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# Mutations in the α-amanitin conserved domain of the largest subunit of yeast RNA polymerase III affect pausing, RNA cleavage and transcriptional transitions

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**The α-amanitin domain or domain f of the largest subunit of RNA polymerases is one of the most conserved of these enzymes. We have found that the C-terminal part of domain f can be swapped between yeast RNA polymerase II and III. An extensive mutagenesis of domain f of C160, the largest subunit of RNA polymerase III, was carried out to better define its role and understand the mechanism through which C160 participates in transcription. One mutant enzyme, C160-270, showed much reduced transcription of a non-specific template at low DNA concentrations. Abortive synthesis of trinucleotides in a dinucleotide-primed reaction proceeded at roughly wild-type levels, indicating that the mutation did not affect the formation of the first phosphodiester bond, but rather the transition from abortive initiation to processive elongation. In specific transcription assays, on the *SUP4* tRNA gene, pausing was extended but the rate of RNA elongation between pause sites was not affected. Finally, the rate of cleavage of nascent RNA transcripts by halted mutant RNA polymerase was increased ~10-fold. We propose that the domain f mutation affects the transition between two transcriptional modes, one being adopted during abortive transcription and at pause sites, the other during elongation between pause sites.**

*Keywords*: α-amanitin/β′-like subunit/pausing/RNA cleavage/RNA polymerase

## Introduction

RNA chain elongation by DNA-dependent RNA polymerases (pol) involves a complex interplay between DNA signals, the nascent RNA chain, factors and the enzyme. Recent observations suggest that RNA polymerases undergo structural changes in response to regulatory signals (Nudler *et al.*, 1994; Chamberlin, 1995). *Escherichia coli* RNA polymerase, for example, adopts alternative conformations depending on the transcribed sequence. The enzyme translocates monotonously along the DNA, as the RNA chain is elongated, until it encounters DNA sequences which temporarily halt the movement of its downstream end. Meanwhile, the upstream edge of the enzyme continues to move, as evidenced by DNA footprinting, and several nucleotides are added to the RNA chain before complete stalling occurs. When the RNA polymerase resumes elongation, its front end leaps ahead by several (up to 18) base pairs (Krummel and Chamberlin, 1992; Nudler *et al.*, 1994). RNA polymerase ternary complexes are capable of cleaving the RNA transcript when halted by the removal of cognate nucleotides, at natural pause sites or in the so-called dead-end complexes where the RNA cannot be chased to a longer form by addition of nucleotides (Suratt *et al.*, 1991; Borukhov *et al.*, 1992, 1993; Whitehall *et al.*, 1994). Cleavage of the RNA has been suggested to help the RNA polymerase to overcome pausing, even though it has been found that TFIIS (a pol II elongation factor) mutants unable to stimulate transcription through pause sites still stimulate RNA cleavage (Cipres-Palacin and Kane, 1994). Cleavage of the RNA in paused or dead-end complexes is thought to restore the distance between the RNA 3′ end and the downstream end of the enzyme to that of the monotonously elongating enzyme (Borukhov *et al.*, 1993). Upon cleavage, the 5′ end of the RNA chain remains tightly associated with the RNA polymerase, suggesting that it is bound to the enzyme through a 5′ tight binding site. A 3′ loose binding site where nucleotides are polymerized or cleaved would hold the RNA in the active site (Altmann *et al.*, 1994).

The functional domains of RNA polymerase subunits and the mechanisms which modulate the elongation rate are still largely unknown. In the β′-like subunits, at least three conserved regions (d, f and g; Thuriaux and Sentenac, 1992) are likely to participate in different catalytic steps of RNA synthesis. Domain d, the most conserved domain of RNA polymerase largest subunits, is implicated in transcription elongation and is probably part of the active site (Dieci *et al.*, 1995). Additionally, mutations that affect start site selection in yeast pol II alter residues in this domain (Berroterran *et al.*, 1994). Domain g is also probably implicated in transcription elongation and start site selection (Borukhov *et al.*, 1991; Hekmatpanah and Young, 1991; Archambault *et al.*, 1992). α-Amanitin-resistant mutations in pol II from mouse (Bartolomei and Corden, 1987, 1995), rat (Crerar *et al.*, 1983), *Drosophila melanogaster* (Chen *et al.*, 1993) and *Caenorhabditis elegans* (Rogalski *et al.*, 1990) are clustered on the largest subunit around the N-proximal part of domain f (see Figure 1 for the positions of these mutations). These observations suggest that at least part of this domain is involved in elongation since the α-amanitin toxin allows the formation of one phosphodiester bond in a dinucleotide-primed reaction, but inhibits further chain elongation by blocking the subsequent translocation step (de Mercoyrol *et al.*, 1989). The toxin binding site is likely to be involved in elongation since the *D.melanogaster*

