Fail-safe termination elements: a common feature of the eukaryotic genome?

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ABSTRACT **A recent scan of the human genome (1) identified ~11 million hairpins. Some have been linked to known sequences, such as viruses, transposable elements, and, more recently, regulating or microRNAs, but the significance, if any, of most sequences that can be predicted to form hairpins remains unknown. Here we show that hairpins that are cleaved by RNase III-like nucleases can induce termination, even with normally polyadenylated transcripts, and that a cleaved hairpin downstream of a normal termination signal can induce fail-safe termination. Because such cleavage sites appear common to intergenic regions, the results raise the possibility that similar fail-safe termination elements are widely distributed in the eukaryotic genome to prevent read-through transcription from disrupting downstream promoter elements or opposing transcription.—Nabavi, S., Nazar, R. N. Fail-safe termination elements: a common feature of the eukaryotic genome? *FASEB J.* 24, 684–688 (2010). www.fasebj.org**

TRANSCRIPT CLEAVAGE AS A TERMINATION MECHANISM IN EUKARYOTES

In bacteria, the 3′ ends of most RNA molecules are produced simply by transcript termination. In eukaryotes, this is operationally replaced by 3′-end processing through endonuclease cleavage or exonuclease trimming. Despite this evolutionary change, termination of transcription appears to remain a critical event, which prevents RNA polymerase from interfering with the expression of downstream genes and releases the RNA polymerase to maintain an adequate pool of enzyme in the cell.

For protein-encoding genes, Pol II termination normally is linked to 3′-end formation. With the exception of replication-dependent histone genes, it is defined by a poly(A) signal, an AAUAAA sequence element, followed by a more variable GU-rich tract (2). This signal serves to recruit *trans*-acting proteins, which participate in transcript cleavage. The polyadenylation reaction that follows acts to protect the nascent mRNA from exonuclease (3) and promotes its export to the cytoplasm (4). The actual cleavage occurs between the AAUAAA and GU-rich elements, commonly after CA nucleotides (5). In yeast, transcription termination actually occurs shortly after the poly(A) signal (6), but in higher eukaryotes, it can occur at varying distances, often involving Pol II pausing mediated by pause sequence elements (7) or even cotranscriptional cleavage (CoTC), which can elicit the actual termination event (8). The need for cleavage is linked to a 5′→3′ exonuclease, which has been hypothesized to rapidly degrade the growing transcript until released from the RNA polymerase. In a somewhat similar fashion, termination of some nonpolyadenylated Pol II transcripts recently also has been linked to a transcript cleavage event in which RNase III-like nuclease cleavage in a downstream hairpin structure induces transcript termination (9). The "reversed torpedoes" model (10) suggests that the cleavage permits a 5′→3′ exonuclease to degrade the transcript in the downstream direction to terminate transcription, while it also permits 3′→5′ cleavage by the exosomal complex to trim the transcript in order to form the mature 3′ end.

In the case of Pol I transcription of pre-rRNA, termination was initially linked to repeated downstream termination sequence elements sometimes referred to as "Sal boxes" (11). These, in turn, have been shown to recruit a *trans*-acting protein factor often referred to as TTF1, which has been shown to act, mechanistically, to pause transcription (12). Most recently, this type of termination again has been linked to an RNase III-like cleavage event (13, 14), which also has been postulated to provide access to 5′→3′ exonuclease and subsequent downstream transcript degradation. In the case of the Pol I transcripts in *Xenopus laevus*, an additional transcription termination site has been reported immediately upstream of the adjacent transcription initiation site in next tandemly arranged rDNA transcriptional units. This has been speculated to serve a regulatory or fail-safe function (15).

