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Drosophila MBF1 is a co-activator for Tracheae Defective and contributes to the formation of tracheal and nervous systems

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SUMMARY

During gene activation, the effect of binding of transcription factors to cis-acting DNA sequences is transmitted to RNA polymerase by means of co-activators. Although co-activators contribute to the efficiency of transcription, their developmental roles are poorly understood. We used *Drosophila* to conduct molecular and genetic dissection of an evolutionarily conserved but unique co-activator, Multiprotein Bridging Factor 1 (MBF1), in a multicellular organism. Through immunoprecipitation, MBF1 was found to form a ternary complex including MBF1, TATA-binding protein (TBP) and the bZIP protein Tracheae Defective (TDF)/Apontic. We have isolated a *Drosophila* mutant that lacks the *mbf1* gene in which no stable association between TBP and TDF is detectable, and transcription of a TDF-dependent reporter gene is reduced by 80%. Although the null mutants of *mbf1* are viable, *tdf* becomes haploinsufficient in *mbf1*-deficient background, causing severe lesions in tracheae and the central nervous system, similar to those resulting from a complete loss of *tdf* function. These data demonstrate a crucial role of MBF1 in the development of tracheae and central nervous system.

Key words: TDF, MBF1, Co-activator, Trachea, CNS, *Drosophila*

INTRODUCTION

Transcriptional regulation plays a major role in the expression of the genomic information during complex biological processes such as differentiation and development. The effect of binding of transcription factors to cis-acting DNA sequences must somehow be transmitted to RNA polymerase to ensure the initiation and maintenance of active transcription. A class of proteins called co-activators serve this role by recruiting the general transcription machinery and by modifying the chromatin structure (Näär et al., 2001; Roeder, 1991). Although sequence-specific DNA-binding transcription factors provide most of the spatial and temporal specificity of expression, the expressional and functional properties of co-activators are also likely to participate in determining the kinetics and efficiency of transcription.

Two classes of transcriptional co-activators have been described to date. The first class comprises proteins that possess or recruit enzymatic activities to modify chromatin proteins, e.g. by acetylation of histones. Resulting alteration in the chromatin structure causes a switch in the 'state' of chromatin between transcriptionally inactive and active. Co-activators of the second class act more directly to recruit the general transcription machinery to a promoter where a transcription factor is bound. Among the latter class are TATA element-binding protein (TBP)-associated factors (TAFs) that are subunits of TFIID, and others that serve as adaptors to mediate the contact between transcription factors and the basal transcriptional complex. Although the importance of the first type co-activators for gene expression has been demonstrated (Akimaru et al., 1997; Brownell et al., 1996; Chakravarti et al., 1996; Grant et al., 1997; Kamei et al., 1996; Ogryzko et al., 1996; Waltzer and Bienz, 1999), little is known about how the second class co-activators function in vivo, or how the two types interact to achieve elaborate regulation of gene expression.

Multiprotein bridging factor 1 (MBF1) was first identified from the silkmoth as a co-factor necessary for transcriptional activation in vitro by a nuclear receptor FTZ-F1 (Li et al., 1994; Takemaru et al., 1997). The ability of MBF1 to bind both FTZ-F1 and TBP suggested a mechanistic model in which MBF1 recruits TBP to a promoter carrying the FTZ-F1-binding site by interconnecting FTZ-F1 and TBP. The MBF1 sequence is highly conserved across species from yeast to human (Takemaru et al., 1997). In the yeast *Saccharomyces cerevisiae*, MBF1 functions as a co-activator of a bZIP transcription factor GCN4, by bridging between GCN4 and TBP in response to amino acid starvation (Takemaru et al., 1998).

To analyze the biological role of MBF1 in a multicellular organism, we characterized its ortholog in *Drosophila*. The *Drosophila mbf1* gene partially rescued the phenotype of a *S. cerevisiae mbf1*$^{-}$ mutant, establishing that both the structure

and the function of MBF1 remained well conserved during evolution. In nuclear extracts of *Drosophila* embryos, we identified a bZIP protein Tracheae Defective (TDF; APT – FlyBase) (Gellon et al., 1997; Su et al., 1999; Eulenberg and Schuh, 1997) as a new partner of MBF1. We show that MBF1 mediates transcriptional activation by TDF through directly binding both TDF and TBP. Although *mbf1* null mutants can survive to adulthood, *tdf* becomes haploinsufficient in *mbf1*$^{-}$ background, resulting in severe tracheal and central nervous system (CNS) defects. These results show that MBF1 acts as a crucial bridging co-activator during tracheal and CNS development.

MATERIALS AND METHODS

Cloning of *Drosophila mbf1* cDNA

mbf1 cDNA was cloned from a *Drosophila* cDNA library prepared from the 3rd instar larval CNS in the lambda gt10 vector (M. J., unpublished). Partial cDNA was initially isolated by screening this library using nested PCR with degenerate antisense primers, corresponding to amino acids 123-129 (ERAIGIK) and 107-114 (YEAGRGIP) in silkmoth (*Bombyx mori*) MBF1 (Takemaru et al., 1997) (Fig. 1A) in combination with a primer derived from the vector. Hybridization screening of the same library with this PCR product was then used to isolate the full-length cDNA. The longest clone was 1562 bp, containing the entire MBF1 open reading frame plus a ~1 kb long 3′UTR, which ends with a poly(A) tail. Subclones of this cDNA were used to construct expression vectors for producing bacterial fusion proteins and FLAG-MBF1 in transgenic flies.

A 4574-bp genomic *Eco*RI fragment encompassing the entire *mbf1* gene was subcloned from the P1 clone DS05624 into pBluescript II (Stratagene) and sequenced on both strands in its entirety (DDBJ/EMBL/GenBank Accession Number, AB031273). Comparison of this genomic sequence with the *mbf1* cDNA reveals that the gene consists of four exons, the first one being non-coding. The clone contains 2.3 kb of DNA upstream of the *mbf1* transcription unit and 54 bp downstream of the *mbf1* mRNA polyadenylation site.

Fly stocks

Using P-element-mediated germline transformation (Rubin and Spradling, 1982), we made a transgenic *Drosophila* line expressing a FLAG epitope-tagged MBF1 (FLAG-MBF1) protein under the control of the constitutive *hsp83* promoter. Expression levels of the tagged MBF1 protein were comparable with those of endogenous MBF1 during embryogenesis and also in the salivary glands. An *mbf1*-null mutant was generated by isolating a P-element insertion in the *mbf1* locus and its subsequent imprecise excision (M. J., M. Okabe, Y. H. and S. H., unpublished). A rescue construct *P[MBF1*$^{+}$*], mbf1* was made by P-element-mediated insertion of the 4574 bp *mbf1* genomic sequence to the *mbf1* mutant chromosome. tdf^{P2} allele corresponds to the P-element insertion line *l(2)k15608* (Torok et al., 1993). $tdf^{P\Delta 3}$ has a deletion that includes the first exon and 1 kb of the first intron of the *tdf* gene (Eulenberg and Schuh, 1997). The *sgP[Gal4]* line has been described by Brand and Perrimon (Brand and Perrimon, 1993). Transgenic lines harboring *fPE-lacZ* or its mutant derivatives have been described elsewhere (Han et al., 1998). Other stocks have been described previously (Lindsley and Zimm, 1992).

