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Use of an *in Vivo* Reporter Assay to Test for Transcriptional and Translational Fidelity in Yeast*

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Eukaryotic RNA polymerase II and *Escherichia coli* RNA polymerase possess an intrinsic ribonuclease activity that is stimulated by the polymerase-binding proteins SII and GreB, respectively. This factor-activated hydrolysis of nascent RNA has been postulated to be involved in transcription elongation as well as removal of incorrect bases misincorporated into RNA. Little is known about the frequency of misincorporation by RNA polymerases *in vivo* or about the mechanisms involved in improving RNA polymerase accuracy. Here we have developed a luciferase reporter system in an effort to assay for base misincorporation in living *Saccharomyces cerevisiae*. The assay employs a luciferase open reading frame that contains a premature stop codon. The inactive truncated enzyme would become active if misincorporation by RNA polymerase II took place at the stop triplet. Yeast lacking SII did not display a significant change in reporter activity when compared with wild-type cells. We estimate that under our assay conditions, mRNAs with a misincorporation at the test site could not exceed 1 transcript per 500 cells. The reporter assay was very effective in detecting the previously described process of nonsense suppression (translational read-through) by ribosomes, making it difficult to determine an absolute level of basal (SII-independent) misincorporation by RNA polymerase II. Although these data cannot exclude the possibility that SII is involved in proofreading, they make it unlikely that such a contribution is physiologically significant, especially relative to the high frequency of translational errors.

Many DNA polymerases contain a nuclease activity that allows them to excise, from newly replicated DNA, bases that are misincorporated with respect to Watson-Crick base pairing rules. With the recognition that many, if not all, multisubunit DNA-dependent RNA polymerases contain a nuclease activity that operates on nascent RNA, came the suggestion that RNA polymerases proofread RNA misincorporation events using this activity (1–3). For bacterial RNA polymerase, this nuclease activity is stimulated by small RNA polymerase-binding proteins called GreA and GreB (4). A similar activity in eukaryotic RNA polymerases is stimulated by a small RNA polymerase II-binding protein called SII (also known as TFIIS) that has been found in all eukaryotes so far investigated. The *greA* and *greB* genes are not essential for *Escherichia coli*; nor is the SII-encoding gene essential for *Saccharomyces cerevisiae* (5, 6).

*In vitro*, these proteins can reactivate RNA polymerase enzymes that lapse into an elongation-incompetent form (7). They do so by activating cleavage of the nascent RNA chain by RNA polymerase. The vast majority of nascent RNA is assembled according to strict Watson-Crick base pairing rules. Hence, these proteins have been considered transcription elongation factors. *In vivo* evidence consistent with this role has been described (8–14).

*In vitro*, misincorporation of nucleotides into RNA by RNA polymerase can be detected by experimental manipulations such as providing a high level of a nucleotide other than that called for by the DNA template. For example, GTP can be incorporated into RNA at a low frequency when poly(dA-dT)/poly(dA-dT) is used as a template for bacterial RNA polymerase (15, 16). *In vitro*, RNA polymerase II poised at a specific template thymine has been shown to incorporate a G residue in lieu of A when ATP is absent (2). Similarly, *E. coli* RNA polymerase will incorporate C instead of U when UTP is absent (17). Both polymerases have been shown to misincorporate a U instead of C on a synthetic template (18). GreA, GreB, and SII stimulate the cleavage of nascent RNA containing misincorporated bases (2, 17, 18). Thus, it has been suggested that these factors could assist in the fidelity of transcription by activating RNA polymerase to excise these misincorporated bases (2, 18). However, there is no direct evidence that RNA polymerases employ this factor-activated nuclease activity *in vivo*. The misincorporation rate of RNA polymerase *in vivo* has not been accurately measured in eukaryotic cells. A frequency of $10^{-5}$ has been estimated for misincorporation in *E. coli*; however, the rate varies as a function of the nucleotide substituted, the identity of the template base, the divalent cation, and presumably, sequence context around the substitution (15, 16, 19).

