DTT / 0.5 % myo-inositol / 0.5 mM PMSF) containing 50 mM KCl. A protion of this material (15 μg protein) was applied to a 0.1 ml column of Mono S PC in a SMART system (Pharmacia) which had been equilibrated with buffer H containing 100 mM KCl. After washing with 1 ml of the same buffer, proteins were eluted with a 1 ml-linear gradient from 100 mM to 500 mM KCl in buffer H and 25 μl fractions were collected. MBF1 eluted at 230 mM KCl (1.5 μg protein) and MBF2 eluted at 320 mM KCl (2 μg protein) were pooled separately. The Mono S step was repeated 2 to 4 times, and pooled MBF1 and MBF2 fractions were dialyzed against buffer H containing 50 mM KCl, frozen in liquid N_2 and stored at -80 °C.

If necessary, MBF1 and MBF2 were further purified by SDS-polyacrylamide gel electrophoresis. Each 2 μg of MBF1 or MBF2 (Mono S fraction) was applied to a 12.5 % SDS-polyacrylamide gel. The 18 kirodaltons (kd) and 22 kd polypeptides were separately recovered from the gel, acetone-precipitated, denatured and renatured as

described (Hager and Burgess, 1980) except that each denatured sample was suspended in 50 µl of buffer H containing 50 mM KCl and renatured by dialysis against the same buffer for 12 hours to remove guanidium hydrochloride instead of dilution.

Purification of BmFTZ-F1 From the Posterior Silk Gland Extract Using DNA Affinity Latex Particles

DNAs. The 33 base pairs (bp) FTZ-F1-binding site was prepared from complementary synthetic oligonucleotides that were gel-purified, 5'-phosphorylated and annealed, and ligated to give oligomers that ranged from 150 to 250 bp as previously described (Kawaguchi, 1989). The oligomer contained tandemy repeated FTZ-F1-binding sites. The FTZ-F1-binding site employed for this purpose corresponds to the *ftz* gene sequence from -300 to -268 relative to the transcription start site. Those with 3'-protruding ends were digested with T4 DNA polymerase (0.5 units/µg of oligomers) in the absence of substrates at 37 °C for 120 seconds to give single-stranded 5'-ends of nucleotide length. After extraction by phenol/chloroform and ethanol precipitation, the oligomers were dissolved in 10 mM potassium phosphate buffer (pH 8.0) and coupled to latex particles.

Preparation of latex particles. GMA-St latex particles were made as described by Inomata et al. (1992). The monomers were glysidyl methacrylate (GMA), styrene St),

and divinylbenzene (DVB). To prepare latex particles, a mixture of 1.8 g GMA, 1.2 g St, 0.04 g DVB, and 110 g distilled water was mixed in a 200-ml three-necked round-bottom flask equipped with a stirrer, a nitrogen gas inlet, and a condenser. Nitrogen gas was bubbled into the mixture to purge oxygen. The system was kept at 70 °C in a water bath. Ten milliliters distilled water containing 0.06 g azobisamidinopropane dihydrochloride was added to initiate soap-free emulsion polymerization. Since the resulting GMA-St copolymer particles had a partially hydrophobic surface due to exposure to polystyrene microdomains, GMA (0.3 g) was added to the reaction mixture 2 hours after the initiation of polymerization, which was continued for further 24 hours in order to cover the whole surface of the particles with poly-GMA. The particles with epoxy groups on the surface were collected by centrifugation, washed three times with water, and used as GMA-St latex particles.

Immobilization of DNA on the latex particles. The GMA-St latex particle 2.5 mg (solid concentration, 13 %;) were washed twice with 500 µl of 10 mM potassium phosphate buffer, pH 8.0, and resuspend in 188 µl of 10 mM potassium phosphate buffer, pH 8.0, then mixed with about 30 µg of the partially single-strand ligated DNA oligomers in 12 µl of 10 mM potassium phosphate buffer. The coupling reaction was carried out at 50 °C for 24 hours. The particles were collected by brief centrifugation

in a microcentrifuge at 15,000 rpm for 30 seconds, washed twice with 500 µl of 2.5 M NaCl, and then suspended in 1 ml of 1 M ethanol amine-HCl, pH 8.0. The suspension was incubated at room temperature for 24 hours to inactivate unreacted epoxy groups on the particulate surfaces. The particles were collected by brief centrifugation and washed once with 500 µl of distilled water, washed three times with 500 µl of storage buffer [10 mM Tris-HCl, pH 8.0 / 0.3 M KCl / 1 mM EDTA / 0.02 % (w/v) NaN_3], suspended in 200 µl of storage buffer, and stored at 4 °C, and used as affinity latex particles for BmFTZ-F1.

Purification of BmFTZ-F1. BmFTZ-F1 was purified using the affinity GMA-St latex particles carrying oligomers of the FTZ-F1 recognition site. The posterior silk gland extract (500 µl) was mixed with 50 µg of poly(dI-dC):poly(dI-dC) and 500 µg of ssDNA for 15 min. at 4 °C. This procedure was necessary for sdecreasing contamination with nonspecific DNA-binding proteins. Latex particles (2.5 mg) with DNA oligomers (10 µg) which were stored in 200 µl of storage buffer, were washed twice with 500 µl extract dialysis buffer (20 mM Hepes-NaOH, pH 7.9 / 1 mM EDTA / 10 % glycerol / 1 mM DTT / 0.5 % myo-inositol) containing 100 mM KCl, resuspend in 200 µl of extract dialysis buffer containing 100 mM KCl, and added to the posterior silk gland extracts for 30 min. on ice with occasional Vortex mixing to allow BmFTZ-F1 binding to the target sequence.

The binding reaction was terminated by a brief centrifugation to separate the particles from the supernatant. The particles were washed five times with 500 µl of extract dialysis buffer containing 100 mM KCl. Then the particles were soaked in 100 µl of extract dialysis buffer containing 1.0 M KCl and stand on ice for 2 min. to elute the BmFTZ-F1 from latex particles. Then add 900 µl of extract dialysis buffer without KCl, stand on ice for 30 min. with occasional Vortex mixing to allow BmFTZ-F1 binding to the target sequence again. After repeat the wash step described as above, the BmFTZ-F1 activity was recovered in the suspernatant. After two cycles of absorption to and elution from the beads, the final eluate was dialyzed against extract dialysis buffer containing 100 mM KCl, frozen in liquid N_2, and stored at -135 °C. The DNA-latex particles were washed twice with 500 µl of 10 mM Tris-HCl, pH 7.9 / 2.5 M NaCl / 1 mM EDTA, washed once with 500 µl of storge buffer, resuspend in 200 µl of storge buffer, store at 4 °C for recycle.

Expression and Purification of rFTZ-F1 and rhTBP

Recombinant FTZ-F1 (rFTZ-F1) was expressed in *Escherichia coli* using the T7 expression system (Studier et al., 1986, Sambrook et al., 1989,). The proteins were recovered from inclusion bodies by denaturation with buffer D (6 M guanidium hydrochloride, 20 mM HEPES,

pH 7.9 / 50 mM KCl / 0.1 mM EDTA / 0.5 mM DTT / 12.5 mM $MgCl_2$ / 0.1 mM ZnSO4 / 0.1 % Nonidet P-40) and renaturation by dialysis against 20 mM HEPES, pH 7.9 / 50 mM KCl / 0.1 mM EDTA / 0.1 mM DTT / 5 mM PMSF / 20 % glycerol). Insoluble materials were removed by centrifugation at 10,000 X g for 15 min. Then the resulting suspernatant were purified using the binding site affinity resin as described above.

Recombinant human (rh) TBP was expressed in *Escherichia. coli* using the T7 expression system and purified according to the procedure developed for purification of recombinant yeast TBP (Usuda et al., 1991). The *Escherichiacoli* cells carrying pAS2D (61 culture) were grown and lysed, and the lysates were passed through a 50 ml DEAE-cellulose column as described previously (Schmidt et al., 1989). The flow-through fractions were precipitated with ammonium sulfate (60 % saturation) and the precipitants were dissolved in 600 μl of buffer G (50 mM Tris-HCl, pH 7.9 / 10 % glycerol / 1 mM EDTA / 1 mM DTT / 1 mM PMSF) contained 0.1 M KCl and then loaded onto Seperose 6 FPLC column (pharmacia) equilibrated with buffer G containing 0.1 M KCl. The TBP activity was examined by gel mobility shift assays. Active fractions with molecular weights of 20-30 kd were pooled and applied to a Mono S PC column in a SMART system (Pharmacia) and the activity was eluted with a 0.1-0.5 M KCl linear gradient in buffer G. The active fractions eluted around 0.3 M KCl were pooled and dialyzed

against TGET (50 mM Tris-HCl, pH 7.9 / 20 % glycerol / 1 mM EDTA / 1 mM DTT) containing 0.1 M KCl (0.1 TGED).

rhTBP carrying 6 consecutive histidine residues (his-tag TBP) was expressed from a cDNA inserted into 6HisT-pE11d as above and purified using Ni-immobilized resin (Novagen) according to the manufacturer's protocol followed by Mono S column chromatography.

FTZ622 labeling with [γ-^{32}P]ATP

FTZ622 consists of a 116 amino acid region in FTZ-F1 encompassing the zinc finger motif and FTZ-F1 box, and binds to DNA with the same specificity as intact FTZ-F1 and BmFTZ-F1 (Ueda et al. 1992). FTZ622PK has six extra amino acids (kinase labeling site) at the C-terminal end of FTZ622, and the binding affinity of FTZ622PK was the same as that of FTZ622. To make the ^{32}P-labeled FTZ622, 50 picomoles of FTZ622PK, expressed on pFTZ622PK, was labeled with 1,000 U of cAMP protein kinase catalytic subunit in a 50 µl reaction mixture containing 20 mM HEPES, pH 7.9 / 100 mM NaCl / 1 mM DTT / 12 mM $MgCl_2$ / 400 µCi of [γ-^{32}P]ATP at 37 °C for 30 min. The reaction mixture was applied to a 0.2 ml column of S-Sepharose equilibrated with buffer E (20 mM HEPES, pH 7.9 / 0.1 mM EDTA / 0.5 M DTT / 10 % glycerol) containing 0.1 M NaCl. The column was washed with buffer E containing 0.4 M NaCl, and the peptides were eluted with buffer E containing 0.6 M NaCl.

In Vitro Transcription

The posterior silk gland extract was prepared as described above. The HeLa nuclear extract was prepared as described by Dignam et al., (1983). BmFTZ-F1-depleted posterior silk gland extracts were prepared by treating the extracts with affinity GMA-St latex particles bearing oligomers of the FTZ-F1-binding site as described above except that poly(dI-dC):poly(dI-dC) and single-stranded DNA were omitted. After centrifugation, the supernatant was used as the factor-depleted extract. *In vitro* transcription was performed as described previously (Hirose and Suzuki, 1988). The standard reaction mixture (12.5 µl) contained 12 mM Hepes (pH 7.9), 7 mM $MgCl_2$, 60 mM KCl, 0.2 mM EDTA , 1.3 mM DTT, 10 % (vol/vol) glycerol, 10 mM creatine phosphate, 600 µM ATP, 600 µM GTP, 600 µM UTP, 50 µM CTP including 5 to 10 µCi of [α-^{32}P]-CTP, 3-5 µl of the extract (final 12 to 13 mg protein per ml) and 125 ng template DNA (final concentration was 10 µg/ml) except that $MgCl_2$ was 4 mM in the HeLa transcription system. When the mixture contained two templates, each DNA was added to 5 µg/ml. Supercoiled plasmid DNAs were used as templates: for the fibroin gene, pFb205 (Hirose et al., 1985); for the Ad_2MLP, pFLBH (Handa and Sharp, 1984); for the wild-type *ftz* gene, pE(HU)5-N; for the mutant *ftz* genes, pE(HU)5-5 and pE(HU)5-15. pE(HU)5 series plasmids were constructed by inserting the *ftz* sequence from -617 to +125 with or without base substitutions in the FTZ-F1-binding site into pEMBL19 (Figure 1A).

