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Transcriptional activation of a moderately expressed *tRNA* gene by a positioned nucleosome

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All of the members of a $tRNA_I^{Gly}$ multigene family from the mulberry silkworm, *Bombyx mori*, have identical coding regions and consequently identical internal promoter elements, but are transcribed at different levels. A moderately expressed copy, $tRNA_I^{Gly}$*-4* from within this multigene family, which was transcribed to 30–50 % of the highly transcribed gene copies harboured two typical TATAA box sequences in the 5′ upstream region at positions − 27 nt and − 154 nt with respect to the + 1 nt of mature tRNA. Deletion of the distal TATAA sequence at − 154 nt brought down the transcription more than 70 %, whereas mutation of the proximal element did not affect transcription. $tRNA_I^{Gly}$*-4* could be readily assembled into chromatin, with a positioned nucleosome in the upstream region, and the assembled nucleosome formed stable complexes with the transcription factors TFIIIC and TFIIIB. Organization of the gene into nucleosomes also enhanced transcription significantly above that of the naked DNA, reaching transcription levels comparable with those of the highly transcribed copies. This nucleosome-mediated enhancement in transcription was absent when the distal TATAA sequences were deleted, whereas mutation of the proximal TATAA element showed no effect. In the absence of the distal TATAA sequences, assembly into the nucleosome inhibited transcription of $tRNA_I^{Gly}$*-4*. TFIIIB bound directly through the distal TATAA sequence at − 154 nt and the positioned nucleosome facilitated its interaction with TFIIIC. The direct binding of TFIIIB to the DNA provided anchoring of the factor to the template DNA which conferred a higher stability on the TFIIIB–TFIIIC–DNA complex. We have proposed a novel mechanism for the nucleosome-mediated stimulation of pol III (RNA polymerase III) transcription of tRNA genes, a model not presented previously.

Key words: RNA pol III transcription, multigene family, positioned nucleosome, moderately expressed gene, transcriptional activation, chromatin immunoprecipitation.

INTRODUCTION

Chromatin structure plays a passive role in transcriptional regulation by compaction of the DNA which prevents the basal transcription machinery from accessing the template. When specific transcription factors bind to the DNA within a nucleosome, a chain of events is initiated that eventually allows the transcription machinery to gain access to the DNA [1,2]. The regulated transcription of a gene thus depends on transcription factors functioning properly in a chromatin context. In order to overcome the repressive effects that arise from chromatin architecture there are remodellers such as the SWI/SNF complex that rearrange nucleosomal structures and facilitate enhanced binding of transcription factors [2].

Transcription of several genes is stimulated by activators that bind to distant enhancer elements located as far as 100 kb from the promoter [3]. Activation often involves a direct interaction between proteins bound to either site while the intervening DNA sequence loops out [4–6]. Packaging of such regions into nucleosomes facilitates transcription by bringing the regulatory elements otherwise separated by long distances into close proximity. For instance, in *hsp26* of *Drosophila melanogaster*, a positioned nucleosome folds the DNA to facilitate interactions between the heat-shock factors bound to the different heat-shock elements [7]. Similarly, a nucleosome juxtaposing enhancer and promoter elements is found in the *Drosophila Adh* gene [8]. The *hsp27* promoter from *D. melanogaster* is yet another example where a positioned nucleosome shortens the distance between the TATA box and two heat-shock elements [9]. In *Xenopus laevis* also, the expression of a vitellogenin B1 gene is enhanced by a positioned nucleosome that creates a static loop, bringing the two regulatory elements into juxtaposition, enabling interaction between the transcription factors [5].

Similar influences have also been demonstrated in the case of two pol III (RNA polymerase III)-transcribed genes. In human U6 snRNA (small nuclear RNA), a nucleosome positioned in the distal promoter region activated transcription [4]. Likewise, a positioned nucleosome between the A and B boxes of the *Saccharomyces cerevisiae* U6 snRNA gene (*SNR6*) activated its transcription by aligning the suboptimally spaced boxes properly [10].

The transcription machinery for tRNA genes is relatively simple and involves the two basal transcription factors, TFIIIB and TFIIIC, apart from pol III [11]. The promoters for the tRNAs are typified by the presence of A and B boxes, located internally within the coding regions. TFIIIC binds first to the promoter elements and recruits the TFIIIB through protein–protein interactions to the transcription initiation region. This complex recruits pol III. The tRNA genes, being mostly constitutive, are known to be devoid of chromatin structure [12–14], although some evidence indicates the contrary [15,16].

The silk glands of the mulberry silk worm, *Bombyx mori*, serve as a good model to study differential gene expression. The fifth instar larval stage is characterized by increased synthesis of silk fibre proteins. In order to optimize the process, a functional

Abbreviations used: ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; MNase, micrococcal nuclease; pol III, RNA polymerase III; PSG, posterior silk gland; snRNA, small nuclear RNA; TBP, TATA-box-binding protein.

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The nucleotide sequence data reported for *Bombyx mori* $tRNA_1^{Gly}$*-4* gene will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AM050560.

adaptation takes place in the PSGs (posterior silk glands) as reflected by increases in the tRNA populations which decode the most frequently represented codons in the fibroin (H) chain mRNA [17,18]. The levels of $tRNA_I^{Gly}$, which decodes the most predominant glycine codon GGU/C present 1350 times in the 5000-nt-long fibroin transcript, goes up several times. In *B. mori*, $tRNA_I^{Gly}$ is encoded by a multigene family comprising 20 copies of $tRNA_I^{Gly}$, of which 11 have been characterized previously by our group [19–23]. All of these gene copies have identical coding regions and consequently the same A and B box sequences, but they are differentially transcribed. Certain *cis*-sequence elements present in the 5′ upstream and 3′ downstream regions of the coding sequences regulated their transcription by exerting positive or negative effects. Based on the *in vitro* transcription patterns, these genes could be classified into groups of highly ($tRNA_I^{Gly}$*-1* and *-11*), moderately ($tRNA_I^{Gly}$*-2*, *-3*, *-4* and *-5*) and poorly ($tRNA_I^{Gly}$*-6*, *-7*, *-8*, *-9* and *-10*) transcribed copies.

A representative gene, $tRNA_I^{Gly}$*-4*, from the moderately transcribed group has been analysed in the present study. We demonstrate the presence of *cis*-regulatory elements and the presence of a positioned nucleosome in the 5′ upstream region of the gene copy that enhanced its transcription.

EXPERIMENTAL

$tRNA_1^{Gly}$ constructs

Several $tRNA_I^{Gly}$ constructs used were from our laboratory stock [19,22]. $tRNA_I^{Gly}$*-4* is a moderately transcribed gene copy (in plasmid pBmH1), comprising 600 bp upstream and 40 bp downstream of the tRNA-coding region. $tRNA_I^{Gly}$*-4 Δ-145* (in plasmid pΔH1) is a deletion derivative of $tRNA_I^{Gly}$*-4*. It was generated from the parental gene by subcloning a DraI fragment (DraI sites located at − 145 nt and + 110 nt) in pBSKS+. This was done to eliminate the TATAA sequence at 145 nt. $tRNA_I^{Gly}$*-11* (in plasmid pBmG1) was the highly transcribed copy with 2.7 kb upstream and 800 bp downstream sequences of the tRNA coding region. The plasmid construct pR8-10 harboured $tRNA_I^{Gly}$*-1* with a 10 bp insertion in the coding region immediately following the B box sequences which did not cause any significant differences in transcription and gave rise to a 10-nt-longer transcript [23]. This construct was used in transcription competition studies to readily distinguish the competing transcripts. A mutant construct (plasmid pmutTATAH1), in which the proximal TATAA sequence at − 27 nt in $tRNA_I^{Gly}$*-4* was mutated to GATATC, was generated from the parental gene by utilizing a mutated primer and cyclic PCR (5′-CAATTTACTATTTGATATCATCATCA-3′). pmutTATAΔ-145 was the deletion derivative of the above mutated copy from which the distal TATAA sequence at − 154 nt was also deleted.

