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A universally conserved region of the largest subunit participates in the active site of RNA polymerase III

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The largest subunits of the three eukaryotic nuclear RNA polymerases present extensive sequence homology with the β′ subunit of the bacterial enzymes over five major co-linear regions. Region d is the most highly conserved and contains a motif, (Y/F)NADFDGD(E/Q)M(N/A), which is invariant in all multimeric RNA polymerases. An extensive mutagenesis of that region in yeast RNA polymerase III led to a vast majority (16/22) of lethal single-site substitutions. A few conditional mutations were also obtained. One of them, *rpc160-112*, corresponds to a double substitution (T506I, N509Y) and has a slow growth phenotype at 25°C. RNA polymerase III from the mutant *rpc160-112* was severely impaired in its ability to transcribe a tRNA gene *in vitro*. The transcription defect did not originate from a deficiency in transcription complex formation and RNA chain initiation, but was mainly due to a reduced elongation rate. Under conditions of substrate limitation, the mutant enzyme showed increased pausing at the intrinsic pause sites of the *SUP4* tRNA gene and an increased rate of slippage of nascent RNA, as compared with the wild-type enzyme. The enzyme defect was also detectable with poly[d(A–T)] as template, in the presence of saturating DNA, ATP and UTP concentrations. The mutant enzyme behavior is best explained by a distortion of the active site near the growing point of the RNA product.

Keywords: pausing/RNA polymerase III/slippage/β′ subunit/transcription/yeast

Introduction

Prokaryotic and eukaryotic RNA polymerases (pol) are complex multisubunit enzymes. The *Escherichia coli* enzyme, which is the best understood in both structural and functional terms, has a four-subunit, catalytically competent core $\alpha_2\beta\beta'$ (Burgess, 1969). In eukaryotes and archaebacteria, the enzyme subunit structure is more complex. A unifying feature is the extensive sequence conservation found in the largest (β′-like) and second largest (β-like) subunits of RNA polymerases from all kingdoms. Each of the two largest subunits contains a series of major conserved regions. These are regions c, d, f, g and h in β′-like subunits (Allison *et al.*, 1985; Mémet *et al.*, 1988) and regions D, E, F, G H and I in β-like subunits (Sweetser *et al.*, 1987; for a review and nomenclature of the conserved regions, see Thuriaux and Sentenac, 1992). One attractive hypothesis, partly based on structural data (Darst *et al.*, 1989, 1991; Schultz *et al.*, 1993), is that the conserved regions of the two largest subunits cooperate in the formation of a cleft where the active site lies. The active site can be viewed, in a broad sense, as the region around the catalytic center comprising the binding pockets for the incoming ribonucleoside triphosphate (NTP), the template fork and the 3′ growing end of the RNA chain. Crosslinking studies have established that residues in conserved regions H and I of the *E.coli* β subunit (Grachev *et al.*, 1989; Mustaev *et al.*, 1991), as well as in the homologous regions of the second largest subunit of yeast RNA pol II (Riva *et al.*, 1990), are close to the 5′ end of the initiator nucleotide. Similarly, the 3′ terminus of the nascent RNA has been shown to contact the β′ subunit in a site which includes part of the conserved region g (Borukhov *et al.*, 1991). These results are consistent with the hypothesis of a joint participation of the two large subunits in the RNA polymerase active site.

One approach to define the domains involved in the RNA polymerase active site is to analyze mutant enzymes carrying amino acid substitutions in conserved regions. Mutations in regions D, F, H and I of the β subunit cause impaired *in vitro* promoter clearance and/or elongation by *E.coli* enzyme (Kashlev *et al.*, 1990; Jin and Gross, 1991; Lee *et al.*, 1991; Mustaev *et al.*, 1991; Sagitov *et al.*, 1993; Jin and Turnbough, 1994). Also, termination-altering mutations were found to occur predominantly in conserved regions D, F and I (Landick *et al.*, 1990). This observation has been extended recently to the β-like subunit of yeast RNA polymerase III, C128 (Shaaban *et al.*, 1995). In some cases, the functional defects have been attributed specifically to a distortion of the active site, causing either an alteration of the nucleotide binding pocket (Jin and Gross, 1991) or a destabilization of the nascent RNA product in the active center (Lee *et al.*, 1991; Sagitov *et al.*, 1993). Mutational analyses of the β′-like subunits are more scarce. A mutant yeast RNA polymerase III carrying a substitution in a Zn^{2+} binding conserved region (region a) is characterized by the absence of a group of essential enzyme-specific subunits and is therefore non-functional (Werner *et al.*, 1992). Region c has a potential helix–turn–helix motif presenting a weak homology with the DNA binding cleft of *E.coli* DNA polymerase I (Allison *et al.*, 1985). This region, together with conserved regions f, g and h of the *E.coli* β′ subunit, has been shown recently to be involved in key nucleic acid contacts by

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rpoc_maize   475   TICLHPLVCKGFNADFDGDQMAVHLPLSLEAQAEARLLMF   514
rpoc_anasp   453   AIQLHPLVCPAFNADFDGDQMAVHVPLSLESQAEARLLML   492
rpoc_ecoli   446   AIQLHPLVCAAYNADFDGDQMAVHVPLTLEAQLEARALMM   485
rpa1_yeast   613   TLRLHYANTGAYNADFDGDEMNMHFPQNENARAEALNLAN   652
rpb1_yeast   467   TFRLNLSVTSPYNADFDGDEMNLHVPQSEETRAELSQLCA   506
rpc1_yeast   497   TFRLNECVCTPYNADFDGDEMNLHVPQTEEARAEAINLMG   536
rpoa_halha   513   TFRLNTVVCPPYNADFDGDEMNMHALQNEEARAEARVLMR   552
rpo1_vaccc   401   TIKISPGIANSQNADFDGDEEWMILEQNPKAVIEQSILMY   440
rpol_klula   840   TIAINPLLCQSFNADFDGDEMNIYGIRNKESIEEMKILAK   879
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Fig. 1. Alignment of region d of various RNA polymerases. Region d from *Zea mays* chloroplast β′ RNA polymerase chain (SWISSPROT name: rpoc_maize), *Anacystis* sp. γ chain (rpoc_anasp), *Escherichia coli* β′ subunit (rpoc_ecoli), *Saccharomyces cerevisiae* A190 (rpa1_yeast), B220 (rpb1_yeast) and C160 subunits (rpc1_yeast), *Halobacterium halobium* A polypeptide (rpoa_halha), vaccinia virus 147 kDa subunit (rpo1_vaccc) and *Kluyveromyces lactis* killer plasmid putative RNA polymerase (rpo1_klula) were aligned. Residues conserved in all region d sequences are shown in bold type. Points indicate non-invariant residues conserved between adjacent sequences, to qualitatively visualize the homology outside the invariant motif.

screening for termination-altering amino acid substitutions (Weilbaecher *et al.*, 1994). This finding is consistent with the close proximity of region g to the RNA 3′ terminus (Borukhov *et al.*, 1991) and with the observation that all known α-amanitin-resistant mutations in RNA polymerase II are located in conserved region f. One of these mutations in *Drosophila* pol II decreases the rate of transcript elongation by the purified enzyme (Coulter and Greenleaf, 1985), in keeping with the fact that α-amanitin affects the translocation step (de Mercoyrol *et al.*, 1989). Accordingly, a mutation in region f of the yeast pol III largest subunit seriously impairs elongation and initiation-to-elongation transition (Thuillier *et al.*, in preparation).