In our studies of U3 snoRNA transcription in *Schizosaccharomyces pombe*, we were able to first demonstrate unequivocally that RNase III-like nuclease cleavage can induce transcript termination (9) when we substituted the highly conserved Pac1 RNase III-like nuclease susceptible 3′-ETS hairpin in a U3 snoRNA transcript to emulate the normal termination event. The fact that the same type of cleavage can induce both Pol I and Pol II termination has caused us to speculate about an additional, more general role for other such potential cleavage sites in the eukaryotic genome. In this respect, we have asked two additional experimental questions; namely, can the ribosomal 3′-ETS structure induce termination in a protein-coding gene without a need for polyadenylation, and can a RNase III-like labile site be demonstrated downstream of an otherwise terminated gene?

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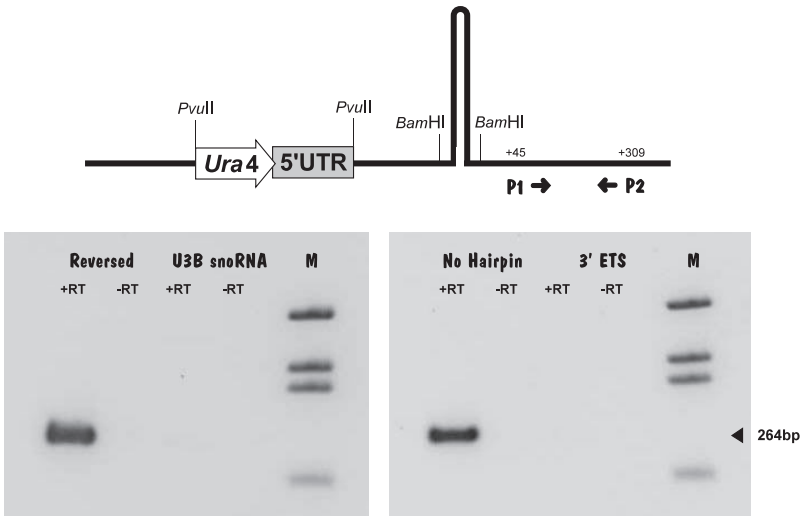

Figure 1. RNase III cleavage can terminate polyadenylated Pol II gene transcription. The upstream and 5′-UTR sequences for the *Ura*4 gene of *S. pombe* was PCR amplified and cloned in the *Pvu*II restriction site of the pFL20 yeast shuttle vector (17) and the Pac1 endonuclease-cleaved 3′-end hairpin of the gene (*snU*32) encoding the U3B snoRNA (left panel) or the rDNA transcriptional unit (right panel) was cloned in the adjacent *Bam*HI site; each recombinant was used to transform *S. pombe* (h$^-$, leu1-32, ura4-D18). RNA was prepared from representative transformants and assayed for the extent of transcription (+RT) by RT-PCR (9) using primers (P1/P2) specific for the region downstream of the cleaved hairpin sequence. Control reactions without reverse transcriptase (−RT) are included together with a fragment length marker (M). Positions of the anticipated fragments are indicated at right.

RNase III-LIKE CLEAVAGE CAN TERMINATE POLYADENYLATED TRANSCRIPTS

To examine the effect of RNase III cleavage on a gene that normally is terminated with polyadenylation, we used the *Ura*4 locus in *S. pombe*, a gene extensively studied in respect to its termination elements (16). As indicated in **Fig. 1**, the upstream region in the *S. pombe Ura*4 gene, together with the 5′-UTR sequence, was cloned into the unique *Pvu*II restriction site of the pFL20 yeast shuttle vector, together with and without the 3′ ETS, Pac1 endonuclease-cleaved hairpin sequence from the rDNA transcriptional unit of *S. pombe* cells. The hairpin sequence was inserted in the unique *Bam*HI site; both sequences were prepared by PCR amplification with specific primers without and with *Bam*HI adapter sequence extensions, respectively. As previously used in our U3 snoRNA expression studies, read-through transcription was assessed by RT-PCR using primers specific to sequence downstream of the *Bam*HI restriction site. As also shown in Fig. 1 (right panel), subsequent gel analyses demonstrated that a transcript was present without the hairpin sequence but clearly absent when Pac1 nuclease cleavage element was inserted in the *Bam*HI site.

