FUNCTIONAL DISSECTION OF RNA POLYMERASE III TERMINATION
USING A PEPTIDE NUCLEIC ACID AS A TRANSCRIPTIONAL ROADBLOCK

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RUNNING TITLE: Pol III transcription termination
SUMMARY

We have shown previously that a T₁₀ peptide nucleic acid (PNA) bound to the transcriptional terminator of a *Saccharomyces cerevisiae* tDNA²⁰e(TAT) gene arrests elongating yeast RNA polymerase (Pol) III at a position that precedes by 20 bp the upstream end of the PNA roadblock. Here, a PNA-binding cassette was placed at various distances downstream of a functional tDNA²⁰e transcriptional terminator (T₆) that is not bound by the T₁₀ PNA, and the effect of the PNA roadblock on RNA 3′-end formation, transcript release and transcription reinitiation was examined. With a PNA roadblock placed as close as 5 bp downstream of the T₆ terminator, Pol III could still reach the termination site and complete pre-tRNA synthesis, implying that the catalytic site-to-front edge (C-F) distance of the polymerase can shorten by more than 10 bp upon recognition of the terminator element. In addition, transcripts synthesized by a PNA-roadblocked, terminating Pol III were found to be released from transcription complexes. Interestingly, however, the same roadblock dramatically reduced the rate of transcription reinitiation. Also, when placed 5 bp downstream of a mutationally inactivated terminator element (T₃GT₂), the PNA roadblock restored transcription termination, thus indicating that the inactivated terminator is compromised in its ability to cause Pol III pausing, but it can still induce C-F distance shortening and transcript release. The latter two activities were found to be further impaired in variants of the inactivated terminator bearing less than three consecutive Ts (T₂₂₂₂, TG₂₂₂₂). The data indicate that RNA polymerase pausing, C-F distance shortening and transcript release arefunctionally distinguishable features of the termination process, and point to the RNA release propensity of Pol III as a major determinant of its remarkably high termination efficiency.
INTRODUCTION

The three eukaryotic transcription machineries seem to have evolved different strategies for transcription termination. Termination by RNA polymerase (Pol) I is relatively complex, minimally requiring a sequence-specific DNA binding protein to arrest RNA chain elongation and a transcript/polymerase release element upstream of a pause site (1). Pol II termination is an even more complex process, being primarily regulated by mRNA 3’-end processing (2). At variance with Pol I and Pol II, Pol III accurately and efficiently recognizes termination signals constituted by a short stretch of T residues in the apparent absence of any additional factor (3). Thus, Pol III termination closely resembles bacterial RNA polymerase termination at intrinsic (rho-independent) terminators, where a GC-rich stem-loop structure in the RNA, followed by a run of T residues in the coding DNA strand, is sufficient to dissociate a ternary elongation complex (4,5). In the case of Pol III, however, factor-independent termination occurs in the absence of a stem-loop RNA structure, and simple clusters of four or more T residues in the coding strand serve as termination signals in most cases (6). Despite these differences, at least some mechanistic features of the termination process seem to be shared by the three different eukaryotic RNA polymerase systems and by the bacterial RNA polymerase as well. In particular, termination generally requires RNA polymerase pausing close to the termination site, and such a pause is crucial to allow sufficient time for destabilization of the transcription elongation complex by a cis-acting release element. At intrinsic bacterial terminators, the dA-dT stretch in the DNA contributes significantly to RNA polymerase pausing, and formation of a stem-loop structure in the RNA destabilizes the elongation complex thus promoting transcript (and polymerase) release (7-9). As mentioned above, termination by eukaryotic Pol I requires a pause-inducing factor (Reb1 in yeast (10); TTF-I in mammals (11)) which binds to a specific site in the terminator region, and an upstream sequence acting as a transcript release element (12) (a releasing factor is additionally needed for rRNA 3’-end formation and release in mammals (13)). Transcription termination by Pol III might as well rely on a similar interplay of pause and release elements. In this case, however, both of these elements are expected to be
superimposed on the simple dT-dA stretch constituting the Pol III termination signal. It is known that Pol III pausing occurs on DNA sequences coding for runs of Us (14,15), concurrently with exoribonucleolytic retraction promoted by the C11 subunit of the enzyme (16,17). The mechanism of RNA release by a Pol III termination complex is still largely unknown, however, and a transcript (or transcription complex) release element has never been functionally identified in the Pol III system.

A convenient approach for studying transcription termination relies on the use of protein roadblocks precisely positioned downstream of termination sequences. By physically contacting a terminating RNA polymerase, such roadblocks can effectively interfere with the termination process, thus providing information on structural transitions undergone by the enzyme during termination. For example, a functional coupling between transcription termination and an RNA polymerase structural rearrangement was suggested previously by the observation that a bacterial termination complex arrested at the termination site by a downstream roadblock is unable to release the RNA chain (18). Protein roadblocks have also been employed for the study of transcription termination by eukaryotic RNA polymerases. In the case of Pol I, the lac repressor has been shown to effectively substitute for Reb1 in inducing the pausing step of termination (19). Reb 1 was also shown to induce Pol III pausing (12). In the case of Pol III, however, a lac repressor positioned just downstream of the termination site did not produce any effect on transcription, except for a 2-3 nt transcript shortening (20). A valuable alternative to DNA-binding protein roadblocks is represented by DNA ligands, such as synthetic polyamides (21,22) and peptide nucleic acids (23-25), which have the advantage of being much smaller than DNA binding proteins, yet highly effective in interfering with transcription in a strictly sequence-specific manner. In particular, we have recently shown that a T10 PNA can efficiently block Pol III elongation by forming a stable triple helix adduct with the poly(dA) tract present on the transcribed strand of class III gene terminators (25). A variant PNA roadblock approach has now allowed us to probe structural rearrangements taking place in a terminating Pol III and to gain insight into previously unexplored mechanistic aspects of Pol III termination.
EXPERIMENTAL PROCEDURES

