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Dual role of the C34 subunit of RNA polymerase III in transcription initiation

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The C34 subunit of yeast RNA polymerase (pol) III is part of a subcomplex of three subunits which have no counterpart in the other two nuclear RNA polymerases. This subunit interacts with TFIIIB70 and is therefore thought to participate in pol III recruitment. To study the role of C34 in transcription, we have mutagenized *RPC34*, the gene encoding C34, and found that mutations affecting growth also altered C34 interaction with TFIIIB70. The two mutant pol III that were purified had catalytic properties indistinguishable from those of the wild-type pol III on a poly[d(A–T)] template, while specific transcription of pol III genes in the presence of general transcription factors was impaired. The defect of the C34-1124 mutant enzyme could be compensated by increasing the amount of pol III present in the reaction, suggesting that the enzyme had a lower affinity for pre-initiation complexes. In contrast, the C34-1109 mutant enzyme was defective in transcription initiation due to impaired open complex formation. These observations demonstrate that the C34 subunit is a major determinant in pol III recruitment by the pre-initiation complex and further acts at a subsequent stage that involves the configuration of an initiation-competent form of RNA polymerase.

Keywords: pre-initiation complex/RNA polymerase III/ transcription initiation

Introduction

Genes in eukaryotes are transcribed by one of three RNA polymerases. Pol I transcribes the 35S precursor of large rRNAs, pol II transcribes mRNAs and some small stable RNAs, and pol III transcribes tRNAs, 5S rRNA and some other small RNAs. Transcription initiation begins with the binding of general transcription factors to the gene promoter, forming the pre-initiation complex, each polymerase having its own set of factors. The pre-initiation complex is then recognized by its cognate RNA polymerase to form the initiation complex in a process that is poorly understood.

Transcription initiation by pol III, except for 5S RNA genes which require TFIIIA, an additional transcription factor, begins with the binding of TFIIIC which then recruits TFIIIB. An initiation complex is then formed by the binding of pol III (White, 1994, and references therein). TFIIIB is the general transcription factor recognized by pol III since a TFIIIB·DNA complex can direct multiple rounds of transcription *in vitro* (Kassavetis *et al.*, 1990). TFIIIB is composed of three polypeptides, TATA-binding protein (TBP; Huet and Sentenac, 1992; Kassavetis *et al.*, 1992b), a general transcription factor required for transcription by all eukaryotic and archaebacterial RNA polymerases (Struhl, 1995, and references therein), TFIIIB90, a 90 kDa subunit which has no equivalent among the other RNA polymerase general transcription factors (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996; Rüth *et al.*, 1996), and TFIIIB70, a 70 kDa protein which is homologous to archaeal general factor TFB (Hausner and Thomm, 1995; Qureshi *et al.*, 1995) and to pol II factor TFIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-De-León *et al.*, 1992). TFIIB is the last general transcription factor to enter the class II pre-initiation complex before pol II (Buratowski *et al.*, 1989), pointing to the possibility that TFIIIB70 might similarly recruit pol III.

Three pol III subunits, C82, C34 and C31, that have no counterpart in the other RNA polymerases (Mosrin *et al.*, 1990; Chiannilkulchai *et al.*, 1992; Stettler *et al.*, 1992), form a subcomplex (Werner *et al.*, 1992, 1993) which might be implicated in transcription initiation. Indeed, mutations in the gene encoding the C31 subunit affect transcription initiation but not the general catalytic properties of the enzyme (Thuillier *et al.*, 1995). The role of the C34 subunit is not known presently, but several lines of evidence suggest that it is also implicated in transcription initiation. Of all pol III subunits, C34 is the one that cross-links the furthest upstream on the promoter DNA in initiation complexes (Bartholomew *et al.*, 1993; Persinger and Bartholomew, 1996). Moreover, antibodies directed against the subunit inhibit *in vitro* transcription of a tRNA template but not non-specific transcription on poly[d(A–T)] (Huet *et al.*, 1985). Finally, the observation that C34 interacts both *in vivo* and *in vitro* with TFIIIB70 has led us to propose that it might be implicated in the recruitment of pol III by the pre-initiation complex (Werner *et al.*, 1993; Khoo *et al.*, 1994).

In this study, using conditional mutations affecting the C34 subunit of pol III, we demonstrate that it plays an essential role in transcription initiation, not only in the recognition of the pre-initiation complex by pol III, but also, more unexpectedly, at the level of open complex formation.

Results

Mutagenesis of RPC34

Three *rpc34* conditional mutations have been described previously (Stettler *et al.*, 1992). We wanted to pursue the characterization of the C34 pol III subunit, first by obtaining tighter conditional growth mutations and,

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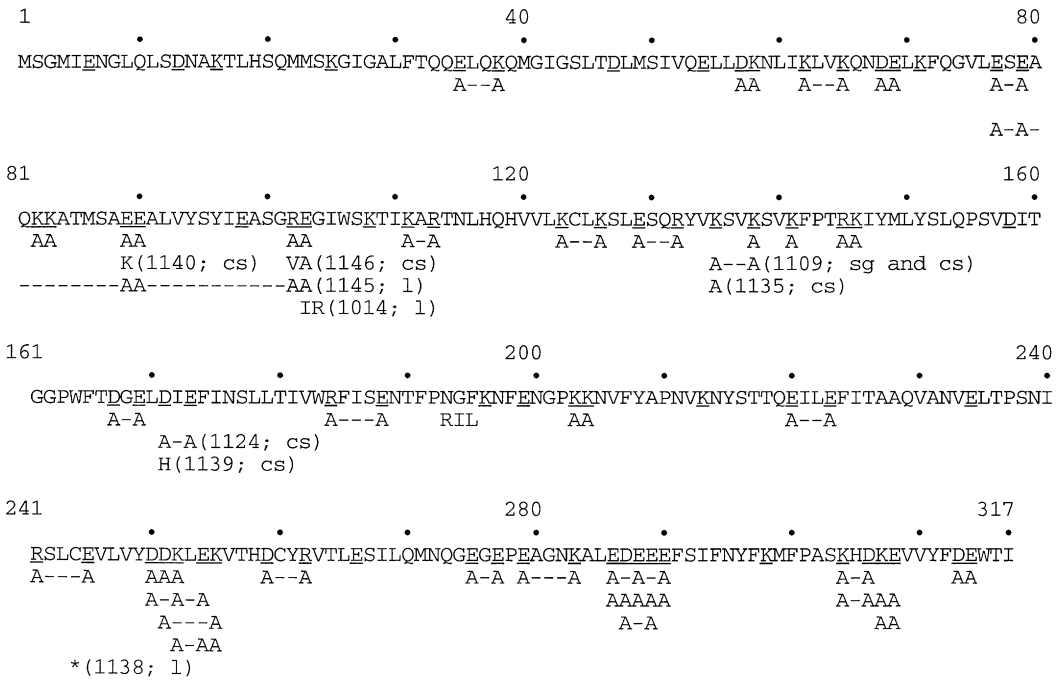


Fig. 1. Charged cluster mutagenesis of the C34 subunit. The mutations which have been introduced into the C34 subunit are indicated beneath the protein sequence. Multiple substitutions are indicated by dashes joining the mutant amino acids when they are not adjacent. * indicates a nonsense mutation. The allele names of the mutations that give rise to a growth phenotype are indicated in parentheses. The phenotypes are coded in the following way: l, lethal; cs, cryosensitive growth at 16°C; sg, slow growth at 24°C.

second, by assaying the effect of the mutations on the interaction of C34 with its partners. For that purpose, we used an *RPC34* allele, *RPC34-1001*, which behaves as the wild-type and allows the fusion of the *RPC34* open reading frame (ORF) in-frame with the GAL4 DNA-binding domain (G_{DB}) or GAL4 activation domain (G_{AD}) to test the effect of mutations in the two-hybrid system (Werner *et al.*, 1993; see below). We thus mutagenized *RPC34-1001* using oligonucleotides following the 'charged cluster analysis' strategy (Wertman *et al.*, 1992). This method targets the residues located at the surface of the protein by changing positively or negatively charged residues to alanine when at least a pair is present within a sequence of five amino acids. Thirty three such mutations were constructed to cover the entire *RPC34* ORF (see Materials and methods; Figure 1). Two other mutations, *rpc34-1145* and *rpc34-1146*, were obtained spuriously during oligonucleotide mutagenesis. Finally, eight mutations were targeted at specific residues among which two (*rpc34-1139* and *rpc34-1140*) altered the same amino acid as the *rpc34-E89K* and *rpc34-D171H* mutations described previously (Stettler *et al.*, 1992). The mutant genes were transformed into strain D57-12C which harbours the *rpc34-Δ::HIS3* deletion complemented by a wild-type copy of the *RPC34* gene borne on a *URA3* plasmid pYS34 (Stettler *et al.*, 1992). The phenotype of the mutations was tested by plasmid shuffling (Boeke *et al.*, 1987), selecting the Ura^- clones that had lost the wild-type resident plasmid.

