Silkworm TFIIIA requires additional class III factors for commitment to transcription complex assembly on a 5S RNA gene

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ABSTRACT

We find striking similarities in promoter structure and requirements for template commitment on 5S RNA and tRNA genes from silkworms. The promoters are nearly the same size (~160 bp) and include flanking as well as internal sequences. To analyze the factor requirements for 5S RNA transcription complex assembly in a completely homologous system, we have isolated a silkworm fraction that is highly enriched for the 5S RNA-specific transcription factor, TFIIIA. Using this fraction, together with the other silkworm fractions, TFIIIB, TFIIIC, TFIIID and RNA polymerase III, we demonstrate that the requirements for 5S RNA transcription complex assembly are very similar to those previously established for a tRNA^{Ala} gene. Specifically, no individual factor fraction is sufficient for commitment of silkworm 5S RNA genes to transcription complex assembly. Rather, combinations of at least three factor fractions are required. Our observation that more than one subset of factors is competent for commitment suggests that silkworm 5S RNA genes further resemble tRNA^{Ala} genes in their ability to use multiple pathways for transcription complex formation.

INTRODUCTION

Transcription of tRNA and 5S RNA genes by RNA polymerase III involves the formation of stable complexes between transcription factors and sequences within the genes. Until recently, transcription of both classes of genes was thought to depend entirely on relatively short internal sequences: the A and B boxes in tRNA genes, and the A and C boxes in 5S RNA genes [reviewed in (1)]. In the case of tRNA genes, however, there is now considerable evidence for the promoter function of sequences outside the A and B boxes [reviewed in (2)]. Furthermore, sequences outside the A and C boxes have been shown to affect promoter activity in the 5S RNA genes of

silkworms (3), *Drosophila* (4,5), *Neurospora* (6–8), yeast (9) and humans (10). Indeed, even in *Xenopus* 5S RNA genes, where the internal promoter was first defined (11,12), flanking sequences contribute to promoter function (13–15).

The multiplicity of factors that are required for 5S RNA and tRNA transcription is consistent with the observed structural complexity of the promoters. It is now recognized that the two fractions that act on both tRNA and 5S genes, TFIIIB and TFIIIC, each contain more than one polypeptide that is essential for transcription [reviewed in (1,2)]. In addition to these two fractions, transcription of 5S RNA genes requires the gene-specific factor, TFIIIA (16,17). The assembly of active transcription complexes from these components has been investigated by analyzing the interactions of the single transcription factor, TFIIIA, as well as the multi-factor fractions, TFIIIB and TFIIIC, with template DNA. These experiments have led to the idea that transcription factors assemble on both 5S RNA and tRNA genes in a strict, linear order, initiated by the stable or metastable interaction of a single factor with the promoter (18–20).

Analysis of tRNA gene transcription in the silkworm system has demonstrated that sequences outside the canonical A and B boxes contain important promoter elements (21–23). Furthermore, fractionation of the silkworm transcription machinery shows that at least three factor fractions are required, in addition to RNA polymerase III, for transcription of tRNA genes. These fractions are designated TFIIIB, TFIIIC and TFIIID (24). An additional component composed of RNA, TFIIIR, is required to reconstitute silkworm class III transcription *in vitro* (25). The RNA with activity has been identified as a particular isoleucine tRNA (26), but since it has recently been shown to play an indirect, protective, role in transcription (27), we no longer consider it a true transcription factor. The other three factor fractions, though resolved from one another, still contain multiple polypeptides, some or all of which may act in class III transcription. Thus, the silkworm system displays complexity in both its *cis*- and its *trans*-acting elements. Moreover, the formation of active transcription complexes on silkworm tRNA genes is not restricted to the single, linear pathway reported in other systems. Instead, transcription complex assembly on a

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silkworm tRNA^{Ala} gene can proceed by at least two pathways, and neither of them is initiated by the stable or metastable interaction of a single factor fraction with the gene. Rather, a combination of two factor fractions is required (24).

To determine the generality of our findings with the silkworm tRNA^{Ala} gene, we asked whether they could be extended to a different kind of polymerase III template. We therefore analyzed the promoter and factor requirements for transcription of a silkworm 5S RNA gene. Transcription of 5S RNA genes requires the gene-specific factor, TFI_{III}A, which had not previously been isolated from insects. Since we wished to avoid the potential complications of a heterologous system, we isolated a fraction that is highly enriched for silkworm TFI_{III}A. 5S RNA transcription activity was reconstituted by combining the TFI_{III}A fraction with the previously described fractions of the silkworm transcription machinery: polymerase III, TFI_{III}B, TFI_{III}C and TFI_{III}D.

