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Nontranscribed spacer sequences promote *in vitro* transcription of *Drosophila* ribosomal DNA

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ABSTRACT

Tandem repeats of ribosomal RNA transcription units in Drosophila melanogaster are separated by a nontranscribed spacer that is comprised in part of serial repeats of a 0.24 kb sequence. DNA sequence analysis shows that such repeats are imperfect copies of a region that includes the site of in vivo rRNA transcription initiation (ca. -240 to +30). Subclones of the rDNA spacer that are copies of the sequence extending from -34 through the initiation site support detectable in vitro transcription in a mixture involving a Drosophila cell-free extract, but accurate in vitro transcription is considerably enhanced when a nontranscribed spacer template includes a copy of the sequence extending upstream of -34. From a comparison of the sequences and transcription template-effectiveness of various rDNA subclones, we infer that a major promoter of RNA polymerase I activity lies between -150 and -30 in the rDNA nontranscribed spacer. The nontranscribed spacer copies of the initiation region are less effective templates for transcription than is the region of in vivo initiation, and there are differences between spacer repeats and the authentic sequence downstream of -240 that may account for this.

INTRODUCTION

Sequences that promote RNA polymerase I transcription of ribosomal DNA have yet to be identified, but it is evident from in vitro transcription of truncated clones of mouse (1) and Drosophila (2) rDNA, and from studies of the transcription of cloned Xenopus rDNA injected into germinal vesicles (3), that information essential for the initiation of rRNA synthesis is present within the region ca. -200 to +100, where transcription begins at +1. The nontranscribed spacers between tandemly repeated copies of the rRNA transcription unit in D. melanogaster are comprised in part of serial repeats of a 0.24 kb sequence defined by Alu I cleavage sites (4). We have found that nontranscribed spacer repeats are imperfect copies of the sequence between about -240 and +30, and show that cloned Alu I segments are templates for in vitro transcription. In addition, we demonstrate that sequences upstream of -30 have a positive influence on transcription.

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MATERIALS AND METHODS

The phage λ(Sep6)Dmr 275 (ref. 5) was provided by M.L. Rebbert and I.B. Dawid. The Hind III segment that contains the entire NTS and ETS was inserted by V.L. Murtif into pBR 313, and the subclone was designated pDmr 275c. The Hind III/Hae III portion of the insert, containing the NTS and the proximal 0.7 kb of the ETS, was recovered and digested with Alu I to produce segments of 1.8 kb (the distal portion of the NTS), 1.0 kb (containing 0.3 kb of the NTS plus 0.7 kb of the ETS), and 0.24 kb (the several Alu I repeats of the NTS). Each segment was blunt-end ligated to pBR 322 that had been digested with Hind III + Bam HI then treated with the Klenow fragment of DNA polymerase plus dNTPs to fill in the ends. Such ligation restores the plasmid Bam HI site, but destroys the Hind III site and the Alu I and Hae III termini of the inserts; there is, however, an Eco RI site in the vector that is 29 base pairs away from the former Hind III site (6). A clone of the 1.0 kb Alu I/Hae III segment was designated pDmr 275c2, and a plasmid containing one of the 0.24 kb Alu I repeats was named pDmr 275c31; pDmr 275c313 contains a head-to-tail dimer of 0.24 kb Alu I segments that was probably generated during ligation (rather than being an authentic dimer resulting from partial Alu I digestion of the Hind III/Hae III segment of pDmr 275c).

DNA sequencing was accomplished by the procedure of Maxam and Gilbert (7) using DNA segments that were 5' end-labelled with polynucleotide kinase and γ^{32}P-ATP at Eco RI or Bam HI sites that flank the rDNA inserts in plasmids. Cell extracts, transcription reactions, and gel electrophoresis were as previously described (2).

RESULTS

We have shown that an extract of D. melanogaster Kc cells effects faithful transcription of truncated segments of homologous rDNA that contain the site of in vivo initiation (2). Unique transcripts of appropriate length were generated when the added template had been digested with Alu I to give 0.3 kb of nontranscribed spacer (NTS) upstream of the in vivo initiation site, and truncated downstream by a restriction enzyme that gives 0.3 kb or more of external transcribed spacer (ETS). The Alu I site 0.3 kb upstream of initiation is one of several in the NTS that define a repeated sequence of 0.24 kb (4) (Fig. 1). We observed that if the in vitro transcription template was only partially digested by Alu I, multiple lengths of transcript that increased in steps of about 0.25 kb were produced, and suggested that the Alu I repeats contain sequences capable of initiating polymerase I transcription (2).

