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Silk Gland-Specific tRNA^{Ala} Genes Interact More Weakly than Constitutive tRNA^{Ala} Genes with Silkworm TFIIIB and Polymerase III Fractions

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Constitutive and silk gland-specific tRNA^{Ala} genes from silkworms have very different transcriptional properties in vitro. Typically, the constitutive type, which encodes tRNA$^{Ala}_{C}$, directs transcription much more efficiently than does the silk gland-specific type, which encodes tRNA$^{Ala}_{SG}$. We think that the inefficiency of the tRNA$^{Ala}_{SG}$ gene underlies its capacity to be turned off in non-silk gland cells. An economical model is that the tRNA$^{Ala}_{SG}$ promoter interacts poorly, relative to the tRNA$^{Ala}_{C}$ promoter, with one or more components of the basal transcription machinery. As a consequence, the tRNA$^{Ala}_{SG}$ gene directs the formation of fewer transcription complexes or of complexes with reduced cycling ability. Here we show that the difference in the number of active transcription complexes accounts for the difference in tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ transcription rates. To determine whether a particular component of the silkworm transcription machinery is responsible for reduced complex formation on the tRNA$^{Ala}_{SG}$ gene, we measured competition by templates for defined fractions of this machinery. We find that the tRNA$^{Ala}_{SG}$ gene is greatly impaired, in comparison with the tRNA$^{Ala}_{C}$ gene, in competition for either TFIIIB or RNA polymerase III. Competition for each of these fractions is also strongly influenced by the nature of the 5′ flanking sequence, the promoter element responsible for the distinctive transcriptional properties of tRNA$^{Ala}_{SG}$ and tRNA$^{Ala}_{C}$ genes. These results suggest that differential interaction with TFIIIB or RNA polymerase III is a critical functional distinction between these genes.

The two classes of genes that encode alanine tRNA in the silkworm, *Bombyx mori*, are distinguished by the distribution of their products among different cells. One class encodes tRNA$^{Ala}_{C}$, which is common to all cell types; the other encodes tRNA$^{Ala}_{SG}$, which is restricted to cells of the silk gland (21, 30). The possibility that selective gene amplification in the terminally differentiated silk gland cells accounts for the extreme tissue specificity of tRNA$^{Ala}_{SG}$ has been ruled out (34). Hence, the two kinds of alanine tRNA genes must have distinctive transcription properties that allow them to be differentially activated in silk gland and non-silk gland cells. The in vitro transcription properties of representatives of these gene classes appear to mirror their distinctive in vivo expression patterns (34, 44). Transcription of a tRNA$^{Ala}_{C}$ gene in vitro is efficient under a wide variety of conditions, consistent with transcription of this gene in all cell types in vivo. In contrast, transcription of the tRNA$^{Ala}_{SG}$ gene is strikingly inefficient under standard conditions in vitro. tRNA$^{Ala}_{SG}$ transcription can approach the efficiency of tRNA$^{Ala}_{C}$ transcription only if more highly concentrated transcription machinery is used.

Previous experiments have defined the critical promoter segment responsible for the distinctive transcriptional properties of the tRNA$^{Ala}_{SG}$ gene. The silkworm tRNA^{Ala} promoter, as delineated by analysis of the tRNA$^{Ala}_{C}$ gene, is large. It includes the coding region of the gene, plus flanking sequences on both sides (39). Although each portion of the tRNA$^{Ala}_{SG}$ gene differs in sequence from the corresponding portion of the tRNA$^{Ala}_{C}$ gene, the 5′ flanking segment of the tRNA$^{Ala}_{SG}$ gene is sufficient to confer the transcription properties characteristic of this gene (44).

A plausible explanation for the difference between tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ transcription rates is that the tRNA$^{Ala}_{SG}$ promoter is defective, in comparison with the tRNA$^{Ala}_{C}$ promoter, in its ability to interact with some component(s) of the basic class III transcription machinery. This defect evidently can be overcome in the silk gland in vivo and in concentrated extracts in vitro. This could be achieved in either of two ways. The concentration of the weakly interacting basal component(s) could be raised to the point of saturating both weak and strong interactions, thereby allowing efficient transcription of both kinds of tRNA genes. Alternatively, a special, gene-specific factor might compensate for the weak interaction with tRNA$^{Ala}_{SG}$ genes.

The silkworm class III transcription machinery has been resolved into at least five fractions that are required for in vitro transcription of tRNA$^{Ala}_{C}$ genes. These are RNA polymerase III plus four transcription factor fractions: TFIIIB, TFIIIC, TFIIID, and TFIIIR (23, 42). As described above, our working hypothesis is that the tRNA$^{Ala}_{SG}$ gene interacts more weakly than the tRNA$^{Ala}_{C}$ gene with one or more of these components. To test this idea, we first determined that tRNA$^{Ala}_{SG}$ genes require the same set of factor fractions that transcribe tRNA$^{Ala}_{C}$ genes and then compared the abilities of these two kinds of genes to interact with individual fractions. Since each of the fractions could contain more than one transcription factor, this approach does not characterize the entire fraction. Rather, it reveals interactions with the component that limits the activity of the fraction.

Interactions between particular transcription components and the genes were measured by a competition assay. Such an assay is useful because it does not require prior knowledge of

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the precise transcriptional roles of the tested components. To focus on the interaction of a particular fraction of the transcription machinery, we designed a mixture in which that fraction was limiting and then compared the abilities of the two kinds of tRNA^{Ala} genes to compete for it. To determine whether inefficient transcription of the tRNA$^{Ala}_{SG}$ gene in vitro can be attributed to a particular fraction, we carried out a systematic series of experiments in which the relative competitive strengths of tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ genes for each fraction were determined. Since all of the distinctive transcriptional properties characteristic of tRNA$^{Ala}_{SG}$ genes are conferred by upstream sequences (44), we suspected that a key discriminatory fraction would interact with upstream promoter elements. Therefore, we also examined the effect of upstream sequence alterations on competition for candidate fractions. By these criteria, both the polymerase III and TFIIIB fractions are important in distinguishing between the two tRNA^{Ala} gene classes. Since others have shown that template association by polymerase requires prior binding of TFIIIB (12), we favor the idea that the primary discriminator between tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ genes is a component of the TFIIIB fraction.

