A novel TATA-box-binding factor from the silk glands of the mulberry silkworm, *Bombyx mori*

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The presence of one or more TATATAA motifs in the flanking sequences of individual members of a multi-gene $tRNA_1^{Gly}$ family from the mulberry silkworm, *Bombyx mori*, negatively modulated the transcription of the gene copies. Characterization of proteins from posterior silk gland nuclear extracts, binding to the TATA TAA motif, identified a novel 43 kD protein, designated here as P43 TATA-box-binding factor (TBF). The protein was purified to homogeneity. P43 TBF binding was highly sequence-specific and showed a 100-fold-higher affinity for binding than the TATA-box-binding protein (TBP). The protein also showed binding to the TATAAA sequence of the *actin5C* promoter. P43 TBF inhibited transcription of all the tRNA genes examined, as well as RNA polymerase II transcription from the *actin5C* promoter. The amino acid sequence of eleven peptides generated from P43 TBF did not share homology with proteins that bind the TATA box, such as TBP, TRF (TBP-related factor) or TLFs (TBP-like factors) reported from other sources. Inhibition of transcription of tRNA genes by P43 TBF could not be reversed by TBP. The inhibitory effect appeared to be exerted through sequestration of the associated transcription factors.

Key words: RNA polymerase III, TATA binding protein, tRNA transcription, transcriptional regulation.

INTRODUCTION

The promoters for eukaryotic tRNA genes consist of two essential and highly conserved regions of approx. 10 bp each, the A- and B-boxes, located within the coding region and generally separated by 30–40 bp [1]. The prevailing models for tRNA gene transcription invoke the function of two multi-subunit transcription factors, TFIIIB and TFIIIC. The binding of TFIIIC to the A and B boxes leads to the binding of TFIIIB immediately upstream of the tRNA-coding region, which in turn recruits RNA polymerase III (pol III) to initiate transcription at the start site [2–4]. The TATA-binding protein (TBP) is recruited to the transcription initiation complex as a component of TFIIIB. Whereas the A- and B-blocks constitute the essential promoter elements of a tRNA gene, variations in the 5′ flanking sequences exert modulatory effects on their transcription in yeast [5–7], fruit flies [8,9], silkworms [10–18], frogs [19–21] and humans [22,23].

There is little or no sequence homology conserved between the 5′ flanking sequences of different tRNA genes, including even those gene copies encoding the same tRNA 'isoacceptor' species. However, the presence of A/T-rich sequences within 20–30 bp of the transcription start site of several tRNA genes occurs consistently. Comparison of the upstream regions of 12 individual tRNAs from yeast revealed an average of 68 % A/T content between nt −1 and −40 [24]. The upstream regions of three copies of human $tRNA^{Val}$ have 70–90 % A/T content [22]. The members of $tRNA_1^{Gly}$ family from *Bombyx mori* possess 63–68 % A/T content within the 100 bp 5′ upstream sequences [14]. Transcription of $tRNA_c^{Ala}$ and $tRNA_1^{Gly}$ from *B. mori* are positively regulated by the A/T-rich motifs present in the immediate upstream up to −40 nt of the coding region [14,25,26].

The tRNA population of differentiated cells is adapted to meet their special protein-synthetic demands, almost exclusively through the transcriptional regulation of the tRNA genes. A classic example of this functional adaptation of tRNA population occurs in the silk glands of the mulberry silkworm *B. mori*. Fibroin, the major constituent protein of the silk fibre, is synthesized at very high levels in the posterior silk glands during the fifth larval instar. The unusual amino acid composition of fibroin H chain (46 % glycine, 33 % alanine, 12 % serine and 5 % tyrosine) is reflected in the distribution of tRNAs in the posterior silk gland, the site of protein synthesis, to achieve optimized fibroin production [27,28]. The large 15 kb mRNA encoding fibroin H comprises 2415 codons for glycine [29], and of these 1352 correspond to GGC/U, which are base-paired with $tRNA_1^{Gly}$. In *B. mori*, $tRNA_1^{Gly}$ constitutes a multi-gene family with an estimated 20 copies, and 11 of these have been characterized previously by our group [13,14,16,17]. On the basis of transcription levels *in vitro* in posterior silk gland nuclear extracts, these copies of $tRNA_1^{Gly}$ could be grouped into three distinct classes. Some of them were highly transcribed ($tRNA_1^{Gly}$ 1 and 11), some were very poorly transcribed ($tRNA_1^{Gly}$ 6, 7, 8, 9 and 10) and the rest were transcribed to intermediary levels ($tRNA_1^{Gly}$ 2, 3, 4 and 5). The *in vivo* transcription of these genes in *B. mori*-derived BmN cell lines also followed essentially the same pattern [30,31]. Since all these gene copies have identical coding regions (and consequently harboured the same identical coding regions), the obvious candidates for mediating the differential transcription are the 3′ and 5′ flanking regions. In fact, sequences present far upstream of the coding region (up to 1 kb away) also modulated transcription of these $tRNA_1^{Gly}$ copies [13,16,17].

A negative regulatory element present between −220 to −300 nt upstream of the tRNA coding sequence in $tRNA_1^{Gly}$-1 has been identified to be a TATATAA motif located at −276 nt in the highly transcribed copy of $tRNA_1^{Gly}$-1 [13]. Multiple copies of this

Abbreviations used: TBF, TATA-box-binding factor; TBP, TATA-box-binding protein; TRF, TBP-related factor; TLF, TBP-like factor; TAF, TBP-associated factor; pol (II/III), RNA polymerase II and III respectively; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; RK fragment, *Eco*RI–*Kpn*I fragment.

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sequence were present in the flanking regions of the poorly transcribed gene copies $tRNA_1^{Gly}$-6 and -7 [16,17]. Mutations in, or deletions of, the TATATAA sequence increased the transcriptional levels *in vitro* substantially. The fact that the TATATAA motif, a perfect polymerase II (pol II) consensus sequence for TBP binding, could exert a negative effect on pol III transcription was somewhat intriguing, since TBP is also required for pol III transcription [32]. These multiple TATATAA elements possibly competed for transcription factors such as TBP or the TBP-associated factors (TAFs), and brought about inhibition by sequestration of these factors.

In this study, we report the purification and characterization of a novel 43 kDa protein, designated here as P43 TBF (TATA-box-binding factor) from the posterior silk gland nuclear extracts, specifically binding to the TATATAA sequences. Supplementation of P43 TBF to the *in vitro* transcription reactions showed a pronounced inhibitory effect on transcription by both pol III and pol II.

EXPERIMENTAL

Plasmid constructs

Individual members of the $tRNA_1^{Gly}$ multi-gene family from *B. mori*, available from our laboratory stocks, were utilized. The gene copies used were $tRNA_1^{Gly}$-1 and -11 (highly transcribed), $tRNA_1^{Gly}$-2 and -4 (medium-level transcription) and $tRNA_1^{Gly}$-6, -7 and -10 (poorly transcribed) [16,17]. *B. mori* $tRNA^{Glu}$ gene was a 420 bp genomic fragment in pBR327, encoding the 72 nt mature $tRNA^{Glu}$ [33]. The plasmid construct pAc5.1 ORF (open reading frame) 42 containing ORF42 from *B. mori* nucleopolyhedrovirus under the control of the *Drosophila* actin promoter (constructed in our laboratory) was used as a template for pol II-mediated transcription. The cDNA clone of *B. mori* TBP carrying the 1.1 kb insert in the expression vector pET 19b [34] was a gift from Dr Karen Sprague (University of Oregon, Eugene, OR, U.S.A.).

In vitro transcription assays

Nuclear extracts from posterior silk glands of *B. mori* in the fifth larval instar were prepared as described previously [13]. Briefly, freshly dissected silk glands (or glands frozen at −70 °C for up to 6 months) were homogenized (Dounce homogenizer) in 10 mM Hepes, pH 7.9, containing 2 M sucrose, 10 % (v/v) glycerol, 15 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.15 mM spermine, 0.15 mM spermidine and 1 mM EDTA. Nuclei were pelleted by centrifugation (25000 rev./min in an SW 28 rotor for 70 min at 4 °C) and lysed in 20 mM Hepes, pH 7.9, containing 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF. The crude lysate was cleared by centrifugation, dialysed against buffer A (20 mM Hepes, pH 7.9, containing 20 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF), and used as crude nuclear extracts for transcription. *In vitro* transcription reactions in a final volume of 25 μl contained 20 mM Hepes, pH 7.9, 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 6 mM creatine phosphate, 50 μM each of ATP, CTP and UTP, 10 μM GTP, 5 μCi of [α-^{32}P]GTP (3000 Ci/mmol), nuclear extract (20 μg of protein) and 4 μg/ml of the supercoiled plasmid DNA template. After incubation at 30 °C for 1 h, the reactions were terminated by the addition of 0.2 % (w/v) SDS and 10 mM EDTA. The samples were extracted once with phenol, and the transcripts in the aqueous layer were precipitated by the addition of 3 vols. of ethanol in the presence of 100 μg/ml glycogen (as a carrier). The precipitate was re-suspended in gel-loading buffer containing 80 % formamide, and subjected to electrophoresis on 7 M urea/8 % (w/v) acrylamide gels.

