2252–2258 Nucleic Acids Research, 1995, Vol. 23, No. 12

The *Xenopus* 9 bp ribosomal terminator (T3 box) is a pause signal for the RNA polymerase I elongation complex

Paul Labhart

The Scripps Research Institute, Department of Molecular and Experimental Medicine, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

Received February 2, 1995; Revised and Accepted April 28, 1995

ABSTRACT

In *Xenopus*, termination by RNA polymerase I (pol I) is mediated by the 9 bp sequence GACTTGCNC and RNA 3′-ends are formed ~15–20 nt upstream of this terminator element. Here I show that this 9 bp element, also called the 'T3 box', is a pause signal for the elongating transcription complex. The two major transcripts in the paused complex have 3′-ends mapping to 15 and 21 nt upstream of the T3 box, remain bound to the template in 0.25% Sarkosyl, are subject to pyrophosphorolysis and can be chased into longer transcripts. Mutations that reduce overall termination also affect pausing, indicating that pausing is a limiting step in the termination process. Oligonucleotide competition experiments, furthermore, suggest that pausing requires a DNA binding factor. The data support a model in which the first step leading to transcription termination by pol I in *Xenopus* is pausing of the elongation complex upstream of the T3 box.

INTRODUCTION

Each *Xenopus* ribosomal gene repeat contains two sites of RNA 3′-end formation and transcription termination: T2 at the 3′-end of the 40S precursor coding sequence and T3 upstream of the gene promoter. Sites T2 and T3 share a short sequence element located 15–20 nt downstream of the RNA 3′-ends and the same element is conserved between *X.laevis* and *X.borealis* (1). Mutagenesis identified the 9 bp signal GACTTGCNC ('T3 box') as the minimal terminator for polymerase I (pol I) (2–5). McStay and Reeder (6) showed that termination at T3 can be competed out with excess T3 sequences, suggesting that termination requires a DNA binding termination factor. Using T3 affinity chromatography the same authors purified a factor, Rib2, that produced a footprint on the T3 site.

We had reported that in oocyte injection assays a mutation in the last (9th) nucleotide of the termination signal (C→G) strongly affected termination activity, while leaving the ability to form RNA 3′-ends intact (4). This uncoupling of RNA 3′-end formation and termination indicated that the RNA 3′-end is formed by some rapid cleavage of the nascent RNA chain, rather than by cessation of RNA synthesis. Interestingly, this mutation of the 9th nucleotide is naturally found at the *X.laevis* T2 site, which efficiently forms RNA 3′-ends of the 40S precursor but allows a considerable level of read-through transcription into the spacer (1,7). *In vitro* T2 behaves like a weak terminator and does not detectably bind Rib2 (6).

The mechanism of termination by pol I is most likely very similar in different eukaryotic organisms. In mammals termination signals are found at similar locations in the ribosomal gene repeat as in *Xenopus* (reviewed in 8). In mouse pol I termination is mediated by the so-called 'Sal box' located 11 bp downstream of the primary RNA 3′-ends (9,10). The cloning of TTF-I, the pol I-termination factor binding to the Sal box, has very recently been reported (11). The organization of termination sites on the yeast ribosomal gene repeat appears to differ from the organization in vertebrates, in that there is no evidence for a T3 homolog upstream of the gene promoter and in that the terminator consists of two distinct sequence elements (12,13). However, similarly to the situation in vertebrates, termination is mediated by a DNA binding protein (Reb1p).

It is clear that the entire process of transcription termination can be separated into several distinct steps. For all three classes of eukaryotic RNA polymerases there is evidence that pausing is the first step leading to transcription termination (13–16). Other subsequent steps would be transcript release, dissociation of RNA polymerase from the DNA and trimming or processing of the RNA 3′-end. Here I show that the 9 bp *Xenopus* pol I termination element is a pause signal for the elongating transcription complex. The results are discussed in the light of a recently proposed model for transcription termination by pol I (13).

MATERIALS AND METHODS

Preparation of immobilized templates

*Hin*dIII-restricted plasmid MH-T3wt (17) was biotinylated with biotin–14-dATP (GIBCO BRL, cat. no. 19524-016) and Klenow DNA polymerase. The biotinylated DNA was separated from the unincorporated nucleotides on Sephadex G-25 and by ethanol precipitation in the presence of 2 M ammonium acetate. After digestion with *Ssp*I the biotinylated DNA was incubated with streptavidin-coated paramagnetic particles (Promega, cat. no. Z5241 or Dynal, cat. no. 112.05) in 20 mM Tris–HCl, pH 8, 0.2 mM EDTA containing either 0.1 M NaCl or 1 M NaCl. After 1–3 h end-over-end rotation at room temperature, the beads were washed twice in the same buffer and finally resuspended in dialysis buffer (20% glycerol, 20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) containing

