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Genetics

In vitro transcription of a silkworm 5S RNA gene requires an upstream signal

(control element/RNA polymerase III)

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ABSTRACT **The RNA polymerase III transcription complex from *Bombyx mori* silkworms, in contrast to that from *Xenopus* frogs, requires a signal that is upstream from the transcription initiation site. We have found that part of the normal 5′ flanking sequence is essential for transcription of *Bombyx* 5S RNA genes by homologous cell-free extracts. The critical region is structurally similar to the upstream signal that directs *Bombyx* tRNA gene transcription. The association of such a control element with two classes of polymerase III templates argues that it acts through a general component of the *Bombyx* transcription apparatus.**

The group of genes encoding tRNA, 5S RNA, and other small RNAs is transcribed by eukaryotic RNA polymerase III. To learn whether common structural elements associated with these genes might account for their recognition by the same transcription apparatus, we have compared *cis*-acting elements that direct initiation by *Bombyx* (silkworm) RNA polymerase III on different *Bombyx* genes. We have determined the transcriptional activities of partially deleted genes to identify required regions, and we have compared the DNA sequences of different wild-type genes to look for structural similarities within these functionally important regions. Previously we established that a short (34-nucleotide) upstream region is essential for silkworm alanine tRNA gene activity *in vitro* (1). Here, we show that a region nearly identical in size, location, and sequence is required for transcription of silkworm 5S RNA genes.

MATERIALS AND METHODS

Construction of Deletion Mutants. The starting material for deletion mutagenesis was a 290-base-pair (bp) *Taq* I restriction fragment containing the 120-bp 5S RNA gene flanked on the 5′ side by 92 bp of silkworm DNA. This fragment was derived from a larger one in pBm13 (2) and was inserted into M13mp8 at the *Acc* I site. The 5′ deletions were generated by cleaving M13mp8 at the *Hin*dIII site and subsequently digesting it with BAL-31 exonuclease (3). Deleted DNA fragments were cut on the 3′ side of the 5S RNA gene with the restriction enzyme *Bam*HI and fractionated on a 5% polyacrylamide gel. Specific size classes of deleted DNA were recovered by electroelution of excised gel pieces (4). In most cases, the 5′ blunt/*Bam*HI 3′ DNA fragments containing the 5S RNA gene were then subcloned into *Hin*cII/*Bam*HI cut M13mp8 vector DNA by standard procedures. To juxtapose different vector sequences, some of the 5′ blunt/*Bam*HI 3′ fragments containing 5S RNA genes were inserted into a derivative of M13mp8 DNA from which 36 bp extending from the *Hin*cII site through the *Hin*dIII site had been removed by treatment with BAL-31. Among the 5S RNA genes inserted into this modified M13mp8, only one was deleted at a position (−9) comparable to the deletion end points of the 5S RNA genes subcloned in standard M13mp8. The end points of deletions were determined by dideoxy-sequencing (5) of single-stranded Δ5S-M13 recombinant phage DNA, and in the case of the −9 deletion, the junction sequence was confirmed by partial chemical cleavage of double-stranded DNA (6).