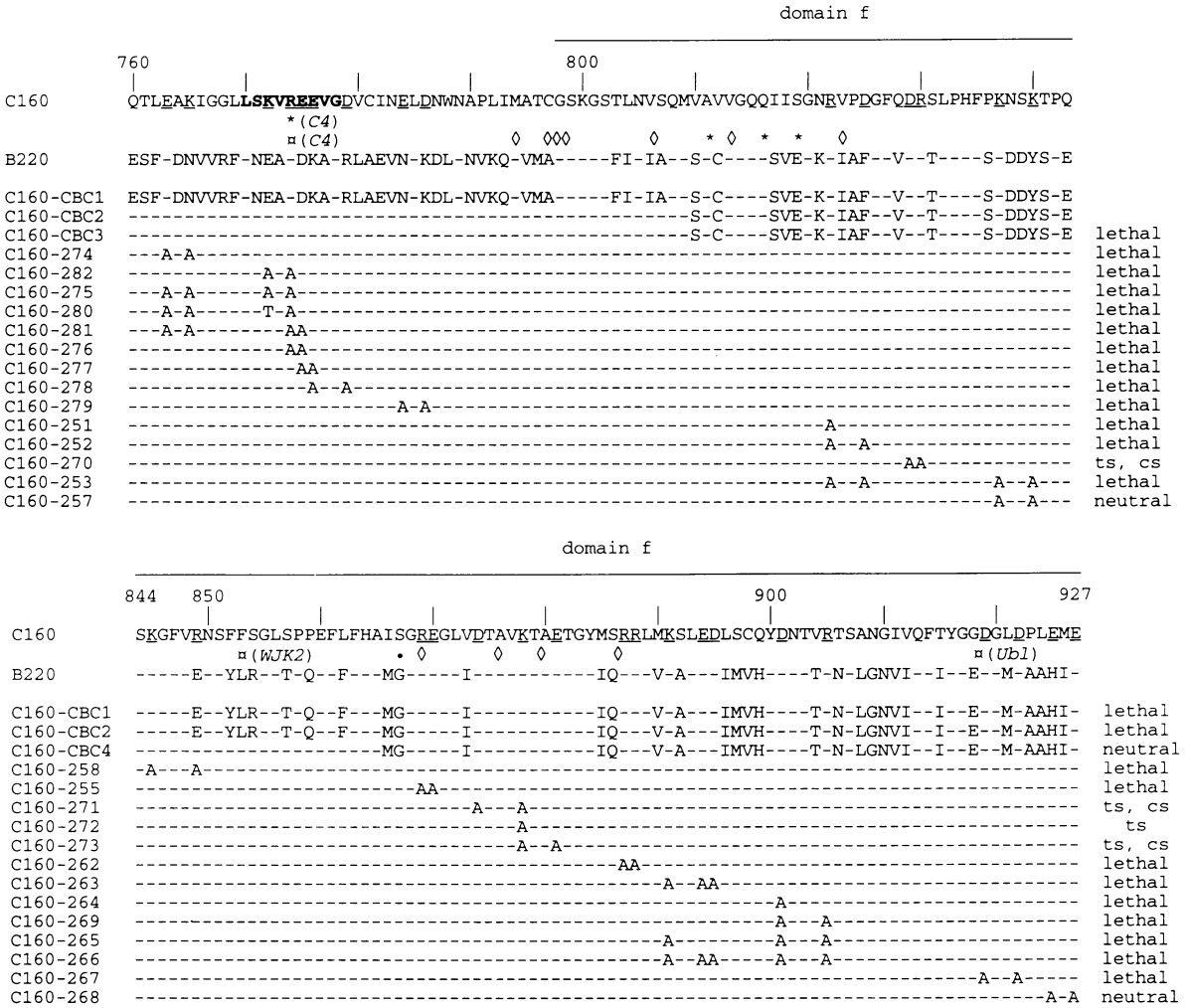



**Fig. 1.** Sequence and phenotype of mutant C160 subunits. The sequence of yeast pol II largest subunit B220, and of each C160 mutant peptide is indicated beneath that of WT C160. – represents residues identical to those of WT C160. The extent of domain f is shown by a bar above the C160 sequence. Mutated charged residues of C160 are underlined. The putative pol III-specific motif is indicated by bold letters. Note that the C160-CBC1 substitution extends to residue 713 of C160 (sequence not shown in the figure). ⋆, location of mutations leading to α-amanitin-resistant pol II (Bartolomei and Corden, 1987, 1995); ¤, location of *D.melanogaster* mutations leading to a Ubx effect (*C4*, *WJK2*, *Ubl*; reviewed in Chen *et al.*, 1993); ◇, position of the mutations in *E.coli* β′ subunit that affect transcription termination (Weilbaecher *et al.*, 1994); •, position of the *rpb1-10* thermosensitive mutation in yeast pol II largest subunit (Scafe *et al.*, 1990); ts, thermosensitive growth at 37°C; cs, cryosensitive growth at 16°C.

mutant pol II *C4*, resistant to α-amanitin, exhibits a reduced elongation rate *in vitro* (Coulter and Greenleaf, 1985). Most of the pol II mutants which exhibit a reduced sensitivity to α-amanitin also induce developmental defects in *D.melanogaster* (Ubx effect; Chen *et al.*, 1993) or pleiotropic effects on myogenic differentiation of rat myoblasts (Crerar *et al.*, 1983). Recently, a large collection of mutants in the β′ subunit of *E.coli* RNA polymerase has been screened for altered termination. The mutations clustered mainly in the conserved domains c, e, f, g and h but also between conserved domains g and h (Weilbaecher *et al.*, 1994).

This report focuses on the role of the evolutionarily conserved domain f of the largest subunit of yeast pol III. Its sequence was altered by oligonucleotide-directed mutagenesis. Whereas most of the mutations led to a lethal phenotype, we found four conditional mutants. RNA polymerase from one of these was extensively studied. The mutant enzyme was capable of forming the first phosphodiester bond at near wild-type (WT) rate, but was affected in the transition from abortive initiation to elongation. Once processive elongation was engaged, the rate of nucleotide addition was the same as that of the WT enzyme. At low template concentrations using synthetic templates, the mutation mimicked the effect of α-amanitin on RNA chain elongation, since it blocked transcription after the formation of one phosphodiester bond. In addition, the mutant enzyme showed increased pausing during transcription of a tRNA gene. Increased pausing did not correlate with increased termination. Finally, the ribonuclease activity of halted mutant ternary complexes was increased ~10 fold relative to WT ternary complexes. These results suggest that transition from abortive initiation to processive elongation, transcription past pause sites and cleavage of the transcript are connected.

## Results

### *Mutagenesis of RPC160*

Figure 1 lists a series of mutants constructed by oligonucleotide-directed mutagenesis that alter the conserved

domain f of C160 (residues 798–927) and a non-conserved region directly N-terminal to this domain. As a first step to identify domains structurally and functionally conserved among the three nuclear enzymes, different parts of the region encompassing domain f were swapped with the homologous sequences of the largest subunit of yeast pol II, B220 (Sweetser *et al.*, 1987), generating hybrid *RPC160-RPB1* genes (see Materials and methods). We obtained the four hybrid proteins described in Figure 1. The sequence substitutions of C160-CBC1, -CBC2 and -CBC3 constructions which replaced respectively residues 713–926, 810–926 and 810–843 of C160 by those of B220 all produced a lethal phenotype. The C160-CBC1 substitution replaces sequences at the amino-terminus of domain f (Figure 1: LSKVREEVG) which were proposed to be pol III-specific (Smith *et al.*, 1989). The C160-CBC4 substitution extended from residues 866–926 and overlapped roughly the C-terminal half of domain f. This domain substitution was phenotypically silent, indicating that the relevant C160 and B220 sequences functioned similarly in pol II and pol III. The sensitivity to α-amanitin of the mutant enzyme was unaltered (data not shown).

To characterize the contribution of the mutated region to the transcription process, we undertook a double alanine scanning mutagenesis of residues 763–927 (Wertman *et al.*, 1992). This region contains a high density of clustered charged residues (Figure 1) which are likely to be exposed to the solvent. Each pair of charged residues (D, E, R, K) present in a stretch of five residues was converted to a pair of alanines. Of the 27 mutations obtained, two, *RPC160-257* and *-268*, were phenotypically silent, while four others, *rpc160-270, -271, -272, -273* were lethal at 37°C. *rpc160-270, -271* and *-273* were also lethal at 16°C. Furthermore, *rpc160-270* altered the cell growth rate at the permissive temperature since the doubling time of the mutant strain at 30°C was 2.7-fold longer than that of the WT strain. The 21 other mutations were non-conditionally lethal (Figure 1). The fact that all the mutations encompassing the putative pol III-specific domain were lethal emphasizes the importance of this domain. Some of the lethal mutations which alter residues conserved between C160 and B220 map in the close vicinity of previously described mutations leading to α-amanitin-resistant pol II in mouse (Bartolomei and Corden, 1987, 1995) or *D.melanogaster* (Chen *et al.*, 1993; see Figure 1). The domain f mutations also lead to a Ultrabithorax-like (Ubl) phenotype in *Drosophila*, leading to the transformation of the fly's halteres into wings. Mutations affecting *E.coli* β′ RNA polymerase subunit domain f that alter transcription termination have also been obtained recently (Weilbaecher *et al.*, 1994).

### *Transcription properties of pol III C160-270 on synthetic templates*

The WT and mutant pol III C160-270, -271, -272 and -273 were purified as described in Materials and methods. The resulting protein fractions, analysed by silver-stained SDS–PAGE, showed similar protein content and were 80% pure with one major contaminating polypeptide of ~12 kDa. The polypeptide composition of the four mutant enzymes was identical to that of the WT although we could not detect the two small common subunits ABC10α and ABC10β using our gel system (data not shown).

*In vitro* transcription activity of the purified mutant enzymes was first globally assayed by counting acid-precipitable RNA synthesized by equal amounts of WT and mutant enzymes at saturating concentrations of poly[d(A-T)] template, ATP and UTP substrates. This assay does not measure the synthesis of short, abortive RNAs. The activities of pol III C160-271, -272 and 273 were similar to that of the WT. In contrast, pol III C160-270, which bears the D829A R830A double mutation, was three times less active than the WT enzyme.

In order to investigate the nature of the functional defect of the C160-270 mutant enzyme, transcription reactions were carried out at different poly[d(A-T)] concentrations and the transcripts, primed by UpA, were analysed by high-resolution PAGE. The WT and mutant enzymes were found to have comparable transcriptional activities at high DNA concentrations (Figure 2A). Both accumulated similar amounts of the abortive trinucleotide UpApU and long chains of poly[r(A-U)]. We measured the elongation rate of WT and C160-270 pol III at high poly[d(A-T)] concentration by analysing the length of synthesized RNA molecules on high-resolution polyacrylamide gels. The elongation rate was found to be ~20 nt/s for both enzymes (data not shown), indicating that the mutation does not affect the rate of translocation and of nucleotide addition. However, elongation of RNA chains was much impaired at low DNA concentrations in the case of pol III C160-270 but the mutant enzyme still accumulated half as much trinucleotide as the WT enzyme (Figure 2A). This reflects a slow transition from abortive to processive transcription only at low template concentration. This suggests that when the mutant enzyme runs off the template in a processive conformation (de Mercoyrol *et al.*, 1990), it can reinitiate in that same conformation, provided that it finds a new template in a sufficiently short time to prevent the return to an abortive transcription conformation (see Discussion).