MATERIALS AND METHODS

Cloned genes used in this work. The wild-type constitutive tRNA^{Ala} gene (tRNA$^{Ala}_{C}$) was inserted as a 437-bp fragment of *B. mori* DNA between the *Pvu*II and *Ava*I sites of pBR322 (17). The 5′ deletion derivative of tRNA$^{Ala}_{C}$ lacking normal sequence upstream of −3 was inserted in the left orientation at the *Eco*RI site of pBR322 (17). Two different versions of the wild-type silk gland-specific tRNA^{Ala} gene (tRNA$^{Ala}_{SG}$) were used for this work. Gene 2 from the cluster of five tRNA$^{Ala}_{SG}$ genes (34) was inserted as a 151-bp fragment at the *Pst*I site of pBR322. Gene 2 or 4 from the cluster was also inserted as a 151-bp fragment at the *Pst*I site of a derivative of pUC13 in which the *Hin*dIII site has been replaced by an *Mlu*I linker (35a). The transcription rates and competitive activities of the two different tRNA$^{Ala}_{SG}$ genes were indistinguishable.

Fractions of the *B. mori* class III transcription machinery. (i) TFIIIB/Pol III fraction. Nuclear extract (22, 39) (40 ml; 4 mg of protein per ml) was loaded onto a 60-ml DEAE-Sephadex (A-50-120; Sigma) column (diameter, 2.5 cm) equilibrated in buffer B (50 mM Tris-HCl [pH 7.5], 20% [vol/vol] glycerol, 1 mM EDTA, 1 mM dithiothreitol [DTT]) plus 250 mM KCl, and the column was washed with 3 column volumes of the same buffer. Protein-containing fractions (detected by the method of Bradford [1]) having an A_{260}/A_{280} ratio of <0.7 were pooled. This pool was loaded onto a 24-ml phosphocellulose column (Whatman P-11). After the column had been washed with 1.1 column volumes of buffer B plus 300 mM KCl, a fraction containing polymerase III, TFIIIB, and TFIIID was obtained by elution with 1 column volume of buffer B plus 450 mM KCl. The peak protein-containing fractions from this step were pooled and dialyzed against a buffer containing 50 mM KCl, 50 mM Tris-HCl (pH 7.0), 20% (vol/vol) glycerol, 0.5 mM EDTA, and 1 mM DTT. To separate polymerase III and TFIIIB from TFIIID, 2 ml of this pool was loaded onto an analytical HR 5/5 Mono S column (Pharmacia) preequilibrated in 0% BS buffer (25 mM KCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 6.7], 10% glycerol, 0.5 mM EDTA, 1 mM DTT). Protein was eluted with a linear gradient (27.6 mM KCl per ml) from 0 to 100% BS buffer (550 mM KCl, 50 mM HEPES [pH 7.8], 10% [vol/vol] glycerol, 0.5 mM EDTA, 1 mM DTT). Individual fractions of 350 μl were collected in the presence of bovine serum albumin (BSA) to achieve a final protein concentration of 1 mg/ml; dialyzed against a buffer containing 75 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10% (vol/vol) glycerol, and 1 mM DTT; and assayed for transcriptional activity. The fraction containing TFIIIB and polymerase III (TFIIIB/Pol III activity) eluted between 45 and 50% of the gradient.

(ii) TFIIIC/D fraction. The fraction containing TFIIIC and TFIIID (the TFIIIC/D fraction) was prepared as previously described (23), except that TFIIIC/D was eluted from phosphocellulose at 750 mM KCl instead of 600 mM KCl. This alteration produces a more highly concentrated form of the TFIIIC/D fraction.

(iii) Separated TFIIIB and RNA polymerase III. TFIIIB and polymerase III were separated from each other by chromatography on Superose 6 (to remove contaminating polymerase from TFIIIB) or heparin-Sepharose (to remove contaminating TFIIIB from polymerase) as previously described (23).

(iv) Transcription factor activity quantitation. During fractionation, transcription activity was monitored as described elsewhere (23). The assays typically employed complementing fractions of intermediate purity in order to avoid unnecessary use of the purest fractions. Although the crude fractions were sometimes contaminated with low levels of the activity being observed, they allowed convenient and reliable monitoring of chromatographic separations.

The transcription activity of each of the isolated fractions used in this work was quantitated by assays (23) in which all components were known to be in excess, except the one being tested. The transcription rates in these assays were reproducible and linearly dependent on the added fraction. They gave the following activities (in units of Cerenkov counts per minute in tRNA$^{Ala}_{C}$ transcripts per microliter of fraction): TFIIIC/D fraction, ~11,000; TFIIIB/Pol III fraction, ~2,000; TFIIIB fraction, ~6,000; RNA polymerase III fraction, ~5,000.

Transcription. (i) Standard reaction conditions. Standard transcription reaction conditions were 600 μM each unlabelled nucleoside triphosphate, 25 μM [α-^{32}P]UTP (5 Ci/mmol), 70 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 8% (vol/vol) glycerol, and 1 mM DTT. Reaction volumes and amounts of template and nonspecific DNA varied in the different experiments, as indicated. Reactions were carried out at room temperature (~22°C) for 2 h and then stopped, and products were fractionated on 8% polyacrylamide gels and autoradiographed as described elsewhere (22). The yield of transcripts was determined by measuring Cerenkov radiation in excised gel pieces.

(ii) Single-round transcription assays. Single-round transcription reactions were performed under the standard conditions, except that the reaction was initiated in the absence of one nucleotide (CTP in the case of the tRNA$^{Ala}_{C}$ gene and UTP in the case of the tRNA$^{Ala}_{SG}$ gene). In addition, tRNA$^{Ala}_{C}$ transcripts were labelled with [α-^{32}P]CTP instead of UTP. Reaction volumes were 20 μl, with 100 ng of the appropriate tRNA^{Ala} gene per reaction. After an incubation period of 60 min, the missing nucleotide was added and the reaction mixtures were adjusted to a final concentration of 100 μg/ml of heparin or 0.5% Sarkosyl. After a second incubation period of 10 min, the transcription reactions were stopped and the products were analyzed by polyacrylamide gel electrophoresis, as described previously (22). Multiple-round reactions with no heparin or Sarkosyl were carried out in parallel for 2 h.

