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Quantitation of RNA editing substrates, products and potential intermediates: implications for developmental regulation

George R. Riley, Peter J. Myler and Kenneth Stuart*

Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, WA 98109, USA

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ABSTRACT

Kinetoplast mitochondrial RNA editing is the developmentally regulated post-transcriptional process of uridine insertion and deletion in mRNAs directed by short guide RNAs (gRNAs), which creates functional mRNAs. Two mechanisms are proposed: transesterification which predicts gRNA/mRNA chimeric intermediates, and enzymatic steps which allow but do not require chimeric intermediates. We quantitated the copy number of apocytochrome *b* (CYb) gRNAs, edited/unedited mRNAs and gRNA/mRNA chimeras in bloodstream and procyclic form cells of *Trypanosoma brucei*. Both forms have 35 copies/cell of two gRNAs. Bloodstream forms contain 15 unedited and edited CYb mRNA molecules/cell while procyclic forms have four times as much unedited and over 10 times as much edited mRNA. Chimera levels are very low, 350–5000-fold lower than unedited mRNA or gRNAs, but are over 10 times more abundant in procyclic than bloodstream forms. These results are consistent with chimeras being editing intermediates if their resolution is rapid in respect to their formation, although they could be non-productive byproducts of the editing reaction. Bloodstream chimera sequences differ from procyclic chimeras. These results indicate that developmental regulation is not by gRNA abundance and suggest that it occurs at the level of gRNA utilization possibly by changing abundance of unedited CYb mRNA.

INTRODUCTION

Mitochondrial (mt) RNA editing in kinetoplastids occurs by site-specific insertion and deletion of uridine residues in many mRNAs. These insertions and deletions eliminate frame-shifts and create initiation and termination codons to make functional mature mRNAs (reviewed, 1,2). RNA editing may be limited to the insertion of as few as four U-residues, as in cytochrome oxidase subunit II (COII), or determine >60% of the mRNA sequence as in ATPase subunit 6 (A6) (3,4). Guide RNAs (gRNAs) are small (~60 nt) RNAs that probably specify the edited sequences of mt RNAs. They complement edited mRNA sequences by a combination of Watson–Crick and G–U base pairing and contain 10–15 uridine residues that are post-transcriptionally added to the 3′ terminus (5–8).

Accumulation of some edited mRNAs is developmentally regulated in *Trypanosoma brucei*. Edited apocytochrome *b* (CYb) and COII mRNAs are abundant in procyclic form (PF) but are nearly undetectable in bloodstream form (BF) cells (9,10). In contrast, edited C-rich (CR) gene 4 and NADH dehydrogenase subunit 8 (ND8) mRNAs are abundant primarily in BF cells (1,11,12). The mechanism of this developmental regulation is unknown, but does not reflect gRNA abundance (13,14).

Two different general mechanisms have been proposed for kinetoplastid mt mRNA editing. One proposed mechanism suggests a series of separate enzymatic steps that has two alternative pathways. This model proposes cleavage of the mRNA by endonuclease, addition of U residues from free UTP by terminal uridylyltransferase (TUTase) and re-ligation of the mRNA fragments by RNA ligase (5). In one pathway, uridines are added to the 3′ terminus of the 5′ mRNA fragment produced by endonucleolytic cleavage. In the other, uridines are added only to the gRNAs which form chimeric intermediates with the 3′ mRNA fragment. The other proposed editing mechanism suggests sequential transesterification reactions with chimeric intermediates (15,16). By this model TUTase only adds the U-tail to the gRNA, whose 3′ hydroxyl group is the nucleophile that attacks at a phosphodiester bond in the mRNA, resulting in the chimera. The chimera is resolved by nucleophilic attack at a chimera phosphodiester bond by the 3′ hydroxyl of the 5′ mRNA fragment, rejoining the mRNA (1,15,16). Since gRNA/mRNA chimeras are present *in vivo* and can be formed *in vitro* in mitochondrial extracts (17–19) and endonuclease, RNA ligase and TUTase activities (18) are present in kinetoplastid mitochondria, it has not yet been determined which of these mechanisms is operating.