PNA and transcription templates
The wild type tDNA^Ile(TAT) template used in this study has been described previously (25,26). Variants of this gene carrying a downstream T10 PNA-binding cassette were obtained by PCR using the oligonucleotide 5’-CAAGCCGAACTCAAAAGGG as a forward primer and the following oligonucleotides as reverse primers:

5’-AAAAAAAAGAGCTCTAGAAAAAAGAGAAAGTGCT for Ile_PNAlink9;
5’-AAAAAAATCTAGAAAAAAGAGAAAGTGCTCGAG for Ile_PNAlink5;
5’-AAAAAAAAGCAAAGAAGAAAGTGCTCGAG for Ile_PNAlink2;
5’-AAAAAAAATCTAGAACAAAGAGAAAGTGCTCGAGG for Ile_PNAT3GT2;
5’-AAAAAAAATCTAGAACCAAGAAGAAAGTGCTCGAGG for Ile_PNAT2GT2;
5’-AAAAAAAATCTAGACACCAGAAGAAAGTGCTCGAGG for Ile_PNATG2TGT

Amplification products were inserted into pUC-derived plasmid vectors and verified by dideoxy-chain termination sequencing. The synthesis and binding properties of the H-T10-d-Lys-NH2 PNA (T10 PNA) were previously described (25).

In vitro transcription
In all transcription experiments, the DNA template, in the form of a supercoiled plasmid, was preincubated with 400 nM T10 PNA in 1 mM Tris/HCl (pH 7.8) at 40°C (60 minutes) in a volume of 11 µl. Multiple and single round transcription assays were then carried out as previously described (25,26) in the presence of a final KCl concentration of 50 mM to minimize PNA dissociation. The size difference between full-length and shortened transcripts in Figures 1B, 2, 5A and 6A was determined by direct comparison with the migration positions of known-size products obtained from a dideoxy-chain termination sequencing reaction run on the same sequencing gel (5-7% polyacrylamide, 7 M urea). Gel filtration analysis of transcription products on Sepharose 2B (Amersham Biosciences) was performed as previously described.
(27). Transcripts were quantified with the MultiAnalyst/PC software (BioRad) using phosphorimages of dried gels obtained with a Personal Imager FX (BioRad).
RESULTS

PNA-induced Pol III arrest.

Figure 1 recapitulates the transcriptional effects of a \( T_{10} \) PNA roadblock positioned on the terminator element of a tRNA\(^{\text{Ile}}\)(UAU) gene, I(TAT)LRI, having a natural termination site made up by 12 consecutive T residues (25). Under multiple round transcription conditions, the overall levels of tDNA\(^{\text{Ile}}\) transcription dramatically decrease in the presence of 400 nM \( T_{10} \) PNA, with the concomitant appearance of truncated transcripts that are considerably shorter than full-length pre-tRNA\(^{\text{Ile}}\) transcripts (Figure 1A, lanes 1 and 2). A precise determination of the difference in size between full-length and shortened transcripts synthesized from the tRNA\(^{\text{Ile}}\) gene (carried out on a sequencing gel; see Materials and Methods for details) revealed that the truncated tDNA\(^{\text{Ile}}\) transcription products are 23 nt shorter than the average full-length tDNA\(^{\text{Ile}}\) transcript\(^2\) (Figure 1B). These truncated transcripts are produced by transcription complexes that have been arrested ahead of the termination site by the PNA roadblock. Taking the upstream border of the roadblock as a landmark for the position of the polymerase front edge, and the RNA 3\(^\prime\)-end as a signature of the position of the catalytic site, the catalytic site-to-front edge (C-F) distance of a PNA-arrested, elongating Pol III can be estimated to be ~20 bp. Full-length transcripts produced in the presence of the PNA (Figure 1A and B, lanes 2) are generated by highly efficient reinitiation of a small number of PNA-free transcription complexes, as demonstrated by the fact that such transcripts almost completely disappear when the PNA-inhibited reaction is limited to a single round by heparin (Figure 1A, lane 4). Under single round transcription conditions (Figure 1A, lanes 3 and 4), the PNA roadblock only causes a transcript shortening effect, but it does not produce any change in the total transcription output, thus indicating that transcriptional inhibition brought about by the PNA is due to an impairment of transcription reinitiation.