(iii) Competition assays. Competition for transcription fractions was measured under the standard transcription reaction conditions with volumes and amounts of extract, fractions, and template appropriate for the particular experiment. Amounts of fractions were chosen such that transcription was limited by one of them. For each different combination of fractions, the

amount of template that saturated the limiting fraction and the range in which additional nonspecific DNA was not inhibitory were determined. The appropriate conditions were as follows: for TFIIIC/D limitation, 1.5 μl of crude silk gland extract, 6 μl of TFIIIB/Pol III fraction, 10 ng of reference template (3′Δ+89 derivative of the $tRNA^{Ala}_{C}$ gene), and additional competitor or vector (pBR322) DNA to a total of 100 ng of DNA per 40-μl reaction volume; for TFIIIB/Pol III limitation, conditions were identical to those for TFIIIC/D limitation, except that 6 μl of TFIIIC/D fraction replaced the TFIIIB/Pol III fraction; for TFIIIB limitation, 9 μl of TFIIIC/D fraction, 9 μl of RNA polymerase III fraction, 3 μl of TFIIIB fraction, 50 ng of reference template, and additional competitor or vector DNA to a total of 270 ng of DNA per 64-μl reaction volume; and for RNA polymerase III limitation, 6 μl of TFIIIC/D fraction, 6 μl of TFIIIB fraction, 2 μl of RNA polymerase III fraction, 50 ng of reference template, and additional competitor or vector DNA to a total of 150 ng of DNA per 62-μl reaction volume.

Competitive strength was calculated after linearization of the data (28). The ratio (T/T_c) of reference transcription rates in the absence (T) and the presence (T_c) of competitor was plotted against the molar ratio of competitor to reference genes. The slope of the resulting straight line gives competitive strength relative to the reference gene. The best-fit line was determined by simple regression by using the CA-Cricket Graph program (Computer Associates). The relative competitive strengths of the $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes were determined from the ratios of their slopes.

RESULTS

The difference in transcription efficiency between $tRNA^{Ala}_{SG}$ and $tRNA^{Ala}_{C}$ genes is explained by the formation of different numbers of transcription complexes. The difference between $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ transcription rates is detected under standard transcription conditions, which allow multiple rounds of transcription by each active complex. Thus, either of two extreme models could explain the inefficiency of $tRNA^{Ala}_{SG}$ genes. $tRNA^{Ala}_{SG}$ genes might form fewer active transcription complexes than $tRNA^{Ala}_{C}$ genes. Alternatively, they might form the same number of complexes, but each complex might fire at reduced frequency. To distinguish between these two models, we measured the number of active transcription complexes formed on each kind of gene. Active complexes were quantitated by measuring the total number of transcripts produced when transcription was restricted to one round. We used transcription conditions that had been previously determined to favor transcription of $tRNA^{Ala}_{C}$ genes over transcription of $tRNA^{Ala}_{SG}$ genes by a factor of ~7 and two different methods (addition of heparin or Sarkosyl) to prevent reinitiation of transcription. Each kind of template was incubated with unfractionated transcription machinery plus the appropriate subsets of three nucleotides such that active transcription complexes would stall at positions +8 and +10 on the $tRNA^{Ala}_{C}$ gene and the $tRNA^{Ala}_{SG}$ gene, respectively. By adding the missing nucleotide in the presence of heparin (14) or Sarkosyl (15, 16), transcripts from both kinds of gene were completed under conditions that did not allow reinitiation. Control experiments verified that the expected oligonucleotides were synthesized by both stalled complexes and that both oligonucleotides were quantitatively elongated to full-length transcripts in the presence of heparin or Sarkosyl (data not shown).

Figure 1 shows that the $tRNA^{Ala}_{C}$ gene gives more transcripts than does the $tRNA^{Ala}_{SG}$ gene when transcription is limited to a single round. The ratio of $tRNA^{Ala}_{C}$ to $tRNA^{Ala}_{SG}$ transcripts is

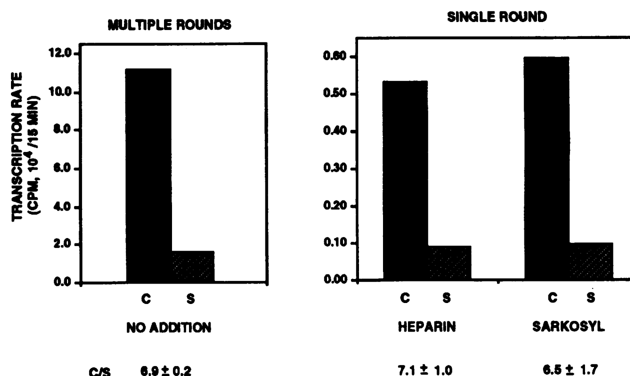

FIG. 1. Single- and multiple-round transcription of $tRNA^{Ala}_{C}$ and RNA^{Ala}_{SG} genes. Transcription directed by a $tRNA^{Ala}_{C}$ gene (C) (black bar) or a $tRNA^{Ala}_{SG}$ gene (S) (hatched bar) was measured under conditions of multiple or single rounds, and results of a representative experiment are shown. Transcription was limited to a single round by the addition of heparin or Sarkosyl, as indicated. The average ratios of $tRNA^{Ala}_{C}$/$tRNA^{Ala}_{SG}$ transcription rates (C/S) and their standard deviations in three to six experiments are given at the bottom of the figure.

about 7 and is the same whether transcription is limited by heparin ($tRNA^{Ala}_{C}$/$tRNA^{Ala}_{SG}$ ratio = 7.1) or Sarkosyl ($tRNA^{Ala}_{C}$/$tRNA^{Ala}_{SG}$ ratio = 6.5). The ratio of single-round transcripts from these two genes is not significantly different from the ratio of their multiple-round transcription rates measured in the same experiment ($tRNA^{Ala}_{C}$/$tRNA^{Ala}_{SG}$ ratio = 6.9). Hence, different efficiencies of active transcription complex formation account for the different transcription rates from these two genes in vitro. Under standard in vitro conditions, the $tRNA^{Ala}_{SG}$ gene forms fewer complexes than does the $tRNA^{Ala}_{C}$ gene, but once formed, complexes on the two genes yield transcripts at the same rate.

