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Genetics

Upstream sequences confer distinctive transcriptional properties on genes encoding silkgland-specific $tRNA^{Ala}$

(upstream control region/RNA polymerase III/transcription)

LISA S. YOUNG, NORIKO TAKAHASHI*, AND KAREN U. SPRAGUE

Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

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ABSTRACT **To understand the molecular basis for the tissue-specific accumulation of alanine tRNAs in silkworms, we have compared the transcriptional properties of genes that encode constitutive and silkgland-specific type alanine tRNA. Genes for each class of alanine tRNA behave very differently when used as templates for homologous *in vitro* transcription. Since these properties are likely related to the activities of the corresponding genes *in vivo*, we wished to identify the *cis*-acting elements responsible for them. We have therefore constructed hybrid silkgland-specific/constitutive genes and have analyzed their capacity to direct transcription *in vitro*. We find a simple pattern: the distinctive transcriptional properties of the two kinds of $tRNA^{Ala}$ genes are the result of different positive signals upstream from their sites of transcription initiation.**

The production of a silk cocoon by the silkworm, *Bombyx mori*, is the result of intense and specialized synthetic activity in cells of the silkgland. A striking feature of the specialization of this tissue is the adjustment of its protein synthetic machinery to match the requirements for translating the major silk protein, fibroin. In particular, the early work of Matsuzaki (1) and Garel *et al.* (2) established that the tRNA population in silkglands becomes highly enriched for the tRNA species cognate to the predominant amino acids in fibroin: glycine (≈40%), alanine (≈20%), serine (≈10%), and tyrosine (≈10%). In the case of alanine tRNA, much of the increase results from the accumulation of a novel $tRNA^{Ala}$ specific to the silkgland (3, 4). Two kinds of $tRNA^{Ala}$ have been detected in the silkworm. One, designated the constitutive type ($tRNA_C^{Ala}$), is found in all silkworm cell types, while the second ($tRNA_{SG}^{Ala}$) is found only in the posterior part of the silkgland, where fibroin is synthesized. Here it makes up 70% of the total alanine tRNA at the time of rapid silk production (3).

To understand how the tissue-specific production of alanine tRNA is achieved, we have cloned silkworm genes encoding alanine tRNA and have found two classes of genes corresponding to the two kinds of $tRNA^{Ala}$ (ref. 5; unpublished data). The coding regions of these two kinds of genes differ from each other at the single position that distinguishes the mature alanine tRNAs, a C → U transition in the anticodon stem (3). The presence of both kinds of genes in the silkworm genome argues that post-transcriptional conversion of C → U is not necessary to explain the appearance of $tRNA_{SG}^{Ala}$ in the silkgland, and it is consistent with the idea that differential transcription of these two gene classes could account for the tissue-specific distribution of the alanine tRNAs. It is important, therefore, to understand the transcriptional properties of both kinds of genes. Previous work has established the boundaries of the region required for full transcriptional activity of a constitutive $tRNA^{Ala}$ gene (6). The control region is surprisingly large [≈160 base pairs (bp)]. It includes coding sequences, as well as flanking sequences both upstream and downstream. Moreover, the large control region provides at least two distinct functions. Internal and downstream sequences are involved in stably binding a required transcription factor(s), whereas the upstream element, though essential for transcriptional activity, does not contribute to this binding (6). In this paper we compare the transcriptional properties of constitutive and silkgland-specific $tRNA^{Ala}$ genes *in vitro*. We show that there are striking differences between them and that the characteristic transcriptional behavior of each kind of gene is conferred by its upstream sequence.