Based on the observation that a \((T_{10} \text{ PNA})_2\)-DNA adduct is highly effective in blocking an elongating Pol III, we devised a strategy aimed at exploiting the PNA roadblock for the study of transcription termination. For this purpose, we prepared a series of tDNA\(^{\text{Ile}}\)(TAT)-derived
constructs bearing a T10 PNA binding cassette (i.e., a stretch of 10 consecutive A residues on the transcribed strand) placed at various distances (from 2 to 9 bp) downstream of a functional T6 termination site (designated as Ile_PNAlink2-9, see Figure 2A). The use of these tDNAIle derivatives to probe Pol III termination relies on the target specificity of the T10 PNA, namely, on its ability to bind a T10 (or longer) sequence element, but not a T6 (or shorter) sequence. In fact, transcription of a natural tDNA bearing such a terminator (e.g., the dT6•dA6 element of tDNA Glu(TTC)) is unaffected by PNA concentrations that severely inhibit transcription of a gene such as tDNAIle(TAT), which has a T12 terminator (25). Therefore, when added to the templates represented in Figure 2A, the T10 PNA will only bind to the dT10•dA10 cassette placed downstream of the T6 terminator, but not to the terminator itself. These templates were transcribed in vitro either in the presence or absence of the T10 PNA, and the resulting RNA products were separated on a sequencing gel (Figure 2B). With the Ile_PNAlink9 and Ile_PNAlink5 templates (lanes 3-6), overall transcription in the presence of the T10 PNA was inhibited by 2-fold and 4-fold, respectively, yet in both cases most of the transcripts were full-length, with a size distribution nearly identical to that observed in the absence of the PNA (cf. lanes 4 and 6 with lanes 3 and 5). Transcription termination at the T6 terminators borne by these templates was otherwise highly efficient; only a few polymerases read through this terminator and produced longer transcripts (a trace of which is visible in the upper half region of lanes 3, 5 and 7), corresponding to termination at the downstream PNA binding cassette (T10) sequence. Such downstream termination products were not formed in the presence of the T10 PNA (lanes 4, 6 and 8). With the Ile_PNAlink2-T10 PNA adduct (lanes 7 and 8), in which the upstream end of the PNA roadblock is only 2 bp behind the last T residue of the T6 terminator, a strong PNA-dependent transcription inhibition was observed (comparable to the inhibition observed for transcription of the unmodified tDNAIle template, lanes 1 and 2), and shortened transcripts appeared in the presence of the PNA (lane 8). The fact that Pol III can complete pre-tRNAIle synthesis despite the presence of a downstream PNA roadblock that is only 5 bp away from the last T residue of the T6 terminator (Ile_PNAlink5 template, lanes 5 and 6) provides strong circumstantial evidence for a terminator-induced conformational change that
allows the enzyme active site to reach a position on the template that is no more than 7 bp away from the upstream end of the roadblock (i.e., the fourth T of the T6 terminator, which corresponds to the average termination site). Indeed, the observation that truncated transcripts (corresponding to upstream-arrested complexes; see below) accumulate to significantly higher levels in the case of the Ile_PNAlink2 template sets a lower limit of ~5 bp for the polymerase C-F distance. Such a distance is clearly at variance with the C-F distance estimated for a PNA-arrested, elongating Pol III on the wild type tRNA^Ile gene (lanes 1 and 2). In the latter case, in fact, the enzyme does not seem to advance beyond a position that is ~20 bp away from the upstream end of the roadblock. In the region immediately preceding the PNA roadblock, the unmodified tDNA^Ile and the Ile_PNAlink5 templates only differ for the presence or absence of the T6 termination sequence (plus the 5 bp linker in the case of Ile_PNAlink5). Therefore, the observed difference in Pol III active site positioning with respect to the upstream end of the roadblock, and thus with respect to the front edge of the enzyme, is likely to reflect a terminator-induced structural rearrangement of the enzyme.

**PNA-induced uncoupling of Pol III termination and reinitiation.**

In the case of the unmodified tRNA^Ile(TAT) gene, the T10 PNA arrests elongating Pol III before it reaches the terminator, thereby strongly inhibiting subsequent recycling (Figure 1). We thus asked whether transcriptional inhibition caused by a PNA roadblock placed downstream of the T6 termination site (i.e., under conditions in which a substantial fraction of transcripts is of full-length size; Figure 2B) is also due to an impairment of Pol III recycling. This point was addressed by limiting transcription of pre-formed PNA-tDNA complexes to a single round. In the experiment in Figure 3, the Ile_PNAlink5 (panel A) and Ile_PNAlink2 (panel B) templates, on which the PNA roadblock had been pre-assembled, were transcribed under conditions allowing either multiple rounds (lanes 1 and 2) or only a single round (lanes 3 and 4) of transcription. With both templates, the PNA reduced the total output of multiple round transcription to levels almost identical (panel B) or close (panel A) to those produced in a single round reaction. Under single round transcription conditions, the PNA had no significant effect
on the total amount of transcripts, nor on their size, except for the appearance of substantial amounts of shortened transcripts in the case of Ile_PNAlink2 (panel B, lane 4) and of lower amounts of similarly sized transcripts also in the case of the Ile_PNAlink5 template (panel A, lanes 2 and 4). Transcript shortening observed with the Ile_PNAlink2 template closely parallels that reported in Figure 2B (where full-length and truncated products were much better resolved because they were fractionated on a sequencing gel). The data thus indicate that a PNA roadblock positioned as close as 5 bp downstream of the termination site does indeed strongly interfere with transcription reinitiation, but it still allows the completion of most pre-tRNA chains.

Transcript release from PNA-arrested Pol III termination complexes.

