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RNA polymerase III mutants in TFIIFα-like C37 that cause terminator readthrough with no decrease in transcription output

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ABSTRACT

How eukaryotic RNA polymerases switch from elongation to termination is unknown. Pol III subunits Rpc53 and Rpc37 (C53/37) form a heterodimer homologous to TFIIFβ/α. C53/37 promotes efficient termination and together with C11 also mediates pol III recycling *in vitro*. We previously developed *Schizosaccharomyces pombe* strains that report on two pol III termination activities: RNA oligo(U) 3′-end cleavage, and terminator readthrough. We randomly mutagenized C53 and C37 and isolated many C37 mutants with terminator readthrough but no comparable C53 mutants. The majority of C37 mutants have strong phenotypes with up to 40% readthrough and map to a C-terminal tract previously localized near Rpc2p in the pol III active center while a minority represent a distinct class with weaker phenotype, less readthrough and 3′-oligo(U) lengthening. Nascent pre-tRNAs released from a terminator by C37 mutants have shorter 3′-oligo(U) tracts than in cleavage-deficient C11 double mutants indicating RNA 3′-end cleavage during termination. We asked whether termination deficiency affects transcription output in the mutants *in vivo* both by monitoring intron-containing nascent transcript levels and ^{14}C-uridine incorporation. Surprisingly, multiple termination mutants have no decrease in transcript output relative to controls. These data are discussed in context of current models of pol III transcription.

INTRODUCTION

The evolutionarily related multisubunit RNA polymerases are responsible for genome transcription in all cells. Eubacteria and archaea each contain a single RNA polymerase comprised of 5 and 12 subunits, respectively, whereas eukaryotic nuclei contain pols I, II and III, in addition to pols IV and V in plants (1,2). Pols II and III appear more closely related to each other than to pol I (3). Pol II transcribes thousands of genes, some of great length, that use a variety of promoters, enhancers and transcription factors to accommodate gene-specific regulation. Pol III transcribes a few hundred genes, the great majority of which are the short tRNAs that bear similar promoters. Pols II and III contain 12 and 17 subunits, respectively, some of which are shared while others are homologous (4,5). The relatively high number of pol III subunits can be accounted for by stable association of what appear to be homologs of general transcription factors in the pol II system (TFII) (3). Pol III contains subcomplexes C82/34 and C53/37 that bear homology to TFIIE and TFIIF, respectively (3,6), as well as C11, a two-domain polypeptide with homology to Rpb9 and TFIIS (7–9). Although the heterodimerization domains of C53 and C37 reside on the pol III surface near the cleft into which incoming DNA approaches the active center (5,6,10,11), parts of C53/37 appear to extend into the active center and are involved in termination and reinitiation, the latter also requiring C11, although how these activities are coordinated is unclear (11–13).

Understanding the mechanisms that contribute to the efficiency of pol III transcription is important because pol III activation occurs in cancer cells, accompanied by elevation of tRNA levels to support proliferative growth (14–17). Calculations based on HeLa cell RNA content and cellular proliferation yield an approximate rate of 50–100 transcripts per gene per minute, reflective of efficient termination and reinitiation. That pol III incorporates the TFIIE- and TFIIF-homologous initiation complexes C82/34 and C53/37 suggests a means to bypass a need for assembling an initiation complex each time pol III needs to fire, and as such it is fitting that C53/37 are required for facilitated recycling (12).

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Data from *in vitro* transcriptions have indicated that the human proteins La, Topo-I, PC4 and NF1 promote termination associated recycling of pol III in human cell extracts (18–23), although the nature of these activities are questionable (reviewed in 24). Sub1, the yeast homolog of PC4, has been shown to promote facilitated recycling in yeast extracts and is required for optimal pol III transcription *in vivo* (25). As described, facilitated recycling by yeast pol III 'requires termination at the natural termination signal' (26). According to current models, the termination mechanism and C11 may reset pol III for facilitated reinitiation (12,20–22,26–28). However, effects of termination deficiency on pol III output has not been subjected to examination *in vivo*. Availability of the pol III termination mutants reported below provided an opportunity to examine this.

Pols I, II and III initiate termination differently (29). The cis-DNA elements that initiate termination by pols I and II are distant from the site at which RNA is released whereas for pol III the oligo(dT) terminator coincides with the site at which transcription ceases and RNA is released, providing opportunity to study active center involvement in termination in a eukaryote.

Pausing by pol III within the oligo(dT) terminator is prerequisite to, and can be uncoupled from, release of the 3′-oligo(U)-terminated transcript (30). Intrinsic RNA 3′-cleavage, mediated by C11, occurs during pausing at U tracts. Pol III that lacks C53/37 and C11, referred to as pol IIIΔ, exhibits oligo(dT) readthrough as well as RNA 3′-cleavage deficiency (12,31); addition of recombinant C53/37 and C11 rescues the termination and RNA 3′-cleavage deficiencies respectively (12). C53/37 decrease the elongation rate of pol III and contribute to pausing at the oligo(dT) terminator although how they do so is unknown (12). Increased elongation rate is associated with terminator readthrough in several polymerase systems, supporting a general 'kinetic coupling' model of termination (32–35). Based on localization of C53/37 on one side of the pol III cleft, it was proposed that this positioning would allow C53/37 to sense the oligo(dT) on incoming DNA (5), presumably acting as brakes to slow elongation.

Genetic screens of *Schizosaccharomyces pombe* strains carrying monomeric or dimeric suppressor-tRNA genes with differently configured terminators led to isolation of two types of C11 mutants, ones that readthrough oligo(dT) terminators and others that impair RNA 3′-oligo(U) cleavage at termination, that map to the Rpb9-like and TFIIS-like domains of C11, respectively (8,9). The monomeric construct reports on deficiency of 3′-oligo(U) cleavage during termination while the dimeric construct reports on propensity of pol III to read through an oligo(dT) terminator. Mutations in the Rpb9-like domain of C11 cause readthrough whereas mutations in the TFIIS-like domain impair RNA 3′-oligo(U) cleavage, reflecting different activities of C11 that are presumably coordinated during termination (9).

Here, we used oligo(dT) terminator readthrough to screen comparably mutagenized C37 and C53 libraries. Although many C37 single point mutants emerged, no C53 mutants with single mutations were obtained. Most of the C37 mutants were in a C-terminal region recently localized near Rpc2p in the pol III active center. We also examined the mutants in the monomeric reporter strain for RNA 3′-end length. A second class of C37 mutants exhibit weaker readthrough but stronger RNA 3′-cleavage deficiency phenotype. Our data indicate shorter 3′-oligo(U) tracts in strong readthrough mutants relative to 3′-cleavage-deficient C11 double mutants analyzed in parallel supporting the idea that RNA 3′-cleavage accompanies RNA release for many transcripts. Additional analyses indicate that deficiency in normal termination occurs without the expected significant decrease in pol III transcript output *in vivo*.

MATERIALS AND METHODS

The suppressor-tRNA genes in *S. pombe* strains are listed in Table 1. The suppressor tRNA in yKR1 contains dimeric construct pDRT at the *leu1* locus, comprised of a wild-type (WT) $tRNA^{Ser}UGA$ followed by a linker and dT(5) terminator, CTAGATTTTT, followed by the suppressor $tRNA^{Ser}UCA$-G37:10 a GAAGATC trailer and 21T terminator, in PstI/SacI sites of pJK148.

Mutagenesis of *rpc37*

Nucleotide analog-based mutagenic polymerase chain reaction (PCR) was used to construct a C37 library as described for C11 (8), using primers C37 FWD: 5′-ACT TAG TATCTCGAG ATGTCCTTTTCAGAAGATC-3′ and C37REV: 5′-ATACTGCACCCGGGCTAAATAAA G GAGTAATCTTC-3′. Briefly, PCR was done with 4 uM 8-oxo-2′-deoxyguanosine-5′triphosphate, 2′-deoxy-*P*-nucleoside-5-triphosphate, 0.2 μM deoxynucleotide triphosphates (dNTPs), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 100 ng of pRep4X-spRpc37 plasmid and 1 uM primers C37FWD and C37REV in 50 μl. Standard PCR using 1 μl of the first PCR product was done in the absence of nucleoside analogues for 15

Table 1. *Schizosaccharomyces pombe* strains used

Strain name	Genotype	References
yAS99	h-ade6-704 ura4-D18 leu1-32	(36–38)
yYH1	h-ade6-704 ura4-D18 leu1-32::[tRNAmSer7T-leu1$^+$]	(8)
yAS68	h-ade6-704 ura4-D18 leu1-32::[tRNAmSer3T-leu1$^+$]	(9)
yAS76	h-ade6-704 ura4-D18 leu1-32::[tRNAmSer5T-leu1$^+$]	This report
yKR1	h-ade6-704 ura4-D18 leu1-32::[DRT5T-leu1$^+$]	This report
yJI1	h-ade6-704 ura4-D18 leu1-32::[DRT6T-leu1$^+$]	(9)

cycles, the product cloned into XhoI/XmaI of pRep4X and transformed into UltraMax DH5α-FT cells (30 plates). Approximately 150 000 transformants were scraped from the plates and plasmid DNA library isolated.

Isolation of *rpc37* mutants

For terminator readthrough screening the C37 library was transformed into *S. pombe* yKR1 and plated on EMM-lacking uracil and containing 10 mg adenine/liter. Approximately 10 000 colonies were screened and plasmid from the transformants showing the suppression phenotype were isolated for retransformation and sequencing.

Suppression assay

Cultures grown overnight in Edinburgh minimal media (EMM) plates lacking uracil and containing 10 mg adenine/L were diluted in fresh medium. Cell growth was monitored by optical density at 600 nm. Five microliters of logarithmically growing cells (OD6001/ml) were spotted onto EMM plates lacking uracil and containing 10 mg/ml of adenine and incubated at 32°C for 3 to 4 days.

Pre-tRNA 3′-oligo(U) length was determined

The same approach was used for endogenous pre-tRNAIleUAU using primer 5′-TCA TGC TTA TAT GAT ACT CCC CTT AGG as described (8). The reverse transcriptase (RT)–PCR products were inserted into TOPO TA cloning vector (Invitrogen); plasmids were isolated from the independent clones and sequenced.

Northern blotting

Total RNA was isolated using guanidinium isothiocyanate. RNA from control strains and mutants were separated on 6% polyacrylamide–urea gel, and transformed to a nylon membrane by using IBlot (Invitrogen, Grand Island, NY, USA). The membrane was UV cross-linked, vacuum dried at 80°C and hybridized with RNA-specific DNA oligos. The blots were exposed to phosphorimager screen and scan with Fuji scanner and quantified. All bands were normalized based on the ratio of U5 RNA.

Growth assays

Overnight cultures of cells were inoculated into EMM plates lacking uracil and further grown at 32°C. Logarithmically growing cells at OD 0.6 were spotted at a cell concentration of 1.0 OD/ml and 10-fold dilutions; 5 μl of each was spotted onto EMM plates lacking uracil and grown at 32°C for 3 days.

^{14}C metabolic labeling

Overnight cultures of cells containing C37-WT, C37–V189D or C37–L41P were inoculated into EMM plates lacking uracil and logarithmically grown at 32°C. Exactly 25 OD of cells from each were harvested and resuspended in 5 ml of EMM plates lacking uracil. A total of 2.5 μCi of ^{14}C-uridine (Perkin-Elmer, Waltham, MA, USA) was quickly added to the cells, mixed and growth continued at 32°C. After 5.0 min, 25 ml of EMM containing excess uridine was added to the growing cells, which were quickly spun down and separated from the supernatant. To the cell pellet, 700 μl of TRIzol reagent was added, flash frozen and used for total RNA isolation. The products were analyzed on a 10% polyacrylamide–urea gel and a Fujifilm phosphorimager and quantitated with Image Reader software.

