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Characterization of RNA polymerase III transcription factor TFIIIC from the mulberry silkworm, *Bombyx mori*

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Fractionation of nuclear extracts from posterior silk glands of mulberry silkworm *Bombyx mori*, resolved the transcription factor TFIIIC into two components (designated here as TFIIIC and TFIIIC1) as in HeLa cell nuclear extracts. The reconstituted transcription of tRNA genes required the presence of both components. The affinity purified TFIIIC is a heteromeric complex comprising of five subunits ranging from 44 to 240 kDa. Of these, the 51-kDa subunit could be specifically crosslinked to the B box of $tRNA_1^{Gly}$. Purified swTFIIIC binds to the B box sequences with an affinity in the same range as of yTFIIIC or hTFIIIC2. Although an histone acetyl transferase (HAT) activity was associated with the TFIIIC fractions during the initial stages of purification, the HAT activity, unlike the human TFIIIC preparations, was separated at the final DNA affinity step. The tRNA transcription from DNA template was independent of HAT activity but the repressed transcription from chromatin template could be partially restored by external supplementation of the dissociated HAT activity. This is the first report on the purification and characterization of TFIIIC from insect systems.

Keywords: *Bombyx mori*; chromatin transcription; histone acetyl transferase activity pol III transcription; transcription factor.

RNA polymerase III (pol III) synthesizes small, untranslated, stable RNAs such as tRNAs, 5S rRNA, and a variety of other cellular and viral RNAs. At least two transcription factors, TFIIIB and TFIIIC are necessary for the transcription of tRNA genes. The basal promoter elements, for pol III transcription are two internal conserved regions comprising of the A and B box sequences, located within the tRNA coding region, which are recognized and bound by TFIIIC to initiate the transcription process [1].

The most detailed and mechanistically elaborate information about transcription factor IIIC is available for yeast followed by the human cell lines [2,3]. In *Saccharomyces cerevisiae,* TFIIIC (scTFIIIC) is a multisubunit protein comprising of six subunits with an aggregate mass of 520 kDa. The largest subunit (138 kDa) binds to the B box and the third largest one (95 kDa) binds to the A box [4,5]. The genes encoding these subunits are essential for yeast cell viability [3].

Human TFIIIC is more complex than the yeast factor and executes additional functions. hTFIIIC can be resolved into two components, TFIIIC1 and TFIIIC2, on ion exchange or oligonucleotide affinity columns [6,7]. The initial recognition of tRNA promoters is achieved by TFIIIC2, which binds to the B box and then serves to recruit TFIIIC1 and TFIIIB [8]. TFIIIC2 from HeLa cells consists of five polypeptides of 220, 110, 103, 90, and 63 kDa of which the largest one binds to the tRNA gene [9]. Regions of homology between the largest TFIIIC subunits, tau 138 from yeast and TFIIIC220 from human, have been demonstrated recently and these conserved residues appear to be of functional significance [10]. TFIIIC102 and TFIIIC63 interact with each other as well as with hTFIIIB90 and TBP, and these subunits form a stable subcomplex in the preinitiation complex [11]. Recombinant hTFIIIC90 and C110 contain intrinsic histone acetyl transferase (HAT) activities and specifically acetylate histones H3 and H4 [12,13]. Highly purified hTFIIIC, at concentrations above that necessary for optimal transcription of naked DNA templates, efficiently relieves nucleosome-mediated repression on chromatin templates [13]. However, no HAT activity has been reported to be associated with scTFIIIC.

So far, the detailed characterization of the pol III transcription factors TFIIIC or TFIIIB from the insect system have not been reported. Fractionation of the silkworm pol III transcription apparatus was attempted previously [14] and a factor TFIIID, that was required in addition to the two known factors TFIIIB and TFIIIC was identified. TFIIID appeared to play a role in the assembly of other factors into transcription complexes. None of these transcription factors have been completely purified or their subunit compositions established. The present study reports the purification and characterization of TFIIIC from the posterior silk glands of the mulberry silkworm, *Bombyx mori*. The oligonucleotide affinity purified swTFIIIC showed five polypeptides ranging in size from 44 to 240 kDa, of which the 51-kDa subunit could be specifically crosslinked to the B box of tRNA genes. The binding affinity of swTFIIIC to the B box was comparable to that of yTFIIIC or hTFIIIC2. The partially purified swTFIIIC fraction contained HAT activity and predominantly acetylated histone H3, but in the final preparation (after DNA

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Abbreviations: HAT, histone acetyl transferase; pol III, RNA polymerase III; EMSA, electrophoretic mobility shift assay.

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affinity purification step), the HAT activity was dissociated from TFIIIC.

EXPERIMENTAL PROCEDURES

DNA–protein interactions

The interaction of nuclear extract proteins with DNA sequence elements was analyzed by electrophoretic mobility shift assays (EMSA). The B box DNA sequences of $tRNA_I^{Gly}$ was generated by annealing of two overlapping complimentary oligonucleotides encompassing the B box sequence (5′-CGGGCGGCCCGGGTTCGATTC-3′ and 5′-GGGCCCAAGCTAAGGCCCGCC-3′) and end filled with Klenow DNA polymerase in the presence of [α-^{32}P]dCTP to generate a 28-bp end labeled probe.

The assay system [15] was contained in a final volume of 15 μL consisting of 0.1–2 μg protein (crude nuclear extract or purified fractions as indicated), 12 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 4 mM Tris HCl (pH 8.0), 0.6 mM EDTA, 0.6 mM dithiothreitol, 5% glycerol and 2 μg double stranded poly (dI-dC). After incubation at 4 °C for 15 min, the radiolabeled DNA fragment (10 000 c.p.m.) harboring the $tRNA_I^{Gly}$ B box sequence was added and the binding was allowed to proceed for another 15 min. In retardation chase experiments, varying amounts of unlabeled specific competitors were also included. The binding reactions were terminated by adding 5 μL of the gel-loading buffer and the samples were subjected to electrophoresis on 5% polyacrylamide gels, at 4 °C.

In vitro transcription assay

Nuclear extracts from posterior silk glands of *B. mori* in the fifth larval instar were prepared as described previously [16]. Briefly, the silk glands (freshly dissected or glands frozen at −70 °C up to 6 months) were homogenized (Dounce homogenizer) in 10 mM Hepes (pH 7.9), containing 2 M sucrose, 10% glycerol, 15 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 0.15 mM spermine, 0.15 mM spermidine and 1 mM EDTA. Nuclei were pelleted by centrifugation and lysed in 20 mM Hepes (pH 7.9), containing 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM phenylmethanesulfonyl fluoride. The crude lysate was cleared by centrifugation, dialyzed against buffer A (20 mM Hepes, pH 7.9 containing 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM phenylmethanesulfonyl fluoride) and used as crude nuclear extracts for transcription. The reactions in a final volume of 25 μL contained 20 mM Hepes (pH 7.9), 60 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 6 mM creatine phosphate, 50 μM each of ATP, CTP and UTP, 10 μM GTP, 5 μCi [α-^{32}P]GTP (3000 Ci·mmol^{-1}), nuclear extract (20 μg protein) and 4 μg·mL^{-1} of the supercoiled plasmid DNA template (pR8, harboring the $tRNA_I^{Gly}$-1 gene) [17]. After incubation at 30 °C for 1 h, the reactions were terminated by the addition of 0.2% SDS and 10 mM EDTA. The samples were extracted once with phenol and the transcripts in the aqueous layer were precipitated by 3 volumes of ethanol in the presence of 100 μg·mL^{-1} glycogen (carrier). The precipitate was resuspended in gel loading buffer containing 80% formamide and subjected to electrophoresis on 7 M urea/8% acrylamide gels.