Identification of FLAG-MBF1 associated proteins

Embryos 0-20 hours after egg laying (AEL) were collected from a population of the *hsp83*-FLAG-MBF1 transgenic line reared at 25°C. Nuclear extracts were prepared from the embryos as described previously (Ueda et al., 1990). For isolation of FLAG-MBF1-associated proteins, nuclear extracts (15 ml) were loaded onto a 2 ml column of anti-FLAG M2-Agarose affinity gel (Sigma). The column was washed extensively with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), until no proteins were detectable in the wash fraction. Bound proteins were then eluted with the FLAG peptide (50 μg/ml) (Sigma). Chromatography was carried out at 4°C.

To identify FLAG-MBF1 associated proteins, the eluted proteins were concentrated by acetone precipitation and then resolved on a preparative 10% SDS-PAGE. The proteins were transferred to a PVDF membrane (Boehringer Mannheim) on a Trans-Blot Semi-Dry apparatus (BioRad). The membranes were subsequently stained with Coommassie Brilliant Blue and selected bands were subjected to N-terminal sequencing. Peptide sequence of 63 kDa band VNKQYSATDLEAFMKIAANWQNSN showed an unambiguous match with that of TDF.

Antibodies

For the production of anti-TDF serum, a 1455 bp cDNA fragment encoding the entire TDF was inserted into a pET-28a vector (Novagen) and the polyhistidine-tagged protein was expressed in *Escherichia coli* BL21 (DE3). The His-TDF protein was purified by Ni^{2+} affinity chromatography and used to generate polyclonal antibodies in rabbit. The serum was used directly for immunostaining at a 1000-fold dilution. For western analyses, it was used at a 10,000-fold dilution. The production of the MBF1 antibody will be described elsewhere (M. J., M. O., Y. H. and S. H., unpublished). This antibody detects a single band of 16 kDa in normal embryos, but not in *mbf1*$^{-}$ embryos (Fig. 3).

Other antibodies include a rabbit anti-*Drosophila* TBP antibody (a gift from Yoshihiro Nakatani); mouse anti-β-galactosidase mAb 40-1a; rabbit anti-β-galactosidase antibody (Cappel); mouse anti-FLAG M2 (Kodak); mouse mAb BP102; and mouse mAb 2A12 that recognizes an unknown luminal component of the trachea (a gift from Shigeo Hayashi).

The secondary antibodies used were as follows: biotinylated anti-mouse IgG; Cy3-conjugated anti-rabbit IgG; Cy3-conjugated anti-mouse IgG (Jackson Laboratory); and Alexa 488 goat anti-rabbit IgG conjugate (Molecular Probes). When biotinylated anti-mouse IgG was used for the 2A12 staining, signals were amplified by adding biotinylated tyramide (NEN Life Science Products) as a substrate, followed by application of the ABC kit (Vector Lab), and finally visualized by Cy3-conjugated streptavidin (Amersham Biosciences).

Immunostaining

For staining of embryos, 0-12 hours or 12-15 hours AEL embryos were collected and fixed as described (Tautz and Pfeifle, 1989). After blocking with 5% normal goat serum/PBS-0.1% Triton X-100, the embryos were incubated with a primary antibody, followed by a secondary antibody.

Staining of larval tissues was performed as described previously (Liu et al., 2000). Larvae were dissected in PBS, fixed in 25 mM PIPES-KOH (pH 7.0), 0.5 mM EDTA, 0.25 mM $MgSO_4$ and 4% formaldehyde for 40 minutes on ice and then permeabilized for 15 minutes at room temperature in PBS containing 0.5% NP-40. In the case of salivary glands, the dissected tissues were first treated with PBS-0.5% NP-40 for 8 minutes before the fixation and the permeabilization step after the fixation was omitted.

Staining of polytene chromosomes using indirect immunofluorescence was performed as described (Shopland and Lis, 1996) except that salivary glands were treated with PBS, 0.5% NP-40 for 5 minutes before fixation. FLAG-MBF1, TBP and TDF were detected with anti-FLAG M2, anti-TBP and anti-TDF antibodies, respectively, and visualized with the mouse Cy3- or rabbit Alexa 488-conjugated secondary antibodies. After staining, samples were washed with PBS, 0.2% NP-40, 0.2% Tween 20 and containing subsequently 300 mM NaCl and 400 mM NaCl.

GST pull-down assay

GST fusion proteins were purified on Glutathione Sepharose 4B beads (Amersham Biosciences). GST pull-down assays were performed as described (Takemaru et al., 1998). Bound proteins were detected on western blots using either the anti-MBF1 or the anti-TBP antibody.

Immunoprecipitation

For co-immunoprecipitation assays, 20 μl of IgG-conjugated Dynabeads (Dynal, Oslo, Norway) containing 2 μg of IgG were incubated at 4°C for 2 hours with 40 μl of a primary antibody or the corresponding preimmune serum and then washed with TBS [50 mM Tris-HCl (pH 7.4), 60 mM NaCl]. Nuclear extracts were prepared from *yw* or *mbf1* embryos (0-20 hours AEL) as described (Ueda et al., 1990) and 30 μl of the nuclear extract was added to the antibody-loaded beads. After incubation at 4°C for 2 hours, the beads were washed with TBS containing 0.01% NP-40. Bound proteins were eluted from the beads with SDS loading buffer, resolved by SDS-PAGE (8% for TBP and TDF, 12.5% for MBF1) and analyzed on a western blot.

In vitro DNA-binding experiments

The TDF-binding consensus was obtained from a pool of oligonucleotides containing 16-nucleotide random sequences in the middle as described (Gogos et al., 1992). Binding reactions were performed as described elsewhere (Pollock and Treisman, 1990). The binding mixture (25 μl) contained buffer E [20 mM Hepes (pH 7.9), 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.1% NP-40, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 0.75 μg/ml aprotinin], 1 μl anti-TDF, 2 μl nuclear extract (0-20 hours AEL), 0.1 pmole of the ^{32}P-labeled oligonucleotide probe, 200 ng poly(dIdC)-poly(dIdC) and 200 ng bovine serum albumin (Sigma). The mixture was incubated on ice for 30 minutes and oligomers bound by TDF were collected on protein A-Sepharose beads. After washing the beads with buffer E, the oligomers were recovered by phenol treatment, amplified using PCR and then used for the next selection cycle. After the fifth round of selection, DNA fragments were subcloned into a T-vector (Novagen) and sequenced. The consensus sequence was deduced from sequences of 54 clones.

Gel mobility retardation assays were performed as described previously (Ueda and Hirose, 1991), except that electrophoresis was carried out on a 4% polyacrylamide gel in 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 380 mM glycine and 5% glycerol. The double-stranded oligomers used for the mobility retardation assay are described below. The probe or functional competitor was 5′-GAGCTCGGATCCGA-ATTCCAATTGGAATCTCGAGAAGCTTGATCA G-3′.

