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Large Ribosomal Protein 4 Increases Efficiency of Viral Recoding Sequences

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Expression of retroviral replication enzymes (Pol) requires a controlled translational recoding event to bypass the stop codon at the end of *gag*. This recoding event occurs either by direct suppression of termination via the insertion of an amino acid at the stop codon (readthrough) or by alteration of the mRNA reading frame (frameshift). Here we report the effects of a host protein, large ribosomal protein 4 (RPL4), on the efficiency of recoding. Using a dual luciferase reporter assay, we found that transfection of cells with a plasmid encoding RPL4 cDNA increases recoding efficiency in a dose-dependent manner, with a maximal enhancement of nearly twofold. Expression of RPL4 increases recoding of reporters containing retroviral readthrough and frameshift sequences, as well as the Sindbis virus leaky termination signal. RPL4-induced enhancement of recoding is cell line specific and appears to be specific to RPL4 among ribosomal proteins. Cotransfection of RPL4 cDNA with Moloney murine leukemia proviral DNA results in Gag processing defects and a reduction of viral particle formation, presumably caused by the RPL4-dependent alteration of the Gag-to-Gag-Pol ratio required for virion assembly and release.

Translation is a tightly regulated process that is dependent upon interpretation of signals in the mRNA for proper initiation, synthesis, and termination of a nascent protein. The ribosomes and initiation and termination factors involved in this process are designed for accurate interpretation of these signals, resulting in a very low error rate during normal cellular translation. Viral mRNAs often contain sequences that can modulate the normal functions of the ribosome. Some animal virus mRNAs, such as those of poliovirus, hepatitis C virus, and cricket paralysis virus, contain RNA structures that direct the assembly of a translation-competent ribosome without the need for a complete set of initiation factors or a 5′ cap (via so-called internal ribosome initiation sequences [IRESs]) (6, 24). Other viral mRNAs contain features that allow modulation of the elongation process. In retroviruses, the *pol* gene, which encodes a polyprotein processed to yield several essential viral replication enzymes (protease, reverse transcriptase, and integrase), lacks its own translation initiation signals and start codon. As a result, Pol is produced only as a fusion product with the upstream Gag polyprotein (28, 30). Efficient production of progeny viruses requires a specific proportion of the replication enzymes to be synthesized and packaged with the structural Gag proteins (matrix [MA], capsid [CA], and nucleocapsid [NC]) and additional proteins (13, 40, 47).

The ratio of Gag to Gag-Pol proteins is specified by the efficiency of either one of two alternative recoding events wherein the *gag* stop codon is not recognized, allowing elongation into the *pol* reading frame about 3 to 5% of the time (39, 40, 42). Many retroviruses (e.g., HIV-1 [52], human T-cell leukemia virus [HTLV] [20], and mouse mammary tumor virus [MMTV] [38]) contain a frameshift element in their mRNA that consists of a slippery sequence upstream of the stop codon and a secondary structure in close proximity to the slippery sequence (23, 29, 44). Slippage of the ribosome on the mRNA during translation shifts the ribosome into a different reading frame, effectively bypassing the stop codon and allowing translation to continue into the downstream Pol reading frame (8, 41). Other retroviruses, including the feline and murine leukemia viruses, utilize stop codon suppression (43, 54, 55): the stop codon is misread by the ribosome as a coding sequence, and near-cognate codon-anticodon pairing allows insertion of an amino acid. As in frameshifting, a secondary mRNA structure immediately downstream of the stop codon is required for this event to occur. For Moloney murine leukemia virus (MoMLV), the structure is an RNA pseudoknot (21, 43, 50). Mutational studies have defined many of the important sequences that are important for the function of the pseudoknot (1, 14, 16, 51). Recently a detailed structure of the pseudoknot was determined by solution nuclear magnetic resonance (NMR), revealing two stems of duplex RNA connected by two single-stranded loops, a single-base loop I and an 18-nucleotide loop II (22). The RNA was found to exist as a mixture of two alternative structures in a pH-dependent equilibrium, and tests of translation *in vitro* showed that the efficiency of readthrough was also pH dependent. The results led to a proposed model in which the protonated form of the RNA was the active conformation for readthrough.

Very little is known about any proteins that may interact with these recoding signals to modulate recoding. To explore this issue, we screened for proteins that affect recoding and identified the large ribosomal subunit protein RPL4 as one such candidate regulator. Here we present a characterization of the effects of this protein on translational recoding events in model retroviral RNAs.

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MATERIALS AND METHODS

Yeast three-hybrid screen. A two-hybrid library of mouse cDNAs derived from NIH 3T3 cells fused to the Gal4 activation domain (3) was tested in a three-hybrid screen described previously (4). The RNA bridge consisted of the MoMLV pseudoknot (PK) RNA fused to the MS2 phage RNA stem-loop. Interactions between RNA bait and protein prey resulted in translation of the reporter genes that were detected by positive X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining.