Determination of equilibrium constant for DNA binding

The equilibrium constants for specific binding reactions are given by the equations:

$$[CD] = [C^\circ] \cdot K_{eq}.[D]/[1 + K_{eq} \cdot [D]\}, \text{and}$$

$$[CD]/[D] = -K_{eq} \cdot [CD] + K_{eq} \cdot [C^\circ],$$

where [D] and [CD] are the free and bound species of the probe, [C°] denotes the total number of DNA binding sites and K_{eq} is the equilibrium constant of the binding reaction [18]. The first equation describes a hyperbola and the second equation a straight line, equivalent to a Scatchard plot. Standard DNA binding reactions were performed with a constant amount of protein sample and varying dilutions of labeled probe. The protein–DNA complex formed in the binding reaction and the unbound probe were quantified in a phosphorimager, following electrophoresis. The equilibrium constant was determined graphically from a plot of [CD]/[D] vs. [CD], according to the second equation where the negative reciprocal of the slope of the line gives the value of K_{eq} and the *x*-intercept the value of [C°].

Purification of swTFIIIC

Posterior silk gland nuclear extracts were fractionated sequentially on phosphocellulose, Q-Sepharose and sequence specific DNA affinity columns for purification of the transcription factor TFIIIC. All fractionation steps were carried out at 4 °C.

Phosphocellulose chromatography. The nuclear extract (3 mg·mL^{-1}, 30 mL) was loaded onto a column of phosphocellulose (1 × 15 cm) in buffer A. The column was washed with the loading buffer and the bound proteins were eluted step wise with buffer A containing 0.35, 0.6 and 1.0 M KCl. Individual fractions, equivalent to 10% of the bed volume were collected and analyzed for B box binding activity. The fractions showing maximal activity were pooled (two to four fractions) and dialyzed against buffer A for 4 h and processed for Q-Sepharose chromatography. The 0.35, 0.6 and 1.0 M KCl fractions are referred to as PC-B, PC-C, and PC-D fractions, respectively.

Q-Sepharose chromatography. The PC-C fraction from the phosphocellulose column was loaded onto a Q-Sepharose column of 2 mL pre-equilibrated with buffer A at a flow rate of 3 column volumes per hour, and washed with 5 column volumes of buffer A. The bound proteins were eluted using a 10-mL linear gradient of KCl (from 0.1 to 0.6 M) in buffer A. Individual fractions from the column were tested for binding to B box and the active fractions were pooled, dialyzed against buffer A for 4 h, and processed for DNA affinity chromatography.

Sequence specific affinity purification of TFIIIC. The B box affinity matrix for purification of TFIIIC was prepared as described by Kadonaga & Tjian [19]. The two oligonucleotides (50 μg each) encompassing the B box sequences of $tRNA_I^{Gly}$ genes of *B. mori* (as described above) were

combined in 50 μL of 70 mM Tris/HCl (pH 7.6) containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine and 1 mM EDTA, heated at 90 °C for 5 min and allowed to cool slowly. The annealed oligonucleotides were 5′ end labeled with polynucleotide kinase (20 U) and 20 mM ATP at 37 °C for 2 h. The oligonucleotides were precipitated with 2 volumes of ethanol, resuspended in 20 μL of 50 mM Tris HCl (pH 7.6) containing 10 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM spermidine. The 5′ end labeled oligonucleotides were ligated using T4 DNA ligase (20 U) in presence of 1 mM ATP at 16 °C for 16 h. Following ligation, the sample was extracted with phenol, precipitated by 2 volumes of ethanol and dissolved in 50 μL of water. Multimerization of the oligonucleotides was checked by agarose gel electrophoresis; multimers ranging from three- to 20-mers of the basic unit, were seen.

The affinity matrix was prepared by covalently coupling the above DNA to CNBr-activated Sepharose (Pharmacia) for 16 h at room temperature on a rotary shaker, followed by treatment with 10 mM potassium phosphate buffer (pH 8.0) containing 0.2 M glycine for 4–6 h to inactivate the unreacted CNBr-derivatized Sepharose. Following this, the DNA coupled matrix was washed with 10 volumes of 10 mM potassium phosphate (pH 8.0), and stored at 4 °C in 10 mM Tris HCl (pH 7.6) containing 0.3 M NaCl, 1 mM EDTA and 0.02% (w/v) NaN_3, till use. The efficiency of DNA coupling to Sepharose (60%) was verified by estimating the amount of DNA present in the input and the unbound fraction subsequent to coupling.

The fractions showing B box binding from Q-Sepharose column were first incubated with 50 μg of poly [(dI-dC)·(dI-dC)] per ml for 30 min to bind all the non-specific DNA binding proteins and then with the affinity matrix at 4 °C for 1 h with gentle swirling. The matrix was washed with vast excess of binding buffer and the bound proteins were eluted using buffer A containing 1 M KCl. The eluate was dialyzed against buffer A for 4 h and stored at −70 °C.

UV crosslinking of proteins to DNA

Labeled DNA probes for UV crosslinking were made by PCR from the appropriate template using specific primers in the presence of 20 μM dCTP, dGTP, and bromodeoxyuridine triphosphate (BrdUTP), 20 μCi of [α-^{32}P]dATP (3000 Ci·mM^{-1}) and *Taq* DNA polymerase (1 U). The labeled PCR product (also containing BrdU) was purified by electrophoresis through a 5% native polyacrylamide gel. The binding reactions contained in a final volume of 15 μL: 0.1 μg protein, 12 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 4 mM Tris HCl (pH 8.0), 0.6 mM EDTA, 0.6 mM dithiothreitol, nonspecific competitor DNA (1000-fold molar excess) and 5% glycerol. The binding was initiated by adding the radiolabeled DNA fragment (10 000 c.p.m.) and allowed to proceed for 15 min at 4 °C. The samples were irradiated with ultraviolet light (254 nm, 7.2 J·cm^{-2}) at 4 °C for crosslinking. Following this, the samples were digested with micrococcal nuclease (5 U) and Dnase I (2 μg) in the presence of 2.5 mM $CaCl_2$ for 30 min at 37 °C, and the nuclease digestion was terminated by the addition of 10 mM EDTA. To identify the DNA binding protein, the polypeptides were separated on an 8% SDS/PAGE and autoradiographed.