Neither the biological sequelae of misincorporation nor the extent to which nucleic acid-stimulating factors participate in proofreading are well understood, particularly in eukaryotes.

Errors in protein synthesis have been described and estimated previously in bacteria and yeast using reporter systems (19–22). Parameters that influence translational fidelity include genetic background, antibiotics, and epigenetic states. Typically, the fidelity of translation has been considered less stringent than that of transcription (19, 23–25).

In an effort to examine the contribution of transcriptional and translational errors and SII's potential role in proofreading, we have designed a reporter system to detect misincorporation events mediated by RNA polymerase II at an artificial stop codon engineered into a plasmid introduced into yeast. Using yeast strains with a deletion or a disruption of the SII-encoding gene (*DST1*), we were unable to find evidence that this cleavage-activating factor participates in proofreading. On the other hand, translational read-through of stop codons was readily detected using pharmacological and genetic approaches.

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and could be readily measured. Rates of SII-independent misincorporation by RNA polymerase appear to be small relative to the higher rates of translational errors such as stop codon bypass.

MATERIALS AND METHODS

Plasmids and Strains—The plasmid pGAL-LUC has been described previously (26) and was obtained from A. Caplan (Mt. Sinai School of Medicine, New York). The sequence (...TACAAAGG...), encoding Lys⁴⁴⁵ and the flanking amino acids (Tyr upstream and Gly downstream), was changed to (...TCTAGGGA...) by site-directed mutagenesis (Gene Dynamics, LLC) to generate the plasmid pLuc-Stop. The mutagenesis generates a novel ArrII restriction site (underlined) that also changes the Tyr codon to Ser and the Lys codon to a stop codon. The stop codon in plc-Stop was then changed at a base at a to AAG, CAG, GAG, TCG, TTG, and TAA to generate the plasmids pGAL-LUC-AAG, pGAL-LUC, pLuc-Stop, pCAG-LUC, pGAG-LUC, pTCG-LUC, pTGG-LUC, and pTTG-LUC, respectively. The pLuc-Δ plasmid was made by religating pLuc-Stop that had been digested with ArrII and SacI after treating the linear DNA with T4 DNA polymerase. The plasmid p2X-Stop was generated by site-directed mutagenesis of the sequence around codon 445 in pLuc-Stop from (...TCC TAG GGA...) to (...TCC TAG GGA...)

The strains used in this study are described in Table I. Cells were transformed with plasmids by the lithium acetate/polyethylene glycol method (27). DY978, DY979, DY980, and DY981 were generated from Z96 (10) by transformation of this stop codon has been shown previously to destroy the translation of the 550-amino acid luciferase open reading frame with a 15-mer of a single oligonucleotide. The probe was labeled to a specific activity of ~10⁸ cpm/μg with Klenow DNA polymerase (Promega, Madison, WI), random hexamer primers (Invitrogen), and [α-³²P]dATP (Amersham Biosciences).

RESULTS

Rationale—We sought to establish an in vivo assay that would provide a positive readout of a transcriptional misincorporation event. S. cerevisiae was selected because of its recently well developed understanding of RNA polymerase II elongation from genetic, biochemical, and molecular biological analyses. Using transformation, we introduced a reporter plasmid into wild-type cells and cells deleted or disrupted for DST1, the gene that encodes transcription elongation factor SII. Yeast is also advantageous, since a large number of cells can be analyzed, and under inducing conditions the GAL1 promoter can be used to generate numerous reporter transcripts per cell, thereby optimizing the ability to detect a rare event. Firefly luciferase was chosen as a reporter, since the activity assay is simple to perform and has a high signal/noise ratio. The lacZ reporter, which has been used for similar purposes in bacteria, may be atypical with respect to transcription and particularly elongation in yeast cells (29–32).