These genetic and biochemical data underscore the role of the conserved regions as important elements of the active site architecture. Remarkably, little is known of the role of the most highly conserved domain, region d, which contains the invariant (Y/F)NADFDGD(E/Q)M(N/A) motif (Mémet *et al.*, 1988). Conditionally defective mutations have been isolated that affect this motif in yeast pol II (Scafe *et al.*, 1990), but the role of this domain remains elusive. On the basis of its remarkable conservation, we suspected that region d would constitute an essential part of the RNA polymerase active site. This prompted us to mutagenize region d of the yeast RNA polymerase III largest subunit. Conservative and non-conservative substitutions for all the evolutionary invariant amino acids were almost invariably lethal. One of these substitutions (N509Y) was partly compensated by a vicinal silent mutation (T506I). This double mutation severely impaired the ability of pol III to transcribe *in vitro* a yeast tRNA gene, due to altered elongation properties, and favored slippage-dependent transcript synthesis. Taken together, these data indicate that the invariant motif directly contributes to the active site of the enzyme.

Results

Mutagenesis of conserved region d of C160 subunit

Region d is the most highly conserved sequence of the β′ homologs of multimeric RNA polymerases. Figure 1 displays an alignment based on the three yeast enzymes, on the eubacterial, chloroplastic, cyanobacterial, archaebacterial and poxviral RNA polymerases and on the putative RNA polymerase of the *Kluyveromyces lactis* killer plasmid. *RPC160* was mutagenized from nucleotide 1489 to 1608 by using a 120 nt long spiked oligonucleotide coding for amino acids 497–536 of C160 (see Materials and methods). The outcome of this mutagenesis is summarized in Figure 2. Fifty three different mutants were obtained, among which 29 were lethal, 17 silent and 7 conditional. Of the 31 mutations affecting a single residue that is invariant between regions d of the three yeast RNA polymerase largest subunits (underlined in Figure 2A), 20 were lethal and four were conditional, while only seven were silent. Conversely, nine out of 12 mutations affecting a single non-conserved residue were silent. Of the 22 different mutations affecting the highly conserved sequence YNADFDGDEMN, only four were silent. These corresponded to conservative replacements that do not change the size of the amino acid side chains (Y508F, F512Y) or reduce this size (A510G and E516D). On the other hand, all the non-conservative substitutions, as well as conservative replacements increasing the size of the side chains (D511E, D513E, G514V, D515E, N518Q), gave either lethal or conditional phenotypes. This suggested that the conserved motif and, presumably, the entire region must have a highly organized structure and are likely to be in close interaction with some other important domain of RNA pol III or with some ligand. Note that the E525P substitution, introducing a potential α-helix breaker, was phenotypically silent.

The mutation *rpc160-206*, which substituted I for M517, led to a conditional phenotype, as did the equivalent mutation (*rpb1-17*) affecting the largest subunit of yeast RNA polymerase II (Scafe *et al.*, 1990), while *rpc160-139*, which introduced the conservative replacement M517L, was lethal. The conditional mutation *rpc160-112* was found to be a double substitution, T506I and N509Y. The T506I mutation alone (*RPC160-170*) was silent, while the N509Y replacement (*rpc160-172*) was lethal. Thus

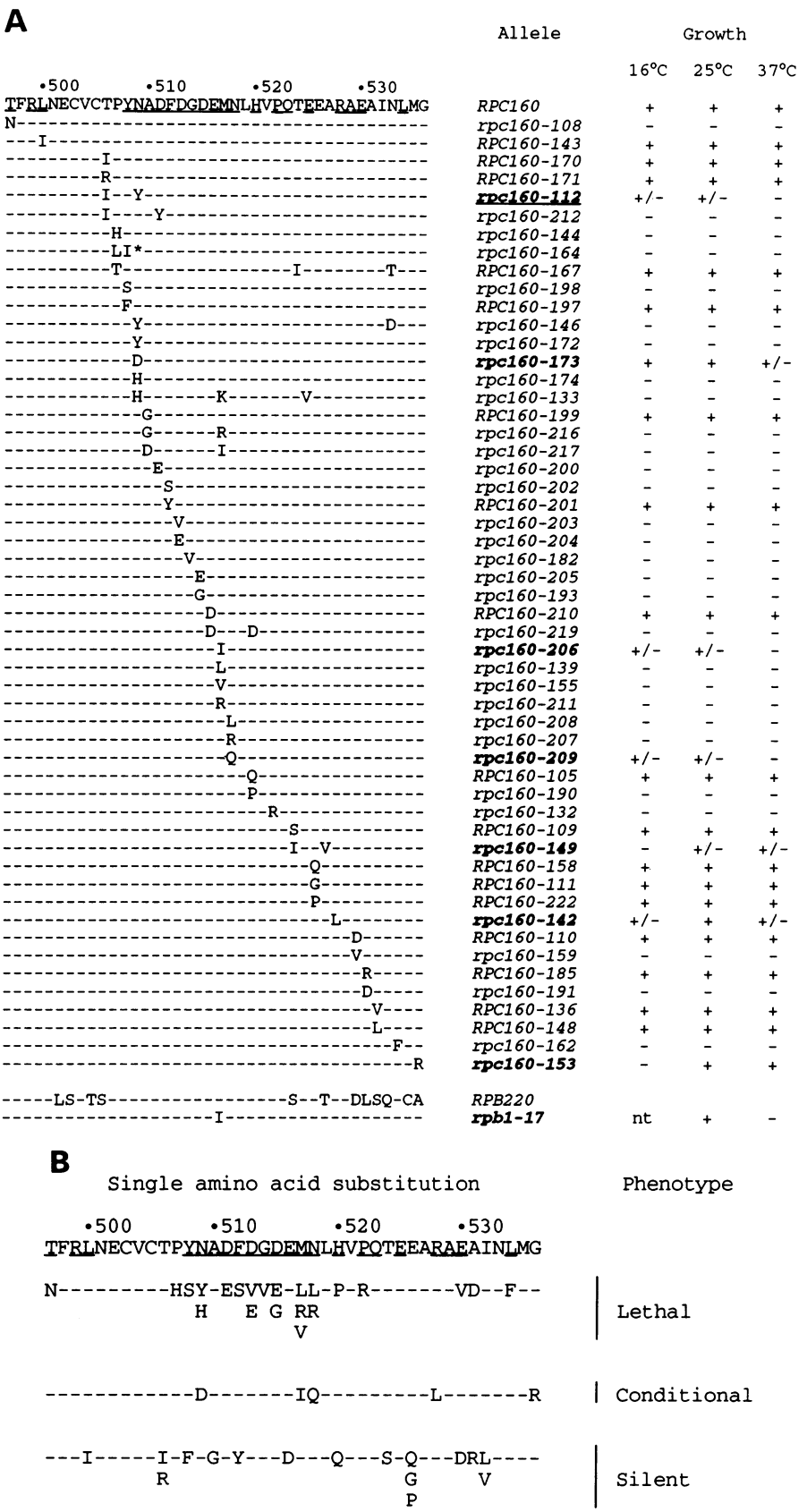

Fig. 2. Mutagenesis of C160 region d. (**A**) Allele numbering and phenotype of mutations affecting C160 region d. The residues invariant between the three yeast RNA polymerases are underlined in the sequence. Residues changed in the mutant C160 sequences are indicated for each *RPC160* allele. The amino acid replacements are indicated in single letter code; –, indicates unchanged residues; *, deleted residue. The sequence of the largest subunit of RNA polymerase II, B220, and of its mutant encoded by *rpb1-17* (Scafe *et al.*, 1990) are indicated at the bottom of the figure. The conditional alleles are set in bold type. The mutation characterized in this work is underlined. The mutant phenotypes are indicated by the following code: +, wild-type growth; +/–, slow growth; –, no growth. (**B**) Single amino acid replacements grouped according to their phenotypes.

the T506I mutation acted as an intragenic suppressor of the N509Y mutation.