We show that the transcription factor and promoter requirements for transcription of silkworm 5S RNA genes are remarkably similar to those for silkworm tRNA genes. The maximum extents of both promoters are nearly equal (~160 bp), and both include flanking as well as internal sequences. Moreover, transcription complex assembly proceeds in a similar manner on both kinds of genes. Specifically, no factor fraction can independently commit a silkworm 5S RNA gene to transcription complex assembly. Combinations of at least three factor fractions are required. As with the silkworm tRNA^{Ala} gene, more than one subset of fractions is competent, however. Thus, there appear to be multiple pathways by which active transcription complexes can form on both 5S RNA and tRNA genes in the silkworm system.

MATERIALS AND METHODS

Cloned genes used for this work

The silkworm 5S RNA gene used in this work is a derivative of the one described previously (3) in which the vector M13 DNA has been modified to ensure efficient termination by RNA polymerase III. To modify the vector, the 452 bp *HincII*–*EcoRI* fragment of pBR322 was inserted into M13mp18 that had been cleaved with *AvaII*, treated with DNA polymerase I Klenow fragment to generate blunt ends, and cleaved with *EcoRI* (designated M13mp18term). Subsequently, the silkworm 5S RNA gene was inserted as a 292 bp *TaqI* fragment (from position –92 to +200 with respect to the transcription initiation site) into the *AccI* site of the modified vector, and designated 5'Δ–92. The orientation was such that the 5' rather than the 3' end of the 5S RNA gene is closest to the *HindIII* site in M13mp18. The resulting gene-containing *BamHI*–*HindIII* fragment was also inserted into M13mp19, creating a construct called 5SRO for reverse orientation. The silkworm alanine tRNA used for this work has been described (22).

Construction of deletion mutations

Deletions were produced by BAL31 exonuclease treatment of either *BamHI*-digested 5'Δ–92 in M13mp18 (downstream deletions), or *HindIII*-digested 5'Δ–92 in M13mp19 (upstream deletions), using established procedures (3,28).

Transcription assays with crude extracts

Extracts derived from *Bombyx* silk glands (29) or ovaries (30) were used to provide the *in vitro* transcription systems for testing the activity of mutant derivatives of 5S RNA and tRNA genes. Transcription reaction conditions and analysis of transcripts were as described (3,29). To maximize detection of mutant phenotypes, non-specific M13mp18 vector DNA was included in each reaction mixture along with a sub-saturating concentration of template (29).

Purification of silkworm TFI_{III}A

TFI_{III}A assay. Biorex chromatography of crude extracts from posterior silk glands was used to generate a concentrated source of the class III transcription components used to complement TFI_{III}A. 40 ml of crude extract (29) was dialyzed against 25 vol of ice-cold Buffer BRX (10 mM MgCl₂, 50 mM Tris–HCl (pH 7.9), 20% glycerol, 1 mM DTT) plus 150 mM KCl for 1 h. The dialyzed extract was loaded onto a 30 ml column (2.5 cm diameter) of Biorex 70 (Biorad) equilibrated in Buffer BRX plus 150 mM KCl, and run at a flow rate of 0.6 ml/min. The column was washed with 1.5 column volumes of the same buffer, followed by successive washes with 1.2 column volumes of Buffer BRX plus 280 mM KCl and Buffer BRX plus 600 mM KCl. 1.5 ml fractions were collected, dialyzed against 50 vol of 50 mM Tris–HCl (pH 7.5), 4 mM MgCl₂, 20% glycerol, 1 mM DTT, 125 mM KCl, and assayed for protein content and the ability to direct transcription of a silkworm tRNA gene. Aliquots of the protein peak resulting from the Buffer BRX plus 600 mM KCl eluate were frozen and stored at –70°C. This fraction was designated BRX600. It was tested for the ability to transcribe tRNA genes but not 5S RNA genes. 5S RNA transcription required the addition of *Xenopus* TFI_{III}A, and was not stimulated by addition of any of the other fractions (TFI_{III}B, TFI_{III}C, TFI_{III}D, or RNA polymerase III). TFI_{III}A assay mixtures contained 3.5–5 μl of the BRX600 fraction and 200 ng of clone 5SRO in a total reaction volume of 40 μl. Transcription was carried out at 22°C for 2 h using the conditions described for crude extracts.

