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Termination of transcription by yeast RNA polymerase I

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ABSTRACT

Analysis of the termination of transcription by yeast RNA polymerase I (Pol I) using *in vitro* run-on experiments in both isolated nuclei and permeabilized cells demonstrated that Pol I does not traverse the whole intergenic spacer separating consecutive 37S operons, but terminates transcription before reaching the 5S rRNA gene, that is within NTS 1. In order to discriminate between processing and termination at the 3'-end generating sites previously identified *in vivo* in NTS 1 (T1, T2 and T3), fragments containing these sites were inserted into the middle of the reporter DNA of an artificial rRNA minigene. RNA isolated from yeast cells transformed with these minigenes was analyzed for the presence of transcripts derived from sequences both up- and downstream of the insert by Northern blot hybridization, reverse transcription analysis and S1 nuclease mapping. In accordance with previously obtained results T1 (+15 to +50) was found to behave as a processing site. T2 (+210) however was concluded to be an efficient, genuine Pol I terminator. In addition to T2, two other terminators were identified in NTS 1: T3A (at +690) and T3B (at +950). Surprisingly, when the 3' terminal part of NTS 2 was tested for its capacity to generate 3'-ends, another terminator (Tp) was found to be present at a position 300 bp upstream of the transcription initiation site of the 37S rRNA operon.

INTRODUCTION

The transcription of rDNA is considered to be the primary target in the overall regulation of ribosome synthesis (1). Consequently, the *cis*- and *trans*-acting factors involved in initiation of transcription by RNA polymerase I (Pol I) have been extensively studied in many laboratories (reviewed in 2 and 3). Recently, however, research has also focussed on the position of and the requirements for termination of transcription by Pol I, the reason being that because of the tandem organization of the rDNA repeats in eucaryotes, the site of termination of transcription relative to that of initiation, could be an important feature in the regulation of transcription of rDNA (4).

Studies in mouse have shown that Pol I termination occurs 565 bp downstream of the 3'-end of the mouse 28S rRNA gene, upstream of a region containing a series of conserved SalI boxes (5). Moreover, Pol I molecules that initiate within the spacer terminate upstream of a SalI box, present at about position −170 relative to the transcription start site of the Pol I operon (6). Pol I promoter activity was found to be enhanced by the presence of this upstream termination site (7, 8). The SalI box itself is sufficient to direct 3'-end formation. However, up- and downstream sequences are required to ensure the accuracy and high efficiency of the termination reaction (9). Recently, Kuhn *et al.* showed that correct 3'-end formation of mouse pre-rRNA in fact involves termination followed by removal of 10 nt leaving a 3'-end at +565 (10). Furthermore, a protein factor has been identified that binds to the SalI box and mediates termination of Pol I transcription (11). Similar

to the situation in mouse, transcription of human rDNA extends a few 100 bp into the 3'-terminal spacer and terminates upstream of a cluster of repeated sequence elements. These elements show partial homology to the SalI boxes (12).

For *Xenopus laevis* on the other hand, it has been demonstrated that Pol I crosses the entire intergenic spacer, terminating only at a site immediately upstream of the next Pol I operon (position −213) (13, 14). This termination site also serves to terminate transcripts initiated at the spacer promoters. The rRNA precursor is rapidly processed at the 3'-end of 28S rRNA as well as 235 bp downstream of this mature 3'-end. The processing products derived from the intergenic spacer sequences are highly unstable and hence rapidly turned over. The presence of the termination site at −213 considerably enhances initiation at the nearby transcription start site (15, 16). Surprisingly, processing (at +235) and termination (at −213) require identical 7 bp long sequences (17). Pol I transcription in *Drosophila melanogaster* has also been shown to traverse the entire spacer (18). Processing of the precursor transcript occurs at the 3'-end of 28S rRNA and at several sites further downstream. However, no termination site has been identified so far, although that some termination of spacer transcription was observed upstream of the promoter of the next rDNA unit (19).

Altogether, it appears that different strategies have evolved for termination of transcription by Pol I in eucaryotes: either termination takes place just downstream of the mature rRNA sequences (mouse and human), or the entire spacer is transcribed and termination occurs upstream of the Pol I transcription initiation site of the next operon (*Xenopus laevis* and (maybe) *Drosophila melanogaster*). Both schemes have important implications for the mechanism involved in the regulation of Pol I transcription. For example, in *Xenopus laevis* the enhancement of promoter activity by the presence of the terminator for pre-rRNA, suggests a functional interdependence between termination and initiation of transcription. This has led to the hypothesis that the efficient transcription of rDNA in *Xenopus laevis* is achieved through recycling of Pol I molecules *via* hand-over from terminator to promoter (readthrough enhancement) (20). However, enhancement of promoter activity by the presence of an upstream terminator may also simply be due to the prevention of promoter occlusion by that terminator, as recent observations in different organisms suggest (21, 22, 23).

The aim of this study was to determine the termination site(s) for yeast Pol I. We have previously reported that in yeast the 3'-ends of both 37S precursor rRNA and mature 26S rRNA are generated by processing (24). The recent observation that processing at the 3'-end of 26S rRNA can occur *in vitro* confirms our *in vivo* data (25). An additional putative processing site, designated T1, was identified slightly downstream from the 3'-end of the 37S pre-rRNA (position +15 to +50, relative to the 3'-end of 26S rRNA). Furthermore we noted two candidates for the Pol I terminator both located in NTS 1 at position +210 (T2,within the Pol I enhancer element (26, 27)) and at about +750 (T3) respectively (24). In order to elucidate the actual site of transcription termination by yeast Pol I we have now extended our studies using both *in vitro* run-on transcription and functional analysis *in vivo* of various parts of the intergenic spacer. The results demonstrate that yeast Pol I molecules are processed at T1 and terminate transcription at one of three sites, including T2, located in NTS 1. Nevertheless, an additional terminator (Tp) was found to be present upstream of the promoter of the next rDNA unit. In contrast to the situation in mouse and *Xenopus laevis*, however, removal of Tp proved to have only a minor negative effect on the level of transcription initiation at the downstream promoter.

MATERIALS AND METHODS

Enzymes and strains

Restriction enzymes were purchased from Bethesda Research Laboratories (Rockville, Maryland, USA) and Pharmacia (Uppsula, Sweden). Polynucleotide kinase, *E. coli* DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 DNA polymerase and MMLV reverse transcriptase were obtained from Bethesda Research Laboratories. SP6 DNA polymerase and DNase RQ1 were from Promega (Madison, Wisconsin, USA). CIAP was obtained from Boehringer (Mannheim, West-Germany). S1 nuclease was from Sigma (St. Louis, MO., USA). Zymolyase-100T was from Seikagaku Kogyo Co (Tokyo, Japan). Helicase was from Industrie Biologique Française (Clichy, France). *E coli* DH1 (F^- *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^-)and JM101 *supE thi* Δ(*lac-proAB*) [F′ *traD36 proAB* $lacI^q$ ZΔM15] were used for construction and propagation of plasmid and M13 phage DNAs, respectively. *S. cerevisiae* L47 (*rna 82.1, leu 2-3, 112 ade 2, his 3Δ1, RNAase* 3^-) was used for *in vitro* run-on transcription studies. *S. cerevisiae* YT 6-2-1L (cir^o, *a, leu 2-3, 112, his 4-519, can 1*) was used for transformation with ARES minigenes.