Purification of C160-112 mutant RNA polymerase III

The tagged C160-112 pol III, corresponding to the *rpc160-112* allele, and an identically tagged wild-type enzyme from an isogenic strain were purified simultaneously by a rapid procedure, including sequential heparin–Ultrogel and DEAE–Sephadex columns (Huet *et al.*, 1985; see Materials and methods). During purification, RNA pol III activities were monitored by a non-specific transcription assay with poly[d(A–T)] as a template. The procedure resulted in 50% homogeneous preparations of both WT and C160-112 pol III, as judged by silver staining of SDS–PAGE gels (data not shown). The final yield was identical for WT and mutant enzymes, thus indicating that the slow growth phenotype of *rpc160-112* and the concomitant reduction of tRNA gene transcription *in vivo* (Hermann-Le Denmat *et al.*, 1994) are not due to a reduced level of pol III in mutant cells.

The subunit compositions of WT and mutant enzymes were indistinguishable in Western blot with antibodies against yeast pol III (Huet *et al.*, 1985; data not shown). Immunoprecipitation of C160-112 pol III with 12CA5 monoclonal antibody directed against the tag of the C160-112 subunit co-precipitated all the other pol III subunits (the ABC10α and β subunits could not be observed with the gel system used). These observations indicated that the mutant phenotype could not be attributed to an assembly or stability defect of the enzyme, as was the case for a mutation in the conserved region a of the yeast pol III largest subunit (Werner *et al.*, 1992).

Functional defects of C160-112 pol III in non-specific transcription assays

C160-112 and WT pol III activities were compared in non-specific transcription assays, using a wide range of template and substrate concentrations. The apparent K_ms of the two enzymes for UTP and ATP were determined by varying the concentration of one of the two substrates while holding the other one at a fixed level. The Lineweaver–Burk double reciprocal plots of the transcription rates obtained by varying the concentration of UTP and ATP respectively are presented in Figure 3A and B. The two enzymes were found to have the same apparent K_m for ATP (panel B), while the apparent K_m for UTP was ~70% higher for the mutant enzyme. In both cases, the mutant V_{max} was about half of the WT V_{max}. When ATP and UTP concentrations were kept constant and the template DNA concentration varied (Figure 3C), the half-saturating DNA concentration was reproducibly found to be the same for the two enzymes (~4 μg/ml). At saturating DNA concentrations, the C160-112 pol III still catalyzed transcription half as efficiently as WT pol III. These data indicated that the C160-112 pol III defect could involve some step distinct from DNA and nucleotide binding. Interestingly, the C160-112 enzyme displayed a wild-type activity when assayed in the presence of a poly[d(G–C)] template (data not shown), whose transcription is presumably limited by the rate of template unwinding-dependent translocation (Bonner *et al.*, 1994), suggesting that the mutation did not affect the ability of the enzyme to unwind DNA.

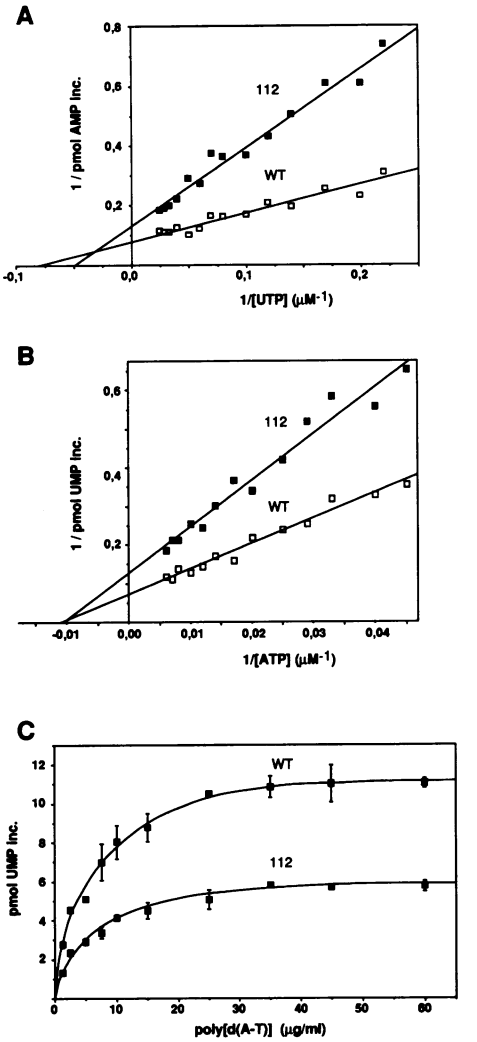

Fig. 3. Evaluation of NTP and DNA affinities for WT and C160-112 pol III. Initial velocities were measured by following the incorporation of [α-^{32}P]ATP (A) or [α-^{32}P]UTP (B and C). For A and B, the reciprocal of these values was plotted as a function of the reciprocal of a varied substrate concentration. (**A**) Evaluation of UTP affinity. The concentration of ATP was held constant at 0.5 mM and the concentration of UTP was varied. The apparent K_m for UTP, calculated as the reciprocal of the intercept on the abscissa, was 12 and 21 μM for WT and C160-112 pol III, respectively. In another independent experiment, the resulting values were 15 and 25 μM, respectively. (**B**) Evaluation of ATP affinity. The concentration of UTP was held constant at 0.3 mM and the concentration of ATP was varied. The apparent K_m for ATP, calculated as in (A), was 85 and 91 μM for WT and C160-112 pol III, respectively. In another independent experiment, the resulting values were 94 and 120 μM, respectively. (**C**) Evaluation of DNA affinity. In the presence of 0.5 mM ATP and UTP, the poly[d(A–T)] concentration was varied. In the same experiment, each DNA concentration was tested twice. Each point represents the average of the two values, and error bars are indicated.

Functional defects of C160-112 pol III on a natural template

The wild-type and mutant enzymes were compared for specific transcription of the yeast *SUP4* tRNA gene in the presence of partially purified transcription factors TFIIIB and TFIIIC. Stable pre-initiation complexes (TFIIIB·TFIIIC·tDNA) were formed, then RNA polymerase was