RESULTS

Our laboratory has used tRNA-mediated suppression to study tRNA biogenesis in *S. pombe*. Pol III terminates tRNA gene transcription with high efficiency (~90%) at an oligo(dT) stretch of 5 Ts in *S. pombe* and this increases at longer oligo(dT) tracts (9,39). Suppression of a premature UGA stop codon in *ade6-704* by suppressor-tRNA^{Ser}UCA decreases accumulation of red pigment, providing an assay that can also be used for genetic screening. Two types of termination reporters were developed that produce suppressor tRNA from either a dimeric or monomeric tRNA gene construct (9) (Figure 1A and B). In the monomeric assay strain yYH1, a 7T terminator resides at the natural position, within a few nucleotides from the 3′-end of the suppressor tRNA sequence (Figure 1B) (39). In yYH1, 3′-oligo(U) length of transcripts terminated at T1 is a major determinant of whether the nascent pre-tRNA is processed to a mature functional suppressor-tRNA (reviewed in 40). In the dimeric system assay strain yJI1 (Figure 1A), a suppressor-tRNA lies downstream of the natural oligo(dT) terminator of the upstream tRNA sequence (9). WT *S. pombe* pol III initiates normally at the upstream tRNA^{Ser} and terminates at the oligo(dT) without transcribing the downstream suppressor-tRNA (9). A suppression phenotype occurs only if pol III fails to terminate at the natural oligo(dT) terminator and transcribes into the suppressor-tRNA which is followed by a 21T failsafe terminator (9). This was used to isolate mutants in the Rpb9-homologous domain of C11 that cause terminator readthrough (9). RNA quantification revealed as expected that the mutant pols III read through a 6T terminator more readily than a 7T terminator (9). To make the dimeric-based screen more sensitive, the 6T terminator was replaced with 5T in strain yKR1. Given that 5T is an efficient terminator in *S. pombe* and one-half of all *S. pombe* tRNA genes have a 5T terminator, more than any other T length class (41), this is a physiologically representative reporter (39).

After isolation from yKR1, the mutant C37 alleles were also assayed for suppression in a second dimeric strain, yJI1, for readthrough of a 6T terminator, and then in the monomeric strain yYH1 for RNA 3′-oligo(U) cleavage function at a 7T terminator from which terminated transcripts are released. This was followed by analysis of the C37 alleles for readthrough transcripts produced *in vivo* from a monomeric gene with either a 5T or 7T terminator.

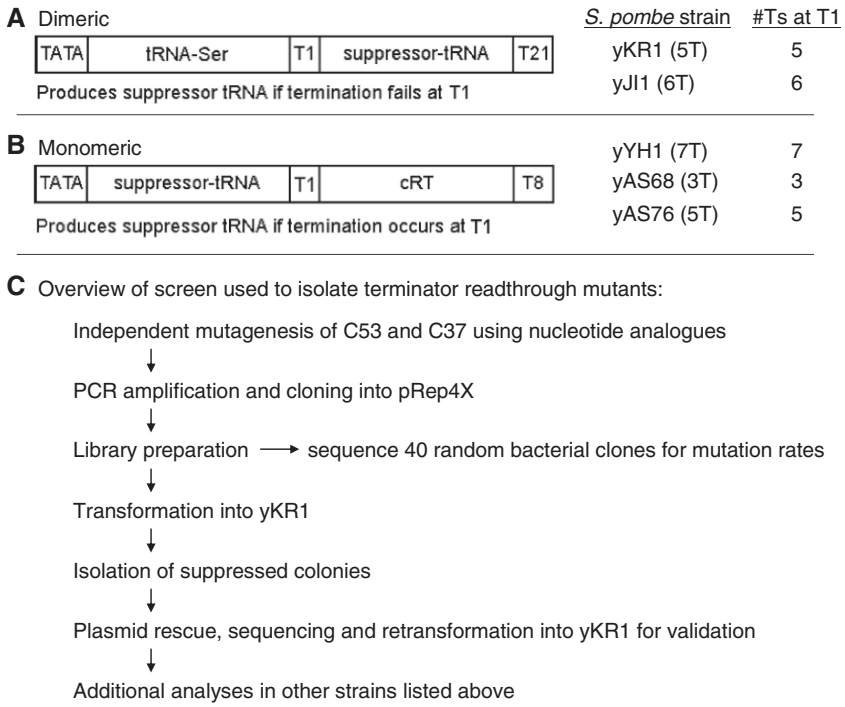

Figure 1. (**A** and **B**) Schematic showing two types of suppressor tRNA gene reporter constructs used in this study, and corresponding strain names with the number of Ts in the monitored terminators. In (**B**), the cRT region is highly complementary to the pre-tRNA sequence as part of the design to block tRNA structure formation and inhibition of processing, as described (39). (**C**) An algorithmic overview describing the terminator readthrough genetic screen process.

Table 2. C37 library mutation rate

	T	A	C	G	Overall
T	—	0.014 (4)	0.020 (6)	0.004 (1)	0.038% (11)
A	0.024 (7)	—	0.01 (3)	0.034 (10)	0.068% (20)
G	0.020 (6)	0.017 (5)	0.007 (2)	—	0.044% (13)
C	0.044 (13)	0.017 (5)	—	0.007 (2)	0.068% (20)

Fractional numbers represent a rate derived from the numbers in parentheses, which reflect actual number of times a nucleotide in column 1 was found mutated to the nucleotide in the other column. Overall rate = Total no. of mutations/(40 clones) × (729 nt/clone) × (100); e.g. for T: $11/(40 \times 729) \times 100 = 0.038\%$.

Isolation of C37 mutants

Nucleotide analog-based mutagenic PCR was used to construct a randomly mutagenized C37 library (8,42) in the pRep4X expression vector. To assess the diversity of the library prior to screening in *S. pombe*, we sequenced 40 randomly chosen bacterial clones. These collectively contained 64 substitutions and 3 insertions or deletions. Each of the four nucleotide identities, A, T, G and C underwent transitional and transversional mutagenesis such that each identity was mutated to all three other identities (Table 2). We also evaluated the rate at which amino acids were changed. Thirty percent of the 40 clones showed no change in amino acid sequence, 22.5% had one, 27.5% had two, and 15% had more than two amino acid mutations, plus 5% with an insertion or deletion. We plotted the distribution of amino acid mutations in the 40 random clones along the length of the C37 polypeptide (Figure 2A). We conclude that the library contained a wide variety of mutations randomly distributed throughout the C37 amino acid sequence (Table 2 and Figure 2A).

We screened ~10 000 colonies for suppression in yKR1, and obtained ~120 suppressed colonies at a rate of ~3.0%. Plasmids from the suppressed colonies were recovered, purified and retransformed into yKR1 (5T terminator) and yJI1 (6T terminator) which verified them. Upon sequencing, 32 contained single amino acid-altering mutations, 43 contained two, 28 contained three or more, 15 contained nonsense mutations alone or in combination with other mutations and 5 contained a single premature stop codon. Some of the single mutation mutants were obtained several times and with different amino acids at the same position (Table 3). These data demonstrate a variety of mutations per codon in the library, multi-fold coverage, and near exhaustive screening of the library. Moreover, not a single WT C37 clone was recovered as a suppressed mutant from our library screening even though WT C37 clones were present at ~30% in the library, indicating specificity of the screening.

Parallel screening with a similarly mutagenized C53 library that bears mutation frequency comparable with the C37 library produced no suppressed mutants from 140 000 colonies (C53–1, Table 4). In a further attempt to isolate C53 readthrough mutants, we created a second library with a higher mutation frequency (C53–2 in Table 4). Twenty mutants were obtained but these all showed only weak phenotype relative to the C37 mutants and all contained more than one mutation (Table 4)

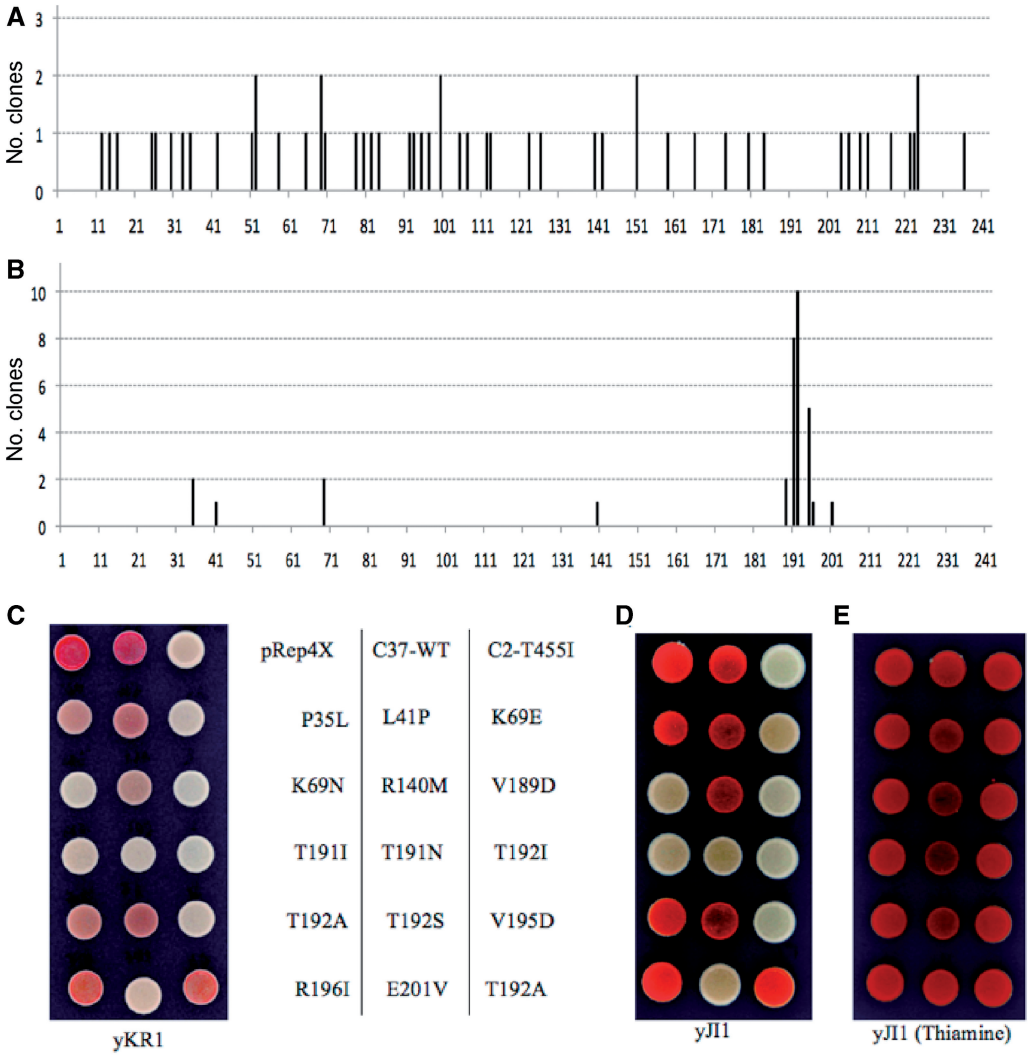

Figure 2. Location of single point mutants of *S. pombe* C37 and their terminator readthrough phenotypes. (**A**) Distribution of amino acid substitutions in the unselected library along the length of C37 (*X* axis). (**B**) Distribution of single amino acid substitution mutants selected for terminator readthrough. (**C**) tRNA-mediated suppression phenotype of C37 single substitution mutants after retransformation of mutated C37 plasmids into yKR1 (T5 terminator). Top row shows the empty vector pRep4X, C37-WT and the *rpc2-mutant* C2–T455I as controls, as indicated to the right. (**D**) The same mutants in yJI1 (T6 terminator). (**E**) The same mutants in yJI1 in the presence of thiamine, which represses expression from the pRep4X-C37 plasmid. (**F**) Sequence alignment of C37s from various yeasts, with positions of single point mutants indicated by asterisks; *gray, mild to moderate; *black, strong readthrough phenotype. Dashed lines above the sequences represent the dimerization domains. Thick horizontal line above sequences represents amino acids deleted/mutated in *S. cerevisiae* C37 (11).

and were not further characterized. Thus, mutation of C37 much more readily caused readthrough than mutation of C53. We therefore focused on the C37 mutants.