Purification of core histones from silkworm

Silkworm core histones were purified from posterior silk gland nuclear pellets. The nuclear pellet (prepared as for *in vitro* transcription assays) was homogenized in 10 mL of buffer B [0.1 M potassium phosphate (pH 6.7), 0.1 mM EDTA, 10% glycerol, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM dithiothreitol] containing 0.63 M sodium chloride and centrifuged at 82 000 ***g*** at 4 °C. The supernatant was treated with 10 mL of previously swollen Biogel-HTP (DNA grade; Bio-Rad) for 3 h. The resin was packed into a column and washed overnight with buffer B containing 0.63 M NaCl to remove any contaminating HAT activity. The core histones were eluted with buffer B containing 2 M NaCl and dialyzed first against buffer C [10 mM potassium phosphate (pH 6.7), 150 mM KCl, 10% glycerol] for 3 h and then against buffer D [20 mM Tris/HCl (pH 7.9), 100 mM KCl, 20% glycerol, 0.1 mM dithiothreitol] for 3 h.

Histone acetyl transferase (HAT) assay

HAT activity was assayed by monitoring the incorporation of the [^{3}H]acetyl group to the core histones from radiolabeled acetyl-CoA either by filter binding or by electrophoretic identification of the labeled acetylated histones. The assay system contained purified silkworm core histones (1 μg) and varying amounts of acetyl transferase in 30 μL reaction mixtures: 50 mM Tris HCl (pH 8.0), 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM EDTA, 10 mM butyric acid, and 125 nCi of [^{3}H]acetyl-coA (10 Ci·mmol^{-1}). After incubation for 30 min at 30 °C, the reaction was stopped by spotting the sample on a Whatman P-81 phosphocellulose paper. The filters were washed three times with gentle swirling for 5 min at 37 °C in 100 mL of 50 mM sodium carbonate buffer (pH 9.2), washed once with acetone at room temperature, dried and the radioactivity incorporated was monitored. The HAT domain of P300, purified after baculoviral expression from insect cells *Sf*21 was used as a positive control for HAT assays.

For determining the substrate histone specificity of the enzyme, the products were precipitated with 27% trichloroacetic acid at 4 °C, washed with acetone, dried and dissolved in 10 μL of SDS/PAGE loading dye. The samples were subjected to electrophoresis on 0.1% SDS/15% acrylamide gels, followed by fluorography to visualize the radiolabeled histones.

In vitro reconstitution of chromatin

Supercoiled plasmid DNA [pR8 harboring the $tRNA_I^{Gly}$ gene(17)] was assembled into chromatin using S190 extracts from *Drosophila* embryos [20]. Briefly, 50 μL of the S190 extract was incubated with 1 μg of silkworm core histones in buffer R[10 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol and 0.2 mM phenylmethanesulfonyl fluoride] in a final volume of 170 μL for 30 min at room temperature. Following this 30 μL of Mg-ATP mix [1 mM Hepes (pH 7.5), 250 mM creatine phosphate, 20 mM ATP, 3.5 mM $MgCl_2$, and 4 μg creatine kinase] and 1 μg of plasmid DNA was added. Chromatin

assembly was allowed to proceed for 4–5 h at 27 °C after which samples of assembled chromatin were used as template for *in vitro* transcription.

RESULTS

Interaction of B box DNA sequences with nuclear proteins

The nuclear extracts derived from posterior silk glands of *B. mori* showed high levels of activity for pol III transcription of tRNA genes. The presence of transcription factor IIIC in these nuclear extracts was initially examined by its B box binding activity. Electrophoretic mobility shift assays (EMSAs) with the labeled B box of $tRNA_I^{Gly}$ sequences and posterior silk glands nuclear extract (in presence of vast excess of nonspecific DNA competitor), identified specific complexes (Fig. 1A). A single complex (complex 1) was detected at lower protein concentrations but on increasing the protein concentration a second, larger size complex (complex 2) also appeared. Both these complexes could be completely chased out by excess unlabeled B box DNA (Fig. 1B, lanes 3–5) or the whole tRNA coding sequence (lanes 6–8), indicating the specificity of the complexes.

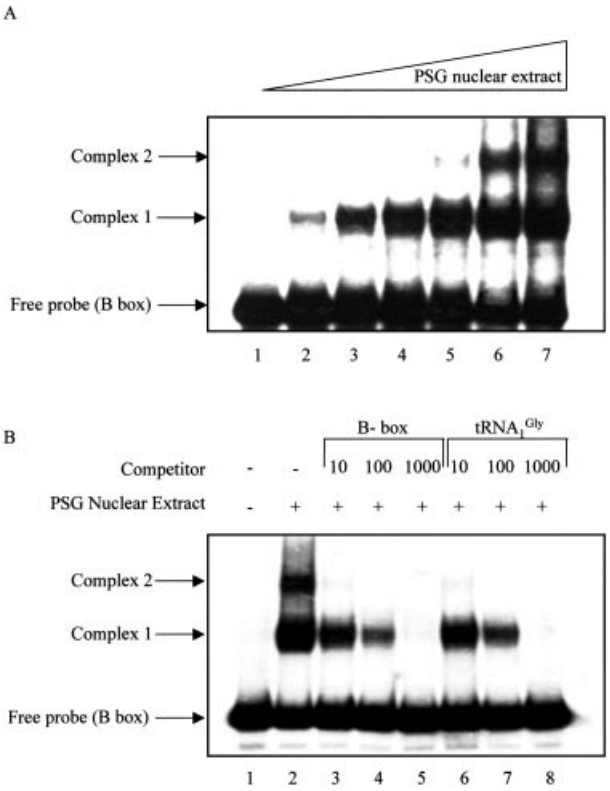

Fig. 1. Binding of posterior silk glands nuclear protein to B box sequences of $tRNA_I^{Gly}$. (A) Effect of varying concentrations of posterior silk glands nuclear proteins on the binding to B box DNA. The DNA binding was performed in presence of large excess of poly (dI-dC). For details of assay see Experimental procedures. Lanes: 1, free probe, 2–7; binding with 0.5, 1, 1.5, 2.0, 2.5 and 3.0 μg of crude nuclear protein. (B) Binding of the nuclear proteins to the B box was competed with increasing amounts of unlabeled B box sequences or a DNA fragment containing the entire coding region of $tRNA_I^{Gly}$. Lanes: 1, free probe; 2, binding with 2 μg of nuclear protein; 3–5, Competition of specific DNA–protein complexes with 10-, 100-, and 1000-fold molar excess of unlabeled B box sequence; 6–8, Competition with $tRNA_I^{Gly}$.

Purification of silkworm TFIIIC

To purify TFIIIC, the posterior silk glands nuclear extract preparation (the same as that used for *in vitro* transcription) was fractionated through three successive chromatographic steps: phosphocellulose P11, Q-Sepharose and B box DNA affinity matrix. The scheme of fractionation is presented in Fig. 2. At each step of purification the fractions containing TFIIIC were identified by EMSA with radiolabeled B box DNA as probe. The fractions were also checked by reconstitution for $tRNA_I^{Gly}$ transcription.

