Insect Molecular Biology (2001) **10**(6), 523–530

Differential expression of individual gene copies from within a *tRNA* multigene family in the mulberry silkworm *Bombyx mori*

L. Srinivasan and K. P. Gopinathan*

Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore – 560012, India

Abstract

In mulberry silkworm *Bombyx mori*, $tRNA_1^{Gly}$ constitutes a multigene family from which the individual members are transcribed at different levels *in vitro* in homologous nuclear extracts. We report here the quantification of functional transcripts of these gene copies *in vivo* in *B. mori*-derived BmN cells based on a suppression assay. The gene copies were converted to encode suppressor tRNAs and co-transfected into cell lines with reporter gene(s) harbouring one or more nonsense mutations and the reporter gene activity was quantified. Individual members of the gene family were transcribed to very high-, medium- and very low-levels, following the same pattern as *in vitro*. All these gene copies were maximally expressed in Bm cells as compared to other insect cell lines.

Keywords: *Bombyx mori*, RNA polymerase III, Transcriptional Regulation, pol III transcription, tRNA genes.

Introduction

Presence of multiple copies of a particular gene, especially those that are transcribed by RNA polymerase I and III is somewhat of a common occurrence in eukaryotes. The end product of expression is RNA in these instances, therefore the requirement for large quantities of the encoded products has to be met by multiple rounds of transcription. The second possible means of achieving large quantities of the product through repeated rounds of translation, as applicable to RNA polymerase II transcripts, is not operative for polymerase I and III transcripts. The enhanced requirement for the gene products is also achieved by having multiple copies of the gene. Besides, the presence of multiple copies provides the option for tissue specific variants. The requirement for large quantities of $tRNA^{Gly}$ in the posterior silk glands of the mulberry silkworm *Bombyx mori* represents one such classical example. This tissue is committed to the synthesis of large quantities of the silk fibre protein, fibroin, which has a highly biased amino acid composition comprising 46% glycine. In the large 15 kb messenger RNA encoding fibroin, 2415 codons are for glycine (Zhou *et al.*, 2000) and of these 1352 are to be GGC/U decoded by $tRNA_1^{Gly}$ species. In order to optimize the synthesis of fibroin in silk glands, a functional adaptation takes place in this tissue during the fifth larval instar when the fibroin synthesis reaches its peak, as reflected by substantial increases in the content of $tRNA_1^{Gly}$ species (Garel, 1976; Patel & Gopinathan, 1991). In *B. mori*, $tRNA_1^{Gly}$ is a multigene family comprising nearly twenty members (Fournier *et al.*, 1993). We have previously characterized eleven of these gene copies that have identical tRNA coding sequences, and therefore the same internal control regions (ICRs), but differ only in the flanking regions (Fournier *et al.*, 1993; Sharma & Gopinathan, 1996a). None of these genes, however, was silk gland specific and they were transcribed in the same pattern by nuclear extracts from different tissues (Fournier *et al.*, 1993). This is in contrast to the situation of $tRNA_1^{Ala}$, which decodes the next most abundant codon (for alanine) in fibroin. There are two variants of $tRNA_1^{Ala}$ of which one is constitutively expressed and the other one is silk gland specific. Strangely, the absence of certain *cis* elements in the 5′ upstream region of the tRNA coding sequences in the silk gland specific copy has been thought to confer tissue specificity to the $tRNA_1^{Ala}$ (Ouyang *et al.*, 2000; Sullivan *et al.*, 1994; Young *et al.*, 1986).

Although investigations have been carried out in the past (Schmutzler & Gross, 1990; Tapping *et al.*, 1993) on differential transcription of genes encoding different tRNA species within the cell (e.g. different isoacceptor species of $tRNA^{Ser}$, or $tRNA_1^{Val}$ and $tRNA_2^{Val}$ as well as their pseudogenes), not much is known about how the individual members from within the same gene family behave. The transcription status of individual members within a multicopy tRNA gene family and the discrimination between

Received 24 January 2001; accepted after revision 4 April 2001. *Corresponding author: Tel.: 91(80) 3600090; fax: 91(80) 3602697; e-mail: kpg@mcbl.iisc.ernet.in

them (e.g. $tRNA_1^{Gly}$–1, $tRNA_1^{Gly}$–2 or $tRNA_1^{Gly}$–3, all of which encode the same isoacceptor species) have been addressed by us *in vitro* using nuclear extracts from the silk glands (Fournier *et al*., 1993; Sharma & Gopinathan, 1996a,b; Taneja *et al*., 1992). Eleven of these gene copies analysed were grouped into three distinct classes based on the *in vitro* transcription in homologous nuclear extracts. Some of them were highly transcribed (e.g. $tRNA_1^{Gly}$–1 and 11) whereas the others were extremely poorly transcribed (less than 10% of the former, e.g. $tRNA_1^{Gly}$–6, 7, 8, 9 and 10) and the rest were transcribed to intermediary levels (30–60% of the first group, e.g. $tRNA_1^{Gly}$–2, 3, 4 and 5). Because all these gene copies had identical coding sequences, clearly they harboured the same ICRs that constitute the essential promoter elements (White, 1994), but differed only in their 3′ and 5′ flanking sequences, which influenced their transcription status.

