Modulation of differential transcription of tRNA genes through chromatin organization

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In higher eukaryotes, *tRNA* multigene families comprise several copies encoding the same tRNA isoacceptor species. Of the 11 copies of a $tRNA_1^{Gly}$ family from the mulberry silkworm *Bombyx mori*, individual members are differentially transcribed *in vivo* in the *B. mori*-derived BmN cell lines and *in vitro* in silk gland nuclear extracts. These genes have identical coding regions and hence harbour identical internal control sequences (the A and B boxes), but differ significantly in their 5′ and 3′ flanking regions. In the present study, we demonstrate the role of chromatin structure in the down-regulation of the poorly expressed copy, $tRNA_1^{Gly}$*-6,7*. Distinct footprints in the 5′-upstream region of the poorly transcribed gene *in vitro* as well as *in vivo* suggested the presence of nucleosomes. A theoretical analysis of the immediate upstream sequence of this gene copy also revealed a high propensity of nucleosome formation. The low transcription of $tRNA_1^{Gly}$*-6,7* DNA was further impaired on assembly into chromatin and this inhibition was relieved by externally supplemented TFIIIC with an associated histone acetyltransferase activity. The inhibition due to nucleosome assembly was absent when the 5′-upstream region beyond −53 nt was deleted or entirely swapped with the 5′-upstream region of the highly transcribed gene copy, which does not position a nucleosome. Footprinting of the *in vitro* assembled $tRNA_1^{Gly}$*-6,7* chromatin confirmed the presence of a nucleosome in the immediate upstream region potentially masking TFIIIB binding. Addition of TFIIIC unmasked the footprints present on account of the nucleosome. Our studies provide the first evidence for nucleosomal repression leading to differential expression of individual members from within a *tRNA* multigene family.

Key words: *Bombyx mori*, chromatin immunoprecipitation (ChIP) assay, chromatin organization, modulation, nucleosome assembly, transcription of tRNA genes.

INTRODUCTION

DNA in eukaryotic cells is packaged into nucleosomes by association with histones and non-histone proteins as higher order chromatin structures. Since nucleosomes can be positioned on certain sequences translationally as well as rotationally, they play important regulatory roles. The specific positioning of nucleosomes modulates the interaction of some of the regulatory factors with their target-binding sites [1–6]. Changes in chromatin organization by nucleosome disruption or displacement can lead to transcriptional activation [7–10]. The presence of organized chromatin structures also helps in bringing sequences and regulatory factors closer [11–13].

Amongst the genes transcribed by RNA pol III (polymerase III), the *5 S rRNA* genes have been studied in detail with respect to their chromatin structure [14–17]. The yeast *U6 snRNA* has also been analysed in the context of chromatin structure [11,18,19]. The binding of transcription factor TFIIIC to the *SNR6* chromatin leads to positioning of a nucleosome between the A and B boxes, which in turn brings them in close proximity to activate transcription [11]. The high-level transcription of the human *U6 snRNA* (*SNR6*) was attributed to a nucleosome positioned upstream of the transcriptional start site between the distal sequence element and the proximal sequence elements by mediating the co-operative binding of specific transcription factors [12,13].

However, much less work has been performed to understand the role of chromatin structure on transcription of tRNA genes. The transcription of *tRNA*s is considered to be relatively resistant to repression by histones. For instance, a yeast $tRNA^{Glu}$ remained active, while the yeast *U6 snRNA* was repressed, on incubation with *Xenopus* egg extract that assembled regularly spaced nucleosomes [18]. Similarly the SUP4 $tRNA^{Tyr}$ remained active in yeast cells after being fused to nucleosome positioning signals that could repress pol II (RNA polymerase II) transcription [20]. On the contrary, a yeast tRNA gene, when incorporated into inactive chromatin through insertion into the silent HMR locus (homothallic mating type locus right) was repressed [21]. In *Xenopus*, regular spacing of nucleosomes on tRNA genes was restricted to transcriptionally inactive erythrocytes but not in tissues that express tRNA [22]. The transcription factors TFIIIB and TFIIIC remained associated with *Xenopus* $tRNA^{Met1}$ in an H1-containing recombinant SV40 (simian-virus-40) minichromosome, in which the somatic *5S RNA* was repressed [23]. The transcription factor TFIIIC with an associated HAT (histone acetyltransferase) activity could relieve the repression of *in vitro* transcription of a human tRNA gene assembled as chromatin [24].

The silk glands of the mulberry silk worm *Bombyx mori* serve as a good model to study tissue-specific and developmental-stage-specific gene expression. The enhanced synthesis of silk fibroin proteins in the PSGs (posterior silk glands) in the fifth instar of larval development is attributed to enhanced transcription as well as translation. The fibroin mRNA levels go up by 100-fold and concomitantly the tRNA population also increases, indicating that both RNA pol II and III transcriptional activities are enhanced [25,26]. In order to optimize the translation process, there is a functional adaptation in the PSG as reflected in the disproportionate increase in those tRNAs, such as $tRNA^{Gly}$, $tRNA^{Ala}$ and $tRNA^{Ser}$, to decode the most frequently represented codons in

Abbreviations used: ChIP, chromatin immunoprecipitation; fibroin H, fibroin heavy; HAT, histone acetyltransferase; HMR locus, homothallic mating type locus right; ICR, internal control region; LMPCR, ligation-mediated PCR; MNase, micrococcal nuclease; NFP, nucleosome formation potential; pol II, polymerase II; PSG, posterior silk gland; TBP, TATA box binding protein.

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the fibroin H (heavy) chain mRNA [25,26]. Glycine constituting nearly 48 % of all amino acids in fibroin H, the frequency of GGC/U codons in the fiboin H mRNA numbering approx. 1350 is most conspicuous [27]. The isoacceptor species of $tRNA_1^{Gly}$ that decodes this triplet is encoded by a multigene family comprising 20 copies of $tRNA_1^{Gly}$, which are differentially transcribed [25,28,29]. All the 11 genes so far characterized from the family have identical coding sequences, but they differ in their upstream and downstream regions. Certain *cis* elements in the flanking regions of these genes regulate their transcriptional status by exerting positive or negative effects [25,28–30]. The *tRNA* genes, in general, are considered to be expressed constitutively but in *B. mori* they are differentially expressed [26,31]. The *B. mori* system, therefore serves as a suitable model to analyse the differential expression of *tRNA*s. All the characterized members of the $tRNA_1^{Gly}$ family from *B. mori* are typical type II genes transcribed by RNA pol III with two ICRs (internal control regions) designated as the A and the B boxes. The transcription machinery comprises the three multisubunit proteins, TFIIIB, TFIIIC and RNA pol III [32,33].

In order to analyse the regulation of the $tRNA_1^{Gly}$ encoding genes with respect to chromatin structure, two representative members of the gene family, i.e., the highly expressed $tRNA_1^{Gly}$*-1* and the poorly transcribed $tRNA_1^{Gly}$*-6,7* were analysed in the present study. The involvement of chromatin structure (isolated *in vivo* or assembled *in vitro*) in modulating the expression of these two *tRNA*s has been demonstrated by structural and functional analyses.

EXPERIMENTAL

tRNA genes

The $tRNA_1^{Gly}$ genes used in the present study were from our laboratory stock [25]. $tRNA_1^{Gly}$*-1* is the highly transcribed copy, comprising 300 bp upstream and 30 bp downstream of the tRNA coding region in plasmid pBSSK+ (plasmid pR8) [30]. $tRNA_1^{Gly}$*-6,7* is a fusion construct of the two tandemly linked genes $tRNA_1^{Gly}$*-6* and $tRNA_1^{Gly}$*-7* present on a single genomic fragment from *B. mori*. This derivatized construct in plasmid pBSKS+ (plasmid clone pΔS1) contained the entire upstream region (0.97 kb) of the $tRNA_1^{Gly}$*-6* and the 3′-downstream region (1.5 kb) of $tRNA_1^{Gly}$*-7*, but was devoid of the linker region between the two gene copies [28]. This construct retained the low transcription profile of $tRNA_1^{Gly}$*-6* or *-7* and was utilized for *in vitro* studies to avoid the presence of two transcripts arising from a single insert in the parental clone [28]. A deletion derivative of $tRNA_1^{Gly}$*-6,7* from which the sequences from the 5′-upstream region beyond −53 nt (which showed a high propensity for nucleosome formation) was generated by PCR using gene specific primers (5′-CGGAATTCCGCATAAATATGGCG-3′ located at −53 nt and 5′-GAATTCAACTTGCAGTCAAC-3′ located at +1570 nt with respect to the +1 of the mature tRNA) and cloned into pBSKS+ (clone designated pΔ-53S1). Another plasmid construct pS1DR8U was generated in which the 5′-upstream sequences of $tRNA_1^{Gly}$*-6,7* was completely replaced with those of the highly transcribed gene $tRNA_1^{Gly}$*-1* by swapping the domains [28].

Purification of transcription factors TFIIIC and TFIIIB

These factors were purified from *B. mori* silk gland nuclear extracts [35] by chromatography on phosphocellulose for TFIIIC and chromatography on heparin–Sepharose following phosphocellulose for TFIIIB. Both the factors were dialysed to a final concentration of 0.1 M KCl.