These nuclear extracts were also used for pol II transcription experiments from the *actin5C* promoter. The pol II transcription reaction contained, in a final volume of 25 μl, 20 mM Hepes, pH 7.9, 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 6 mM creatine phosphate, 500 μM each of ATP, CTP, UTP and GTP, nuclear extract (20 μg of protein) and 8 μg/ml of the supercoiled plasmid DNA template. After incubation at 30 °C for 1 h, the reactions were terminated by the addition of 0.2 % SDS and 10 mM EDTA, and the transcripts were isolated as for pol III transcriptions. The pol II-generated transcripts were identified using primer-extension analysis for the Bm ORF42 transcript using ^{32}P-end-labelled primer (ATCTCGAGTGTG-TTCGCG) and reverse transcriptase, in the presence of all four dNTPs. The primer-extended products were analysed on a 7 M urea/6 % (w/v) acrylamide gel by electrophoresis.

Electrophoretic mobility shift assay (EMSA)

DNA fragments harbouring the TATATAA sequence were derived either from the flanking regions of $tRNA_1^{Gly}$ copies or by the annealing of two complimentary oligonucleotides. The TATATAA sequence present upstream of $tRNA_1^{Gly}$-1 was released as a 150 bp *Eco*RI–*Kpn*I fragment (RK fragment). The TATATAA sequence present in the far upstream region in $tRNA_1^{Gly}$-6 and -7 (at −871 nt with respect to +1 of $tRNA_1^{Gly}$-6) was released as a 45 bp *Sac*I fragment (*Sac*45 fragment) [16]. The *actin* promoter sequence containing the TATAAA element was released as a 300 bp *Nco*I–*Eco*RI fragment from the plasmid vector pAc5.1/V5 (obtained from Invitrogen). The synthetic oligonucleotides 1 (5′ GATCGAACTTTGCTTATATAAAAATATACC 3′) and 2 (5′ GATCGGTATATTTTTATATAAGCAAAGTTC 3′) used both in the EMSAs and in the preparation of affinity matrix were designed on the basis of the upstream sequence of $tRNA_1^{Gly}$-1.

The assay system for the EMSAs [35] contained, in a final volume of 15 μl, 0.1–2 μg of protein (crude nuclear extract or purified fractions as indicated), 12 mM Hepes, pH 7.9, 40 mM KCl, 5 mM $MgCl_2$, 4 mM Tris/HCl, pH 8.0, 0.6 mM EDTA, 0.6 mM DTT, 5 % (v/v) glycerol and 2 μg of double-stranded poly(dI-dC). After incubation at 4 °C for 15 min, the radiolabelled DNA fragment (10000 c.p.m.) harbouring the TATATAA sequence was added, and the binding reaction was allowed to proceed for a further 15 min. In retardation–chase experiments, varying amounts of the unlabelled specific competitors were also included. The binding reactions were terminated by adding 5 μl of the gel-loading buffer, and the samples were electrophoresed on 5 % polyacrylamide gels at 4 °C.

Expression and purification of silkworm TBP

Escherichia coli BL-21(DE3) cells transformed with the TBP cDNA construct were grown at 37 °C in Luria–Bertani broth containing 100 μg/ml ampicillin, and were induced by the addition of 500 μM isopropyl β-D-thiogalactoside during the mid-exponential phase of growth. After 2 h, the cells were harvested and lysed by sonication in 0.1 M NaCl/HMGN buffer [25 mM Hepes (pH 7.6)/12.5 mM $MgCl_2$/10 % glycerol/0.1 % Nonidet P40/4 mM 2-mercaptoethanol/0.1 mM PMSF]. The supernatant fraction was loaded on to a 5 ml heparin–Sepharose column (Pharmacia) equilibrated in 0.1 M NaCl/HMGN buffer. The column was washed with 25 ml of loading buffer, and the bound proteins were step-eluted with 0.4 M and 1 M NaCl/HMGN buffers. TBP was eluted with 1 M NaCl, and the peak fractions were pooled and loaded on to a 0.5 ml Ni^{2+}–Sepharose column (Novagen) pre-equilibrated with 0.7 M NaCl/HMGN

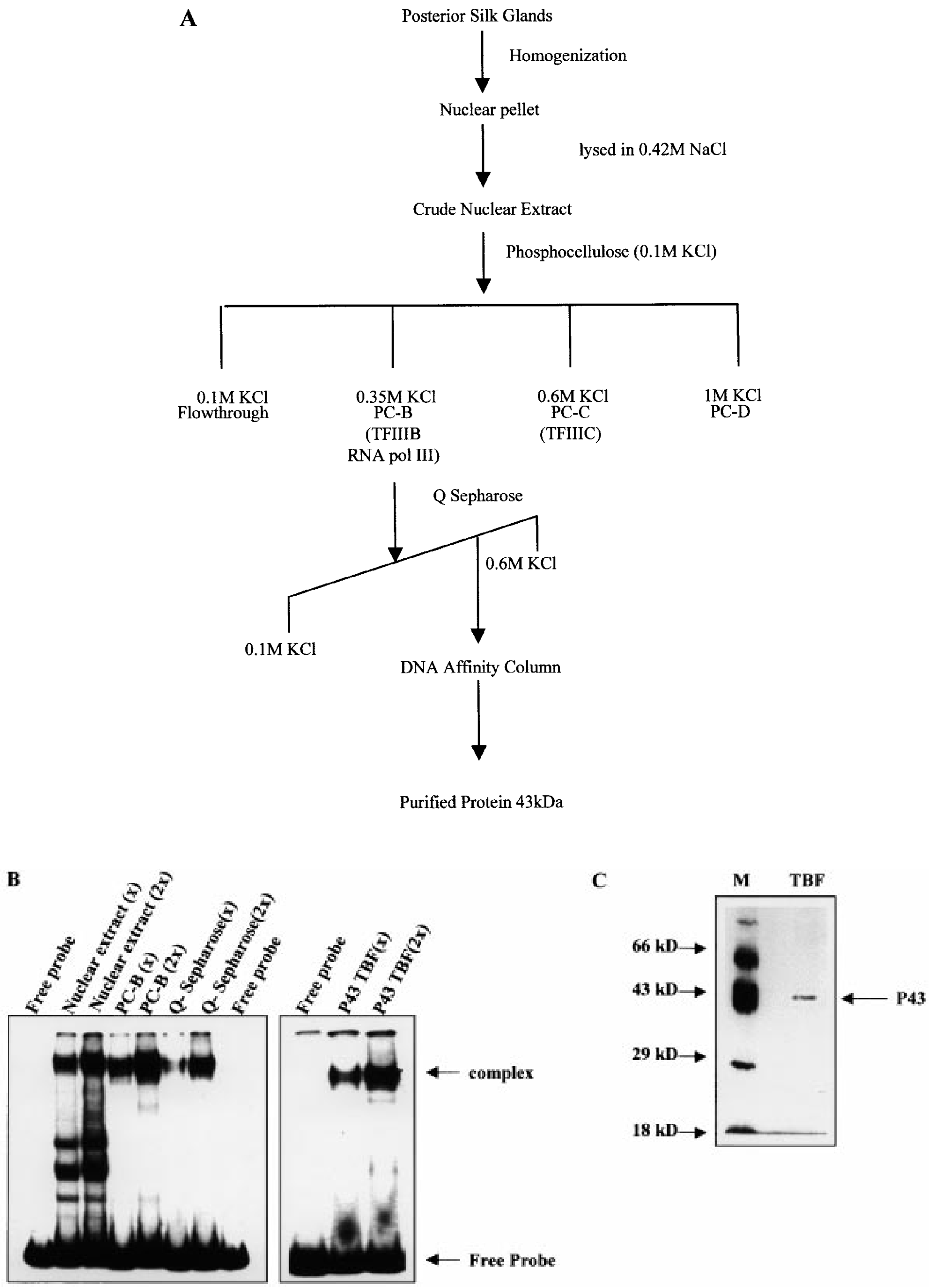

Figure 1 Purification of TATATAA-sequence-specific-binding protein

(**A**) Schematic presentation of the purification strategy for the TATATAA-sequence-binding protein from *B. mori* posterior silk gland nuclear extracts. (**B**) Individual fractions at each fractionation step were analysed for sequence-specific DNA binding using the 150 bp fragment (-150 to -300 nt upstream of the $tRNA_1^{Gly}$-1 coding region; see Figure 2A, top panel). The probe (RK fragment containing one TATATAA sequence) was isolated as the *Kpn*I–*Eco*RI restriction fragment from $tRNA_1^{Gly}$-1 and was end-labelled using Klenow DNA polymerase in the presence of [α-^{32}P]dATP. For assay conditions of EMSA, see the Experimental section. Lanes were loaded as indicated on the Figure. PC-B, Q-Sepharose and TBF refer to the fractions emerging from phosphocellulose, Q-Sepharose, and the protein eluted from affinity chromatography respectively. 1 × and 2 × refer to the concentrations of the samples used. (**C**) shows the electrophoretic pattern on SDS/PAGE of TBF (150 ng) eluted from the affinity matrix. The proteins were stained by silver nitrate [44]. A single band of 43 kDa is detected.

buffer. The column was washed with binding buffer, and the bound proteins were eluted with 0.7 M NaCl/HMGN buffer containing 500 mM imidazole, and dialysed against 0.1 M NaCl/HMGN buffer for 5 h. The purity of TBP was confirmed by SDS/PAGE, and checked for its activity by restoration of $tRNA_1^{Gly}$ transcription in nuclear extracts that were immuno-depleted of anti-TBP antibodies.