Competition for the TFIIIB/Pol and the TFIIIC/D fractions. To understand why the $tRNA^{Ala}_{SG}$ gene is inefficient in transcription complex formation, we first asked whether $tRNA^{Ala}_{SG}$ genes require the same fractions of the transcription machinery as do $tRNA^{Ala}_{C}$ genes. Figure 2 shows that transcription of $tRNA^{Ala}_{SG}$ genes requires each of the protein fractions known to act on $tRNA^{Ala}_{C}$ genes: TFIIIC/D, TFIIIB, and RNA polymerase III. The requirement for TFIIIC and TFIIID individually was not tested. Transcription of $tRNA^{Ala}_{SG}$ genes also requires the RNA factor, TFIIIR (data not shown). Since the two genes require the same transcription fractions, it is possible that their different efficiencies result from different affinities for one or more components within these fractions.

To determine whether the $tRNA^{Ala}_{SG}$ gene is impaired in its interaction with particular components, we first tested its ability to compete for each of two crude fractions that together contain all of the silkworm class III transcription machinery. One fraction (TFIIIB/Pol) contains polymerase plus TFIIIB, and the other (TFIIIC/D) contains TFIIIC plus TFIIID. Both fractions contain TFIIIR. Recent work in our laboratory indicates that the role of TFIIIR in in vitro transcription is indirect. It appears to serve a protective function by preventing damage to the template (7). Since the requirement for TFIIIR does not vary among templates (data not shown), we think it unlikely that TFIIIR is an important discriminator between $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes. Therefore, in the present work, we avoided possible complicating indirect effects by performing all experiments in the presence of saturating concentrations of TFIIIR.

The competitive strengths of $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes

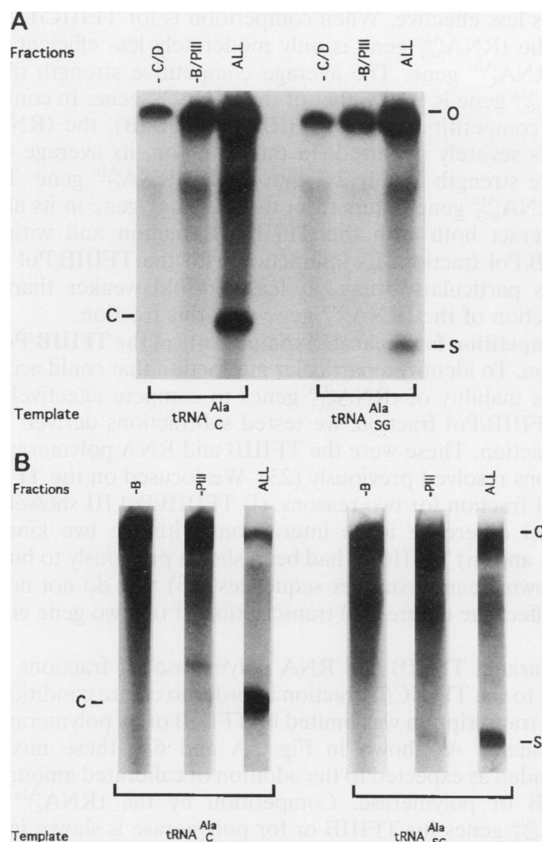

FIG. 2. tRNA$^{Ala}_{C}$ and RNA$^{Ala}_{SG}$ genes require the same factor fractions for transcription. Transcription directed by a tRNA$^{Ala}_{C}$ gene or a tRNA$^{Ala}_{SG}$ gene was carried out under standard conditions, in the absence of either the TFIIIC/D fraction (−C/D) or the TFIIIB/Pol III fraction (−B/P III) or in the presence of 6 μl of TFIIIC/D fraction plus 2 μl of TFIIIB/Pol III fraction (ALL) (A), or in the absence of either TFIIIB (−B) or RNA polymerase III (−PIII) or in the complete system, 3 μl of TFIIIB fraction, 6 μl of TFIIIC/D fraction, and 6 μl of polymerase III fraction (ALL) (B). Transcription products after resolution on polyacrylamide gels are shown. The gel origin (O) and positions of tRNA$^{Ala}_{C}$ (C) and tRNA$^{Ala}_{SG}$ (S) transcripts are indicated.

