In vivo transcription of rDNA spacers in *Drosophila*

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ABSTRACT
     The sequence-nonhomologous rDNA spacers of D. melanogaster and D.
virilis exhibit remarkably similar organizations and transcription
capabilities. In spacers of both species, tandemly repeated sequences are
present upstream of the beginning of the pre-rRNA coding region, and the
repeats are capable of transcriptional activity. Short, nuclear
transcripts homologous to the spacer repeats have been identified by S1
nuclease protection experiments and by northern blot analyses. Although
spacer transcripts are rare in steady state RNA populations, the presence
of multiple promoter elements may suggest a regulatory role for rDNA
spacers.

INTRODUCTION
     The tandemly repeated ribosomal RNA genes in multicellular eukaryotes
are expressed via ca. 8 kb primary transcripts that are processed to yield
18S, 5.8S, and 28S rRNAs. The region separating sequences that encode the
primary transcript has been called the nontranscribed spacer (NTS), but it
is now evident that the NTS can be an expressed component of rDNA in vivo.
In Xenopus laevis, transcripts homologous to the rDNA spacer have been
detected (1), and repetitive elements within the spacer have been
postulated to enhance the frequency of initiation of transcription (2-5).
Drosophila cells also contain transcripts homologous to rDNA spacers (6).
Characterizations of spacer transcripts in two species of Drosophila, D.
melanogaster, and D. virilis, are reported here.
     The rDNA spacers of D. melanogaster and D. virilis exhibit remarkably
similar organizations despite the lack of sequence homology between them
(7; unpublished sequence data). In both, the region immediately upstream
of the external transcribed spacer (ETS) contains between five and twelve
ca. 0.24 kb repeats (7,8), and we show here that the repeats support the
production of transcripts with 5' ends that are identical or analogous to
those of the rRNA precursors. Spacer repeats of D. melanogaster contain a

copy of the sequence spanning the ETS initiation site, and the repeats have
been shown to promote polymerase I transcription in vitro (9). Transcripts
generated both in vitro and in vivo begin at a sequence identical to the
precursor initiation site.

In D. virilis, the site of initiation of spacer transcription differs
in sequence from the precursor initiation site. However, since the end of
the last repeat abuts the beginning of the ETS, the two transcribed
sequences have identical upstream sequences. In neither species are spacer
transcripts that span the ETS boundary detected; thus, spacer transcripts
terminate upstream of the ETS initiation site.

MATERIALS AND METHODS

D. virilis (Pasadena) was originally obtained from the Yale Stock
Collection. D. melanogaster (Oregon R [Stanford]) was obtained from S.
Artavanis-Tsakonas. RNA was prepared according to Dawid et al. (10). RNA
was isolated from cultured cell lines of D. virilis and D. melanogaster (KC
line) according to the procedure of Jolly and Thomas (11). The plasmid
pDmr275c is a subclone of λ(Sep 6)Dmr275 (12); the Hind III segment that
contains the entire NTS and ETS was inserted into pBR313. Other plasmids
are described in citations given.

Unless indicated otherwise, probes for S1 nuclease protection were 5'
end labeled using polynucleotide kinase or 3' end labeled using the Klenow
fragment of DNA polymerase. End labeled fragments were made single
stranded either by electrophoresis in and recovery from neutral
polyacrylamide gels (13) or by digestion with exonuclease III (P-L
Biochemicals). The 5' labeled D. melanogaster probes, probes 9 and 11 in
fig. 1B, were labeled to equal specific activities in the following way:
Equimolar amounts of the plasmids E3 and c2, identified in the legend to
fig. 1, were combined, digested with Dde I, and 5' end labeled using
polynucleotide kinase. Dde I fragments containing appropriate sequences of
each plasmid were then isolated by electrophoresis in and recovery from low
melting temperature agarose gels. After secondary digestion of the E3 Dde
I fragment with Msp I, the 197 nucleotide E3 segment (probe 9 in fig. 1B)
and the 488 nucleotide c2 segment (probe 11 in fig. 1B) were run in and
recovered from strand separation gels. Since the Dde I sites were labeled
together (to equal specific activities), and since equal cpm of each probe
were used in hybridizations, any variability in the recovery of each
segment was inconsequential.

S1 nuclease protection assays were performed as follows: For hybridization, RNA (approximately 3 μg) and single stranded, end labeled DNA (approximately 50 ng and 5-50K cpm) were combined in 30 μl of 40% formamide, 0.6 M NaCl, 60 mM NaCitrate, 33 mM Na_2HPO_4-KH_2PO_4 pH 7.0, and incubated for at least 8 hours at 37°. For digestion by S1 nuclease, hybridizations were diluted to 0.25 ml in 20 mM NaCl, 30 mM NaOAc, 5 mM $ZnSO_4$ pH 4.5.   50-300 U S1 nuclease (P-L Biochemicals or New England Nuclear) were added, and reactions were incubated at room temperature for 1 hour. Digestions were stopped by addition of 50 μl of 0.5 M Tris pH 9.5, 0.1 M EDTA, and, after addition of 20 μg of E. coli tRNA as carrier, reactions were precipitated in ethanol prior to electrophoresis in denaturing polyacrylamide gels. All gel electrophoresis and filter hybridizations were performed essentially according to Maniatis et al. (14).

RESULTS AND DISCUSSION

Identification of the 5' end of the ETS in D. virilis rDNA.

The 5' end of the rRNA precursor was identified in S1 nuclease protection experiments. Total nuclear RNA of D. virilis embryos or tissue culture cells was hybridized to single stranded fragments of coding strands that were 5' end labeled at sites downstream of the NTS repeats. A 2.1 kb Alu I/Hinf I fragment of pDvE2-1/H6 (7), 5' end labeled only at the Hinf I end, was made single stranded by exonuclease III digestion and used as a hybridization probe for S1 nuclease protection (fig. 1A, probe 6). The Alu I/Hinf I fragment contains all the spacer repeats of pDvE2-1/H6 and 260 bp of sequence downstream of the repeats.

