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Multiple repeated units in *Drosophila melanogaster* ribosomal DNA spacer stimulate rRNA precursor transcription

(RNA polymerase I/promoter duplications/transient gene expression)

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ABSTRACT **_Drosophila melanogaster_ ribosomal DNA (rDNA) transcriptional units are separated by nontranscribed spacer (NTS) segments consisting of tandemly arranged repeats 95, 330, and 240 base pairs long. NTS sequences stimulate transcription from the rRNA precursor (pre-rRNA) promoter. Primer extension analysis of RNA from cells cotransfected with plasmids carrying NTS sequences of various lengths shows that the activity of the pre-rRNA promoter is directly correlated with the number of 240-base-pair repeats; NTS sequences upstream of these units also stimulate pre-rRNA transcription. The NTS effect might depend upon transcription from duplicated promoters present within the 240- and 330-base-pair repeats. The strength of the pre-RNA promoter correlates in each construct with the level of spacer transcription. The action of spacer sequences, although able to take place over a large distance, is not independent of orientation: stimulation of pre-rRNA transcription is abolished in plasmids carrying inverted NTS segments. Removal of a putative transcription termination site located upstream of the pre-rRNA promoter has no effect on pre-rRNA initiation nor does it substantially alter spacer enhancement.**

In eukaryotic cells nearly half of all transcription is contributed by rRNA synthesis. Ribosomal DNA genes (rDNA) are repeated 100–1000 times per haploid genome, clustered in tandem arrays at one or a few chromosomal sites (1, 2). Species-specific factors are required, in addition to RNA polymerase I, for accurate promoter recognition (3). DNA sequences sufficient for faithful transcription initiation are generally contained within 50–150 base pairs (bp) upstream of the rRNA precursor start site (pre-rRNA promoters; refs. 4 and 5). Remote control elements located in the so-called nontranscribed spacers (NTSs), the intergenic segments that separate adjacent transcriptional units, affect the rate of pre-rRNA transcription in several species. Sequences >2 kilobases upstream of the pre-rRNA start site positively regulate the expression of *Saccharomyces cerevisiae* (6, 7) and rat (8) rDNA. These elements are reminiscent of polymerase II gene enhancers, as they act in either orientation and at variable distance from the promoter region. In *Xenopus*, arrays of enhancer-like sequences (60/81-bp elements) are interspersed within the NTS with copies of the pre-rRNA promoter (9–12). While the 60/81-bp elements alone are able to stimulate the activity of the pre-rRNA promoter (10–12), transcription from the duplicated promoters is required for full spacer enhancement (13–14).

Multiple copies of the promoter region are found in the NTSs of *Drosophila melanogaster* (15–19), *Drosophila virilis* (20, 21), *Drosophila orena*, and *Drosophila hydeii* (21) rDNA. We have shown (22) that transcription from the *D. melanogaster* pre-rRNA promoter is influenced by spacer sequences upstream of position −180 *in vivo*. Here we report the results of a more systematic analysis of the relationship between spacer structure and promotion of polymerase I transcription in the fruit fly.