To explore the developmental regulation of editing we quantitated CYb gRNAs, edited and unedited mRNAs, and gRNA/mRNA chimeras. CYb was chosen since its editing is developmentally regulated in *T.brucei*, its editing is limited to 34 U residue insertions in the 5′ end of the message (20), gRNAs for its first editing sites are known (13), and partially edited CYb mRNAs and gRNA/mRNA chimeras have been characterized

* To whom correspondence should be addressed

(13). Our data indicate that chimeras are present at very low levels, consistent with their being intermediates if chimera resolution is rapid in respect to formation. These data further indicate that gRNA abundance is not a factor in developmental regulation of editing, rather that regulation may be controlled at the level of gRNA utilization, perhaps affected by the abundance of unedited mRNA.

MATERIALS AND METHODS

Isolation of organisms and nucleic acids

The growth and isolation of procyclic *Trypanosoma brucei* IsTaR 1 (derived from EATRO 164) *in vitro* and its BF and dyskinetoplastic forms (DK) in rats is previously described (21,22). RNA was isolated by guanidinium–acid phenol extraction (23). The efficiency of recovery of RNA on a per cell basis was determined by adding a radiolabelled synthetic RNA transcript to the guanidinium–phenol immediately prior to extraction. The average RNA recovery from BF was $5.2 \pm 0.1 \times 10^{-13}$ g total RNA per cell ($n = 2$), from PF was $9.3 \pm 3.1 \times 10^{-13}$ g total RNA per cell ($n = 3$). No degradation of the tracer RNA was detected.

Oligonucleotide primers and probes

The following oligonucleotides were used for PCR amplification and hybridization. Underlined nucleotides indicate added restriction enzyme sites.

5′gCYb(558): 5′-CGGATCCAAGACAATGTGAATTTTTAGG-3′.
5′gCYb(560): 5′-CGGATCCGTAAAAGACAATGTAGATTTC-3′.
3′gCYb(558): 5′-ATTATTCCCTTTATCACCTA-3′.
3′gCYb(560A): 5′-TTATCCTCCCCATTACTCAG-3′.
3′gCYb(560B): 5′-TTATCCTCCCTATTACTCAG-3′.
CYb-CS2: 5′-CCGGATCCATATATTCTATATAAACAACC-3′.
CYb-CS3: 5′-CGGAATTCACACTCCACAAATTATTTGCAATGC-3′.
CYb-CS4: 5′-GGAATTCATATATGTACACTTCTTATCAC-3′.
CYbD: 5′-CCTGACATTAAAAGACCCTTTCTTTTTTCTC-3′.
CYbR: 5′-CCTGACATTAAAAGACAACACAAATTTCTAAA-3′.

Northern blot analysis

RNA gel electrophoresis and Northern blots were as previously described (13). Ten μg of total cellular RNA and a 5-fold dilution series of synthetic 1200 nt unedited or edited CYb RNA were separated on 1% agarose gels for analysis of mRNAs. Twenty μg of total cellular RNA and a 5-fold dilution series of synthetic CYb gRNA were separated on 6% polyacrylamide–urea gels for analysis of gRNAs. Multiple non-saturated autoradiograms of each blot were analyzed with a BioRad Model 620 densitometer. The RNA copy number per cell was calculated from the moles of RNA measured per μg total cellular RNA and the RNA recovery per cell for both BF and PF cells.

Quantitative PCR

Chimeras were quantitated by reverse transcription-PCR amplification (24). CYb cDNA was synthesized from 5 μg of total cellular BF, PF and DK RNA with 10 pmol of CYb-CS4 primer (complementary to CYb mRNA 173 nt 3′ to the editing domain). The primer and RNA were heated to 70°C for 10 min in 10.5 μl water, chilled to 0°C, then incubated with Superscript MMLV reverse transcriptase (Life Technologies) in 20 μl according to the manufacturer's specifications, at 37°C for 10 min followed by 42°C for 20 min. RNA was hydrolyzed by addition of 15 μl of 150 mM NaOH and incubation at 65°C for 1 h. Fifteen μl of 1 M Tris–HCl pH 8.0 was added and the cDNA precipitated with ammonium acetate, using 20 μg of glycogen as carrier. Control quantitations using synthetic chimeric RNA transcripts indicate that reverse transcription is quantitative under these conditions (see below). DK RNA, which contains no mt sequences, serves as a negative control.