It should also be noted that as was the case for prior C11 screening (8), the present screening as well as further analyses occurred in cells that have a chromosomal copy of WT C37. This strategy has an advantage as it theoretically allows identification of important mutations that when mutated might otherwise be lethal in the absence of the endogenous WT protein. Potential limitations are noted in the Discussion.

C37 mutants cluster in a region previously localized near Rpc2p in the pol III active center

Only 10 amino-acid positions were mutated in the 33 single mutants, represented by 14 unique mutations, and 27 of these were clustered at positions 189 to 201 (Figure 2B). This distribution is remarkably distinct from the single mutations found throughout C37 in the unselected library (Figure 2A). Twenty-three of the 33 readthrough mutants were at T191 and T192, replaced by various amino acids, and V195, many of which were independent isolates obtained multiple times (Table 3).

After isolation, the mutant alleles were retransformed into reporter strain yKR1, along with WT C37 and empty vector pRep4X, to confirm that their suppression phenotype was due to the C37 allele and not mutation at a chromosomal locus. Different C37 alleles produced different levels of suppression, from mild (pink) to strong (white) as exemplified by L41P and T191I, respectively (Figure 2C).

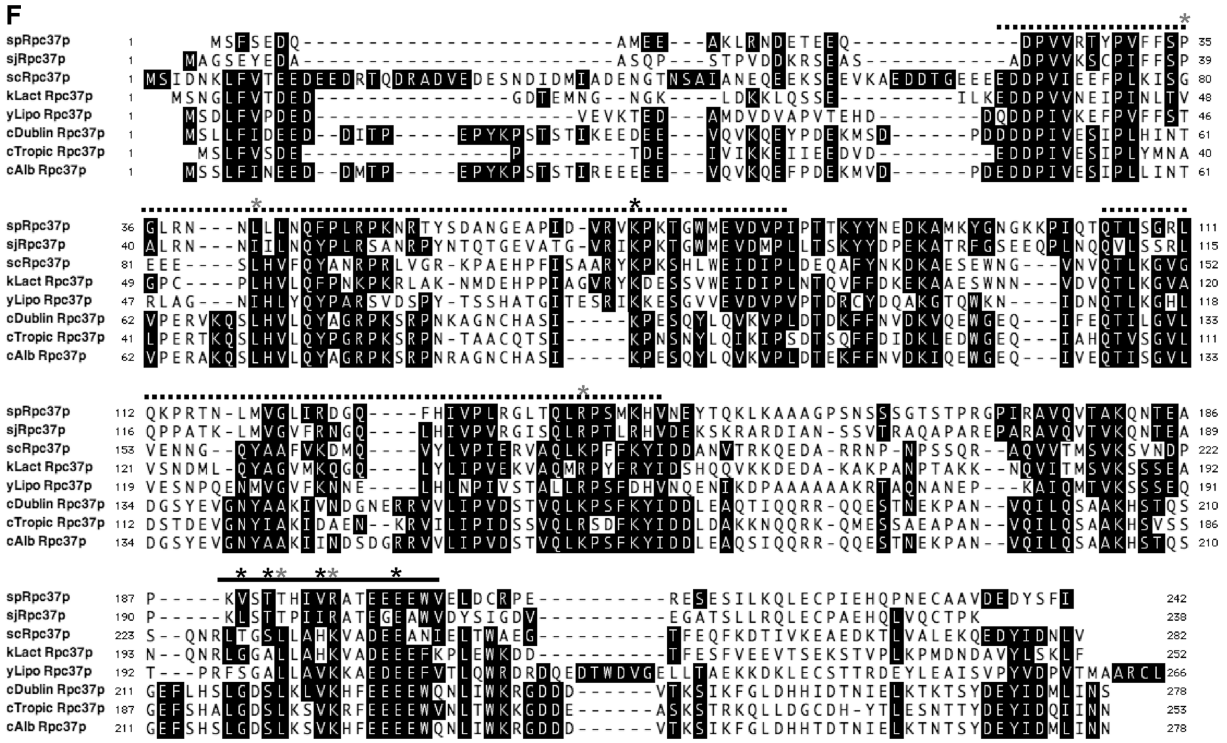

Figure 2. Continued.

Table 3. Single mutation C37 mutants obtained from yKR1 and phenotype in yKR1, yJI1 and yYH1 (see text)

Mutation	No. of mutants obtained	Suppression dimeric; 5T, yKR1	Suppression dimeric; 6T, yJI1	Suppression monomeric, yYH1
P35L	1	Moderate	None	Weak
L41P	1	Moderate	None	Strong
K69E	1	Strong	Strong	Weak
K69N	1	Strong	Moderate	None
R140M	1	Moderate	None	Weak
V189D	2	Strong	Strong	Weak
T191I	7	Strong	Strong	Weak
T191N	1	Strong	Strong	Weak
T192I	6	Strong	Strong	Weak
T192A	3	Moderate	Weak	Weak
T192S	1	Moderate	None	Weak
V195D	5	Strong	Strong	Weak
R196I	1	Moderate	None	Weak
E201V	1	Strong	Strong	Weak
Q177-X	2	Moderate	None	Strong
Q220-X	3	Moderate	None	Strong

Comparison of mutants in strains bearing different length oligo(dT) tracts in the dimeric tRNA reporter is a way to gauge their relative readthrough strength *in vivo* (9). Therefore, to test whether the different levels of suppression seen in yKR1 reflected different readthrough strengths of the C37 alleles, we compared the suppression phenotypes of the 14 unique single mutants in yKR1 and yJI1 strains, which bear 5T and 6T terminators (Figure 1A), along side a previously characterized terminator-readthrough mutant in Rpc2p, C2–T455I (9) (Figure 2C and D). This revealed that the eight mutants that produced strong suppression in yKR1 also produced suppression in yJI1, whereas the mutants with moderate to weak suppression in yKR1 produced less suppression in yJI1, consistent with the stronger test terminator in yJI1 (Figure 2C and D). Although most of the strong phenotype associated mutations were clustered at positions 189–201, the exception, K69 also produced strong phenotype in the single mutants, K69E and K69N.

The *nmt1* promoter driving expression from pRep4X-C37 is repressible by thiamine (43), and as shown in Figure 2D suppression was repressed by thiamine (Figure 2E). This confirmed that suppression was due to the C37 alleles driven by the *nmt1* promoter.

Sequence alignment shows that most mutations mapped beyond the C53–C37 dimerization domain in a conserved region that includes *S. cerevisiae* C37 residues previously localized near Rpc2p in the pol III active center (11) (Figure 2F). For example, V189 of *S. pombe* corresponds to T228 of *S. cerevisiae* C37, which was reactive with Rpc2p near the active site (11).

We also examined C37 mutant alleles with two mutations after retransforming them into yKR1 (Figure 3A). This revealed that strong suppression occurred only when a mutation was at K69, T191, T192 or E201, which also cause strong phenotype as single mutants, as well as S190, which was not isolated as a single mutation mutant (Figure 3A). Of the 43 clones with two mutations, 32 contained one at either K69, S190, T191 and T192 but none contained mutations at two of these positions. Thus, the double mutation mutant data are important because it

Table 4. Library screening of Rcp37 and Rpc53 library

Library	Mutation rate[a]	No. of clones screened	Suppression rate	Single mutation clones	Two mutation clones	Multiple mutation clones	Other mutant clones	Notes
C37	0.57% or 1/176	10 000	3%	32	43	28	17	Strong
C53–1	0.53% or 1/188	140 000	≤0.01%	—	—	—	—	Not detected
C53–2	1.0% or 1/90	50 000	0.1%	None	6	14	—	Weak

[a]Mutation rate = Total mutation found/(total clone sequence × no of amino acids in protein) × 100. For Rpc37: Mutation Rate = 59/(43 × 242) × 100 = 0.57%. Rpc37 and Rpc53 (1) library contains very similar rate of mutation but Rpc53 (2) is created with higher rate of mutation to further conform that no suppression in C53 was not due to inadequate mutants in the library.

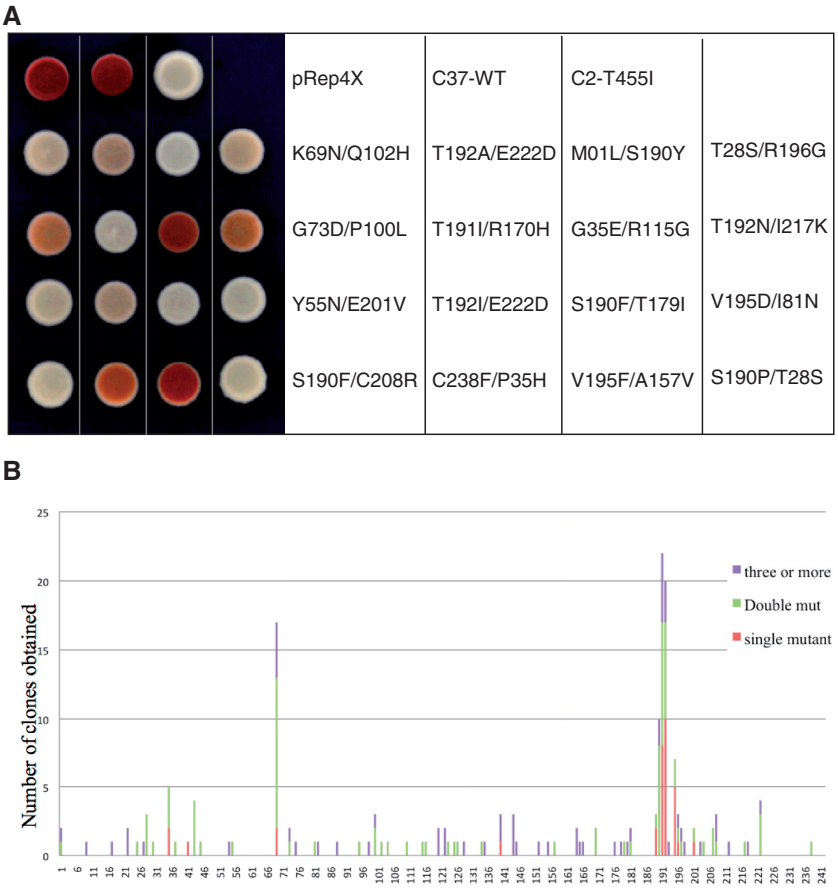

Figure 3. Multiple mutation mutants also cluster in the C-terminal region. (**A**) tRNA-mediated suppression phenotype of C37 double substitution mutants after retransformation of the mutated C37 plasmid into yKR1. The top row shows the empty vector pRep4X, C37-WT and the *rpc2-mutant* C2-T455I as controls, as indicated to the right of the panel. (**B**) Plot of mutations in C37 readthrough mutants with ≥1 mutation along the C37 length (*X* axis).

confirms the clustering pattern observed for single mutation mutants (Figure 3B).