Purification methods. All procedures were carried out at 4–10°C. 0.5 ml of crude extract (20–25 mg/ml protein) from *Bombyx* pupal ovaries (3) was filtered through a 0.2 μ filter (Millex GV) and applied to a 25 ml Superose 12 gel filtration column (Pharmacia) equilibrated in Buffer C (5 mM MgCl₂, 50 mM Tris–HCl (7.5), 2 mM DTT, 10% glycerol) with 0.125 M or 1.0 M KCl, and run at a flow rate of 0.3 ml/min. The column was washed with 25 ml of the same buffer, and 0.35 ml fractions were collected. The fractions from columns run in 0.125 M KCl buffer were assayed for TFI_{III}A with no further manipulation. The fractions from the columns run in 1.0 M KCl buffer were dialyzed against 50 vol of ice-cold Buffer C with 100 mM KCl for 2 h before being assayed for TFI_{III}A activity. Fractions with TFI_{III}A activity in the excluded volume from a Superose 12 gel filtration column equilibrated in 0.125 M KCl were pooled, and 4–7 ml of the pool (0.3–0.5 mg/ml protein) were applied to a 1 ml Mono S analytical column (Pharmacia) equilibrated in Buffer C with 0.125 M KCl (flow rate of 0.5 ml/min). The column was washed with the same buffer until the OD₂₈₀ of the eluate had returned to the baseline level, and then washed with Buffer C containing 0.38 M KCl (usually 8–12 column volumes). TFI_{III}A activity was eluted with a linear gradient of KCl in buffer C at a rate of increase

of 85 mmol/ml from 0.38 to 1.0 M KCl. Fractions were collected in tubes containing BSA (final concentration 0.4 mg/ml), and were then dialyzed against Buffer C plus 75 mM KCl until the conductivity was equal to that of the dialysis buffer. The fractions were assayed for TFIIIA activity, frozen, and stored at -70°C . For footprinting reactions, TFIIIA-containing fractions from multiple Mono S column runs were concentrated 10-fold by Amicon pressure filtration, assayed for TFIIIA activity, and stored at -70°C .

SDS-PAGE analysis of proteins. The proteins in the fractions from Mono S chromatography (0.35 ml each) were precipitated in 10% trichloroacetic acid (TCA), and fractionated on a discontinuous SDS-PAGE gel. After electrophoresis, the gel was fixed, and stained with 0.1% silver nitrate (31). The concentration of silkworm TFIIIA in the Mono S fractions with peak transcriptional activity was estimated at 1×10^{-10} M, based on the intensity of silver staining of gel-fractionated protein, in comparison with known amounts of *Xenopus* TFIIIA run in parallel. The amounts of either silkworm or *Xenopus* TFIIIA used in particular experiments correspond to total TFIIIA, without correction for the fraction of protein that was active.

Other class III transcription factor fractions

Preparation of transcription factor fractions designated TFIIIB, TFIIIC, TFIIID, TFIIIR and RNA polymerase III have been described (24,25,27). Protein concentrations for the fractions used for these experiments were: TFIIIB, 0.6 mg/ml; TFIIIC, 80 $\mu\text{g}/\text{ml}$; and TFIIID, 10 $\mu\text{g}/\text{ml}$.

Gel retardation analysis and DNase I footprinting

Gel retardation assay. Gel retardation analysis was carried out essentially as described (32). A 340 bp DNA fragment (*EcoRI*–*PstI* fragment of clone 5SRO) containing the silkworm 5S RNA gene was labeled using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and the Klenow fragment of *E. coli* DNA polymerase I to a specific activity of $1\text{--}2 \times 10^3$ c.p.m./fmol. Binding reactions using the silkworm TFIIIA fraction contained 5 μl fraction, $1\text{--}5 \times 10^{-10}$ M DNA fragment, 50 $\mu\text{g}/\text{ml}$ poly(dI–dC), and 40 $\mu\text{g}/\text{ml}$ BSA, in a total volume of 15 μl Buffer B [20 mM Tris–HCl (pH 7.5), 7 mM MgCl_2 , 50 mM KCl, 10% glycerol, 4 mM DTT, 10 mM ZnCl_2]. Binding reactions with purified *Xenopus* TFIIIA were carried out under the same conditions, except that no poly(dI–dC) was added to the reaction. The binding reactions were incubated for 20 min at 22°C , and loaded onto a 6% polyacrylamide gel. The gel and running buffer contained 0.025 M Tris–base, 0.2 M glycine; 5% glycerol (v/v). The gel was pre-run at 4°C and 300 V for 60 min, and electrophoresis was at 4°C and 250 V for 3–5 h. Bound and free DNA were visualized by autoradiography of wet or dried gels. To test specificity, specific (340 bp *EcoRI*–*PstI* fragment of 5SRO) or non-specific (375 bp *EcoRI*–*BamHI* fragment of pBR322) competitor fragments were mixed with the labeled probe in the indicated molar ratios, before addition of TFIIIA.