MATERIALS AND METHODS

rDNA Clones. pDmNTS was obtained by inserting a *Taq* I fragment that includes the NTS and 34 bp of external transcribed spacer (ETS) from pDmrY22 (23) into the *Acc* I site of pEMBL8CAT (24). *Eco*RI and *Sma* I sites are at the 5′ end of the insert; *Pst* I and *Hin*dIII sites are at the 3′ end between ETS and vector sequences. pDmA8, pDmA5, pDmA3, pDmA2, and pDm-306 were obtained by ligating a pEMBL8CAT digested with *Sma* I and *Hin*dIII to a partial *Alu* I digest of an *Eco*RI–*Hin*dIII fragment from pDmNTS. pDm-360 was constructed by inserting a *Dde* I–*Taq* I (position −360 to position +34) fragment from pDmNTS into pEMBL8CAT digested with *Sma* I and *Acc* I. pDm-180 was obtained by the insertion of a *Nde* I–*Hin*dIII fragment from pDm-360 into pEMBL8CAT digested with *Sma* I and *Hin*dIII. pDm-72 was constructed from a derivative of pDmra51 (23) in which an *Eco*RI site is located upstream of residue −72 in the NTS. A segment extending from this site to the *Taq* I site at residue +34 in the ETS was inserted between the *Eco*RI and *Acc* I sites of pEMBL8CAT. pDmΔ plasmids were derived from the homologous clones digested at the *Pst* I and *Hin*dIII sites and religated. This procedure generates an 8-bp deletion (see Fig. 2*C*). pDm-180Δ carries a *Nde* I–*Nco* I fragment from pDMrCAT1 (22) that extends from position −180 within the NTS to the *Nco* I site within the chloramphenicol acetyltransferase (CAT) gene, inserted between the pEMBL8CAT *Sma* I and *Nco* I sites. pDmN1, pDmN4, and pDmN7 were obtained by ligation of a partial *Nhe* I digest of pDmA8 digested with *Pst* I. pDmRI-II/-306 was obtained upon ligation of pDmNTS cleaved with *Nhe* I. pDmN7/72 was derived by inserting a *Eco*RI–*Hin*dIII fragment from pDm-72 into the *Hin*dIII site of pDmN7. The cloning procedure introduces 4 bp into the transcribed region of this construct. pDmN7inv/-180 derives from a triple ligation event where two *Eco*RI–*Hin*dIII fragments from pDmN7 and pDm-180, including in a 5′→3′ direction the NTS region III and the interval from position −180 to position +34 of the rDNA unit, are inserted into the *Hin*dIII site of pEMBL8-CAT. pDmRI-IIinv/-306 was derived upon ligation of pDmRI-II/-306 digested with *Sma* I and *Nhe* I. Incompatible termini were made blunt-ended by T4 polymerase prior to ligation.

DNA Transfections and RNA Analysis. *D. melanogaster* Schneider II cells, seeded at a density of 1–2 × 10^6 cells per ml, were transfected as reported (25). Forty-eight hours later,

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Abbreviations: ETS, external transcribed spacer; NTS, nontranscribed spacer; CAT, chloramphenicol acetyltransferase; neo, neomycin; rDNA, ribosomal DNA.

cells were harvested and total RNA was purified as described (22). S1 nuclease analyses were carried out as reported (25). Uniformly ^{32}P-labeled probes were prepared as described (24), by using Mp19rCAT or Mp19RSVneo single-stranded DNA as templates. Mp19rCAT contains a 0.66-kilobase *Eco*RI fragment from pDm-360 that extends from position −360 within the NTS to the *Eco*RI site within the CAT gene (26). Mp19RSVneo contains a 2.2-kilobase *Bgl* II–*Pst* I fragment that includes the Rous sarcoma virus (RSV) promoter and part of the neomycin (neo) gene (26). Primer extension analysis was carried out by hybridizing 20 μg of total RNA to the CAT primer described in Fig. 2. Samples were processed as reported (27). Reaction products were resolved on 6% polyacrylamide/8 M urea gels.

RESULTS

Construction and Assay of rDNA Templates Altered in the NTS. The *D. melanogaster* rDNA NTS consists of three repetitive regions, each comprised of tandemly arranged homologous repeats assembled from a few basic sequence motifs (ref. 19 and Fig. 1). The 330- and 240-bp repeats in regions II and III include segments homologous to the pre-rRNA promoter region (refs. 16–19 and Fig. 1).

The *Drosophila* NTS influences the rate of expression of the pre-rRNA promoter *in vivo* (22). To further investigate this phenomenon, rDNA templates altered in their spacer content (Fig. 1*A*) were transfected into Schneider II cells with rDNA constructs (pDmΔ clones, Figs. 1*A* and 2*C*), which direct the synthesis of homologous but distinguishable transcripts to provide an internal reference. The influence of NTS sequences is magnified in competition experiments (ref. 22 and Fig. 2*A*). The ratio of transcripts from pDmNTS and pDm-180Δ, two constructs that substantially differ in their spacer complement (Fig. 1*A*), increases as a nearly linear function of the amount of contransfected DNAs (Fig. 2*A*). With 1 μg of DNA, transcripts initiated from pDmNTS are 20–30 times more abundant than those from pDm-180Δ (Fig. 2*A*); this ratio, as well as the extent of expression, does not vary linearly as the amount of transfected DNA is increased to 10 μg (data not shown; see Fig. 2*B*). The level of hybrid rRNA–CAT transcripts originating from the two plasmids is not affected by differences in their primary sequences (Fig. 2*C*), as a similar ratio is obtained with the reciprocal pair pDmNTSΔ and pDm-180 (Fig. 2*B*). The difference in the transcriptional efficiency of these constructs increases at higher DNA inputs probably because competition for limiting transcriptional components results in the preferential utilization of pre-rRNA promoters preceded by a complete NTS.