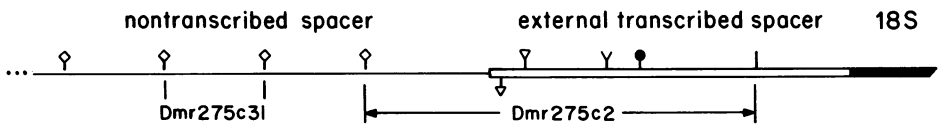

Fig. 1. Map of the external transcribed spacer and a portion of the nontranscribed spacer of D. melanogaster rDNA. Symbols for restriction enzyme cleavage sites are: ⚲ - Alu I; ⚳ - Dde I; ⊥ - Hae III; ⚹ - Hinf I; ⚲ - Hha I; ⚳ - Taq I. Also indicated are segments that were subcloned from λDmr 275 (ref. 5): a clone of the 1 kb Alu I/Hae III segment was designated pDmr 275c2, and one containing a copy of the 0.24 kb Alu I repeats was named pDmr 275c31; pDmr 275c313 (not shown) contains a dimer of the Alu I repeats.

To examine this further, we subcloned Alu I repeats, including a tandem dimer of the 0.24 kb unit, as well as the 1 kb Alu I/Hae III segment that contains the site of in vivo initiation (Fig. 1). We sequenced the 0.24 kb Alu I insert of pDmr 275c31, the entire dimer of the Alu I repeat in the clone pDmr 275c313, and the upstream region of the Alu I/Hae III segment (in pDmr 275c2) that had not been reached by Long et al. (5) in their description of the site of in vivo initiation. We also evaluated the template effectiveness of each of these subclones in the Kc cell extract.

Figure 2 is a comparison of the sequences of Dmr 275c2 and Dmr 275c31/ Dmr 275c313. The portion of Dmr 275c2 that is transcribed in vivo (5) and in vitro (2) is doubly underlined; mismatches between Dmr 275c2 and the Alu I repeats in Dmr 275c31 and Dmr 275c313 are singly underlined beneath the c31/ c313 sequence, and gaps are indicated by dashes in one or the other sequence. The Alu I repeats contain essentially perfect copies of the sequence from -30 to +30. They also contain copies of the sequence extending from -305 (the Alu I site of c2) to -151, and sequences that are largely related to the region between -114 and -30. The homologies are diagrammed in Figure 3, which shows that the nontranscribed spacer repeats in D. melanogaster rDNA are derived from the sequence between about -240 and +30, and extend leftward from about -240. Differences between the NTS repeats and the authentic NTS/ETS junction are due to both substitutions and deletions/insertions.

The results of in vitro transcription using pDmr 275c2, pDmr 275c31 and pDmr 275c313 as templates are shown in Figure 4. The orientation of insert in all three of the plasmids is such that the Sal I site in the pBR 322 vector is 275 base pairs (6) downstream of the 3' end of the insert with respect to the direction of transcription, so plasmids were linearized with Sal I to provide truncated templates for run-off transcription. In pDmr 275c2, the distance from the initiation site to the Sal I site is about 975 base pairs;

Fig. 2. A comparison of the sequences of inserts in pDmr 275c2, pDmr 275c31 and pDmr 275c313. The two Alu I repeats in Dmr 275c313 and the single Alu I segment in Dmr 275c31 are identical, and a dimer is shown in the figure. The limits of Dmr 275c31 are indicated by ▲. The numbering in the figure refers to the Dmr 275c2 sequence, and +1, which is the site of *in vivo* and *in vitro* transcription initiation, was first identified by Long *et al.* (5). Underlines and dashes are explained in the text. The ● at -272 indicates the position of NTS copies of the site of transcription initiation, 34 base pairs downstream of Alu I sites.