Chromatin assembly

Chromatin lacking histone H1 was assembled on the plasmids pR8 and pΔS1, in the presence of the core histones and S190 extract from *Drosophila* embryos [34]. In a typical assembly reaction, 1–2 μg of each of the plasmid DNAs were incubated for 5 h at 27 °C with 0.9–1.8 μg of core histones and 1.2 mg of S190 protein in 200 μl of 10 mM Hepes/KOH buffer (pH 7.5), containing 7 mM $MgCl_2$, 30 mM NaCl, 3 mM ATP, 8.5 mM β-glycerophosphate, 30 mM phosphocreatine and 1 μg/ml creatine kinase. Transcription factors TFIIIC and TFIIIB, purified from *B. mori* silk gland nuclear extracts, were added 30 min before the end of assembly process. The periodicity of the assembled nucleosomes on the templates was examined each time by MNase (micrococcal nuclease) digestion followed by electrophoresis on agarose gels.

Chromatin structure analysis *in vitro*

In vitro footprinting was performed using 125 ng of chromatin assembled *in vitro* or the naked DNA, by digestion with MNase [$(7–65) \times 10^{-3}$ unit for chromatin and $(2–8) \times 10^{-4}$ unit for naked DNA] at room temperature (24 °C) for 10 min. DNase I digestions for the same amount of chromatin and naked DNA were performed using 1 μg or 1 ng of the enzyme respectively for 1 min at room temperature. TFIIIC when required was added 30 min before the end of the assembly reaction. The partial digestion products were deproteinized by phenol extraction and subjected to primer extension using $Vent^R$ (exo–) DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) and 5′-end ^{32}P-labelled primers. The primers were designed for the upstream regions at position −276 nt for $tRNA_1^{Gly}$*-1* [R8 primer 1 (top strand); 5′-AATATACCGTGACAGAAT-3′] and at −190 nt for $tRNA_1^{Gly}$*-6,7* [S1 primer 3 (top strand); 5′-GAATGGTGCCGTCCAC-3′]. The samples were deproteinized again after primer extension reaction and the products were analysed by electrophoresis on 7 M urea/6 % (w/v) polyacrylamide gels. The profiles of the footprints were generated using the Image Gauge Program.

Chromatin analysis *in vivo*

In vivo analysis of chromatin from PSGs was performed as described for the fibrohexamerin gene [36]. The silk glands from the fifth instar stage were powdered under liquid nitrogen and digested with 25 μg of DNase I for 15 min at room temperature in 35 mM Hepes buffer (pH 7.4), containing 150 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$ and 2 mM $CaCl_2$. The reaction was terminated by the addition of 20 mM NaCl, 20 mM EDTA, 1 % SDS and 10 μg of proteinase K and the DNA was precipitated with ethanol. Primer extension was performed on the above DNA using 5′-end ^{32}P-labelled gene specific primers (S1 primer 3, as mentioned above) and *Taq* DNA polymerase for 20 cycles (denaturation at 95 °C for 4 min, annealing at 50 °C for 2 min and extension at 72 °C for 3 min). The products were deproteinized by extraction with phenol and analysed by electrophoresis on 7 M urea/6 % polyacrylamide gels.

Southern hybridization

The chromatin as well as genomic DNA extracted from PSG was digested with MNase and subsequently with BglI (a unique site for BglI is present at +33 nt of both the *tRNA*s). After the digestion, the samples were electrophoresed on 1.2 % (w/v) agarose gel and transferred on to nylon membrane (Hybond N, Amersham Biosciences, Piscataway, NJ, U.S.A.) [37]. Hybridization was performed using either the 0.35 kb $tRNA_1^{Gly}$*-1* probe (EcoRI–BglI fragment from clone pR8) or the 0.38 kb $tRNA_1^{Gly}$*-6,7* probe

(DraI–BglI fragment from clone pΔS1) labelled with [α-^{32}P]-dATP, in 6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at 60 °C. The membranes were washed successively with 1 × SSC, 0.5 × SSC and 0.1 × SSC containing 0.1 % SDS.

Indirect end-labelling analysis

For indirect end-labelling analysis, the *in vitro* assembled chromatin or naked DNA (125 ng) was digested with MNase, deproteinized and then restricted with endonucleases (EcoRI and XbaI for *tRNA$_1^{Gly}$-1* and EcoRI for *tRNA$_1^{Gly}$-6,7*), the recognition sites for which are present upstream and downstream of the respective genes. Southern blots of these fragments were probed with an end-labelled 150 nt EcoRI–KpnI fragment of *tRNA$_1^{Gly}$-1* (from plasmid pR8 corresponding to the −150 to −300 nt upstream region of the gene) or an 81 nt EcoRI–SacI fragment of *tRNA$_1^{Gly}$-6,7* (from clone pΔS1 corresponding to −976 to −895 nt upstream region of the gene). Hybridization and washing conditions were the same as described previously.

LMPCR (ligation-mediated PCR)

LMPCR analysis [38,39] was performed on chromatin isolated from PSG and digested with DNase I (as mentioned in the previous section). Primer extension was performed initially for three cycles followed by ligation of the annealed linker (100 pmol) at 16 °C. Subsequent amplification steps were performed using Vent (exo+) DNA polymerase for 18 cycles [38] and later for three cycles using 5′-end ^{32}P-labelled primer. The primers used for the analysis of the upstream region of *tRNA$_1^{Gly}$-6,7* were the primers S1 (5′-GAGATATCACATTAAGCGTA-3′) at position −786, S2 (5′-CAATGCAGTGCATTTCTTC-3′) at −662 nt and S3 (as described previously for *in vitro* analysis) at the −190 nt position. The linker primers were the partially double-stranded oligonucleotides, 5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and 5′-GAATTCAGATC-3′, annealed to each other [40]. After amplification, the samples were deproteinized by phenol extraction and subjected to electrophoresis on 7 M urea/6 % polyacrylamide gels.

Theoretical analysis of nucleosomal positioning

The NFP (nucleosomal formation potential) of the two *tRNA$_1^{Gly}$* genes was analysed theoretically using a program that calculates the NFP of a particular sequence derived by discriminant analysis of dinucleotide frequencies with the trained set of nucleosome positioning sequences in the nucleosomal DNA database. The NFP profile may be transformed to the interval [0; +1] so that the value +1 corresponds to the best prediction, and 0 to the worst. The dinucleotide relative abundance distance was chosen as additional restriction for input data to exclude the sequences with a poor dinucleotide content [41,42]. The output data values were represented in a graphical form, where the *x*-axis denotes the nucleotide number and the *y*-axis denotes the NFP.

In vitro transcription assays

Nuclear extracts from PSG of *B. mori* in the fifth larval instar were prepared as described previously [28,30]. The protein contents were determined by dye-binding method [37]. *In vitro* transcription reactions contained in a final volume of 30 μl, 20 mM Hepes (pH 7.9), 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 6 mM phosphocreatine, 50 μM each of ATP, CTP and UTP, 10 μM GTP, 5 μCi of [α-^{32}P]GTP (3000 Ci/mmol), suboptimal concentrations of the crude nuclear extract (4 μg of protein) and 100 ng of DNA template (naked DNA or chromatin) and incubated at 30 °C for 1 h. Increasing amounts of purified TFIIIC fraction were added wherever indicated to the freshly assembled chromatin and incubated for 20 min at 30 °C before the addition of the nuclear extract for transcription. The transcription reactions were terminated by the addition of 0.2 % (w/v) SDS, 10 mM EDTA and 100 μg/ml glycogen and analysed by electrophoresis on 7 M urea/8 % polyacrylamide gels.

ChIP (chromatin immunoprecipitation) assay

For ChIPs [43], TFIIIB fraction (purified from *B. mori* PSG nuclear extracts; 6 μg of protein) was added together with TFIIIC to the DNA template at 4.5 h during the 5 h assembly process. After the assembly, the chromatin was digested with MNase as described previously. The samples were diluted 10-fold with dilution buffer (16.7 mM Tris, pH 8.0, 0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA and 167 mM NaCl) and precleared by treatment with Protein A–agarose beads at 4 °C for 1 h. The supernatant was recovered by a brief spin at 4000 ***g*** for 30 s and immunoprecipitated using a polyclonal cross-reactive antibody directed to the TBP (TATA box binding protein; raised against the C-terminal domain of TBP) component of TFIIIB. Protein A–agarose beads were added to the samples after 1 h incubation and left overnight at 4 °C with shaking. The immunocomplexes were recovered by centrifugation of the agarose beads. The supernatant from the control reaction (identical to the above except that no antibody was added) was used as the input. The beads were washed sequentially with a low-salt solution (150 mM NaCl, 0.1 % SDS, 1 % Triton X-100 and 2 mM EDTA in 20 mM Tris, pH 8), high-salt solution (500 mM NaCl, 250 mM LiCl, 1 % Nonidet P40, 1 % sodium deoxycholate and 1 mM EDTA in 10 mM Tris, pH 8) and finally twice with 10 mM Tris (pH 8) containing 1 mM EDTA. The immunocomplexes bound to the agarose beads were eluted using 0.2 % SDS and 0.1 M sodium bicarbonate. The samples were deproteinized by phenol extraction and the DNA in the aqueous phase was slot blotted on to a Hybond N+ nylon membrane (Amersham Biosciences). To serve as a control for the reaction, similar immunoprecipitation was also performed with the naked DNA incubated for 4.5 h with S190 extract and subsequently for 30 min with TFIIIC and TFIIIB. The blots were probed using either the *tRNA$_1^{Gly}$-1* probe (the EcoRI–XbaI fragment from clone pR8 corresponding to −300 to +30 nt beyond the tRNA coding region) or with *tRNA$_1^{Gly}$-6,7* probe (the DraI fragment from clone pΔS1 corresponding to −284 to +33 nt beyond the tRNA coding region).