We constructed a selectable centromeric plasmid (pLuc-Stop) that contained the inducible GAL1 promoter driving transcription of the 550-amino acid luciferase open reading frame with a premature stop codon replacing lysine 445 (Fig. 1). Introduction of this stop codon has been shown previously to destroy the protein’s activity, and it has been used as a reporter in E. coli to assess transcription on mismatch-containing DNA (33, 34). Nine different RNA polymerase II misincorporation events are possible at the introduction of UAG stop codon triplet: A, G, or C instead of U at position 1; U, C, or G instead of A in position 2; and A, C, or U instead of G at position 3 (Fig. 1). One of these would yield another stop codon (UAG → UAA). The others would result in transcripts that would lead to the substitution of Gln, Glu, Leu, Ser, Trp, or Tyr (two events) for Lys (Fig. 1).

One of the changes would restore a codon for lysine (AAG), the

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triplicate samples of 0.5 OD unit of cells were collected at the indicated
culture was treated with galactose (2% (w/v) final concentration), and

and

was

Assay—Cells from a saturated culture were diluted to an

° C for 2 h and

prehybridized for a minimum of 3 h at 42 °C in 5× SSC (1× SSC: 0.15
m NaCl, 0.015 sodium citrate), 5× Denhardt’s solution, 50% (v/v) form-

amid-1% (w/v) SDS, and 100 μg/ml salmon sperm DNA. Filters were

hybridized under the same conditions with 10⁻⁸ cpm of ³²P-labeled DNA probe for 15–18 h. The filters were washed twice at 22 °C in 2× SSC, 0.1% SDS for 5 min each and twice in 0.2× SSC, 0.1% SDS for 5 min each, followed by two 0.2× SSC, 0.1% SDS washes at 42 °C for 20 min each. The washed filters were exposed to X-Omat film and quan-
titated with a Fuji BAS1000 phosphorimaging system. The luciferase

probed for pGAL-LUC using the oligonucleotides 5′-TTCATCTTCAG

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Alternatively, the level of enzyme activity of the truncated luciferase polypeptide.
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misincorporation events would be required on the same tran-
scription to obtain functional full-length luciferase, an event
with a very low probability of occurrence. Cells containing either
of these plasmids expressed only background activity (Fig. 2A).

To test whether the truncated protein had residual enzyme
activity, two control plasmids were created. In one, the lucifer-
ase coding sequence downstream of the stop codon at position
445 was severely compromised in its ability to generate
luciferase, producing a level of activity ~300-fold lower
than Lys 445 after 30 min of galactose induction (Fig. 2A, com-
pare Lys with Stop; note broken y axis scale). Longer induction
times resulted in the additional accumulation of luciferase
exhibiting ~10^5 relative light units in a standard assay (data
not shown). Similar activity levels were measured in cells with
a plasmid containing the UAA stop codon (Fig. 2, UAA). Activity
from the pLuc-Stop plasmid could result from a residual
level of enzyme activity of the truncated luciferase polypeptide.
Alternatively, “leaky” read-through of the stop codon-containing
mRNA by the translation machinery and/or a basal level of
misincorporation by RNA polymerase II could yield full-length
active protein from the mutation-containing plasmid.

Luciferase Activity in Cells Harboring Reporter Plasmids—
Yeast cells harboring a reporter with an intact luciferase reading
frame were able to generate luciferase activity that was
inducible upon exposure to galactose (Lys, Fig. 2A) (26). A strain bearing
the plasmid encoding a UAG stop codon at position 445 was severely compromised in its ability to generate
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active protein from the mutation-containing plasmid.

Yeasts Deleted for the SII Gene Do Not Show Enhanced
Luciferase Activity from the pLuc-Stop Reporter—To test
whether SII plays a role in misincorporation, we transformed
the stop codon-containing luciferase reporter plasmid into a
strain of yeast deleted for the DST1 open reading frame. If SII
enhanced the fidelity of transcription, cells lacking it would
show an increased level of transcripts containing misincorporated
bases that should code for active luciferase. However, after a 3-h galactose induction, these cells generated luciferase
activity comparable with, or slightly less than, that seen for
cells expressing wild-type SII (Fig. 2B).