Transfections of mammalian cells. Cells were plated at densities of 10,000 per well for 96-well plates or 250,000 per well for 6-well plates the day before transfection. Cells were transfected with Fugene 6 transfection reagent (Roche) at a ratio of 3 μl Fugene 6 to 1 μg DNA and harvested for dual-luciferase assays, quantitative PCR (qPCR), or Western blotting 24 h posttransfection. For assays conducted in 96-well plates, cells were transfected with 100 ng of reporter DNAs and 60, 120, or 240 ng of DNAs expressing ribosomal proteins, in triplicate. Transfection mixes contained 340 ng of DNA per well, with 1.0 μl Fugene and Optimem serum-free medium for a total volume of 15 μl/well. For assays conducted in 6-well plates, cells were transfected with 1 μg of reporter DNA and 1, 3, or 6 μg ribosomal protein expression vector. Transfection mixes contained 7 μg of total DNA, with 21 μl Fugene 6 in Optimem serum-free medium for a total volume of 100 μl/well. All transfections were adjusted with empty vector DNA to contain the same total amount of DNA per well.

Dual-luciferase assay. Lysates were assayed for firefly and *Renilla* luciferase activity successively on an automated plate reader. Cell lysates from transfections were transferred to opaque 96-well plates. For transfections done in the 96-well format, cells were lysed with 20 μl passive lysis buffer, and 15 μl from each well was transferred to opaque 96-well plates. For assays performed in 6-well plates, cells were lysed with 150 μl passive lysis buffer, and 20 μl from each well was transferred to opaque 96-well plates. Dual-luciferase assays were performed with a Promega dual-luciferase kit with buffers diluted 1:4 or 1:2 with distilled water (dH_2O). Assays were read on a Berthold or Omega plate reader using 100 μl of luciferase reagent and reading for 10 to 12 s. Values from each interval of firefly and *Renilla* luciferase were summed in the Omega Mars data analysis program and exported to a spreadsheet program (Excel or Numbers) for analysis. Percent recoding was calculated as [(fluc wild-type/rluc wild-type) × (rluc control/fluc control)] × 100, where "fluc" represents firefly luciferase signal, "rluc" represents renilla luciferase signal, "wild-type" represents signals from expression of plasmids containing the native recoding sequence and "control" represents signals from plasmids designed to eliminate recoding elements and allow maximal expression of both luciferases.

Plasmid DNAs. Recoding sequences containing a stop codon and the requisite downstream sequences of the *gag-pol* junctions of MoMLV (MoMLV-PK) and HIV-1 (HIV1-FS), the UGA C readthrough sequence of Sindbis virus (Sindbis-RT), the scrambled MoMLV pseudoknot (ScrPK), and MoMLV pseudoknot mutants MoMLV-UGA, MoMLV-UAA, MoMLV-U38C, and MoMLV-U38A, and the corresponding nonrecoding (no stop codon) control sequences were cloned into p2luc (17) (a gift from G. Grentzmann, University of Utah) between the *Renilla* and firefly luciferase coding sequences. Mouse RPL4 and RPS3a cDNAs were cloned into pcDNA4-myc-His-A and pFlag-2 M. Myc-tagged ribosomal proteins and human RPL4 cDNAs were cloned into pcDNA3.1-myc-His-A. Cloning was completed using sequence and ligation-independent cloning (SLIC) protocols adapted from reference 34. All cloning products were verified by DNA sequencing.

qPCR assay for mRNA levels. Primers were designed using Primer3 software to specifically detect mouse RPL4 mRNA, spanning the junction of exons 3 and 4. HEK 293A cells were transfected with the mRPL4 expression vector, and mRNA was extracted using TRIzol according to the manufacturer's instructions and reverse-transcribed into cDNA with a Superscript III first-strand synthesis kit for RT-PCR (Invitrogen). cDNA was mixed with Fast Start SYBR green master mix (Roche) according to the manufacturer's instructions and analyzed for levels of mRPL4 and 18S rRNA using Ambion Quantum RNA universal 18S primers. Assays were run on a 7500 fast real-time PCR system (Applied Biosystems).

Assays for production and release of virion particles. To monitor effects of mRPL4 on virus production, 293T cells were transfected with 1 μg pNCS DNA, containing a complete MoMLV provirus, and 1, 3, or 5 μg DNAs encoding mRPL4 or mRPS3a and lysed with RIPA (radioimmunoprecipitation assay) buffer containing protease inhibitors 48 h posttransfection. Supernatants of transfected cells were clarified through a 0.45-μm filter. Virus was pelleted from clarified supernatants by ultracentrifugation through 20% sucrose–phosphate-buffered saline (PBS) at 25,000 rpm in a Beckman SW-55 rotor for 2 h and recovered in 40 μl sample buffer containing sodium dodecyl sulfate (SDS). Protein levels of cell lysates were normalized by a Bradford assay.

Western blots. Flag-tagged mRPS3a and mRPL4 were immunoprecipitated with rabbit anti-Flag antibodies conjugated to protein G beads from lysates of 293T cells transfected with N-terminal Flag-tagged expression constructs. Beads were boiled for 15 min with 1× sample buffer, run on 12% polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes activated with methanol and equilibrated in phosphate transfer buffer. Western blots were probed with mouse anti-Flag and visualized with horseradish peroxidase-labeled anti-mouse antibody. Lysates of 293T cells transfected with the MoMLV infectious clone pNCS were normalized for total protein concentration via Bradford assay and boiled for 5 min with 5× sample buffer. Purified virions were boiled for 5 min with 1× sample buffer. Proteins were separated on 12% polyacrylamide gels, transferred to PVDF membranes activated with methanol, and equilibrated in phosphate transfer buffer. Western blots were probed with (R187) rat anti-CA monoclonal antibody, mouse anti-actin (Sigma 1978) for loading controls for cell lysates, and species-appropriate fluorescent antibodies from Li-Cor. Bands were visualized on an Odyssey imaging system (Li-Cor).