Of the 33 mutations constructed according to the 'charged cluster analysis' scheme, only two displayed a conditional phenotype. Mutant *rpc34-1109* displayed reduced growth at the permissive temperature of 24°C and ceased to grow at 16°C. Mutant *rpc34-1124* grew nearly as well as the wild-type at 24°C but showed only marginal growth at 16°C (Figure 2). Curiously, the RE102-103AA change was silent while the RE102-103VA change (*rpc34-1146*) displayed a cryosensitive phenotype. Since, of all the conditional mutations, *rpc34-1109* had the most drastic effect, we separated the K135A and K138A amino acid replacements and found that only the former (K135A in *rpc34-1135*) had a detectable effect, though it was less pronounced than when combined with K138A. Other mutations on either side of *rpc34-1135* were phenotypically silent, showing that K135 is critical for C34 function. The phenotype of *rpc34-1139* and *rpc34-1140* (identical to mutations *rpc34-D171K* and *rpc34-E89K*; Stettler *et al.*, 1992) are also shown in Figure 2 for comparison. Quite strikingly, none of the mutations were thermosensitive at 37°C.

Three mutations, *rpc34-1014*, *-1138* and *-1145*, were lethal: *rpc34-1014* resulted from a EG103-104IR double substitution, overlapping the conditional *rpc34-1146* mutation, *rpc34-1145* had six amino acid changes and *rpc34-1138* a deletion of the 74 C-terminal amino acids. This latter mutation, which shows that the C34 C-terminus is required for the function of the subunit, was constructed because multiple mutations that changed up to five contiguous acidic residues in that region had no phenotypic effect (Figure 1). The low number of lethal mutations was unexpected since a 'charged cluster mutagenesis' of region f of C160 pol III subunit yielded 21 lethal and four conditional mutants out of a total of 27 (Thuillier *et al.*, 1996). This double alanine scanning mutagenesis and previous attempts at obtaining conditional mutations affecting C34 through hydroxylamine mutagenesis (Stettler *et al.*, 1992) suggest that the C34 subunit is

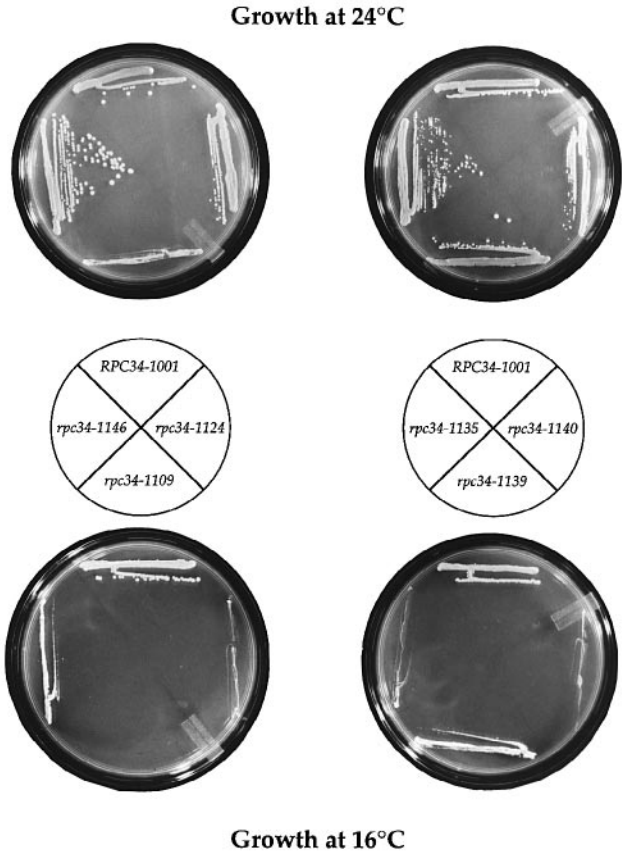


Fig. 2. Crysosensitive growth of mutant *RPC34* strains. Wild-type (*RPC34-1001*) or mutant (*rpc34-1109*, *-1124*, *-1135*, *-1139*, *-1140*, *-1146*) strains were grown for 4 days at 24°C or 7 days at 16°C on YPD rich medium.

very resistant to amino acid substitutions that lead to thermosensitivity.

The C34 subunit interacts with C31 and C82 subunits of pol III and with the TFIIIB70 subunit of TFIIIB, as evidenced by two-hybrid experiments (Werner *et al.*, 1993). To explore the physiological relevance of these interactions, we tested whether the phenotype of the lethal or conditional mutations could be due to weakened protein–protein contacts between C34 and its partners. Each C34 mutant allele was cloned in the pACT2 vector to yield G_{AD}::C34-1### (where ### represents three digits) fusions. These were tested in the two-hybrid system against G_{DB}::C31, G_{DB}::C82 and G_{DB}::TFIIIB70 fusions cloned in vector pAS2 after growth at 30°C (see Materials and methods). In each case, the correct expression of the G_{AD}::C34-1### fusion was tested using antibodies directed against C34 (Huet *et al.*, 1985) in order to eliminate the possibility that some of the *rpc34* mutations might reduce the level of expression and/or the stability of the fusion protein. Strikingly, all the mutations that showed a growth defect affected the interaction with TFIIIB70 (Table I). The two lethal mutations (*rpc34-1138* and *rpc34-1145*) that were tested were also severely affected in their interaction with the C31 and C82 subunits of pol III. The altered interactions were observed even though the cells were grown at 30°C, the permissive temperature for the mutations. Two possibilities might explain this observation. First, we observed that the *rpc34-1124* and *-1139* mutants, though growing normally at 30°C, showed reduced *in vivo* transcription of tRNAs, indicating that the mutation already exerted its effect even at the permissive temperature (see below). Second, in pol III, the mutant C34 subunit is part of a multiprotein complex that might stabilize its conformation and allow its interaction with TFIIIB70. This is probably not the case in the two-hybrid assay that reflects direct interactions between protein pairs overproduced in the cells. Whatever the case, our observations strongly suggested that the decreased interaction between C34 mutant subunits and TFIIIB70 were responsible for the growth phenotype of the mutant strains.

Table I. Two-hybrid interations between mutant C34 proteins and C31, C82 and TFIIIB70

RPC34 allele	Growth phenotype[b]	Two-hybrid interaction[a]		
		C31[c]	C82[d]	TFIIIB70[c]
RPC34-1001	wild-type	++	++	++
rpc34-1109	Sg, Cs	++	+	–
rpc34-1124	Cs	++	++	–
rpc34-1135	Cs	++	+	–
rpc34-1138	lethal	+	–	–
rpc34-1139	Cs	++	++	–
rpc34-1140	Cs	++	++	–
rpc34-1145	lethal	+	+/–	–
rpc34-1146	Cs	++	++	+

[a]++ represented the wild-type level of *lacZ* activation as determined by the β-galactosidase overlay assay on patches of cells growing at 30°C on minimal medium. This assay is linear in response to the β-galactosidase activity (Werner *et al.*, 1993). + represented intermediate levels of coloration, +/–very light blue colour and – white colour. For comparison, interaction between wild-type C34 and C31 led to the production of 123 U of β-galactosidase, to 145 U with C82 and to 246 U with TFIIIB70 (Werner *et al.*, 1993); the background level was <5 U of β-galactosidase.
[b]Cs, cryosensitive growth on YPD medium at 16°C; Sg, slow growth on YPD medium at 24°C; lethal, no growth on 5-fluoroorotic acid medium at 24°C.
[c]Tested in strain Y526.
[d]Tested in strain Y190.

Transcription properties of mutant pol III

Pol III transcription in *rpc34-1109*, *-1124*, *-1139* and *-1146* mutant strains was assayed *in vivo* by labelling the RNAs with tritiated uracil as described previously (Gudenus *et al.*, 1988; Stettler *et al.*, 1992; Hermann-Le Denmat *et al.*, 1994). All mutants showed, as expected, a reduced level of transcription of tRNAs at 16°C, the restrictive temperature for growth (data not shown). Moreover, mutants *rpc34-1109*, *-1124* and *-1139* already displayed reduced tRNA transcription at 30°C, the permissive temperature.