Quantitative PCR (24) was done in 100 μl with one-fifth of a cDNA synthesis (1 μg RNA equivalent), 20 μM each dNTP, 100 μCi [α-^{32}P]dATP (800 Ci/mmol, NEN/Dupont), 250 μM each primer, 2.5 U *Taq* polymerase (Boehringer Mannheim), 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl. Initial denaturation was for 2 min, denaturation was 94°C for 1 min, annealing for 30 s, and elongation 72°C for 1.25 min. Annealing in the first two cycles was at 42°C, followed by 30 cycles with annealing at 52°C. Primers were CYb-CS3, an internal primer 65 nt 3′ to the editing domain in the CYb mRNA, and either 5′gCYb(558) or 5′gCYb(560) which are specific for the 5′ ends of their respective gRNAs. Primer choice is critical: 5′gCYb(558) and CYb-CS2 (14 nt 3′ to the editing domain) amplify spurious sequence of the size expected for chimera product (not shown) in DK cDNA, which contains no mitochondrial DNA. Two μl samples were taken from each PCR reaction after 25 s extension at 72°C. These samples were mixed with molecular weight markers and separated on 4.5% acrylamide or PCR Purity Plus (J. T. Baker) PAGE–TBE gels (25). The gels were stained for 15 min with 0.5 μg/ml ethidium bromide (25); the chimera product band and an equivalently-sized background gel-slice above the product were excised; the gel-slices were dried overnight at 80°C and counted by liquid scintillation. The $\log_{10}$ values of linear data points were analyzed by linear regression (24) which indicated a PCR efficiency from 75–94% in the various reactions. The fit of the linear regression gave r^2 values of 0.997–0.999. Controls containing either of two different synthetic chimeric RNAs mixed with DK RNA indicate that this method is quantitative (not shown).

Cloning chimeras

Unlabelled gCYb(558) and gCYb(560) PCR products amplified from BF RNA using reaction conditions described above were cloned into the *Bam*HI and *Eco*RI sites of pBluescript II SK(–) (Stratagene) to validate the identity of the PCR product and to ensure that it was heterogeneous, reducing the likelihood that it might result from contaminating material previously cloned. DNA sequence was determined from both strands using a *Taq* dye primer cycle sequencing kit on a model 373A DNA sequencer (ABI).

RESULTS

mRNA and gRNA quantitation

Densitometric comparison of the signal in total cellular RNA (Fig. 1A and B, lanes 1–3) to synthetic CYb RNAs (lanes 4–8) indicates ~50 attomol each of unedited and edited CYb mRNA per μg cellular RNA in BF, ~15 copies per cell (Table 1). PF cells contain more than four times as much unedited message (120 attomol/μg total RNA, 66 copies/cell) as BF cells, and 12 times