Recombinant plasmids

Plasmids pMY57, pMY60, pML2, pARES2, pARES10, and pARES10.1 deletion derivates have been described previously (24, 28, 29, 30). In addition, pARES10.1Δ−149/+111 and pARES2 are outlined in Fig. 2. All rDNA coordinates are given relative to the 3′-end of the 26S rRNA gene, unless indicated otherwise. *A: Construction of M13 and SP6 clones* (*cf.* Fig. 1A). Spacer fragments were cloned in M13mp9, mp10 or mp11 resulting in the following constructs: *clone A* contains the BglII-EcoRI fragment (+18 to +100) from pARES10.1Δ−149/+18 in M13mp10 (cloned between BamHI and EcoRI); *clone B* contains the EcoRI-HindIII fragment (+100 to +270) from pARES10.1Δ−149/+18 in M13mp11 (cloned between EcoRI and HindIII); *clone C* contains the HindIII-HpaI fragment (+270 to +393) from pARES 10 in M13mp10 (cloned between HindIII and SmaI); *Clone D* contains the HpaI-Sau3A fragment (+393 to +721) from pARES10 in M13mp11 (cloned between SmaI and BamHI); *Clone E* contains the Sau3A-TaqI fragment (+721 to +1027) isolated from pARES10 as Sau3A-HindIII fragment (the latter site is present in the vector), in M13mp11 (cloned between BamHI and HindIII); *clone F* contains the MspI-HaeIII fragment (+1595 to +1739) from pMY57 in M13mp10 (cloned between AccI and SmaI). The *ETS clone* contains the BglII-HindIII fragment (+128 to +509, both coordinates are relative to the transcription start site) from pMY57 in M13mp9 (cloned between BamHI and HindIII). *pML2*ΔB was made by deleting the 2636 bp BclI-BclI fragment (−2963 to −327) from the 26S rRNA region in pML2. *B: Construction of ARES minigenes* (*cf.* Fig. 2, 4, 5, 7 and 8). 3′-End generating fragments (see below) for construction of *pT1, pT2, pT3, pTp and pTp.1* were first subcloned in M13mp18 (containing a XhoI linker in the KpnI site, and digested with BamHI, or SmaI and XhoI). Next they were isolated as XhoI-SalI fragments from these M13mp18 subclones, and cloned into the unique XhoI site of either pARES10.1Δ−149/+111 (T1- and T2-containing fragments) or pARES2 (T3(B)-, Tp- and Tp.1-containing fragments). By checking the orientation of the inserts we obtained pT1, pT1R, pT2, pT3, pTp and pTp.1. As *T1*-containing fragment we used the BglII-Sau3A fragment (−70 to +100) from pARES10.1Δ−149/−70; as *T2*-containing fragment we isolated the Sau3A-BamHI fragment from a M13 subclone containing the EcoRI-HpaI fragment (+100 to +393) between the EcoRI and SmaI site; as *T3(B)*-containing fragment we chose the Sau3A-Sau3A fragment (+721 to +1183) from pMY57; as *Tp*-containing fragment we isolated the EcoRV-XhoI fragment (−379 to +128, both

coordinates are relative to the transcription start site) from pMY57, containing a XhoI linker into the BglII site (+128); as *Tp.1*-containing fragment we isolated a fragment similar to the latter, except that it contained a BamH1 linker inserted into the SmaI site (−207, relative to the transcription start site). For construction of *pARES12.2* and *pARES12.9* we used pBR322 subclones, containing EcoR1-EcoR1 fragments of different rDNA units (+100 to +44, the latter coordinate being relative to the transcription start site), kindly provided by Dr. Øyen (31). From these clones we isolated EcoR1-pVUII fragments (+100 to +1003), that were cloned into SP64 (digested with EcoRI and pVUII), and afterwards isolated as BamHI-pVUII fragments (+100 to +1003) to insert them into pARES10.1Δ−149/+111, digested with BglII (+111) and pVUII (+1003), thereby giving rise to pARES12.2 and pARES12.9. For construction of *pARES13* we first subcloned the pVUII-HindIII fragment (+1003 to +509, the latter coordinate is relative to the transcription start site) from pMY57 in pUC18 (digested with HincII and HindIII). Next we isolated the insert as BamH1-HindIII fragment to ligate it with the large vector-containing BglII-HindIII fragment (+111 to +1040, the latter site is present in the vector) of pARES10.1Δ−149/+111, giving rise to pARES13. *pARES14* was constructed from pARES10.1Δ−149+111 by deleting the HindIII-HindIII fragment (+270 to +1040, the latter coordinate is present in the vector).

In vitro transcription

In vitro run-on transcription was performed both in isolated nuclei and in permeabilized cells. Nuclei were isolated according to Ide and Saunders (32) using a Percoll gradient (37%). Yeast cells were permeabilized with sarkosyl essentially according to Elion and Warner (27). Transcription in isolated nuclei was performed according to Kempers-Veenstra *et al.* (29). For transcription in permeabilized cells we used the protocol of Elion and Warner (27). Transcripts were labeled with [α-^{32}P]UTP (3000 Ci/mmol, Amersham) for 10 min at 25° C in the presence of 1 mg/ml α-amanitine.The transcription time of 10 min was chosen as being the optimum to ensure enough incorporation of [α-^{32}P]UTP on the one hand, and to have as little as possible degradation of RNA on the other hand. After transcription, RNA was isolated and precipitated with ethanol. RNA was fragmented by incubation in 25 mM Na_2CO_3 for 10 min at 50°C. After addition of Tris-HCl, pH 7.4 to 40 mM and HCl to 25 mM, RNA was precipitated. Next RNA was treated with 50 U/ml DNase I for 15 min at 37°C, followed by phenol extraction and ethanol precipitation. After hybridization of the RNA to filters containing single-stranded DNA of spacer subclones (see below), filters were incubated with 10 μg/ml RNAase A in 0.5×SSC for 20 min at room temperature. *In vitro* transcription of SP6 DNA was performed according to Melton *et al.* (33). SP6 transcripts were treated the same way as run-on RNA.

Miscellaneous techniques

Yeast transformation was performed by the method of Beggs (34). RNA from transformed cells was usually isolated according to Kraig *et al.* (35). For large scale RNA isolation we used the method described by Verbeet *et al.* (36). Northern blot hybridization was performed as described by Thomas (37). Probes for the analysis of RNA from pARES10.1Δ−149/+111 derivatives were obtained by cloning either the 280 bp EcoRI-XhoI fragment (upstream probe) or the 170 bp XhoI-HindIII fragment (downstream probe), isolated from M13mp9 containing a 450 bp *Spirodela* chloroplast DNA fragment (28), in M13mp19 (digested with EcoRI and SalI or SalI and HindIII, respectively). Probes for the analysis of RNA of pARES2 derivatives were obtained in the same way, but now using M13mp18 as a vector, since, compared to pARES10.1Δ−149/+111 derivatives,

the orientation of the *Spirodela* marker DNA in pARES2 derivatives is reversed. Consequently, this also implies that the upstream probe for analysis of RNA of the latter plasmid is the 170 bp fragment, and that the downstream probe encompasses the 280 bp fragment. Slot blot hybridization was performed according to Kempers-Veenstra *et al.* (38). Labeling of probes, strand separation, S1 nuclease mapping and primer extension were carried out as described before (29). Sequencing reactions were performed according to Maxam and Gilbert (39).

RESULTS

Transcription by yeast RNA polymerase I continues in NTS 1 but not NTS 2

In order to determine the extent to which yeast RNA Pol I transcription proceeds beyond the 3'-end of the 26S rRNA gene we performed *in vitro* run-on experiments in both isolated nuclei and in cells permeabilized by treatment with sarkosyl. Since it is well documented that in isolated nuclei transcription by Pol I initiates correctly, such nuclei presumably constitute a suitable *in vitro* system for analyzing termination. On the other hand, permeabilized cells can be prepared much more rapidly, thus reducing the possibility of loss or inactivation of transcription termination factors. In both systems transcription was carried out in the presence of 1 mg/ml α-amanitine to block transcription by polymerases II and III. [α-^{32}P]UTP-labeled transcripts were analyzed by hybridization to a number of probes covering the whole NTS 1 part of the intergenic spacer, as well as a probe derived from the middle of NTS 2 (*cf.* Fig. 1A). In addition, a probe corresponding to part of the ETS was used as a positive (qualitative) control for Pol I transcription of the 37S rRNA operon (*cf.* Fig. 1A)

In order to be able to correlate the intensity of the hybridization signals with the degree of transcription in the region of the NTS in question, the following precautions were taken. Firstly, the average length of the RNA was reduced by treatment with sodium bicarbonate to about 200 nt in order to avoid competition between probes of different sizes for the same transcript. Secondly, non-hybridizing RNA tails were removed after hybridization by treatment with RNAase. Finally, reference signals were obtained by hybridizing a fragmented [α-^{32}P]UTP labeled SP6 transcript of the complete intergenic spacer (cf. Fig. 1A) to the set of probes under the same conditions. In this way corrections can be made for factors such as the lenght of probe, U-content of the hybridizing RNA and relative stability of the hybrids, all of which will influence the relative intensities of the hybridization signals obtained with the *in vitro* Pol I transcripts. Fig. 1B demonstrates that it is essential to take the results of such control hybridizations into account when interpreting the experimental data, since no linear correlation exists between the intensity of a given hybridization signal and the size of the probe in question (*cf.* the signals of probes C and E which differ in size by a factor 2).

