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Silkworm TFIIIB Binds both Constitutive and Silk Gland-Specific tRNA^{Ala} Promoters but Protects Only the Constitutive Promoter from DNase I Cleavage

LISA S. YOUNG,[1] NANCY AHNERT,[1] AND KAREN U. SPRAGUE[1,2]*

Institute of Molecular Biology[1] and Department of Biology,[2] University of Oregon, Eugene, Oregon 97403-1229

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We have identified a complex between TFIIIB and the upstream promoter of silkworm tRNA^{Ala} genes that is detectable by gel retardation and DNase I footprinting. Formation of this complex depends on the integrity of previously identified upstream promoter elements and on the presence of other silkworm transcription factors, either TFIIID or a fraction that contains both TFIIIC and TFIIID. We have used this complex to compare the interactions of TFIIIB with two kinds of tRNA^{Ala} genes whose different in vitro transcription properties are conferred by the upstream segments of their promoters. These are the tRNA$_C^{Ala}$ genes, which are transcribed constitutively, and the tRNA$_{SG}^{Ala}$ genes, which are transcribed only in the silk gland. We find that TFIIIB binds tRNA$_{SG}^{Ala}$ genes with lower affinity than it binds tRNA$_C^{Ala}$ genes. In addition, the TFIIIB complexes formed on tRNA$_{SG}^{Ala}$ genes differ qualitatively from those formed on tRNA$_C^{Ala}$ genes. Both the transcriptional activity of tRNA$_{SG}^{Ala}$ complexes and the ability of the complexes to protect upstream DNA from DNase I digestion are reduced.

Transcription of tRNA genes is regulated to tailor tRNA populations to the special protein-synthetic demands of differentiated cells. A dramatic example occurs in the silkworm, *Bombyx mori*, where the distribution of tRNAs in the mature silk gland matches the amino acid composition of the gland's chief protein product, silk fibroin: 44% glycine, 29% alanine, and 12% serine (26, 47). The corresponding enrichment of the cognate tRNAs (5, 15, 29) and aminoacyl-tRNA synthetases (6, 8, 27, 33) in the posterior silk gland represents extreme specialization of the protein-synthetic machinery, which can be tolerated because little other synthetic activity is required of the terminally differentiated silk gland cells. Spiders have apparently adopted a similar strategy to maximize the production of silk for webs (4). These examples are particularly easy to recognize because the biosynthetic activities of silk glands are limited and the amino acid composition of silk is distinctive, but it is likely that compositional adjustment of tRNA populations is a widespread phenomenon. For instance, the distribution of tRNAs changes to match the requirements of globin biosynthesis during reticulocyte differentiation (18). Thus, analysis of the mechanisms underlying the accumulation of certain tRNAs in the *B. mori* silk gland has the potential to illuminate general control mechanisms.

We have focused on the accumulation of alanine tRNA in silk glands because much of the increase in the amount of this tRNA is due to the production of a novel, silk gland-specific type, a fact that makes the system particularly amenable to analysis. Only two types of silkworm alanine tRNA have been identified: one that is found in all cell types, including the silk gland (tRNA$_C^{Ala}$), and a second that is found only in the silk gland (tRNA$_{SG}^{Ala}$) (32, 48). These tRNAs differ in primary sequence and are encoded by two distinct groups of genes. Each group is composed of about 20 copies per haploid genome, and the possibility of silk gland-specific amplification of tRNA$_{SG}^{Ala}$ genes has been ruled out (56). Since both types of tRNA are stable, control is at the level of transcription. tRNA$_C^{Ala}$ genes are transcribed constitutively and account for the tRNA^{Ala} required by ordinary cells; tRNA$_{SG}^{Ala}$ genes are transcribed exclusively in the silk gland, where their output adds to that of the tRNA$_C^{Ala}$ genes, bringing the total concentration of tRNA^{Ala} to high levels.

Previous analysis of representative cloned copies of tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes revealed in vitro transcriptional properties that are consistent with the pattern of tRNA accumulation from these genes in vivo. Specifically, tRNA$_C^{Ala}$ genes direct in vitro transcription efficiently in a variety of homologous transcription systems—including nuclear extracts of both silk gland and non-silk gland (ovary) origin—whereas tRNA$_{SG}^{Ala}$ genes are as efficient as tRNA$_C^{Ala}$ genes only in concentrated nuclear extracts from silk glands (68, 69). Analysis of mutant tRNA^{Ala} genes containing chimeric C-SG promoters established that although the full tRNA$_C^{Ala}$ promoter is extensive (61), a short ~30-bp segment upstream of the transcription initiation site is sufficient to confer tRNA$_C^{Ala}$ transcriptional properties on a tRNA$_{SG}^{Ala}$ gene, and vice versa (69).

To understand their distinctive properties, we want to know why tRNA$_{SG}^{Ala}$ genes are transcribed inefficiently in transcription systems that permit high levels of tRNA$_C^{Ala}$ transcription. The result of mixing extracts that efficiently transcribe tRNA$_{SG}^{Ala}$ genes with other extracts that do not argued that inefficient tRNA$_{SG}^{Ala}$ transcription is caused by the absence of a positive effector rather than by the presence of a repressor (69). Moreover, since DNA is not assembled into chromatin in any of these extracts (68), it is unlikely that differential in vitro transcription of tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes is mediated through chromatin structure. Single-round transcription assays showed that the difference between tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ transcription rates is quantitatively accounted for by the numbers of active transcription complexes formed on each kind of template (52). The tRNA$_{SG}^{Ala}$ transcription complexes are less abundant but are functionally indistinguishable from the tRNA$_C^{Ala}$

* Corresponding author. Mailing address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229. Phone: (541) 346-6094. Fax: (541) 346-5891.

complexes. Once formed, complexes on both genes direct multiple rounds of transcription at the same rate.

To identify the components responsible for differential transcription complex formation on tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes, we created a systematic set of assay systems in which transcription was limited in turn by RNA polymerase III or by one of the silkworm transcription factor fractions. We compared the abilities of tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes to compete with a reference gene for the limiting component in each case (52). These experiments showed that, compared with the tRNA$_C^{Ala}$ gene, the tRNA$_{SG}^{Ala}$ gene was somewhat impaired in its ability to compete for all components but that it was a particularly weak competitor for TFIIIB or RNA polymerase III. Inefficient competition for polymerase III may be a consequence of impaired interaction with TFIIIB, since in *Saccharomyces cerevisiae*, TFIIIB binding is a prerequisite for incorporation of polymerase into an active transcription complex (23). Thus, on the basis of the indirect evidence provided by competition experiments, TFIIIB appeared to be an important discriminator between the two kinds of silkworm tRNA^{Ala} genes.

We have now used gel retardation and DNase I footprints to compare levels of TFIIIB binding to tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes directly. We find that TFIIIB binds to the upstream promoter element of the tRNA$_C^{Ala}$ gene and that both quantitative and qualitative features distinguish the interaction of TFIIIB with the tRNA$_{SG}^{Ala}$ gene. As assayed by gel retardation, a concentration of TFIIIB higher than that required to bind the tRNA$_C^{Ala}$ gene is required to bind the tRNA$_{SG}^{Ala}$ gene. Moreover, unlike TFIIIB complexes formed on the tRNA$_C^{Ala}$ gene, TFIIIB complexes formed on the tRNA$_{SG}^{Ala}$ gene do not protect the upstream promoter from DNase I digestion, and only a subset of them is transcriptionally active.

MATERIALS AND METHODS

Cloned genes and DNA fragments used in this work. The tRNA^{Ala} genes were inserted into a derivative of pUC13 (pUC13M) in which the *Hin*dIII site had been replaced by an *Mlu*I linker. The wild-type tRNA$_C^{Ala}$ gene and mutant derivatives containing substitutions in upstream promoter elements (35) were inserted at the *Eco*RI site in the orientation that puts the polylinker cloning sites upstream of the tRNA transcription start site. The tRNA$_{SG}^{Ala}$ gene referred to throughout this work was actually a chimeric tRNA$_{SG}^{Ala}$-tRNA$_C^{Ala}$ gene (69) that was inserted in the same orientation between the *Eco*RI and *Pst*I sites of the same vector. A mutant derivative of this chimera altered from positions +1 through +3 in order to make the primary transcript from the chimera identical to that from the tRNA$_C^{Ala}$ gene was constructed by recombinant PCR (20) and used in the experiments whose results are reported in Fig. 7. This sequence change has no detectable effect on the transcriptional properties of the chimeric gene.