We had previously analysed the *in vivo* transcription of one copy each of a highly and poorly transcribed $tRNA_1^{Gly}$ *in vivo* in *B. mori*-derived BmN cells based on an RNase protection assay (Sharma *et al*., 1997). However, this assay did not quantify the transcript levels and only monitored the total but not the functional transcripts. Now we have quantitatively assessed whether the differential transcription pattern of $tRNA_1^{Gly}$ copies seen *in vitro* is true *in vivo*. To achieve quantification of the *in vivo* transcripts and to distinguish them from the endogenous tRNA transcripts, we have designed and developed sensitive and functional assays based on suppression. Nonsense mutations (TGA) were introduced in reporter genes such as *luciferase* and *green fluorescent protein*, and simultaneously the anticodon region of individual members of $tRNA_1^{Gly}$ family were mutated to generate suppressor tRNA (two mutations from GGC to TGA). Co-transfection of the mutant reporter gene and the Su^+ *tRNA* into cell lines, followed by assays for the reporter gene activity allowed us to quantify the differential expression of the individual members of the same *tRNA* gene family *in vivo.*

Results and discussion

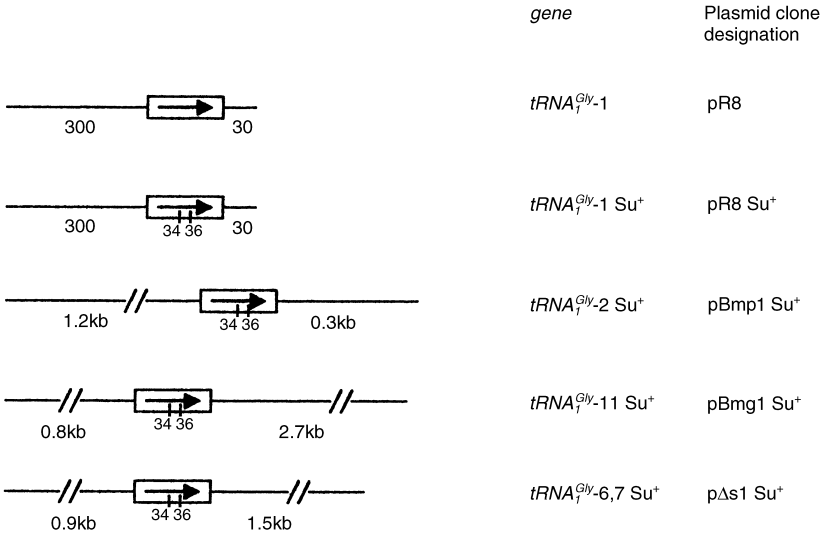
In vitro *and* in vivo *transcription of* $tRNA_1^{Gly}$ *copies*

The $tRNA_1^{Gly}$ copies, $tRNA_1^{Gly}$–1, 2, 11 and 6, 7 (Fig. 1, top panel) analysed here belong to three different groups based on *in vitro* transcription in homologous nuclear extracts (Fournier *et al*., 1993; Sharma & Gopinathan, 1996a). $tRNA_1^{Gly}$–1 and 11 were highly transcribed (taken as 100%) whereas $tRNA_1^{Gly}$–2 was transcribed to medium levels (30–60%) and $tRNA_1^{Gly}$–6, 7 to very low levels (2–5%). All of them had identical tRNA coding sequences but differed in their 3′ and 5′ flanking regions (Fournier *et al*., 1993). These tRNA gene copies were converted to Su^+ tRNA by mutating two bases (at +34 and +36 nt in the anticodon region) of the tRNA gene. The four suppressor *tRNAs* generated (in pR8 Su^+, pBms1 Su^+, pBmp1 Su^+ and pBmg1 Su^+) were analysed for *in vitro* transcription using posterior silk gland nuclear extracts (Fig. 1, bottom panel). The mutants were transcribed as efficiently as the parental gene copies, establishing that the introduction of suppressor mutations (2 base change at the anticodon position) did not have any adverse effects on *in vitro* transcription.

We checked their transcription levels *in vivo* in BmN cells following transfection (Fig. 2). Because the antisense RNA corresponding to the suppressor tRNAs was used as the probe in the RNase protection assay, the endogenous tRNA transcripts break down to 34 and 35 nt fragments on treatment with RNase, whereas the transcript coming from the transfected genes should give rise to a full length protected fragment of 71 nt. In addition to the fully processed 71 nt transcripts, however, bands corresponding to unprocessed and partially processed transcripts of 78–83 nt were also seen (Fig. 2). In most of the *tRNA* copies analysed here, transcription was initiated at –4 and –5 nt and terminated at +76 to +78 nt (5–7 nt after the 3′ end of the tRNA, and corresponding to the run of T residues; Taneja *et al*., 1992) and the primary transcript size should vary between 81 and 84 nt in agreement with the results obtained. The protected bands due to the endogenous transcripts present in the untransfected cells, as predicted, corresponded to 34–35 nt. The endogenous tRNA transcripts were mostly present in the processed form (lane 1). It is evident that $tRNA_1^{Gly}$–1 Su^+ and 11 Su^+ were transcribed to very high levels (lanes 2 and 4) and $tRNA_1^{Gly}$–6, 7 Su^+ to barely detectable levels (lane 5) whereas $tRNA_1^{Gly}$–2 Su^+ was transcribed to intermediary levels (lane 3). These results reveal that the suppressor $tRNA_1^{Gly}$ copies were efficiently transcribed in BmN cells and the differential expression pattern of these gene copies was the same as that seen in nuclear extracts of posterior silk glands of *B. mori*.