Two classes of C37 mutants: oligo(dT) readthrough and 3′-oligo(U) lengthening

Five clones with moderate suppression contained only a nonsense mutation, two at Q177 and three at Q220 (Table 3). The stop at Q220 would truncate 23 amino acids, similar to *S. cerevisiae* C37 bearing a 27 amino acid deletion that yields pol IIIΔ which lacks RNA 3′-cleavage activity and exhibits terminator readthrough (11,12). We therefore examined these mutants for suppression in the RNA 3′-cleavage-sensitive strain yYH1. Side-by-side comparison with other mutants in both the RNA 3′-cleavage-sensitive and readthrough-sensitive strains is required to demonstrate the striking contrast with which they partition these phenotypes (Figure 4A). Q177x and Q220x exhibit moderate suppression in yKR1, none in yJI1 (as expected from moderate phenotype in yKR1), but strong suppression in yYH1, similar to C37–L41P and C11–C102S (Figure 4A),

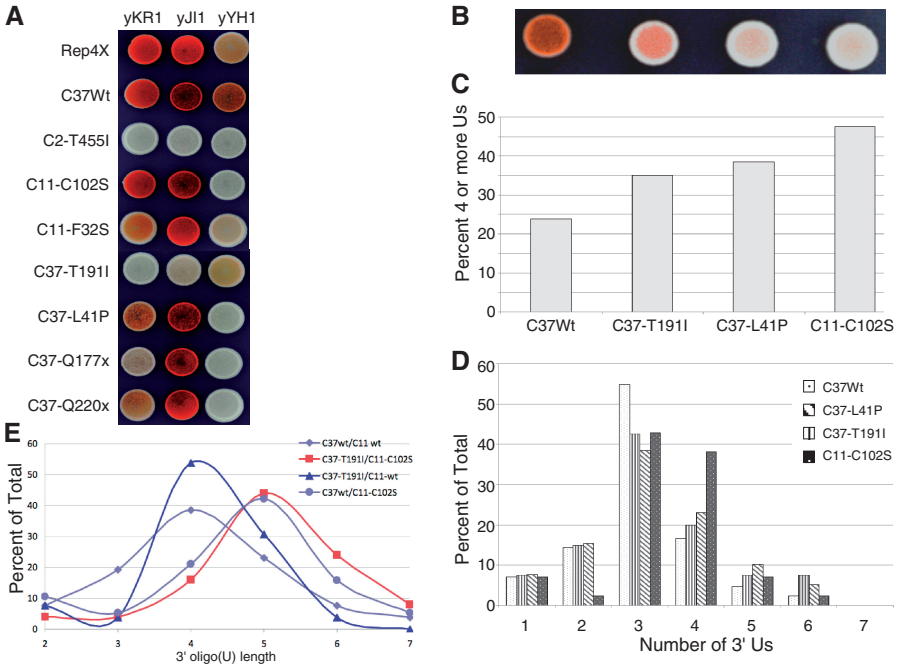

Figure 4. C37 mutants contain 3′-oligo(U) lengthened nascent pre-tRNAs. (**A**) Side-by-side analysis of mutants for terminator readthrough phenotype in yKR1, yJI1 (5T and 6T) as well as 3′-oligo(U) lengthening-associated phenotype in yYH1. A range of C37 mutants as well as C2- and C11-mutants as controls are shown for comparison. (**B**) Direct comparison of 4 strains for suppression on yYH1, as indicated under (**C**), which shows the fraction of pre-tRNASerUCA with ≥4 3′Us for each of these. (C) Nascent pre-tRNASerUCA was isolated from total RNA by a 3′-end ligation followed by gene-specific amplification strategy described previously (8). Individual clones were sequenced and the number of 3′-terminal Us counted. (**D**) The percent of total with 1–6 Us were plotted as indicated (none contained 7 or more, not shown). The total numbers for the four pools of sequences were 42, 40, 39 and 42. (**E**) 3′-oligo(U) lengths for endogenous pre-tRNAIleUAU in C37/C11 double mutants. *Y* axis reflects percentage of the total transcripts with the length plotted.

the latter a RNA 3′-cleavage-deficient mutant (8). These data suggest that C37–Q177x, -Q220x and -L41P cause RNA 3′-oligo(U) lengthening as in cleavage-deficient C11 mutants (8) (below).

In striking contrast to the suppression pattern for Q177x, Q220x and L41P, is the C-terminal cluster mutant T191I, which exhibits strong suppression in yKR1 and yJI1 but much less, albeit detectable suppression in yYH1 (Figure 4A). A similar pattern was observed for C37–V189D (not shown). Adjusting adenine content of the media sensitizes the suppression assay and confirmed that C37–T191I exhibits slightly more suppression than WT in yYH1 but less than L41P (Figure 4B). These data suggest that the C37–T191I class mutants exhibit more terminator readthrough than 3′-oligo(U) lengthening whereas Q177x, Q220x and L41P exhibit more 3′-oligo(U) lengthening than readthrough. Thus, we isolated two classes of phenotypic C37 mutants. We also note that while C37–T191I and C37–L41P are diametric in these phenotypes, this is in contrast to Rpc2–455I, which reproducibly exhibits high levels of both phenotypes (8,9) (Figure 4A). These data support the idea that these phenotypes reflect associated but distinguishable activities that are associated with pol III termination.

Elongated 3′-oligo(U) in C37–L41P accounts for suppression in the RNA 3′-oligo(U) cleavage-sensitive strain, yYH1

A strong phenotype of C37–L41P in yYH1 while weak in yJI1 is similar to C11–C102S (Figure 4A), the C11 cleavage mutant that produces 3′ elongated oligo(U) (8,9). In yYH1, whose suppressor-tRNA gene requires termination at a 7T terminator, the majority of nascent suppressor pre-tRNAs bear only three or fewer Us on their 3′-ends (8). tRNA-mediated suppression is increased in the C11–C102S cleavage mutant because increasing length to ≥4Us increases affinity of the pre-tRNAs and thereby their effective competition for a limiting amount of *S. pombe* La protein which promotes tRNA maturation (8). We examined 3′-oligo(U) length of the nascent suppressor pre-tRNAs, with WT and cleavage-deficient C11–C102S as controls. We cloned and sequenced ~40 nascent pre-tRNA cDNAs from each mutant and tabulated the percentage with ≥4 Us at the 3′-end (Figure 4C). This showed 23% and 47% of nascent pre-tRNA transcripts with ≥4 Us for WT and C11–C102S respectively, in good agreement with previous results (8). At 38%, C37–L41P was increased relative to WT (Figure 4C). C37–T191I produced lower percentage of nascent transcripts with ≥4 Us as compared with L41P, consistent with its intermediate suppression phenotype relative to WT and C37–T191I (compare with Figure 4B). These data support the idea that increase in the fraction of pre-tRNAs with 3′-oligo(U) length of ≥4 Us is accompanied by and correlated with suppression in yYH1 (8) and suggest that certain C37 mutants may influence 3′-oligo(U) length more than others. This provides novel evidence that the C37 point mutants characterized here may affect pol III active site-mediated RNA 3′-oligo(U) cleavage during termination.

Plotting oligo(U) length distribution as in Figure 4D revealed that C37–T191I and C37–L41P appeared more similar to WT than to C11–C102S. This showed that C37 mutant pols III produce nascent pre-tRNAs with near normal 3′-oligo(U) length, centered at 3Us, upon termination within a 7T terminator. Previous data indicated that RNA 3′-cleavage occurs during termination (8). The normal length of RNA 3′-oligo(U) was not necessarily expected for C37–T191I because as a strong readthrough mutant with expected fast elongation it might terminate farther into the oligo(dT) tract than WT pol III thereby producing longer 3′-oligo(U).

RNA 3′-cleavage accompanies transcript release at termination

To evaluate the potential for C11-mediated cleavage in the C37–T191I readthrough mutant, we created C11/C37 double mutants and examined 3′-oligo(U) length of nascent pre-tRNA transcripts released at a normal terminator. For this, we examined nascent intron-containing endogenous pre-tRNA^{Ile}UAU, comparing C37–T191I mutants carrying either WT or cleavage-deficient C11–C102S (Figure 4E). First, it should be noted that it has been known from multiple studies that the 3′-oligo(U) tracts of nascent pol III transcripts are heterogenous in length (reviewed in 40). C11-mediated 3′-oligo(U) metabolism during termination in the context of C37-WT pol III has been examined before (8). Pol III containing the cleavage-active WT C11 (C11-WT) produces 3′-oligo(U) tracts that are shortened by 1–2 nt relative to the cleavage-deficient pol III mutant containing C11–C102S (8). For the present study, the critical comparison is 3′-oligo(U) length in C37–T191I carrying C11-WT versus cleavage-deficient C11–C102S. Figure 4E shows that the peak of 3′-(U) length of nascent pre-tRNA^{Ile}UAU transcripts from C37–T191I/C11-WT reflects shorter 3′-(U) tracts than in C37–T191I/C11–C102S. Since C11-WT versus C11–C102S is the only variable in this experiment it can be reasonably concluded that the 3′-oligo(U) length difference is attributed to the cleavage activity of C11. Thus, although C37–T191I exhibits increased read through of oligo(dT) terminators, its nascent transcripts are nonetheless subjected to C11-mediated 3′-end shortening when it terminates at a normal oligo(dT) terminator.

Suppressor-tRNA readthrough transcripts in the mutants

It was reassuring that tRNA-mediated suppression could be used to reflect the relative readthrough of terminators of varying T length by the mutants (Figure 2C versus 2D). However, although *S. pombe* and other yeast contain a small number of dimeric tRNA genes similar to our reporter genes in yKR1 and yJI1 (44,45), the vast majority are monomeric, with a natural oligo(dT) terminator just downstream of the 3′-end of the tRNA sequence (41,46). Therefore, we wanted to confirm readthrough of oligo(dT) terminators using the monomeric tRNA gene. For this, we used northern blotting to detect readthrough transcripts from the monomeric suppressor tRNA allele in yAS76 whose naturally positioned 5T terminator is followed by ~100 bp before a strong 8T downstream terminator (Figure 1B) (9,39). By probing for sequence between the natural and downstream terminators we can detect a specific transcript band and assess readthrough of the monomeric tRNA gene natural terminator (Figure 5A) (39). yAS68 (lane 1) is a control with the same monomeric suppressor-tRNA as in yAS76 but with 3T in place of the 5T terminator (Figure 1B) (9). yAS99 (lane 2) lacks a suppressor-tRNA gene and serves as negative control (9). *rpc2-mutant* C2-T455I (lane 3) is a positive control for readthrough (9). As expected, yAS99, empty vector, and WT C37 showed only background levels of readthrough transcript (Figure 5A, lanes 2, 4 and 5). The mutants that exhibit strong suppression in yKR1 showed more readthrough transcripts than the weak readthrough mutant C37–L41P (Figure 5, compare lanes 6–11 with lane 12).