Purification of transcription factors TFIIIC and TFIIIB

The transcription factors TFIIIB and TFIIIC were purified from the *B. mori* silk gland nuclear extracts as described previously [24]. The TFIIIC preparation was the phosphocellulose fraction. For TFIIIB, the phosphocellulose fraction was purified further by chromatography on a heparin–Sepharose column to separate the activity from that of pol III [25]. All of the fractions were dialysed to a final concentration of 0.1 M KCl.

Chromatin assembly

Chromatin lacking histone HI was assembled on the plasmids pBmH1, pΔH1 and pBmG1 in the presence of the core histones and the S-190 extract from *Drosophila* embryos [26]. 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 protein of S-190 in 200 μl of 10 mM Hepes buffer, pH 7.5, containing 7 mM $MgCl_2$, 30 mM NaCl, 3 mM ATP, 8.5 mM α-glycerophosphate, 30 mM creatine phosphate and 1 μg/ml creatine kinase. The transcription factors TFIIIC and TFIIIB were added either 30 min before the assembly reaction or at the end of the assembly process (30 min before the termination). 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 carried out using 125 ng of chromatin assembled *in vitro* or the naked DNA by digestion with MNase [$(7–70) \times 10^{-3}$ 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. The digested products were deproteinized by phenol extraction and were subjected to primer extension using $Vent_R$® (exo-) DNA polymerase (New England Biolabs) and 5′ ^{32}P-end-labelled primer. The primer was designed for the upstream region at position − 183 nt [H1Primer 2 (top strand, 5′-ACCTAACTGAAGTATTGACGG-3′)]. The samples were deproteinized following the primer-extension reaction, and the products were analysed by electrophoresis on 7 M urea/6 % polyacrylamide gels. The profiles of the footprints were generated using the Image Gauge program of the PhosphorImager (Molecular Dynamics).

Theoretical analysis of nucleosomal positioning

The nucleosomal formation potential of the $tRNA_I^{Gly}$*-4* gene was analysed theoretically using a program that calculates the nucleosome formation potential of a particular sequence derived by discriminant analysis of dinucleotide frequencies with the trained set of nucleosome positioning sequences in the nucleosomal DNA database [27,28]. The dinucleotide relative abundance distance was chosen as an additional restriction for input data to exclude the sequences with a poor dinucleotide content [27,28]. The output values were represented graphically.

In vitro transcription assays

Nuclear extracts from PSGs of *B. mori* in the fifth larval instar were prepared as described previously [20,21]. The protein contents were determined by the dye-binding method [29]. *In vitro* transcription reactions were carried out 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 creatine phosphate, 50 μM each of ATP, CTP and UTP, 10 μM GTP, 5 μCi of [α-^{32}P]GTP (3000 Ci/mmol), PSG nuclear extract (4–8 μg of protein) and 100 ng of DNA template (naked DNA or chromatin), incubated at 30 °C for 1 h. Increasing amounts of purified TFIIIC fraction were added to the freshly assembled chromatin wherever indicated and were incubated for 20 min at 30 °C before the addition of the nuclear extract. For single-round transcriptions, incubations were carried out initially for 10 min in the absence of non-radioactive GTP and a further 50 min after adding 10 μM GTP and heparin (100 μg/ml) [30]. Competition for transcription factors was performed as in the standard transcription reactions at suboptimal concentrations of nuclear extract (4 μg of protein) so that the transcription factors were limiting and the transcription levels could be enhanced by external supplementation with the transcription factors TFIIIC or TFIIIB. The $tRNA_I^{Gly}$ templates

used in competition were the moderately transcribed *tRNA*$_I^{Gly}$*-4* and the highly transcribed *tRNA*$_I^{Gly}$*-1* (in plasmid pR8-10, which harboured *tRNA*$_I^{Gly}$*-1* with a 10 nt insertion, giving rise to a longer transcript without compromising on transcriptional activity). The transcription reactions were terminated by the addition of 0.2 % (w/v) SDS, 10 mM EDTA and 100 μg/ml glycogen and were analysed by electrophoresis on 7 M urea/8 % polyacrylamide gels.

Heparin-resistant TFIIIB complex formation

The stability of the interaction between TFIIIB and the *tRNA*$_I^{Gly}$*-4* gene was examined by the formation of heparin-resistant complexes [30]. A 700 bp HindIII fragment from plasmid pBmH1 (containing full-length *tRNA*$_I^{Gly}$*-4*) or a 255 bp DraI fragment from the same plasmid (from -145 nt to $+110$ nt with respect to the *tRNA*$_I^{Gly}$*-4* coding region) were radioactively labelled by end-labelling or random priming and were used as probes [29]. The binding reaction contained, in a 20 μl volume, radiolabelled probe (60 000 c.p.m.), 4 μg of poly(dG-dC) · (dG-dC), 100 ng of pBR322 DNA, 70 mM KCl, 4 mM $MgCl_2$, 13 % (v/v) glycerol, 3 mM dithiothreitol and 30 mM Tris/HCl, pH 7.5, 6 μg of TFIIIC and/or 6 μg of TFIIIB. After incubation for 1 h at 4 °C, 20 μg/ml heparin was added and the incubation was continued for 5 min. The complex formation was analysed by electrophoresis on non-denaturing 4 % polyacrylamide gels and visualized using a PhosphorImager.

Footprinting of TFIIIB on *tRNA*$_1^{Gly}$*-4* gene

A 300 bp DNA probe for footprinting was generated by digesting the *tRNA*$_I^{Gly}$*-4* gene with RsaI and EcoRI and end-filling with [α^{32}-P]dATP (3000 Ci/mmol). The end-labelled fragment (20 000 c.p.m.) was incubated with TFIIIC and TFIIIB (4 μg each) in 25 μl of 80 mM NaCl, 3 mM dithiothreitol and 10 % (v/v) glycerol in 40 mM Tris/HCl, pH 8.0, for 30 min at 4 °C. DNaseI digestions were carried out using 3 ng and 6 ng of the enzyme for 30 s in the presence of 7 mM $MgCl_2$ and 5 mM $CaCl_2$. The reactions were terminated by the addition of 10 mM Tris/HCl, pH 8.0, 3 mM EDTA and 0.2 % SDS. The samples were deproteinized by extraction with phenol and analysed by electrophoresis on 7 M urea/6 % polyacrylamide gels.