Reaction mixture were incubated at 30 °C for 60 min. The reactions were stopped by addition of 75 µl of 0.1 M NaOAc, PH5.5 / 1 % NaDodSO4 containing 50 µg of tRNA. The samples were extracted with phenol / chloroform. RNA synthesized *in vitro* was precipitated with ethanol, dissolved in 50 µl of a mixture containing 10 mM PIPES, (pH 6.4), 0.1 mM EDTA, 50 % formamide, and 5 µg of probe DNA. Then heated the mixture at 70 °C for 5 min., and quickly chilled on ice. Phage DNA of flFb38. (Hirose et al., 1985), M13XH11 (Handa and Sharp, 1984), and M13Ftz10 were used as hybridization partners for the fibroin gene, the Ad2MLP, and the *ftz* gene, respectively. M13Ftz10 was constructed by inserting the 0.9 kirobase (kb) HindIII-EcoRI fragment from pE(HU)5-N into M13mp19. The protected bands of faithful transcripts should have the following sizes: 318 bp (*ftz* gene), 510 bp (fibroin gene), 197 bp (Ad2MLP). After addition of NaCl to 0.4 M, the mixture were incubated at 37 °C for 60 min. Then it was diluted 10-fold with S1 buffer containing 50 mM sodium acetate (pH 4.6), 0.3 M NaCl, 1 mM ZnSO4, and 20 µg/ml of heat-denatured salmon sperm DNA and incubated with 120 unitu/ml of nuclease S1 (Sigmar) at 37 °C for 30 min. The protected transcript was recovered by ethanol precipitation, dissolved in a solution containing 10 mM Tris-HCl (PH 8.0), 10 mM NaCl, 1 mM EDTA, 5 % sucrose, 0.025 % bromphenol blue, and 0.025 % xylene cyanol, and analyzed by electrophoresis through a 6 % polyacrylamide gel.

Gel Mobility Shift Assay

The TATA element probe carrying the *ftz* gene sequence from -44 to -14 was prepared from complementary oligonucleotides that were gel-purified, labeled with polynucleotide kinase and [γ-^{32}P]ATP, and annealed. The binding of rhTBP to the TATA element was performed in reaction mixtures (10 µl) containing 12mM Hepes-NaOH, pH 7.9, 60 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 12 % glycerol, 2 % polyvinyl alcohol (Sigma, average molecular weight 10,000), 0.5 mg/ml poly(dG-dC):poly(dG-dC) (Pharmacia), 5 mg/ml BSA and 5 fmols of the probe. Samples were incubated at 30 °C for 30 min. Electrophoresis was carried out at 30 °C and 120V for 2.5 hours on a 1 % agarose gel in 0.5 X TBE containing 0.5 mM DTT and 2 mM $MgCl_2$. DNA-binding assays using the FTZ-F1 recognition site probe were carried out as described previously (Ueda and Hirose, 1991).

Gel Filtration

Protein samples in 50 µl of buffer H containing 200 mM KCl were applied on a 2.4 ml column of Superose 12 PC, eluted with the same buffer at a rate of 40 µl/min, and 60 µl fractions were collected using a SMART system. Each fraction was analyzed by SDS-polyacrylamide gel electrophoresis. Retention times of MBF1 and MBF2 were quite reproducible. Marker proteins used to calculate molecular mass were bovine serum albumin (BSA) (66 kd), ovalbumin (43 kd), chymotrypsinogen (25 kd), and lysozyme (14 kd).

Protein-binding assay using his-tag TBP

His-tag TBP, MBF1 and MBF2 used in these assays had been dialyzed against buffer H containing 50 mM KCl but lacking DTT. His-tag TBP was incubated at 4 °C for 30 min. with various proteins to allow protein-protein interactions in mixtures (10 μl) containing 12 mM Hepes-NaOH, pH 7.9, 60 mM KCl, 4 mM $MgCl_2$, 12 % glycerol, 2 % polyvinyl alcohol and 0.25 mg/ml BSA. After incubation, Ni-immobilized resin (Novagen, 2.5 μl packed volume) in 15 μl of wash buffer (12 mM Hepes-NaOH, pH 7.9, 60 mM KCl, 4 mM $MgCl_2$, 12 % glycerol) was added to the mixtures and the binding of his-tag TBP to the resin was carried out at 4 °C for 30 min. with gentle agitation. The resin was collected by centrifugation, washed twice with 250 μl of the wash buffer containing 20 mM imidazole-HCl, pH 7.9, and the bound proteins were eluted with 20 μl of 300 mM imidazole-HCl, pH 7.9 and resolved on an SDS-polyacrylamide gel.

Results

BmFTZ-F1 Activates In Vitro Transcription of the ftz Gene by Binding to the FTZ-F1 Recognition Site

To study mechanism involved in transactivation by BmFTZ-F1, we used *in vitro* transcription assay. Because the target genes of BmFTZ-F1 in the silkworm *Bombyx mori* are unknown, I used the *Drosophila ftz* gene as a model template. It carries the *ftz* gene sequence from nucleotide position -617 to +124 relative to the transcription initiation site (Figure 1A). Single binding site for FTZ-F1 is present at around 280 bp upstream from the initiation site. I also prepared mutant templates carrying base substitutions within the FTZ-F1 recognition site (mutants 5-5 and 5-15). These mutations abolished the binding of FTZ-F1 and BmFTZ-F1, and markedly reduced expression of the *ftz-lacZ* fusion gene in transformed *Drosophila* embryos (Ueda et al., 1990; Ueda and Hirose, 1991). After the *in vitro* transcription reaction, RNA was analyzed by a modified nuclease S1 assay (Hirose et al., 1985). Transcripts from the *ftz* promoter should yield a protected band of 318 base.

When the wild-type template was incubated with the posterior silk gland extract containing BmFTZ-F1, the *ftz* gene was transcribed as efficiently as the fibroin gene added as an internal control (Figure 1B, lane 1).

The mutant templates 5-5 and 5-15 gave only weak bands compared with the fibroin gene transcripts (Figure 1B, lanes 2 and 3). I compared transcription activities of the wild-type and mutant templates for more than five times and figure 1B is one of them. In each case, band intensities of the transcripts were quantitated using a FUJIX Bioimage Analyzer. When I used the posterior silk gland extract from which BmFTZ-F1 had been depleted by treatment with latex beads coupled with the FTZ-F1-binding site DNA, transcription of the *ftz* gene was severely reduced while that of the fibroin gene was unaffected (Figure 1C, lane 2). Addition of the purified BmFTZ-F1 to the depleted extract restored the *ftz* gene transcription to the level before depletion (Figure 1C, lanes 3 and 4). These results show that BmFTZ-F1 activates transcription of the *ftz* gene in the posterior silk gland extract by binding to the FTZ-F1 recognition site.

Transactivation by BmFTZ-F1 Requires an Additional Component in the Posterior Silk Gland Extract

Though the nuclear extract prepared from HeLa cells supported transcription of the *ftz* gene, the mutant templates 5-5 and 5-15 gave the same level of transcripts as the wild type in this extract (Figure 2A). This may be explained by the lack of a FTZ-F1-like factor in the extract. However, addition of purified BmFTZ-F1 to this extract did not result in transcriptional activation of the *ftz* gene

(Figure 2B). These results suggest that some components present in the posterior silk gland extract but absent in the HeLa nuclear extract are required for transactivation by BmFTZ-F1.

In searching for such components, I fractionated the posterior silk gland extract by chromatography on a Heparin-Sepharose column. Every even-numbered fraction was added to transcription reactions containing the HeLa nuclear extract and purified BmFTZ-F1. As shown in Figure 2C, the fractions eluting at around 0.45 M NaCl (fraction numbers 12 and 14) activated transcription of the *ftz* gene without affecting transcription from the adenovirus 2 major late promoter (Ad_2MLP). Other fractions were incapable of supporting selective activation of the *ftz* gene transcription, though the fractions eluting at around 0.35 M NaCl (fraction numbers 8 and 10) activated transcription from all promoters tested thus far (e.g. Ad_2MLP, *ftz* gene, *fibroin* gene, *Drosophila hsp* 70 gene) in the absence of BmFTZ-F1 (data not shown). These latter fractions contain at least TFIID, TFIIE and RNA polymerase II as revealed by complementation assays using partially purified general transcription factors from HeLa cells (data not shown). The Heparin-Sepharose fractions 12 and 14 did not activate transcription of the *ftz* gene on the mutant template 5-5 and 5-15 (data not shown). The selective activation of the *ftz* gene transcription by these fractions was not completely dependent on the exogenously added BmFTZ-F1 because

these fractions contained a small amount of the transactivator. However, the residual BmFTZ-F1 was removed by further purification on a hydroxyapatite column and the activation became absolutely dependent on the exogenous transactivator (see the section below). These results suggest that the Heparin-Sepharose fractions 12 and 14 contain a mediator activity that is required for transactivation by BmFTZ-F1.

Two Polypeptides Mediate Transactivation by BmFTZ-F1

Using the complementation assay containing the HeLa nuclear extract and purified BmFTZ-F1, the mediator activity in Heparin-Sepharose fractions 2-15 was purified according to the purification scheme outlined in Figure 3A. More than 97 % of the proteins in the pooled Heparin-Sepharose fractions retained on the hydroxyapatite column while the activity was recovered in the flow-through fraction. Approximately 94 % of the proteins in the pooled hydroxyapatite fractions retained on the Mono Q column but the activity was again recovered in the flow-through fraction. The pooled Mono Q fractions contained polypeptides of 18 kd and 22 kd besides other proteins (data not shown). These two polypeptides were separated each other and from other proteins by Mono S column chromatography and I obtained apparently homogeneous preparations of individual polypeptides (Figure 3B). Both 18 kd and 22 kd polypeptides are required to mediate

transactivation by BmFTZ-F1 (see below), and I called them MBF1 and MBF2, respectively.

When MBF1 and MBF2 were added to the HeLa transcription system supplemented with purified BmFTZ-F1, they activated transcription of the *ftz* gene without affecting transcription from the Ad2MLP (Figure 4A, compare lanes 3 and 2). BmFTZ-F1 was required for the selective activation of the *ftz* gene by MBF1 and MBF2 (Figure 4A, compare lanes 3 and 1). *Drosophila* FTZ-F1 was able to replace BmFTZ-F1 in this assay (data not shown). MBF1 alone had no effect on the transcription (Figure 4A, lane 4). MBF2 alone enhanced transcription from both the *ftz* promoter and Ad2MLP (Figure 4B). This enhancement was observed in the presence (data not shown) or absence of BmFTZ-F1 (Figure 4B). Activation of the Ad2MLP by MBF2 is weak at the concentration used in the mediator experiments (25 ng) and becomes prominent at 50 ng. MBF1 suppresses this activity of MBF2. Thus, in the presence of 25 ng MBF2, addition of increasing amounts of MBF1 resulted in decreasing levels of transcription from the Ad2MLP and the activation by MBF2 was canceled by 25 ng of MBF1 (data not shown). A small increase in transcripts from the Ad2MLP by addition of Heparin-Sepharose fraction number 14 (Figure 2C, lanes 4 and 5) is probably due to excess MBF2 over MBF1 in this fraction. Even in the presence of MBF1, MBF2 and BmFTZ-F1, no activation of the *ftz* gene transcription was detectable on the mutant template 5-5 (Figure 4A,

compare lanes 5 and 6) or 5-15 (data not shown). These experiments were carried out using the apparently homogeneous preparations of 18 kd and 22 kd polypeptides (Figure 3B). However, there is a formal possibility that the mediator activity resides in other contaminating polypeptides, if any, which escaped from the silver staining due to their minor contents. To test the possibility, the 18 kd and 22 kd polypeptides were individually recovered from a preparative SDS-polyacrylamide gel, renatured and assayed for their abilities to mediate transactivation by BmFTZ-F1. The gel-purified 18 kd and 22 kd polypeptides supported the selective activation of the *ftz* gene transcription in a dose dependent manner (Figure 4C). From these results, I conclude that the mediator activity is contained in the 18 kd and 22 kd polypeptides.