The outcome of a representative experiment using isolated nuclei is depicted in Fig. 1C. It can be clearly seen that Pol I transcription continues for at least several hundreds of basepairs beyond the 3'-end of the 26S rRNA gene since a distinct hybridization signal is obtained with probe D (positions +393 to +721, all coordinates are given relative to the 3'-end of the 26S rRNA gene, unless stated otherwise). Transcription does terminate upstream of the 5S rRNA gene, however, since no signal (above background; see signals obtained with M13 mp10 and mp11 in Fig. 1C) is visible at the position of probe E (positions +721 to +1027). Consistent with this conclusion, no transcripts complementary to probe F (positions +1595 to +1739) could be detected. Comparison of the hybridization patterns

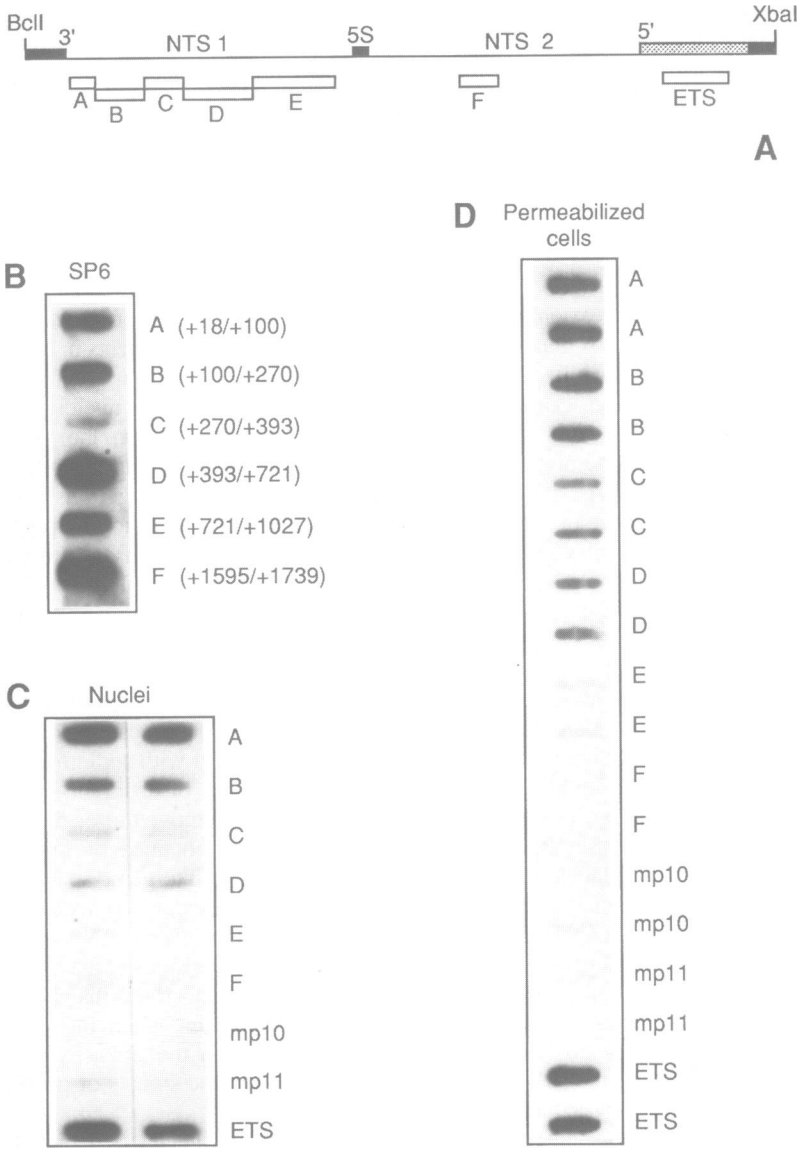

Fig. 1. *Run-on transcription in isolated nuclei and permeabilized cells.*
A: Map of part of the rDNA insert present in pML2ΔB (from BclI to XbaI), encompassing the entire intergenic spacer (NTS 1, 5S rRNA gene and NTS 2). The inserts of the M13 clones A to F, and ETS are also shown. The 5'- and 3'-terminal parts of the rRNA operon are indicated by black bars (rRNA coding sequences) and a stippled bar (external transcribed spacer, ETS). *B*: Hybridization pattern of *in vitro* synthesized SP6 RNA of pML2ΔB with clones A to F. The coordinates of the rDNA inserts present in these clones are also given (relative to the 3'-end of 26S rRNA). *C*: Hybridization pattern of run-on RNA synthesized *in vitro* in isolated nuclei in the presence of 1 mg/ml α-amanitine with the M13 subclones. *D*: Hybridization pattern of run-on RNA synthesized *in vitro* in permeabilized cells in the presence of 1 mg/ml α-amanitine with the M13 subclones.

shown in Figs. 1B and 1C demonstrates that the ratio of the signals obtained with the probes B and A is significantly lower for the run-on RNA (Fig. 1C) than is the case for the control RNA (Fig. 1B). A similar conclusion can be drawn with respect to the D/C ratio, though in this case the reduction is less severe. These results lead us to conclude that attenuation of Pol I transcription in isolated nuclei occurs at at least two sites within NTS 1. One of these sites is located between positions +18 and +270, the other between +270 and +721. Although no hybridization signal is observed with probe E we cannot completely exclude the possibility that a small number of Pol I molecules bypass the upstream attenuation signals and terminate somewhere in region E.

The results of a typical *in vitro* run-on experiment using permeabilized cells are depicted in Fig. 1D. The data are in general agreement with those obtained using isolated nuclei. Pol I transcription is seen to continue beyond the 3'-end of the 37S rRNA operon for several hundreds of basepairs and terminates upstream of the 5S rRNA gene. No transcription of NTS 2 sequences is detectable. A major difference with the results obtained in isolated nuclei, however, is that the B/A signal ratio in the latter type of experiment is only slightly reduced relative to that shown by the control (compare Figs. 1D to 1C and 1B). Thus, contrary to the situation in isolated nuclei, there is no (obvious) transcription attenuation in the region between position +18 and +270 in permeabilized cells. The reason for this discrepancy between the results obtained with the two *in vitro* systems is not clear. Possibly the presence of sarkosyl in the permeabilized cell system interferes with recognition of the first attenuation signal. Several reports describing interference of sarkosyl with transcription can indeed be found in the literature (41, 42). The most likely cause for this interference is thought to be a disturbance of protein-DNA interaction. Thus, sarkosyl might decrease the efficiency of the first attenuation site either by affecting its recognition by Pol I directly or by disturbing its interaction with an ancillary factor required for attenuation.

Taken together, the results described above strongly suggest that termination of transcription by yeast Pol I occurs in NTS 1, though it might still be possible that very low amounts of highly unstable RNA species were not detected in our *in vitro* assay. Furthermore, our findings are basically in line with our previous data on 3'-end formation by yeast Pol I *in vivo*, which indicated the presence of a processing site (T1) at position + 15 to + 50, as well as terminators (T2 and T3) at +210 and around +750 respectively (24). However, the full characterization of transcription termination by Pol I clearly requires a more detailed mapping of these 3'-end generating sites within NTS 1, combined with a functional assay that discriminates between processing and bona fide termination. Such an analysis is described in the following section.

Functional analysis of 3'-end generating sites in NTS 1

Mapping and functional analysis of the 3'-end generating sites in NTS 1 was carried out using the ARES minigene system which has already proven its worth in our previous studies on Pol I transcription in yeast (24, 28, 29). A fragment containing a potential 3'-end generating site was cloned into the middle of the reporter DNA of a minigene and RNA isolated from yeast cells transformed with the minigene was then analyzed by Northern hybridization using specific probes for reporter sequences both up- and downstream of the inserted fragment. If the fragment contains a processing site, the (resulting) processed transcripts will be detected by either type of probe. If, however, a terminator is present in the fragment only the upstream probe will detect a specific minigene transcript. Readthrough transcripts will hybridize to both probes (*cf.* Fig. 3A). For functional analysis

