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Unusual transcription termination of the ribosomal RNA genes in *Ascaris lumbricoides*

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We studied termination of transcription of the ribosomal RNA genes in *Ascaris lumbricoides*, the first representative in the phylum of nemathelminthes analysed so far. RNase protection experiments *in vivo* reveal that the 3′ end of the precursor rRNA coincides with the end of mature 26S rRNA. Promoter-containing miniplasmids are able to direct unique 3′ end formation *in vitro* at a site identical to that observed *in vivo*, whereas deletion of these sequences abolishes 3′ end formation throughout the entire spacer. A nuclear run-on experiment *in vitro* confirms the drop of polymerase I concentration downstream of this site. The termination site for polymerase I transcription of the rDNA operon in *A.lumbricoides* is therefore unique, and located at the very end of the 26S rRNA gene.

Key words: Ascaris lumbricoides/nematode/Pol I transcription termination/rDNA

Introduction

Eukaryotic ribosomal RNA genes are transcribed by polymerase I as long precursor molecules which are processed into mature 18S, 5.8S and 28S rRNAs. Cytological and biochemical studies led for a long time to the assumption that the end of mature 28S rRNA coincides with the transcription termination site of the ribosomal precursor rRNA (see Reeder *et al.*, 1987, for review). However, the report of mouse rRNA precursors, extending into the intergenic spacer (Grummt *et al.*, 1985a), revealed this model to be incorrect. Reinvestigations of other species, specifically of *Xenopus laevis* (Labhart and Reeder, 1986; De Winter and Moss, 1986), *Drosophila melanogaster* (Tautz and Dover, 1986) and yeast (Kempers-Veenstra *et al.*, 1986) confirmed these results. Nowadays it is believed that polymerase I transcription does not terminate at the end of the large rRNA gene but rather extends beyond this site into the intergenic spacer (see Baker and Platt, 1986; Reeder *et al.*, 1987, for review). Although similarities in termination events between different species were reported, differences do exist, reflecting species specificity of polymerase I transcription initiation (Grummt *et al.*, 1982). Mouse (Grummt *et al.*, 1985b; Grummt *et al.*, 1986; Miwa *et al.*, 1987), rat (Kermekchiev and Grummt, 1987) and human (Bartsch *et al.*, 1987; Safrany *et al.*, 1989; Parker and Bond, 1989) rRNA genes have an authentic termination site, 565 bp, 560–565 bp and 360 bp, respectively, downstream of the mature large rRNA gene. In these three species, several additional terminators are present in the spacer. *X.borealis* seems to have a real termination site near position +300 with respect to the 28S rDNA, followed by a second one further downstream (P.Labhart, personal communication). *X.laevis* shows a diverged mode of *X.borealis* transcription termination: a point mutation in a terminator-like sequence (T2) downstream of the 28S rRNA gene abolishes transcription termination. In *X.laevis*, most polymerase I molecules transcribe through the entire spacer until an authentic terminator (T3) is reached, located ~200 bp in front of the next rDNA coding region (Moss, 1983; De Winter and Moss, 1986; Labhart and Reeder, 1986, 1987, 1990). Additional termination sites in front of the next rDNA operons are found in all mammalian and amphibian species investigated so far. The regulatory sequences mediating transcription termination are located a few nucleotides downstream of the termination site in conserved sequence boxes (see Reeder *et al.*, 1987, for review). *D.melanogaster* represents a special case since no termination site has been found so far (Tautz and Dover, 1986). The polymerases read through the whole spacer producing long and stable transcripts. Also, no full stop termination site seems to be present in front of the next rDNA transcription unit. In yeast, a terminator is located 210 bp downstream of the mature rRNA gene, followed by two other terminators further downstream. A 3′ end generating site was found in front of the next rDNA promoter, but seems not to be very efficient (Kempers-Veenstra *et al.*, 1986; van der Sande *et al.*, 1989).

Some years ago we initiated an analysis of the organization of the rDNA operon in *A.lumbricoides*. We reported the presence of two rDNA size classes, differing from each other mainly by a 486 bp long insertion element in the intergenic spacer (Back *et al.*, 1984a; Briner *et al.*, 1987). In addition, some copies of the main size class are interrupted in the 26S rDNA region by type I-like intervening sequences of variable lengths (Back *et al.*, 1984b; Neuhaus *et al.*, 1987). In comparison with higher eukaryotic organisms, the spacers of the ribosomal genes of *A.lumbricoides* are very short (~1.9 kb and 2.3 kb) and devoid of any short repeated elements and promoter duplications (Briner *et al.*, 1987; E.Müller, in preparation). A homologous S-100 extract and a whole cell *in vitro* transcription system developed in our laboratory (Briner *et al.*, 1987) provides a powerful tool for the study of transcription termination. Here we report experimental data which show that termination of polymerase I transcription in *Ascaris*, unlike in all other investigated species, takes place at the end of the 26S rRNA gene. The sequences mediating termination are located, at least partially, upstream of the end of the 26S rRNA coding region. Functional analysis revealed no evidence for the existence of other termination sites located further downstream in the spacer or in front of the next rDNA operon.

Results

RNase protection mapping of the 3′ end of in vivo rRNA

In order to determine the longest detectable transcription product of the ribosomal RNA genes *in vivo*, we performed an RNase protection mapping of total RNA in comparison with sucrose gradient purified 40S precursor rRNA. The [α-^{32}P]UTP labelled T3 RNA polymerase transcript of a pBS M13$^+$ clone was used as a probe. It contains the last 188 bp of the 26S rRNA gene as well as 247 bp of the adjacent intergenic spacer (Figure 1A). The experiments for both total RNA from oocytes and 40S larval rRNA yield an identical digestion pattern (Figure 1B). The largest protected rRNA species (~190 nt long) share the same 3′ end, which maps, as determined by mung bean mapping experiments, to the guanosine marking the 3′ end of the 26S rRNA transcript (cf. Figure 1A; E.Müller, in preparation). Internal cutting of the hybrids observed below the main band is probably due to the combined digestion with RNase A and RNase T1 (cf. Labhart and Reeder, 1986). Furthermore, no full length protection of the labelled rRNA probe is detected, thus no stable transcription products are present extending beyond the downstream *Alu*I site (position +247). In conclusion, these results either point to a termination site localized at the end of the 26S rRNA gene or, alternatively, to a 3′ end formation in the intergenic spacer (IGS) followed by a very rapid processing of longer transcripts.

