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Transcriptional Silencing of a $tRNA_1^{Gly}$ Copy from within a Multigene Family Is Modulated by Distal *cis* Elements*

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Individual copies of $tRNA_1^{Gly}$ from within the multigene family in *Bombyx mori* could be classified based on *in vitro* transcription in homologous nuclear extracts into three categories of highly, moderately, or weakly transcribed genes. Segregation of the poorly transcribed gene copies 6 and 7, which are clustered in tandem within 425 base pairs, resulted in enhancement of their individual transcription levels, but the linkage itself had little influence on the transcriptional status. For these gene copies, when fused together generating a single coding region, transcription was barely detectable, which suggested the presence of negatively regulating elements located in the far flanking sequences. They exerted the silencing effect on transcription overriding the activity of positive regulatory elements. Systematic analysis of deletion, chimeric, and mutant constructs revealed the presence of a sequence element TATATAA located beyond 800 nucleotides upstream to the coding region acting as negative modulator, which when mutated resulted in high level transcription. Conversely, a TATATAA motif reintroduced at either far upstream or far downstream flanking regions exerted a negative effect on transcription. The location of *cis*-regulatory sequences at such farther distances from the coding region and the behavior of TATATAA element as negative regulator reported here are novel. These element(s) could play significant roles in activation or silencing of genes from within a multigene family, by recruitment or sequestration of transcription factors.

The expression of tRNA genes is controlled by two internal control regions (ICRs),[1] the A-box and the B-box located within the coding sequences (1). These highly conserved intragenic regions constitute the essential promoter elements of a tRNA gene (2, 3). Although the transcription of *tRNAs* depends on the ICRs, the sequences preceding the gene influence both the choice of initiation site and the efficiency of transcription (4, 5). In eucaryotes, most *tRNAs* exist as multigene families and the expression of individual members of each family may be differentially regulated (6). Since the ICRs are highly conserved among different copies, the more variable flanking sequences qualify to be the obvious candidates for bringing about differential regulation.

The involvement of both the 5′- and 3′-flanking sequences in modulating expression of tRNA genes has been reported in yeast (7–9), *Drosophila* (10, 11), silkworm (12–16), *Xenopus* (17, 18), mouse (19, 20), and human (21, 22). However, such regulatory elements are generally located in the immediate vicinity up to 100 bp upstream (8, 12, 13, 23) or about 50 bp downstream (24, 25) of the coding region. The presence of potential regulatory elements upto 300 nucleotides 5′ upstream to the coding region in a $tRNA_1^{Gly}$ copy from *Bombyx mori* has been reported previously by us (15).[2] Effects of regulatory sequences can be strictly position-dependent, and moving them by even a few base pairs relative to the coding region can change transcription efficiency dramatically (17).[2]

In *B. mori*, fibroin, the major constituent protein of the silk fiber, is synthesized at very high levels in the posterior silk gland during the fifth larval instar. The unusual amino acid composition of fibroin (glycine, 46%; alanine, 26%; serine, 12%; tyrosine, 5%) demands an uneven distribution of tRNA populations to optimize the fibroin production (26, 27). While the overall tRNA content in posterior silk glands increases by at least 10-fold when fibroin synthesis is at its peak, the tRNAs cognate to the four abundant amino acids in fibroin account for about 70% of this increase. Understandably $tRNA_1^{Gly}$ is most predominant, because the large 15-kb messenger RNA for the fibroin heavy chain contains about 2400 codons corresponding to glycine, of which nearly 50% are decoded by this tRNA species (28). $tRNA_1^{Gly}$ constitutes a multigene family with an estimated 20 copies in *B. mori*, and 10 of them have been cloned and characterized previously (16). All these copies have identical coding sequences but differ in their flanking regions and show varying levels of transcription. None of these genes appear to be silk gland-specific.

In this communication, we have attempted to decipher the mechanisms by which some copies are rendered silent, by comparative transcription analysis of a set of $tRNA_1^{Gly}$ genes belonging to the extreme groups (highly or poorly transcribed). We demonstrate here the involvement of negative regulatory *cis* elements located at distances much farther upstream or downstream to the coding region than believed earlier.

EXPERIMENTAL PROCEDURES

In Vitro Transcription Assays—Crude nuclear extracts from posterior silk glands of *B. mori* in the fifth larval instar were prepared as described previously (15). In brief, freshly dissected out posterior silk glands were homogenized in buffer (2 M sucrose, 10% glycerol, 10 mM HEPES (pH 7.9), 15 mM KCl, 0.5 mM DTT, 0.5 M PMSF, 0.15 M sperm-

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[1] The abbreviations used are; ICR, internal control region; bp, base pair(s); kb, kilobase(s); DTT, dithiothreitol; nt, nucleotide(s); PMSF, phenylmethylsulfonyl fluoride; Pol II and Pol III, RNA polymerases II and III, respectively; TBP, TATA-binding protein; *tRNA(s)*, gene(s) encoding tRNA.

[2] S. Sharma and K. P. Gopinathan, unpublished data.