Purification of TBF

Phosphocellulose chromatography

The posterior silk gland nuclear extract was loaded on to a column of phosphocellulose (1 cm × 15 cm) in buffer A (see above). The column was washed with the loading buffer, and the bound proteins were eluted using a step gradient (0.35 M, 0.6 M and 1 M) of KCl. Individual fractions were tested for the presence of TBF using EMSAs.

Q-Sepharose chromatography

The TBF-containing fraction (0.35 M KCl fraction) from the phosphocellulose column was dialysed against buffer A and loaded on to a Q-Sepharose column (2 ml) pre-equilibriated with buffer A at a flow rate of 3 column-vols./h. The column was washed with 5 column-vols. of buffer A, and the bound proteins were eluted using a 10 ml linear gradient of KCl (from 0.1–0.6 M) in buffer A. The fractions containing TBF were pooled, dialysed against buffer A for 4 h, and then processed for DNA-affinity chromatography.

DNA-affinity chromatography

The DNA-affinity matrix was prepared using the double-stranded oligonucleotides [generated by annealing of oligonucleotide 1 (conjugated to biotin at the 5′ end) and the complementary oligonucleotide 2]. The annealed oligonucleotides were coupled with 0.1 ml of streptavidin–agarose beads for 30 min at 20–22 °C. The efficiency of coupling was checked by estimating the amount of DNA in the input and the unbound fractions. Almost 100 % coupling efficiency was achieved. The non-specific binding sites in the affinity matrix were blocked by incubation with three column-vols. of buffer A containing 0.25 mg BSA/ml for 30 min at 20–22 °C. The agarose beads (linked to the double-stranded oligonucleotides via the biotin–streptavidin interaction) were used as an affinity matrix for the purification of TBF.

The fraction containing TBF from the previous step was incubated initially with 50 μg of poly(dI-dC)·(dI-dC)/ml for 30 min to bind all the non-specific DNA-binding proteins. This sample (final volume 0.2 ml) was incubated with the affinity matrix at 4 °C for 1 h with gentle swirling. The suspension was briefly centrifuged, and the matrix was washed with 10 ml of binding buffer. The bound proteins were eluted using buffer A containing 1 M KCl, dialysed against buffer A for 4 h, and then stored as aliquots at -70 °C.

Determination of the equilibrium constant for DNA binding

The equilibrium constants for specific binding reactions are provided by the following equations [36]:

$$[CD] = [C^o] \cdot K_{eq} \cdot [D]/(1 + K_{eq} \cdot [D]) \quad (1)$$

and

$$[CD]/[D] = -K_{eq} \cdot [CD] + K_{eq} \cdot [C^o] \quad (2)$$

where [D] and [CD] are the free and bound species of the probe respectively, $[C^o]$ denotes the total number of DNA binding sites and K_{eq} is the equilibrium constant of the binding reaction. Eqn (1) describes a hyperbola, whereas eqn (2) is a straight-line plot, equivalent to a Scatchard plot. Standard DNA-binding reactions were performed with a constant amount of protein sample and various dilutions of labelled probe. The protein–DNA complex formed in the binding reaction and the unbound probe were quantified in a PhosphorImager apparatus following electrophoresis. The equilibrium constant was determined graphically from a plot of [CD]/[D] against [CD], according to eqn (2), where the negative reciprocal of the slope of the line yields the value of K_{eq} and the intercept on the y-axis gives the value of $[C^o]$.

RESULTS

Purification of the factor binding to TATATAA

We have reported previously that EMSAs with a DNA fragment derived from $tRNA_1^{Gly}$-1 (-150 to -300 nt upstream of the tRNA-coding region, designated as RK fragment and harbouring a single TATATAA motif; see Figure 2A) [17] and nuclear extracts derived from posterior silk glands identified specific complexes. These labelled complexes could be 'chased out' in the presence of an excess of unlabelled competitor. We examined the binding of silkworm TBP (purified recombinant protein) to the RK fragment, but this did not reveal any stable complex formation under the experimental conditions used.

The binding factor was purified from the posterior silk gland nuclear extracts using the strategy depicted in Figure 1(A). The essential features of the purification scheme included the use of two conventional chromatographic columns (phosphocellulose and Q-Sepharose) and the use of a specific DNA-affinity matrix. In the first step, posterior silk gland nuclear extracts were fractionated on a phosphocellulose P11 column. The pol III transcription machinery separates into two fractions, one containing pol III in addition to the transcription factor TFIIIB (PC-B fraction), and the other containing TFIIIC (PC-C fraction) [37]. The PC-B fraction that showed binding to the probe was purified further by chromatography on a Q-Sepharose column. The eluate showing the maximum binding (the 0.38 M KCl fraction) was purified further on the oligonucleotide affinity matrix. For this step, the fractions from the Q-Sepharose column were incubated first with poly(dI-dC) · (dI-dC) to eliminate the non-specific DNA-binding proteins present in the fraction, and then applied to specific DNA matrix (double-stranded oligonucleotides linked to agarose beads via streptavidin–biotin interactions). The pre-treatment of the samples with non-specific DNA [poly (dI · dC)] before the specific oligonucleotide affinity column step improved the purity of the final samples. The bound proteins were eluted at high salt concentrations.

The purification profile of the TBF is shown in Figure 1(B). Although multiple complexes were detected with crude nuclear extract, only a single specific complex was detected from the phosphocellulose chromatography step onwards. SDS/PAGE analysis (Figure 1C) of the active fraction from the affinity column revealed a single polypeptide of 43 kDa. That the DNA-binding activity was associated with this purified band was confirmed by EMSAs using the protein eluted from the gel following SDS/PAGE (Figure 2B; lanes 8–10).The TATATAA-binding activity of fractions generated during the course of purification is shown in Table 1. Total protein content was reduced from 70 mg in the crude nuclear extract to 4 μg in the purified fraction.

This purified protein was subjected to protein microsequence analysis, and partial amino acid sequences of the 12 peptides generated from this protein are presented in Table 2. The peptide sequences did not match with any of the known TBFs, such as TBPs, TRF (TBP-related factor) or TLFs (TBP-like factors) from other sources. Extensive database searches did not reveal any matches to these peptides. This protein thus appears to be a novel TBF, which we have designated P43 TBF.

P43 TBF binds specifically to the TATATAA sequence

To characterize further the purified P43 TBF and establish the target DNA specificity, TATATAA sequence in the RK fragment was mutated to GATATCA (mutant available in the lab stocks [17]; Figure 2A). EMSAs performed with the parental, as well as the mutant, RK fragment (Figure 2B) revealed that P43 TBF

