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Localization of DNA sequences promoting RNA polymerase I activity in *Drosophila*

(*in vitro* transcription/BAL-31 nuclease deletions)

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ABSTRACT **We used BAL-31 nuclease to delete sequences that surround the transcription initiation site of *Drosophila* ribosomal DNA. A series of deletions was used as templates for *in vitro* transcription in a *Drosophila* cell-free system to identify sequences that influence the activity of RNA polymerase I. Sequences that lie upstream of the site of transcription initiation (nucleotide +1) affect ribosomal RNA synthesis. We show that the major promoter of polymerase I involves the sequence −43 to −27 and that the region between nucleotides −18 and +20 contains sequences capable of sustaining a low level of accurate transcription.**

Nontranscribed spacer sequences upstream of the site of rRNA transcription initiation affect RNA polymerase I activity in diverse eukaryotes. Sequences influencing the *in vitro* expression of truncated mouse ribosomal DNA (rDNA) templates lie between 74 and 39 base pairs (bp) upstream of the start of transcription (1). In *Xenopus*, polymerase I regulatory sequences have been mapped to within 145 bp upstream and 16 bp downstream of the initiation site (2). In *Drosophila* rDNA, sequences having a major role in the promotion of transcription lie between 240 and 30 bp upstream of the site of transcription initiation, but only 34 bp upstream and *ca.* 30 bp downstream are sufficient for low levels of accurate *in vitro* RNA synthesis (3). Thus, DNA sequences relevant to the quantity and quality of *Drosophila* rDNA expression may be separable into at least two components.

In this paper we further delimit rDNA sequences that promote or sustain accurate *in vitro* transcription of *Drosophila melanogaster* rDNA. Using BAL-31 nuclease-deleted subclones of rDNA as templates for *in vitro* transcription, we have found that (*i*) the major promoter of polymerase I activity involves the sequence −43 to −27, where the site of transcription initiation is at nucleotide +1, and (*ii*) the region between nucleotides −18 and +20 is sufficient to sustain a low level of accurate transcription.

RESULTS

To locate sequences that are involved in the promotion of polymerase I transcription, we have constructed a series of recombinant plasmids that contain decreasing amounts of nontranscribed spacer (NTS) sequences adjacent to the external transcribed spacer (ETS) and then have evaluated template activity in an extract of *D. melanogaster* Kc cells (4). The cell extract gives specific and accurate transcription of truncated segments of cloned *D. melanogaster* rDNA. When prepared as described, extracts have no appreciable RNA polymerase II or III activity, they are species specific with respect to the source of template, and *in vitro* transcription initiations are essentially limited to copies of the site of *in vivo* transcription initiation. The plasmid pDmr275c2 contains a segment of *D. melanogaster* rDNA that extends from an *Alu* I cleavage site at nucleotide −305 to a *Hae* III site at about +680 (Fig. 1). The plasmid has been described by Kohorn and Rae (3), but the essential information is that the rDNA segment was inserted between the *Hin*dIII and *Bam*HI sites in pBR322 with the ETS *Hae* III site fused to the vector *Bam*HI site, so that the direction of transcription is toward the *Bam*HI site.

pDmr275c2 was treated with *Eco*RI and then BAL-31. *Eco*RI linkers were added, and the mixture was digested with *Bam*HI and *Eco*RI. The deleted rDNA inserts were purified by gel electrophoresis and then ligated to the *Eco*RI and *Bam*HI sites of pBR322. After transformation of *Escherichia coli*, cloned NTS-deletion plasmids were analyzed by restriction digestion of DNA isolated from minilysates, and a series of plasmids having decreasing lengths of nontranscribed spacer was created.

Upon identification by DNA sequence determination of the precise point to which the NTS had been deleted in a plasmid, a sample was linearized for run-off transcription by digestion with *Sal* I. The enzyme cleaves the pBR322 vector once at a site 275 bp downstream of the BamHI site or about 950 bp downstream of the site of transcription initiation. The transcription of 0.4 μg of NTS-deletion clones was evaluated in the *Drosophila* cell-free system (Fig. 2). Each transcription reaction included 0.2 μg of pDmr275c2/*Ava* I as an internal control template to ensure that failure of any deletion plasmid to support *in vitro* transcription was not a peculiarity of a reaction mixture. The *Ava* I cleavage site is about 1,700 bp downstream of the site of transcription initiation.

