Large-scale detection of in vivo transcription errors

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Accurate transmission and expression of genetic information are crucial for the survival of all living organisms. Recently, the coupling of mutation accumulation experiments and next-generation sequencing has greatly expanded our knowledge of the genomic mutation rate in both prokaryotes and eukaryotes. However, because of their transient nature, transcription errors have proven extremely difficult to quantify, and current estimates of transcription fidelity are derived from artificial constructs applied to just a few organisms. Here we report a unique cDNA library preparation technique that allows error detection in natural transcripts at the transcriptome-wide level. Application of this method to the model organism Caenorhabditis elegans revealed a base misincorporation rate in mRNAs of $\approx 4 \times 10^{-6}$ per site, with a very biased molecular spectrum. Because the proposed method is readily applicable to other organisms, this innovation provides unique opportunities for studying the incidence of transcription errors across the tree of life.

Errors in biological processes are at the very heart of the evolution of life. Indeed, mutations caused by DNA replication errors are ultimately essential for species adaptation in the face of changing environments. Despite the important role of adaptive mutation for evolution, most mutations are deleterious, especially when they affect protein-coding sequences (1). As a consequence, selection is expected to enhance the fidelity of replication (2–4). However, because errors can occur at any step of the protein synthesis process, even nonmutated sequences can produce nonfunctional proteins. Indeed, misincorporations by RNA polymerase (transcription errors) and erroneous tRNA recruitment (translation errors) may often lead to the synthesis of misfolded, nonfunctional proteins with potentially harmful consequences (5, 6). Therefore, selection is expected to enhance the fidelity of each of these processes. However, the error rates that can be tolerated by different organisms remain unclear, and several hypotheses have been proposed for the limits to the fidelity of replication and transcription (7–10).

Two fundamental differences with DNA mutations may reduce the strength of selection against transcription and translation errors. First, unlike DNA mutations, the latter are not permanently transmitted to daughter cells. Second, individual loci generally produce multiple transcripts with relatively short half-lives (11), so that each error is present in only a fraction of the proteins produced. Therefore, it has been suggested that the strength of selection against transcription and translation errors might be less intense than that operating at the level of genome replication (9).

With the recent improvement in sequencing techniques, detection of mutations is now commonly achieved by next-generation sequencing of mutation accumulation lines (12–15), providing ample opportunities for developing and testing theories on the evolution of mutation rates. However, because of their transient nature, transcription and translation errors have remained difficult to detect. The few attempts to measure transcription error rates have relied on indirect techniques involving reporter constructs and/or in vitro template copying (16–20). Reporter constructs measure transcription errors at only a small number of sites and are often convoluted with translation errors (18, 19), and in vitro methods use experimental conditions that may be quite different from the intracellular environment. Thus, it is not surprising that previous estimates of transcription error rates vary by orders of magnitudes even within the same organism (16–20), although a rough overall average value of $10^{-6}$ per nucleotide has been suggested (21). Likewise, measurements of translation error rates are still sparse and can be hard to disentangle from transcription errors (18, 19, 22–24).

Although large-scale analysis of translation errors might require a breakthrough in mass spectrometry techniques, one can imagine that the large amount of RNA-sequencing (RNA-seq) data now routinely obtained by next-generation sequencing could help in detecting transcription errors. Indeed, after mapping RNA-seq reads to a reference genome, transcription errors will appear as mismatches between mRNA reads and the reference genome. Therefore, the billions of RNA-seq reads deposited in public databases probably contain thousands of transcription errors within their sequences. Unfortunately, such a naive approach cannot be used with traditional RNA-seq data, because mismatches caused by transcription errors are not accurately distinguishable from the potentially much more numerous sequencing errors, not to mention errors introduced by reverse transcription during cDNA synthesis [RT (reverse transcription) errors]. In principle, bar-coding of nucleic acid molecules before sequencing can facilitate the discrimination of sequencing errors from real mutations (25). Here we describe a unique method for identifying transcription errors by sequencing multiple cDNAs originating from the same mRNA molecule, using a bar-coding strategy to trace back the origin of individual cDNAs.