RESULTS

Chromatin assembly on *tRNA$_1^{Gly}$* genes

tRNA$_1^{Gly}$-1 is highly transcribed and *tRNA$_1^{Gly}$-6* and *-7* or *tRNA$_1^{Gly}$-6,7* are poorly transcribed *in vitro* by silk gland nuclear extracts. The plasmid pR8 (harbouring *tRNA$_1^{Gly}$-1*) gives rise to one predominant (precursor) and one minor (processed) form of tRNA transcripts, whereas pS1 (harbouring *tRNA$_1^{Gly}$-6* and *-7* in tandem) gives rise to two precursor transcripts corresponding to *tRNA$_1^{Gly}$-6* and *-7* respectively (larger than the *tRNA$_1^{Gly}$* transcript) and one processed form (Figures 1A and 1B; see [29,30]). Plasmid pΔS1 (*tRNA$_1^{Gly}$-6,7*) gives rise to a single precursor and processed tRNA transcript. The transcription of *tRNA$_1^{Gly}$-6,7* was only 3–8 % of that of *tRNA$_1^{Gly}$-1* as naked DNA templates (Figure 1B). The other two constructs utilized in later chromatin analysis were pS1DR8U, which had the entire upstream region of *tRNA$_1^{Gly}$-6,7* replaced with the upstream of *tRNA$_1^{Gly}$-1* (therefore designated as *tRNA$_1^{Gly}$-1,7*), and a deletion derivative (pΔ-53S1) with a deletion

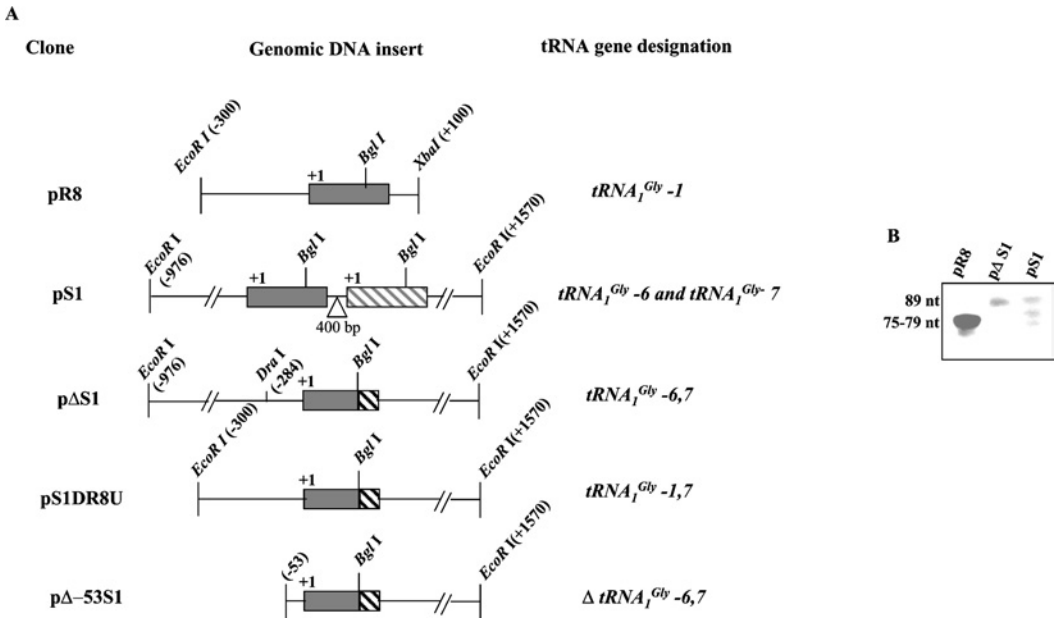

Figure 1 Transcription of *tRNA$_1^{Gly}$-1* and *tRNA$_1^{Gly}$-6,7* genes from *B. mori*

(**A**) Plasmid constructs containing *tRNA$_1^{Gly}$*. The boxed areas represent the *tRNA$_1^{Gly}$* encoding sequences. The plasmid clone pR8 harbours *tRNA$_1^{Gly}$-1* and the flanking regions marked are with respect to the +1 position of *tRNA$_1^{Gly}$*. The plasmid construct pΔS1 contains *tRNA$_1^{Gly}$-6,7* that was derived by fusion of *tRNA$_1^{Gly}$-6* and *7* genes (in the parental construct pS1) by eliminating the 400 bp region between them and had identical sequence of the coding region. The coordinates are marked with respect to +1 of the *tRNA$_1^{Gly}$-6* and *tRNA$_1^{Gly}$-7* in plasmid pS1. The locations of some of the relevant restriction sites are also indicated. A deletion construct pΔ-53S1 was generated by deletion of the upstream region of *tRNA$_1^{Gly}$-6,7* up to −53 nt, and a construct pS1DR8U was generated by replacing the upstream sequences of *tRNA$_1^{Gly}$-6,7* with that of *tRNA$_1^{Gly}$-1* still retaining the downstream regions. (**B**) *In vitro* transcription patterns of the different *tRNA$_1^{Gly}$* constructs in PSG nuclear extracts. See text for details of the transcription assays. The size range of the labelled transcripts are marked on the side.

up to −53 nt with respect to the tRNA coding region in *tRNA$_1^{Gly}$-6,7* (therefore designated as Δ*tRNA$_1^{Gly}$-6,7*) (Figure 1A).

Plasmid DNAs containing either *tRNA$_1^{Gly}$-1* (highly transcribed) or *tRNA$_1^{Gly}$-6,7* (poorly transcribed) were assembled into regularly spaced chromatin using a *Drosophila* embryo S190 extract and the core histones. The nucleosome assembly *in vitro* was verified by the formation of MNase-resistant nucleosomal DNA ladders (Figure 2A). The presence of nucleosomal ladders was evident in both instances. These *in vitro* assembled chromatin samples were used for transcription or MNase and DNase I digestion studies, described later.

An indirect end labelling analysis was also performed with the chromatin assembled *in vitro* to see the protection at a long range. The *tRNA$_1^{Gly}$-1* assembled as chromatin or as naked DNA showed identical patterns indicative of the absence of positioned nucleosomes on the gene (Figure 2B, left panel, compare lanes 1 and 2). On the other hand, *tRNA$_1^{Gly}$-6,7* chromatin showed a protection especially in the high molecular size ranges in chromatin as compared with the naked DNA indicating the presence of positioned nucleosomes (Figure 2B, right panel, compare lanes 1 and 2). On digestion with MNase alone the presence of a MNase ladder was seen in both instances (Figure 2B, lane 3, both panels).

The accessibility of a unique BglI site present within these genes was exploited as a preliminary assay to monitor the formation of chromatin *in vivo*. An identical pattern for *tRNA$_1^{Gly}$-1* in the genomic DNA as well as PSG chromatin restricted first with MNase and subsequently with BglI indicated that the BglI site was equally accessible in both instances (Figure 2C, left panel, lanes 2 and 3). Digestion of genomic DNA with BglI alone gave rise to a single prominent band at approx. 2 kb due to *tRNA$_1^{Gly}$-1*. The bands present in the upper regions of the gel were due to the other copies of *tRNA$_1^{Gly}$*, which will also be recognized by the probe used. The additional band present in genomic DNA and chromatin samples digested with MNase and BglI could be due to the presence of an MNase sensitive site in the vicinity of the BglI site. The chromatin digested with MNase alone gave a nucleosome ladder indicative of intact chromatin structure *in vivo* (Figure 2C, left panel, lane 4). In the case of *tRNA$_1^{Gly}$-6,7*, on the other hand, BglI digestion of the naked DNA gave rise to two bands of approx. 2 kb and 470 bp (Figure 2C, right panel, lane 1). The *tRNA$_1^{Gly}$-6* and *tRNA$_1^{Gly}$-7* are closely linked and are separated by a 400 bp region between the two gene copies (shown in Figure 1A). There were distinct differences in the pattern of BglI digestion between chromatin and naked DNA in the case of *tRNA$_1^{Gly}$-6*. Clearly, the BglI site (located at +33 nt within the tRNA coding region) was not accessible for digestion, when the gene was associated with chromatin (compare lanes 2 and 3 in the right panel). The cleavage pattern indicated a spacing of approx. 190 bp and therefore it was likely that the cuts occurred in the linker DNA between positioned nucleosomes (lane 2).