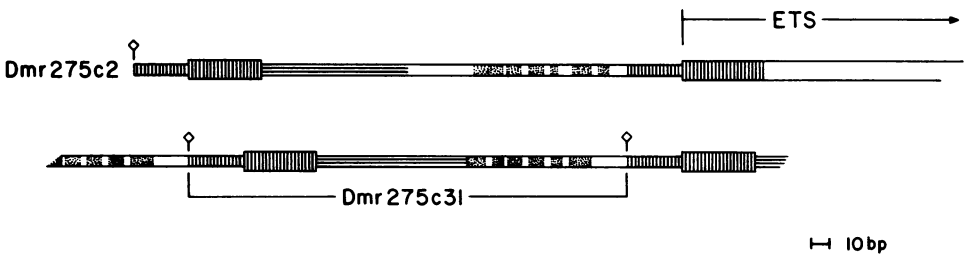

Fig. 3. Relatedness of the Alu I nontranscribed spacer segment in pDmr 275c31 and the Alu I/Hae III nontranscribed/external transcribed spacer segment in pDmr 275c2. Extensive homologies are indicated by horizontal or vertical hatching, and stippled regions are less well matched between the clones. Clear areas have little or no homology, or are unique to one or the other clone.

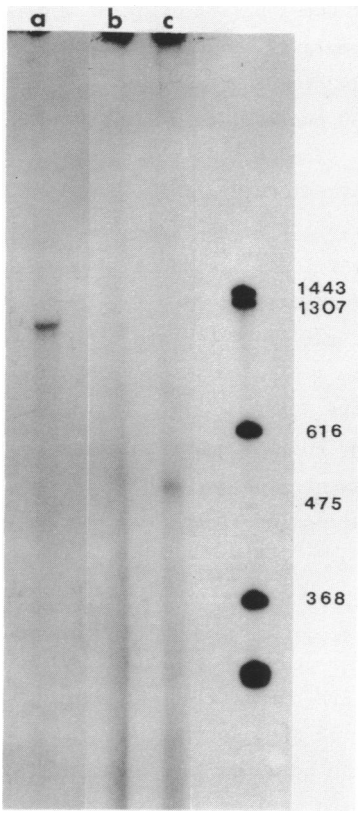

Fig. 4. Template activity of various rDNA subclones for in vitro RNA polymerase I transcription. The plasmids pDmr 275c2 (lane a), pDmr 275c31 (lane b) and pDmr 275c313 (lane c) were treated with Sal I and added to aliquots of an extract of D. melanogaster Kc cells plus other components of an in vitro transcription mixture that has been described (2). The effectiveness of a reaction mixture is sensitive to the concentration of added DNA, and this was optimized for each plasmid preparation. Transcription was evaluated by acrylamide gel electrophoresis of RNA labelled by the incorporation of ^{32}P-GMP. Gels were run under denaturing conditions, and the marker in lane d is denatured Taq I segments of pBR 322 that had been end-labelled; segment sizes are given in base pairs (6). The display of transcripts of the various rDNA subclones is discussed in the text. The invisibility of discrete RNA in lane b is not due to degradation nor an inhibitor in the template preparation.

transcripts of this plasmid were rather homogeneous and of the expected size (Fig. 4a). In pDmr 275c31 and pDmr 275c313, the distance from the copy, or the nearest copy, of the initiation site to the Sal I site is 480 base pairs. With pDmr 275c31 as a template, *in vitro* transcription produced RNA that could not be evaluated in acrylamide gel displays of transcript sizes (Fig. 4b). However, discrete transcription products of about the expected size were obtained when the dimer of the 0.24 kb Alu I NTS repeat in pDmr 275c313 served as template (Fig. 4c).

While transcripts of pDmr 275c31 could not be discerned in gels such as that in Figure 4, there are nevertheless accurate initiations. RNA transcribed from truncated pDmr 275c31 was annealed with purified coding strand from an Eco RI/Bam HI digest of the plasmid, and the mixture was treated with nuclease S_1. Protected ^{32}P-RNA was run in a denaturing acrylamide gel along with the sequence ladder of Dmr 275c31 that was 5' end-labelled at the Bam HI site (5' end-labelling the coding strand of the template). Figure 5 shows that protected RNA comigrates with DNA that extends from the Bam HI site, through the sequence that is a copy of the first 30 nucleotides of *in vivo* transcription, to the nucleotides that include the site of *in vivo* initiation (initiation begins at the first T in the sequence TCCATCCG; ref. 5).

A major difference between Dmr 275c31 and Dmr 275c2 is that sequences between -240 and -30, while largely present in Dmr 275c31, are *downstream* of the copy of the initiation site in this clone (Figs. 2 and 3). This disorientation is evidently partly responsible for the poor template activity of Dmr 275c31 (Fig. 4b), as dimerization of the Alu I repeat, in pDmr 275c313, restores upstream sequences and improves the quality of transcripts (Fig. 4c). In fact, there is a second copy of the sequence -30 to +30 in Dmr 275c2 (at -301 to -241) that is homologous with the initiation region in Dmr 275c31, but from which there is no detected *in vitro* transcription (there is but the single *ca*. 975 nucleotide band in Fig. 4a).