Figure 2B and C show the influence of ATP concentration on RNA synthesis at low poly[d(A-T)] concentration (0.5 ng/μl). ATP is the second nucleotide incorporated in a UpA-primed reaction (Figure 2B). UpApU and UpApUpA were identified as abortive products, since they could not be chased into longer RNA chains upon addition of a 100-fold excess of unlabelled nucleotides (Figure 2D). At high ATP concentrations, WT pol III synthesized long poly[r(A-U)] chains (Figure 2B and C). Increasing the ATP concentration also stimulated the formation of abortive UpApUpA tetranucleotide and inhibited the synthesis of the abortive trinucleotide UpApU. In contrast, pol III C160-270 was unable to synthesize long RNA chains and the formation of the tetranucleotide was very much reduced. However, the amounts of trinucleotide produced by the mutant pol III, even though lower (~30% less), paralleled that produced by the WT enzyme. This suggests that ATP concentration influenced the equilibrium between the abortive and elongating forms of the enzyme. The decreased incorporation of UTP into UpApU was not due to competitive inhibition by ATP since UpApU synthesis was not affected by another non-complementary substrate like GTP (data not shown).

All four mutant pol III were insensitive to α-amanitin, as was the WT enzyme (data not shown). Nevertheless, the effect of mutation *rpc160-270* is reminiscent of that

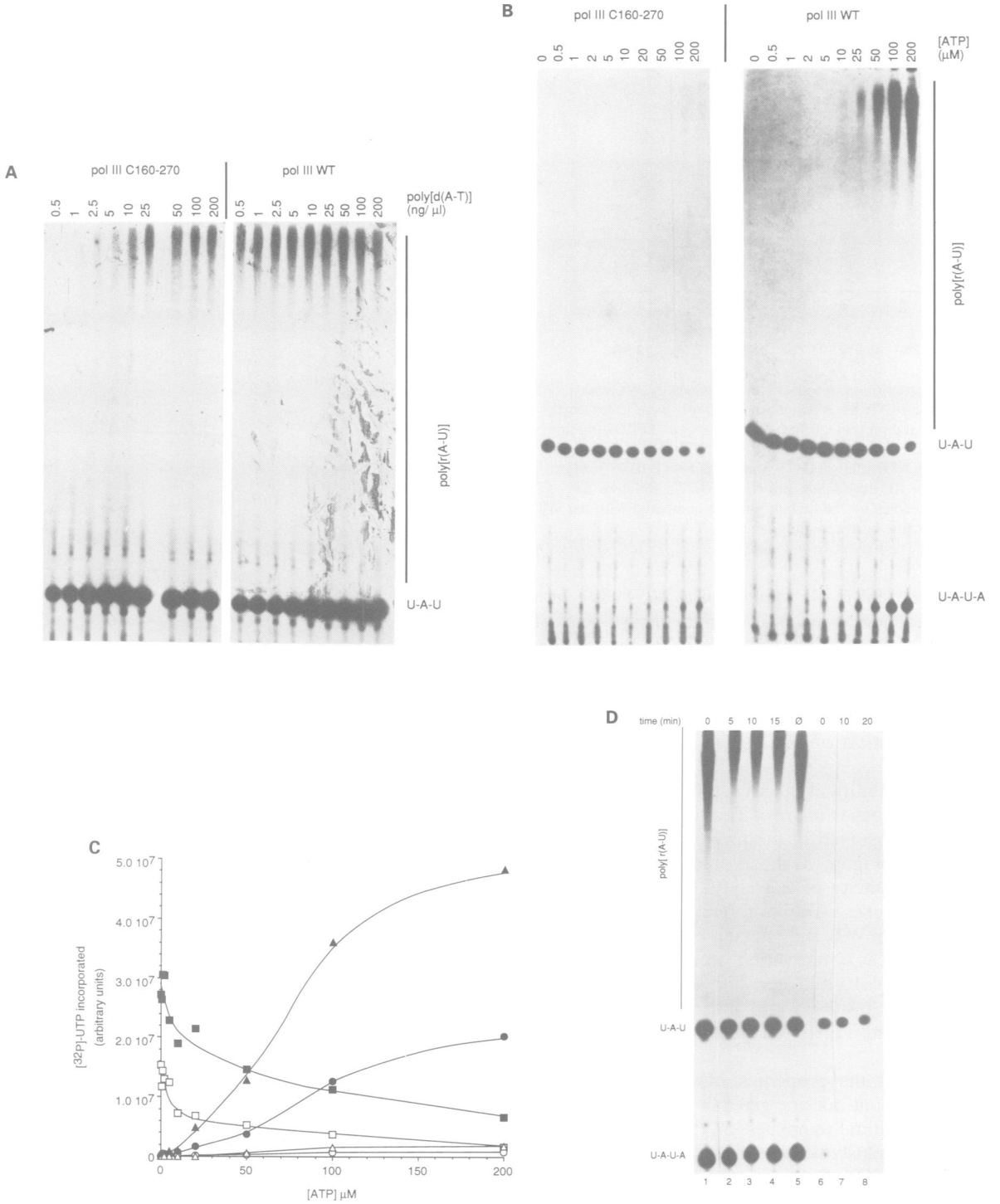



**Fig. 2.** Non-specific transcription on a poly[d(A-T)] template by WT and mutant pol III. (**A**) Non-specific transcription with various concentrations of template DNA. Transcription reactions were performed at 30°C for 30 min in the presence of 0.9 mM UpA, 20 µM UTP (10 µCi), 100 µM ATP and various concentrations of poly[d(A-T)] as indicated. Equal quantities of pol III (30 ng) were added. Since the mean length of poly[d(A-T)] chains is ~50 000 nt long and the purity of the enzyme 50%, one can compute that the molar ratio of pol III to template was varied between 85 and 0.2 in this experiment. The products of the transcription reactions were resolved on 20% polyacrylamide–1% bisacrylamide denaturing gels. The reaction products are indicated on the right side of the figure. UpApUpA is not visible on this autoradiogram. (**B**) Non-specific transcription with various ATP concentrations. Transcription reactions were carried out as described in (A) except that poly[d(A-T)] concentration was 0.5 ng/µl and ATP concentration was varied as indicated. (**C**) PhosphorImager quantification of the data of (B). Filled symbols, WT pol III; open symbols, C160-270 pol III; ■ or □, UpApU trinucleotide; ● or ○, UpApUpA tetranucleotide; ▲ or △, poly[r(A-U)] RNA. (**D**) Identification of the abortive RNA products. Poly[d(A-T)] template was transcribed by WT pol III as described in (A). After 15 min, cold ATP and UTP were added to 2 mM (lanes 1–4) and incubation continued for the indicated periods of time. In lane 5 (labelled Ø) cold nucleotides were omitted and the reaction was incubated for an additional 15 min. In lanes 6–8, the initial incubation was performed in the absence of ATP to identify the trinucleotide. For details of UpApU and UpApUpA identification and migration characteristics, see de Mercoyrol *et al.* (1990).

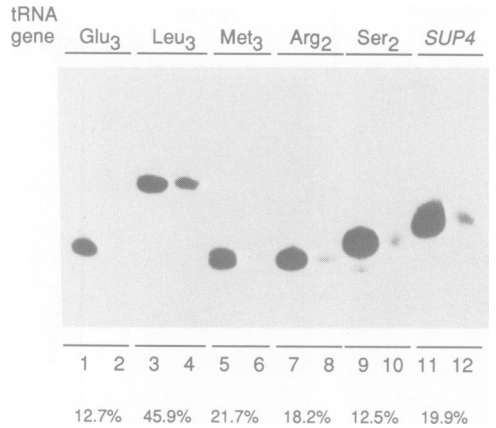



**Fig. 3.** Specific transcription of various tRNA genes by WT and mutant pol III. The tRNA gene templates (25 fmol) were transcribed for 1 h at 25°C in the presence of a heparin–Ultrogel TFIIIB fraction (0.68 µg), a DEAE–Sephadex TFIIIC fraction (90 ng), pol III WT or C160-270 (50 ng), ATP, CTP, GTP (0.6 mM) and UTP (0.03 mM). Odd lanes, WT pol III; even lanes, pol III C160-270. Relative transcription efficiencies by the mutant enzyme compared with the WT are indicated under the corresponding lanes.

of α-amanitin on pol II (de Mercoyrol *et al.*, 1989). In a UpA-primed reaction, α-amanitin permits the synthesis of stoichiometric amounts of UpApU relative to the enzyme and blocks translocation. Mutation *rpc160-270* similarly inhibits the catalytic accumulation of UpApUpA.