RESULTS

To identify cellular proteins that might modulate readthrough, we conducted a yeast three-hybrid screen for host proteins that could interact with the MoMLV pseudoknot RNA. This screen tethers an RNA bridge to the reporter gene via a LexA binding protein fusion and allows detection of RNA binding proteins in a library. The RNA bridge consisted of the pseudoknot fused to tandem MS2 coat protein binding sites expressed in plasmid pIIIA/MS2-2 (a gift from M. Wickens, University of Wisconsin, Madison, Wisconsin) (46). The DNA was introduced into yeast strain L40-coat, constitutively expressing an MS2 coat protein-LexA DNA binding domain fusion protein, along with a pooled plasmid library consisting of mouse cDNAs fused to the Gal4 activation domain (3, 5). Colonies were screened for LacZ expression by colony filter staining with X-Gal. Out of more than 10^6 colonies screened, approximately 200 candidates were identified. Of these initial isolates, two clones showed very high reporter activity and were chosen for further study. One encoded a C-terminal fragment of large ribosomal protein 4 (mRPL4) and the other the small ribosomal protein 3a (mRPS3a). Both mRPL4 and mRPS3a also demonstrated high nonspecific binding to control RNAs.

mRPL4 enhances readthrough at the MoMLV *gag-pol* junction. To test for effects mRPL4 might have on readthrough efficiency, we employed a dual-luciferase assay (DLA) in a two-plasmid reporter system (18). The region containing the MoMLV *gag* stop codon and pseudoknot sequence (MoMLV-PK) was cloned into plasmid p2luc between *Renilla* and firefly luciferase coding sequences, thus allowing expression of the downstream firefly luciferase only when the readthrough event had occurred. To control for effects on overall translation, and to allow normalization

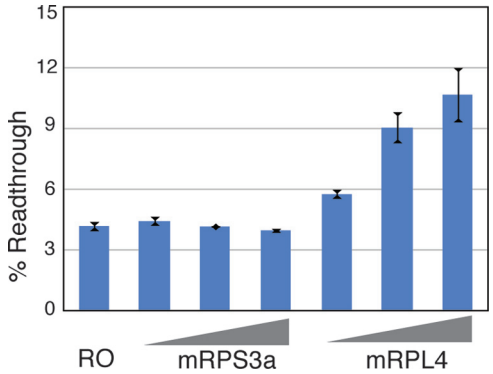

FIG 1 mRPL4 effects on readthrough of MoMLV *gag-pol* junction. Dual luciferase analysis of readthrough of MoMLV pseudoknot and control reporter constructs and 60 ng, 120 ng, or 240 ng/well of expression plasmids for mRPS3a or mRPL4, transfected into HEK 293A cells in 96-well format. RO (reporter only) indicates transfection of cells with MoMLV pseudoknot and control luciferase constructs and 240 ng of empty expression vector to determine basal readthrough. Error bars correspond to standard deviations of averages derived from three wells transfected with either wild-type or control reporter.

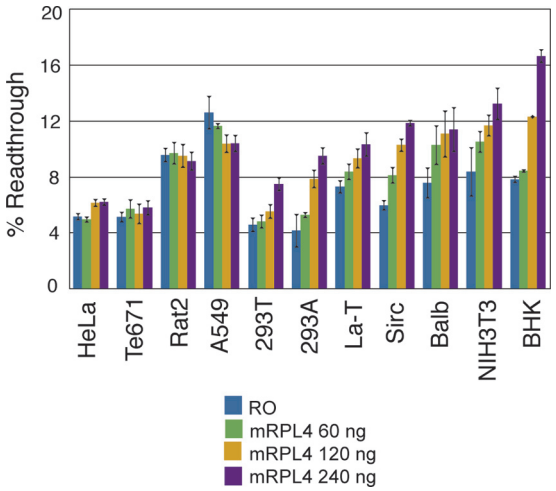

FIG 2 mRPL4 effects on MoMLV *gag-pol* readthrough in various cell lines. Dual-luciferase analysis of readthrough of MoMLV pseudoknot and control reporter constructs transfected into rodent, rabbit, and human cell lines was carried out with increasing amounts of mRPL4 cDNA expression construct. Data are averages for two to four experiments for each cell line.

of basal readthrough levels independently of cell line or transfection efficiency, we also constructed a control plasmid that contained the same MoMLV sequence with a glutamine codon replacing the wild-type stop codon. This control plasmid was transfected in parallel on separate cells in each experiment. Cell lysates were prepared 24 h posttransfection and assayed successively for the two luciferase enzymes. The efficiency of readthrough was determined by measuring the ratio of firefly to *Renilla* luciferase and normalizing to the ratio obtained with the glutamine codon full-readthrough control DNA (see Materials and Methods). A series of plasmids containing various lengths of sequences upstream of the *gag* stop codon and after the pseudoknot structure were tested in this system. A plasmid containing 13 codons before the stop codon and 6 codons downstream from the pseudoknot (G ACC CTA GAT GAC **TAG** GGA GGT CAG GGT CAG GAG CCC CCC CCT GAA CCC AGG ATA ACC CTC AAA GTC GGG GGG CAA CCC GTC; the *gag* stop codon is in bold) gave readthrough efficiencies similar to those obtained with larger inserts and was used as our standard wild-type construct.