Suppression of nonsense mutation introduced in gfp *and* luciferase

We extended the results to analyse the efficiency of transcription of these individual gene copies encoding the same tRNA species by a functional assay for the transcript. Because Su^+ mutations were introduced into the transfecting tRNA gene, if transcribed and processed correctly they should be able to suppress the nonsense mutation in a reporter gene. In mammalian cells $tRNA^{Ser}$ and $tRNA^{Trp}$ have been mutagenized to function as opal (UGA) suppressors (Capone *et al*., 1985, 1986). We checked the suppression of nonsense codon (TGA) in *gfp* on a qualitative basis. The Gly10-stop *gfp* was expressed from the polyhedrin promoter of the baculovirus, thus it was necessary to infect the BmN cells with BmNPV following transfection. The parental *gfp* (wt) was expressed well in BmNPV infected BmN cells (Fig. 3, top panel). Cells transfected with Gly10-stop *gfp* did not show any fluorescence but when co-transfected with



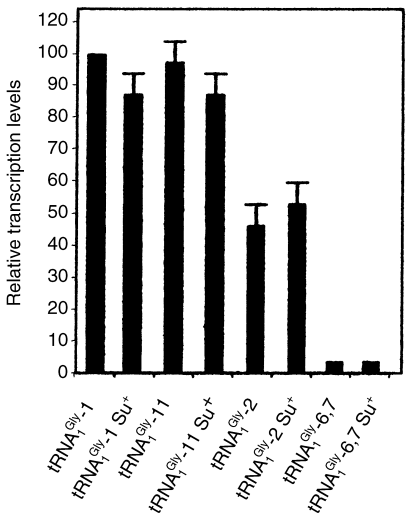


Figure 1. *In vitro* transcription of wild-type (wt) and suppressor $tRNA_1^{Gly}$ constructs. The $tRNA_1^{Gly}$ gene copies used in this study are shown in the top panel. The Su^+ mutants of $tRNA_1^{Gly}$ –2, 11 and 6, 7 were derived from their corresponding parental constructs and were therefore identical to the parental copies except for the two base alterations at positions 34 and 36. The individual copies of $tRNA_1^{Gly}$ were transcribed *in vitro* in homologous nuclear extracts from posterior silk glands using 100–200 ng of template DNA (at equimolar concentrations of the template). The transcript signals were quantified through phosphoimager and the quantification was based on at least three independent transcription experiments and with different batches of nuclear extracts. Lanes as marked.

Su^+ $tRNA_1^{Gly}$–1 showed significant fluorescence (Fig. 3, middle and bottom panels). The results indicate that the suppressor $tRNA_1^{Gly}$–1 efficiently suppressed the UGA stop codon and that the gene was transcribed and processed properly in BmN cells. No fluorescence was detected in cells on co-transfection with $tRNA_1^{Gly}$ –6, 7 Su^+, due to the poor transcription efficiency of the latter.

Although the *gfp* expression was more qualitative in nature, the approach provided an easier means for monitoring the exogenous transcripts *in vivo*, as compared to the RNase protection assay reported by us earlier (Sharma *et al*., 1997). Moreover the present approach could be extrapolated to quantify the functional transcripts. To avoid virus infection that is necessary to turn on the polyhedrin promoter, the quantification of suppression was done using the reporter gene (*luc* harbouring Gly20-stop mutation), under the viral *ie*1 promoter that is constitutively recognized in BmN cells. Figure 4(a) shows the levels of luciferase activity and the restoration of luciferase activity due to suppression of nonsense codon when co-transfected with the Su^+ mutants of $tRNA_1^{Gly}$. Suppression efficiency was 35–75% when increasing concentrations of $tRNA_1^{Gly}$–1 Su^+ were co-transfected (1, 2 and 4 μg, respectively), which was significantly above the endogenous levels of suppression (about 10%) seen in BmN cells. $tRNA_1^{Gly}$ –6, 7 Su^+ showed an increase in luciferase levels from 10% of the background levels to 13–15%. Clearly, the suppression due to $tRNA_1^{Gly}$ –1 Su^+ was much higher than that seen from $tRNA_1^{Gly}$ –6, 7 Su^+, indicating higher levels of transcription of the former *in vivo*.

The single mutant Gly20-Stop luciferase showed 10% of the wild-type activity due to endogenous suppression