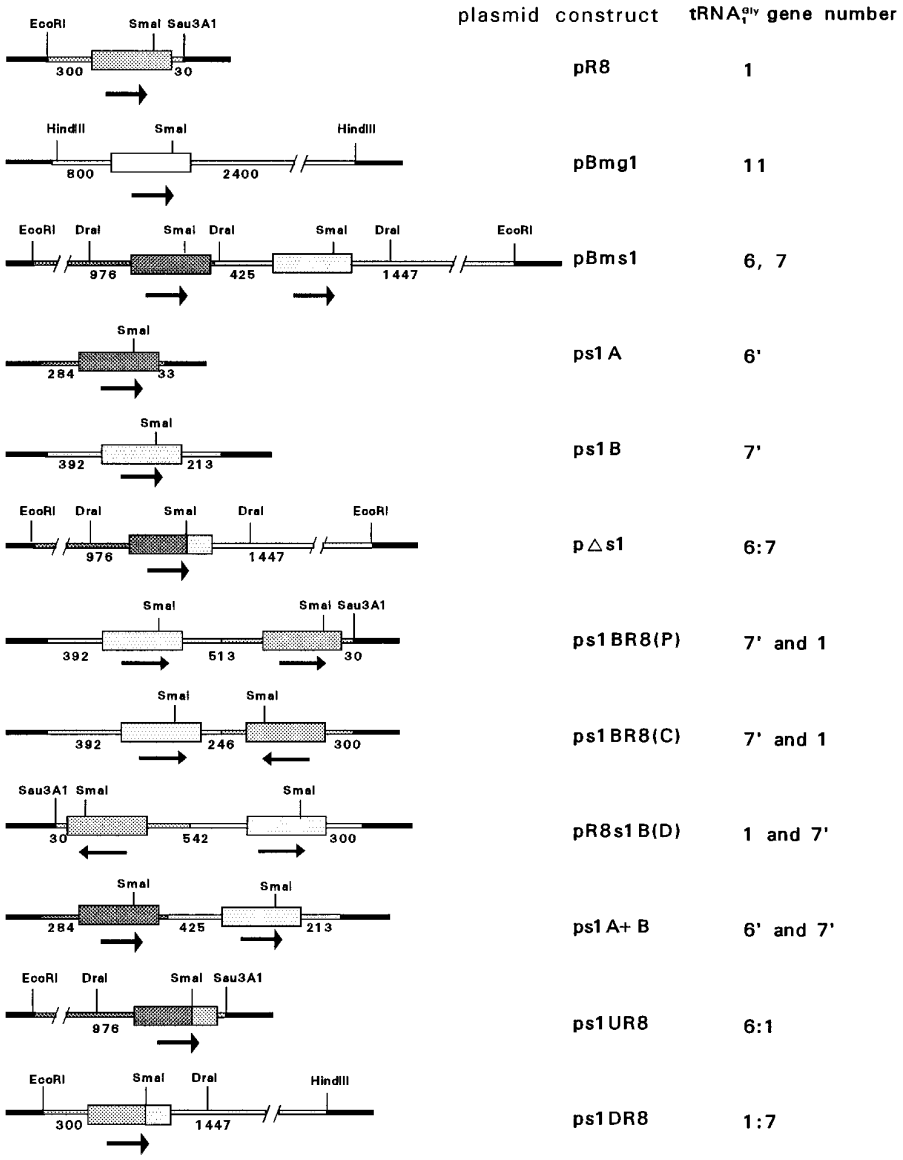

FIG. 1. ***tRNA$_1^{Gly}$* constructs used in this study.** The plasmid clones pR8, pBms1, and pBmg1 have been described under methods. The *tRNA$_1^{Gly}$*-6 and -7 present in the parental clone pBms1 were isolated from each other using the *Dra*I sites indicated and individually cloned into the *Sma*I site of both pTZ 18 and 19 to generate ps1A and ps1B. The flanking regions of both gene copies 6 and 7 with respect to each other have been disrupted during this subcloning. The *tRNA$_1^{Gly}$*-6 and -7 were fused in frame (without altering the original sequence) to generate the hybrid gene 6:7 (in clone pΔs1) making use of the *Sma*I sites in pBms1. This hybrid gene (6:7) has identical tRNA coding sequence but retains the entire 5′ upstream of gene 6 and 3′ downstream of gene 7. The constructs ps1BR8(P) and ps1BR8(C) were generated by mobilizing the entire *tRNA$_1^{Gly}$* insert upto −300 nt in pR8 into ps1B such that the two gene copies (1 and 7) are placed in tandem and in orientations parallel to or converging from each other. The construct pR8 s1B(D) was generated by swapping the *tRNA$_1^{Gly}$*-1 from the deletion clone pKX in which the sequences from −150 nt to −300 nt upstream to the coding region was removed, onto ps1B. In ps1A+B, the gene copies 6 and 7 were brought back together with the same linking sequences between them as in the parental clone pBms1 but devoid of the far 5′ upstream and 3′ downstream flanking sequences. ps1UR8 and ps1DR8 were constructed by swapping domains between the parental clones pR8 and pBms1 to generate the fused genes 6:1 or 1:7. These hybrid genes retained identical tRNA coding sequences but harbored either the entire 5′ upstream of gene 6 or 3′ downstream of gene 7 while retaining the other part from gene 1.

ine, 0.15 M spermidine, and 1 mM EDTA, (10 ml/g tissue)). The homogenate was layered on a cushion of the above buffer and centrifuged at 25,000 rpm for 1 h at 4 °C in a Beckman SW28 Ti rotor. The pelleted nuclei were lysed by gently stirring with 1 ml of lysis buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and centrifuged at 25,000 × *g* for 30 min at 4 °C. The clear supernantant was dialyzed for 4 h against the dialysis buffer (20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and used as crude nuclear extracts for *in vitro* 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), crude nuclear extract (25 μg of protein), and 4–8 μg/ml supercoiled plasmid DNA template. After incubation at 30 °C for 1 h, the reactions were terminated by the addition of 0.2% SDS, 10 mM EDTA, and 100 μg/ml glycogen (carrier) and processed as described previously (15). Quantification of transcription was carried out by laser densitometric scanning of the autoradiographs at different exposures to ensure the sensitivity and range of detection.

DNA Sequencing—DNA sequencing was carried out by the dideoxy method using the T7 sequencing kit (Pharmacia Biotech Inc.).

Deletion and Hybrid Gene Constructs—The parental clones pR8 and pBms1 harboring *tRNA$_1^{Gly}$*-1, -6, and -7 from *B. mori* were those described previously (16). The clone pBmg1 (a gift from A. Fournier) carried a 3.2-kb insert of *B. mori* chromosomal DNA harboring a copy of glycyl tRNA gene, designated here as *tRNA$_1^{Gly}$*-11. All other constructs were generated by exploiting the restriction sites (marked in Fig. 1) in the appropriate clones either within the coding region or in the flanking regions. For subcloning, the vectors pTZ18/19 (Pharmacia) or pBS KS+ (Stratagene) were used.

Mutagenesis—Site-directed mutagenesis of the TATATAA element was done by Kunkel's method (29). An oligodeoxyribonucleotide, 5′-CCATTTAAGGAAGATATCAATAAAATAGAG-3′ was used to mutagenize the two highly conserved nucleotides of TATAT AA to GATAT CA, generating a new restriction site (*Eco*RV). The mutants were initially screened by digestion of plasmid DNAs with *Eco*RV and confirmed by sequencing.

RESULTS

tRNA Genes—We have described previously 10 copies of *tRNA$_1^{Gly}$* from *B. mori*, serially designated as *tRNA$_1^{Gly}$*-1 to -10 (16). An additional copy of the gene, *tRNA$_1^{Gly}$*-11 present in clone pBmg1, as well as copies of *tRNA$_1^{Gly}$*-1, -6, and -7, their deletion derivatives and domain-swapped, or hybrid constructs generated for detailed analysis in the present study are listed in Fig. 1.