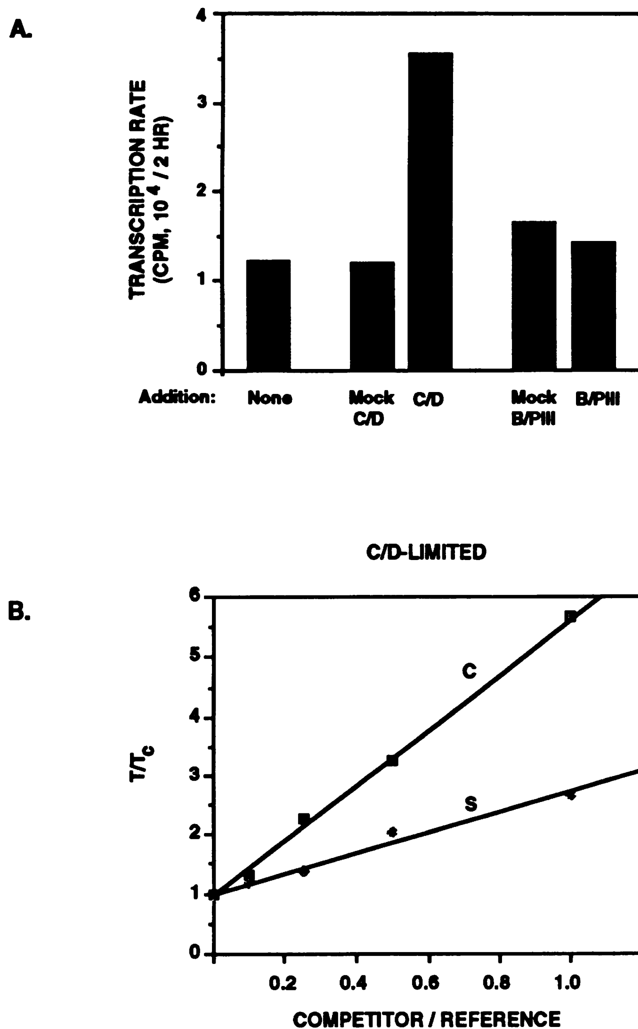

FIG. 3. Competition for TFIIIC/D. (A) Demonstration that TFIIIC/D is limiting. The base reaction mixture contained 1.5 μl of extract supplemented with 6 μl of TFIIIB/Pol III fraction and 100 ng of reference gene (tRNA$^{Ala}_{C}$ 3′Δ+89) as the template. Transcription rates are shown with no addition (None) or with the following additions: 9 μl of TFIIIC/D fraction (C/D) or its buffer equivalent (mock C/D) or with 8 μl of TFIIIB/Pol III fraction (B/PIII) or its buffer equivalent (mock B/PIII). The amounts of added fractions were the maximum compatible with the volume and salt concentration of the base reaction mixture. (B) Competition for TFIIIC/D (C/D) by tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ genes. The ability of increasing amounts of either the tRNA$^{Ala}_{C}$ gene (C) or the tRNA$^{Ala}_{SG}$ gene (S) to reduce transcription of the reference gene in a representative experiment is shown. Competition data were linearized by plotting the ratio (T/T_c) of the reference transcription rate without competitor (T) to the rate with competitor (T_c) against the molar ratio of competitor to reference genes. In the experiment shown, the ratio of tRNA$^{Ala}_{SG}$ to tRNA$^{Ala}_{C}$ competitive strength is 0.34. The average ratio for seven experiments was 0.3 ± 0.1.

were quantitated by determining the ability of each of them to compete with a reference gene for a limiting transcription component. To distinguish reference transcripts from competitor transcripts, we used as a reference a mutant tRNA$^{Ala}_{C}$ gene that produces a longer transcript than either the wild-type tRNA$^{Ala}_{C}$ gene or the tRNA$^{Ala}_{SG}$ gene (17). Because this mutant is slightly impaired in its ability to compete for unfractionated transcription machinery (its competitive strength is twofold below that of a wild-type tRNA$^{Ala}_{C}$ gene [39]), we measured competition over a range of relatively low competitor/reference molar ratios (0.10 to 2.0) that would detect both weak and strong competition. In all of the reaction mixtures, the concentrations of total DNA and protein were kept constant by the addition of nonspecific DNA (pBR322) and protein (BSA).

If two genes are to compete for a defined fraction of the transcription apparatus, conditions must be such that transcription is limited by a component of that fraction. We created these conditions by supplementing unfractionated machinery with subsets of the complete apparatus derived by fractionation. To achieve TFIIIC/D limitation, a small amount of crude silk gland nuclear extract was mixed with excess TFIIIB/Pol fraction. Likewise, to achieve TFIIIB/Pol limitation, crude extract was supplemented with TFIIIC/D fractions. To verify that these limitations had in fact been achieved, the mixtures were tested by supplementation with calibrated amounts of either TFIIIB/Pol or TFIIIC/D. Figures 3A and 4A show such tests. Addition of the TFIIIC/D fraction to the TFIIIC/D-limited mixture (Fig. 3A) stimulated transcription, whereas

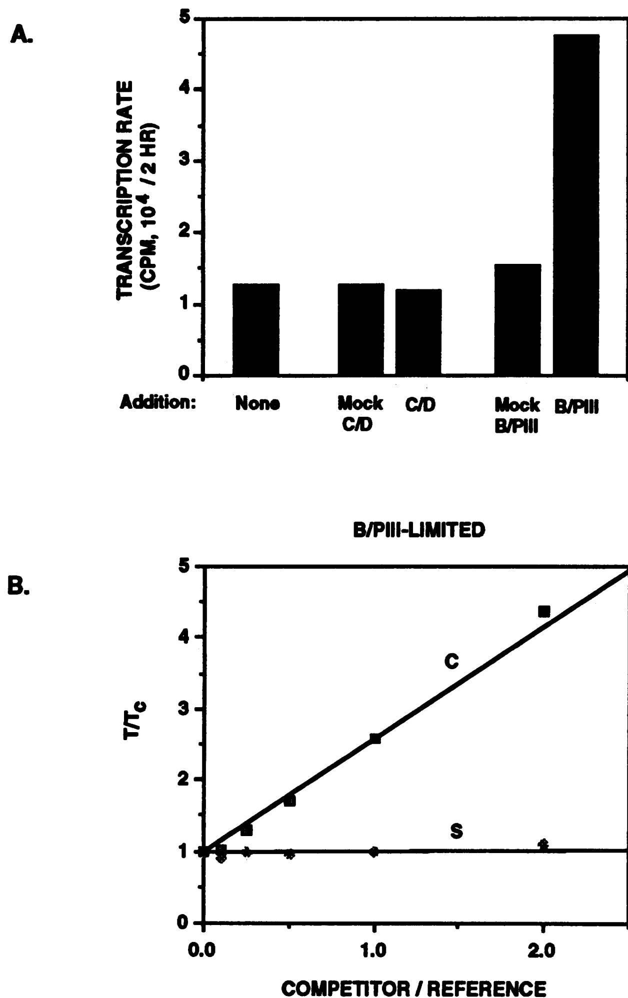

FIG. 4. Competition for TFIIIB/Pol III. (A) Demonstration that TFIIIB/Pol III is limiting. The base reaction mixture contained 1.5 μl of extract supplemented with 6 μl of TFIIIC/D fraction and 100 ng of the reference gene. Transcription rates are shown with no addition (None) or with the following additions: 6 μl of TFIIIC/D fraction (C/D) or its buffer equivalent (Mock C/D) and 11 μl of TFIIIB/Pol III fraction (B/PIII) or its buffer equivalent (Mock B/PIII). The amounts of added fractions were the maximum compatible with the volume and salt concentration of the standard transcription mixture. (B) Competition for TFIIIB/Pol III by $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes. Abbreviations and treatment of the data are as described in the legend to Fig. 3B. In the experiment shown, the ratio of $tRNA^{Ala}_{SG}$ to $tRNA^{Ala}_{C}$ competitive strength is 0.003. The average ratio for six experiments was 0.02 ± 0.02. B/PIII, TFIIIB/Pol III.

addition of the TFIIIB/Pol fraction had no effect. In contrast, the TFIIIB/Pol-limited mixture (Fig. 4A) was stimulated by TFIIIB/Pol but not by TFIIIC/D. Each of the supplementing fractions had been previously titrated to establish its activity when other components were in excess (data not shown). This ensured that the amount of each fraction added would give detectable stimulation, if stimulation were possible.