MATERIALS AND METHODS

tRNA Genes Used in This Work. The transcriptional properties of the wild-type $tRNA_C^{Ala}$ (≡ $tRNA_2^{Ala}$) gene and the wild-type $tRNA_{SG}^{Ala}$ (≡ $tRNA_1^{Ala}$) gene and the hybrids derived from them were determined by using cloned gene-containing DNA fragments inserted into bacteriophage M13 (7) as transcription templates. The wild-type $tRNA^{Ala}$ gene was on a 158-bp fragment (−34 to +124 with respect to the transcription initiation site) inserted at the *Eco*RI site of M13mp9. The wild-type $tRNA_{SG}^{Ala}$ gene was on a 157-bp fragment (−30 to +127 with respect to transcription initiation) inserted at the *Pst* I site of M13mp9. Hybrids involving exchange of 3′ flanking sequences with respect to the middle (+43) of the coding region were constructed by cleavage at the unique *Kpn* I site that is present in both genes at +52 and at the unique *Bgl* II site at position 6930 in M13mp9, with subsequent religation of the appropriate fragments. To join the upstream region of a $tRNA_C^{Ala}$ gene to the middle of a $tRNA_{SG}^{Ala}$ gene, a circular DNA heteroduplex was formed between the coding strand of a $tRNA_{SG}^{Ala}$ gene and the noncoding strand of a $tRNA_C^{Ala}$ gene. The $tRNA_{SG}^{Ala}$ gene was carried on a methylated single-stranded DNA circle derived from M13 phage particles, while the $tRNA_C^{Ala}$ gene was on a linear strand of unmethylated DNA produced by melting the linearized replicative form of M13 DNA isolated from an *Escherichia coli* K-12 *dam*$^-$ host (*dam-3*[F′ *proB lacZ*::Tn*5*]). After transfection of the heteroduplex molecules into *E. coli* JM103, the single-base-pair mismatch in the middle of the genes was corrected (at a frequency of ≈1:20 transformants) in favor of the methylated chain (C·A to T·A), while the large heterologies created by structural differences in the 5′ and 3′ flanking regions remained unchanged. To join the upstream region of the $tRNA_{SG}^{Ala}$ gene to the middle of the $tRNA_C^{Ala}$ gene, the $tRNA_{SG}^{Ala}$ gene was cleaved with *Kpn* I and then digested to a limited extent in the presence of a single nucleotide (dCTP) with *E. coli* DNA polymerase (Klenow fragment). Digested

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Abbreviations: $tRNA_C^{Ala}$, constitutive alanine tRNA; $tRNA_{SG}^{Ala}$, silkgland-specific alanine tRNA; bp, base pair(s).

*Present address: Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

DNA was denatured, annealed with excess single-stranded 5'-CCS-3' DNA (defined in *Results*), and, after repair of the gap with DNA polymerase and DNA ligase, transfected into *E. coli* JM103. The structures of all genes used in this work were established by dideoxy sequence analysis (8).

***In Vitro* Transcription.** The crude nuclear extracts used to catalyze transcription were prepared from *Bombyx* posterior silkglands as previously described (6, 9) with the following modifications: Extracts 1, 2, and 3 (Fig. 1) and others like them were reproducibly made by washing the nuclear pellets with increasing volumes of salt-containing buffer (1, 2.5, or 4 times the pellet volume, respectively). In our standard protocol (6), the volume of this wash is 4 times that of the pellet. The concentrations of total DNA and template optimal for transcription reactions were determined as described (6). Typical 20-μl reaction mixtures contained $tRNA^{Ala}$ genes (at 0.25 nM) on supercoiled M13mp9 replicative form DNA as transcription templates, additional M13 replicative form DNA (to a total of 0.08–0.15 μg, optimized for each extract), 5 μl of extract, 20–120 mM KCl (specified in figure legends), 4 mM $MgCl_2$, 4 mM phosphocreatine, 30 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, 10% (vol/vol) glycerol, 600 μM each of UTP, ATP, and CTP, and 25 μM [α-^{32}P]GTP (1–4 Ci/mmol). Reactions proceeded at room temperature (22°C) for 2 hr and then were stopped, and the products were applied without prior fractionation to a polyacrylamide gel. Autoradiography and quantitation of the products were as described (9).

The structure of the 98-nucleotide primary *in vitro* transcription product of the $tRNA_C^{Ala}$ gene was determined previously (5). We have recently characterized the transcript produced *in vitro* from the $tRNA_{SG}^{Ala}$ gene, and we find a 91-nucleotide primary transcript. Its structure was deduced by analysis of fingerprints produced after digestion with T1 or pancreatic ribonuclease (10). Secondary and tertiary digestion with base-specific and base-nonspecific nucleases established the structure of the primary nuclease digestion products. This analysis, coupled with the use of different nucleoside [α-^{32}P]triphosphates to label the transcript, allowed unambiguous identification of oligonucleotides derived from the ends of the molecule. The site of transcription initiation was verified by examining the mononucleotide products of complete digestion of the intact primary transcript (5). These data show that at least 90% of the transcripts have the structure indicated in Fig. 3.