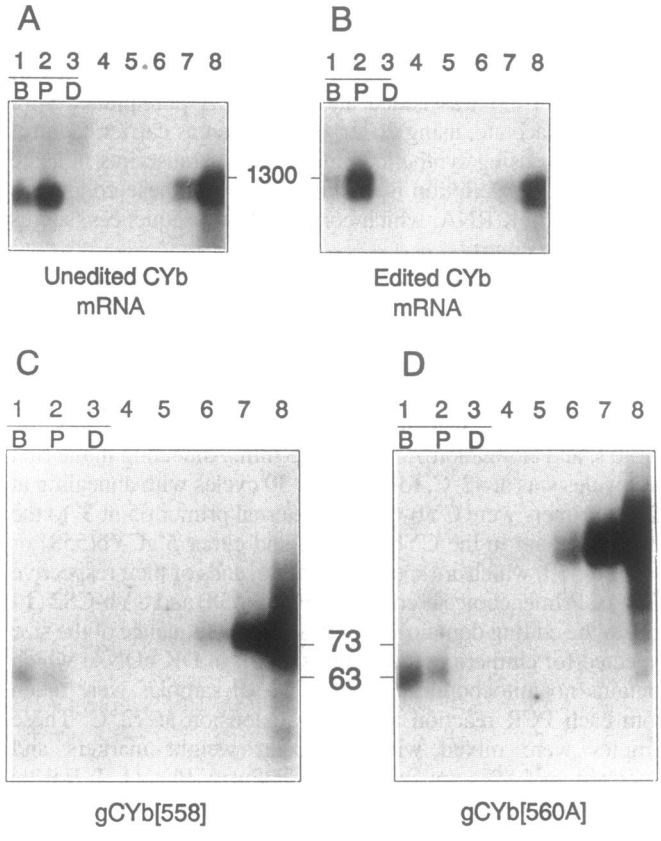

Figure 1. Quantitation of CYb mRNA and gRNA. Specific RNAs were quantitated by electrophoresing 10 μg total RNA in formaldehyde–agarose gels (A and B) or 20 μg total RNA in PAGE–urea gels (C and D), blotting, and probing northern blots with specific oligonucleotides. (**A**) Total BF (B), PF (P) and DK (D) RNAs probed with an oligonucleotide specific for unedited CYb mRNA (CYbD). Lanes 4–8 contain a 6-fold dilution series of 2.3–3000 attomol (10^{-18}) of synthetic unedited CYb RNA. (**B**) Total RNAs probed with an oligonucleotide specific for edited CYb mRNA (CYbR). Lanes 4–8, as in A, 2.6–3400 attomol of synthetic edited CYb RNA. (**C**) Total RNAs probed with an oligonucleotide specific for gCYb(558). Lanes 4–8, as in A, 96–120 000 attomol of synthetic gCYb(558) transcript. (**D**) Total RNAs probed with an oligonucleotide specific for gCYb(560A). Lanes 4–8, as in A, 100–130 000 attomol of synthetic gCYb(560A) RNA.

as much edited mRNA (340 attomol/μg total RNA, 188 copies/cell).

Two gRNAs, gCYb(558) and gCYb(560A), both of which specify identical editing of the same domain of the CYb mRNA, are present in the same amount (~35 copies/cell) in both BF (110 attomol/μg cellular RNA) and PF (65 attomol/μg total RNA)

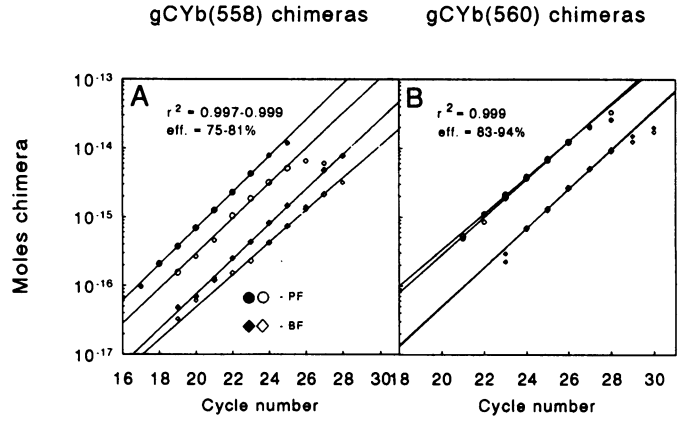

Figure 2. PCR quantitation of CYb gRNA/mRNA chimeras. Moles of chimera PCR product determined for each PCR cycle plotted with the calculated regression line. (**A**) Two separate experiments with gCYb(558) chimeras. (**B**) Two separate experiments with gCYb(560) chimeras. The PCR cycle efficiency and data fit (r^2 values) calculated from the linear regression are indicated.

(Fig. 1C and D). Thus, these gRNAs together are at about five times the concentration of unedited CYb mRNA in BF and about the same concentration in PF RNA (Table 1).

Chimera quantitation

CYb chimera levels were below the limit of detection by Northern blot and thus were quantitated by PCR. Figure 2 shows two independent experiments each for gCYb(558) (panel A) and gCYb(560) (panel B) chimeras in BF and PF RNAs. These experiments determined incorporation of radiolabelled nucleotides over a number of amplification cycles and used linear regression to determine the quantity of chimera RNA (24) (see Materials and Methods). PCR efficiencies ranged from 75 to 94% in different reactions. DK RNA, which lacks kDNA, gave no product thus showing product specificity. Supplementation of DK RNA with known amounts of either of two different synthetic chimera RNAs showed that this method is quantitative (data not shown). These studies showed that gCYb(558) and gCYb(560) chimeras are present at 0.06 and 0.019 attomol/μg of total BF RNA respectively, or substantially less than one copy per cell. This is 800–2500-fold lower than the levels of unedited CYb mRNA in BF cells. Chimera levels are about 10–15 times higher in PF with gCYb(558) and gCYb(560) chimeras at 0.339 and 0.168 attomol/μg total PF RNA respectively, still less than one copy per cell. This is 350–700-fold lower than unedited mRNA in PF cells.

Table 1. Cyb gRNA, mRNA and chimeric RNA quantities

		gRNAs[a]		mRNAs[b]		chimeric RNAs[a]	
		(558)	(560A)	Unedited	Edited	(558)	(560A)
BF	attomol/μg	110 ± 27	110 ± 24	46 ± 13	50 ± 4	0.060 ± 0.017	0.019 ± 0.013
	copy/cell	34 ± 9	35 ± 9	15 ± 5	16 ± 2	0.019 ± 0.006	0.006 ± 0.005
PF	attomol/μg	65 ± 12	64 ± 28	120 ± 11	340 ± 55	0.339 ± 0.098	0.168 ± 0.069
	copy/cell	36 ± 15	36 ± 22	66 ± 26	188 ± 78	0.190 ± 0.092	0.094 ± 0.054

[a]Averages of three quantitations.
[b]Averages of two quantitations.

