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

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Abstract

In mulberry silkworm Bombyx mori, tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Gly} species (Garel, 1976; Patel & Gopinathan, 1991). In B. mori, tRNA\textsuperscript{Gly\textsubscript{1}} 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\textsuperscript{Ala\textsubscript{1}}, which decodes the next most abundant codon (for alanine) in fibroin. There are two variants of tRNA\textsuperscript{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\textsuperscript{Ala\textsubscript{1}} (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\textsuperscript{Ala}, or tRNA\textsuperscript{Gly} and tRNA\textsuperscript{Glu} 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...
them (e.g. tRNA\textsubscript{Gly} \textsuperscript{–1}, tRNA\textsubscript{Gly} \textsuperscript{–2} or tRNA\textsubscript{Gly} \textsuperscript{–3}, all of which encode the same isoacceptor species) have been addressed by \textit{us in vitro} using nuclear extracts from the silk glands (Fournier \textit{et al.}, 1993; Sharma \& Gopinathan, 1996a,b; Taneja \textit{et al.}, 1992). Eleven of these gene copies, analysed were grouped into three distinct classes based on the \textit{in vitro} transcription in homologous nuclear extracts. Some of these were highly transcribed (e.g. tRNA\textsubscript{Gly} \textsuperscript{–1} and 11) whereas the others were extremely poorly transcribed (less than 10\% of the former, e.g. tRNA\textsubscript{Gly} \textsuperscript{–6}, 7, 8, 9 and 10) and the rest were transcribed to intermediary levels (30 – 60\%). 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 \textit{in vivo} transcription of one copy each of a highly and poorly transcribed tRNA\textsubscript{Gly} \textsuperscript{+} in \textit{B. mori}-derived BmN cells based on an RNase protection assay (Sharma \textit{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\textsubscript{Gly} \textsuperscript{+} copies seen \textit{in vitro} is true \textit{in vivo}. To achieve quantification of the \textit{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 anticyodon region of individual members of tRNA\textsubscript{Gly} \textsuperscript{+} family were mutated to generate suppressor tRNA (two mutations from GC to TGA). Co-transfection of the mutant reporter gene and the Su\textsuperscript{+} 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 \textit{in vivo}.

\textbf{Results and discussion}

\textit{In vitro and in vivo transcription of tRNA\textsubscript{Gly} \textsuperscript{+} copies}

The tRNA\textsubscript{Gly} \textsuperscript{+} copies, tRNA\textsubscript{Gly} \textsuperscript{–1}, 2, 11 and 6, 7 (Fig. 1, top panel) analysed here belong to three different groups based on \textit{in vitro} transcription in homologous nuclear extracts (Fournier \textit{et al.}, 1993; Sharma \& Gopinathan, 1996a). tRNA\textsubscript{Gly} \textsuperscript{–1} and 11 were highly transcribed (taken as 100\%) whereas tRNA\textsubscript{Gly} \textsuperscript{–2} was transcribed to medium levels (30–60\%) and tRNA\textsubscript{Gly} \textsuperscript{–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 \textit{et al.}, 1993). These tRNA gene copies were converted to Su\textsuperscript{+} tRNA by mutating two bases (at +34 and +36 nt in the anticyodon region) of the tRNA gene. The four suppressor tRNAs generated (in pR8 Su\textsuperscript{+}, pBms1 Su\textsuperscript{+}, pBmp1 Su\textsuperscript{+} and pBmg1 Su\textsuperscript{+}) were analysed for \textit{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 anticyodon position) did not have any adverse effects on \textit{in vitro} transcription.

We checked their transcription levels \textit{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 –6 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 \textit{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\textsubscript{Gly} \textsuperscript{–1} Su\textsuperscript{+} and 11 Su\textsuperscript{+} were transcribed to high levels (lanes 2 and 4) and tRNA\textsubscript{Gly} \textsuperscript{–6}–7 Su\textsuperscript{+} to barely detectable levels (lane 5) whereas tRNA\textsubscript{Gly} \textsuperscript{–2} Su\textsuperscript{+} was transcribed to intermediary levels (lane 3). These results reveal that the suppressor tRNA\textsubscript{Gly} \textsuperscript{+} 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 \textit{B. mori}.