To investigate the effect of mutations in the C34 subunit on the *in vitro* transcription properties of pol III, the enzyme was purified from mutant *rpc34-1109* and *-1124* strains in parallel with that from a wild-type strain. These two mutants were chosen since they showed the more drastic conditional phenotype. The purified enzymes, analysed by silver-stained SDS–PAGE, showed a normal subunit composition and were 80% pure, with one major contaminating polypeptide of ~12 kDa which was also present in the corresponding purified wild-type enzyme fraction (data not shown).

In vitro transcription activity of the purified mutant

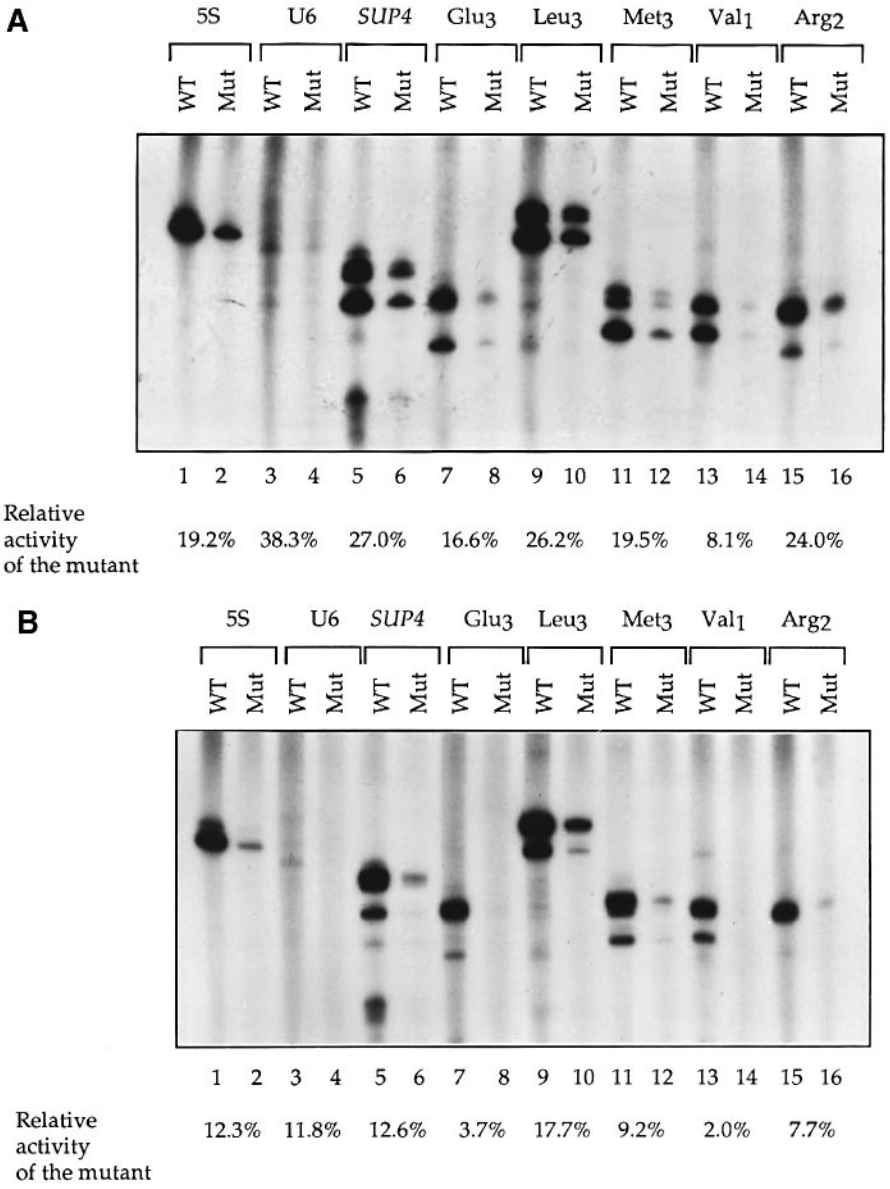


Fig. 3. Specific transcription of various pol III genes by C34-1109 mutant enzyme. Various tRNA genes (*SUP4*, Glu_3, Leu_3, Met_3, Val_1, Arg_2), U6 snRNA or 5S rRNA genes (100 ng template) were transcribed using wild-type (WT) or C34-1109 (Mut) pol III (50 ng) in the presence of a purified TFIIIB fraction (1.5 μg), TFIIIC (90 ng) and, for the 5S RNA gene, recombinant TFIIIA. The relative activity of the mutant pol III compared with the wild-type enzyme is indicated below in percent. (**A**) Transcription for 1 h at 24°C. (**B**) Transcription for 1 h at 16°C.

enzymes was assayed by counting acid-precipitable RNA synthesized by equal amounts of wild-type and mutant enzymes at saturating concentrations of poly[d(A–T)] template, ATP and UTP substrates. The activity of the two mutant enzymes was indistinguishable from that of the wild-type at both 24 and 16°C. This assay does not measure the synthesis of short, abortive RNAs since they are not precipitated. In the case of C34-1109, reactions were performed using poly[d(A–T)] as template, a UpA dinucleotide primer, ^{32}P-labelled UTP and various concentrations of ATP to observe abortive transcription (Thuillier *et al.*, 1996). Irrespective of the ATP or poly[d(A–T)] concentrations, the amount of abortive tri- and tetranucleotides or long RNA chains synthesized by the mutant enzyme was always identical to that produced by the wild-type enzyme (data not shown). This behaviour is similar to that of mutant pol III in which the C31 subunit, essential for transcription initiation, was mutated (Thuillier *et al.*, 1995), but is in marked contrast with mutations that affect the catalytic function of the enzyme due to mutations in the largest subunit of the enzyme (Dieci *et al.*, 1995; Thuillier *et al.*, 1996).

The effect of the *rpc34-1109* mutation on pol III transcription was next investigated in specific multiple round transcription assays using various templates. Transcription was performed in the presence of purified TFIIIB and TFIIIC fractions as well as TFIIIA for transcription of the 5S RNA template. At 24°C, C34-1109 pol III already showed 60–90% reduction in activity depending on the template (Figure 3A). Reducing the incubation temperature to 16°C, the restrictive temperature for the *rpc34-1109* mutation, further diminished the activity of the enzyme to between 18 and 2% of that of the wild-type, depending on the template (Figure 3B), showing a