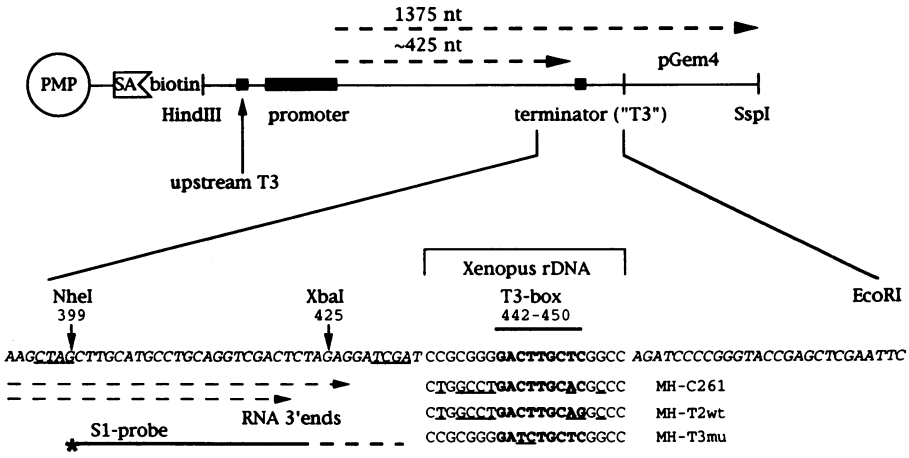

Figure 1. Diagram of the immobilized minigene construct. The plasmid MH-T3wt (17) was attached to paramagnetic particles (PMP) by a streptavidin (SA)–biotin linkage to the *Hin*dIII site upstream of the promoter. The map is not to scale. The 9 bp elements in both the upstream T3 and the downstream terminator are indicated by small black boxes. The terminated and run-off transcripts and their predicted sizes are indicated above the map. The nucleotide sequence of the multiple cloning site of pGem4 and of the various synthetic terminators cloned into its *Bam*HI site are shown below. The 20 bp corresponding to natural *Xenopus* rDNA sequences are bracketed and the 9 bp terminator element (T3 box) is printed in bold. Note that despite having only 20 bp of T3 sequences, the entire termination site on the present construct is referred to as 'T3' throughout the paper. The nucleotides in MH-T2wt and in the mutants that differ from the MH-T3wt sequence are underlined. A *Hin*dIII and *Bam*HI site present in the original construct were destroyed by filling-in the recessed ends followed by religation, resulting in two 4 bp insertions (underlined). The numbering of the nucleotides is relative to the initiation site at the promoter. The last nucleotide transcribed from a minigene truncated with *Xba*I and the nucleotide in the *Nhe*I site that was labeled for the preparation of the S1 probe are indicated by arrows. The location of the transcripts in the paused complex and the 3′-end of the S1 probe are shown below.

100 μg/ml BSA and 0.02% NaN_3. The amount of DNA bound was estimated by proteinase K–SDS extraction of a small aliquot and gel electrophoresis alongside known amounts of DNA.

Preparation of competitor DNA

Oligonucleotide competition experiments were performed essentially as described (6). Oligonucleotides were 24 bases long and were designed such that after annealing of complementary pairs the double-stranded oligonucleotides had a 5′-GATC-3′ single-stranded extension at both ends. Phosphorylated and annealed oligonucleotides were ligated to an average length of ~1 kb. The non-coding strands of the three competitors used in the experiment of Figure 6 were (mutated nucleotides underlined): T3wt, GATCCGCGGGGACTTGCTCGGCCA; LS11, GATCCGCGG GGACTTGCTCAGATA; T3mu, GATCCGCGGGGATCTGC-TCGGCCA.

Transcription of immobilized templates

The S-100 cell extract was prepared and fractionated on DEAE–Sepharose as described (6). Immobilized template DNA (100–400 ng/40 μl reaction) was washed once in 100 μl of dialysis buffer and transcribed in the DEAE–Sepharose fractions as described (17), except that the concentration of the unlabeled nucleotides was 10 times less than standard (i.e. 50 μM each ATP, UTP and GTP and 10 μM CTP) and 2 mM 6-dimethylaminopurine was present in all reactions to inhibit a transcription-repressing kinase (17). At the end of the reaction the DNA was separated from the soluble components by magnetic separation, the supernatant was removed, treated with proteinase K–SDS and the RNA was extracted with phenol/chloroform. The DNA–beads were washed twice with 200 μl of a buffer containing 0.25% Sarkosyl, 90 mM KCl, 20 mM HEPES, pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF. The RNA was extracted from the washed beads by treatment with proteinase K–SDS and phenol/chloroform extraction. RNA was analyzed on 4 or 5% denaturing polyacrylamide gels using standard procedures. The 'transcription buffer' used for incubating the beads for pyrophosphorolysis and for the chase experiment (Fig. 4) consisted of 90 mM KCl, 6 mM $MgCl_2$, 20 mM Hepes, pH 7.9, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF. The nucleotide concentrations during the chase were 400 μM ATP, GTP and UTP and 40 μM CTP.

Appropriately exposed autoradiographs were scanned with an Apple One Scanner and analyzed using Ofoto 1.1 and NIH Image 1.44 software. To determine the relative amounts of RNA in the bands the measured densities were corrected for the length of the RNA in the bands.

S1 protection assay

To map the RNA 3′-ends at T3, a 333 bp *Nhe*I fragment was labeled with [α-^{32}P]dCTP and Klenow DNA polymerase. The two strands were separated by denaturation in 0.3 N NaOH and electrophoresis in a native 5% polyacrylamide gel in 50 mM Tris–borate/EDTA buffer. The strand complementary to the RNA was eluted from the gel. The location of the labeled 3′-end of the probe is indicated in Figure 1. S1 protection assays were performed as described previously (1).