The data in Figure 5A and another blot were quantified using U5 RNA (synthesized by pol II) on the same blots (Figure 5B) as a loading control. We set yAS68 to represent 100% readthrough, intentionally loading less total RNA to maintain linearity of detection (9). Quantitation is shown in Figure 5C, reflecting readthrough of a T5 terminator. Most mutants produced more readthrough transcripts than vector or WT, consistent with their readthrough phenotype. The expected exception was C37–L41P, which exhibits relatively weak readthrough phenotype.

Readthrough of endogenous tRNA gene terminators with evidence of gene-specific termination defects

The substantial readthrough transcripts observed from the suppressor-tRNA gene suggested that we might also detect readthrough of natural terminators of endogenous tRNA genes. We surveyed the genome for tRNA genes whose natural terminators were followed by a not too distant downstream tract of ≥5Ts so that we could detect a distinct readthrough band of a predicted size. Probes specific to the regions downstream of the natural terminators detected readthrough transcripts from the three tRNA genes examined, tRNA^{Lys}CUU, tRNA^{Pro}CGG and tRNA^{Ile}UAU (Figure 5D–F). As expected, endogenous readthrough transcripts were observed in C37 mutants and the *rpc2-mutant* but not the controls. Intriguingly, the relative abundance of these transcripts and the RT transcripts in (A) appear to vary in a gene-specific or terminator context-specific manner in different mutants (Discussion). We also uncovered evidence for tRNA gene-specific effects of the termination mutants (Figure 5D–F). This was observed in the differential pattern of RT transcripts from the four tRNA genes we examined as can be appreciated by comparing the relative amounts of RT transcripts in lanes 3, 7 and 9 of Figure 5A, D, E and F). In any case, the presence of readthrough transcripts in the mutants but not controls confirmed that the pol III termination mutants are defective at natural endogenous tRNA gene terminators and therefore exhibit generalized widespread pol III termination deficiency.

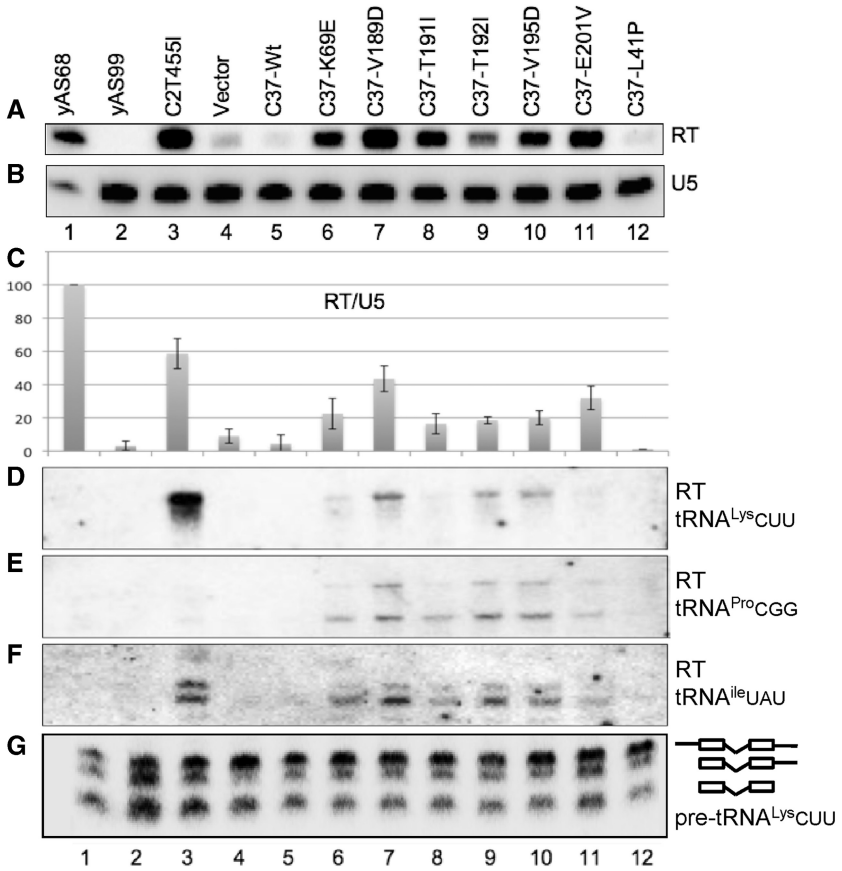

Figure 5. Detection of 5T terminator readthrough transcripts in C37 mutants by northern blotting. RNAs isolated from various mutants and controls; the same blot was probed sequentially (after stripping previous probe, not shown) as indicated below. Lanes 1 and 2 contain RNA from positive and negative control strains (see text); lanes 3–12 contain RNA from yAS76 (monomeric tRNA gene with 5T terminator), and the various mutants indicated above the lanes. (**A**) The blot was probed for the cRT region downstream of the T5 terminator of the reporter tRNA gene in yAS76 to monitor readthrough transcripts specifically. (**B**) Probed for U5 RNA, synthesized by pol II to serve as loading control. (**C**) The data in (A) and (B) were quantified and the ratio of RT:U5 was plotted after setting yAS68 (lane 1) to 100% (see text); *Y* axis shows relative amounts. (**D–F**) Probed for the sequences downstream of the terminators of the $tRNA^{Lys}CUU$, $tRNA^{Pro}CGG$ and $tRNA^{Ile}UAU$ gene as indicated. (**G**) Probed for the intron of the pre-$tRNA^{Lys}CUU$ (see text).

The termination mutants show no apparent decrease in overall pol III transcription

As noted in the 'Introduction' section, pol III can efficiently recycle on *in vitro*-assembled transcription complexes to support multiround transcription in a process referred to as facilitated reinitiation (18–23,26–28). In the yeast *in vitro* system, facilitated recycling 'requires termination at the natural termination signal' (26) as well as pol III initiation factors and the pol III subunits C11 and C53/37 (12,47). According to current understanding, termination deficiency could plausibly affect transcription by two mechanisms; via effects on single round transcription and on facilitated recycling. With a single passage of pol III, readthrough of a natural tRNA terminator should manifest as an increase in readthrough transcript with corresponding decrease in the normally terminated nascent pre-tRNA, independent of reinitiation. A prediction of this effect would be that the ratio of readthrough to primary nascent pre-tRNA transcripts should increase as termination deficiency decreases. By the second mechanism, if termination promotes reinitiation (12,47), defects that impair termination may further decrease tRNA transcript output because in growing yeast most of this should be due to reinitiation. Thus, if facilitated recycling is impaired due to termination deficiency, total output should be diminished in a termination mutant relative to WT. In contrast to this would be effects of the first mechanism only, that total output (primary nascent pre-tRNA + readthrough transcript) would not be diminished even in the presence of demonstrable termination deficiency.

We examined nascent intron-containing pre-tRNAs, which because of their rapid processing, reflect differences in pol III transcription rates *in vivo* (48–51). Northern blots were probed with an intron probe for nascent pre-$tRNA^{Lys}CUU$, used as a standard in several studies (8,36,37,52–56). This probe detects three pre-$tRNA^{Lys}CUU$ bands, of which the uppermost is the nascent transcript that contains a 5′ leader, a 3′-oligo(U)-containing trailer, and intron, whereas the lower two are processing intermediates (36,52,53,55) (see right of Figure 5G). We found no consistent variation between the amounts of readthrough observed in Figure 5A and nascent pre-$tRNA^{Lys}CUU$ levels in the

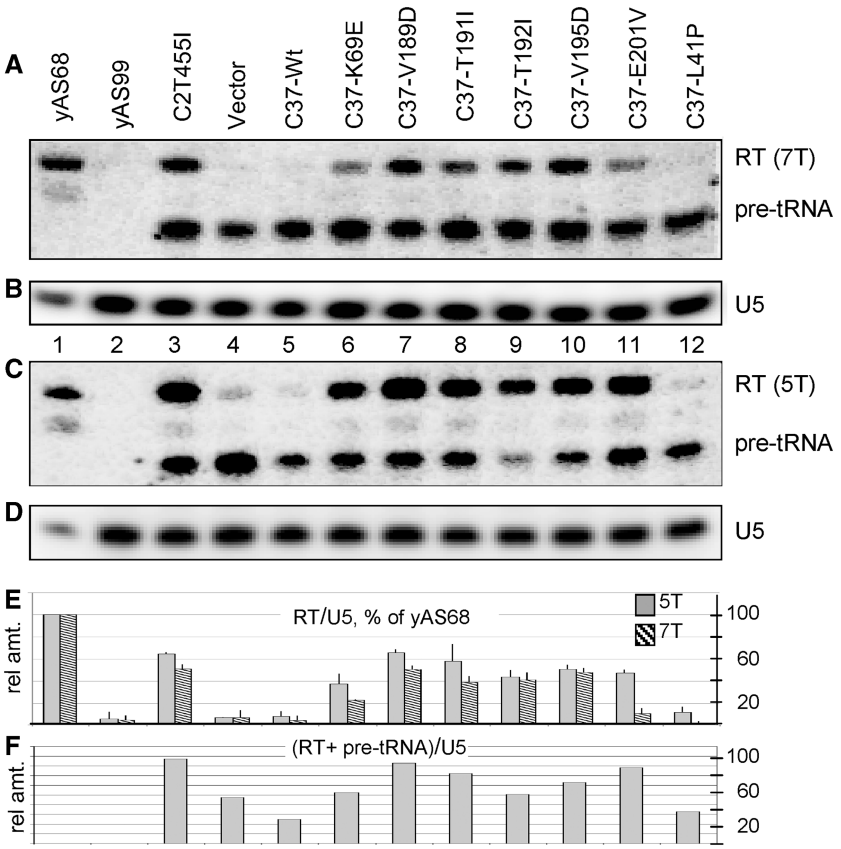

Figure 6. Assessment of termination mutants for transcription output of single tRNA genes by northern blotting. RNAs isolated from mutants and controls; the same blot was probed sequentially (after stripping previous probe, not shown). Lanes 1 and 2: RNA from positive and negative control strains (see text); lanes 3–12: RNA from yYH1 which carries a tRNA reporter gene with a 7T terminator, and the various mutants indicated above the lanes. (**A**) The blot was probed for the intron in the pre-tRNA reporter which detects nascent pre-tRNA as well as readthrough transcripts. (**B**) Probed for U5 RNA synthesized by pol II, as loading control. (**C** and **D**) The same as (A) and (B) except the reporter pre-tRNA gene had a 5T terminator. (**E**) Quantitation of readthrough (RT) transcripts calibrated to yAS68 (3T terminator) after correction for loading by U5 RNA, units are indicated to the right. Filled gray bars reflect the 5T and cross-hatched bars reflect the 7T terminator data in (C) and (A) respectively. (**F**) Quantitation of (RT + pre-tRNA)/U5 from (C and D).

mutants (Figure 5G). For example, C37–V189D (lane 7) produced more readthrough of $tRNA^{Lys}CUU$ than any other C37 mutant (Figure 5D) but with no significant decrease in the nascent pre-$tRNA^{Lys}CUU$ (Figure 5G) relative to the other C37 mutants, the vector and C37-WT (compare lanes 4, 5 and 7).

We next examined both the naturally terminated nascent pre-tRNA from a 7T terminator and the readthrough transcript that results at a downstream 8T terminator, with a probe that simultaneously detects both on the same blot (Figure 6A), followed by separate probing of the blot for U5 as loading control (Figure 6B). Again, we found no consistent variation in the amounts of readthrough and nascent pre-tRNA in the readthrough mutants when compared with C37-WT and vector controls (Figure 6A). The *rpc2-mutant* C2-T455I produced a relatively large amount of readthrough but this was not accompanied by decrease in the pre-tRNA as compared with C37-WT and vector controls (lanes 4 and 5) or C-37-E201V which showed less readthrough (lane 11). C37-V195D produced a relatively large amount of readthrough transcript (lane 10) that was not accompanied by decrease in pre-tRNA as compared to the controls (lanes 4 and 5). Likewise, low readthrough levels in C37–E201V (lane 11) was not accompanied by more nascent pre-tRNA.