RESULTS

Transcriptional Properties of Constitutive and Silkgland-Specific $tRNA^{Ala}$ Genes *in Vitro*. To examine the transcriptional properties of $tRNA_C^{Ala}$ and $tRNA_{SG}^{Ala}$ genes, we have used single constitutive and silkgland-specific $tRNA^{Ala}$ genes isolated on small *Bombyx* genomic DNA fragments. We have previously estimated that there are on the order of 20–30 copies of $tRNA_C^{Ala}$ genes in the silkworm genome (5). Preliminary work suggests that the number of $tRNA_{SG}^{Ala}$ genes in the silkgland is roughly comparable (D. Underwood and K.U.S., unpublished observations). Our structural and functional analysis of these two groups indicates that the genes we have chosen for study here are representative of each of their classes. The transcriptional properties of the 10 $tRNA_C^{Ala}$ genes and 4 other constitutively expressed *Bombyx* RNA polymerase III templates that have been examined are all very similar to each other and are like the properties of the $tRNA_C^{Ala}$ gene described here (refs. 9, 11, and 12; unpublished observations). Complete sequence analysis of five genes from this group (including both $tRNA_C^{Ala}$ genes and three other types of templates) has revealed obvious structural similarities in their flanking regions (refs. 9, 11, and 12; unpublished observations). The transcriptional properties of four silkgland-specific type $tRNA^{Ala}$ genes have been analyzed extensively and are indistinguishable. The sequences of these genes differ from each other at only 2 out of 28 5' flanking nucleotides and 4 out of 35 3' flanking nucleotides. Detailed restriction mapping indicates that the remaining $tRNA_{SG}^{Ala}$ genes are structurally similar.

Since silkgland-specific genes appear to be tightly clustered in the silkworm genome (unpublished data), we have limited the amount of flanking sequence included in this analysis to the boundaries of adjacent genes. The sequences flanking the constitutive $tRNA^{Ala}$ gene were chosen to approximate the sizes of regions flanking the silkgland-specific gene and to include both upstream and downstream elements known to influence constitutive gene transcription (6).

Transcription of cloned genes was catalyzed by crude extracts from *Bombyx* silkgland nuclei. Previous work in our laboratory showed that differences in transcriptional activity of genes can be obscured by the use of concentrations of templates that saturate the transcription apparatus (6). We found that DNA binding inhibitory substances in crude transcription extracts commonly prevent transcription from the small amounts of template DNA that must be used to achieve subsaturating concentrations. To ensure a sensitive comparison of silkgland-specific and constitutive $tRNA^{Ala}$ genes, we have used subsaturating amounts of template in the presence of sufficient nonspecific DNA to titrate inhibitory substance(s) (6, 13). We have not found differences between the two kinds of tRNA genes either in the amount of nonspecific DNA required to eliminate inhibition or in the template concentration that gives maximal transcription rates.

Fig. 1*A* compares the activities of constitutive and silkgland-specific $tRNA^{Ala}$ genes in three extracts prepared by systematically varying the extraction volume. In contrast to the behavior of the constitutive gene, the ability of the silkgland-specific gene to direct transcription is extremely sensitive to this variation in extracts. Different silkgland extracts that are nearly equal in their transcriptional activity on $tRNA_C^{Ala}$ genes vary widely in their activity on $tRNA_{SG}^{Ala}$