According to a previous study of transcription termination by E. coli RNA polymerase, roadblock-induced arrest of the bacterial enzyme on a Rho-independent terminator sequence results in suppression of RNA chain release at the termination site (18). We thus reasoned that, similar to the bacterial enzyme situation and consistent with the observed inhibition of transcription reinitiation, a terminating Pol III impeded in its downstream movements by the PNA roadblock might be incapable of releasing the completed transcript. We tested this possibility by investigating the physical state (either free or transcription complex-bound) of the transcripts produced in PNA-inhibited reactions by molecular filtration on Sepharose 2B, a gel-permeation matrix that can resolve released RNA from RNA molecules that are still part of arrested ternary complexes (14,25). Figure 4A shows the results of such an analysis carried out with the Ile_PNAlink5 template. In the PNA-inhibited reaction (“input” lane) transcripts of different sizes were produced. The predominant species (>90% of the total RNA) was represented by full-length transcripts, while the less abundant species corresponded to the same truncated transcripts that are also visible in Figure 2B (lane 6) and Figure 3A (lanes 2 and 4). When the products of this reaction were subjected to gel filtration analysis, all of the truncated transcripts turned out to be associated with paused Pol III ternary complexes, thus eluting in the void volume of the column (lanes corresponding to fractions 4-9), while full-length transcripts
were all recovered in a released form (lanes corresponding to fractions 10-40). A quantitative analysis of the phosphorimage in Figure 4A, and of the results of similar fractionation experiments carried out with the unmodified tDNA\textsuperscript{1le} and Ile\textsubscript{PNA}link2 templates, is reported in Figure 4B. With the unmodified tDNA\textsuperscript{1le} template, on which Pol III is arrested in the elongation mode, the majority (60%) of the transcripts synthesized in the presence of the T\textsubscript{10} PNA were truncated and associated with paused Pol III ternary complexes, while the remaining transcripts (resulting from highly efficient reinitiation by a small number of PNA-free transcription complexes; see Figure 1A) were full-length and released. In the case of Ile\textsubscript{PNA}link5, instead, 90% of the transcripts were full-length and released, thus indicating that the PNA roadblock, even when it is only 5 bp away from the terminator, does not interfere with RNA release. With the Ile\textsubscript{PNA}link2 template, an increase in the levels of shortened, unreleased transcripts was observed, but again all of the full-length transcripts were recovered in a released form. Full-length, yet unreleased transcripts were never detected in several gel filtration experiment replicates conducted with the Ile\textsubscript{PNA}link5 and Ile\textsubscript{PNA}link2 templates (Figure 4 and data not shown). Thus, at variance with the roadblock response behavior previously documented for bacterial RNA polymerase (18), transcript release by eukaryotic Pol III does not appear to require a roadblock-sensitive conformational change.

**PNA-mediated polymerase pausing and transcript release at mutationaly inactivated Pol III terminators**

Eukaryotic transcription termination is thought to generally require both a pause signal (halting polymerase progression) as well as a sequence element (usually A/T-rich) promoting transcript release (12). In the case of Pol III, both of these elements may be viewed as overlapping and functionally interconnected subcomponents of the short T-stretch that acts as a transcriptional terminator. The fact that Pol III can be forced to pause on any given sequence (regardless of its ability to act as a transcriptional terminator) by a downstream PNA roadblock, prompted us to test whether RNA release can occur on a modified terminator sequence no longer capable of supporting termination. To this end, we introduced a point mutation into the Ile\textsubscript{PNA}link5
template that, by changing its T6 terminator sequence into T3GT2, was expected to inactivate it. As shown in Figure 5A, termination at the T3GT2 sequence was indeed very inefficient, and most transcription termination events occurred at the downstream T10 sequence, thus producing transcripts ~10 nt longer than those synthesized from the unmodified Ile_PNAlink5 template (cf. lanes 3 and 1). In the presence of the T10 PNA, which binds to the dT10-dA10 sequence, such longer transcripts disappeared, and only early terminated transcripts, with a size distribution approximating that observed with the unmodified Ile_PNAlink5 template, were produced (cf. lanes 4 and 2). Under these conditions, most Pol III molecules thus appear to be arrested within the T3GT2 region, which corresponds positionally to the T6 region of the Ile_PNAlink5 template. Low amounts of even shorter transcripts also appeared in PNA-containing, Ile_PNAT3GT2-supported reactions (marked by an arrowhead in Figure 5A). Such shortened transcripts, which were also detected among the products of PNA-inhibited reactions programmed with the unmodified Ile_PNAlink5 template (lane 2; see also Figure 2B), likely originate from a small subset of transcription complexes arrested upstream of both the T6 and the T3GT2 element. As revealed by a densitometric quantification of the results reported in Figure 5A and of those obtained from two additional independent experiments (not shown), the T10 PNA causes a ~2-fold reduction in Ile_PNAT3GT2 transcription. The physical state (released or transcription complex-associated) of the transcripts produced from the Ile_PNAT3GT2 template in the presence of the T10 PNA was then analyzed by gel filtration. As shown in Figure 5B, full-length transcripts ending at the T3GT2 sequence were all recovered in gel-included fractions in a released form (Figures 5B, lanes 7-10), while shortened transcripts were found within gel-excluded fractions (lanes 2-5), as expected for RNAs associated with arrested ternary complexes. Therefore, PNA-induced pausing of Pol III on the T3GT2 sequence results in complexes that are prone to transcript release. By comparison, a nearly complete transcript retention was observed with PNA-roadblocked complexes arrested on the unmodified tRNAIle(TAT) gene (25), thus indicating that RNA release is not a common feature of any PNA-arrested ternary complex.