DNase I protection (footprinting) assay. Binding reactions and analysis were as described for gel retardation, except that the reaction mixture was doubled and, before loading on a gel, it was incubated with 10 ng DNase I for 60 s. DNA in the bound and free positions on the gel was eluted, freed of protein by phenol

extraction and ethanol precipitation and fractionated on a 5% sequencing gel (28).

Template commitment assays

The protocol for measuring template commitment is shown schematically in Figure 7A, and has been described in detail (24). Conditions for binding were as for transcription, except that the reaction was carried out for 20 min at 22°C in a total volume of 8–28 μl containing 10 μM unlabeled UTP, 75 ng (15 fmol) of 5SRO (gene 1) and the appropriate factor fractions or mock buffers. The second incubation period (60 min, 22°C) was initiated by the addition of 75 ng of 3' Δ +118 (gene 2) plus the remaining fractions and appropriate buffer components. Transcription was measured during a final incubation period (60 min, 22°C) initiated by the addition of 10 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ 800 Ci/mmol to a total volume of 43–50 μl . An amount of each fraction that just saturated 5 μl TFIIIA, was used: 5 μl each TFIIIB, TFIIIC, TFIIID, TFIIIR; 3 μl RNA polymerase III.

RESULTS

The silkworm 5S RNA promoter

The promoter of the silkworm tRNA^{Ala} gene includes at least 161 bp (29). To compare the extent of the silkworm 5S RNA gene promoter, we constructed a series of deletions that resected the 5S RNA gene from either the 5' or the 3' side. We determined the ability of the resulting mutant genes to direct transcription in unfractionated extracts. To maximize detection of subtle phenotypes, we avoided saturating the transcription machinery with template (29). As shown in Figure 1A, deletions from the 5' side establish the existence of an essential upstream promoter element whose upstream boundary is between –32 and –26. Thus, under conditions that detect mutant phenotypes more sensitively, we have confirmed our previous results (3). Deletions from the 3' side show that the downstream boundary of the 5S RNA promoter is between +134 and +128. Thus, the silkworm 5S RNA promoter is approximately the same size as the silkworm tRNA^{Ala} promoter, including at least 156 bp. The previously published deletion analysis of the silkworm tRNA^{Ala} promoter (29) is plotted in Figure 1A to facilitate comparison of tRNA and 5S RNA promoters in the silkworm system. Both promoters clearly extend beyond the classical promoter elements indicated by black rectangles. They also resemble each other in the distribution of transcriptionally important sequences. In both cases, sequences that are essential for transcription are located upstream, whereas sequences that are stimulatory, but not essential, are downstream.

Silkworm TFIIIA

We anticipated that analysis of 5S transcription complex assembly would require TFIIIA, in addition to the factors that allow tRNA transcription. Since none of the fractions previously resolved from silkgland nuclear extracts contained TFIIIA activity, we developed a method for isolating this factor from extracts of silkworm oocytes. We chose oocytes because, although oocyte and silkgland extracts are about equally active in tRNA transcription, oocyte extracts are much more active than silkgland extracts in 5S RNA transcription. To assay TFIIIA activity during purification, we took advantage of the natural inefficiency of 5S RNA transcription in silkgland extracts, and