Deletion of NTS regions I and II does not abolish this selective advantage (Fig. 3*A*). Progressive removal of 240-bp repeats from region III concomitantly reduces the competitive strength of the adjacent pre-rRNA promoter, regardless of the reference template (Fig. 3). A nearly linear correlation between the length of region III and the efficiency of the pre-rRNA promoter emerges from the relative transcription ratios (Fig. 3*D*). It seems, therefore, that there is no threshold spacer length required to activate the ETS promoter, but rather that individual repeats contribute similarly to increase pre-rRNA transcription.

The results shown in Fig. 3 indicate that much of the effect of the NTS is due to sequences in region III. However, analysis of pDmRI-II/-306, a clone where the left-hand portion of the spacer is attached to residue −306 (Fig. 1), reveals

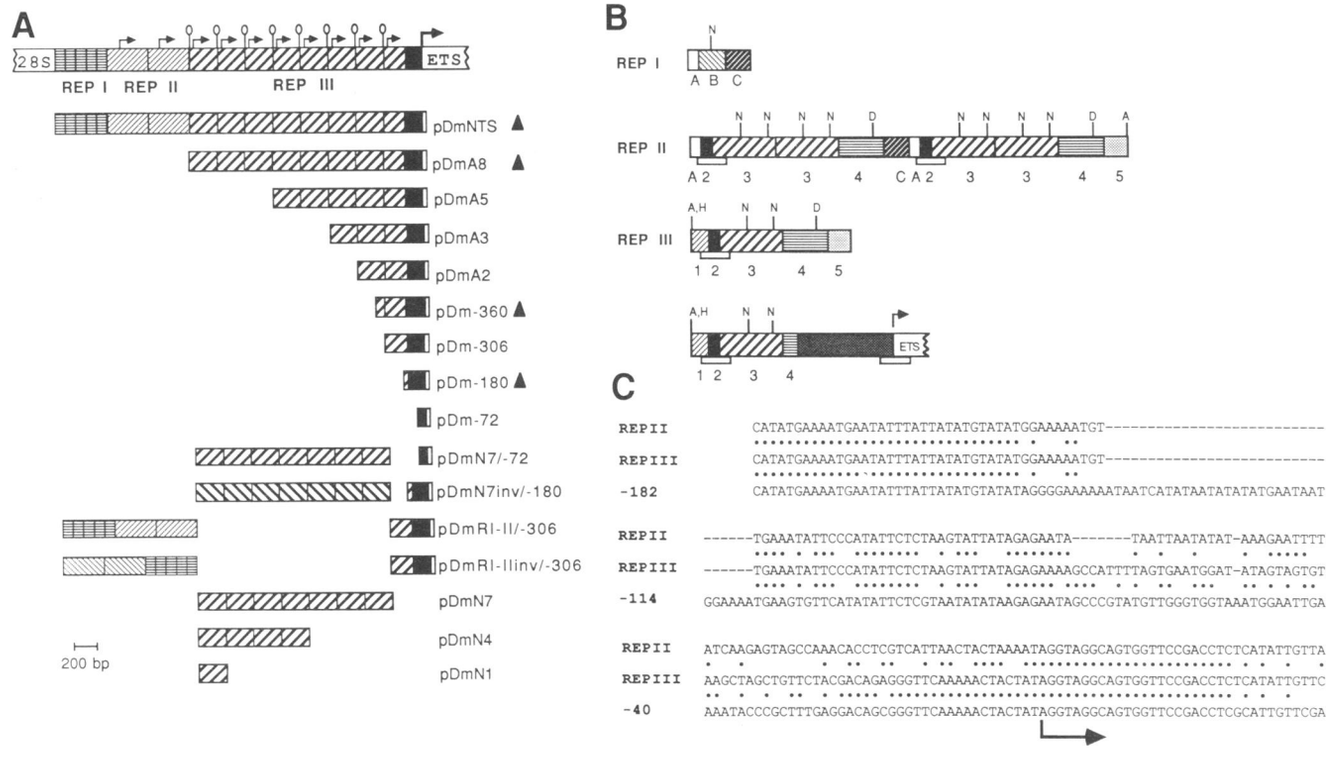

FIG. 1. (*A*) NTS regions of the *D. melanogaster* clone pDmrY22 (23) and of all the clones utilized in this study are shown. Regions I, II, and III are comprised of repeats of 95 bp, 330 bp, and 240 bp, respectively. Arrows, NTS and pre-rRNA promoters; open circles, overlapping *Alu* I and *Nhe* I restriction sites; open boxes, ETS sequences, when present, extend to position +34; solid triangles, constructs from which reference templates (pDmΔ clones) were derived. (*B*) Structure of NTS repeats. In pDmrY22, NTS region I contains five 95-bp repeats. The two 330-bp repeats contained in region II are both shown as they have different termini (19). Region III contains seven 240-bp repeats; a truncated repeat precedes the pre-rRNA promoter region. Sequence motifs (A, B, C, 1, 2, 3, 4, 5) are as in Simeone *et al.* (19). Arrow, pre-rRNA start site; open bars, promoter duplications; letters above repeats, restriction sites (A, *Alu* I; D, *Dde* I; H, *Nhe* I; N, *Nde* I). (*C*) Homology between the pre-rRNA promoter and NTS sequences. Dots indicate identical nucleotides. Residue −182 is within sequence motif 3. The DNA sequences shown are from ref. 19.