3′ end formation in vitro

As an alternative way to study transcription termination, we have analysed the 3′ end formation of rRNA in our *Ascaris* cell-free *in vitro* transcription system (Briner *et al.*, 1987). Different miniplasmids, carrying variable fragments surrounding the 3′ end of the 26S rRNA gene downstream of the polymerase I promoter of plasmid HHi661 (Figure 2A), were incubated in their supercoiled form and under standard transcription conditions (Briner *et al.*, 1987). Constructs with the downstream fragment in correct orientation are able to form a unique 3′ end (Figure 2B, lanes HpEE, HpAA, HpRE) which maps, in agreement with the *in vivo* results, to the guanosine located at the end of the 26S rRNA gene (results not shown). The clone HpER, however, containing the fragment of clone HpRE in opposite orientation, is not able to terminate transcription, but generates a run-off rRNA when incubated in the linearized form. Thus the sequences involved in 3′ end formation depend on their orientation relative to the direction of transcription. The standard incubation time of 1 h allows a steady state level of transcription on exogenous rDNA plasmids to be reached, but may be too long to allow the detection of putative precursors, post-transcriptionally cleaved before the end of the reaction. In order to uncover such possible nascent rRNA molecules, we stopped *in vitro* transcription of plasmid HpEE (cf. Figure 2A) at variable time points during the 1 h incubation. The 610 nt long rRNA transcripts, extending to the 3′ end of mature 26S rRNA, accumulate with increasing incubation time, but no longer discrete rRNA products can be observed (Figure 3). A transcription independent processing of longer precursor rRNA can also be excluded because there are no endonucleolytic processing factors present in the extracts. This was demonstrated with artificial T3 RNA polymerase transcripts which were recovered at their original size after a 1 h incubation (results not shown).

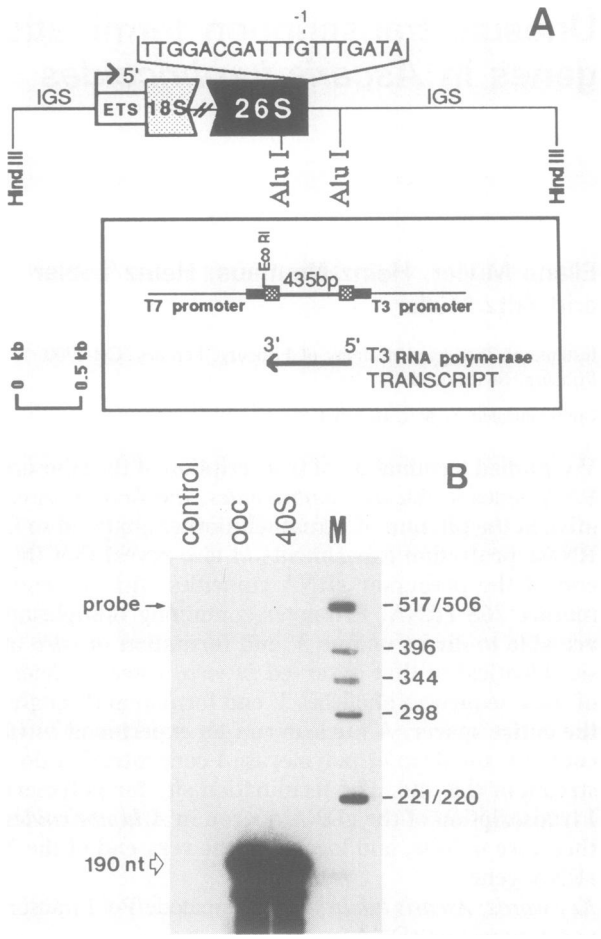

Fig. 1. RNase protection mapping of total RNA and 40S precursor rRNA *in vivo*. (**A**) Structure of an 8.8 kb ribosomal *Hin*dIII repeat of *A.lumbricoides* (cf. Back *et al.*, 1984a). For the RNase protection mapping, the 435 bp *Alu*I–*Alu*I fragment, covering the last 188 bp of the 26S rRNA gene and the first 247 bp of the IGS, was ligated into the vector pBS M13$^+$. This clone was linearized in the 5′ polylinker (▩) with *Eco*RI and transcribed with T3 RNA polymerase in the presence of [α-^{32}P]UTP (800 mCi/mmol; Amersham). ETS: external transcribed spacer. (**B**) Total RNA from oocytes (**ooc**) and 40S precursor rRNA from larvae (**40S**) were hybridized to the labelled rRNA transcript before digestion with RNase A and RNase T1. The digestion products were analysed on a 4% polyacrylamide gel. The band of ~190 nt is the longest transcript and is protected by both RNA species. Over-exposition of the gel confirms the absence of longer and less abundant transcripts. The 3′ end of the transcripts, determined in mung bean mapping experiments (E.Müller, in preparation), is indicated by position −1 (G) in the sequence shown in (**A**). As a control, an aliquot of the labelled rRNA transcript was treated without adding any rRNA. Lane **M** shows *Hin*fI digested pBR322 as a size marker.

It is still conceivable, however, that processing of longer primary transcripts occurs within the transcription complex and is coupled to elongation.