DNA fragments used for protein binding were prepared either by 3′ end labelling of plasmid restriction fragments or by PCR amplification of the desired fragment in the presence of a labelled primer. As noted in the figure legends, the restriction fragments containing the wild-type tRNA$_C^{Ala}$ gene or its mutant derivatives were either 400 bp long (extending from positions −91 to +309 relative to the transcription start site) and labelled with [α-^{32}P]CTP at positions −88 and −90 or 261 bp long (extending from positions −40 to +221) and labelled with [α-^{32}P]ATP at positions −37, −38, +218, and +219. Restriction fragments containing the tRNA$_{SG}^{Ala}$ gene were 250 bp long (from positions −29 to +221) and were labelled with [α-^{32}P]ATP at positions +218 and +219. PCR fragments containing either the tRNA$_C^{Ala}$ gene (262 bp) or the tRNA$_{SG}^{Ala}$ gene (261 bp) were generated from primers whose 5′ ends were at positions −91 and +171 or positions −90 and +171, respectively. In both cases, the upstream primer was 5′ end labelled with polynucleotide kinase before being used in the amplification reaction. Labelled restriction fragments were isolated by elution from polyacrylamide gels, and PCR fragments were freed of unincorporated primers by passage through a spin column (Chroma Spin-100; Clontech). All fragments were purified by phenol extraction and ethanol precipitation and then quantitated spectroscopically before use. As an additional check, the DNA fragments were also quantitated on gels by comparison with ethidium bromide-stained standards. A preparation typically yielded 2 to 3 μg (8 to 20 pmol) of fragment, with a specific radioactivity of 2×10^3 to 7×10^3 dpm/fmol.

Fractions of the *B. mori* class III transcription machinery. TFIIIC/D was isolated as described elsewhere (52). TFIIIB was isolated from the DEII fraction by a modification of a method described previously (34) that yields highly concentrated TFIIIB activity, free of detectable RNA polymerase III. The DEII fraction (40 ml) was concentrated 20-fold by Amicon pressure filtration at 60 lb/in^2. Recovery of TFIIIB activity after concentration was 90 to 100%. Low levels of RNA polymerase III activity present in the concentrate were removed by gel filtration on Superose 6, as described elsewhere (34). Fractions whose TFIIIB activity was ≥80% of the peak activity were pooled and stored as aliquots at −70°C. Separated TFIIIC, TFIIID, and RNA polymerase III were obtained and checked for cross-contamination as described elsewhere (34).

Assays. (i) DNA binding. Standard 20-μl binding reaction mixtures contained 2.5 to 5 fmol of labelled DNA fragment, 2 μg of dG-dC, 5 μl of TFIIIC/D and, if included, 5 μl of TFIIIB. Variations from the standard conditions are indicated in the figure legends. In reaction mixtures that contained separated factors, 2.5 μl of TFIIIB, 2.5 μl of TFIIIC, and 2.5 μl of TFIIID were incubated with 10 fmol of labelled DNA fragment and 4 μg of dG-dC in a total volume of 20 μl. The final concentrations of buffer components in all reaction mixtures were 70 mM KCl, 30 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 10% glycerol, and 3 mM dithiothreitol. Reactions were initiated by addition of the protein fraction(s) and, unless otherwise indicated, proceeded for 60 min at room temperature (~22°C). Reaction products were fractionated and visualized as described elsewhere (67), except that the gels were run longer (the xylene cyanol marker dye was run 9 cm) and a 2.5-cm-thick cushion of 10% polyacrylamide was included below the 3.5% polyacrylamide gel to facilitate retention of unbound DNA.

(ii) DNase I footprinting. Direct footprinting of the protein-DNA complexes formed in binding reactions was done after 60 min of incubation by treating each reaction mixture with 25 ng of DNase I (Worthington) for 2 min at room temperature. After purification by phenol extraction and ethanol precipitation, the DNA fragments were resolved on standard 8% polyacrylamide sequencing gels and visualized by autoradiography in the presence of an intensifying screen. When gel-resolved complexes were footprinted, the binding reaction mixture was treated with DNase as described for direct footprinting but digestion was stopped by adding EDTA to a final concentration of 5 mM and immediately fractionating the sample on the 3.5%–10% polyacrylamide step gel described in the paragraph above. Protein-DNA complexes were detected autoradiographically and the DNA fragments were isolated and run on sequencing gels, as described previously (67).

(iii) Transcription. Standard transcription reaction conditions were as previously described (52), with 2.5 fmol of template (5 ng of plasmid) and 0.2 μg of nonspecific DNA in a 41-μl reaction volume. Template DNA was typically supercoiled, but linear and supercoiled templates are transcribed with equal efficiency by both crude and fractionated silkworm transcription machineries. Single-round transcription assays were performed under the same conditions, with the addition of heparin, as described elsewhere (67), except that both tRNA$_C^{Ala}$- and tRNA$_{SG}^{Ala}$-promoted reactions were initially stalled by omission of the same nucleotide, CTP, and both transcripts were labelled with [α-^{32}P]UTP. This protocol eliminates potential differences in stalled complex formation due to differences in the location of the complexes and was made possible by using an altered chimeric tRNA$_{SG}^{Ala}$-tRNA$_C^{Ala}$ gene whose primary transcript is identical to that of a tRNA$_C^{Ala}$ gene.

RESULTS

TFIIIB binding to the tRNA$_C^{Ala}$ gene is detectable by gel retardation. To perform detailed analysis of TFIIIB binding to tRNA^{Ala} genes, we developed a gel retardation assay that detects TFIIIB addition to a complex consisting of a tRNA^{Ala} gene plus TFIIIC and TFIIID. The silkworm machinery required for tRNA transcription includes three transcription factor fractions: TFIIIB, TFIIIC, and TFIIID (34). Since each of these fractions is devoid of RNA polymerase III activity, reconstitution of transcription activity requires complementation with a fourth fraction that contains silkworm RNA polymerase III (34). Previous experiments showed that, in combination, either TFIIIC and TFIIID or TFIIIB and TFIIID can form complexes with the tRNA$_C^{Ala}$ gene that are detectable by a template commitment assay (34). Until now, however, only TFIIIC/D complexes had been detected by gel retardation (67). Figure 1a shows that complexes formed in the presence of TFIIIB plus TFIIID are readily detectable by gel retardation as well and that the mobility of these complexes is clearly different from that of the TFIIIC/D complexes. As anticipated from previous template commitment assays, none of the transcription factor fractions individually forms significant amounts of retarded complex. The small amounts of fast-moving complex formed by certain combinations of fractions are not functionally relevant, since variations in the amount of this complex do