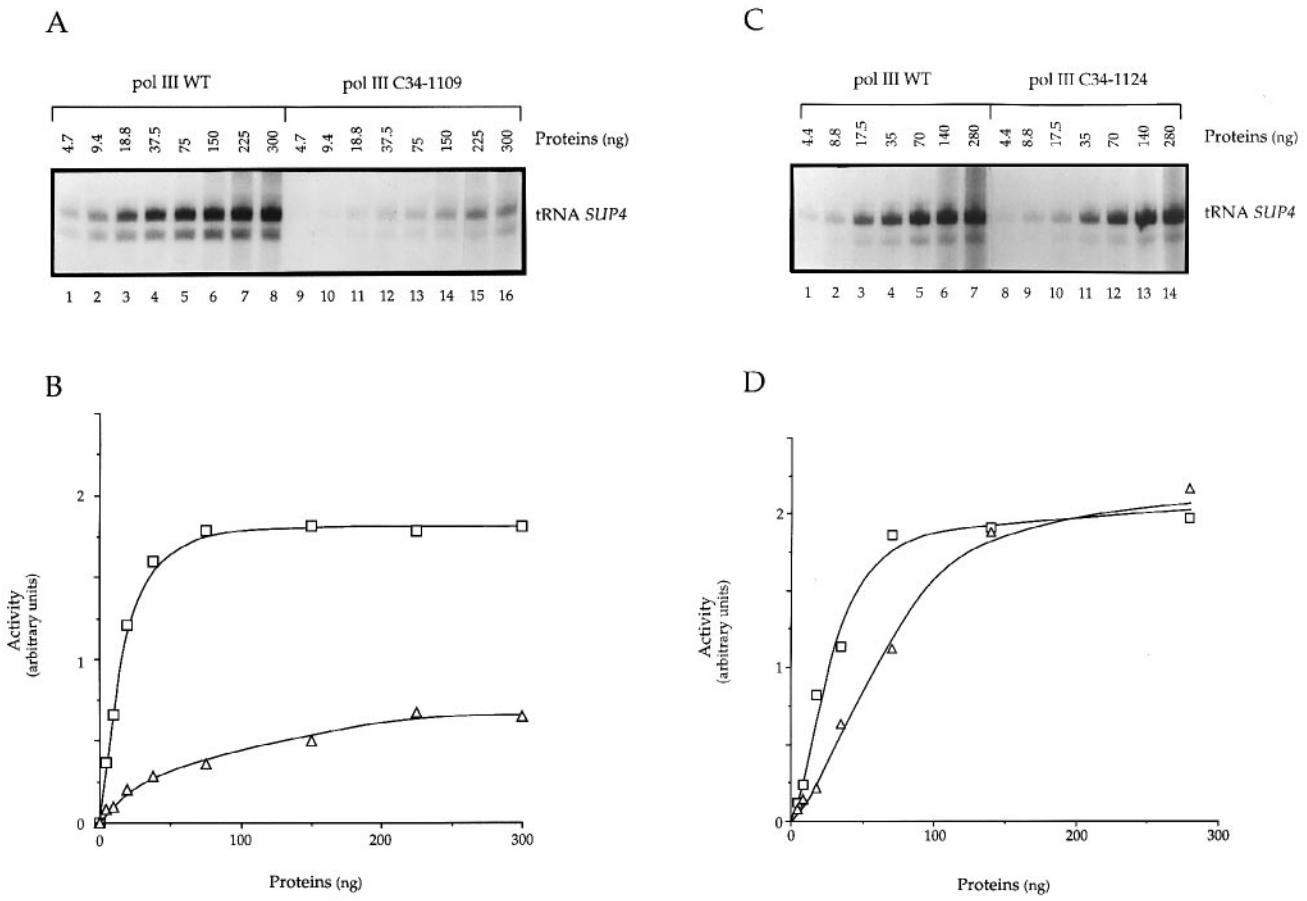


Fig. 4. Transcription by wild-type, C34-1109 and C34-1124 pol III as a function of enzyme concentration. The *SUP4* gene was transcribed in the presence of TFIIIB and TFIIIC at 24°C as described in Figure 3 with varying amounts of wild-type or mutant pol III as indicated. (**A**) Autoradiographs of the RNA products synthesized by wild-type and C34-1109 pol III. The amount of pol III added is indicated above the autoradiograph. (**B**) PhosphorImager quantification of the data shown in (A). □ Wild-type pol III; △ C34-1109 pol III. (**C**) Autoradiographs of the RNA products synthesized by wild-type and C34-1124 pol III. The amount of pol III added is indicated above the autoradiograph. (**D**) PhosphorImager quantification of the data shown in (C). □ Wild-type pol III; △ C34-1124 pol III.

strong parallel between the *in vivo* growth phenotype and specific *in vitro* transcription activity.

If a mutation affected the association of pol III with the pre-initiation complex, increasing the enzyme concentration should correct the transcription initiation defect. Indeed, saturating amounts of C34-1124 pol III restored transcription to the wild-type level (Figure 4C and D). Therefore, the *rpc34-1124* mutation essentially affected the recruitment of pol III, since, when the enzyme was recruited, transcription occurred efficiently. This observation was in keeping with the expected role of C34 in pol III recognition of the pre-initiation complex via its interaction with TFIIIB70. On the contrary, increasing the concentration of C34-1109 pol III did not compensate for the reduced specific transcription initiation. As shown in Figure 4A and B, at nearly saturating concentrations of enzyme, the activity of the mutant enzyme was still around 3-fold lower than that of the wild-type.

This observation suggested that the transcription complexes formed but were impaired in a subsequent step leading to RNA chain initiation. If this hypothesis was correct, then increasing the incubation time during which the initiation complex is formed should correct the defect observed in C34-1109 pol III. Pre-initiation complexes were thus formed by incubating the *SUP4* template with affinity-purified TFIIIC, recombinant TFIIIB70, TBP and a fraction containing the TFIIIB90 subunit of TFIIIB, then transcription was initiated by the addition of wild-type or mutant pol III and ATP, CTP and labelled UTP. The formation of the 17 nucleotide RNA was analysed at different times to estimate the rate of transcription initiation. As shown in Figure 5A and B, C34-1109 pol III initiated transcription more slowly than the wild-type enzyme at 24°C, but reached 84% of the wild-type level after 10 min incubation. This experiment indicated that if given enough time, C34-1109 pol III was able to form roughly the same number of initiation complexes as the wild-type enzyme.

We investigated the transcription elongation properties of the C34-1109 mutant enzyme. Similar amounts of mutant and wild-type ternary complexes stalled at nucleotide 17 on a *SUP4* template were formed by incubating TFIIIC, TBP, TFIIIB70, B″ fraction (containing TFIIIB90), C34-1109 or wild-type pol III with ATP, CTP and labelled UTP for 15 min at 24°C. As indicated above, this incubation period was sufficient for the C34-1109 to form >84% of the 17 nucleotide RNA transcript formed by the wild-type pol III. The rate of synthesis of full-length RNA was then followed by analysing the RNA chain pattern at different time points after the addition of GTP and heparin,