No further termination site is present in the spacer downstream of the 3′ end of the 26S rRNA gene

Deletion of the sequences surrounding the end of the 26S rRNA gene may allow us to distinguish between a true termination event at the 3′ end of this gene and a rapid processing coupled with transcription. If a terminator is located at the end of the 26S rRNA coding region, transcription termination will be abolished or another cryptic

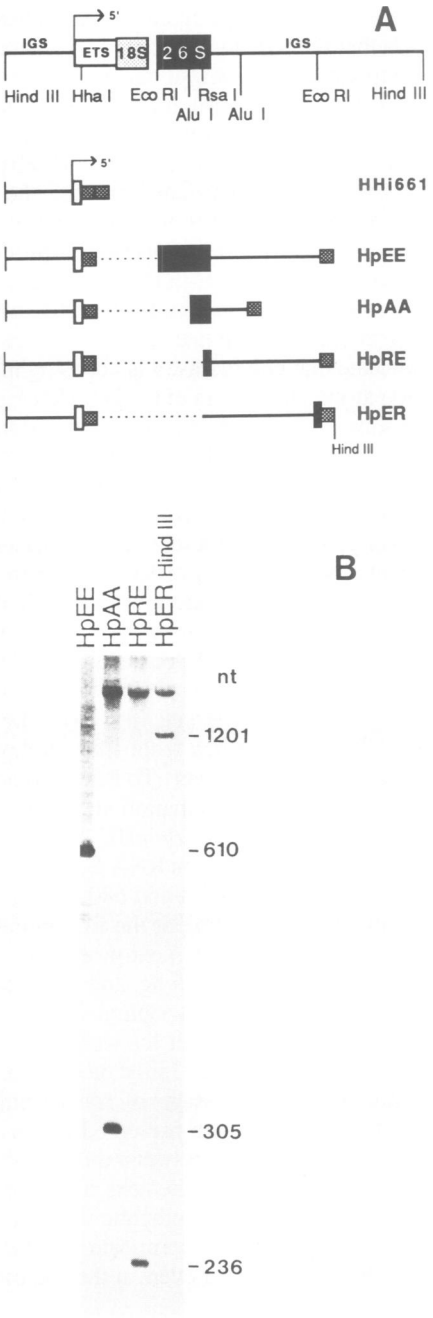

Fig. 2. 3′ end formation *in vitro*. (**A**) Different fragments spanning the end of the 26S rRNA gene and portions of the adjacent IGS were ligated into the polylinker (▩) of clone HHi661. This construct contains an rDNA promoter element extending 572 bp upstream to the *Hin*dIII site and 82 bp downstream to the *Hha*I site, with respect to the transcription initiation site. **HpEE** contains an *Eco*RI fragment (493 bp of the 26S rRNA gene and 944 bp of the IGS), **HpAA** an *Alu*I fragment (188 bp of the 26S rRNA gene and 247 bp of the IGS) and **HpRE** an *Rsa*I−*Eco*RI fragment (119 bp of the 26S rRNA gene and 944 bp of the IGS). The clone **HpER** carries the spacer fragment of clone HpRE, but inserted in opposite orientation. ETS: external transcribed spacer. (**B**) The supercoiled miniplasmids HpEE, HpAA and HpRE were transcribed in a homologous whole-cell extract *in vitro* under standard transcription conditions (Briner *et al.*, 1987). The miniplasmid HpER was digested with *Hin*dIII in the downstream polylinker before incubation. The autoradiograph of the 4% polyacrylamide gel shows distinct transcripts with lengths of 610 nt for **HpEE**, 305 nt for **HpAA** and 236 nt for **HpRE**. On the other hand, **HpER** yields a run-off transcript of 1201 nt which extends through the whole IGS to the *Hin*dIII site in the downstream polylinker, *Hin*fI digested pBR322 was used as a size marker.

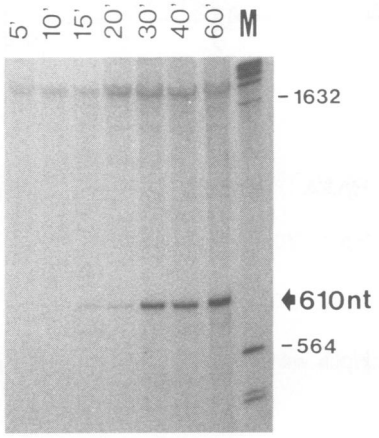

Fig. 3. *In vitro* transcription of the rDNA containing miniplasmid HpEE for variable time points. The miniplasmid HpEE (cf. Figure 2A) was transcribed *in vitro* for various periods of time between 5 and 60 min. The different transcription products were run on a 4% polyacrylamide gel. The 610 nt rRNA transcripts (cf. Figure 2B), which extend to the 3′ end of 26S rRNA, accumulate with increasing incubation time. No longer discrete transcripts can be observed. The band at ~1700 nt is due to the labelling of endogenous RNA and is always present in the control if the extracts are incubated without exogenous DNA templates (cf. control lane in Figures 4 and 5). Lane **M** shows *Hin*dIII digested λ^+ and *Hin*fI digested pBR322 as size markers.

termination site will be unmasked. However, if these sequences code for a processing signal, their absence will prevent the trimming of a longer transcript so that the real termination site may be uncovered, unless termination cannot occur without processing. To this end, we introduced deletions at the end of the 26S rRNA gene by digesting the *Alu*I−*Alu*I fragment (cf. Figure 1A) with exonuclease III from the 26S rRNA gene into the adjacent spacer (Figure 4A). The extent of the deletions was determined by sequencing and the truncated fragments were inserted downstream of the polymerase I promoter of plasmid HHi 661 (cf. Figure 2A) and transcribed in the *in vitro* extract. The original clone HpAA, as a control, produces an RNA of 305 nt (cf. also Figure 2B), as well as read-through transcripts, or a 569 nt long run-off product upon linearization of the template with *Hin*dIII (Figure 4B). The quantity of these read-through products depends on the amount of plasmid DNA added to the extract and varies in different preparations (cf. also Figure 2B, lanes HpEE and HpRE). The reason for this phenomenon, which has been previously described for the mouse system (Grummt *et al.*, 1985b), is that limited amounts of termination factors are available in the extracts. The linearized clone Hp▲−70, containing the last 70 bp of the 26S rRNA gene, forms a correct 3′ end (189 nt, Figure 4B) as well as a 476 nt run-off transcript. The miniplasmid Hp▲+41, containing only spacer sequences, and suprisingly, also clone Hp▲−20, which carries the last 20 bp of the 26S rRNA gene, are not able to mediate 3′ end formation. The total amount of transcribed rRNA accumulates at the top of the gel. *Hin*dIII digestion of the templates converts the read-through transcripts to run-off rRNAs of 329 nt and 391 nt, respectively. In summary, our results localize the 5′ boundary of sequences necessary for correct 3′ end formation between positions −70 and −20 with respect to the end of the 26S rRNA gene. Furthermore, the data show that in the absence of these sequences,

