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Transcription of individual *tRNA*$_1^{Gly}$ genes from within a multigene family is regulated by transcription factor TFIIIB

Akhila Parthasarthy and Karumathil P. Gopinathan

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India

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Correspondence
K. P. Gopinathan, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India
Fax: +91 80 2360 2697
Tel: +91 80 2360 0090
E-mail: kpg@mcbl.iisc.ernet.in

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Members of a *tRNA*$_1^{Gly}$ multigene family from the silkworm *Bombyx mori* have been classified based on their transcriptions in homologous nuclear extracts, into three groups of highly, moderately and poorly transcribed genes. Because all these gene copies have identical coding sequences and consequently identical promoter elements (the A and B boxes), the flanking sequences modulate their expression levels. Here we demonstrate the interaction of transcription factor TFIIIB with these genes and its role in regulating differential transcriptions. The binding of TFIIIB to the poorly transcribed gene *tRNA*$_1^{Gly}$*-6,7* was less stable compared with binding of TFIIIB to the highly expressed copy, *tRNA*$_1^{Gly}$*-1*. The presence of a 5′ upstream TATA sequence closer to the coding region in *tRNA*$_1^{Gly}$*-6,7* suggested that the initial binding of TFIIIC to the A and B boxes sterically hindered anchoring of TFIIIB via direct interactions, leading to lower stability of TFIIIC–B-DNA complexes. Also, the multiple TATATAA sequences present in the flanking regions of this poorly transcribed gene successfully competed for TFIIIB reducing transcription. The transcription level could be enhanced to some extent by supplementation of TFIIIB but not by TATA box binding protein. The poor transcription of *tRNA*$_1^{Gly}$*-6,7* was thus attributed both to the formation of a less stable transcription complex and the sequestration of TFIIIB. Availability of the transcription factor TFIIIB in excess could serve as a general mechanism to initiate transcription from all the individual members of the gene family as per the developmental needs within the tissue.

In eukaryotes, nuclear gene transcriptions are accomplished by three different RNA polymerases, RNA pol I, pol II and pol III [1,2]. The promoters for class III genes transcribed by RNA pol III, with the exception of the *snRNA*s, generally lack a TATA box but still require TATA box binding protein (TBP) for transcription [3–5]. The genes encoding tRNAs have promoter elements located within the coding region of the genes (designated as the A and B boxes), and require two basal factors, TFIIIB and TFIIIC [6], which are multisubunit proteins [7–10]. TFIIIC binds to the A and B boxes first, followed by recruitment of TFIIIB in the immediate upstream region (through protein–protein interaction) and finally the RNA pol III [11–13]. TFIIIB consists of three subunits, B-double prime 1 (Bdp1; 90 kDa), TFIIB-related factor 1 (Brf1; 60 kDa) and TBP in yeast, or two forms, TFIIIBα (comprising TBP, Brf2 and Bdp1 required for transcription of U6-type RNA pol III promoters) [14] and TFIIIBβ (comprising TBP, Brf1 and Bdp1 required for transcription of tRNA and VA1-type RNA pol III promoters) [15], in humans. In the absence of TATA box sequences in these promoters, recruitment of TBP to the transcription site is achieved by interactions between the associated factors [16,17]. TFIIIB is analogous to the pol II-specific factor,

Abbreviations
Bdp1, B-double prime 1; Brf1, TFIIB-related factor 1; EMSA, electrophoretic mobility shift assay; PC-B/C, phosphocellulose B/C; pol II/III, RNA polymerase II/III; PSG, posterior silk glands; TBP, TATA box binding protein; TF, transcription factor.

TFIID, although the mechanisms by which these factors are recruited to the promoters differ [15,18]. In pol II transcription, sequence-specific binding of the TBP component TFIID to DNA nucleates the transcription, whereas TFIIIB is normally recruited to the initiation site via interactions of one of its protein subunits with TFIIIC which is already bound to the DNA.

In the mulberry silkworm, *Bombyx mori*, the $tRNA_I^{Gly}$ genes occur as a multigene family of about 20 members that are differentially transcribed to high, moderate or low levels *in vitro* in homologous nuclear extracts or *in vivo* in *B. mori*-derived cell lines [19,20]. These gene copies have identical coding sequences and consequently the same A and B boxes, but they differ in their 5′ and 3′ flanking regions. Although transcription of tRNA genes depends on the internal promoters, the sequences flanking the gene evidently influence the efficiency of transcription [21–24]. Because sequences binding to TFIIIC are identical in all $tRNA_I^{Gly}$ copies, the factor that can show variability in binding to these genes is most likely to be TFIIIB. When TATAA sequences are present in the gene promoter, TFIIIB binds directly to DNA even in the absence of TFIIIC [25]. Recruitment of RNA pol III to the template requires prior binding of TFIIIB. All individual members of the $tRNA_I^{Gly}$ family from *B. mori* analysed to date contain perfect TATAA sequences or AT-rich sequences that resemble TBP binding sites at different locations in the flanking regions. The TATAA- and TATA-like sequences immediately upstream of the tRNA coding region (within the first 50 nucleotides) are essential for transcription, but such sequences when present in the far-upstream regions reduced transcription levels [21,23,24]. This implies that if more copies of TATAA elements are present in the flanking regions of the gene, TFIIIB may bind to these sequences independent of TFIIIC, resulting in sequestration of the factor and lower transcription levels. Differential transcription of the $tRNA_I^{Gly}$ genes could, therefore, be mediated through differences in their zabilities to form stable transcription complexes and the amounts of transcription factors available.

Results

Transcription of different $tRNA_1^{Gly}$ copies

The different $tRNA_I^{Gly}$ gene constructs (showing high, moderate and low transcription levels in homologous nuclear extracts) used in this study are shown in Fig. 1.

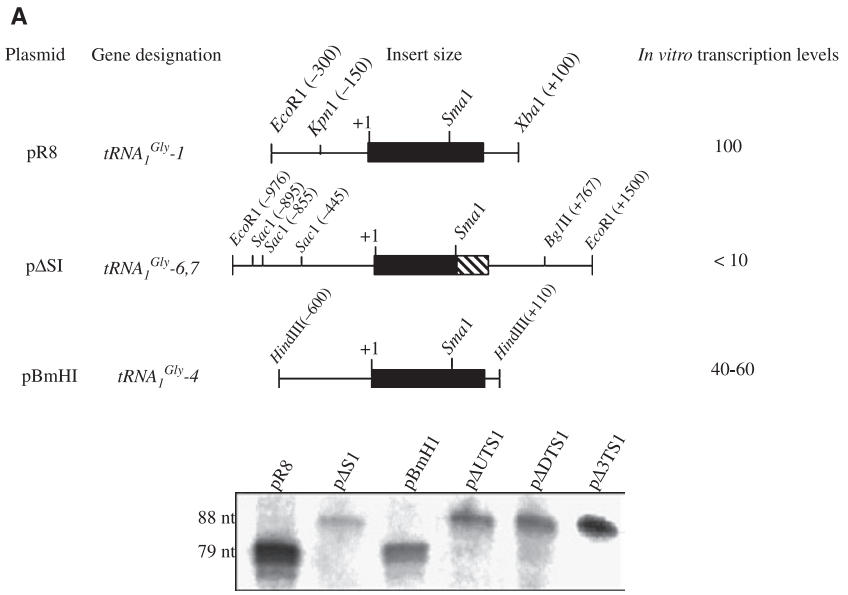

Fig. 1. $tRNA_I^{Gly}$ gene constructs used and their *in vitro* transcription status. All the plasmid constructs were in pBSSK+ vector. The tRNA encoding regions (70 nucleotides, shown in boxes) are identical in all gene copies. $tRNA_1^{Gly}$*-6,7* is shown as a combination of filled and striped boxes to indicate that it was derived by fusion of $tRNA_1^{Gly}$*-6* and $tRNA_1^{Gly}$*-7* genes but was identical in sequence to others. The coordinates for flanking regions are marked with respect to +1 nucleotide of mature tRNA. The plasmid constructs pΔUTS1, pΔDTS1 and pΔ3TS1 harbour, respectively, the $tRNA_1^{Gly}$*-6,7* derivatives from which the 5′ upstream sequences beyond –445 or the downstream sequences beyond +767 or both the upstream (from –445) and downstream (from +767) sequences were deleted. The *in vitro* transcription of these gene copies in PSG nuclear extracts is shown at the bottom and the quantified transcription levels as the percentage of $tRNA_1^{Gly}$*-1* taken as 100, are indicated on the right-hand side of the upper panel.