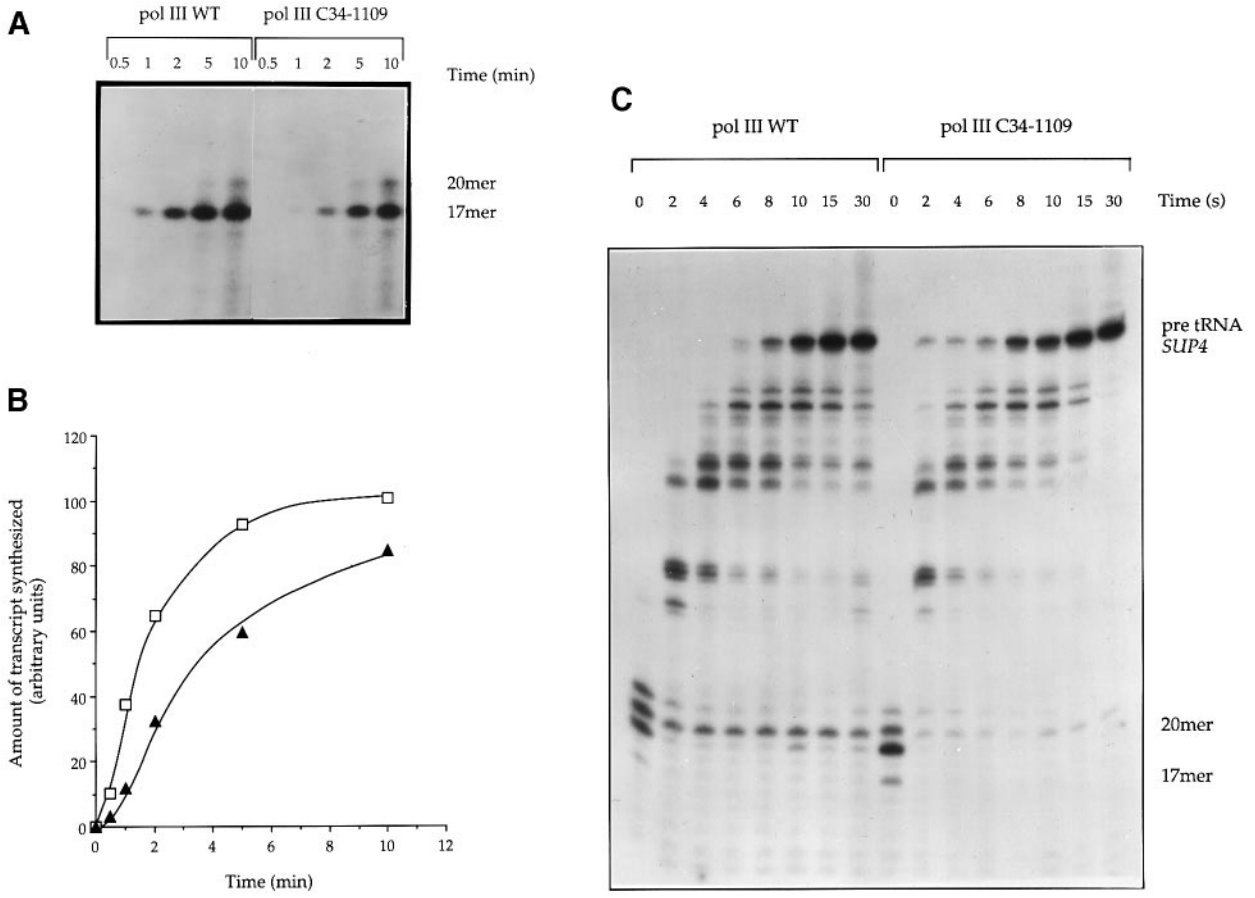


Fig. 5. Transcription initiation and elongation by wild-type or mutant pol III. (**A**) Transcription initiation on the *SUP4* tRNA gene was started by the addition of wild-type or mutant pol III to pre-initiation mixtures containing the *SUP4* DNA template, TBP, TFIIIB70, B″ fraction, TFIIIC, ATP, CTP and ^{32}P-labelled UTP as indicated above. The reactions were performed for various periods, as indicated. The 17 and 20 nucleotide RNA products are indicated on the right side of the panel. (**B**) PhosphorImager quantification of the data shown in (A). □ Wild-type pol III; ▲ C34-1109 pol III. (**C**) Transcription elongation assays were performed by first forming ternary complexes halted at nucleotide 17 by incubating wild-type or mutant pol III for 15 min at 24°C in the presence of the *SUP4* DNA template, 40 ng of recombinant TBP, 50 ng of recombinant TFIIIB70, 400 ng of a B″ fraction (containing TFIIIB90), 50 ng of affinity-purified TFIIIC, 500 μM ATP, 500 μM CTP and 3 μM ^{32}P-labelled UTP. Transcription elongation was then allowed to resume by the addition of GTP (500 μM) and heparin (300 μg/ml) to prevent re-initiation. Reaction products were analysed by electrophoresis after 2, 4, 6, 8, 10, 15 and 30 s elongation.

which prevents pol III recycling. As shown in Figure 5C, the pause pattern was similar for the wild-type and mutant enzymes. Unexpectedly, the mutant pol III synthesized full-length transcripts faster than the wild-type enzyme since full-length *SUP4* pre-tRNA appeared after 2 s in the first case and 6 s in the second (Figure 5C). This observation at least proved that an elongation defect could not account for the slower rate of RNA synthesis by the mutant pol III.

Recently, Dieci and Sentenac (1996) observed that transcription initiation by a recycling pol III was faster than the first initiation step, suggesting that there is a facilitated recycling pathway for the enzyme. We thus asked whether recycling might also be affected in pol III with mutant C34 subunit. This was tested by performing multiple round transcription assays on *SUP4* DNA starting with ternary complexes stalled at position 17 in the absence of GTP. The number of transcription cycles during a short incubation period was then determined after addition of GTP with heparin (single round transcription) or without heparin (multiple rounds; Thuillier *et al.*, 1995, 1996). The average time needed by the C34-1109 enzyme to complete one cycle was 45 s at early time points, which was significantly longer than the 30 s required by the wild-type enzyme (Figure 6). Furthermore, the measured cycling time increased to >60 s for the mutant pol III, while it remained constant for the wild-type enzyme (Figure 6B). The behaviour of the mutant showed that the C34 subunit plays a role in both the first initiation event and the facilitated recycling pathway.

Altogether, these experiments suggested that C34-1109 pol III was impaired at a step subsequent to pre-initiation complex recognition and prior to elongation, i.e. open complex formation and/or promoter clearance. Since open complex formation is strongly dependent on temperature (Kassavetis *et al.*, 1992a), we assayed transcription initiation on a supercoiled *SUP4* template, as a function of temperature, again allowing the reaction to proceed for 15 min. As shown in Figure 7, contrary to what was observed for the wild-type pol III, transcription by the C34-1109 enzyme was very inefficient at low temperatures (4–10°C). The transition temperature (to reach 50% of total transcripts) was 15°C for the mutant pol III as compared with 5°C for the wild-type enzyme. The same experiment was done using a linear template and also revealed a higher transition temperature for the mutant

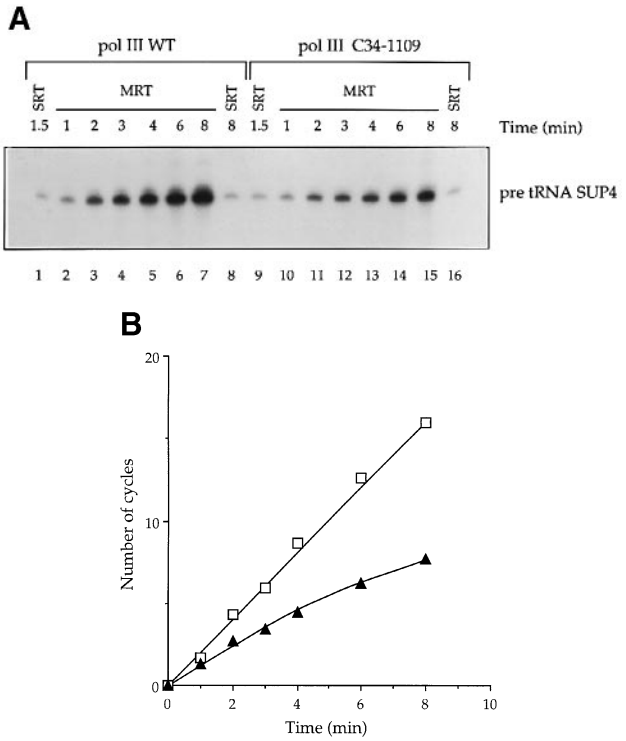


Fig. 6. Kinetics of transcript accumulation by re-initiating wild-type and C34-1109 pol III. (**A**) Wild-type or mutant pol III were incubated at room temperature for 15 min in the presence of the *SUP4* tRNA gene, 40 ng of recombinant TBP, 50 ng of recombinant TFIIIB70, 400 ng of a B″ fraction (containing TFIIIB90), 50 ng of affinity-purified TFIIIC, 500 μM ATP, 500 μM CTP and 30 μM ^{32}P-labelled UTP to form a halted ternary complex complex at position 17. Elongation of the 17mer RNA was allowed to resume for 1.5–8 min by addition of 600 μM GTP and 300 μg/ml heparin, which enabled only a single round of transcription (SRT), or GTP alone, which allowed multiple rounds of transcription (MRT). Lanes 1–8, wild-type pol III; lanes 9–16, C34-1109 pol III. (**B**) PhosphorImager quantification of the data shown in (A). □ Wild-type pol III; ▲ C34-1109 pol III.

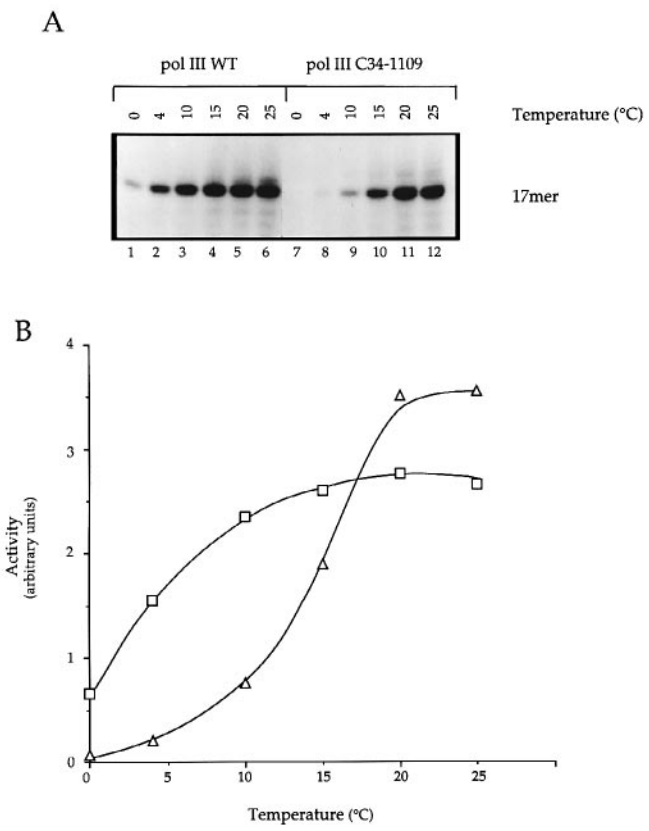


Fig. 7. Transcription initiation by wild-type and C34-1109 pol III as a function of temperature. Stable pre-initiation complexes were formed on the *SUP4* tRNA gene at 24°C as described in Materials and methods, and then incubated further at the temperature indicated for 10 min. Wild-type or mutant pol III were then added together with 500 μM ATP, 500 μM CTP and 10 μM ^{32}P-labelled UTP equilibrated at the same temperature. Synthesis of the 17mer was allowed to proceed for 15 min at the temperature indicated. The reaction products were separated on a 15% polyacrylamide–7 M urea gel. (**A**) Autoradiographs of the RNA products. The position of the 17 nucleotide transcript is indicated. (**B**) PhosphorImager quantification of the data shown in (A). □ Wild-type pol III; △ C34-1109 pol III.

pol III as compared with the wild-type enzyme (data not shown).