RESULTS

To investigate whether the *Xenopus* pol I elongation complex pauses at T3, *in vitro* transcription reactions were carried out using ribosomal minigenes that were immobilized on paramagnetic particles by a streptavidin–biotin linkage. The structure of the minigene template is shown in Figure 1. The biotin label

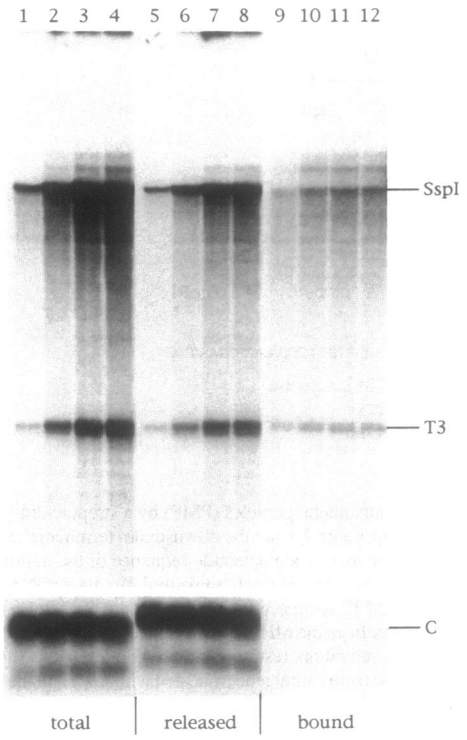

Figure 2. Demonstration of a Sarkosyl-resistant, paused complex at T3. Immobilized MH-T3wt was transcribed in recombined DEAE–Sepharose fractions for 30 (lanes 1, 5 and 9), 60 (lanes 2, 6 and 10), 90 (lanes 3, 7 and 11) or 120 min (lanes 4, 8 and 12). From each reaction RNA was extracted from 1/10 of the total suspension (lanes 1–4), from 1/10 of the soluble fraction (lanes 5–8) and from 9/10 of the Sarkosyl-washed beads (lanes 9–12). T3, transcripts with 3′-ends at T3; *Ssp*I, run-off transcripts to the end of the template; C, endogenous, soluble RNAs of ~80 nt that become end-labeled during the transcription reaction by a nucleotidyl transferase and that are shown as a control for washing efficiency.

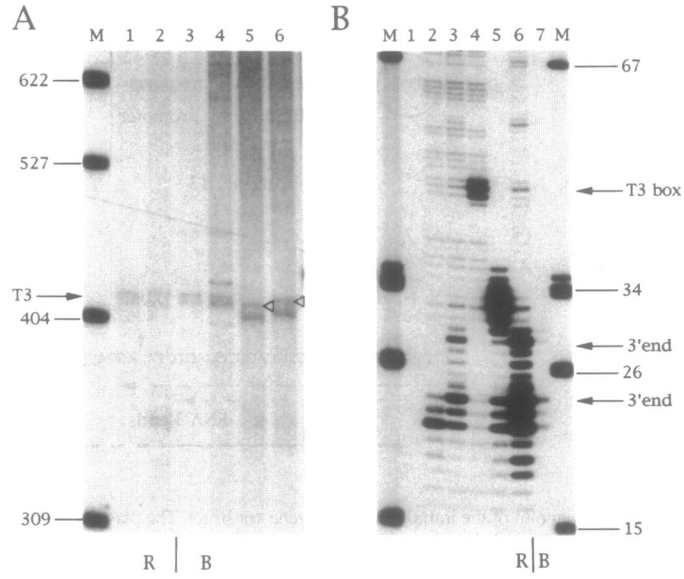

Figure 3. Mapping of the 3′-ends of the RNA in the paused complex. (**A**) Released (R, lanes 1 and 2) and template-bound (B, lanes 3 and 4) RNA was run alongside 425 nt run-off transcripts produced from the same minigene truncated with *Xba*I (lane 6). A 421 nt run-off was produced by truncating a minigene bearing a 4 bp deletion with *Xba*I (lane 5). Separate experiments with templates truncated at additional sites (not shown) indicated that the real run-off transcripts are the fainter bands (indicated with arrowheads) above the stronger sub-terminal RNA bands. Transcription reactions were either in S-100 extract (lanes 1 and 3) or recombined DEAE–Sepharose fractions (lanes 2 and 4). M, 5′-end-labeled *Hpa*II digest of pBR322. The length (in nucleotides) of the marker fragments are given to the left. (**B**) S1 protection assays using the 3′-end-labeled probe shown in Figure 1. The following RNA samples were analyzed: lane 1, *E.coli* RNA control; lane 2, read-through control using RNA synthesized from MH-T3wt by T7 RNA polymerase; lane 3, RNA from non-immobilized MH-T3wt; lane 4, RNA from MH-T3mu (the location of the mutated nucleotides in the T3 box gives rise to an S1 signal, indicated by an arrow, due to the divergence between the RNA and the probe); lane 5, RNA from a minigene lacking the two 4 bp insertions underlined in Figure 1 (the location of the downstream deletion gives rise to a strong S1 signal at ~32 nt due to the divergence between the RNA and the probe); lane 6 (R), released RNA from immobilized MH-T3wt; lane 7 (B), template-bound RNA. The two S1 signals corresponding to the major 3′-ends of both the T3-terminated and the T3-paused RNA are indicated by arrows. M, marker lanes as in (A).

was incorporated at a *Hin*dIII site 266 bp upstream of the start site of transcription. Transcripts initiating at the promoter and terminating at T3 were predicted to be ~425 nt long, run-off transcripts to the *Ssp*I site were 1375 nt long. After *in vitro* transcription in the presence of [α-^{32}P]CTP the soluble components of the reaction were separated from the beads. The template–beads were washed in a buffer containing 0.25% Sarkosyl, conditions to which elongating pol I transcription complexes have been shown to be resistant (5,18). Similar Sarkosyl buffers have been used to wash and purify pol II elongation complexes (19,20). RNA was extracted from both the soluble fraction (released RNA) and the washed beads (template-bound RNA) and analyzed by gel electrophoresis. Any discrete RNA band in the template-bound fraction is interpreted to represent paused pol I elongation complexes.