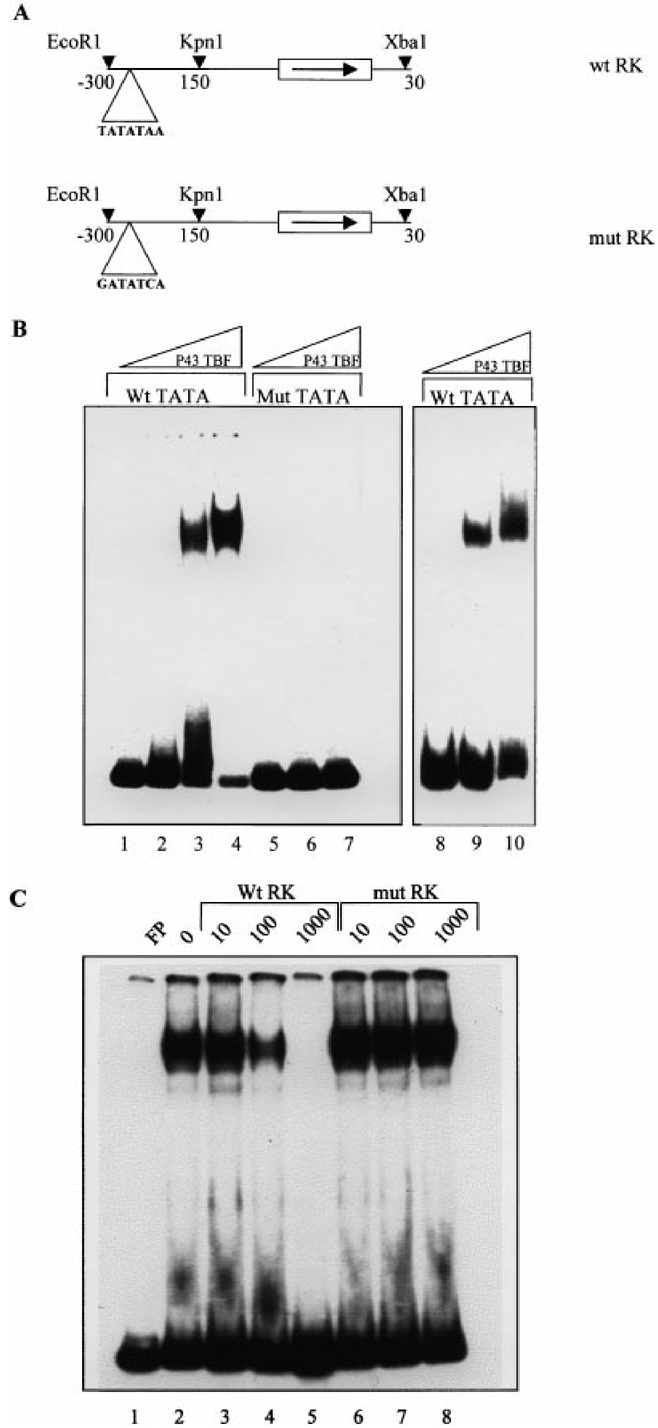

Figure 2 Specfic binding of P43 TBF to the TATATAA sequence

(**A**) The position of RK fragment (parental type or mutant) on the $tRNA_1^{Gly}$-1 construct. The sequence TATATAA is present at −276 nt with respect to the tRNA-coding region. In the mutant RK fragment, the sequence TATATAA was mutated to GATATCA. (**B**) EMSA with parental and mutant RK fragments (Wt TATA and Mut TATA respectively) using purified P43 TBF, showing the increasing concentrations of P43 TBF that were used for binding to the end-labelled probes (10000 c.p.m.). Lanes: 1, free probe (wild-type RK); 2, 3 and 4, binding of the fragment with 2.5, 5 and 10 ng of P43 TBF respectively; 5, free probe (mutant RK); 6 and 7, binding of mutant RK fragment with 5 and 10 ng of P43 TBF respectively. EMSA with the parental RK fragment and the P43 TBF eluted from a preparative acrylamide gel following electrophoresis (5 and 10 ng of protein) is shown in the right panel (lanes 8–10). The protein was eluted from the gel and renatured by dialysing out the SDS and treating with guanidinium chloride, followed by dialysis. (**C**) Competition of DNA binding. The binding of radiolabelled RK fragment (10000 c.p.m.) with 10 ng of P43 TBF was performed, and the complex was 'chased' using increasing amounts of either unlabelled parental RK fragment or mutant RK fragment. Lanes: 1, free probe (wt RK); 2, binding in the presence of 100 ng of P43 TBF; 3–5, chase using 10, 100- and 1000-fold molar excess of unlabelled parental RK fragment; 6–8, chase using 10, 100- and 1000-fold molar excess of mutant RK fragment.

Table 1 Quantification of TATATAA binding factor purification by DNA-binding activity

TATATAA box binding activity of fractions generated during the purification was measured by EMSAs. One unit of activity is defined as the amount of TATATAA binding factor necessary to bind 1 fmol of the 150 bp RK fragment.

Column	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold purification
Nuclear extract	70	62500	892	–
Phosphocellulose	22.2	36038	1623	1.8
Q-Sepharose	0.72	12628	17538	19.6
DNA-affinity	0.004	4000	1000000	1121

Table 2 Sequences of peptides generated from protein microsequence analysis of P43 TBF

The peptide sequences shown below have been determined from two independent batches of the purified protein P43. The protein sequencing was carried out at the University of Virginia Biomedical Research Facility. Peptides were generated by tryptic digest of P43 TBF and the sequence of each peptide was analysed by MS. 'X' denotes either isoleucine or leucine; '?' designates a single unknown residue, and ... designates an unspecified number of unknown residues.

Peptide no.	Measured molecular mass (Da)	Peptide sequence
1	678.6	VXSMTK
2	935.5	EAXTVFEK
3	1303.7	??EEHDXDYR
4	1359.7	??PSDTVP ... K
5	1443.9	KGDDQEDTGFGFK
6	1478.7	FXDDFESXHXSR
7	1536.7	??XAAYSSVDGEYK
8	1589.8	... EXAGESV ... QK
9	1691.9	??EESENXTWDXVR
10	1692.6	??XAAYSSVDGEYKR
11	2052.3	??MEVFEX??XADXDENK

binding to the parental fragment was completely abolished when **T**ATATA**A** was mutated to **G**ATATC**A** (mutations are in bold). The binding-competition experiments performed using the parental TATATAA and the mutant sequences confirmed these results (Figure 2C). Even at a 1000-fold molar excess, when the parental sequence completely chased out the labelled complexes, the mutant sequence did not have any effect on the complex formed with the parental sequences.

Having established that the binding is specific to the TATATAA sequence, we also analysed the binding of P43 TBF to the TATATAA sequences present in the flanking region of $tRNA_1^{Gly}$-6 and -7 [16], as well as the TATA sequence present in *actin5C* promoter, which is a typical pol II promoter. The position of the TATATAA sequence (harboured in the *Sac*45 fragment) with respect to the $tRNA_1^{Gly}$-6 and -7 coding regions, and the location of the TATA box in the *actin* promoter, are shown in Figure 3(A). EMSAs performed with *Sac*45 fragment and the *actin* promoter fragment revealed the presence of complexes proportional to the increasing amounts of P43 TBF added (Figure 3B). The specificity of binding to these fragments was also

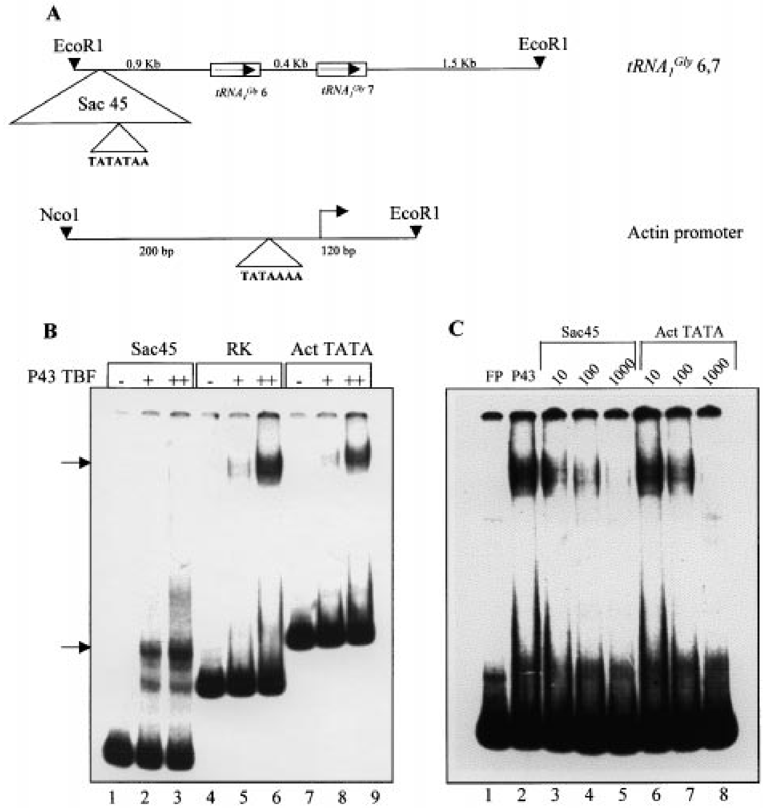

Figure 3 Binding of P43 TBF to TATA motifs present in other genes

(**A**) The position of *Sac*45 fragment harbouring the TATATAA sequence on the *tRNA*$_1^{Gly}$-6,7 construct is shown. The tRNA-coding region is marked with an arrow. The location and sequence of *actin* promoter TATA box in construct pAc5.1 V5 is shown. (**B**) EMSAs with the *Sac*45 and RK (150 bp) fragments, and the 300 bp *actin* promoter region (Act TATA). All three fragments were end-filled in the presence of [α-^{32}P]dATP, and were used as probes for EMSAs (10000 c.p.m. of each probe). Lanes: 1–3, binding with *Sac*45 probe; 4–6, binding with RK probe; 7–9, binding with *actin5C* TATA box as probe. The positions of the complex are indicated by arrows. (**C**) The complex between the wild-type RK fragment and P43 TBF (10 ng) was chased with excess unlabelled *Sac*45 fragment or unlabelled *actin5C* TATA fragment. Lanes: 1, free probe (labelled RK fragment); 2, binding in the presence of purified P43; 3–5, chase using 10-, 100- and 1000-fold molar excess of unlabelled *Sac*45 fragment; 6–8, chase using 10-, 100- and 1000-fold molar excess of unlabelled *actin* promoter fragment.

confirmed by competition experiments in which they were used as competitors for binding to the labelled RK fragment. The P43 TBF–RK fragment complex was competed out with increasing concentrations of either the *Sac*45 or *actin* promoter fragments with equal efficiency, reaching almost complete competition at 1000-fold molar excess of the competitor (Figure 3C). P43 TBF therefore appears to be a general TBF involved in the regulation of transcription.

Equilibrium constant of P43 TBF binding to TATATAA sequences

EMSA, which separates DNA probe–protein complex from unbound probe, provides a simple assay for studying the kinetics of specific DNA–protein interactions [36]. Figure 4(A) shows the result of a titration experiment in which P43 TBF complex formation was measured as a function of increasing concentrations of the DNA probe. Since the P43 TBF used for the binding reaction was pure, addition of excess carrier DNA to quench the non-specific binding of other proteins was not required. Therefore the binding reactions did not contain any DNA other than the specific probe. The equilibrium constant K_{eq} for P43 TBF binding determined from the quantification of binding data from Figure 4(A) was 4.0×10^{11} M^{-1}, and the total number of binding sites [C^o] was 3.04×10^{-10} M (Figure 4B).