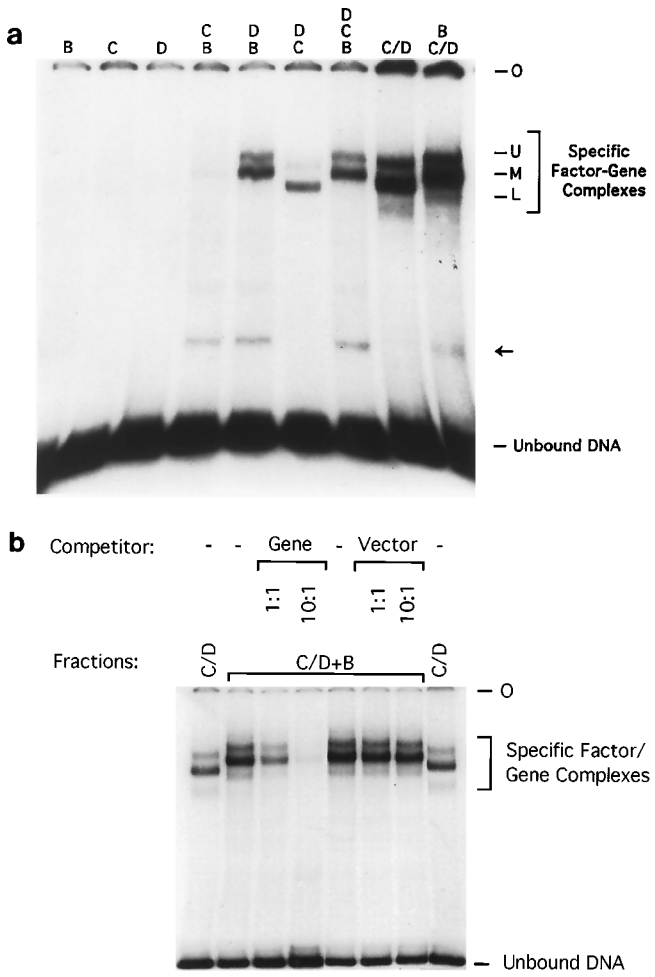

FIG. 1. TFIIIC/D and TFIIIB/C/D form distinctive and specific gel retardation complexes with the $tRNA_C^{Ala}$ gene. (a) TFIIIB binding is detectable by formation of TFIIIC/D-DNA and TFIIIB/C/D-DNA complexes having different mobilities. The transcription factor fractions indicated by letters at the top of the figure (TFIIIB [B], TFIIIC [C], TFIIID [D], and TFIIIC/D [C/D]) were incubated, singly or in combination, with 10 fmol of a labelled 262-bp PCR fragment (2.3×10^3 dpm/fmol) containing the wild-type $tRNA_C^{Ala}$ gene. The TFIIIC/D fraction contains TFIIIC and TFIIID activities resolved from TFIIIB and RNA polymerase III but not from each other. The resulting protein-DNA complexes were visualized by autoradiography after resolution by polyacrylamide gel electrophoresis. Complexes with three different mobilities are indicated: U (upper), M (middle), and L (lower). The complexes shown in the rightmost two lanes were formed by using a single fraction (TFIIIC/D) to supply both TFIIIC and TFIIID activities. The positions of the gel origin (O), specific factor-gene complexes, functionally irrelevant complexes (arrow), and unbound DNA are marked. (b) Input DNA is assembled into gene-specific TFIIIB/C/D complexes. A 2.5-fmol amount of a labelled 262-bp $tRNA_C^{Ala}$ gene-containing PCR fragment (2.3×10^3 dpm/fmol) was incubated with either TFIIIC/D (C/D) alone or TFIIIC/D plus TFIIIB (C/D+B) in the presence of a 0:1 (−), 1:1, or 10:1 mole ratio of unlabelled competitor to labelled probe. The competitor was either a 269-bp gene-containing restriction fragment extending from position −91 to +178 (left lanes) or a 253-bp nonspecific restriction fragment corresponding to positions 3480 through 3733 in pBR322 (right lanes). The amounts of TFIIIB/C/D-DNA complex detected in the presence of competitor relative to those detected in its absence were 56 and 18% for 1:1 and 10:1 specific competitor/probe mole ratios, respectively, and 94 and 95% for 1:1 and 10:1 nonspecific competitor/probe mole ratios, respectively. The positions of the gel origin (O), specific factor-gene complexes, and unbound DNA are shown.

not correlate with transcriptional activity. Figure 1a also shows the complexes that form when all three transcription factor fractions are allowed to bind. The resulting TFIIIB/C/D complexes clearly have lower mobility than the TFIIIC/D complexes do, but at this resolution they are indistinguishable from the TFIIIB/D complexes. Results like those in Fig. 1a suggested that the mobility difference between the TFIIIC/D and TFIIIB/C/D complexes could serve as an assay for TFIIIB binding. To provide these factors in a more concentrated form, we wished to use a less highly resolved source of TFIIIC and TFIIID. Figure 1a shows that a single fraction supplying both activities (TFIIIC/D) gives the same pattern of complexes as do the resolved TFIIIC and TFIIID fractions. Figure 1b shows that the TFIIIB/C/D complex formed by combining the TFIIIC/D fraction with TFIIIB is specific to $tRNA^{Ala}$ gene sequences. It is eliminated by competition with gene-containing DNA but not with unrelated vector DNA. Since all of the silkworm transcription factor fractions we used are devoid of RNA polymerase III activity, it is unlikely that the reduced mobility caused by the TFIIIB fraction is due to the binding of polymerase III rather than a transcription factor(s).

Input $tRNA_C^{Ala}$ template is efficiently assembled into protein-DNA complexes that are detectable by gel retardation and that are transcriptionally active when supplied with RNA polymerase. Before the complexes visualized by gel retardation could serve as probes of $tRNA_C^{Ala}$ and $tRNA_{SG}^{Ala}$ promoter function, their relationship to functional transcription complexes had to be established by satisfying the following three criteria. (i) The amount of retarded complex should match the amount of active transcription complex formed under the same conditions. (ii) The location of the complexes, as indicated by DNase I footprints, should coincide with transcriptionally important sequences. (iii) Addition of TFIIIB to the TFIIIC/D complex should depend on the integrity of known upstream promoter elements (35).

To make quantitative comparisons between the amounts of retarded complex and active transcription complex, we performed binding reactions and single-round transcription assays in parallel, using reaction conditions that were as nearly identical as possible. In particular, the reaction volumes, buffer compositions, and concentrations of template and transcription factor fractions were identical. The TFIIIC/D fraction was limiting. Of course, the transcription reaction mixture contained an additional component, the RNA polymerase III fraction, which was present in excess. The only other differences between the binding and transcription reaction mixtures were the physical form of the template (linear and supercoiled, respectively) and the amount and kind of nonspecific DNA. Experiments performed in parallel established that linear and supercoiled templates are transcribed with equal efficiency under the conditions used (data not shown). Moreover, addition of standard amounts of plasmid DNA to binding reaction mixtures or poly(dG-dC) to transcription reaction mixtures has a negligible effect (data not shown).

Transcription was initiated with a subset of nucleoside triphosphates and then restricted to one round by the addition of heparin along with the missing nucleoside triphosphate. Under these conditions, >90% of the 8-nucleotide nascent transcripts are correctly elongated and terminated (52, 58). Thus, the molar amount of transcript is a direct measure of the molar amount of active transcription complex.

The transcription reaction conditions used for these experiments were previously determined to give efficient assembly (60 to 100%) of labelled DNA into gene-specific protein-DNA complexes like those shown in Fig. 1b. We have tested two different preparations of TFIIIB and five of TFIIIC/D and find that TFIIIC/D-DNA and TFIIIB/C/D-DNA complexes typically are resolved into patterns consisting of two or three bands. The heterogeneity is apparently due to the formation of structurally distinct TFIIIC/D complexes. All of these com-

TABLE 1. Quantitative comparison of gel retardation complexes and active transcription complexes

Complex	fmol of complex[a] formed on 2.5 fmol of TFIIC/D template	
	prepn 1	prepn 2
Gel retardation		
Upper	0.3	0.4
Middle	0.9	1.0
Lower	0.5	0.2
Active	0.9	1.3

[a] The values shown are representative of multiple determinations that differed by less than 0.05 fmol for the gel retardation complexes and by less than 0.15 fmol for the transcriptionally active complexes. Active complexes were measured as fmol of tRNA transcripts produced per 2.5 fmol of template in single-round transcription assays. The standard conditions for binding and transcription, given in Materials and Methods, were modified as follows: for both reactions, the reaction volume was 32 μl and the buffer composition was that of a standard transcription reaction mixture.

plexes appear to be substrates for TFIIIB addition, since all are further retarded by TFIIIB. The relative proportions of these bands vary, but the middle band of the 3-band pattern is always most abundant and the 2-band pattern is correlated with the disappearance of the lowest band.

To determine whether any of these complexes could account for the amount of transcription activity detected in the presence of excess RNA polymerase III, each of the bands in the 3-band patterns (upper, lower, and middle, as indicated in Fig. 1a) formed by two different preparations of the TFIIIC/D fraction was separately quantitated. Table 1 shows these results compared with the amounts of active transcription complex measured in parallel. There is good agreement between the molar amounts of active transcription complex and the main (middle) retarded complex for both preparations of the TFIIIC/D fraction. The fact that a large proportion of the input template is accounted for by either active complexes (40 to 50%) or gel retardation complexes (60 to 70%) argues that the quantitative agreement is not fortuitous. Thus, it is likely that the main band detected by gel retardation corresponds to transcriptionally active complexes. It is possible that the two minor bands also correspond to transcriptionally active complexes, although neither is sufficiently abundant to account for all of the measured activity and large variations in the amount of the lowest band (not shown) do not correlate with changes in the amount of transcriptionally active complexes.

TFIIIB protects promoter sequences from DNase I digestion. DNase I footprints were used to determine the location of the TFIIIC/D and TFIIIB/C/D complexes with respect to transcriptionally important sequences. Figure 2 (leftmost lanes) shows that TFIIIC/D protects most of the wild-type tRNA$_C^{Ala}$ gene, with strong protection extending from position +136 to approximately +10 and weaker protection extending upstream to position −11. The strongly protected sequences correspond to the downstream promoter of this gene (61), and the result agrees generally with a previous analysis of TFIIIC/D binding to the gene (67). The slight 5′ extension of the TFIIIC/D footprint in the present work (from position −1 to −11) is probably the result of using fractions in which the transcription factor activity is more highly enriched. The addition of TFIIIB has two effects on the TFIIIC/D footprint: (i) it intensifies protection downstream of the transcription initiation site, and (ii) it extends protection upstream. Most obviously, TFIIIB protects sequences between positions −11 and −34, a region that provides essential promoter function to this gene (25). Thus, the location of both the TFIIIC/D and the TFIIIB/C/D complexes is consistent with their involvement in transcription.