Significance

Gene expression requires accurate copying of the DNA template into messenger RNA by RNA polymerases. Errors occurring during this transcription process can lead to the production of nonfunctional proteins, which is likely to be deleterious. Therefore, natural selection is expected to enhance the fidelity of transcription. However, very little is known about the transcription error rates of different organisms. Here we present a unique method for the detection of transcription errors by replicated high-throughput sequencing of cDNA libraries. Applying this method to the model organism Caenorhabditis elegans, we report a large-scale analysis of transcription errors. Future applications of this method should allow a rapid increase in our knowledge of evolutionary forces acting on transcription fidelity.


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Results
A Unique Library Preparation Technique. To accurately identify transcription errors in RNA-seq data, we developed a unique cDNA library preparation technique. We start by tagging fragmented mRNAs at their 5’ ends with bar codes made of random 8-mers (Fig. 1A). The tagged RNA fragments are then attached to beads and reverse transcribed three times (Fig. 1B). After each round of reverse transcription, the newly generated cDNAs are washed away (Fig. 1C) and characterized by Illumina paired-end sequencing. To simplify the following discussion, we denote a series of reads originating from a unique molecule of fragmented mRNA as a family. Two reads are considered as belonging to the same family if they share the same bar code and have identical 5’ and 3’ breakpoints introduced during the process of mRNA fragmentation.

The rationale for identifying transcription errors in such samples is as follows. Errors that are already present in the fragmented mRNAs (i.e., transcription errors) will be copied by the reverse transcriptase and incorporated into each newly generated cDNA, therefore appearing as a mismatch in every read of a family (Fig. 1D, leftmost family). In contrast, individual reads in the family may contain unique RT and sequencing errors, which will generally occur as singletons (Fig. 1D, blue and green stars). Provided that the sequencing and RT error rates are low enough, the probability of observing the same sequencing or RT error in all three reads would be negligible, and only errors present in the original mRNA molecule could produce the pattern shown in the leftmost family of Fig. 1D. Ideally, every single cDNA generated should be sequenced, so that each family contains three pairs of reads (size 3 family). However, this would require extremely deep sequencing, and in practice, only a fraction of the cDNA library is sequenced (see below), so that most families contain reads from only one or two cDNAs (size 1 and size 2 families). Although size 1 families cannot be used to disentangle transcription errors from other types of errors, we show that size 2 families are sufficient to accurately call transcription errors and that the number of size 2 and size 3 families obtained from a single run of HiSeq sequencing is sufficient to find dozens of transcription errors in Caenorhabditis elegans.

Estimating Sequencing Error Rates. Every base call in an Illumina sequencing run is given a quality score, which is an indication of the probability that the base call is erroneous. In principle, the product of these probabilities at a given position within a family can be used as an estimation of the probability of observing a false positive caused by multiple sequencing errors. Assuming that all three possible erroneous base calls at a given position are equiprobable, the probability of observing the same erroneous base call in two different reads would be $3 \times \left(\frac{1}{16}\right)^{2}$, where $p$ is the probability of an erroneous base call. By setting a threshold on the quality scores ($p$) of the sites analyzed, we can control the rate at which sequencing errors introduce false positives into our analysis.

To evaluate whether the Illumina quality scores do indeed yield correct estimates of the probability of erroneous base calls, we developed our own method of estimating sequencing error rates. With paired-end sequencing of short mRNA fragments, most pairs of reads contain a region of overlap that is sequenced from both ends. Within this region, sequencing errors are revealed as different base calls between the left and right reads. Assuming that the erroneous read is the one containing a different base call than that in the reference genome, we can directly discriminate erroneous from correct base calls and therefore estimate the sequencing error rate for the different values of quality scores. This analysis clearly showed that the Illumina quality scores tend to overestimate the probability of erroneous base calls (Table S1) and therefore can safely be used to estimate an upper limit to the number of false positives introduced by sequencing errors.