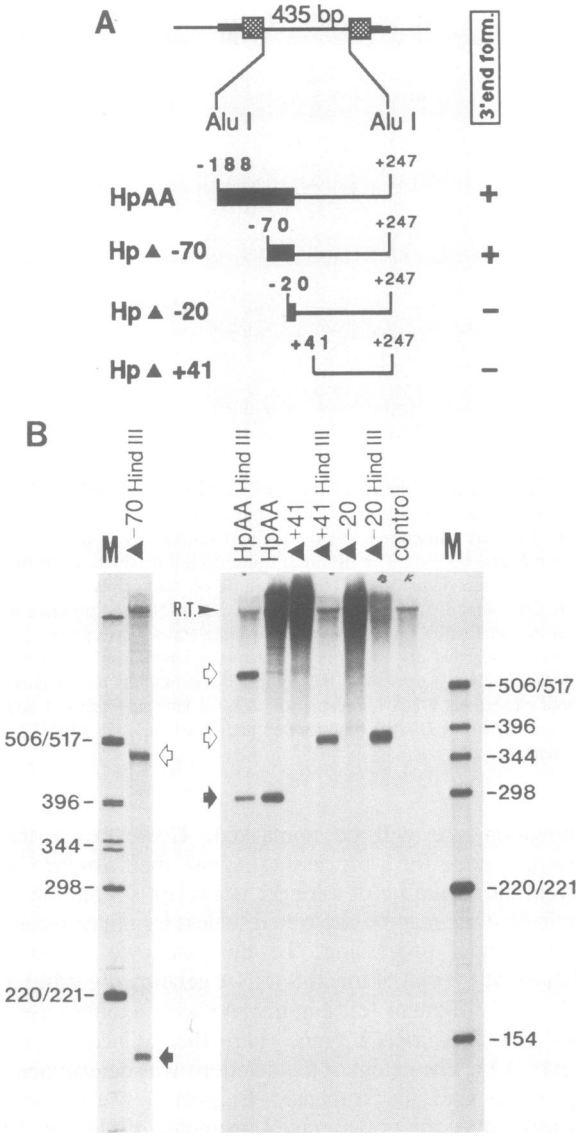

Fig. 4. *In vitro* transcription of miniplasmids carrying 3′ deletions at the end of the 26S rRNA gene. (**A**) The *Alu*I−*Alu*I fragment (cf. Figure 1A) was truncated with exonuclease III from the 3′ end towards the 5′ end and the digestion products were inserted into the polylinker (▩) of the promoter containing clone HHi661 (for further details see Materials and methods). In clone Hp▲−70, the insert starts at position −70 and in clone Hp▲−20 at position −20 with respect to the 3′ end of the 26S rRNA gene. Clone Hp▲+41 completely lacks the 3′ end of the 26S rRNA gene as well as the first 41 bp of the remaining IGS. (**B**) The *in vitro* transcription products of clones HpAA, Hp▲+41 and Hp▲−20 were separated on a 6% polyacrylamide gel. The transcripts of clone Hp▲−70 were analysed on a 4% polyacrylamide gel. Clones **HpAA** and **Hp▲−70** are able to direct unique 3′ end formation. Their transcription products of 305 or 189 nt are indicated by black arrows and the run-off transcripts of 569 nt and 476 nt by white arrows, respectively. Transcription of the supercoiled miniplasmids **Hp▲−20** and **Hp▲+41** generate only read-through transcripts (R.T.), accumulating at the top of the gel. Upon digestion of the miniplasmids with *Hin*dIII, the read-through transcripts are converted into run-off rRNAs of 391 and 329 nt. The run-off transcripts are indicated by a white arrow. As a control, the same amount of extract was incubated under transcription conditions but without adding any template DNA. Lane **M** shows *Hin*fI digested pBR322 as size marker.

polymerase I fails to terminate transcription at any other point between the end of the 26S gene and the *Alu*I site (position +247) in the IGS.

In a further series of experiments, we addressed the question of whether the IGS does contain a termination signal further downstream of the *Alu*I site at position +247. To do this we inserted different fragments that together span the whole spacer into the promoter containing plasmid HHi661 (Figure 5A). The linearized clones HpHH777 and HpXH1389, covering the left-hand part of the spacer, provide a run-off transcript of 918 nt or 1530 nt, respectively, and no shorter discrete transcription products are detected (Figure 5B). However, in the latter case, the run-off rRNAs are preceded by a multitude of smaller fragments, but none of them clearly dominates above the background. This might indicate that polymerase I is not as tightly bound as in the region around the 3′ end of the 26S rRNA gene and falls off at various sites, rather than at a distinct termination site. The spacer fragment of HpHX1146 covers the right-hand part of the spacer and extends beyond the next initiation site. This clone therefore contains the promoter of plasmid HHi661 upstream and a second one adjacent to the spacer fragment downstream. In order to test transcription initiation at these two sites separately, we cross-digested the clone with *Bam*HI and *Hin*dIII. As expected, the upstream promoter generates a run-off rRNA extending to the *Bam*HI site (108 nt) and the downstream promoter a run-off transcript extending to the *Hin*dIII site (587 nt). The run-off transcript of ~355 nt originates from transcription in the region of the downstream promoter. To test the spacer fragment for the presence of a termination site, we incubated the plasmid without cutting with *Bam*HI. The presence of a termination site should generate an RNA from the upstream promoter which is between 108 nt and 680 nt long. To our surprise, the polymerases starting at the first initiation site do not terminate but read over the next promoter. The produced run-off RNA is 1287 nt long and extends to the *Hin*dIII site. Unexpectedly, transcription initiation at the downstream promoter occurs with the same efficiency as with the *Bam*HI digested template. Initiation therefore seems not to be impaired by the passage of transcribing polymerases. In conclusion, the data presented above give no indication for a termination site between the end of the 26S rRNA gene and the beginning of the next rDNA promoter. This points to the presence of an authentic terminator at the end of the 26S rRNA gene, unless termination further downstream does require a processing event at the end of the 26S rRNA gene.