### *In vitro transcription of tRNA genes by pol III C160-270*

We examined the ability of pol III C160-270 to transcribe specifically tRNA genes *in vitro*. Transcription of various tRNA genes was assayed in the presence of TFIIIC and TFIIIB fractions. Figure 3 shows a comparison of the transcription efficiencies of WT and C160-270 pol III on different tRNA genes. The difference in transcription efficiency between WT and mutant enzymes using the same amount of enzyme varied from 2-fold for the $tRNA_3^{Leu}$ gene to 10-fold for the $tRNA_3^{Glu}$ gene or $tRNA_2^{Ser}$. Similar results were obtained with affinity-purified TFIIIC and TFIIIB reconstituted from its components.

We investigated the properties of pol III C160-270 which could account for its general low efficiency of transcription on natural templates. *SUP4* tRNA gene was used since it is transcribed with an average efficiency by pol III C160-270. Furthermore, a single round of transcription can be performed easily, and the *in vitro* transcription of this gene by yeast pol III has been extensively studied (Bardeleben *et al.*, 1994; Matsuzaki *et al.*, 1994). The results shown in Figure 3 indicate that the transcription rate of pol III C160-270 on the *SUP4* gene was 5-fold lower than that of pol III WT. Transcription of the *SUP4* tRNA gene was performed at various concentrations of UTP, up to 0.5 mM. The apparent $K_m$ for UTP was 25 µM for pol III WT in accordance with previous data (Szafranski and Smagowicz, 1992) and 75 µM for pol III C160-270. The transcriptional defect of pol III C160-270 was not fully relieved at high nucleotide concentrations. The rate of transcription by the mutant enzyme remained 3-fold lower than that of the WT (Figure 4).

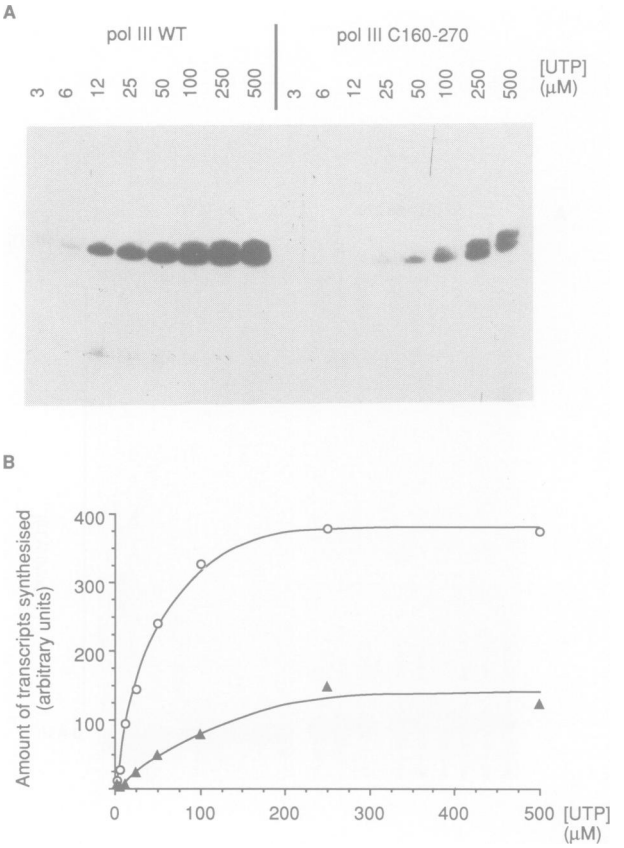



**Fig. 4.** Measurement of the apparent $K_m$ for UTP in specific transcription assays. WT or mutant pol III were incubated at room temperature for 15 min in the presence of the *SUP4* tRNA gene, reconstituted TFIIIB, TFIIIC, ATP, CTP and various concentrations of labelled UTP to form a complex halted at nucleotide 17. Elongation of the transcript was allowed to resume by incubation for 5 min with 0.6 mM GTP. Specific radioactivity was kept constant (0.33 µCi/µmol). The two bands correspond to the two natural *SUP4* termination sites. (**A**) Autoradiogram of the transcription products separated on a 6% urea–polyacrylamide gel. The concentration of UTP is indicated. (**B**) PhosphorImager quantification of the experiment shown in (A). ○, WT pol III; ▲, C160-270 pol III.

Thus the apparent specificity constant $K_m/V_{max}$ was not significantly altered.

We then compared the time required by the mutant and WT pol III to perform one transcription cycle. Halted 17mer transcripts were synthesized by incubating the enzyme with the necessary factors and 3 nt (GTP was omitted) for 15 min, then GTP was added either alone or together with heparin to prevent reinitiation. The transcription products were then electrophoresed and quantitated (Figure 5). The mean number of transcription cycles as a function of time was computed by dividing the amount of transcript synthesized in the multiple-round transcription experiment (without heparin) by that synthesized in the single-round experiment (with heparin). We calculated that the time required for one transcription cycle was 25 s for the WT enzyme and 190 s for the mutant, under the same conditions. Since the C160-270 enzyme was able to interact as productively as the WT pol III with the transcription factors in single-round transcription assays (compare lanes 1 and 9, and lanes 8 and 16), the experiment described above essentially monitored the rate at which RNA polymerases perform

multiple rounds of transcription, i.e. initiation, elongation, termination of the RNA transcripts and recycling of the enzyme.

We next investigated the time-course of the elongation phase of transcription. Halted 17 nt-labelled transcripts were first synthesized in the presence of ATP, CTP (500 μM) and labelled UTP (3 μM); the ternary complexes were then purified on a Sepharose CL-2B column. Transcription was resumed by addition of ATP, CTP, GTP (600 μM) and unlabelled UTP (30 μM) and aliquots were removed at different time to analyse the rate of transcript elongation by gel electrophoresis (Figure 6). Since the elongation is performed with unlabelled nucleotides, the intensity of each transcript can be directly compared with the amount of the labelled 17 nt RNA initially present in the purified ternary complex and prevents the observation of the transcripts synthesized during subsequent rounds of transcription. A 20 nt transcript elongated by slippage of the RNA during abortive initiation (Dieci *et al.*, 1995) is synthesized by both enzymes though at a slightly higher level by the C160-270 enzyme. This 20 nt transcript has a 3 nt extension at the RNA 5′ end and thus its 3′ end is identical to that of the 17 nt RNA (Dieci *et al.*, 1995). Both the 20 nt and the 17 nt RNAs are shortened by 2 nt at their 3′ end when incubated in the absence of nucleotide and with $MgCl_2$, which is the case during the gel filtration step since trace amounts of $MgCl_2$ remain from the labelling reaction. However, since the slipped transcript is a minor species and since the 15 nt can be chased into the 17 nt transcript, transcription elongation can be followed using these purified ternary complexes.