Figures 3B and 4B compare the abilities of the $tRNA^{Ala}_{C}$ and the $tRNA^{Ala}_{SG}$ genes to compete with the reference gene for either the TFIIIC/D fraction or the TFIIIB/Pol fraction. Both genes are capable of competing with the reference gene for each of these fractions. In both cases, however, the $tRNA^{Ala}_{SG}$ gene is less effective. When competition is for TFIIIC/D (Fig. 3B), the $tRNA^{Ala}_{SG}$ gene is only moderately less efficient than the $tRNA^{Ala}_{C}$ gene. The average competitive strength of the $tRNA^{Ala}_{SG}$ gene is ~30% that of the $tRNA^{Ala}_{C}$ gene. In contrast, when competition is for TFIIIB/Pol (Fig. 4B), the $tRNA^{Ala}_{SG}$ gene is severely impaired. In this situation, its average competitive strength is only 2% that of the $tRNA^{Ala}_{C}$ gene. Thus, the $tRNA^{Ala}_{SG}$ gene differs from the $tRNA^{Ala}_{C}$ gene in its ability to interact both with the TFIIIC/D fraction and with the TFIIIB/Pol fraction. Its interaction with the TFIIIB/Pol fraction is particularly weak, at least 50-fold weaker than the interaction of the $tRNA^{Ala}_{C}$ gene with this fraction.

Competition for separated components of the TFIIIB/Pol III fraction. To identify a particular interaction that could account for the inability of $tRNA^{Ala}_{SG}$ genes to compete effectively for the TFIIIB/Pol fraction, we tested subfractions derived from this fraction. These were the TFIIIB and RNA polymerase III fractions resolved previously (23). We focused on the TFIIIB/Pol III fraction for two reasons. (i) TFIIIB/Pol III showed the greatest difference in its interactions with the two kinds of genes, and (ii) TFIIIC/D had been shown previously to bind to the downstream promoter sequences (43) that do not noticeably affect the differential transcription of the two gene classes (44).

Separated TFIIIB and RNA polymerase III fractions were added to the TFIIIC/D fraction in order to create conditions in which transcription was limited by TFIIIB or by polymerase III individually. As shown in Fig. 5A and 6A, these mixtures responded as expected to the addition of calibrated amounts of TFIIIB or polymerase. Competition by the $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes for TFIIIB or for polymerase is shown in Fig. 5B and 6B. In both cases, the $tRNA^{Ala}_{SG}$ gene is an extremely weak competitor. It has only 3% of the competitive strength of the $tRNA^{Ala}_{C}$ gene for either TFIIIB or polymerase. Thus, the relative capacities of the two genes to compete for TFIIIB or for polymerase alone are the same as their relative abilities to compete for a fraction that contains both TFIIIB and polymerase.

TFIIIB and RNA polymerase III act through the upstream promoter element. The competition experiments described above show that the $tRNA^{Ala}_{SG}$ gene interacts very weakly with at least two components of the silkworm class III transcription machinery. These are the TFIIIB and the RNA polymerase III fractions. We wished to determine whether either of these weak interactions is likely to be responsible for the unusual transcriptional properties of the $tRNA^{Ala}_{SG}$ gene. Since these transcription properties are conferred by upstream sequences (44), the most straightforward prediction is that the components of the transcription machinery that discriminate between the two genes act through upstream sequences. We therefore compared the abilities of the wild-type $tRNA^{Ala}_{C}$ gene and a derivative lacking the upstream promoter (5'Δ-3) to compete for TFIIIB or for RNA polymerase III. Figure 7 shows that the interactions of TFIIIB and of RNA polymerase III with the $tRNA^{Ala}_{C}$ gene are greatly impaired when upstream sequences are altered. Thus, since each of these fractions requires normal upstream sequences for proper interaction with the template, each could contain a component that makes a critical distinction between $tRNA^{Ala}_{SG}$ and $tRNA^{Ala}_{C}$ genes.

DISCUSSION

To investigate the molecular basis of the differential transcription displayed by constitutive and silk gland-specific $tRNA^{Ala}$ genes in vitro, we have compared the abilities of these genes to form active transcription complexes and to

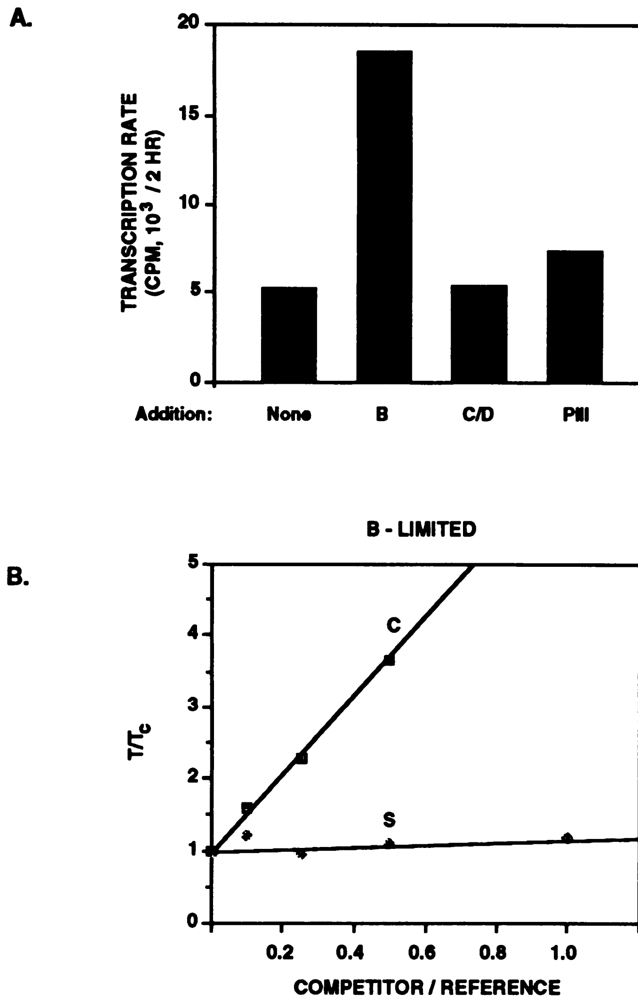

FIG. 5. Competition for TFIIIB. (A) Demonstration that TFIIIB is limiting. The base reaction mixture contained 3 μl of TFIIIB fraction, 9 μl of RNA polymerase III fraction, 9 μl of TFIIIC/D fraction, and 100 ng of the reference gene. Transcription rates are shown with no addition (None) or with the following additions: 3 μl of TFIIIB fraction (B), 7 μl of TFIIIC/D fraction (C/D), or 7 μl of RNA polymerase III fraction (PIII). Addition of buffer equivalents of these fractions did not significantly alter the transcription rate (data not shown). (B) Competition for TFIIIB by tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ genes. Abbreviations and treatment of the data are as described in the legend to Fig. 3B. In the experiment shown, the ratio of tRNA$^{Ala}_{SG}$ to tRNA$^{Ala}_{C}$ competitive strength is 0.03. The average ratio for two experiments was 0.035. B, TFIIIB.