Nuclear run-on transcription assay

Termination of transcription at the end of the 26S rRNA gene implies a release of polymerases at this site. In order to test this hypothesis, we performed a nuclear run-on transcription assay. Nuclei from oogonia, the stages used for the preparation of the homologous *in vitro* transcription extracts, were isolated and pretreated wtih pancreatic RNase in order to digest endogenous RNAs. RNA molecules were then elongated *in vitro* by short incubation of the nuclei in presence of [α-^{32}P]UTP and 200 μg/ml α-amanitin. The transcription products were hybridized to a Southern blot of different rDNA fragments (Figure 6A). As expected, fragments A and B, containing the end of the 26S rRNA gene, do hybridize clearly to the labelled run-on transcripts (Figure 6B). Fragment C, containing only the last 20 nt of the 26S rRNA gene, and fragment D, starting at position +41 just downstream of the 26S rRNA gene, yield no signal.

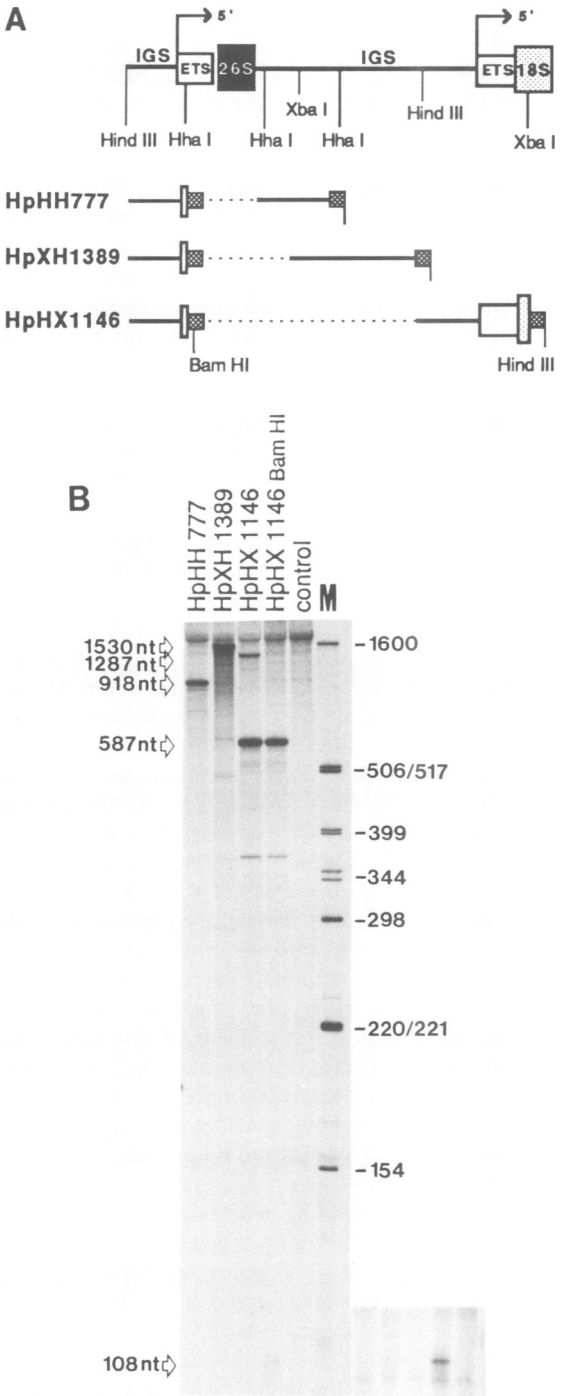

Fig. 5. *In vitro* transcription of miniplasmids containing different spacer fragments. (**A**) Different restriction fragments, together covering the entire spacer, were individually cloned downstream of the promoter element of clone HHi661 (cf. Figure 2A). Clone HpHH777 contains a *Hha*I fragment (777 bp from position +107 to position +883 in the IGS), HpXH1389 an *Xba*I−*Hin*dIII fragment (1389 bp of the IGS) and HpHX1146 a *Hin*dIII−*Xba*I fragment (572 bp of the IGS, 413 bp of the ETS and 161 bp of the 18S rDNA). (**B**) For transcription *in vitro*, the clones were linearized with *Hin*dIII in the downstream polylinker and HpHX1146 additionally with *Bam*HI and *Hin*dIII. The transcription products were run on a 4% polyacrylamide gel. The run-off products are indicated by white arrows. As a control, the same amount of the extract was incubated under transcription conditions but without adding any template DNA. A longer exposition of the lower part of the gel renders the faint transcript at 108 nt prominent. Lane **M** shows *Hin*fI digested pBR322 as a size marker.

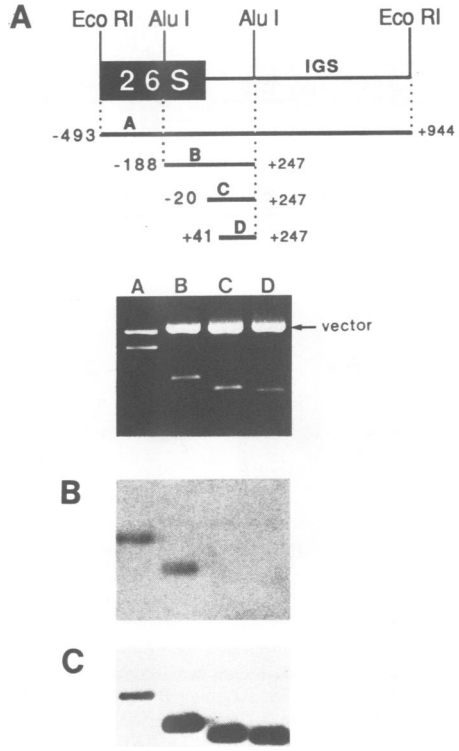

Fig. 6. Nuclear run-on transcripts *in vitro*. (**A**) Ethidium bromide-stained agarose gel (1%) of the different restriction fragments shown by lines at the top. Fragments C and D are cloned exonuclease III digestion products of fragment B. The figures at both ends of the fragments indicate the nucleotide numbers with respect to the 3′ end of the 26S rRNA gene. (**B**) The DNA of the gel presented in (**A**) was transferred to a nitrocellulose membrane and hybridized with the labelled nuclear run-on products transcribed *in vitro*. (**C**) As a control, the same filter was subsequently hybridized with an rRNA probe transcribed by T7 RNA polymerase from the pBS M13$^+$ clone containing the *Alu*I−*Alu*I fragment (cf. Figure 1A) and covering the entire region B. The weaker hybridization signal for fragment A is due to a lower DNA content.