***In Vitro* Transcription Assays.** *Bombyx* transcription extracts were prepared from the nuclei of 11-day pupal ovaries that had been freed of other silkworm tissues by dissection in 1× NaCl/Cit (standard saline citrate; 0.15 M NaCl/0.015 M Na citrate, pH 7.5). All solutions were kept ice cold and all manipulations were done on ice or at 4°C. Ovary tissue was rinsed twice and once, respectively, in slightly modified versions of the DB and RB solutions described by Wu and Zubay (7). The modifications were as follows: substitution of $MgCl_2$ for $Mg(OAc)_2$ and addition of 7 mM 2-mercaptoethanol to RB. Ovary tissue was allowed to stand 20 min in an equal volume of modified RB solution diluted 1:4 and was then homogenized with 5 strokes of a loose pestle in a Dounce homogenizer. The homogenate was spun at 1000 × *g* for 10 min, and the pellet (containing nuclei and cell debris) was resuspended in a volume of 0.3 M KCl/50 mM Tris·HCl, pH 7.5/4 mM $MgCl_2$/7 mM 2-mercaptoethanol/20% (vol/vol) glycerol equal to one-half the original tissue volume. The resulting suspension was allowed to stand for 60 min with occasional stirring. It was then spun at 120,000 × *g* for 30 min. Aliquots of the resulting supernatant were stored at −70°C and used as transcription extracts. Typical 20-μl transcription reaction mixtures contained 2–60 fmol of template in a total of 0.3 μg of DNA/5 μl of extract/65 mM KCl/4 mM $MgCl_2$/15 mM phosphocreatine/10 mM Tris·HCl, pH 7.5/3.5 mM 2-mercaptoethanol/600 μM each GTP, ATP, and CTP/25 μM [α-^{32}P]UTP (1–4 Ci/mmol; 1 Ci = 37 GBq). Reactions were carried out at room temperature (≈22°C) for 3 hr. Reaction mixtures were made 2 M urea/4% $NaDodSO_4$/0.2 M 2-mercaptoethanol, heated at 65°C for 15 min, and then loaded without prior fractionation on an 8% polyacrylamide gel containing 6 M urea/0.1% $NaDodSO_4$. Transcription products were detected by autoradiography of gels at −60°C with DuPont Cronex Lightning Plus intensifying screens. Exposure times were 2–3 hr.

Xenopus extracts were S-30 extracts prepared by the method of Wu and Zubay (7). The conditions for transcription by *Xenopus* extracts were as described for *Bombyx* extracts except that 10 μl of extract and 0.5 μg of DNA (0.1 pmol of template) were used in each 20-μl reaction mixture.

RESULTS AND DISCUSSION

To learn whether upstream regions are needed for silkworm 5S RNA gene activity, we constructed a series of partially deleted genes. Progressive amounts of 5′ flanking and coding

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Abbreviation: bp, base pair(s).

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sequences of a silkworm 5S RNA gene were removed and replaced with unrelated sequences derived from a bacteriophage vector, M13mp8 (8). Fig. 1 diagrams these constructions. We examined the effect of the deletions/substitutions by comparing the activity of wild-type and mutant genes in homologous *in vitro* transcription extracts derived from *Bombyx* ovaries. As shown in Fig. 2, substitution of all but 30 bp of normal 5′ flanking DNA does not affect transcriptional activity. In contrast, sequence alterations closer to the gene decrease activity. Mutant genes with 9 bp or less of normal flanking sequence are completely inactive.

In principle, this loss of activity could be due to either removal of a required positive signal or the fortuitous provision of a negative effector in the substituted DNA. To distinguish between these possibilities, we have used two different replacement sequences in the vicinity of −9. (Compare the −6 and −9 sequences in Fig. 3.) Since both of these substitutions abolish transcription (Fig. 2), it is likely that a positive signal upstream from the normal gene has been lost in the inactive mutants.

What is this signal? Is it a particular sequence within the required region upstream of the 5S RNA gene? Structural comparison has revealed identical sequences within upstream regions of this 5S RNA gene and two *Bombyx* $tRNA^{Ala}$ genes. We have previously reported (2) that the most significant sequence homologies outside the coding regions of these genes are within the 30 bp preceding each of them. Three blocks of conserved sequence (T-A-T-A-T, A-A-T-T-T-T, and T-T-C) occur at nearly the same positions (±1 nucleotide) in front of each gene. Fig. 3 shows the location of these sequence blocks in the *Bombyx* 5S RNA gene analyzed here. The transcriptional properties of partially deleted 5S RNA genes suggest that these sequences are important and that more than one of them may be needed for full activity (Fig. 2). The shortest deletion that affects activity (−26) alters the most distal conserved oligonucleotide (T-A-T-A-T) and lowers transcriptional activity to 66% of the wild-type level. Deletions that remove the A-A-T-T-T-T block as well (deletion −9, for example) result in complete inactivation.

Mutant genes with deletions ending between these points retain successively fewer of the conserved nucleotides and might be expected to support intermediate levels of transcription. Two mutant genes (retaining 21 and 17 bp of normal 5′ flanking sequences) do behave in this manner (Fig. 2).