Open and closed complexes exhibit a different sensitivity to heparin (Kassavetis *et al.*, 1992a; Dieci and Sentenac, 1996). We therefore compared the heparin sensitivity of pre-formed wild-type and mutant initiation complexes. Mutant or wild-type pol III was pre-incubated for 15 min with TFIIIB·TFIIIC·DNA complexes at 24°C then assayed for 17mer synthesis by the addition of ATP, CTP and labelled UTP in the presence of various heparin concentrations (this second incubation was performed for 20 min). As shown in Figure 8, mutant initiation complexes were clearly more sensitive to heparin than wild-type complexes. The concentration of heparin required to achieve 50% inhibition of mutant complexes was intermediate between that required to inhibit free pol III (0.25–0.5 μg/ml; Kassavetis *et al.*, 1992a) and open complexes (5 μg/ml; Dieci and Sentenac, 1996).

The cold and heparin sensitivities of the mutant enzyme were strongly suggestive of a defect at the level of open complex formation but could also stem from an uncharacterized effect of the *rpc34-1109* mutation. To eliminate this latter possibility, the formation of the open complex was investigated directly using $KMnO_4$ footprinting to probe the accessibility of T residues in the transcription bubble (Kassavetis *et al.*, 1992a). Pre-initiation complexes were first formed by incubating end-labelled *SUP4* template with TFIIIC and reconstituted TFIIIB. Wild-type or C34-1109 mutant RNA polymerase were then incubated at 19°C with the pre-initiation complexes for variable periods of time before a brief treatment with $KMnO_4$. The reactivity of T residues at positions –2 to –9 reflected open complex formation. As shown in Figure 9, the reactivity of these T residues was very much reduced in the case of mutant pol III, indicating a deficiency in open complex formation even after extensive incubation, in keeping with the strong temperature dependence and heparin sensitivity of mutant pre-initiation complexes.

To confirm further that the lack of promoter opening was due to the impaired transition from the closed to the open complex conformation and not from a poor association of the mutant pol III with the pre-initiation complex, we reasoned that adding nucleotides to closed initiation complexes in $KMnO_4$ footprinting experiments should shift the equilibrium towards open complex formation and transcription initiation. Indeed, adding ATP and CTP during the incubation period increased the $KMnO_4$ foot-

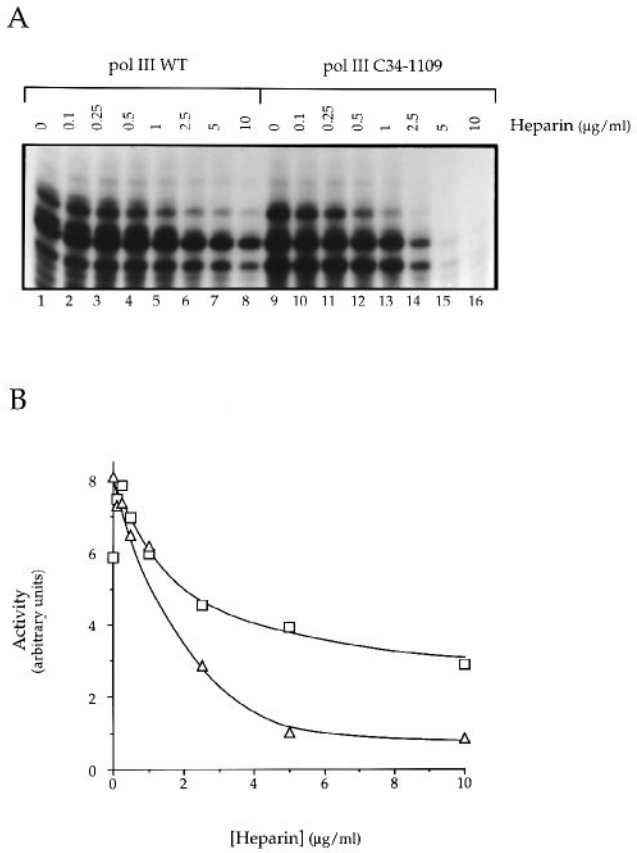


Fig. 8. Heparin sensitivity of initiation complexes. Pre-initiation complexes were formed on the *SUP4* tRNA gene as described in Materials and methods. Then 100 ng of wild-type or mutant pol III were added and the incubation continued for 15 min at 24°C to form the initiation complexes. Then 500 μM each of ATP and CTP and 3 μM ^{32}P-labelled UTP were added, together with varying concentrations of heparin, as indicated. Synthesis of the 17mer was allowed to proceed for 20 min at 24°C. The 17mer, the 20mer slippage product and the 15mer cleavage product (Dieci *et al.*, 1995) were separated on a 15% polyacrylamide–7 M urea gel. (**A**) Autoradiographs of the RNA products. (**B**) PhosphorImager quantification of the trancripts (sum of 15, 17 and 20mer intensities) observed on the autoradiogram shown in (A). □ Wild-type pol III; △ C34-1109 pol III.

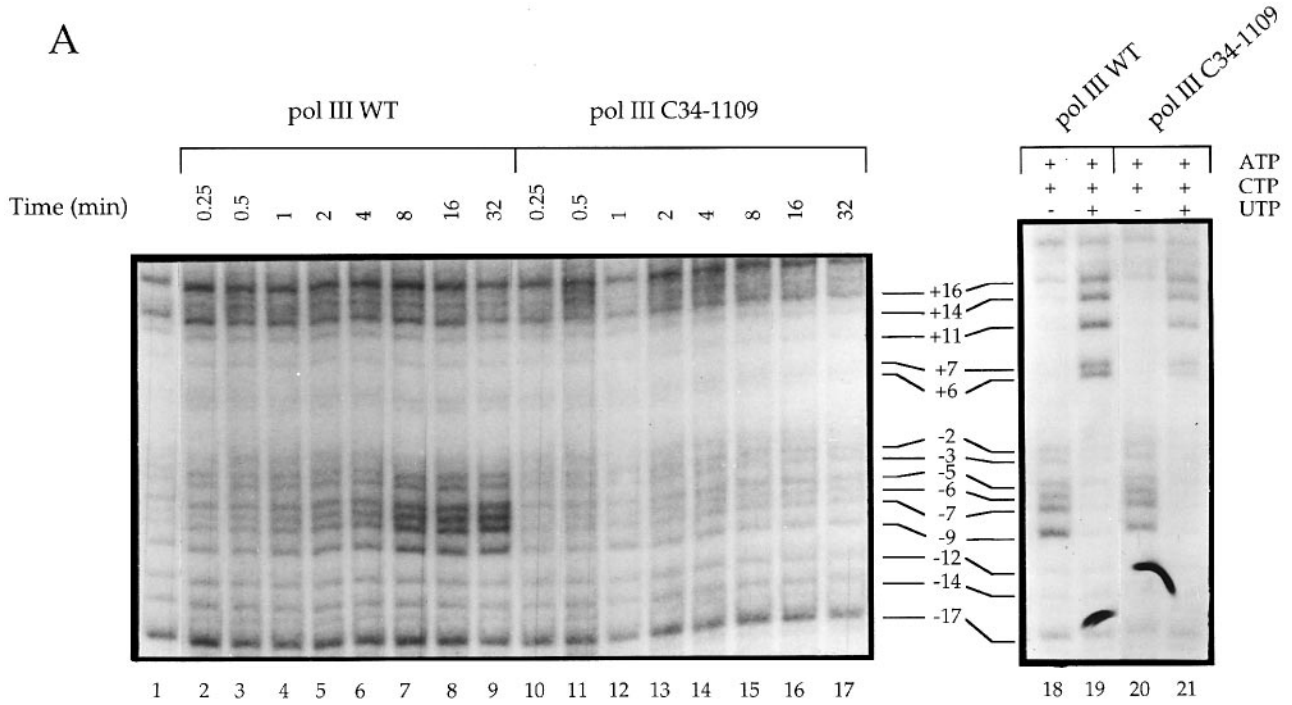
printing of the mutant pol III to a level similar to that of the wild-type enzyme (Figure 9). Adding the third nucleotide, UTP, which allowed the formation of a stable elongating ternary complex, displaced the transcription bubble similarly in the mutant and the wild-type, and the intensity of the footprint generated by the mutant reached 65% that of the wild-type which is well above the 5% value observed when no nucleotide was added. This experiment confirmed that the lack of $KMnO_4$ reactivity of DNA in the absence of nucleotides was due to a deficiency of the mutant enzyme in forming the open complex.