The TFIIIB footprint requires the integrity of upstream promoter elements. To test the functional relevance of the footprint extension caused by TFIIIB, we determined the sensitivity of this footprint to specific mutations in the upstream promoter. These mutations were chosen from the group that had previously defined two short AT-rich sequences as the key promoter elements in the upstream promoter (35). The sequences of the wild-type promoter elements (located at positions −29 to −25 and −20 to −15) are indicated at the left of Fig. 2. Our choice of mutants to analyze was aimed at distinguishing the effect of functionally relevant protein binding from that of fortuitous binding by proteins with a high degree of affinity for AT-rich DNA. In the particular distal-element mutant we analyzed, the wild-type TATAT sequence is replaced with a sequence that is GC rich (CGGCT) but that is not deleterious to transcription. Figure 2 shows that the upstream protection by TFIIIB is only slightly weakened by this mutation and is still clearly detectable. In contrast, a similar mutation in the proximal element (AATTTT replaced with AGGACG) reduces transcription by ~10-fold and also completely eliminates upstream TFIIIB-dependent protection. It also eliminates the intensified downstream protection seen on the wild-type gene in the presence of TFIIIB. Substitution of both elements in the double mutant lowers transcription to undetectable levels and, like mutation of AATTTT alone, eliminates protection by TFIIIB, both upstream and downstream. Thus, in each of these mutants, upstream protection parallels the promoter activity of the template DNA but is not strictly correlated with the AT richness of the promoter.

The tRNA$_{SG}^{Ala}$ gene binds TFIIIB with lower affinity than does the tRNA$_C^{Ala}$ gene. Having identified and characterized the gel retardation complexes and DNase I footprints that correspond to TFIIIB binding the tRNA$_C^{Ala}$ gene, we used both gel retardation and DNase I footprinting to compare the interactions of TFIIIB with tRNA$_C^{Ala}$ versus tRNA$_{SG}^{Ala}$ upstream promoters. To facilitate this comparison, the tRNA$_{SG}^{Ala}$ upstream promoter was analyzed in the context of a chimeric gene in which the 5′-flanking sequences were derived from a tRNA$_{SG}^{Ala}$ gene, whereas the remainder of the template was a tRNA$_C^{Ala}$ gene. This chimera possesses the transcriptional properties of a wild-type tRNA$_{SG}^{Ala}$ gene (69), and we will refer to it hereafter as a tRNA$_{SG}^{Ala}$ gene. It allowed us to compare templates that were identical except for their upstream promoters. To provide approximate standards for variations in TFIIIB binding, we also analyzed the tRNA$_C^{Ala}$ upstream promoter mutants whose effects on TFIIIB footprints are shown in Fig. 2. Comparison of Fig. 2 and 3 shows that in these mutants, TFIIIB-mediated gel retardation, transcription activity, and TFIIIB footprints are affected in parallel. Figure 3 shows that mutation of the distal promoter element (TATAT) has little or no deleterious effect on formation of the TFIIIB-containing complex, mutation of the proximal element (AATTTT) reduces the amount of this complex noticeably, and mutation of both elements eliminates it. Figure 3 shows that, like the tRNA$_C^{Ala}$ mutant genes, the tRNA$_{SG}^{Ala}$ gene is less efficient than the wild-type tRNA$_C^{Ala}$ gene at directing the addition of TFIIIB to a TFIIIC/D-gene complex. Under conditions in which all of the wild-type tRNA$_C^{Ala}$ gene-containing DNA is assembled into a TFIIIB/C/D complex, only about one-half of the tRNA$_{SG}^{Ala}$ gene-containing DNA is in a complex of similar mobility. The remaining DNA is in a complex whose mobility resembles that of a TFIIIC/D-gene complex. Interestingly, the pattern of complexes formed by the tRNA$_{SG}^{Ala}$ gene under these conditions is very similar to the pattern formed on the mutant tRNA$_C^{Ala}$ gene that carries a

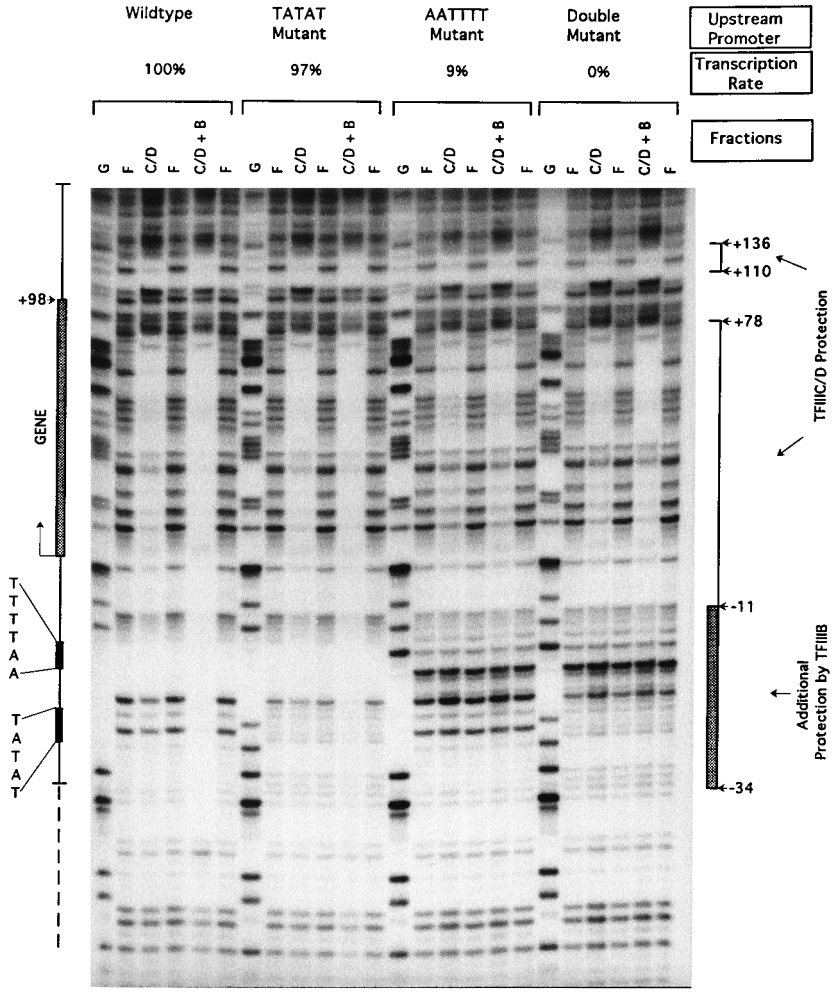

FIG. 2. TFIIIB protects sequences upstream of a wild-type $tRNA_C^{Ala}$ gene. DNA fragments (400 bp) containing either the wild-type $tRNA_C^{Ala}$ gene or mutant derivatives were labelled at the 5′ end of the coding strand, and 5-fmol amounts (3.0×10^3 dpm/fmol) were treated with DNase I either in the absence of other proteins (F) or after preincubation with TFIIIC/D (C/D) or TFIIIC/D plus TFIIIB (C/D + B). Lanes marked G contain the products of partial chemical cleavage of the same DNA fragments at G residues. (Top) The kind of gene (wild type or mutant) and its transcriptional activity relative to that of a wild-type $tRNA_C^{Ala}$ gene are shown. The replacements for wild-type sequences in the mutants were CGGCT for TATAT, AGGACG for AATTTT, and both of these in the double mutant, as shown in Fig. 3b. These mutants differ from similar ones described previously (35) in containing no other changes from wild type in their 5′-flanking sequences. The transcription activities are based on determinations in standard (not concentrated) silk gland nuclear extracts. (Left) The full extent of promoter sequences (solid line) and the locations of the primary transcript (shaded rectangle), the transcription start site (arrow), the upstream promoter elements of the wild-type $tRNA_C^{Ala}$ gene (black rectangles), and vector sequences (dashed line) are shown. (Right) Sequences protected by TFIIIC/D alone (solid line) and the additional sequences protected by TFIIIC/D plus TFIIIB (shaded rectangle) are shown. Numbers correspond to nucleotide positions either upstream (−) or downstream (+) of the transcription initiation site.

defective proximal promoter element (AATTTT). We do not know whether the faster protein-DNA complex corresponds to bound TFIIIC/D alone or to TFIIIC/D plus a subset of TFIIIB components. In either case, it is clear that there is one or more TFIIIB components whose binding kinetics or affinity discriminates between the two kinds of $tRNA^{Ala}$ genes.