Cells lacking SII are defective in transcriptional induction of
a number of genes (10, 12, 35). To ensure that the luciferase
activity determinations were not biased by a difference in lu-
ciferase mRNA levels between the DST1 deletant and cells
wild-type for DST1, we measured the amount of transcript by
Northern blotting (data not shown). When luciferase activity

FIG. 1. Experimental design of the luciferase reporter assay. The potential products of misincorporation and the respective codons that would result are shown at the lower right.

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misincorporation by RNA polymerase II could yield full-length
active protein from the mutation-containing plasmid.

To test whether the truncated protein had residual enzyme
activity, two control plasmids were created. In one, the lucifer-
ase coding sequence downstream of the stop codon at position
445 was deleted (pLUC-Δ). This plasmid could only yield truncated
protein regardless of the efficiency of transcriptional or translational read-through. The second control plasmid (p2X-
Stop) had a second stop codon engineered in tandem with the
first. Thus, two sequential stop codon read-through events or
misincorporation events would be required on the same
transcript to obtain functional full-length luciferase, an event
with a very low probability of occurrence. Cells containing either
of these plasmids expressed only background activity (Fig. 2A,
Luc-Δ and 2× Stop). Western blotting showed that the amount
of each of the truncated proteins generated in cells carrying
pLuc-Δ or p2X-Stop was similar to that produced by cells with
full-length luciferase (data not shown). This suggests that
the majority of the enzyme activity generated by the pLuc-Stop
plasmid comes from translational read-through of the stop
codon or transcriptional misincorporation and not from resid-
ual activity of the truncated proteins.

To test if active luciferase can be produced by the possible
amino acid substitutions expected from transcriptional misin-
corporation (Fig. 1), we generated a family of plasmids with the
cognate single base mutations that change the stop codon to
Leu, Trp, Gln, Glu, or Ser. These were compared with lucifer-
ase encoding the natural lysine residue at position 445. Plas-
mids were introduced individually into wild-type yeast and the
level of inducible luciferase activity was measured after galac-
tose induction. The luciferase enzymes produced by these sub-
stitutions were as active as that containing lysine 445 (shown for
the Gln substitution in Fig. 2; data not shown for the other
substitutions). We conclude that active luciferase enzyme
can be produced by eight of the nine misincorporation events at the
DNA-encoded stop codon, and we should be able to score them
using this luciferase reporter system. (The ninth possible
change, which yields a UAA stop codon, would not be
informative.)

Figure 1: Experimental design of the luciferase reporter assay. The potential products of misincorporation and the respective codons that would result are shown at the lower right.
was normalized to the abundance of luciferase mRNA in each sample, the values were indistinguishable for SII-containing and SII-lacking strains (Fig. 2C).

Messenger RNAs containing a premature stop codon can be substrates for the nonsense-mediated mRNA decay pathway (36). To compare the mRNA degradation rates of the luciferase mRNAs bearing the stop codon, lysine codon, or tryptophan codon at position 445, we measured mRNA half-life by inhibiting transcription by the addition of glucose and by quantifying the mRNA decay rate. All of these mRNAs showed similar half-lives (25–30 min), indicating that differential mRNA turnover cannot account for differences in the yield of luciferase activity for mRNAs encoding wild-type, missense-containing, or prematurely truncated luciferases (Fig. 3).

From these results, we conclude that there is no evidence that SII affects misincorporation by RNA polymerase II, at least as assessed in this assay system.