Presence of a positioned nucleosome in the upstream region of *tRNA$_1^{Gly}$-6,7*

In order to confirm whether the BglI site within *tRNA$_1^{Gly}$-6,7* was inaccessible due to the presence of chromatin in that region, the *tRNA$_1^{Gly}$-6,7* after *in vitro* assembly into chromatin was footprinted by MNase and DNase I digestions. A primer designed in the immediate upstream region (−190 nt) of the tRNA coding region in *tRNA$_1^{Gly}$-6,7* was used for the primer extension (Figure 3, top, left panel). Naked DNA incubated with the S190 extract before the digestions (but no nucleosome assembly) was included as a control to rule out any non-specific protections conferred by the extract (Figure 3, top panels, lanes as marked). Signs of protection from MNase digestion were evident in *tRNA$_1^{Gly}$-6,7* assembled as chromatin, when compared with the naked DNA (left panel, compare lanes 1 and 2). The protected regions were distinct, as shown in the scanning profiles of these digests (Figure 3,

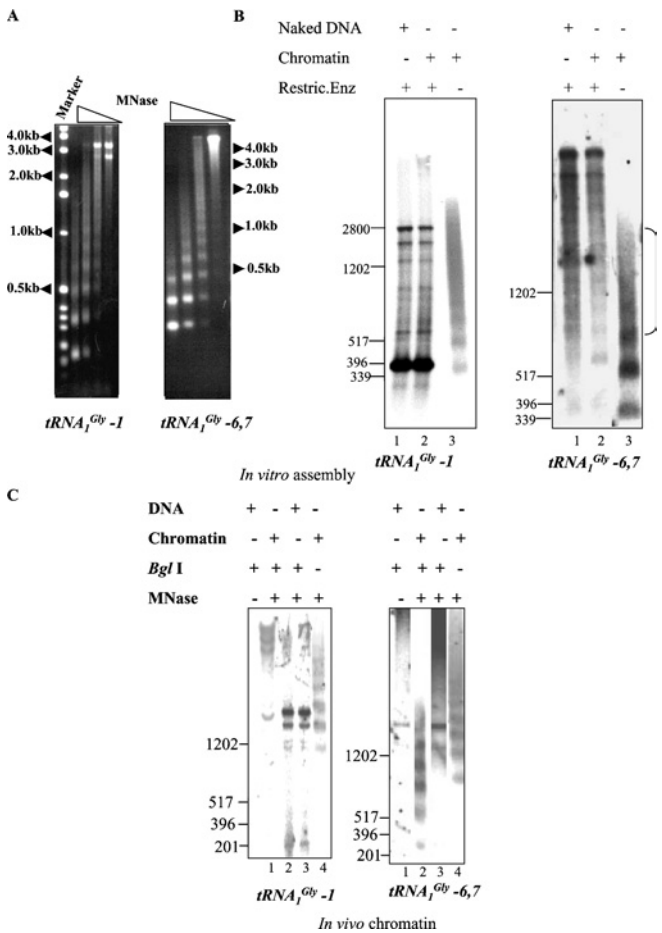

Figure 2 Chromatin assembly and indirect end labelling analysis of *tRNA$_1^{Gly}$* chromatin

(**A**) *In vitro* assembly of chromatin on *tRNA$_1^{Gly}$* genes (in plasmids pR8 and pΔS1) was performed using the *Drosophila* S190 extracts. The assembled chromatin was digested with increasing concentrations of MNase and verified for the presence of ladders, by electrophoresis on 1.2% agarose gels. The ethidium bromide staining patterns are shown. (**B**) Indirect end labelling analysis of *in vitro* assembled *tRNA$_1^{Gly}$* chromatin. The naked DNA as well as the *in vitro* assembled chromatin of *tRNA$_1^{Gly}$-1* or *tRNA$_1^{Gly}$-6,7* were digested with MNase followed by digestion with EcoRI and XbaI (for the former) and EcoRI alone (for the latter) (lanes 1 and 2, both panels). The samples were subjected to electrophoresis and transferred to Hybond (N+) membranes and probed with either end labelled *tRNA$_1^{Gly}$-1* (left panel) or with *tRNA$_1^{Gly}$-6,7* (right panel). Controls for MNase digested chromatin (lane 3) are also included for both panels. (**C**) Restriction site accessibility on *tRNA* chromatin. Chromatin or genomic DNA isolated from PSG were digested with MNase and BglI (lanes 2 and 3, both panels) and subjected to electrophoresis on 1.2% agarose gels. Genomic DNA digested with BglI alone (lane 1, both panels) and chromatin digested with MNase alone (lane 4, both panels) were also included as controls. The samples were transferred to Hybond Nylon (N+) charged membrane after electrophoresis and probed with either the *tRNA$_1^{Gly}$-1* (left panel) or *tRNA$_1^{Gly}$-6,7* (right panel).

bottom, profile of naked DNA shown as dotted lines and chromatin shown as continuous lines). The protected regions mapped from -118 to -66 nt and -55 to $+12$ nt. The addition of TFIIIC to the assembled chromatin unmasked the footprints due to the presence of nucleosomes and showed a pattern similar to that of the naked DNA (lanes 3 and 4). However, TFIIIC binding itself showed protection from $+20$ nt and extended to the downstream region, and the pattern was similar in both chromatin and naked DNA (left panel, lanes 3 and 4). The TFIIIC preparation used in the present study had an associated HAT activity, which was possibly responsible for the displacement of nucleosomes leading to the removal of footprints.

DNase I digestion of *tRNA$_1^{Gly}$-6,7* chromatin also showed protection at positions -109, -100, -89, -67, -57 and -29 nt in the immediate upstream region within the same protected area mapped in MNase digestion (Figure 3, top, middle panel, lanes 1 and 2). The scanning profile of the DNase I digested chromatin and naked DNA is also shown for better clarity (Figure 3, bottom right; scanning profile for naked DNA shown in dotted lines and chromatin in continuous lines. A similar analysis of *tRNA$_1^{Gly}$-1* using the appropriate primer did not show any distinct nucleosomal footprint (Figure 3, top; right panel). There were only some minor differences, but a few hypersensitive sites were apparent in chromatin as compared with the naked DNA. These footprinting patterns were independently reproduced at least three times.

The presence of nucleosomes *in vivo*

The primer extension of chromatin isolated from PSG and digested with DNase I using the same primers also showed distinct protected regions on *tRNA$_1^{Gly}$-6,7* chromatin as compared with the genomic DNA (Figure 4A, left panel). The more sensitive LMPCR analysis on the *in vivo* chromatin once again confirmed the presence of the protected sites in this region (Figure 4A, right panel, compare lanes 1 and 2). On the contrary, a similar analysis with *tRNA$_1^{Gly}$-1* showed almost identical patterns for chromatin as well as genomic DNA (Figure 4B, compare lanes 1 and 2).

Theoretical analysis of nucleosome positioning over *tRNA$_1^{Gly}$* genes

Based on a theoretical programme, the NFP of the two *tRNA$_1^{Gly}$* genes was analysed and the output is depicted graphically (Figure 4C). Both *tRNA$_1^{Gly}$-1* and *tRNA$_1^{Gly}$-6,7* were examined from -300 to $+100$ nt to maintain uniformity of the immediate upstream regions analysed. Sequences with very poor dinucleotide frequencies were omitted and the graph was plotted as a measure of the nucleosome potential (on *y*-axis) versus the nucleotide number (on *x*-axis). *tRNA$_1^{Gly}$-6,7* showed NFP values > 0.9 at numerous positions in the immediate upstream region indicating that these sequences had high NFPs (Figure 4C, lower panel). In contrast, *tRNA$_1^{Gly}$-1* showed only moderate NFP values with a maximum of only 0.8 and that too restricted to a single spot indicating that this gene copy had a lower NFP.

Transcription competence of chromatin templates of *tRNA$_1^{Gly}$* genes

After detecting the presence of nucleosome in the immediate upstream region of *tRNA$_1^{Gly}$-6,7*, the transcriptional competence of the assembled chromatin was determined *in vitro*. Suboptimal concentrations of PSG nuclear extracts were used for transcription, so that the effects of external supplementation of transcription factors could be clearly visualized. The transcription of *tRNA$_1^{Gly}$-1* gene was not significantly affected, whether used as free DNA or after chromatin assembly (Figure 5, upper panel, lanes 2 and 3). The external addition of TFIIIC fraction with associated HAT activity [24,33] had no effect on its transcription (lanes 4 and 5), indicating that the TFIIIC present in the extract was sufficient to relieve the chromatin-mediated inhibition, if any, on *tRNA$_1^{Gly}$-1*. In contrast, the already low transcription of *tRNA$_1^{Gly}$-6,7* (only 3–8% of *tRNA$_1^{Gly}$-1* transcription, even when naked DNA templates were used), was almost completely abolished on assembly into chromatin (Figure 5, middle panel, compare lanes 2 and 3). The addition of TFIIIC relieved the chromatin-mediated repression as revealed by the reversal of inhibition (lanes 4–6). Controls with just S190 extracts were always