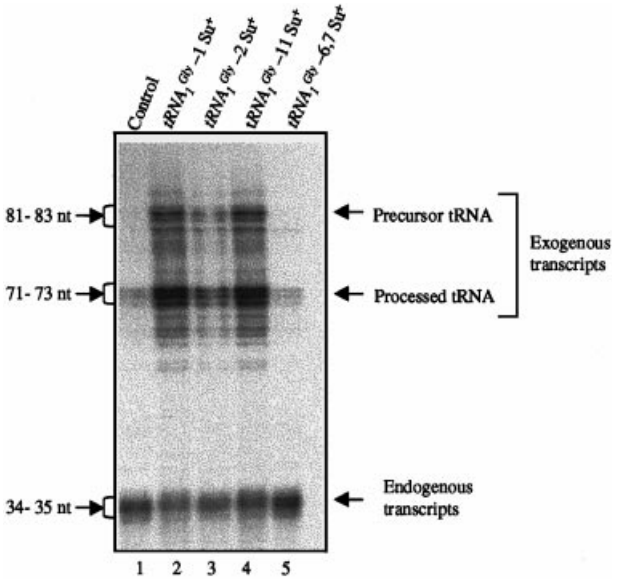


Figure 2. *In vivo* expression of $tRNA_1^{Gly}$ copies. RNA was isolated from BmN cells following transfection with the different $tRNA_1^{Gly}$ Su^+ constructs as indicated. Radiolabelled antisense strand corresponding to the suppressor tRNA was generated by *in vitro* transcription from the plasmid pR8 Su^+ DNA and was used as the probe for the RNase protection assay. Because the antisense RNA used as the probe harboured two mutations in the anticodon region, the endogenous $tRNA_1^{Gly}$ transcripts will be fragmented to two fragments whereas the Su^+ tRNA transcripts arising from the transfected genes will give rise to a full size protected fragment. For details of assay see text under methods. Lanes: 1, Control, cells transfected with plasmid $pBSKS^+$ DNA; lanes 2–5, RNA from cells transfected with Su^+ $tRNA_1^{Gly}$ –1, 2, 11 and 6, 7, respectively.

activity, therefore a second mutation Gly23-stop was introduced into *luc* in order to reduce the background. The double mutant of *luc* (Gly20-stop and Gly23-stop) when transfected into BmN cells showed practically no background luciferase activity (Fig. 4b). The suppression by the different suppressor $tRNA_1^{Gly}$ copies when co-tranfected with the double mutant *luc* is presented in Fig. 4(b). $tRNA_1^{Gly}$ –1 Su^+ was able to restore about 42% of the wild-type activity, whereas $tRNA_1^{Gly}$ –6, 7 Su^+ showed less than 3% suppression, $tRNA_1^{Gly}$ –2 Su^+ restored 16% of the wild-type activity (40% of $tRNA_1^{Gly}$ –1 Su^+ efficiency) and $tRNA_1^{Gly}$ –11 Su^+ restored approximately 40% of the wild-type activity (the same as $tRNA_1^{Gly}$ –1 Su^+). The transcript levels of different $tRNA_1^{Gly}$ copies confirmed their differential expression *in vivo* and the values were in agreement with the *in vitro* transcription levels seen in homologous posterior silk gland nuclear extracts.

Although the single mutation in *luc* was suppressed to 65% (above the background levels) by $tRNA_1^{Gly}$ –1 Su^+ (see Fig. 4a), the presence of the second nonsense mutation evidently reduced the suppression efficiency to 42%, well in agreement with the predicted level of suppression (i.e. 65% of 65%). In contrast, $tRNA_1^{Gly}$ –6, 7 Su^+ showed barely detectable levels of luciferase activity (less than 3%) indicating that this gene is indeed transcribed poorly *in vivo*.

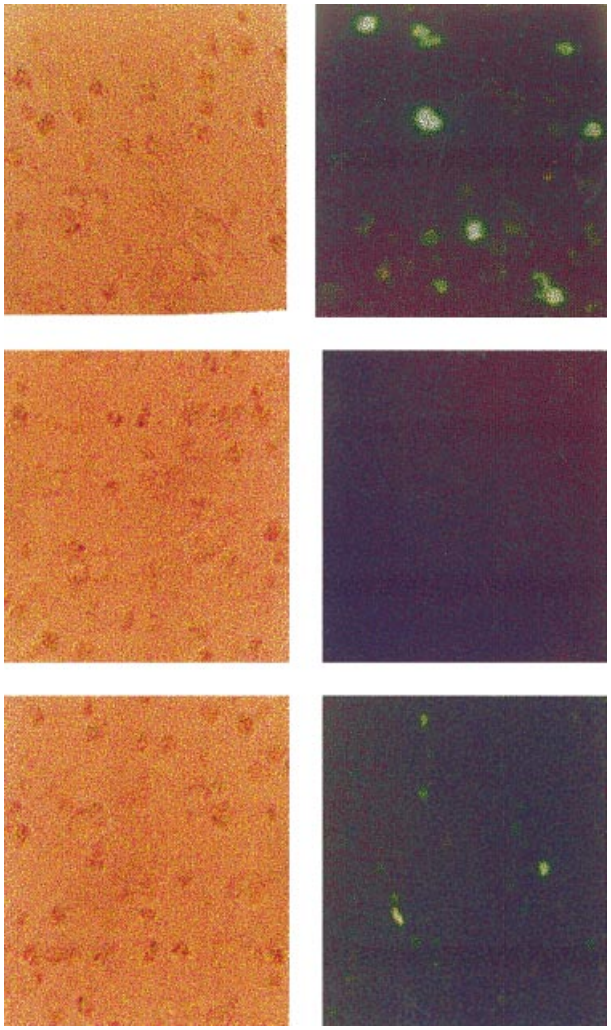


Figure 3. Functional assay for $tRNA_1^{Gly}$ –1 Su^+ transcripts. The suppression of UGA mutation in *gfp* was monitored following transfection of the reporter gene, *gfp* Gly10-stop and $tRNA_1^{Gly}$ –1 Su^+ in BmN cells. The cells were infected with BmNPV (multiplicity of infection, 10) 12 h following transfection and at 48 h post-infection the expression was checked. Cells were photographed under visible light (left panels) and UV light (right panels). Top row, cells transfected with wild-type *gfp*; middle row, cells co-transfected with Gly10-stop *gfp* and wt $tRNA_1^{Gly}$ –1; and last row, cells co-transfected with Gly10-stop *gfp* and $tRNA_1^{Gly}$ –1 Su^+.