To determine whether it is the rate or the extent of TFIIIB binding to $tRNA_C^{Ala}$ and $tRNA_{SG}^{Ala}$ genes that is different, we measured the amount of TFIIIB complex formed on each gene after incubation of preformed TFIIIC/D-gene complexes with TFIIIB for various periods of time. Since previous experiments (data not shown) had established that the TFIIIB/C/D complexes formed on both kinds of gene are stable during gel electrophoresis, the amount of complex observed on the gel corresponds to the amount originally present in the binding reaction mixture. To reduce possible complications caused by differential binding of TFIIIC/D to the distinctive $tRNA_C^{Ala}$ and $tRNA_{SG}^{Ala}$ sequences between positions −1 and −11, we used conditions in which TFIIIC/D saturated both templates. The experiment was carried out with several different concentrations of TFIIIB, and in no case was there a detectable difference in the rate of TFIIIB addition to the two genes. Data obtained at the concentration of TFIIIB used in the experiment shown in Fig. 3 are plotted in Fig. 4 and show that the rate of association of TFIIIB with both genes is rapid. Binding is essentially complete within 5 min. Although the $tRNA_{SG}^{Ala}$ gene is not saturated with TFIIIB at this point, additional incubation for a total of 60 min does not increase the amount of TFIIIB/C/D complex. Thus, it appears to be the extent, rather than the rate, of TFIIIB binding that distinguishes $tRNA_{SG}^{Ala}$ from $tRNA_C^{Ala}$ genes.

If the difference in extents of TFIIIB binding to the two $tRNA^{Ala}$ genes simply reflects a difference in their affinities for TFIIIB, it should be possible to increase the extent of binding to $tRNA_{SG}^{Ala}$ genes by increasing the concentration of TFIIIB. To test this prediction, TFIIIB binding to both genes was

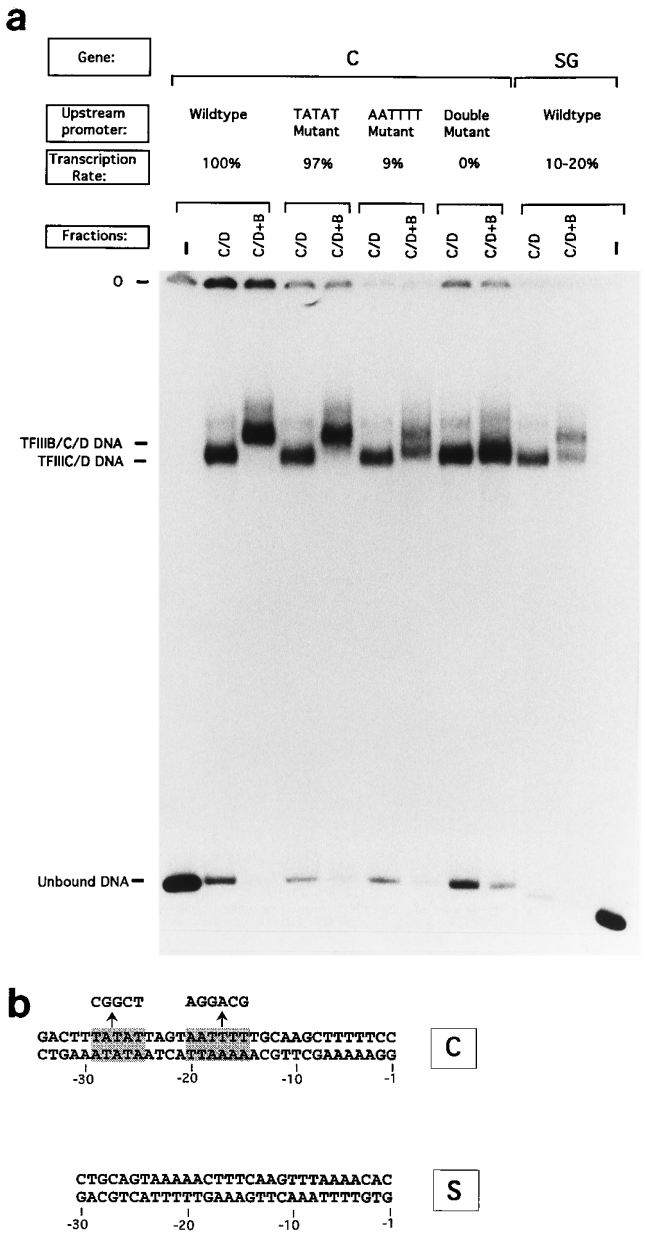

FIG. 3. Mutant tRNA$_C^{Ala}$ genes and a tRNA$_{SG}^{Ala}$ gene bind TFIIIB less efficiently than does a wild-type tRNA$_C^{Ala}$ gene. (a) The gel retardation complexes formed by TFIIIC/D (TFIIIC/D-DNA) or by TFIIIC/D plus TFIIIB (TFIIIB/C/D-DNA) with 5 fmol of labelled restriction fragments containing wild-type or mutant tRNA$_C^{Ala}$ genes (261 bp) or tRNA$_{SG}^{Ala}$ genes (250 bp) are shown. The specific radioactivity of the tRNA$_C^{Ala}$ gene-containing fragments was twice that of the tRNA$_{SG}^{Ala}$ gene-containing fragments (7.1×10^5 versus 3.6×10^5 dpm/fmol). The kind of gene, its transcriptional activity relative to that of a wild-type tRNA$_C^{Ala}$ gene, and the transcription factor fractions included in the binding reaction mixture, TFIIIC/D (C/D) or TFIIIC/D plus TFIIIB (C/D+B), are shown at the top. The mutants are the same as those used in the experiment shown in Fig. 2, and their sequences are shown in panel b. The transcriptional activity of the tRNA$_{SG}^{Ala}$ gene is based on determinations done with standard silk gland nuclear extracts, as well as with the transcription factor fractions used for gel retardation and footprinting. Also shown are the positions of the gel origin (O), TFIIIB/C/D-DNA and TFIIIC/D-DNA complexes, and unbound DNA. (b) Sequences upstream of the tRNA$_C^{Ala}$ (C) and tRNA$_{SG}^{Ala}$ (S) genes are shown. The TATAT and AATTTT tRNA$_C^{Ala}$ promoter elements are shaded, and the noncoding-strand substitutions contained by mutants are shown above the wild-type sequence.

measured as a function of TFIIIB concentration. In all cases, TFIIIB was added to templates in the presence of a saturating concentration of TFIIIC/D and an incubation period (60 min) sufficient to allow binding equilibrium was used. As shown in Fig. 5, higher concentrations of TFIIIB do indeed drive a greater proportion of tRNA$_{SG}^{Ala}$ genes into complexes having TFIIIB/C/D-like mobility. In fact, at the highest concentrations tested (10 or 12 μl of TFIIIB per reaction mixture), all of the input DNA is assembled into such complexes.

TFIIIB addition to the tRNA$_{SG}^{Ala}$ gene does not protect upstream sequences from DNase I digestion. To determine whether TFIIIB interaction with the two kinds of tRNA^{Ala} genes might differ qualitatively, as well as quantitatively, we compared the abilities of bound TFIIIB to protect these two templates from DNase I digestion. Because it was crucial that the comparison be made under conditions in which both genes were saturated with TFIIIB, we used two different methods to obtain the DNase I footprints. One was by analyzing the unfractionated products from a binding reaction in which saturation was verified in a parallel gel retardation assay. The other was by analyzing DNA eluted from gel-fractionated protein-DNA complexes that exhibited the mobility of TFIIIB/C/D-DNA complexes. The results from the two analyses were indistinguishable and are shown in Fig. 6. Surprisingly, although TFIIIB binds the tRNA$_{SG}^{Ala}$ gene, as indicated by gel retardation, it does not protect upstream promoter sequences from digestion by DNase I. Despite variations in the conditions of digestion, we have not detected any evidence of upstream protection. Neither does TFIIIB increase TFIIIC/D-mediated protection of downstream sequences, as it does with the tRNA$_C^{Ala}$ gene.

Some TFIIIB/C/D-tRNA$_{SG}^{Ala}$ gene complexes lack transcription activity. The DNase I footprinting data show that the TFIIIB/C/D complexes that form on the two kinds of tRNA^{Ala} genes are structurally distinct. To determine whether these complexes differ functionally as well, we used single-round transcription assays to compare the numbers of active transcription complexes formed on tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes under conditions in which both genes are quantitatively assembled into TFIIIB/C/D complexes. As shown in Fig. 7, the number of active transcription complexes formed on the tRNA$_{SG}^{Ala}$ gene is only about one-sixth the number formed on the tRNA$_C^{Ala}$ gene. Thus, even though high concentrations of TFIIIB can drive complex formation on the tRNA$_{SG}^{Ala}$ gene, only a subset of the resulting complexes is actually competent for transcription.