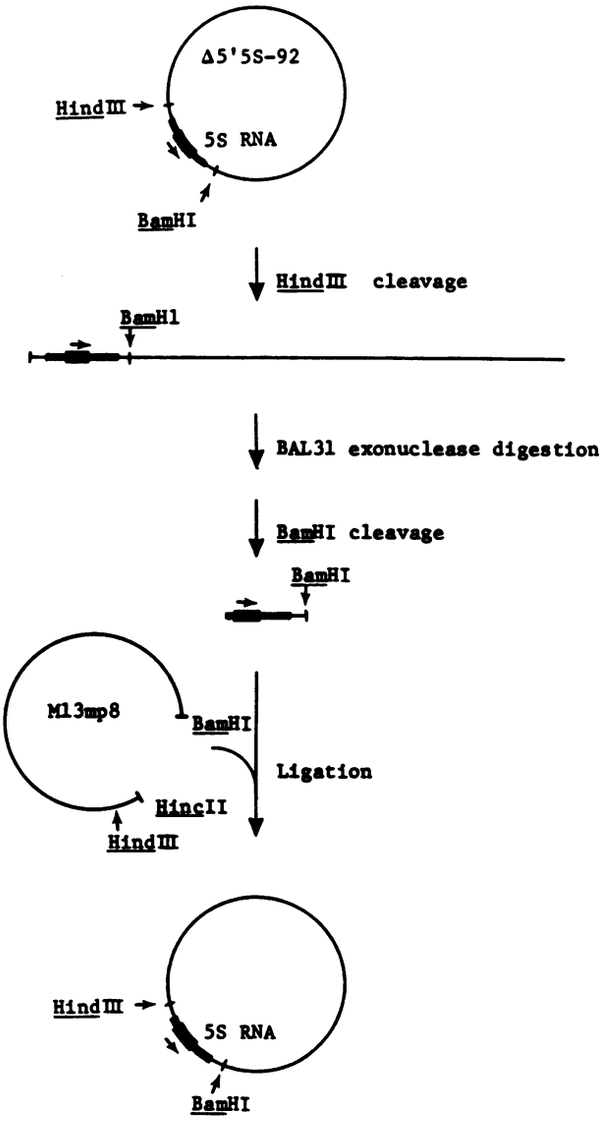

FIG. 1. Construction of 5′ deleted 5S RNA genes. Thin lines represent bacteriophage M13 DNA; thick lines represent *Bombyx* DNA. The thickest bars correspond to the coding region of the *Bombyx* 5S RNA gene, and the arrow associated with these bars denotes the direction of transcription.