Transcription of $tRNA_1^{Gly}$-*6,7* (poorly transcribed gene) was < 10% that of $tRNA_1^{Gly}$-*1* (highly transcribed). However, the transcription levels for the gene reach 30–50% that of $tRNA_1^{Gly}$-*1* when the 5′ upstream, 3′ downstream, or both negative regulatory sequences were deleted (in constructs pΔUTS1, pΔDTS1 and pΔ3TS1, respectively). Transcription of $tRNA_1^{Gly}$-*4* (moderately transcribed gene) was almost 40–60% that of $tRNA_1^{Gly}$-*1*. $tRNA_1^{Gly}$ *−6,7* transcripts were slightly longer due to differences in the transcription initiation and termination sites of the gene [22].

Fractionation of the *B. mori* posterior silk glands nuclear extract

Transcription factors TFIIIB and TFIIIC were partially purified from posterior silk gland (PSG) nuclear extracts (Fig. 2A). TFIIIC (0.6 M KCl fraction from a phosphocellulose column) and TFIIIB (0.3 M KCl fraction from a heparin–Sepharose column) activities were separated and were active in transcriptional reconstitution (Fig. 2B). Plasmid pR8 (harbouring $tRNA_1^{Gly}$-*1*), when transcribed with crude nuclear extracts, mostly gave rise to one predominant primary tRNA transcript. Occasionally, processed forms of the tRNA transcript were seen, but the tRNA processing activity of the crude nuclear extracts varied from batch to batch. The reconstitution assay was carried out with the phosphocellulose fractions, PC-B and PC-C as well as with the heparin–Sepharose fractions. The reactions were maximally active at 6 μg of both PC-B and PC-C (Fig. 2B; lane 4) and at 4 μg of TFIIIB and RNA pol III fractions (0.3 and 0.4 M KCl eluates from the heparin–Sepharose column) in presence of 6 μg TFIIIC (lane 9). Fractionation of the PC-B fraction on heparin–Sepharose (to separate TFIIIB and RNA pol III activities) resulted in some loss of transcriptional activity. The PC-C or PC-B fractions alone (lanes 2, 3) or the heparin–Sepharose fractions individually (lanes 5–8) did not show transcriptional activity. Evidently, the fractions were devoid of mutual contamination. In every fractionation the quantities of fractions had to be optimized because use of larger amounts of any individual fraction tended to result in inhibition of transcription. Recombinant *B. mori* TBP was purified as a His-tag fusion protein from a cDNA clone (Fig. 2C, lane 2) showing cross-reactivity with anti-αTBP serum (human) raised against the C-terminal region of human TBP (lane 3, showing western blot).

The phosphocellulose and heparin–Sepharose fractions were also tested for sequence-specific DNA binding in gel retardation assays using a labelled fragment containing the TATATAA sequence (Fig. 2D, left). Because TBP is present as a component of TFIIIB, the TFIIIB-containing fraction (0.3 M KCl eluate from heparin–Sepharose) was predicted to bind to the probe. As a positive control TBP binding to this element was also included in the binding assays (lane 3). Clearly, the TFIIIB fraction showed binding (lane 2) and, as anticipated, a higher mobility shift compared with the TBP complex. TFIIIC (lane 4) or the RNA pol III fraction (0.4 M KCl eluate) from heparin–Sepharose (lane 5) did not show any complex formation. TFIIIB–DNA complexes were competed out by increasing concentrations (10 and 100×) of the unlabelled fragment (Fig. 2D, right, lanes 3 and 4), but not by the fragment from which the TATATAA sequences were mutated to GATATCA, at the same concentrations (lanes 5 and 6). These competition experiments confirmed the binding specificity of TFIIIB to the TATATAA sequences.

Stability of transcriptional complexes on $tRNA_1^{Gly}$-*6,7*

In order to analyse whether the stability of the transcription complexes on the two representative $tRNA_1^{Gly}$ gene copies contributed to the differences in their transcription levels, the dissociation of TFIIIB complexes in the presence of heparin was examined. Because heparin strips off the TFIIIC complexes as well as the weakly interacting TFIIIB complexes, the amounts of TFIIIB–promoter complexes that remain after heparin stripping provide a measure of its stable interaction [12,13]. Formation of TFIIIC/TFIIIB complexes on the two different $tRNA_1^{Gly}$ copies is shown in Fig. 3. TFIIIB and TFIIIC alone showed binding to both $tRNA_1^{Gly}$-*1* and $tRNA_1^{Gly}$-*6,7* (Fig. 3A; lanes 2 and 3 in both panels). The TFIIIC complex showed further compaction and a shift on the addition of TFIIIB (lane 4, both panels). Heparin dissociated the complex formed with TFIIIC alone from both *tRNA* genes (lane 5, both panels). However, a stable undissociated TFIIIB complex on $tRNA_1^{Gly}$-*1* was evident even when heparin was present (lane 6, left), whereas this complex in the poorly transcribed gene $tRNA_1^{Gly}$-*6,7* was completely dissociated (lane 6, right). These results indicated that the interaction of TFIIIB with $tRNA_1^{Gly}$-*1* was more stable than the interaction with $tRNA_1^{Gly}$-*6,7*. Quantification of the ratio of heparin-resistant complexes to the TFIIIB/C–DNA complexes in the absence of heparin (from three separate experiments and at two concentrations of heparin, 10 and 20 $\mu g \cdot mL^{-1}$) revealed a ratio of 0.33 for $tRNA_1^{Gly}$-*1* and a low ratio of 0.053 for $tRNA_1^{Gly}$-*6,7,* suggesting weak or unstable complex formation in $tRNA_1^{Gly}$-*6,7*. The