Mutant competitor was 5′-GAGCTCGGATCCGAATTCCCCTTG-TGATCTCGAGAAGCTTGATCAG-3′. Underlined regions indicate the recognition sequence of TDF and its mutant derivative respectively.

TDF binding site-dependent reporter assays

Reporter constructs were fusions of the *lacZ* gene with three tandemly repeated copies of either the functional TDF-binding site (*TDS-lacZ*) or the mutated site (*TDMS-lacZ*) (Fig. 4A), inserted into the hsCaSpeR-AUG-β-gal vector. The functional and mutant binding sites were those used in the DNA binding assays (above). These constructs were introduced into the *yw* host by the P-element transformation method of Rubin and Spradling (Rubin and Spradling, 1982). A line carrying the *TDS-lacZ* reporter at the cytological position 34F on the second chromosome was used for most experiments. The position of the reporter gene was determined by circularization of *Sau*3AI-digested genomic DNA with DNA ligase, followed by sequencing of PCR-amplified fragment with P-element primers.

To examine the effect of the loss of *tdf* function on the reporter expression, the *TDS-lacZ* transgene was introduced into the tdf^{P2} chromosome. The *TDS-lacZ* expression in $mbf1^{-}$ and heterozygous tdf^{P2} background were examined in *TDS-lacZ/CyO; mbf1* and *TDS-lacZ*, tdf^{P2}*/CyO; mbf1,* respectively. β-Galactosidase activities in embryonic extracts were measured using a Galacto-Light Plus System (Applied Biosystems) and a luminometer LUMAT LB9507 (Berthold Technologies). For scoring of X-gal-stained embryos, we excluded embryos that died earlier and only counted embryos that developed to stage 16.

To misexpress TDF in the salivary glands, a *UAS-tdf* transgene was controlled by a Gal4 driver *sgP[Gal4]* on the X chromosome. *sgP[Gal4]* is one of the enhancer trap lines isolated by Brand and Perrimon (Brand and Perrimon, 1993) and is active in the salivary gland cells from embryonic through larval stages. To localize TDF on its binding site in vivo, the *TDS-lacZ* reporter was introduced into the *UAS-tdf* chromosome and the y^{+} *sgP[Gal4]/Binscinsy* females were crossed with the *yw:UAS-tdf, TDS-lacZ* males. After culturing at 18°C, y^{+} third-instar larvae were dissected and stained with anti-TDF serum and the anti-β-gal mAb 40-1a.

Genetic analyses

To analyze genetic interactions between *mbf1* and *tdf*, we made two double mutant stocks, homozygous for *mbf1* deletion: $tdf^{P\Delta 3}$*/CyOP[ftz-lacC]; mbf1* and tdf^{P2}*/CyOP[ftz-lacC]; mbf1*. To compensate for the *mbf1* deletion, the rescue construct *P[MBF1⁺]* was introduced into the *mbf1* mutant chromosome. A double mutant $tdf^{P\Delta 3}$*/CyO; P[MBF1⁺], mbf1* was also prepared and used for rescue experiments. To rescue the CNS phenotype of the *tdf* mutant, *UAS-tdf* was introduced into the $tdf^{P\Delta 3}$ chromosome. *elav-Gal4;* $tdf^{P\Delta 3}$*/CyO* females were crossed with *UAS-tdf,* $tdf^{P\Delta 3}$*/CyO* males, then embryos were collected at 12-15 hours AEL and stained with the mAb 2A12 and mAb BP102 antibodies. The ability of *Drosophila* MBF1 to rescue the yeast *mbf1* disruptant was tested using aminotriazole sensitivity assay. Yeast MBF1 mediates transcriptional activation by GCN4 that governs induction of many genes in the amino acid synthetic pathways; hence, a yeast *mbf1* disruptant is sensitive to aminotriazole, an inhibitor of the *His3* gene product (Takemaru et al., 1998).

RESULTS

Characterization of *Drosophila* MBF1

A cDNA encoding *Drosophila* MBF1 was cloned from a larval CNS library. The predicted protein of 145 amino acids had 44, 64 and 83% identity to MBF1 from yeast, human and silkmoth, respectively (Fig. 1A). MBF1 consists of two structural domains: a well-structured C-terminal half that binds the general transcription factor TBP; and a flexible N-terminal half that participates in binding to various activators (Ozaki et al., 1999). The region conserved in *Drosophila* MBF1 includes both of these functional domains (Fig. 1A) (Ozaki et al., 1999). Expression of *Drosophila mbf1* cDNA partially rescued the yeast *mbf1* mutant phenotype upon amino acid starvation (Fig. 1B), indicating that the ability to bind partner transcription factors is also conserved between yeast and *Drosophila*.

In situ hybridization revealed that a large amount of maternal *mbf1* mRNA was deposited to the egg (data not shown). Likewise, MBF1 protein was present in preblastoderm embryos and was later expressed in many tissues, including the CNS and the trachea (Fig. 1C). Widespread expression of MBF1 was also seen in post-embryonic stages, with particularly high levels in the larval salivary glands, gonads (Fig. 1C) and adult gonads (data not shown).

To address the role of MBF1 in vivo, we generated a null mutant of *mbf1* (see Materials and Methods). This allele lacks

a 2082 bp DNA segment encompassing the entire coding region and most of the 3′-noncoding region of *mbf1* (from 211 bp upstream of the initiation codon to 857 bp downstream of the stop codon). *mbf1*$^{-}$ animals derived from *mbf1*$^{-/+}$ parents were viable, with no detactable loss in viability. *mbf1* mutant females, however, had a maternal effect phenotype with incomplete penetrance; a variable fraction of progeny from *mbf1*$^{-}$ homozygous females died before stage 5, regardless of whether they were mated to *mbf1*$^{+}$or *mbf1*$^{-}$ males. As escapers developed to adults with no obvious morphological defects, a stable *mbf1* homozygous line could be maintained under laboratory conditions. No MBF1 protein was detectable in the *mbf1* mutant by western analyses (Fig. 3A, lane 2; Fig. 3B, lane 2). Based on the requirement of MBF1 as an co-activator for FTZ-F1-dependent transcription in vitro, we had expected to see a *ftz-f1*-like segmentation defect among the dead *mbf1* homozygous embryos. However, no such pair-rule phenotype was observed. Furthermore, we were unable to detect any decrease in the expression of a FTZ-F1-dependent reporter gene in *mbf1*$^{-}$ mutant embryos (Fig. 1D). These results showed that MBF1 is not a crucial co-activator for FTZ-F1-dependent transcription in vivo. This led us to search for new partners of MBF1.