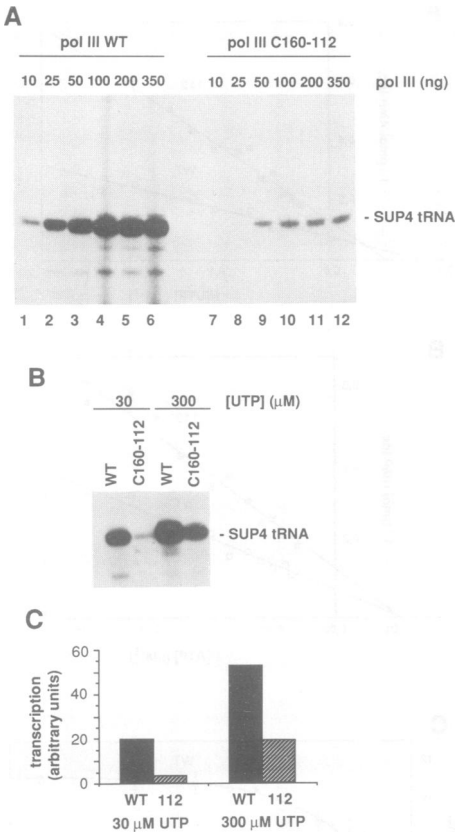

Fig. 4. Transcription of yeast *SUP4* tRNA gene by WT and C160-112 pol III. (**A**) Varying pol III concentration. The yeast *SUP4* tRNA gene was pre-incubated for 20 min with partially purified TFIIIB and TFIIIC, then increasing amounts of either WT or C160-112 pol III were added. The incubation was continued for 10 min, to allow the formation of complete, pol III-containing initiation complexes, then transcription was started by the addition of NTPs (500 μM ATP, CTP and GTP, 30 μM UTP) and allowed to proceed for 5 min in the presence of 10 μCi of [α-^{32}P]UTP (800 Ci/mmol). The reaction products were separated on a 6% polyacrylamide–7 M urea gel. The position of the complete tRNA gene primary transcript (SUP4 tRNA) is indicated. (**B**) Varying UTP concentration. Transcription was performed as described for (A), in the presence of 75 ng of either WT or C160-112 pol III. The ATP, CTP and GTP concentration was 500 μM, while the UTP concentration was either 30 μM (plus 4 μCi of [α-^{32}P]UTP) or 300 μM (plus 40 μCi of [α-^{32}P]UTP), as indicated. The reaction products were separated on a 6% polyacrylamide–7 M urea gel. The position of the complete tRNA gene primary transcript (SUP4 tRNA) is indicated. (**C**) Histogram representation of the results in (B). The transcription rate values are expressed in arbitrary units derived from PhosphorImager quantification.

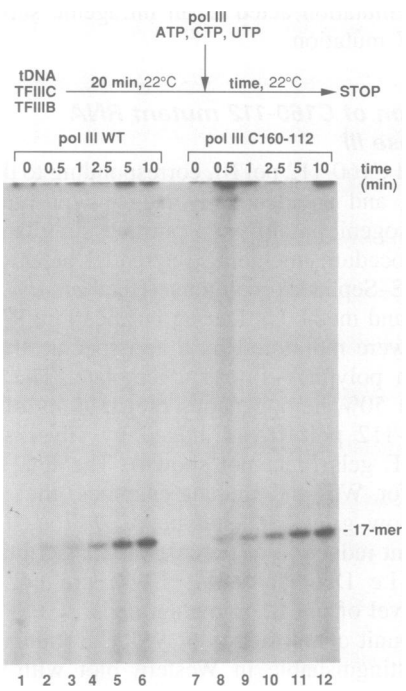

Fig. 5. Kinetics of transcription initiation by WT and C160-112 pol III. The *SUP4* tRNA gene was pre-incubated for 20 min with partially purified TFIIIB and TFIIIC to allow the formation of stable pre-initiation complexes. The mixture was then split into two parts, each of which received either WT or C160-112 pol III (2 ng/μl) together with ATP (500 μM), CTP (500 μM), UTP (30 μM) and 30 μCi of [α-^{32}P]UTP for each time point. At the indicated times, samples were taken from the mixtures and transferred to tubes chilled in dry ice to stop the reaction. The products were separated on a 15% polyacrylamide–7 M urea gel. The position of the 17 nt transcript is indicated. The upper part of the figure shows a scheme of the incubation protocol.

added, together with the four NTPs, and allowed to perform multiple rounds of the transcription cycle. In our *in vitro* transcription system, when a preformed tDNA·IIIB·IIIC·pol III transcription complex is allowed to begin transcription by the addition of NTPs, the wild-type rate of transcript accumulation is linear during the first 5 min of incubation and then decreases. Multiple-round transcription assays were thus performed for 5 min. The mutant pol III was invariably 6–7 times less active than WT pol III at limiting or saturating enzyme concentrations (Figure 4A, compare lanes 1–6 with 7–12), the half-saturating concentration being ~1 ng/μl for both enzymes (lanes 3 and 9 for WT and C160-112 pol III, respectively). These results indicated that the C160-112 pol III defect was not simply due to a reduced affinity for the pre-initiation complex. However, the mutant defect was partly reduced by increasing the concentration of UTP from 30 μM to nearly saturating levels (the other NTPs were always saturating; UTP was the labeling nucleotide). As shown in Figure 4B and C, raising the UTP concentration from 30 to 300 μM produced a 6-fold increase in transcription by C160-112 pol III, while it stimulated transcription by WT pol III only 2.7-fold. At the highest UTP concentration, C160-112 pol III still remained one third as active as the WT enzyme.

C160-112 pol III is not affected in transcription initiation

To compare the efficiency of WT and C160-112 pol III in performing the initiation step, we took advantage of the sequence of the yeast *SUP4* tRNA gene:

$$\ldots T^{-3}T^{-2}C^{-1}\underline{A}^{+1}A^{+2}C^{+3}A^{+4}A^{+5}T^{+6}T^{+7}A^{+8}A^{+9}A^{+10}T^{+11}$$
$$A^{+12}C^{+13}T^{+14}\overline{C}^{+15}T^{+16}C^{+17}G^{+18}G^{+19}T^{+20}A^{+21}G^{+22}C^{+23}\ldots$$

In the absence of GTP, a halted, stable ternary complex is formed, containing a 17 nt long, nascent RNA product (Kassavetis *et al.*, 1989). A preformed, stable pre-initiation complex (tDNA·TFIIIC·TFIIIB) was incubated with RNA pol III, ATP, CTP and α-^{32}P-labeled UTP, and the time course of the formation of the 17mer product was analyzed (Figure 5). Even in the presence of a sub-optimal UTP concentration (30 μM), equal amounts of mutant or wild-

type enzymes produced the same number of ternary complexes with exactly the same time kinetics (Figure 5, compare lanes 1–6 with lanes 7–12). Under the conditions of this experiment, synthesis of the 17mer RNA was not the rate-limiting step. If RNA polymerase was allowed to associate with tDNA·TFIIIC·TFIIIB complexes, before the addition of ATP, CTP and UTP, 17mer synthesis was completed in a few seconds by both WT and mutant enzymes (data not shown). Since synthesis time was negligible, the time needed for formation of the 17mer product reflected essentially the time required for pol III association to the pre-initiation complex and DNA melting. Thus, the defect of the mutant enzyme in multiple rounds of transcription did not originate from a deficiency in transcription complex formation and chain initiation.

Transcript slippage by C160-112 pol III in the early phases of RNA synthesis

Since five U residues must be incorporated during the synthesis of the 17mer transcript, we examined the effect of lowering the UTP concentration from 30 to 3 μM on the 17mer synthesis. Initiation complexes containing the WT pol III were able to complete 17mer synthesis in <30 s under these conditions (Figure 6A, lane 1 and data not shown). In contrast, C160-112 pol III-containing complexes could not produce any 17mer transcript in 30 s (Figure 6A, lane 2), and only after 10 min (lane 6) had they completed the synthesis of the expected 17mer. This extremely reduced rate of 17mer synthesis was associated with the appearance of a 20mer, representing 45% of total reaction products after 10 min (lane 6). A much smaller amount of the 20mer was also produced by WT pol III (lane 1). Since the 20mer synthesis strongly depended on lowering the UTP concentration, it could not be explained as a consequence of incorrect initiation by the mutant pol III at a site 3 nt upstream of the normal start site, which would have involved an improbable UU initiation event, strongly unfavored by the very low UTP concentration. Similarly, readthrough past the +17 position (due, for example, to an increased misincorporation of non-G nucleotides at positions +18 and +19) also seemed unlikely, as it should not depend on lowering the UTP concentration. The UTP dependence could be explained, however, if the 20mer transcript derived from a 'slippage' event during the early phase of RNA synthesis. The transcript slippage process described for T7 and bacterial RNA polymerases (Parker, 1986; Martin *et al.*, 1988; Borukhov *et al.*, 1993) involves the upstream movement of a short, nascent RNA along the template strand without translocation of the enzyme. It seems to require the presence, around the +1 site, of either a short stretch of identical residues (Martin *et al.*, 1988) or a short direct repeat allowing minimal base pairing of the slipped transcript with the DNA template strand immediately upstream of the initiation site (Borukhov *et al.*, 1993; Severinov and Goldfarb, 1994). The sequence of the yeast *SUP4* tRNA gene around the site of initiation (...C^{-1} $A^{+1}A^{+2}C^{+3}A^{+4}A^{+5}$...) meets the latter requirement. At low UTP concentrations, the rate of UMP incorporation at position +6 could be extremely reduced in the case of the mutant, and this could provide enough time for the AACAA nascent transcript to slip three positions upstream along the template strand. Further elongation of this