At the phosphocellulose chromatography step, pol III transcription activity was separated into two fractions, the PC-B fraction containing polymerase III as well as the transcription factor TFIIIB and the PC-C fraction containing TFIIIC activity. Only the PC-C fraction showed binding to the B box sequences (Fig. 3A). The fractions were also analyzed for pol III transcription activity by reconstitution

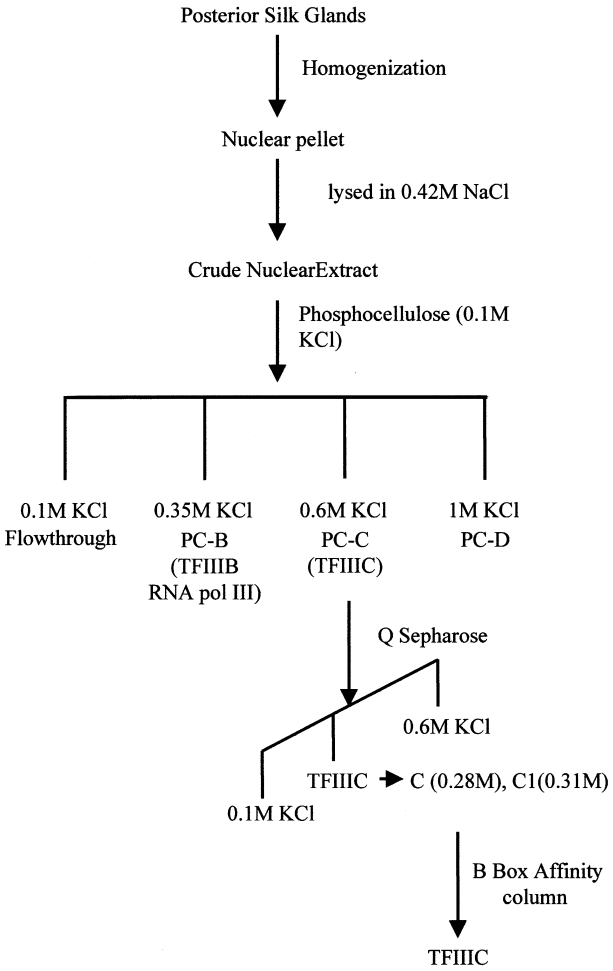

Fig. 2. Purification strategy for silkworm TFIIIC. A schematic presentation of the purification strategy for TFIIIC from *B. mori* posterior silk gland nuclear extracts is shown.

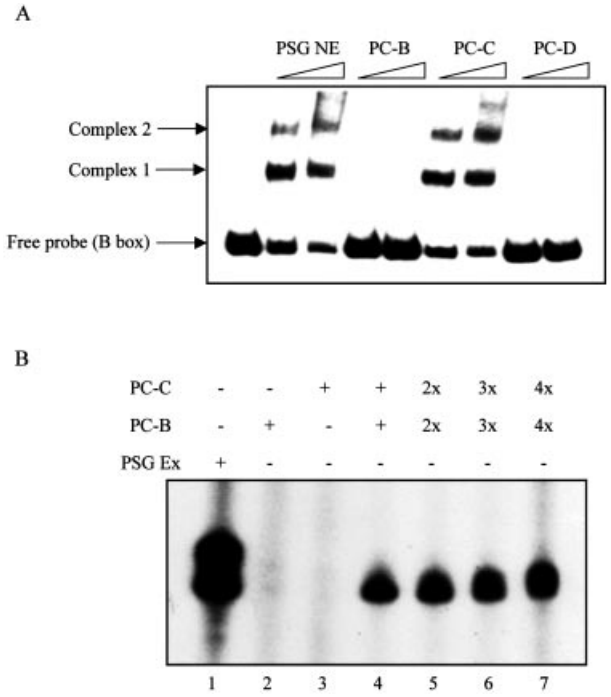

Fig. 3. DNA binding and transcription activity of Phosphocellulose fractions. (A) EMSA was carried out with $tRNA_I^{Gly}$ B box and different phosphocellulose fractions as indicated. Equal amounts of protein (at two different concentrations) from each fraction were used in binding. Lanes as marked. (B) Transcription activity of phosphocellulose fractions. Transcription of $tRNA_I^{Gly}$-1 was assayed either with PC-B or PC-C fractions alone (lanes 2 and 3, respectively) or with varying concentrations of both fractions (lanes 4–7). Lane 1, transcription by crude nuclear extract. 100 ng of $tRNA_I^{Gly}$-1 was used as template.

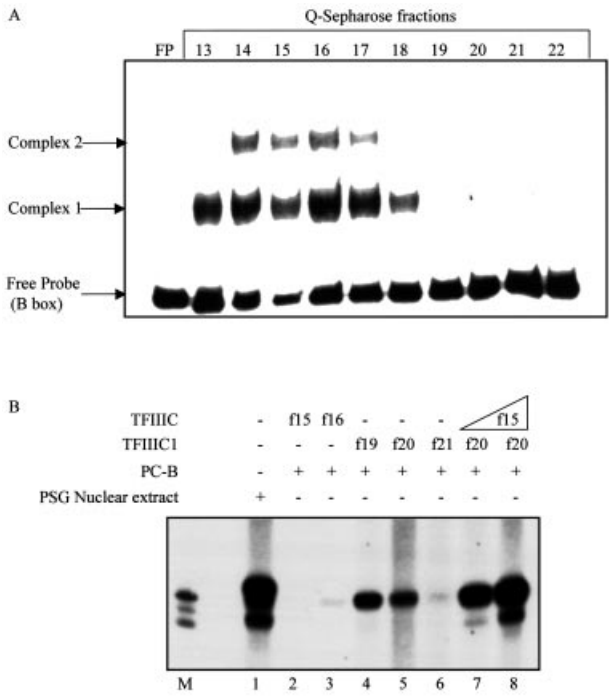

Fig. 4. B box binding and transcription activity of Q-Sepharose fractions. (A) Individual fractions from the Q-Sepharose column were assayed for B box binding activity in EMSA. For details of assay see Experimental procedures. Lanes: 1, free probe ($tRNA_I^{Gly}$ 1 B box); 2–11, binding with equal amounts of Q-Sepharose fractions 13–22 as marked. (B) Transcription of $tRNA_I^{Gly}$-1 was assayed with the fractions from Q-Sepharose column in the presence of 1 μg of the PC-B fraction which provided the TFIIIB and pol III activities. Fractionation of PC-C through a Q-Sepharose column separated the TFIIIC activity into two fractions, TFIIIC and TFIIIC1. Transcription reconstitution of $tRNA_I^{Gly}$-1 was carried out with C and C1 fractions alone or in combination. Lanes: M, Marker, pTZ plasmid DNA digested with *Hinf* I and end labeled; 1, transcription of $tRNA_I^{Gly}$-1 with crude posterior silk glands nuclear extract; 2 and 3, reconstituted transcription with fractions 15 and 16 from Q-Sepharose; 4, 5 and 6, reconstituted transcription with fractions 19, 20 and 21; 7 and 8, reconstituted transcription with fraction 20, PC-B and two concentrations of fraction 15 (TFIIIC).