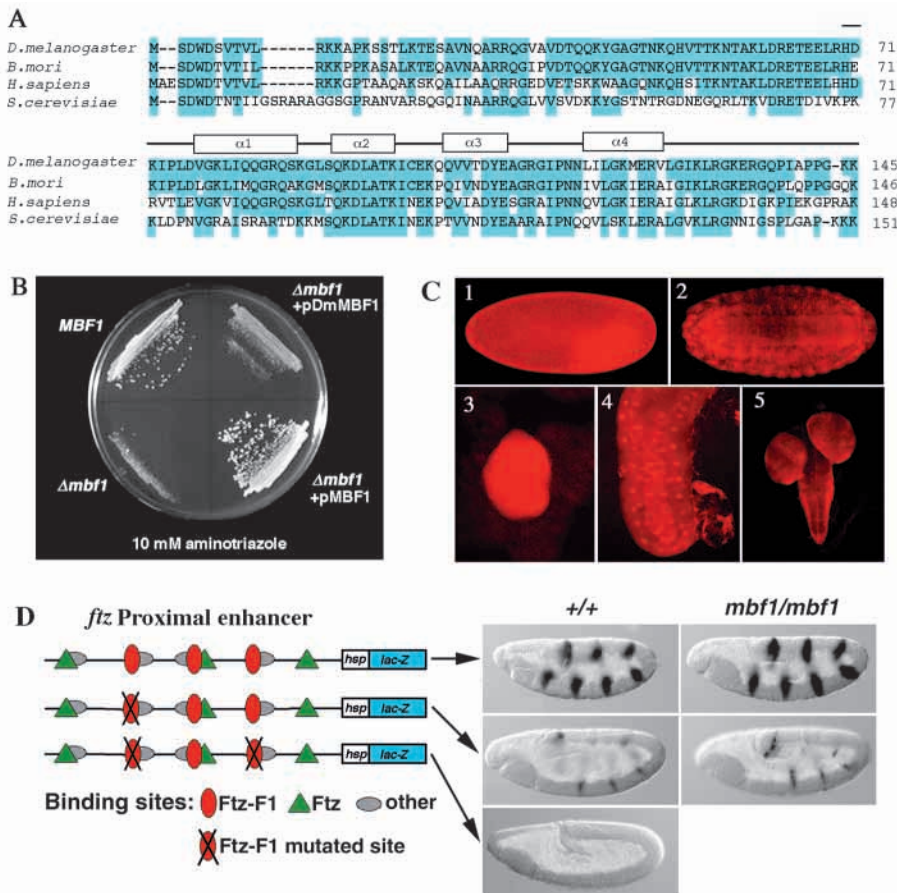


Fig. 1. Molecular cloning, expression and functional analyses of *Drosophila* MBF1. (A) Deduced amino acid sequences of fly, silkmoth, human and yeast MBF1. Residues that are identical to those of fly MBF1 are shaded. Thick bar represents the well-structured domain; α1-α4 denote four amphipathic helices. (B) *Drosophila mbf1* partially rescues sensitivity of yeast *mbf1* disruptant to aminotriazole. Indicated *Saccharomyces cerevisiae* strains were tested for aminotriazole sensitivity. Yeast *MBF1* (*trp1-Δ1 ura3-52 leu2-P1*) and *Δmbf1* (*trp1-Δ1 ura3-52 leu2-P1 Δmbf1::LEU2*), and a yeast genomic *MBF1* construct pMBF1 have been described previously (Takemaru et al., 1998). pDmMBF1 carries *Drosophila mbf1*$^{+}$ cDNA in place of the yeast *MBF1* coding region in pMBF1. As a control, these four yeast strains showed essentially the same growth in the absence of aminotirazole (data not shown). (C) Expression of MBF1 as revealed by immunostaining with anti-MBF1 antibody. (1) Syncytial blastoderm stage embryo. (2) Stage 16 embryo. Staining of CNS along the midline and tracheal staining on the margin of the embryo. (3-5) Dissected tissues from a 3rd instar larva: (3) strong staining in both the somatic and germ cells of the developing testis; (4) nuclear staining in the polyploidal salivary gland; and (5) CNS. (D) Expression of a FTZ-F1-dependent reporter gene in *mbf1*$^{+}$ and *mbf1*$^{-}$ homozygous embryos. Expression of a transgene *fPE-lacZ* carrying the *ftz* proximal enhancer and the *hsp70* minimal promoter fused to *lacZ* (upper panels) or its mutant derivatives (middle and bottom panels) was analyzed by X-gal staining. Mutation in FTZ-F1-binding sites but not in the *mbf1* locus affected the reporter gene expression. The *fPE-lacZ* is silent in *ftz-f1* mutant background (Yussa et al., 2001).

Identification of TDF as an MBF1-associated protein

To isolate new partners of MBF1 in vivo, we used the transgenic line expressing FLAG-MBF1 and pulled out MBF1-associated factors from embryonic nuclear extracts. Complexes including MBF1 were captured on anti-FLAG antibody beads, eluted with the FLAG peptide and resolved by SDS-PAGE. While the nuclear extract of a strain lacking FLAG-MBF1 yielded no specific protein bands (Fig. 2A, lane 1), many proteins were recovered from the FLAG-MBF1 transgenic embryos (Fig. 2A, lane 2). As anticipated, TBP was identified among the MBF1-associated proteins (Fig. 2B, lane 4). The above procedure is also expected to pull down TAFs; indeed some of the bands had sizes corresponding to *Drosophila* TAFII32, TAFII60, TAFII110 and TAFII150, but we did not examine them further.

We focused on the most prominent protein at 63 kDa that could not be accounted for by any TAF. Its N-terminal amino acid sequence identified it as the bZIP protein TDF (Gellon et al., 1997; Eulenberg and Schuh, 1997). This was supported by western analysis with an anti-TDF antibody (Fig. 2B, lane 6). As yeast MBF1 has been known to interact with the bZIP protein GCN4 (Takemaru et al., 1998), TDF appeared as a good candidate for a new functional partner of MBF1. Indeed, GST pull-down assays using bacterially expressed purified proteins showed that MBF1 bound directly to TDF (Fig. 2C) and to TBP (Fig. 2D). Yeast GCN4 binds MBF1 with its bZIP domain (Takemaru et al., 1998). Similarly, the bZIP domain of TDF was sufficient for the binding of *Drosophila* MBF1 (Fig. 2C).