A similar experiment also was undertaken using the Pac1 nuclease-cleaved hairpin immediately downstream of the *S. pombe* U3 snoRNA gene. This cleavage previously has been shown to induce Pol II termination of the U3 snoRNA transcript (9). In the present case, the hairpin sequence again was inserted in the *Bam*HI restriction site, but both orientations since past analyses (9) have shown that only the normal orientation is cut by the Pac1 endonuclease. Again, as also shown in Fig. 1(left panel), no downstream transcript was evident with the normal orientation, but read-through was once more clearly evident (reversed) when this sequence was present in the opposite orientation. The results show that the Pac1 nuclease-cleaved hairpin structure can induce the termi-

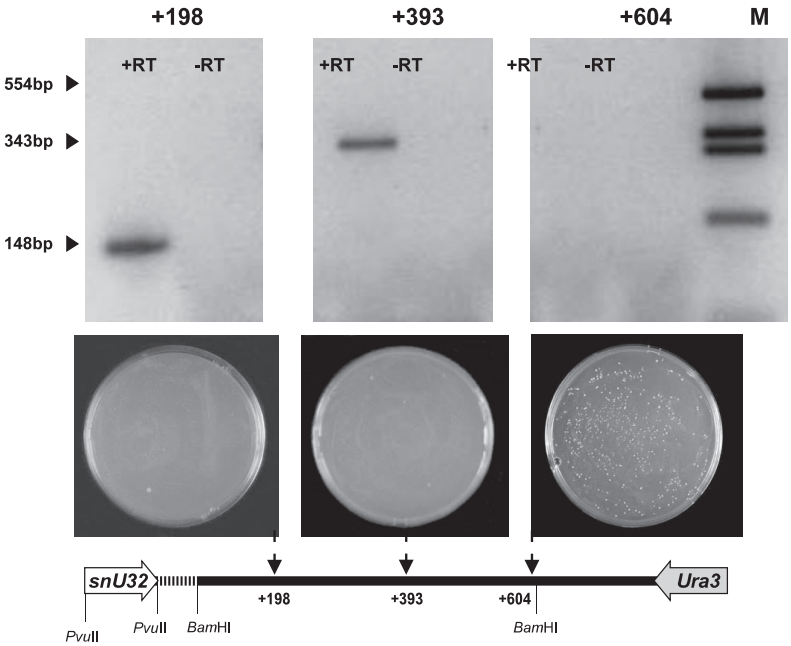

Figure 2. A downstream fail-safe termination element in the *S. pombe snU*32 locus. Upstream and coding sequences for the U3B snoRNA were PCR amplified and cloned in the *Pvu*II restriction site of the pFL20 yeast shuttle vector (9), and downstream sequences beginning at +50 and ending at +198, +393, or +604 were cloned in the adjacent *Bam*HI site, upstream of the selectable marker gene (*Ura*3). Recombinants were used to transform *S. pombe* (h$^-$, leu1–32, ura4-D18); example plates are shown (bottom panels). RNA was prepared from one of the many transformants with the largest downstream sequence (+50 to 604) and assayed for the extent of transcription by RT-PCR using a common upstream primer and primers ending at +198, +393 and +604, respectively. Products (+RT) were fractionated on a 2% agarose gel together with a fragment length marker (M). Reactions without reverse transcriptase (−RT) are included as controls. Positions of the anticipated fragments are indicated at left.

nation of transcription, even with a normally polyadenylated gene. With either the 3′-ETS or the U3B snoRNA hairpin structure, the termination efficiency was sufficiently high that no read-through was detected.