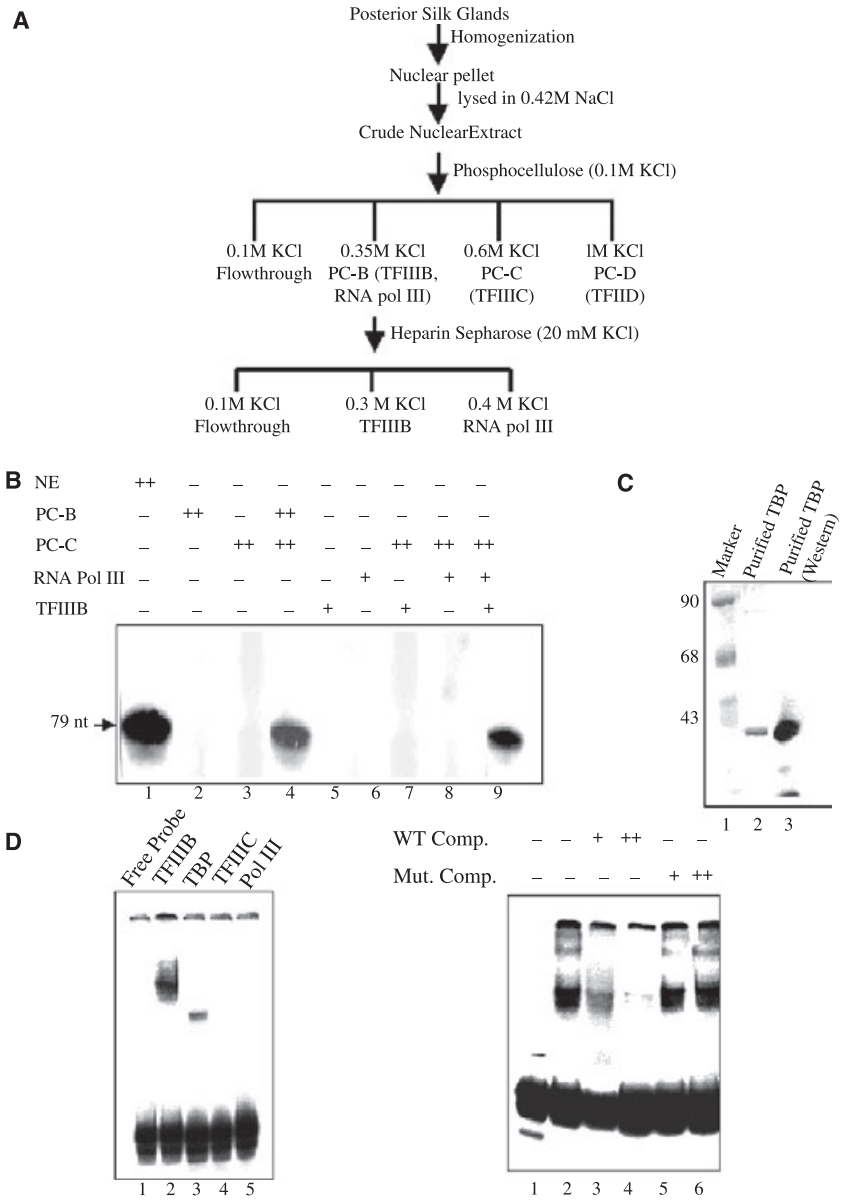

Fig. 2. Purification of TFIIIB and TBP. (A) Schematic presentation of TFIIIB purification from PSG nuclear extract. Nuclear extracts were prepared from freshly dissected silk glands of *B. mori* larvae in the fifth instar (day 2 or 3) or from glands kept at –80 °C for up to a month. For more details, see text. (B) *In vitro* transcription reconstitution with purified TFIIIB. The *in vitro* transcription reaction was performed using $tRNA_1^{Gly}$*-1* as template and varying concentrations of phosphocellulose (PC-C containing TFIIIC, and PC-B containing TFIIIB as well as RNA pol III) either alone (lanes 2, 3) or combined (lane 4). The heparin–Sepharose column fractions (0.3 and 0.4 M KCl eluates containing TFIIIB and polymerase III, respectively) were also tested for reconstitution either alone (lanes 5–8) or combined (lane 9) with a fixed concentration of TFIIIC fraction. All these fractions containing different salt concentrations were dialysed against 0.1 M KCl prior to these additions (+ and ++ denote 4 and 6 μg protein). Lane 1, transcription with unfractionated nuclear extract (NE). (C) Purification of recombinant TBP. Bacterially expressed recombinant *B. mori* TBP was purified as a His-tag fusion protein by adsorption and elution from Ni-NTA affinity matrix and subjected to SDS/PAGE. Lane 1, size markers; lane 2, purified TBP (37 kDa protein); lane 3, western blot of the purified TBP using antibodies against the C-terminal region of human TBP. (D) Gel retardation assay. EMSA was performed to examine the presence of TFIIIB in the fractions by complex formation (for details of the assay, see text). The labelled probe used was the *Eco*RI/*Kpn*I fragment from the $tRNA_1^{Gly}$*-1* construct pR8 (shown in Fig. 1) which harboured the TATATAA sequence. (Left) Binding of different fractions. TFIIIB fraction from the heparin–Sepharose column (lane 2); TBP (purified recombinant TBP from *B. mori*), taken as the positive control (lane 3); PC-C fraction containing TFIIIC (lane 4); RNA pol III fraction from heparin-Sepharose (lane 5). (Right) Binding competition with increasing concentrations of the unlabelled fragment (lanes 3 and 4, 10 and 100×, respectively); same fragment from which the TATATAA sequence was mutated to GATATCA (lanes 5 and 6, 10 and 100×, respectively).

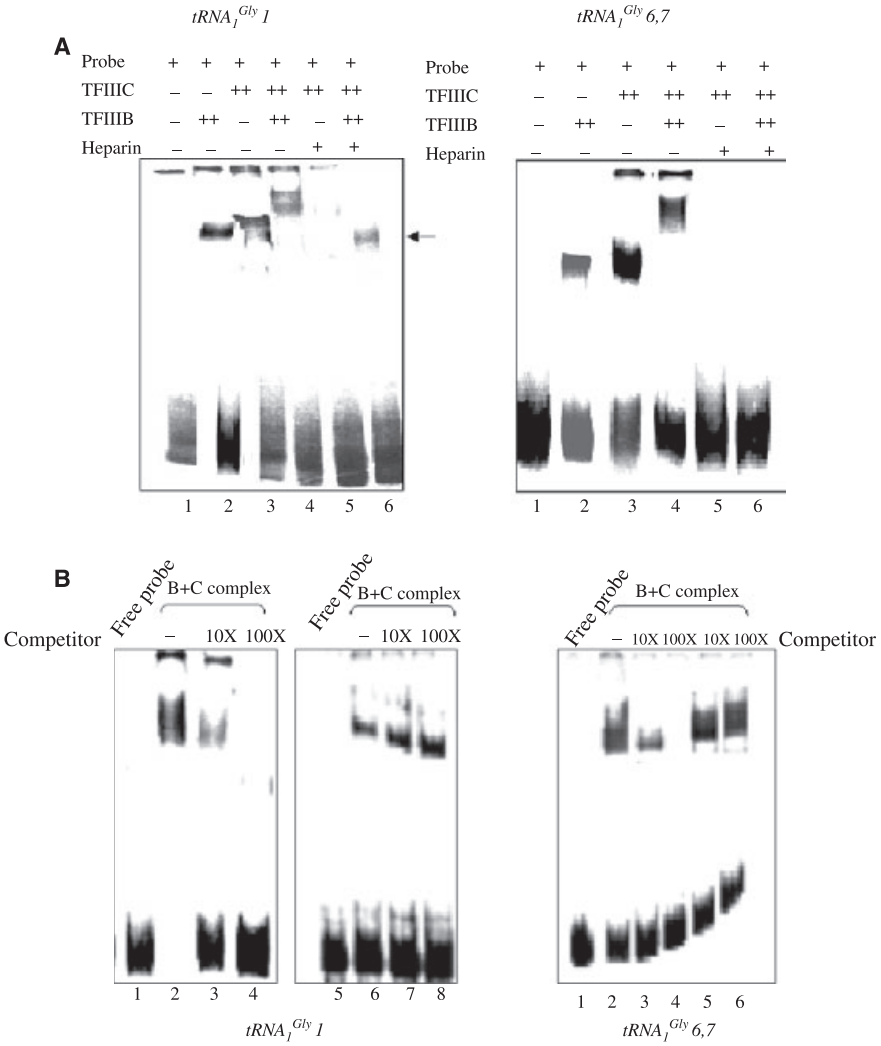

Fig. 3. Formation of heparin-resistant complexes on the $tRNA_1^{Gly}$ genes. (A) The stability of the transcription complexes on the $tRNA_1^{Gly}$ genes was tested by their ability to form TFIIIC/TFIIIB complexes in the presence of heparin. Radioactively labelled *fontshapeit*$tRNA_1^{Gly}$*-1* (400 bp *Eco*RI/*Xba*I fragment from pR8) or $tRNA_1^{Gly}$*-6,7* (370 bp *Dra*I fragment from the parental plasmid pS1 from –260 to +110 with respect to $tRNA_1^{Gly}$*-6*) were incubated with fractions containing TFIIIC and TFIIIB. The stability of the DNA–TFIIIC complex and DNA–TFIIIC–TFIIIB complex on $tRNA_1^{Gly}$*-1*(left) and $tRNA_1^{Gly}$*-6,7* (right) was examined by including heparin (20 μg·mL^{-1}) in the binding reaction (lanes 5, 6, both panels). The complex formation was analysed by electrophoresis on 4% polyacylamide (nondenaturing) gels and visualized in a Phosphorimager. Lanes as marked. The heparin-resistant complex on $tRNA_1^{Gly}$*-1* (left) is marked by an arrow; ++ denotes 6 μg of protein. (B) The specificity of complex formation was examined by the competition with 10 and 100× molar excess of unlabelled specific probe or a nonspecific 600 bp DNA fragment corresponding to the *lef2* gene from BmNPV. Monitoring of the complex formation was done as in Fig. 3A. Panels and lanes as marked.

instability of the $tRNA_1^{Gly}$*-6,7*–TFIIIB complex may contribute to the poor transcription of this gene. The specificity of TFIIIC/TFIIIB complex formation on both the genes is evident from the binding competition analysis (Fig. 3B; left, $tRNA_1^{Gly}$*-1*; right, $tRNA_1^{Gly}$*-6,7*). At a 100× molar excess of unlabelled probe, the complex was entirely chased out (left and right, lane 4), whereas a 100× molar excess of a nonspecific competitor did not chase the complex (left, lanes 7, 8; right, lanes 5, 6).