ChIP (chromatin immunoprecipitation) assay

For ChIP assays [31], TFIIIB fraction (6 μg) was added together with TFIIIC (6 μg) to the DNA template either 30 min before the assembly or at 4.5 h during the 5 h assembly process. After assembly, the chromatin was digested with MNase as described above. The samples were diluted 10-fold with dilution buffer (16.7 mM Tris/HCl, pH 8.0, 0.01 % SDS, 2 % Triton X-100, 1.2 mM EDTA and 167 mM NaCl) and were pre-cleared 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 was immunoprecipitated using a polyclonal cross-reactive antibody directed against the TBP (TATA-box-binding protein) component of TFIIIB. After 1 h, Protein A–agarose beads were added to the samples and left overnight at 4 °C with shaking. The immune complexes were recovered by centrifugation of the agarose beads at 4000 ***g*** for 30 s. The supernatant from the control reaction (identical with the above reaction mixture, except that no antibody was added) was used as the input. The beads were washed sequentially with a low-salt solution (150 mM NaCl in 20 mM Tris/HCl, pH 8, containing 0.1 % SDS, 1 % Triton X-100 and 2 mM EDTA) and high-salt solution (500 mM NaCl in the same buffer), followed by 10 mM Tris/HCl, pH 8, containing 250 mM LiCl, 1 % Nonidet P40, 1 % sodium deoxycholate and 1 mM EDTA and finally twice with 10 mM Tris/HCl, pH 8, containing 1 mM EDTA. The immune complexes 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. To serve as a control for the reaction, parallel immunoprecipitations were performed using the naked DNA template incubated for 4.5 h with TFIIIC and TFIIIB. The blots were probed using ^{32}P-labelled *tRNA*$_I^{Gly}$*-4* (the HindIII fragment from clone pBmH1 corresponding to -600 nt to $+113$ nt beyond the tRNA-coding region).

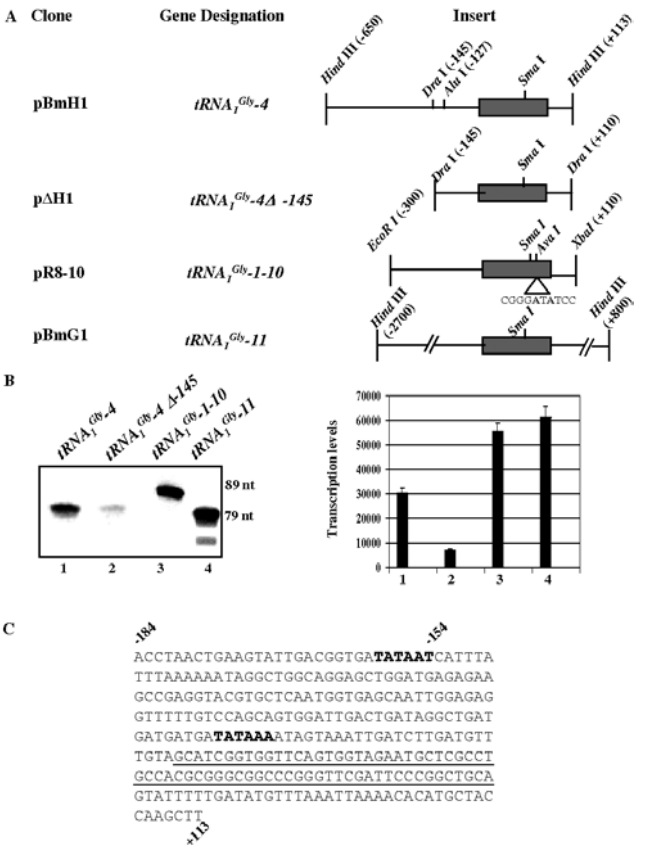

Figure 1 ***tRNA*$_1^{Gly}$ gene constructs and their *in vitro* transcription status**

(**A**) All of the plasmid constructs were in pBSSK+ vector. The 70 nt tRNA-coding region (shown as shaded boxes) is identical in all gene copies. The co-ordinates for flanking regions are marked with respect to +1 nt of mature tRNA. For details of the constructs, see text. (**B**) *In vitro* transcription patterns in PSG nuclear extracts. The sizes of the labelled transcripts are marked and their quantifications are shown. (**C**) The sequence of the moderately expressed *tRNA*$_I^{Gly}$*-4* gene (GenBank® accession number AM050560; shown up to -184 nt with respect to the start site). The two TATA sequence elements at -27 nt and -154 nt are indicated in bold. The coding region is underlined.

RESULTS

tRNA$_1^{Gly}$ gene constructs

The *tRNA*$_I^{Gly}$ gene copies used in the present study are shown schematically in Figure 1. All copies of the *tRNA*$_I^{Gly}$ genes from *B. mori* have identical coding regions [19]. *tRNA*$_I^{Gly}$*-4* present in the plasmid construct pBmH1 comprising 600 bp upstream and 40 bp downstream of the tRNA-coding region was transcribed approx. 30–40 % compared with the highly expressed gene copy *tRNA*$_I^{Gly}$*-11* and gave rise to a single predominant transcript of

79 nt (Figure 1B, left-hand panel). The transcription of *tRNA$_1^{Gly}$-11* (in plasmid pBmG1) was approx. 20 % higher than that of *tRNA$_1^{Gly}$-1* used in most of our previous analyses.

tRNA$_1^{Gly}$-11 gave rise to one predominant transcript of 79 nt and a smaller less predominant processed transcript (Figure 1B, left-hand panel). The other construct pR8-10 shown harboured *tRNA$_1^{Gly}$-1-10*, *tRNA$_1^{Gly}$-1* with a 10 bp insertion within the coding region immediately following the B box [23]. This derivatized *tRNA$_1^{Gly}$-1-10* was transcribed at levels comparable with that of the parent, but gave rise to a larger transcript of 89 nt (owing to the decameric oligonucleotide insertion; Figure 1B, left-hand panel). This construct facilitated the differentiation of the transcripts arising from the different gene copies in mixed template experiments. The quantifications are given in the right-hand panel of Figure 1(B).

The analysis of the *tRNA$_1^{Gly}$-4* flanking sequences indicated the presence of two AT-rich sequences (TATAA) at -27 and -154 nt with respect to the $+1$ of the mature tRNA (Figure 1C). An upstream deletion beyond -145 nt which eliminated the distal TATAA sequence (plasmid pΔH1) brought down the transcription of the gene by more than 60–70 % (Figure 1B; compare the first two lanes).

Chromatin assembly on *tRNA$_1^{Gly}$-4*

The *tRNA$_1^{Gly}$-4* could be efficiently assembled into nucleosomes *in vitro* using the *Drosophila* S190 extract and the core histones. The assembled chromatin when digested with MNase showed the presence of nucleosome ladders (see Supplementary Figure at http://www.BiochemJ.org/bj/396/bj3960439add.htm). Chromatin was also assembled on *tRNA$_1^{Gly}$-4 Δ-145*, the mutant construct, and the highly expressed *tRNA$_1^{Gly}$-11* gene in a similar manner.

Presence of a positioned nucleosome in the immediate upstream region of *tRNA$_1^{Gly}$-4*

Analysis for the presence of the nucleosome in the near upstream region of *tRNA$_1^{Gly}$-4* by footprinting using MNase and DNase I is presented in Figure 2(A). Naked DNA incubated with the S190 extract (but without nucleosome assembly) was included as a control to rule out any non-specific protection conferred by the S190 extract. There were distinct regions of protection in both the MNase- and DNase I-treated chromatin compared with the naked DNA (Figure 2A; left- and right-hand panels respectively; compare lanes 1 and 2 in each panel). The protected regions in chromatin from MNase digestion mapped from -94 to -43 nt and from -18 to $+70$ nt (with respect to the $+1$ of the tRNA-coding region). The DNase I digestions also indicated the presence of clear footprints at several locations between -16 and $+22$ nt, -61 and -33 nt, and at positions -93, -79 and -70 nt.

The scanning profiles of the MNase- and DNase I-digested chromatin in comparison with the naked DNA are also shown (Figure 2B). The protected regions mapped by MNase digestions (predominantly between -94 and -43 nt, and -18 and $+70$ nt) are indicated (bold underlines).