We next wanted to test whether the ratio of readthrough transcript to primary nascent pre-tRNA transcript would increase as termination deficiency decreases as would be expected from a 5T versus a 7T terminator. Figure 6A shows transcripts from a tRNA gene 7T terminator, and Figure 6C from the same gene with a 5T terminator. This revealed a higher ratio of RT to pre-tRNA in Figure 6C relative to 6A, confirming an expected increase in RT transcript at the expense of nascent pre-tRNA as termination efficiency decreases. Greater readthrough of 5T was also apparent by quantification after control for loading by U5 RNA (Figure 6E). These data confirmed that our northern blotting approach reassuringly reflected quantitative differences in termination efficiency for the 7T versus 5T terminator.

Surprisingly, quantitation of triplicate samples for total transcripts (RT + pre-tRNA) revealed more in some of the terminator readthrough mutants than in the controls after correction by U5 (Figure 6F) (see below and the 'Discussion' section). Thus, we readily observe relative

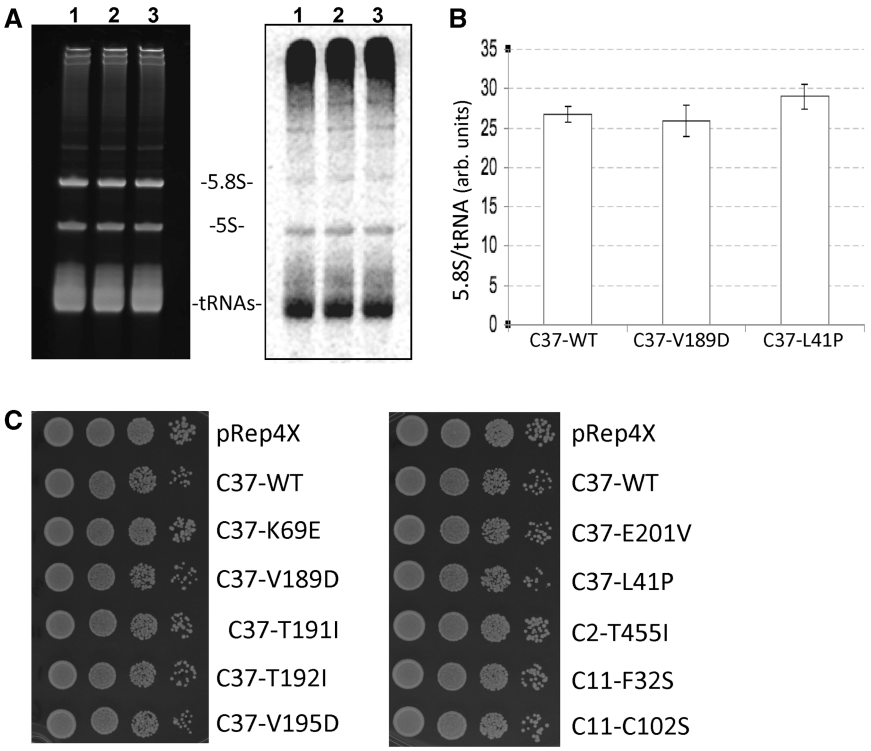

Figure 7. Characterization of C37 termination mutants by metabolic labeling of tRNA and growth in minimal media. (**A**) An equal number of logarithmically growing cells were incubated for 5.0 minutes in ^{14}C-uridine, washed and RNA was purified. Left panel shows ethidium bromide-stained denaturing 6% polyacrylamide gel; the right panel shows autoradiogram of the same gel. Lanes 1, 2 and 3 are C37-WT, -V189D and -L41P, respectively. (**B**) Quantification of duplicate gels as in (A); levels show arbitrary levels (*Y* axis) of the ratio of ^{14}C in 5.8S to tRNA newly synthesized RNA for the three strains shown A. (**C**) Serial dilution spot assays to compare growth in minimal media of the various mutant strains indicated.

increase in RT transcript as termination deficiency decreases from a 7T versus 5T terminator as expected on a single tRNA gene, but no decrease in total transcription output in the termination mutants.

^{14}C-Uridine labeling reveals no decrease in tRNA synthesis or growth on minimal media in the pol III termination mutants

Others have used metabolic labeling to demonstrate impaired tRNA synthesis in mutants (57). We incubated logarithmically growing cells for 5 minutes in ^{14}C-uridine to label newly synthesized tRNA. No significant difference between WT and C37 mutants was found (Figure 7A). Quantification of duplicate experiments revealed no significant deficiency of tRNA synthesis in the C37 mutants (Figure 7B). We also observed no significant difference after pulse chase labeling (not shown).

To further characterize the mutants, we examined them for growth on minimal media. The C37 mutants exhibited no significant growth deficiency relative to WT (Figure 7C).

DISCUSSION

We employed an *in vivo* screen that uses a functional suppressor-tRNA to produce a biosynthetic colorimetric phenotype if pol III fails to terminate properly. In this system, pol III initiates correctly but fails to terminate at a normally positioned terminator at the 3′-end of the first tRNA of a dimeric tRNA gene and thereby transcribes the downstream suppressor tRNA. A library of randomly mutagenized C37 screened for mutants defective in termination yielded strong (white) and weak (pink) phenotype mutants. The mutants were then examined for readthrough phenotype in a more stringent terminator test strain, yJI1, which not only confirmed their defects but also helped reflect the relative strengths of their termination deficiencies.

Readthrough (RT) transcripts from a 5T terminator were readily detected at levels that generally correlated with the relative suppression phenotypes of the C37 mutants. Our northern data also showed that as expected, the mutants produced more RT transcripts from a 5T than a 7T terminator, as reflected both by the ratio of RT to nascent pre-tRNA and the ratio of RT to U5 snRNA, a pol II transcript. The cumulative data using two dimeric tRNA gene reporters with 5T and 6T terminators as well as monomeric tRNA gene reporters with 5T and 7T terminators solidly support the expectation that the longer T tracts are stronger terminators than the shorter, noteworthy here because this validates the system and thereby suggests that it also reflects the relative termination deficiencies of the mutants.

Two classes of C37 mutants reflect two pol III termination-related activities

Twenty-seven of the 32 single point substitution mutants of C37 were clustered in a short span comprising residues

189–201, and most of these showed strong readthrough phenotypes with significant amounts of readthrough transcripts. Amino acids in this region, which is conserved in yeasts, were recently found to reside in the active center of pol III near the catalytic site. Although a conserved feature of the downstream part of this region is an array of acidic side chains, only one of these was found mutated in the single mutants, at position 201. Eighteen mutations were found at the threonines at positions 191 and 192, replaced by multiple other side chains, including serine in one case. An additional two mutants were at position 189 which is analogous to the Thr-228 residue that was physically mapped to the active center of *S. cerevisiae* pol III (11). The large number of point mutants with robust phenotypes that concentrated in this region together with the previous physical mapping (11) provide strong evidence that this is the region of C37 that mediates its major effects on termination. In addition, the relative paucity of termination mutants found in C53 (this report) provide compelling evidence to suggest that the observed termination effect that is attributed to the C53/37 heterodimer (12) have been narrowed down to the 189–201 region of C37.

Although residues M214 and S215 of *S. cerevisiae* C37 were found to crosslink to the transcription initiation factor, Bdp1, we did not obtain any single mutants in the analogous *S. pombe* C37 residues, V178 and T179. Of the 189 mutations recovered in all of our mutants, only two (1%) were in these, V178A and T179I. Presumably this paucity reflects that as expected, mutations that impair initiation are not likely to appear in our mutants.

The second class of C37 mutants differ from the first in their lower numbers, weaker readthrough phenotypes and readthrough transcripts, and localization to the dimerization domains responsible for interaction with C53 (Figure 2F). These mutations presumably affect the stability with which the C37 subunit maintains contact with C53 and pol III. Indeed five of these Class 2 mutants had mutations that created premature stop codons that caused truncation of the C-terminal tail of C37, similar in position to *S. cerevisiae* C37 truncation mutants that lead to loss from pol III of C53/37 as well as C11 and its RNA 3′-cleavage activity. Consistent with this, and in contrast to the class 1 mutants, these have strong phenotype in yYH1 attributable to longer 3′-oligo(U) length on their pre-tRNAs, consistent with deficiency of C11-mediated cleavage.

In vitro, WT pol III and pol IIIΔ exhibit stochastic termination as only a fraction of each terminates at the 5T terminator and a fraction reads through; the difference is that pol IIIΔ has greater propensity than pol III-WT to read through (12, also see Supplementary Data in 13, 31). Although the mutants' pols III characterized here exhibit different amounts of readthrough, a significant fraction of each ($\geq$50%) also terminates at the normal 5T terminator. This allowed examination of the mutants for suppression in the monomeric reporter strain yYH1. Increase in suppression in yYH1 results from either of two mechanisms that increase levels of mature suppressor tRNA: (i) lengthening of the terminal 3′-oligo(U) tract due to decreased RNA 3′-end cleavage which leads to better competition of the suppressor pre-tRNA for La-dependent maturation (8), and (ii) overall increase in suppressor pre-tRNA transcriptional output, as occurs with increased copy number of the suppressor tRNA gene or overexpression of Brf1 (38,58). We observed strong suppression phenotypes in yYH1 for two C-terminal truncation mutants that are similar to *S. cerevisiae* C37 truncation mutants that lead to loss of C11 and its RNA 3′-cleavage activity. Interestingly however, C37–L41P also exhibited strong suppression in yYH1, suggesting 3′-oligo(U) lengthening. We examined C37–L41P and C37–T191I for the 3′-oligo(U) length of their transcripts released from the functional 7T terminator in yYH1. This provided evidence for two classes of C37 mutants that differ in their relative phenotypes in the readthrough and 3′-oligo(U) length sensitive strains. This is significant because it shows that the C37 point mutants affect two different pol III activities, recognition of oligo(dT) as a pause/termination site and RNA 3′-cleavage. The different classes of C37 point mutants collectively exhibit both characteristics of pol IIIΔ, terminator readthrough and RNA 3′-cleavage deficiency although the latter is less severe than it is in pol IIIΔ.

We note that our findings here reflect only on pol III activities as occur *in vivo* and have not been complemented by biochemical analysis because at present we do not have a functional *S. pombe*-derived *in vitro* system that can be used for this purpose. As noted in the Results section there are advantages of obtaining results in cells that also have a chromosomal copy of WT C37. However, this approach also imposes limitations including the potential for a variable mixture of mutant and WT subunits in the pols III of different mutants.

Catalytic center sensitivity to C37 during pol III termination

A model suggests that upon sensing incoming oligo(dT) from a position on one of the jaws of pol III, C53/37 act analogously to brakes, causing deceleration and switch from a fast to a slow stepping mode conducive for termination, with C11 somehow inducing a conformational change in pol III required for facilitated reinitiation (5,10,12,29). Recent advances indicate that while the dimerization domains are in a peripheral position near the incoming DNA cleft, other parts of the C53/37 polypeptides extend to the active center (11,13). That the *S. pombe* C37 cluster region identified here corresponds to *S. cerevisiae* C37 in the active center suggests that the termination action of C37 reflected here is mediated through the pol III catalytic center (5).