TFIIIB alone also showed binding to both $tRNA_1^{Gly}$*-1* and $tRNA_1^{Gly}$*-6,7* (Fig. 4A, left) and this complex could be supershifted with anti-TBP serum (lane 3 in each). Evidently, the AT-rich elements present in the immediate vicinity of the transcription start sites in both these genes independently bound TFIIIB and

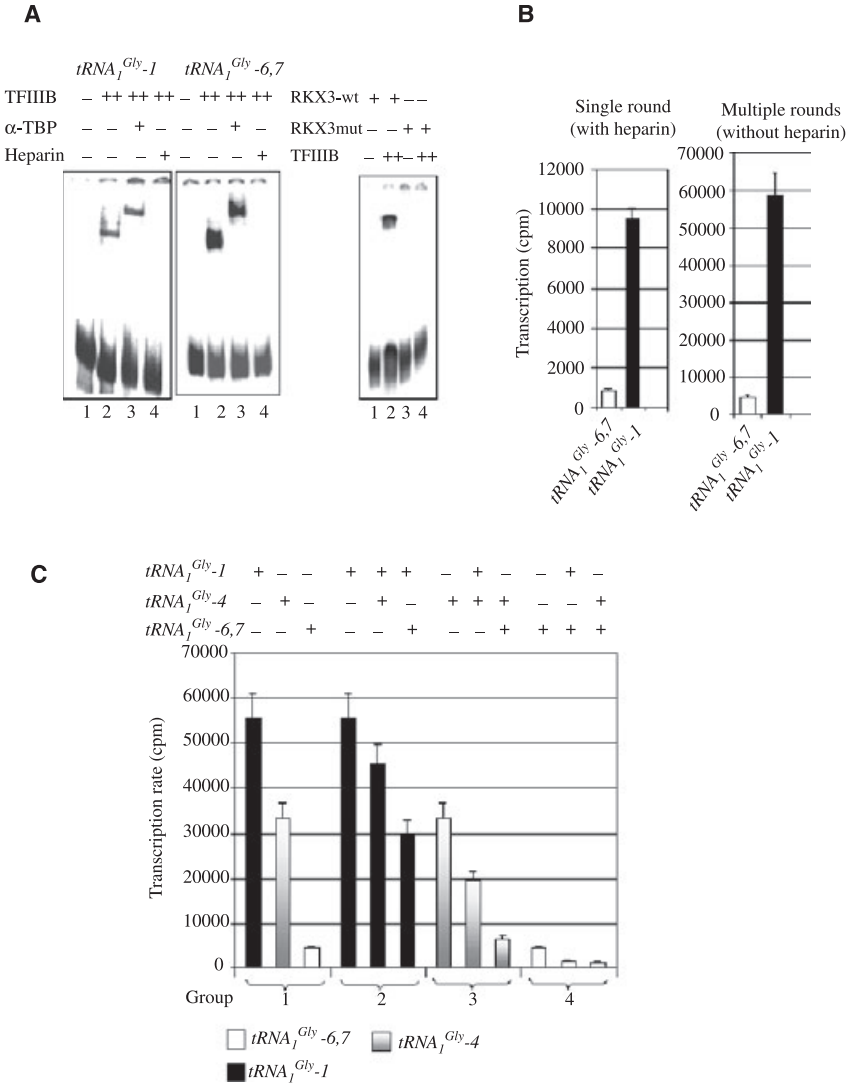

Fig. 4. Sequestration of transcription factors by *tRNA*$_1^{Gly}$*-6,7*. (A) Binding of TFIIIB alone (in the absence of TFIIIC) to the two genes. (Left) *tRNA*$_1^{Gly}$*-1*and *tRNA*$_1^{Gly}$*-6,7*. TFIIIB binding to a derivative of *tRNA*$_1^{Gly}$*-1* with a single TATATAA element in the upstream region (in plasmid construct pRKX3) [24] or the same construct in which the TATATAA sequence was mutated to GATATCA (pRKX3mut) was also carried out (right). For experimental details, see text. Lanes as marked. (B) Single- (in the presence of heparin) and multiple-round (in the absence of heparin) transcriptions of the two *tRNA*$_1^{Gly}$ genes. Multiple-round transcriptions were carried out at 30 °C for 1 h in presence of all the four nucleotides, whereas for single-round transcriptions, incubations were initially carried out for 10 min in the absence of nonradioactive GTP and a further 50 min after the addition of 100 μg·mL^{-1} heparin and 10 μM GTP. The incubation time for single-round transcriptions was standardized to 10 min after trying out different incubation times. The transcriptions from three independent experiments (with error bars) are presented. (C) Competition between *tRNA*$_1^{Gly}$*-1*, *tRNA*$_1^{Gly}$*-6,7* and *tRNA*$_1^{Gly}$*-4* in *in vitro* transcription. The *in vitro* transcription (quantification from Phosphorimager) of the three genes alone (grouped as 1) or in the presence of the other as a competing template (shown in groups; 2 for *tRNA*$_1^{Gly}$*-1*, 3 for *tRNA*$_1^{Gly}$*-4* and 4 for *tRNA*$_1^{Gly}$*-6,7*) The transcripts arising from each of the *tRNA*$_1^{Gly}$ genes were differentially quantified. Filled bars, *tRNA*$_1^{Gly}$*-1*; unfilled bars, *tRNA*$_1^{Gly}$*-6,7*; shaded bars, *tRNA*$_1^{Gly}$*-4*. The average of three independent experiments is presented.

these complexes were dissociated in the presence of heparin in both cases (lane 4). This binding was via direct interactions of the TBP component of TFIIIB with the TATA sequences and was not anchored via interactions with TFIIIC. The stable binding (heparin-resistant complex formation) also required the presence of TFIIIC (Fig. 3A). Independent binding of TFIIIB was again confirmed using another construct, a derivative of *tRNA*$_1^{Gly}$*-1* with a single TATA box at −130 with respect to +1 nucleotide of the coding region (construct pRKX3; Fig. 4A, right) [24]. TFIIIB bound efficiently to the probe (lanes 1, 2) and binding was

completely abolished when the TATATAA sequence was mutated to GATATCA (lanes 3, 4). These results were also consistent with the observation that TFIIIB alone was not sufficient to initiate transcription despite being able to bind independently to the DNA via the TATA sequences (Fig. 2B, compare lanes 2 and 4). Prior binding of TFIIIC, which presumably anchored the stable binding of TFIIIB, was important for transcription.

The deductions from the binding assays were also confirmed by performing single-round transcriptions with these two gene copies (Fig. 4B). Transcription of $tRNA_1^{Gly}$-*6,7* was lower than that of $tRNA_1^{Gly}$-*1* to a similar extent in both single- and multiple-rounds of transcription (Fig. 4B), confirming that the lower efficiency of $tRNA_1^{Gly}$-*6,7* was in the initial formation of transcription complexes.

Competition for transcription factors

To analyse whether $tRNA_1^{Gly}$-*6,7* was less efficient in its interaction with different components of the transcription machinery, competition assays were designed based on their ability to compete for transcription factors with the other $tRNA_1^{Gly}$ copies. Competition between $tRNA_1^{Gly}$-*1* and $tRNA_1^{Gly}$-*6,7*, as well as with another gene copy, $tRNA_1^{Gly}$-*4* (a moderately expressed gene), in the presence of limiting amounts of transcription factors was therefore analysed (Fig. 4C). Transcription levels of $tRNA_1^{Gly}$-*4* were $\approx$ 40–60% that of $tRNA_1^{Gly}$-*1* and $<$ 10% that of $tRNA_1^{Gly}$-*6,7* (Fig. 4C, first three bars grouped together). Transcripts from $tRNA_1^{Gly}$-*6,7* and $tRNA_1^{Gly}$-*1* could be differentially quantified due to differences in their sizes (each initiated and terminated at slightly different sites; Fig. 1) [22] (AP & KPG, unpublished observations). However, because there was only a marginal difference between the transcript sizes of $tRNA_1^{Gly}$-*4* and $tRNA_1^{Gly}$-*1*, a derivative of $tRNA_1^{Gly}$-*1* which had a 10 nucleotide insertion immediately after the B box (plasmid pR8-10) and gives rise to a transcript 10 nucleotides longer than the wild-type $tRNA_1^{Gly}$-*1* without compromising its transcription activity [19], was utilized to differentiate and quantify these transcripts. $tRNA_1^{Gly}$-*4* partially competed with $tRNA_1^{Gly}$-*1* and reduced its transcription by $\approx$ 15%. $tRNA_1^{Gly}$-*6,7*, however, competed more effectively and reduced the transcription level of $tRNA_1^{Gly}$-*1* by $\approx$ 45% at the same molar concentrations of the two templates (compare the bars grouped together in 2). Likewise, transcription of $tRNA_1^{Gly}$-*4* was inhibited $\approx$ 35% by competing $tRNA_1^{Gly}$-*1* and much more effectively ($\approx$ 75–80%) by $tRNA_1^{Gly}$-*6,7*. Thus, $tRNA_1^{Gly}$-*6,7* appeared to be a more effective competitor for $tRNA_1^{Gly}$-*1* or $tRNA_1^{Gly}$-*4*, indicating that the former was effectively sequestering some essential transcription factors. This observation correlated well with the presence of additional TATAA sequences in the flanking regions of $tRNA_1^{Gly}$-*6,7*. Conversely, both $tRNA_1^{Gly}$-*1* and $tRNA_1^{Gly}$-*4* showed somewhat similar inhibition of transcription to $tRNA_1^{Gly}$-*6,7*. The lower transcription levels of $tRNA_1^{Gly}$-*6,7*, therefore, were due to not only inefficient transcription complex formation but the *cis* elements present in the flanking regions capable of sequestration of transcription factors.