To investigate whether the introduction of mRPL4 into cells affected readthrough, human embryonic kidney 293A cells were transfected with plasmids expressing mouse RPL4 along with the MoMLV-PK or control reporter plasmids, and lysates were prepared and assayed for luciferase activities. The results showed a dose-dependent increase in the amount of readthrough that correlated with the amount of mRPL4 DNA transfected (Fig. 1). A DNA construct expressing small ribosomal protein 3a (mRPS3a), a second hit in the yeast three-hybrid screen, was tested in parallel. Transfection with the mRPS3a DNA did not result in an increase in readthrough. The raw values for firefly and *Renilla* luciferases indicate that the transfection of ribosomal cDNAs did not significantly affect the overall translation levels of reporter constructs (see Fig. S1 in the supplemental material). Attempts at reducing the levels of mRPL4 by RNA interference (RNAi)-mediated knockdown were unsuccessful due to toxic effects on the cells (data not shown).

mRPL4 effects on readthrough are cell line dependent. To survey the range of cells in which the enhancement of readthrough occurred, we tested a number of cell lines derived from several species and tissue types. mRPL4 increased readthrough in many, but not all, cell lines tested (Fig. 2). Differences were not attributable to species or lineage of cell types. Some cell lines of both mouse and human origin responded to mRPL4 transfection with increased readthrough of the reporter, indicating that the effect seen in 293A cells was not attributable solely to a mouse protein being overexpressed in a human line. mRPL4 did not increase readthrough in several human cell lines tested (HeLa, Te671, and A549), nor in Rat2 fibroblasts, nor in other mouse lines tested (data not shown). Variations in absolute values of luciferase signals indicated that transfection efficiencies or expression levels did vary from cell to cell (see Fig. S2 in the supplemental material), but there was no significant correlation between basal reporter levels and responsiveness to mRPL4. Many responding and nonresponding lines showed similar basal levels, and all signals were well above background and within the linear range of the assay.

The basal levels of readthrough varied over a wide range among the cell lines surveyed (Fig. 2). Among the nonresponding lines, basal readthrough ranged from 5% to more than 10%; among the responding cell lines, basal readthrough varied from 3% to 7%. Thus, the lines in both groups varied over a twofold range in the levels of basal readthrough. All cell lines were tested with mRPS3a as a negative control and showed no increase in readthrough (data not shown). The variety in responses suggests that readthrough may be modulated by other unknown cellular factors and is not purely a *cis*-acting function of the pseudoknot-containing viral mRNA.

Mouse and human RPL4 enhance MoMLV *gag-pol* readthrough. RPL4 is a highly conserved protein across kingdoms, and the overall sequence is very similar among mammals. The N-terminal region from amino acids 1 to 350 is nearly identical between mice and humans (residues 170 and 208 are glutamate and methionine, respectively, in mice and leucine and isoleucine in humans). The C-terminal 17 amino acids, as well several other lysine-containing regions close to the C terminus, are identical. To

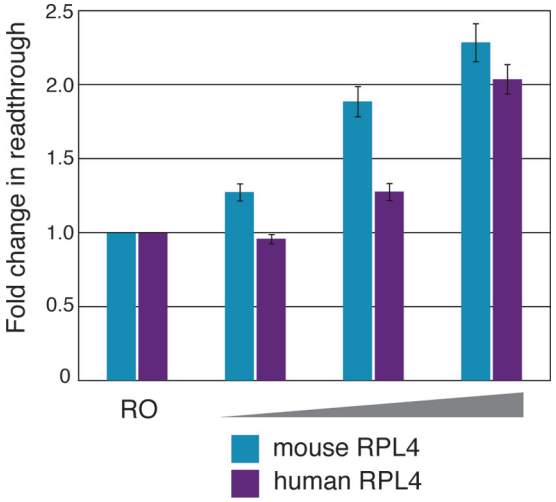

FIG 3 mRPL4 and hRPL4 induced increase of MoMLV *gag-pol* readthrough. Fold change in readthrough of MoMLV pseudoknot and control reporter constructs transfected into HEK 293A cells with 60 ng, 120 ng or 240 ng/well of mouse RPL4 (mRPL4) or human RPL4 (hRPL4) cDNA expression constructs is shown. Data are from a single representative experiment. Fold change was calculated as readthrough level in transfected samples divided by basal readthrough level of control cells.

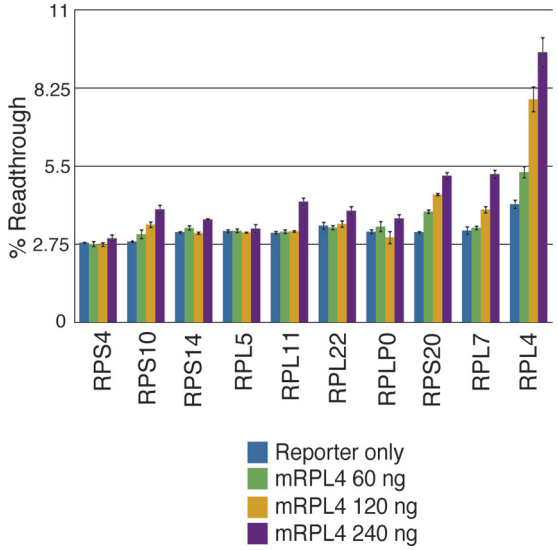

FIG 4 Survey of effect of additional ribosomal proteins on MoMLV *gag-pol* readthrough. Readthrough percentages based on dual-luciferase assay in HEK 293A cells transfected with various mouse ribosomal protein cDNAs. Data are from a representative experiment of at least three assays with each protein. Error bars correspond to standard deviations of averages derived from triplicate luciferase readings of cell lysates.