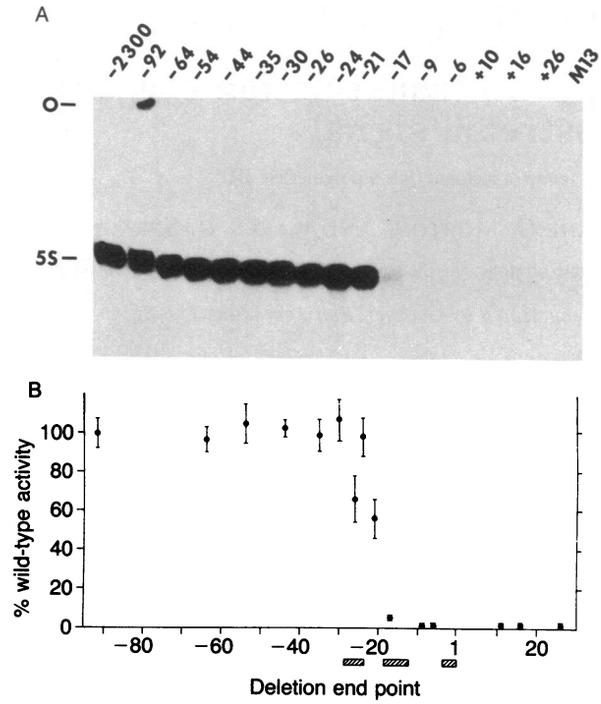

FIG. 2. *In vitro* transcription of 5′ deletion derivatives of a silkworm 5S RNA gene. Double-stranded RF 5S-M13 recombinant DNAs were used as templates for transcription by extracts of *Bombyx* ovary nuclei. (*A*) Products of transcription are shown after fractionation by polyacrylamide gel electrophoresis and detection by autoradiography. Each lane is labeled with the end point of the deletion in the template DNA. The 5S RNA transcript produced *in vitro* from the wild-type 5S RNA gene has been characterized previously (2). RNA sequence data (not shown) established that transcripts from mutant and wild-type genes are identical. (*B*) Transcriptional activity of each DNA template relative to a wild-type (−2300) 5S RNA gene is plotted as a function of the position of the deletion end point. Transcriptional activities were determined by measuring Cerenkov radiation in gel pieces containing 5S RNA transcripts. The value reported for each gene represents the mean (±SD) of four or more determinations, using at least two different preparations of DNA. In each case, mutant and wild-type genes were compared in the same experiment. Positions of conserved sequences that precede *Bombyx* genes are indicated as hatched bars below the abcissa.

5'Δ	Transcriptional Activity	-50 -40 -30 -20 -10 I (C)
WT	100%	GTGGTTGGTTACATTCTGTAAATATATAGCTTAATTTTAACTTTCTATTCG
-44	103%	CAGGTCGGTTACATTCTGTAAATATATAGCTTAATTTTAACTTTCTATTCG
-35	99%	GCTTGGCTGCAGGTCCTGTAAATATATAGCTTAATTTTAACTTTCTATTCG
-30	107%	GCCAAGCTTGGCTGCAGGTCAATATATAGCTTAATTTTAACTTTCTATTCG
-26	66%	CAGTGCCAAGCTTGGCTGCAGGTCTATAGCTTAATTTTAACTTTCTATTCG
-24	98%	GCCAGTGCCAAGCTTGGCTGCAGGTCTAGCTTAATTTTAACTTTCTATTCG
-21	56%	ACGGCCAGTGCCAAGCTTGGCTGCAGGTCCTTAATTTTAACTTTCTATTCG
-17	5%	AACGACGGCCAGTGCCAAGCTTGGCTGCAGGTCATTTTAACTTTCTATTCG
- 6	<3%	CGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGGCTGCAGGTCCTATTCG
- 9	<3%	CGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTTCTATTCG

FIG. 3. Comparison of the DNA sequences flanking wild-type and partially deleted 5S RNA genes on the 5′ side. Sequence of the noncoding DNA strand is shown. Top sequence is that of the wild-type gene. Sequences of deletion derivatives of the 5S RNA gene are aligned below. Nucleotide positions are numbered with respect to the transcription initiation site (+1) of the 5S RNA gene. Normal silkworm DNA sequences are shown in large letters; juxtaposed M13mp8 DNA sequences are in smaller letters. Deletion end points and transcriptional activities of the mutant genes are shown on the left. Sequences common to the 5S RNA gene and two silkworm $tRNA^{Ala}$ genes are underlined. The (C) above the most distal conserved sequence block indicates that this sequence sometimes reads "T-A-C-T-A-T" (2).

However, a mutant gene with 24 bp of *Bombyx* 5′ flanking sequence is a more active template than is a gene with 26 bp of normal upstream sequence. We do not understand the basis for the relative activities of the genes with deletion end points at −24 and −26. Possibly, transcriptional efficiency can be influenced by the particular sequence that replaces *Bombyx* DNA in this region. In any case, although more detailed mutagenesis will be required to establish exactly which nucleotides are critical, it is clear that a short upstream region (containing sequences conserved among several *Bombyx* RNA polymerase III templates) is essential for transcription of the 5S RNA gene. It is not unreasonable to suppose that it is the conserved sequences within this region that provide the positive upstream signal for transcription of silkworm 5S RNA genes.

Our results emphasize the importance of 5′ flanking signals for transcription of a silkworm 5S RNA gene by the silkworm RNA polymerase III complex. The behavior of this silkworm gene is strikingly different from that of its counterpart in *Xenopus* frogs. Transcription of *Xenopus* 5S RNA genes by *Xenopus* RNA polymerase III does not require a particular sequence 5′ to the gene. Instead, transcriptional control is exerted by sequences located entirely within the 5S RNA gene coding region (9, 10). We do not yet know whether internal coding regions contribute control functions in *Bombyx* 5S RNA genes. Nonetheless, it is clear that transcription of *Bombyx* 5S RNA genes by *Bombyx* RNA polymerase III requires 5′ flanking signals, whereas transcription of *Xenopus* 5S RNA genes by *Xenopus* RNA polymerase III does not.