To identify the component that was responsible for the low transcription efficiency of $tRNA_1^{Gly}$-*6,7*, competition analyses were also carried out in the presence of externally supplemented, purified components. In initial experiments, partially purified fractions of TFIIIB and TFIIIC (the PC-B and PC-C fractions, respectively; Fig. 5A) were used. TFIIIC did not rescue the transcription of either $tRNA_1^{Gly}$-*1* or $tRNA_1^{Gly}$-*6,7* to any significant extent (compare lanes 3, 4 and 5; Fig. 5A) but the external supplementation of PC-B (containing both TFIIIB and RNA pol III activities) showed efficient rescue of transcription of both $tRNA_1^{Gly}$-*1* and $tRNA_1^{Gly}$-*6,7* (compare lanes 3–6 and 7). In fact, the transcription of $tRNA_1^{Gly}$-*6,7* was even better than that seen in crude nuclear extracts, although it was still only $\approx$ 15–20% that of $tRNA_1^{Gly}$-*1*. To confirm whether it was TFIIIB or RNA pol III limiting transcription of $tRNA_1^{Gly}$-*6,7*, external supplementation studies were performed again using TFIIIB or RNA pol III fractions which were separated from each other (after heparin–Sepharose fractionation) (Fig. 5B). The near complete inhibition of $tRNA_1^{Gly}$-*1* by the competing $tRNA_1^{Gly}$-*6,7* (lane 3), was rescued very efficiently by increasing concentrations of TFIIIB (lanes 5 and 6) but not by pol III (lane 4). Transcription of $tRNA_1^{Gly}$-*6,7* was also enhanced in the presence of externally supplemented TFIIIB (compare lane 5 and with lanes 3 and 4). Evidently, $tRNA_1^{Gly}$-*1* showed better efficiency in making use of the externally added TFIIIB.

Upstream and downstream elements in $tRNA_1^{Gly}$-*6,7* were responsible for sequestration of transcription factors

Deletion of the upstream and downstream regions containing the TATA box from $tRNA_1^{Gly}$-*6,7* led to much higher transcription levels, reaching almost 30–40% of the transcription levels of $tRNA_1^{Gly}$-*1* (Fig. 1). In order to confirm whether the downregulation of transcription by $tRNA_1^{Gly}$-*6,7* was due to the sequestration of TFIIIB, these two deletion derivatives (plasmids pΔUTS1 and pΔDTS1|), as well as a construct harbouring both deletions (plasmid pΔ3TS1), were used in

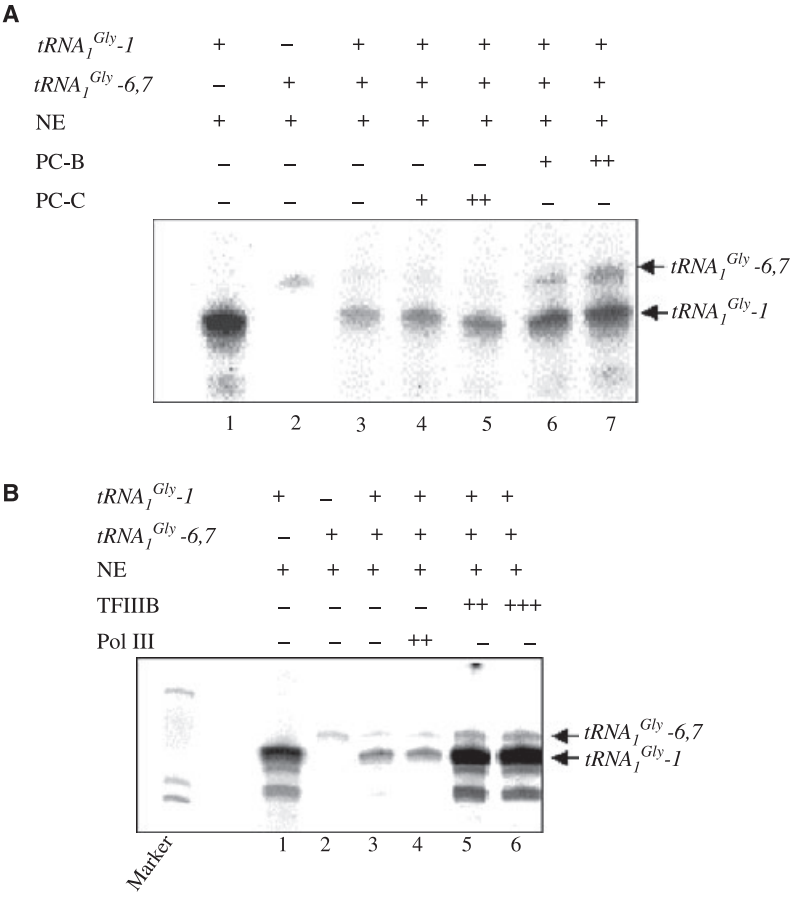

Fig. 5. Competition for TFIIIB by $tRNA_1^{Gly}$ genes. (A) Competition in transcription between $tRNA_1^{Gly}$*-1* and $tRNA_1^{Gly}$*-6,7* under limiting concentration of crude nuclear extracts (lane 3) and the effect of external supplementation with partially purified TFIIIC (phosphocellulose fraction, PC-C; lanes 4, 5) or TFIIIB (PC-B, which also contains RNA pol III; lanes 6, 7) are presented. For details of the transcription assay see text. Suboptimal concentrations of nuclear extract (4 μg protein) were utilized to observe the effect of external supplementations. For PC-C and PC-B fractions + and ++ correspond to 4 and 6 μg protein, respectively. The transcripts were detected in a Phosphorimager following electrophoresis on 7 M urea/8% polyacrylamide gels. Lanes as marked. (B) A similar competition analysis was performed with supplementation of TFIIIB (0.3 M KCl fraction from heparin–Sepharose; lanes 5, 6) separated from RNA pol III (0.4 M KCl fraction from heparin–Sepharose; lane 4). For the TFIIIB and RNA pol III fractions + and ++ correspond to 4 and 6 μg of protein. The transcripts were detected in Phosphorimager following electrophoresis on 7 M urea/8% polyacrylamide gels. The marker lane, pTZ DNA *Hin*fI digest.

competition assays with $tRNA_1^{Gly}$*-1*. The downstream- or upstream-deleted derivatives of $tRNA_1^{Gly}$*-6,7* (indicated by ** and *, respectively, in Fig. 1) did not significantly inhibit the transcription of $tRNA_1^{Gly}$*-1*, unlike the parental gene (Fig. 6A,B; compare with Fig. 5). Furthermore, deletion of both these regions made it noninhibitory to the transcription of $tRNA_1^{Gly}$*-1* (Fig. 6C). Quantification of the transcription levels is presented on the right-hand side of each panel. The results again indicated that the negative regulatory sequences present in the flanking regions of the former were indeed responsible for the sequestration of TFIIIB (Fig. 6). Conversely, transcription of all these deletion derivatives was significantly inhibited by $tRNA_1^{Gly}$*-1* and the inhibition could be reversed by external supplementation of TFIIIB. These observations lend support to the concept that $tRNA_1^{Gly}$*-1* had a greater affinity for the transcription factor.

To confirm that the component responsible for sequestration of the factors was indeed the TATAA box-containing region, TATATAA sequences [a 40 bp *Sac*I fragment of pΔS1 present at −895 nucleotides in plasmid pSac40 and a 150 bp *Eco*RI/*Kpn*I fragment from pR8 present at −300 in plasmid pRK (Fig. 1) or the same fragment from which the TATATAA sequence was mutated to GATATCA] were used for competitions. Transcription of $tRNA_1^{Gly}$*-1* was 50% inhibited in the presence of fragments containing the TATATAA sequence, but not by the mutated GATATCA sequence (Fig. 7A; lanes 3, 4, 6, 7 and 9). Inhibition by TATATAA-containing fragments was reversed by supplementation of the TFIIIB fraction to almost 100% of original levels (lanes 5 and 8). These results confirmed the role of TATATAA sequences in the sequestration of TFIIIB presumably by binding to the TBP component of TFIIIB.