As described by Matsuzaki *et al.* (1994), WT pol III paused at several defined sites before completing transcription. The first full-length transcripts were observed after ~8 s, and after 20 s transcription was essentially complete (Figure 6). Strikingly, transcription by C160-270 pol III was much slower and pausing was enhanced. For example, the first pause site which occurs at G19 was still apparent after 40 s in the reactions performed with the C160-270 pol III while after 4 s the corresponding transcript had been completely chased with the WT enzyme. The pausing pattern by the mutant enzyme was similar to that of the WT. Though additional transcripts are apparent, these derived from the 20 nt 'slipped' transcript. For example, the 22 nt transcript corresponds to the G19 pause. Independent elongation experiments, that confirmed this analysis, were performed

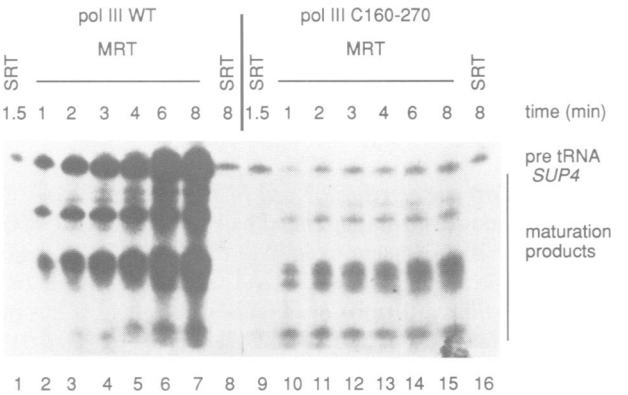



**Fig. 5.** Kinetics of *SUP4* tRNA transcript accumulation by WT and mutant pol III. WT or mutant pol III was incubated at room temperature for 15 min in the presence of the *SUP4* tRNA gene, a heparin–Ultrogel TFIIIB fraction, TFIIIC, ATP, CTP and labelled UTP to form a halted complex at nucleotide 17. Elongation of the ternary complexes was allowed to resume by incubation for 1.5–8 min either with 0.6 mM GTP and 0.3 mg/ml heparin which enabled only one round of transcription (SRT, single-round transcription) or with 0.6 mM GTP alone which allowed multiple rounds of transcription (MRT).

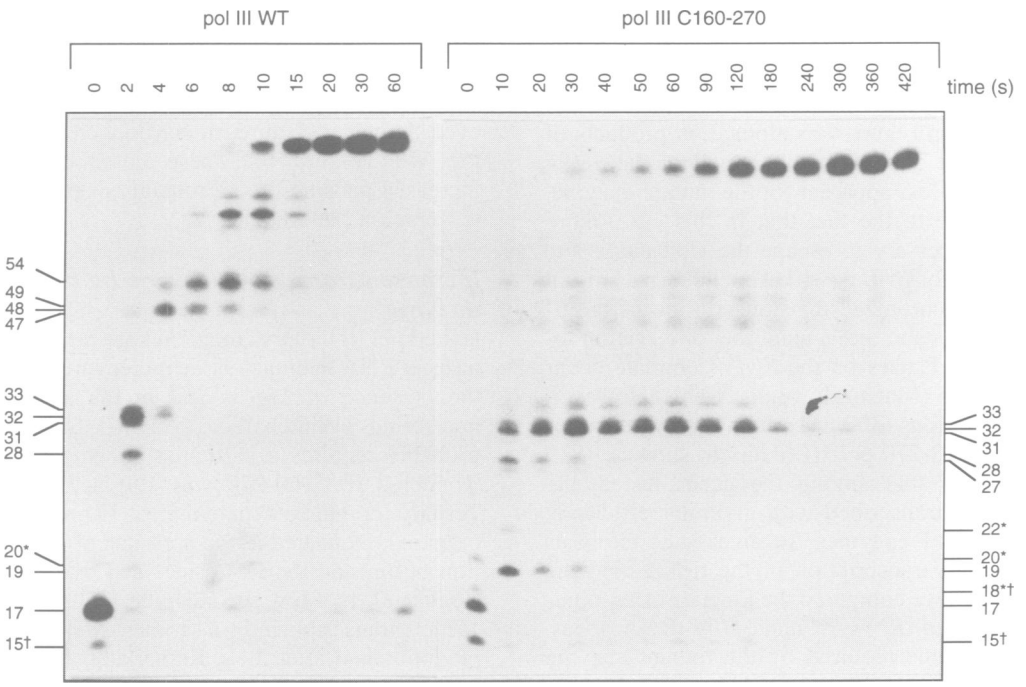



**Fig. 6.** Kinetics of elongation on the *SUP4* tRNA gene by WT and mutant pol III. 17 nt WT or C160-270 pol III-labelled ternary complexes were isolated, then incubated with unlabelled nucleotides for various periods of time, as indicated. The positions of pause sites are indicated on the side of the figure (see Matsuzaki *et al.*, 1994). *, transcripts derived from slippage; †, cleaved transcripts (see text).

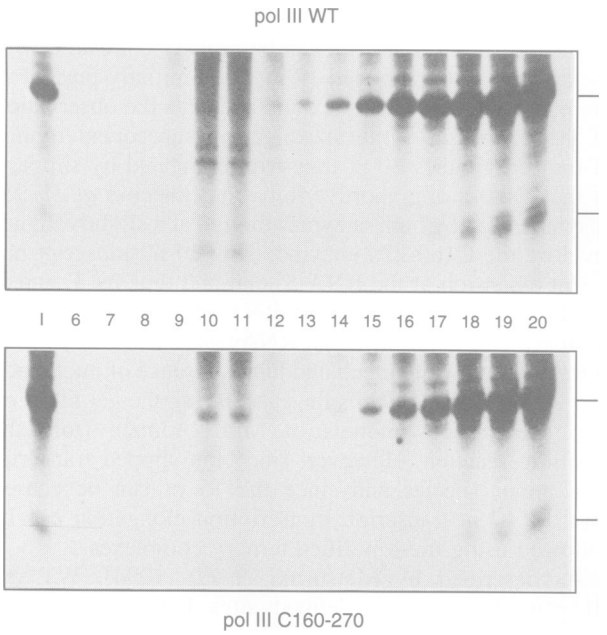



**Fig. 7.** Pausing and termination properties of WT and mutant pol III. Standard transcription mixture containing *SUP4* template and WT or C160-270 pol III were fractionated on Sepharose CL-2B. Each fraction was analysed for RNA by gel electrophoresis. Fraction numbers are indicated between the two autoradiograms. I, input. Fractions 9–12 contain ternary complexes. Fractions 13 and following contain released RNA transcripts. The RNA released at the minor (lower) or major (upper) terminators are indicated on the side of the figure.

by first synthesizing the 17 nt ternary complex at 30 mM UTP concentration, then resuming elongation by the addition of GTP and heparin to prevent recycling of pol III (data not shown). This method does not allow comparison of the intensity of the various transcripts since labelled UTP is present in the elongation mix. However, this experimental protocol prevents the synthesis of the 'slipped' transcript and cleavage of the products. In this case, the WT and C160-270 elongation patterns are superimposable, confirming the identification of the additional bands visible in Figure 6 as elongation products of the slipped transcript. Pauses in the 3′ half of the *SUP4* transcription unit are less apparent for the mutant enzyme. This is probably due to the fact that in this case, more than 3 min were necessary to escape the U32 pause site compared with 4 s for WT pol III. Extended pausing at U32 probably desynchronized the transcription of the 3′ end of the template and prevented the observation of downstream pauses. Pauses on the *SUP4* template occur mainly at U residues (Matsuzaki *et al.*, 1994). However, high UTP concentrations (300 μM) did not prevent extensive pausing by C160-270 pol III (data not shown).

Figure 3 revealed that among the genes tested, the $tRNA_3^{Leu}$ gene was transcribed with a similar efficiency by the mutant and WT enzymes. To investigate the basis of the more efficient transcription of the $tRNA_3^{Leu}$ gene by pol III C160-270, we compared the kinetics of accumulation of transcripts on $tRNA_3^{Leu}$ and *SUP4* tRNA genes. We found that the time required by the mutant enzyme for the synthesis of one transcript of $tRNA_3^{Leu}$ was shorter than for *SUP4* tRNA (100 s versus 190 s). The fact that the *SUP4* tRNA gene contains three stretches of UUU whereas the $tRNA_3^{Leu}$ template only contains one (starting

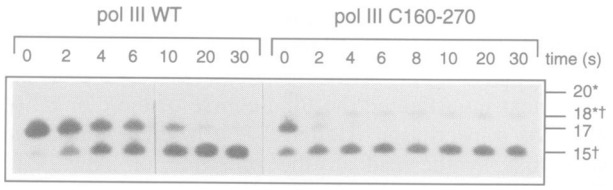



**Fig. 8.** Transcript cleavage by halted ternary complexes. WT or C160-270 pol III 17 nt-labelled ternary complexes were isolated on Sepharose CL-2B columns then incubated in transcription buffer containing 8 mM $MgCl_2$ in the absence of nucleotides, for various time lengths. The size of the different transcripts is indicated on the right side of the figure. *, transcripts derived from slippage; †, cleaved transcripts.

at the residue corresponding to +6 of the mature tRNA) may account in part for the different transcription efficiency of the two templates.