MBF1 and MBF2 Constitute a Heterodimer

When the Mono Q fraction was subjected to gel filtration on a column of Superose 12 using a buffer containing 0.15 M or 0.2 M KCl, the 18 kd and 22 kd polypeptides were eluted in the same fractions and I was unable to separate them. However, these polypeptides were resolved easily on the same column if I used the buffer containing 0.5 M KCl (data not shown). It is possible that these polypeptides interact each other at KCl concentrations below 0.2 M. To test the possibility, I applied MBF1 and MBF2 to the Superose 12 column either separately or after mixing them and eluted with

the buffer containing 0.2 M KCl. When analyzed separately, MBF1 and MBF2 were eluted in the fractions expected from their molecular mass (Figure 5, the top and middle panels). On the contrary, these proteins were eluted simultaneously in earlier fractions corresponding to molecular mass of approximately 35 kd if analyzed together (Figure 5, the bottom panel). These results suggest that MBF1 and MBF2 form a heterodimer at physiological salt concentration.

Neither MBF1 nor MBF2 Binds Directly to the ftz Promotor

To test the possibility that MBF1 and/or MBF2 bind to a specific DNA sequence in the *ftz* promoter, I added MBF1 or MBF2 to gel mobility shift assays containing an oligonucleotide bearing the DNA fragments from the *ftz* promoter in the presence (Figure 6A) or absence (Figure 6B) of 4 mM $MgCl_2$. BmFTZ-F1 was able to form a slow migrating complex with the probe DNA containing the FTZ-F1 recognition site both in the presence (Figure 6A, lane 7 and 11) or absence (Figure 6B, lane 7 and 11) of 4 mM $MgCl_2$. In the absence of BmFTZ-F1, neither MBF1 nor MBF2 made association with any DNA fragments from the *ftz* promoter on the template DNA (Figure 6A and 6B). No protein•DNA complex was detectable even if the binding mixture contained both MBF1 and MBF2 (data not shown).

MBF1 Stabilizes BmFTZ-F1 Binding to Its Recognition Site

To examine interactions between the mediators and BmFTZ-F1, I added MBF1 and MBF2 to gel mobility shift assays containing BmFTZ-F1 and an oligonucleotide bearing the FTZ-F1 recognition site. In the absence of BmFTZ-F1, neither MBF1, MBF2 (figure 6A and 6B) nor a combination of them (data not shown) made association with any DNA fragments carrying the *ftz* gene sequence on the template DNA. BmFTZ-F1 was able to form a slow migrating complex with the probe DNA (Figure 7A, lane 1). Addition of MBF1, MBF2, or both did not result in further retardation of the BmFTZ-F1•DNA complex (Figure 7A). However, significantly larger amounts of BmFTZ-F1•DNA complex were detected in the presence of MBF1 than its absence (Figure 7A, compare lanes 2, 3 and 1). Essentially the same results were obtained in the presence of MBF1 and MBF2 (Figure 7A, lanes 6 and 7). MBF2 alone had no effect on the complex formation (Figure 7A, lanes 4 and 5). These results suggest that MBF1 interacts with BmFTZ-F1 and promotes the formation of BmFTZ-F1•DNA complex. Because purified BmFTZ-F1 is not stable and gradually loses its DNA-binding activity even if it was stored at -135 °C, usually I use a fresh batch with full activity for the experiments within two weeks after preparation.

The observed effect of MBF1 could be due to either an increase in the rate of complex formation or stabilization

of the complex once formed. To test these possibilities, I examined the time course for the complex formation in the presence or absence of MBF1. No significant difference in the rate of complex formation was observed in the presence or absence of MBF1 (the time required for appearance of half the final number of complexes was 3 minutes in both cases). Instead, MBF1 increased the final number of complexes to form (Figure 7B). Next, I analyzed the stability of the complex. In the absence of MBF1, the BmFTZ-F1•DNA complex once formed was unstable and disappeared with a half life of about 30 min. On the contrary, no dissociation of the complex was detectable within 60 min. in the presence of MBF1 (Figure 7C). Further addition of MBF2 did not alter the results (data not shown). Essentially the same results were obtained using bacterially expressed and purified FTZ-F1 in place of BmFTZ-F1 (data not shown). From these results, I conclude that MBF1 stabilizes the BmFTZ-F1 (or FTZ-F1)•DNA complex by reducing the dissociation rate of the complex. Upon addition of MBF1, no qualitative changes were observed in the DNase I footprinting patterns by BmFTZ-F1 (data not shown).

Both MBF1 and MBF2 are Required to Form a Complex Containing BmFTZ-F1 and TBP

MBF1 and MBF2 mediate transactivation by BmFTZ-F1 (Figure 4A). It is possible that the mediators may serve as a bridge between BmFTZ-F1 and general transcription

machinery. To test the possibility, protein-protein interactions between the mediators and TBP were analyzed by adding MBF1 and MBF2 to gel mobility shift assays consisting of recombinant human (rh) TBP and a DNA fragment carrying the TATA element of the *ftz* gene. In the absence of TBP, neither MBF1 nor MBF2 produced a complex with the probe DNA (Figure 8A, lanes 2-4). When the probe DNA was incubated with TBP and electrophoresed in the presence of 2 mM $MgCl_2$, I observed a slow migrating complex (Figure 8A, lane 5). Only a marginal increase in the mobility of the TBP•TATA element complex was observed upon addition of MBF2 (Figure 8A, compare lanes 6 and 5, and also lanes 11 and 10). However, addition of MBF1 resulted in small but significant retardation of the TBP•TATA element complex (Figure 7A, compare lanes 7 and 5). Further addition of MBF2 did not change the results (Figure 8A, compare lanes 8 and 7). I repeated the assay for many times using different preparations of TBP and MBF1 including the gel purified 18 kd polypeptides, and found that the observed supershift by MBF1 is quite reproducible. These results suggest that MBF1 makes a direct contact with TBP.

The studies described above demonstrate that MBF1 stabilizes the BmFTZ-F1 (or FTZ-F1)•DNA complex and also binds to TBP and MBF2. This raises a possibility that BmFTZ-F1 (or FTZ-F1) can interact with TBP through these mediators. To test the possibility, I added the FTZ622 polypeptides bearing the DNA-binding region of FTZ-F1

to gel mobility shift assays containing rhTBP and the TATA element of the *ftz* gene. FTZ622 consists of a 116 amino acid region in FTZ-F1 encompassing the zinc finger motif and FTZ-F1 box, and binds to DNA with the same specificity as intact FTZ-F1 and BmFTZ-F1 (Ueda et al. 1992). I used FTZ622 in these experiments in place of BmFTZ-F1, because large amounts of bacterially expressed FTZ622 polypeptides were available in pure form. The amino acid sequence of FTZ622 is almost identical to the corresponding sequence of BmFTZ-F1 [91 % identity, 96 % similarity (Ueda et al. 1992)]. FTZ622 alone did not make association with the probe DNA (Figure 8, lane 9). Addition of FTZ622 to the binding reaction containing TBP, TBP plus MBF2, or TBP plus MBF1 did not change the mobility of TBP•TATA element complex or MBF1•TBP•TATA element complex (Figure 8, compare lanes 10 and 5, lanes 11 and 6, lanes 12 and 7, respectively). However, when I added FTZ622 to the mixture containing TBP, MBF1 and MBF2, further retardation of the protein•DNA complex occurred (Figure 8, compare lanes 13 and 8). These results suggest that both MBF1 and MBF2 are essential to form a complex containing FTZ-F1 and TBP. The presence of FTZ622 in this complex was confirmed by antibody binding. Thus, further addition of an antibody against FTZ-F1 to the mixture resulted in supershift of the protein•DNA complex (Figure 8, compare lanes 16 and 15), while preimmune serum did not cause the supershift (compare lanes 17 and 15). In the absence of

FTZ622, the anti-FTZ-F1 antibody did not affect the mobility of protein•DNA complex (Figure 8, compare lanes 18 and 14).

In order to get independent evidence for the interaction of BmFTZ-F1 (or FTZ-F1) with TBP through these mediators, I prepared TBP carrying 6-consecutive histidine residues (his-tag TBP). After incubation of his-tag TBP with mediators and FTZ622 or BmFTZ-F1, proteins interacted with TBP were collected using Ni-immobilized resin and resolved on an SDS-polyacrylamide gel. As shown in Figure 9A, radioactive FTZ622 was recovered from the resin only when his-tag TBP was incubated with MBF1, MBF2 and ^{32}P-labeled FTZ622 (Figure 9A, lane 4). Even in the presence of MBF1 and MBF2, radioactive FTZ622 was not detectable if his-tag TBP was omitted from the incubation mixture (Figure 9A, lane 5). Essentially similar results were obtained with BmFTZ-F1 as visualized on a Western blot (Figure 9B). From these results, I conclude that both MBF1 and MBF2 are required to form the specific complex containing BmFTZ-F1 (or FTZ-F1) and TBP.

Discussion

Using a functional *in vitro* transcription assay, I have identified and purified two factors (MBF1 and MBF2) that mediate transactivation by BmFTZ-F1. MBF1 makes direct contacts with TBP and MBF2, and stabilizes the BmFTZ-F1 (or FTZ-F1)•DNA complex. Both MBF1 and MBF2 stabilize TBP binding to the TATA element and are required to form the complex containing FTZ-F1, TBP and the promoter.

Based on these data, I propose a following model for transactivation by BmFTZ-F1 (Figure 10). TBP associated with TAFs binds to the TATA element in the core promoter. BmFTZ-F1 binds to the FTZ-F1 recognition site in the regulatory element located away from the core promoter. MBF1 and MBF2 form a heterodimer and serve as bridging molecules that connect BmFTZ-F1 and TBP. The resulting juxtaposition of the regulatory element with the core promoter is further stabilized by the actions of MBF1 to TBP and BmFTZ-F1, which in turn allows induced level of transcription by RNA polymerase II and general transcription factors. In the absence of MBF1, MBF2 activated BmFTZ-F1-independent transcription from both the *ftz* promoter and Ad_2MLP. It is possible that MBF2 stimulates transcription through direct interactions with some component of the basal transcription machinery. Alternatively, it may titrate a negative acting factor.

In any case, BmFTZ-F1 and MBF1 can recruit MBF2 to form an active preinitiation complex on a promoter carrying the FTZ-F1-binding site.

The original view of a mediator was that it may not affect transcription without a transactivator. MBF2 is not consistent with the view. However, I considered it as a component of mediator because both MBF1 and MBF2 are required to mediate transactivation by BmFTZ-F1. At least four other protein factors essential for mediating transactivation, PC1, NC1, Dr2, and TFIID subunit (p230), have been shown to affect basal level of transcription (Kokubo et al.; 1993; Meisterernst et al., 1991; Merino et al., 1993).