Probe 6 was hybridized to nuclear RNA, and the hybrids were digested with S1 nuclease. Protected DNA fragments of 260 nucleotides were observed in autoradiograms of denaturing polyacrylamide gels in which the products of digestion were displayed (fig. 2A). The protected fragments of probe 6 comigrated with segments of the adjacent sequence ladder that extended, from the labeled Hinf I end, upstream through non-repetitive sequence to the junction between non-repetitive sequences and the end of the NTS repeats. Therefore, the rRNA precursor has a 5' terminus near the end of the spacer repeats. Taking an adjustment of 1.5 nucleotide into account (12; fragments of the chemically degraded sequence ladder migrate 1.5 nucleotide faster than the corresponding S1-digested fragment), the larger of the two bands in lanes a and b represents a protected fragment with its
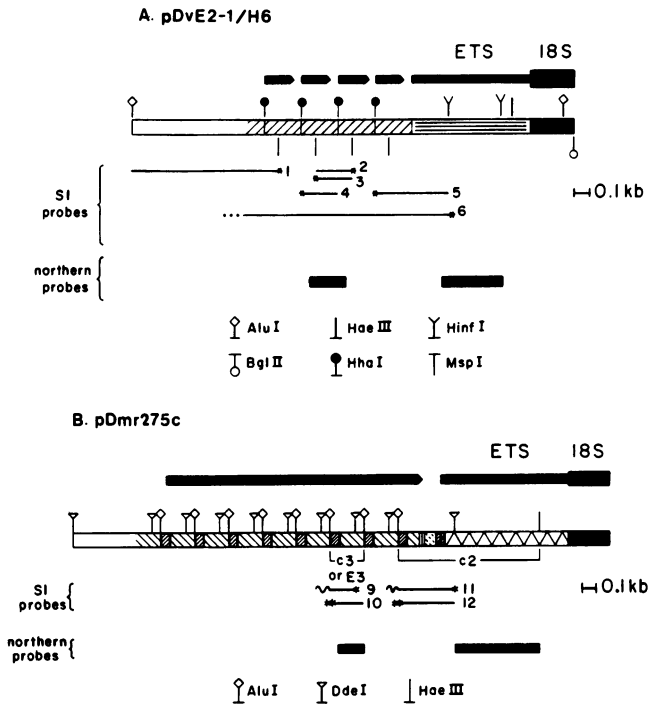
A. pDvE2-1/H6

B. pDmr275c

Fig. 1.   Maps of transcribed regions in D. virilis (pDvE2-1/H6; 7) and D. melanogaster (pDmr275c). Arrows above the restriction maps indicate the direction of transcription and transcript boundaries.   (While it is shown in panel A that there is transcription of each of the identical copies of the D. virilis spacer repeat, this has not been proven; it is formally possible that, say, only the first or the last of the repeats is transcribed in vivo.)   Thin lines beneath each map indicate single-stranded, end labeled DNA fragments (coding strand) used as probes in S1 protection studies, where the asterisk designates the labeled end.   Bold lines at the bottom of each panel represent fragments used as nick-translated probes in northern blot hybridizations.

In panel B, brackets immediately below the restriction map denote subclones used to construct the end labeled probes represented below the brackets. The segment c3 (Dmr275c3) is an Alu I fragment (single spacer repeat) and the segment c2 (Dmr275c2) is an Alu I/HaeIII fragment, both of which were from pDmr275c, and were blunt-end ligated into the filled-in Hind III and Bam HI sites of pBR322 (9).   E3 is also a subcloned Alu I repeat, originating from pDmra56 (10), and constructed in the same way as was c3. Wavy lines represent pBR322 vector sequences included in probes.   Double asterisks represent probes labeled by T4 DNA polymerase.

3' terminus at the 'T' of AGTA... within the sequence ladder of the coding strand segment (fig. 2A).   Therefore, the ETS begins within two nucleotides of AT... of the non-coding strand, shown as the beginning of the thick underline in fig. 3A.   Identical results were obtained with nuclear RNA of

embryos (fig. 2A, lane a) and with nuclear RNA of tissue culture cells that were treated with toyocamycin (fig. 2A, lane b) in order to inhibit processing (15). This suggested that the size of the protected fragments reflected the position of the end of the primary transcript, rather than an end produced by rapid processing.

When the D. virilis transcription start site was aligned with that of D. melanogaster (12; see fig. 3B), comparison of upstream sequences revealed limited homologies (16). For this comparison, sequences were aligned by initiation sites (indicated by vertical arrows in fig. 3), which were designated as +1. (This numbering differs from that shown in fig. 3A, where numbers are based on the spacer repeat, and transcription begins at the third nucleotide.) There are four oligonucleotides of 5 bp or longer that are present at identical positions, between -110 and -7, in spacers of the two species. These are between -110 and -103 (AATGAAGT), between -56 and -52 (TGGTA), between -41 and -36 (AAAATA), and between -12 and -7 (AAAAC). The spacer transcript of D. virilis, which is characterized in the following section, contains an additional homology with the D. melanogaster sequence between -3 and +3 (TATAGG). (Since the ETS and spacer transcripts of D. virilis have identical upstream sequences, the other four homologies are also applicable to spacer transcripts.) However, D. virilis templates are not transcribed in a D. melanogaster in vitro transcription system (17). Furthermore, the AT-richness of spacer sequences (approximately 70% AT; 7) tends to increase the probability of coincidental homologies.

Probes constructed using fragments downstream of the ETS initiation site were used to localize the 5' end of the 18S gene. This analysis indicated that the ETS is approximately 0.80 kb in length (data not shown).

Characterization of spacer transcripts in D. virilis.

S1 nuclease protection assays. Experiments in which spacer repeat sequences were used as hybridization probes revealed the existence of transcripts homologous to the spacer repeat. Transcripts 172 nucleotides in length are present in nuclear RNA of both D. virilis embryos and tissue culture cells, and protection assays showed that the 5' terminus of the transcript is near the beginning of the repeated sequence.