Transcription from templates assembled as chromatin

Since the presence of nucleosome was detected in the immediate upstream region of *tRNA$_1^{Gly}$-4*, the transcriptional competence of the assembled chromatin was determined. The transcription of *tRNA$_1^{Gly}$-4* was increased more than 2-5-fold in the chromatin context as compared with transcription of naked DNA (Figure 3A, top-left-hand panel, compare lanes 2 and 3) and this was restored to naked DNA levels when the chromatin structure was

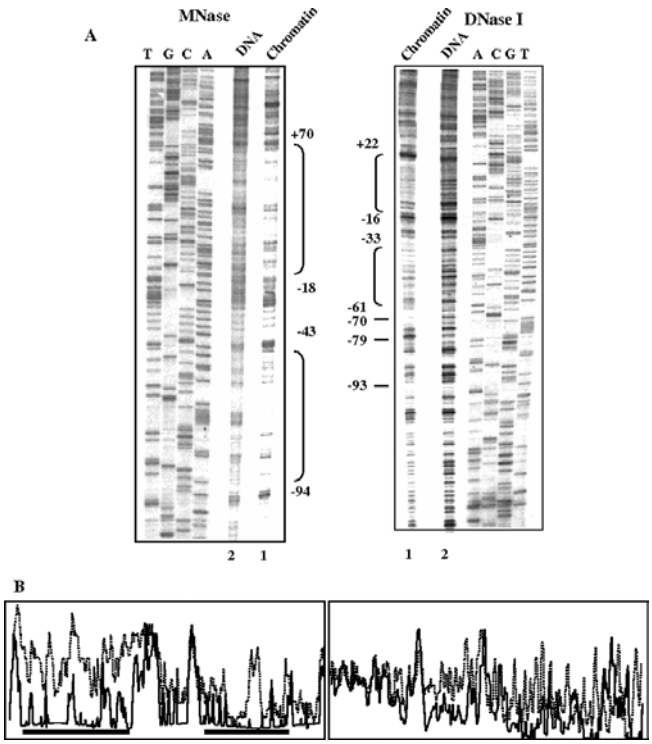

Figure 2 Nucleosome protection in the immediate upstream region of *tRNA$_1^{Gly}$-4*

(**A**) Primer extension analysis was carried out on chromatin assembled on *tRNA$_1^{Gly}$-4* after digestion with MNase (left-hand panel) or DNase I (right-hand panel) using a ^{32}P 5′-end-labelled primer positioned at -183 nt. 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 presence of the nucleosome from both MNase and DNase I digestions are indicated at the side. The MNase footprintings were carried out using four different concentrations of the enzyme, but only those showing the best footprints of four typical digestions are presented. (**B**) The profiles of the nucleosome footprints on *tRNA$_1^{Gly}$-4*, generated using the Image Gauge program (left: MNase footprint; right: DNase I footprint). Broken and continuous lines correspond to DNA and chromatin respectively.

disrupted by additional amounts of TFIIIC possessing HAT (histone acetyltransferase) activity (Figure 3A, top-left-hand panel, compare lanes 4 and 5 with lane 2). The quantifications are an average for three independent experiments. These results indicated that the nucleosome positioned on the gene was exerting a positive effect on transcription, enhancing the levels comparable with those of the highly transcribed copies. In contrast, the already low transcription of the upstream-deleted derivative of *tRNA$_1^{Gly}$-4* was completely abolished when assembled as chromatin (Figure 3A, top-right-hand panel, compare lanes 2 and 3). However, external supplementation of TFIIIC rescued the transcription to the same levels as the naked DNA (Figure 3A, top-right-hand panel, compare lanes 4 and 5 with lane 2). These observations indicated that same nucleosome assembly in the absence of the more upstream sequences (inclusive of the TATAA element) repressed transcription. The positioned nucleosome therefore exerted a positive effect in the parental construct possibly by bringing the distal TFIIIB-binding sequences closer to the transcription start site. Mutation of the proximal TATAA sequence to GATATC in *tRNA$_1^{Gly}$-4* did not influence its transcription as naked DNA templates (Figure 3A, bottom panel, lane 1). Moreover, the increased transcription on the assembled chromatin with this TATAA mutant construct was similar to that of the parental gene (Figure 3A, bottom panel, compare lanes 1 and 2) and, once again, supplementation with additional amounts of

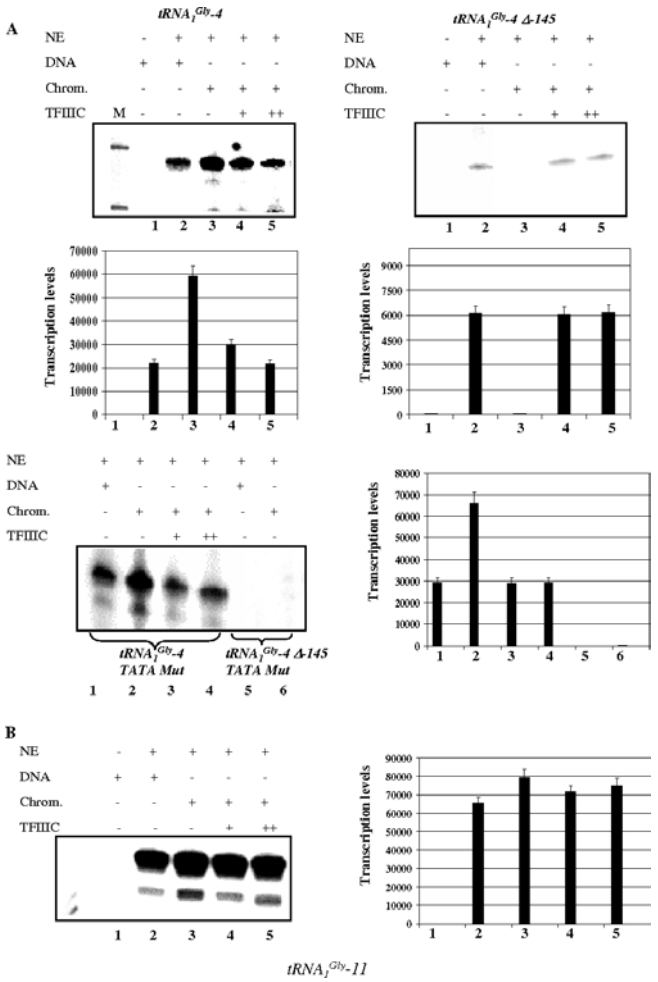

Figure 3 ***In vitro*** **transcription of assembled chromatin of** ***tRNA*** $_1^{Gly}$ **-4**

(**A**) *In vitro* transcription of full-length *tRNA* $_1^{Gly}$ *-4* (top-left-hand panel), its upstream deleted derivative (top-right-hand panel), a mutant construct in which the proximal TATAA was mutated to GATATC, or this mutant from which the distal TATAA element was deleted (bottom panel) are shown. The transcription from the naked DNA templates (lanes 2, top panels; lanes 1 and 5, bottom panel) or after nucleosome assembly (lanes 3, top panels; lanes 2 and 6, bottom panel) are presented. Controls without PSG nuclear extract (NE) are shown in lane 1 in top panels. Suboptimal concentrations of PSG nuclear extracts were used so that the effects of external supplementation of transcription factors could be readily visualized. Partially purified TFIIIC was supplemented to the transcription reaction (lanes 4 and 5, top panels; lanes 3 and 4, bottom panel) to see the relieving of chromatin (Chrom.)-mediated repression/stimulation. + for nuclear extract corresponds to 4 μg of protein; + and ++ for TFIIIC denotes 4 and 6 μg of protein. Lane M, pTZ HinfI marker. The quantification of transcription is shown beneath the top panels or to the right of the bottom panel. (**B**) *In vitro* transcription assay for a highly expressed gene, *tRNA* $_1^{Gly}$ *-11*, after chromatin assembly. Lane 1, control (without PSG nuclear extract); lane 2, naked DNA transcription; lanes 3–5, transcription of chromatin templates in the absence or presence of externally supplemented TFIIIC.