RNase III-LIKE CLEAVAGE SITES IN INTERGENIC REGIONS

Although not normally transcribed, intergenic regions often contain sequences that can be predicted to form extensive secondary structures if single stranded (1). Some of this type of structure can strongly resemble substrate for an RNase III-like nuclease (18, 19) an observation that raises the possibility that in times of termination, failure or partial read-through due to spontaneous mutation or other factors, the structure could induce fail-safe termination as a result of RNase III cleavage, as illustrated in Fig. 1. Again, our studies of U3 snoRNA gene expression in *S. pombe* caused us to examine downstream, intergenic sequence with respect to potential secondary structure, and a number of hairpin structures could be predicted with RNA-fold software (20). To explore the possibility of fail-safe termination sites, extended downstream regions also were examined in the absence of normal termination. As indicated in **Fig. 2**, the normal proximal Pac1 nuclease cleaved hairpin was deleted, and constructs with extended downstream sequences were tested for transformation efficiency when cloned in a conflicting orientation to the *Ura*3 selection marker gene and for read-through transcription when cloned in the opposite orientation, so transcription from the *Ura*3 gene would not be interrupted and transformants could be isolated (9). As also shown in Fig. 2, the results fully supported the presence of a fail-safe termination signal in the most distal extension between nucleotides + 393 and +604, a region that could be predicted to contain a hairpin structure that resembled a Pac1 nuclease substrate (**Fig. 3**). With the two shorter extensions, +50→+198 and + 50→+393, the transformation frequency was drastically reduced and read-through transcription was detected readily by RT-PCR. In strong con-