Figure 1. The promoters of silkworm 5S and tRNA^{Ala} genes are similar in size and position. (A) The transcription activities of deletion mutants of a silkworm 5S RNA gene (transcribed in crude extracts), relative to that of a full-length gene are plotted in boldface (squares) against the deletion endpoint, along with comparable data for mutants of a tRNA^{Ala} gene (circles) previously published in (29). Deletions entering the genes from the upstream end (5'Δ) are shown as closed symbols, whereas deletions entering the genes from the downstream end (3'Δ) are shown as open symbols. Numerical data for the 5S deletion mutants are: 5'Δ-49, 104 ± 7% (n = 4); 5'Δ-44, 99 ± 6% (n = 5); 5'Δ-36, 107 ± 6% (n = 4); 5'Δ-26, 88 ± 4% (n = 4); 5'Δ-24, 90 ± 8% (n = 5); 5'Δ-21, 76 ± 7% (n = 4); 5'Δ-17, 9 ± 4% (n = 5); 5'Δ-6, 2 ± 2% (n = 4); 3'Δ+193, 93 ± 9% (n = 5); 3'Δ+174, 104 ± 6% (n = 7); 3'Δ+135, 93 ± 13% (n = 16); 3'Δ+128, 83% (n = 2); 3'Δ+118, 67 ± 11% (n = 15); 3'Δ+104, 63 ± 12% (n = 16); 3'Δ+97, 49 ± 16% (n = 4); 3'Δ+93, 32 ± 12% (n = 12); 3'Δ+91, 19% (n = 2); 3'Δ+86, 10 ± 10% (n = 4); 3'Δ+76, 10 ± 10% (n = 3); 3'Δ+68, 2 ± 4% (n = 4). The transcribed region (open rectangle), positions of canonical promoter elements (black boxes), and direction of transcription are shown diagrammatically below the graph. The limits of the *Bombyx* TFIIIA footprint (results shown in Fig. 5) are bracketed below the 5S gene. (B) The sequences of the 5S and tRNA^{Ala} genes are shown juxtaposed with the endpoints of selected 5' and 3' deletion mutants. The transcriptional phenotype of each mutant is indicated parenthetically after the deletion endpoint.

reduced this activity still further by ion exchange chromatography on Biorex 70. Figure 2 shows the differential transcription of tRNA and 5S RNA genes by a step-elution fraction from such a column. Figure 2 also demonstrates the utility of this fraction as a TFIIIA assay, by showing that 5S RNA transcription is specifically stimulated by highly purified *Xenopus* TFIIIA (the generous gift of David Setzer).

In designing a purification protocol for silkworm TFIIIA, we exploited our observation that TFIIIA activity in unfractionated extracts migrates anomalously during gel filtration chromatography in the presence of 5 mM MgCl₂ and relatively low concentrations of KCl (125 mM). Specifically, under these conditions, most of the TFIIIA activity appears in the void volume, in contrast to its position in the included volume when chromatographed in 5 mM MgCl₂ plus 1 M KCl. Presumably, the anomalous migration in low concentrations of KCl reflects aggregation or association of TFIIIA with other components in the extracts. This unusual elution position is useful because it

separates TFIIIA from other class III transcription factors that contaminate TFIIIA chromatographed in 1 M KCl (principally, TFIIIB and TFIIID). Accordingly, we used gel filtration chromatography in 5 mM MgCl₂ and 125 mM KCl as the first step in TFIIIA purification. The material obtained from the void volume was further purified by gradient elution from a cation exchange (Mono S) column. TFIIIA eluted from this column as a single peak of 5S RNA gene-specific transcription activity at 0.68 M KCl, and was free of detectable RNA polymerase III, TFIIIB, TFIIIC or TFIIID.

The protein content and transcriptional activity of the TFIIIA fraction purified in this manner is shown in Figure 3. Individual fractions from the Mono S column contain a prominent 33.5 kDa polypeptide (indicated by an arrow), whose abundance correlates with 5S-specific transcription activity. The size of this polypeptide is within the range (25–50 kDa) we had previously estimated for silkworm TFIIIA activity by density gradient sedimentation of extracts (data not shown). To determine whether these fractions

Figure 2. Specificity of the assay for TFIIIA activity. The 0.6 M KCl step fraction from Biorex 70 chromatography of crude silkgland extract was used to transcribe a silkworm 5S RNA or a silkworm tRNA^{Ala} gene. The fraction was supplemented (+) or not (-) with 0.5 nM TFIIIA purified from *Xenopus* oocytes.