### *Termination properties of pol III C160-270*

The consequences of exaggerated pausing by pol III C160-270 on termination were examined using the *SUP4* gene. This template contains the UUUAU sequence (nt 52–56) which constitutes a weak termination site, and a strong natural terminator UUUUUUUUGUUUUUU (nt 101–115). Termination was investigated by chromatographic analysis of the transcription mixture on a CL-2B column (Figure 7). This size exclusion column separates the truly terminated (released) transcripts, which are retained on the column (fractions 14–20), from transcribing ternary complexes that are excluded in the void volume (fractions 9–12). Each fraction was analysed for RNA by gel electrophoresis (Figure 7). The ratio of transcripts terminated at the weak and the strong *in vivo* termination sites by the WT and C160-270 pol III was found to be identical (0.11 as measured with a PhosphorImager). In addition, uncompleted, paused transcripts were only present in ternary complexes. Moreover, in the elongation experiment, the paused transcripts synthesized by C160-270 were chased to full-length in the elongation reactions, even though it required much longer incubation than for the WT (Figure 6). These observations showed that increased pausing by the mutant enzyme did not alter the termination pattern.

### *Increased transcript cleavage by halted mutant enzyme*

Halted pol III ternary complexes are capable of cleaving the nascent RNA in dinucleotide increments when incubated in the presence of $MgCl_2$ and in the absence of cognate nucleotides (Whitehall *et al.*, 1994). Since RNA cleavage probably requires a particular enzyme conformation, we wondered if C160-270 was still active in this respect. Ternary complexes containing 17 nt transcripts were prepared, incubated in the presence of $MgCl_2$ in transcription buffer and the RNA analysed by gel electrophoresis (Figure 8). In a few seconds, the WT enzyme cleaved the 17mer transcript into a 15mer that accumulated and predominated after 20 s. Remarkably, transcript cleavage was 10-fold faster with the mutant enzyme. The cleaved transcript was already abundant in ternary complexes and all 17mer RNAs were cleaved in ~2–4 s. Again, a 20 nt slipped and the corresponding 18 nt cleaved transcripts

can be observed in the preparation of the labelled ternary complexes (time 0 s).

## Discussion

Domain f of C160, the largest subunit of yeast pol III, is one of the most conserved of RNA polymerases largest subunits (Mémet *et al.*, 1988). All mutations that alter α-amanitin sensitivity of pol II from different organisms map to this region (Bartolomei and Corden, 1987, 1995; Chen *et al.*, 1993). A *D.melanogaster* pol II α-amanitin-resistant mutation has also been shown to alter its elongation properties (Coulter and Greenleaf, 1985). Recently, mutations that increase termination by *E.coli* RNA polymerase have been found to occur in conserved regions of β′ subunit including in region f (Weilbaecher *et al.*, 1994). We have observed that exchanging the C-terminal half of domain f of C160 for that of B220, the largest subunit of pol II, did not affect pol III function. This, together with the observation that many conditional and lethal mutations map to this subdomain, shows that its function is essential and conserved between pol II and pol III. The mutant pol III, C160-270, harbouring a D839A R840A double substitution, did not exhibit an altered rate of phosphodiester bond formation. In contrast, the mutant enzyme was impaired in its switch from abortive transcription to elongation. It also showed increased pausing during tRNA gene transcription and increased cleavage rate of the RNA transcripts in halted ternary complexes. The phenotype of the mutant enzyme suggests that these three aspects of transcription are connected.

The non-specific transcription activity of C160-270 was reduced by lowering the DNA template concentration. One striking feature was that the synthesis of the first phosphodiester bond in a dinucleotide-primed reaction, during the abortive phase of transcription initiation, proceeded at a near WT rate but few enzymes entered a processive elongation mode. However, once the mutant enzyme elongated, its rate of nucleotide addition was the same as that of the WT enzyme. This indicated that the mutation affected the transition from abortive to processive transcription rather than the catalysis of phosphodiester bond formation. Kinetic analyses suggested a model where the enzyme switches from an abortive conformation to a processive conformation (de Mercoyrol *et al.*, 1990). This model could well explain the DNA concentration dependence of chain elongation by the mutant enzyme. Indeed, if the enzyme terminates transcription in a processive conformation and is able to reinitiate in that same conformation, increasing the DNA concentration should stimulate transcription, provided that the time to find a template is shorter than that required for the mutant enzyme to return to the abortive conformation. The synthesis of trinucleotides was inhibited in both the WT and C160-270 enzymes by increasing the concentration of the next nucleotide to be incorporated. In the WT this stimulated the abortive synthesis of the tetranucleotide and of the synthesis of long transcripts. In contrast, the mutant enzyme produced only very low amounts of tetranucleotide and of long RNA chains at low template concentrations. The lowered ability of the mutant enzyme to accumulate the tetranucleotide products is reminiscent of the effect of α-amanitin on pol II, which allows the stoichiometric synthesis of trinucleotides in a dinucleotide-primed reaction but blocks the catalytic accumulation of trinucleotide and further translocation (de Mercoyrol *et al.*, 1989). However, we did not determine whether the tetranucleotide was produced in a stoichiometric amount by C160-270 pol III.

The transcription cycle of the *SUP4* tRNA gene was slowed down by a factor of 7.6 at a concentration of 30 μM UTP. In contrast, a mutation in the C31 subunit of pol III affecting transcription initiation displayed only a 1.2-fold increase in cycling time under the same experimental conditions (Thuillier *et al.*, 1995). The time required for the synthesis of a *SUP4* tRNA was increased similarly in single-round experiments. This effect on transcript elongation was mainly due to increased pausing. Indeed, the duration of the pauses was markedly increased. Finally, RNA products of intermediate size between pause sites have not been observed (Figure 6 and data not shown) which also indicates that in the actively transcribing conformation the mutant enzyme adds nucleotides as fast as the WT one. These observations again argue in favour of the existence of two enzyme conformations. One would allow the rapid synthesis of the RNA, while the other would be adopted at or near pause sites. The mutant enzyme would thus be affected in the transition from the paused to the processive conformation. This transition could be the same as the one occurring during the switch from abortive to processive initiation. Increased pausing was also apparent with the *D.melanogaster* C4 α-amanitin-resistant pol II, though only a 2-fold reduction in elongation rate was reported and the experiments were not devised to observe increased pausing (Coulter and Greenleaf, 1985). Interestingly, the apparent $K_m$ for UTP of the C4 pol II and C160-270 were increased while the specificity constant $K_m/V_{max}$ was unchanged for the pol III mutant. On the *SUP4* tRNA gene, the pauses occur at tracts of Us (Matsuzaki *et al.*, 1994). However, increasing the UTP concentration did not relieve the pausing defect of the mutant. These observations suggest that the mutation produces its effect at a stage past nucleotide binding. It has been proposed that elongation rate and/or pausing might affect termination efficiency (McDowell *et al.*, 1994). This was not the case for pol III C160-270 since the efficiency of termination was similar to that of the WT. Specific transcription by pol III C160-270 of different tRNA genes was variously affected. This could reflect the number and strength of the pause sites present in the different templates. One might thus suggest that the developmental defects observed for the *D.melanogaster* Ubl or C4 mutations are related to the fact that the expression of some of the loci involved in development are regulated by strong pause sites within the transcribed sequence, reducing the amount of mRNA produced by the mutant pol II below a critical level. We have recently described a mutation in C160 region d, the most conserved domain of RNA polymerase largest subunits (Dieci *et al.*, 1995). The mutant enzyme, as C160-270, showed increased pausing during tRNA gene transcription. This phenotype was however partially suppressed by increasing UTP concentration. Additionally, the region d mutant enzyme synthesized much more slipped transcript compared with the WT and C160-270 enzyme, arguing that

regions d and f play different roles in the polymerase activity.