compare the activity of human and mouse RPL4, we generated an expression construct for the human version (hRPL4) and transfected it into 293A cells with the DLA reporter constructs. Readthrough was also increased by hRPL4, although to a lesser degree than by mRPL4 (Fig. 3). The raw levels of firefly and *Renilla* luciferase were affected similarly by mouse and human RPL4 (see Fig. S3A and B in the supplemental material).

mRPL4 enhancement of readthrough is distinctive among ribosomal proteins. To address whether the increase in readthrough with mRPL4 was a general consequence of overexpression of a ribosomal protein, we repeated the dual-luciferase assay with additional ribosomal proteins. The proteins chosen included several with known extraribosomal functions, typically positioned on the surface of the ribosome, and also some with native positions deep within the ribosome. Transfection of 293A cells with cDNAs encoding these proteins resulted in no significant increase in readthrough, in marked contrast to the level of enhancement seen with mPRL4 (Fig. 4). Constructs for mRPS20 and mRPL7 induced smaller, less striking increases in readthrough.

In an attempt to verify that the various ribosomal protein cDNAs were expressed, plasmids encoding C-terminally Myc-tagged versions of all proteins were constructed and tested in the DLA system. Addition of the C-terminal Myc tag to mRPL4 abolished its enhancement of readthrough and reduced the slight increase seen with mRPS20 and mRPL7. Furthermore, expression of the Myc-tagged proteins, with the exception of mRPL22, was undetectable by Western blotting. The addition of the tag may inhibit proper folding of the proteins and promote their subsequent degradation. Alternatively, the expression of many of these ribosomal proteins is known to be tightly regulated (31, 37, 49, 57), and feedback controls may prevent the accumulation of high protein levels *in vivo*. With the exception of the acidic stalk protein RPLP0, transfection of overexpression constructs of all the proteins did not cause decreases in the raw reporter luciferase values (see Fig. S4 in the supplemental material) or lead to apparent cell toxicity.

To monitor the expression levels of the key ribosomal proteins, we prepared constructs encoding N-terminally Flag-tagged versions of mRPL4 and mRPS3a. The proteins expressed from these constructs were also hard to detect, though immunoprecipitation followed by Western blotting allowed their visualization (Fig. 5A). Tests of the activity of these constructs to promote readthrough, however, showed that the addition of this tag also abolished enhancement. In an attempt to avoid the use of tags, we obtained several commercial antibodies specific for human and mouse RPL4 and examined lysates by Western blotting. We could not detect a significant increase in RPL4 protein levels in response to transfection of increased amounts of DNA constructs expressing either mouse or human RPL4, again suggesting that the proteins are tightly regulated (data not shown).

As a surrogate for RPL4 protein, we examined mRPL4 RNA levels in human 293A cells transfected with mRPL4 DNA by q-RT-PCR. Primers were designed to match a region unique to mouse RPL4 in order to specifically detect transfected mouse RPL4 and not endogenous human RPL4. Transfection of increasing levels of mRPL4 cDNA resulted in proportional increases in levels of mRPL4 mRNA accumulating in the cells (Fig. 5B). Therefore, we can at least be confident that mRPL4 transcripts are produced from the plasmid used. Similar experiments in NIH 3T3 cells resulted in an increase in mRNA levels over the basal levels in untransfected cells of at least fourfold (data not shown).

Since we observed an increase in mRNA, and because of the difficulty in visualizing overexpression of protein, we considered the possibility that the effect on readthrough could be an RNA-induced effect. RPL4 regulates its own transcription in bacteria (59, 60), and we hypothesized that the increased levels of transcript may not lead to increased levels of protein and that the RNA itself could interact with the translation machinery to enhance