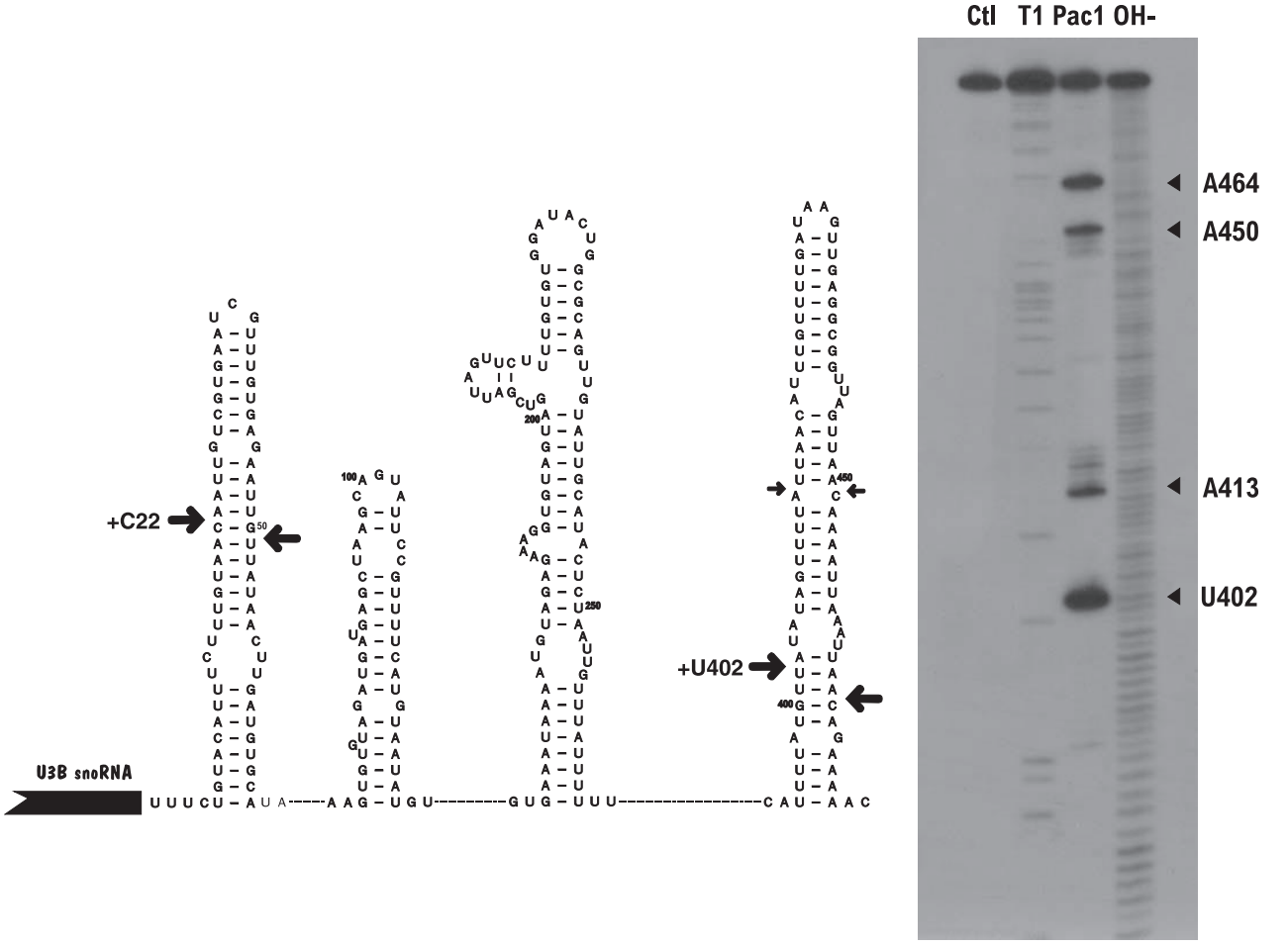

Figure 3. A Pac1 endonuclease cleavage site corresponds with the downstream fail-safe termination element. An estimate (23) of a read-through transcript predicts 3 significant hairpin structures beyond the previously described (9) Pac1 nuclease-cleaved (+C22) 3′ end-termination hairpin in the snU32 locus of *S. pombe* (left panel). RNA corresponding to the last hairpin, putatively identified as the fail-safe element (see Fig. 2), was prepared by transcription *in vitro* (21), labeled at the 5′ end with [γ^{32}P] ATP, and digested with Pac1 endonuclease (9, 22). Digest (Pac1) was fractionated on an 8% sequencing gel together with undigested RNA (Ctl) and T1 ribonuclease (T1) or base-cleaved (OH^-) samples as nucleotide markers. Major cleavage site (U402) together with any secondary and complementary cleavage sites are identified at right.

trast, essentially, a normal transformation frequency was evident with the most distal extension (+50→+604), and read-through transcription was no longer evident.

To confirm the presence of such a Pac1 nuclease cleavage site, RNA representing this region was prepared by *in vitro* transcription using T7 RNA polymerase and treated with Pac1 nuclease. As shown in Fig. 3, cleavage clearly was evident, consistent with transcript termination, which was observed *in vivo* (Fig. 2). Both a major (U402) and a minor (A413) site were detectable. A similar search for a natural Pac1 nuclease cleavage sites also was conducted downstream of the *Ura*4 gene locus in *S. pombe*. Structure analyses based on computer-generated modeling (20) suggested many potential hairpins, but the presence of actual Pac1 nuclease cleavage sites, if any, remained unclear. Again, RNA representing regions downstream of the *Ura*4-encoding sequence were prepared by *in vitro* transcription and treated with Pac1 nuclease. As shown in **Fig. 4**, a number of cleavage sites again clearly were evident in all three of the regions that were examined. At least 8 significant cleavage sites were evident in the first 500 nucleotides (G_{120}-C_{613}) downstream of the normal transcript. A further 4 sites were evident in the next 500-nucleotide region (A_{543}-G_{1040}), and 4 more were evident even 1500 nucleotides (T_{1386}-C_{1837}) downstream of the normal transcript.

Fail-safe termination as a possibility in eukaryotic gene expression originally was raised in studies of rRNA synthesis, when repeating termination signals were recognized in the 3′ ETS of the genes encoding the rRNAs in many divergent organisms, beginning with *Xenopus* (23). The significance of such repeated signals was further underscored when Reeder and coworkers (15) recognized an additional, isolated termination signal, just upstream of the site of transcription initiation in *Xenopus laevis*. A similar site in *Saccharomyces cerevisiae* subsequently was reported to be very effective in blocking polymerase that might begin to move in the wrong direction (24). In addition, read-through at a single termination site can be observed as a routine event; for example, in *S. pombe* only 90% of the transcripts are terminated at the first termination site, with 90% of the remaining transcripts being terminated further at the next site (25). Read-through appears equally or is even more common with many mRNA transcripts (26) and may be a reason why mRNA termination sites were so difficult to define over many years of study. Although interpreted differently, of special interest in this respect may be two recent studies by Chanfreau and coworkers (27, 28), who demonstrated that in *S. cerevisiae*, Rnt1 endonuclease (RNase III-like) depletion resulted in extended polycistronic mRNA species and cellular toxicity but suggested that Rnt1p played a role in RNA surveillance through multiple ribonucleolytic pathways.