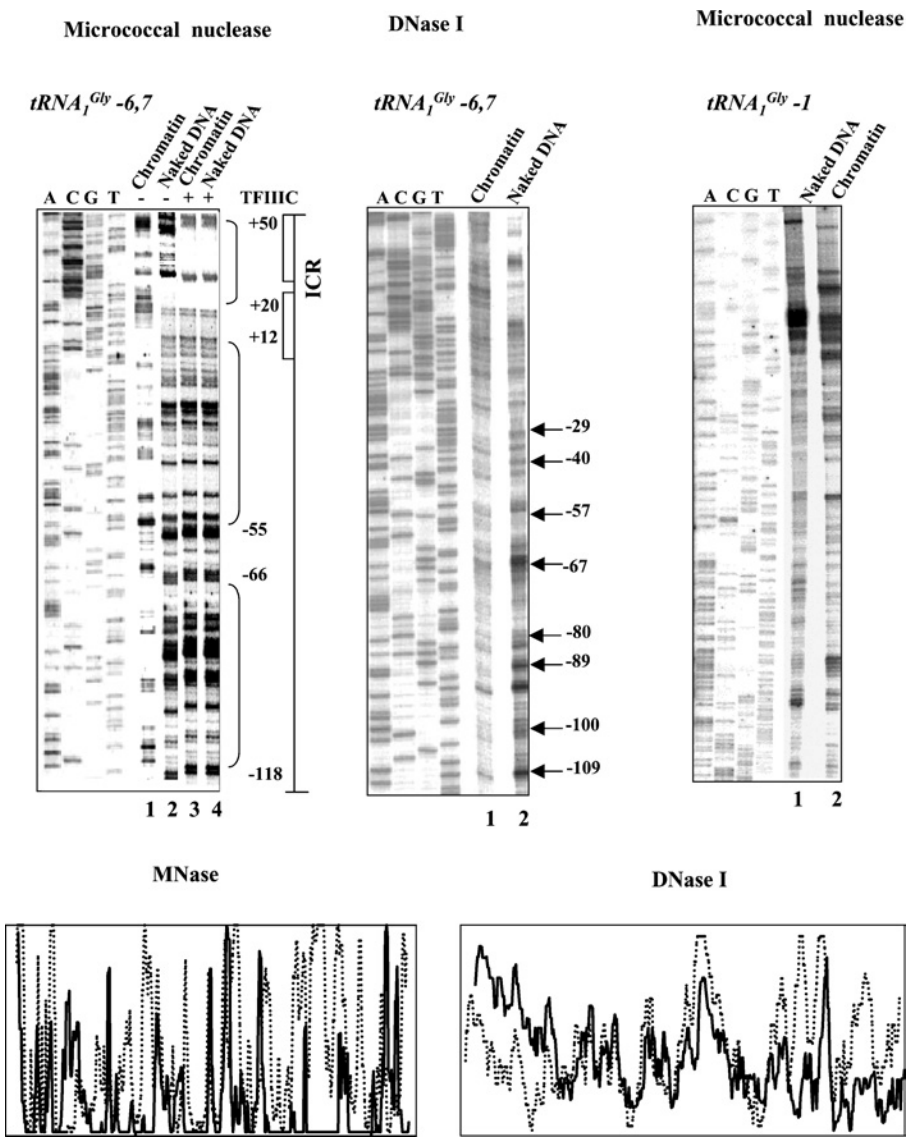

Figure 3 Nucleosome protection in the immediate upstream region of *tRNA$_1^{Gly}$-6,7*

Primer extension analysis was performed with chromatin assembled on *tRNA$_1^{Gly}$-6,7* after digestion with MNase (top left panel), or with DNase I (top middle panel) using a ^{32}P-labelled 5′ primer positioned at − 190 nt (S1 primer 3). The sequencing ladders of the region generated using the same primer are also shown (lanes A, C, G and T). The protected regions due to the presence of the nucleosome from MNase digestion in the absence or presence of TFIIIC are indicated on the right side (left panel). The protected sites in DNase I digested chromatin as compared with the naked DNA (right panel) are marked by arrows and numbered with respect to the sequence. Primer extension analysis was also performed on chromatin assembled *in vitro* on *tRNA$_1^{Gly}$-1* and digested with MNase using ^{32}P-end labelled primer (R8 primer 1) at position − 300 nt (top right panel). The sequencing ladder was generated with the same primer, shown in lanes marked A, C, G and T. The MNase footprintings were performed using four different concentrations of the enzyme, but only those showing the best footprints in typical digestions are presented. The profiles of the nucleosome footprints on *tRNA$_1^{Gly}$-6,7* generated using the Image Gauge program are presented in the bottom (left: MNase footprint; right: DNase I footprint). Dotted lines correspond to naked DNA and black lines correspond to chromatin.

included in these experiments to ensure that by themselves (in the absence of the PSG nuclear extract) they did not transcribe either of these gene copies (lane 1, both panels). To confirm whether the inhibitory effect was indeed due to the presence of the positioned nucleosomes, a deletion derivative of *tRNA$_1^{Gly}$-6,7* (in plasmid pΔ-53S1), in which the deletion extended up to − 53 nt in the 5′-upstream region eliminating the nucleosome positioning sequences, was also examined. This deletion derivative of *tRNA$_1^{Gly}$-6,7* showed no chromatin mediated repression on transcription (Figure 5, bottom panel, lanes 4–6). Alternatively, a domain swapped construct in which the entire 5′-upstream region of *tRNA$_1^{Gly}$-6,7* replaced with that of the highly expressed *tRNA$_1^{Gly}$-1* was also utilized to confirm this inference. This *tRNA$_1^{Gly}$-1,7* derivative also did not show chromatin-mediated repression (Figure 5, bottom panel, lanes 1–3). Interestingly, in both these instances, the transcription from the naked DNA template itself had considerably increased as compared with the parental gene *tRNA$_1^{Gly}$-6,7* and amounted to almost 40–60 % of that of *tRNA$_1^{Gly}$-1*.

From these results, it was inferred that the nucleosome present immediately upstream to the start site of *tRNA$_1^{Gly}$-6,7* prevented the formation of an active transcription complex on this gene copy and thereby caused repression. The presence of nucleosome might prevent the efficient binding of TFIIIB, which is recruited to the immediate upstream region following the binding of TFIIIC [44,45].

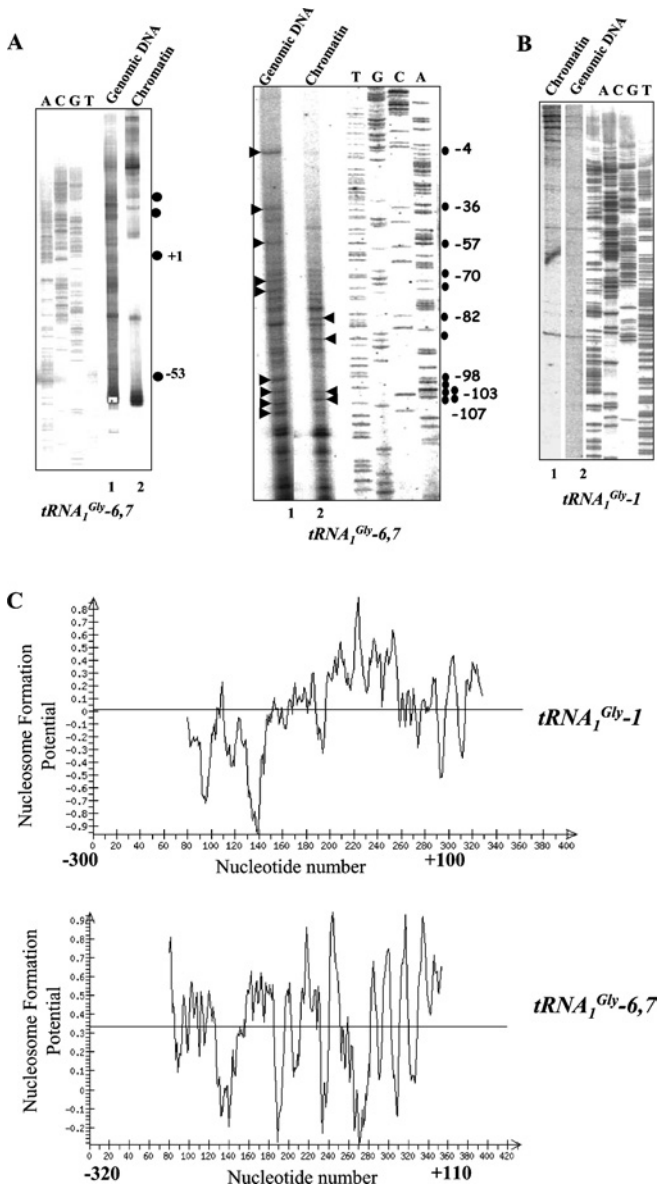

Figure 4 Nucleosomal protection on chromatin isolated *in vivo*

(**A**) The primer extension analysis of genomic DNA or *in vivo* chromatin digested with DNase I was performed using the S1 primer 3 located at position −190 nt (left panel). The sequence of the same region was generated using the primer (as shown in lanes A, C, G and T). The protected or hypersensitive sites in chromatin as compared with genomic DNA are indicated and numbered. The right panel shows the LMPCR performed using the primers, located at −786 and −662 nt and isotopic labelling using the primer at −190 nt (S1 primer 3) in the upstream region of $tRNA_1^{Gly}$*-6,7* on genomic DNA or *in vivo* chromatin digested with DNase I. The sequence ladder of the region using the same primer are marked as A, C, G and T. The protected or hypersensitive sites in chromatin in comparison with genomic DNA are marked by arrows and numbered. (**B**) The primer extension analysis of $tRNA_1^{Gly}$*-1 in vivo* chromatin or genomic DNA was performed using a primer R8 primer 1 (located at −300 nt). The sequencing ladders using the same primer are shown as A, C, G and T. (**C**) NFP. Sequences of the $tRNA_1^{Gly}$*-1* or $tRNA_1^{Gly}$*-6,7* from −300 to +100 nt for the former and −320 to +110 nt for the latter were analysed using a theoretical programme that calculates NFP of the input sequences. The upper panel represents $tRNA_1^{Gly}$*-1* and the lower panel represents $tRNA_1^{Gly}$*-6,7*. The output results are presented as nucleotide number versus NFP.