The protein-protein interactions in the model were based on the results of gel filtration, gel mobility shift assays and protein-binding assays using his-tag TBP. I confirmed these interactions by using binding of histidine-tagged FTZ-F1 to Ni-immobilized resin. TBP was recovered from the resin only when histidine-tagged FTZ-F1 was incubated with MBF1, MBF2 and TBP or TFIID fraction partially purified from HeLa cells (data not shown). These results also suggest that FTZ-F1 can interact with TBP through MBF1 and MBF2 when TBP is present in the TFIID complex. In these protein-protein interactions, heterodimerization between MBF1 and MBF2 occurred in the absence of $MgCl_2$ but formation of the complex containing TBP, MBF1, MBF2 and BmFTZ-F1 (or FTZ-F1) required $MgCl_2$. This may explain why I was unable to

detect further retardation of the BmFTZ-F1•DNA complex upon addition of MBF1 and MBF2 in gel mobility shift assays without $MgCl_2$ (Figure 7A).

Three classes of mediators or coactivators have been reported (Lewin, 1990; Roeder, 1991; Gill and Tjian, 1992). The TAFs are a group of proteins that are required for activation by many DNA-binding transcription factors such as SP1, CTF and NTF-1. They are tightly associated with TBP and can only be removed from the TFIID by a guanidine treatment (Tanese et al., 1991; Dynlacht et al., 1991; Hoey et al., 1993). Neither MBF1 nor MBF2 appears to be TAFs because these mediators can be separated from TFIID by chromatography on a Heparin-Sepharose column. The second group consists of adaptors or mediators that are required for induced but not basal level of transcription in a reconstituted yeast transcription system (Flanagan et al., 1991; Berger et al., 1991). These mediators are also not tightly associated with TBP but further biochemical studies are necessary to clarify the nature of these activities. In the third group, the viral activator E1A has been shown to interact directly with both TBP and a DNA-binding transcription factor such as ATF-2 or Oct-4, and mediate transactivation (Liu and Green, 1990; Lee et al., 1991; Horikoshi et al., 1991; Schöler et al., 1991). Another viral activator VP16 also binds to TBP and TFIIB, and is expected to play a similar role as E1A (Stringer et al., 1990; Lin and Green, 1991). The cellular activity termed USA has been shown to interact directly to TBP and mediate

transactivation by USF and SP1 (Meisterernst et al., 1991). Another cellular protein Dr2 binds to TBP and inhibits basal level of transcription Dr2 is required for high levels of transcription in response to an activator (Merino et al.,1993). These viral and cellular proteins (or activity) share some common features with MBF1 and MBF2. For example, they all make a loose association with TBP. Most of them have been shown or expected to serve as bridge molecules that link the regulatory protein to the general transcription factors. The present study reveals that MBF1 can stabilize protein•DNA interactions at the regulatory element. I propose that mediators play a crucial role in the regulation of transcription by establishing the stable juxtaposition of the two promoter elements.

The embryonal long terminal repeat-binding protein, ELP, is a murine homologue of FTZ-F1. It recognizes and binds to the same DNA sequence as FTZ-F1 and BmFTZ-F1 (Tsukiyama et al.,1992). ELP suppresses transcription of the Molony leukemia virus long terminal repeat (Tsukiyama et al., 1989) while FTZ-F1 and BmFTZ-F1 activate transcription of the *ftz* gene (Ueda et al., 1990; the present study). What determines these opposite effects on transcription? It is possible that these sequence-specific DNA-binding proteins have only a mediator-binding domain but lack an activation (or silencing) domain. When positive mediators as shown in the present study bind to them, they will behave as positive regulators. If mediators with

inhibitory effect bind to them, they will suppress transcription in a sequence-specific manner. Indeed, Ueda and Hirose isolated an unknown gene of *Drosophila* which seems to be negatively regulated by FTZ-F1. It carries the FTZ-F1-binding site in the upstream region and is expressed in the developmental stages of embryo when FTZ-F1 is absent but is repressed when FTZ-F1 is present (H.Ueda and S. Hirose, unpublished). Activation of a set of genes and simultaneous repression of other group of genes by a nuclear hormone receptor appear to be important for the control of sequential gene activation during development (Ashburner et al., 1990).

Acknowledgments

I wish to express my deep gratitude to Prof. S. Hirose for his supports, helpful discussions and supervision. I thank Prof. A. Ishihama for his advice and encouragement. I also thank Drs. H. Ueda, S. Hayashi and S. Hirose for critical reading of the manuscript. I thank Drs. H. Ueda, S. Hayashi, T. Ohta, G.-C. Sun and other members in the S. Hirose's Laboratory for discussions .

I thank Drs. H. Handa for providing a cDNA clone encoding hTBP, C. Wu for a gift of anti-FTZ-F1 antibody. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and by the Joint Studies Program of the Graduate University for Advanced Studies to H.Ueda and S. Hirose. I was supported by a fellowship from the Fujisawa Foundation.

References

Ashburner, M. (1990) Puffs, genes, and hormones revisited. Cell 61: 1-3

Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg, and L. Guarente (1990) Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: Evidence for transcriptional adaptors. Cell 61: 1199-1208

Dignam, J. D., P. L. Martin, B. S. Shastry, and R. G. Roeder (1983) Eukaryotic gene transcription with purified components. Meth. Enzymol. 101: 582-598

Dynlacht, B. D., T. Hoey, and R. Tjian (1991) Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. Cell 66: 563-576

Flanagan, P. M., R. J. Kelleher III, M. H. Sayre, H. Tschochner, and R. D. Kornberg (1991) A mediator required for activation of RNA polymerase II transcription *in vitro*. Nature 350: 436-438

Gill, G. and R. Tjian (1992) Eukaryotic coactivators associated with the TATA box binding protein. Curr. Opin. Genet. Dev. 2: 236-242

Grueneberg, D. A., S. Natesan, C. Alexandre, and M. Z. Gilman (1992) Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum response factor. Science 257: 1089-1095

G.-.C. Sun, S. Hirose and Ueda, H. (1993). Intermittent expression of BmFTZ-F1, a member of the nuclear hormone receptor superfamily during development of the silkworm *Bombyx mori*. Develop. Biol. in press

Hager, D. A. and R. R. Burgess (1980) Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: Results with sigma subunits of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. 109: 76-86

Handa, H. and P. A. Sharp (1984) Requirement for distal upstream sequences for maximal transcription *in vitro* of early region IV of adenovirus. Mol. Cell. Biol. 4: 791-794

Hirose, S. and Y. Suzuki (1988) *In vitro* transcription of eukaryotic genes is affected differently by the degree of DNA supercoiling. Proc. Natl. Acad. Sci. USA 85: 718-722

Hirose, S., M. Tsuda, and Y. Suzuki (1985) Enhanced transcription of fibroin gene *in vitro* on covalently closed circular templates. J. Biol. Chem. 260: 10557-10562

Hoey, T., R. O. J. Weinzierl, G. Gill, J.-L. Chen, B. D. Dynlacht, and R. Tjian (1993) Molecular cloning and functional analysis of *Drosophila* TAF110 reveal properties expected of coactivators. Cell 72: 247-260

Hoffmann, A., E. Sinn, T. Yamamoto, J. Wang, A. Roy, M. Horikoshi, and R. G. Roeder (1990) Highly conserved core domain and unique N terminus with presumptive regulatory motifs in a human TATA factor (TFIID). Nature 346: 387-390

Horikoshi, M., M. F. Carey, H. Kakidani, and R. G. Roeder (1988) Mechanism of action of a yeast activator: Direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell 54: 665-669

Horikoshi, M., T. Hai, Y.-S. Lin, M. R. Green, and R. G. Roeder (1988) Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54: 1033-1042

Horikoshi, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann (1991) Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. Proc. Natl. Acad. Sci. USA 88: 5124-5128

Inomata, Y., H. Kawaguchi, M. Hiramoto, T. Wada, and H. Handa (1992) Direct purification of multiple ATF/E4TF3 polypeptides from HeLa cell crude nuclear extracts using DNA affinity latex particles. Anal. Biochem. 206: 109-114

Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg (1992) Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. Cell 70: 477-489

Johnson, P. and S. McKnight (1989) Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58: 799-839

Kawaguchi, H., Asai, A., Ohtsuka, Y., Watanabe, H., Wada, T., and Handa, H., (1989) Purification of DNA-binding transcription factors by their selective adsorption on the affinity latex particles. Nucl. Acids Res. 17:6229-6240.

Lavorgna, G., H. Ueda, J. Clos, and C. Wu (1991) FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. Science 252: 848-851

Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk (1991) Adenovirus E1A activation domain binds the basic repeat in the TATA Box transcription factor. Cell 67: 365-376

Lewin, B. (1990) Commitment and activation at pol II promoters: A tail of protein-protein interactions. Cell 61: 1161-1164

Lieberman, P. M. and A. J. Berk (1991) The Zta transactivator protein stabilizes TFIID association with promoter DNA by direct protein-protein interaction. Genes Dev. 5: 2441-2454

Lin, Y.-S. and M. R. Green (1991) Mechanism of action of an acidic transcriptional activator *in vitro*. Cell 64: 971-981

Liu, F. and M. R. Green (1990) A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. Cell 61: 1217-1224

Maldonado, E., I. Ha, P. Cortes, L. Weis, and D. Reinberg (1990) Factors involved in specific transcription by mammalian RNA polymerase II: Role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex. Mol. Cell. Biol. 10: 6335-6347

Meisterernst, M., A. L. Roy, H. M. Lieu, and R. G. Roeder (1991) Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. Cell 66: 981-993

Mitchell, P. J. and R. Tjian (1989) Transcriptional regulation in cells by sequence-specific DNA binding proteins. Science 245: 371-378

Ptashne, M. and A. A. F. Gann (1990) Activators and targets. Nature 346: 329-331

Pugh, B. F. and R. Tjian (1990) Mechanism of transactional activation by Sp1: Evidence for coactivators. Cell 61: 1187-1197

Roeder, R. G. (1991) The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. Trends Biochem. Science 16: 402-408

Sambrook, J., E. F. Fritsch, and T. Maniatis. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sawadogo, M. and R. G. Roeder (1985) Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43: 165-175

Sawadogo, M. and A. Sentenac (1990) RNA polymerase B(II) and general transcription factors. Annu. Rev. Biochem. 59: 711-754

Schmidt, M. C., Kao, C. C. Pei, R. and Berk, A. J. (1989) Yeast TATA-box transcription factor gene. Proc. Natl Acad. Sci. USA, 86, 7785-7789

Schöler, H. R., T. Ciesiolka, and P. Gruss (1991) A nexus between Oct-4 and E1A: Implications for gene regulation in embryonic stem cells. Cell 66: 291-304

Stringer, K. F., C. J. Ingles, and F. Greenblatt (1990) Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature 345: 783-786

Studier, F. W., and B. A. Mpffatt (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189: 113-130

Tanese, N., B. F. Pugh, and R. Tjian (1991) Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev. 5: 2212-2224

Tsuda, M. and Y. Suzuki (1981) Faithful transcription initiation of fibroin gene in a homologous cell-free system reveals an enhancing effect of 5' flanking sequence far upstream. Cell 27: 175-182

Tsukiyama, T., O. Niwa, and K. Yokoro (1989) Mechanism of suppression of the long terminal repeat of Moloney Leukemia Virus in mouse embryonal carcinoma cells. Mol. Cell. Biol. 9: 4670-4676

Tsukiyama, T., H. Ueda, S. Hirose, and O. Niwa (1992) Embryonal long terminal repeat-binding protein is a murine homologue of FTZ-F1, a member of the steroid receptor superfamily. Mol. Cell. Biol. 12: 1286-1291

Ueda, H. and S. Hirose (1990) Identification and purification of a *Bombyx mori* homologue of FTZ-F1. Nucl. Acids Res. 18: 7229-7234

Ueda, H. and S. Hirose (1991) Defining the sequence recognized with BmFTZ-F1, a sequence specific DNA binding factor in the silkworm, *Bombyx mori*, as revealed by direct sequencing of bound oligonucleotides and gel mobility shift competition analysis. Nucl. Acids Res. 19: 3689-3693

Ueda, H., S. Sonoda, J. L. Brown, M. P. Scott, and C. Wu (1990) A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. Genes Dev. 4: 624-635

Ueda, H., G.-C. Sun, T. Murata, and S. Hirose (1992) A Novel-DNA binding motif abuts the zinc finger domain of

insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein. Mol. Cell. Biol. 12: 5667-5672

Usuda, Y., A. Kubota, A. J. Berk, and H. Handa (1991) Affinity purification of transcription factor IIA from HeLa cell nuclear extracts. EMBO J. 10: 2305-2310

Figure Legends

Figure 1. Binding-Site Dependent Activation of *In Vitro* Transcription by BmFTZ-F1.