In Vitro Transcription Analysis—The 11 copies of *tRNA$_1^{Gly}$* from *B. mori* have been analyzed by transcription i*n vitro* in homologous nuclear extracts derived from posterior silk glands (Fig. 2, *a* and *b*). Each of the gene copies gave rise to two transcripts corresponding to an approximately 75-nt precursor and 71-nt mature transcript (Fig. 2*a*). However, the presence of

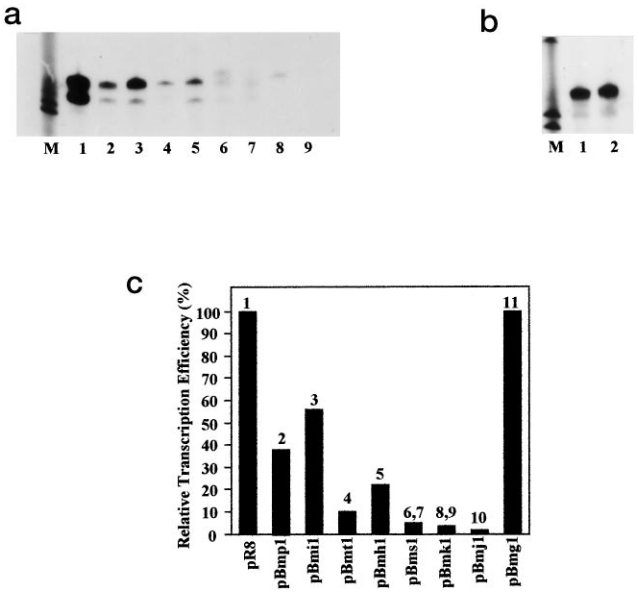

FIG. 2. ***In vitro* transcription of $tRNA_1^{Gly}$ copies.** The individual copies of $tRNA_1^{Gly}$-1 to -11 were analyzed by transcription *in vitro* in homologous nuclear extracts from posterior silk glands (*a* and *b*). The template concentration used was 100 ng of plasmid DNA in each case. *a*: *lanes 1–5*,$tRNA_1^{Gly}$-1 to -5, respectively; *lane 6*, $tRNA_1^{Gly}$-6 and -7 (present together on a single plasmid); *lane 7*, $tRNA_1^{Gly}$-8 and -9 (present on a single plasmid); *lane 8*, $tRNA_1^{Gly}$-10; *lane 9*, no template; *lane M*, molecular size marker, pTZ18, DNA digested with *Hin*fI (this marker has been used in all experiments). *b*: *lanes 1* and *2*, $tRNA_1^{Gly}$-11 and -1, respectively. *c*, quantitation of the transcription levels was carried out by laser densitometric scanning of the autoradiographs. The names of the individual plasmid constructs (described in Ref. 15 and this study) as well as the $tRNA_1^{Gly}$ copy numbers are indicated in figure.

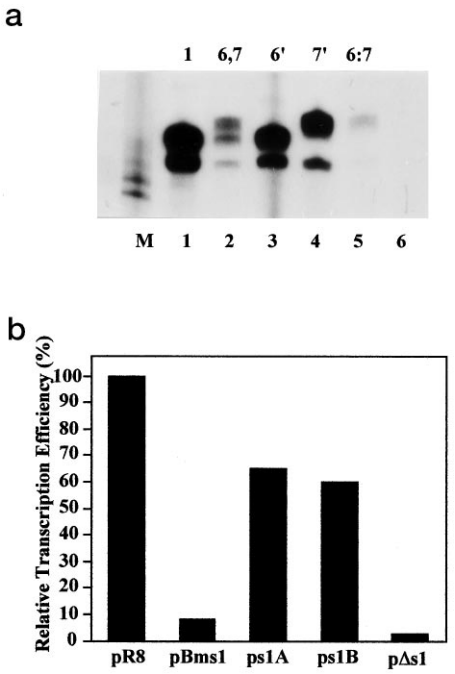

FIG. 3. **Transcription of linked, unlinked and chimeric gene constructs.** *a*, the *in vitro* transcription of individual genes 6 and 7 when unlinked from each other or fused in frame within the coding region (designated 6:7). *Lane M*, size markers; *lane 1*, $tRNA_1^{Gly}$-1 (in pR8); *lane 2*, $tRNA_1^{Gly}$-6 and -7 (in pBms1); *lane 3*, $tRNA_1^{Gly}$-6 (in ps1A); *lane 4*, $tRNA_1^{Gly}$-7 (in ps1B); *lane 5*, $tRNA_1^{Gly}$-6:7 (in pΔs1); *lane 6*, no template. *b*, quantitation of the transcriptions in *A*; lanes as marked.

a slightly longer transcript, about 80 nt, was discernible in *lane 6* (see later sections) and *8*. $tRNA_1^{Gly}$-11, analyzed here for the first time, was transcribed to very high levels comparable with $tRNA_1^{Gly}$-1 (Fig. 2*b*). The quantitation of transcription efficiencies of these 11 gene copies relative to the highly transcribed $tRNA_1^{Gly}$-1 is presented in Fig. 2*c*. The variability in the levels of transcription of individual copies was highly consistent (+5%) in several independent experiments using different batches of nuclear extracts and plasmid template DNA preparations. In every individual analysis the transcription levels were normalized to that of $tRNA_1^{Gly}$-1 taken as 100%.

Based on the *in vitro* transcriptions, evidently, the genes could be classified into three groups, *viz*. those which are transcribed to very high levels ($tRNA_1^{Gly}$-1 and -11), medium to high levels ($tRNA_1^{Gly}$- 2, -3, -4, and -5), and low to undetectable levels ($tRNA_1^{Gly}$-6, -7, -8, -9, and -10). Since all these gene copies had identical coding sequences (16),[2] and consequently the same ICRs, their differential transcription could be attributed to the flanking sequences, upstream or downstream. The differences in transcription levels were not just due to the differences in the size of inserts in various constructs, because the gene copies 10 and 11 (present on 3-kb genomic fragments in each case, in plasmids pBmj1 and pBmg1, respectively) belonged to the opposite groups in terms of transcriptional efficiency. Neither did the plasmid vector sequences exert any noticeable influence on transcription, because the same $tRNA_1^{Gly}$ copy in different vector backgrounds showed identical levels of transcription *in vitro*.

Comparative studies were therefore made using the highly transcribed $tRNA_1^{Gly}$-1, and the poorly transcribed copies 6 and 7, to examine the influence of the flanking sequences on transcriptional modulation.

Transcription Analysis of $tRNA_1^{Gly}$-6 and -7—The $tRNA_1^{Gly}$-6 and -7 are located in tandem and in the same orientation on a single chromosomal segment, but separated from each other by 425 nt (clone pBms1, Fig. 1). This construct gave rise to three weak but distinct transcripts, two of them corresponding to the precursor tRNA forms of gene copies 6 (75 nt) and 7 (81 nt), respectively, and the third (71 nt) corresponding to the mature transcript (*lane 6*, Fig. 2*a*). Comparison of their transcription with that of $tRNA_1^{Gly}$-1 revealed that at optimal template concentration, the highest levels of transcription of both the gene copies 6 and 7 together (in pBms1) were around 2–10% of the former (in pR8). Even at much higher molar equivalents of $tRNA_1^{Gly}$-6 and -7 template, the transcription levels never exceeded 5–10% of that of $tRNA_1^{Gly}$-1.