P43 TBF inhibits *in vitro* transcription from both pol II and pol III promoters

The effect of P43 TBF on *in vitro* pol III transcription of $tRNA_1^{Gly}$ gene copies was analysed by supplementing the *in vitro* transcription assays using posterior silk gland nuclear extracts with P43 TBF. Figure 5(A) shows the effect of P43 TBF on the transcription of $tRNA_1^{Gly}$-1. Transcription from $tRNA_1^{Gly}$-1 gives rise to two distinct transcripts, corresponding to an approx. 81 nt primary transcript (precursor) and a 71 nt mature (processed) transcript. With increasing concentrations of P43 TBF, transcription was decreased markedly, and was completely inhibited in the presence of 40 nM P43 TBF (Figures 5A and 5B). The inhibition of transcription was consistent, and the levels of inhibition were identical with different batches of nuclear extract and purified P43 TBF. The inhibition of the transcription due to P43 TBF was also confirmed using the protein eluted from the gel following SDS/PAGE (Figure 5A, lanes 6–8).

The effect of P43 TBF on the transcription of other $tRNA_1^{Gly}$ gene copies was also analysed (Figure 5C). The gene copies examined belonged to the three different categories of expression, namely $tRNA_1^{Gly}$-1 and -11 (highly transcribed), $tRNA_1^{Gly}$-2 and -4 (medium-level transcription) and $tRNA_1^{Gly}$-6, -7 and -10 (poorly transcribed). Supplementation of 30 nM of the purified protein

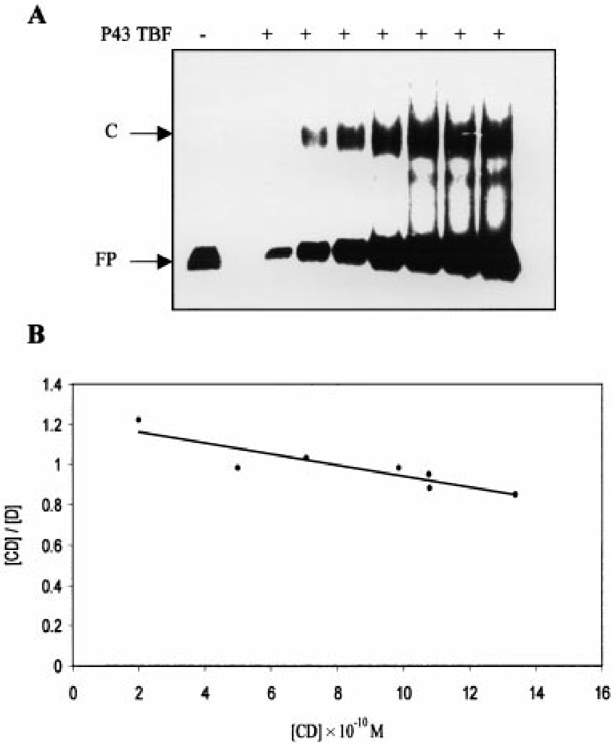

Figure 4 Equilibrium constant for the binding of P43 TBF with RK fragment

EMSAs were performed with increasing amounts of labelled RK fragment and a fixed amount (10 ng) of purified P43 TBF. (**A**) A typical autoradiogram of a titration gel. The protein–DNA complex formed and the unbound probe remaining were quantified in a PhosphorImager following electrophoresis. The Scatchard analysis of the binding data (for details, see the Experimental section) is presented in (**B**). The negative reciprocal of the slope of the line gave the value of K_{eq} of binding of P43 TBF to the TATATAA sequence and the *y*-axis intercept, [C^0], which corresponds to the total number of DNA-binding sites.

resulted in complete inhibition of *in vitro* transcription of all the $tRNA_1^{Gly}$ genes tested.

The effect of P43 TBF on the *in vitro* transcription of a $tRNA_1^{Gly}$-1 construct, where the TATATAA box sequences have been deleted, as well as the transcription of a different tRNA gene, $tRNA^{Glu}$ from *B. mori*, was analysed. On supplementation with increasing concentrations of P43 TBF, the transcription of both the genes were markedly decreased (Figure 5D). The quantification of transcription is presented in Figure 5(E). As we reported previously [17], transcription from the $tRNA_1^{Gly}$-1 construct lacking the TATATAA sequences was found to be 30–50 % greater than that of the parental construct. The transcription from both these constructs were equally susceptible to inhibition by the externally added P43 TBF, and more than 90 % inhibition was seen at a concentration of 40 nM protein.

Having established that *in vitro* supplementation of P43 TBF to crude nuclear extracts causes inhibition of tRNA gene transcription, the effect of this protein on pol II transcription was analysed. The posterior silk gland nuclear extracts from *B. mori* showed appreciable levels of pol II transcription from the *Drosophila actin5C* promoter. The amount of nuclear protein required for maximum transcription from the *actin* promoter was standardized. Maximal transcriptional activity was achieved in the presence of 15 μg of nuclear protein, and a specific transcript of approx. 350 nt, corresponding to the inserted fragment (Bm ORF42), under the expression of the *actin* promoter was obtained. The effect of P43 TBF on transcription from the *actin* promoter is shown in Figure 6(A). Addition of 40 nM P43 TBF caused approx. 60 % inhibition of transcription from the *actin* promoter (Figure 6B). To confirm that the transcript arising from the *actin* promoter was indeed due to pol II, we also analysed the sensitivity of the transcription to α-amanitin. Among eukaryotic polymerases, pol II is most sensitive to α-amanitin (50 % inhibition at 25 ng/ml) and pol III shows an intermediate level of sensitivity (50 % inhibition at 20 μg/ml), whereas pol I is resistant. Addition of 50 ng/ml of α-amanitin to *in vitro* transcription reactions completely abolished the transcription from the actin promoter (Figure 6A, lanes 7–10), indicating that the transcription from the *actin* promoter was indeed due to pol II.

P43 TBF inhibited both pol II and pol III transcription; however, the inhibition was more pronounced in the case of pol III. TBP is a common factor involved in transcription by both polymerases, being a component of TFIIIB and TFIID. The nuclear extracts, when supplemented with increasing concentrations of recombinant TBP, caused a decrease in the *in vitro* transcription levels of $tRNA_1^{Gly}$-1, and the transcript was almost undetectable after supplementation with excess TBP (Figure 7A, lanes 1–6). The sequestration of TAFs is a possible cause for this inhibition by excess TBP. The nuclear extracts, when immunodepleted of TBP with TBP-specific antibodies, showed a marked reduction in the levels of $tRNA_1^{Gly}$-1 transcription (Figure 7A, lanes 7 and 8). The transcription by these immunodepleted extracts was restored upon addition of purified recombinant silkworm TBP (lanes 9–12). Evidently, TBP was essential for transcription. In order to see if inhibition caused by P43 TBF was due to direct competition with TBP, we examined whether externally supplemented TBP could reverse the inhibitory effect of P43 TBF (Figure 7B). Supplementation of TBP did not reverse the inhibition caused by P43 TBF. On the contrary, addition of TBP augmented the inhibitory effect of P43 TBF (lanes 4–6). We also analysed the effect of P43 TBF on transcription of $tRNA_1^{Gly}$-1 with nuclear extracts immunodepleted of TBP antibodies (Figure 7C). Transcription by the immunodepleted extracts was not restored by supplementation with purified P43 TBF (lanes 3–5). However, as expected, recombinant TBP restored the transcription of $tRNA_1^{Gly}$-1 (lanes 6 and 7). These results support the hypothesis that both TBP and P43 TBF were competing for other associated factors necessary for transcription.

DISCUSSION

pol III transcription of tRNA genes, in general, is considered to be less stringently regulated in tissue-specific terms than is pol II transcription, since they form part of the 'housekeeping' functions. However, tissues such as the silk-glands of *B. mori* might provide exceptions to this rule, where a functional adaptation as reflected by enhanced synthesis of certain species of tRNA (e.g. $tRNA^{Gly}$ and $tRNA^{Ala}$) takes place to optimize the committed synthesis of the silk proteins [27,28]. In such instances, more regulatory circuits might be operative to achieve preferential and enhanced transcription of specific tRNA genes, as demanded by tissues with a committed function. The presence of *cis*-regulatory elements, and the *trans*-acting factors capable of interacting with them, can both contribute to such regulation of pol III transcription of tRNA genes.