TFIIIC resulted in the loss of enhanced chromatin transcription (Figure 3A, bottom panel, lanes 3 and 4). Deletion of the distal TATAA from this mutated construct abolished the transcription of the gene completely either as naked DNA or as chromatin template (Figure 3A, bottom panel, lanes 5 and 6). The distal TATAA sequence thus appeared to be more crucial for transcription than the proximal TATAA sequence.

To establish that the chromatin effect on *tRNA* $_1^{Gly}$ *-4* transcription was genuine and specific, transcriptions were also carried out with a highly expressed gene *tRNA* $_1^{Gly}$ *-11* following nucleosome assembly. The transcription of *tRNA* $_1^{Gly}$ *-11* was not significantly affected, and the differences between the naked DNA

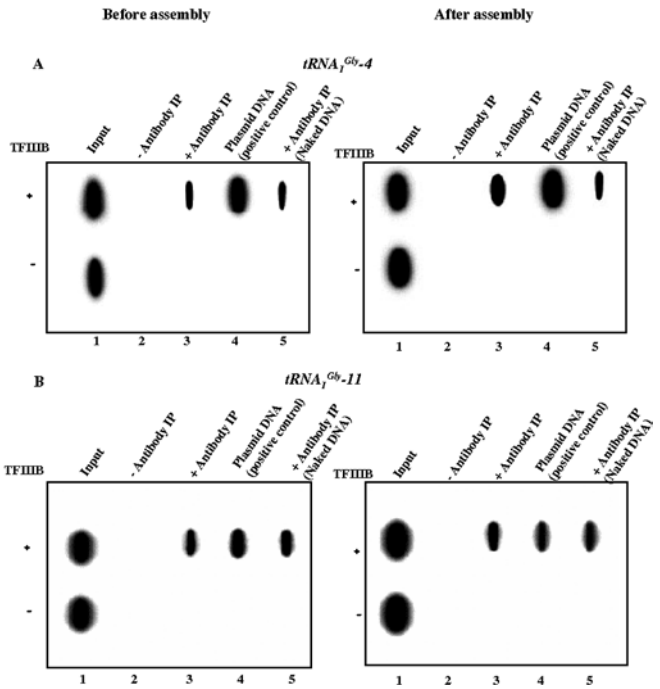

Figure 4 Efficiency of TFIIIB binding to ***tRNA*** $_1^{Gly}$ **assembled as chromatin**

In vitro ChIP assay was carried out using the chromatin assembled *in vitro* on *tRNA* $_1^{Gly}$ *-4* (**A**) or *tRNA* $_1^{Gly}$ *-11* (**B**), TFIIIB was added either before (left-hand panels) or after (right-hand panels) the assembly in the presence of TFIIIC. The bound TFIIIB was pulled-down using anti-TBP antibody. The immunoprecipitates (IP) were adsorbed on to Protein A–agarose beads, and the DNA in the fractions was probed with *tRNA* $_1^{Gly}$ *-4* or *tRNA* $_1^{Gly}$ *-11*. Lanes 1, input DNA; lanes 2, control (no antibody); lanes 3, chromatin immunoprecipitated with anti-TBP antibody; lanes 4, plasmid DNA directly probed without ChIP procedures included as a control to monitor the hybridization efficiency; lanes 5, immunoprecipitated naked DNA–TFIIIB complex.

and chromatin transcriptions were only 20–25 % (Figure 3B, lanes 2 and 3), which were much less compared with the chromatin effect on *tRNA* $_1^{Gly}$ *-4*. Even in this case, additional amounts of TFIIIC (with associated HAT activity [24,25]) brought the chromatin transcription to those of naked DNA levels (Figure 3B, lanes 4 and 5). Controls with just the *Drosophila* S190 extract were always included in these experiments to ensure that by itself (in the absence of the PSG nuclear extract) it had no transcriptional activity (lane 1 in Figures 3A, top panels, and 3B).

TFIIIB binding is enhanced in a chromatin context

In order to confirm further that the positive effect of the nucleosome on transcription was through TFIIIB binding, ChIP analysis was performed. A polyclonal antibody directed against TBP, a component of the TFIIIB, was used for immunoprecipitation. There was a 2–5-fold increase in TFIIIB binding (as evident by the amount of DNA that was pulled-down with the antibody) when TFIIIB/TFIIIC were added to the assembled chromatin on *tRNA* $_1^{Gly}$ *-4* (Figure 4A, compare lanes 3). This was in agreement with the fact that transcription was more efficient when the template was in a chromatin context. A similar analysis was also performed on the highly expressed *tRNA* $_1^{Gly}$ *-11* gene, which did not show any nucleosomal effect on transcription. The TFIIIB pull-down in the case of *tRNA* $_1^{Gly}$ *-11* was very similar, irrespective of whether or not the nucleosome was assembled (Figure 4B, compare lanes 3). In the control experiments, when external TFIIIB was not added, the pull-down was absent, confirming that there was no binding of or precipitation due to components

of the S190 extract (Figure 4B, lower rows in each panel). The controls with no antibody were also included (Figure 4, lanes 2) which clearly showed that there was no non-specific binding to the Protein A–agarose. Controls in which the factors were added on to the naked DNA of both the gene copies (without chromatin assembly) were also included. As anticipated, the pull-downs with the antibody were identical, irrespective of whether TFIIIB/TFIIIC were added before or after incubation with S190 extracts (but no chromatin assembly) in both instances (Figure 4, lanes 5). A positive control to monitor the hybridization, each consisting of the plasmid DNAs (50 ng), were also included (Figure 4, lanes 4). A representative experiment of three independent experiments is shown.

Stability of transcription complexes on *tRNA$_1^{Gly}$-4*

The stability of the transcription complexes on *tRNA$_1^{Gly}$-4* was examined by analysing the formation of heparin-resistant TFIIIB–DNA complexes on the parental and the upstream-deletion derivative of *tRNA$_1^{Gly}$-4*. Each of them showed binding to TFIIIC (Figure 5A, lanes 2) and, on addition of TFIIIB, this complex showed a further shift in mobility (Figure 5A, lanes 3). The TFIIIC binding was completely dissociated in the presence of heparin (Figure 5A, lanes 4). However, the TFIIIB–TFIIIC complex was still detectable for *tRNA$_1^{Gly}$-4* in the presence of heparin (Figure 5A, left-hand panel, lane 5), whereas this complex was unstable and fully dissociated from the upstream deleted *tRNA$_1^{Gly}$-4* (Figure 5A, right-hand panel, lane 5). The undissociated complex was due to the TFIIIB that was still bound to the gene even after the TFIIIC was stripped out. These results correlated with the involvement of the distal upstream sequence in the transcription of *tRNA$_1^{Gly}$-4*. Evidently, the TFIIIB–TFIIIC complexes on each of the constructs were specific because they were successfully competed out by 10- and 100-fold excess of the unlabelled DNA (Figure 5B, lanes 2–4), but not by a non-specific competitor DNA (Figure 5B, lanes 5 and 6). A mutation of the proximal TATAA sequence, but still retaining the distal TATAA, showed efficient binding to both TFIIIC and TFIIIB (Figure 5C, left-hand panel, lanes 2 and 3). However, the deletion of the distal TATAA showed binding to TFIIIC, but the binding of TFIIIB was abolished (Figure 5C, right-hand panel, lanes 2 and 3).