In order to check whether the low transcription of the gene copies 6 and 7 could be due to their linkage in close proximity, they were separated from each other (clones ps1A harboring $tRNA_1^{Gly}$-6 and ps1B harboring $tRNA_1^{Gly}$-7) and analyzed. When unlinked from each other, individually they showed higher levels of transcription, approaching 60–65% of the levels of $tRNA_1^{Gly}$-1 (*lanes 3* and *4* in Fig. 3, *a* and *b*).

The transcripts from both $tRNA_1^{Gly}$-6 (clone ps1A) and -7 (clone ps1B) were initiated at −4 with respect to the +1 nt of mature $tRNA_1^{Gly}$ as deduced from primer extension analysis (data not shown) just as in the case of $tRNA_1^{Gly}$-1 (15). However, the transcript from gene copy 7 terminated 6 nt farther downstream, compared with gene copies and 6, and hence gave rise to the longer (81 nt) precursor transcript.

In the process of separating out the two gene copies present together in the parental clone pBms1, although the coding region and the immediate flanking regions (up to −284 nt 5′ upstream and 33 nt 3′ downstream in $tRNA_1^{Gly}$-6 and −392 nt 5′ upstream and 213 nt 3′ downstream in $tRNA_1^{Gly}$-7) were unaltered and remained identical to those present in the parental construct, the far upstream and downstream regions as well as the sequences linking them were altered.

Significance of Gene Linkage on Transcription—The increase

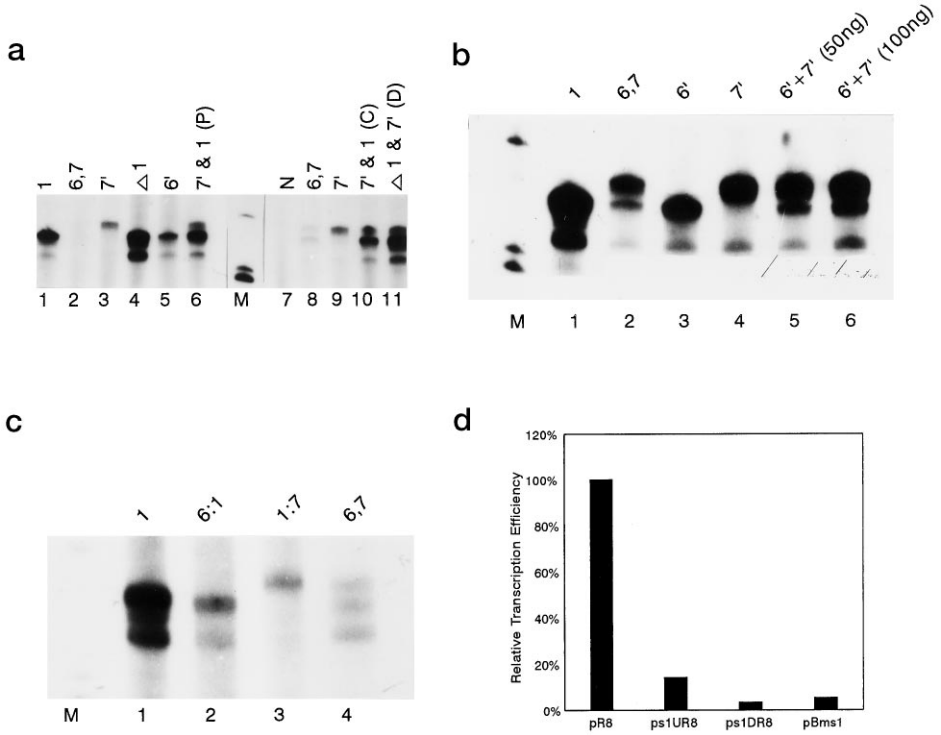

FIG. 4. **Role of gene context on transcription.** Transcription of the chimeric gene constructs where $tRNA_1^{Gly}$-6 and -7 were linked back together as in the parental clone (but devoid of farther upstream and downstream flanking regions) or $tRNA_1^{Gly}$-7 placed in proximity to the highly transcribed $tRNA_1^{Gly}$-1 in different orientations, as indicated in Fig. 1, is presented. *a*: *lane 1*, $tRNA_1^{Gly}$-1 (in pR8); *lanes 2* and *8*, $tRNA_1^{Gly}$-6 and -7 (in pBms1); *lanes 3* and *9*, $tRNA_1^{Gly}$-7 (in ps1B); *lane 4*, $tRNA_1^{Gly}$-1 (in pKX, a deletion mutant of $tRNA_1^{Gly}$-1 in which sequences 5′ upstream from −150 to −300 nt harboring a negative element are removed which results in higher transcription (15); *lane 5*, $tRNA_1^{Gly}$-6 (in ps1A); *lane 6*, $tRNA_1^{Gly}$-7:1 (in ps1BR8[P]); *lane 7*, no template (control); *lane 10*, $tRNA_1^{Gly}$-7:1 (in ps1BR8[C]); *lane 11*, $tRNA_1^{Gly}$-1:7 (in pR8 s1B[D]). All the transcriptions were carried out at 100 ng of template DNA (in a final volume of 25 μl) except in *lanes 3* and *9*, where the template DNA concentration used was only 50 ng. *b*: *lane 1*, $tRNA_1^{Gly}$-1 (in pR8); *lane 2*, $tRNA_1^{Gly}$-6 and -7 (in pBms1); *lane 3*, $tRNA_1^{Gly}$-6 (in ps1A); *lane 4*, $tRNA_1^{Gly}$-7 (in ps1B); *lane 5*, $tRNA_1^{Gly}$-6 and -7 (in ps1A+B, 50 ng of template DNA); *lane 6*, $tRNA_1^{Gly}$-6 and -7 (in ps1A+B, 100 ng of template DNA); *lane M*, marker. *c*, down-regulation of $tRNA_1^{Gly}$-1 by the far flanking sequences of $tRNA_1^{Gly}$-6 and -7. Transcription from plasmids ps1UR8 and ps1DR8 (constructs obtained by swapping domains of $tRNA_1^{Gly}$-1 with those of $tRNA_1^{Gly}$-6 and -7 in pBms1). *Lane M*, marker; *lane 1*, $tRNA_1^{Gly}$ (in pR8); *lane 2*, $tRNA_1^{Gly}$-6:1 (in ps1UR8); *lane 3*, $tRNA_1^{Gly}$-1:7 (in ps1DR8); *lane 4*, $tRNA_1^{Gly}$-6 and -7 (in pBms1). *d*, quantitation of transcriptions in *c*; lanes as marked.