Our data indicate that C37 affects terminator readthrough and RNA 3′-end lengthening. The relative levels of these in the mutants of three different pol III subunits C2, C11 and C37 is quite variable (9,59). These data argue that these activities are related to each other and elongation rate but occur by separate mechanics during termination. The C11 RNA 3′-cleavage mutants make this point well because despite their cleavage

deficiency no terminator readthrough was observed *in vitro or* in *vivo* (8,9). C37–T191I appears to be at the other extreme with strong terminator readthrough but less 3′-end shortening activity. Nonetheless, detectable perturbation of both of these activities in C37 mutants provide evidence that this subunit is intimately involved in function of the active center during termination. Two classes of mutants with different relative levels of readthrough and 3′-end shortening activities may reflect that C37 can affect active center function by two mechanisms.

A mutagenized C11 library produced a multitude of cleavage-deficient mutants whereas the same library yielded terminator-readthrough mutants at a much lower rate (8,9). By contrast, for C37 we obtained a much higher rate of readthrough mutants while independent screening of C37 in the RNA cleavage-sensitive strain, yYH1, yielded far fewer mutants and these were mostly C-terminal truncations similar to Q220-x (not shown). These observations lead to the idea that while the TFIIS-homologous domain of C11 is a direct and major determinant of intrinsic RNA cleavage during termination, C37 can affect this activity. By contrast, C37 would appear to be a major determinant of the ability of pol III to pause long enough at a terminator to allow transcript release, whereas the Rpb9-homologous domain of C11 can mediate this to a lesser extent, perhaps indirectly via effects on C37 (8,9).

Evidence of tRNA gene-specific effects of termination mutants

We also uncovered evidence for terminator-specific effects of the termination mutants (Figure 5D–F). This was observed in the differential pattern of RT transcripts from the four tRNA genes, we examined as can be appreciated by comparing the relative amounts of RT transcripts in lanes 3, 7 and 9 of Figure 5A, D, E and F). We note that the analysis of three endogenous tRNA genes for readthrough was confounded by multiple bands for each (Figure 5D–F). The multiplicity might be explained by either posttranscriptional processing to generate various intermediates, and/or successive readthrough of consecutive oligo(dT) tracts in downstream DNA, or a combination of these possibilities. In support of the latter, some of the natural terminators are followed by downstream oligo(dT) tracts of varying lengths, in some cases equal to the oligo(dT) length of the natural terminator. In support of the former is the ability of tRNase Z to process tRNA readthrough transcripts (in a context-dependent manner) (60–62).

Recently, Wu *et al.* (11) showed that deletion of a short internal tract in the C-terminal region of *S. cerevisiae* C37 caused terminator readthrough *in vitro*. In that case ~10% readthrough of the SUP4 tRNA T1 terminator was observed for C37 mutants deleted of a 5 amino acid internal tract or 27 amino acid truncation, *S. cerevisiae* C37 Δ(226–230) and Δ(256–282). The work here is to be distinguished from the previous study as our mutants were isolated from a random library as opposed to site directed, occurred as single substitutions, and were characterized *in vivo* both for terminator readthrough of different length terminators and for 3′-oligo(U) shortening-activity at a normal 7T terminator. It is remarkable that our cluster of mutants of which C37–V189D caused ~40 and ~22% readthrough of 5T and 7T terminators, respectively (Figure 6E), overlap the *S. cerevisiae* C37 226–230 region (Figure 2F).

Additional novel aspects of our work are that mutants pol III with substantial propensity for readthrough of even a strong, i.e. 7T, terminator produce nascent transcripts with relatively short oligo(U) tracts, and do not decrease overall transcription despite clear increases in readthrough transcription of the natural terminator. We believe that it is significant that the C37–T191I mutant has short 3′-oligo(U) tracts similar in length to WT. This was unexpected if 3′-oligo(U) length reflects elongation into the oligo(dT) tract since C37–T191I is expected to be faster than WT and therefore more likely to extend farther into the 7T terminator. That the C37–T191I pol III mutant exhibits shorter 3′-oligo(U) tracts than the RNA 3′-cleavage-deficient C11–C102S mutant suggests that it has not lost its functional C11 subunit. This is consistent with the findings of Wu *et al.* who found that a five residue deletion of *S. cerevisiae* C37 that includes the amino acid that corresponds to T191 maintained stable association with pol III (11). C37–T191I produces nascent transcripts with 3′-oligo(U) tracts that are significantly shorter than the 7T terminator from which they were released. This suggests that normal termination including transcript release involves 3′-end cleavage as occurs with polymerase retraction or backtracking (63). The cumulative data suggest that terminator pausing and retraction reflect distinguishable components of a multistep process of termination, and that the mutants characterized here probably change the balance among pausing, retraction, elongation and transcript release (63).

How does pol III termination affect overall transcription output?

A significant finding of this study was that pol III terminator readthrough was not associated with apparent decrease in overall pol III transcription in the mutants. In principle, this is not surprising because significant decrease in transcription output would likely be associated with growth deficiency and such mutants would not have been selected by our screen. Indeed, the mutants show no growth deficiency in minimal media (Figure 7C). It may be argued that the types of mutations in the C37 189–201 cluster region and C2–T455I, which appear to affect the catalytic center, may not be of the type that would decrease overall transcription according to a facilitated recycling model. However, the possibility that other types of termination mutants might do so, remains open, e.g. ones that alter conformational changes in the pol III jaw or a B-reader like region. If so, the mutants characterized here would reflect uniquely on a distinct aspect of termination. An alternative reconciliation might be that although the C37 and C2 mutants do

indeed exhibit termination deficiency, they nonetheless do terminate at a strong downstream terminator, producing distinct readthrough transcript bands; maybe it is the downstream termination event that resets pol III for facilitated recycling. Still other rationales may account for why our mutants show no apparent decrease in overall transcription output. Nonetheless, it must also be noted that if two processes are mechanistically linked and dependent on each other, as has become accepted for termination and recycling, it may be expected that mutations that reduce the efficiency of one process will decrease the dependent process. Yet, by all accounts, the termination mutants characterized here that do indeed reduce termination efficiency show no overall transcription output by pol III. The data reported here, which are the first to look for a link between termination and overall transcription efficiency *in vivo* do not support the observed *in vitro* phenomenon of facilitated reinitiation (26,27). Although termination-dependent facilitated recycling may occur *in vivo,* this pathway may not be rate limiting under the conditions we have grown the yeast. We suspect that reinitiation can occur by more than one pathway.

Quantitation of nascent intron-containing pol III transcripts showed that some of the termination mutants had increased their pol III transcription output from the suppressor-tRNA gene (Figure 6F). A more direct and global assessment was performed by a 5.0 minute metabolic labeling of tRNA by ^{14}C-uridine, which showed no difference between the mutants and WT (Figure 7A and B). A potential explanation for the discrepancy is that the readthrough transcripts observed by northern blot may not be efficiently converted to the mature tRNA observed by metabolic labeling. Another possibility is that the mutants exhibit tRNA gene-specific effects and that the suppressor-tRNA is an exception in its increased output relative to most other tRNA genes. This may be relevant since it was recently reported that terminator strength can be a strong determinant of pol III occupancy on tRNA genes (64). In any case, the mutants showed no significant decrease in pol III output despite readily observable significant decrease in termination efficiency. These findings suggest that current models of pol III transcription termination and its role in transcription rate *in vivo* according to a termination-dependent facilitated recycling model, be re-evaluated.

As noted in the Introduction, factors reported to promote pol III termination and recycling are La, NF1, topoisomerase I, PC4 and its homolog, Sub1p (18–20,22,25). It will be interesting to determine if the levels of overall pol III transcription in the termination mutants characterized here will be affected by compromising them for the *S. pombe* homologs of these factors.

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REFERENCES

1. Roeder,R.G. and Rutter,W.J. (1969) Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature*, **224**, 234–237.
2. Haag,J.R. and Pikaard,C.S. (2011) Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. *Nat. Rev. Mol. Cell Biol.*, **12**, 483–492.
3. Carter,R. and Drouin,G. (2010) The increase in the number of subunits in eukaryotic RNA polymerase III relative to RNA polymerase II is due to the permanent recruitment of general transcription factors. *Mol. Biol. Evol.*, **27**, 1035–1043.
4. Geiger,S.R., Lorenzen,K., Schreieck,A., Hanecker,P., Kostrewa,D., Heck,A.J. and Cramer,P. (2010) RNA polymerase I contains a TFIIF-related DNA-binding subcomplex. *Mol. Cell*, **39**, 583–594.
5. Fernandez-Tornero,C., Bottcher,B., Rashid,U.J., Steuerwald,U., Florchinger,B., Devos,D.P., Lindner,D. and Muller,C.W. (2010) Conformational flexibility of RNA polymerase III during transcriptional elongation. *EMBO J.*, **29**, 3762–3772.
6. Vannini,A., Ringel,R., Kusser,A.G., Berninghausen,O., Kassavetis,G.A. and Cramer,P. (2010) Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell*, **143**, 59–70.
7. Chedin,S., Riva,M., Schultz,P., Sentenac,A. and Carles,C. (1998) The RNA cleavage activity of RNA polymerase III is mediated by an essential TFIIS-like subunit and is important for transcription termination. *Genes Dev.*, **12**, 3857–3871.
8. Huang,Y., Intine,R.V., Mozlin,A., Hasson,S. and Maraia,R.J. (2005) Mutations in the RNA polymerase III subunit Rpc11p that decrease RNA 3′ cleavage activity increase 3′-terminal oligo(U) length and La-dependent tRNA processing. *Mol. Cell Biol.*, **25**, 621–636.
9. Iben,J.R., Mazeika,J.K., Hasson,S., Rijal,K., Arimbasseri,A.G., Russo,A.N. and Maraia,R.J. (2011) Point mutations in the Rpb9-homologous domain of Rpc11 that impair transcription termination by RNA polymerase III. *Nucleic Acids Res.*, **39**, 6100–6113.
10. Fernandez-Tornero,C., Bottcher,B., Riva,M., Carles,C., Steuerwald,U., Ruigrok,R.W., Sentenac,A., Muller,C.W. and Schoehn,G. (2007) Insights into transcription initiation and termination from the electron microscopy structure of yeast RNA polymerase III. *Mol. Cell*, **25**, 813–823.
11. Wu,C.C., Lin,Y.C. and Chen,H.T. (2011) The TFIIF-like Rpc37/53 dimer lies at the center of a protein network to connect TFIIIC, Bdp1, and the RNA polymerase III active center. *Mol. Cell Biol.*, **31**, 2715–2728.
12. Landrieux,E., Alic,N., Ducrot,C., Acker,J., Riva,M. and Carles,C. (2006) A subcomplex of RNA polymerase III subunits involved in transcription termination and reinitiation. *EMBO J.*, **25**, 118–128.
13. Kassavetis,G.A., Prakash,P. and Shim,E. (2010) The C53/C37 subcomplex of RNA polymerase III lies near the active site and participates in promoter opening. *J. Biol. Chem.*, **285**, 2695–2706.
14. White,R.J. (2005) RNA polymerases I and III, growth control and cancer. *Nat. Rev. Mol. Cell Biol.*, **6**, 69–78.