Figure 2 shows an experiment in which total RNA, released RNA and template-bound RNA were analyzed after 30, 60, 90 and 120 min transcription. As expected, the amount of total RNA, both T3-terminated and run-off transcripts, increased steadily during the 2 h reaction (lanes 1–4). Analysis of the released RNA (lanes 5–8) gave a very similar result, both quantitatively and qualitatively, showing that the majority of the total RNA was released into the reaction. Analysis of the template-bound RNA (lanes 9–12) showed that this fraction contained an RNA species that co-migrated with the released T3-terminated RNA, indicating that elongating pol I transcription complexes paused near or at the T3 terminator. The amount of RNA in the paused complex barely increased after 30 min transcription, consistent with the notion that the RNA present in the complex paused at T3 is a precursor to the released RNA. Complexes paused downstream of T3 and at the end of the template gave rise to a smear in the gel lane extending to the position of the run-off transcript. To obtain signals of similar intensity, only 1/10 of the total and of the released RNA from a single reaction was analyzed (lanes 1–8) and was compared with 9/10 of the bound RNA (lanes 9–12) from the same reaction. The similar intensities of the T3 signals in lanes 1, 5 and 9 therefore indicate that after 30 min transcription the fraction of RNA present in the complex paused at T3 was about 10% of the RNA terminated at T3. After a 2 h reaction this fraction dropped to 2–3%.

To map the RNA 3′-ends in the paused complex, released (Fig. 3A, lanes 1 and 2) and template-bound (lanes 3 and 4) RNA was

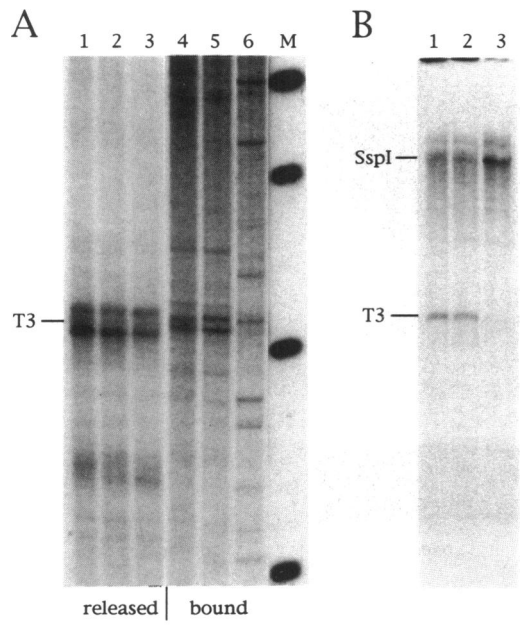

Figure 4 .The paused complex at T3 is elongation competent. (**A**) Pyrophosphorolysis experiment. Released (lanes 1–3) or template-bound (lanes 4–6) RNA was directly analyzed (lanes 1 and 4) and incubated in transcription buffer (lanes 2 and 5) or in transcription buffer containing 5 mM sodium pyrophosphate (lanes 3 and 6). Incubation was for 30 (lanes 2 and 3) or 15 min (lanes 5 and 6). M, end-labeled *Hpa*II digest of pBR322. See Figure 3A for the size of the marker fragments. (**B**) 'Chase experiment'. Lane 1, template-bound (Sarkosyl-resistant) transcripts without further incubation; lane 2, template-bound transcripts after incubation for 30 min in transcription buffer without nucleotides; lane 3, template-bound transcripts after incubation for 30 min in transcription buffer containing nucleotides.

run on a denaturing 4% polyacrylamide gel alongside run-off transcripts produced from the same minigene template truncated at an *Xba*I site 17 bp upstream of the T3 box (lane 6; see also Fig. 1). An additional *Xba*I run-off was produced from a minigene that had a 4 bp deletion (lane 5; see also Fig. 1). It can be seen that both the RNA in the complex paused near T3 and the released T3-terminated RNA ran as a doublet on the gel, but that the two RNA species in the two fractions did not precisely co-migrate (see also Fig. 4A). The RNA in the paused complex co-migrated approximately with the two *Xba*I run-offs, whose predicted lengths are 421 and 425 nt and which would place the paused complex 17–21 bp upstream of the T3 box. The location of the RNA 3′-ends in the paused complex was also determined with an S1 protection assay (Fig. 3B). The single-stranded DNA probe was labeled at its 3′-end at position 399 on the minigene template (see Fig. 1). The two major protected fragments were 23 and 29 nt long (lanes 3, 6 and 7). An additional S1 signal at 21 nt was also seen with the T7 read-through control (lane 2) and thus probably did not represent an RNA 3′-end. This result indicated that the RNA 3′-ends mapped to nucleotide positions 421 and 427 on the minigene or 15 and 21 bp upstream of the T3 box, in good agreement with the result using the *Xba*I run-offs. Interestingly, the S1 assay did not reveal a difference between the template-bound RNA and the released RNA (lanes 6 and 7), raising the possibility that the different migration of the released and bound transcripts might be due to RNA modification.

To establish further that the RNA 3′-ends upstream of the T3 box in the DNA-bound fraction represented paused elongation complexes, the experiments shown in Figure 4 were performed. First, it was investigated whether the bound RNA was subject to pyrophosphorolysis. Pyrophosphorolysis is the reverse of the polymerization reaction and can only occur if the RNA 3′-end is associated with the active site of the RNA polymerase (20–22). As shown in Figure 4A, sodium pyrophosphate (5 mM) had no effect on RNA released at T3 (compare lanes 3 with 1 and 2), while it resulted in drastic changes in the RNA pattern observed in the template-bound fraction (compare lanes 6 with 4 and 5). These changes included the disappearance of T3-paused transcripts and the appearance of several new RNA species (lane 6). Additional experiments did not identify the products of pyrophosphorolysis of the T3-paused transcripts, since all the major bands visible after pyrophosphorolysis (as in lane 6) were seen with both the wild-type and a mutant T3 terminator (not shown). While the details of the pyrophosphorolysis reaction remain to be worked out, the sensitivity of the T3 bands in the template-bound fraction to pyrophosphorolysis demonstrates that the T3-paused RNA is present in a paused ternary complex.