Discussion

The C34 pol III subunit, together with C82 and C31, belongs to a complex of three polypeptides which have no counterpart in the other two RNA polymerases (Mosrin *et al.*, 1990; Chiannilkulchai *et al.*, 1992; Stettler *et al.*, 1992; Werner *et al.*, 1992, 1993). DNA–protein cross-linking studies of TFIIIB·pol III·DNA initiation complexes have established that of all detectable pol III subunits, C34 was the one located the most upstream at the level of the start site (Bartholomew *et al.*, 1993; Persinger and Bartholomew, 1996). Moreover, anti-C34 antibodies inhibit pol III-specific transcription *in vitro* (Huet *et al.*, 1985) and C34 interacts with the TFIIIB70 subunit of TFIIIB (Werner *et al.*, 1993; Khoo *et al.*, 1994). All these observations suggested that C34 is implicated in transcription initiation (Werner *et al.*, 1993). We now demonstrate that the interaction between C34 and TFIIIB70 is a major determinant in the recognition of the pol III pre-initiation complex by its cognate enzyme. More unexpectedly, we found that the C34 subunit influences the formation of the open promoter complex. Altogether, our results show that C34 plays an essential role in transcription initiation.

Our mutagenic analysis of the C34 subunit of pol III showed that all mutations that affected the growth of the mutant strains also impaired the ability of C34 to interact with TFIIIB70. This result shows that this interaction is essential for the function of pol III and suggests that C34 is at least one of the critical subunits that specifically recognizes the pre-initiation complex for the recruitment of the enzyme. This conclusion was supported further by the observation that one mutant pol III, C34-1124, which had a wild-type activity in non-specific transcription assays but showed impaired *in vitro* specific transcription of the *SUP4* tRNA gene, could be rescued by increasing the mutant enzyme concentration. The possibility that the effect of the *rpc34-1124* mutation is not due to its weakened interaction with TFIIIB70 but to some indirect effect through C82 or C31 subunits is less likely. Indeed, direct interaction between C34 and TFIIIB70 has been demonstrated by GST pull-down experiments both for the *Saccharomyces cerevisiae* subunits and for their human homologues (Khoo *et al.*, 1994; Wang and Roeder, 1997), while no interaction was detected between the human homologues of C31 and C82 and TFIIIB70 (Wang and Roeder, 1997). Moreover, of all the 15 pol III subunits investigated, only C34 interacted with TFIIIB70 in the two-hybrid system (Werner *et al.*, 1993, and unpublished results). Finally, the C34-1124 subunit did not show any interaction defect with either C31 or C82, lending further support to the notion that its defect resulted from its weakened interaction with TFIIIB70.

The second mutant pol III, C34-1109, also displayed a reduced affinity with TFIIIB70 but had an unexpected defect in transcription initiation. In addition to its role in the recognition of the pre-initiation complex, C34 subunit appears also to be involved in a subsequent step of transcription initiation. Several observations led us to this conclusion. (i) Contrary to a mutant of the large subunit that affects this step (Thuillier *et al.*, 1996), abortive transcription by C34-1109 mutant pol III was normal. Moreover, the rate of transcription elongation was slightly faster than that measured for the wild-type enzyme, suggesting that the defect in C34-1109 pol III occurred at an early step. (ii) Increasing the amount of mutant enzyme in multiple round transcription assays did not correct the transcription defect, contrary to what was observed for the C34-1124 pol III. (iii) On the other hand, given enough time, the same number of productive transcription initiation



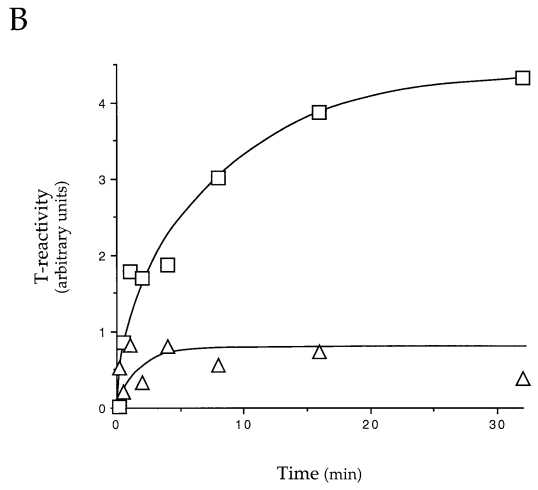


Fig. 9. Open complex formation by wild-type and C34-1109 pol III. The *SUP4* tRNA gene (4 fmol), 5′ end-labelled on the non-transcribed strand, was incubated with 24 ng of recombinant TBP, 24 ng of recombinant TFIIIB70, 240 ng of a B″ fraction (containing TFIIIB90), 30 ng of affinity-purified TFIIIC for 30 min at 24°C to form a stable pre-initiation complex and then transferred to 19°C. Then 100 ng of wild-type or mutant pol III were added for the specified times prior to a 1 min $KMnO_4$ treatment at the same temperature. When nucleotides were added (ATP at 200 μM; CTP and UTP at 100 μM) transcription was allowed to proceed for 30 min at 19°C and followed by a 30 s $KMnO_4$ treatment. (**A**) Autoradiograph: lane 1, no pol III; lanes 2–9, wild-type pol III; lanes 10–17, C34-1109 pol III; lane 18, wild-type pol III in the presence of ATP and CTP; lane 19, wild-type pol III in the presence of ATP, CTP and UTP; lane 20, C34-1109 pol III in the presence of ATP and CTP; lane 21, C34-1109 pol III in the presence of ATP, CTP and UTP. The location of T residues between –17 and +16 with respect to the site of transcription initiation (+1) is shown. (**B**) PhosphorImager quantification of the data shown in (A) lanes 2–17 using the total hyperreactivity to $KMnO_4$ between T-9 and T-2. T-14 reactivity was used for calibration. □ Wild-type pol III; △ C34-1109 pol III.

complexes could be formed with both wild-type and mutant C34-1109 pol III. Thus the mutant was affected at some critical step of transcription initiation. (iv) The initiation reaction (as measured by synthesis of the 17mer) by the mutant pol III was strikingly dependent on the temperature. We thus hinted that the temperature dependence of transcription initiation by the mutant pol III could be related to open complex formation. (v) C34-1109 pol III was found to be more sensitive to heparin in initiation complexes than the wild-type enzyme, suggesting again that open complex formation was affected. (vi) The accessibility of T residues in the transcription bubble of mutant pol III was reduced in the absence of nucleotides. Based on $KMnO_4$ reactivity, the number of open complexes formed by the mutant enzyme was roughly five times lower than that formed by the wild-type. However, when nucleotides were present, the $KMnO_4$ footprinting reaction with the mutant pol III became comparable with that of the wild-type enzyme, indicating that the equilibrium had been shifted from a closed to an open complex conformation. Altogether, these observations indicated that the defect of C34-1109 did not stem from a defect of association with the pre-initiation complex but from an altered isomerization step required to shift the enzyme into an initiation-competent configuration. A proper C34–TFIIIB70 interaction appears to be critical to promote or facilitate this functional transition.

What is then the significance of the decreased interaction between C34-1109 and TFIIIB70 observed by the two-hybrid method? One possibility is that, while C34 contacts specific residues of TFIIIB70 during pre-initiation complex recognition, the contacts between the two proteins could be extended or modified afterwards during promoter opening and have to be disrupted at the promoter clearance step. Pol III containing the C34-1109 mutant subunit is probably not affected in its initial interaction with the pre-initiation complex but could be altered in the way in which it interacts with TFIIIB70 during later steps. This possibility is supported by the fact that the C34-1109 mutant subunit, but not other C34 mutant polypeptides

like C34-1124, was still capable in two-hybrid experiments of interacting with the TFIIIB70 C-terminus (amino acids 252–596) while it did not interact detectably with the whole protein (J.-C.Andrau, S.Shaaban and M.Werner, unpublished observation).

The mechanism of interaction between C34 and TFIIIB70 and its role in pol III transcription is probably conserved in higher eukaryotes. A protein similar to TFIIIB70 exists in mammalian cells (Wang and Roeder, 1995). Moreover, Wang and Roeder (1997) have cloned three cDNAs encoding proteins with significant similarity to C82, C34 and C31. The three human recombinant polypeptides could be assembled to form a subcomplex *in vitro*, confirming our previous observations showing that the three yeast subunits dissociate from a mutant enzyme affected in the N-terminal zinc-binding domain of the largest subunit (Werner *et al.*, 1992) and that these three subunits interact both genetically and in two-hybrid assays (Chiannilkulchai *et al.*, 1992; Werner *et al.*, 1993; Thuillier *et al.*, 1995). Quite strikingly, the region and the residues which are mutated in the C34-1109 and C34-1124 subunits are conserved in the human pol III subunit. Finally, human pol III devoid of the three subunits similar to C82, C34 and C31 (termed core pol III by Wang and Roeder) was able to transcribe non-specific DNA (contrary to what was observed for the yeast mutant pol III; Werner *et al.*, 1992), but not the VA1 gene. However, specific transcription by human pol III was restored when the three missing subunits were added back to the 'core pol III', showing that in this case also these subunits played a role in specific transcription, possibly at the enzyme recruitment and isomerization steps as shown here.