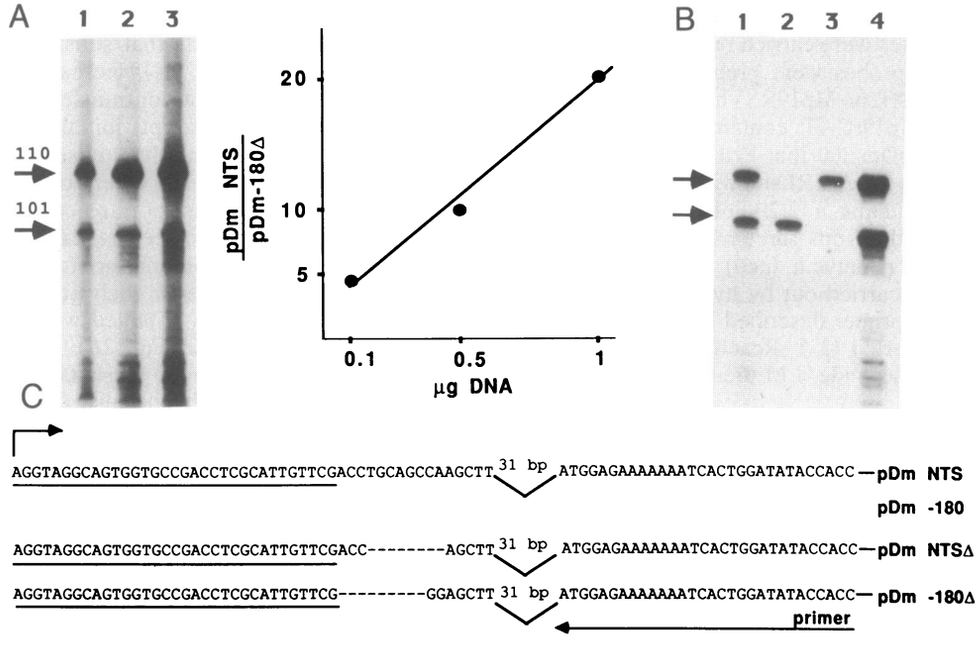

FIG. 2. (*A*) Primer extension analysis of RNA from Schneider II cells transfected with 0.1 (lane 1), 0.5 (lane 2), and 1 (lane 3) μg of both pDmNTS and pDm-180Δ. Arrows indicate pDmNTS and pDm-180Δ transcripts and length is in bases. Carrier DNA was added to the transfection mixtures to a concentration of 20 μg. Ratio of transcription signals at various DNA inputs is shown. (*B*) Primer extension analysis of RNA from cells cotransfected with 10 μg of each of the following clones: pDmNTS and pDmNTSΔ (lane 1), pDm-180 and pDmNTSΔ (lane 2), pDmNTS and pDm-180Δ (lane 3), or pDm-180 and pDm-180Δ (lane 4). (*C*) Boundaries of rDNA and vector sequences in pDm plasmids. Underlined sequence, ETS residues; arrow, pre-rRNA start site. The CAT sequence complementary to the oligonucleotide used as primer is shown. The same junction between ETS and CAT sequences is present in pDmNTSΔ, pDmA8Δ, and pDm-360Δ. All clones containing the pre-rRNA promoter have the same junction as pDmNTS and pDm-180; pDmN7/−72 contains an additional 4 bp due to the cloning procedure.

that sequences upstream of region III also stimulate polymerase I transcription (Fig. 4*A*). The 330-bp repeats from region II are closely related in sequence to the 240-bp repeats (Fig. 1*B*). Therefore, it is likely that the effect is exerted by a similar sequence(s). Transcription from pDmRI-II/-306 is ≈5 times higher than from pDm-306 (Fig. 4*A*); however, pDmRI-II/-306 is a weaker template than pDmNTS and pDmA8 (Fig. 4). Taking into account the different number of region II and III repeats in each template, it seems that sequence elements within the 330- and 240-bp repeats influence pre-rRNA transcription to a similar extent.