DISCUSSION

We have identified a silkworm TFIIIB-DNA complex that corresponds to the addition of TFIIIB to TFIIIC/D bound to the downstream promoter of a silkworm tRNA$_C^{Ala}$ gene. The TFIIIB-tRNA$_C^{Ala}$ gene complex is detectable by additional gel electrophoretic retardation of the TFIIIC/D complex and by extension of the TFIIIC/D DNase I footprint into 5′-flanking sequences. The functional relevance of this complex is indicated by the following three findings. (i) The amount of gel retardation complex matches the amount of active transcription complex, measured under the same conditions. (ii) TFIIIB binding protects known promoter sequences from cleavage by DNase I. (iii) Addition of TFIIIB to a TFIIIC/D-gene complex depends on the integrity of upstream promoter elements. We have also identified a gel retardation complex formed by incubating a tRNA$_C^{Ala}$ gene with TFIIIB and TFIIID. This complex appears to correspond to the stable interaction responsible for template commitment by the combination of TFIIIB and TFIIID (34). Further characterization will be required to determine whether and how the TFIIIB/D complex differs from

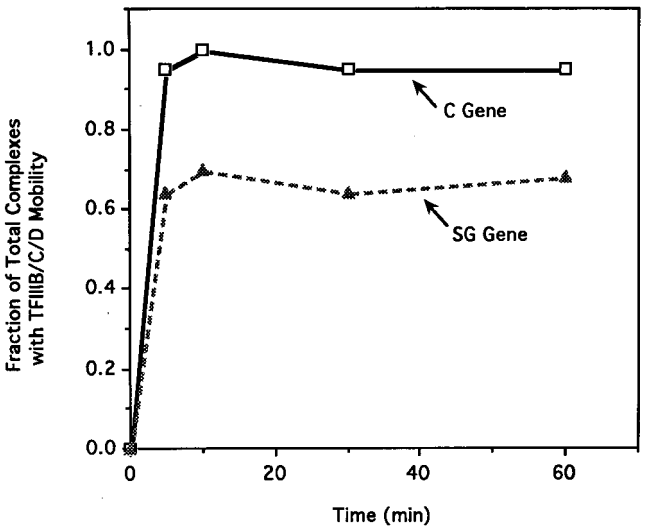

FIG. 4. Rates of TFIIIB binding to $tRNA^{Ala}_{SG}$ and $tRNA^{Ala}_{C}$ genes are indistinguishable. The rate of formation of gel retardation complexes having the electrophoretic mobility of TFIIIB/C/D-$tRNA^{Ala}_{C}$ gene complexes was measured at a single concentration of TFIIIB (5 μl/32-μl reaction mixture) from the same preparation of TFIIIB used in the experiment shown in Fig. 5. The vertical axis shows the fraction of the total gel retardation complexes having TFIIIB/C/D mobility, and the horizontal axis shows the time after TFIIIB binding was initiated by addition of TFIIIB to TFIIIC/D complexes that had been preformed during a 60-min incubation with 2.5 fmol of PCR fragment containing either the $tRNA^{Ala}_{C}$ gene (262 bp, 2.3×10^3 dpm/fmol) (C) or the $tRNA^{Ala}_{SG}$ gene (SG) (261 bp, 2.0×10^3 dpm/fmol).

the TFIIIB/C/D complex, since it is not distinguishable at the low resolution provided by gel mobility assays. We know that TFIIIC is required to reconstitute transcription with TFIIIB, TFIIID, and polymerase III (34), but we do not have direct evidence that it contributes DNA-binding polypeptides and, if so, how many. TFIIIB/D and TFIIIB/C/D complexes could be similar in size and shape but differ by a small number of polypeptides.

Detection of TFIIIB binding to polymerase III templates by gel retardation or DNase I footprinting has been reported previously only for *S. cerevisiae* (21, 23, 24), despite the chromatographic resolution of TFIIIB activity in a variety of other systems: mammalian cells (43, 57, 66), *Xenopus laevis* (16, 46), *Drosophila melanogaster* (3), and *Acanthamoeba castellanii* (30). Two kinds of experiments provide indirect evidence for TFIIIB binding in nonyeast systems, however. (i) Template commitment assays show that TFIIIB is stably sequestered by preformed TFIIIC-template complexes (1, 45). (ii) Transcription is severely impaired if exotic proteins are permitted to bind within upstream regions corresponding to the binding site of yeast TFIIIB (9, 28, 31, 54). The principal difficulty in detecting TFIIIB-DNA complexes directly may be due to low concentrations of the required factors. In keeping with this idea, a complex whose functional significance is not yet known, but whose gel mobility and DNase I footprint are consistent with TFIIIB binding, has been detected as a minor constituent of the complexes formed on a *Xenopus* 5S RNA gene by unfractionated extracts of *Xenopus* oocytes (36). An additional impediment to identifying TFIIIB-DNA complexes is that since TFIIIB binding depends on the binding of other multicomponent transcription factors—TFIIID or TFIIIC/D in silkworms and TFIIIC in other systems—the resulting protein-DNA complexes are large enough to challenge the resolving power of gel electrophoresis (42, 50). We attribute our success to the following two factors. (i) We used fractions of the silkworm transcription machinery in which the transcription factor activity was sufficiently enriched to allow specific protein-DNA complexes to be readily distinguished from nonspecific complexes. (ii) We deliberately sought gel electrophoresis conditions that allow resolution of large protein-DNA complexes. The silkworm TFIIIB-$tRNA^{Ala}_{C}$ promoter complexes we have identified are remarkably similar to yeast TFIIIB-tRNA promoter complexes. In both cases, approximately 40 bp of 5′-flanking DNA is protected from DNase I digestion and the addition of TFIIIB intensifies the protection of downstream sequences by other transcription factors (23, 24).

Using gel retardation and DNase I footprinting, we find that TFIIIB interacts differently with $tRNA^{Ala}_{C}$ genes and with $tRNA^{Ala}_{SG}$ genes. As judged by gel retardation, TFIIIB is capable of binding both kinds of gene, but TFIIIB concentrations higher than those required to saturate the $tRNA^{Ala}_{C}$ gene are required to saturate the $tRNA^{Ala}_{SG}$ gene. Strikingly, bound TFIIIB does not protect sequences upstream of the $tRNA^{Ala}_{SG}$ gene from DNase I digestion, as it does sequences upstream of the $tRNA^{Ala}_{C}$ gene. Moreover, when all of the input $tRNA^{Ala}_{SG}$ genes are assembled into TFIIIB/C/D-like complexes, only 17% of these complexes are transcriptionally active. This value is an upper limit since it is based on a comparison of the numbers of active complexes formed on $tRNA^{Ala}_{SG}$ and on $tRNA^{Ala}_{C}$ genes, assuming that all of the $tRNA^{Ala}_{C}$ complexes are active. If they are not, then the fraction of active $tRNA^{Ala}_{SG}$ complexes may be as low as 10% on the basis of the ratio of active complexes (0.5 fmol) to total input template (5 fmol).

There are several possible explanations for the failure of TFIIIB to efficiently activate the $tRNA^{Ala}_{SG}$ upstream promoter and to protect it from DNase I digestion. First, although the TFIIIB-containing complexes on both genes have the same electrophoretic mobility, they may have different polypeptide compositions. At one extreme, the gel retardation complex that appears to correspond to TFIIIB binding the $tRNA^{Ala}_{SG}$ gene could represent the binding of completely different proteins, unrelated to TFIIIB. This possibility seems unlikely, since such proteins would have to interact quite specifically—that is, bind the $tRNA^{Ala}_{SG}$ gene, but not the $tRNA^{Ala}_{C}$ gene, in a TFIIIC/D-dependent manner (68)—yet fail to exclude DNase I.

A more conservative possibility is that only a subset of the TFIIIB components that bind the $tRNA^{Ala}_{C}$ gene is also capable of binding the $tRNA^{Ala}_{SG}$ gene, a subset whose incorporation into the transcription complex depends primarily on protein-protein interactions with TFIIIC/D bound downstream and is sufficient to further retard the complex on gels. If essential transcription factors that make close contact with DNA upstream of the $tRNA^{Ala}_{C}$ gene do not bind the $tRNA^{Ala}_{SG}$ gene, then the lack of transcription activity and the accessibility to DNase I can be explained.