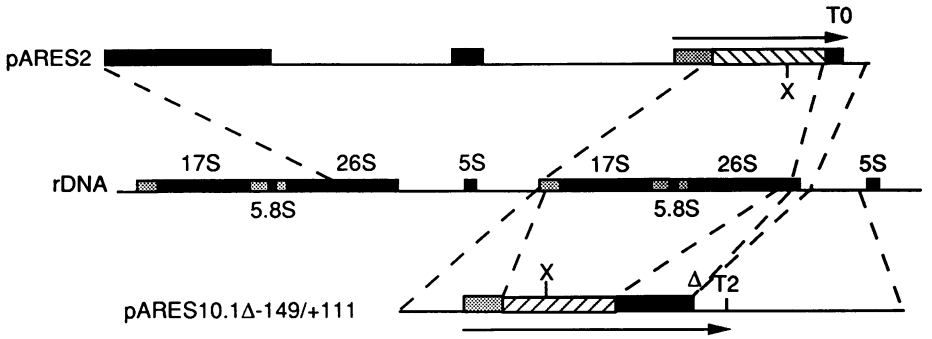

Fig. 2. *Parent minigenes used in this study.*
In the middle, two rDNA repeats are schematically represented. Above and below the two parent minigenes used in our study are shown. Stippled lines indicate which rDNA sequences constitute these plasmids. The promotorfragment in pARES2 ranges from −4328 to +128, and in pARES10.1Δ−149/+111 from −207 to +128 (coordinates relative to the transcription start site). The 3′-end generating fragment in pARES2 extends from −36 to +101, and in pARES10.1Δ−149/+111 from −527 to +1027 with a deletion (Δ) from −149 to +111 (coordinates relative to the 3′-end of 26S rRNA). Black bars and stippled bars: see legend to Fig. 1. The orientation of the 450 bp marker DNA fragment in the two plasmids is different (indicated by hatched bars). × = XhoI restriction site, located 170 bp (pARES10.1Δ−149/+111) or 280 bp (pARES2) downstream of the 5′-end of marker sequence. Arrows represent the minigene transcripts of pARES2 and pARES10.1Δ−149/+111, with 3′-ends at T0 and T2, respectively.

of T1 and T2, fragments covering positions −70 to +100 and +100 to +393 of NTS 1 respectively were inserted into the unique XhoI site present in the reporter segment of plasmid pARES10.1Δ−149/+111 (Fig. 2) giving rise to plasmids pT1 and pT2 (Fig. 5). Functional analysis of site T3 was carried out using a 462 bp fragment extending from position +721 to +1183. In order to facilitate detection of the readthrough transcript, this fragment was cloned into pARES2 instead of pARES10.1Δ−149/+111, however (Fig. 2). The readthrough RNA of the T3 derivative of the latter plasmid would be about 1550 nt long and therefore difficult to detect against the background due to aspecific hybridization with the chromosomal 17S rRNA. The readthrough transcripts of pT3 (Fig. 4B), derived from pARES2, on the other hand, would have a lenght of about 1100 nt. Finally, plasmid pT1R was constructed by insertion of the −70/+100 fragment into pARES10.1Δ −149/+111 in the reverse orientation.

The results of the Northern analysis using the upstream probe are shown in Fig. 3B. Although there is significant (aspecific) hybridization with plasmid DNA, as well as 26S and 17S rRNA, signals due to minigene transcripts can clearly be observed for all four types of transformants. The lengths of the upstream transcripts of plasmids pT1 (ca 450 nt) and pT2 (ca 500 nt) agree with formation of 3′-ends at site T0 (3′-end of 26S rRNA, also present in the −70/+100 insert of pT1) and T2 (+210) respectively. Moreover, using 3′ S1 nuclease mapping we confirmed the position of these 3′-ends (data not shown). The length of the upstream transcript derived from plasmid pT3 (ca 550 nt), however, indicates 3′-end formation to have occured at a position about 200 nt downstream of the one deduced for T3 from our previous *in vivo* analysis using deletion mutant pARES10.1Δ−149/+282, which lacks T0, T1 and T2 (Fig. 4A; 24) Consequently, we decided to map the 3′-end generating site in the pT3 transformant as well as in the deletion mutant more accutately, using the probes indicated in Figs. 4A and B. The results demonstrate that 3′-end formation

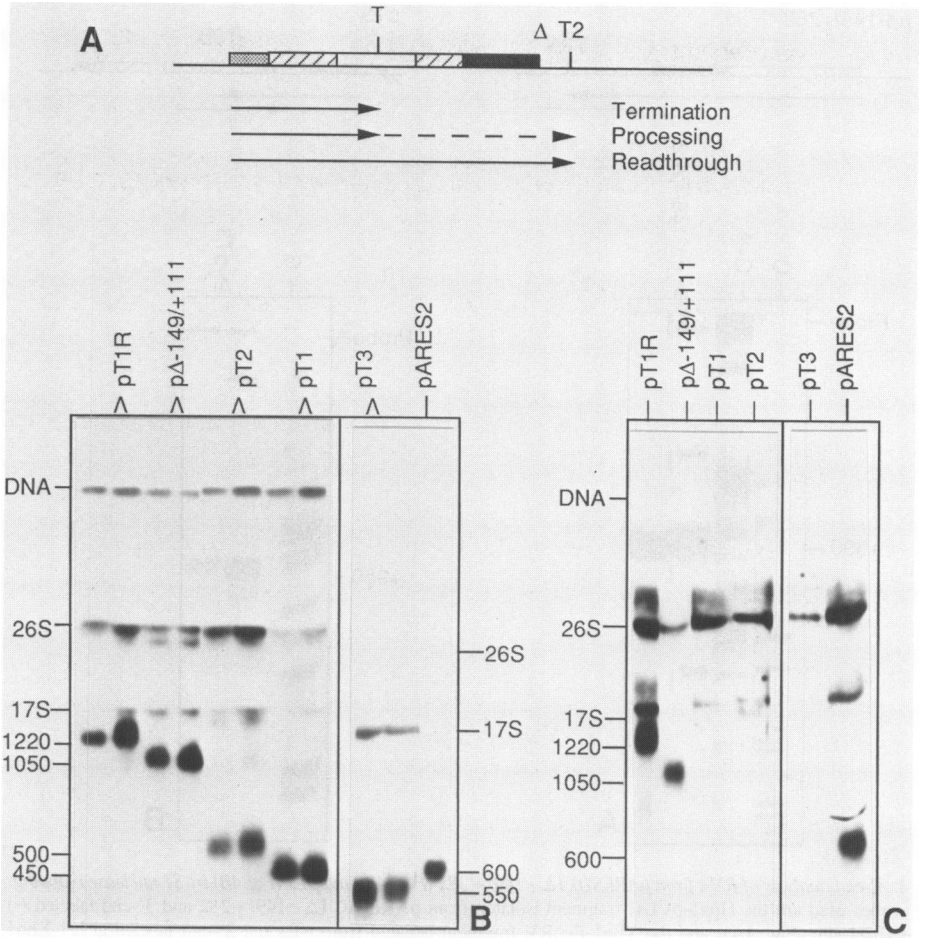

Fig. 3. *Functional analysis of 3'-end generating sites in NTS 1.*
A: Assay system for the functional analysis of 3'-end generating sites, outlined using a pARES10.1Δ−149/+111 derivative as an example. See text for explanation. *B* and *C*: Northern blot hybridization of RNA isolated from yeast cells transformed with either pARES2, pARES10.1Δ−149/+111, pT1, pT1R, pT2 or pT3, using either the upstream probe (Fig. 3B), or the downstream probe (Fig. 3C). These probes (see Materials and Methods) differ for pARES10.1Δ−149/+111 derivatives (left panels in Figs. 3B and 3C) and pARES2 derivatives (right panels in Figs. 3B and 3C). The length of each minigene transcript is given next to the autoradiograph. 20 μg of RNA was fractionated on a 1.6% agarose gel and blotted onto a Hybond filter.

in the previously constructed deletion mutant occurs predominantly at a position +690 (Fig. 4A), and not +750 as previously concluded. This site, now called T3A, is not included in the insert present in pT3. However, the pT3 insert does contain a 3'-end generating site (T3B) which maps mainly at position +950 (Fig. 4B). Hence a total of four 3'-end generating sites is identified in NTS 1 by analysis of the various minigene constructs.