in the transcription of the gene copies 6 and 7 on isolating them from each other suggested that either the close linkage or those sequences that were eliminated or altered during their separation played a role in modulating their expression. If indeed the lower levels of transcription of the two genes in pBms1 were due to the gene linkage, the proximal presence of either gene copy 6 or 7 to the hyperexpressed $tRNA_1^{Gly}$-1 should have influenced the transcriptional efficiency of the latter. To test this possibility, dual gene constructs harboring the gene copies 1 and 7 were made (Fig. 1). We preferred gene copy 7 for such chimeric constructs, because the longer precursor transcript arising from this could be readily distinguished from that of gene copy 1. The three hybrid constructs generated for this purpose had the two gene copies in orientations, converging (in ps1BR8(C)), diverging (in pR8 s1B(D)) or parallel (in ps1BR8(P)), and in locations upstream or downstream with respect to each other (see Fig. 1). The *in vitro* transcription of these constructs revealed no significant changes in the levels of transcription of either copy, indicating that the gene context (*i.e.* the linkage or the orientation of the gene copies with respect to each other) had little influence on transcription (compare *lanes 6* and *10* with *lanes 1* and *3* and *lane 11* with *lanes 3* and *4* in Fig. 4*a*).

Even when the two gene copies 6 and 7 were brought back together to be placed in context of each other and restoring the original sequences that linked them (ps1A+B, see Fig. 1), there were no significant changes in their individual expression levels (Fig. 4*b, lanes 5* and *6*). This construct, ps1A+B, differed from the parental plasmid pBms1 harboring the same two gene copies 6 and 7 only by the absence of the 5′ far upstream flanking sequences (−284 to −976 nt with respect to +1 of $tRNA_1^{Gly}$-6) and 3′ far downstream sequences (+278 to +1518 nt with respect to +1 of $tRNA_1^{Gly}$-7).

Role of Far Upstream and Downstream Flanking Sequences on Transcription—From the foregoing results, it was clear that deletion of far upstream or downstream sequences had resulted in 10–20-fold stimulation in transcription of gene copies 6 and 7 and that the linkage itself had little influence. In order to confirm that the down-regulation seen in the parental gene construct, pBms1, was indeed due to far upstream or downstream sequences, three more hybrid genes were constructed (plasmids pΔs1, ps1UR8, and ps1DR8 in Fig. 1).

In the deletion construct pΔs1, the gene copies 6 and 7 in the parental clone pBms1 were fused in frame to generate a single copy of the gene (*i.e.* without altering the tRNA coding sequence) by removing parts of coding regions from both the gene copies as well as the intervening sequences. This hybrid gene, designated 6:7, retaining the entire 5′- and 3′-flanking regions (0.9 and 1.5 kb, respectively) was barely transcribed (Fig. 3*a, lane 5*) resembling the parental copies 6 and 7 in pBms1. As anticipated, the transcript from 6:7 was longer (81 nt), corresponding to the one arising from gene copy 7, because the transcription termination of the hybrid gene was derived from gene 7. The very low transcription levels of gene 6:7 thus confirmed the presence of *cis*-acting negative regulatory elements in those far upstream or downstream sequences, which

were eliminated during the separation of $tRNA_1^{Gly}$-6 and -7 (in clones ps1A and ps1B, respectively).

In order to further confirm the presence of negative regulatory elements, two other hybrid constructs, ps1UR8 and ps1DR8, generated by swapping the domains between $tRNA_1^{Gly}$-1 and -6 or -7 were exploited. In these constructs, either the entire 5′ upstream (300 nt) of $tRNA_1^{Gly}$-1 was replaced with the 976-nt sequences of gene copy 6 while retaining the 30-bp 3′-flanking sequences, in ps1UR8, or the complete 300-nt 5′-flanking sequences of $tRNA_1^{Gly}$-1 were retained while replacing the 30-nt downstream sequences with the 1.5-kb 3′-flanking sequences of gene copy 7, in ps1DR8 (Fig. 1). These hybrid genes, designated, respectively, as $tRNA_1^{Gly}$-6:1 or -1:7, had identical tRNA coding sequences but differed only in their flanking regions with respect to the parental copies. There was drastic reduction (by 10–15-fold) of transcription in both cases (Fig. 4, *c* and *d, lanes 2* and *3*, respectively), and the extent of inhibition was more pronounced in $tRNA_1^{Gly}$-1:7 (Fig. 4*c, lane 3*). Evidently, *cis*-acting negative regulatory elements are present in the far upstream sequences of the gene copy 6 as well as the far downstream sequences of the gene copy 7.

Presence of Negative Regulatory Elements in the Far Flanking Sequences of $tRNA_1^{Gly}$-6 and -7—In the immediate 5′ upstream sequences up to 100 nt of the $tRNA_1^{Gly}$ genes, we could not identify any characteristic regulatory elements other than a few possible sequence motifs such as TTC, TATAT, and ATTT, present in all of them, irrespective of their transcriptional status (16). The complete nucleotide sequence of the 3-kb chromosomal DNA fragment harboring both genes 6 and 7 present in the parental clone pBms1 was therefore determined (EMBL accession number Z49226). The sequences were generally AT-rich. The presence of a typical TATA box sequence element, TATATAA, at multiple locations, both upstream and downstream to the coding regions of the two *tRNA* copies 6 and 7, was conspicuous. Such a sequence motif (TATATAA) negatively influenced transcription of the highly transcribed $tRNA_1^{Gly}$-1 by about 35–40% (30).

In order to check the influence of these TATATAA motifs on transcription of $tRNA_1^{Gly}$-6 and -7, a systematic deletion analysis was carried out. Three deletion constructs were generated such that the sequences containing either far upstream (−871 nt with respect to +1 nt of $tRNA_1^{Gly}$-6) or far downstream (+1019 nt and +1054 nt with respect to +1 nt of $tRNA_1^{Gly}$-7), and both far upstream and downstream TATATAA motifs were deleted in constructs pΔUTs1, pΔDTs1, and pΔ3Ts1, respectively (Fig. 5*a*). The deletion of the far flanking regions encompassing the TATATAA elements along with their flanking sequences in either upstream or downstream locations resulted in the enhancement of transcription (*lanes 3–5* in Fig. 5, *b* and *c*). The transcription enhancement was mere pronounced in pΔ3Ts1 where both the upstream and downstream far localized elements were removed. In this case, the levels of transcription were the same or even higher than that of $tRNA_1^{Gly}$-1 (*lane 5*, Fig. 5*b*).