Estimating the Reverse Transcriptase Error Rate. Sequencing errors are not the only possible source of false positives. Reverse transcription of mRNA fragments into cDNAs introduces errors into the newly generated cDNAs at a certain rate. In the extreme case in which the same error is introduced at the same position into every cDNA in a family, these parallel RT errors would be mistaken for a transcription error and contribute false positives
to our analysis. To estimate the number of false positives contributed by parallel RT errors, we sought to estimate the reverse transcriptase error rate. With the exception of extremely rare cases where the same RT error occurs in multiple cDNAs within a family, RT errors should be characterized by mismatches present in only one cDNA of families of size 2 or 3. To avoid contamination by sequencing errors, we focused on sites for which the probability of an erroneous base call (computed as described above) was less than 1 × 10⁻⁶. Note that this number is less than the probability corresponding to the best possible quality score, so that only positions covered by both reads in a pair can be used here. RT error base substitutions were found at a rate of 1.14 × 10⁻⁶ (±6.4 × 10⁻⁷, 95% confidence interval). For each of the 12 possible base substitutions, we computed the conditional RT error rate and obtained the full molecular spectrum of RT error base substitutions (Fig. S1). The most common type of RT error corresponds to G → A base substitutions, which occurs at a conditional rate of 1.29 × 10⁻⁶ (Fig. S1). Therefore, the probability of observing the same G → A RT error in two cDNAs from the same family is (1.29 × 10⁻⁶)² = 1.65 × 10⁻¹². For all other possible base substitutions, the probability of observing the same error twice in two cDNAs is less than 10⁻¹⁸.

Thus, because the transcription error rate is expected to be on the order of 10⁻⁶ to 10⁻², it is unlikely that RT errors contribute a significant number of false positives as long as the analysis is restricted to families of size 2 and more. This is confirmed by our observation that within 17,300 size 3 families, we never observed a case where two cDNAs contained a base substitution while the third one contained the same base call as in the reference genome, which would be the signature of parallel RT errors.

Transcription Error Rate and Spectrum of Caenorhabditis elegans. To demonstrate the feasibility of the proposed technique, we applied it to the transcriptome of C. elegans. We obtained cDNAs from both wild-type (N2) nematodes and an RNA-editing deficient strain (RB886) to disentangle transcription errors from RNA-editing events (26). Because some transcription errors might result in degradation of the corresponding mRNAs by nonsense-mediated mRNA decay (NMD) (27), we also obtained cDNAs from a NMD-deficient strain (VC1305). The three corresponding cDNA libraries were prepared according to the method described in Fig. 1 and sequenced on an Illumina HiSeq 2000, producing a total of 766,868,947 101-nt-long paired reads. Reads were aligned to the reference genome, carefully filtered for coding regions, and grouped into families as defined previously. Reads that mapped to the mitochondrial genome were discarded to avoid confusing transcription errors from faithful transscripts at any potential heteroplasmic sites. This yielded a total of 38,411,057 size 1, 168,094 size 2, and 14,905 size 3 families (Table S2). Informativ families (i.e., size 2 and size 3) represent only about 0.5% of all families, which probably reflects the fact that only a fraction of the total cDNAs generated were sequenced. Although deeper sequencing would certainly help increase the fraction of informative families, we show here that the transcription error rate can still be estimated without the need of extremely deep sequencing.

For every family, we built a consensus sequence by selecting only the aligned positions that fulfilled three criteria: (i) in a family of size 2 or 3, (ii) the same base call for all reads, and (iii) a probability of all base calls to be erroneous of <10⁻⁶ (computed from the quality score). After filtering for potentially polymorphic sites (see below), consensus sites with a base call different from the reference genome were considered as transcription errors. We found a total of 6, 25, and 52 transcription errors in the wild-type, RNA editing-deficient, and NMD-deficient strains, respectively, yielding base substitutional transcription error rates of 2.2 × 10⁻⁶, 3.3 × 10⁻⁶, and 5.2 × 10⁻⁶ per site, respectively (Tables S3 and S4). These three rates are not significantly different from each other (P > 0.1 for all two-by-two comparisons, χ² test), indicating that the transcription errors recovered in this analysis are not likely to be by-products of RNA-editing processes and that the removal of error-containing transcripts by NMD, if any, is below our statistical power of detection. Because only the small fraction of error events that produce a premature stop codon can be detected by NMD, the expected increased error rate in the NMD-deficient strain is likely to be on the order of only a few percent and will require a much deeper analysis to be detected. Although we found that 2 out of the 52 base substitutions in the NMD-deficient strain produced a premature termination codon (PTC), this was not significantly different from the 1 out of 31 PTC-creating errors observed in the combined data from the two NMD-capable strains (P > 0.1, Fisher’s exact test).