An independent way to demonstrate that an RNA is present in a ternary complex is to test whether it can be chased into longer RNAs (Fig. 4B). At the end of standard transcription reactions DNA–beads were washed in Sarkosyl buffer, which should remove most proteins except for pol I, and thus could be expected to also remove putative factors that are responsible for the pausing. We had reported previously that termination at T3 is indeed abolished in 0.25% Sarkosyl (5). If such Sarkosyl-washed elongation complexes were incubated in transcription buffer lacking nucleotides the amount of T3-paused and run-off RNA did not change (lanes 1 and 2). However, if (unlabeled) nucleotides were present during the incubation, the amount of T3-paused RNA decreased, while the amount of run-off RNA increased (lane 3). This observation strongly indicates that some of the paused complexes at T3 can be chased to the end of the template and that they therefore represent elongation-competent complexes.

Previous studies had shown that the minimal terminator is contained within the 9 bp sequence GACTTGCNC (2–4). The minigene used in the present study contained only 20 bp of natural *Xenopus* T3 sequences, including the T3 box and 7 bp of upstream flanking nucleotides (see Fig. 1). *In vitro* termination on the present minigene MH-T3wt was comparable to termination on a related minigene that had a T3 site with 45 upstream flanking rDNA nucleotides, including the sequences where the RNA 3′-ends are formed (data not shown; a co-transcription of these two minigenes is shown in 23). Thus the sequences where the RNA 3′-ends in the paused complex are located have little effect on the termination efficiency, confirming previous data (3,5). To further delineate the sequences required for termination and for pausing at T3 in the present *in vitro* system, mutations within or in the vicinity of the T3 box were tested (see Fig. 1). In addition to non-functional T3mu, the weaker terminator T2wt and its 'up-mutant' C261 were used (4). Analysis of the released RNA synthesized from these four constructs showed that the amount of RNA terminated at these various terminators decreased in the order T3wt > C261 > T2wt > T3mu (Fig. 5, lanes 1–4). Termination efficiencies (defined as percentage of total released RNA that is terminated at T3 or its mutants) were measured to be 62, 41, 14 and 0%, respectively. Analysis of the template-bound RNA (lanes 6–9) showed that the pausing efficiencies (defined

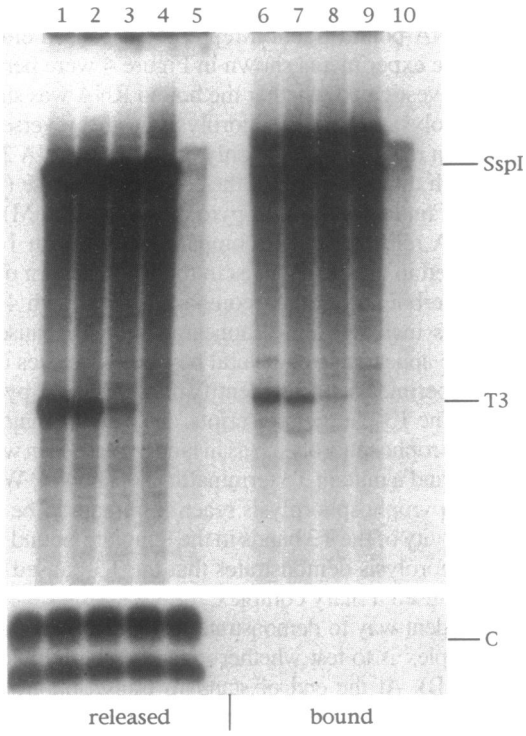

Figure 5. Sequence requirement for termination and pausing. Immobilized minigenes bearing various terminators were transcribed *in vitro* and released RNA (lanes 1–4; 1/10 of the total reaction) and template-bound RNA (lanes 6–9; 9/10 of the total reaction) were analyzed. The terminators were: lanes 1 and 6, T3wt; lanes 2 and 7, C261; lanes 3 and 8, T2wt; lanes 4 and 9, T3mu. The sequences of these variant terminators are shown in Figure 1. The analysis of a promoter mutant (LS–142/–133; 28) in lanes 5 and 10 shows that virtually all of the run-off transcripts are promoter-dependent. The RNA bands are labeled as in Figure 2. Note that termination and pausing is similarly affected by mutations in or near the T3 box.