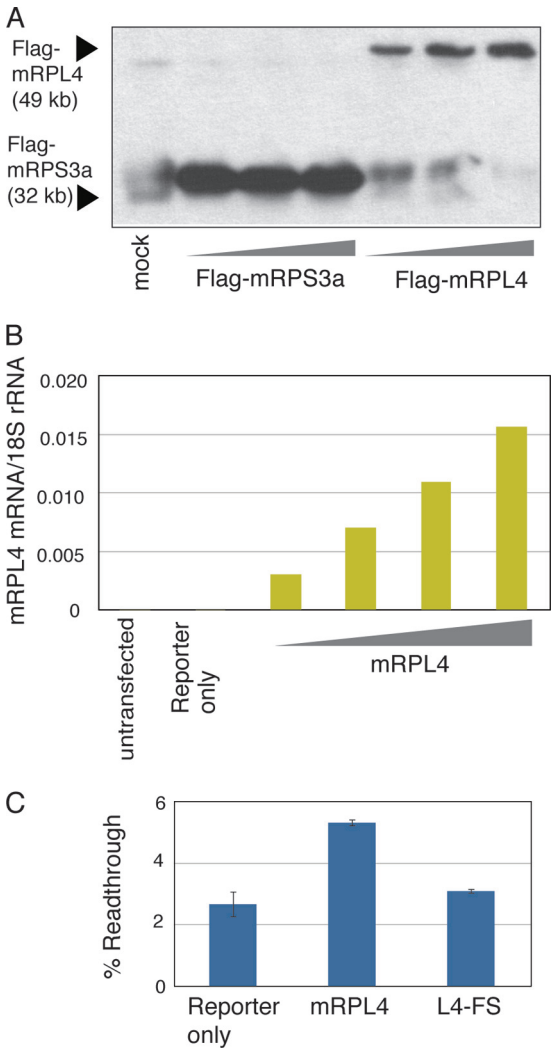

FIG 5 Expression of mRPL4 mRNA. (A) Western blot analysis of lysates of 293T cells transfected with N-terminal Flag-tagged mRPS3a and mRPL4 cDNAs, probed with anti-Flag antibody. (B) qPCR results of mRPL4 levels after cDNA transfection of 293A cells, normalized to 18S rRNA. Cells were transfected with 1, 3, 6, or 9 μg/well of mRPL4 cDNA expression construct. (C) Dual luciferase analysis of MoMLV readthrough in HEK 293A cells transfected with mRPL4 cDNA expression construct or the L4-FS construct and the MoMLV pseudoknot reporter pair. The data are from a representative experiment. Error bars correspond to standard deviations of averages derived from triplicate luciferase readings of cell lysates.

readthrough. We designed an mRPL4 frameshift (L4-FS) mutant by deleting the 11th nucleotide from the coding sequence, thus altering the reading frame downstream of the deletion. This produces an mRNA which retains the overall secondary structure of the native sequence but, because of the shift, does not encode the mRPL4 protein. Transfection of the L4-FS DNA into 293A cells did not significantly increase readthrough over basal levels (Fig. 5C). Thus, increased readthrough is likely caused not by the mRPL4 transcript but by the mRPL4 protein itself.

mRPL4 enhances translation of other functional viral recoding sequences. We next explored the range of recoding events that were susceptible to mRPL4 enhancement. Recoding also occurs in other contexts in the translation of viral proteins. In the case of HIV-1, Gag-Pol is synthesized by a −1 frameshift; the HIV-1 RNA does not contain a pseudoknot but forms a stem-loop downstream of the recoding site (25, 48). The genome of the alphavirus Sindbis virus utilizes a leaky termination sequence, a UGA stop codon followed by a cytidine, to control the ratio of its structural proteins (17, 32, 33). We tested for the effects of overexpression of mRPL4 on both the HIV-1 frameshift and the Sindbis virus readthrough sequences in the dual-luciferase assay. A reporter plasmid for HIV-1 RNA (HIV1-FS) was constructed containing the slippery sequence, the stop codon, and 62 downstream nucleotides, including the proposed extended stem-loop mRNA structure, inserted between the luciferase reporters. A control plasmid was constructed with correction of the reading frame to eliminate the need for frameshift. The HIV1-FS construct induced frameshifting and downstream reporter expression at about 6% of the level of the control. To test Sindbis virus readthrough, we cloned a small fragment containing the UGA C tetranucleotide code between the reporters (Sindbis-RT); no other viral sequences were included, eliminating the possibility of virus-specific secondary mRNA structure. A control plasmid replaced the UGA stop codon with CGA to allow maximum translation of the reporter. The UGA C tetranucleotide sequence promoted suppression of termination at a significant rate, about 3% of the control, which is comparable to the low end of MoMLV readthrough.

Transfection of mRPL4 expression construct increased HIV-1 frameshift in a dose-dependent manner, similar to its effects on MoMLV readthrough, indicating that the effects of mRPL4 are not limited to suppression of termination or dependent on a specific pseudoknot structure (Fig. 6A). mRPL4 also increased readthrough for the Sindbis virus recoding sequence when transfected at high levels (Fig. 6A). Since Sindbis virus recoding is not dependent on a secondary structure in this context, the mRPL4 effect may be due in part to an interaction that affects decoding or interferes with termination. Analogous tests with reporters using the frameshift sequences for Mason-Pfizer monkey virus and mouse mammary tumor virus yielded similar results (data not shown), suggesting that the effect of mRPL4 overexpression is broad.

In an attempt to test whether mRPL4 affected simple termination, we generated a construct containing a scrambled pseudoknot (ScrPK) that retains the nucleotide composition of the native sequence but eliminates the predicted secondary structure. However, the raw values of luciferase induced by this reporter construct were below the levels reliably above background (see Fig. S5D in the supplemental material), suggesting that the mRNA was not translated at a significant level. We suspect that the low levels of expression from this construct may be due to rapid turnover of the mRNA by the nonsense-mediated decay (NMD) pathway. This notion is consistent with recent work from our lab demonstrating that readthrough transcription is sufficient to inhibit NMD and promote increased mRNA stability (19). mRPL4 did not increase the luciferase reporter levels into the meaningful range of the assay (Fig. 6A); counts below 1,000 units are not considered reliably above background. The raw values of luciferase induced by HIV-FS (see Fig. S5B) and Sindbis-RT (see Fig. S5C) were comparable to those induced by the MoMLV-PK construct (see Fig. S5A).