Spacer Transcription. The NTS effect might depend upon transcription from the duplicated spacer promoters (Fig. 1*C*). Spacer-initiated transcripts read through the downstream repeats generating RNA ladders (15, 18, 20). S1 nuclease analysis of RNA from cells transfected with 3′ deletions derived from pDmA8 (Fig. 1) shows that transcripts initiate from all the spacer promoters in region III (Fig. 5*A*). Adopting a different S1 mapping strategy, it is possible to show that transcripts from each spacer promoter are produced at comparable levels (Fig. 5*B*). Although in this experiment only spacer transcripts that read through the ETS promoter are detected, it is noteworthy that the intensity of band c, corresponding to transcripts initiated within spacer

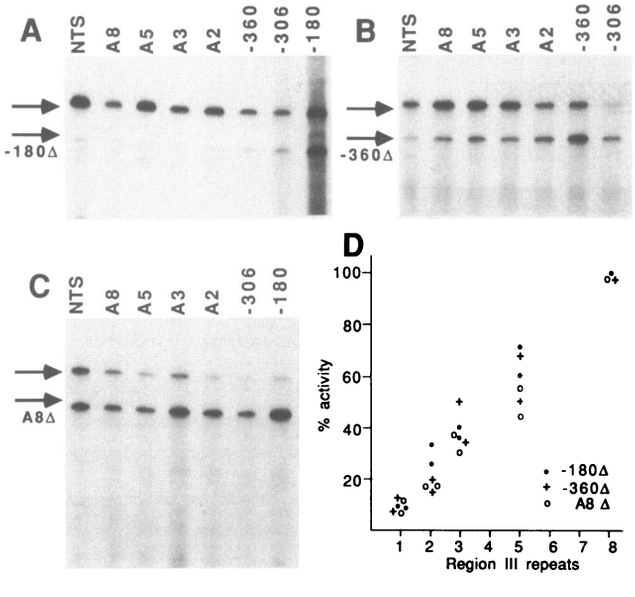

FIG. 3. Primer extension analysis of RNA from cells cotransfected with 10 μg of the indicated DNAs and 10 μg of pDm-180Δ (*A*), pDm-360Δ (*B*), or pDmA8Δ (*C*). (*D*) The relative transcriptional activity of each template is shown versus the number of region III repeats present. Autoradiograms were traced in a densitometer, and the ratio of signals of pDmA8 versus pDm-180Δ (•), pDm-360Δ (+), or pDmA8Δ (○) was taken as 100%. Each point represents the result of independent experiments. Densitometric values were adjusted by taking into account differences in the molecular size of individual plasmids.