The comparative efficiency of these transcription complexes in fostering transcription of the highly transcribed *tRNA$_1^{Gly}$-1* copy (with a 10 nt insertion) and moderately transcribed *tRNA$_1^{Gly}$-4* was examined by single- and multiple-round transcriptions. This gene was taken rather than *tRNA$_1^{Gly}$-11* because it gave rise to a 10 nt longer transcript and thus could be distinguished from the transcript arising from *tRNA$_1^{Gly}$-4*. The transcriptions of the *tRNA$_1^{Gly}$-1* and *tRNA$_1^{Gly}$-4* were nearly identical in single-round transcriptions (Figure 6A, right-hand panel), whereas *tRNA$_1^{Gly}$-4* was transcribed less in multiple-round transcriptions, normally between 40 and 60% of the *tRNA$_1^{Gly}$-1* transcription levels (Figure 6A, left-hand panel). These results indicated that the initial binding of the transcription factors to the genes was equally efficient and therefore the transcription complex stability on *tRNA$_1^{Gly}$-4* was less, resulting in lower transcription levels in multiple rounds.

When *tRNA$_1^{Gly}$-1* and *tRNA$_1^{Gly}$-4* were present together in the transcription assays, the transcription levels of each were substantially reduced (Figure 6B, compare lane 3 with lanes 1 and 2). External supplementation of TFIIIC or pol III did not rescue this inhibition (Figure 6B, lanes 4 and 6 respectively), but addition of TFIIIB substantially increased the transcription almost to the same levels to which they were transcribed individually (Figure 6B, compare lane 5 with lanes 1 and 2). In fact, the recovery of

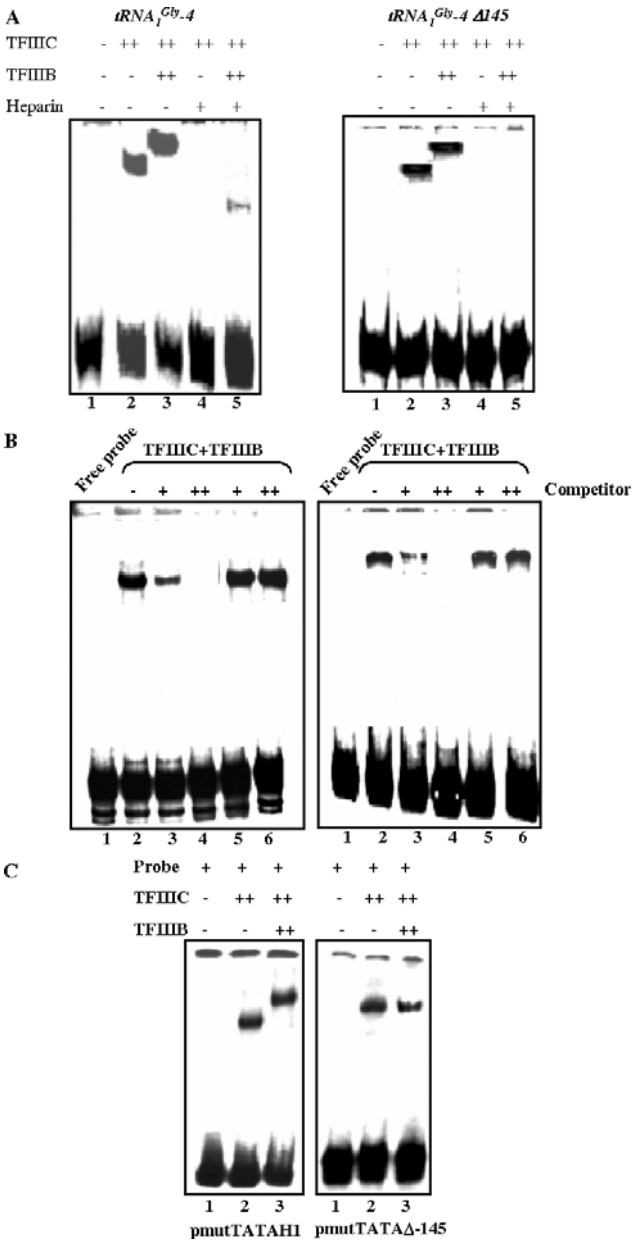

Figure 5 Stability of transcription complexes on the *tRNA$_1^{Gly}$-4* gene

(**A**) The stability of the transcription complexes on the *tRNA$_1^{Gly}$-4* gene was tested by its ability to form TFIIIC–TFIIIB complexes in the presence of heparin. Radioactively labelled probes (*tRNA$_1^{Gly}$-4* as a 700 bp HindIII fragment from plasmid pBmH1 from −650 to +113 nt) or the upstream-deleted derivative of *tRNA$_1^{Gly}$-4* as a 255 bp DraI fragment (from −145 to +110 nt of *tRNA$_1^{Gly}$-4*) were incubated with TFIIIC and TFIIIB. The stability of DNA–TFIIIC and TFIIIB–TFIIIC–DNA complexes on each of the probes was examined by the addition of 20 μg/ml heparin in the binding reaction (lanes 4 and 5). Complex formation was analysed by electrophoresis on 4% polyacylamide gels, non-denaturing gels and visualized using a PhosphorImager. ++ denotes 6 μg of protein. (**B**) The specificity of complex formation was examined by competition with 10- and 100-fold molar excess (marked as + and ++) of specific unlabelled probe or non-specific probes. All the binding reactions contained 100 ng of pBR322 DNA and a 630 bp genomic fragment from a baculovirus BmNPV as the non-specific DNA. (**C**) TFIIIC and TFIIIB complexes on *tRNA$_1^{Gly}$-4* gene from which the proximal TATAA was mutated (to GATATC) as well as the construct having a deletion of the distal TATAA along with the mutation was analysed by electrophoresis on 4% non-denaturing polyacrylamide gels.

tRNA$_1^{Gly}$-4 transcription was even more pronounced, consistent with the presence of two TATAA sequences that are capable of binding to TFIIIB in the flanking region.

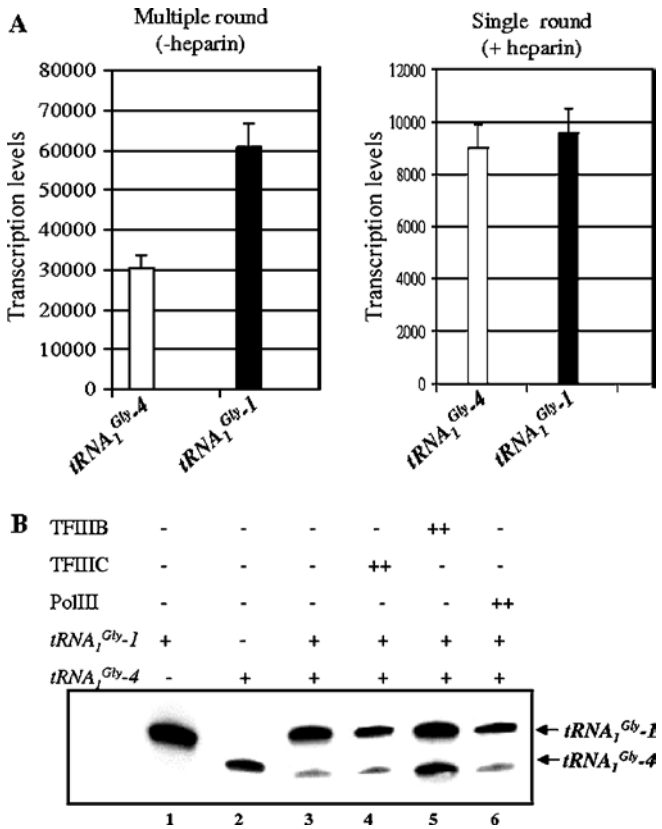

Figure 6 Single- and multiple-round transcription of *tRNA$_1^{Gly}$-1* and *tRNA$_1^{Gly}$-4*