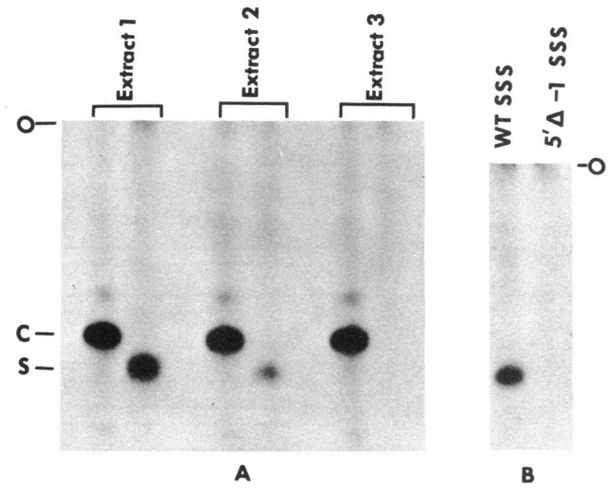

FIG. 1. (*A*) *In vitro* transcription products from wild-type constitutive (left lane of each pair) and silkgland-specific (right lanes) $tRNA^{Ala}$ genes in three different silkgland extracts. Products are displayed on a 10% polyacrylamide gel. O, origin; C, $tRNA_C^{Ala}$; S, $tRNA_{SG}^{Ala}$. Each extract was tested for activity with template DNA at 0.25 nM and additional nontemplate DNA to a total of 0.1 μg. Reactions were carried out at 50 mM KCl. (*B*) *In vitro* transcription products from the wild-type silkgland-specific gene and a 5' deletion mutant (endpoint at position −1) transcribed in extract 1. Reactions were carried out as described for *A*.

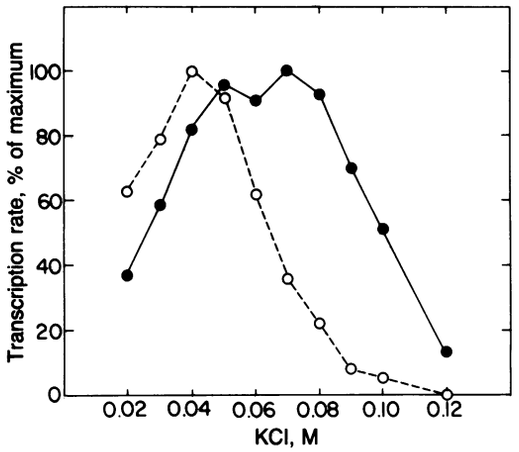

FIG. 2. Salt dependence for transcription of constitutive (●) and silkgland-specific (○) $tRNA^{Ala}$ genes. Reactions were carried out with extract 2 (Fig. 1). Data are normalized to the maximal amount of transcription activity observed for each gene. In similar experiments, we found that the salt dependence characteristic of each gene was obtained in extracts 1 and 3 (Fig. 1) as well (data not shown).

genes. Fig. 1*A* illustrates this range. Between the least active and most active of the three extracts, the transcriptional activity of the $tRNA_C^{Ala}$ gene increases by less than 2-fold, whereas that of the $tRNA_{SG}^{Ala}$ gene increases by 30-fold. The observed transcriptional variability of the silkgland-specific gene is consistent with the expected behavior of a gene whose expression is modulated in a tissue-specific fashion. Such a gene should be sensitive to transcriptional activators or repressors that do not affect transcription of constitutively expressed genes.

In addition to quantitative differences in template efficiency, the two kinds of genes have different salt requirements for transcription. We compared the rates of transcription directed by both genes in crude extracts at different concentrations of KCl. As shown in Fig. 2, the curve depicting the optimal salt concentration for transcription of the constitutive gene has a broad peak with a center at around 0.07 M. This value is typical of the salt optima for activity of other *Bombyx* RNA polymerase III templates *in vitro* [5S RNA and other $tRNA_C^{Ala}$ genes, for example (D. Rivier and K.U.S., unpublished observations). In contrast, the $tRNA_{SG}^{Ala}$ gene is transcribed poorly at 0.07 M KCl and functions best at a lower KCl concentration (≈0.04 M). The salt dependence characteristic of the $tRNA_{SG}^{Ala}$ gene is observed in all silkgland extracts and is independent of the efficiency of $tRNA_{SG}^{Ala}$ gene transcription.

Identification of Sequences Responsible for the Characteristic Transcriptional Properties of $tRNA_C^{Ala}$ and $tRNA_{SG}^{Ala}$ Genes. The sequences of constitutive and silkgland-specific $tRNA^{Ala}$ genes differ in three places (Fig. 3): the 5′ and 3′ flanking regions of the two genes are obviously dissimilar, and the two coding regions differ by the single nucleotide that distinguishes the mature alanine tRNAs. All three sites of structural heterogeneity lie within the large region known to be required for full transcriptional activity of the constitutive $tRNA^{Ala}$ gene. Therefore, all three are candidates for elements that differentially influence transcription of the two kinds of genes. To determine which part (or parts) of the $tRNA^{Ala}$ genes is actually responsible for the transcriptional phenotype(s) observed, we constructed a series of constitutive/silkgland-specific hybrid genes. In this series, the three sequence elements that differ between the two genes were interchanged precisely to create all eight possible combinations.