Pol III, as pol II and *E.coli* RNA polymerase, has an intrinsic ribonuclease activity (Suratt *et al.*, 1991; Izban and Luse, 1992; Reines, 1992; Whitehall *et al.*, 1994). In *E.coli* RNA polymerase, pol II and possibly pol III this activity is probably exerted by the enzyme active site (Rudd *et al.*, 1994; Whitehall *et al.*, 1994; Orlova *et al.*, 1995). The present finding that a mutation which increases the ribonuclease activity of pol III lies in the C160 subunit lends further support to the notion that the enzyme itself is responsible for this activity and suggests that the nuclease site may lie in the β′-like subunit. The increase in the ribonucleolytic activity of pol III C160-270 was unexpected, since it is supposed to reset paused or dead-end ternary complexes in an elongating conformation and thus help the enzyme to transcribe through the pause sites. It is possible that the mutation distorts the active site of pol III in a way which activates the ribonucleolytic activity, but slows down the conformation change required to transcribe past pause sites. It is less likely that increased cleavage was an indirect consequence of increased pausing because a distinct mutation in C160, also causing increased pausing, did not affect cleavage (Dieci *et al.*, 1995). Interestingly, Cipres-Palacin and Kane (1994) have obtained mutations in the pol II elongation factor TFIIS which still activate the RNA cleavage but do not stimulate transcription through pause sites.

Mutations in the β subunit of *E.coli* RNA polymerase also impair the transition from initiation to elongation (Kashlev *et al.*, 1990; Lee *et al.*, 1991; Sagitov *et al.*, 1993). In contrast to what was observed for pol III C160-270, the defect of the E813K mutant *E.coli* RNA polymerase, which affected both promoter clearance and pausing, could be compensated by increasing the nucleotide concentration (Sagitov *et al.*, 1993). Other mutations map in the segment K1064–L1073 of conserved domain I (Kashlev *et al.*, 1990; Lee *et al.*, 1991) which is located near the nucleotide binding site (Grachev *et al.*, 1989) These mutations seem to distort the active centre of the enzyme in a way that uncouples the elongation of the RNA chain and the propagation of the RNA polymerase along the template.

Recent experiments with *E.coli* RNA polymerase suggest that it transcribes RNA in two distinct modes (Feng *et al.*, 1994; Nudler *et al.*, 1994). In the 'monotonous mode' the enzyme moves along the DNA template one nucleotide at a time in register with the addition of ribonucleotides. The footprint of the enzyme moves continuously and the distance between its 5′ and 3′ boundaries and the active site, measured by the length of the RNA, is constant. As the RNA polymerase reaches a pause site, however, the enzyme enters an 'inchworming mode'. The footprint shrinks, due to a decrease in the distance between the downstream border of the enzyme and the active site. Transcribing past the last nucleotide of the pause site provokes a leap forward of several nucleotides (up to 18) of the 3′ boundary of the enzyme (Krummel and Chamberlin, 1992; Nudler *et al.*, 1994). It should be noted that some sequences which are not pause sites also induce the 'inchworming mode'. The RNase activity of RNA polymerase (Suratt *et al.*, 1991; Borukhov *et al.*, 1993; Rudd *et al.*, 1994) entails a reverse 'inchworming mode' where the downstream border of the enzyme does not move while the upstream edge translocates in register with the 3′ end of the shortened transcript (Lee *et al.*, 1994). These observations have led to a model proposing that RNA polymerase has a 3′ loose RNA binding site and a 5′ tight RNA binding site. At pause sites and during abortive transcription, the 3′ loose RNA binding site would be progressively filled in until further transcription is blocked. To progress through pause sites, the RNA polymerase would empty the 3′ loose binding site and leap forward. Similar movements would also happen during the transition from abortive to processive transcription (Chamberlin, 1995). Several arguments suggest that this model also applies to yeast pol III: (i) photocross-linking studies have shown that pol III undergoes extensive conformational changes between initiation and elongation (Bartholomew *et al.*, 1993); (ii) the kinetics of *SUP4* transcript elongation are best explained by models in which pol III can repeatedly switch between rapidly and slowly stepping states (Matsuzaki *et al.*, 1994); (iii) pol III is capable under nucleotide-limiting conditions of synthesizing 'slipped' transcripts, and mutations in the enzyme can accelerate this process (Dieci *et al.*, 1995). The production of 'slipped' transcripts is best explained by the existence of a loose and a tight RNA binding site (Nudler *et al.*, 1995); (iv) pol III has a ribonuclease activity which is increased by a mutation in C160 (Whitehall *et al.*, 1994 and this work) and (v) the C160-270 enzyme is affected in pausing, transition from abortive to elongating transcription simultaneously which suggests, as does the model, that these various aspects of transcription are connected. We would thus like to propose that the conformation changes required for the transition from abortive initiation to elongation, the transcription past pause sites and transcript cleavage are controlled by domain f.

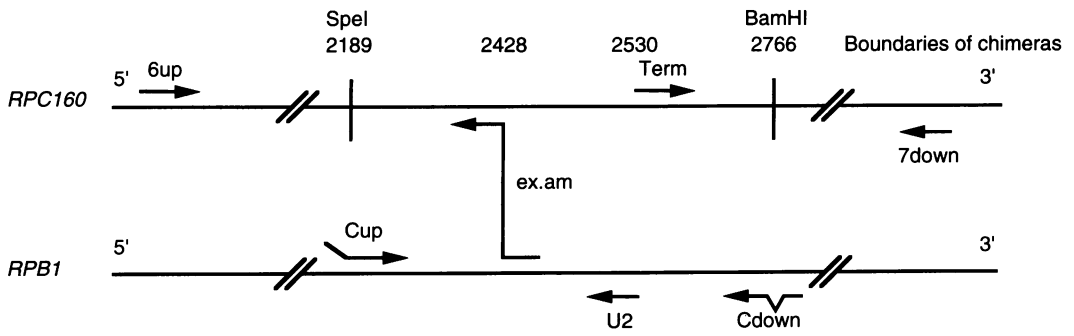
## Material and methods

### *Strains and plasmids construction*

Standard molecular biology techniques were described previously (Sambrook *et al.*, 1989). Yeast genetic techniques and media were described by Sherman (1991).

Strain MW636 is a derivative of MW420 (Werner *et al.*, 1992) containing the *trp1-Δ63* allele instead of *trp1-Δ1* and the *leu2-Δ1* allele instead of *LEU2*. MW420 was crossed with YPH102 (Sikorski and Hieter, 1989) to obtain the *TRP1 leu2-Δ1* segregant MW634 (*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 rpc160-Δ1::HIS3 pC160-6[URA3 CEN4 RPC160]*). MW634 was crossed with YPH499 (Sikorski and Hieter, 1989) to obtain the *trp1-Δ63 leu2-Δ1* segregant MW636 (*MATa ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63 rpc160-Δ1::HIS3 pC160-6[URA3 CEN4 RPC160]*). All the mutagenized *RPC160* alleles were tested by plasmid shuffling for their capacity to complement the chromosomal *rpc160-Δ1::HIS3* deletion of yeast strain MW636.