This inference was further confirmed by immunodepletion of TFIIIB using a polyclonal antibody directed against TBP (Fig. 7B). The transcription of either gene alone (lanes 2 and 3) or together (lanes 4–9) is shown here. The presence of both genes led to inhibition of transcription to 70% (lane 4), which was rescued by the addition of the TFIIIB fraction to almost 90% of the parent (lane 5). This rescue of transcription was abolished by immunodepletion of the TFIIIB using a TBP antibody (lanes 7 and 8; compare with

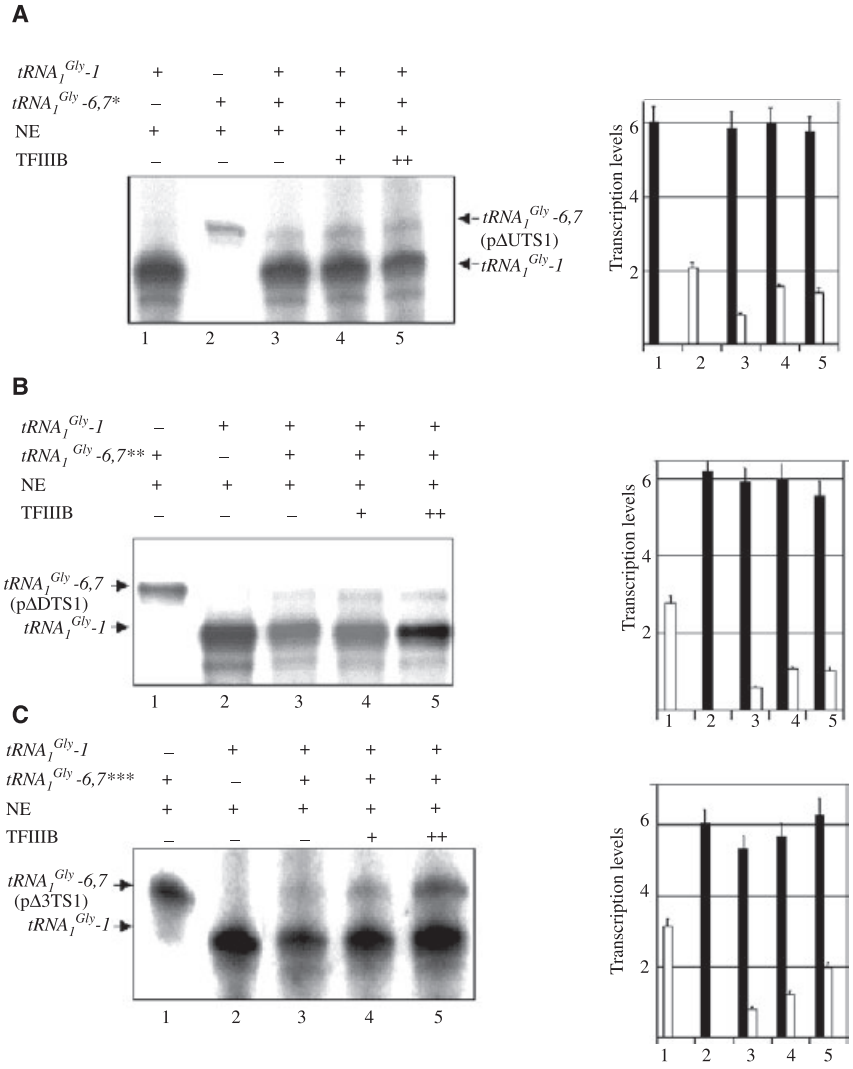

Fig. 6. Competition of $tRNA_1^{Gly}$-*1* transcription by deletion derivatives of $tRNA_1^{Gly}$-*6,7*. The transcription competition assays were carried out with $tRNA_1^{Gly}$-*1* and the upstream deletion derivatives of $tRNA_1^{Gly}$-*6,7* marked with a * (clone pΔUTS1) in (A) or its downstream deletion marked ** (clone pΔDTS1) in (B) or a construct with both the upstream and downstream regions deleted, marked *** in (C) in thye presence of increasing concentrations of TFIIIB (lanes 4, 5 in all panels). Transcriptions were performed with 4 μg of the extract and the transcripts were detected in Phosphorimager (+ and ++ in the case of TFIIIB represents 4 and 6 μg of protein). The quantification of the transcripts (done in Phosphorimager) in each of the lanes are shown on the right-hand side of the panels. Black bars represent $tRNA_1^{Gly}$-*1* and white bars represent $tRNA_1^{Gly}$-*6,7*.

lane 5). Mock immunodepletion using preimmune serum, performed as a control, showed no effect (lane 6). Inhibition brought about by immunodepletion of TBP was reversed by the external supplementation of TFIIIB to 90% the original levels (compare lanes 9 and 10 with lane 7). The rescue of transcription inhibition seen by the addition of TFIIIB (Fig. 7C, lane 5; compare with lane 4) was absent when TBP alone was added (lane 6). Moreover, the inhibition brought about by immunodepletion using TBP antibodies was not reversed by external supplementation of TBP (lanes 7, 8), unlike TFIIIB supplementation (lane 9). These results indicated that the impairment in transcription was due to sequestration of the whole TFIIIB rather than the TBP component alone. We infer, therefore, that both weak binding to TFIIIB and the sequestration of TFIIIB contributed to lower transcription levels of $tRNA_1^{Gly}$-*6,7*.

Discussion

The $tRNA_1^{Gly}$ genes of *B. mori* constitute a multigene family from which individual members are differentially transcribed *in vitro* in homologous nuclear extracts or *in vivo* in *B. mori*-derived BmN cells [19,20]. The genes do not show any tissue specificity [22] but their expression is regulated developmentally because substantial quantities of $tRNA_1^{Gly}$ transcripts accumulate in the silk glands of *B. mori* during the fifth instar larval stage in order to optimize silk fibroin synthesis [26,27]. Because of the presence of a large number of glycine codons in heavy-chain fibroin (1350 codons in the 15 kb fibroin H mRNA are decoded by $tRNA_1^{Gly}$), there is excessive requirement for $tRNA_1^{Gly}$ to achieve optimal translation of the message. In such circumstances of a high demand for $tRNA_1^{Gly}$, transcription from a single gene may not be adequate to meet