15. Geiduschek,E.P. and Kassavetis,G.A. (2006) Transcription: adjusting to adversity by regulating RNA polymerase. *Curr. Biol.*, **16**, R849–851.
16. Willis,I.M. and Moir,R.D. (2007) Integration of nutritional and stress signaling pathways by Maf1. *Trends Biochem. Sci.*, **32**, 51–53.
17. Marshall,L., Goodfellow,S.J. and White,R.J. (2007) Diminished activity of RNA polymerase III selectively disrupts tissues with the most actively dividing cells. *PLoS Biol.*, **5**, e286.
18. Wang,Z., Bai,L., Hsieh,Y. and Roeder,R.G. (2000) Nuclear factor 1 (NF1) affects accurate termination and multiple-round transcription by human RNA polymerase III. *EMBO J.*, **19**, 6823–6832.
19. Wang,Z. and Roeder,R.G. (1998) DNA topoisomerase I and PC4 can interact with human TFIIIC to promote both accurate termination and transcription reinitiation by RNA polymerase III. *Mol. Cell*, **1**, 749–757.
20. Fan,H., Sakulich,A.L., Goodier,J.L., Zhang,X., Qin,J. and Maraia,R.J. (1997) Phosphorylation of the human La antigen on serine 366 can regulate recycling of RNA polymerase III transcription complexes. *Cell*, **88**, 707–715.
21. Maraia,R.J. (1996) Transcription termination factor La is also an initiation factor for RNA polymerase III. *Proc. Natl Acad. Sci. USA*, **93**, 3383–3387.
22. Gottlieb,E. and Steitz,J.A. (1989) The RNA binding protein La influences both the accuracy and the efficiency of RNA polymerase III transcription in vitro. *EMBO J.*, **8**, 841–850.
23. Gottlieb,E. and Steitz,J.A. (1989) Function of the mammalian La protein: evidence for its action in transcription termination by RNA polymerase III. *EMBO J.*, **8**, 851–861.
24. Schramm,L. and Hernandez,N. (2002) Recruitment of RNA polymerase III to its target promoters. *Genes Dev.*, **16**, 2593–2620.
25. Tavenet,A., Suleau,A., Dubreuil,G., Ferrari,R., Ducrot,C., Michaut,M., Aude,J.C., Dieci,G., Lefebvre,O., Conesa,C. *et al.* (2009) Genome-wide location analysis reveals a role for Sub1 in RNA polymerase III transcription. *Proc. Natl Acad. Sci. USA*, **106**, 14265–14270.
26. Dieci,G. and Sentenac,A. (1996) Facilitated recycling by RNA polymerase III. *Cell*, **84**, 245–252.
27. Maraia,R.J., Kenan,D.J. and Keene,J.D. (1994) Eukaryotic transcription termination factor La mediates transcript release and facilitates reinitiation by RNA polymerase III. *Mol. Cell. Biol.*, **14**, 2147–2158.
28. Cabart,P., Lee,J. and Willis,I.M. (2008) Facilitated recycling protects human RNA polymerase III from repression by Maf1 in vitro. *J. Biol. Chem.*, **283**, 36108–36117.
29. Richard,P. and Manley,J.L. (2009) Transcription termination by nuclear RNA polymerases. *Genes Dev.*, **23**, 1247–1269.
30. Campbell,F.E. Jr and Setzer,D.R. (1992) Transcription termination by RNA polymerase III: uncoupling of polymerase release from termination signal recognition. *Mol. Cell. Biol.*, **12**, 2260–2272.
31. Chedin,S., Ferri,M.L., Peyroche,G., Andrau,J.C., Jourdain,S., Lefebvre,O., Werner,M., Carles,C. and Sentenac,A. (1998) The yeast RNA polymerase III transcription machinery: a paradigm for eukaryotic gene activation. *Cold Spring Harb. Symp. Quant. Biol.*, **63**, 381–389.
32. Hagler,J., Luo,Y. and Shuman,S. (1994) Factor-dependent transcription termination by vaccinia RNA polymerase. Kinetic coupling and requirement for ATP hydrolysis. *J. Biol. Chem.*, **269**, 10050–10060.
33. Burns,C.M. and Richardson,J.P. (1995) NusG is required to overcome a kinetic limitation to Rho function at an intragenic terminator. *Proc. Natl Acad. Sci. USA.*, **92**, 4738–4742.
34. Burns,C.M., Richardson,L.V. and Richardson,J.P. (1998) Combinatorial effects of NusA and NusG on transcription elongation and Rho-dependent termination in *Escherichia coli*. *J. Mol. Biol.*, **278**, 307–316.
35. Jin,D.J., Burgess,R.R., Richardson,J.P. and Gross,C.A. (1992) Termination efficiency at rho-dependent terminators depends on kinetic coupling between RNA polymerase and rho. *Proc. Natl Acad. Sci. USA*, **89**, 1453–1457.
36. Intine,R.V.A., Sakulich,A.L., Koduru,S.B., Huang,Y., Pierstorrf,E., Goodier,J.L., Phan,L. and Maraia,R.J. (2000) Control of transfer RNA maturation by phosphorylation of the human La antigen on serine 366. *Mol. Cell.*, **6**, 339–348.
37. Huang,Y., Bayfield,M.A., Intine,R.V. and Maraia,R.J. (2006) Separate RNA-binding surfaces on the multifunctional La protein mediate distinguishable activities in tRNA maturation. *Nat. Struct. Mol. Biol.*, **13**, 611–618.
38. Iben,J.R., Epstein,J.A., Bayfield,M.A., Bruinsma,M.W., Hasson,S., Bacikova,D., Ahmad,D., Rockwell,D., Kittler,E.L., Zapp,M.L. *et al.* (2011) Comparative whole genome sequencing reveals phenotypic tRNA gene duplication in spontaneous *Schizosaccharomyces pombe* La mutants. *Nucleic Acids Res.*, **39**, 4728–4742.
39. Hamada,M., Sakulich,A.L., Koduru,S.B. and Maraia,R. (2000) Transcription termination by RNA polymerase III in fission yeast: a genetic and biochemical model system. *J. Biol. Chem.*, **275**, 29076–29081.
40. Maraia,R.J. and Lamichhane,T.N. (2011) 3′ processing of eukaryotic precursor tRNAs. *Wiley Interdiscip. Rev. RNA.*, **2**, 362–375.
41. Iben,J.R. and Maraia,R.J. (2012) Yeast tRNAomics: tRNA gene copy number variation and codon use provide bioinformatics evidence of a new wobble pair in a eukaryote. *RNA*, **18**, 1358–1372.
42. Zaccolo,M., Williams,D.M., Brown,D.M. and Gherardi,E. (1996) An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. *J. Mol. Biol.*, **255**, 589–603.
43. Maundrell,K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*, **123**, 127–130.
44. Mao,J., Schmidt,O. and Soll,D. (1980) Dimeric transfer RNA precursors in *S. pombe*. *Cell*, **21**, 509–516.
45. Schmidt,O., Mao,J., Ogden,R., Beckmann,J., Sakano,H., Abelson,J. and Soll,D. (1980) Dimeric tRNA precursors in yeast. *Nature*, **287**, 750–752.
46. Orioli,A., Pascali,C., Quartararo,J., Diebel,K.W., Praz,V., Romascano,D., Percudani,R., van Dyk,L.F., Hernandez,N., Teichmann,M. *et al.* (2011) Widespread occurrence of non-canonical transcription termination by human RNA polymerase III. *Nucleic Acids Res.*, **39**, 5499–5512.
47. Ferrari,R., Rivetti,C., Acker,J. and Dieci,G. (2004) Distinct roles of transcription factors TFIIIB and TFIIIC in RNA polymerase III transcription reinitiation. *Proc. Natl Acad. Sci. USA*, **101**, 13442–13447.
48. Cormack,B.P. and Struhl,K. (1992) The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *Cell*, **69**, 685–696.
49. Upadhya,R., Lee,J. and Willis,I.M. (2002) Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol. Cell*, **10**, 1489–1494.
50. Reina,J.H., Azzouz,T.N. and Hernandez,N. (2006) Maf1, a new player in the regulation of human RNA polymerase III transcription. *PLoS ONE*, **1**, e134.
51. Karkusiewicz,I., Turowski,T.W., Graczyk,D., Towpik,J., Dhungel,N., Hopper,A.K. and Boguta,M. (2011) Maf1, repressor of RNA polymerase III, indirectly affects tRNA processing. *J. Biol. Chem.*, **286**, 39478–39488.
52. Van Horn,D.J., Yoo,C.J., Xue,D., Shi,H. and Wolin,S.L. (1997) The La protein in *Schizosaccharomyces pombe*: a conserved yet dispensable phosphoprotein that functions in tRNA maturation. *RNA*, **3**, 1434–1443.
53. Intine,R.V., Dundr,M., Misteli,T. and Maraia,R.J. (2002) Aberrant nuclear trafficking of La protein leads to disordered processing of associated precursor tRNAs. *Mol. Cell*, **9**, 1113–1123.
54. Bayfield,M.A., Kaiser,T.E., Intine,R.V. and Maraia,R.J. (2007) Conservation of a masked nuclear export activity of La proteins and its effects on tRNA maturation. *Mol. Cell. Biol.*, **27**, 3303–3312.
55. Bayfield,M.A. and Maraia,R.J. (2009) Precursor-product discrimination by La protein during tRNA metabolism. *Nat. Struct. Mol. Biol.*, **16**, 430–437.
56. Cherkasova,V., Bahler,J., Bacikova,D., Pridham,K. and Maraia,R.J. (2011) Altered nuclear tRNA metabolism in La-deleted *S. pombe* is accompanied by a nutritional stress

response involving Atf1p and Pcr1p that is suppressible by Xpo-t/Los1p. *Mol. Biol. Cell*, **23**, 480–491.
57. Lefebvre,O., Ruth,J. and Sentenac,A. (1994) A mutation in the largest subunit of yeast TFIIIC affects tRNA and 5 S RNA synthesis. Identification of two classes of suppressors. *J. Biol. Chem.*, **269**, 23374–23381.
58. Huang,Y., McGillicuddy,E., Weindel,M., Dong,S. and Maraia,R. (2003) The fission yeast TFIIB-related factor limits RNA polymerase III to a TATA-dependent pathway of TBP recruitment. *Nucleic Acids Res.*, **31**, 2108–2116.
59. Bobkova,E.V., Habib,N., Alexander,G. and Hall,B.D. (1999) Mutational analysis of the hydrolytic activity of yeast RNA polymerase III. *J. Biol. Chem.*, **274**, 21342–21348.
60. Adeniyi-Jones,S., Romeo,P.H. and Zasloff,M. (1984) Generation of long read-through transcripts in vivo and in vitro by deletion of 3′ termination and processing sequences in the human $tRNAi^{met}$ gene. *Nucleic Acids Res.*, **12**, 1101–1115.
61. Allison,D.S. and Hall,B.D. (1985) Effects of alterations in the 3′ flanking sequences on *in vivo* and *in vitro* expression of the yeast *SUP4*-o $tRNA^{Tyr}$ gene. *EMBO J.*, **4**, 2657–2664.
62. Haussecker,D., Huang,Y., Lau,A., Parameswaran,P., Fire,A.Z. and Kay,M.A. (2010) Human tRNA-derived small RNAs in the global regulation of RNA silencing. *RNA.*, **16**, 673–695.
63. Nudler,E. (2012) RNA polymerase backtracking in gene regulation and genome instability. *Cell*, **149**, 1438–1445.
64. Canella,D., Bernasconi,D., Gilardi,F., Lemartelot,G., Migliavacca,E., Praz,V., Cousin,P., Delorenzi,M. and Hernandez,N. (2012) A multiplicity of factors contributes to selective RNA polymerase III occupancy of a subset of RNA polymerase III genes in mouse liver. *Genome Res.*, **22**, 666–680.