Detection of Active Luciferase Enzyme by Induction of Translational Misreading—A crucial question is whether this assay would detect transcriptional misincorporation if it took place, especially if translational read-through of the stop codon takes place at high frequency. There are no known genetic changes that lead to an increase or decrease in misincorporation by RNA polymerase II that could serve as a positive control for an error-prone polymerase. Nor are there experimental perturbations proven to alter misincorporation by RNA polymerases in...
vivo. As an alternative test of the assay’s sensitivity, we resorted to the use of a drug that enhances the frequency of translational read-through of a stop codon. The antibiotic paromomycin has been shown to induce stop codon read-through in yeast as well as in other organisms (see Ref. 37). Wild-type cells harboring pLuc-Stop were induced with galactose to express luciferase and then treated with the antibiotic paromomycin or left untreated. A strong, time-dependent increase in luciferase activity was observed after treatment of wild-type cells with paromomycin compared with untreated cells (Fig. 4, ○). After 7.5 h, the paromomycin-treated cells showed a 6-fold increase in luciferase activity. Note as well that after this long induction period, untreated yeast with a disruption of DST1 again displayed luciferase activity comparable with wild-type cells with the pLuc-Stop plasmid (Fig. 4, ■). This latter result provides further confirmation of the data shown in Fig. 2B. Here, however, a different laboratory strain of yeast was used in which the DST1 gene was disrupted by a hisG cassette (10). The paromomycin experiment shows that the assay was able to detect the induction of full-length active luciferase from transcripts containing a stop codon at position 445.

Certain strains designated \( \Psi^+ \) show a prion phenotype in which the translational termination factor Sup35p becomes aggregated (reviewed in Refs. 38 and 39). Such strains demonstrate nonsense suppression in the absence of known suppressor mutations that is probably due to loss of translation terminating activity. Since our assay appears to be measuring translational read-through, we were interested in testing the effect of the \( \Psi^- \) phenotype upon translational read-through of the stop codon-containing luciferase reporter. pLuc-Stop was introduced into a known \( \Psi^- \) strain and a cognate \( \Psi^+ \) strain, the cells were induced with galactose, and cells were assayed for luciferase activity. The \( \Psi^- \) strain expressed 8–32-fold more enzyme activity than the \( \Psi^+ \) strain depending upon the time of galactose induction (Fig. 5). These results are an independent genetic confirmation of the pharmacological data of Fig. 4, showing that this assay is a sensitive measure of stop codon read-through. The magnitude of luciferase expression in the known \( \Psi^- \) strain was comparable with that found in DY2014, the strain used above to measure pLuc-Stop expression (Fig. 2A), suggesting that our analysis thus far has taken place in \( \Psi^- \) strains, although their \( \Psi^+ \) phenotype has not been otherwise tested. The plasmid encoding truncated luciferase (pLuc-Δ), which cannot yield full-length luciferase by either translational or transcriptional errors, was equivalently inactive in the \( \Psi^- \) and \( \Psi^+ \) strains (data not shown).

Sensitivity of the Luciferase Assay—To assess the sensitivity of the luciferase assay under the conditions employed here, we set up a titration curve using wild-type luciferase purified to near homogeneity. The assay was linear over 6 orders of magnitude of enzyme concentration (Fig. 6A). We could reliably measure the signal from 4 fg (39,000 molecules) of luciferase, which was greater than 2 S.D. values above background (Fig. 6A, solid line). To ensure that the activity of purified luciferase was not compromised after cell lysis by inhibitors in the extract, we performed a mixing experiment in which varying amounts of purified luciferase was added to a standard amount of cell extract made from yeast devoid of a luciferase gene (Fig. 6A, dashed line). Signal from the extract containing recombinant purified luciferase was comparable with, and even slightly higher on a per molecule basis than, purified protein alone. This indicates that the enzyme is stable in a yeast cell extract. From this standard curve, we determined that the relative light unit output per molecule of wild type luciferase is \( 8 \times 10^{-7} \). The basis of the use of this assay to score for SII-dependent misincorporation requires the detection of an increase in luciferase reporter activity over that detected in cells bearing the pLuc-Stop plasmid. To determine the sensitivity with which we could observe full-length luciferase against the pLuc-Stop signal, we repeated the mixing experiment, this time adding increasing amounts of purified recombinant luciferase to an extract from cells with pLuc-Stop plasmid that had been induced with galactose (Fig. 6B). From this graph, we conservatively (>11 S.D. values) estimate that we can detect \( 12 \times 10^6 \) molecules of wild-type luciferase over the basal luciferase activity of this extract (Fig. 6B, inset).