(A) Structure of the three DNA templates used in the *in vitro* transcription reactions. The wild-type template carries DNA sequence of the *Drosophila ftz* gene from -617 to +124 relative to the transcription initiation site (+1). It has a single binding site for FTZ-F1 at around 280 base pairs upstream from the initiation site. The mutant template carries two (5-15) or four (5-5) base substitutions in the FTZ-F1 recognition site as indicated. After the transcription reaction, RNA was analyzed by a modified nuclease S1 assay as described in Experimental Procedures. Transcripts from the *ftz* gene should yield a protected band of 318 base.

(B) Transcription in the posterior silk gland extract. The wild-type (lane 1) and the mutant templates 5-5 (lane 2) and 5-15 (lane 3) were transcribed in the posterior silk gland extract. Each reaction also contained the fibroin gene template as an internal control. Transcripts from the fibroin gene (510 base) and the *ftz* gene (318 base) are indicated by arrows.

(C) Transcription in the BmFTZ-F1-depleted posterior silk gland extract. The wild-type *ftz* and the fibroin genes were transcribed in the posterior silk gland extract (lane 1) or the same extract from which BmFTZ-F1 had been depleted

(lanes 2-5). Indicated amounts of purified BmFTZ-F1 were added into the depleted extract in lanes 3-5.

Figure 2. Transcriptional Activation by BmFTZ-F1 Requires Another Component in the Posterior Silk Gland Extract.

(A) Transcription in the HeLa nuclear extract. The wild-type (lane 1) and the mutant templates 5-5 (lane 2) and 5-15 (lane 3) were transcribed in the HeLa nuclear extract. DNA carrying the Ad2MLP was added to the reaction as an internal control. Transcripts from the *ftz* gene (318 base) and the Ad2MLP (197 base) are indicated by arrows.

(B) Transcription in the HeLa nuclear extract supplemented with purified BmFTZ-F1. The wild-type *ftz* gene and the Ad2MLP were transcribed in the HeLa nuclear extract supplemented with 0 (lane 1), 25 (lane 2), 50 (lane 3) and 100 ng (lane 4) of purified BmFTZ-F1.

(C) Transcriptional activation by BmFTZ-F1 in the presence of components in the posterior silk gland extract. The wild-type *ftz* gene and the Ad2MLP were transcribed in the HeLa nuclear extract supplemented with 50 ng of purified BmFTZ-F1. The transcription reactions in lanes 2-5 also contained indicated amounts of the Heparin-Sepharose fraction derived from the posterior silk gland extract.

Figure 3. Purification of the Mediators of BmFTZ-F1.

(A) Purification scheme for the mediators. Shown is the strategy used for fractionation of the mediators from the posterior silk gland whole cell extract (psgWCE). BmFTZ-F1 was also purified from the eluate of the Heparin-Sepharose column by two cycles of absorption to and elution from latex beads carrying oligomers of the FTZ-F1 recognition site. For details, see Experimental Procedures.

(B) SDS-polyacrylamide gel electrophoresis of purified MBF1 and MBF2. Each 20 ng of purified MBF1 (lane 1) or MBF2 (lane 2) was electrophoresed on a 12.5 % SDS-polyacrylamide gel and visualized by silver staining.

Figure 4. Functional Analyses of MBF1 and MBF2 by *In Vitro* Transcription.

(A) MBF1 and MBF2 support transcriptional activation by BmFTZ-F1. The wild-type (lanes 1-4) or the mutant template 5-5 (lanes 5-6) as well as the Ad2MLP template were transcribed in the HeLa nuclear extract. The transcription reactions also contained 50 ng of purified BmFTZ-F1 and each 25 ng of purified MBF1 and MBF2 as indicated.

(B) MBF2 also activates transcription from the Ad2MLP in the absence of MBF1. The wild-type *ftz* gene (lanes 1-3) or the Ad2MLP (lanes 4-6) was transcribed in the HeLa nuclear extract in the absence (lanes 1 and 4) or presence of 25 (lanes 2 and 5) or 50 ng (lanes 3 and 6) of purified MBF2.

(C) Gel purified 18 kd and 22 kd polypeptides mediate transactivation by BmFTZ-F1. Each 2 μg of purified MBF1 or MBF2 was resolved by SDS-polyacrylamide gel electrophoresis. The 18 kd and 22 kd polypeptides were separately recovered from the gel and, after renaturation, used for transcription of the wild-type *ftz* gene and the Ad_2MLP in the HeLa nuclear extract with or without 50 ng of purified BmFTZ-F1 as indicated.

Figure 5. MBF1 and MBF2 Form a Complex in the Absence of DNA.

Each 1 μg of purified MBF1 or MBF2 was subjected to gel filtration on a Superose 12 column in a SMART system either separately (top and middle panels) or after mixing and incubation on ice for 15 min. (bottom panel) as described in Experimental Procedures. Ten microliter of each fraction was analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and visualized by silver staining. Only fractions with retention times from 30 to 45 min. were shown. O, C, and L represent the elution positions of ovalbumin (43 kd), chymotrypsinogen (25 kd), and lysozyme (14 kd), respectively.

Figure 6. Neither MBF1 nor MBF2 Binds to DNA.

(A) The binding of MBF1 or MBF2 to a specific DNA sequence in the *ftz* promoter was analyzed by a gel mobility shift assay in the presence of 4 mM $MgCl_2$. Each binding mixture containing 25 ng of purified MBF1 or MBF2 or

BmFTZ-F1 as indicated was incubated at 25 °C for 30 min. with ^{32}P-labeled various DNA fragments derived from the *ftz* promoter. The specific protein•DNA complexes (Bound) were separated from the free probe (Free) by 1 % agarose gel electrophoresis.

(B) The binding of MBF1 or MBF2 to the specific DNA sequence in the *ftz* promoter was analyzed as in (A) except that the binding mixture was incubated in the absence of $MgCl_2$.

Figure 7. MBF1 Stabilizes BmFTZ-F1 Binding to Its Recognition Site.

(A) Effect of MBF1 and MBF2 on BmFTZ-F1•DNA interactions. The binding of purified BmFTZ-F1 to a ^{32}P-labeled FTZ-F1-binding site oligonucleotide was analyzed by a gel mobility shift assay. Each binding mixture containing 10 ng of purified BmFTZ-F1 and indicated amounts of purified MBF1 and/or MBF2 was incubated at 25 °C for 30 min. The specific protein•DNA complexes (Bound) were separated from the free probe (Free) by 1 % agarose gel electrophoresis.

(B) Time course of BmFTZ-F1 binding to DNA. The radioactive FTZ-F1-binding site probe was incubated with 10 ng of purified BmFTZ-F1 in the presence (closed circles) or absence (open circles) of 25 ng of purified MBF1. After 2, 4, 8, 16 and 30 min. of incubation, the specific protein-DNA complexes were resolved by agarose gel electrophoresis and their radioactivities were measured.

(C) Stability of the BmFTZ-F1•DNA complex. The radioactive FTZ-F1-binding site probe was preincubated with 10 ng of purified BmFTZ-F1 in the presence (closed circles) or absence (open circles) of 25 ng of purified MBF1 for 15 min. After addition of 20-fold excess non-radioactive probe, the incubation was continued and, at indicated times, the specific protein-DNA complexes were anlyzed as in (B).

Figure 8. Interactions of MBF1, MBF2 and FTZ622 with rhTBP.

^{32}P-labeled oligonucleotide bearing the TATA element of the *ftz* gene was incubated with various combinations of proteins. When indicated, the incubation mixture contained 6 ng of purified rhTBP, 25 ng of purified MBF1, 25 ng of purified MBF2 and/or 0.5 ng of FTZ622. α-FTZ-F1 and pre-serum represent rabbit antiserum against FTZ-F1 and its preimmune serum, respectively. The specific protein-DNA complexes were resolved by electrophoresis on 1 % agarose gels containing 2 mM $MgCl_2$. The samples in lanes 1-13 were electrophoresed on the same gel but those in lanes 14-18 were on the other gel.

Figure 9. Analyses of protein-protein interactions using his-tag TBP.

(A) Formation of complex containing FTZ622 and rhTBP. [^{32}P]FTZ622 (2 ng) was incubated with various combinations of proteins and subjected to protein-binding assay using

his-tag TBP. When indicated, the incubation mixture contained 50 ng of purified his-tag TBP, 25 ng of purified MBF1 and/or 25 ng of purified MBF2. The proteins bound to Ni-immobilized resin were resolved on an SDS-12.5 % polyacrylamide gel and [^{32}P]FTZ622 was detected by autoradiography.

(B) Formation of complex containing BmFTZ-F1 and rhTBP. The same as in (A) except that 100 ng of BmFTZ-F1 was used in place of FTZ622. The bound proteins were resolved on an SDS-8 % polyacrylamide gel and BmFTZ-F1 was visualized on a Western blot using rabbit anti-FTZ-F1 antibody and goat anti-rabbit IgG alkaline phosphatase conjugate (Promega).

Figure 10. Model for transcriptional activation through MBF1 and MBF2.

MBF1 and MBF2 serve as a bridge that connects between BmFTZ-F1 and TBP. These interactions lead to stable juxtaposition of the regulatory element with the core promoter as a prelude to induced level of transcription.