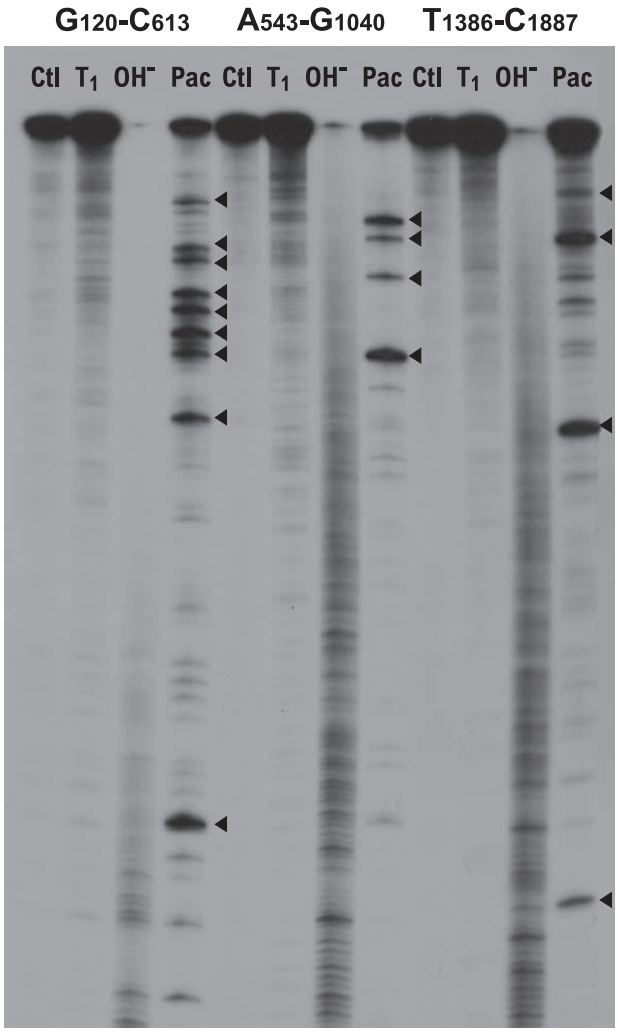

Figure 4. Pac1 endonuclease cleavage sites in downstream transcripts of the *S. pombe Ura*4 locus. RNA corresponding to 3 regions (G_{120}-C_{613}, A_{543}-G_{1040}, and T_{1386}-C_{1837}) downstream of the *Ura*4 encoding sequence was prepared by transcription *in vitro*, labeled at the 5′ end with [γ^{32}P] ATP, and digested with Pac1 endonuclease (9). Digest (Pac1) was fractionated on an 8% sequencing gel together with undigested RNA (Ctl) and T1 ribonuclease (T1) or base-cleaved (OH^-) samples as nucleotide markers. Major cleavage site (U402) together with any secondary and complementary cleavage sites are indicated with arrowheads at right.

CONCLUSIONS

The present study illustrates that RNase III cleavage, which now has been implicated directly in specific instances of both Pol I and Pol II transcript termination, can induce termination in a Pol II transcript, which normally is initiated by polyadenylation and also appears as a natural fail-safe termination site in a nonpolyadenylated transcript. As illustrated in **Fig. 5**, with similar putative RNase III cleavage sites being widely present in genomes, it seems highly likely that, intentionally or not, they can serve a fail-safe role and may act to prevent downstream disruption by read-through transcription. It seems equally possible, particularly given the past reported fail-safe site associated with rRNA transcriptional units, that such sites may even act to regulate transcription in some instances. Individual examples will require extensive investigation, which could also include more direct assays of transcription complexes based on chromatin immunoprecipitation to distinguish clearly transcription termination from RNA instability. In the interim, the present results serve to raise this as yet