Control hybridization with a T7 RNA polymerase transcript (Figure 6C) confirms that the difference in the hybridization signals is representative of a significant drop in the polymerase I distribution immediately downstream of the end of the 26S rRNA gene. The weaker signal for fragment A is due to a lower amount of DNA on the filter (cf. Figure 6A). Nuclear run-on transcripts were also hybridized to a fragment covering 500 bp of the 3′ region of the spacer, and as a control, to fragments containing the transcription initiation region (Figure 7A). No hybridization signal is detected for the spacer fragment B, whereas a strong signal is observed for fragment A (Figure 7B), covering the whole external transcribed spacer and 161 bp of the 18S rRNA gene. For fragment C, containing as few as 82 bp of the external transcribed spacer, hybridization is also readily detectable. The absence of polymerase I transcripts downstream of the 26S rRNA gene clearly suggests that transcription terminates at the end of the large rRNA gene of *Ascaris*.

Discussion

In all eukaryotic organisms which have been used for detailed rDNA expression studies so far, transcription extends into

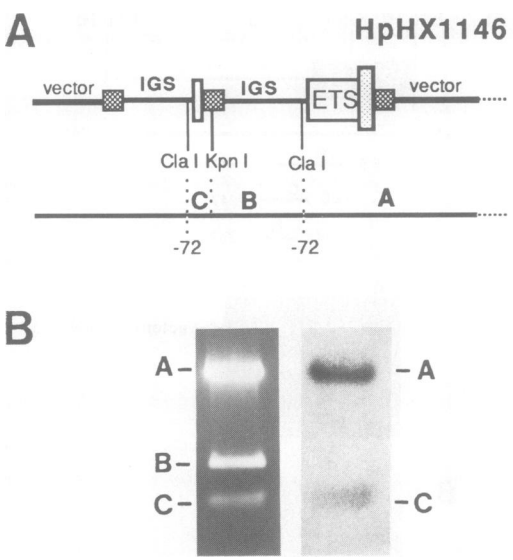

Fig. 7. Hybridization of nuclear run-on transcripts to fragments covering the 3′ part of the spacer and the initiation region. (**A**) Clone HpHX1146 (cf. Figure 5A) was digested with *Kpn*I and *Cla*I. Three digestion products are generated: fragment C, containing the last 72 bp of the IGS and 82 bp of the ETS; fragment B, covering the last 500 bp of the IGS and fragment A, containing the remaining part of the plasmid construct with 413 bp of the ETS, 161 bp of the 18S rDNA and vector DNA. (**B**) The digestion products described in (A) were run on an agarose gel (1%), transferred to a nitrocellulose membrane and hybridized with the labelled nuclear run-on products transcribed *in vitro*.

the IGS, and the 3′ end of the large ribosomal RNA is formed subsequently by processing of the primary transcript (see Reeder *et al.*, 1987, for review). The presence of corresponding rRNA precursors was detected in most cases by appropriate S1-mapping experiments *in vivo* (e.g. Grummt *et al.*, 1985a; De Winter and Moss, 1986; Labhart and Reeder, 1986; Tautz and Dover, 1986; Miwa *et al.*, 1987; Parker and Bond, 1989; Safrany *et al.*, 1989; Walker *et al.*, 1990). The same approach used for *Ascaris*, however, shows that the 3′ end of the 40S precursor rRNA coincides with the end of mature 26S rRNA. Even far more sensitive RNase protection mapping experiments reveal no evidence for the existence of longer transcripts.

Our present data demonstrate that 3′ end formation of the *Ascaris* 26S rRNA is fundamentally different from the processing event occurring at the end of the large ribosomal RNA of all other organisms investigated so far. The *in vitro* transcription system of, for example, the mouse produces transcripts extending to the first termination site within the IGS. The lack of processing factors in the extracts prevents their post-transcriptional cleavage into mature 28S rRNA (Grummt *et al.*, 1985b; 1986). 3′ end formation in our *Ascaris* cell-free extract, on the other hand, takes place at the end of the 26S rRNA. Artificial rRNA transcripts are not processed in the *Ascaris in vitro* extracts (data not shown), indicating that 3′ end formation of the *Ascaris* 26S rRNA is coupled to transcription. That 3′ end formation is mediated exclusively by distinct signals located at the end of the 26S rRNA gene, is supported by experiments with miniplasmids of *Ascaris* rDNA, where the sequences surrounding the 3′ end of the gene have been deleted. The absence of these sequences renders the polymerase I incapable of terminating transcription *in vitro* at any point within the spacer between two rDNA transcription units. Obviously, no other transcription termination site exists further downstream of the end of the 26S rRNA gene. Deletion of the 3′ end of the 26S rRNA gene in yeast, on the other hand, revealed two termination sites, hidden by a rapid processing event occurring under normal transcription conditions (Kempers-Veenstra *et al.*, 1986).

Nuclear run-on experiments further confirm the absence of transcription products immediately downstream of the end of the *Ascaris* 26S rRNA gene, whereas continuous transcription is reported at or adjacent to the processing site at the end of the large ribosomal subunit in the mouse (Grummt *et al.*, 1985b), *Xenopus* (Labhart and Reeder, 1986), *Drosophila* (Tautz and Dover, 1986) and yeast (van der Sande *et al.*, 1989). In *Xenopus laevis*, the processing site at the end of the 28S gene (T1) is followed by a second one (T2) located 235 bp downstream in the IGS. Transcription continues beyond this site through the whole spacer to the termination site T3 in front of the next promoter. At T2 a rather efficient transcription dependent processing occurs so that longer precursors are underrepresented *in vivo*. Two pieces of evidence suggest that the site at the end of the *Ascaris* 26S rRNA gene differs from T2 in *Xenopus*. First, our data show that there is no functional terminator in the spacer and in front of the next rDNA promoter. A processing site similar to T2 at the end of the 26S rRNA gene in *Ascaris* should cause the polymerase I to continue transcription throughout the whole spacer and into the next transcription unit. However, we never detected any transcripts in the 3′ region of the spacer in nuclear run-on experiments. Second, a site with the properties of T2 in rRNA genes was reported exclusively for *X.laevis*; even the closely related *X.borealis* contains a functional terminator in this region (P.Labhart, personal communication). The evolutionary specialization of *X.laevis* may therefore represent the exception rather than the rule in regulating transcription termination for polymerase I. In summary and in comparison with the findings of corresponding experiments in other species, our results strongly argue that transcription termination of *Ascaris* rDNA occurs at a unique site located at the end of the 26S rRNA gene.