There is an Msp I cleavage site at nucleotide 84 of the 226 bp repeated sequence (figs. 1A and 3A). Therefore, Msp I digestion of the spacer insert of pDvE2-1/H6 generates multiple copies of a 226 bp fragment, as well as two flanking fragments that each contain about one half of a
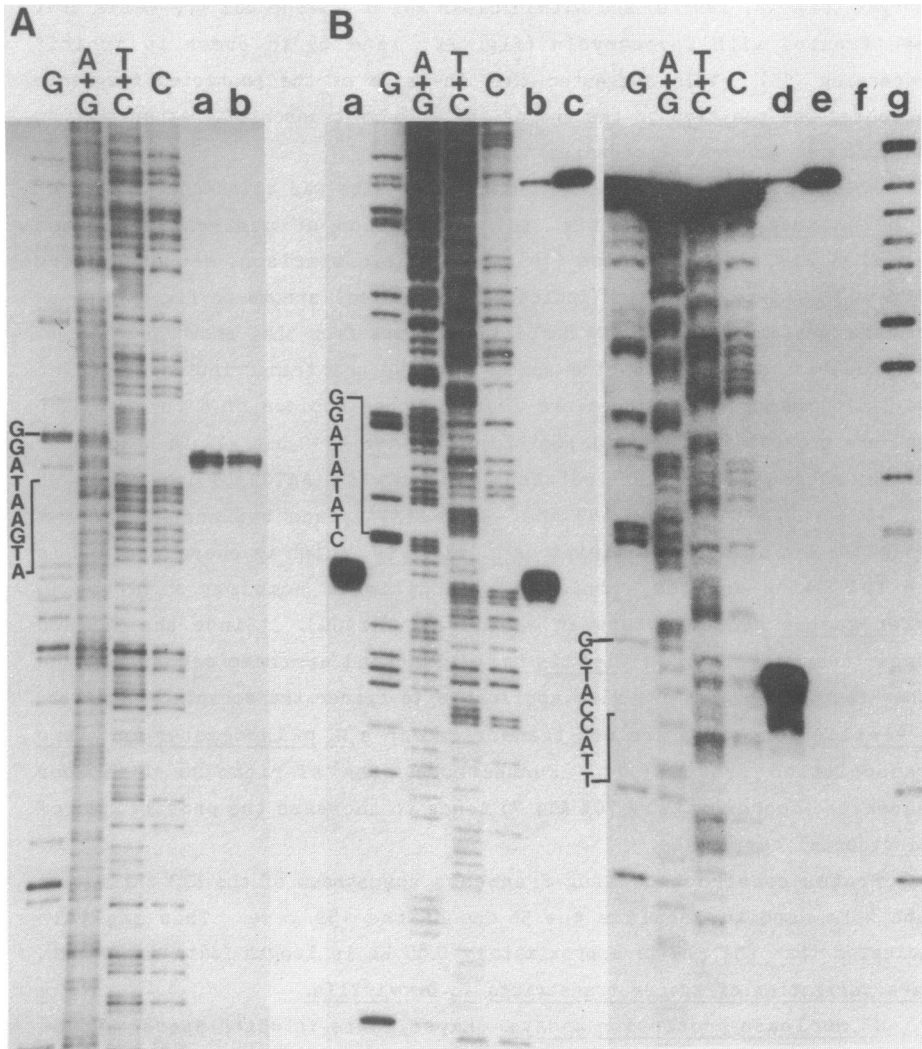
Fig. 2. S1 nuclease protection assays of ETS and NTS transcription in D. virilis. Panel A identifies the 5' end of the ETS using probe 6 (fig. 1A). The autoradiograph of the bottom half of an 80 cm (5%) sequencing gel is shown, in which the first four lanes show Maxam and Gilbert (13) sequence reactions of probe 6, lane a shows the 260 nucleotide fragments of probe 6 protected by nuclear RNA of embryos, and lane b shows the (identical) fragments protected by nuclear RNA of tissue culture cells that were treated with toyocamycin in order to inhibit processing. The short sequence indicated to the left of the ladder is the complement of the CCTAT/TCAT that is the junction of the repeated and non-repetitive portions of the spacer upstream of the 18S coding region. This junction is in line 3 of fig. 3A.

Panel B identifies 5' and 3' ends of transcripts that are homologous to the spacer repeats. Hybridizations with probes 1, 2, and 3 involved nuclear RNA from tissue culture cells. Probes 1 and 2 are 5' labeled at Msp I ends (see fig. 1A), and sequences of both are identical for 126 nucleotides from the labeled ends. The 82 nucleotide protected fragments of probe 1 (lane a) and probe 2 (lane b) were each displayed adjacent to the sequence ladder of probe 1. The indicated short sequence is the junction of two adjacent 226 bp NTS repeats. The sequence is complementary to that given at the beginning of line 1 in fig. 3A. Lane c is a minus S1 control of probe 1 (226 nucleotides), containing 1/10 the cpm used in the hybridization of lane b. The next four lanes contain the sequence ladder of probe 3, which was 3' end labeled at the Msp I site of the spacer repeat and had an initial length of 226 nucleotides. The indicated portion of the sequence is complementary to that shown from 171 to 179 in line 2 of fig. 3A. Lane d shows the 90 nucleotide protected fragment of probe 3. Lane e is a minus S1 control of probe 3, containing 1/10 the cpm used in the hybridization of lane d. Lane f is a minus RNA control, in which an amount of probe equal to that displayed in lane e was used in a mock hybridization and S1 digestion with E. coli tRNA. Lane g contains Msp I-digested pBR322.

spacer repeat and upstream or downstream adjacent sequences (fig. 1A). DNA sequencing has shown that each copy of the repeat is identical in pDvE2-1/H6 (16), so that internal and flanking fragments served as equivalent probes with respect to repeat sequences. Msp I fragments were 5' end labeled and made single stranded in order to construct probes 1 and 2 (fig. 1A). When these probes were hybridized to nuclear RNA, S1-resistant hybrids of 82 nucleotides were formed (fig. 2B, lanes a and b). The protected fragments were displayed adjacent to the sequence ladder of the upstream flanking fragment, probe 1; the protected fragments of both probe 1 and probe 2 comigrated with segments of the sequence ladder extending from the labeled Msp I site to the beginning of the repeated sequence (fig. 3A). Protected fragments aligned most closely with the 'T' in the coding strand sequence ladder that corresponds to the 'A' that is the third nucleotide of the repeated sequence given as the non-coding strand in fig. 3A (beginning of the thin underline in fig. 3A). This indicated that spacer transcripts have 5' termini at approximately the third nucleotide of the repeat, and they extend past the Msp I site at nucleotide 84.