DISCUSSION

We have shown that in *D. melanogaster*, tandem repeats of the Alu I NTS segment contain sites of transcription initiation and 5' flanking sequences that influence *in vitro* transcription. In species of *Xenopus*, the rDNA NTS contains complex repetitive regions, among which can be a few copies of the region of the 5' end of the ETS (8-10). These copies may serve as sites of infrequent transcription initiation, as short "prelude" spacer transcripts have been observed occasionally by electron microscopy, and they correspond

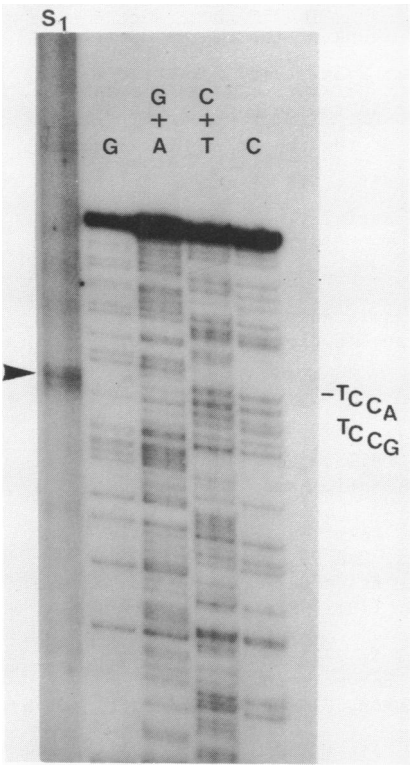

Fig. 5. Accuracy of *in vitro* transcription initiations on the plasmid pDmr 275c31, containing a single 0.24 kb Alu I segment of the nontranscribed spacer. The fidelity of *in vitro* transcription could not be estimated from Fig. 4b, so it was evaluated in a nuclease S_1 protection assay. The rDNA segment in pDmr 275c31 was separated from most of the vector by digestion of the plasmid with Bam HI and Eco RI. The fragment was strand-separated and the putative coding strand was isolated. The DNA was annealed with ^{32}P-RNA synthesized *in vitro* from pDmr 275c31, and the product was digested with nuclease S_1. RNA that was protected from digestion by hybridization with DNA was sized in a denaturing gel by comparison with a sequence ladder of sense strand DNA that had been 5' end-labelled at the same site in the transcribed region (Bam HI) that served as one end of the fragment used for protection. In this way, the 5' end of *in vitro* transcripts could be mapped to within a few nucleotides of the site in Dmr 275c31 that is a copy of authentic transcription initiation (the first T in the sequence TCCATCCG; ref. 5).

in position to the NTS copies of the initiation region (11, 12). From the composition of *X. laevis* rDNA subclones that have been observed to act as transcription templates upon injection into germinal vesicles, a promoter of RNA polymerase I activity must lie between -320 and +113 (3). If prelude sequence transcripts indeed involve the NTS copies of the authentic initiation region, then the context of a promoter is -145 to +4 (9). *In vitro* transcription of mouse rDNA subclones has been accomplished (1, 13), and efficient transcription is obtained with a segment truncated to include the sequence -170 to +150 (ref. 1).

Using subclones of *D. melanogaster* rDNA, we have found that the NTS contains several repeats of the region that includes the beginning of the ETS, and that these copies can serve as templates for *in vitro* transcription. Templates that contain the reduplicated region of the ETS and only 30 base pairs of 5' flanking NTS do support accurate initiations (in pDmr 275c31), but substantial *in vitro* transcription requires the presence of additional upstream sequences that lie between -240 and -30 (in pDmr 275c2 and pDmr 275c313). NTS

repeats have not been observed to be templates for *in vivo* transcription (14, 15), and the NTS repeats and the immediate 5' flank of the ETS do differ in sequence. With respect to the numbering in Dmr 275c2 (Fig. 2), these diffrences lie between -150 and -30; the greatest is the absence of the sequence -150 to -115 in Dmr 275c31 and in both copies of the repeat in Dmr 275c313. We are currently investigating the role of specific NTS sequences in the promotion of *D. melanogaster* RNA polymerase I activity.

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