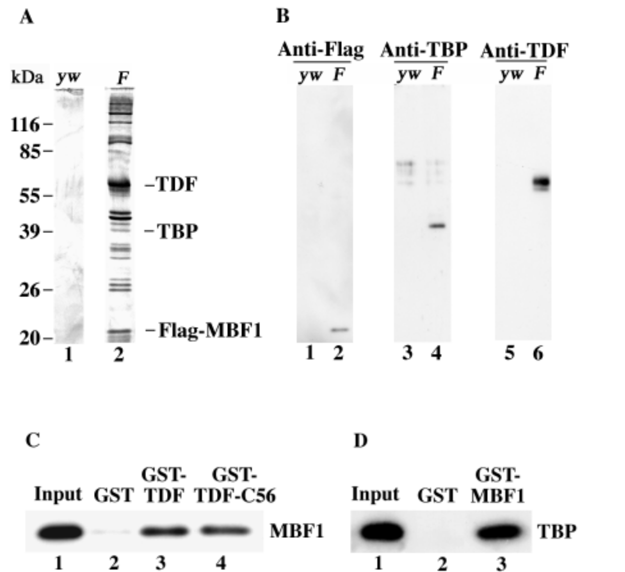


Fig. 2. Isolation of MBF1-associated proteins. (A) FLAG-MBF1-associated proteins. Proteins from nuclear extracts of the FLAG-MBF1-expressing transgenic line F (lane 2) were captured on an anti-FLAG affinity column, separated by 10% SDS-PAGE and silver stained. Nuclear extracts of the parental *yw* line (lane 1) served as a control. Positions of molecular weight markers are shown on the left. (B) Western analyses of the captured proteins. The same samples as in A were resolved on SDS-PAGE, blotted onto membranes and probed with the indicated antibodies. (C) GST pull-down assay for interaction between MBF1 and TDF. Bacterially expressed and purified His-MBF1 (100 ng) was incubated with 200 ng of either GST (lane 2), GST-TDF (lane 3) or the GST-TDF-C56 fusion with the N-terminally truncated TDF (lane 4). The bound His-MBF1 was resolved by 15% SDS-PAGE and detected with the anti-MBF1 antibody. Lane 1 contains 1/10 of input His-MBF1. (D) GST pull-down assay for interaction between MBF1 and TBP. Bacterially expressed and purified His-TBP (100 ng) was incubated with 200 ng of either GST (lane 2) or GST-MBF1 (lane 3). The bound TBP was resolved by 8% SDS-PAGE and detected with the anti-TBP antibody. Lane 1 contains 1/10 of input His-TBP.

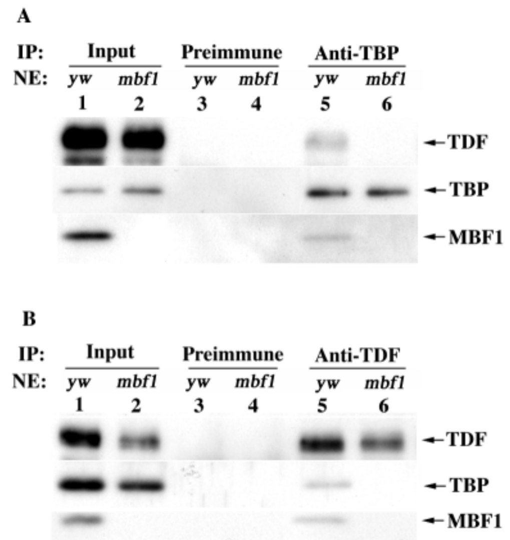


Fig. 3. Co-immunoprecipitation of TDF, MBF1 and TBP. Western blots of nuclear proteins from *yw* (*mbf1*$^+$) or *mbf1* mutant embryos immunoprecipitated using the anti-TBP (A) and anti-TDF (B) antibodies (IP) were probed with anti-TDF (top), anti-TBP (middle) or anti-MBF1 (bottom) antibodies. Western blots of input proteins are shown in the left columns.

If MBF1 bridges TDF and TBP, association between these two proteins should depend on the presence of MBF1. To examine the requirement of MBF1 for tethering TBP to TDF in vivo, we carried out co-immunoprecipitation using nuclear extracts prepared from *mbf1*$^+$ or *mbf1*-null mutant embryos. From the *mbf1*$^+$ nuclear extract, MBF1 and TDF were co-immunoprecipitated with the anti-TBP antibody, but not with the pre-immune serum (Fig. 3A, lanes 3 and 5). By contrast, when we started from the extract from *mbf1*$^-$ embryos, TDF was not co-immunoprecipitated with the anti-TBP antibody (Fig. 3A, lanes 2 and 6). In a reciprocal test, MBF1 and TBP were co-immunoprecipitated with the anti-TDF antibody from the *mbf1*$^+$, but not from the *mbf1*$^-$ nuclear extracts, or from the *mbf1*$^+$ extract using the pre-immune serum (Fig. 3B, lanes 3, 5 and 6). These results demonstrate that MBF1 forms a bridge between TDF and TBP and that in its absence, a stable association of TDF and TBP does not take place.

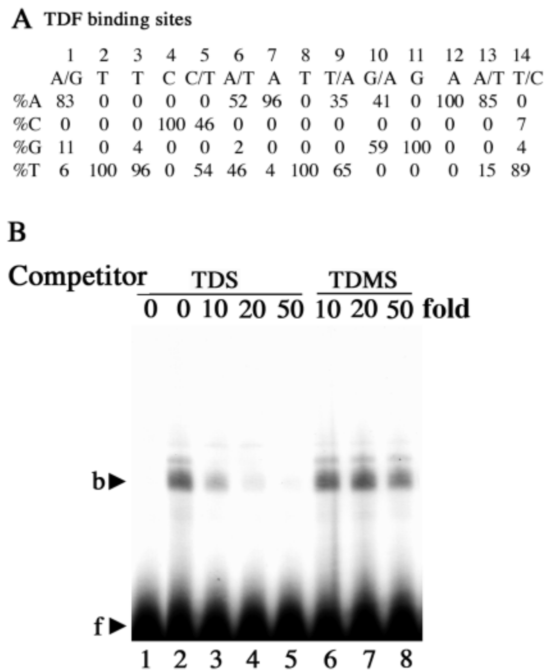
TDF binds DNA in a sequence-specific manner

The physical interaction between MBF1 and TDF, as well as the presence of bZIP domain in TDF suggests that TDF is a transcription factor that uses MBF1 for transcriptional regulation of its target genes. To test this possibility, we first determined the binding sequence of TDF. The full-length TDF protein or its C-terminal 56 amino acids harboring the bZIP domain were expressed in bacteria and purified. In a preliminary gel mobility retardation experiment, both the full-length and the C-terminal proteins showed DNA-binding activities (data not shown). The optimal binding sequences were selected from a pool of random oligonucleotides (see Materials and Methods). The consensus sequence deduced from the selected oligonucleotides was (A/G) TTC (C/T)(A/T) AT (T/A) (G/A) GA (A/T)(T/C) (Fig. 4A).

The specificity of TDF binding to the consensus DNA sequence was verified by a competition assay. Incubation of ^{32}P-labeled double-stranded DNA carrying the sequence 5′-ATTCCAATTGGAAT-3′ with the full-length TDF protein yielded a slowly migrating complex. The complex formation was efficiently competed with the unlabeled oligonucleotide of the same sequence (Fig. 4B, lanes 2-5) but not with a mutant oligonucleotide carrying four base substitutions (Fig. 4B, lanes 6-8). Essentially the same results were obtained with the C-terminal 56 amino acid fragment of TDF (data not shown). These data indicate that TDF is a sequence-specific DNA binding protein that recognizes a 14 bp palindrome (A/G) TTC (C/T) (A/T) AT (T/A) (G/A) GA (A/T) (T/C).