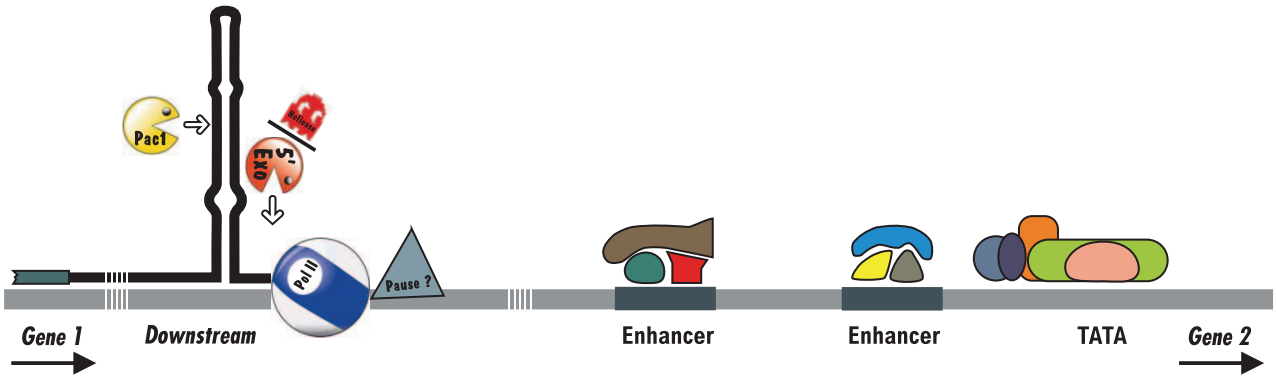

Figure 5. Failsafe termination in the eukaryotic genome. RNase III-like cleavage of hairpin structures downstream of normal termination signals can act to terminate run-on transcription before the polymerase disrupts distal promoter complexes.

unrecognized but potentially important and widely distributed feature of the eukaryotic genome.