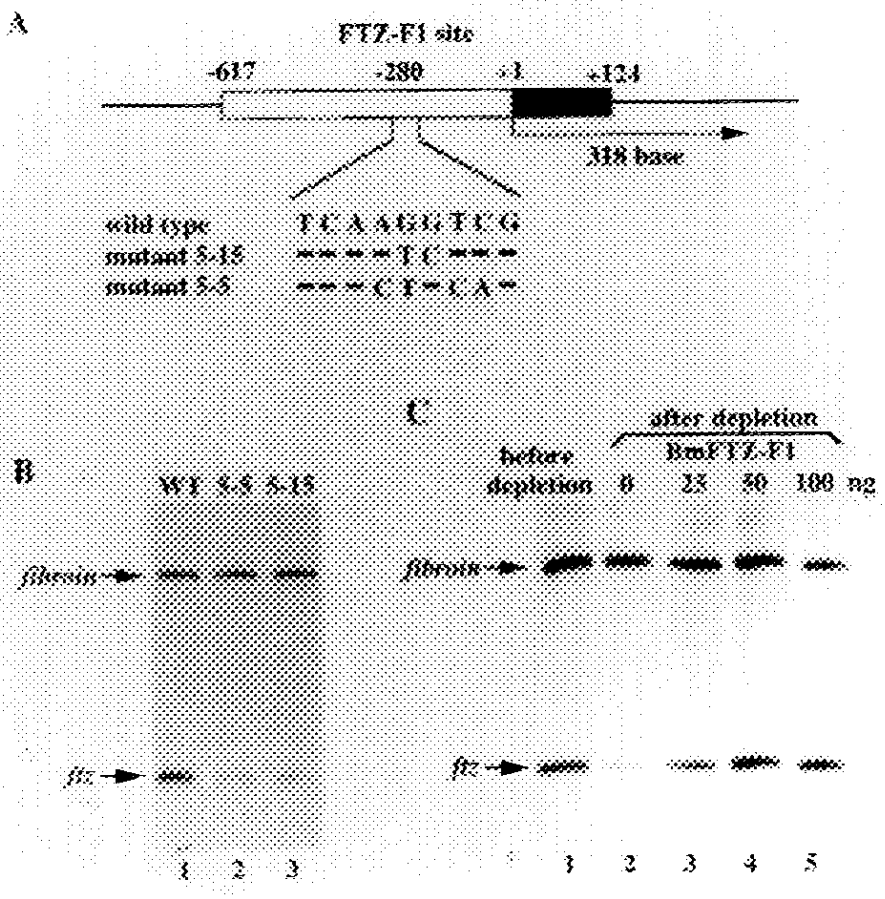

A
FTZ-F1 site
-617
-280
+1
+124
338 base
wild type
mutant 5-15
mutant 5-5
B
W1
5-5
5-15
fibroin
ftz
1
2
3
C
before depletion
after depletion
BmFTZ-F1
0
23
30
100 ng
fibroin
ftz
1
2
3
4
5

Figure 1

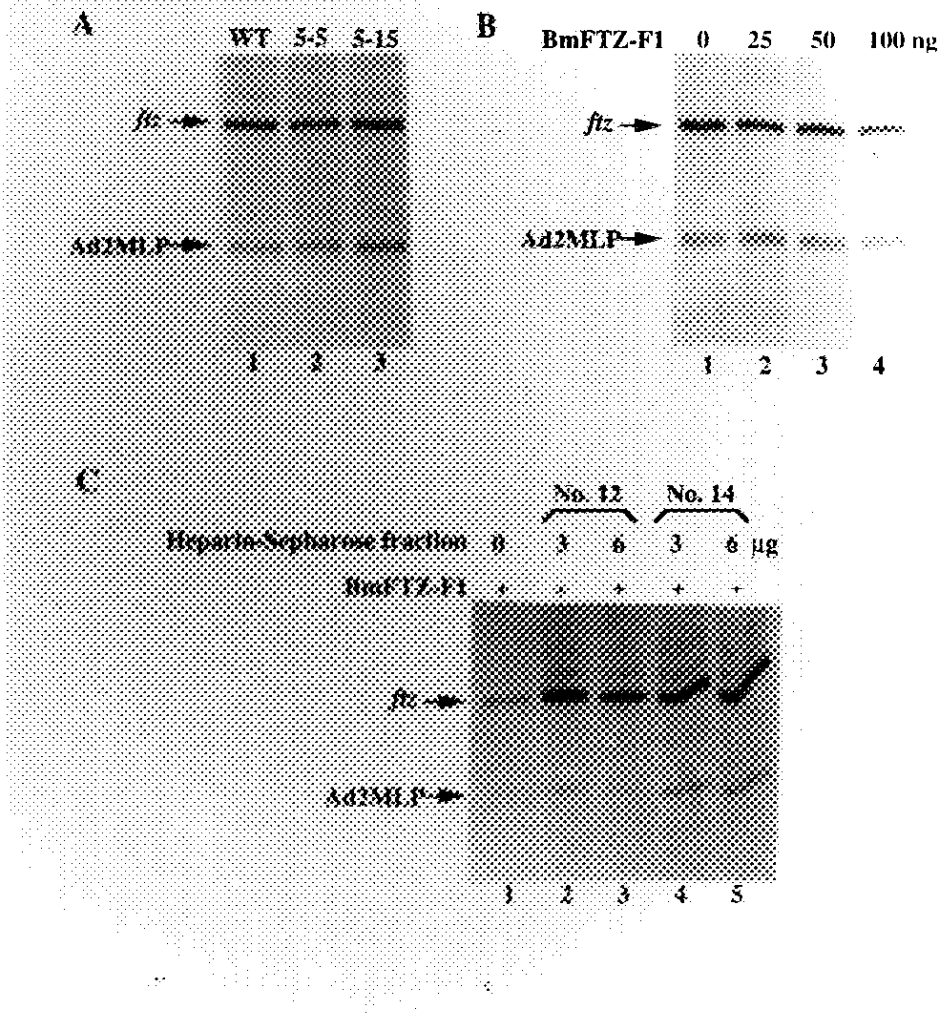

A
WT
5-5
5-15
ftz
Ad2MLP
1
2
3
B
BmFTZ-F1
0
25
50
100 ng
ftz
Ad2MLP
1
2
3
4
C
No. 12
No. 14
Heparin-Sepharose fraction
0
3
6
3
6
μg
BmFTZ-F1
+
-
+
+
+
ftz
Ad2MLP
1
2
3
4
5

Figure 2

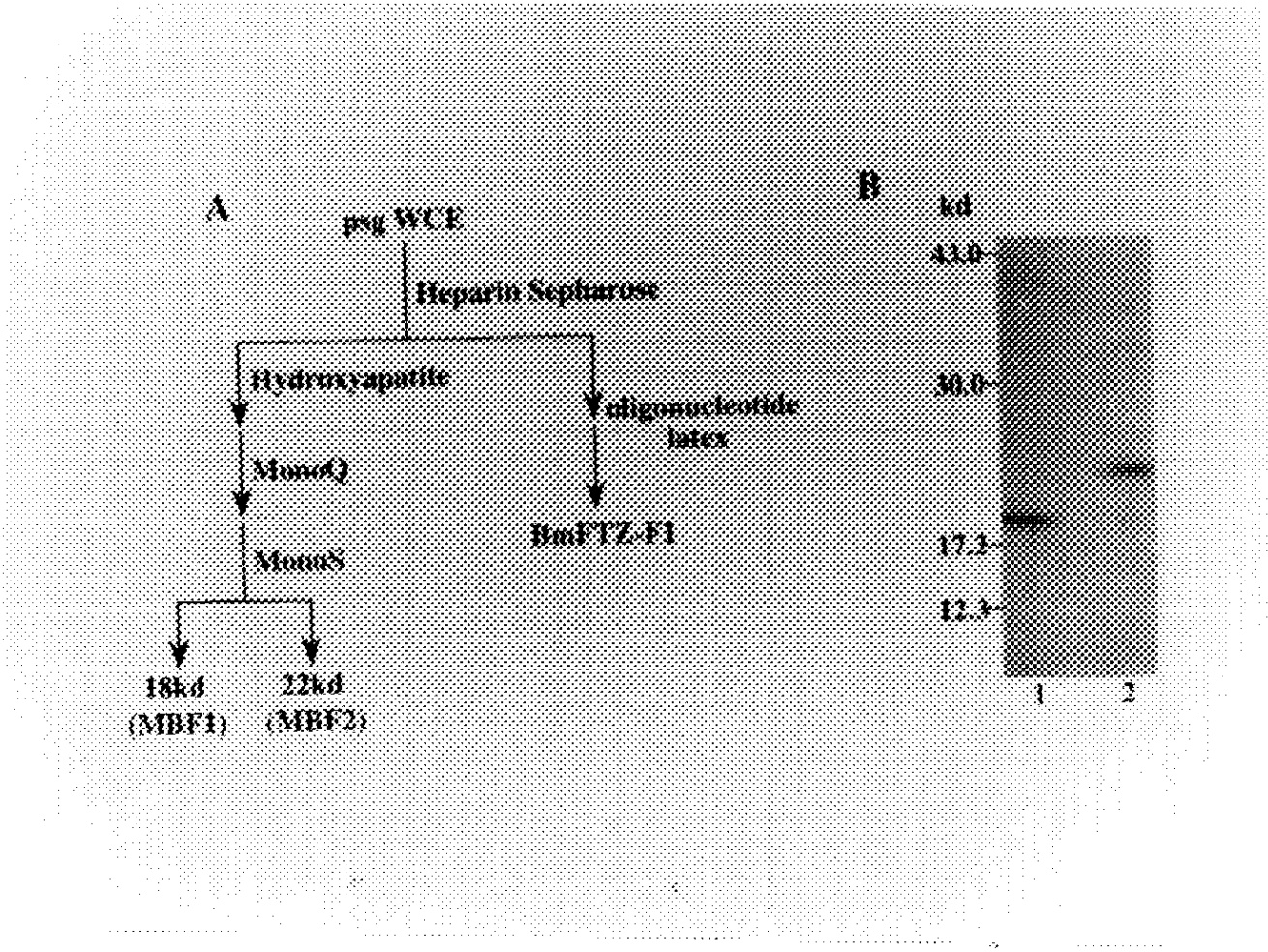

A
psg WCE
Heparin Sepharose
Hydroxyapatite
oligonucleotide latex
Mono Q
BmFTZ-F1
Mono S
18kd (MBF1)
22kd (MBF2)
B
kd
43.0
30.0
17.2
12.3
1
2

Figure 3

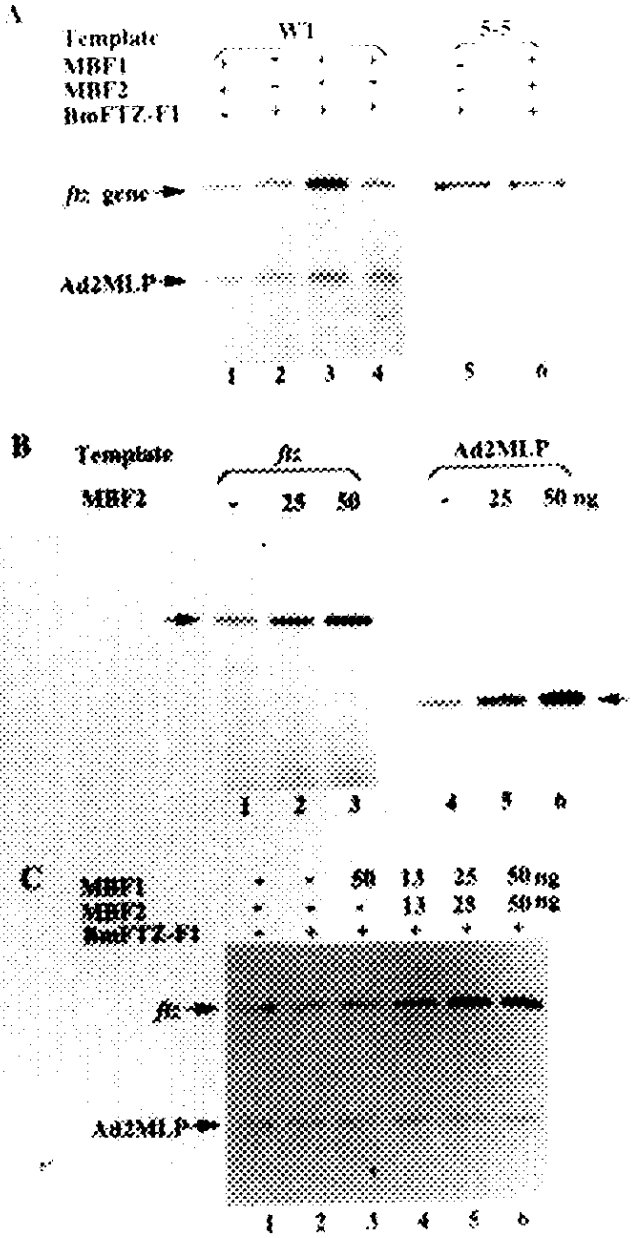

A
Template
WT
5-5
MBF1
MBF2
BmFTZ-F1
ftz gene
Ad2MLP
1 2 3 4 5 6
B
Template
ftz
Ad2MLP
MBF2
- 25 50
- 25 50 ng
1 2 3 4 5 6
C
MBF1
MBF2
BmFTZ-F1
50 13 25 50 ng
13 25 50 ng
ftz
Ad2MLP
1 2 3 4 5 6