The hybrid genes are represented schematically in Fig. 3. We use the symbolism 5′-CCC-3′ and 5′-SSS-3′ to indicate wild-type genes that are of the fully constitutive or silkgland-specific type, respectively. Hybrids are indicated by the appropriate combinations of C and S. For example, a hybrid composed of the 5′ flanking region of a constitutive gene and the coding and downstream regions of a silkgland-specific gene is designated 5′-CSS-3′. The transcriptional activity of these genes was measured in extract 2 (Fig. 1). A comparison of the activities of the hybrid genes with the wild-type parents is shown in Fig. 4. The simple pattern observed indicates that substitution of the appropriate 5′ flanking region is sufficient

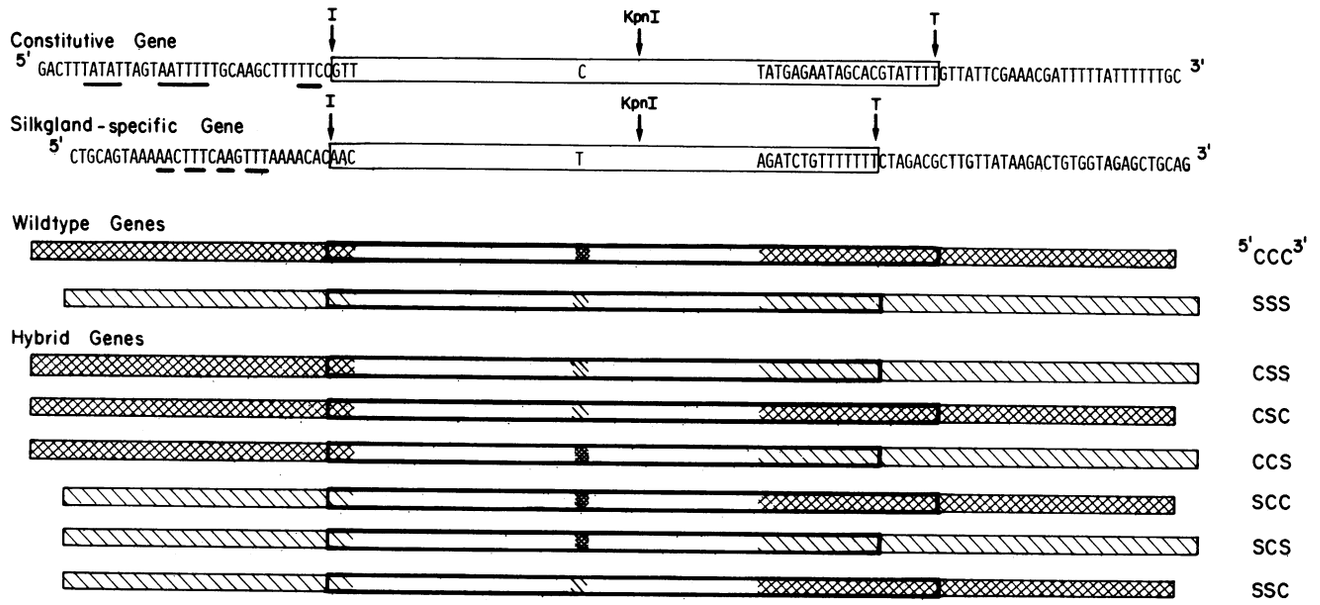

FIG. 3. Diagram of wild-type constitutive and silkgland-specific genes and their hybrid derivatives. Sequences that differ between the two genes are shown, as well as the positions of transcription initiation (I) and termination (T). Oligonucleotides that exist upstream (5′) from several other *Bombyx* genes ($tRNA_C^{Ala}$, $tRNA^{Gly}$, 5S RNA) transcribed by RNA polymerase III are underlined. Regions of DNA unique to the constitutive type gene are designated by crosshatching, while those unique to the silkgland-specific gene are designated by hatching. The nomenclature that describes the structure of each hybrid (e.g., 5′-CSS-3′) is defined in the text.