Expression of $tRNA_1^{Gly}$ *in Sf-21 and S2 cells*

The individual members of the $tRNA_1^{Gly}$ family did not exhibit any tissue specificity *in vitro* transcriptions using nuclear extracts derived from *B. mori* silk glands, fat bodies or ovarian tissues (Taneja *et al.*, 1992; Fournier *et al.*, 1993). We examined the transcription of these genes in another lepidopteran cell line, Sf-21 from *Spodoptera frugiperda* and a dipteran cell line, S2 from *Drosophila*. The transcription was initially checked *in vitro* using nuclear extracts from Sf-21 cells, which showed less than 10% compared to homologous PSG nuclear extracts (data not shown). Therefore, we analysed the expression of these

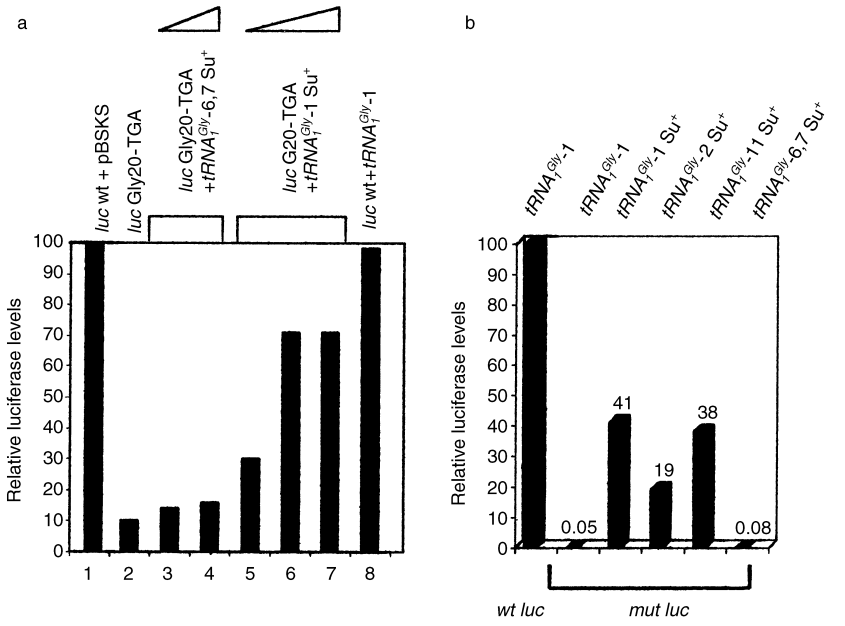


Figure 4. Quantification of *in vivo* transcription. (a) Suppression of single mutation in *luc*. The transcription levels of different $tRNA_1^{Gly}$ copies *in vivo* were quantified by the functional suppressor assay. BmN cells were co-transfected with Gly20-stop *luc* and individual members of $tRNA_1^{Gly}$ Su^+. The ability to suppress the UGA codon (in the reporter) was quantified by the luciferase assay and presented as the percentage luciferase activity compared to wt *luc*. Luciferase activity was assayed by quantifying the light emission on addition of the substrate D-luciferin, in a luminometer. Lanes: 1, wt *luc* (+ plasmid vector pBSKS$^+$, 2 μg); 2, Gly20-stop *luc* (+ plasmid vector pBSKS$^+$, 2 μg); 3,4, Gly20-stop *luc* and $tRNA_1^{Gly}$ –6, 7 Su^+ (2 μg and 4 μg, respectively); 5–7, Gly20-stop *luc* and $tRNA_1^{Gly}$ –1 Su^+ (1 μg, 2 μg and 4 μg, respectively); 8, wt *luc* and $tRNA_1^{Gly}$ –1 Su^+. Quantification of transcription is from three independent experiments. (b) Suppression of double mutant *luc*. Assay conditions as in (a) except that *luc* double mutant Gly20-stop and Gly23-stop was used in place of the single mutant. Lanes as marked. Quantification of transcription is from three independent experiments.