Binding of TFIIIB is impaired in the presence of nucleosome

The ICRs to which TFIIIC binds initially before the recruitment of TFIIIB are identical in both the gene copies. Since the nucleosome present in the immediate upstream region of $tRNA_1^{Gly}$*-6,7* was located at the point of assembly of the transcription complexes, it was possibly hindering the TFIIIB binding [44,45]. In order to confirm this, ChIP was attempted using a polyclonal antibody directed against the TBP component of TFIIIB. Purified preparations of TFIIIB were added to the templates after the chromatin assembly. If TFIIIB is bound, the DNA will be co-immunoprecipitated with TBP antibody. The immunoprecipitated DNA in the ChIP assay was analysed by hybridization with gene copy-specific probes (Figure 6). The DNA pulled down by the antibody in the case of $tRNA_1^{Gly}$*-6,7* (lower panel) was much less compared with the pull down in the case of $tRNA_1^{Gly}$*-1* (upper panel), although equal inputs of both the genes were utilized (Figure 6, compare lanes 1 and 3 in both panels). This pull down was absent when no external TFIIIB was added, establishing that there was no binding and precipitation due to the components of the S190 extract. The controls with no antibody also did not show precipitation with either of the genes, clearly indicating that there was no non-specific binding to the Protein A–agarose (lane 2, both panels). A control immunoprecitation was also included in

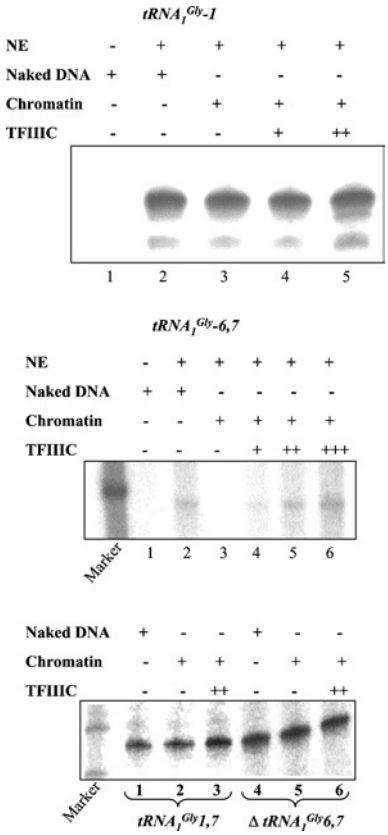

Figure 5 Transcription of chromatin templates

In vitro transcription of $tRNA_1^{Gly}$*-1* (top panel) or $tRNA_1^{Gly}$*-6,7* (middle panel) assembled *in vitro* as chromatin was performed using suboptimal concentrations of PSG nuclear extracts (lane 3, both panels) or as naked DNA templates (lane 2, both panels). A control, where the DNA was incubated with the S190 extract (but not the PSG nuclear extract) to ensure that there was no endogenous transcription due to S190 extract was also included (lane 1). Purified preparations of TFIIIC were added to the transcription reaction (lanes 4 and 5) to see the relieving of chromatin repression. The similar transcriptions were performed with a deleted construct of $\Delta tRNA_1^{Gly}$*-6,7*, from which the upstream region beyond −53 nt (with respect to the mature tRNA start; bottom panel, lanes 4–6) or with $tRNA_1^{Gly}$*-1,7*, a construct in which the 5′-upstream region of $tRNA_1^{Gly}$*-6,7* was swapped with that of $tRNA_1^{Gly}$*-1* but retaining the entire downstream region of $tRNA_1^{Gly}$*-6,7* (bottom panel, lanes 1–3), in the presence or absence of TFIIIC. For details of transcription assay, see text. + for the nuclear extract corresponds to 4 μg protein, which was suboptimal for transcriptions; +, ++ and +++ for TFIIIC denotes 4, 6 and 8 μg of protein respectively.

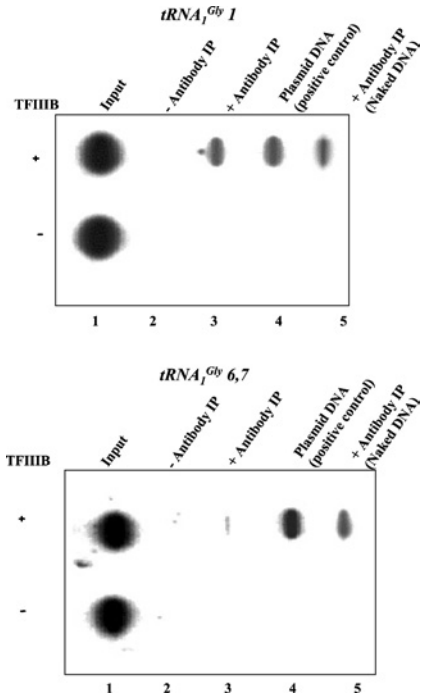

Figure 6 Efficiency of TFIIIB binding to *tRNA$_1^{Gly}$* chromatin detected by ChIP assays

In vitro ChIP assay was performed using the chromatin assembled *in vitro* on *tRNA$_1^{Gly}$-1* (top panel) or *tRNA$_1^{Gly}$-6,7* (bottom) to which the transcription factor TFIIIB was added after the assembly in the presence of suboptimal concentrations of TFIIIC. The bound TFIIIB was pulled down using a TBP antibody (cross-reactive to silkworm TBP). The immunoprecipitated materials were adsorbed to Protein A–agarose beads and the DNA in the fractions was probed with *tRNA$_1^{Gly}$-1* or *tRNA$_1^{Gly}$-6,7*. The assay was performed in the presence as well as absence of TFIIIB to account for any component of the S190 extract binding to these genes. Lanes: 1, the input DNA; 2, control (no TBP antibody); 3, chromatin immunoprecipitated (IP) with TBP antibody; 4, plasmid DNA directly probed without ChIP procedures, included as a positive control; 5, immunoprecitated as naked DNA–TFIIIB complex, to compare the binding of the factors in the chromatin context.

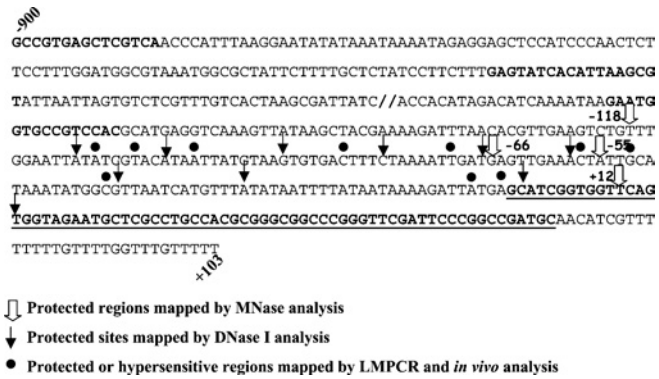

Figure 7 Protected nucleosomal regions in the immediate upstream region of *tRNA$_1^{Gly}$-6,7*

The *tRNA$_1^{Gly}$-6,7* coding sequences (bold underlined) are presented along with the 5′-upstream sequences up to − 900 nt. The sequences marked in bold correspond to the primer positions (S1, S2 and S3). The protected regions determined by MNase digestion are indicated by white arrows and numbered. The protected regions deduced by DNase I digestion are marked by black arrows. The other protected or hypersensitive regions (detected by *in vivo* analyses) are shown as black circles.

which the factors were added to the naked DNA of these gene copies and as anticipated the pull down with the antibody was identical for both (lane 5, both panels). Another control for hybridization efficiency was included using 50 ng of each of the plasmid DNAs (lane 4, both panels). The results from the ChIP assays confirmed that the assembled chromatin structure was responsible for preventing the binding of TFIIIB, which correlated to the lowered transcription of *tRNA$_1^{Gly}$-6,7*.

The protection of the nucleosome mapped in the present study by both *in vitro* and *in vivo* analyses was in the immediate upstream region of *tRNA$_1^{Gly}$-6,7* and is summarized in Figure 7. The boundaries of MNase protection extended from − 118 to + 12 nt with respect to the *tRNA* coding sequence (shown in bold and underlined). The DNase I mapping of *in vitro* chromatin and the LMPCR analysis indicated the presence of protected or hypersensitive sites within this region (white arrows, MNase protection; black arrows, DNase I protection; black circles, *in vivo* analysis). The region from − 118 to − 53 nt positioned the nucleosome strongly, because the deletion of this region abolished the negative effect of the nucleosome.

DISCUSSION

In higher eukaryotes, several genes are present in multiple copies, so that their expression levels can be modulated based on the developmental needs or tissue-specific requirement for the gene product. Of the large number of copies of *5 S rRNA* present in *Xenopus*, 23 000 are of the somatic type and approx. 120 are of the oocyte type and they differ in sequences in only approx. 5 or 6 nt in the entire coding region of 125 nt. Both somatic-type- and oocyte-specific *5 S rRNA* genes are transcribed by RNA pol III and their expression is regulated by the availability of the transcription factor TFIIIA [16,46]. The other RNA-encoding genes such as *7SK* or *VA5* transcribed by RNA pol III are also present in several thousand copies, but little is known about their transcriptional regulation. The RNA-encoding genes including large ribosomal RNAs (*28 S* and *18 S rRNA*) and *tRNA*s are also present in multiple copies, usually in the range of 10–100. Evidently it is desirable for a cell to have multiple copies of these genes, since, the end products of their expression being RNA, a second level of amplification of the gene product achieved through multiple rounds of translation of individual mRNAs in the case of RNA pol II transcripts, is not operative for RNA pol I or pol III transcripts. The only way to achieve the desired high levels of the gene product, consequently, is through multiple rounds of transcriptions. Therefore the presence of multiple copies of a particular gene will be helpful for the cell at times of enhanced need for these gene products.