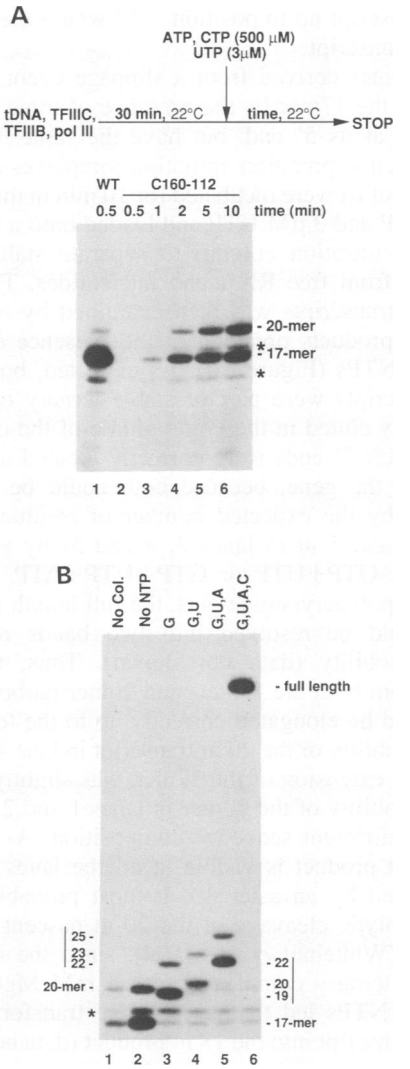

Fig. 6. Slippage by C160-112 pol III. (**A**) 17mer synthesis by WT and C160-112 pol III in the presence of a limiting UTP concentration. Complete transcription initiation complexes were formed on the *SUP4* tRNA gene in the presence of 2 ng/μl of either WT (lane 1) or C160-112 (lanes 2–6) pol III. After the addition of ATP (500 μM), CTP (500 μM) and [α-^{32}P]UTP (3 μM, 30 μCi for each time point), samples were taken at the indicated times and transferred to tubes chilled in dry ice. The reaction products were separated on a 15% polyacrylamide–7 M urea gel. The positions of 17 and 20 nt transcripts are indicated. Asterisks indicate the positions of 18 and 15 nt minor products derived from nucleolytic degradation of the 20mer and 17mer, respectively (Whitehall *et al.*, 1994). The upper part of the figure shows a scheme of the incubation protocol.
(**B**) Controlled RNA chain extension by purified ternary complexes containing the 17mer and 20mer. On the *SUP4* tRNA gene, complete transcription initiation complexes were formed containing C160-112 pol III. RNA synthesis was then allowed to proceed for 10 min in the presence of 500 μM ATP and CTP and 3 μM [α-^{32}P]UTP. Ternary complexes were purified by gel filtration on a Sepharose CL-2B column. In lane 2 (No NTP) the purified ternary complexes were incubated in the absence of NTPs and $MgCl_2$. The complexes (lane 2) were allowed to resume elongation by the addition of 500 μM GTP (lane 3, 2 nt extension), GTP and UTP (lane 4, 3 nt extension), GTP, UTP and ATP (lane 5, 5 nt extension) or all four NTPs (lane 6, full-length transcript synthesis), together with 5 mM $MgCl_2$. In lane 1 (No Col.) the transcript products before gel filtration are shown. The positions of the 20mer and of its partial elongation products are indicated on the left. The positions of the 17mer and its products are indicated on the right. The asterisk indicates the position of a 18 nt product deriving from nucleolytic degradation of the 20mer (see A).

slipped transcript up to position +17 would then produce the 20 nt transcript.

If the 20mer derived from a slippage event, it should differ from the 17mer by the presence of three additional nucleotides at its 5′ end, but have the same 3′ end. To confirm this interpretation, initiation complexes containing C160-112 pol III were incubated for 10 min in the presence of ATP, CTP and 3 μM UTP and loaded onto a Sepharose CL-2B gel filtration column to separate stable ternary complexes from free RNA and nucleotides. The 3′ end of nascent transcripts was then examined by analysis of elongation products obtained in the presence of defined subsets of NTPs (Figure 6B). As expected, both 17 and 20 nt transcripts were part of stable ternary complexes, because they eluted in the void volume of the column. In addition, their 3′ ends were correctly located at the +17 position of the gene, because both could be elongated identically by the expected number of residues (respectively 2, 3 and 5 nt in lanes 3, 4 and 5) by addition of GTP alone, GTP+UTP or GTP+UTP+ATP. On lower percentage polyacrylamide gels, the full length product in lane 6 could be resolved into two bands of slightly different mobility (data not shown). Thus, transcripts deriving from both the 17mer- and 20mer-harboring complexes could be elongated correctly up to the termination site. The mobility of the 20 nt transcript in lane 4, deriving from -GGU extension of the 17mer, was slightly different from the mobility of the 20mer in lanes 1 and 2, probably due to the different sequence composition. As in Figure 6A, a 18 nt product is visible in all the lanes of Figure 6B (indicated by an asterisk). It most probably derived from nucleolytic cleavage of the 20 nt nascent transcript by pol III (Whitehall *et al.*, 1994), since the incubation of purified ternary complexes with 5 mM $MgCl_2$ in the absence of NTPs led to the complete transformation of the 20 nt transcript into the 18 nt product (data not shown). Under the same conditions, the 17 nt nascent transcript was completely converted to a 15 nt product. Thus, the mutant enzyme was not apparently affected in its ability to catalyze nucleolytic cleavage of nascent RNA. The fact that the 20mer and the 17mer could be shortened identically further indicated that they had the same 3′ end.