The sequences regulating transcription termination of *Ascaris* rDNA have not yet been determined precisely but our data show that their 5′ boundary lies between positions −70 and −20 with respect to the end of the 26S rRNA gene. Hence, they are located, at least partially, upstream of the termination site within an evolutionary highly conserved primary structure of the gene. An upstream sequence requirement was also suggested for T2 in yeast (Mestel *et al.*, 1989), whereas in *Xenopus* and mammalian species, termination is mediated by conserved sequence boxes located immediately downstream of the termination sites (see Reeder *et al.*, 1987, for review). Short thymidine stretches on the coding strand are not uncommon for polymerase I dependent termination sites, and this is also the case for the two other eukaryotic RNA polymerase classes (see Platt, 1986, for review). It is therefore attractive to speculate that the T triplets present immediately upstream and downstream of the termination site in *Ascaris* may serve as part of the termination signal.

The intergenic spacer of *Ascaris* rDNA is very small (~2.3 kb) and contains neither short repeated sequences, nor promoter duplications (Briner *et al.*, 1987; E.Müller,

in preparation), nor a full stop terminator in front of the next rDNA unit. A short spacer without repeated elements was also shown to occur in the free-living nematode *Caenorhabditis elegans* (Ellis *et al.*, 1986). While spacer length and sequence organization of the rDNA of both nematodes contrast with the situation reported for most other species, they are nevertheless surprisingly similar to those found in yeast rDNA (Skryabin *et al.*, 1984). Here, as well as in the two nematode species, all possible functions of the IGS must be encoded in a rather short region. Thus, the long IGS of many other eukaryotes is not an essential general feature of the rRNA gene, and models ascribing important enhancer function to their repeated spacer elements (see Moss *et al.*, 1985, for review) are not in accord with the findings in *Ascaris, Caenorhabditis* and yeast.

A model like the 'ribomoter' proposed for yeast (Kempers-Veenstra *et al.*, 1986; Johnson and Warner, 1989), seems to be more appropriate since it excludes the interference of the spacer with the transcription process. A physical association of the ends of the rRNA transcription unit brings the beginning of the gene (initiation site) and the end of the gene (termination site and enhancer) in close vicinity to each other and the polymerases may pass directly from the end to the start of the transcribed region. In *Ascaris*, transcriptional regulation of rRNA synthesis may occur in a similar fashion. Preliminary *in vitro* competition assays indicate that the region surrounding the termination site binds a transcription factor. Fragments containing the termination region as a competitor not only inhibit termination on a promoter-containing plasmid, but also abolish initiation to nearly the same extent, whereas neutral plasmid sequences show no such effects (data not shown). Thus it seems possible that the same factor binds to the termination as well as to the promoter region and that an enhancer-like element is present, as in yeast, close to the termination site. Such transcription factors binding simultaneously to both regions were in fact recently isolated from human and frog (Learned *et al.*, 1986; Bell *et al.*, 1989; Dunaway, 1989; Pikaard *et al.*, 1989). This interesting hypothesis, however, needs to be investigated in more detail.

Transcription termination at the end of the 26S rRNA gene in *A.lumbricoides* and most likely also in *C.elegans* probably represents a specialization that occurred during evolution. This hypothesis is supported by the comparative sequence analyses for two coding regions of the ribosomal gene in *C.elegans* (Ellis *et al.*, 1986). They show that *C.elegans* has diverged greatly from yeast, *D.melanogaster* and the vertebrates. Interestingly, *C.elegans* and *D.melanogaster* have diverged even more from yeast than the vertebrates. Moreover, the divergence of the primary structure during evolution parallels the functional specificity of the different polymerase I transcription systems (Grummt *et al.*, 1982). It is thus not surprising that regulation of rRNA 3′ end formation in *A.lumbricoides* and probably also in *C.elegans*, as well as in *D.melanogaster*, differs considerably from that in yeast and vertebrates.

Materials and methods

Plasmid constructions

The original clone used for all subcloning experiments is pAlr8 (Back *et al.*, 1984a) which contains a single repeating unit of the main size class of *Ascaris* rDNA. All miniplasmids tested in the *in vitro* assay were constructed by inserting different restriction fragments into the polylinker of clone HHi661, downstream of a promoter element (cf. Figure 2A). The 3′ deleted *AluI*–*AluI* fragments described in Figures 4A and 6A were created by progressive digestion with exonuclease III following the method of Henikoff (1984). The truncated fragments were sequenced using the chemical method of Maxam and Gilbert (1980).

RNase protection mapping

Total RNA from oocytes and larvae of *A.lumbricoides* was isolated following the method of Pilgrim *et al.* (1988). The larval RNA was fractionated on a sucrose gradient and the rRNA precursors preceding the peak of 26S rRNA were collected. This fraction will be referred to as '40S' precursor rRNA. The *AluI*–*AluI* fragment, cloned in a pBS M13$^+$ vector (Stratagene, San Diego; cf. Figure 1A), was transcribed with T3 RNA polymerase in the presence of [α-^{32}P]UTP (800 Ci/mmol; Amersham), as suggested by the supplier, and subsequently digested with RNase-free DNase. The labelled transcription products were separated on a 4% polyacrylamide gel and the full length transcripts were recovered by elution (Maniatis *et al.*, 1982) and used as probe. The RNase protection mapping conditions were applied as described by Melton *et al.* (1984) with some minor adaptations: the labelled rRNA probe was hybridized to 15 μg of total RNA and 5 μg of 40S precursor rRNA for 14 h at 50°C. Non-hybridizing RNA tails were digested with 50 μg/ml RNase A and 3 μg/ml RNase T1.

In vitro transcription assay

Whole cell extracts were prepared from oogonia and used essentially under the same transcription conditions as reported previously (Briner *et al.*, 1987). Each assay was in a total volume of 12.5 μl, contained 0.3–0.5 μg of plasmid DNA and was incubated for 1 h unless otherwise specified.