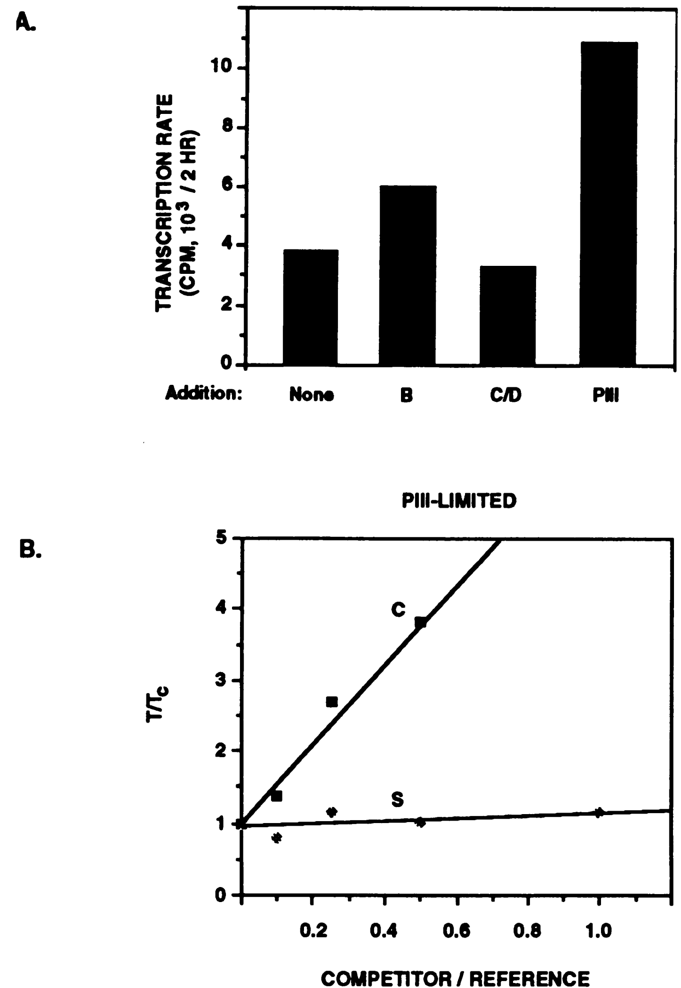

FIG. 6. Competition for RNA polymerase III. (A) Demonstration that RNA polymerase III is limiting. The base reaction mixture contained 6 μl of TFIIIC/D fraction, 6 μl of TFIIIB fraction, 2 μl of RNA polymerase III fraction, and 100 ng of the reference gene. Transcription rates are shown with no addition (None) or with the following additions: 10 μl of TFIIIB fraction (B), 6 μl of TFIIIC/D (C/D) fraction, or 10 μl of RNA polymerase III fraction (PIII). Addition of the buffer equivalents of these fractions did not significantly alter the transcription rate (data not shown). (B) Competition for Pol III by tRNA$^{Ala}_{C}$ and tRNA$^{Ala}_{SG}$ genes. Abbreviations and treatment of the data are as described in the legend to Fig. 3B. In the experiment shown, the ratio of tRNA$^{Ala}_{SG}$ to tRNA$^{Ala}_{C}$ competitive strength is 0.03. The average ratio for two experiments was 0.035. PIII, Pol III.

compete for the limiting component in defined fractions of the silkworm transcription machinery. Although these two templates require the same set of fractions for transcription, the genes differ markedly in their abilities to assemble these fractions into active transcription complexes. The difference in complex-forming ability is sufficient to account for all of the difference in transcription rates under standard assay conditions. A plausible explanation for this phenomenon is that the tRNA$^{Ala}_{SG}$ gene interacts less efficiently than does the tRNA$^{Ala}_{C}$ gene with some component of the basal transcription apparatus, RNA polymerase III or a class III transcription factor. We therefore used competition experiments to determine whether a particular fraction of the silkworm transcription machinery contains a component that discriminates sharply between the two genes.

The tRNA$^{Ala}_{SG}$ gene is slightly impaired (~3-fold) in its ability to compete for TFIIIC/D but is severely impaired (~50-fold) in its ability to compete for TFIIIB or polymerase. We have shown that effective competition for TFIIIB and RNA polymerase III by the tRNA$^{Ala}_{C}$ gene requires normal upstream sequences. In contrast, competition for TFIIIC/D was shown previously to depend on sequences downstream of the tRNA$^{Ala}_{C}$ gene transcription initiation site (43). Since upstream sequences are known to determine the transcriptional properties of tRNA$^{Ala}_{SG}$ and tRNA$^{Ala}_{C}$ genes (44), it is likely that polymerase III or a component of the TFIIIB fraction discriminates more strongly between these genes than does TFIIIC/D.