Fig. 2 shows an autoradiogram of RNA isolated from transcription reactions containing DNA templates that differed in the amount of 5′ flanking NTS. Lane 1 shows RNA transcribed from the nondeleted template Dmr275c2, which has 305 bp of nontranscribed spacer adjacent to the transcribed region. Deletion of sequences to nucleotide −150 or −60 (where initiation is at nucleotide +1) had neither a quantitative nor a qualitative effect on transcription (lanes 2 and 3). Deletion of 17 more bp, to −43 (lane 4), resulted in a slight reduction in the amount of RNA synthesis. However, transcription could not be detected in RNA gels when 5′ sequences down to nucleotide −27 or −18 were absent (lanes 5 and 6, respectively).

Although transcripts from rDNA templates lacking the sequences upstream of nucleotide −18 could not be visualized in gel displays of RNA isolated from a transcription mixture, accurately initiated transcripts were produced at low levels. These transcripts were detected in an S1 nuclease protection experiment, as shown in Fig. 3. Purified coding strand containing 98

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Abbreviations: rDNA, ribosomal DNA; NTS, nontranscribed spacer; ETS, external transcribed spacer; bp, base pairs.

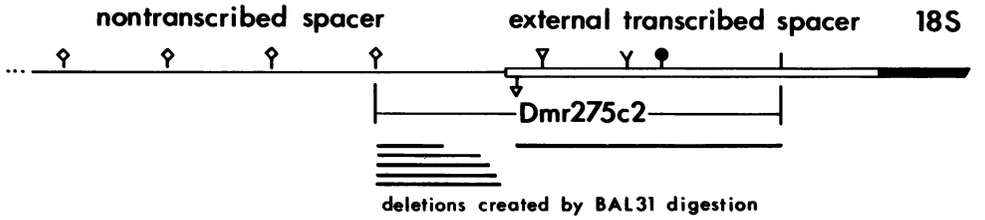

FIG. 1. A map of the region of *D. melanogaster* rDNA that includes the site of transcription initiation. Dmr275c2 was inserted between the *Bam*HI and *Hin*dIII sites of pBR322. Bold lines indicate regions that were removed by using BAL-31. Plasmid (20 μg) was digested with either *Eco*RI or *Bam*HI, and fragments were precipitated with ethanol and resuspended in 98 μl of water; 100 μl of 24 mM $CaCl_2$/24 mM $MgCl_2$/0.4 M NaCl/40 mM Tris·HCl, pH 8/2 mM Na_2 EDTA was added along with 5 units of BAL-31 (New England BioLabs). The reaction was incubated at 25°C; every 5 min 20 μl was removed, combined with 0.1 vol of 0.2 M EGTA, and then kept at 4°C. Fractions were pooled, the mixture was extracted with phenol, and DNA was precipitated with ethanol. Samples were treated with the Klenow fragment of *E. coli* DNA polymerase and four unlabeled nucleoside triphosphates to ensure that molecules had flush ends. The deleted rDNA segments were cloned through pBR322 as described in the text. ⇧, *Alu* I; ⇩, *Dde* I; ⊥, *Hae* III; ⏊, *Hha* I; ⇓, *Hin*fI; ⇩, *Taq* I.

bp of ETS and 305 bp of NTS (the *Alu* I/*Dde* I fragment indicated in Fig. 3) was hybridized (liquid hybridization) to labeled RNA synthesized in a transcription mixture containing a template having only 18 bp of NTS flanking the ETS. The hybridization mixture was treated with S1 nuclease, then denatured, and electrophoresed along with the sequence ladder of the *Alu* I/*Dde* I fragment, 5′-labeled at the *Dde* I site. Lane 2 of Fig. 3 shows protected RNA transcribed from the plasmid with an NTS truncated to nucleotide −18. It comigrated with RNA that was transcribed from the nondeleted template pDmr275c2 and subjected to the same hybridization and S1 nuclease treatment (Fig. 3, lane 1). The site of *in vivo* transcription initiation on *D. melanogaster* rDNA has been identified by Long *et al.* (7) as the first T in the sequence 3′ T-C-C-A-T-C-C-G 5′ of the coding strand, and we have shown that RNA synthesized *in vitro* from a template that is the equivalent of Dmr275c2 initiates at the same point (4). Thus, although the run-off transcripts of a template having only 18 bp of NTS do not accumulate to an extent that is readily detectable, there are nevertheless low levels of accurate transcription initiation.