In the case of *tRNA*s, the transcription machinery is relatively simple and starts with the sequence-specific binding of TFIIIC to the B box located within the coding region of the gene. This multisubunit protein through one of the subunits interacts with TFIIIB to locate it in the vicinity of the transcription start site, which in turn recruits the RNA pol III. However, this mechanism does not account for how the machinery differentially picks up the different *tRNA* genes (e.g. *tRNA^{Gly}* from *tRNA^{Ala}*), or the different genes encoding various isoacceptor species of the same tRNA (*tRNA$_1^{Gly}$* from *tRNA$_2^{Gly}$*), since each of them varies in the internal control sequences located within the coding region. Moreover, when multiple copies of genes encoding the same isoacceptor species of tRNA are present (e.g. *tRNA$_1^{Gly}$* in silkworm *B. mori* comprising of 20 copies), whether all or some individual copies within the gene family are selectively expressed is also not clear. In general, the tRNAs are considered to be house-keeping functions and therefore constitutively expressed. In the silk glands of *B. mori* for example, however, enhanced requirements of certain tRNA

isoacceptor species are warranted to optimize the committed function of the tissue (the synthesis of the silk fibre proteins). The major silk structural protein, fibroin H chain with a highly biased amino acid composition comprising nearly 48 % of all amino acids as glycine, requires large quantities of $tRNA_1^{Gly}$, which decodes the most abundant codon (1350 Gly codons decoded by $tRNA_1^{Gly}$ are present in this single messenger, with a total of 5000 codons). We have previously shown that of the 20 copies of $tRNA_1^{Gly}$ present in the silkworm, their expressions are differentially regulated both *in vitro* in silk gland nuclear extracts or *in vivo* in *B. mori* derived BmN cells [29,47]. These genes could be classified into three groups of highly transcribed (e.g. $tRNA_1^{Gly}$-*1,11*), moderately transcribed (e.g. $tRNA_1^{Gly}$-*2,3,4,5*) or barely transcribed (e.g. $tRNA_1^{Gly}$-*6,7,9,10*) genes. All of them harboured the same A and B box sequences, but the flanking regions of these genes influenced their transcription levels [25,28,48]. Certain TATA sequence-binding proteins like the P43 TBF from silk glands of *B. mori* also bind to these flanking AT-rich sequences and exert inhibitory effect on transcription [49]. However, there was no apparent tissue specificity associated with the expression of $tRNA_1^{Gly}$ copies although they might still be regulated developmentally. In the normal course of development, when the $tRNA_1^{Gly}$ species are mostly involved in the maintenance of housekeeping functions, transcription from the highly expressed copies alone might be sufficient and the other gene copies could be down-regulated or completely shut off. However, in the fifth instar of larval development, when there is demand for large excesses of a particular type of tRNA as in the PSGs and a large excess of all transcription factors are available, transcription from all the gene copies can be initiated.

When the *tRNA*s are constitutively expressed, the need for chromatin repression does not arise. In a multigene family, comprising differentially transcribed members, however, such a regulation could be operating to optimize the needs of the cell according to the developmental requirements and based on the availability of the transcription factors. The silk glands of *B. mori* serve as a suitable model to analyse the highly expressed *tRNA*s in comparison with those which are poorly expressed within the same gene family.

In the present study, we have analysed the effect of chromatin structure on the transcription status of these individual copies and focused on two of them from within the multigene family, one of which is highly expressed ($tRNA_1^{Gly}$-*1*) and the other is poorly expressed ($tRNA_1^{Gly}$-*6,7*). Sequences in the immediate 5′-upstream region of the poorly expressed gene showed a high NFP. This was also confirmed by locating the presence of a positioned nucleosome in the immediate upstream regions *in vitro* and *in vivo*. The protection observed in both the MNase and DNase I analyses gave a preliminary indication that the nucleosome might be positioned both translationally as well as rotationally on the gene. Contrary to this, the highly expressed gene did not show any distinct chromatin structure and the corresponding flanking sequences were also low in NFP. Addition of TFIIIC having a HAT activity relieved the chromatin structure and the nucleosomal footprint. The footprints due to TFIIIC binding alone were similar both in chromatin and naked DNA. Thus the positioning of the nucleosome could be a virtue of the flanking sequences, since both the $tRNA_1^{Gly}$ genes have identical internal control sequences. This was consistent with the previously published observations, that although tRNA genes were able to override certain nucleosome positioning signals, they were rendered inactive after insertion into the silent HMR locus in yeast [23]. Likewise the regular spacing of nucleosomes on tRNA genes in *Xenopus* was restricted to such transcriptionally inactive tissues as the erythrocytes [22].

The mapping of the nucleosome-protected region of $tRNA_1^{Gly}$-*6,7* between −118 and −66 nt and between −55 and +12 nt from both *in vitro* and *in vivo* analyses revealed that the regions essential for the binding of the basal transcription factors TFIIIC and TFIIIB were masked. This was further validated by the transcriptions of the gene as chromatin (under conditions where transcription factors were limiting). The transcription of the highly transcribed $tRNA_1^{Gly}$-*1* was not noticeably affected, whereas the already low transcription of the poorly transcribed $tRNA_1^{Gly}$-*6,7* was completely inhibited, when assembled as chromatin. Since, the nucleosome covered the region implicated in the binding of TFIIIC and TFIIIB, the addition of a HAT activity was anticipated to relieve the repression by the nucleosome [24]. The acetylation of the histones in the chromatin of $tRNA_1^{Gly}$-*6,7* could lead to opening of the structure and thus enhance binding of the transcription factors. In fact, TFIIIC (having HAT activity) addition to the $tRNA_1^{Gly}$-*6,7* assembled as chromatin unmasked the nucleosomal footprints, but the footprints due to TFIIIC binding itself were identical in both naked DNA and chromatin. As predicted, the addition of increasing amounts of TFIIIC relieved the repression of $tRNA_1^{Gly}$-*6,7*, whereas it had no effect on $tRNA_1^{Gly}$-*1* transcription. Consistent with these observations, deletion of the immediate upstream region of $tRNA_1^{Gly}$-*6,7*, where the positioned nucleosome was resident, resulted in the absence of repression after nucleosome assembly. This conclusion was further supported by the observation that when the entire upstream region of $tRNA_1^{Gly}$-*6,7* was replaced with that of $tRNA_1^{Gly}$-*1* (which does not harbour nucleosome positioning sequences and hence is not subject to chromatin mediated repression), the modified gene construct $tRNA_1^{Gly}$-*1,7* (in which only those sequences were exchanged) was not inhibited following chromatin assembly. From these results, we infer that the 5′-upstream region between −118 and −55 nt of $tRNA_1^{Gly}$-*6,7* is a major determinant for nucleosome positioning.

Since TFIIIB binding is through interactions with the immediate upstream regions after recruitment of TFIIIC, the inefficient binding of TFIIIB to the gene in the chromatin context could account for the poor transcription of $tRNA_1^{Gly}$-*6,7*. A ChIP was performed to monitor the binding of TFIIIB to the genes in the context of chromatin. The TFIIIC-dependent binding of TFIIIB to $tRNA_1^{Gly}$-*1* chromatin was as efficient as that of the naked DNA. However, TFIIIB binding was severely impaired in the case of $tRNA_1^{Gly}$-*6,7* when assembled into chromatin, although the binding of the factor to the naked DNA was the same for both gene copies. These results concurred with those of the transcription patterns from the chromatin templates, further supporting the model that the chromatin structure was obstructing the binding of the essential transcription factors.

Repression mediated by positioned nucleosomes operates in a wide variety of genes [2,4,50]. Positive or negative effects of positioned nucleosomes on genes transcribed by RNA pol III have been previously reported in the case of *5S RNA* and *U6 RNA* genes. A preference of factor binding over the nucleosomal structure is mainly attributed to their transcription levels. This can also be extended to a situation, such as in the $tRNA_1^{Gly}$ multigene family in *B. mori*, where the presence of nucleosomes keeps the transcription levels from several gene copies in check without interfering with the basal levels maintained through the other gene copies. However, in specific developmental stages, when the requirements for $tRNA^{Gly}$ is high, the presence of excess of transcription factors like TFIIIC could relieve the repression and transcription can be initiated from all the gene copies. Such a chromatin-mediated transcriptional regulation could operate in conjunction with other mechanisms, such as formation of unstable complexes on the poorly transcribed genes and sequestration of

essential transcription factors. There is also some preliminary evidence to suggest that the instability of the transcription complexes on *$tRNA_1^{Gly}$-6,7* could be alleviated by additional supplementation of TFIIIB (A. Parthasarthy and K. P. Gopinathan, unpublished work). Even in the case of *$tRNA^{Ala}$*, which has variants of silk-gland-specific and constitutively expressed copies, the TBP–TATA interactions are a major determinant for the differences in transcription due to critical differences in the interaction of the flanking sequences with TFIIIB [35].

A combination of theoretical and structure–function studies have thus helped in demonstrating a basic mode of repression mediated by chromatin in a tRNA multigene family. A developmental stage regulation depending on the availability of transcription factors therefore could be a general mechanism for all tRNA multigene families comprising differentially transcribed members.