In order to identify 3' termini of spacer transcripts, coding strand probes labeled at their 3' ends were analyzed. Probes were constructed in which spacer sequences were 3' end labeled either at the Msp I site at nucleotide 84, or at the Hha I site at nucleotide 18 (fig. 1A, probes 3, 4, and 5). Protection assays with all 3' labeled probes indicated the existence of transcript termini at nucleotide 174 of the repeated sequence, which is 52 nucleotides before the end of the repeated sequence. When the

A. D. virilis: pDvE2-1/H6

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               HhaI                                                                    MspI
...CCTAT(ATAGGGAGTGGTGTCGCGCAAGGCATGTCATATGAAAAATTTTAAATACGGTAAATTCATATGACTGTGGACTGTTGACCTTGCCGGCATAAGTCAATTA
                       30                             60                             90

TGTTTATAAAAGGAGAATAATGAAGTTATAAAAGTGTATTATTAAATTAGTACATGAAGACATTAAGGTGAATGGTAGCATTTGGAAAAAATATCGCCAT
                   120                            150                            180

TATAGATGATGTGTCAAAAAACCTAT)n TCATGGTGAGCAGTGTGTGCTCATCACATTACGCTGAAAGCGTATAATTTTAAAAGTTTGTCATATACTCGT
                   210                               30                             60

TATTTGAGTTAGATATCGTTATTTGTTATGTTGTATAACATTTGGCTTACGATATTAAGAAAAAAAGATAATTGTGAAACTTTCAAGCCAAGATGTGTTG
                   90                             120                            150

TTAGTAATAATTTTATTGTTACTACATCGTGTAAACAAATGGGTTTCCTTTTATCGTAAAATTTAACA
                   180                            210                            240
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B. D. melanogaster: pDmr275c3 / pDmr275c2

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                                                                                    Dde I (+152)
c3: ATGAAAATAAATATTTATTATATGTATAT---------------------------------GGAAAAATGTTGAAATATTCCCATATTCTCTAAGTATTATAGAGAAAAGC

c2: ATGAAAATGAATATTTATTATATGTATATAGGGGAAAAAATAATCATATAATATATATGAATAATGGAAAA----TGAAGTGTTCATATATTCTCGTAATATATAAGAGAATAGC
             -150                           -120                           -90

          AluIAluI                                         ↓                Dde I (+152)
c3: C---ATTTTA-GTG---AATGGATATTAGTAGTGTAAGCTAGCTGTTTTACGACAGAGGGTTCAAAAACTACTATAGGTAGGCAGTGGTTGCCGAC // TCTCTAAGTATTATA

c2: CCGTATGTTGGGTGGTAAATGGA-AT------TGAAAATACCCGCTTTGAGGACAGCGGGTTCAAAAACTACTATAGGTAGGCAGTGGTTGCCGACCTCGCATTGTTCGAAATAT
          -60                           -30                           +1                +30
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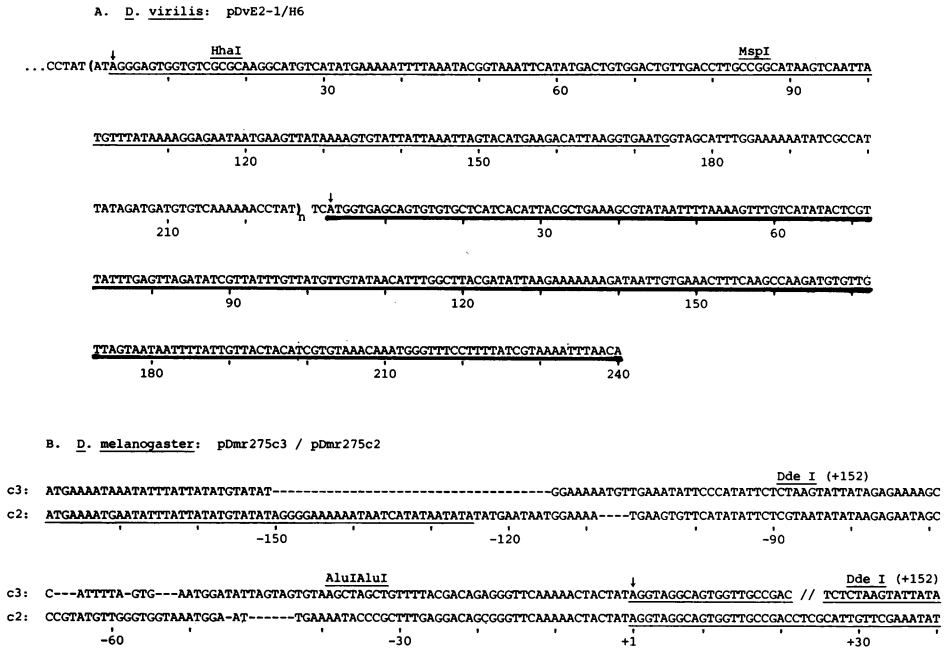
Fig. 3.   Spacer repeat and ETS sequences of D. virilis (A) and D.
melanogaster (B). In panel A, the sequence of the 226 bp spacer repeat is
enclosed in parentheses with a subscript 'n'. The enclosed sequence
corresponds to one of the hatched blocks within the restriction map of fig.
1A. The repeated sequence is followed by the nonrepetitive sequence of the
beginning of the ETS. Sequences protected in S1 nuclease protection assays
(see fig. 2) are underlined. The thin underline delineates the boundaries
of a spacer transcript, and the bold underline indicates the 5' end of the
ETS.   The 5' ends of both the spacer repeat transcript and the ETS
transcript are at approximately the third nucleotide past the end of the
adjacent upstream repeat (indicated by vertical arrows). Sequencing was
performed according to ref. 13.
In panel B, a portion of the previously published (9) spacer repeat
sequence (c3) is aligned above sequence of the ETS and adjacent upstream
regions (c2).   Sequences have been aligned according to NTS and ETS
initiation sites (both designated as +1), and gaps have been introduced in
order to maximize homology. Underlined sequences indicate 5' (at +1;
indicated by vertical arrow) and 3' (at -125) ends of protected regions.
Since spacer transcripts in D. melanogaster extend through multiple
repeats, all c3 sequences are potentially protected. The c2 sequence from
approximately -124 to -1 was never protected in S1 nuclease assays.

3' labeled Msp I fragment, probe 3, was used in protection assays, a

protected fragment of 90 nucleotides was observed (fig. 2B, lane d).

Comparison of the size of the protected fragments with the adjacent

sequence ladder of probe 3 showed that they comigrated with nucleotide 174 of the repeated sequence (90 bp downstream of the Msp I site at 84 bp).

Protection assays using probes in which the Hha I site at 18 bp was 3' end labeled (fig. 1A, probes 4 and 5) also indicated that spacer transcripts end at nucleotide 174 of the repeat (analyses with probes 4 and 5 yielded protected fragments of 156 nucleotides; data not shown). The distance of 66 nucleotides between the Hha I and Msp I sites provided an overlap between segments used to map 5' and 3' termini. Therefore, transcripts that protected the 3' labeled Hha I probes (probes 4 and 5) must span the Msp I site at 84 bp. Since the Msp I site was used in independent analyses to map both 5' and 3' ends (probes 1, 2, and 3), the 66 bp overlap provides evidence that both termini belong to a single transcript. Protection assays using 5' labeled probes indicated that transcripts begin at approximately the third nucleotide of the repeated sequence, and assays with 3' labeled probes mapped the ends of transcripts to nucleotide 174, which is 52 nucleotides before the end of the spacer repeat. These analyses predict the existence of nuclear transcripts of spacer repeats that are 172 nucleotides in length.