In order to narrow down the sequence motif within the 440-bp sequences in pΔs1, which when deleted gave rise to enhancement in transcription (in *pΔUTs*, Fig. 5*a*), a 40-bp fragment from within this deleted sequence harboring the TATATAA element was reintroduced into the latter clone, such that this motif was reinstated in the upstream region (but at the same time devoid of the 400 bp previously present in the parental copy). This construct, designated p40ΔUTs1 (Fig. 6*a*) was barely transcribed (Fig. 6, *lane 4*). The insertion of the above 40-bp fragment thus brought down the transcription level to the same as that of the gene copy in pΔs1 (compare *lane 4* with *lanes 2* and *3*), indicating further the role of TATATAA sequences in bringing down the transcription.

In order to confirm that the enhancement in transcription in the deletion constructs was indeed due to the elimination of only the TATATAA sequence, this element was selectively mutagenized. The TATATAA motif located in the far upstream region of the construct ps1UR8 harboring $tRNA_1^{Gly}$-6:1 was mutagenized to GATATCA. This construct was chosen for the mutagenic analysis so that the influence of a single TATATAA element could be analyzed without interference from the identical elements localized downstream as in pΔs1 or pBms1. The *tRNA* copy present in this chimeric construct ps1UR8 was transcribed at very low levels amounting only to 10% of $tRNA_1^{Gly}$-1 (*lane 3*, Fig. 7). The mutagenized derivative, pmuts1UR8, harboring the mutation in far upstream localized TATATAA was transcribed to very high levels (comparable with that of wild-type $tRNA_1^{Gly}$-1) (*lane 4*, Fig. 7). Evidently the TATATAA motif was responsible for down-regulating the gene copies.

In an alternate approach, a completely randomized synthetic DNA fragment harboring the TATATAA sequence was exploited to analyze the specific effect of the element on transcription. Such a fragment was reintroduced to a gene copy from which the negatively regulating region was deleted, and the effect of insertion of this sequence on transcription was analyzed. The 150-bp randomized sequence containing the TATATAA element corresponded to the region −150 nt to −300 nt upstream to $tRNA_1^{Gly}$-1 in clone pR8, which was shown to harbor the negative element (30). The above DNA fragment was generated synthetically and inserted into pΔDTs1, in which the downstream sequences harboring the two TATATAA sequences of pΔs1 were deleted such that the TATATAA sequences are now located 800 nt downstream to the tRNA coding region (Fig. 6*b*). When this construct pΔran150DTs1 was analyzed the transcription level was found to be considerably decreased (Fig. 6, *lane 5*), resembling the original parent construct pΔs1 (*lane 2*) in contrast to the deletion derivative (*lanes 5* and *6*). The above results are consistent with the conclusion that the TATATAA element was responsible for bringing down the transcription.

DISCUSSION

The $tRNA_1^{Gly}$ constitutes a multigene family in *B. mori*, with an estimated copy number of 20. We have compared here, under identical conditions, the transcription *in vitro* of 11 individual members of this multigene family in homologous posterior silk gland nuclear extracts. These gene copies showed wide variations in the efficiency of transcription. Based on the *in vitro* transcription, they could be classified into three groups, showing very high, medium, or very low levels of transcription. All the $tRNA_1^{Gly}$ copies had identical coding sequences and consequently absolute identity of their internal conserved regions, the A- and B-boxes. Evidently, therefore, the flanking sequences should be responsible for modulating their levels of transcription. Such modulations could be due to the presence or absence of positive as well as negative elements, alone and in combination to achieve the expressions ranging from very high levels to the complete silencing.

We have examined in detail two sets of $tRNA_1^{Gly}$ copies belonging to the highly transcribed ($tRNA_1^{Gly}$-1) and barely transcribed ($tRNA_1^{Gly}$-6 and -7) categories. Under optimal conditions, the *in vitro* transcriptions of $tRNA_1^{Gly}$-6 and -7 together accounted for only 2–10% or less of the transcription of $tRNA_1^{Gly}$-1.

The possibility that the low level of transcription of gene copies 6 and 7 is due to their close linkage on a single DNA fragment was suggested initially because when the two genes were unlinked, individually they were transcribed to high lev-