Fig. 3B also demonstrates that hardly if any readthrough transcripts accumulate in cells transformed with pT1, pT2, and pT3, indicating that these 3'-end generating sites, are

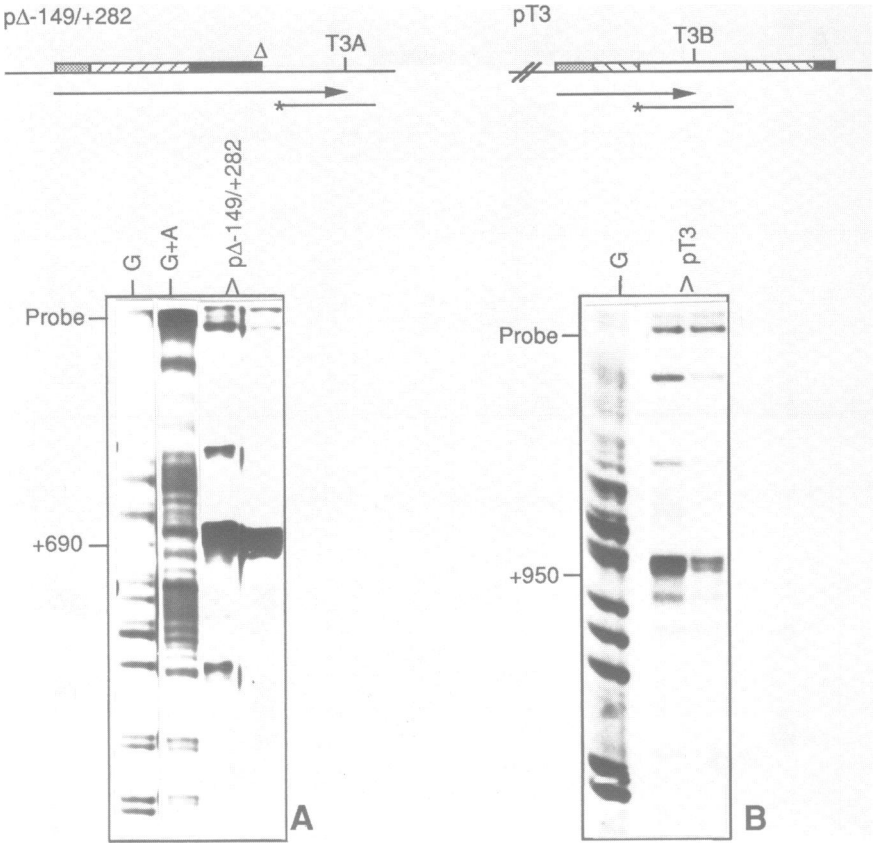

Fig. 4. *3'-end analysis of RNA from pARES10.1Δ−149/+282 (Fig. 4A) and pT3 (Fig. 4B) by S1 nuclease mapping.* The probes used are the Hpa1-pVUII fragment isolated from pARES10.1Δ−149/+282 and 3'-end labeled (*) at the Hpa1 site (Fig. 4A), and the Xho1-EcoRV fragment isolated from pT3 and 3'-end labeled at het Xho1 site (Fig. 4B). 25 μg of RNA was hybridized to either of the probes. Samples were treated with 25 units (left lane) or 75 units (right lane) of S1 nuclease. Lanes A and A+G show chemical modifications of the probes according to Maxam and Gilbert (39).

highly efficient. The one construct that does cause accumulation of readthrough transcripts is pT1R, in which has the 'T1 fragment' was cloned in the opposite orientation. Thus, 3'-end formation at T0, as well as T1, is orientation dependent.

Fig. 3C shows the outcome of a Northern blot hybridization using the downstream probe. No fragments corresponding to possible processing products of pT1, pT2 and pT3, which should range in size from ca 550 to ca 750 nt, can be detected in this blot. We do observe the transcripts of the control minigenes and the readthrough RNA of pT1R, however. Although this result might be interpreted to indicate termination of transcription at T1, T2 and T3B, one also has to consider the possibility that the 'downstream fragments' resulting from processing may be very unstable and hence not detectable by the relatively insensitive Northern analysis. A precedent can be found in the very unstable processed spacer transcripts in *Xenopus laevis* (13, 14). Therefore we decided to turn to the more

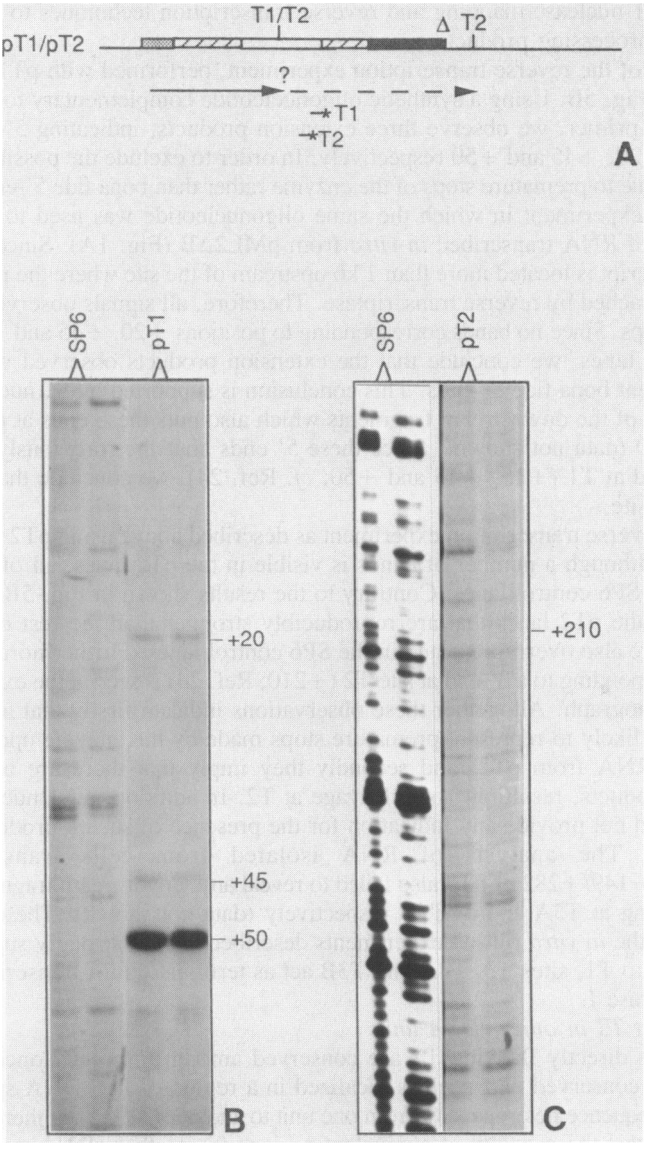

Fig. 5. *Primer extension analysis of RNA of pT1 and pT2.*
A: General representation of the type of experiment. See text for explanation. *B* and *C*: Primer extension analysis of pT1 RNA (Fig. 5B) and pT2 RNA (Fig. 5C). In both experiments, RNA isolated from two independent transformants was used. Primers used are 5′-end labeled (*) oligonucleotides that are either complementary to sequences in pT1 extending from +89 to +108 (relative to the 3′-end of 26S rRNA) (Fig. 5B), or complementary to sequences in pT2 ranging from +291 to +310 (Fig. 5C). Note that this latter primer can also anneals to the few (if any) RNA molecules that bypass the second T2 at the 3′-end of the minigene. *In vitro* synthesized SP6 RNA (5 and 1 ng, respectively) of pML2ΔB was used as a control in both experiments.Total extension reactions were performed.

sensitive 5′ S1 nuclease mapping and reverse transcription techniques to detect possible downstream processing products.

The results of the reverse transcription experiment, performed with pT1 derived RNA are shown in Fig. 5B. Using a synthetic oligonucleotide complementary to positions +89 to +108 as a primer, we observe three extension products, indicating 5′-end formation at positions +20, +45 and +50 respectively. In order to exclude the possibility that these products are due to premature stops of the enzyme rather than bona fide 5′-ends, we carried out a control experiment in which the same oligonucleotide was used to prime reverse transcription of RNA transcribed *in vitro* from pML2ΔB (Fig. 1A). Since the 5′-end of this SP6 transcript is located more than 1 kb upstream of the site where the primer anneals, it cannot be reached by reverse transcriptase. Therefore, all signals observed must be due to artificial stops. Since no bands corresponding to positions +20, +45 and +50 are visible in the control lanes, we conclude that the extension products observed with pT1 RNA indeed represent bona fide 5′-ends. This conclusion is supported by S1 nuclease mapping of the 5′-ends of the downstream fragments which also puts these ends at positions +20, +45 and +50 (data not shown). Since these 5′ ends abut the (previously identified) 3′ ends generated at T1 (+15, +45 and +50; *cf.* Ref. 24), we conclude that T1 is indeed a processing site.