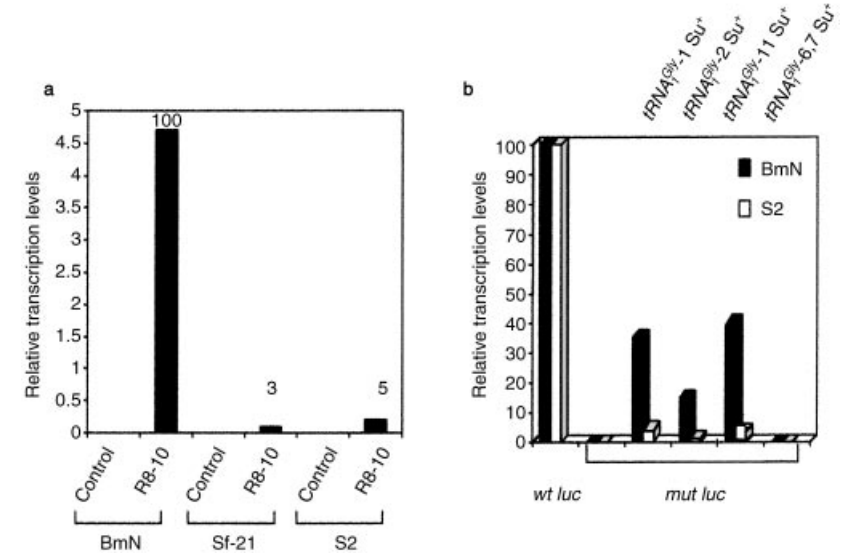


Figure 5. Expression of $tRNA_1^{Gly}$ in other insect cell lines. The expression of $tRNA_1^{Gly}$ in BmN, Sf-21 and S2 cell lines were assayed by RNase protection assay (a) or functional suppressor assay (b). For RNase protection assay, oligotagged $tRNA_1^{Gly}$ –1 where a 10 nt insertion was made within the coding region between a and b boxes (clone pR8-10), was transfected into BmN, Sf-21 and S2 cells. The expression of $tRNA_1^{Gly}$ was estimated by RNase protection using the antisense probe generated from the linearized plasmid pR8-10 DNA by *in vitro* transcription with T3 RNA polymerase in the presence of α-^{32}P GTP. Total RNA isolated 36 h after transfection was assayed. Lanes as marked. (b) Functional suppression assay. BmN and S2 cells were co-transfected with double mutant *luc* (Gly20-stop, Gly23-stop) and individual $tRNA_1^{Gly}$ Su^+ copies. Luciferase activity was quantified at 36 h post-transfection by the light emission assay in a luminometer. The data presented are the averages of two independent experiments.

gene copies *in vivo* in the cell lines by the RNase protection and suppression assays.

For the RNase protection analysis, the oligotagged copy $tRNA_1^{Gly}$ –1 (as in plasmid pR8-10 with a 10 nt insertion between the A and B boxes, Sharma *et al.*, 1997) was used in order to distinguish the transcript arising from the transfected gene from the endogenous tRNA transcripts. The antisense corresponding to the oligotagged $tRNA_1^{Gly}$ –1 was used as the probe. We had previously established that the 10 nt insertion did not affect transcription of this gene but the processing of the primary transcript was not efficient (Sharma *et al.*, 1997). The three cell lines, BmN, Sf-21 and S2 were transfected with the oligotagged $tRNA_1^{Gly}$ –1. The transcription of $tRNA_1^{Gly}$ –1 (in R8-10, oligotagged construct) in these cell lines is shown in Fig. 5(a). Highest levels of transcripts were seen in BmN cells (88–91 nt RNase protected species arising from the transfected $tRNA_1^{Gly}$ –1 gene). Only weak signals due to $tRNA_1^{Gly}$ –1 transcripts were detected in Sf-21 and S2 cells (3–8% efficiency) compared to BmN cells. The differences in exogenous tRNA transcript levels seen between BmN and S2 or Sf-21 cells were unlikely to be due to the differences in transfection efficiencies of these cells, because under the same conditions the transfection of β-*gal* or *luc* reporters (under the control of constitutively expressed actin or viral *ie1* promoters) showed comparable expression levels. Because

the tRNA transcripts are relatively stable, the accumulated tRNA levels shown here may indeed reflect the transcription status of these genes.

Although the expression of $tRNA_1^{Gly}$ –1 itself was poor in S2 cells, the transcription efficiency of the other members of the family was also analysed by the suppression assay (Fig. 5b). Consistent with the results of RNase protection analysis, $tRNA_1^{Gly}$ –1 Su^+ restored only 2–3% of the wild-type luciferase activity in S2 cells (against 40% efficiency in BmN cells), amounting to 5% transcription efficiency of the gene, $tRNA_1^{Gly}$ –1. Once again, $tRNA_1^{Gly}$ –6, 7 Su^+ did not show detectable levels of suppression, whereas $tRNA_1^{Gly}$ –2 Su^+ and –11 Su^+ restored 1.25% and 3%, respectively, of the wild-type activity (Fig. 5b). The expression of $tRNA_1^{Gly}$ copies was most efficient in *B. mori* derived cell line. Nevertheless, the pattern of differential expression *viz*, $tRNA_1^{Gly}$ –1 and –11 as the highly transcribed copies, $tRNA_1^{Gly}$ –6, 7 as very poorly transcribed copy and $tRNA_1^{Gly}$ –2 occupying an intermediary position (40–50% of the former copies) was maintained.