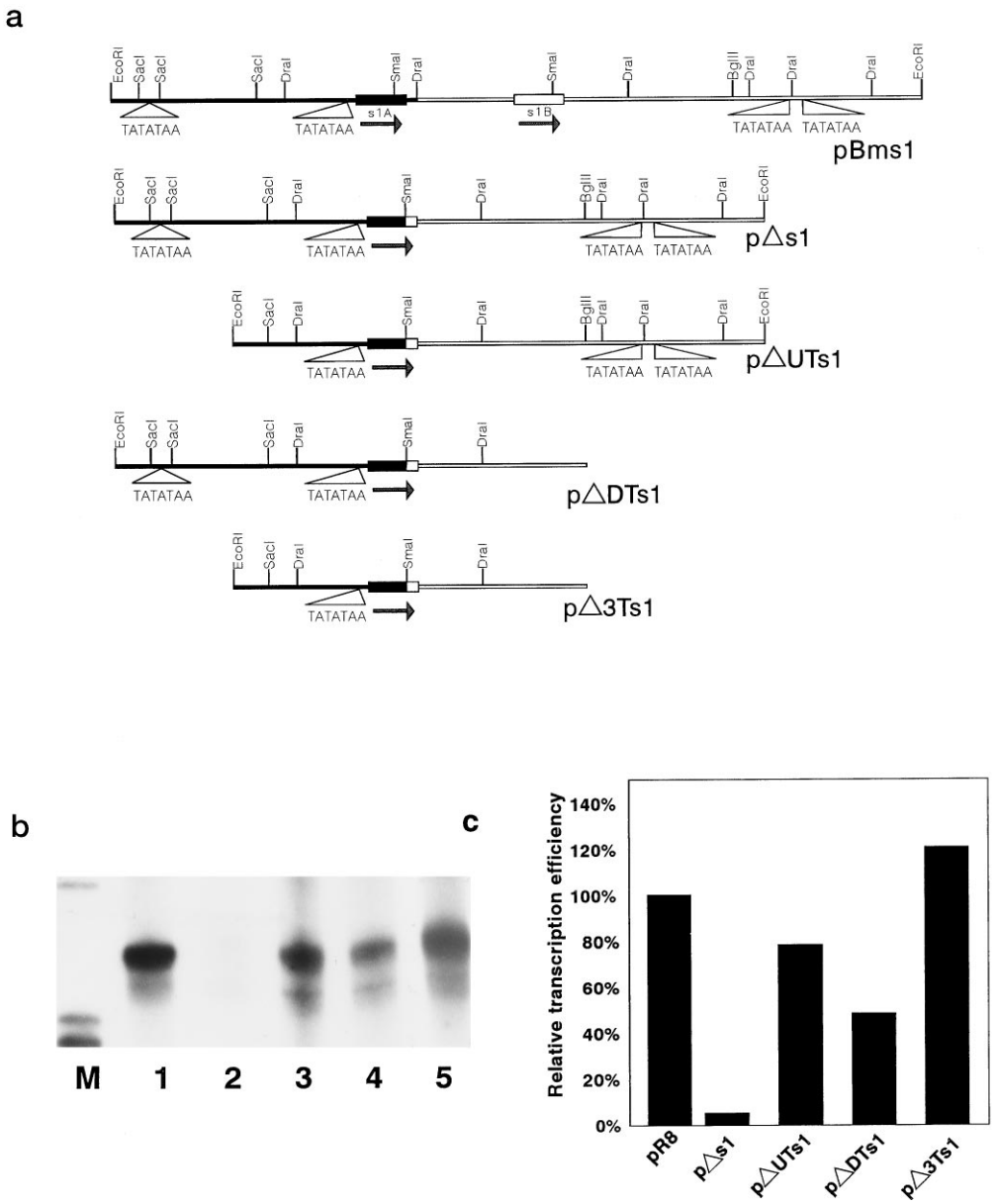

FIG. 5. **Effect of deletion of far flanking sequences on transcription.** *a*, constructs harboring deletions of far flanking sequences in pΔs1. *b, In vitro* transcription of the deletion constructs. *Lane M*, marker (radiolabeled pTZ18 DNA digested with *Hin*fI); *lanes 1–5*, transcription of $tRNA_1^{Gly}$-1 and -6:7 and deletion derivatives in clones pΔUTs1, pΔDTs1, and pΔ3Ts1 (at equimolar template concentration), respectively. *c*, quantitation of transcription.

els (60–65% of the $tRNA_1^{Gly}$-1). If indeed the close linkage of the genes was responsible for the low transcriptions, bringing either of the gene copies 6 or 7 in proximity to the highly transcribed $tRNA_1^{Gly}$-1 should have resulted in the lowering of the transcription of the latter. However, when the gene copies 1 and 7 were brought together in tandem on the same DNA fragment, there was barely any effect on the transcription of either gene copies. This lack of interference was independent of the orientation of the linked gene copies, whether they were placed in parallel, converging, or diverging modes. Therefore, the linkage itself seemed to exert very little influence on transcription.

Nevertheless, one could argue that the disruption of intervening sequences linking the gene copies 6 and 7 in pBms1 during their separation from each other was responsible for reduced transcription. This possibility was also ruled out, because when the two isolated genes were brought back together (in clone ps1A+B), just as they were in the parental clone pBms1, the higher levels of transcription of the individual copies were still retained. The difference between this construct ps1A+B and the parental clone pBms1 was that the latter contained additional 5′ upstream (−284 to −976 nt with respect to +1 nt of $tRNA_1^{Gly}$-6) and 3′ downstream (+278 to +1518 nt with respect to +1 nt of $tRNA_1^{Gly}$-7) sequences. These flanking regions should, therefore, be harboring the negative regulatory elements involved in silencing the gene copies, 6 and 7. The presence of such regulatory elements was confirmed by analyzing several additional $tRNA_1^{Gly}$ constructs. For instance, a deletion construct (pΔs1) harboring a single copy of the gene $tRNA_1^{Gly}$-6:7, generated by fusion of the gene copies 6 and 7 from the parental clone pBms1 and thus harboring the entire 5′- and 3′-flanking sequences (but devoid of the linker region between the gene copies), gave rise to extremely low levels of transcription. The presence of negative regulatory sequences in both 5′- and 3′-flanking regions was also evident because either of the regions alone when swapped with the corresponding domains of $tRNA_1^{Gly}$-1 (in plasmid constructs ps1UR8 or ps1DR8) resulted in near complete abolition of the transcrip-

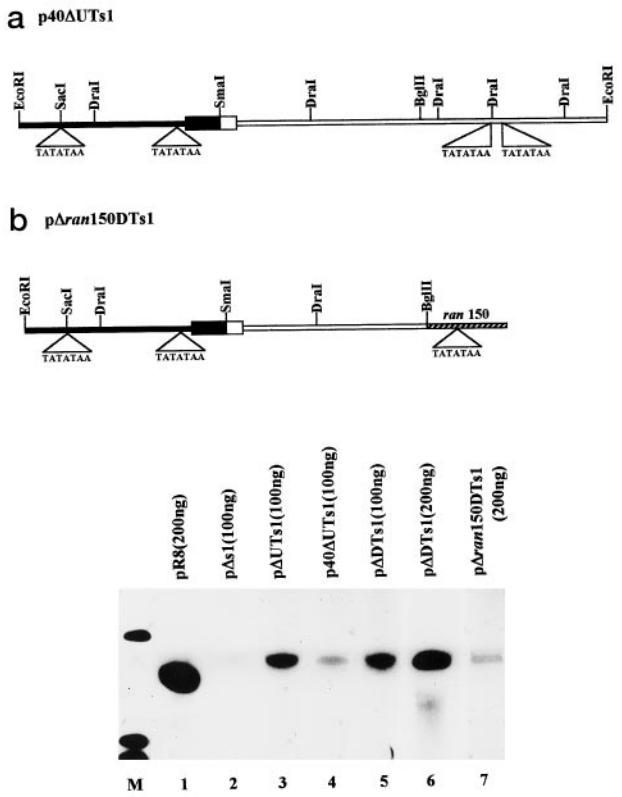

FIG. 6. **Reinsertion of TATATAA harboring DNA fragments to the deletion derivatives.** *a*, a 40-bp fragment harboring the TATATAA element located at −871 nt upstream of $tRNA_1^{Gly}$ in clone pΔs1 was excised out as a *Sac*I restriction fragment and introduced into the 5′ upstream region of pΔUTs1 (in which 440-bp sequences, including the 40-bp *Sac*I fragment, were deleted). The transcription of this construct, 40 pΔUTs1 (*lane 4*), in comparison with the parental copy of pΔs1 (*lane 2*) and the entire 440 bp upstream deletion construct pΔUTs1 (*lane 3*) is presented. *Lane 1*, transcription of $tRNA_1^{Gly}$ in pR8 included as reference. *Lane M*, molecular size markers. The *numbers* in *parentheses* indicated next to the clone names denote the template DNA concentration in nanograms used in 25 μl of transcription assay. *b*, a 150-bp synthetic DNA fragment, harboring the sequences from the region −150 to −300 nt upstream of $tRNA_1^{Gly}$-1 (in clone pR8) completely randomized except for retaining the TATATAA sequence motif, was generated through polymerase chain reaction using six overlapping oligodeoxynucleotides (36–42-mers). This synthetic DNA fragment was inserted downstream to the tRNA coding region in plasmid construct pΔDTs1 in which about 750 bp downstream sequences starting from the *Bgl*II site (harboring the two TATATAA elements present in the parental clone pΔs1) were deleted. The newly generated construct, pΔran150DTs1, now harbored the TATATAA element at 800 nt downstream to the tRNA coding region. The transcription of this construct, pΔran150DTs1 (*lane 7* at 200 ng template DNA), in comparison with its parental deletion construct pΔDTs1 (*lanes 5* and *6*, at template DNA concentrations of 100 and 200 ng, respectively) is presented.