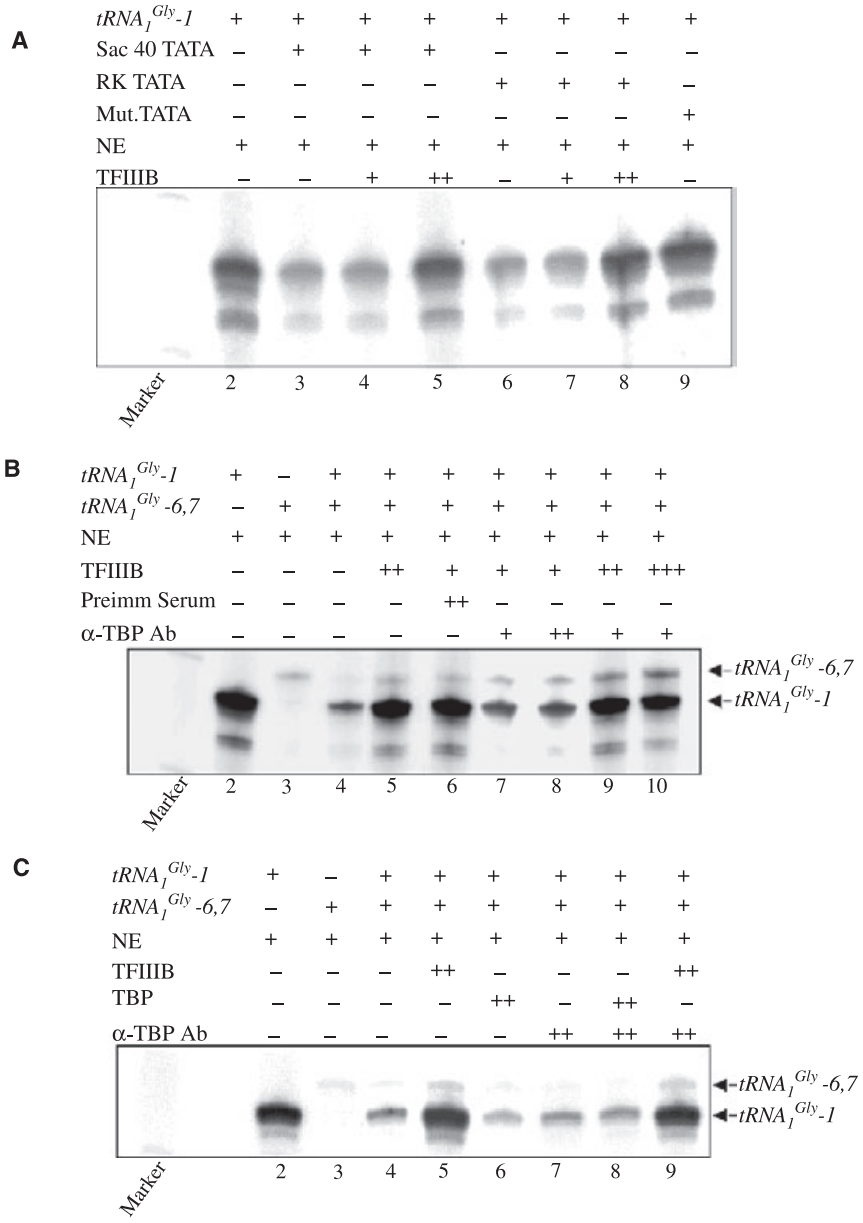

Fig. 7. Sequestration of TFIIIB by interactions with the TATA sequences in the flanking regions of $tRNA_1^{Gly}$ genes. (A) Competition by DNA fragments containing TATATAA sequences. Transcription of $tRNA_1^{Gly}$*-1* was carried out in the presence of increasing concentrations of a 40 bp fragment containing the TATATAA sequence upstream of the coding region in $tRNA_1^{Gly}$*-6,7* (*Sac*I fragment from pΔS1, Fig. 1) (lanes 3–5) or the 150 bp fragment containing the TATATAA sequence upstream of the coding region in $tRNA_1^{Gly}$*-1* (*Eco*RI/*Kpn*I fragment from plasmid pR8, Fig. 1) (lanes 6–8) or the latter from which the TATATAA sequence was mutated to GATATCA (lane 9), with or without externally supplemented TFIIIB (4 and 6 μg protein corresponding to + and ++ ; lanes 5 and 8). The transcripts were visualized in Phosphorimager following electrophoresis on urea–acrylamide gels. (B) Immunodepletion of TFIIIB. $tRNA_1^{Gly}$*-1* competitions were performed with $tRNA_1^{Gly}$*-6,7* after immunodepletion of TFIIIB using a polyclonal antibody directed against the TBP component of TFIIIB. The rescue of transcription by externally supplemented TFIIIB (lane 5; compare with lane 4) was abolished by the anti-TBP serum (lanes 7, 8). Inhibition was again rescued by increasing concentrations of TFIIIB (lanes 9, 10). Samples treated with preimmune serum were included as control for nonspecific antibody reaction (lane 6). Lanes 2 and 3 contained, respectively, $tRNA_1^{Gly}$*-1* or $tRNA_1^{Gly}$*-6,7* alone. Lanes 4–10 contained both templates. (C) External supplementations of TBP (recombinant TBP from *B. mori*; 6 μg protein) were carried out after immunodepletion of the TFIIIB from the nuclear extracts. Lanes 2 and 3 contained either $tRNA_1^{Gly}$*-1* or $tRNA_1^{Gly}$*-6,7* as a template. Lanes 4–9 contained both templates. Individual lanes as marked.

the cellular needs. The presence of multiple copies of $tRNA_I^{Gly}$ in *B. mori* may meet this requirement but it is still not clear whether these transcripts arise from multiple gene copies. In the normal course of development, when $tRNA_1^{Gly}$ species are mostly involved in the maintenance of housekeeping functions, transcription from the highly expressed copies alone might be sufficient and other gene copies could be downregulated or completely shut off. By contrast, when there is demand for large excesses of a particular type of tRNA, as in the PSG, and sufficient quantities of transcription factors are available, transcription from all the gene copies would be desirable. Thus, the regulation of expression of individual members from within a multigene family like $tRNA_I^{Gly}$ may depend on the developmental stage and the overall availability of transcription factors.

We made a comparative analysis of two $tRNA_I^{Gly}$ gene copies, which belonged to the highly and poorly transcribed groups. The lower stability of TFIIIB complex on $tRNA_I^{Gly}$*-6,7* appeared to be a major reason for its low level of transcription. All the $tRNA_I^{Gly}$ copies had conserved intragenic sequences characteristic of classical pol III promoters, but differed in their 5′- and 3′-flanks. They also harboured perfect TATA box sequences in the flanking regions. Certain TATA sequence-binding proteins like P43 TBF from the silk glands of *B. mori* bind to these sequences and exert an inhibitory effect on transcription [28].

The TATATAA sequence elements present in the $tRNA_I^{Gly}$ genes influenced their transcription either positively or negatively in a position-dependent manner and removal of negative sequences enhanced their transcription considerably [24] (AP & KPG, unpublished observations). Both $tRNA_I^{Gly}$*-1* and $tRNA_I^{Gly}$*-6,7* bind TFIIIB without prior binding of TFIIIC, but unlike TFIIIC-independent transcription in yeast, they were transcriptionally incompetent in the absence of TFIIIC in silkworm. The TFIIIB–*tRNA* interactions were directed through the TBP component with the AT-rich elements but the complexes were readily dissociated in the absence of TFIIIC. The TATA sequences present elsewhere in the flanking regions of these genes could also bind TFIIIB, leading to its sequestration from the transcription initiation site. Our analysis was mostly confined to the upstream TATA sequences because the downstream element in $tRNA_I^{Gly}$*-6,7* was significantly distant from the TFIIIC binding region.

In $tRNA_I^{Gly}$*-6,7* binding of TFIIIB, even in the presence of TFIIIC, was dissociated by heparin, unlike TFIIIB/TFIIIC binding to the highly transcribed $tRNA_I^{Gly}$*-1*. Although a perfect TATA sequence is present in the immediate upstream region of $tRNA_I^{Gly}$*-6,7* (at position −26 with respect to the +1 of tRNA) proper positioning of the TATA sequences appeared necessary for the formation of stable complexes. The mere presence of the TATA sequence alone was not sufficient to support formation of stable transcription complexes. The location of TATA sequences at −34 in $tRNA_I^{Gly}$*-1* was optimal to achieve high levels of transcription. It has been shown previously that the DNA in transcriptionally active TFIIIB–promoter complexes is bent sharply at approximately −30 nucleotides, in the middle of the TFIIIB binding site [29,30]. Thus, the TATA sequences present in $tRNA_I^{Gly}$*-1* and $tRNA_I^{Gly}$*-6,7* which are positioned on the different phases of the DNA helix allow TFIIIB binding in an active state (i.e. capable of interacting with TFIIIC to stabilize binding) in the case of $tRNA_I^{Gly}$*-1* and in an inactive state (incapable of efficient interaction with TFIIIC leading to unstable binding) in the case of $tRNA_I^{Gly}$*-6,7*. This conclusion was also supported by the observation that even when a vast excess of TFIIIB was supplemented for *in vitro* transcriptions when both templates were present, $tRNA_I^{Gly}$*-6,7* transcription was still only ≈15–20% that of $tRNA_I^{Gly}$*-1*. In the later stages of *B. mori* development, transcription from more $tRNA_I^{Gly}$ copies may be warranted to optimize translation of the fibroin messenger. The presence of excess quantities of transcription factors like TFIIIB would facilitate transcription from all the gene copies. In fact, such a regulatory mechanism through the availability of transcription factor TFIIIA is known to operate in the differential expression of oocyte-specific and somatic cell-specific 5*S* RNA genes transcribed by RNA pol III in *Xenopus* [31]. Differential transcription of oocyte-specific *tRNA* has been attributed to TFIIIC in this organism [32]. In *Drosophila*, as well as in humans, differences in TFIIIB have been reported to be responsible for transcriptional regulation associated with growth restriction or cell-cycle control [33–35].

Competition analysis to identify the limiting component of the transcription machinery confirmed sequestration of the transcription factor by $tRNA_I^{Gly}$*-6,7*. It has been shown previously that for both $tRNA^{Ala}$ and $tRNA^{Gly}$ genes in *B. mori*, certain AT-rich elements present in the upstream regions modulated their transcription [22,23,36]. In the case of $tRNA^{Ala}$, which has variants of silk gland-specific and constitutively expressed copies, the critical differences in the interaction of the flanking sequences with TFIIIB were major determinants for the differences in transcription [37–39]. The transcription competition experiments carried out here confirmed that the poorly transcribed $tRNA_I^{Gly}$*-6,7* harboured sequences that were sequestering components of the basal transcription machinery

and making them unavailable for transcription. The 'TATATAA' sequence present in the flanking regions of $tRNA_1^{Gly}$-*6,7* was responsible for the sequestration of TFIIIB by directly binding to the factor via interactions with TBP, independent of TFIIIC. From samples immunodepleted with anti-TBP sera, the reduced transcription could be restored to original levels by external supplementation of TFIIIB, but not TBP. Evidently, TFIIIB, and not free TBP, was the limiting component in the nuclear extracts.

This study established that transcriptional inhibition was achieved through sequestration of the basal transcription factor TFIIIB, as well as the formation of unstable transcription complexes. In *Drosophila*, a transcription factor TRF, rather than TBP, has been reported to be involved in RNA pol III transcriptions [40,41]. However, all our efforts to identify such a factor in *B. mori* by PCR using primers based on the TRF sequences or western blots of different tissue extracts of *B. mori* using *Drosophila* anti-TRF sera have been unsuccessful. We believe that TBP and not TRF is involved as the component of TFIIIB in RNA pol III transcription in *B. mori* and the presence of TRF could be exclusive to *Drosophila*. The recent characterization of the cDNA encoding Brf1 from *B. mori* [42] has revealed that individual domains of Brf had considerable similarity to the *Drosophila* counterpart (55%). However, the domain II, which interacts with TBP in most cases but with TRF in *Drososphila*, was divergent in *B. mori*. The *Bombyx* Brf domain II was more similar to human Brf, suggesting that the silkworm protein could indeed be interacting with TBP because TRF was absent.