The 20 different copies of $tRNA_1^{Gly}$ in *B. mori* are differentially transcribed both *in vitro* and *in vivo*, and hence form a good model system for the analysis of tRNA gene regulation. The

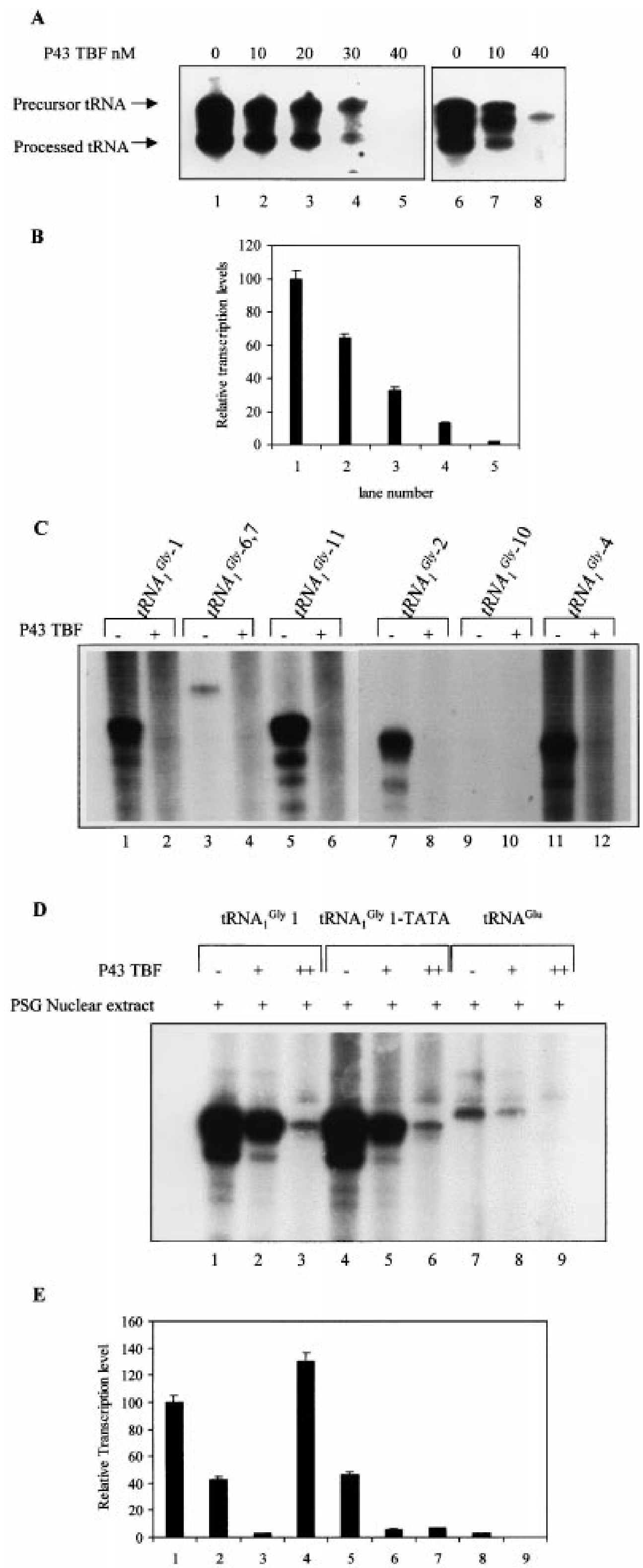

Figure 5 Effect of P43 TBF on pol III transcription

P43 TBF was supplemented to *in vitro* transcription reactions. For details of the *in vitro* transcription assays with posterior silk gland nuclear extracts, see the Experimental section. Various amounts of P43 TBF were added to the nuclear extracts, as indicated, and incubated for 10 min in the presence of transcription buffer and NTP mix. To initiate the transcription reaction, 100 ng of the plasmid template was added and the samples were incubated for 60 min at 30 °C. (**A**) Effect of supplementation of increasing amounts of P43 TBF to the *in vitro* transcription of $tRNA_1^{Gly}$-1. Distinct transcripts, corresponding to the precursor tRNA (81 nt) and processed tRNA (71 nt), are shown. *In vitro* transcription of $tRNA_1^{Gly}$-1 in the absence of supplemented P43 TBF (lane 1), and in the presence of 10, 20, 30 and 40 nM P43 TBF (lanes 2–5 respectively) is presented. The panel to the right (lanes 6–8) shows the inhibition of pol III transcription $tRNA_1^{Gly}$ by P43 TBF eluted out

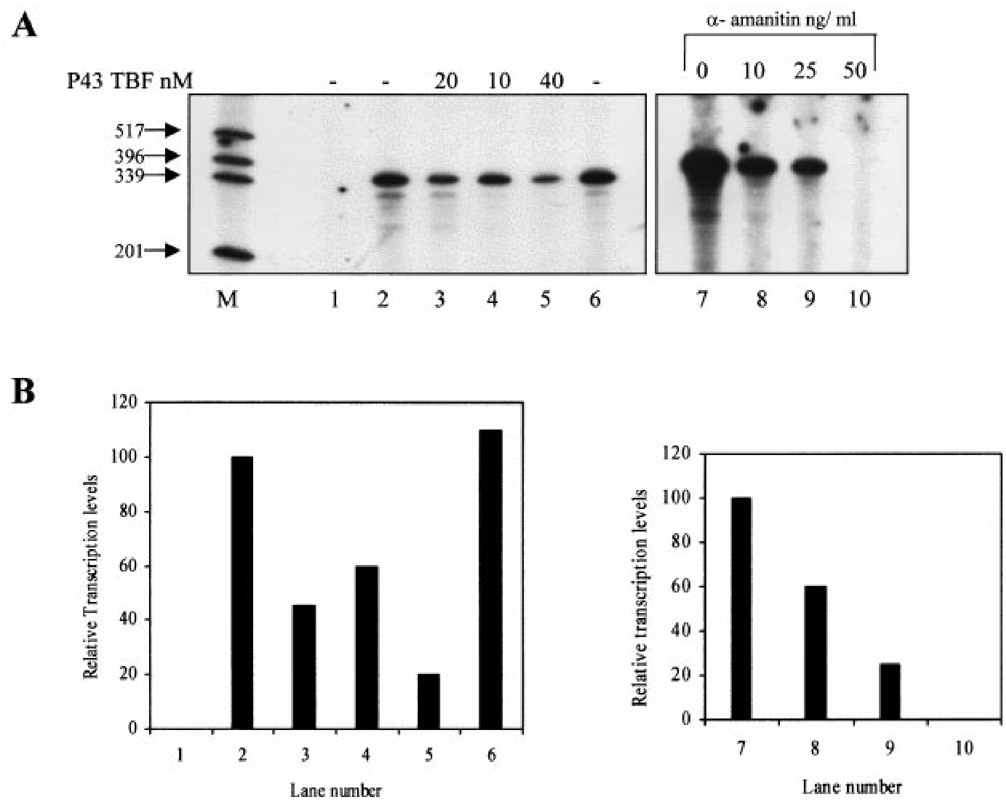

Figure 6 Effect of P43 TBF on pol II transcription

The effect of P43 TBF on transcription by pol II from the *Drosophila actin* promoter was analysed *in vitro* using the posterior silk gland nuclear extracts. The transcripts generated were detected by primer-extension analysis using a primer specific for the reporter gene (ORF42 from BmNPV cloned under the *actin* promoter), and situated 350 nt from the transcription start site. (**A**) The effect of increasing concentrations of P43 TBF on transcription from the actin promoter. Lanes: M, molecular-size marker (pTZ 18 DNA digested with *Hin*f I and end-labelled); 1, control (no template DNA); 2 and 6, *in vitro* transcription from actin template in the absence of supplemented P43 TBF; and 3, 4 and 5, in the presence of 20, 10 and 40 nM of P43 TBF respectively; 7–10, *in vitro* transcription from actin template, in the absence (lane 7) or presence (lanes 8–10) of 10, 25 or 50 ng/ml α-amanitin. Quantification of transcription (by PhosphorImaging) is shown in (**B**).

upstream sequences of $tRNA_1^{Gly}$ copies, located up to -100 nt with respect to the tRNA-coding region, are highly A/T-rich [14]. Transcription of the different $tRNA_1^{Gly}$ copies is negatively modulated by the presence of the TATATAA sequence in the far-flanking regions of the coding region [13,16,17]. However, similar sequences (with changes in one or two nucleotides) were also present within the first 100 nt in 5′ flanking regions of many $tRNA_1^{Gly}$ copies. For instance, in addition to the far-flanking TATATAA sequence, which exerted the negative effect on transcription, $tRNA_1^{Gly}$-1 and $tRNA_1^{Gly}$-6 harboured perfect TATATAA sequences at nt positions -86 and -26 respectively. These 'near-TATATAA' elements, when deleted, resulted in a loss of transcription [13,17]. Moreover, the distal TATATAA sequence, when moved closer to the transcription start site, acted as a positive modulator for transcription in the absence of other positive regulatory elements [17].

Although most regions upstream of the *tRNA* gene do not harbour TATA-box sequences, TBP is an essential component of the pol III transcription machinery, as in the case of pol II and pol I transcription [38]. The recruitment of TBP to the pol III transcription initiation sites is generally achieved as a component of the TFIIIB, by interaction with TFIIIC. Some of the pol III-transcribed genes, such as that for the U6 snRNA, however, harbour TATA sequences in the upstream region and are known to recruit TBP directly via interaction with these elements [1].