Northern blot analysis. Transcripts of approximately 172 nucleotides were detected in northern blot analyses in which a spacer repeat probe was hybridized to nuclear RNA of tissue culture cells (fig. 4A, lanes c and d). Identical patterns of hybridization were observed to nuclear RNA of untreated tissue culture cells and to nuclear RNA of cells that were treated with toyocamycin in order to inhibit RNA processing. In both cases, hybridization was to a single band at approximately 172 nucleotides, and to a lower molecular weight smear of less than 100 nucleotides. The origin of this smear is unclear, but since spacer transcripts are not present in cytoplasmic RNA (lane e), it may represent degradation products of the 172 nucleotide transcript. The smear of hybridization was not observed to embryo RNA (lane b), and this may reflect different kinetics of spacer transcription or transcript degradation in the two cell types.

In addition to the band at ca. 172 nucleotides, a second band was observed at ca. 0.37 kb in hybridizations of the spacer repeat to embryo RNA (fig. 4A, lane b). The larger band is approximately the size predicted for transcription through two spacer repeats. Transcripts initiating at the beginning of one repeat, and terminating at nucleotide 174 of an adjacent repeat would have a length of about 0.40 kb (224 + 174 = 398). Therefore, the larger band may represent transcripts of two adjacent
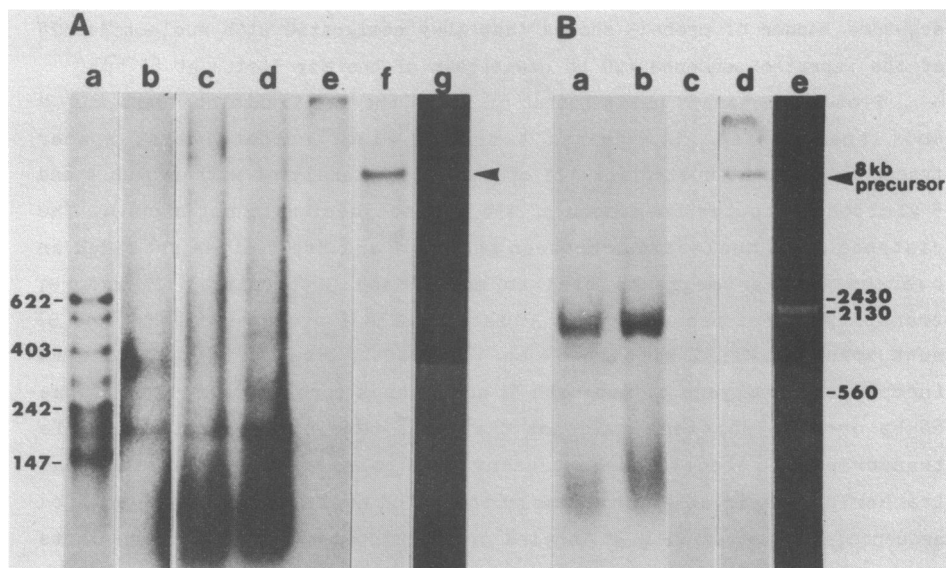
Fig. 4. Northern blot analyses of spacer transcripts in D. virilis (panel A) and D. melanogaster (panel B). In panel A, northern blot filters of D. virilis RNA were hybridized either to the 226 bp Msp I spacer repeat fragments (lanes b-e) or to a 350 bp Hinf I fragment from within the ETS (lane f) (see fig. 1A). Lanes a-e are from a 2.5% agarose gel containing glyoxalated RNA samples (3 μg/lane). Lanes f-g are from a formaldehyde step gel (3 μg/lane), in which the top half was 1% agarose and the bottom half was 2% agarose. Lane a, pBR322/Msp I; lane b, nuclear RNA of embryos; lane c, nuclear RNA of tissue culture cells; lane d, nuclear RNA of tissue culture cells that were treated with toyocamycin; lane e, cytoplasmic RNA of tissue culture cells; lane f, nuclear RNA of embryos, hybridized to the ETS-specific probe; lane g, replicate gel lane stained with ethidium bromide; the band in lane g are 18S and 28S and 28S rRNA.
In panel B, RNA of D. melanogaster (3 μg/lane) was electrophoresed in a formaldehyde step gel, in which the top half was 1% agarose and the bottom half was 2% agarose. Northern blots were hybridized either to a 200 nucleotide Mnl I fragment of c3 (lanes a-c) or to a 600 nucleotide Dde I/Hae III fragment of c2 (lane d) (see fig. 1B). Lane a, nuclear RNA of embryos; lane b, nuclear RNA of tissue culture cells; lane c, cytoplasmic RNA of tissue culture cells; lane d, nuclear RNA of tissue culture cells hybridized to the ETS-specific (c2) probe; lane e, replicate gel lane stained with ethidium bromide; the stained bands in lane e are 18S and 28Sα and 28Sβ rRNA. Some sizes of denatured λ/Hind III marker are indicated.

repeats, in which case termination within a repeat is not obligatory. This interpretation is based solely on the approximate size of the longer transcripts that have homology with the rDNA spacer, and their existence was not predicted by results of S1 protection experiments. Although lanes a-e of figure 4A originated from a single gel that contained equal amounts

of RNA in lanes b-e (3 μg), lane b represents an exposure that was much longer than the one shown in the other lanes. The autoradiogram of lane b was exposed to the northern hybridization filter approximately 10 times longer than the autoradiogram of the other lanes. Therefore, spacer transcripts are much more abundant in nuclear RNA of the cultured cell line than they are in nuclear RNA of embryos.

In no case was there hybridization of the spacer repeat to high molecular weight RNA (compare fig. 4A, lane f). Therefore, spacer repeat sequences are not transcribed as part of the rRNA precursor, and the small transcripts are not a consequence of rapid processing. The absence of transcripts proceeding from the NTS into the ETS was also observed in S1 protection assays using probe 6 (see fig. 1A), in which there were no fragments protected by toyocamycin-containing RNA that were longer than 260 nucleotides (the distance from the beginning of the ETS to the labeled Hinf I end of probe 6). Therefore, spacer transcripts appear to occur as discrete transcripts of one or a few repeats.

Characterization of spacer transcripts in D. melanogaster.