If the slippage event in the case of C160-112 pol III (Figure 6A) was mainly a consequence of the extremely reduced rate of U incorporation at position +6, it should be possible to induce the formation of the 20 nt product by WT pol III in the absence of UTP. In the experiment shown in Figure 7A, transcription initiation complexes containing either WT (lanes 1–7) or C160-112 (lanes 8–14) pol III were pre-incubated for different periods of time with ATP and CTP only, to produce the AACAA nascent transcript. Then UTP was added to a final concentration of 30 μM, in order to allow all the initiated transcripts to be elongated rapidly up to position +17. Under these conditions, the amounts of 17mer and 20mer products observed after each pre-incubation time were taken to represent the amounts of normal and 'slipped' transcripts formed during the pre-incubation period. The 20 nt product became visible after a 2 min pre-incubation of WT pol III with ATP and CTP (Figure 7A, lane 3). After a 10 min pre-incubation, it represented 50% of total products (lane 5). Interestingly, a quantification of the 20mer and 17mer synthesis by WT and C160-112 enzymes

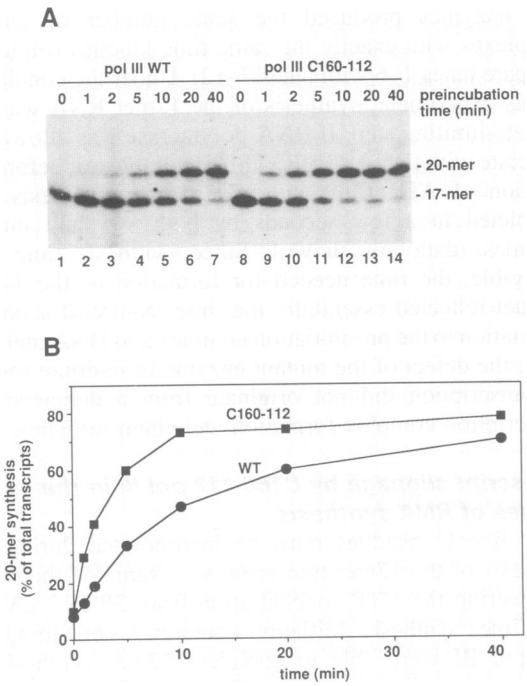

Fig. 7. Kinetics of slippage by WT and C160-112 pol III. (**A**) Complete transcription initiation complexes were formed on the *SUP4* tRNA gene in the presence of either WT pol III (lanes 1–7) or C160-112 pol III (lanes 8–14). ATP and CTP (500 μM each) were added and the incubation continued for the indicated periods of time. Then UTP was added to 30 μM (together with 30 μCi of [α-^{32}P]UTP) and the incubation continued for 2 min, to allow all the initiated RNA chains to be extended up to position +17. Lanes 1 and 8 represent control reactions in which ATP, CTP and UTP were added simultaneously to the reaction mixtures. The reaction products were separated on a 15% polyacrylamide–7 M urea gel. The positions of 17 and 20 nt products are indicated. (**B**) Reaction products in (A) were quantitated as described in Materials and methods. The percent of 20 nt transcript with respect to total products was plotted as a function of the pre-incubation time in the presence of ATP and CTP only.

showed that slippage was markedly (4–5 times) more rapid in the case of the mutant enzyme (see Figure 7B, and compare lanes 4 and 9 in Figure 7A).

Reduced elongation rate of C160-112 pol III

Altogether, the above data indicated that the C160-112 pol III can correctly perform the steps leading to transcription initiation, but is somehow affected at the level of chain elongation. To look in more detail at its elongation properties, WT or C160-112 pol III ternary complexes containing the 17 nt nascent transcript were allowed to resume elongation by the addition of GTP, in the presence of 30 μM UTP (Figure 8A) or 300 μM UTP (Figure 8B) together with heparin to inhibit re-initiation (Kassavetis *et al.*, 1989) and the time course of the elongation reaction was analyzed. Nascent transcripts in both wild-type and mutant ternary complexes could be quantitatively chased into full length transcripts (Figure 8B, compare lanes 1 and 5 with lanes 4 and 9, respectively). In this experiment, the intensity of the 17mer and of the full length transcript are directly comparable, since elongation was performed after a 10-fold isotopic dilution of the [α-^{32}P]UTP. In the experiment shown in Figure 8A, the full length transcript bands representing complete elongation of all nascent transcripts (lanes 7 and 13, for example) were much more

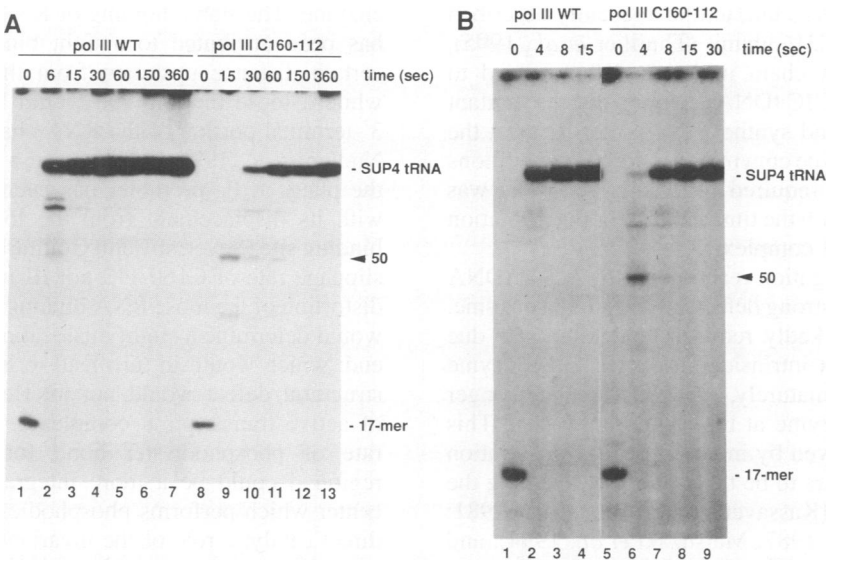

Fig. 8. Kinetics of transcription elongation on the *SUP4* tRNA gene by WT and C160-112 pol III. (**A**) Elongation at low UTP concentration. Complete transcription initiation complexes were formed on the yeast *SUP4* tRNA gene by pre-incubation with partially purified TFIIIB and TFIIIC, and 2 ng/μl of either WT or C160-112 pol III. ATP (500 μM), CTP (500 μM) and UTP (30 μM) were then added (together with 30 μCi of [α-^{32}P]UTP for each time point) in order to allow the formation of stable ternary complexes containing the 17mer. Elongation was re-started by the addition of GTP (500 μM) together with heparin (0.3 mg/ml) to block reinitiation of transcription. At the indicated times, samples were taken from the mixtures and transferred to tubes chilled in dry ice, to stop the reaction. For lanes 1 and 8, the samples were taken before the addition of GTP. The reaction products were separated on a 15% polyacrylamide–7 M urea gel. The positions of the 17mer and of the complete tRNA gene primary transcript (SUP4 tRNA) are indicated. The black arrow indicates the position of a 50 nt intermediate reaction product. (**B**) Elongation at high UTP concentration. The experiment was the same as in (A), with the exception that, at the moment of GTP addition, the UTP concentration was adjusted to 300 μM.

intense than the 17mer bands (lanes 1 and 8), because of the lack of isotopic dilution. Nevertheless, accurate quantification of nascent and full length transcripts showed that, even in this case, all the 17mer pool had been chased into full length transcripts by both RNA polymerases. Thus, ternary complexes containing C160-112 pol III were able to perform a complete elongation cycle, without premature dissociation due to a reduced processivity. However, at 30 μM UTP, the elongation process took ~6 s in the case of WT pol III and >30 s in the case of C160-112 enzyme (Figure 8A, compare lane 2 with lanes 9–11). Elongation by yeast RNA polymerase III on the *SUP4* tRNA gene is characterized by the existence of several intrinsic pause sites where the rate of nucleotide addition is considerably reduced, with most of the slowest steps involving U→A, U→U and A→U additions (Matsuzaki *et al.*, 1994). Interestingly, the decreased elongation rate of the C160-112 enzyme was found to correspond to an increased half-life of several paused complexes during the elongation process. For example, the paused complex whose corresponding 50 nt transcript is marked by an arrow in Figure 8A was detectable only after 6 s elongation with WT pol III, and had completely disappeared after 15 s. In the case of C160-112 pol III, the same paused complex was predominant after 15 s and still detectable after 60 s. In agreement with the results of multiple-round transcription assays, the elongation defect of the mutant enzyme was partially relieved at high UTP concentrations (300 μM). Under these conditions, the mutant enzyme elongated only about half as rapidly as the wild-type (Figure 8B, compare lanes 2 and 6). In this case, the 50 nt paused transcript could be observed only after a 4 s elongation by C160-112 pol III (Figure 8B, lane 6) and disappeared after 15 s. When the same kinetics of chain elongation were carried out in the presence of 300 μM UTP and a limiting concentration (10 μM) of GTP, whose addition to growing RNA chains is generally not involved in pausing (Matsuzaki *et al.*, 1994), the results were very similar to those in Figure 8B (data not shown).