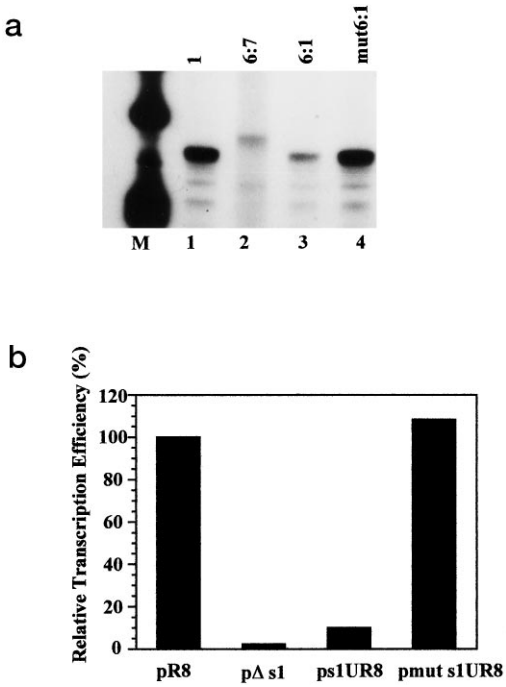

FIG. 7. ***In vitro* transcription of $tRNA_1^{Gly}$-6:1 in TATATAA-mutated ps1UR8.** *a*:, *Lane M*, marker; *lanes 1–4*, transcription of $tRNA_1^{Gly}$-1, -6:7, −6:1, and mut6:1 (with a mutated TATATAA element), respectively. The transcriptions were carried out at equimolar template concentrations. *b*, quantitation of transcription.

tion of the latter. The negative elements present in the 3′-flanking regions of $tRNA_1^{Gly}$-7 thus appeared to override the effect of positive elements resident in the immediate 5′ upstream sequences of $tRNA_1^{Gly}$-1 and brought down the transcription of the latter. The silencing of gene activity by negative elements may serve as an important means for modulating Pol III transcription.

A pertinent question that can be raised is whether these differential expression patterns of individual tRNA genes hold good *in vivo*. However, it is not possible to discriminate the tRNA transcripts arising from the individual genes *in vivo* by the standard procedures such as primer extensions or RNase protection assays, because all of them have identical coding sequences and yield the same size transcripts. As an alternative, therefore, we used "oligotagged" copies of some of these genes and monitored their transcription in the *B. mori*-derived BmN cell lines following transfection.[3] Our results clearly established that the *in vivo* transcription levels followed the same pattern as seen *in vitro*. What then is the relevance of this differential expression of tRNA genes *in vivo*? The expression of *tRNAs* is generally believed to be housekeeping function but in the highly specialized situations like the posterior silk gland of *B. mori* (that undergoes a functional adaptation to optimize the synthesis of large quantities of fibroin in a development stage specific manner), many such gene copies, which are otherwise rendered silent, may have to be turned on to meet the additional demands. Considering the nature of the *cis*-acting negative regulatory element, it is conceivable that the availability of transcription factors can dictate their transcriptional status (see the following section), especially in the absence of any tissue specifically expressed copy of $tRNA_1^{Gly}$ in *B. mori* (16, 28). While the posterior silk gland of *B. mori* represents an extreme manifestation of functional adaptation, such a situation may prove to be of common occurrence in the specialized tissues of multicellular eucaryotes.

The presence of regulatory sequences located at such farther upstream or downstream regions for a set of Pol III transcribed genes described here is rather novel, although the location of regulatory sequences, like enhancers and silencers, at considerable distances (10–15 kb or even more) is commonly seen in the case of Pol II transcribed genes. The sequence analysis of the entire chromosomal DNA insert fragment revealed that it was AT-rich in nature and contained typical TATA-like elements in both upstream and downstream flanking regions. A sequence motif "TATATAA" located about 270 nt 5′ upstream to the coding region of the highly transcribed $tRNA_1^{Gly}$-1 down-regulated transcription of this gene (30). There were four such typical elements in the flanking regions of $tRNA_1^{Gly}$-6 and -7. Of the two TATATAA sequence motifs present in the upstream region of $tRNA_1^{Gly}$-6, one was present in the immediate 5′

[3] S. Sharma and K. P. Gopinathan, manuscript in preparation.

vicinity and the other one at −871 nt with respect to the tRNA coding region. The 3′ downstream TATATAA sequences were located beyond 1 kb downstream to the coding region of $tRNA_1^{Gly}$-7. These far upstream and downstream elements negatively influenced the transcriptional status of the gene copies 6 and 7, because their deletion resulted in enhancement of transcription. Reintroduction of a 40-bp DNA fragment harboring TATATAA sequences to the upstream deleted construct or a completely randomized 150-bp sequence harboring the TATATAA motif to the downstream deleted construct resulted in the inhibition of transcription in both cases. Moreover mutagenesis of a single TATATAA motif located at −871 nt upstream in the parental construct resulted in the restoration of transcription. How do the regulatory elements, located so far upstream or downstream to the coding regions, modulate the transcription of *tRNA* by Pol III is not known, although mechanisms similar to those postulated for Pol II transcribed genes involving DNA structural alterations as well as protein-protein interactions are likely to be operative. Our general premise now is that these TATATAA elements compete for transcription factors such as TBP (or the TATA-associated factors and other components of TFIIIB which associate with TBP) and bring about inhibition by sequestration of these factors, especially under conditions when they are limiting, as in nuclear extracts *in vitro*. A single such element brings about inhibition by 35–40% as in $tRNA_1^{Gly}$-1 (30) or by more than 90% combinatorially with three such elements as in $tRNA_1^{Gly}$-6 and -7. The sequestration effect is position-dependent, because the TATATAA element located in the immediate vicinity of the transcription start site enhances transcription (as in $tRNA_1^{Gly}$-6) presumably by additional recruitment of TBP, over and above that achieved through TFIIIB binding. One obvious question whether the inhibitory effect could be reversed by supplementation of TBP could not be answered due to the squelching effect of TBP, resulting in depletion of factors.[2] Thus by regulating the availability of transcription factors *in vivo*, differential regulation of gene expression from within multigene families can be achieved.

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