We further investigated whether there is any strict DNA sequence requirement for transcript
release by roadblocked Pol III. To explore this possibility, two additional variants of the Ile_PNAlink5 template were constructed, in which the T6 terminator element was replaced by either T2G2T2, a sequence containing a total of four T residues (instead of five as in T3GT2) all occurring as pairs, or TG2TGT, a sequence with only three, unpaired T residues. The effect of the T10 PNA on the transcription of these templates, compared to that exerted on Ile_PNAT3GT2 transcription, is shown in Figure 6A. In the absence of the PNA, the frequency of termination events taking place at T2G2T2 (12%; lane 3) and TG2TGT (10%; lane 5) was lower than that of transcription termination at T3GT2 (18%; lane 1). Regardless of these differences in sequence-dependent termination efficiency, however, most polymerase molecules read-through these mutated terminator elements and terminated at the downstream T10 string. In contrast, in the presence of the T10 PNA, much lower levels of downstream (T10) terminated transcripts —originating from a small number of PNA-free (and thus reinitiation competent) complexes— were observed with the three mutated terminator elements (lanes 2, 4 and 6). Transcript patterns produced in PNA-inhibited reactions were qualitatively similar for the three templates. In fact, besides residual T10 terminated transcripts, they all contained transcripts ending at the mutated T6 terminators, plus lower amounts of shortened transcripts (ending at ~20 bp upstream of the T10 PNA roadblock) with the same size as those previously observed in PNA-supplemented reactions programmed with the unmodified Ile_PNAlink5 template (see Figure 2B, lane 6). The latter two types of transcription products likely reflect the occurrence of two different kinds of PNA-arrested transcription complexes. Complexes of the first type can reach the mutated T6 region, notwithstanding the presence of the downstream PNA roadblock, whereas an earlier arrest of transcription takes place in the case of the second type of complex. These two complexes were very differently affected by T6 element mutations. In fact, compared with T3GT2, the most evident effect of a reduced T content and proximity, as in T2G2T2 and TG2TGT, was a strong decrease in the number of transcription complexes that could reach the mutated T6 region, and a consequent increase in the relative abundance of upstream arrested complexes (from 22% in the case of Ile_PNAT3GT2 to 48% in the case of Ile_PNATG2TGT; cf. lanes 2 and 6 in Figure 6A; see also Figure 6B). Moreover, as shown by the gel-filtration analysis data
reported in Figure 6C, the shortened transcripts generated from Ile_PNATG2TGT in the presence of the PNA roadblock were found to be selectively associated with gel-excluded, stable ternary complexes (lanes 2 to 5), while all transcripts ending at the mutated terminator region (as well as most of the T10-terminated species) were recovered in the form of gel-included, released RNA molecules (lanes 7 to 13). Reducing the content and proximity of T residues in the T3GT2 sequence thus results in a loss of C-F distance shortening and termination at the mutated T6 region.
DISCUSSION

A synthetic DNA-binding ligand positioned downstream of an RNA polymerase III transcription terminator has been used to study some basic, but as yet largely unknown, aspects of transcription termination. This approach allowed us to reveal and dissect, for the first time, structural rearrangements and functional steps occurring during transcription termination by Pol III. The most interesting features highlighted by our analysis are a terminator sequence-dependent structural change within RNA polymerase III, that manifested itself as a shortening of the catalytic site-to-front edge (C-F) distance of the enzyme, a strict functional coupling between termination and reinitiation, and the existence of distinct sequence requirements for polymerase pausing and transcript release. These issues are discussed below in the framework of current transcription termination models.

Structural rearrangements accompanying termination site recognition by Pol III

At any given template position, a transcription elongation complex may adopt different conformations and proceed through various alternative reaction pathways: it can move forward along the template with concomitant elongation of the RNA chain; it can slide backward along the RNA and DNA chains shifting the RNA:DNA hybrid and the transcription bubble; or it can dissociate from the template thus leading to transcription termination (4,28,29). When an elongating RNA polymerase is artificially halted (e.g. by omission of the next-required NTP), reaction pathways alternative to elongation may become predominant. In particular, a halted elongation complex tends to repeatedly slide backward and forward at certain positions, a behavior that can be visualized as irregularities in the DNA footprinting of an elongation complex halted at sequential sites on the DNA (30). Such irregularities led to hypothesize a discontinuous mode of transcription elongation, in which the polymerase, instead of progressing monotonically along the template, alternates between strained and relaxed conformations in a sequence-dependent manner (31,32). A transition from a strained to a relaxed conformation was also proposed to take place during termination by bacterial RNA polymerase (18). Subsequent
studies, however, suggested that the apparent contraction/expansion of the polymerase might actually reflect oscillations of the stalled complex due to reversible “backtracking”, thus excluding an internal structural flexibility of the enzyme as a fundamental requirement for elongation and termination (5). Our results, instead, argue in favor of the existence of some kind of structural plasticity within Pol III. We find, in fact, that the introduction of a terminator element (a run of 6 T residues) in a region ~5 bp upstream the PNA-DNA adduct causes a ~13 bp shortening of the C-F distance previously measured for a Pol III hitting the PNA roadblock during elongation. Such an effect can be easily accounted for by a T₆-dependent structural change within the transcription complex allowing the Pol III catalytic site to get closer to the roadblock. Alternatively, the PNA-bound DNA might penetrate into the polymerase up to a position that is only 5-10 nt away from the catalytic site. If the Pol III active site happens to be in touch with a functional termination sequence, termination occurs; instead, if the enzyme comes upon a sequence that is not relevant for termination, it might retract to an upstream position. Such a sequence-dependent retraction might in turn reflect an elongation-specific conformational state of the transcription complex that is altered upon terminator recognition.