(Fig. 3B). Neither PC-C nor PC-B alone were able to foster transcription (lanes 2, 3) and were therefore relatively free of cross contamination with each other. When combined in different proportions, these fractions regained $tRNA_I^{Gly}$ transcription activity (lanes 4–7). The reconstituted $tRNA_I^{Gly}$-1 transcription with PC-B and PC-C fractions gave rise to a single transcript (lanes 4–7) corresponding to the fully processed form of tRNA as compared to the crude nuclear extracts (lane 1) which gives rise to the precursor and processed transcripts corresponding to $tRNA_I^{Gly}$. The identities of these transcripts have been established previously [16] by primer extension analysis. The enrichment in the tRNA transcript processing activity at this stage was lost during the subsequent steps of fractionation. Because the PC-B fraction was free of TFIIIC activity, it was used without further fractionation as a source of TFIIIB and pol III, in the subsequent analysis.

The PC-C fraction (containing TFIIIC) was fractionated through the strong anion-exchange column Q-Sepharose. The proteins eluting between 250 and 280 mM KCl showed the B box binding activity (Fig. 4A). When these fractions were analyzed for reconstituted transcription of $tRNA_I^{Gly}$-1 in the presence of PC-B (TFIIIB and pol III), it did not support transcription (Fig. 4B; lanes 2 and 3) indicating that another factor that gets separated from TFIIIC in the Q-Sepharose column is possibly involved in $tRNA_I^{Gly}$ transcription. This possibility was tested by analyzing all the fractions from the Q-Sepahrose by individually supplementing them to the reconstituted transcription assay, containing the TFIIIC (280 mM KCl) and the PC-B fractions. The transcription of $tRNA_I^{Gly}$-1 was restored in the presence of the 310 mM KCl fraction (Fig. 4B; lanes 7 and 8). These results suggested the presence of another transcription factor, like the swTFIIID [14] or the human transcription factor TFIIIC1 [21] in the 310 mM fraction. We designate this fraction (310 mM fraction) as TFIIIC1, which by itself was able to foster transcription of $tRNA_I^{Gly}$ by the PC-B fraction (Fig. 4B; lanes 4 and 5). However, these transcription levels were increased significantly when the transcription was carried out in the presence of supplemented B box binding, 280 mM KCl fraction (designated as TFIIIC, compare lanes 7, 8 to lane 5). The low level of transcription seen with TFIIIC1 (310 mM KCl fraction) alone in the presence of PC-B was due to the cross

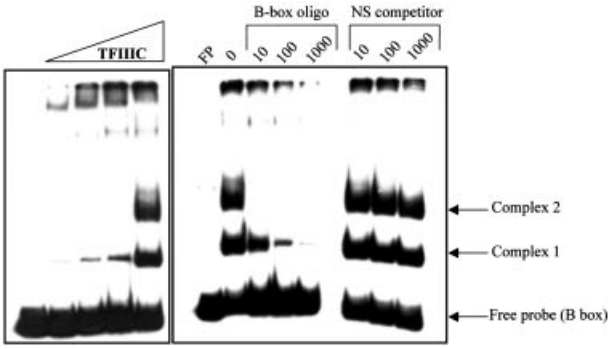

Fig. 5. Binding of swTFIIIC to $tRNA_I^{Gly}$ B box. Binding with increasing concentrations of TFIIIC (50, 100, 150 and 200 ng) as indicated (left panel). Binding of TFIIIC to the B box fragment was competed with increasing amounts of unlabeled B box fragment or a nonspecific DNA fragment (right panel). The nonspecific DNA used here was a 150-bp *Eco*RI–*Kpn*I restriction fragment from the far upstream region of the $tRNA_I^{Gly}$-1 gene clone, in addition to poly (dI-dC). Lanes: 1, free probe; 2, binding with 200 ng of TFIIIC; 3–5, Competition with 10-, 100-, and 1000-fold molar excess of unlabelled B box fragment; and 6–8, Competition with nonspecific DNA fragment.

contamination with TFIIIC in this fraction because this activity of TFIIIC1 (fostering transcription by itself in the absence of added TFIIIC) was not consistent in all batches of purification (see Fig. 6B; lane 2).

The TFIIIC fraction was further purified by oligonucleotide affinity chromatography using the multimerized B box DNA sequences as the affinity matrix. Prior to loading on to the specific DNA affinity matrix, the TFIIIC fraction was incubated with nonspecific DNA [poly(dI·dC). poly(dI·dC)] to partition the nonspecific DNA binding proteins present in the fraction. The bound proteins from the DNA affinity matrix were eluted using a high salt (1.0 M KCl) buffer.

Two prominent complexes (marked 1 and 2 in Fig. 5, left panel) were seen with increasing concentrations of the affinity purified TFIIIC and $tRNA_I^{Gly}$ B box. As seen with crude nuclear extracts or the PC-C fractions, the second complex appeared only at higher protein concentrations. Both complexes were specifically competed out with excess unlabeled B box oligonucleotides but not with unlabeled nonspecific competitor (Fig. 5, right panel) confirming the binding specificity of TFIIIC to the B box sequence.

The affinity-purified TFIIIC preparation when analyzed on a 0.1% SDS/8% acrylamide gels, showed five protein bands of 240, 125, 68, 51 and 44 kDa (Fig. 6A). Of these, the 51-kDa band was at least twice as intense as the others indicating that the 51-kDa protein either associated with a stoichiometry of more than 1 or there was more than one polypeptide of 51-kDa in the TFIIIC preparation.

The transcriptional activity of affinity-purified swTFIIIC was assayed by transcription reconstitution *in vitro*, using $tRNA_I^{Gly}$ as template (Fig. 6B). Clearly the transcription was achieved only when affinity purified TFIIIC was supplemented to reconstituted transcription system comprising of PC-B (RNA pol III and TFIIIB) and TFIIIC1 fractions. With increasing concentrations of TFIIIC, initially there was an increase followed by a reduction in transcription activity at the highest level tested. The inhibitory effect on transcription resulting from an excess of TFIIIC could be