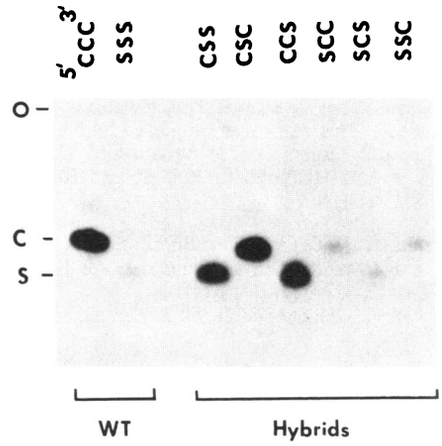

FIG. 4. Transcriptional activity of wild-type constitutive and silkgland-specific genes and their hybrid derivatives. Products are displayed on a 10% polyacrylamide gel. Reactions were carried out with extract 2 (Fig. 1) at 70 mM KCl to discriminate clearly between the activities of the parental genes but permit detection of possible intermediate activities of the hybrid genes. The average rates of transcription directed by the genes were calculated from five or more independent determinations using at least two different preparations of DNA. The mean rates (±SD) in nM transcripts per hr are as follows: CCC, 15.2 ± 1.3; SSS, 3.6 ± 0.8; CSS, 13.5 ± 1.6; CSC, 12.8 ± 1.1; CCS, 16.6 ± 1.4; SCC, 2.9 ± 1.9; SCS, 2.0 ± 0.8; SSC, 2.7 ± 1.1.

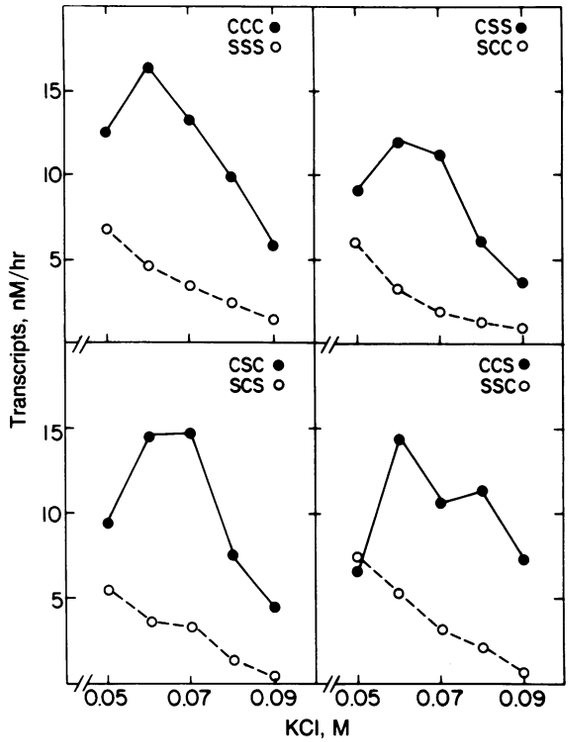

FIG. 5. Salt dependence for transcription of wild-type constitutive and silkgland-specific genes and their hybrid derivatives. Transcription reactions were catalyzed by extract 2 (see Fig. 1). Data for this experiment are not normalized as they are in Fig. 2.

to confer on either gene the template activity of the other. Thus, upstream sequences are responsible for the quantitative difference in the ability of constitutive and silkgland-specific type $tRNA^{Ala}$ genes to direct transcription. In addition, we examined the effects of salt on transcription of the same series of hybrid genes. The results, summarized in Fig. 5, demonstrate that the salt dependence of transcription of each hybrid gene is also conferred by the upstream region of the gene. Thus, 5′ flanking sequences are the major determinant of both of the characteristic transcriptional properties of these genes.

Since we know that the 5′ flanking region of constitutive genes contains an essential transcription signal (11, 14), we wondered whether the distinctive behavior of genes with the silkgland-specific upstream sequence results from the absence of any signal there or the substitution of a different one. Possibly, silkgland-specific $tRNA^{Ala}$ genes lack an upstream control element and depend for activity on a factor that overcomes the requirement for it. To test directly for a signal upstream of $tRNA^{Ala}_{SG}$ genes, we replaced the entire 5′ flanking region (−1 to −30) of the $tRNA^{Ala}_{SG}$ gene with unrelated DNA from bacteriophage M13. As shown in Fig. 1*B*, this substitution abolishes transcription from the $tRNA^{Ala}_{SG}$ gene. Transcripts are undetectable at a sensitivity of at least 0.1% of wild type. We conclude, therefore, that the upstream region of the $tRNA^{Ala}_{SG}$ gene provides an essential signal but that this signal differs significantly from the one associated with constitutive genes.