Interestingly, the equilibrium between retracted and termination-prone conformational states of the transcription complex was found to be strongly influenced by small variations in the T content of the terminator element. When the stretch of 6 T residues was interrupted by a G (T₃GT₂), the contracted state still prevailed and PNA-induced termination occurred; however, a further reduction in the content and proximity of T residues (as in T₂G₂T₂ and TG₂TGT) shifted the balance towards the retracted, termination-incompetent state. The shortening of the C-F distance observed in the presence of a downstream PNA roadblock thus appears not to be an exclusive consequence of the recognition of a fully functional terminator element: even an inactivated terminator (such as T₃GT₂) can induce shortening, provided that at least a T₃ stretch is present. On the same note, in experiments in which the T₁₀ PNA roadblock was bound to the terminator elements of different class III genes, thus blocking the elongating Pol III before it reached the terminator sequence, the C-F distance was found to vary considerably (25). The most striking in this regard is the case of a tDNAArg(CCT) gene, in which no run of
consecutive T residues is present in the region immediately preceding the termination (and PNA roadblock-positioning) sequence, yet the C-F distance in the PNA-roadblocked complex appears to be only ~7 bp (data not shown). Therefore, sequence-dependent rearrangements of Pol III, albeit quite evident in cases such as that of the tDNA^{IIle(TAT)} gene, cannot be easily predicted simply on the basis of linear sequence information, and may also involve a more complex interplay between different features of the elongation complex, such as the relative phasing of the elongating Pol III and the (PNA)$_2$-DNA adduct as well as TFIIIC-Pol III interactions near the arrest site. A C-F distance shortening is also a feature of E. coli RNA polymerase arrested at an intrinsic terminator by a downstream protein roadblock (18). In that case, however, hindering the forward movement of the enzyme was shown to result in an inhibition of RNA chain release, as if a transition to a more extended enzyme conformation were required to complete termination (including transcript release) after RNA 3’-end formation. At variance with this bacterial polymerase model, our data indicate that once the synthesis of a transcript containing a 3’-terminal run of Us has been completed, transcript release can take place without any major forward movement of the terminating Pol III complex.

**Distinguishing between the pause site recognition, transcript release, and reinitiation requirements of Pol III**

Pol III is known to pause when it encounters oligo(dT) stretches on the coding DNA strand (14,15). The PNA roadblock approach enabled us to force Pol III to pause on interrupted oligo(dT) clusters (T$_3$GT$_2$, T$_2$G$_2$T$_2$, TG$_2$TGT) that are inactive as terminators. When followed by a PNA roadblock, the T$_3$GT$_2$ sequence acted as a transcript release element for Pol III, thus allowing the reconstitution of an effective termination site from individual pause (i.e., the PNA roadblock) and release elements. Noteworthily, when the sequence of the oligo(dT) cluster was altered so as to further reduce the content and proximity of T residues, the C-F distance shortening effect and the associated transcript release tended to disappear. The data thus suggest that the oligo(dT)$_n$ cluster (with n≥5) required for termination by yeast Pol III is essential for polymerase pausing, but not for C-F distance shortening and RNA chain release, and that
sequence requirements for the latter processes might be looser. Importantly, however, C-F distance shortening and associated transcript release are not general (and possibly artifactual) features of any PNA-arrested ternary complex, as inferred from the lack (or the strong impairment) of such transactions in PNA-roadblocked complexes arrested on the unmodified tDNA^Ile(TAT) gene (25) and on the Ile_PNAT_G2T2 and Ile_PNATG2TGT templates. Therefore, pausing by itself is not sufficient to induce transcript release, the latter process displaying a marked dependence on the presence of clusters of at least 3 T residues, and concomitantly involving C-F distance shortening.

Oligo(dT)-induced pausing seems to be a common feature of most RNA polymerases, possibly related to the low strength of DNA:RNA hybrids at dA:rU stretches, and to the consequent reduction of the lateral stability of the enzyme (28,33,34). However, Pol III is peculiar in its ability to terminate transcription at DNA sequences coding for runs of 5 or more Us, in the absence of any additional ternary complex-destabilizing feature (such as a stem-loop RNA structure). Based on the present data, we propose that the facility of transcription termination by Pol III mainly derives from a marked propensity of this enzyme towards RNA release. Support to this idea also comes from the observation that RNA release by Pol III, at variance with transcript release by the bacterial enzyme (18), is not impeded by a roadblock placed just downstream of the terminator. As a consequence of its RNA release propensity, Pol III must have evolved a special ability to negotiate its path along the template with DNA-bound proteins encountered during transcription, in order to reduce the probability of unprogrammed pausing events that might result in accidental transcript release. Indeed, while transcribing class III genes with internal promoter elements, Pol III breaches through the bulky and stable TFIIC-DNA complex without any significant delay in elongation (14,35). Elongating RNA polymerase II, instead, is prone to arrest by template bound proteins, and the elongation factor TFIIS is required to enable it to proceed through various kinds of blockage, probably by stimulating transcript cleavage by the arrested complex (36). It is unlikely that cooperation between multiple RNA polymerase molecules associated to the same transcription unit contributes to roadblock overcoming by Pol III, as recently reported for E. coli RNA polymerase (37), because a typical
class III gene (with a length of ~100 bp) can hardly be imagined to accommodate more than one transcribing polymerase. A feature that might be crucial for endowing Pol III with its unique ability to overcome protein-mediated elongation pause or arrest, instead, is the incorporation within this enzyme of a TFIIS-like function, provided by the Pol III-specific and stably associated C11 subunit (17).