A pair of pseudoknot mutants was also examined for their responses to mRPL4. These constructs contain point mutations within pseudoknot sequences which have been shown to alter the normal frequency of readthrough, presumably by changing the

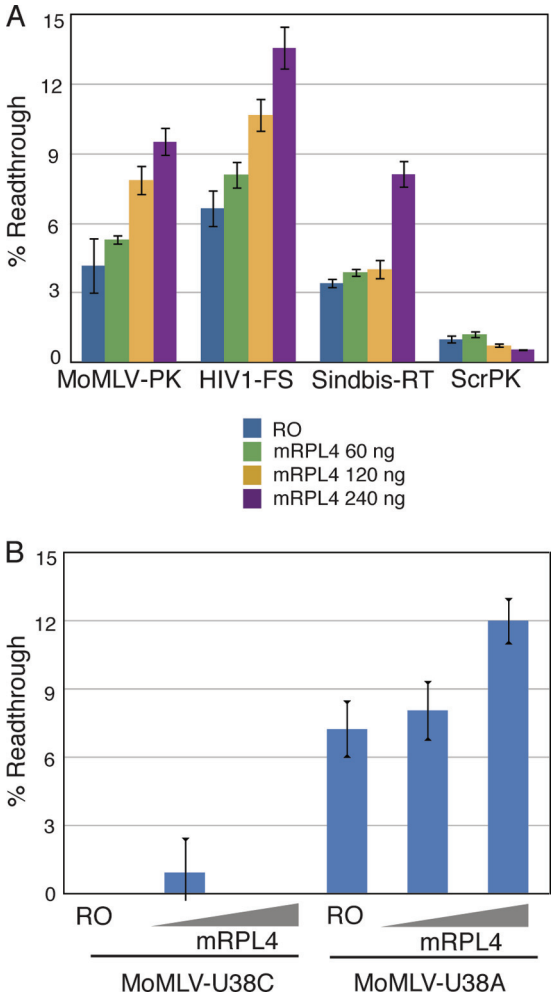

FIG 6 Effect of mRPL4 on other viral recoding and mutant MoMLV pseudoknot sequences. Comparison of readthrough of MoMLV readthrough (MoMLV-PK), HIV-1 frameshift (HIV1-FS), Sindbis virus readthrough (Sindbis-RT), and scrambled MoMLV pseudoknot readthrough (ScrPK) in 293A cells cotransfected with dual luciferase reporters and 60 ng, 120 ng, or 240 ng/well of mRPL4 cDNA expression construct. Data are from a representative experiment. Error bars correspond to standard errors of averages of three wells. (B) Readthrough levels based on dual-luciferase assay of U38C hypoactive and U38A hyperactive MoMLV pseudoknot mutant and control reporter constructs cotransfected with 1, 3, or 6 μg of mRPL4 cDNA construct in a six-well format. Data are from a representative experiment. Error bars correspond to standard deviations of averages derived from triplicate luciferase readings of cell lysates.

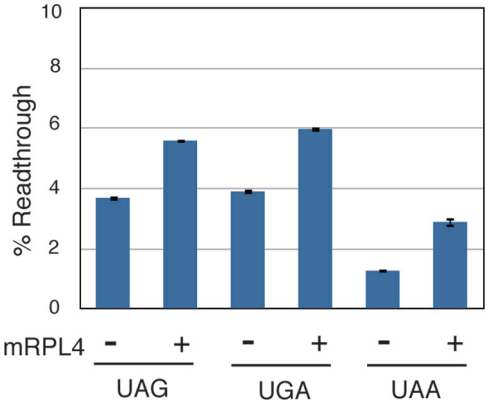

FIG 7 Effect of mRPL4 on alternate stop codon readthrough. Shown are readthrough percentages in HEK 293A cells transfected with 1 μg dual luciferase reporter constructs containing the wild-type MoMLV pseudoknot with the native UAG stop codon, alternate UGA or UAA stop codons, or the CAG control and 6 μg/well of mRPL4 cDNA expression construct. Data are from a representative experiment. Error bars correspond to standard deviations of averages derived from triplicate luciferase readings of cell lysates.

stability of the pseudoknot structure or the ability of ribosomal or other host factors to interact with the viral mRNA. The U38C mutant has been shown to be hypoactive, yielding reduced readthrough; the U38A mutant, in which the same base is changed to an A, is a hyperactive mutant (1). The levels of luciferase induced by the hypoactive mutant were too low to reliably quantify the readthrough efficiency, and mRPL4 did not bring the levels into the useful range of the assay, as with the scrambled PK reporter (Fig. 6B; also, see Fig. S5E in the supplemental material). mRPL4 did increase the readthrough of the hyperactive pseudoknot mutant U38A to even higher levels, inducing increases in the readthrough efficiency comparable to those obtained with the wild-type sequence (Fig. 6B). Thus, the mRPL4 increase is independent of the basal readthrough level of the pseudoknot and is not limited to any ceiling detected so far.

mRPL4 increases readthrough of all stop codons. Previous studies on the MoMLV *gag-pol* junction showed that readthrough could occur with any of the three stop codons (15). The Gag-Pol fusion protein was translated via readthrough of UAA and UGA stop codons in the context of the MoMLV pseudoknot, indicating that MoMLV readthrough is not dependent on the identity of the stop codon. We confirmed these findings and also confirmed that the basal levels of readthrough did vary with the various stop codons. The construct containing the UAA stop codon showed the lowest basal readthrough. UAA is known to be most resistant to readthrough in general, even with the addition of readthrough-promoting drugs (12, 36). We transfected 293A cells with dual luciferase constructs containing the alternate stop codons in front of the MoMLV pseudoknot sequence, with and without mRPL4. We observed that mRPL4 increased the incidence of readthrough in all three cases (Fig. 7).