A similar reverse transcription experiment as described above using pT2 RNA is shown in Fig. 5C. Although a number of bands is visible in the pT2 lanes, all of these are also present in the SP6 control lanes. Contrary to the results shown in Fig. 5B, however, the few bands in the pT2 lanes that are reproducibly stronger than the rest of the bands in these lanes, are also overrepresented in the SP6 control lanes. Furthermore, no extension product corresponding to a 3′-end at site T2 (+210; Ref. 24) is seen at the expected position in the autoradiograph. Altogether these observations indicate firstly that all bands in the pT2 lanes are likely to represent premature stops made by the enzyme upon transcribing readthrough RNA from pT2, and secondly they imply that there are no downstream processing products, resulting from cleavage at T2. In addition, a S1 nuclease mapping experiment did not provide any indication for the presence of such a product either (data not shown). The analysis of RNA isolated from cells transformed with pARES10.1Δ−149/+282 or pT3 also failed to reveal any 'downstream fragments' resulting from processing at T3A and/or T3B respectively (data not shown). These results taken together with the *in vitro* run-on experiments described above, strongly support the view that, contrary to T1, sites T2, T3A and T3B act as terminators for transcription by yeast RNA polymerase I.

Termination at T2 in other rDNA units

The sequences directly flanking T2 are conserved among different cloned yeast rDNA units, but this conserved part itself is localized in a region of the rDNA spacer showing considerable sequence heterogeneity from one unit to another (31, 43). It therefore appeared neccessary to test the capacity of equivalent regions from other rDNA units to generate 3′ ends at the corresponding site. To this end we replaced the 3′ terminal part of pARES10.1Δ−149/+111 (from +111 to +1003) by similar fragments of two other units that display sequence variation around T2 (Fig. 6A). Northern analysis showed that each of these constructs (pARES12.2 and pARES12.9) produces transcripts comparable in size to the one transcribed from pARES10.1Δ−149/+111 (data not shown) indicating that 3′-end formation at or near T2 has occurred in both constructs. An S1 nuclease mapping experiment located the 3′-ends of the pARES12.2 and pARES12.9 transcripts at precisely

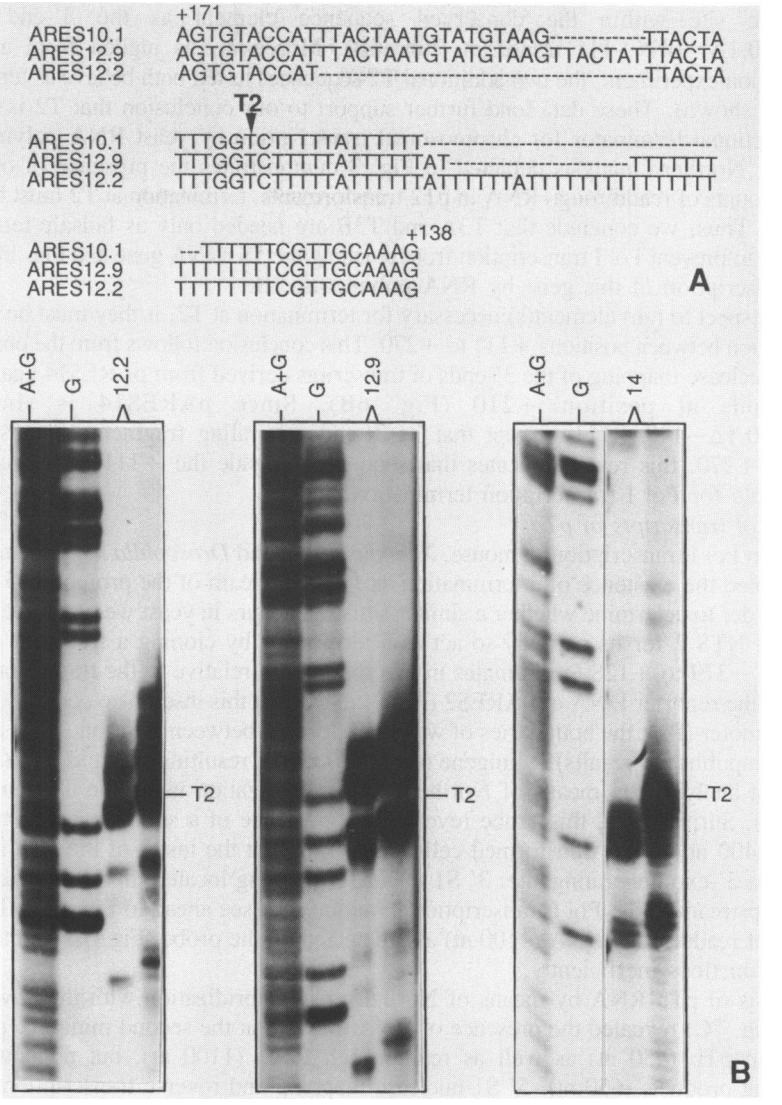

Fig. 6. *Mapping of T2 in different rDNA units.*
A: Sequence comparison of the region flanking T2 in three independently cloned rDNA units of *Saccharomycetoideae*. The upper lane shows the sequences surrounding T2 in pARES10.1Δ−149/+111 and the lower lanes represent a similar part of the NTS 1 found in two other cloned rDNA units, and contained in pARES12.2 and pARES12.9 respectively. Lines indicate that the sequence is continuous. *B*: 3′-end analysis of RNA of pARES12.2 (i), pARES12.9 (ii) and pARES14 (iii) by S1 nuclease mapping. Used probes: (i and ii) BamHI-HpaI fragments, 3′-end labeled at the BamH1 site and isolated from SP64 subclones containing part of the 3′-end generating fragments contained in pARES12.2 and 12.9.; (iii) AccI-HincII fragment, 3′-end labeled at the AccI site, and isolated from pARES14. In all 3′ S1 nuclease mapping experiments a doublet of bands is seen, of which the upper represents T2 (24). Samples were treated with 25 units (left lanes) or 8.3 units (right lanes) of S1 nuclease. See also the legend to Fig. 4.

the same site within the conserved sequence element as the 3'-end of the pARES10.1Δ−149/+111 transcript (Fig. 6B). Moreover, as jugded from a reverse transcription experiment, the two additional T2 sequences tested both behave as terminators (data not shown). These data lend further support to our conclusion that T2 is likely to be a functional terminator for chromosomal transcription by yeast RNA polymerase I. Since the Northern analysis depicted in Fig. 3 demonstrates the presence of only very small amounts of readthrough RNA in pT2 transformants, termination at T2 must be highly efficient. Thus, we conclude that T3A and T3B are needed only as failsafe terminators in NTS 1 to prevent Pol I transcription from reaching the 5S rRNA gene and thus interfering with transcription of this gene by RNA polymerase III.

With respect to (an) element(s) necessary for termination at T2, it/they must be confined to the region between positions +111 to +270. This conclusion follows from the observation that S1 nuclease mapping of the 3'-ends of transcripts derived from pARES14 again places these ends at position +210 (Fig. 6B). Since pARES14 is similar to pARES10.1Δ−149/+111, except that its 3'-end generating fragment extends to only position +270, this result indicates that sequences outside the +111/+270 region are dispensable for Pol I transcription termination.

Analysis of transcripts of pTp

Studies on Pol I transcription in mouse, *Xenopus laevis* and *Drosophila melanogaster* have demonstated the existence of a termination site just upstream of the promoter (5, 13, 14, 19). In order to determine whether a similar situation occurs in yeast we tested the relevant region of NTS 2 for its capacity to act as a terminator by cloning a fragment covering positions −379 to +128 (coordinates in this section are relative to the transcription start site) into the reporter DNA of pARES2 (Fig.2). Note that this insert also contains a second Pol I promoter (P2), the boundaries of which are located between position −142 and +18 (29 and unpublished results). Minigene transcripts of the resulting plasmid pTp (Fig. 7A) were first analyzed by means of Northern blot hybridization using the upstream probe (Fig. 7B). Surprisingly, this probe revealed the presence of a short upstream transcript of about 400 nt in pTp transformed cells, indicating that the insert of the pTp minigene contains a 3'-end generating site. 3' S1 nuclease mapping located this site, called Tp, at 300 bp upstream of the Pol I transcription initiation site (see ahead to Fig. 8B). The large amount of readthrough RNA (1100 nt) also detected by the probe (Fig. 7B) demonstrates that Tp functions inefficiently.

Analysis of pTp RNA by means of Northern blot hybridization with the downstream probe (Fig. 7C) revealed the presence of RNA initiated at the second minigene promoter present in pTp (450 nt) as well as readthrough RNA (1100 nt), but no downstream processing products (650 nt). 5' S1 nuclease mapping and reverse transcription analysis (data not shown) also failed to detect any 'downstream fragments', corresponding to transcripts processed at Tp, indicating that Tp acts as a terminator (albeit an inefficient one) rather than a processing site.