We have purified a novel TBP, P43 TBF, from the posterior silk gland nuclear extracts of *B. mori*. Microsequence analysis of a few peptides generated from this protein did not show matches to any in the database entries. It is now well established that, besides TBP, metazoans also encode homologues of TBP, designated as TRFs. Two classes of TRF have been identified so far [39]. The shared homology of TBP and TRFs is confined to their DNA-binding core repeat domains. TRF1, reported only from *Drosophila* (dTRF1), is involved in both pol II and pol III transcription and is able to bind to TATA boxes present in pol II-associated promoters [40,41]. It also plays a major role in pol III transcription in *Drosophila*, and is found to be associated with the *Drosophila* TFIIIB [41]. In contrast, TRF2s, also termed

from the preparative acrylamide gel after electrophoresis (see the legend to Figure 2A). (**B**) Quantification of transcripts was accomplished using the PhosphorImager apparatus, and is presented as a bar chart. (**C**) Effect of P43 TBF on the *in vitro* transcription of various $tRNA_1^{Gly}$ copies. For details of transcription assays, see the Experimental section. P43 TBF (40 nM) was added to each of the transcription reactions. The gene copies tested were $tRNA_1^{Gly}$-1 (lanes 1 and 2), $tRNA_1^{Gly}$-6,7 (lanes 3 and 4), $tRNA_1^{Gly}$-11 (lanes 5 and 6), $tRNA_1^{Gly}$-2 (lanes 7 and 8), $tRNA_1^{Gly}$-10 (lanes 9 and 10) and $tRNA_1^{Gly}$-4 (lanes 11 and 12). (**D**) Effect of P43 TBF on the transcription of $tRNA^{Glu}$ and $tRNA_1^{Gly}$-1 devoid of the TATATAA motif. *In vitro* transcription of $tRNA_1^{Gly}$-1 with the posterior silk gland nuclear extract, in the absence (lane 1) or the presence (lanes 2–3) of increasing concentrations (15 and 30 nM) of P43 TBF. Lanes 4–6, *in vitro* transcription of $tRNA_1^{Gly}$-1 devoid of TATATAA in the absence (lane 4) or presence (5–6) of increasing concentrations of P43 TBF; lanes 7–9, *in vitro* transcription of $tRNA^{Glu}$ in the absence (lane 7) or presence (8–9) of increasing concentrations of P43 TBF. (**E**) Quantification of transcription in (**D**) by PhosphorImaging.

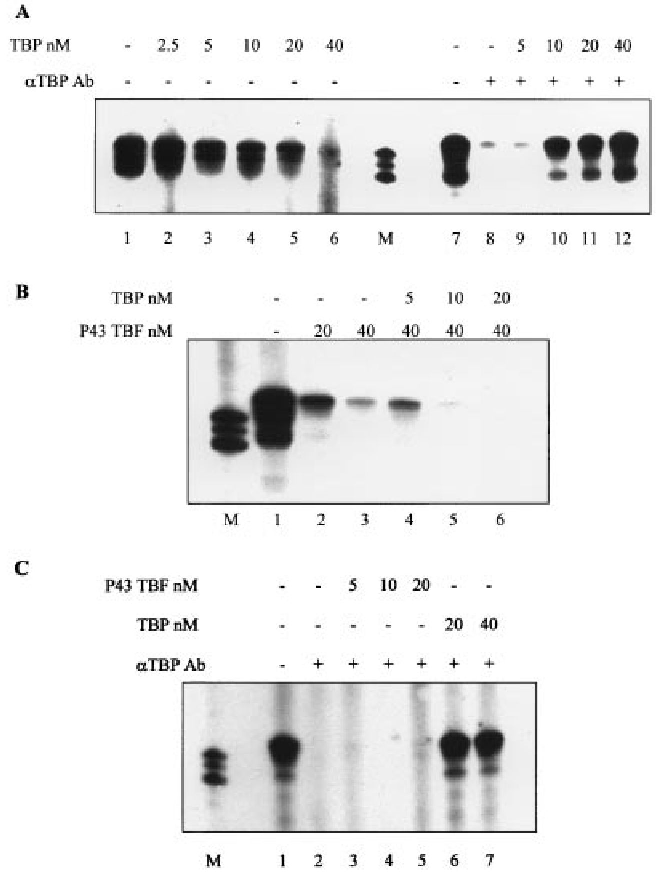

Figure 7 Effect of TBP on $tRNA_1^{Gly}$-1 transcription and transcription inhibition by P43 TBF

Silkworm TBP, expressed in *E. coli* as a His-tag fusion protein, was purified using a Ni^{2+}-nitrilotriacetic acid column. Purified TBP and P43 TBF were supplemented to the transcription assays, as indicated. (**A**) TBP was added to *in vitro* transcription reactions of $tRNA_1^{Gly}$-1 in untreated nuclear extracts (lanes 1–6) or nuclear extracts immunodepleted of TBP antibodies (lanes 7–12). Extracts were incubated with TBP monoclonal antibodies (58C9; Santa Cruz Biotechnology) for 1 h before use in the transcription reaction. Lane M, molecular-size marker. (**B**) Recombinant TBP was added (as indicated) to *in vitro* transcription reactions after the addition of P43 TBF. Lanes: M, molecular-size markers; 1–3, *in vitro* transcription of $tRNA_1^{Gly}$-1 in the presence of increasing amounts of P43 TBF; 4–6, transcription in the presence of 40 nM P43 TBF and increasing amounts of TBP, as indicated. (**C**) Effect of P43 TBF on $tRNA_1^{Gly}$-1 transcription by TBP-immunodepleted extracts. Increasing amounts of P43 TBF (lanes 3–5) or recombinant TBP (lanes 6 and 7) were added to posterior silk gland nuclear extracts immunodepleted of TBP antibodies (lane 2). Lane 1, *in vitro* transcription of $tRNA_1^{Gly}$-1 with posterior silk gland nuclear extract; M, molecular-size markers.

TLFs as reported in many systems, do not show binding to the TATA boxes [39,42]. P43 TBF, described in the present study, is qualified as a distinct TBF, which appears to resemble TRF1 in its property of binding to TATA boxes. So far, all our efforts to clone the gene encoding P43 TBF, on the basis of degenerate oligonucleotide primers designed from the peptide sequences either by genomic PCR or reverse transcription-PCR, have been unsuccessful. Although TBP has been cloned from the silkworm [34], no homologues of TRF1 or TRF2 have been reported to date.

The binding of P43 TBF to the TATATAA boxes was highly sequence-specific, and mutation of the sequence to GATATCA abrogated the binding. However, the protein was able to bind to the TATA box (TATAAA) sequence of a canonical pol II promoter (*Drosophila actin5C*). Thus P43 TBF could be a general TATA-binding factor. Analysis of the equilibrium constant for the binding of P43 TBF to a specific DNA sequence revealed it to be a much stronger DNA-binding protein ($K_{eq} = 4.0 \times 10^{11}\ M^{-1}$) than silkworm TBP or yeast TBP, for which K_{eq} values of $2.4 \times 10^{9}\ M^{-1}$ and $3 \times 10^{9}\ M^{-1}$ respectively were determined for binding to the adenovirus major late promoter TATA sequence [34,43].

Supplementation of P43 TBF inhibited both pol II and pol III transcription *in vitro*. It was possible that the protein competed with the TBP-binding sequences and brought about inhibition. However, the inhibition by P43 TBF was independent of the presence of the cognate DNA-binding site. For instance, transcription from $tRNA_1^{Gly}$-1 construct, in which the TATATAA sequence was deleted, was inhibited to the same extent as the parental copy. Moreover, transcriptional inhibition by P43 TBF was not reversed on addition of TBP. On the other hand, there was a further decrease in transcription by externally supplemented TBP. In fact, externally supplemented TBP by itself brought about inhibition of transcription, and evidently was not limiting in crude nuclear extracts. However, removal of TBP by antibody treatment abolished pol III transcription, and the inhibition could be reversed by externally added TBP, but not by P43 TBF. In the absence of the P43 TBF clone, we could not generate antibodies against the protein to analyse the direct effect of its depletion from the nuclear extracts, on transcription.

From the above results, it is clear that P43 TBF, in addition to binding to the cognate sequence elements, inhibited transcription by interacting with some other components of the transcription machinery. It is likely that some other transcription factor that associates with TBP (e.g. the subunits of TFIIIB or some of the TAFs necessary for pol II/pol III transcription) become limiting due to sequestration by the externally supplement P43 TBF (or TBP), resulting in inhibition of transcription. We conclude that when P43 TBF is bound at a distal region from the transcription start site, or when available in excess, this sequesters some essential transcription factor.