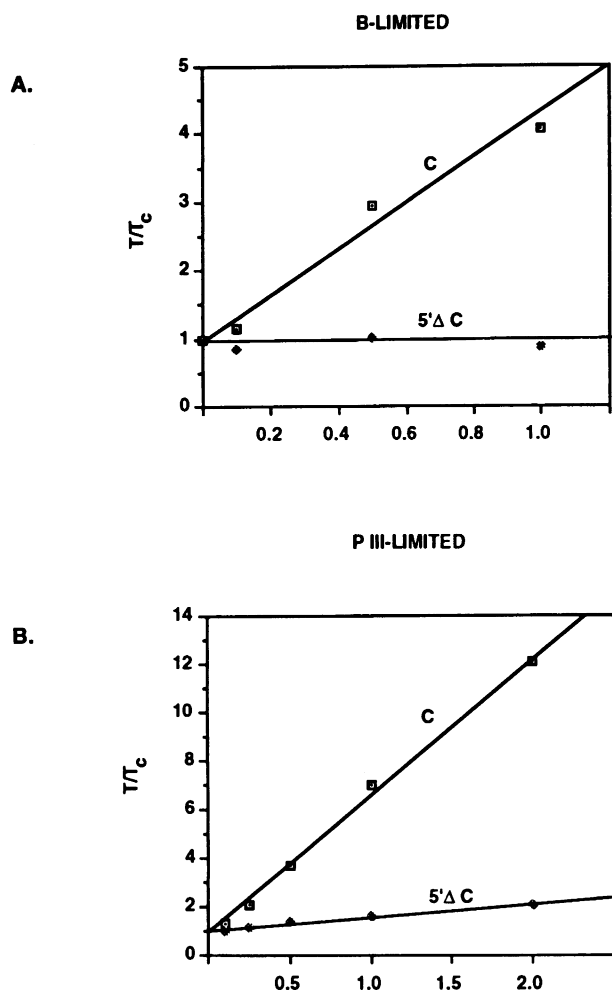

FIG. 7. Effect of 5′ flanking sequences on competition for TFIIIB or RNA polymerase III. Competition for TFIIIB (B-limited) (A) or for RNA polymerase III (P III-limited) (B) by a wild-type $tRNA^{Ala}_{C}$ gene (C) or a mutant derivative lacking normal sequences upstream of −3 (5′ΔC) is plotted. Other abbreviations and treatment of the data are as described in the legend to Fig. 3B. In the experiment shown, the ratios of mutant to wild-type competitive strength were 0.003 for TFIIIB and 0.18 for RNA polymerase.

The fact that competition by the $tRNA^{Ala}_{SG}$ gene for TFIIIB is impaired to the same extent as is competition for RNA polymerase III suggests that interactions of the gene with these two components could be related. Since studies of transcription complex assembly in *S. cerevisiae* show that incorporation of RNA polymerase III requires prior template association of TFIIIB (12), it is possible that the impaired interaction of the $tRNA^{Ala}_{SG}$ gene with RNA polymerase III is a consequence of its poor interaction with TFIIIB. Thus, it is reasonable to hypothesize that a component of the TFIIIB fraction is the primary discriminator between $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes.

Are there known TFIIIB components that might play this role? At present, we think that the TATA-binding protein, TBP, is a strong candidate. TBP is required for transcription of tRNA genes in organisms as diverse as humans and yeasts (3, 29, 37) and has been found in the TFIIIB fraction of both systems (13, 18, 32, 36). More compelling is our finding that the key functional elements of the $tRNA^{Ala}_{C}$ upstream promoter are two AT-rich sequences that resemble TBP binding sites (TATA boxes) in class II promoters (24). The sequence upstream of $tRNA^{Ala}_{SG}$ genes differs markedly from that of the $tRNA^{Ala}_{C}$ upstream promoter (44). Thus, a simple hypothesis is that TBP interacts directly with these two templates but does so differentially, thereby favoring the formation of complete, active transcription complexes on the $tRNA^{Ala}_{C}$ gene.

It is possible, of course, that the means of discrimination between $tRNA^{Ala}_{C}$ and $tRNA^{Ala}_{SG}$ genes is more complex. In other systems, the interaction of TBP with tRNA genes is influenced by accessory proteins (TAFs) (13, 18, 32, 36). In the silkworm system, one or more of these could potentially interact differently with the two genes and thus act as the discriminator(s). A silkworm homolog of the yeast TFIIB-related factor might play this role (2, 19). A direct test of this idea is not currently possible, however, since yeast TFIIIB components do not replace the present silkworm TFIIIB fraction (41). The critical component could also be something that is not physically associated with TBP. TFIIIE, a recently resolved component of standard yeast TFIIIB fractions, is a candidate for such an activity (5). Another possibility is TFIIA, a class II transcription factor that has recently been shown to function in human class III transcription (20, 35).

A few other examples of regulated transcription by RNA polymerase III are known, and it will be interesting to learn whether they are mechanistically similar to silkworm $tRNA^{Ala}$ regulation. In *Xenopus* frogs both 5S RNA and certain tRNA genes display oocyte-specific expression (31, 40). The biological rationale presumably is to supply oocytes with the machinery for rapid protein synthesis in early development. The in vitro transcription properties of all of the oocyte-specific genes are very different from those of their constitutively expressed counterparts. Interestingly, these properties are strongly dependent on sequences upstream of the coding region (11, 25). In *Drosophila melanogaster*, regulation of a variety of RNA polymerase III templates by phorbol esters can be detected both in vitro and in vivo (8). Although this effect has not been shown to be mediated by upstream sequences, such a mechanism is plausible because of the observed dependence of *Drosophila* class III transcription on 5′ flanking sequences (4, 6, 10, 27, 28).

The transcription components that mediate regulation in these and other systems are under investigation. In *Xenopus laevis*, differential expression of oocyte-specific tRNA and OAX genes has been attributed to changes in the transcriptional activation properties of the TFIIIC fraction (26). In *D. melanogaster*, differences in the TFIIIB fraction are responsible for induction by phorbol esters (9). In mammals, regulation of TFIIIB activity in response to growth conditions (33) and embryonic development (38) has been observed.

Our long-range goal is to understand how the interactions that cause inefficient transcription of silkworm $tRNA^{Ala}_{SG}$ genes under certain conditions in vitro are overcome under other conditions. We are considering two kinds of models. In one model, transcription of $tRNA^{Ala}_{SG}$ genes is turned up by quantitative changes in the amount or activity of a basal transcription factor such as TBP or a TBP-associated polypeptide. Consistent with this possibility is our observation that the silk gland extracts that transcribe the $tRNA^{Ala}_{SG}$ gene efficiently have high levels of TFIIIB activity (41). We do not yet know whether TBP levels, specifically, are elevated. In the second model, a qualitatively different component, perhaps a silk gland-specific transcription factor, compensates in a gene-specific manner for the defective interaction of $tRNA^{Ala}_{SG}$ genes with the basal machinery. Current experiments, aimed at defining the polypeptide(s) responsible for inefficient $tRNA^{Ala}_{SG}$

transcription, will set the stage for investigation of the compensatory mechanism.

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