Even though a (PNA)\textsubscript{2}-DNA adduct downstream of a Pol III terminator does not impair transcript completion and release, it strongly inhibits transcription reinitiation. Efficient reinitiation on class III genes depends on the recapture of a terminating polymerase by the same transcription unit through a facilitated recycling process that requires the terminator element and components of the preinitiation complex (38,39)\textsuperscript{4}. Inhibition of reinitiation by a downstream roadblock provides further evidence for the strict functional coupling between Pol III termination and reinitiation. One intriguing possibility is that such a coupling entails a post-termination step involving temporary contacts between Pol III and the DNA region downstream of the terminator, and that the PNA roadblock inhibits Pol III recycling by hindering such contacts. Alternatively, the roadblock might interfere with a Pol III conformational change that is required for reinitiation.
ACKNOWLEDGMENTS

We thank André Sentenac and Christophe Carles for insightful discussions. This study was supported by the Italian Ministry of Education, University and Research (FIRB 2003 Program) and by the Human Frontier Science Program Organization (grant RGY0011/2002-C to G.D.).
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FOOTNOTES

1 The abbreviations used are: Pol, RNA polymerase; PNA, peptide nucleic acid; bp, base pairs; nt, nucleotide(s); T\textsubscript{10} PNA, H-T\textsubscript{10}-d-Lys-NH\textsubscript{2} PNA; C-F, catalytic site-to-front edge.

2 Transcription termination at T runs generally results in transcript size micro-heterogeneity due to the incorporation of different numbers of 3’-terminal U residues (14); a migration position corresponding to the middle region of the terminated transcript ladder (as in Figure 1B, lane 1) was thus chosen as a reference to determine the transcript size difference.

3 The extent of this reduction differs from the 4-fold transcription inhibition observed with the Ile_PNAlink5 template. Although the cause of such a difference has not been investigated in detail, the lesser inhibition of Ile_PNAT\textsubscript{3}GT\textsubscript{2} transcription might be due, at least in part, to the slightly lower reinitiation efficiency supported by this template.

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FIGURE LEGENDS

Figure 1. PNA-induced Pol III elongation arrest.
(A) The tDNA_{Ile}^{TAT} template was preincubated with either 400 nM T\textsubscript{10} PNA (lanes 2 and 4) or buffer (lanes 1 and 3), and stalled elongation complexes, containing a 7-nt long nascent RNA, were allowed to form by incubation in a reaction mixture lacking CTP. Transcription was then allowed to resume by the addition of CTP, either alone so to allow for multiple rounds of transcription (lanes 1-2, MR) or together with heparin (100 µg/ml) to limit transcription to a single round (lanes 3-4, SR). Reaction products were separated by denaturing PAGE and revealed by phosphorimaging. The migration positions of full-length and shortened pre-tRNA\textsubscript{Ile} transcripts are indicated on the right. (B) In vitro transcription reactions were conducted as in panel A (lanes 1 and 2) but transcripts were run on a sequencing gel along with molecular size markers. The migration positions of full-length and shortened transcripts are indicated on the right. The sequence of the 3′-terminal region of tDNA\textsubscript{Ile}^{TAT} is shown on the left, with the natural T\textsubscript{12} terminator element in bold. Sequence positions corresponding to full-length and shortened transcripts are indicated.

Figure 2. Transcriptional effects of a T\textsubscript{10} PNA roadblock placed downstream of a Pol III terminator
(A) Sequences of the terminator regions of the wild type tDNA\textsubscript{Ile}^{TAT} template (Ile) and of tDNA\textsubscript{Ile} variants bearing a T\textsubscript{10} PNA binding cassette (underlined) at 9 bp (Ile\textsubscript{PNAlink9}), 5 bp (Ile\textsubscript{PNAlink5}) or 2 bp (Ile\textsubscript{PNAlink2}) downstream of a functional T\textsubscript{6} terminator element (replacing the natural T\textsubscript{12} tDNA\textsubscript{Ile} terminator). (B) The templates in panel A were preincubated in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5 and 7) of the T\textsubscript{10} PNA and used to program multiple round transcription reactions. Transcripts were resolved on a sequencing gel and revealed by phosphorimaging. Full-length pre-tRNA\textsubscript{Ile} transcripts are bracketed; shortened transcripts are indicated by arrowheads. Residual transcription values (mean ± standard error) measured in at least three independent experiments conducted with the different constructs in
the presence of the T10 PNA are reported below the gel phosphorimage.

**Figure 3.** Impaired transcription reinitiation on the Ile_PNAlink5 and Ile_PNAlink2 templates in the presence of the T10 PNA

The Ile_PNAlink5 template (panel A) or the Ile_PNAlink2 template (panel B) were preincubated with 400 nM T10 PNA (lanes 2 and 4) or buffer (lanes 1 and 3), then stalled elongation complexes, containing a 7-nt long nascent RNA, were allowed to form by the omission of CTP from the reaction mixture. Transcription was then allowed to resume by the addition of CTP, either alone (lanes 1-2, MR) or together with heparin (100 µg/ml) to limit transcription to a single round (lanes 3-4, SR). The transcriptional output of each reaction, relative to the output of the uninhibited (PNA-less) multiple round transcription reactions in lanes 1 (arbitrarily set to 100), is reported below each lane (Txn).