For the construction of the C160–B220 chimeras, we first constructed pC160-Δ7, a derivative of the *RPC160*-bearing plasmid pC160-7 (Werner *et al.*, 1992), to remove the *Bam*HI and *Spe*I sites present in the polylinker. This was done by cutting the vector at the polylinker *Not*I site and digesting ~100 nt with exonuclease III and nuclease S1 (Promega). The ends were then filled in with the Klenow enzyme and the linear DNA was self-ligated. pC160-CBC1 to -CBC4 were obtained by replacing the 577 nt long *Spe*I–*Bam*HI *RPC160* fragment, encompassing nt 2189–2766 of its open reading frame, with various *Spe*I–*Bam*HI PCR fragments bearing *RPC160–RPB1* fusions (Figure 1). PCR reactions were carried out according to the manufacturer's specifications (Perkin-Elmer-Cetus). Oligonucleotides and templates are listed in Figure 9. The PCR fragment used for the construction of pCBC1 was amplified from the *RPB1* sequence of pRP19 (Nonet *et al.*, 1987) homologous to the 577 bp sequence of *RPC160* between the *Spe*I and *Bam*HI sites,




Oligonucleotides

| | |
|---|---|
| Cup: | 5'-GCGCGACTAGTAAAGAAAGTTTTGGATGT-3' |
| Cdown: | 5'-TGCGCAGGATCCATACCATC-3' |
| 6up: | 5'-CCCCAGTTGTGATAAACTTAGACGC-3' |
| 7down: | 5'-GCTCCAGTCTTGCCTTACGATA-3' |
| U2: | 5'-CTCTGGGGAGTAATCATC-3' |
| Term: | 5'-TCCAAAGGTTTTGTAAGAAA-3' |
| Ex.am: | 5'-TGTCCTACACAAGCTGACATCTGTGAAACATTTAATGTGG-3' |

**Fig. 9.** Oligonucleotides used for gene fusions. Oligonucleotides are drawn as arrows parallel to the sequence they are complementary to, the arrow pointing towards the 3′ end. The tail of oligo Cup contains a *Spe*I site and the mismatch in Cdown creates a *Bam*HI site. Nucleotide coordinates are given relative to the beginning of the *RPC160* open reading frame.

with oligonucleotide 'Cup' containing the *Spe*I site and oligonucleotide 'Cdown' containing the *Bam*HI site. The PCR fragments used for the construction of pCBC2 and pCBC4 were obtained by PCR-driven gene fusion of the *RPB1* sequence with the *RPC160* sequence according to Yon and Fried (1989). Two templates were used in the same PCR reaction: pC160-Δ7 and pRP19. The oligonucleotides were '6up' and 'Cdown'. The 'linker oligonucleotide', 'Ex.am', was present in limiting amounts. pCBC4 was obtained serendipitously in the course of the PCR gene fusion. The most plausible explanation was synthesis of single-stranded DNA from oligonucleotide '6up' and priming of its 3′ end on pRP19 in regions of extensive sequence homology between *RPB1* and *RPC160*. The PCR fragment used for construction of pCBC3 was obtained by ligating blunt-ended PCR fragments amplified from pCBC2 with oligonucleotides '6up' and 'U2′ and from pC160-Δ7 with oligonucleotides '7down' and 'Term'. The hybrid fragment was cut with *Spe*I and *Bam*HI and cloned as described above. The region between the *Spe*I and *Bam*HI sites of the chimeras was sequenced on both strands.

Plasmid pC160-240 bears the *RPC160-240* allele of *RPC160* which codes for a C160 derivative with three repeats of the influenza haemagglutinin epitope, recognized by the 12CA5 antibody, after the N-terminal Met. The sequence of the N-terminus of C160-240 is thus MGVDYPYDVPDYALDYPYDVPDYALDYPYDVPDYALD (E.Lukhtanov and M.Werner, unpublished results). Plasmids pC160-251 to -268 and pC160-269 to -282 were obtained by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987) of *RPC160* and *RPC160-240* alleles borne on plasmids pC160-7 and pC160-240, respectively, leading to the substitutions listed in Figure 1. The sequence of the oligonucleotides was chosen according to the rationale described by Wertman *et al.* (1992). Alleles *rpc160-265, -266, -275, -280, -281* and *-253* were obtained by using two oligonucleotides. Alleles *rpc160-272, -264* and *-265* which contain only one mutated residue most probably result from oligonucleotide displacement by the DNA polymerase during *in vitro* synthesis of the mutant DNA strand. The 200 bp regions encompassing the expected mutations were sequenced on both strands.

### Protein purification

Yeast strains were grown in 6 l YPD and collected in late log phase ($A_{600}$ = 1) yielding ~30 g of cells (wet weight). Pol III was purified as described previously (Thuillier *et al.*, 1995) with the following modifications. Mono Q chromatography was replaced by a DEAE–Sephadex chromatography step. Proteins were eluted by a five column volume 200–500 mM ammonium sulphate gradient. Finally, the active fractions were concentrated on a 0.5 ml heparin column (loaded at 200 mM and eluted at 700 mM ammonium sulphate).

TFIIIC was purified by phosphocellulose, heparin Ultrogel, DEAE–Sephadex and for some experiments DNA affinity (Gabrielsen *et al.*, 1989). TFIIIB was purified by phosphocellulose and heparin Ultrogel chromatography (Moenne *et al.*, 1990). Recombinant TBP (Burton *et al.*, 1991), TFIIIB70 (Colbert and Hahn, 1992) and B″ fraction (Kassavetis *et al.*, 1992) were purified as described previously. The B″ fraction used for multiple-round transcription experiments was chromatographed on a Q Sepharose column (Huet and Sentenac, 1992)

The protein content of the different fractions was analysed by silver-stained SDS–PAGE (Blum *et al.*, 1987) and Western blotting as described previously (Thuillier *et al.*, 1995).

### In vitro transcription assays

To monitor pol III activity during purification, assays were carried out as described previously (Huet *et al.*, 1985). Analysis of the pattern of transcription products obtained with poly[d(A-T)] template was described previously (Thuillier *et al.*, 1995).

Multiple-round transcription assays using plasmid template were essentially performed as described previously (Moenne *et al.*, 1990) except that 20 mM Tris–HCl, pH 8.0, was used as buffer. The reaction was stopped by the addition of SDS to 1% final concentration, and the samples were treated with one volume phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v). Synthesized RNAs were ethanol-precipitated twice in the presence of 20 μg carrier RNA. Plasmid templates used were pRS316-*SUP4* (S.Shaaban, personal communication) bearing the *SUP4* gene, pUC-Glu (Gabrielsen and Øyen, 1987) bearing the $tRNA_3^{Glu}$ gene, pGE2WT (Baker and Hall, 1984) bearing the $tRNA_3^{Leu}$ gene, pY6 bearing the $tRNA_3^{Met}$ gene (Olah and Feldmann, 1980), pY41 and pY44 bearing respectively the $tRNA_2^{Arg}$ and $tRNA_2^{Ser}$ genes (Baker *et al.*, 1982).

For single-round transcription or kinetic assays, pol III was incubated 15 min at room temperature with transcription factors and DNA template before adding ATP, CTP to 0.6 mM and labelled UTP (10 μCi) to 0.03 mM. This results in the synthesis of a 17 nt-long RNA using the *SUP4* gene and a 5 nt-long RNA using the $tRNA_3^{Leu}$ gene. Transcription was allowed to resume by the addition of GTP to 0.6 mM for kinetic studies of multiple-round transcription or by the addition of GTP to 0.6 mM plus heparin to 0.3 mg/ml for single-round transcription.

Elongation and cleavage assays were performed using ternary complexes prepared as described above, except that labelled UTP was at a concentration of 3 μM. The complexes were purified by size-exclusion chromatography on 1 ml CL-2B columns as described previously (Steinberg and Burgess, 1992; Dieci *et al.*, 1995). Transcript elongation was analysed by resuming elongation by 17 nt halted ternary complexes by the addition of ATP, CTP, GTP (600 μM) and UTP (30 or 300 μM). Reactions were stopped at regular intervals and the reactions products were analysed on 15% denaturing polyacrylamide gels. Cleavage reactions were initiated by the addition to the labelled ternary complexes of transcription buffer containing $MgCl_2$ to bring its final concentration to 8 mM. Cleavage products were analysed as described for the elongation reactions. Termination was analysed by fractionation of multiple-round transcription assays on Sepharose CL-2B as described above. Transcripts

were quantitated using a PhosphorImager with Image Quant software (Molecular Dynamics).

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