Although further in-depth analysis may reveal significant differences between these three strains, our results indicate that their transcription error rates are similar enough that the combined data from all three strains should be representative of the overall C. elegans transcription error rate. This leads to an overall transcription error rate estimate of 4.1 × 10⁻⁶ (±1.4 × 10⁻⁶, 95% confidence interval), whereas U = 18 watts of families with size 2 or more. This is in agreement with previous observations from a random distribution (P = 0.2, χ² test). In addition, within coding regions, transcription errors were observed at the first, second, and third positions of codons at frequencies not significantly different from the random expectation of one-third at each position (21, 21, and 23 errors at positions 1, 2, and 3, respectively; P = 0.9, χ² test).

An important advantage of our method over reporter constructs and in vitro assays is the specific identification of the types of errors that occur, which reveals the full molecular spectrum of transcription errors. We found no significant difference between the three molecular species (W > 18 watts of families with size 2 or more. This is in agreement with previous observations from a random distribution (P = 0.2, χ² test). In addition, within coding regions, transcription errors were observed at the first, second, and third positions of codons at frequencies not significantly different from the random expectation of one-third at each position (21, 21, and 23 errors at positions 1, 2, and 3, respectively; P = 0.9, χ² test).

Insertions and Deletions. In addition to generating base substitution errors, DNA-dependent RNA polymerases can also erroneously skip (deletion) or add (insertion) extra nucleotides in the nascent transcripts. As above, before measuring the RNA polymerase insertion/deletion (indel) error rate, we first need to control the rate of false-positive indels introduced by multiple sequencing or RT errors. We again used the overlapping regions of paired-end reads to detect sequencing indel errors. Using the same method as applied to base substitution errors, we estimated the sequencing indel error rate to be 1.7 × 10⁻⁶ (±1.4 × 10⁻⁶, 95% confidence interval). This indicates that sequencing indels introduce false positives at a rate of (1.7 × 10⁻⁶)² = 2.9 × 10⁻¹⁰ for families with two informative reads and less than 10⁻¹⁰ for families with more than two informative reads.

Indels introduced by the reverse transcriptase (RT indels) at the same position in two cDNAs also have the potential to create
false positives. We searched for RT indels, reasoning that indels present in both reads of a pair but not in the other reads from the same family are caused by RT errors. The resulting RT indel error rate is $8.8 \times 10^{-6}$ ($\pm 1.4 \times 10^{-6}$, 95% confidence interval). Therefore, assuming that RT indels are randomly distributed, the probability of observing the same RT indel in two reads of a family is $(8.8 \times 10^{-6})^2 = 7.8 \times 10^{-11}$. Given these observations, indels present in all reads of size 2 and size 3 families were considered as being present in the mRNA and therefore corresponding to transcription indels. We found a total of 26 indels (18 insertions and 8 deletions; Table S5), yielding a transcription indel rate of $1.2 \times 10^{-5}$ ($\pm 1.6 \times 10^{-6}$, 95% confidence interval). One potential consequence of indels in mRNAs is creation of a frameshift in the ORF, leading to the appearance of premature stop codons in the transcript. Because at least some transcripts containing premature termination codons are expected to be degraded by NMD, the indel error rate estimate that we provide is likely biased downward, except in the NMD-deficient strain. However, although the indel error rate measured in the NMD-deficient strain is slightly higher than the average from the two other strains, this difference was not statistically significant ($1.5 \times 10^{-6}$ vs. $1.0 \times 10^{-6}$ for NMD-deficient and NMD-capable strains, respectively; $P = 0.5$, $\chi^2$ test). All of the transcription indels reported here are one nucleotide in length (see Table S6 for a list of transcription indels). We also observed that transcription indels tend to occur in homopolymeric nucleotide runs (Table S6). It has been previously reported that genomic indels in C. elegans are dominated by insertions and tend to occur in homopolymeric nucleotide runs (31, 32), again suggesting that strong parallels exist in the types of errors generated by DNA and RNA polymerases.