**Figure 4.** Effect of a downstream PNA roadblock on RNA release by Pol III

(A) A scaled up transcription reaction, programmed with the Ile_PNAlink5 template previously incubated with 400 nM T10 PNA, was blocked with 20 mM EDTA, and loaded onto a 1 ml Sepharose-2B column. Fourty fractions (50 µl each) were collected, and transcripts contained in the indicated fractions (3-40) were analyzed by denaturing PAGE; an aliquot of the unfractionated reaction mixture was analyzed in parallel (input). Transcripts in the last lane correspond to a pool of fractions 23 to 40. The migration positions of full-length and shortened transcripts are indicated on the left. (B) Bar plot representation of the amounts of released transcripts (empty bars) and ternary complex-associated RNAs (filled bars) derived from the gel filtration analysis of PNA-inhibited reactions conducted as in panel A with the indicated templates. Data are expressed as the percentage of the total amount of transcripts recovered after gel filtration. Only shortened transcripts were found associated with gel-excluded ternary complexes, whereas released (gel-included) transcripts were all full-length.

**Figure 5.** Rescue of an inactivated Pol III terminator by a downstream positioned PNA
A) Two different templates, Ile_PNAlink5 (lanes 1-2) or Ile_PNAT3GT2 (lanes 3-4) were pre-incubated with either 400 nM T10 PNA (lanes 2 and 4) or buffer (lanes 1 and 3), and used to program multiple round transcription reactions. Transcripts were resolved on a sequencing gel and revealed by phosphorimaging. The migration positions of transcripts terminated at either the T6 or T10 sequence are indicated on the left; the position of transcripts ending at the mutated T6 terminator (mutT6) is indicated on the right. The arrowhead on the right points to shortened RNAs. (B) A scaled up transcription reaction, programmed with the Ile_PNAT3GT2 template previously incubated with 400 nM T10 PNA, was subjected to Sepharose-2B gel filtration analysis. Forty fractions (50 µl each) were collected and transcripts contained in the indicated fractions were analyzed by denaturing PAGE; transcripts in lane 10 correspond to a pool of fractions 15 to 40, in which most of the gel-included RNA molecules were eluted. An aliquot of the unfractionated reaction mixture was analyzed in parallel (input). The migration positions of transcripts terminated at either the T10 or the T3GT2 (mutT6) sequence are indicated. Gel-excluded, shortened transcripts associated with ternary complexes (lanes 2-6) are indicated on the left (shortened).

Figure 6. Sequence-dependence of RNA release by roadblocked transcription complexes

(A) The Ile_PNAT3GT2, Ile_PNAT2G2T2 and Ile_PNATG2TGT templates were pre-incubated with either 400 nM T10 PNA (lanes 2, 4 and 6) or buffer (lanes 1, 3 and 5), and used to program multiple round transcription reactions. Transcripts were resolved on a sequencing gel and revealed by phosphorimaging. The migration positions of transcripts terminated at either the most downstream T10 terminator (T10), the mutated T6 elements (mutT6) or at a position ~20 bp upstream of the roadblock (shortened) are indicated on the left. (B) The bar plot, derived from quantification of the phosphorimage in panel A, shows the levels of transcripts terminated at the mutated T6 element (filled bars) and of shortened RNAs (empty bars) produced in reaction mixtures containing the Ile_PNAT3GT2, the Ile_PNAT2G2T2 or the Ile_PNATG2TGT template. The reported values are representative of two independent
experiments. (C) A scaled up multiple round transcription reaction, programmed with the Ile_PNATG2TGT template previously incubated with 400 nM T10 PNA, was fractionated by gel filtration chromatography on Sepharose-2B. Transcripts contained in the indicated fractions (50 µl each) were analyzed on a sequencing gel; transcripts in lane 13 correspond to a pool of fractions 16 to 25. An aliquot of the unfractionated reaction mixture was analyzed in parallel (input). The migration positions of transcripts terminated at the downstream T10 terminator (T10), at the TG2TGT sequence (mutT6), and of shortened transcripts associated with ternary complexes (shortened), are indicated on the left. The elution profile of a labeled 51-nt oligonucleotide (M), used as a marker of gel-included molecules, is shown below.
A

Ile  ...CTTCTCCTTTTTTTTTTTTTTTTTT...
Ile_PNAlink9  ...CTTCTCCTTTTTTTCTAGAGCTCTTTTTTTTTTTT...
Ile_PNAlink5  ...CTTCTCCTTTTTTTCTAGATTTTTTTTTTTTT...
Ile_PNAlink2  ...CTTCTCCTTTTTTCTGTTTTTTTTTTTTT...

B

| Template       | Residual transcription upon $T_{10}$PNA inhibition |
|----------------|-----------------------------------------------------|
| Ile            | 10%±1.2%                                            |
| Ile_PNAlink9   | 46%±6.4%                                            |
| Ile_PNAlink5   | 24%±6.5%                                            |
| Ile_PNAlink2   | 15%±3.7%                                            |
Functional dissection of RNA polymerase III termination using a peptide nucleic acid as a transcriptional roadblock
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J. Biol. Chem. published online February 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311295200

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