Nuclear run-on transcription assay

Oogonia were disintegrated in a B-dounce homogenizer and the nuclei were isolated and RNase digested, following the method described by Schibler *et al.* (1983). In vitro transcription was performed after the protocol of Collart *et al.* (1987), in the presence of [α-^{32}P]UTP (800 Ci/mmol; Amersham) and 200 μg/ml α-amanitin for 4 min at 26°C. Treatment and isolation of the transcripts, hybridization conditions and washing of the filters were done following the same protocol, with the only modification that hybridization was performed at 60°C for 42 h.

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References

Back,E., Müller,F. and Tobler,H. (1984a) *Nucleic Acids Res.*, **12**, 1313–1332.

Back,E., Van Meir,E., Müller,F., Schaller,D., Neuhaus,H., Aeby,P. and Tobler,H. (1984b) *EMBO J.*, **3**, 2523–2529.

Baker,S.M. and Platt,T. (1986) *Cell*, **47**, 839–840.

Bartsch,I., Schoneberg,C. and Grummt,I. (1987) *Mol. Cell. Biol.*, **8**, 3891–3897.

Bell,S.P., Pikaard,C.S., Reeder,R.H. and Tjian,R. (1989) *Cell*, **59**, 489–497.

Briner,G., Müller,E., Neuhaus,H., Back,E., Müller,F. and Tobler,H. (1987) *Nucleic Acids Res.*, **15**, 6515–6538.

Collart,M.A., Belin,D., Vassalli,J.-D. and Vassalli,P. (1987) *J. Immunol.*, **139**, 949–955.

De Winter,R.F.J. and Moss,T. (1986) *Nucleic Acids Res.*, **14**, 6041–6051.

Dunaway,M. (1989) *Genes Dev.*, **3**, 1768–1778.

Ellis,R.E., Sulston,J.E. and Coulson,A.R. (1986) *Nucleic Acids Res.*, **14**, 2345–2364.

Grummt,I., Roth,E. and Paule,M.R. (1982) *Nature*, **296**, 173–174.

Grummt,I., Sorbaz,H., Hofmann,A. and Roth,E. (1985a) *Nucleic Acids Res.*, **13**, 2293–2304.

Grummt,I., Maier,U., Ohrlein,A., Hassouna,N. and Bachellerie,J.-P. (1985b) *Cell*, **43**, 801–810.

Grummt,I., Kuhn,A., Bartsch,I. and Rosenbauer,H. (1986) *Cell*, **47**, 901–911.
Henikoff,S. (1984) *Gene*, **28**, 351–359.
Johnson,S.P. and Warner,J.R. (1989) *Mol. Cell. Biol.*, **9**, 4986–4993.
Kempers-Veenstra,A.E., Oliemans,J., Offenberg,H., Dekker,A.F., Piper,P.W., Planta,R.J. and Klootwijk,J. (1986) *EMBO J.*, **5**, 2703–2710.
Kermekchiev,M.B. and Grummt,I. (1987) *Nucleic Acids Res.*, **15**, 4131–4143.
Labhart,P. and Reeder,R.H. (1986) *Cell*, **45**, 431–443.
Labhart,P. and Reeder,R.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 56–60.
Labhart,P. and Reeder,R.H. (1990) *Genes Dev.*, **4**, 269–276.
Learned,R.M., Learned,T.K., Haltiner,M.M. and Tjian,R.T. (1986) *Cell*, **45**, 847–857.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p. 178.
Maxam,A.H. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Melton,D.A., Krieg,P.A., Rebagliati,M.R, Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Mestel,R., Yip,M., Holland,J.P., Wang,E., Kang,J. and Holland,M.J. (1989) *Mol. Cell. Biol.*, **9**, 1243–1254.
Miwa,T., Kominami,R., Yoshikura,H., Sudo,K. and Muramatsu,M. (1987) *Nucleic Acids Res.*, **15**, 2043–2058.
Moss,T. (1983) *Nature*, **302**, 223–228.
Moss,T., Mitchelson,K. and De Winter,R. (1985) In Maclean,N. (ed.), *Oxford Surveys on Eukaryotic Genes, Vol. 2: The Promotion of Ribosomal Transcription in Eukaryotes*. Oxford University Press, Oxford, pp. 207–250.
Neuhaus,H., Müller,F., Etter,A. and Tobler,H. (1987) *Nucleic Acids Res.*, **15**, 7689–7707.
Parker,K.A. and Bond,U. (1989) *Mol. Cell. Biol.*, **9**, 2500–2512.
Pikaard,C.S., McStay,B., Schultz,M.C., Bell,S.P. and Reeder,R.H. (1989) *Genes Dev.*, **3**, 1779–1788.
Pilgrim,D. (1988) *The Worm Breeder's Gazette* (Columbia: Univ. of Missouri-Columbia), **10**, 154.
Platt,T. (1986) *Annu. Rev. Biochem.*, **55**, 339–372.
Reeder,R.H., Labhart,P. and McStay,B. (1987) *BioEssays*, **6**, 108–112.
Safrany,G., Kominami,R., Muramatsu,M. and Hidvégi,E.J. (1989) *Gene*, **79**, 299–307.
Schibler,U., Hagenbuchle,O., Wellauer,P.K. and Pittet,A.C. (1983) *Cell*, **33**, 501–508.
Skryabin,K.G., Eldarov,M.A., Larionov,V.L., Bayev,A.A., Klootwijk,J., de Regt,V.C.H.F., Veldman,G.M., Planta,R.J., Georgiev,O.I. and Hadjiolov,A.A. (1984) *Nucleic Acids Res.*, **12**, 2955–2968.
Tautz,D. and Dover,G.A. (1986) *EMBO J.*, **5**, 1267–1273.
van der Sande,C.A.F.M., Kulkens,T., Kramer,A.B., de Wijs,I.J., van Heerikhuizen,H., Klootwijk,J. and Planta,R.J. (1989) *Nucleic Acids Res.*, **17**, 9127–9146.
Walker,K., Wong,W.M. and Nazar,R.N. (1990) *Mol. Cell. Biol.*, **10**, 377–381.

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