Another possibility is that TFIIIB complexes on both kinds of genes contain the same polypeptides but differences in DNA conformation prevent close contact with upstream sequences in the case of the $tRNA^{Ala}_{SG}$ gene. This suggestion is consistent with the fact that the distribution of A tracts upstream of the two genes (49) predicts intrinsically different DNA conformations. Such a model readily explains accessibility to DNase I digestion but does not immediately explain the lack of transcription activity. Potentially, DNA conformation could affect recruitment of RNA polymerase or successful completion of an early phase of transcription initiation.

Finally, it is formally possible that TFIIIB, even at a high concentration, does not actually saturate the $tRNA^{Ala}_{SG}$ gene under transcription or footprinting conditions. Our data do not

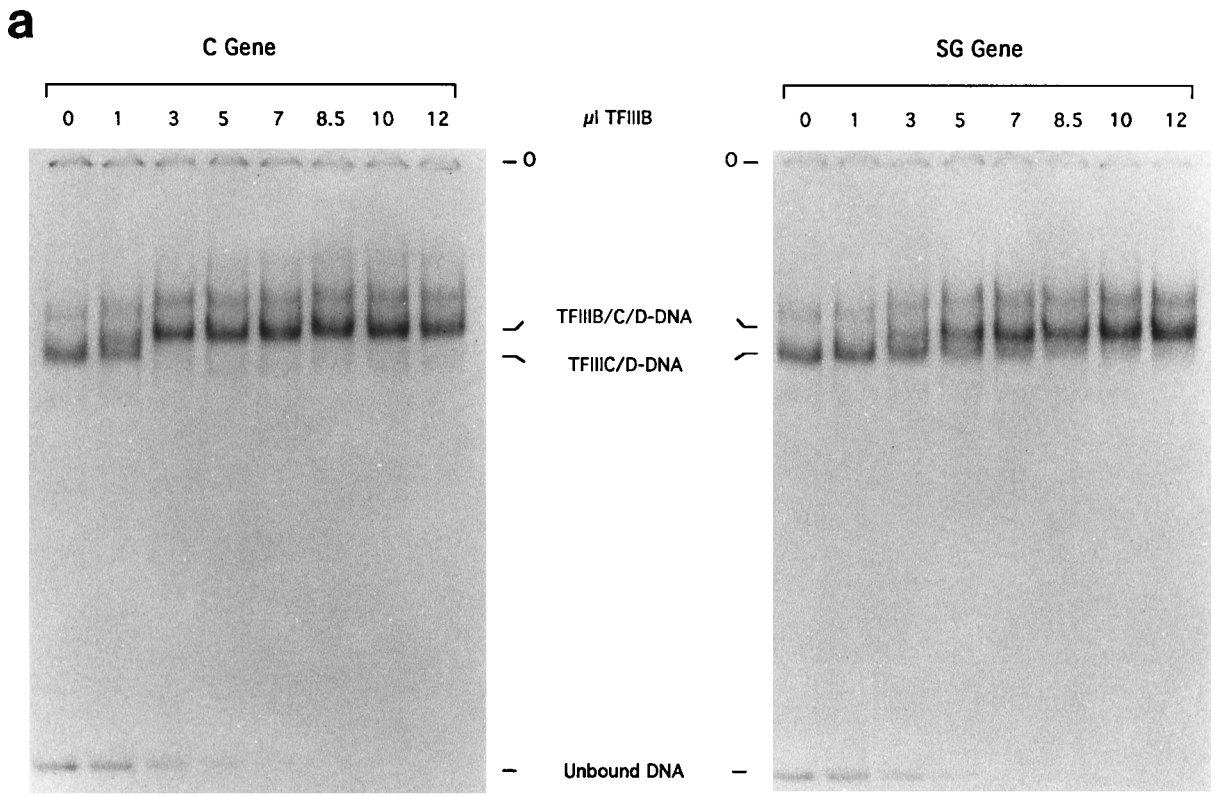

FIG. 5. A higher concentration of TFIIIB is required to drive TFIIIB/C/D complex formation on a tRNA$^{Ala}_{SG}$ gene than on a tRNA$^{Ala}_{C}$ gene. (a) The fraction of total protein-DNA complexes having TFIIIB/C/D electrophoretic mobility was measured after various amounts of TFIIIB plus a constant amount of TFIIIC/D had been incubated for 60 min with 2.5 fmol of either the 262-bp tRNA$^{Ala}_{C}$ gene-containing PCR fragment (2.3×10^3 dpm/fmol) (C gene) or the 261-bp tRNA$^{Ala}_{SG}$ gene-containing PCR fragment (2.0×10^3 dpm/fmol) (SG gene). This period of time had previously been shown to be sufficient for equilibrium binding of either TFIIIC/D alone or TFIIIC/D plus TFIIIB. The amount (in microliters) of TFIIIB fraction (μl TFIIIB) included in the binding reaction mixture is shown at the top. Also indicated are the positions of the gel origin (O), TFIIIB/C/D-DNA and TFIIIC/D-DNA complexes, and unbound DNA. (b) Graphical representation of the data shown in panel a. TFIIIC/D-DNA and TFIIIB/C/D-DNA complexes were separately quantitated by using an Ambis 4000 radioanalytic scanner.

exclude the possibility that the extensive binding that is detected by gel retardation occurs only after the binding reaction conditions have been altered by gel electrophoresis.

Whatever the precise mechanism, our results suggest that the capacity of TFIIIB to discriminate between the two kinds of tRNA^{Ala} genes accounts for their different transcriptional activities in vitro and is the basis of the regulatory strategy that keeps the tRNA$^{Ala}_{SG}$ genes silent in non-silk gland cells. In vivo, additional mechanisms, such as changes in chromatin structure, could stabilize or magnify the effect of differential TFIIIB binding. For example, *Xenopus* oocyte and somatic 5S RNA genes are differentially susceptible to the repressive effect of H1-rich chromatin (2). The basis of differential H1 sensitivity is apparently a difference in the rates at which the two kinds of 5S RNA genes are assembled into stable transcription complexes (44, 63, 64). Since their rates of assembly are lower, oocyte genes are more likely than somatic genes to be preempted by the competing reaction, assembly into stable chromatin.

Analysis of other systems shows that TFIIIB components can be regulators. A general loss of class III transcription caused by specific reduction in TFIIIB activity occurs in actively growing cells during the mitotic phase of the cell cycle (16, 59), in cells undergoing arrest induced by entry into stationary phase or treatment with protein synthesis inhibitors (7, 55), and also in differentiating embryonal carcinoma cells (60). TFIIIB activity can also increase in response to certain stimuli—for example, in cultured *Drosophila* cells treated with phorbol esters (13, 14).

Although no regulatory mechanisms have been elucidated in detail, some specific information is emerging. For instance, the cycloheximide-induced loss of yeast polymerase III transcription activity has been specifically attributed to reduced amounts or activities of two TFIIIB components, the 70-kDa polypeptide and the B″ fraction (7). The MPF-induced mitotic