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REFERENCES

1 White, R. J. (1998) RNA Polymerase III Transcription, Springer-Verlag, New York, NY
2 Bartholomew, B., Kassavetis, G. A., Braun, B. R. and Geiduschek, E. P. (1990) The subunit structure of *Saccharomyces cerevisiae* transcription factor TFIIIC probed with a novel photocrosslinking agent. EMBO J. **9**, 2197–2205
3 Bartholomew, B., Kassavetis, G. A. and Geiduschek, E. P. (1991) Two components of *Saccharomyces cerevisiae* transcription factor IIIB (TFIIIB) are stereospecifically located upstream of a tRNA gene and interact with the second largest subunit of TFIIIC. Mol. Cell. Biol. **11**, 5181–5189
4 Gabrielsen, O. S. and Sentenac, A. (1991) RNA polymerase III and its transcription factors. Trends Biochem. Sci. **57**, 873–914
5 Shaw, K. J. and Olson, M. V. (1984) Effect of altered 3′-flanking sequences on the *in vitro* expression of a *Saccharomyces cerevisiae* $tRNA^{Tyr}$ gene. Mol. Cell. Biol. **4**, 657–665
6 Raymond, K. C., Raymond, G. J. and Johnson, J. D. (1985) *In vivo* modulation of yeast tRNA gene expression by 5′ flanking sequences. EMBO J. **4**, 2649–2656
7 Leveillard, T., Kassavetis, G. A. and Geiduschek, E. P. (1993) Repression and redirection of *Saccharomyces* tRNA synthesis from upstream of the transcription start site. J. Biol. Chem. **268**, 3594–3603

8 DeFranco, D., Sharp, S. and Soll, D. (1981) Identification of regulatory sequences contained in the 5′ flanking region of *Drosophila* lysine $tRNA_2$ genes. J. Biol. Chem. **256**, 12424–12429
9 Dingermann, T., Burke, D. J., Sharp, S., Schaack, J. and Soll, D. (1982) The 5′ flanking sequences of *Drosophila* $tRNA^{Arg}$ genes control their *in vitro* transcription in *Drosophila* cell extracts. J. Biol. Chem. **257**, 14738–14744
10 Sprague, K. U., Larson, D. and Morton, D. (1980) 5′- flanking sequences are required for activity of silkworm alanine tRNA genes in homologous *in vitro* transcription systems. Cell **22**, 171–178
11 Larson, D., Bradford-Wilcox, J., Young, L. S. and Sprague, K. U. (1983) A short 5′ flanking region containing conserved sequences is required for silkworm alanine tRNA gene activity. Proc. Natl. Acad. Sci. U.S.A. **80**, 3416–3420
12 Young, L. S., Takahashi, N. and Sprague, K. U. (1986) Upstream sequences confer distinctive transcriptional properties on genes encoding silkgland specific $tRNA^{Ala}$. Proc. Natl. Acad. Sci. U.S.A. **83**, 374–378
13 Taneja, R., Gopalkrishnan, R. and Gopinathan, K. P. (1992) Regulation of glycine tRNA gene expression in the posterior silk glands of the silkworm *Bombyx mori*. Proc. Natl. Acad. Sci. U.S.A. **89**, 1070–1074
14 Fournier, A. F., Taneja, R., Gopalkrishnan, R., Prudhomme, J. C. and Gopinathan, K. P. (1993) Differential transcription of multiple copies of a silk worm gene encoding $tRNA_1^{Gly}$. Gene **134**, 183–190
15 Sullivan, H. S., Young, L. S., White, C. N. and Sprague, K. U. (1994) Silk gland specific $tRNA^{Ala}$ genes interact more weakly than constitutive $tRNA^{Ala}$ genes with silkworm TFIIIB and polymerase III fractions. Mol. Cell. Biol. **14**, 1806–1814
16 Sharma, S. and Gopinathan, K. P. (1996) Transcriptional silencing of a $tRNA_1^{Gly}$ copy from within a multigene family is modulated by distal *cis* elements. J. Biol. Chem. **271**, 28146–28153
17 Sharma, S. and Gopinathan, K. P. (1996) Role of TATATAA element in the regulation of a $tRNA_1^{Gly}$ gene expression in *Bombyx mori* is position dependent. J. Mol. Biol. **262**, 396–406
18 Ouyang, C., Martinez, M. J., Young, L. S. and Sprague, K. U. (2000) TATA-binding protein–TATA interaction is a key determinant of differential transcription of silkworm constitutive and silk gland specific $tRNA^{Ala}$ genes. Mol. Cell. Biol. **20**, 1329–1343
19 Hipskind, R. A. and Clarkson, S. G. (1983) 5′ flanking sequences that inhibit *in vitro* transcription of a *Xenopus laevis* tRNA gene. Cell (Cambridge, Mass.) **34**, 881–890
20 Gouilloud, E. and Clarkson, S. G. (1986) A dispersed tyrosine tRNA gene from *Xenopus laevis* with high transcriptional activity *in vitro*. J. Biol. Chem. **261**, 486–494
21 Carbon, P. and Krol, A. (1991) Transcription of the *Xenopus laevis* selenocysteine $tRNA^{(Ser)Sec}$ gene. A system that combines an internal B-box and upstream elements also found in U6 snRNA genes. EMBO J. **10**, 599–606
22 Arnold, G. J., Schmutzler, C., Thomann, U., Van Tol, H. and Gross, H. J. (1986) The human $tRNA^{Val}$ gene family: organization, nucleotide sequences, homologous transcription of three single-copy genes. Gene **44**, 287–297
23 Tapping, R. I., Syroid, D. E., Bilan, P. T. and Capone, J. P. (1993) The 5′ flanking sequence negatively modulates the *in vivo* expression and *in vitro* transcription of a human tRNA gene. Nucleic Acids Res. **21**, 4476–4482
24 Huibregtse, J. M. and Engelke, D. R. (1989) Genomic footprinting of a yeast tRNA gene reveals stable complexes over the 5′ flanking region. Mol. Cell. Biol. **9**, 3244–3252
25 Palida, F. A., Hale, C. and Sprague, K. U. (1993) Transcription of silkworm $tRNA_c^{Ala}$ gene is directed by two AT rich upstream sequence elements. Nucleic Acids Res. **21**, 5878–5881
26 Trivedi, A., Young, L. S., Ouyang, C., Johnson, D. L. and Sprague, K. U. (1999) A TATA element is required for tRNA promoter activity and confers TATA binding protein responsiveness in *Drosophila* Schneider 2 cells. J. Biol. Chem. **274**, 11369–11375
27 Garel, J. P. (1976) Quantitative adaptation of isoacceptor tRNAs to mRNA codons of alanine, glycine and serine. Nature (London) **260**, 805–806
28 Patel, C. V. and Gopinathan, K. P. (1991) Development stage specific expression of fibroin in the silkworm *Bombyx mori* is regulated transcriptionally. Indian J. Biochem. Biophys. **28**, 521–530
29 Zhou, C. Z., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C., Yang, T., Jacquet, M., Janin, J., Duguet, M., Perasso, R. and Li, Z. G. (2000) Fine organization of *Bombyx mori* fibroin heavy chain gene. Nucleic Acids Res. **28**, 2413–2419
30 Sharma, S., Sriram, S., Patwardhan, L. and Gopinathan, K. P. (1997) Expression of individual members of a $tRNA_1^{Gly}$ multigene family *in vivo* follows the same pattern as *in vitro*. Gene **194**, 257–266
31 Srinivasan, L. and Gopinathan, K. P. (2001) Differential expression of individual gene copies from within a *tRNA* multigene family in the mulberry silkworm *Bombyx mori*. Insect Mol. Biol. **10**, 523–530
32 Taggart, A. K. P., Fisher, T. S. and Pugh, B. F. (1992) The TATA binding protein and associated factors are components of Pol III transcription factor TFIIIB. Cell (Cambridge, Mass.) **71**, 1015–1028
33 Corlet, J., Clarkson, S. G., Fournier, A. and Guerin, M. A. (1986) Sequence of glutamic acid tRNA from *Bombyx mori*. Nucleic Acids Res. **14**, 1916
34 Ouyang, C. and Sprague, K. U. (1998) Cloning and characterization of the TATA-binding protein of the silkworm *Bombyx mori*. Gene **221**, 207–213
35 Ausubel, I., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1993) In Current Protocols in Molecular Biology, Wiley Inc., New York, NY
36 Boulanger, P. A., Yoshinaga, S. K. and Berk, A. J. (1987) DNA-binding properties and characterization of human transcription factor TFIIIC2. J. Biol. Chem. **262**, 15098–15105
37 Ottonello, S., Rivier, D. H., Diilottle, G. M., Young, L. S. and Sprague, K. U. (1987) The properties of a new RNA polymerase III transcription factor reveal that transcription complexes can assemble by more than one pathway. EMBO J. **6**, 1921–1927
38 White, R. J. and Jackson, S. P. (1992) The TATA-binding protein: a central role in transcription by RNA polymerases I, II and III. Trends Genet. **8**, 284–288
39 Berk, A. J. (2000) TBP-like factors come into focus. Cell (Cambridge, Mass.) **103**, 5–8
40 Hansen, S. K., Takada, S., Jacobson, R. H., Lis, J. T. and Tijan, R. (1997) Transcription properties of a cell type specific TATA-binding protein TRF. Cell (Cambridge, Mass.) **91**, 71–83
41 Takada, S., Lis, J. T., Zhou, S. and Tijan, R. (2000) A TRF1:BRF complex directs *Drosophila* RNA polymerase III transcription. Cell (Cambridge, Mass.) **101**, 459–469
42 Rabenstein, M. D., Zhou, S., Lis, J. T. and Tijan, R. (1999) TATA box-binding protein (TBP)-related factor 2 (TRF2), a third member of the TBP family. Proc. Natl. Acad. Sci. U.S.A. **96**, 4791–4796
43 Hoopes, B. C., LeBlanc, J. F. and Hawley, D. K. (1992) Kinetic analysis of yeast TFIID–TATA box complex formation suggests a multi-step pathway. J. Biol. Chem. **267**, 11539–11547
44 Schoenle, E. J., Adams, L. D. and Sammons, D. W. (1984) Insulin induced rapid decrease of a major protein in fat cell plasma membranes. J. Biol. Chem. **259**, 12112–12116

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