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REFERENCES

1. Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y., and Bentwich, Z. (2005) Identification of hundreds of conserved and nonconserved human microRNAs. *Nat. Genet.* **37,** 766–770
2. Greger, I. H., and Proudfoot, N. J. (1998) Poly(A) signals control both transcriptional termination and initiation between the tandem GAL10 and GAL7 genes of Saccharomyces cerevisiae. *EMBO J.* **17,** 4771–4779
3. Bernstein, P., and Ross, J. (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability. *Trends Biochem. Sci.* **14,** 373–377
4. Huang, Y., and Carmichael, G. G. (1996) Role of polyadenylation in nucleocytoplasmic transport of mRNA. *Mol. Cell. Biol.* **16,** 1534–1542
5. Sheets, M. D., Ogg, S. C., and Wickens, M. P. (1990) Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Res.* **18,** 5799–5805
6. Hyman, L. E., and Moore, C. L. (1993) Termination and pausing of RNA polymerase II downstream of yeast polyadenylation sites. *Mol. Cell. Biol.* **13,** 5159–5167
7. Ashfield, R., Enriquez-Harris, P., and Proudfoot, N. J. (1991) Transcriptional termination between the closely linked human complement genes C2 and factor B: common termination factor for C2 and c-myc? *EMBO J.* **10,** 4197–4207
8. Dye, M. J., and Proudfoot, N. J. (1999) Terminal exon definition occurs cotranscriptionally and promotes termination of RNA polymerase II. Mol. *Cell* **3,** 371–378
9. Nabavi, S., and Nazar, R. N. (2008) Nonpolyadenylated RNA polymerase II termination is induced by transcript cleavage. *J. Biol. Chem.* **283,** 13601–13610
10. Nabavi, S., and Nazar, R. N. (2007) "Reverse torpedoes" model for the termination and processing of U3 snoRNA. *FASEB J.* **21**, 811.8
11. Grummt, I., Maier, U., Ohrlein, A., Hassouna, N., and Bachellerie, J. P. (1985) Transcription of mouse rDNA terminates downstream of the 3′ end of 28S RNA and involves interaction of factors with repeated sequences in the 3′ spacer. *Cell* **43,** 801–810
12. Kuhn, A., Bartsch, I., and Grummt, I. (1990) Specific interaction of the murine transcription termination factor TTF I with class-I RNA polymerases. *Nature* **344,** 559–562
13. El Hage, A., Koper, M., Kufel, J., and Tollervey, D. (2008) Efficient termination of transcription by RNA polymerase I requires the 5′ exonuclease Rat1 in yeast. *Genes Dev.* **22,** 1069–1081
14. Kawauchi, J., Mischo, H., Braglia, P., Rondon, A., and Proudfoot, N. J. (2008) Budding yeast RNA polymerases I and II employ parallel mechanisms of transcriptional termination. *Genes Dev.* **22,** 1082–1092
15. Bakken, A., Morgan, G., Sollner-Webb, B., Roan, J., Busby, S., and Reeder, R. H. (1982) Mapping of transcription initiation and termination signals on *Xenopus laevis* ribosomal DNA. *Proc. Natl. Acad. Sci. U. S. A.* **79,** 56–60
16. Aranda, A., and Proudfoot, N. (2001) Transcriptional termination factors for RNA polymerase II in yeast. *Mol. Cell.* **7,** 1003–1011
17. Losson, R., and Lacroute, F. (1983) Plasmids carrying the yeast OMP decarboxylase structural and regulatory genes: transcription regulation in a foreign environment. *Cell* **32,** 371–377
18. Danin-Kreiselman, M., Lee, C. Y., and Chanfreau, G. (2003) RNAse III-mediated degradation of unspliced pre-mRNAs and lariat introns. *Mol. Cell.* **11,** 1279–1289
19. Ge, D., Lamontagne, B., and Elela, S. A. (2005) RNase III-mediated silencing of a glucose-dependent repressor in yeast. *Curr. Biol.* **15,** 140–145
20. Mathews, D. H., Turner, D. H., and Zuker, M. (2007) RNA secondary structure prediction. *Curr. Protoc. Nucleic Acid Chem.* **11,** 11.2
21. Hitchen, J., Ivakine, E., Melekhovets, Y. F., Lalev, A., and Nazar, R. N. (1997) Structural features in the 3′ external transcribed spacer affecting intragenic processing of yeast rRNA. *J. Mol. Biol.* **274,** 481–490
22. Rotondo, G., and Frendewey, D. (1996) Purification and characterization of the Pac1 ribonuclease of *Schizosaccharomyces pombe. Nucleic Acids Res.* **24,** 2377–2386
23. Sollner-Webb, B., and Reeder, R. H. (1979) The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in *X. laevis. Cell* **18,** 485–499
24. Elion, E. A., and Warner, J. R. (1984) The major promoter element of rRNA transcription in yeast lies 2 kb upstream. *Cell* **39,** 663–673
25. Shwed, P. S., and Nazar, R. N. (1999) Terminator element mutations affect both the efficiency and position of RNA polymerase I termination in *Schizosaccharomyces pombe. Nucleic Acids Res.* **27,** 2883–2888
26. West, S., Zaret, K., and Proudfoot, N. J. (2006) Transcriptional termination sequences in the mouse serum albumin gene. *RNA* **12,** 655–665
27. Zer, C., and Chanfreau, G. (2005) Regulation and surveillance of normal and 3′-extended forms of the yeast aci-reductone dioxygenase mRNA by RNase III cleavage and exonucleolytic degradation. *J. Biol. Chem.* **280,** 28997–29003
28. Lee, A., Henras, A. K., and Chanfreau, G. (2005) Multiple RNA surveillance pathways limit aberrant expression of iron uptake mRNAs and prevent iron toxicity in *S. cerevisiae. Mol. Cell* **19,** 39–51

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