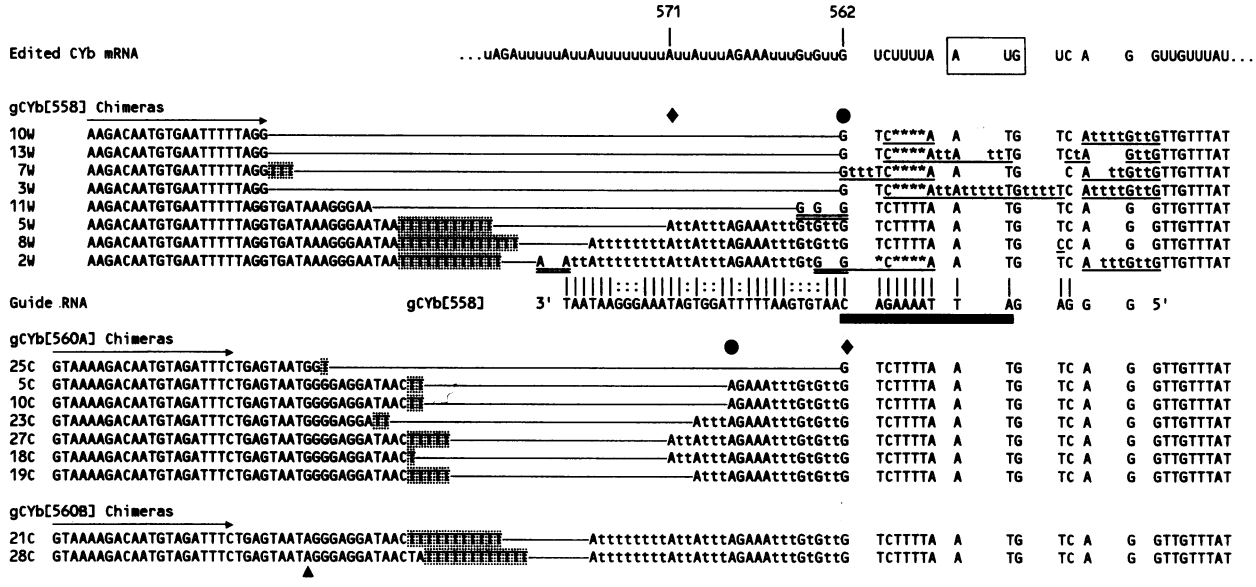

Figure 3. Sequence of BF CYb gRNA/mRNA chimeras. BF chimeras containing gCYb(558) and gCYb (560) were cloned to authenticate the PCR product being quantitated. Clones are aligned 5′ to 3′, with the gRNA sequence on the left and mRNA on the right. gCYb(558) and its homology are shown 3′ to 5′ below clone 2W to illustrate disruption of the anchor duplex (indicated by the solid bar). Edited CYb mRNA sequence is shown at the top of the figure, with the downstream AUG boxed. Uridine tails of the gRNAs are shaded. Upper case Ts indicate encoded uridines, lower case Ts indicate uridines inserted by editing, and asterisks represent encoded uridine residues removed by editing. Sites which are normally edited, but are unedited in these chimeras are double-underlined and editing downstream of the consensus first editing site is single-underlined. A single anomalous C residue is underlined in clone 8W. Predominant gRNA linkage sites in PF and BF chimeras are indicated by ♦ and ● respectively. The single base difference between gCYb(560) A and B is indicated by an arrowhead below the gCYb(506B) chimera sequence. The primers are indicated by arrows over the sequence. Sixty-six nt of identical unedited downstream sequence is omitted for clarity. The previously published sequences of clones 21C and 28C (13) are included for comparison.

Cloning chimeras

The gCYb(558) and gCYb(560) chimeras cloned and sequenced from BF PCR product (Fig. 3) showed typical chimera structure with gRNAs linked, usually by a U-tail, to editing sites in the mRNA. However, the BF chimeras differ from gCYb(558) and gCYb(560) PF chimeras previously cloned (13) in gRNA/mRNA linkage, in unusual editing 3′ to the normal editing domain and in all chimeras but one having edited sequence.