\textbf{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\textsuperscript{+} 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\textsubscript{Gly} \textsuperscript{+} and tRNA\textsubscript{Gly} \textsuperscript{–} have been mutagenized to function asopal (UGA) suppressors (Capone \textit{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...
Differential expression of tRNA<sup>Gly</sup>-1

Su<sup>+</sup>tRNA<sup>Gly</sup>-1 showed significant fluorescence (Fig. 3, middle and bottom panels). The results indicate that the suppressor tRNA<sup>Gly</sup>-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<sup>Gly</sup>-6, 7 Su<sup>+</sup>, 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 ief 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<sup>+</sup> mutants of tRNA<sup>Gly</sup>-1. Suppression efficiency was 35–75% when increasing concentrations of tRNA<sup>Gly</sup>-1 Su<sup>+</sup> 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<sup>Gly</sup>-6, 7 Su<sup>+</sup> showed an increase in luciferase levels from 10% of the background levels to 13–15%. Clearly, the suppression due to tRNA<sup>Gly</sup>-1 Su<sup>+</sup> was much higher than that seen from tRNA<sup>Gly</sup>-6, 7 Su<sup>+</sup>, 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.
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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 back-ground luciferase activity (Fig. 4b). The suppression by the different suppressor tRNA\textsubscript{Gly} copies when co-transfected with the double mutant luc was presented in Fig. 4(b). tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+} was able to restore about 42% of the wild-type activity, whereas tRNA\textsubscript{Gly}\textsuperscript{−6, 7} Su\textsuperscript{+} showed less than 3% suppression, tRNA\textsubscript{Gly}\textsuperscript{−2} Su\textsuperscript{+} restored 16% of the wild-type activity (40% of tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+} efficiency) and tRNA\textsubscript{Gly}\textsuperscript{−11} Su\textsuperscript{+} restored approximately 40% of the wild-type activity (the same as tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+}). The transcript levels of different tRNA\textsubscript{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\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+} (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\textsubscript{Gly}\textsuperscript{−6, 7} Su\textsuperscript{+} showed barely detectable levels of luciferase activity (less than 3%) indicating that this gene is indeed transcribed poorly in vivo.

Figure 2. In vivo expression of tRNA\textsubscript{Gly} copies. RNA was isolated from BmN cells following transfection with the different tRNA\textsubscript{Gly} Su\textsuperscript{+} constructs as indicated. Radiolabelled antisense strand corresponding to the suppressor tRNA was generated by in vitro transcription from the plasmid pR8 Su\textsuperscript{+} 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\textsubscript{Gly} transcripts will be fragmented to two fragments whereas the Su\textsuperscript{+} 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\textsuperscript{+} DNA; lanes 2–5, RNA from cells transfected with Su\textsuperscript{+} tRNA\textsubscript{Gly}\textsuperscript{−1, 2, 11 and 6, 7}, respectively.

Figure 3. Functional assay for tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+} transcripts. The suppression of UGA mutation in gfp was monitored following transfection of the reporter gene, gfp Gly10-stop and tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+} 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\textsubscript{Gly}\textsuperscript{−1}; and last row, cells co-transfected with Gly10-stop gfp and tRNA\textsubscript{Gly}\textsuperscript{−1} Su\textsuperscript{+}.

Expression of tRNA\textsubscript{Gly} in Sf-21 and S2 cells

The individual members of the tRNA\textsubscript{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
Differential expression of tRNA<sup>Gly</sup> in vivo in the cell lines by the RNase protection and suppression assays.

For the RNase protection analysis, the oligotagged copy tRNA<sup>Gly</sup> –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<sup>Gly</sup> –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<sup>Gly</sup> –1. The transcription of tRNA<sup>Gly</sup> –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<sup>Gly</sup> –1 gene). Only weak signals due to tRNA<sup>Gly</sup> –1 transcripts (1–8% 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

**Figure 4.** Quantification of in vivo transcription. (a) Suppression of single mutation in luc. The transcription levels of different tRNA<sup>Gly</sup> copies in vivo were quantified by the functional suppressor assay. BmN cells were co-transfected with Gly20-stop luc and individual members of tRNA<sup>Gly</sup> Su+. The ability to suppress the USA 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<sup>+</sup>); 2, Gly20-stop luc (+ plasmid vector pBSKS<sup>+</sup>); 3, Gly20-stop luc + tRNA<sup>Gly</sup> (1 μg); 4, tRNA<sup>Gly</sup> (1 μg, 2 μg and 4 μg respectively); 5–7, Gly20-stop luc and tRNA<sup>Gly</sup> <sup>–1</sup> Su+ (1 μg, 2 μg and 4 μg respectively); 8, wt luc and tRNA<sup>Gly</sup> <sup>–1</sup> 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<sup>Gly</sup> in other insect cell lines. The expression of tRNA<sup>Gly</sup> 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<sup>Gly</sup> –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<sup>Gly</sup> 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<sup>–</sup>32P 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<sup>Gly</sup> 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.
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\(^{\text{Gly}}\) \(-1\) 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\(^{\text{Gly}}\) \(-1\) 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\(^{\text{Gly}}\) \(-1\) Su\(^{+}\) restored 1.25% and 3%, respectively, of the wild-type activity (Fig. 5b). The expression of tRNA\(^{\text{Gly}}\) copies was most efficient in B. mori derived cell line. Nevertheless, the pattern of differential expression viz., tRNA\(^{\text{Gly}}\) \(-1\) and \(-11\) as the highly transcribed copies, tRNA\(^{\text{Gly}}\) \(-6\) and \(-7\) as very poorly transcribed copy and tRNA\(^{\text{Gly}}\) \(-2\) occupying an intermediary position (40–50% of the former copies) was maintained.