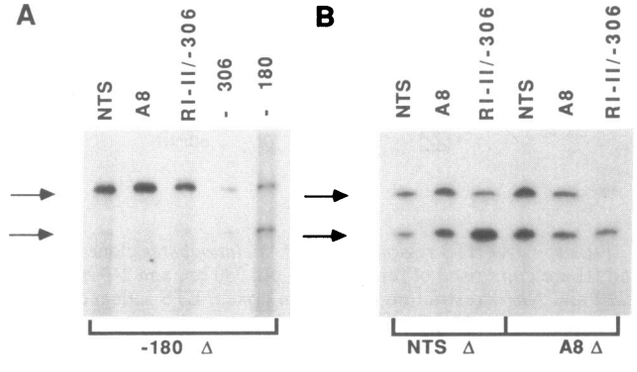

FIG. 4. Primer extension analysis of RNA from cells cotransfected with 10 μg of DNA as indicated and 10 μg of either pDm-180Δ (*A*) or pDmNTSΔ and pDmA8Δ (*B*).

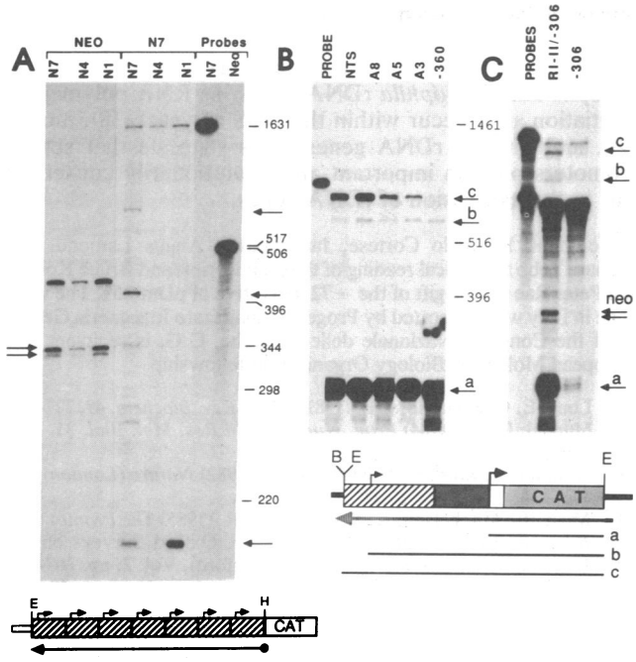

FIG. 5. Transcription from spacer promoters. (*A*) Total RNA (20 μg) from cells cotransfected with 10 μg of RSVneo (25) and with 10 μg of pDmN7, pDmN4, or pDmN1, separately hybridized with a uniformly labeled neo probe and with a 5'-end-labeled probe from pDmN7 (see lower diagram) was processed by S1 nuclease mapping. Arrows on the left and right side indicate transcripts from the Rous sarcoma virus and the NTS promoters, respectively. Upper bands in the three neo lanes represent read-through transcripts. Faint bands in lane pDmN4 are clearly visible on the original autoradiogram. (*B*) Total RNA (20 μg) was hybridized to a uniformly labeled rDNA/CAT probe (see lower diagram) and processed by S1 nuclease. Transcripts from the pre-rRNA promoter and the first or further upstream spacer promoters are denoted as a, b, and c, respectively. (*C*) Total RNA (20 μg) from cells cotransfected with RSVneo and the indicated DNAs were hybridized to the neo and rDNA/CAT probes described and subjected to S1 mapping. Numbers refer to the size in base pairs of restriction fragments used as markers. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

sequences upstream of the first spacer promoter, parallels the number of NTS promoters in each template. Therefore, differences in the level of pre-rRNA initiation in constructs bearing spacers of variable length (see Fig. 3) might depend upon the amount of spacer-initiated transcription (Fig. 5*B*; see *Discussion*). The latter is likely to be underestimated in this type of experiment, as only transcripts reading through the gene promoter are measured; moreover, spacer transcripts are rather unstable *in vivo* (20). Read-through transcripts originating from spacer sequences upstream of the first spacer promoter are also detected in cells transfected with pDmRI-II/-306 (Fig. 5*C*).