To establish a 3′ boundary of the polymerase I promoter, we used BAL-31 to delete ETS sequences progressively from the *Bam*HI site in the plasmid Dmr275c2 (Fig. 1). The mixture of BAL-31-treated plasmids was then digested with *Eco*RI, and the deleted ETS sequences were purified by gel electrophoresis. The fragments were ligated to pBR322 that had been treated with *Eco*RI and *Bam*HI, BAL-31 and *Bam*HI ends of molecules were filled in by using the Klenow fragment of *E. coli* DNA polymerase, and then circularization was accomplished by blunt-end ligation. Clones selected from an examination of transformant minilysates were subjected to DNA sequence analysis and were linearized by digestion with *Sal* I for use as templates for run-off transcription. Templates that contain only 20 bp of ETS (and 305 bp of NTS) were found to support readily detectable levels of accurate RNA synthesis (Fig. 2, lane 7).

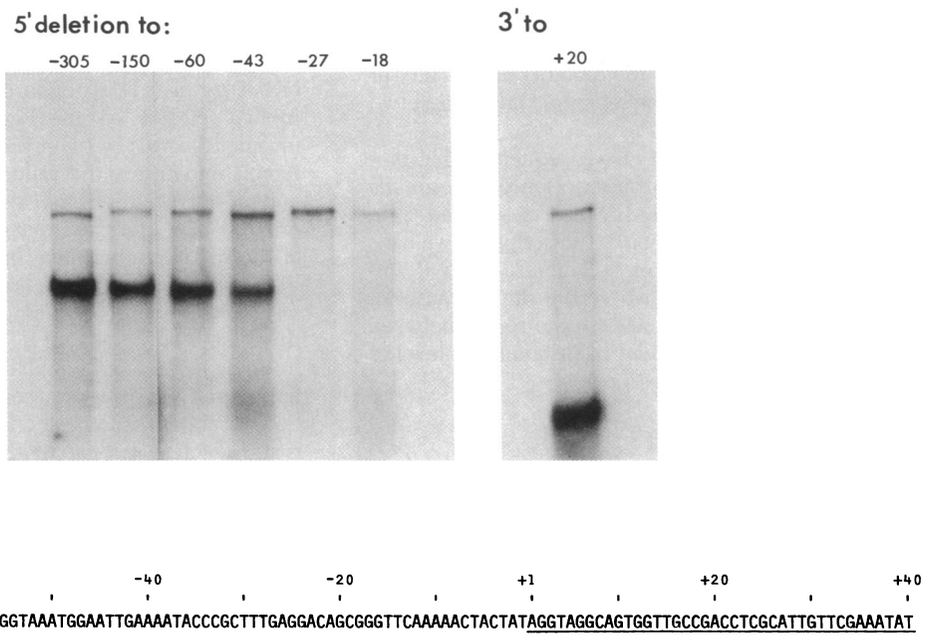

FIG. 2. Transcription of BAL-31-deleted rDNA templates. Conditions for *in vitro* transcription were as described (4). Each lane displays RNA isolated from a reaction that contained 0.4 μg of BAL-31-deleted template linearized by *Sal* I digestion. Numbers above each lane indicate the extent of the deletion. Each reaction also included 0.2 μg of pDmr275c2/*Ava* I to produce a larger RNA as an internal control (see the text). Care was taken to ensure that the absolute amounts of deleted and control templates were constant for all reactions. The transcripts were subjected to electrophoresis in 2% agarose/formaldehyde gels (5) and examined by autoradiography. Below the autoradiogram is the DNA sequence of the noncoding strand of Dmr275c2, extending 80 bp upstream and 40 bp downstream of initiation. Bold underlines indicate lengths found to contain sequences important for polymerase I transcription.