Discussion

Region d is the most conserved motif in the largest subunit of multimeric RNA polymerases (Mémet *et al.*, 1988). Our mutational study confirmed the critical role of this region, since amino acid substitutions are predominantly lethal or conditionally lethal. This is especially true for the invariant motif (Y/F)NADFDGD(E/Q)M(N/A), where even highly conservative substitutions such as D511E, D513E and D515E are fully lethal. Analysis of the *in vitro* properties of one mutant enzyme, C160-112, revealed the participation of region d in the RNA polymerase active site, specifically at the growing point of the RNA chain. This conclusion stems from the behavior of the mutant enzyme in non-specific as well as in specific transcription assays.

Under non-specific transcription conditions, in the presence of saturating concentrations of poly[d(A–T)] and NTPs, the C160-112 pol III catalyzed transcription half as efficiently as WT pol III. The two enzymes showed the same basal affinity for DNA and ATP, and the apparent K_m for UTP was only slightly (70%) higher in the case of mutant pol III. These results suggested an intrinsic defect in the catalysis of the polymerization reaction. The enzyme defect was most prominent with a tRNA gene as template in the presence of the class III transcription

factors TFIIIB and TFIIIC but, unlike a recently described pol III mutant in the C31 subunit (Thuillier *et al.*, 1995), it did not affect RNA chain initiation. When added to preformed TFIIIB·TFIIIC·tDNA complexes, the mutant pol III could initiate and synthesize a 17mer RNA at the same rate as the wild-type enzyme (Figure 5), in conditions under which the time required for 17mer synthesis was negligible compared with the time needed for the formation of initiation-competent complexes.

Analysis of the elongation reaction on the *SUP4* tDNA template revealed two strong defects of the mutant enzyme. First, there was a markedly reduced elongation rate due to increased pausing at intrinsic pause sites. The enzyme did not terminate prematurely, but paused much longer than the wild-type enzyme at the same pause sites. This defect was partly relieved by increasing the concentration of UTP. Pausing appears to be the major factor setting the mean elongation rate (Kassavetis and Chamberlin, 1981; Yager and von Hippel, 1987; Matsuzaki *et al.*, 1994), and there is a direct link between the extent of pausing and nucleotide concentration (Kassavetis and Chamberlin, 1981; Jin and Gross, 1991). It has been suggested that polymerization is temporarily blocked at pause sites due to a distortion of the active site (Sagitov *et al.*, 1993). Along this line, one could imagine that the double mutation in C160-112 pol III causes an alteration of the active site, which in turn generates a defect in phosphodiester bond formation most prominent at pause sites. The effect of such an alteration would be less evident on poly[d(A–T)], due to the absence of pause sites (Jin and Gross, 1991). The similar activity of mutant and wild-type enzymes on poly[d(G–C)] argues against a translocation defect, since, on this template, the transcription rate is supposed to be limited by the rate of template unwinding-dependent translocation (see similar consideration in Bonner *et al.*, 1994).

The second remarkable defect of the C160-112 pol III was its propensity to catalyze a slippage reaction during the early phase of RNA synthesis. Slippage was detected at limiting substrate concentrations and, therefore, is likely to reflect the same alteration of the active site as the prolongation of pausing. Slippage by the wild-type enzyme could also be induced by artificially blocking the elongation complex at position +5 of the *SUP4* tRNA gene. Remarkably, however, under the same conditions, slippage by the mutant enzyme was 4–5 times faster than by the wild-type (Figure 7), as if the corresponding active site rearrangements were favored by the mutation. This was not accompanied by an increased instability of ternary complexes and release of the nascent RNA reaching position +5, since equal amounts of UTP-chaseable ternary complexes were present for the two enzymes at each time point (see Figure 7A). There was also no premature RNA release when the C160-112 pol III was stalled at position +17, nor significant alterations of its *in vitro* termination properties (G.Dieci, unpublished). Thus, the primary effect of the *rpc160-112* mutation is a distortion of the active site favoring slippage and impairing pause site clearance without reducing the stability of ternary complexes.

It has been proposed recently that RNA binding during transcription depends primarily on RNA–RNA polymerase interactions involving two RNA binding sites on the enzyme. The stable holding of RNA in ternary complexes has been attributed to a tight binding site located at a certain distance upstream from the RNA growing end, while a loose binding site would be in contact with the 3′-terminal portion of the RNA chain (Chamberlin, 1994; Nudler *et al.*, 1994). Slippage of a 4 nt long transcript at the phage λ P_L promoter has been proposed to coincide with its displacement from the loose to the tight RNA binding site (Severinov and Goldfarb, 1994). The increased slippage rate of C160-112 pol III may thus derive from a distortion of the loose RNA binding site. Such an alteration would determine a slight mislocation of the RNA growing end, which would in turn lead to increased pausing. This structural defect would not interfere with the formation of active transcription complexes, but would reduce the rate of phosphodiester bond formation. Alternatively, region d could be a more integral part of the catalytic center which performs phosphodiester bond formation. A direct catalytic role of the invariant aspartic acid residues of region d seems possible (cf. Joyce and Steitz, 1994). Due to its proximity, N509 could directly influence the performance of the catalytic center. Increased slippage and pausing could be linked to an intrinsic deficiency in the catalysis of phosphodiester bond formation. It should be noted that mutations touching the catalytic center of a simpler enzyme, T7 RNA polymerase, produce mutant enzymes in which a defect in phosphodiester bond formation is associated with increased synthesis of aberrant transcripts deriving from slippage (Bonner *et al.*, 1994).

A mutant yeast RNA pol III carrying a substitution in the conserved region f of C160 has been characterized recently in our laboratory (Thuillier *et al.*, in preparation). Like C160-112 pol III, it exhibits increased pausing on the *SUP4* tRNA gene. However, no increased slippage has been observed in this case, arguing that regions d and f of the largest subunit contribute differently to the RNA polymerase active site. The functional consequences of the *rpc160-112* mutation are also distinct from those of previously described mutations in β-like subunits. Some of these mutations induce destabilization and increased release of the nascent RNA from the active site, resulting in promoter clearance and elongation defects (Kashlev *et al.*, 1990; Lee *et al.*, 1991; Mustaev *et al.*, 1991; Sagitov *et al.*, 1993; Jin and Turnbough, 1994), or reduce the basal affinity of the enzyme for purine nucleotides (Jin and Gross, 1991). Several β-like subunit mutations, grouped in conserved regions, have been isolated on the basis of an altered termination phenotype (Landick *et al.*, 1990; Shabaan *et al.*, 1995). However, none of the β-like subunit conserved regions has been implicated directly in the catalytic act of phosphodiester bond formation. Recently, termination-altering mutations in the bacterial β′ subunit have also been isolated (Weilbaecher *et al.*, 1994). None of them mapped in region d. All these observations support the idea that the conserved regions of the two large subunits jointly participate in the RNA polymerase active center through functionally distinct contributions.