To determine whether the low efficiency of Tp was due to an insufficient amount of flanking sequences in pTp, we constructed pARES13 (Fig. 8A). In this minigene the 3'-terminal part of pARES10.1Δ−149/+111 (Fig. 2) is replaced by a fragment (−1464 to +509) containing considerably more Tp flanking sequences than the 3'-end generating fragment in pTp (−379 to +128). The efficiency of 3'-end formation at Tp in pARES 13 was analyzed by means of a S1 nuclease mapping (Fig. 8B). As shown in Fig. 8B, Tp maps at 300 bp upstream of the transcription start site. Comparison of the relative

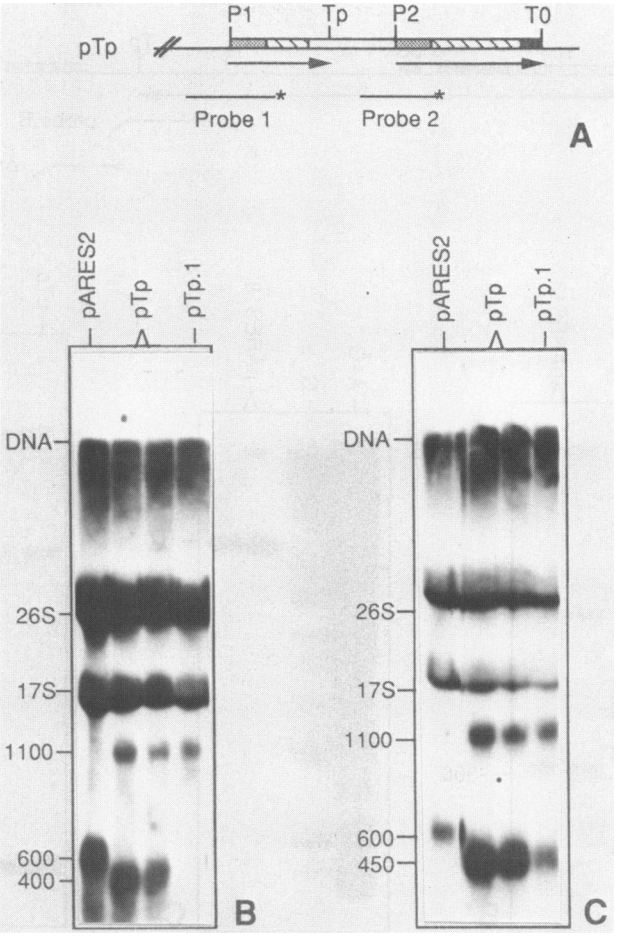

Fig. 7. *Northern analysis of RNA of pTp and pTp.1.*
A: Map of pTp. The positions of the first and the second minigene promoter (P1 and P2), as well as that of Tp and T0 are indicated. Probes used for the 5′ S1 nuclease mapping shown in Fig. 8D are also depicted. *B* and *C*: Northern blot hybridization of RNA from yeast cells transformed with either pARES2, pTp or pTp.1 with either the upstream probe (Fig. 7B) or the downstream probe (Fig. 7C). See also the legend to Fig. 3.

intensities of the signals representing 3′-end formation at Tp and readthrough RNA respectively, shows, however, that also in pARES13 only 60% of the Pol I molecules reaching Tp actually generates a 3′-end at this site. Thus, the fact that Tp functions inefficiently in our minigene system, is unlikely to be caused by an insufficient amount of flanking sequences.

Next we asked ourselves whether polymerases that bypass Tp, encounter other termination sites before reaching the transcription start site. As demonstrated by different S1 nuclease mapping experiments using pARES13 derived RNA, however, there are no further 3′-end generating sites present between Tp and the transcription start site of the 37S operon. E.g.

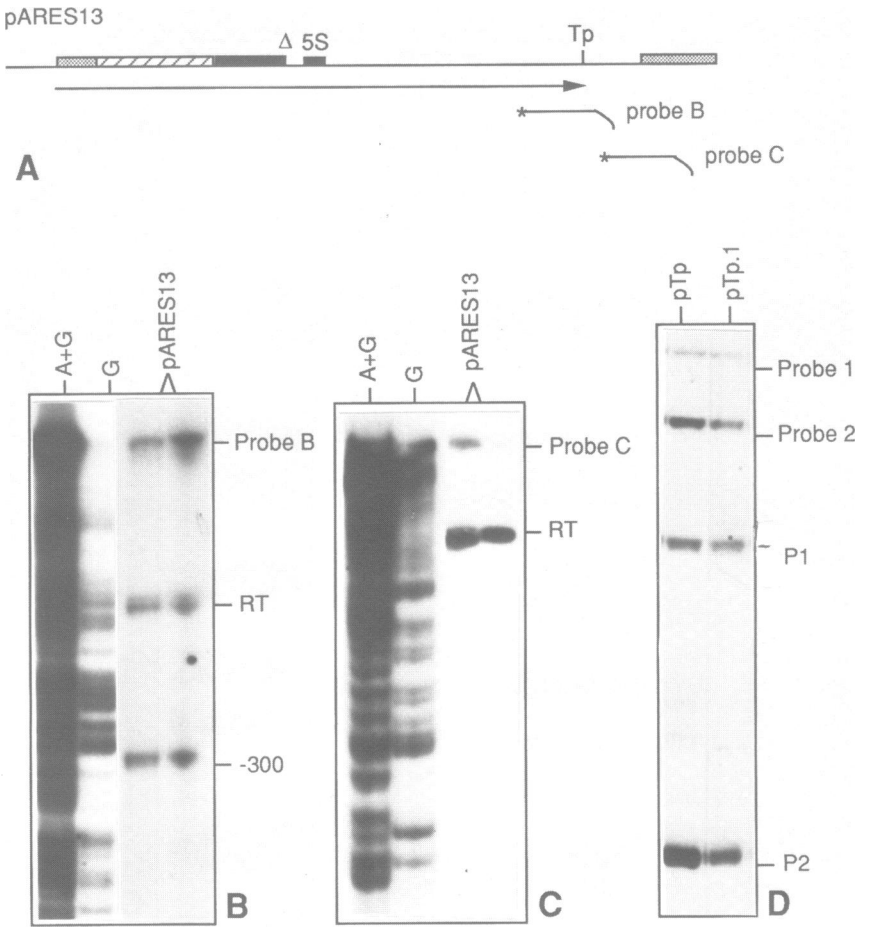

Fig. 8. *Efficiency of Tp, and effect of Tp on the promoter.*
A: Map of pARES13. Note that in this construct 5S rRNA sequences are transcribed by Pol I as part of the minigene transcript. Since, however, it is not known whether the 5S rRNA gene in pARES13 is transcribed by Pol III, we cannot draw any conclusion about transcription interference between Pol I and Pol III in pARES13. *B* and *C*: 3′-end analysis of pARES13 RNA using S1 nuclease. Used probes are (B) the Fok1-HaeIII fragment, 3′-end labeled at the Fok1 site and isolated from a M13 subclone containing most of the 3′-end generating fragment of pARES13, or (C) the BstEII-Xho1 fragment, 3′-end labeled at the BstEII site and isolated from pARES2. The 5′ terminal part of both probes is non homologous to the template. Thereby the readthrough signals (RT) become distinguisable from the bands representing left-over of the probes. See also the legend to Fig. 4. *D*: 5′-end analysis of pTp RNA and pTp.1 RNA using S1 nuclease mapping. The probes used, depicted in Fig. 7A, are (1) the SmaI-ClaI fragment, 5′-end labeled at the ClaI site, and isolated from pTp, and (2) the SmaI-EcoRV fragment, 5′-end labeled at the EcoRV site, also isolated from pTp. The probes differ in sequence and lenght at their 5′ terminal part to make them promotor specific and the protected fragments they give rise to distinghuisable from one another. A mixture of both probes was hybridized to pTp RNA or pTp.1 RNA; samples were subsequently threated with 75 units of S1 nuclease. P1: transcription initiation at the first promotor in the minigenes (internal standard), P2: transcription initiation at the second promotor of the constructs. Only the ratio of signals P1 and P2 between the two lanes may be compared.

in the experiment depicted in Fig. 8C, the probe used, extending to position 128 downstream of the start site, reveals only the presence of readthrough RNA and no signals due to RNA molecules with a 3'-end mapping between Tp and the start site. This implies that in pARES13, as is the case in pTp (*cf.* readthrough RNA in pTp lanes in Fig. 7B) Pol I molecules that bypass Tp read into the downstream promoter.