**Potential Sources of False Positives.** To validate the results presented in this study, artifacts (other than convergent sequencing/RT errors) that might produce the illusion of transcription errors have to be ruled out. Somatic mutations can produce transcripts whose sequences in the affected cell lineages would differ from the reference genome and would be viewed as transcription errors in our analysis. However, in order for 5% of our inferred base substitution transcription errors to actually be somatic mutation-derived false positives, somatic mutations would have to occur at a frequency of $\sim 2 \times 10^{-3}$ per site per cell, which would correspond to a rate of $\sim 2 \times 10^{-8}$ mutations per site per cell division (assuming an average of 10 cell divisions from the embryo to the adult worm). This is $\sim 30x$ the estimated mutation rate per cell division in the C. elegans germ line (9). Based on the observation that somatic mutations rates per cell division are less than $30x$ that of the germ line in humans (9), we can reasonably infer that a difference of more than $30x$ in C. elegans is unlikely and therefore that somatic mutations do not contribute more than 5% of false positives to our estimation of the transcription error rate.

Although C. elegans is self-fertilizing, polymorphic sites also have the potential to produce faithful transcripts that would be mistaken for transcription error-containing mRNAs compared with the reference genome. However, such aberrations are easily detected because 100% (50% if heterozygous) of the families covering such positions would show a mismatch. Therefore, we retained only sites covered by at least 20 families and for which more than 95% of the families support the reference genome base call. We also sequenced the genomic DNA from the same worms that were used to search for transcription errors and mapped these genomic reads against the genomic regions surrounding each of the 83 transcription errors found in this study (Materials and Methods). Out of the 3,547 genomic DNA reads mapped, only 2 supported a base call matching the inferred transcription error, which is exactly the number expected simply from sequencing errors in the genomic DNA reads based on our previous estimate of sequencing error rates (Materials and Methods). This strongly suggests that the transcription errors inferred in our study are not false positives caused by genomic mutations.

We also used genomic DNA reads to search for the presence of inferred transcription indels within the DNA of the worms used in this analysis. We found that 5 out of the 531 mapped genomic DNA reads spanning the position of an inferred transcription indel contained the inferred transcription indel. Based on our estimation of the sequencing indel error rate ($1.7 \times 10^{-5}$ per site), we expect to observe zero ($531 \times 1.7 \times 10^{-5} = 0.01$) such indel-containing genomic DNA reads. The excess of indel-containing genomic DNA reads might be partially explained by an elevated sequencing indel error rate within homopolymeric nucleotide runs. Indeed, these five genomic DNA reads map to only two inferred indels, both of them falling within a large homopolymeric run (9 and 11 nt). However, it is also possible that a fraction of the transcription indels inferred in our study are false positives caused by indels present at low frequency in the genomic DNA of the worms sequenced in this analysis. Therefore, although we are confident that our analysis provides an accurate estimation for the upper limit of the transcription indel error rate.

**Fig. 2.** Molecular spectrum of transcription errors. The values shown in this graph are conditional error rates (i.e., $A \to U$ gives the probability of a U to be inserted at a position where an error-free mRNA should contain an A) for all ($n = 96$) transcription errors detected. Error bars represent the 95% confidence interval of the error rate.

**Fig. 3.** Comparison of the genomic and transcription base substitution spectra. The genomic base substitution data are from ref. 41. The total numbers of base substitutions are $n = 448$ for genomic mutations and $n = 96$ for transcription errors. Because genomic mutations are not polarized (an A-to-G mutation is equivalent to a T-to-C mutation), transcription errors are merged into groups of complementary mutations to compare them to genomic mutations. This graph shows, for all possible types of transcription and genomic base substitutions, the fraction of base substitutions of a given type. For example, the blue bar A $\to$ G shows that $\sim 24\%$ of all transcription errors are A $\to$ G or T (U) $\to$ C errors.
rate, the actual rate might be lower than 1.2 × 10^{-6} indels per site.

Finally, RNA editing (26, 33, 34), a site-specific posttranscriptional process that can deaminate mRNA adenosines to inosine, which is then recognized as a guanosine, could be confused with transcription errors. However, if RNA editing was interfering with our analysis, we would observe an abnormally high level of A → G transcription errors, which is not the case (Fig. 2). Also, we did not observe any significant difference in the transcription error rate and pattern in the editing-deficient strain compared with the wild-type and the NMD-deficient strains (Table S4). It is very unlikely that other types of RNA editing exist in C. elegans [with the exception of very rare C → U changes (35)], and even if such a mechanism existed, our requirement that 95% of the sequenced mRNAs show the same base call as in the reference genome would remove all sites that are systematically edited.