DISCUSSION

The transcriptional properties of constitutive and silkgland-specific $tRNA^{Ala}$ genes are strikingly different *in vitro*. Comparison of the transcriptional properties of wild-type genes and hybrid derivatives containing interchanged flanking and internal segments has identified the upstream region as the critical distinguishing element. Obvious structural differences exist between the upstream region of the silkgland-specific $tRNA^{Ala}$ gene and other genes transcribed by RNA polymerase III in silkworms. A group of three oligonucleotides has been found at nearly identical positions upstream of several silkworm genes encoding tRNA and 5S RNA (see $tRNA^{Ala}_{C}$ underlined sequences in Fig. 3). In every case tested ($tRNA^{Ala}_{C}$, $tRNA^{Gly}$, and 5S RNA), these sequences occupy a short (≈35 bp) region that has been shown to be essential for transcriptional activity (9, 11, 12). Since we now know that upstream sequences confer distinctive transcriptional properties on the $tRNA^{Ala}_{SG}$ gene, we think that this region may also play a regulatory role in the tissue-specific control of $tRNA^{Ala}$ gene transcription. Silkgland-specific $tRNA^{Ala}$ genes lack two of the canonical oligonucleotides entirely and have two copies of an altered form of the third one. We do not yet know whether this remnant of a feature characteristic of constitutive genes provides the upstream signal in silkgland-specific type genes or whether some other part of the $tRNA^{Ala}_{SG}$ 5′ flanking region is critical.

In this paper, we have deliberately focused attention on the upstream region of the silkgland-specific $tRNA^{Ala}$ gene because this element confers unique transcriptional properties. It is likely, however, that silkgland-specific type genes also possess a downstream control element. Two observations support the existence of such an element and suggest that it functions in transcription factor binding to these genes as it does in constitutive genes. First, substitution with silkgland-specific internal and downstream sequences does not impair the template activity of otherwise constitutive genes. Second, silkgland-specific and constitutive $tRNA^{Ala}$ genes compete with each other for a transcription factor(s) that binds to downstream $tRNA^{Ala}_{C}$ gene sequences (unpublished observations). Our findings are consistent with a model in which at least two elements direct transcription of alanine tRNA genes. We imagine that the output of both kinds of genes is modulated by the large downstream region in response to changes in transcription factor concentration or activity. In contrast, interactions in the upstream region affect each gene class in a distinctive fashion and determine which class of genes is expressed.

At first glance, the low transcriptional activity of the silkgland-specific $tRNA^{Ala}$ gene in certain silkgland extracts may seem anomalous since this gene is expected to be active in silkglands *in vivo*. An alternative interpretation of our data is that the variable activity of the silkgland-specific $tRNA^{Ala}$ gene *in vitro* is indicative of its capacity to be regulated *in vivo*. Low activity *in vitro* could be due either to the presence of a gene-specific inhibitor or to suboptimal levels of an activator in some extracts. Three kinds of experiments suggest that differences in $tRNA^{Ala}_{SG}$ gene activity in different extracts are due to variation in the concentration of an activator rather than an inhibitor. First, since increasing the template concentration does not alter the relative transcriptional efficiencies of the two kinds of genes in any extract, it is unlikely that subsaturating levels of a DNA binding inhibitor are repressing silkgland-specific gene activity. Second, mixtures of transcription extracts that differ in activity on $tRNA^{Ala}_{SG}$ genes catalyze transcription of these genes at the intermediate rates expected from simple dilution of the more active extracts. The results are independent of template concentration over a 10-fold range. Therefore, it is unlikely that either a DNA binding inhibitor in excess or an inhibitor that does not act stoichiometrically is reducing $tRNA^{Ala}_{SG}$ gene transcription. Finally, preliminary experiments with fractionated *Bombyx* silkgland transcription apparatus indicate that the components sufficient for high rates of constitutive gene transcription are inactive on $tRNA^{Ala}_{SG}$ genes. Addition of a silkgland fraction that does not further stimulate constitutive gene transcription is required for transcription of silkgland-specific $tRNA^{Ala}$ genes. The goal of our current work is to identify the critical component in this fraction and to learn how it acts. Conceivably, changes in the concentration, activity, or tissue distribution of such a component accountfor the presence of the silkgland-specific $tRNA^{Ala}$ in the silkgland and its absence in other tissues.

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