With respect to a number of recent studies on the relevance of promoter occlusion (21, 22, 23), it is noteworthy that in the Northern blot hybridization of RNA isolated from pTp transformants using the downstream probe (Fig. 7C) minigene transcripts initiated at the second promoter of pTp (450 nt) are clearly observed, in addition to readthrough RNA (1100 nt). We interprete this observation as an indication that incoming Pol I transcription at the second minigene promoter in pTp does not prevent transcription initiation at this promoter, though one can argue that there is no direct proof that in pTp tranformants, readthrough transcription and transcription initiation at the second minigene promoter occur on the same molecules.

In conclusion, our results strongly suggest that in yeast, similar to the situation in other organisms, a terminator (Tp) is present upstream of the promoter. However (at least in our testsystem) a considerable amount of polymerases does not recognize Tp and subsequently reads into the downstream promoter.

Effect of Tp on the promoter

In *Xenopus laevis* and mouse the presence of the terminator upstream of the promoter has been shown to stimulate transcription initiation by Pol I (7, 8, 15, 16). For this reason we compared the extent of transcription initiation at the additional promoter present in the pTp minigene to that at the same promoter in a derivative (pTp.1), in which termination at Tp has been abolished by inserting a linker into the SmaI site (−207) downstream of Tp. Pol I transcription initiated at the first promoter in pTp.1 produces only readthrough RNA as shown by Northern hybridization using the upstream probe (*cf.* Fig. 7B). Apparently the linker insertion at SmaI destroys an element essential for termination at Tp. Quantitative comparison of transcription initiation at the second minigene promoter in pTp and pTp.1 was carried out by using transcription initiation at the first promoter of these genes as an internal standard. To this end we performed a 5' S1 nuclease mapping using two probes simultaneously (Fig. 7A). The first one measures the level of transcription initiation at the first promoter (P1), the second one that at the downstream promoter (P2). The result of such an experiment is depicted in Fig. 8D. By comparing the P1/P2 ratio between the two lanes in Fig. 8D, we infer that termination at Tp indeed has a slight stimulatory effect (ca 25 %) on transcription initiation. This, of course, is a rather limited effect with respect to the results obtained with other organisms. In both *Xenopus laevis* and mouse, the terminator found adjacent to the promoter is considered to be an essential element of this promoter, since deletion of the terminator sequences causes a drop in the level of transcription initiation of 50 to 100 % (7, 8, 15, 16).

DISCUSSION

In this paper we describe the identification of termination sites for yeast RNA polymerase I. A summary of the results is given in Fig. 9. For our study, we have followed different strategies, since the identification of termination sites is hampered by the fact that (i) 3' terminal extensions of primary transcipts, once processed, are turned over very rapidly, and (ii) 3'-ends formed by termination are indistinguishable from those generated by processing.

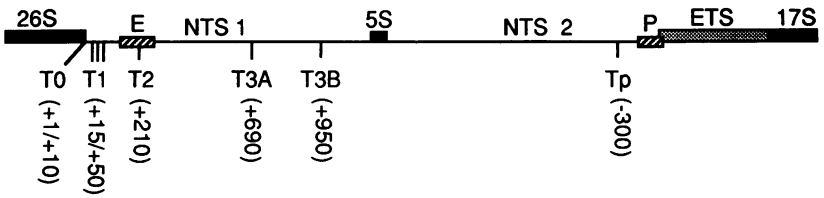

Fig. 9. *Schematic representation of all 3'-end generating sites.*
The position of all 3'-end generating sites within the yeast intergenic spacer is indicated. Coordinates in NTS 1 are relative to the 3'-end of 26S rRNA, and in NTS 2 relative to the transcription start site. T0 and T1 are processing sites, whereas T2 (within the enhancer: E, hatched bar), T3A, T3B and Tp are termination sites. P (hatched bar) represents the promoter. See also the legend to Fig. 1.

As no faithful template-dependent *in vitro* transcription system for yeast has been described as yet, we performed *in vitro* run-on transcription in both isolated nuclei and permeabilized cells. Although small differences between the results of the two systems were observed, both sets of results provided evidence that termination of transcription by yeast Pol I occurs upstream of the 5S rRNA gene within NTS 1.

Next, we have tested previously identified 3'-end generating sites in NTS 1 (24) for their capacity to act as a terminator. To this end, fragments containing a 3'-end generating site were cloned into the middle of the reporter DNA of an ARES minigene. Using different techniques (Northern blot hybridization, S1 nuclease mapping and reverse transcription analysis) we analyzed RNA of yeast cells transformed with such minigenes for the presence of transcripts both up- and downstream of the inserted 3'-end generating sequences. With respect to T1, we identified downstream processing products which 5'-ends (+20, +45, +50) (almost) perfectly match the 3'-ends generated at this site (+15, +45, +50; Ref. 24). We therefore conclude that T1 is a processing site. With respect to T2 (+210), on the other hand, the analysis did not reveal any downstream processing product resulting from cleavage at this position. This result together with the fact that 3'-end formation at T2 is highly efficient and furthermore also takes place in different rDNA units at a similar position, strongly supports the view that T2 is a functional terminator for transcription by yeast RNA polymerase I. Apart from T2, we identified two other terminators T3A (+690) and T3B (+950) in NTS 1, that might function as fail safe terminators to prevent those Pol I molecules that do not recognize T2 from reaching the 5S rRNA gene.

Comparison of the regions surrounding T2, T3A and T3B did not reveal any sequence conservation that might mask (an) *cis*-acting element(s) required for transcription termination by Pol I. Our results indicate that as far as T2 (+210) is concerned, the sequence elements for termination of transcription at this site must be confined to a region encompassing +111 to +270. Recent studies performed by Mestel *et al.* (44) suggest that these requirements may even be located between position +120 to +211. Taking into consideration the *cis*-acting elements identified for termination by Pol I in other organisms (e.g. the 18 bp SalI box in mouse (5) or the 7 bp T3 box in *Xenopus laevis* (16)), it is likely that also in yeast the real sequence motif that is responsibe for termination at T2, is much smaller. Its identification, however, has to await further analysis.

Altogether, both our *in vitro* results and *in vivo* analysis indicate that there is no Pol I transcription beyond the 5S rRNA gene in NTS 2. Also, contrary to the situation in e.g. *Xenopus laevis* and mouse (reviewed in Ref. 3), no (*in vivo*) spacer promoters have been identified within the yeast intergenic spacer. Nevertheless, in addition to the termination

sites in NTS 1, we have also identified a terminator in NTS 2 located shortly upstream of the Pol I promoter, which we have designated Tp (−300). Whereas the NTS 1 terminators are very efficient, Tp appears to be rather inefficient. Furthermore, termination at Tp does not have a significant enhancing effect on transcription initiation at the downstream promoter, as is the case in *Xenopus laevis* and mouse (7, 8, 15, 16).

We are currently investigating in what way, if any, termination at Tp is involved in the regulation of Pol I transcription. As stated above, at present there are no candidate transcripts that can account for 3'-end formation at Tp, that is at least under normal growth conditions. Possibly, spacer transcription terminating at Tp occurs in response to alterations in growth conditions (e.g. medium upshift). Such a mechanism might contribute to the rapid increase in Pol I transcription observed under such circumstances (1).

Regulation of Pol I transcription in yeast

The experiments described in this paper demonstrate that, contrary to the situation in *Xenopus laevis* (13, 14) and *Drosophila melanogaster* (18), yeast Pol I does not transcribe the entire spacer separating two consecutive rDNA units. Instead, similar to Pol I transcription in mouse and human (5, 12), termination occurs just downstream of the mature rRNA sequences. The main yeast Pol I terminator is T2, which is located within the previously identified Pol I enhancer element (26, 27). This striking result further supports the model for yeast Pol I transcription that we have previously proposed (24). In this so-called 'ribomotor' model each Pol I transcription unit forms a loop causing juxtaposition of its Pol I promoter and the Pol I terminator/enhancer element. Pol I molecules that terminate at T2, are immediately transferred back to the promoter by the action of the enhancer. Consequently, Pol I molecules once they have initiated transcription do not re-enter the free pool, but are efficiently recycled. In a slightly different version of the model the terminator/enhancer element of one rDNA unit is juxtaposed to the promoter of the next one causing hand-over of Pol I molecules from one transcription unit to another. Experiments are in progress to discriminate between these alternatives, and to further prove the validity of the model.

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