The NTS of D. melanogaster also has an organization in which a sequence of approximately 0.24 kb is tandemly repeated (8), and the Alu I repeats of the D. melanogaster spacer also code for nuclear transcripts. The repeats of D. melanogaster differ from those of D. virilis in that they each contain a copy of the sequence from -30 to +30, where the ETS begins at +1 (9; compare lines 3 and 4 in fig. 3B). The spacer transcripts begin at the nucleotide of the repeated sequence that is the same as +1 of the ETS, and in subsequent discussion of transcripts, positive numbers referring to the repeated sequence will use this nucleotide as +1.

S1 nuclease protection assays. The 5' and 3' termini of in vivo spacer transcripts in D. melanogaster were identified through S1 nuclease protection experiments that were similar in design to those described for D. virilis. In order to distinguish spacer transcripts from ETS transcripts, the 5' labeled end of spacer probes was downstream of the initiation site duplication, within sequences specific to spacer repeats. Therefore, protected fragments observed in autoradiograms were the result of hybrids involving sequences that are limited to the spacer. Since the repeats of a single spacer in D. melanogaster exhibit small sequence heterogeneities (17), labeled probes were constructed using plasmids containing subcloned single Alu I repeats. Several subcloned Alu I repeats differing from one another by a few single base pair substitutions and
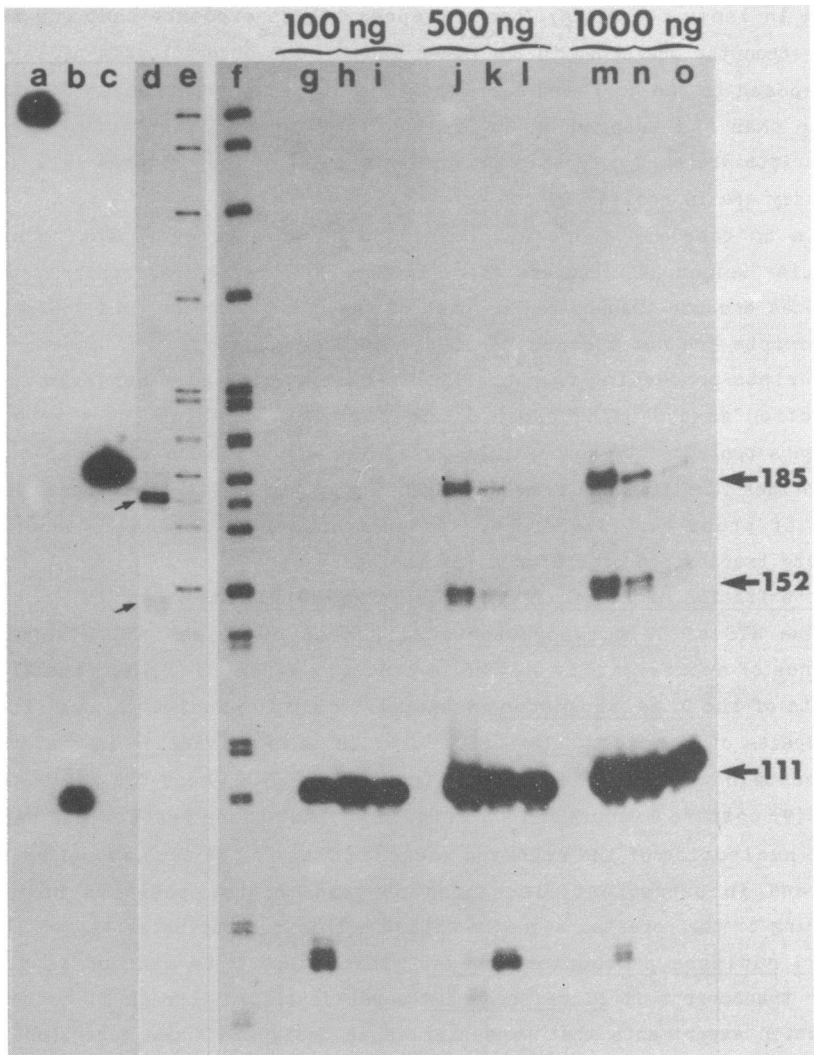
Fig. 5. Mapping 5' ends of spacer transcripts in D. melanogaster and quantitative S1 analysis of steady state transcript abundance. Probe 9 was prepared from a 197 nucleotide Msp I/Dde I fragment of E3, and probe 11 was prepared from a 488 nucleotide Dde I fragment of c2 (see fig. 1B). The probes were 5' end labeled to identical specific activities (see Materials and Methods). Individual hybridizations and controls (lanes a-d) each contained 75,000 cpm of a single probe. Comparative hybridizations (lanes g-o) contained 75,000 cpm (about 50 ng) of each probe and either 100 ng, 500 ng, or 1000 ng nuclear RNA of embryos. Lane a, probe 11, -S1 control; lane b, the 111 nucleotide protected fragment of probe 11; lane c, probe 9, -S1 control; lane d, the 185 nucleotide and 152 nucleotide protected fragments of probe 9; lanes e and f, pBR322/Msp I; lanes g-i, probes 9 and

11 were hybridized to 100 ng of RNA and digested with 100 U (lane g), 200 U
(lane h), or 400 U (lane i) of S1 nuclease; lanes j-1, probes 9 and 11 were
hybridized to 500 ng of RNA and digested with 100 U (lane j), 200 U (lane
k), or 400 U (lane 1) of S1 nuclease; lanes m-o, probes 9 and 11 were
hybridized to 1000 ng of RNA and digested with 100 U (lane m), 200 U (lane
n), or 400 U (lane o) of S1 nuclease.

deletions were analyzed. Spacer transcripts were initially detected using
probe 9 (fig. 1B), which was 5' end labeled at a Dde I site at nucleotide
152 of the spacer repeat; each probe contained a short length of pBR322
vector sequence at its unlabeled 3' end. The length of the vector tail
varied, depending upon the method of construction of each probe, and upon
the orientation of the spacer insert within the vector. The presence of
heterologous vector sequences within hybridization probes provided an
unambiguous assay for total protection of spacer sequences, since digestion
of the vector tail resulted in the production of fragments of a novel size.
Identical results were observed with all subclones tested.