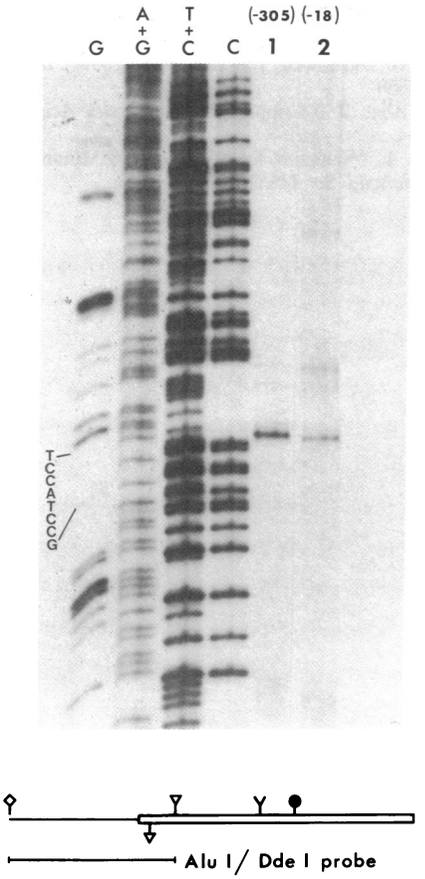

FIG. 3. Detection of accurate but low levels of *in vitro* transcription. A *Sal* I digest of pDmr275c2 and of a plasmid having a 5′ deletion to nucleotide −18 served as templates for *in vitro* transcription. (*Lower*) The ^{32}P-labeled RNA was purified and then hybridized with the isolated coding strand of an *Alu* I/*Dde* I segment that spans the site of *in vivo* initiation as indicated. (*Upper*) The hybrids were then treated with S1 nuclease and denatured. Hybridization and S1 nuclease digestion conditions have been described (4). Protected labeled RNA produced from pDmr275c2 (lane 1) and the plasmid with the 5′ deletion to −18 (lane 2) were run in an 8% polyacrylamide/urea gel (6). The length of the protected RNAs was compared with the sequence ladder of the *Alu* I/*Dde* I segment 5′-end-labeled at the *Dde* I site (6). S1 nuclease-resistant RNA from both transcription reactions migrates along with the length of DNA from the *Dde* I site to that of *in vivo* initiation, the first T in the sequence 3′ T-C-C-A-T-C-C-G 5′ (4, 7).

DISCUSSION

Evidence has been given that sequences upstream of the site of polymerase I transcription initiation influence the expression of *Drosophila* rDNA (3). It is now possible to distinguish two regions that influence rRNA synthesis in the *Drosophila* cell-free system. Data derived from transcription of BAL-31-deleted rDNA templates are summarized in the lower part of Fig. 2.

Deletion of sequences upstream of nucleotide −43 has a small or no effect on transcription as it compares with that from a template that is intact to −305. However, a very large reduction in template activity results from deletion of the next 16 bp, so that sequence between nucleotides −43 and −27 must make a major contribution to the promotion of polymerase I transcription.

Whereas the region upstream of nucleotide −27 is necessary for *in vitro* RNA synthesis that is readily evident in gel electropherograms, templates that contain as few as 18 bp of NTS support low levels of accurate transcription that are detectable with the highly sensitive S1 nuclease assay. Because templates from which all but 20 bp of ETS have been deleted support substantial accurate run-off transcription, the region −18 to +20 contains information that directs the polymerase to initiate accurately, albeit relatively infrequently. Thus, the region −18 to +20 may be considered to contain a polymerase phasing signal.

DNA sequences that influence the activity of RNA polymerase I have also been located with degrees of precision in *Mus musculus* and *Xenopus laevis*. Using cloned segments of mouse rDNA truncated to various extents by digestion with restriction enzymes, together with a homologous *in vitro* transcription system, Grummt (1) found that removal of all but 74 bp of the NTS did not affect the detectability of run-off transcripts in an RNA gel, but that the digestion of plasmid with an enzyme that cleaves between nucleotides −40 and −39 resulted in no observable transcript. No attempt to detect transcripts at higher sensitivity was reported. By injecting into *Xenopus borealis* oocytes various clones of *X. laevis* rDNA resectioned by restriction enzyme digestion and using an S1 nuclease assay for transcription, Moss (2) was able to infer that DNA sequences essential for polymerase I activity lie between nucleotides −145 and +16. The deletion of certain sequences within this span resulted in no detectable transcription, but it is not known whether the deletions *per se* or the consequent rearrangements of remaining rDNA sequences were responsible for reductions in template activity.

The expression of rDNA in cell-free extracts is species specific in both *Drosophila* and mammalian systems (4, 8), although this is evidently not so for transcription in *Xenopus* germinal vesicles (2, 9, 10). DNA sequences that surround sites of the initiation of rRNA precursor synthesis and almost certainly include regulatory elements have been reported for a wide variety of eukaryotes, from yeast (11, 12) and *Tetrahymena* (13) through *Drosophila* (7) and *Xenopus* species (14) to mouse, rat, and human (e.g., see ref. 15). The most striking outcome of comparisons that have been made among these spatially homologous regions of rDNA is that there are few conserved sequences among even closely related organisms and virtually none between highly diverged eukaryotes. In particular, no consensus sequence can be drawn for elements regulating polymerase I activity as there can be for the so-called "CAAT" and "TAATA" boxes that are upstream of genes transcribed by RNA polymerase II in diverse eukaryotes.

Indeed, the best that can be done to date in relating polymerase I transcription signals among unrelated eukaryotes is to note that Grummt's (1) localization of an important sequence between nucleotides −74 and −39 in mouse rDNA overlaps our identification of an important sequence between −43 and −27 in *Drosophila* rDNA. However, the two organisms have no sequence homology in this region.

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