Discussion

A Unique Method for Detecting Transcription Errors. In this study, we have described a unique cDNA library preparation technique and the associated bioinformatic analyses that allow for detection of transcription errors in RNA-seq data. To demonstrate the feasibility of our method, we applied it to the transcriptome of C. elegans and detected dozens of transcription errors, yielding a base substitution transcription error rate of ∼4 × 10^{-6}. Our limitation of our method is that it cannot discriminate misincorporations by the RNA polymerase from posttranscriptional RNA modifications. The observation that the most common type of transcription error is a C → U base substitution suggests that a fraction of the errors observed could be due to cytosine deamination, rather than base misincorporation by the RNA polymerase. However, even if this were true, it would still be the case that the total error rate (misincorporation + posttranscriptional modifications) in mRNAs at the time of translation is being counted. The Messenger RNA fragmentation in C. elegansMessenger RNA fermentation. Messenger RNA was fragmented using the RNA Fragmentase system (Catalog ID: E61465; New England BioLabs), per manufacturer’s specifications. Fragmented mRNA (in 100 μL water) was ethanol-precipitated with 2 μL of 5 mg/mL Glycogen (Ambion; Life Tech), 10 μL of 5M sodium acetate pH 5.3, and 300 μL of absolute ethanol (chilled). Samples were ethanol precipitated at −80 °C for 1 h, followed by spinning at 15,000 × g for 45 min at 4 °C. Pellets were washed twice with chilled 70% ethanol (vol/vol) and resuspended in a final volume of 5 μL RNase-free water.

Sequencial adaptor ligation. The 5′ adaptor used in these libraries is the standard Illumina TruSeq Small RNA 5′ Adaptor. Ligation of the 3′ adaptor was done in accordance with the Illumina TruSeq Small RNA Sample Prep kit (Illumina). Specifically, 5 μL of fragmented mRNA was mixed with 1 μL of the Illumina TruSeq 3′ adaptor and incubated at 70 °C for 2 min before being placed on ice. We then added 5 μL of the TruSeq Fragmentation buffer and 1 μL of T4 RNA ligase 2 truncated (NEB) followed by incubation at 28 °C for 1 h. Reaction was stopped with 1 μL stop solution followed by incubation at 28 °C for 15 min before being placed on ice. The 5′ adapter is a modified version of the Illumina TruSeq Small RNA 5′ adaptor (Integrated DNA Technology). The modification includes a 5′ Biotin and eight N bases on the 3′ end of the oligonucleotide (5′-BioGurUrCrArGurUrCrArGurUrCrArGurUrCrArGurUrCrArGurCrGurCrGurCrGurCrGurCrGurCrN3′). Ligation included 1 μL of the modified RAS adapter (100 μM stock), 1 μL of ATP as supplied by Illumina, and 1 μL of T4 RNA Ligase 2 (NEB). This reagent mix was added to the 3′ adapted mRNA, and the sample was incubated at 28 °C for 1 h followed by incubation at 4 °C. Sequentially adapted libraries were then purified using RNA MinElute columns (Qiagen) per the manufacturer’s protocol and eluted in 10 μL RNase-free water. Samples were stored overnight at −80 °C.

Reverse transcription cycling. To capture the products of each RT reaction in separate indexed libraries, we used the biotinylated 5′ ends of the constructs to allow for separation of the RT products after each reaction and transfer of template RNA to a subsequent round of RT. For the first round of reverse transcription the sample was mixed with 1 μL of Illumina TruSeq Small RNA primer and incubated at 70 °C for 2 min, followed by placing the sample on ice. An addition of RT reaction mix and incubation at 25 °C for 30 min. During the incubation, 50 μL of Dyna M270 streptavidin beads (Life Tech) were prepared for RNA use as described in the manufacturer’s protocol by washing the beads for 2 min with an equal volume of Diethylpyrocarbonate.
We generated transcription error-containing DNA reads containing the inferred base substitution because of sequencing error, we extracted, for all of the mapped reads, their base call quality score at the position of the inferred base substitution and converted the base quality into the probability that the base call is wrong according to the observed error rate reported in Table S1. We then summed these probabilities for all mapped reads and divided the total number by 3, assuming that all three possible errors at a given position are equiprobable.

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