Figure 3. A 33.5 kDa protein co-elutes with TFIIIA activity from MonoS. Fractions from the 0.38 M–1.0 M KCl gradient applied to a Mono S column were assayed for TFIIIA activity, using the Biorex 600 fraction described in the legend to Figure 2 (see Methods), and for protein content by SDS-PAGE and silver-staining. Lane V was loaded with 30 μ l of the pool of Superose 12 void fractions that was the input for the Mono S column. Lane S was loaded with 30 μ l of the material that eluted from Mono S with 0.38 M KCl. Ten μ l of each Mono S gradient fraction was used in the activity assay; 350 μ l was used for the silver-stained gel. The position of the 33.5 kDa protein (arrow) and molecular weights and positions of marker proteins are indicated.

had the additional properties expected of TFIIIA, we used a combination of gel mobility shift and DNase I footprinting to characterize the interaction of their constituent proteins with the silkworm 5S RNA gene. The Mono S fractions with the highest TFIIIA activity from several column runs were pooled, concentrated by pressure filtration (Materials and Methods) and incubated with a labeled DNA fragment that contains a silkworm

Figure 4. Formation of specific complexes between silkworm or *Xenopus* TFIIIA and the silkworm 5S RNA gene. (A) 5 μ l of concentrated *Bombyx mori* silkworm TFIIIA, giving a final concentration of ~ 0.3 nM, (lane B) or 3 nM *Xenopus* TFIIIA (lane X) were used in binding reactions with 2 fmol of a 340 bp fragment containing a silkworm 5S RNA gene. The positions of the bound complex and the unbound fragment after electrophoresis on a nondenaturing gel are indicated. (B) Specificity of the silkworm TFIIIA-gene interaction was shown by determining the relative amount of bound radioactive probe fragment that remained when increasing amounts of unlabeled competitor fragments were included in a binding reaction that contained 7 fmol of probe. Competitor fragments used were the 340 bp gene fragment (specific) or a fragment of pBR322 of the same length (non-specific).

5S RNA gene. Gel retardation analysis revealed a protein–DNA complex that migrates more slowly than the DNA fragment itself (Fig. 4A). This is a specific complex, since it can be competed with unlabeled gene-containing DNA fragments in a concentration range in which non-specific DNA fragments have little effect (Fig. 4B). The protein–DNA complex formed with the silkworm protein migrates slightly faster than the complex formed with *Xenopus* TFIIIA, consistent with putative silkworm TFIIIA being smaller than *Xenopus* TFIIIA [33.5 kDa compared to 38–40 kDa (16,33–35)].

A footprint of the silkworm protein was obtained by treating the binding reaction mixture with DNase, and then separating bound from unbound DNA fragments by non-denaturing gel electrophoresis. Figure 5 compares footprints on the silkworm 5S RNA gene produced by purified *Xenopus* TFIIIA or by the silkworm fraction. The protection patterns are strikingly similar. Both proteins protect the same segment of the gene, positions +45 to +96 on the non-coding strand (compare lanes B and X in Fig. 5). The only differences in the protection patterns correspond to two DNase I hypersensitive sites, indicated by arrows. These are at position +61 for *Xenopus* TFIIIA, and at position +75 for the

Figure 5. Silkorm TFIIIA binds to the same region of the 5S RNA gene as does *Xenopus* TFIIIA. Binding reaction mixtures identical to those of Figure 4 were prepared and treated with DNase I before loading onto a non-denaturing gel. DNA fragments were eluted from silkorm (Bombyx TFIIIA) or *Xenopus* (Xenopus TFIIIA) TFIIIA-DNA complexes, and from the unbound position (U). A gene fragment cleaved at G or C+T residues provided size standards. The extent of the coding region of the gene and the TFIIIA footprints is shown diagrammatically at the right, with the positions of hypersensitive sites indicated.

Figure 6. Five separate fractions are required for silkorm 5S RNA transcription in vitro. The silkorm 5S RNA gene was transcribed in the presence of all the transcription factor fractions (lanes ALL), or in the absence of the indicated factor. Silkorm TFIIIA was used in all cases.

silkorm fraction. Thus, on the basis of the template specificity of its transcription activity and the location of its DNA binding site, we conclude that the transcriptional activity in this silkorm fraction corresponds to TFIIIA.

Figure 6 shows 5S RNA transcription in a system reconstituted from silkorm fractions, TFIIIA, TFIIIB, TFIIIC, TFIID and RNA polymerase III. A saturating level of TFIIR activity was present in all reactions. These results show that we have resolved the silkorm class III transcription machinery into five components, each of which is required for transcription of a silkorm 5S RNA gene.