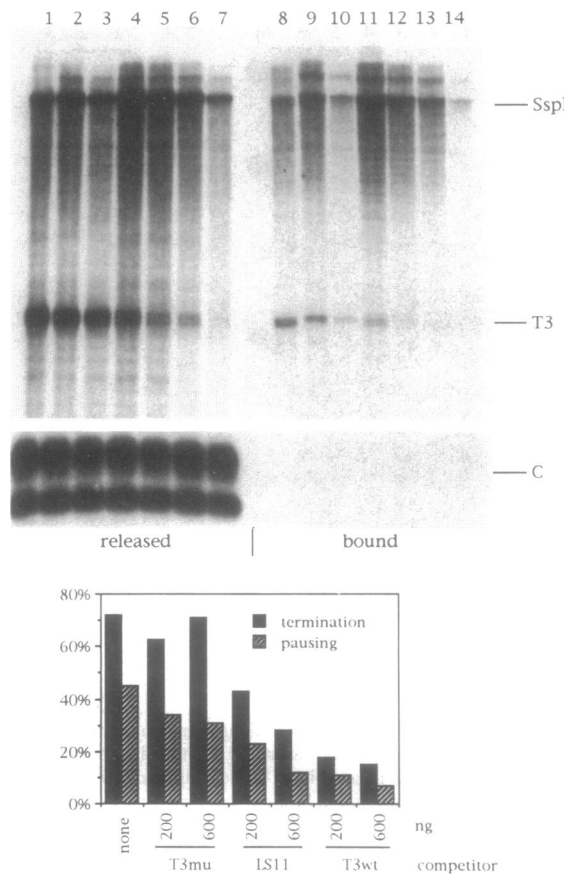

Figure 6. Evidence for the requirement for a DNA binding factor for pausing at T3. Transcription reactions were performed in the presence of polymerized double-stranded oligonucleotides as competitor DNA and the released RNA (lanes 1–7; 1/10 of the total reaction) and template-bound RNA (lanes 8–14; 9/10 of the total reaction) were analyzed. The competitors were: lanes 1 and 8, none; lanes 2 and 9, 200 ng T3mu; lanes 3 and 10, 600 ng T3mu; lanes 4 and 11, 200 ng LS11; lanes 5 and 12, 600 ng LS11; lanes 6 and 13, 200 ng T3wt; lanes 7 and 14, 600 ng T3wt. The RNA bands are labeled as in Figure 2. The bar graph below shows the termination efficiencies (black bars) and pausing efficiencies (hatched bars) determined by densitometry of the autoradiograph. Note that the two values are similarly affected by the competitors.

here as percentage of total template-bound RNA that is in complexes paused at T3 or its mutants) also decreased in the order T3wt > C261 > T2wt > T3mu (measured at 39, 22, 10 and 0%, respectively), thus correlating well with the termination efficiencies at these various terminators. These results suggest that the weak and intermediate *in vitro* termination efficiencies of T2wt and C261, respectively, are due to accordingly reduced pausing efficiencies at these sites.

A previous study showed that termination at the *Xenopus* T3 site is sensitive to excess T3 sequences, suggesting that a DNA binding termination factor is involved (6). To test whether pausing would require a DNA binding factor, I investigated the effect of three different competitors on the termination efficiency and the pausing efficiency (as defined above). Figure 6 shows that termination efficiency was unchanged in the presence of the mutant T3mu oligonucleotide as a competitor (lanes 2 and 3), while T3wt oligonucleotides reduced the termination efficiency (lanes 6 and 7; see also bar graph below). In addition to its effect on termination efficiency, the T3wt competitor also reduced total transcription, due to its separate effect on promoter function (24). A previous mutagenesis of the T3 site showed that the promoter and terminator functions can be separated by a mutation just downstream of the T3 box, which was called LS11 (24). It can be seen in Figure 6 (lanes 4 and 5) that the LS11 competitor did not affect total transcription, but still reduced the termination efficiency, albeit to a lesser degree than T3wt. Figure 6 (lanes 8–14) shows the analysis of the template-bound transcripts in these competition reactions. It can be seen that the competitors had similar relative effects on pausing efficiency as on termination efficiency. Both the T3wt and the LS11 competitors led to less T3-paused complexes relative to the total amount of template-bound RNA (lanes 11–14; see also bar graph). The finding that pausing efficiency is affected by competitor DNA containing the termination signal indicates that a DNA binding factor is required for the pausing step at T3. Furthermore, the parallel effects on termination and pausing suggest a causal link between these two processes.

Chromatography of the S-100 extract on DEAE–Sepharose was reported to separate the basic initiation factors from an activity that mediates termination at T3 (6). This termination factor fractionates into the DEAE flow-through fraction (DEAE FT), while the initiation factors can be eluted from the column with a buffer

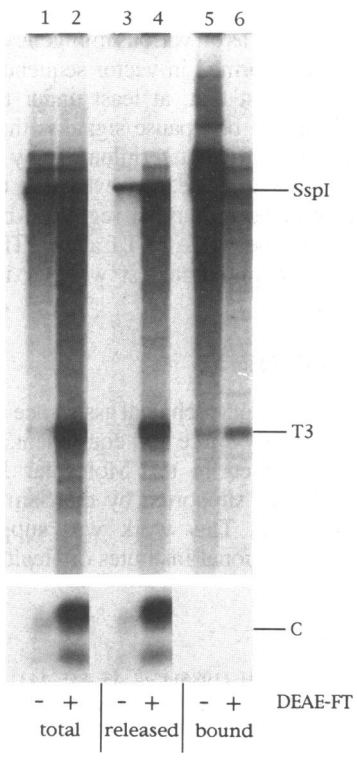

Figure 7. An activity in DEAE FT stimulates termination but does not mediate pausing at T3. Transcription reactions were performed with the DEAE 0.35 fraction alone (lanes 1, 3 and 5) or in the presence of the DEAE FT fraction (lanes 2, 4 and 6). Total RNA (lanes 1 and 2; 1/10 of the total reaction), released RNA (lanes 3 and 4; 1/10 of the total reaction) and template-bound RNA (lanes 5 and 6; 9/10 of the total reaction) were analyzed. The RNA bands are labeled as in Figure 2. Note that DEAE FT leads to more released RNA, but that there is pausing at T3 even in its absence (lane 5).