Results of an assay using probe 9 prepared from plasmid E3 are shown
(fig. 5). The probe was a 197 nucleotide coding strand Dde I/Msp I
fragment labeled at its 5' Dde I end (see fig. 1B, probe 9). The probe
contained 185 nucleotides of spacer sequence and 12 nucleotides of pBR322
sequence. S1 nuclease protection assays in which probe 9 was hybridized to
nuclear RNA of either embryos or Kc cells gave protected fragments of 152
nucleotides and 185 nucleotides (fig. 5, lane d). When the protected
fragments were displayed adjacent to the sequence ladder of the probe (not
shown), the 152 nucleotide fragment comigrated with the ladder segment
extending from the labeled Dde I site to the initiation site of the spacer
repeat (ie., the 'T' in the coding strand sequence ladder that corresponds
to the 'A' that is +1 in the non-coding strand sequence of fig. 3B), and
the 185 nucleotide fragment aligned with the junction between insert and
vector. The fragments of 152 nucleotides indicated the existence of RNAs
that have 5' termini at +1 of the repeated sequence and extend downstream
beyond the Dde I site at 152 nucleotides. The fragments of 185 nucleotides
represented complete protection of the spacer sequences and removal of the
pBR322 vector sequences at the unlabeled 3' end of probe 9. Since total
protection of spacer sequences was not due to DNA contamination of nuclear
RNA (as evidenced by RNase+ controls, data not shown), existence of the 185
nucleotide fragment suggested that spacer transcripts may proceed through
more than one repeat.

As in D. virilis, S1 protection of a D. melanogaster coding strand

probe that was 5' end labeled at a site within the ETS and extended into
the NTS (fig. 1B, probe 11) resulted in a single protected fragment that
extended from the labeled end to the beginning of the ETS (fig. 5, lane b).
This indicated that NTS transcripts do not proceed into the ETS, so that
transcripts initiating within the NTS must terminate upstream of the ETS
initiation site. In order to include all sequences upstream of the ETS in
an analysis of 3' termini, 3' end labeled probes were constructed from both
pDmr275c3 and pDmr275c2 (fig. 1B, probes 10 and 12). Segments c3 and c2
are identical in sequence for about 160 bp from the Alu I site, at which
point c2 contains a 35 bp sequence that is missing in c3 (documented in
ref. 9, and indicated by vertical lines in fig. 1B, and by a length of
dashes in fig. 3B). This is followed by about 90 bp of incomplete homology
between the two segments (9). The orientation in pBR322 of both segments
is the same, with the single Cla I site of pBR322 9 bp away from the 3' end
of the coding strand of the insert of each plasmid (9 bp away from the Alu
I sites that are the beginnings of the c3 and c2 inserts). 3' labeled
probes were constructed from both plasmids by labeling the 3' end of each
coding strand, from the Cla I site, using T4 DNA polymerase. After
secondary digestion with Dde I (c2) or Bam HI (c3), the labeled strands
were run in and recovered from a strand separation gel (fig. 1B, probes 10
and 12). The probes consisted of the coding strand of each plasmid labeled
over a short distance from the 3' Cla I end into each rDNA insert.

When the c3 probe (probe 10) was used in S1 protection assays with
nuclear RNA of D. melanogaster embryos, the major S1-digested fragment was
approximately 238 nucleotides in length (fig. 6, lanes g-i). This
corresponds to complete protection of the spacer sequences within the 247
nucleotide probe and removal of the 9 nucleotide vector tail. There is
also a prominent band at 247 nucleotides, the original size of the probe.
This band is apparent at equal intensity in lane j (-RNA control), and is
due to contamination of the full-length probe, including vector sequences,
by the non-coding strand. Since the spacer segment within probe 10 is
fully protected, transcripts do not terminate within the repeated sequence.

Evidence of the existence of c2-specific transcripts was apparent from
assays using probe 12. RNA protection of the 3' labeled c2 probe from S1
nuclease resulted in a band of approximately 180 nucleotides (fig. 6;
indicated by arrowhead in lanes c-e). This indicated the existence of
protected fragments extending from the labeled end of the rDNA insert to a
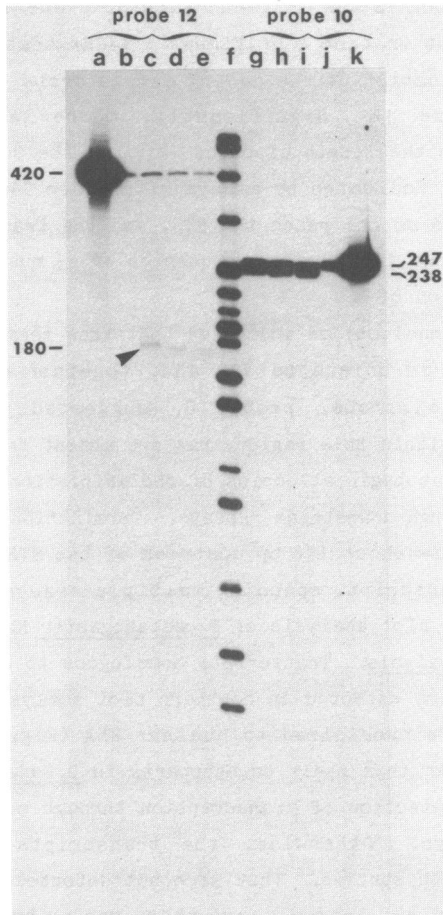point within the 35 bp unique to c2 (see fig. 3B). There is also a band at

Fig. 6. Mapping 3' ends of spacer transcripts in D. melanogaster. Spacer segments of c2 and c3 were 3' labeled by T4 DNA polymerase from the single Cla I site of pBR322. Probes 10 and 12 each contained 9 nucleotides of vector sequence at their labeled 3' ends, and were labeled over a short distance into each spacer insert (see fig. 1B). All S1 hybridizations and control reactions contained equal cpm. Each probe was hybridized to nuclear RNA of embryos, and digested with either 50, 100, or 150 U of S1 nuclease. Lane a, probe 12, the c2 probe, -S1 control; lane b, probe 12, -RNA control; lane c, protected fragments of probe 12 after digestion with 50 U S1 nuclease (indicated by arrowhead); lane d, protected fragments of probe 12 after digestion with 100 U S1 nuclease; lane e, protected fragments of probe 12 after digestion with 150 U S1 nuclease; lane f, pBR322/Msp I; lane g, protected fragments of probe 10, the c3 probe, after digestion with 50 U S1 nuclease; lane h, protected fragments of probe 10 after digestion with 100 U S1 nuclease; lane i, protected fragments of probe 10 after digestion with 150 U S1 nuclease; lane j, probe 10, -RNA control; lane k, probe 10, -S1 control.