Figure 4

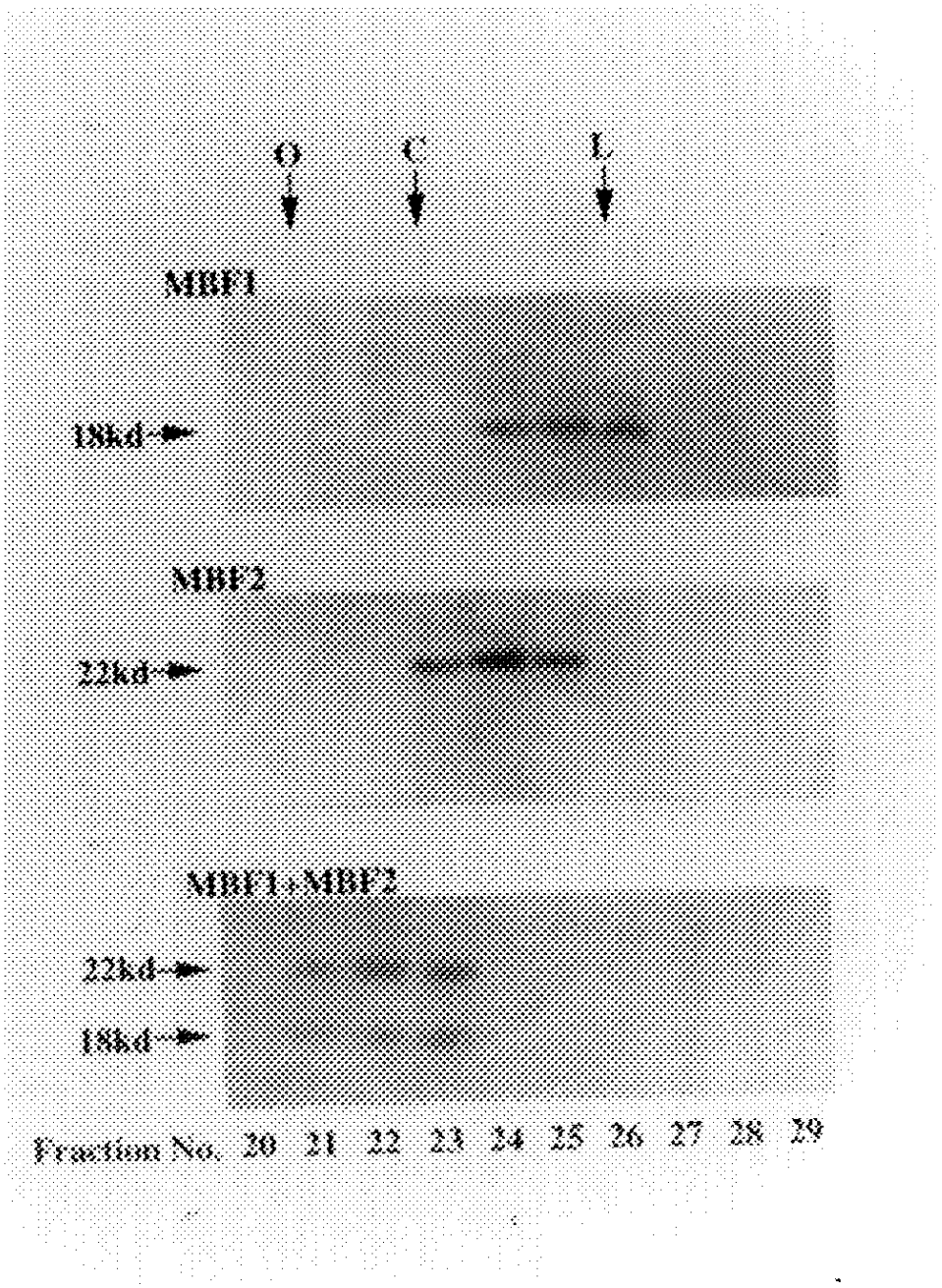
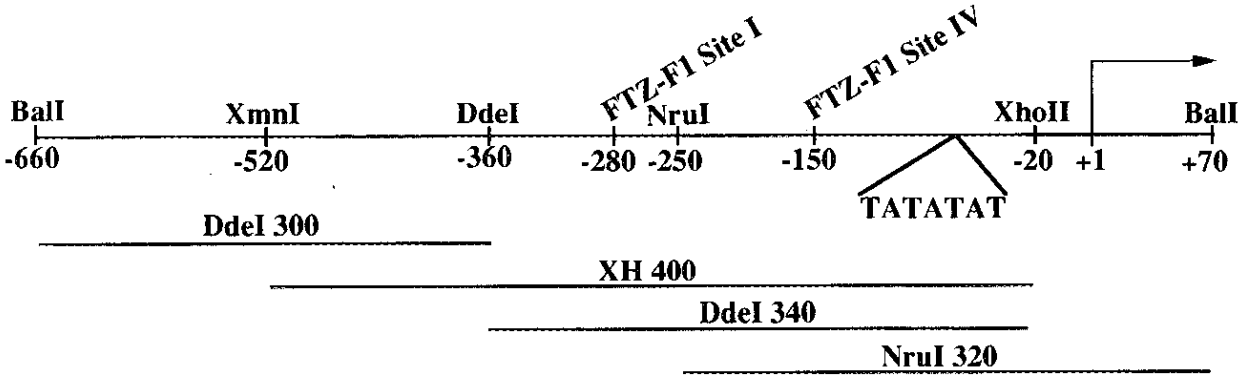
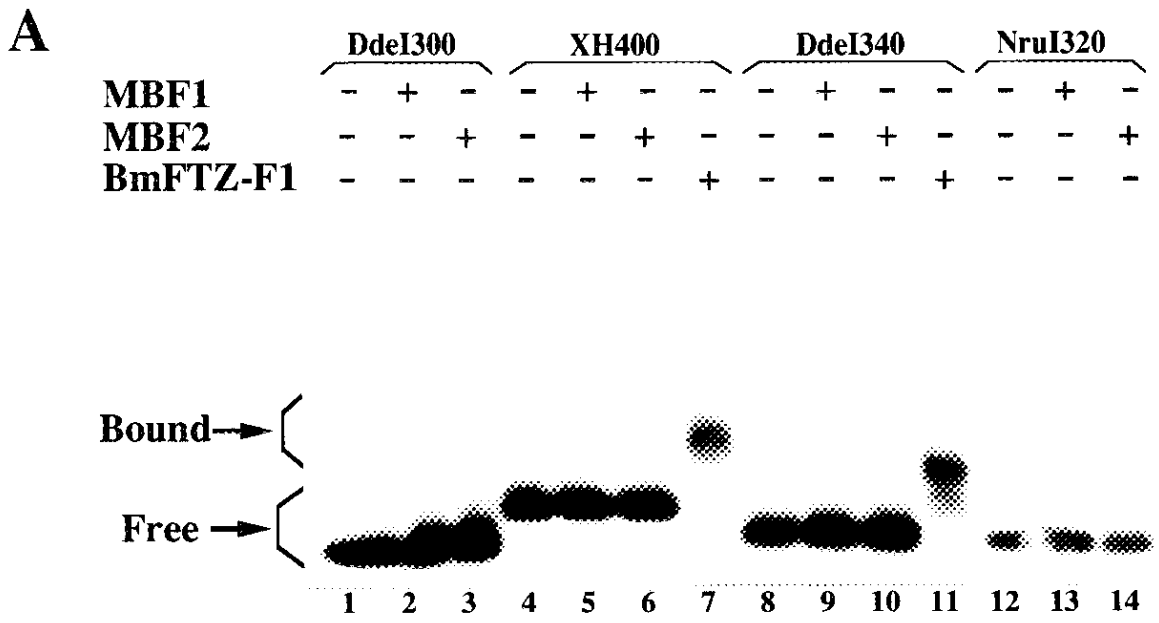

O
C
L
MBF1
18kd
MBF2
22kd
MBF1+MBF2
22kd
18kd
Fraction No. 20 21 22 23 24 25 26 27 28 29

Figure 5


BalI
XmnI
DdeI
FTZ-F1 Site I
NruI
FTZ-F1 Site IV
XhoII
BalI
-660
-520
-360
-280
-250
-150
-20
+1
+70
TATATAT
DdeI 300
XH 400
DdeI 340
NruI 320


A
DdeI300
XH400
DdeI340
NruI320
MBF1
MBF2
BmFTZ-F1
Bound
Free
1 2 3 4 5 6 7 8 9 10 11 12 13 14

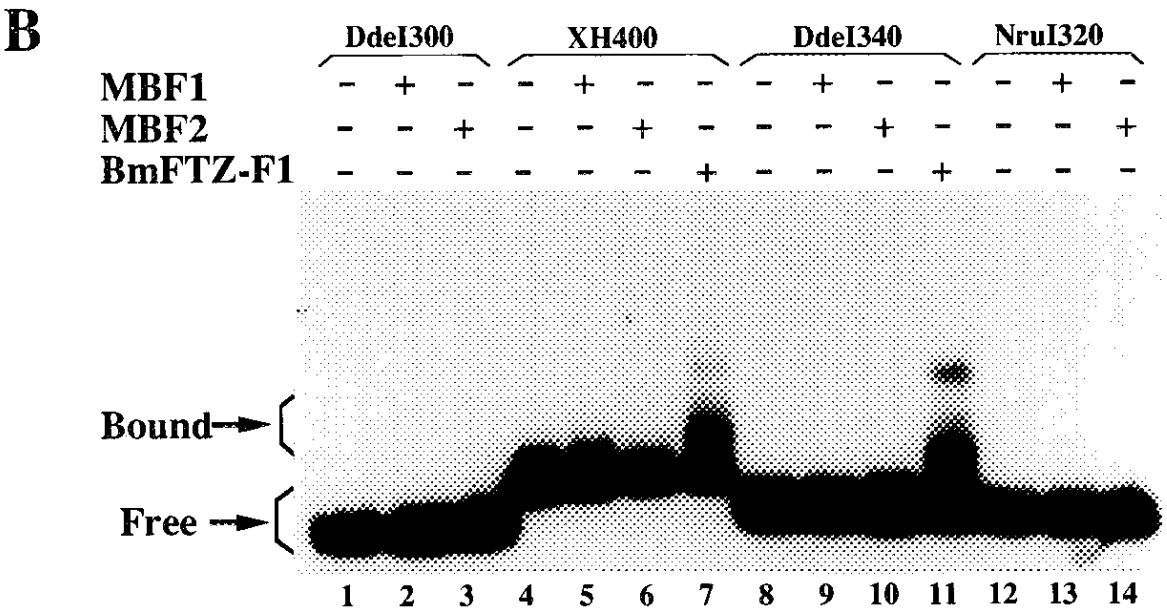
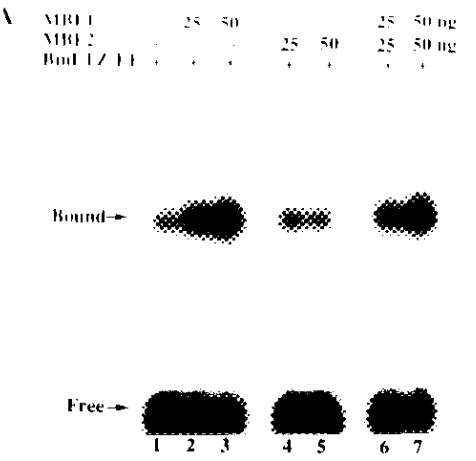
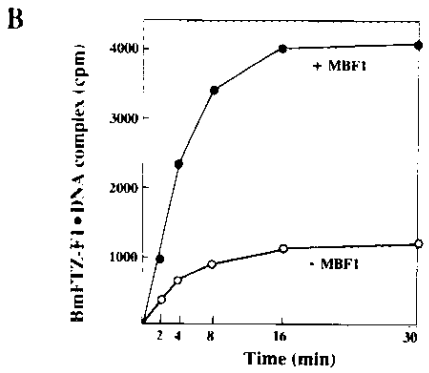