Experimental procedures

$tRNA_1^{Gly}$ genes

Plasmid constructs harbouring the $tRNA_1^{Gly}$ genes from *B. mori* (Fig. 1) were from our laboratory stock [22]. Plasmid clone pR8 contains $tRNA_1^{Gly}$-*1*, which is highly transcribed and comprises sequences 300 bp upstream and 30 bp downstream of the coding region, in plasmid pBSSK+ [21]. Clone pRKX3, a derivative of $tRNA_1^{Gly}$-*1* has a single TATA element at −130 bp and is transcribed to the same levels as the parent. pmutRKX3 has the single TATATAA element of pRKX3 mutated to GATATCA. $tRNA_1^{Gly}$-*6,7* (in plasmid clone pΔS1) is a fusion construct of two genes $tRNA_1^{Gly}$-*6* and $tRNA_1^{Gly}$-*7* present in a single genomic fragment of *B. mori*, which, after fusion, contains the 970 bp upstream sequences of $tRNA_1^{Gly}$-*6* and the 1.5 kb downstream sequences of $tRNA_1^{Gly}$-*7*, but lacks the 400 bp region between the two gene copies [23]. This construct retained the low transcriptional activity of the two parental gene copies ($tRNA_1^{Gly}$-*6* and $tRNA_1^{Gly}$-*7*) and was used to avoid the presence of two transcripts arising from the single genomic fragment in the parental clone. Upstream deletions of $tRNA_1^{Gly}$-*6,7* were made using the *Sac*I sites at positions −895 and −445 with respect to +1 nucleotide of the tRNA coding region (plasmid clone pΔUTS1) and the downstream deletions were generated utilizing the *Bgl*II site at +767 with respect to the start of the tRNA coding region (pΔDTS1). A combination of these restriction sites was used to generate the construct pΔ3TS1 which lacked both the upstream and the downstream sequences. Plasmid construct pBmH1 harbouring the moderately transcribed $tRNA_1^{Gly}$-*4* was used in competition studies. *B. mori* TBP was expressed from the construct in pET19b after transformation into *Escherichia coli* BL21 (DE3), upon induction with 1 mM isopropyl thio-β-D-galctoside. The expressed protein containing a fused N-terminal histidine tag was purified by binding to and elution from Ni-NTA-affinity matrix [43].

Nuclear extract preparation and fractionation of the *B. mori* transcription machinery

Nuclear extracts from the PSG of *B. mori* in the fifth larval instar (day 2) were prepared as described previously [21]. Nuclear extract (3 mg protein·mL^{-1}) was loaded onto a 15 mL phosphocellulose column (Whatman P-11, Forham Park, NJ) equilibrated in buffer A (20 mM Hepes pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 M EDTA, 0.5 mM dithiothreitol and 0.5 mM phenylmethanesulfonyl fluoride). The column was washed with 3 vol. of the same buffer and the fraction containing RNA pol III and TFIIIB was obtained by elution with one column volume of buffer A containing 0.35 M KCl [11]. The TFIIIC fraction was eluted from the phosphocellulose column at 0.6 M KCl, whereas the TBP-containing pol II component TFIID was eluted at 1.0 M KCl. The 0.35 M KCl fraction, containing RNA pol III and TFIIIB was fractionated further to separate the two activities. The 0.35 M KCl fraction was dialysed against buffer A containing 0.02 M KCl and passed through a heparin–Sepharose column (5 mL) equilibrated with buffer A containing 0.02 M KCl. After washing the column with three column volumes of loading buffer, the TFIIIB component was eluted in one column volume of buffer A containing 0.3 M KCl, whereas polymerase III was eluted in buffer A containing 0.4 M KCl [11,44]. Total proteins were estimated by the dye-binding method [45].

In vitro transcriptions

In vitro transcription reactions in a final volume of 30 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

[^{32}P]GTP[αP] (3000 Ci·mmol^{-1}), 100–200 ng of template DNA and nuclear extract (6 μg protein) or the reconstituted fractions comprising of 4–6 μg protein each of partially purified TFIIIB (0.3 M KCl eluate from the heparin–Sepharose column), RNA polymerase III (0.4 M KCl fraction from heparin–Sepharose column) and TFIIIC (0.6 M KCl fraction from the phosphocellulose column). The reactions were incubated at 30 °C for 1 h in the absence of heparin for multiple-round transcriptions. For single-round transcriptions incubations were carried out initially for 10 min in the absence of nonradioactive GTP and a further 50 min after adding 10 μM GTP and heparin (100 μg·mL^{-1}). The reactions were terminated by the addition of 0.2% (w/v) SDS, 10 mM EDTA and 100 μg·mL^{-1} glycogen, and analysed by electrophoresis on 7 M urea/8% polyacrylamide gels and visualized using a Phosphorimager (Bioimage Analyser FLA 5100, Fuji Photofilm Co, Ltd, Tokyo, Japan).

Competition for transcription factors was done as in the standard transcription reactions at suboptimal concentrations of nuclear extract (4 μg protein) such that the transcription factors were limiting and the transcription levels could be visibly enhanced by external supplementation of the transcription factor. External supplementations were made by adding TFIIIC, polymerase or TFIIIB fractions (4 or 6 μg protein) and 100 ng of each of the competing templates. The three $tRNA_I^{Gly}$ templates used here were the moderately transcribed $tRNA_I^{Gly}$*-4*, the highly transcribed $tRNA_I^{Gly}$*-1* and the poorly transcribed $tRNA_I^{Gly}$*-6,7*. A 40 bp region containing the TATATAA sequence present at −895 upstream of the $tRNA_I^{Gly}$*-6,7* in plasmid pΔS1 (EMBL Accession no. Z49226) was also used for competitions (isolated as a *Sac*I restriction fragment of 40 nucleotides from the pΔS1 construct; Fig. 1). In addition a 150 bp fragment harbouring the TATATAA element from $tRNA_I^{Gly}$*-1* (*Eco*RI/*Kpn*I fragment from plasmid pR8; Fig. 1) or this region from which the TATATAA sequence was mutated to GATATCA, were also used for competitions.

For antibody depletion studies, the nuclear extracts supplemented with external TFIIIB were incubated with polyclonal antibodies against TBP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h on ice, and the supernatants after pull down with protein A–agarose were used in transcription reactions. The cross-reactivity of the anti-TBP serum against silkworm TBP was established by western blotting.

Electrophoretic mobility shift assays (EMSA)

Gel retardation assays (EMSA) were carried out in a final volume of 20 μL containing 6 μg of the purified TBP, TFIIIC, TFIIIB or RNA pol III, in 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 dithiothrietol, 5% glycerol and 2 μg double-stranded poly(dI-dC). After incubation of 15 min at 4 °C, the radiolabelled DNA probe (20 000 c.p.m.) harbouring the TATATAA sequence (the 150 bp *Eco*RI/*Kpn*I fragment from plasmid pR8) was added and binding was allowed to proceed for another 15 min. Binding reactions were terminated by the addition of gel loading buffer and electrophoresed on 6% polyacylamide gels at 4 °C and visualized in Phosphorimager. In binding competition experiments, 10 or 100× concentration of unlabelled fragment either wild-type or from which the TATATAA sequence was mutated were included.

Heparin-resistant TFIIIB complex formation

The stability of the interaction between TFIIIB and the $tRNA_I^{Gly}$ genes was examined by the formation of heparin-resistant TFIIIB complexes. A 400 bp *Eco*RI/*Xba*I fragment from plasmid pR8 containing $tRNA_I^{Gly}$*-1* or the 370 nucleotide fragment from the parental plasmid pS1 (as a *Dra*I fragment from −260 to +110 beyond the coding region of $tRNA_I^{Gly}$*-6* gene) were radioactively labelled by end-labelling [45]. The binding reaction contained in 20 μL, radiolabelled DNA (60 000 c.p.m.), 4 μg poly(dG-dC), 100 ng of pBR322 DNA, 6 μg of TFIIIC and 6 μg of TFIIIB, 70 mM KCl, 4 mM $MgCl_2$, 13% glycerol, 3 mM dithiothreitol and 30 mM Tris/HCl (pH 7.5). After incubation for 1 h at 4 °C, 20 μg·mL^{-1} of heparin was added and the incubation was continued for 5 min. The complex formation was analysed by electrophoresis on nondenaturing 4% polyacrylamide gels and visualized in a Phosphorimager.

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