Materials and methods

Plasmid and strain constructions

The growth phenotype of the oligonucleotide-directed *RPC160* mutations (see Figure 2A) was tested by a plasmid shuffling assay based on the

ability to complement null insertion or deletion mutations of *RPC160* in strains MW409 [*MAT***a** *ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160::HIS3* pC160-6 (*URA3 CEN4 RPC160*)], MW420 [*MATα ade2-101 ura3-52 trp1-Δ1 lys2-801 his3-Δ200 rpc160Δ-1::HIS3* pC160-6 (*URA3 CEN4 RPC160*); Werner *et al.*, 1992] or MW636 [*MAT***a** *ade2-101 lys2-801 leu2-Δ1 his3-Δ200 ura3-52 trp1-Δ63 rpc160-Δ1::HIS3* pC160-6 (*URA3 CEN4 RPC160*); Thuillier *et al.*, in preparation].

Plasmid pC1607 (*TRP1 CEN4 RPC160*) was randomly mutagenized on domain d using a spiked oligonucleotide from nucleotide 1489 to 1608 of RPC160, encoding amino acids 497–536 of the C160 pol III subunit. Independent plasmid clones (2500) were obtained, pooled and transformed in strain MW409 on a minimal medium supplemented with caseine hydrolysate, adenine sulfate and uracil, thus selecting for growth without tryptophan (see Werner *et al.*, 1992). The presence of uracil allows the loss of the wild-type *RPC160 CEN4 URA3*-bearing plasmid pC160-6. Yeast transformants (15 000) were replica plated on 5FOA medium (Boeke *et al.*, 1984) and grown at the permissive temperature of 25°C to select against the *URA3* clones bearing the pC160-6 plasmid. The resulting clones were replica plated on YPD-rich medium. The mutated plasmids were isolated from the clones unable to grow at low (16, 25°C) or high (37°C) temperature and retested by the same plasmid shuffling procedure in strain MW409, since it is common to find thermosensitive mutations unlinked to the mutagenized plasmid after yeast transformation. Only one thermosensitive mutation, *rpc160-112*, was found by this procedure. Plasmids were then randomly selected from the mutant pool, sequenced around region d and tested by plasmid shuffling. Except for mutation *RPC160-170* to *rpc160-174* and *rpc160-112*, all 26 mutations having an allele number up to *rpc160-191* were obtained through this procedure. Twenty six other mutations were constructed by mutagenesis with oligonucleotides designed to generate single-site amino acid replacements at selected positions of region d. To assess the possibility that the observed phenotype might result from mutations outside the sequenced region, 104 mutagenized plasmid clones which had no mutation in region d were tested for their ability to complement *rpc160* null alleles. All but two were able to complement the null mutation at 16, 25 or 37°C, documenting the rarity of spurious mutations outside region d. Moreover, 21 out of the 53 mutations (including the conditional mutations *rpc160-112*, *rpc160-173*, *rpc160-206* and *rpc160-209*) were isolated several times and invariably displayed the same phenotype.

Epitope-tagged WT or C160-112 pol III were generated on plasmid pC160-7 by inserting the *Sal*I site-containing sequence GGAGTCGAC between nucleotides 3 and 4 of the *RPC160* open reading frame and then introducing the sequence TCGACTACCCATACGACGTCCCAG-ACTACGCTCTCGACTACCCATACGACGTCCCAGACTACGCTCT-CGACTACCCATACGACGTCCCAGACTACGCTC into the *Sal*I site. The resulting construct coded for a C160 derivative with three repeats of the influenza hemagglutinin epitope YPYDVPDYA, recognized by the 12CA5 monoclonal antibody. A tagged version of C160-112 was then constructed by oligonucleotide mutagenesis. Strains MW671 and MW1067 were obtained by first transforming strain MW636 by plasmids pC160-240 (containing tagged *RPC160*) and pC160-249 (containing tagged *rpc160-112*) respectively, then shuffling out the *URA3 RPC160*-bearing plasmid pC160-6 as previously described (Werner *et al.*, 1992; Thuillier *et al.*, in preparation).

RNA polymerase III purification

Wild-type and mutant RNA polymerase III were purified from MW671 and MW1067 strains respectively. Cells were grown at 24°C in YPD up to OD_{600} = 2.0. The purification procedure started from 30 g of cells (wet weight) and was carried out in parallel for wild-type and mutant pol III. It consisted of a combination of heparin–Ultrogel and DEAE–Sephadex A-25 chromatographies, as already described (Huet *et al.*, 1985; Chiannilkulchai *et al.*, 1992), with the difference that the active fractions from the DEAE–Sephadex column (10 ml, 0.15 mg) were concentrated on a 0.4 ml heparin–Ultrogel column. In the final preparations (0.6 ml, 0.1 mg), both WT and C160-112 pol III were 50–70% pure, as judged by silver staining of SDS–PAGE gels, with a major contaminant of 13 kDa.

RNA polymerase III non-specific transcription assays

Non-specific polymerization assays were carried out at 30°C for 15 min in a 20 µl reaction mixture containing 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, 2.5 mM $MnCl_2$, 100 mM $(NH_4)_2SO_4$, 75 ng pol III and variable poly[d(A–T)], UTP and ATP concentrations, in the presence of 2–5 µCi of [α-^{32}P]UTP or [α-^{32}P]ATP (Amersham, 800 Ci/mmol). For the K_m determinations of Figure 3, reactions contained 0.3 mM UpA dinucleotide primer (Sigma). The reaction products were TCA-precipitated, filtered (Millipore HA filters, 0.45 µm) and quantitated by scintillation counting of dried filters. To monitor RNA polymerase activity during purification, the same assays were performed in 50 µl mixtures containing 1 µg of poly[d(A–T)], 1 mM ATP and 0.5 mM UTP.

RNA polymerase III specific transcription assays

tRNA gene transcription by purified RNA polymerase III was assayed in the presence of a partially purified heparin–Ultrogel TFIIIB fraction (1.5 µg; Moenne *et al.*, 1990) and of affinity-purified yeast TFIIIC (0.05 µg; Gabrielsen *et al.*, 1989). The reaction mixtures (40 µl) contained 20 mM Tris–HCl pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol (v/v), 0.1 mM EDTA, 125 ng of pRS316-SUP4 plasmid containing the yeast *SUP4* tRNA gene (S.Shaaban, personal communication) and variable concentrations of ribonucleoside triphosphates (Boehringer Mannheim, molecular biology grade) and [α-^{32}P]UTP (indicated in figure legends). pRS316-SUP4 was first pre-incubated for 20 min with TFIIIB and TFIIIC, to allow the formation of a stable pre-initiation complex, then pol III and nucleotides were added. Reaction products were purified by phenol extraction and double ethanol precipitation and separated on 0.8 mm thick 6 or 15% polyacrylamide gels (20:1, w/w, acrylamide:bisacrylamide) containing 7 M urea. Transcripts were quantitated using a PhosphorImager with Image Quant software (Molecular Dynamics).

Ternary complex purification

In vitro transcription reactions were carried out as described above, but the reaction mixture was scaled up to 110 µl. Sepharose CL-2B chromatography was performed essentially as described in Steinberg and Burgess (1992), on 1 ml columns, and 50 µl fractions were collected. Ternary transcription complexes reproducibly eluted in fractions 8–11, while released transcripts started to elute at fractions 14–15.

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