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REFERENCES

1 Beato, M. and Eisfeld, K. (1997) Transcription factor access to chromatin. Nucleic Acids Res. **25**, 3559–3563
2 Wolffe, A. P. (1991) Developmental regulation of chromatin structure and function. Trends Cell Biol. **1**, 61–66
3 Wolffe, A. P. (1994) Nucleosome positioning and modification: chromatin structures that potentiate transcription. Trends Biochem Sci. **19**, 240–244
4 Wolffe, A. P. (1999) Chromatin: Structure and Function, 3rd edn, Academic Press, New York
5 Workman, J. L. and Kingston, R. E. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. **67**, 545–579
6 Wu, C. (1997) Chromatin remodeling and the control of gene expression. J. Biol. Chem. **272**, 28171–28174
7 Kadonaga, J. T. (1998) Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell (Cambridge, Mass.) **92**, 307–313
8 Owen-Hughes, T. and Workman, J. L. (1996) Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted trans-displacement of histones. EMBO J. **15**, 4702–4712
9 Studitsky, V. M., Kassavetis, G. A., Geiduschek, E. P. and Felsenfeld, G. (1997) Mechanism of transcription through the nucleosome by eukaryotic RNA polymerase. Science **278**, 1960–1963
10 Svaren, J., Klebanow, E., Sealy, L. and Chalkley, R. (1994) Analysis of the competition between nucleosome formation and transcription factor binding. J. Biol. Chem. **269**, 9335–9344
11 Shivaswamy, S., Kassavetis, G. A. and Bhargava, P. (2004) High-level activation of transcription of the yeast U6 snRNA gene in chromatin by the basal RNA polymerase III transcription factor TFIIIC. Mol. Cell. Biol. **24**, 3596–3606
12 Stunkel, W., Kober, I. and Seifart, K. H. (1997) A nucleosome positioned in the distal promoter region activates transcription of the human U6 gene. Mol. Cell. Biol. **17**, 4397–4405
13 Zhao, X., Pendergrast, P. S. and Hernandez, S. (2001) A positioned nucleosome on the human U6 promoter allows recruitment of SNAPc by the Oct-1 POU domain. Mol. Cell **7**, 539–549
14 Bogenhagen, D. F., Wormington, W. M. and Brown, D. D. (1982) Stable transcription complexes of *Xenopus* 5S RNA genes: a means to maintain the differentiated state. Cell (Cambridge, Mass.) **28**, 413–421
15 Chipev, C. C. and Wolffe, A. P. (1992) Chromosomal organization of *Xenopus laevis* oocyte and somatic 5S rRNA genes *in vivo*. Mol. Cell. Biol. **12**, 45–55
16 Felts, S. J., Weil, P. A. and Chalkley, R. (1990) Transcription factor requirements for *in vitro* formation of transcriptionally competent 5S rRNA gene chromatin. Mol. Cell. Biol. **10**, 2390–2401
17 Howe, L. and Ausio, J. (1998) Nucleosome translational position, not histone acetylation, determines TFIIIA binding to nucleosomal *Xenopus laevis* 5S rRNA genes. Mol. Cell. Biol. **18**, 1156–1162
18 Burnol, A.-F., Margottin, F., Huet, J., Almouzni, G., Prioleau, M.-N., Mechali, M. and Sentenac, A. (1993) TFIIIC relieves repression of U6 sn RNA transcription by chromatin. Nature (London) **362**, 475–477
19 Martin, M. P., Gerlach, V. L. and Brow, D. A. (2001) A novel upstream RNA polymerase III promoter element becomes essential when the chromatin structure of the yeast U6 RNA gene is altered. Mol. Cell. Biol. **21**, 6429–6439
20 Morse, R. H., Roth, S. Y. and Simpson, R. T. (1992) A transcriptionally active tRNA gene interferes with nucleosome positioning *in vivo*. Mol. Cell. Biol. **12**, 4015–4025
21 Schnell, R. and Rine, J. (1986) A position effect on the expression of a tRNA gene mediated by the SIR genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**, 494–501
22 Bryan, P. N., Hofstetter, H. and Birnstiel, M. L. (1981) Nucleosome arrangement on tRNA genes of *Xenopus laevis*. Cell (Cambridge, Mass.) **27**, 459–466
23 Lassar, A. B., Hamer, D. H. and Roeder, R. G. (1985) Stable transcription complex on a class III gene in a minichromosome. Mol. Cell. Biol. **5**, 40–45
24 Kundu, T. K., Wang, Z. and Roeder, R. G. (1999) Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Mol. Cell. Biol. **19**, 1605–1615
25 Fournier, A., Taneja, R., Gopalkrishnan, R., Prudhomme, J. C. and Gopinathan, K. P. (1993) Differential transcription of multiple copies of a silk worm gene encoding *$tRNA^{Gly}_1$*. Gene **134**, 183–190
26 Garel, J. P., Fournier, A. and Daillie, J. (1973) Functional and modulatory adaptation of tRNA in the silk gland of *Bombyx mori* L. to fibroin biosynthesis. Hoppe Seylers Z. Physiol. Chem. **354**, 600
27 Zhou, C. Z., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C., Yang, T., Jacquet, M., Janin, J., Duguet, M., Perasso, R. et al. (2000) Fine organization of *Bombyx mori* fibroin heavy chain gene. Nucleic Acids Res. **28**, 2413–2419
28 Sharma, S. and Gopinathan, K. P. (1996) Transcriptional silencing of a *$tRNA_1^{Gly}$* copy from within a multigene family is modulated by distal *cis* elements. J. Biol. Chem. **271**, 28146–28153
29 Sharma, S. and Gopinathan, K. P. (1996) Role of TATATAA element in the regulation of *$tRNA_1^{Gly}$* gene expression in *Bombyx mori* is position dependent. J. Mol. Biol. **262**, 396–406
30 Taneja, R., Gopalkrishnan, R. and Gopinathan, K. P. (1992) Regulation of glycine tRNA gene expression in the posterior silk glands of the silkworm *Bombyx mori*. Proc. Natl. Acad. Sci. U.S.A. **89**, 1070–1074
31 Young, L. S., Takahashi, N. and Sprague, K. U. (1986) Upstream sequences confer distinctive transcriptional properties on genes encoding silkgland-specific *$tRNA^{Ala}$*. Proc. Natl. Acad. Sci. U.S.A. **83**, 374–378
32 Geiduschek, E. P. and Kassavertis, G. A. (2001) The RNA polymerase III transcription apparatus. J. Mol. Biol. **310**, 1–26
33 Srinivasan, L. and Gopinathan, K. P. (2002) Characterization of RNA polymerase III transcription factor TFIIIC from the mulberry silkworm, *Bombyx mori*. Eur. J. Biochem. **269**, 1780–1789
34 Bulger, M. and Kadonaga, J. T. (1994) Biochemical reconstitution of chromatin with physiological spacing. Methods Mol. Genet. **5**, 241–261
35 Ouyang, C., Martinez, M. J., Young, L. S. and Sprague, K. U. (2000) TATA-binding protein-TATA interaction is a key determinant of differential transcription of silkworm constitutive and silk gland-specific *$tRNA^{Ala}$* genes. Mol. Cell. Biol. **4**, 1329–1343
36 Horard, B., Julien, E., Nony, P., Garel, A. and Couble, P. (1997) Differential binding of the *Bombyx* silk gland-specific factor SGFB to its target DNA sequence drives posterior-cell-restricted expression. Mol. Cell. Biol. **17**, 1572–1579
37 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbour Laboratory Press, Plainview, NY
38 Garrity, P. A. and Wold, B. J. (1992) Effects of different DNA polymerases in ligation-mediated PCR: enhanced genomic sequencing and *in vivo* footprinting. Proc. Natl. Acad. Sci. U.S.A. **89**, 1021–1025
39 Quivy, J. P. and Becker, P. B. (1993) An improved protocol for genomic sequencing and footprinting by ligation-mediated PCR. Nucleic Acids Res. **21**, 2779–2781
40 Mueller, P. R. and Wold, B. (1989) *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246**, 780–786
41 Ioshikhes, I. and Trifonov, E. N. (1993) Nucleosomal DNA sequence database. Nucleic Acids Res. **21**, 4857–4859
42 Levitsky, V. G., Podkolodnaya, O. A., Kolchanov, N. A. and Podkolodny, N. L. (2001) Nucleosome formation potential of eukaryotic DNA: calculation and promoters analysis. Bioinformatics **17**, 998–1010
43 Kundu, T. K., Palhan, V. B., Wang, Z., An, W., Cole, P. A. and Roeder, R. G. (2000) Activator-dependent transcription from chromatin *in vitro* involving targeted histone acetylation by p300. Mol. Cell **3**, 551–561

44 Kassavetis, G. A., Riggs, D. L., Negri, R., Nguyen, L. H. and Geiduschek, E. P. (1989) Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. Mol. Cell. Biol. **9**, 2551–2566
45 Kassavetis, G. A., Braun, B. R., Nguyen, L. H. and Geiduschek, E. P. (1990) *S. cerevisiae* TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell (Cambridge, Mass.) **60**, 235–245
46 Tremethick, D., Zucker, K. and Worcel, A. (1990) The transcription complex of the 5 S RNA gene, but not transcription factor IIIA alone, prevents nucleosomal repression of transcription. J. Biol. Chem. **265**, 5014–5023

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47 Srinivasan, L. and Gopinathan, K. P. (2001) Differential expression of individual gene copies from within a tRNA multigene family in the mulberry silkworm *Bombyx mori*. Insect Mol. Biol. **10**, 523–530
48 Sharma, S., Sriram, S., Patwardhan, L. and Gopinathan, K. P. (1997) Expression of individual members of a $tRNA^{Gly}_{1}$ multigene family *in vivo* follows the same pattern as *in vitro*. Gene **194**, 257–266
49 Srinivasan, L. and Gopinathan, K. P. (2002) A novel TATA-box-binding factor from the silk glands of the mulberry silkworm, *Bombyx mori*. Biochem. J. **363**, 3–13
50 Tyler, J. K. and Kadonaga, J. T. (1999) The 'dark side' of chromatin remodeling: repressive effects on transcription. Cell (Cambridge, Mass.) **99**, 43–46