TDF is a sequence-specific activator of transcription

We then tested whether TDF regulates transcription from a promoter carrying its binding site. A reporter gene was constructed in which three tandemly repeated TDF-binding sites (TDS) were placed upstream of a *hsp70* basal promoter (–40 ~ +90)-*lacZ* fusion gene (*TDS-lacZ*; Fig. 5A). We also made another reporter gene containing three tandemly repeated mutant binding sites (*TDMS-lacZ*). Transgenic fly lines


A TDF binding sites

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	A/G	T	T	C	C/T	A/T	A	T	T/A	G/A	G	A	A/T	T/C
%A	83	0	0	0	0	52	96	0	35	41	0	100	85	0
%C	0	0	0	100	46	0	0	0	0	0	0	0	0	7
%G	11	0	4	0	0	2	0	0	0	59	100	0	0	4
%T	6	100	96	0	54	46	4	100	65	0	0	0	15	89

Fig. 4. DNA-binding properties of TDF. (A) Consensus binding sequence of TDF. After five cycles of immunoselection of TDF-bound PCR amplified random oligonucleotides, the recovered DNA was cloned into a T-vector and sequenced. Percentages indicate frequencies at which each nucleotide appeared at a given position from 1 to 14 in the total of 54 sequenced clones. The second line represents the deduced consensus sequence. (B) Electrophoretic mobility shift assay for DNA binding of TDF. Ten fmoles of ^{32}P-labeled DNA probe and 25 ng of His-TDF in 10 µl of binding mixture were incubated at 25°C for 60 minutes. The DNA-TDF complex (b) was separated from the free probe (f) by electrophoresis. Lanes 1 and 2, binding reaction without any competitor; lane 1 received a mock-purified fraction from bacteria carrying an empty vector in place of His-TDF. In lanes 3-8, samples contained fold excess amounts of the unlabeled competitor DNA: either the functional TDS (lanes 3-5), or the mutant TDMS (lanes 6-8) sequence.

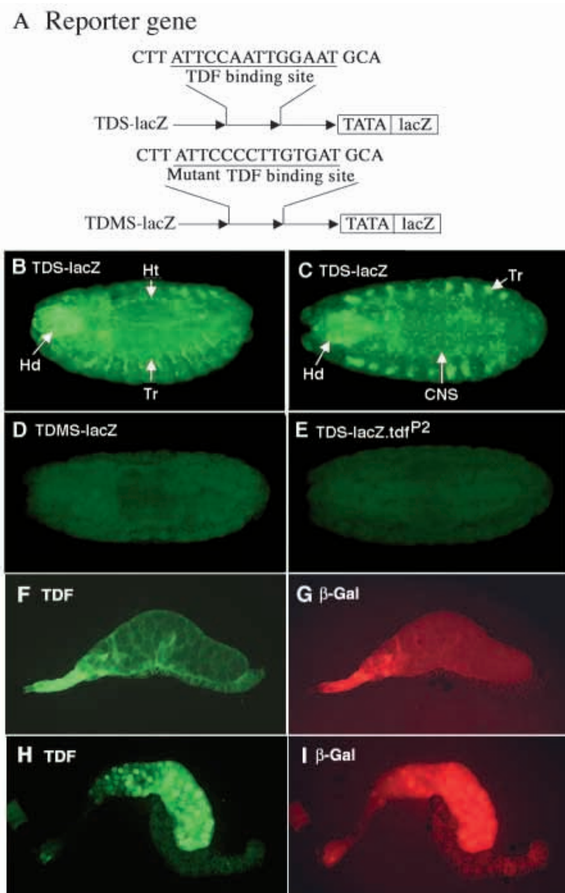


Fig. 5. Reporter assay for TDF-dependent transcriptional activation. (A) Schematic illustration of the *lacZ* reporter constructs driven by either the functional (TDS) or the mutant (TDMS) TDF-binding elements. (B-E) Staining of stage 16 embryos with an anti-β-galactosidase antibody. (B,C) The reporter gene *TDS-lacZ* was expressed in the tracheae (Tr), head (Hd), heart precursor cells (Ht) and CNS of a *tdf* $^{+}$ line. (D) The mutant reporter *TDMS-lacZ* was silent in the *tdf* $^{+}$ line. (E) *TDS-lacZ* was not expressed in the *tdf*^{P2} mutant. (F-I) Activation of *TDS-lacZ* upon ectopic expression of TDF in the posterior salivary gland of a third instar larva. Salivary glands from control *TDS-lacZ* (F, G) or *sgP[Gal4]/+; UAS-tdf, TDS-lacZ/+* (H,I) larvae were stained with anti-TDF (F,H) or anti-β-galactosidase (G,I) antibodies.

carrying these reporter genes were analyzed for *lacZ* expression by staining with anti-β-galactosidase antibody. *TDS-lacZ* was expressed in the tracheae, heart precursor cells, head and CNS of stage 16 embryos (Fig. 5B,C), i.e. in the pattern of the endogenous TDF protein (Eulenberg and Schuh, 1997; Gellon et al., 1997; Su et al., 1999) (Q.-X. L., M. J., H. U., Y. H. and S. H., unpublished). By contrast, the *TDMS-lacZ* reporter with mutated binding sites showed no activity (Fig. 5D). Expression of *TDS-lacZ* was not detectable in the *tdf* loss-of-function mutants *tdf*^{P2} (Fig. 5E) and *tdf*$^{P\Delta 3}$ (data not shown). Conversely, when TDF was ectopically expressed in the posterior salivary gland cells, it induced expression of the *TDS-lacZ* reporter in these cells (Fig. 5I). Such induction of *lacZ* was not observed when we used the line carrying *TDMS-lacZ* (data not shown). These results clearly show that TDF is required and sufficient to activate transcription by binding to its specific recognition sequence TDS.

If MBF1 and TDF form a complex to activate the reporter gene, both proteins should localize to TDS. When TDF was ectopically expressed in the salivary glands of a transgenic line harboring a *TDS-lacZ* insertion at the polytene chromosome position 34F, TDF was detected at the *TDS-lacZ* insertion site. Visualization of MBF1 using FLAG-MBF1 strain revealed that MBF1 also accumulates at 34F, colocalizing with TDF (Fig. 6A). The localization of MBF1 to the TDS site at the position 34F occurred only when TDF was expressed in the salivary gland. These data support the idea that MBF1 is recruited to TDS owing to the sequence-specific DNA-binding activity of TDF.

MBF1 plays a role in the TDF-dependent activation

As animals that lack MBF1 can survive to adulthood while null mutants of TDF are embryonic lethal, TDF must be able to carry out its function even when no stable association with TBP occurs. This suggests that, in the absence of MBF1,