Inverting the Orientation of NTS Sequences. The effect that NTS sequences have on pre-rRNA transcription is polar. This conclusion is drawn from the analysis of the transcriptional activity of plasmids in which a 240-bp repeat array (pDmN7inv/-180) or spacer regions I–II (pDmRI-II/-306) are oppositely oriented with respect to the pre-rRNA promoter (Fig. 1*A*). In both constructs the stimulatory effect of spacer sequences is abolished (compare lanes A8 and N7inv/-180 in Fig. 6*A* and lanes RI-II/-306 and RI-IIinv/-306 in Fig. 6*B*, respectively). These results seem to rule out the possibility that elements within the NTS repeated units act as canonical enhancers. We cannot prove that pre-rRNA transcription is not influenced by cis-acting elements that work at a distance from the gene promoter only in one orientation; however, the polarity observed is consistent with the hypothesis that the

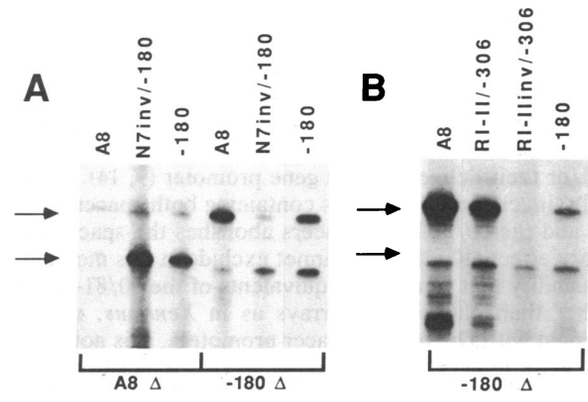

FIG. 6. Template activity of constructs containing inverted NTS sequences form region III (*A*) or region I–II (*B*). RNA from cells cotransfected with the plasmids as indicated at the top and the reference templates as indicated at the bottom were processed by primer extension. Arrows mark the elongated products.

spacer effect involves transcription from the duplicated promoters toward the pre-rRNA promoter. It is of interest to note that the presence in cis of inverted spacer segments actually reduces pre-rRNA initiation, as transcripts from either pDmN7inv/-180 of pDmRI-IIinv/-306 are less abundant by a factor of 2–4 than those from pDm-180.

DISCUSSION

In *D. melanogaster*, ribosomal transcriptional units are separated by long internally repetitive DNA segments consisting of 95-, 330-, and 240-bp units (Fig. 1). Spacer sequences influence to a significant extent the level of pre-rRNA transcription in this organism (22). The effect, measurable on separately transfected constructs, is magnified in competition experiments (ref. 22 and Fig. 2). The level of pre-rRNA transcription is related, in a nearly linear fashion, to the number of 240-bp repeats that precede the pre-rRNA promoter (Fig. 3). Spacers devoid of these units still enhance pre-rRNA initiation (Fig. 4), and the related nature of 330- and 240-bp repeats (Fig. 1) suggests that a transcriptional advantage is conferred by the same sequence type(s). Spacer sequences are able to act at a distance from the gene promoter; the hierarchical rank between rDNA templates carrying a different number of spacer repeats implies that distal and proximal units in the 240-bp array influence pre-rRNA transcription to a similar extent. This remote type of control is reminiscent of the effect exerted on promoter activity by enhancer elements; however, spacer sequences act in an orientation-dependent manner, as their influence is abolished in constructs where the pre-RNA promoter is preceded by inverted copies of either regions I–II or III (Fig. 6). Though not directly proven, this polar effect might depend upon transcription from the promoters included within the 240- and 330-bp repeats (Fig. 1 *B* and *C*). In agreement with this hypothesis, the level of spacer transcription (Fig. 5) is correlated, in each construct, with the competitive strength of the pre-rRNA promoter. The rate of pre-rRNA initiation in plasmids containing spacer promoters might be higher as polymerase molecules loaded at these sites are delivered to the gene promoter (9); alternatively, transcription from upstream promoters may favor template activation, maintaining an open chromatin conformation that facilitates binding of limiting transcription factors at the promoter region. The two mechanisms are not mutually exclusive and might operate in conjunction.