(**A**) The binding stability of transcription factors to *tRNA$_1^{Gly}$-4* was determined by single- (in the presence of heparin) and multiple-round (in the absence of heparin) transcription. Multiple-round transcriptions were performed at 30 °C for 1 h in the presence of all four nucleotides. For details of single-round transcriptions, see the text. Results are means ± S.D. for three independent experiments. (**B**) Transcriptional competition between *tRNA$_1^{Gly}$-4* and *tRNA$_1^{Gly}$-1* under limiting concentrations of crude nuclear extracts (lane 3) and the effects of external supplementation with partially purified TFIIIC (phosphocellulose fraction; lane 4) or TFIIIB (heparin–Sepharose fraction; lane 5) and pol III (lane 6) are presented. Suboptimal concentrations of nuclear extract (+ corresponds to 4 μg of protein; lanes 3–6 were employed so that the effects of external supplementations were clearly discernable. For TFIIIC, TFIIIB and pol III fractions, ++ corresponds to 6 μg of protein. The transcripts were detected using a PhosphorImager following electrophoresis on a 7 M urea/8 % polyacrylamide gel.

Binding of TFIIIB to *tRNA$_1^{Gly}$-4*

Since the nucleosome was enhancing TFIIIB binding to *tRNA$_1^{Gly}$-4*, footprinting analysis was performed in the presence of the transcription factors (both TFIIIB and TFIIIC) to decipher the binding regions of TFIIIB. As expected, TFIIIC alone bound to the A and the B boxes (Figure 7, lane 3), the footprints extending up to the immediate upstream region. TFIIIB binding in the presence of TFIIIC extended the footprint to − 170 nt upstream (Figure 7, lanes 4 and 5) with strong protection in the region of the distal TATAA sequence (− 150 nt region) and weaker protection in the proximal TATA sequence region (− 30 nt region). This indicated that the TATAA element at − 27 nt was not as extensively covered by TFIIIB binding as the distal element, which showed more efficient masking. A reduction of transcription on deletion of the upstream TATAA as well as no significant change in transcription on mutation of the proximal TATAA also correlated with this observation. Thus, of the two TATAA box sequences present in the 5′ upstream region of *tRNA$_1^{Gly}$-4*, the distal one located at − 154 nt showed a more pronounced effect on TFIIIB binding as

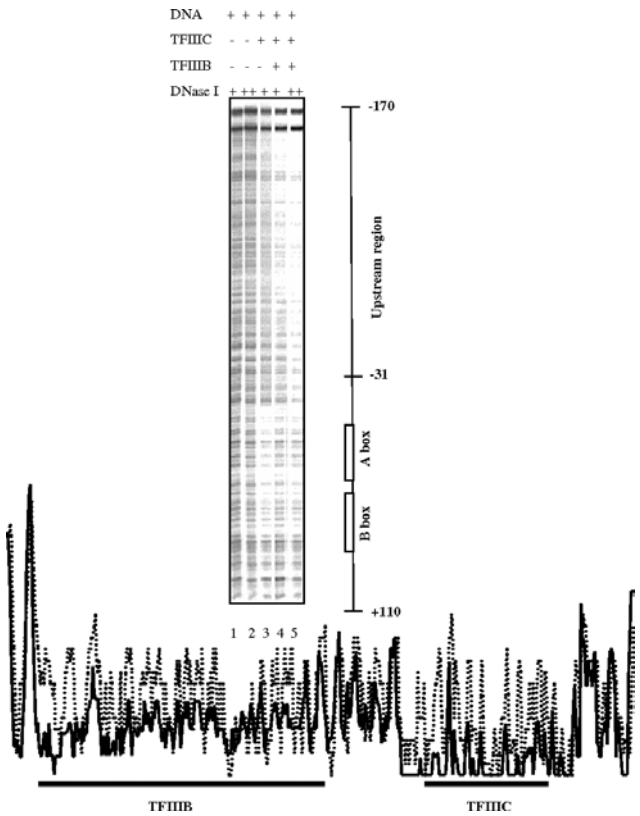

Figure 7 Binding of TFIIIB to *tRNA$_1^{Gly}$-4*

TFIIIB binding to *tRNA$_1^{Gly}$-4* directed by TFIIIC was analysed by DNase I footprinting. An end-labelled RsaI–EcoRI fragment from plasmid pBmH1 (− 198 to + 110 nt with respect to *tRNA$_1^{Gly}$-4*) was used as the probe. Lanes 1 and 2 represent the digestions of the fragment in the absence of the protein using two different amounts (3 and 6 ng) of DNase I. DNase I footprinting in the presence of TFIIIC alone (lane 3) and TFIIIC and TFIIIB together (lanes 4 and 5) are shown. The footprinted region is marked schematically on the right. The exact positions were mapped using sequencing ladders. The profiles of these footprints on *tRNA$_1^{Gly}$-4*, generated using the Image Gauge program are presented in the bottom. Broken and continuous lines correspond respectively to the footprint in the absence or presence of TFIIIC and TFIIIB. The strongly footprinted areas are underlined.

well as on the transcription of the gene. Evidently, the placement of a nucleosome in the 5′ upstream of the tRNA-coding region brought the distal TATAA sequence close to the transcription start site to facilitate better interaction with the TFIIIC that was already bound to the initiator regions.

DISCUSSION

The *B. mori* system serves as an ideal model to analyse differential gene expression. The multicopy family of *tRNA$_1^{Gly}$* genes in *B. mori* contains members which are transcribed from high and moderate to very low levels. The differential transcriptions of these genes were primarily attributed to their flanking regions, since all the members of this multigene family had identical coding regions [19–22], and some typical TATAA box sequences were strongly implicated in this process. A protein P43 TBF (TATA-box-binding factor) isolated from silk glands of *B. mori* also bound specifically to this sequence and exerted inhibitory effects on transcription [32]. In addition, the presence of a positioned nucleosome in the 5′ upstream region of the poorly transcribed gene *tRNA$_1^{Gly}$-6,7* also contributed to its lowered transcription [25]. While the high and very low transcription of the gene copies were due to the presence of *cis* regulatory elements in the flanking regions and/or

through chromatin organization, the mechanism governing the moderate transcription of others which are generally in the range 40–60 % (of the highly transcribed $tRNA_I^{Gly}$-*1* copy) has not been investigated so far. In the present study, we chose $tRNA_I^{Gly}$-*4* as the model for the analysis.