The present results established that the different members of the $tRNA_1^{Gly}$ multigene family from *B. mori* are differentially transcribed *in vivo* and *in vitro* and the highest levels of transcription are seen in homologous nuclear extracts or cell lines. Because the different $tRNA_1^{Gly}$ copies have identical coding regions, and consequently the same ICRs, the flanking sequences evidently are responsible for modulating their expression. Detailed analysis of the flanking sequences of the different $tRNA_1^{Gly}$ copies (Taneja *et al.*, 1992; Sharma & Gopinathan, 1996a,b) have identified both positive as well as negative regulatory elements located at significant distances from the coding region, exerting control on RNA polymerase III transcription. The presence of sequences TATAT, AATTTT and TTC within 40 bp upstream of $tRNA_1^{Gly}$ acted as positive regulatory elements in the highly transcribed gene copies (Taneja *et al.*, 1992; Fournier *et al.*, 1993), whereas the presence of one or more typical TATA box-like sequences led to substantial reduction in the transcriptional levels. The occurrence of multiple copies of TATATAA sequences in the flanking regions of the poorly transcribed $tRNA_1^{Gly}$ 6, 7, as far as 1 kb upstream or downstream of the tRNA coding region was responsible for the lower levels of transcription, because deletion or mutations of TATATAA motifs resulted in enhancement of transcription (Sharma & Gopinathan, 1996a,b). The binding of TBP (the TATA box binding protein) is essential for RNA polymerase III transcription also, therefore the negative influence of the TATA box sequences on the transcription was somewhat surprising. However, in RNA polymerase III transcription of tRNA genes TBP is recruited to transcription start site as a component of TFIIIB by protein–protein interactions, rather than through direct DNA binding. We therefore presume that these sequences sequester the TBP or other related factors and bring about the reduction in transcription. Thus, the presence of *cis* regulatory elements in the flanking regions of the different copies of the tRNA genes influence their transcription status either by recruitment of specific activators or by sequestration of transcription factors. The nature of these activators and or repressors if any, is not known at present. The pattern of differential expression of $tRNA_1^{Gly}$ with the same relative strength was also maintained in *Drosophila* cell line suggesting that the factors involved in modulating differential transcription are conserved between *B. mori* and *Drosophila*.

Experimental procedures

tRNA genes and reporter gene

Individual members of $tRNA_1^{Gly}$ multigene family from *B. mori* available in our laboratory stocks were utilized. The gene copies used were (Fig. 1), $tRNA_1^{Gly}$ –1 and 11 (highly transcribed), $tRNA_1^{Gly}$ –2 (medium level transcription) and $tRNA_1^{Gly}$ –6, 7 (poorly transcribed) (Sharma & Gopinathan, 1996a,b). The reporter genes used were firefly *luciferase* (*luc*) or the *green fluorescent protein* (*gfp*) from the jellyfish *Aqueorea victoria.*

Mutagenesis

The four tRNA genes chosen in the present study were mutated at positions +34 and +36 within the coding region such that the anticodon region **G**C**C** (+34 to +36) corresponding to the glycine codon GGC/U was converted to **T**C**A** to decode the nonsense codon TGA (see Fig. 1, top panel). Mutagenesis of the $tRNA_1^{Gly}$ copies was done by the double primer PCR based method (Weiner *et al.*, 1994). Two complimentary oligonucleotides (GAATGCTCGCCT-TCAACGCGGGCGGC and GCCGCCCGCGTTGAAGGCGAG-CATTC) corresponding to the tRNA anticodon region, harbouring the mutation TGA (in lieu of the original sequence GGT) were used to PCR amplify the plasmid pR8 DNA containing the $tRNA_1^{Gly}$ –1. After amplification the samples were digested with *Dpn1* (10 units) at 37 °C for 1 h to degrade the parental wild-type DNA template and then transformed into competent cells of *Escherichia coli*, strain DH5α. The presence of the mutation was confirmed by sequencing the plasmid DNA isolated from the transformants (pR8 Su^+). A similar strategy was used to generate Su^+ mutants of $tRNA_1^{Gly}$ –2, 11 and 6, 7 except that the 5′ regions of these genes up to the B box encompassing the anticodon region were first mobilized individually into plasmid vector pBS SK^+, and following mutagenesis, moved back to their respective parental constructs. This two-step approach was necessary because of the large size of the flanking region. Each of the mutated genes was confirmed by DNA sequencing.

The reporter genes, both *luc* and *gfp* were mutated in the N terminal region (at amino acid positions 20 and 8, respectively) to harbour stop codon TGA in place of GGA encoding glycine. A second TGA codon was introduced at Gly in position 23 of the *luc*. Site directed mutagenesis of *gfp* was carried out (Kunkel, 1985) using primer P1 (GAACTTTTCACTTGAGTTGTCCC), to generate the stop codon TGA at Gly10 (GGA). The *gfp* mutant harbouring Gly10-TGA was subcloned under the polyhedrin promoter of *Autographa californica* multiple nucleopolyhedrosis virus (AcM-NPV), which is expressed at high levels in BmN cells infected with

Bombyx mori nucleopolyhedrosis virus (BmNPV, Palhan *et al.*, 1995). Mutation in *luc* was generated by megaprimer mutagenesis method using the mutagenic primer (GGAACCGCTTGAGAGCAACTG) to convert the GGA encoding Gly 20 to TGA, in combination with vector primer (sequencing forward primer for pTZ, GTAAAACGACGGCCAGT). The 100 bp product harbouring the mutation was purified and used as a megaprimer together with pTZ reverse primer (AACAGCTATGACCATG), for a second round of amplification of the plasmid DNA. The PCR amplified, full length product was gel purified, cloned in plasmid pBS SK^+ and the presence of the mutation (Gly20-TGA) was confirmed by sequencing. A second mutation of GGA encoding Gly23 to TGA was also introduced into the gene by repeating the PCR megaprimer mutagenesis with second mutagenic primer (CAGTTGCTCTCAAGCGGTTCAATCCTC) to convert Gly23 to TGA.