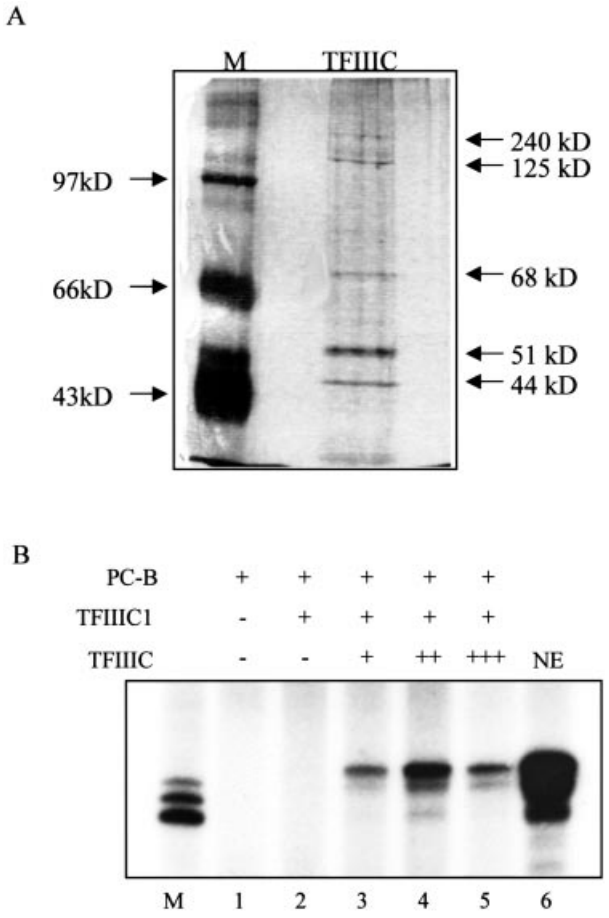

Fig. 6. Subunit composition and transcription activity TFIIIC (A). SDS/PAGE gel analysis of silkworm TFIIIC. Polypeptides from the B box affinity column step were separated by 8% SDS/PAGE and visualized by silver staining. The subunit sizes are marked on the side. Lane M, molecular size markers. (B). Transcription of $tRNA_I^{Gly}$-1 was assayed with purified TFIIIC in the presence of 1 μg of the PC-B fraction (which provided the TFIIIB and pol III activities), TFIIIC1 (0.5 μg of protein in the 310 mM KCl eluate from Q-Sepharose) and varying amounts of TFIIIC, as indicated. Lanes: M, Marker pTZ plasmid DNA digested with *Hinf* I and end labeled; 1, reconstituted transcription with PC-B alone; 2, reconstituted transcription with TFIIIC1, and PC-B; 3–5 reconstituted transcription in the presence of 50, 100 and 200 ng of affinity purified TFIIIC; 6, transcription of $tRNA_I^{Gly}$-1 with crude posterior silk glands nuclear extract.

due to sequestration of other components from the transcription machinery. The levels of reconstituted transcription using the purified components never reached the levels of transcription with the crude nuclear extracts from posterior silk glands. Quantification of the reconstituted transcription by phosphorimaging showed that highest levels reached were only 10–15% of that from crude nuclear extracts. It is likely that part of the activity was lost due to inactivation of some component or due to the removal of some stimulatory factor(s) required for $tRNA_I^{Gly}$ transcription, because the specific B box binding activity increased from 14 $U \cdot mg^{-1}$ proteins in the crude nuclear extracts to 4673 $U \cdot mg^{-1}$ protein in the affinity purified fraction (one unit is defined as the amount of TFIIIC necessary to bind 1fmol of B box sequence probe).

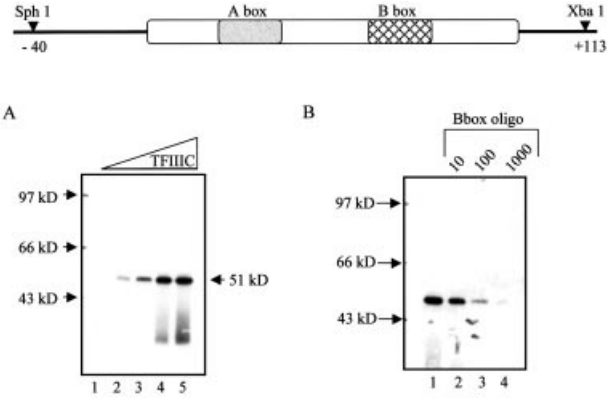

Fig. 7. Crosslinking of swTFIIIC to the $tRNA_I^{Gly}$ promoter. A. The 150-bp DNA fragment encompassing the entire tRNA coding region as well as the ICRs (shown in top panel), radiolabeled with [α-^{32}P]dATP and BrdU (to facilitate UV crosslinking), was used as probe for TFIIIC binding. Standard binding reactions were performed (in presence of 1000-fold molar excess of nonspecific DNA fragments, described in Fig. 5 legend) using the affinity-purified TFIIIC and the samples were subsequently irradiated with UV. Excess free DNA was removed by DNase digestion, and the polypeptides were separated on SDS/PAGE (0.1% SDS/8% acrylamide). Lanes: 1, control (crosslinking in the absence of any protein); 2–5, crosslinking in the presence of increasing concentrations of TFIIIC. (B) UV crosslinking was performed in the presence of excess unlabeled B box DNA as specific competitor. Lanes: 1, crosslinking in the absence of specific competitor; 2–4, crosslinking in the presence of 10-, 100- and 1000-fold molar excess of competitor. The positions of the molecular mass markers are indicated.

The 51-kDa subunit of silkworm TFIIIC binds to the tRNA B box

Because TFIIIC is a sequence-specific DNA binding protein, one or more of its subunits must come into close contact with the DNA recognition site. In order to determine which of the five polypeptides present in the affinity-purified TFIIIC preparation was directly involved in DNA binding, DNA protein crosslinking experiments were carried out. After UV crosslinking the TFIIIC bound to the labeled probe (the entire $tRNA_I^{Gly}$ coding region with −40 bp 5′ upstream and 30 bp 3′ downstream flanking sequences, shown in the top panel of Fig. 7), the samples were digested with DNase I and analyzed by SDS/PAGE (Fig. 7A). A single polypeptide of 51 kDa was crosslinked to the $tRNA_I^{Gly}$-1, in the entire range of TFIIIC concentration tested. The binding of the 51-kDa band to the $tRNA_I^{Gly}$-1 B box was specific because it was efficiently competed out in the presence of excess unlabeled B box oligonucleotides (Fig. 7B). A 1000-fold excess of B box oligonucleotides completely chased out the complex of 51-kDa protein and the $tRNA_I^{Gly}$-1.