**DISCUSSION**

Our initial efforts at designing an in vivo transcriptional misincorporation assay in yeast provided no evidence for a role of SII in RNA polymerase II proofreading. Since this is negative
evidence, we cannot exclude the possibility that SII and the nuclease activity it activates are involved in transcriptional fidelity in vivo. The results, however, suggest that the SII-activated nuclease activity and misincorporation itself are not biologically robust processes in yeast and that they may be masked by a higher rate of translational errors.

A more direct measurement of misincorporation, such as nucleotide sequencing of cDNAs or RT-PCR products derived from cells containing or lacking SII, would be technically forbidding, since the best estimates of in vivo RNA polymerase misincorporation frequencies suggest that it is low \(\left(10^{-3}\right)\) (19, 23, 24, 40). Furthermore, base changes due to misincorporation by RNA polymerase II would have to be detectable against a comparable background of misincorporation by reverse transcriptase and DNA polymerase during reverse transcription and PCR. As a positive indicator of the efficacy of the assay presented here, we induced translational misreading of a reporter transcript in yeast using an antibiotic known to alter stop codon read-through. In prior work, paromomycin increased translation errors by \(20\)-fold at 200 \(\mu\)M in yeast (20).

Our ability to detect an effect of paromomycin in this reporter assay indicates that we can observe rate changes of this size. Similarly, experiments on yeast with a known nonsense suppression phenotype \((\Psi^-)\) support the idea that the assay is robust in its ability to detect nonsense codon read-through by the translation machinery.

We can use the data presented herein to make two estimates. The first is the maximal level of RNA polymerase misincorporation that would be required if the activity from pLuc-Stop in a DST1 cell is assumed to be entirely due to misincorporation by RNA polymerase II. The second is the maximal number of misincorporation-containing transcripts that would be detectable in a standard reaction.

Since luciferase truncated at codon 445 is inactive (Fig. 2A), we can estimate a maximal frequency of transcriptional misincorporation that would be needed to account for all of the enzyme activity derived from pLuc-Stop plasmid. Although it is possible, and perhaps likely, that little or none of this activity is due to misincorporation, this would provide an upper limit.

Using our standard curve for purified authentic wild-type luciferase activity (Fig. 6A, solid line), we calculate that a molecule of Lys\(^{445}\) expressed from the plasmid, which also contains a Tyr\(^{444}\) \(\rightarrow\) Ser substitution due to creation of a restric-
tion site during cloning (data not shown). This allows us to estimate that the pLuc-Stop extract contains at most $1.1 \times 10^7$ molecules ($90,000$ relative light units in a 4-h induction) of luciferase. This is in comparison with $4 \times 10^{10}$ luciferase molecules measured in yeast expressing luciferase from pGAL-LUC-AAG with the natural lysine 445. Thus, a maximal frequency of misincorporation by RNA polymerase II of $2.8 \times 10^{-4}$ can be estimated by dividing the maximal number of active luciferase molecules derived from pLuc-Stop, $1.1 \times 10^7$, by the number of total luciferase enzymes present in the pGAL-LUC-AAG extract, $4 \times 10^{10}$. The actual frequency would be lower if activity from the pLuc-Stop plasmid was due to other causes, one of which would be stop codon read-through.

The deletion or disruption of SII did not change the yield of luciferase reporter activity in two independent dst1 

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Use of an \textit{in Vivo} Reporter Assay to Test for Transcriptional and Translational Fidelity in Yeast

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