The present results established that the different members of the tRNA\(^{\text{Gly}}\) 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\(^{\text{Gly}}\) copies have identical coding regions, and consequently the same IGRs, the flanking sequences evidently are responsible for modulating their expression. Detailed analysis of the flanking sequences of the different tRNA\(^{\text{Gly}}\) copies (Taneja et al., 1992; Sharma & Gopinathan, 1992; Weiner et al., 1994). Two complimentary oligonucleotides (GAATTCTGCCGT-TCAACGGGCCGCGC and GCCGGCCCGGCTGAAGGGCAG-GATT) corresponding to the tRNA anticodon region, harbouring the stop codon TGA (in lieu of the original sequence GGT) were used to PCR amplify the plasmid pBR DNA containing the tRNA\(^{\text{Gly}}\) \(-1\). After amplification the samples were digested with Dpn I (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 DH5\(\alpha\). The presence of the mutation was confirmed by sequencing the plasmid DNA isolated from the transformants (pBR Su\(^{-}\)). A similar strategy was used to generate Su\(^{+}\) mutants of tRNA\(^{\text{Gly}}\) \(-2\), \(-11\) and \(-6\) except that the 5’ regions of these genes were cloned into 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 four tRNA genes chosen in the present study were mutated at positions +34 and +36 within the coding region such that the anticodon region GCC (+34 to +36) corresponding to the glycine codon GCCU was converted to TCA to decode the nonsense codon TGA (see Fig. 1, top panel). Mutagenesis of the tRNA\(^{\text{Gly}}\) copies was done by the double primer PCR based method (Weiner et al., 1994). Two complimentary oligonucleotides (GAATTCTGCCGT-TCAACGGGCCGCGC and GCCGGCCCGGCTGAAGGGCAG-GATT) corresponding to the tRNA anticodon region, harbouring the mutation TGA (in lieu of the original sequence GGT) were used to PCR amplify the plasmid pBR DNA containing the tRNA\(^{\text{Gly}}\) \(-1\).

Experimental procedures

tRNA genes and reporter gene

Individual members of tRNA\(^{\text{Gly}}\) multigene family from B. mori available in our laboratory stocks were utilized. The gene copies used were (Fig. 1), tRNA\(^{\text{Gly}}\) \(-1\) and \(-11\) (highly transcribed), tRNA\(^{\text{Gly}}\) \(-2\) (medium level transcription) and tRNA\(^{\text{Gly}}\) \(-6\), \(-7\) (poorly transcribed) (Sharma & Gopinathan, 1996a,b). The reporter genes used were firefly luciferase (luc) or the green fluorescent protein (gfp) from the jellyfish Aequorea 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 GCC (+34 to +36) corresponding to the glycine codon GCCU was converted to TCA to decode the nonsense codon TGA (see Fig. 1, top panel). Mutagenesis of the tRNA\(^{\text{Gly}}\) copies was done by the double primer PCR based method (Weiner et al., 1994). Two complimentary oligonucleotides (GAATTCTGCCGT-TCAACGGGCCGCGC and GCCGGCCCGGCTGAAGGGCAG-GATT) corresponding to the tRNA anticodon region, harbouring the mutation TGA (in lieu of the original sequence GGT) were used to PCR amplify the plasmid pBR DNA containing the tRNA\(^{\text{Gly}}\) \(-1\). After amplification the samples were digested with Dpn I (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 DH5\(\alpha\). The presence of the mutation was confirmed by sequencing the plasmid DNA isolated from the transformants (pBR Su\(^{-}\)). A similar strategy was used to generate Su\(^{+}\) mutants of tRNA\(^{\text{Gly}}\) \(-1\) and \(-6\) except that the 5’ regions of these genes were cloned into 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 (GAAC TTTTCA CTT CA TTC) corresponding to the tRNA anticodon region, harboring the stop codon TGA at Gly10 (GGA). The gfp mutant harbouring Gly10-TGA was subcloned under the polyhedrin promoter of Autographa california multiple nucleopolyhedrosis virus (AcMNPV), 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 (GGAAACGGCTGGAGCAACTG) to convert the GGA encoding Gly23 to TGA, resulting in luc activity being silent (CAAGCGCAAGCG). 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 (CACATGCTCAACAGC-GF2TAGCTGC) 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 mM 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, dialysed against 20 mM HEPES (pH 7.9), containing 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 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 mM 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 MgCl2, 0.1 mM EDTA, 6 mM creatine phosphate, 50 μM each of ATP, CTP and UTP, 10 μM GTP, 5 μCi [32P] GTP (3000 Ci/mm), 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 precipitated with 100 μg/ml of ethanol. The pellet was resuspended in 10 μl of gel loading dye (containing 80% formamide and marker dyes) and subjected to electrophoresis on a 7% 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 EDTA and 15 mM MgCl2, 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).

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References

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