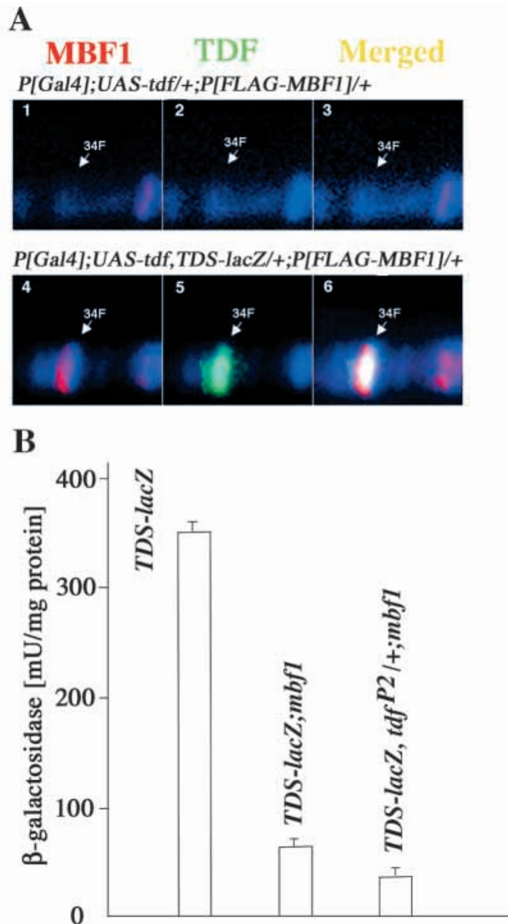


Fig. 6. MBF1 participates in the TDF-dependent activation. (A) Colocalization of TDF and MBF1 to the transgenic reporter *TDS-lacZ* on the polytene chromosome. Salivary gland polytene chromosomes were prepared from a third instar larva of a *sgP[Gal4]; UAS-tdf, TDS-lacZ/+; P[FLAG-MBF1]/+* line (4-6) or the same line without *TDS-lacZ* (1-3) and stained with anti-TDF (2 and 5) and anti-FLAG (1 and 4) antibodies. Blue signals represent DAPI staining. Panels 3 and 6 show the merged signals. The arrow marks the locus harboring the *TDS-lacZ* transgene. (B) MBF1 is required for full activation by TDF in vivo. Extracts were prepared from stage 16 embryos of the indicated lines and their β-galactosidase activities were measured. Shown are average specific activities of six independent assays with standard errors.

transcriptional activation by TDF may be reduced, but not abolished. To measure the transcriptional activity of TDF, we quantitated the expression of the *TDS-lacZ* reporter in normal and MBF1-deficient embryos (Fig. 6B). We found that β-galactosidase expression from *TDS-lacZ* was reduced by 80% when MBF1 was absent. This effect was reverted by expressing a transgenic MBF1 (data not shown). The reduction was even more pronounced when only one copy of the tdf^{+} gene was present, while removing one copy of the tdf^{+} gene in $mbf1^{+}$ background had no effect on *TDS-lacZ* reporter activity. This indicates that the loss of MBF1 has a quantitative effect on TDF-mediated transcriptional activation. Interestingly, when a population of stage 16 $mbf1^{-}$ embryos carrying *TDS-lacZ* gene was stained for β-galactosidase expression, there was a considerable individual variation in the expression levels; about 15% of embryos had no detectable levels of β-galactosidase expression, while the rest exhibited varying levels. Such variation was not observed in $mbf1^{+}$ embryos. It is possible that MBF1 function becomes essential for TDF-mediated transcription under conditions that are not completely controlled in our experiments.

Role of TDF and MBF1 in the development of the tracheae and CNS

The involvement of MBF1 in TDF-mediated transcription suggests that loss of *mbf1* may also affect a *tdf* mutant phenotype. In addition to the tracheal system (Eulenberg and Schuh, 1997; Gellon et al., 1997), TDF is strongly expressed in the CNS (Eulenberg and Schuh, 1997). Although a defect in neural function has been reported in a *tdf* mutant (Takasu-Ishikawa et al., 2001), the role of TDF in the CNS development remained to be investigated. We found that $tdf^{P\Delta3}$ embryos had breakages in the CNS axon tracts and tracheal networks (Fig. 7C,D). CNS and tracheal phenotypes were individually rescued when TDF was expressed in all neurons or in all tracheal cells, respectively, indicating that these phenotypes represent independent requirement for TDF in two tissue types, rather than one being a secondary consequence of another (Fig. 7E,F) (Eulenberg and Schuh, 1997) (Q.-X. L., M. J., H. U., Y. H. and S. H., unpublished). The breakages in the CNS and tracheal networks were also observed in the *mbf1* mutant, although the penetrance of these phenotypes was only 2-3% (Fig. 7G,H).

Although the developmental defect caused by the lack of MBF1 was rather subtle, *mbf1* mutation exhibited a strong genetic interaction with *tdf* mutants. Animals that were heterozygous for the $tdf^{P\Delta3}$ allele had no detectable defects in either the CNS or tracheae (Fig. 7A,B). However, when MBF1 was removed from the $tdf^{P\Delta3}$ heterozygotes, we observed a significant increase in the number of defective embryos (17-18% in Fig. 7I,J). Moreover, the degree of defects was also enhanced: increase in the number of breakages and in their size (Fig. 7I,J versus Fig. 7G,H). This effect was completely reverted by expressing MBF1 from a transgene carrying the genomic *mbf1* locus (Fig. 7K-N). These genetic interactions between *mbf1* and *tdf* strongly suggest that MBF1 participates in TDF-mediated transcription during normal development of the tracheae and CNS.

DISCUSSION

MBF1 mediates TDF-dependent transcriptional activation during development of tracheae and CNS

We have analyzed the biological function of an evolutionarily conserved transcription co-activator MBF1 through identifying its partner protein TDF. MBF1 bound directly to TBP and TDF and colocalized with TDF on a TDF-binding site experimentally placed on a polytene chromosomal locus. Co-immunoprecipitation experiments demonstrated that a TDF-MBF1-TBP ternary complex occurred and that MBF1 was required for bridging between TDF and TBP. In the absence of MBF1, the association between TDF and TBP was weakened and TDF-dependent expression of a reporter gene was reduced by 80%. Loss of *mbf1* function resulted in developmental defects similar to those seen in *tdf* mutants and affected the proper formation of the tracheae and CNS. These phenotypes

were enhanced when the gene dose of *tdf* was halved. Based on these biochemical and genetic data, we conclude that *Drosophila* MBF1 functions as a co-activator of TDF during the development of the tracheae and CNS.

Two pathways for transcriptional activation by TDF

The relationship between *Drosophila* MBF1 and TDF is similar to that between yeast MBF1 and its partner transcription factor GCN4 (Takemaru et al., 1998). Just as yeast MBF1 contacts GCN4 through its bZIP domain, *Drosophila* MBF1 binds the bZIP domain of TDF. Moreover, the lack of GCN4-dependent activation in yeast *mbf1* mutant can be partially restored by expressing *Drosophila* MBF1. The sequence and functional conservation between yeast and *Drosophila* MBF1 indicates that the interaction with bZIP proteins is a conserved feature of the bridging factor MBF1.

Genetic studies of *mbf1* in yeast and *Drosophila* suggest that MBF1-associated transcription factors have two pathways for activation. In addition to the MBF1-mediated recruitment of TBP via its bZIP domain (Takemaru et al., 1998), GCN4 also recruits the SAGA complex with its N-terminal activation domain and effects transcription through chromatin modification (Grant et al., 1997). Likewise, *Drosophila* TDF has a region similar to the glutamine-rich transactivation domain (Eulenberg and Schuh, 1997) and may employ an activation pathway independent of recruiting TBP through MBF1. Such pathway may account for the residual expression of the *TDS-lacZ* reporter gene in the absence of MBF1. Although MBF1 is essential for GCN4-dependent transcription of its target gene *HIS3* (Takemaru et al., 1998), low level of TDF-dependent transcription of the *TDS-lacZ* gene can still occur in the absence of MBF1. This suggests that the relative importance of the two pathways is different between GCN4 and TDF. The DNA-binding domain of FTZ-F1 carries a basic region homologous to those in bZIP proteins and binds MBF1 through this region (Takemaru et al., 1997). However, loss of *mbf1* showed no effect on FTZ-F1-dependent transcription in vivo, suggesting that the activation by FTZ-F1 relied solely on the pathway through its transactivation domain. In our in vitro transcription system, the transactivation domain does not seem to be functional because FTZ622 polypeptide bearing only the DNA-binding domain of FTZ-F1 showed the same transcriptional activity as the intact FTZ-F1 (Takemaru et al., 1997). This may explain the difference in the MBF1 requirement between FTZ-F1-dependent transcription in vivo and in vitro.