Figure 7. Multiple silkorm transcription factor fractions are required to form stable or metastable complexes with the silkorm 5S RNA gene. The protocol employed in these experiments is capable of detecting stable or metastable complexes, and is diagrammed in (A). Gene 1 was the wild-type 5S RNA gene; gene 2 was a mutant 5S RNA gene that lacks the normal terminator and therefore produces a longer transcript. The fractions not present in the initial incubation mixture were added simultaneously with gene 2. (B) Silkorm TFIIIA was tested for the ability to form a stable or metastable complex with the silkorm 5S RNA gene. The positions of the transcripts from gene 1 and gene 2 are indicated, and the factors included in the pre-incubation mixture are shown above the lanes. (C) Various subsets of the transcription machinery were tested as in (B) for their ability to form stable or metastable complexes with the silkorm 5S RNA gene.

The formation of transcription complexes

Transcription complexes on 5S RNA genes have been proposed to assemble via a single, linear pathway that is initiated by the metastable interaction of TFIIIA with the A and C boxes. The resulting complex is thought to be stabilized by the addition of TFIIIC, and only then is TFIIIB able to join the complex (18,19). This pathway was deduced principally from template-commitment assays, in which various subsets of transcription factors are exposed to a saturating amount of template. A second kind of template is then added, along with the remaining factors, and transcription from both templates is measured. If transcription is from the first template only, then one or more factors in the first incubation mixture must have become committed to the first template. Two levels of stability in such commitment assays have been distinguished operationally (18). Factor-template interactions are said to be metastable if they can withstand challenge by the second template only when the remainder of the transcription machinery is added simultaneously. They are called stable if the pre-incubated factors can remain sequestered when challenged with a second template in the absence of the other transcription components.

Figure 8. More than one subset of transcription factors can commit a silkworm 5S RNA gene to transcription complex assembly. All of the possible combinations of transcription factor fractions were tested in template commitment assays. The ratio of transcripts from gene 1 to gene 2 in each reaction was compared to this same ratio of transcripts in a control reaction in which the full set of factors was pre-incubated with gene 1. The result is plotted as the relative ability (%) of each factor combination to bias transcription in favor of gene 1. Error bars are the standard deviations of multiple trials.

To determine how transcription complexes form on the silkworm 5S RNA gene, we measured the capacity for template commitment of silkworm transcription fractions, either singly or in combination. The protocol we used is diagrammed in Figure 7A. In this protocol, the remainder of the transcription machinery is added simultaneously with the second template. Therefore, this assay detects the least stable interactions that have been described in other systems. Examples of the raw data we obtained are shown in Figure 7B and C, and a quantitative compilation of all of the data is given in Figure 8. None of the individual fractions, including the TFI_{IIA} fraction, formed detectable complexes with the 5S RNA gene under these conditions (Figs 7 and 8).

Since no single fraction was able to form even a metastable complex, we tested various combinations to determine the minimum set, or sets, that could do so. Figures 7 and 8 show that two different combinations of factors (TFI_{IIA}/C/D and TFI_{IIA}/B/D) are sufficient for a level of template commitment that is at least 80% that of the complete system. Both of these combinations contain three factor fractions. Combinations of two fractions alone give only modest levels of commitment (15–20%). It is clear that TFI_{IIA} is absolutely required for commitment, since any combination of two or three fractions that lacks TFI_{IIA} is also incapable of template commitment. TFI_{IID}, while not essential, also makes a major contribution (compare the level of template commitment by TFI_{IIA}/B/C with those of TFI_{IIA}/B/D and TFI_{IIA}/C/D in Fig. 8). The addition of either TFI_{IIB} or TFI_{IIC} to the combination of TFI_{IIA} and TFI_{IID} allows high levels of commitment. The simplest interpretation of this result is that there are at least two complexes that can efficiently commit silkworm 5S RNA genes to transcriptional activity. It is formally possible that although TFI_{IIB} and TFI_{IIC} differ in their content of required transcription components, they supply the same template commitment component, and thus yield indistinguishable committed complexes. In either case, it is clear that an effective complex requires combinations of factors, indicating that template commitment involves interactions among factors.