420 nucleotides, which is the original size of probe 12. A band of the same size is apparent in lane b of figure 6 (-RNA control), and is once again due to contamination of the coding strand probe by the non-coding strand (compare lane j). Significantly, there is no band at 411 nucleotides, which is the length of probe 12 minus the 9 nucleotide vector tail. Therefore, as indicated by assays with probe 11 (see fig. 5, lane b), spacer transcripts do not reach the ETS, and the transcripts that give total protection of the Alu I repeat sequences of c3 must terminate within the heterologous region of c2.

The band at 180 nucleotides indicated that this terminus is within the 35 bp sequence that is unique to c2, and, together with full-length protection of the c3 probe, probe 10, suggested the existence of termination signals within this region that are absent from c3. Therefore, spacer transcripts that begin at copies of the initiation site within Alu I repeats continue through downstream repeats. Termination occurs within the 35 bp sequence that is about 140 bp upstream of the ETS initiation site. The existence of transcripts spanning multiple spacer repeats is also suggested by northern blot analysis of D. melanogaster RNA.

Northern blot analysis. Transcripts homologous to the spacer repeats of D. melanogaster were detected in northern blot analyses in which spacer repeat fragments were hybridized to nuclear RNA (fig. 4B). The spacer transcripts are longer than their counterparts in D. virilis, and this is consistent with the detection of transcription through multiple repeats in S1 protection assays. Otherwise, the transcripts exhibit similar characteristics in both species. They were not detected as a component of cytoplasmic RNA (fig. 4B, lane c), and there was no hybridization of an NTS specific probe to RNA in the size range of rRNA precursor, providing additional evidence that spacer repeats are not transcribed as part of the primary transcript (compare lane d).

In lanes containing nuclear RNA of embryos and tissue culture cells, hybridization of the spacer probe was to a diffuse band of about 2 kb (fig. 4B, lanes a and b). As in D. virilis, there was also hybridization to a lower molecular weight smear. The predicted size of a transcript of nine tandem copies of the Alu I repeat is approximately 2 kb. The predominant spacer size class in the Oregon R strain used in these analyses is one that accommodates approximately nine copies of the repeat, as deduced from Southern blot analyses of genomic DNA (data not shown). With respect to the ca. 2 kb spacer transcript, this suggested that when spacer

transcription occurs, transcription begins at the initiation site of the first Alu I repeat, continues through all downstream repeats, and stops upstream of the ETS initiation site. This is consistent with the termination site at about -140 that was mapped by S1 protection analyses. Spacer transcripts of D. melanogaster differ from those of D. virilis in this respect, since in D. virilis each copy of the repeat must contain signals sufficient for termination of spacer transcription.

There is a sequence of 10 bp that is present in the vicinity of the 3' ends of both spacer transcripts (GGAAAAAATA), and this decanucleotide is present once per repeat in D. virilis and only within the 35 bp unique to c2 in D. melanogaster (see fig. 3). However, the sequence is approximately 10 bp downstream of the 3' terminus in D. virilis and approximately 10 bp upstream of the transcript end that was localized in D. melanogaster. Although it is the longest sequence common to spacers of both species, no functional significance can presently be ascribed to it. An identical sequence is not present at the termination site of 28S gene transcription, although 28S genes of both species also end near runs of As (16; 18). Furthermore, within a single species, 3' termini of spacer transcripts and 28S transcripts are within non-homologous sequences.

Quantitative S1 analysis. The abundance of spacer transcripts in steady state populations of D. melanogaster nuclear RNA was estimated by quantitative comparisons of S1 nuclease protection experiments (19). The number of 5' ends of spacer transcripts was estimated relative to the number of 5' ends of authentic rRNA precursor molecules. This analysis was facilitated by the presence of non-homologous Dde I sites in an NTS repeat and in the ETS, which were used to prepare probes for each transcript that were labeled to equal specific activities (see Materials and Methods). Equal cpm of probes 9 and 11 were combined for hybridization to nuclear RNA of embryos. In order to obtain meaningful estimates of the levels of RNA homologous to each probe, it was important that each DNA probe was in excess relative to complementary RNA sequences. Therefore, hybridizations were performed with a series of decreasing RNA concentrations in order to assure DNA excess in some samples.

Each set of hybridizations was digested with three different concentrations of S1 nuclease, and the products were displayed on sequencing gels prior to visualization by autoradiography (fig. 5). The protected fragments (lanes g-o) were a summation of the protection patterns seen with each DNA probe alone (lanes a-d). Protection of the E3 segment

(probe 9) by spacer transcripts gave protected fragments of 152 and 185 nucleotides, as previously discussed. The smaller fragments represented protection by transcripts with 5' ends at the initiation site, while the larger ones were the result of total protection of spacer sequences in the probe by transcripts spanning multiple repeats. Fragments in the 185 nucleotide class were not included in the subsequent quantification, since they do not reflect 5' ends of transcripts. The sole protected fragment of the c2 segment (probe 11) was 111 nucleotides in length, which is the distance between the beginning of the ETS and the Dde I site within the ETS. In lanes g-o, the intensity of the protected bands was seen to increase with increasing RNA concentration, suggesting that DNA probe was in excess in hybridizations with less RNA.

Within a single set of hybridizations, the protected band of probe 11 was much darker than those of probe 9, indicating that ETS transcripts are more abundant than spacer transcripts. In order to estimate relative steady state levels of each transcript, gel lanes were fractionated into 1 cm slices, and these were counted by scintillation spectrometry. Within a single set of hybridizations (ie., lanes j-l in fig. 5), a ratio was established between the number of cpm in the peak representing the 111 nucleotide ETS band and the number of cpm in the peak corresponding to the 152 nucleotide band that reflects 5' ends of spacer transcripts. The results of several such quantifications indicated that in steady state RNA populations transcripts containing 5' ends of ETS transcripts are approximately 100 times more abundant than are transcripts containing 5' ends of spacer transcripts.

Spacer transcripts are a relatively rare component of nuclear RNA, and their absence from cytoplasmic RNA (fig. 4) suggests that they do not serve a function related to that of the rRNAs. However, it is striking that similar functional capabilities have been maintained in spite of divergence of spacer sequences. In both Drosophila and Xenopus laevis (5), rDNA spacers contain promoter duplications that are transcribed at a disproportionately low frequency compared to authentic gene transcripts. Therefore, it is proposed that it is not the actual production of transcripts that is important; rather, the presence of promoter capabilities upstream of pre-rRNA initiation sites increases the efficiency of initiation of transcription, thereby providing a selective advantage that encourages their maintenance.

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