Determination of equilibrium constant of TFIIIC with $tRNA_I^{Gly}$ B box

EMSA, which separates DNA probe–protein complex from unbound probe, provides a simple assay for studying Specific DNA–protein interactions [18]. Figure 8A shows the result of a titration experiment in which TFIIIC DNA

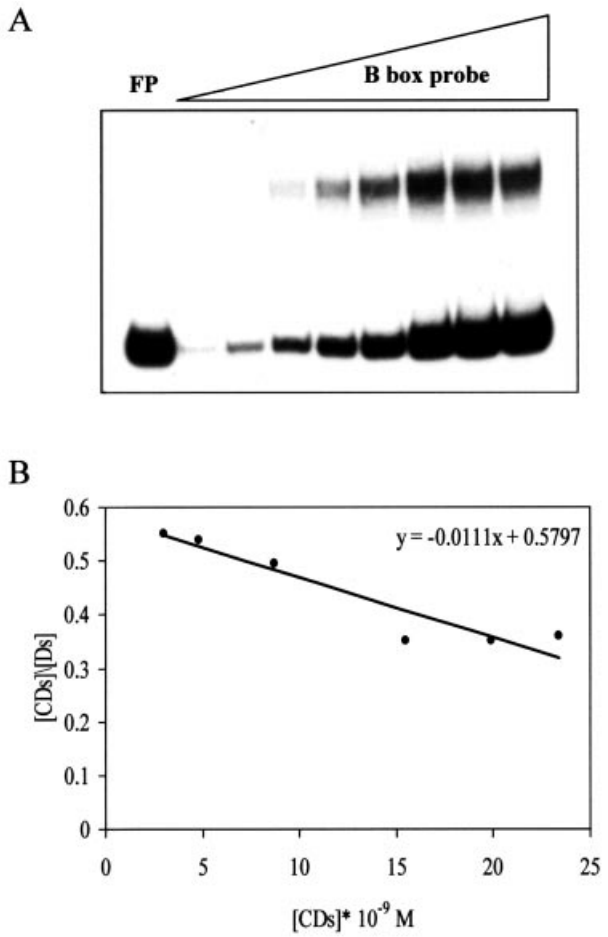

Fig. 8. Equilibrium constant for the binding of swTFIIIC with B box DNA. (A) EMSAs were performed with increasing amounts of labeled B box and 100 ng of affinity purified swTFIIIC. A typical autoradiogram of a titration gel is presented. (B) The DNA–protein complex formed in the binding reaction and the unbound probe were quantified in a phosphorimager, following electrophoresis. The binding data was analyzed by Scatchard plots. The negative reciprocal of the slope gives the binding constant for TFIIIC binding to $tRNA_I^{Gly}$ B box.

complex formation was monitored at increasing concentrations of the labeled B box sequence. Because only the affinity purified TFIIIC was used for the binding reaction, addition of excess carrier DNA to quench the nonspecific binding due to other proteins was not required. Therefore, the binding reaction mixtures did not contain any DNA other than the labeled probe. The equilibrium constant for TFIIIC binding was determined from the binding data (shown in Fig. 8A) by the graphical analysis (Fig. 8B). In this plot, the slope of the line gives the negative reciprocal of the binding constant. The binding constant for swTFIIIC to $tRNA_I^{Gly}$ B box was calculated to be 9.01×10^{10} M^{-1}.

Histone acetyl transferase (HAT) activity of swTFIIIC

The presence of any associated HAT activity with the swTFIIIC preparation was analyzed during different stages of purification of the transcription factor. The silkworm core histones, purified from posterior silk glands of *B. mori* larvae on the second day of the fifth instar, were used as

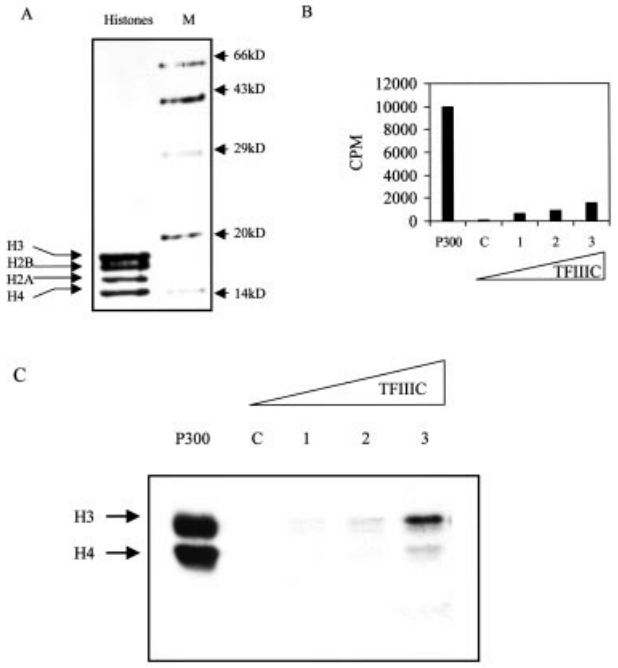

Fig. 9. Histone acetyltransferase activity of partially purified swTFIIIC. (A). The core histones purified from posterior silk glands nuclei. The positions of individual histones are indicated. Lane M corresponds to protein molecular mass marker. (B) Filter binding assays were performed with 1 μg of silkworm core histones, [^{3}H]acetyl-CoA, and increasing concentrations of partially purified TFIIIC (280 mM KCl eluate from Q-Sepharose; lanes, 1–3). HAT domain of recombinant P300 was used as a positive control for the assay and the control in which no TFIIIC was added, is marked C. (C) Substrate specificity of silkworm TFIIIC. The acetylated histones were separated on a 15% SDS/PAGE to examine the substrate specificity of swTFIIIC. Lanes 1–3, acetylation of silk gland histones with increasing concentrations of swTFIIIC; C, control (no TFIIIC); P300, positive control.

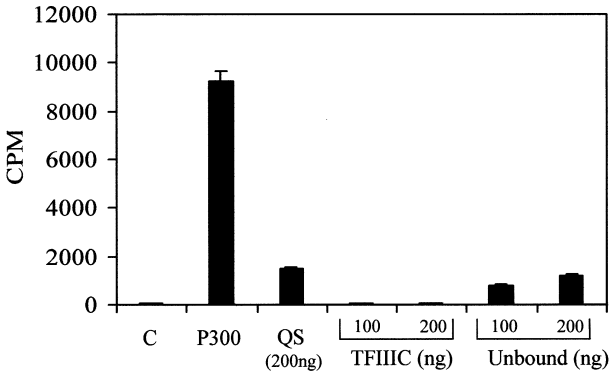

Fig. 10. HAT activity of affinity purified TFIIIC. Filter binding assays were performed with 1 μg of silkworm core histones, [^{3}H]acetyl-CoA, and indicated amounts of the partially purified TFIIIC (Q-Sepharose fraction), affinity purified TFIIIC, and the unbound fraction of the B box oligo affinity column. Recombinant HAT domain P300 was used as a positive control for the assay; C, control (no enzyme added).

substrate for acetylation (Fig. 9A). The presence of four core histones was clearly seen. HAT assays using the partially purified TFIIIC fraction (280mMKCl fraction) from the Q-Sepharose column is presented in Fig. 9B. The HAT domain from recombinant P300 (expressed in *Sf*21 insect cells) was used as a positive control in these assays, which showed a high level of HAT activity (Fig. 9B; lane P300). The partially purified swTFIIIC showed a modest but significant level of HAT activity (Fig. 9B; lanes 1–3), much above the background levels (lane C, mock assay with no TFIIIC added). The histone substrate specificity of partially purified swTFIIIC was assessed by electrophoretic resolution of radiolabeled proteins. Of all the four core histones, swTFIIIC acetylated predominantly H3; and weak acetylation of histone H4 and H2B was also seen (Fig. 9C; lane 2–5). As reported previously [22], the HAT domain of P300 fragment predominantly acetylated both H3 and H4 (Fig. 9C; lane marked P300).