B
DdeI300
XH400
DdeI340
NruI320
MBF1
MBF2
BmFTZ-F1
Bound
Free
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 6


A
MBF1 25 50 25 50 ng
MBF2 25 50 25 50 ng
BmFTZ-F1 + + + + + +
Bound
Free
1 2 3 4 5 6 7


B
BmFTZ-F1•DNA complex (cpm)
4000
3000
2000
1000
+ MBF1
- MBF1
2 4 8 16 30
Time (min)

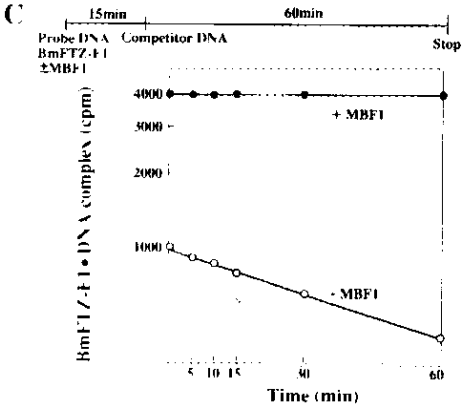

C
15min
60min
Probe DNA Competitor DNA
BmFTZ-F1
±MBF1
Stop
BmFTZ-F1•DNA complex (cpm)
4000
3000
2000
1000
+ MBF1
- MBF1
5 10 15 30 60
Time (min)

Figure 7

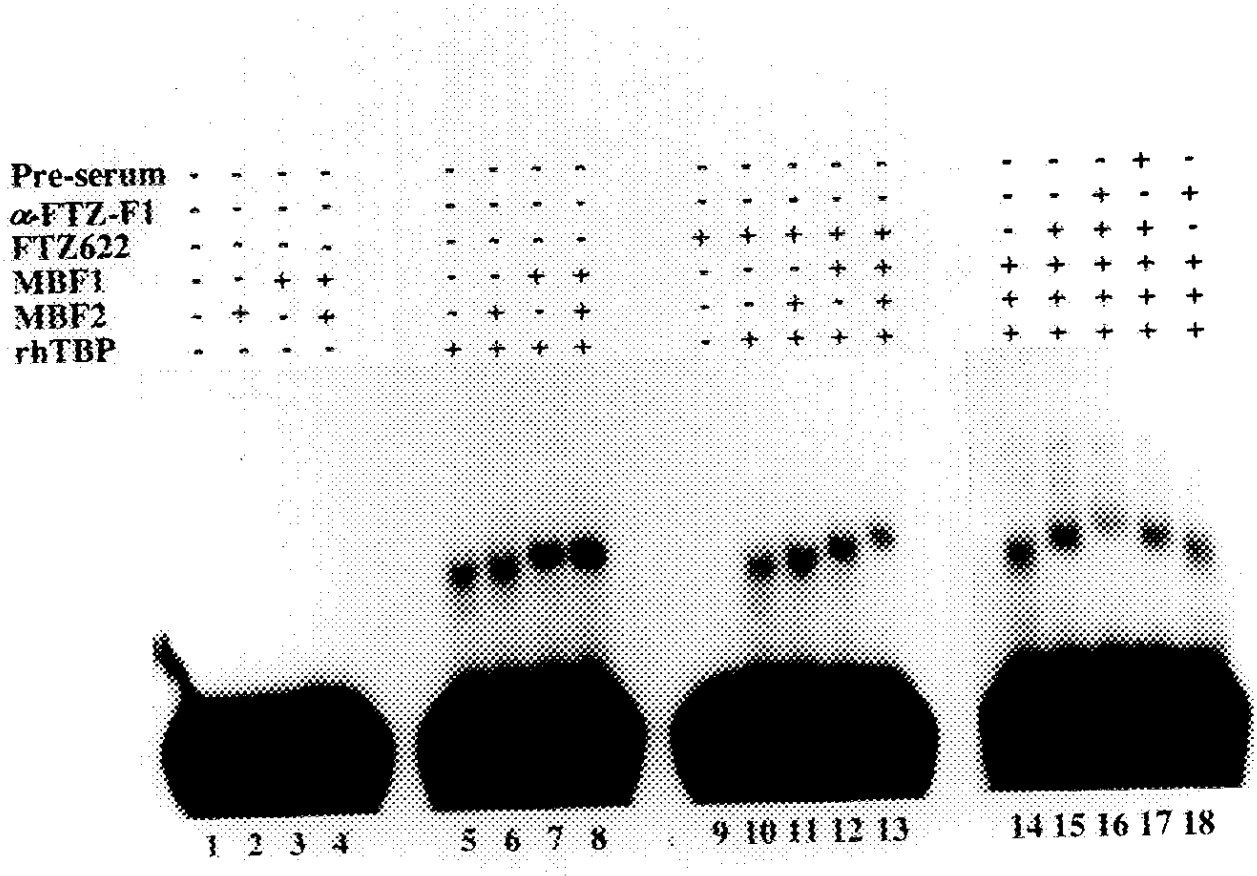

Pre-serum
α-FTZ-F1
FTZ622
MBF1
MBF2
rhTBP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 8

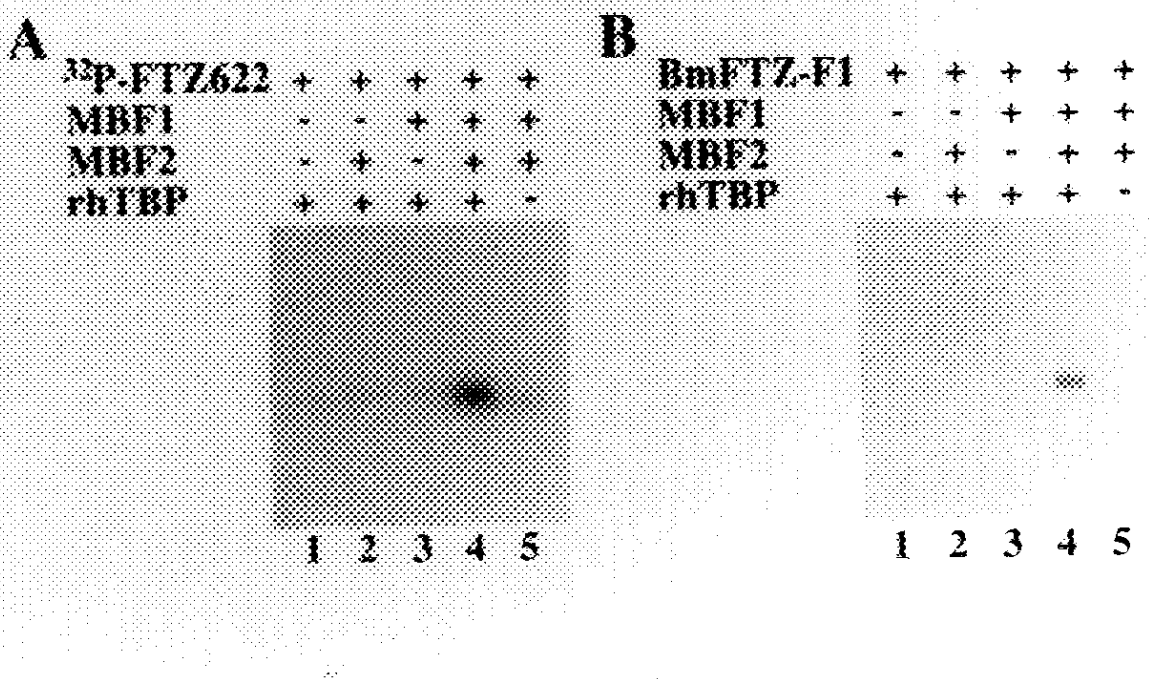

A
32P-FTZ622 + + + + +
MBF1 - - + + +
MBF2 - + - + +
rhTBP + + + + -
1 2 3 4 5
B
BmFTZ-F1 + + + + +
MBF1 - - + + +
MBF2 - + - + +
rhTBP + + + + -
1 2 3 4 5

Figure 9

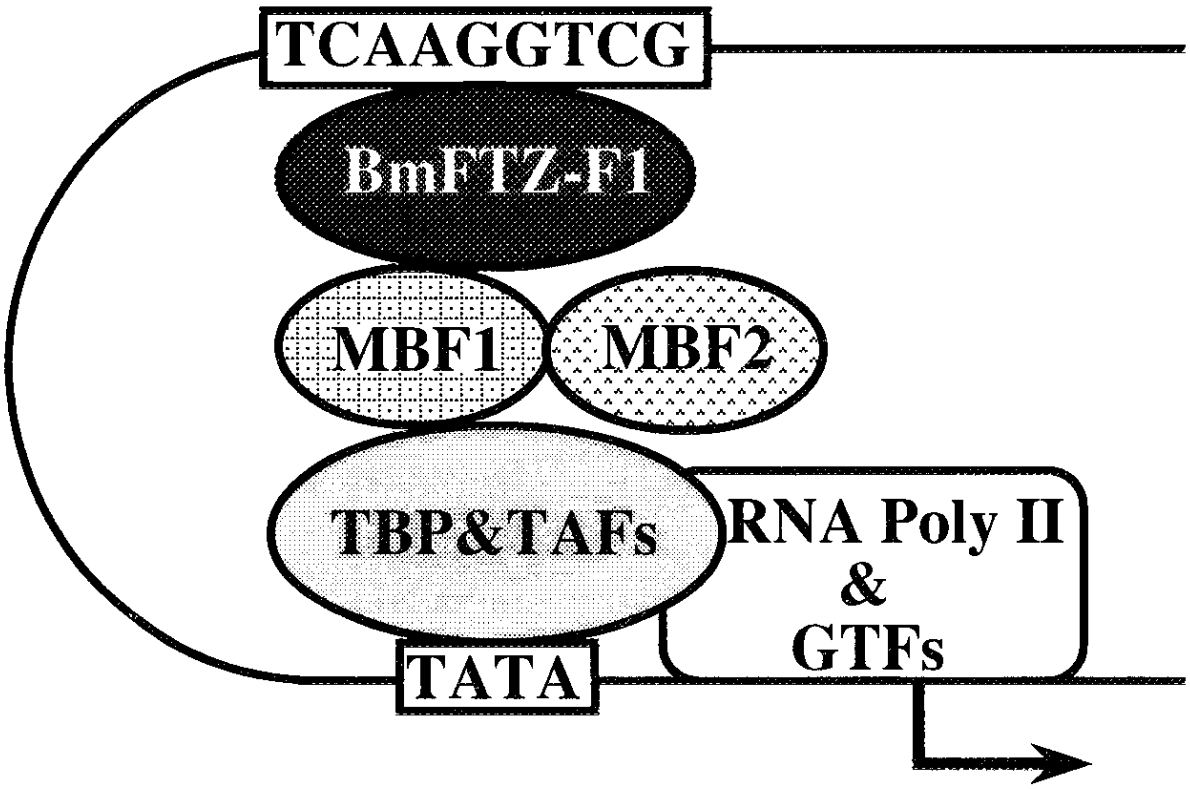


Figure 10