It is possible that the role of MBF1 becomes more critical under certain circumstances, when rapid induction of gene expression is demanded by environmental conditions. The expression of the *TDS-lacZ* reporter in *mbf1*⁻ background varied considerably from embryo to embryo, suggesting that certain conditions that are uncontrolled in our experiments may render transcription particularly dependent on the MBF1-mediated pathway. In the natural environment, there are many stimuli that alter gene expression profile: UV radiation, poison agents, nutrient starvation and so on. Therefore, direct recruitment of TBP by MBF1 may become essential for rapid activation of transcription under such conditions. In agreement with this idea, the yeast *mbf1* disruptant is viable under normal culture conditions, but sensitive to amino acid starvation (Takemaru et al., 1998).

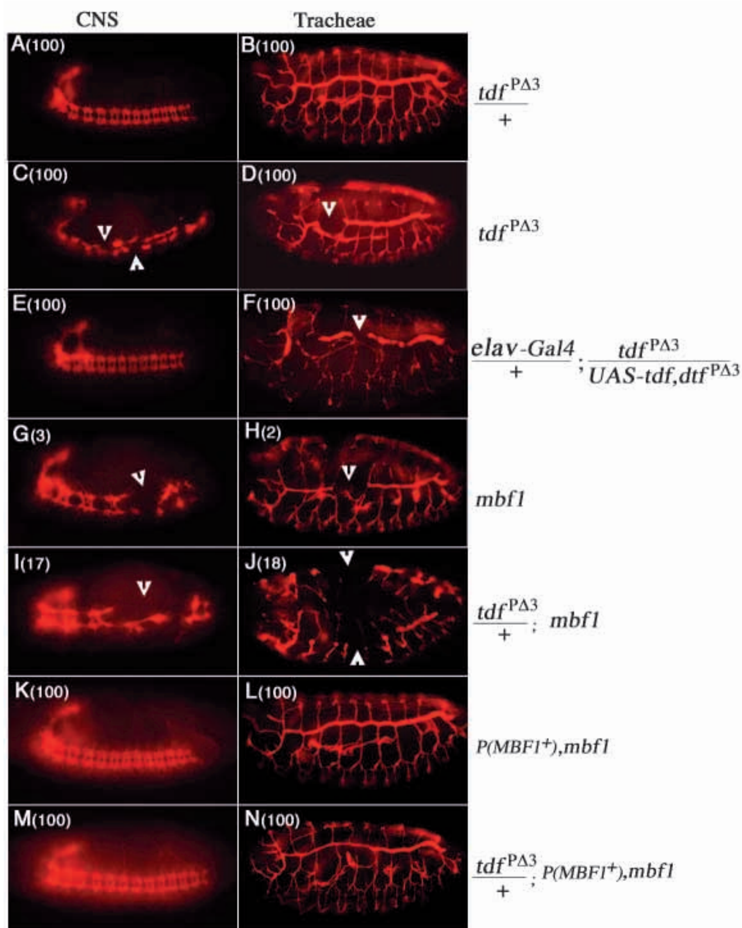


Fig. 7. CNS and tracheal defects in *tdf* and *mbf1* mutants and genetic interaction between the two genes. CNS and tracheal lumen networks were stained in stage 16 embryos of the indicated genotypes with the mAb BP102 (left) and the mAb 2A12 (right), respectively. Arrowheads represent the defects in the CNS and tracheal network formation. The penetrance of each phenotype (%) is shown in the parentheses. *P[MBF1⁺]* is a rescue construct placed on the *mbf1* chromosome, providing a wild-type copy of the gene. The tracheal defects in $tdf^{P\Delta3}$ was variable from embryo to embryo. (D) A mild phenotype seen in some embryos. We also observed more severe phenotype in other embryos (data not shown, but see Fig. 1E by Eulenerg and Schuh (Eulenerg and Schuh, 1997). This could be due to variable and partial rescue of the lack of zygotic *tdf* function by maternally supplied *tdf,* because the lack of maternal *tdf* enhances the zygotic *tdf* phenotype (Eulenberg and Schuh, 1997). Total numbers of examined embryos were: A, 138; B, 136; C, 98; D, 112; E, 89; F, 108; G, 156; H, 98; I, 120; J, 132; K, 118; L, 128; M, 116; N, 121.

The biological role of MBF1

Studies on MBF1 homologs also support the idea that MBF1 may function when gene expression is required in response to developmental or environmental signals. Rat MBF1 has been isolated as a calmodulin-associated peptide 19 (CAP-19) (Smith et al., 1998) and human MBF1 (Kabe et al., 1999) has been identified as

endothelial differentiation-related factor 1 (EDF1) (Dragoni et al., 1998). EDF1/MBF1 is downregulated when endothelial cells are induced to differentiate. Interestingly, EDF1/MBF1 binds to calmodulin in the cytoplasm under low Ca^{2+} conditions but the two proteins dissociate when intracellular Ca^{2+} is high. The released EDF1/MBF1 is then phosphorylated and shuttled into the nucleus, where it binds TBP (Mariotti et al., 2000). Nuclear translocation of MBF1 has also been observed at a specific stage of molting in the silkworm *B. mori* (Liu et al., 2000). Considering the Ca^{2+} elevation upon exposure to the molting hormone ecdysteroid (Biyasheva et al., 2001), these data raise an intriguing possibility that MBF1 is involved in Ca^{2+}-induced gene activation. Although in this study we have analyzed only the developmental roles of MBF1 associated with TDF function, *Drosophila* MBF1 may also be involved in other biological processes, such as stress response, homeostasis and longevity.

Several lines of evidence suggest that *Drosophila* MBF1 has partners other than TDF. MBF1 is expressed in a wide spatiotemporal pattern, including tissues and stages where TDF is absent. Although TDF is not expressed in the salivary gland, immunolocalization of MBF1 on salivary gland chromosome revealed a large number of loci associated with MBF1 (Q.-X. L., M. J. and S. H., unpublished). Furthermore, FLAG-tagged MBF1 pulled down many proteins besides TDF. Although *mbf1*-null mutants were viable under laboratory conditions, *tdf* became haploinsufficient in $mbf1^{-}$ genetic background, clearly indicating the importance of MBF1 in the expression of the genomic information. This finding opens a way to identify new partners of MBF1 through genetic screening for loci that exhibit dominant phenotypes in the absence of MBF1. Characterization of MBF1 partners will contribute to our knowledge on how co-activators mediate specific biological events.

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