Finally, one could speculate that similar reaction steps occur during the formation of pol II initiation-competent complexes. The general pol II transcription initiation factor TFIIB is similar to TFIIIB70 except for a C-terminal extension that doubles the size of TFIIIB70 (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-De-León *et al.*, 1992). Although several studies have shown that pol II interacts directly with TFIIB (Buratowski *et al.*, 1989; Bushnell *et al.*, 1996), the RAP30 subunit of TFIIF is also required for the formation of the pol II initiation complex through its interaction with both TFIIB and pol II (Flores *et al.*, 1991; Killeen *et al.*, 1992). The large C-terminal extension of TFIIIB70 does not bear any resemblance to RAP30, but might function in a similar way. This hypothesis is supported by the fact that, though GST pull-down experiments suggested that TFIIIB70 interacts with C34 through its N-terminus (Khoo *et al.*, 1994), our unpublished data (J.-C.Andrau, S.Shaaban and M.Werner, unpublished observations), based on two-hybrid assays and the use of conditional point mutations in TFIIIB70, indicate that the C-terminus of the factor plays an essential role in C34–TFIIIB70 interaction. Additionally, like RAP30 (Tan *et al.*, 1994), the C-terminus of TFIIIB70 bears a cryptic DNA-binding domain, lending further support to their similar function (Huet *et al.*, 1997). Interestingly, pol I appears to use a different system for transcription initiation since biochemical studies have not uncovered a factor of the TFB/TFIIB/TFIIIB70 family (Comai *et al.*, 1994; Keys *et al.*, 1994; Lalo *et al.*, 1996; Yamamoto *et al.*, 1996) and since searches for similar proteins in the now complete yeast genome sequence failed to reveal more members of this family of proteins.

In summary, we have obtained mutations in the C34 pol III-specific subunit affecting growth. All these mutations impaired the interaction between C34 and TFIIIB70. The *in vitro* analysis of transcription showed that C34–TFIIIB70 interaction is essential during initiation, both for the recognition of the pre-initiation complex step and, more unexpectedly, at a later stage during promoter opening.

Materials and methods

Strains and plasmids construction

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

In order to facilitate the manipulation of the *RPC34* gene fragment, we have used the *RPC34-1001* derivative of *RPC34* which has two *Bam*HI sites, one 8 bp upstream (at position –8) and the other 9 bp downstream of the C34 ORF (at position 963; Werner *et al.*, 1993) cloned in vector pRPC34-1001 (*TRP1 RPC34-1001 CEN4*). This allowed us to construct transcriptional and translational fusions in different vectors. The *RPC34-1001* allele was tested for its ability to complement at different temperatures an *rpc34-Δ::HIS3* deletion by plasmid shuffling in *S.cerevisiae* strain D57-12C [S.Stettler, S.Labarre and P.Thuriaux, personal communication; *MATα ade2-101 lys2-801 ura3-52 his3-Δ200 trp1-Δ1 rpc34-Δ::HIS3* pYS34(*CEN URA3 RPC34*)]. All further mutations were performed by oligonucleotide mutagenesis of plasmid pRPC34-1001 (Kunkel *et al.*, 1987). Their growth phenotype was tested by plasmid shuffling in strain D57-12C.

To test two-hybrid interactions of mutant C34 pol III subunits, the 970 bp *Bam*HI fragment of pRPC34-1001 and its derivatives, containing the complete *RPC34* ORF, were cloned in the cognate site of the pACT2 vector (Harper *et al.*, 1993) directing the production of G_{AD}–C34 fusions. These constructions were labelled pACT-C34-1###. These fusions were tested against G_{DB} fusions with either C31, C82 pol III subunits or the TFIIIB70 subunit of TFIIIB. The pAS-C31 construction was done by cloning the 773 bp *Bam*HI *RPC31-1001* fragment from plasmid pRPC31-1001 (Werner *et al.*, 1993) into the *Bam*HI site of vector pAS2 (Harper *et al.*, 1993). The pAS-C82 vector was constructed by cloning the 1987 bp *Bam*HI fragment of pMA-C82 (Werner *et al.*, 1993) bearing *RPC82-1001* into the *Bam*HI site of pAS2. The pAS-TFIIIB70 vector producing the G_{DB}–TFIIIB70 fusions has been described previously (Chaussivert *et al.*, 1995). All interactions were tested in *S.cerevisiae* strain Y526 (*MAT**a** ura3-52::GAL1-lacZ::URA3 his3-200 ade2-101 leu2-3, 112 trp1-901 gal4-542 gal80-538*; Bartel *et al.*, 1993) or Y190 (*MAT**a** gal4-Δ gal80-Δ his3-Δ200 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL-lacZ LYS2::GAL(UAS)-HIS3 cyh^r*; Harper *et al.*, 1993).

Protein purification and in vitro transcription assays

Pol III, TFIIIC, TFIIIB, B″ fraction of TFIIIB, recombinant TBP and TFIIIB70 were prepared as described previously (Thuillier *et al.*, 1996). References and a description of the plasmids bearing the different pol III gene templates used can be found in Thuillier *et al.* (1996), except for the 5S-bearing plasmid pBS-5S (Camier *et al.*, 1995), the U6-containing plasmid pB6 (Burnol *et al.*, 1993) and the $tRNA_1^{Val}$ plasmid pY7 (Baker *et al.*, 1982).

Transcription assays were performed as described previously (Thuillier *et al.*, 1996), except for the elongation assay. This was performed using ternary complexes halted at nucleotide 17 prepared by incubating 100 ng of wild-type or mutant pol III for 15 min at 24°C in the presence of the *SUP4* DNA template, 40 ng of recombinant TBP, 50 ng of recombinant TFIIIB70, 400 ng of a B″ fraction (containing TFIIIB90), 50 ng of affinity-purified TFIIIC, 500 μM ATP, 500 μM CTP and 3 μM ^{32}P-labelled UTP. The ternary complexes were dispatched in a microplate, and transcription was allowed to resume at different times (30, 15, 10, 8, 6, 4 and 2 s before the end of the reaction) by the addition, using a multidelivery pipette, of GTP (500 μM) and heparin (300 μg/ml) to prevent re-initiation. Reactions were all stopped simultaneously 2 s after starting the last reaction by the addition of 50 μl of stop mix (EDTA 40 mM, SDS 10%) using an 8-channel pipette. The reactions products were analysed on a 15% denaturing polyacrylamide gel.

The heparin sensitivity assay was realized as described by Dieci and Sentenac (1996), except that the initiation complex was pre-formed before the addition of heparin together with ATP, CTP and UTP.

Permanganate footprinting of the transcription bubble

The DNA probe used was a 270 bp *Bam*HI–*Hin*dIII fragment carrying the *SUP4* tRNA gene. Transcription factors TFIIIC and TFIIIB were incubated at 24°C during 30 min with 4 fmol of 5′-end-labelled DNA probe in 19 µl of buffer containing 20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM EDTA, 5% glycerol, 0.5% polyvinyl alcohol and 5 µg/ml of bovine serum albumin (BSA). Pol III was then added and incubation continued for various times. When nucleotides were included (ATP at 200 µM; CTP and UTP at 100 µM), the reaction was performed for 30 min. $KMnO_4$ was then added as an 11× stock providing 23 mM reagent. After 30 s or 1 min, the reactions were quenched by the addition of 2 µl of β-mercaptoethanol, mixing and the addition of 180 µl of buffer containing 10 mM Tris–HCl (pH 8.0), 3 mM EDTA and 0.2% SDS. The reaction products were extracted with phenol–chloroform–isoamylalcohol (25:25:1) and DNA was precipitated with ethanol (in the presence of 20 µg calf thymus DNA as carrier) and treated with piperidine (Maxam and Gilbert, 1980) prior to electrophoresis on 8% polyacrylamide gels containing 7 M urea. Hyperreactivity at the transcriptional start for each sample was quantified using a PhosphorImager with Image Quant software (Molecular Dynamics).

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