Half the BF gCYb(558) chimeras have the gRNA linked to potential editing site 562 (● in Fig. 3), while the others are linked to edited sequence at more upstream sites. BF gCYb(560) chimeras are linked to mature edited mRNA sequence at sites 569(●)–572. In contrast, in previously cloned PF gCYb(558) chimeras (13), the gRNA was most often linked to fully edited mRNA sequence at potential editing site 571 (♦) while in gCYb(560) chimeras the gRNA was most often linked to unedited sequence at the 3′-most editing site, 562 (♦).

Five BF gCYb(558) chimeras show unusual U-insertion and deletion (underlined) in sites downstream of potential editing site 562, which is the 3′-most editing site found in mature CYb mRNA. This unusual editing is particularly interesting because it disrupts the 10 nt Watson–Crick anchor duplex (indicated by the bar in Fig. 3), which is predicted to form between gCYb(558) and unedited CYb mRNA, and is thought to provide gRNA specificity (8,13,19), suggesting that the unusual downstream editing of the mRNA took place after gCYb(558)/CYb mRNA recognition and association. Two partially edited PF CYb cDNA clones with similar unusual editing have been sequenced (13,26; Corell, Riley and Stuart, unpublished results), indicating that this editing is not a phenomenon entirely restricted to BF chimeras. In the case of clones 13W and 3W, this unusual editing disrupts an AUG codon 9 nt 3′ of potential editing site 562 (boxed in Fig. 3), which could theoretically serve as an initiation codon in place of the upstream AUG created by editing of site 580 (not shown) (13,20). BF gCYb(558) chimeras also contain unedited mRNA sites (double underlined in 11W and 2W). The significance of the single C replacement of an encoded U residue in 8W (underlined) is unknown. The gCYb(560) chimeras do not show any unusual editing downstream of the first consensus editing site. Two of the chimeras, 21C and 28C, can base pair with the entire guiding domain of the gRNA, suggesting that the gRNA has completed its editing of the message.

DISCUSSION

This study shows that CYb gRNAs have the same abundance in BF and PF cells. Thus, developmental regulation of editing is not controlled by gRNA abundance. Edited CYb mRNA is about 12 times more abundant in PF than in BF. The developmental regulation is probably even greater, since stumpy and intermediate BF edit CYb mRNA (10) and lower levels of editing have been seen previously in BF (9), suggesting a >12-fold difference between PF and slender BF. Unedited CYb mRNA is four times more abundant in PF than in BF, perhaps as a result of greater transcription and/or mRNA stability. This increased level of unedited substrate mRNA may be a factor in the developmental regulation by enhancing gRNA/mRNA interaction, kinetically favoring editing.

gRNA/mRNA chimeras were found to be rare, occurring at levels substantially less than a single copy per cell. Assuming chimera resolution is rapid in comparison to their formation, their low abundance is consistent with their being editing intermediates. However, the possibility that chimeras have other significance can not be discounted, since their low abundance may reflect infrequent side reactions not part of the normal editing process. For example, if the U-tail of the gRNA is not constrained in the enzymatic step model, RNA ligase might fortuitously ligate the 3′ fragment of the mRNA to the gRNA U-tail rather than the 5′ fragment of the mRNA.

The correlation between 10–15 times higher chimera abundance in PF and the 12-fold higher abundance of edited mRNA implies that chimera abundance may be an index of editing activity which is also consistent with chimeras being editing intermediates. However, it could simply reflect an increased gRNA/mRNA interaction during editing in PF producing more side products. Direct experiments are required to determine whether chimeras are editing intermediates.

The similar levels of gRNAs and unedited pre-mRNA in BF and PF and the greater abundance of chimeras in PF suggests that developmental regulation of editing may be at the level of gRNA utilization. This might be due to its association with or utilization by the editing machinery, and/or its increased participation in the editing process due to increased levels of unedited mRNA. In addition, specific factors may regulate utilization of critical gRNAs or may regulate the stability of edited and unedited mRNA. Additional developmental regulation may occur at upstream secondary gRNAs. gCYb(558) chimeras are two to three times more abundant than gCYb(560) chimeras although their gRNAs have similar abundance, indicating a difference in the CYb gRNAs' utilization. This may reflect the longer anchor sequence (10 versus 8 nt) of gCYb(558). The differences in gRNA/mRNA linkages with both CYb gRNAs between BF and PF chimeras also imply that there is stage-specific difference in gRNA utilization.

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