DISCUSSION

Our results show that the promoter for the silkworm 5S RNA gene includes sequences outside of the coding region and is similar in extent to the silkworm tRNA promoter (29). This is in contrast to the relatively simple picture of transcription by RNA polymerase III suggested by early work on both tRNA and 5S RNA genes. For tRNA genes, two short sequences (the A and B boxes), binding sites for two transcription factors (TFI_{IIB} and TFI_{IIC}), were thought to be the key promoter elements. For 5S RNA genes, the lack of a B box homolog was thought to necessitate a third factor, TFI_{IIA}, to recruit TFI_{IIC} to the template.

Recent work, including the results reported here, suggests that this picture of the mechanism of transcription by RNA polymerase III is too simple. Considering 5S RNA genes in particular, there are now multiple examples (3,4,6–9,14,15) in which sequences outside the A and C boxes contribute to promoter function. Thus, the large silkworm promoter that we have analyzed may represent the general case for 5S RNA promoter structure. In addition, the number of factors known to be required for class III transcription has increased, as the complexity of the classical TFI_{IIB} and TFI_{IIC} fractions has become apparent. In yeast, the TFI_{IIB} fraction contains at least two separable polypeptides that can be readily cross-linked to the template (36), and the most highly purified TFI_{IIC} fractions contain at least four such polypeptides (37). In addition, a fraction called TFI_{IIIE} that is distinct from both TFI_{IIB} and TFI_{IIC} has been resolved in yeast (38). Finally, it is likely that the general class III transcription machinery includes factors previously recognized by their activity in class II transcription systems. TATA binding protein (TBP) is known to be such a factor (39–41), and TFI_{IIA} is an excellent candidate for another (42).

In the silkworm system, 5S RNA promoters resemble tRNA promoters in size, and also in the way they direct transcription complex assembly. In both cases, no single factor fraction forms a complex with the gene that can be detected in template commitment assays. This includes the fraction containing silkworm TFI_{IIA}. Our gel mobility shift and footprint results show that, although silkworm TFI_{IIA} alone can bind a 5S RNA gene, the resulting interaction is either not stable enough, or does not proceed to a sufficient extent, to commit the template to transcriptional activity. Our results are similar to those in a yeast system, where template commitment assays revealed only a slight bias due to TFI_{IIA} alone (43). Thus, the ability to bind template independently is not equivalent to the capacity to form stable or metastable complexes with the template. This behavior is shared with at least one other class III transcription factor fraction. Specifically, gel filtration experiments indicate that *Xenopus* TFI_{IIC} alone can associate with a 5S gene (44), and footprints show that human TFI_{IIC} alone binds a human 5S RNA gene (45,46), but in both of these systems, TFI_{IIC} fails to commit templates in the absence of TFI_{IIA}.

In our experiments, TFI_{IIA} is necessary, but not sufficient, for template commitment. What do the additional required components provide? Our data cannot distinguish between thermodynamic and kinetic roles, but they do indicate that the effect of the other components is roughly additive. The degree of template commitment increases as the number of factor fractions increases. A straightforward interpretation of the data is that different template commitment values correspond to the number of specific protein–DNA contacts that are possible with various

combinations of fractions. We expect that the final, active transcription complex makes numerous contacts across a large area of DNA within and around the silkworm 5S gene. The similarity of the silkworm tRNA and 5S RNA promoter profiles, as delineated by mutagenesis, suggests that the 5S RNA transcription complex may resemble the complex described for a silkworm tRNA gene. DNase I footprinting (47) shows that a complex involving only fractions TFIIC and TFIID extends from -1 to +136 on a silkworm tRNA^{Ala} gene. The silkworm 5S RNA transcription complex may also resemble the extensive active complex revealed by footprinting on a yeast 5S RNA gene (20).

The lack of template commitment by TFIIA alone suggests a way of thinking about 5S RNA transcription complex assembly that has interesting regulatory implications. In the traditional model of linear assembly, TFIIA binding is the critical determinant of ultimate transcriptional activation. In contrast, our results suggest that since 5S RNA transcription complexes can form in more than one way, and since a combination of transcription factors nucleates binding, variation in the concentration of any of several different factors has the potential to control 5S RNA transcription complex assembly. This possibility is supported by the observation that *in vitro* transcription of *Xenopus* oocyte 5S RNA genes is sensitive to changes in the concentration of TFIIC, as well as TFIIA (48,49), and that oocyte 5S RNA genes have a lower affinity for TFIIC than do their somatic counterparts (50).

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