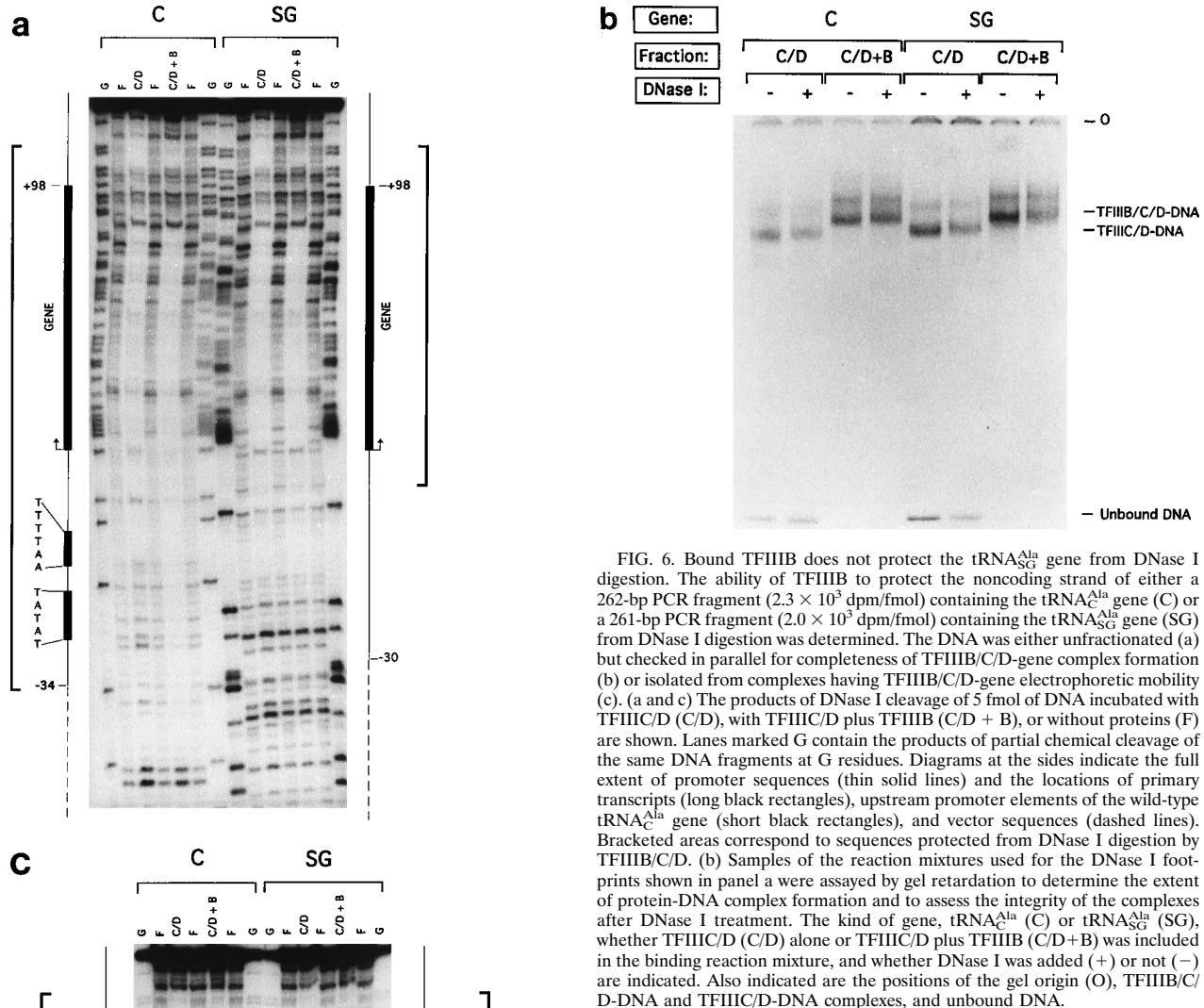

FIG. 6. Bound TFIIIB does not protect the $tRNA^{Ala}_{SG}$ gene from DNase I digestion. The ability of TFIIIB to protect the noncoding strand of either a 262-bp PCR fragment (2.3×10^3 dpm/fmol) containing the $tRNA^{Ala}_{C}$ gene (C) or a 261-bp PCR fragment (2.0×10^3 dpm/fmol) containing the $tRNA^{Ala}_{SG}$ gene (SG) from DNase I digestion was determined. The DNA was either unfractionated (a) but checked in parallel for completeness of TFIIIB/C/D-gene complex formation (b) or isolated from complexes having TFIIIB/C/D-gene electrophoretic mobility (c). (a and c) The products of DNase I cleavage of 5 fmol of DNA incubated with TFIIIC/D (C/D), with TFIIIC/D plus TFIIIB (C/D + B), or without proteins (F) are shown. Lanes marked G contain the products of partial chemical cleavage of the same DNA fragments at G residues. Diagrams at the sides indicate the full extent of promoter sequences (thin solid lines) and the locations of primary transcripts (long black rectangles), upstream promoter elements of the wild-type $tRNA^{Ala}_{C}$ gene (short black rectangles), and vector sequences (dashed lines). Bracketed areas correspond to sequences protected from DNase I digestion by TFIIIB/C/D. (b) Samples of the reaction mixtures used for the DNase I footprints shown in panel a were assayed by gel retardation to determine the extent of protein-DNA complex formation and to assess the integrity of the complexes after DNase I treatment. The kind of gene, $tRNA^{Ala}_{C}$ (C) or $tRNA^{Ala}_{SG}$ (SG), whether TFIIIC/D (C/D) alone or TFIIIC/D plus TFIIIB (C/D+B) was included in the binding reaction mixture, and whether DNase I was added (+) or not (−) are indicated. Also indicated are the positions of the gel origin (O), TFIIIB/C/D-DNA and TFIIIC/D-DNA complexes, and unbound DNA.

inactivation of class III transcription appears to involve transcription factor modification, since it can be mimicked by phosphorylation of TFIIIB (16). Finally, phorbol ester-induced TFIIIB activation is accompanied by increases in the level of TATA-binding protein (TBP) (14).

The relevance of these examples to silkworm $tRNA^{Ala}$ regulation is not yet clear, since in these cases changes in ubiquitous TFIIIB components cause general, rather than gene-specific, effects. Gene-specific regulation could be achieved by quantitative changes in such unspecialized factors if the response threshold for the regulated genes were above the factor concentration required to saturate the unregulated genes. Alternatively, gene-specific regulation may require that the changes in TFIIIB be qualitative rather than quantitative. For example, different TBP-TAF complexes may be required for transcription of constitutive and regulated class III templates. Although in *S. cerevisiae* the same components of TFIIIB are competent for transcription from two very different promoters, U6 and tRNA (22), there are more specialized requirements in human transcription systems. A particular TBP-TAF complex, SNAPc, is required for transcription of human U6 genes, but not for the tRNA-like VAI genes (19). Interestingly, although SNAPc contains TBP, it binds specifically to a non-TATA sequence (41). Presumably, other polypeptides in the SNAPc

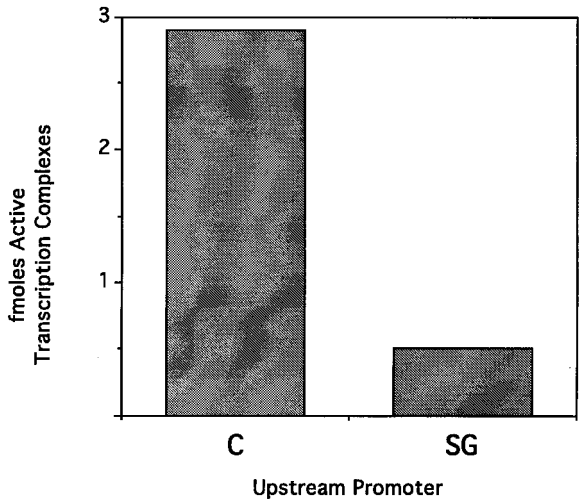

FIG. 7. TFIIIB/C/D complexes formed on tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes differ in transcriptional activity. Single-round transcription assays were performed with 5-fmol each of tRNA$_C^{Ala}$ and tRNA$_{SG}^{Ala}$ genes in supercoiled plasmids, by using concentrations of TFIIIB and TFIIIC/D that give quantitative assembly of both input DNAs into complexes with the electrophoretic mobility of TFIIIB/C/D-DNA complexes. The number of transcriptionally active complexes was quantitated by direct scintillation counting of transcripts from the tRNA$_C^{Ala}$ (C) and tRNA$_{SG}^{Ala}$ (SG) genes.

complex contact DNA directly and thereby serve to recruit TBP to this site.

A variant protein complex, analogous to SNAPc, could be required for transcription of tRNA$_{SG}^{Ala}$ genes to provide the close contacts with the upstream promoter that are missing in the TFIIIB-tRNA$_{SG}^{Ala}$ gene complexes we have analyzed. Although the TFIIIB in these complexes was derived from silk glands, its level of transcriptional activity is lower on the tRNA$_{SG}^{Ala}$ gene than on the tRNA$_C^{Ala}$ gene. This is not surprising, since all but the most concentrated silk gland extracts and fractions have this property (68, 69), which could result from dilution either of a general TFIIIB component or of a silk gland-specific factor that is required for tRNA$_{SG}^{Ala}$ but not tRNA$_C^{Ala}$ transcription.

We have considered whether other examples of gene-specific class III regulation are mechanistically related to tRNA$_{SG}^{Ala}$ regulation. For instance, do common mechanisms account for silk gland-specific enrichment of both glycine and alanine tRNAs? The *B. mori* tRNA^{Gly} genes do not encode a distinctive tissue-specific tRNA, but individual genes differ greatly in template efficiency in vitro and could be regulated independently through flanking sequence elements (11, 12, 53). In *X. laevis*, there are a variety of class III genes that have both constitutive and tissue-specific counterparts (10, 40, 51, 65). The best-studied are the somatic and oocyte-specific 5S rRNA and tRNA^{Tyr} genes (37–39, 51, 62, 64). Although direct evidence is lacking, differential interaction with TFIIIB might contribute to regulation of these genes, since their distinctive in vitro transcription properties are conferred by 5′-flanking sequences that are appropriately positioned for TFIIIB binding (17, 31, 38, 39, 51).

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