The influence of *Drosophila* NTS sequences on pre-rRNA transcription might be due to multiple components, as shown for *Xenopus laevis* rDNA spacers. Here, spacer promoters are interspersed and separated from the gene promoter by arrays of enhancer sequences (the 60/81-bp elements) in turn homologous to the region from position −114 to position

−72 of the gene promoter. These repeats are capable of enhancing transcription in either orientation and at variable distance from the pre-rRNA promoter (10–12). The integrity of at least one spacer promoter is, however, required for full enhancement (13, 14). It has been suggested that transcription facilitates binding of factors at the 60/81-bp elements and/or their delivery to the gene promoter (9, 14). Interestingly, inversion of segments containing both spacer promoters and the 60/81-bp enhancers abolishes the spacer stimulatory effect (10, 28). We cannot exclude at this moment the possibility that functional equivalents of the 60/81-bp units, rather than clustered in arrays as in *Xenopus*, are interspersed with *Drosophila* spacer promoters. It is notable that the gene interval from position −110 to position −72 is reiterated in the 240- and 330-bp repeats (Fig. 1*C*). Although the *Xenopus* enhancers are copies of an essential promoter region (29), removal of sequences to position −72 does not affect *Drosophila* pre-rRNA transcription *in vivo* (Fig. 7, see below). This is in accord with *in vitro* studies that restrict promoter sequences to the 43 nucleotides preceding the pre-rRNA start site, where an upstream domain influencing the rate of transcription (from position −43 to position −27) precedes a polymerase phasing signal sufficient to direct faithful initiation (30). The poor homology of *Drosophila* spacer repeats with the −43/−27 promoter element (Fig. 1*C*) seems to rule out that a factor binding at this site also interacts with spacer repeats, although transcription factors that recognize sequences bearing limited similarities have been described (31).

Whether initiated at the NTS promoters or reading through from upstream rDNA units, polymerase I transcription terminates in mouse (32, 33) and *Xenopus* (9, 34) at specific sites located about 200 bp upstream of the pre-RNA start site. Termination presumably avoids impingement of incoming transcription complexes onto the pre-rRNA promoter and might increase the efficiency of pre-rRNA initiation, as polymerases stopped at these sites could be directly transferred to the pre-rRNA promoter (9, 32–35). Alterations of the terminator site reduce pre-rRNA initiation in mouse (32, 33) and *Xenopus* (14, 36). In *D. melanogaster*, spacer transcripts read through adjacent repeats, and a unique site of 3′-end formation has been mapped to about −140 bp from the pre-rRNA start site (20). An extensive deletion around this region has no effect on pre-rRNA initiation, nor does it substantially alter spacer enhancement (Fig. 7, lanes −72 and N7/−72). These results imply either that the putative terminator is not essential for optimal promoter activity as in other systems or that polymerase I transcription does not terminate within the *D. melanogaster* spacer at this or any other preferred site, as proposed on the basis of results from nuclear run-on experiments (37). Terminators and promoters are juxtaposed in mouse and *Xenopus* rDNA genes (32–36). It is possible that in *D. melanogaster*, given the smaller size of the promoter region (30), polymerase I termination occurs nearer to the pre-rRNA start site. In this context, it is worth mentioning that in *D. virilis* rDNA genes the 3′ end of transcripts initiated from tandemly duplicated spacer promoters lies ≈50 bp upstream of the adjacent initiation site (20).

The occurrence of upstream promoters is not restricted to *Xenopus* and *Drosophila* rDNA genes, as RNA polymerase I initiation sites occur within the NTS of yeast (38), mouse (39), and rat (40) rDNA genes. This suggests that spacer promoters play an important and evolutionarily conserved part in the expression of rDNA genes.

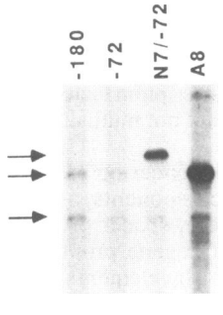

FIG. 7. Primer extension analysis of RNA from cells cotransfected with 10 μg of the indicated DNAs and 10 μg of pDm-180Δ. Transcripts from pDmN7/−72 are longer because the cloning procedure introduced 4 bp at the ETS–CAT junction in this plasmid.

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