The affinity-purified fraction of swTFIIIC, however, did not show any associated HAT activity (Fig. 10). Most of the HAT activity was recovered in the fraction that was not bound to the DNA affinity matrix (Fig. 10; lanes marked). These results suggested that the HAT activity seen with the TFIIIC fraction was only weakly or nonspecifically associated and was dissociated from it during the affinity purification step.

Because swTFIIIC lacked an intrinsic HAT activity, the effect of external supplementation of HAT activity on $tRNA_I^{Gly}$ transcription from reconstituted chromatin template was examined. Chromatin was reconstituted *in vitro* using the *Drosophila* S190 extract [20] in the resence of purified core histones from silkworm. The *in vitro* transcription by posterior silk glands nuclear extract was abolished from the chromatin template compared to the high levels of transcription seen with the DNA template (Fig. 11). The HAT activity present in the unbound fraction from B box DNA affinity step of TFIIIC purification, when supplemented to the nuclear extract, was able to partially restore the transcription from the chromatin templates. However, addition of the fraction showing HAT activity

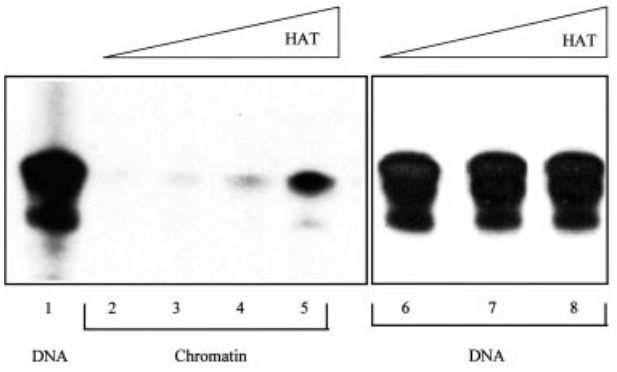

Fig. 11. Transcription of chromatin template. The plasmid clone pR8 harboring $tRNA_I^{Gly}$ was assembled into chromatin using the *Drosophila* S190 extract and assessed for transcription activity using posterior silk gland nuclear extracts. Lanes: 1, transcription from DNA template; 2, transcription from *in vitro* assembled chromatin; 3–5, transcription from *in vitro* assembled chromatin in the presence of increasing amounts of HAT activity; lanes 6–8, transcription from naked DNA template in presence of increasing amounts of HAT activity.

had no effect on the transcription from the naked DNA template (Fig. 11, lanes 6–8). Thus, HAT activity is essential for $tRNA_1^{Gly}$ transcription from chromatin template.

DISCUSSION

The posterior silk glands of *B. mori* are specialized organs involved in the massive production of the silk protein fibroin during the fifth instar of larval development. To meet such high protein synthetic demands, posterior silk gland cells undergo a functional adaptation to meet the cellular needs of specialized tRNAs [23,24]. Consequently, together with high levels of RNA polymerase II activity, there is concomitant increase in pol III activity.

Amongst the two transcription factors involved in tRNA gene transcription, TFIIIC is of particular importance because of its role in the regulation of the overall levels of transcription by pol III in a number of systems. For instance the increased pol III activity seen in adenoviral infected HeLa cells is accounted for by an increase in TFIIIC activity mediated by the E1A protein of adenovirus [25]. Furthermore, TFIIIC activity is also increased in cases of ovarian cancers, a situation where increased levels of pol III activity are seen [26]. High levels of TFIIIC were present in the pol III nuclear extracts from the posterior silk glands of *B. mori*.

sw TFIIIC is a heteromeric complex of at least five polypeptides of sizes 240, 125, 68, 51 and 44 kDa, which showed sequence specific binding to the B box sequences of $tRNA_1^{Gly}$ genes and also fostered *in vitro* transcription activity. Both yeast and human TFIIIC are shown to be large proteins with multiple polypeptides [1]. The human TFIIIC is more complex and could be fractionated into C1 and C2 components. The fractionation of swTFIIIC activity also divided the TFIIIC activity into two components, which we have designated as TFIIIC and TFIIIC1. The B box binding activity was associated with the TFIIIC fraction but this fraction alone was not sufficient to foster transcription of $tRNA_1^{Gly}$ genes in the reconstituted system. However, the transcription activity could be restored by supplementation of the TFIIIC1 fraction. Ottonello *et al.* [14] had previously reported an activity called TFIIID that was required for tRNA gene transcription. This factor apparently played a role in the assembly of other factors into transcription complexes but TFIIID by itself did not show binding to the tRNA promoter. Similarly human TFIIIC1is also reported to enhance the binding of TFIIIC2 to the B box of pol III promoters [21]. We have not established the identity of TFIIIC1 reported here to either of these.

The amount of the 51-kDa polypeptide in swTFIIIC preparation was at least twice as much as compared to the rest of the polypeptides. The 51-kDa polypeptide of TFIIIC could be specifically crosslinked to the B box of $tRNA_1^{Gly}$ genes suggesting it to be a bona fide subunit of TFIIIC. However, in the absence of any confirmatory genetic data, we have yet to establish that the identified polypeptides are indeed components of TFIIIC based on the biochemical fractionation alone. For instance, a predominant 55-kDa protein present in *Xenopus* TFIIIC preparations purified through B box DNA affinity chromatography, which shows binding to B box sequences, turned out to be a Y-box binding factor (designated YB3) [27] and not a component of TFIIIC. Therefore, the assignment of TFIIIC subunits shown here is only provisional. The binding affinity of swTFIIIC (9.01×10^{10} M^{-1}) to the B box of $tRNA_1^{Gly}$ genes was very similar to that reported for yeast (1.0×10^{10} M^{-1} for the $tRNA^{Tyr}$ gene) [28] or human TFIIIC with (4.2×10^{10} M^{-1} for the VA1 promoter) [18].

Yeast as well as human TFIIIC preparations relieve chromatin mediated repression of preassembled class III gene transcription *in vitro* [13,29]. hTFIIIC was subsequently shown to have an intrinsic HAT activity associated with the 230-, 110- and 90-kDa subunits [13]. Due to its associated HAT activity, hTFIIIC can bind alone to the A and B boxes of a tRNA gene within a chromatin template. However, no HAT activity has been reported to be associated with yTFIIIC. Transcription of chromatin templates requires the targeted acetylation of the histones bound at the promoter region. Our analysis of HAT activity of silkworm TFIIIC suggested that the HAT activity was weakly associated with the TFIIIC fraction but gets separated at the final step of sequence-specific DNA affinity purification. However, the transcription of $tRNA_1^{Gly}$ that was subjected to repression by chromatin assembly could be partially overcome by external supplementation of the HAT activity associated with TFIIIC fraction. This HAT activity did not show any effect on the transcription from naked DNA template. swTFIIIC therefore is capable of directing nucleosomal transcription by associating with a HAT activity present in the cell, if not as an intrinsic component of the TFIIIC itself.

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