containing 0.35 M KCl (DEAE 0.35 fraction). I wondered whether this activity in DEAE FT was responsible for the pausing at T3. To reduce possible contamination of DEAE 0.35 with the termination activity in DEAE FT, the DEAE 0.35 fraction was reapplied to a DEAE–Sepharose column and this second column was washed with an additional salt step containing 0.2 M KCl. The analysis of total RNA (1/10 of the reaction) synthesized during a standard 2 h reaction in the absence and presence of DEAE FT is shown in Figure 7 (lanes 1 and 2). It can be seen that transcription in DEAE 0.35 alone produced predominantly run-off transcripts (lane 1) and that reconstitution of the complete system with DEAE FT restored efficient termination at T3 (lane 2). Analysis of the released RNA (1/10 of the reaction) and the template-bound RNA (9/10 of the reaction) is shown in lanes 3–6. Interestingly, the majority (65–70%) of the high molecular weight RNA formed in the absence of DEAE FT remained bound to the template (lanes 3 and 5). However, a significant number of complexes paused at T3 were reproducibly observed (lane 5), suggesting that pausing *per se* does not require DEAE FT. In the presence of the DEAE FT (lanes 4 and 6) <5% of the transcripts were bound to the template. While the amount of T3-paused RNA was increased in the presence of DEAE FT, this increase was only 2- to 3-fold (lanes 5 and 6). On the other hand, the amount of transcripts terminated and released at T3 increased more than 30-fold (lanes 3 and 4). The result suggests that the apparent termination activity in DEAE FT is not responsible for pausing, but increases the efficiency of termination at some different step, possibly at the level of transcript release.

DISCUSSION

It might be a general rule that the elongation complex has to pause before termination of transcription can occur. A paused RNA polymerase can either remain paused, continue transcription, retract by pyrophosphorolysis of the nascent RNA or release the transcript and dissociate from the template (25). It could therefore have been expected that pausing would play a role during transcription termination by pol I and a recent study with yeast pol I (13), discussed in more detail below, confirmed this prediction. The ribosomal terminator in *Xenopus* has been well characterized, but if and where pausing would occur at this terminator had not been investigated. The *Xenopus* pol I terminator is a 9 bp sequence that works in only one orientation and directs the formation of RNA 3′-ends ~15–20 nt upstream. Thus pol I could either pause as it attempts to transcribe the 9 bp element and the observed RNA 3′-ends would be the result of a 15–20 nt trimming of the primary transcript or the observed RNA 3′-ends could represent the very site at which pol I pauses. The present results favor the latter scenario. In the present experiments, in which the template-bound transcripts represent a momentary picture of ongoing transcription reactions, a paused complex is seen which contains nascent RNAs with 3′-ends mapping to 15 and 21 bp upstream of the 9 bp signal. These RNAs are still part of a ternary complex, since they are bound to the template in a Sarkosyl-resistant way, they are subject to pyrophosphorolysis and the Sarkosyl-washed complexes can continue transcription. The finding that the level of RNA present in the paused complex remains fairly constant during the transcription reaction, while the amount of released RNA gradually increases, as well as the observations that mutations and competitor DNA that affect pausing similarly affect the entire termination process, strongly suggest, but do not prove, that the RNA in the paused complex is a precursor to the released RNA.

The present results indicate that it is this pausing step that requires a DNA binding factor, while an activity present in the DEAE FT fraction appears to be involved in some different step during the termination process. These findings extend a previous study by McStay and Reeder (6), who showed that pol I termination in *Xenopus* can be competed out by excess T3 oligonucleotides, indicating that termination requires a DNA binding protein. On a T3–DNA affinity column, a factor called Rib2 was purified (6) that produced a footprint on the terminator and on flanking upstream sequences. In addition, the same authors showed that for efficient termination at T3, DEAE FT was required. It is important to note, however, that Rib2 was not directly purified from DEAE FT and, since Rib2 was functionally inactive, it could not be demonstrated whether Rib2 was responsible for the transcriptional effects of the DEAE FT fraction. Therefore, the present finding that the activity in DEAE FT appears not to be involved in pausing does not contradict the notion that Rib2 is the factor causing pol I to pause at T3. To conclusively answer the question whether Rib2 is the pausing factor, a method for the preparation of transcriptionally active Rib2 will have to be developed. Additional experiments (unpublished data) show that the putative 'release factor' in DEAE FT

does not bind to the T3 sequence, thus further confirming that it is distinct from Rib2.

At present the significance of the two RNA 3′-ends in the paused complex is not understood and they might represent two different conformations or two different locations on the DNA of the paused pol I. It is tempting to speculate, however, that the 15 and 21 nt distance represents the distance between the active site of pol I and its leading edge, which met some kind of obstacle at the T3 box. This notion is consistent with data on *Escherichia coli* RNA polymerase and pol II, where the distance between the leading edge of the polymerase and the catalytic site has been found to be 17–19 nt (26,27). The distance can become shortened to 10–12 nt in an arrested complex or during inchworm-like forward movements. In the present model the obstacle encountered by pol I could either be the DNA binding termination factor itself or a particular DNA structure (e.g. a bend) induced by the termination factor.