Of the two typical TATAA box sequences present in the 5′ upstream region of this gene copy, the distal one located at − 154 nt played a more significant role in the transcriptional regulation than the one located proximally at − 27 nt of the tRNA-coding region. Deletion of the distal TATAA sequence element brought down the transcription of the gene by more than 60–70 %, whereas mutation of the proximal TATAA sequence did not noticeably influence the transcription. We have shown previously that the positive influence of the TATAA box sequences on tRNA gene transcription was position-dependent [22,30]. For instance, a TATAA sequence located at − 26 nt of $tRNA_I^{Gly}$-*6,7* had no positive effect on its transcription, whereas the same sequence located at − 34 nt in $tRNA_I^{Gly}$-*1* distinctly served as a positive element. It appeared that the TATAA sequences within 30 nt immediately upstream of the tRNA-coding sequences were fully or partially covered by TFIIIC binding which was sterically blocking the direct binding of TFIIIB to the gene. Although TFIIIB could be recruited to the transcription complex indirectly through interaction with TFIIIC in the absence of the TFIIIB-binding sequence (TATAA boxes), such complexes appeared to be unstable and readily dissociated. On the other hand, the presence of an optimally located TATAA sequence as seen in $tRNA_I^{Gly}$-*1* (at position − 34 nt) or $tRNA_I^{Gly}$-*11* (at position − 41 nt) provided a direct anchoring of the TFIIIB to the DNA close to the transcription initiation site, and conferred stability to the TFIIIB–TFIIIC–DNA complex. Even in $tRNA_I^{Gly}$-*4*, the presence of the proximal TATAA sequence at − 27 nt allowed only approx. 30 % of the transcription of the parental gene, in the absence of the distal TATAA element. Conversely, when the latter sequence was present, mutation of the proximal TATAA sequence had practically very little effect on transcription. In confirmation of these observations, the stability of the TFIIIB–DNA complex on the deletion derivative of $tRNA_I^{Gly}$-*4* was also found to be weak (as deduced from its dissociation in presence of heparin). Evidently the distal TATAA sequence stabilized TFIIIB binding to the gene. Moreover, the footprints of TFIIIC on the gene extended up to the immediate upstream region, whereas the footprints due to TFIIIB extended up to − 170 nt and beyond the distal TATAA sequence. In this instance, since the positive element was located at − 154 nt and therefore significantly far from the range of TFIIIC-binding region, how did the two transcription factors interact with each other if the sequence-specific TFIIIB binding was independent of its interaction with TFIIIC? Alternatively, if TFIIIB was recruited through its interaction with TFIIIC, how did it access the anchoring TATAA sequence on the DNA which was located at a distance? Answers to these questions came from the nucleosome-assembly studies.

In the full-length $tRNA_I^{Gly}$-*4*, assembling the gene as chromatin resulted in more than 2–5-fold stimulation of transcription. On the other hand, when the distal TATAA sequences were deleted, chromatin assembly resulted in complete abolition of the already lowered transcription of the deletion derivative. Therefore, clearly, the distal TATAA element was involved in fostering transcription of this gene copy and exerted an even stronger positive influence than the proximal element. The positioning of the nucleosome in the 5′ upstream region between the TFIIIC- and the distal TFIIIB-binding sites brought the two transcription factors closer together, permitting their interaction which resulted in higher levels of transcription. Therefore the nucleosome was bridging the transcription factors and stabilizing their interactions. The

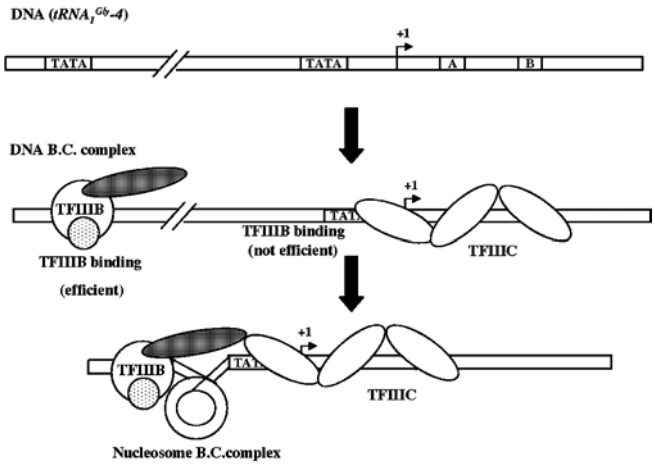

Figure 8 Model for nucleosome-mediated transcriptional stimulation of $tRNA_I^{Gly}$-4

The binding of TFIIIB to the distal TATAA (at − 154 nt) was more efficient as compared with its binding to the proximal TATAA (at − 27 nt) presumably due to steric hindrance caused by TFIIIC binding. The positioned nucleosome in the immediate upstream region facilitated efficient interactions between TFIIIC and TFIIIB by looping the region of DNA between the two. When the TFIIIB- and TFIIIC-binding sites are optimally positioned, the presence of nucleosome showed only marginal effects on transcription. For further explanation, see the text.

TFIIIB bound to the distal TATAA sequence in $tRNA_I^{Gly}$-*4* was brought closer to TFIIIC already bound to the transcription start site region through the positioned nucleosome, resulting in higher transcription (see the model shown in Figure 8). On supplementation with additional TFIIIC (with associated HAT activity), the enhanced transcription from the nucleosome fell back to that of the naked DNA levels suggesting that the latter caused nucleosome unwinding and thus lowered the transcription. Our model for chromatin stimulation of the tRNA gene transcription (Figure 8) is supported further by the observation [25] that the positioning of a nucleosome in the region where TFIIIC- and TFIIIB-binding sites are optimally placed showed less pronounced effects only on the transcription of $tRNA_I^{Gly}$-*1* and $tRNA_I^{Gly}$-*11* genes.

Packaging of DNA into nucleosomes and higher-order structures causes structural looping to bring distant sequences closer together. For instance, gene activation from distal GAL4 regulatory elements is facilitated by packaging of the template into chromatin by bringing GAL4-VP16 (viral protein 16 from herpes simplex virus) closer to the transcriptional machinery of the start site [33]. Packaging has also been reported to confer an advantage over short distances *in vitro*. Although nucleosomes are classically known to be repressive in nature, they can exert profound effects on transcription in a positive manner through such compaction. The evidence for association of tRNA genes with chromatin has been contradictory [12–14,34,35]. We have shown previously the association of a positioned nucleosome on the poorly transcribed $tRNA_I^{Gly}$*6,7* that caused substantial reduction of transcription of this gene copy by preventing TFIIIB binding, whereas the absence of positioned nucleosomes contributed to enhanced transcription of the highly transcribed $tRNA_I^{Gly}$-*1* [25]. In the absence of proper positioning of the proximal TATA element in $tRNA_I^{Gly}$-*4*, the distal TATA was important for the proper transcription of the gene. The fact that this gene copy when assembled into nucleosome structures gave rise to transcript levels comparable with those of $tRNA_I^{Gly}$-*1* or $tRNA_I^{Gly}$-*11* suggests that within the silk gland tissues, even the moderately transcribed $tRNA_I^{Gly}$ copies could be contributing to

the large $tRNA_1^{Gly}$ pools in the functional adaptation process. Stimulation of pol III transcription of tRNA genes through nucleosome assembly is seen as a novel mode of regulation of tRNA gene expression.

We thank the Department of Science and Technology, Government of India, for financial support. We also thank the Indian Council of Medical Research and the Department of Biotechnology (Government of India) for infrastructure support to the department. We are grateful to Dr Purnima Bhargava, Centre for Cellular and Molecular Biology, Hyderabad, India, for providing the *Drosophila* S190 extract. We thank Dr Sreekumar, Central Sericulture Research and Training Institute, Central Silk Board, Mysore, India, and Dr Ponnuvelu, Central Silk Board, Hosur, India, for providing *B. mori* larvae. A.P. was a recipient of a Senior Research Fellowship of the Council of Scientific and Industrial Research (CSIR), Government of India and K.P.G. is an Emeritus Senior Scientist of the Indian National Science Academy.

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Received 21 December 2005/1 March 2006; accepted 9 March 2006
Published as BJ Immediate Publication 9 March 2006, doi:10.1042/BJ20052029