To determine whether this difference reflects distinct properties of these two transcription complexes, we have compared the transcriptional activities of intact and partially deleted 5S RNA genes in *Xenopus* and *Bombyx* transcription extracts. Fig. 4 shows that in *Xenopus* extracts, wild-type *Bombyx* genes are as active as wild-type *Xenopus* genes. This result is likely a consequence of the fact that *Bombyx* genes possess an internal sequence similar to the one that suffices for transcriptional control of *Xenopus* 5S RNA genes (2, 9, 11). Considering the entire coding region, the *Bombyx* 5S RNA gene is 79% homologous to somatic-type 5S RNA genes from *Xenopus laevis* or *Xenopus borealis*. Within the critical *Xenopus* control region (+50 to +83), 82% of the nucleotides in frog and silkworm genes are identical. Support for the idea that internal *Bombyx* gene sequences direct transcription in *Xenopus* extracts comes from the behavior of the *Bombyx* 5S RNA gene deleted at +26. This mutant template, although transcriptionally silent in *Bombyx* extracts, is fully active in *Xenopus* extracts (Fig. 4). This result shows that the *Xenopus* transcription machinery is not directed by an upstream signal provided by the wild-type *Bombyx* 5S RNA gene. Additional evidence of a difference between the *Bombyx* and *Xenopus* transcription apparatus comes from the finding that even wild-type *Xenopus* 5S RNA genes are completely inactive in *Bombyx* extracts (Fig.

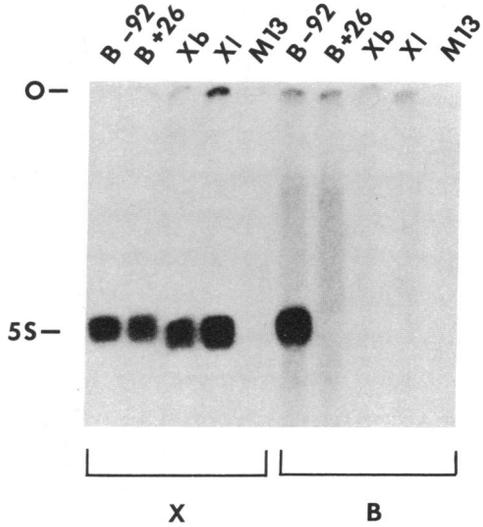

FIG. 4. *In vitro* transcription of *Bombyx* and *Xenopus* 5S RNA genes in *Bombyx* or *Xenopus* extracts. The DNA templates were as follows: wild-type (B −92) and partially deleted (B +26) *B. mori* 5S RNA genes; wild-type *X. borealis* (Xb) and *X. laevis* (Xl) somatic-type 5S RNA genes; bacteriophage M13mp8 without a 5S RNA gene insert (M13). The five templates shown on the left were incubated with *Xenopus* oocyte extracts (X); the five templates on the right were incubated with *Bombyx* extracts (B). Transcription products were fractionated on a polyacrylamide gel and detected by autoradiography.

4). Since the sequences upstream of these *Bombyx* and *Xenopus* genes differ (the 40-bp 5′ flanking regions are only 40% homologous; see refs. 2 and 11), lack of an appropriate upstream signal may be sufficient to prevent transcription of *Xenopus* genes by the *Bombyx* polymerase III complex.

Our results indicate that different organisms make use of RNA polymerase III transcription signals that vary in sequence and location. Species-specific transcription signals have also been found in tRNA genes from silkworms (12, 13), *Drosophila* (14, 15), and yeast (16). Within a single organism, however, different RNA polymerase III templates may share common signals. We have shown that the activity of two classes of *Bombyx* polymerase III templates, genes encoding 5S RNA and tRNA, depends on very similar upstream sequences. Likewise, certain parts of *Xenopus* 5S RNA and tRNA gene control regions are interchangeable (17). It is likely that the signals flanking *Bombyx* genes are recognized by a part of the *Bombyx* transcription apparatus that acts on both tRNA and 5S RNA genes. This component could be RNA polymerase III itself, or it could be a transcription factor. We would like to know whether the same *cis*-acting elements control all genes transcribed by RNA polymerase III in *Bombyx*, and whether they act by promoting local changes in DNA secondary structure or by providing double-stranded recognition sites.

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