Site T2 at the 3′-end of the *Xenopus* 40S precursor rRNA coding region has been shown to be partially impaired by a naturally occurring mutation in the 9 bp element, but its behavior has been and remains puzzling in many aspects. *In vivo* and in oocyte injection experiments this mutation abolishes the ability of the signal to terminate transcription, while RNA 3′-end formation is little affected (1,2,4,5). These observations led us to conclude that the RNA 3′-ends had to be the result of a rapid processing event (4). Interestingly, the *in vitro* behavior of T2 is somewhat different, in that RNA 3′-end formation is also reduced as compared with T3 (6). Thus T2 *in vitro* behaves quite simply like a weaker terminator. In the present study this behavior of T2 and its up-mutant C261 was used to demonstrate a correlation between termination efficiency and pausing at a ribosomal terminator. At least *in vitro*, pausing thus appears to be a limiting step during the termination process. It is conceivable that site T2 displays different characteristics *in vivo*, because a different step, for example RNA 3′-end formation or RNA release, is limiting. Furthermore, it is clear that a full understanding of the events at T2 and T3 will not be possible without knowing the fate of pol I after pausing and transcript release.

The present results have to be discussed in the light of a model for termination by pol I that was recently published by Lang *et al.* (13). Based on observations with a defined, purified system for yeast pol I termination, these authors proposed that terminators consist of a pause element that, with the help of a DNA binding factor, causes the elongation complex to pause and a release element that is required to efficiently release the transcript in the paused complex. Efficient termination only occurs if the pausing element causes the elongation complex to halt over a release element. The present data indicate that the pausing aspect of this model is clearly also applicable to pol I termination in *Xenopus*. However, our studies with *Xenopus* have so far failed to provide any evidence for the existence of a release element. Termination occurs with comparable efficiencies at *X.laevis* T3 and *X.borealis* T2 and T3, which all have very different sequences upstream of the 9 bp element, as well as on various minigene constructs, where the RNA 3′-ends are formed in vector sequences (2,5; see also 23). These data suggest that, at least under the experimental conditions used, the T3 box pause signal is the only *cis*-acting element required for efficient termination by pol I. The data presented in Figure 7 indicate, however, that the efficiency of *Xenopus* pol I termination may be regulated by a *trans*-acting factor present in the crude DEAE FT fraction. The purification of this factor will be required to test whether it is involved in transcript release.

ACKNOWLEDGEMENTS

I thank K. Pak for excellent technical assistance and Dr F. Chisari for the use of laboratory space and equipment. Oligonucleotide synthesis was performed in the Molecular Biology Service Laboratory, which was supported by the Sam and Rose Stein Charitable Foundation. This work was supported by grant GM46938 from the National Institutes of Health.

REFERENCES

1 Labhart,P. and Reeder,R.H. (1986) *Cell*, **45**, 431–443.
2 Labhart,P. and Reeder,R.H. (1987) *Mol. Cell. Biol.*, **7**, 1900–1905.
3 Firek,S., Read,C., Smith,D.R. and Moss,T. (1989) *Mol. Cell. Biol.*, **9**, 3777–3784.
4 Labhart,P. and Reeder,R.H. (1990) *Genes Dev.*, **4**, 269–276.
5 Labhart,P. and Reeder,R.H. (1990) *Nucleic Acids Res.*, **18**, 5271–5277.
6 McStay,B. and Reeder,R.H. (1990) *Mol. Cell. Biol.*, **10**, 2793–2800.
7 De Winter,R.F.J. and Moss,T. (1986) *Nucleic Acids Res.*, **15**, 6041–6051.
8 Reeder,R.H., Labhart,P. and McStay,B. (1987) *BioEssays*, **6**, 108–112.
9 Bartsch,I., Schoneberg,C. and Grummt,I. (1988) *Mol. Cell. Biol.*, **8**, 3891–3897.
10 Kuhn,A. and Grummt,I. (1989) *Genes Dev.*, **3**, 224–231.
11 Evers,R., Smid,A., Rudloff,U., Lottspeich,F. and Grummt,I. (1995) *EMBO J.*, **14**, 1248–1256.
12 Lang,W. and Reeder,R.H. (1993) *Mol. Cell. Biol.*, **13**, 649–658.
13 Lang,W.H., Morrow,B.E., Ju,Q., Warner,J. and Reeder,R.H. (1994) *Cell*, **79**, 527–534.
14 Proudfoot,N.J. (1989) *Trends Biochem. Sci.*, **14**, 105–110.
15 Campbell,F.E. and Setzer,D.R. (1992) *Mol. Cell. Biol.*, **12**, 2260–2272.
16 Eggermont,J. and Proudfoot,N.J. (1993) *EMBO J.*, **12**, 2539–2548.
17 Labhart,P. (1994) *Mol. Cell. Biol.*, **14**, 2011–2020.
18 Schnapp,A. and Grummt,I. (1991) *J. Biol. Chem.*, **266**, 24588–24595.
19 Izban,M.G. and Luse,D.S. (1991) *Genes Dev.*, **5**, 683–696.
20 Wang,D. and Hawley,D. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 843–847.
21 Reines,D., Ghanouni,P., Li,Q.Q. and Mote,J. (1992) *J. Biol. Chem.*, **267**, 15516–15522.
22 Rudd,M.D., Izban,M.G. and Luse,D.S. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 8057–8061.
23 Labhart,P. (1994) *FEBS Lett.*, **356**, 302–306.
24 McStay,B. and Reeder,R.H. (1990) *Genes Dev.*, **4**, 1240–1252.
25 Von Hippel,P.H. and Yager,T.D. (1992) *Science*, **255**, 809–812.
26 Gu,W., Powell,W., Mote,J. and Reines,D. (1993) *J. Biol. Chem.*, **268**, 25604–25616.
27 Nudler,E., Goldfarb,A. and Kashlev,M. (1994) *Science*, **265**, 793–796.
28 Reeder,R.H., Pennock,D., McStay,B., Roan,J., Tolentino,E. and Walker,P. (1987) *Nucleic Acids Res.*, **15**, 7429–7441.