After mutagenesis, *luc* harbouring Gly20-TGA, as well as both Gly20-TGA and Gly23-TGA mutations, were subcloned under the immediate early promoter of BmNPV to enable luciferase expression in insect cell lines.

In vitro *transcription assays*

Nuclear extracts from PSG of *B. mori* at the fifth larval instar were prepared as described previously (Taneja *et al.*, 1992). Briefly, freshly dissected silk glands (or glands frozen at –70 °C up to 6 months) were homogenized (Dounce homogenizer) in 10 mM HEPES (pH 7.9), containing 2 M sucrose, 10% glycerol, 15 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.15 mM spermine, 0.15 mM spermidine and 1 mM EDTA. Nuclei were pelleted by centrifugation and lysed in 20 mM HEPES (pH 7.9), containing 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF. The crude lysate was cleared by centrifugation, dialysed against 20 mM HEPES (pH 7.9), containing 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF and used as nuclear extract for transcription. *In vitro* transcription reactions in a final volume of 25 µl contained: 20 mM HEPES (pH 7.9), 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 6 mM creatine phosphate, 50 µM each of ATP, CTP and UTP, 10 µM GTP, 5µCi [α-^{32}P] GTP (3000 Ci/mmol), nuclear extract (20 µg protein) and 4 µg/ml of the supercoiled plasmid DNA template. After incubation at 30 °C for 1 h, the reactions were terminated by the addition of 0.2% SDS and 10 mM EDTA. The samples were extracted once with phenol and the transcripts in the aqueous layer were precipitated by three volumes of ethanol in the presence of 100 µg/ml glycogen (carrier). The precipitate was resuspended in gel loading buffer containing 80% formamide and subjected to electrophoresis on 7 M urea –8% acrylamide gels.

Transfection in cell lines

The insect cell lines used were BmN, a *B. mori* derived ovarian cell line, Sf-21, the lepidopteran cell line from *Spodoptera frugiperda* and S2, the dipteran cell line from *Drosophila*. BmN and Sf-21 cell lines were grown in TC-100 supplemented with 10% foetal calf serum (FCS) and S2 cells were grown in Schneiders medium supplemented with 10% FCS to a density of $1–2 \times 10^6$ cells in 35 mM dishes or T 25 culture flasks. Transfections were carried out by the lipofection technique (Palhan *et al.*, 1995). The DNA–liposome complex was prepared by mixing the indicated amounts of DNA (1–5 µg) along with lipofectin (6 µg/million cells) in 200 µl incomplete TC100 medium (without FCS) and incubated for 10 min at room temperature. The DNA–liposome complex was mixed with 1×10^6 cells in 800 µl of incomplete medium and incubated at 27 °C for 8 h. Following this, the cells were washed and recovered in complete medium containing 10% FCS for 36 h.

RNase protection assay

The total RNA from the cells was isolated by the guanidinium isothiocyanate method (Chomczynski & Sacci, 1987). Antisense RNA probe (400 nt) was generated by *in vitro* transcription of the cloned $tRNA_1^{Gly}$–1 Su^+ (linearized plasmid DNA from pR8 Su^+, see Fig. 1) using T3 RNA polymerase in the presence of [α-^{32}P] GTP. Total RNA (5 µg) isolated from the transfected cells (36 h post-transfection) was mixed with 5×10^5 cpm of antisense riboprobe and coprecipitated using 2.5 volume of ethanol. The pellet was resuspended in 30 µl of hybridization mix (80% formamide, 40 mM PIPES, 1 mM EDTA and 400 mM NaCl) and kept overnight at 50 °C. Following hybridization, 300 µl of RNase digestion mix (300 mM NaCl, 10 mM EDTA, 20 µg/ml RNase A and 100 U/ml RNase T1) was added and incubated at 30 °C for 1 h. The reaction was terminated by the addition of 20 µl of 10% SDS and 10 µg Proteinase K and further incubated at 37 °C for 30 min. The samples were extracted with phenol-chloroform and precipitated with 2.5vol ethanol in the presence of 20 µg glycogen. The RNA pellet was resuspended in 10 µl of gel loading dye (containing 80% formamide and marker dyes) and subjected to electrophoresis on a 7 M urea- 8% polyacrylamide gel. The gels were dried and autoradiographed.

Luciferase assay

To monitor the reporter gene activity, non-invasive luciferase assays were done 24–36 h post-transfection. The cells from 35 mm dishes were suspended in 50 µl of assay buffer (30 mM Tricine, 3 mM ATP, 10 mM DTT and 15 mM $MgCl_2$, pH 7.8) and samples were assayed for luciferase activity using a luminometer after direct injection of the substrate, D-luciferin (200 µM). The luciferase activity is expressed as relative light units (RLU).

Acknowledgements

We thank Dr S. Srikumar and Dr K. S. Ponnuvel of CSR&TI (Central Silk Board, Mysore) for generous supply of silkworm larvae and the DNA sequencing facility of our institute for assistance. We also thank the Department of Science and Technology, Government of India for financial support. L.S. is a recipient of a research fellowship from Council for Scientific and Industrial Research.

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