mRPL4-mediated increase in *gag-pol* readthrough impairs viral replication. In the cases of HIV-1 and the L-A double-stranded RNA element in yeast, deviation from the normal ratio of Gag to Gag-Pol expression is known to negatively impact virus assembly and release, perhaps by disturbing the packing of Gag proteins into the assembling virion (10, 47). We investigated the effect of mRPL4 overexpression on virion production in cells transfected with the wild-type MoMLV proviral DNA. 293T cells were transfected with provirus DNA, and increasing amounts of DNA constructs expressing the mRPL4 or mRPS3a cDNA. Cultures were lysed after 48 h, and intracellular viral proteins in the lysates were assessed by Western blotting with anti-CA antibody. The major viral protein detected was the precursor protein Pr65gag, and intracellular levels of the Gag-Pol precursor were too low to be detected directly. Overexpression of the ribosomal proteins showed no significant effect on the levels of Pr65gag precursor. Low levels of processed CA protein were detectable in the intracellular lysates, and overexpression of mRPL4 showed inhibition of formation of processed CA, suggestive of inhibition of virion assembly (Fig. 8A). To directly monitor virion release, the

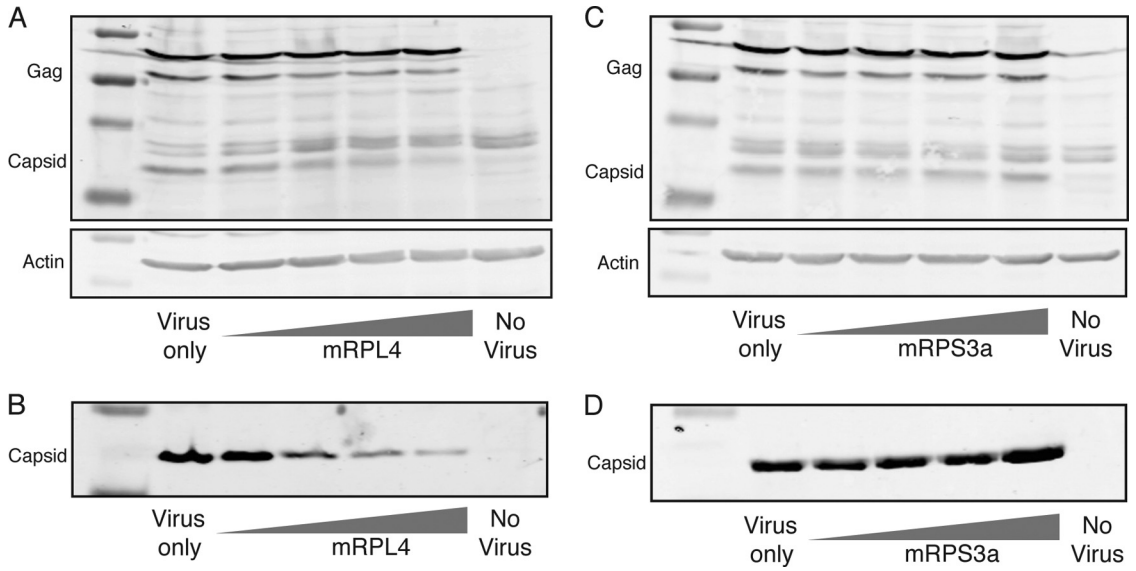

FIG 8 Effect of mRPL4 on viral replication. (A) Western blot analysis of 293T cells cotransfected with 1 μg pNCS (MoMLV provirus) and 0.5, 1, 2, or 4 μg of mRPL4 cDNA construct and probed with rat anti-CA polyclonal antibody. (B) Western blot analysis of 293T cells cotransfected with 1 μg pNCS and 0.5, 1, 2, or 4 μg of mRPS3a cDNA expression construct and probed with rat anti-CA polyclonal antibody. (C) Western blot analysis of pelleted virus from 293T cells cotransfected with 1 μg pNCS and 0.5, 1, 2, or 4 μg of mRPL4 cDNA expression construct and probed with rat anti-CA polyclonal antibody. (D) Western blot analysis of pelleted virus from 293T cells cotransfected with 1 μg pNCS and 0.5, 1, 2, or 4 μg of mRPS3a cDNA expression construct and probed with rat anti-CA polyclonal antibody.

culture medium was harvested, virions were pelleted, and the levels of virion-associated CA proteins were similarly assessed by Western blotting. Overexpression of mRPL4 showed a strong and dose-dependent inhibition of CA levels in the medium (Fig. 8B). At the highest levels of mRPL4 cDNA, virus yields were reduced as much as 10-fold. In contrast, overexpression of the control mRPS3a protein had no effect on CA levels compared to the basal levels seen with virus alone (Fig. 8C and D). These results suggest that overexpression of mRPL4 altered the ratio of Gag to Gag-Pol expression sufficiently to inhibit the proper assembly and release of virion particles.

DISCUSSION

Decoding, the proper pairing of tRNAs with their cognate codons in the mRNA, occurs in the decoding center of the ribosome, located in the small subunit. This process is thought to be governed predominately by the 18S rRNA (16S in prokaryotes); specific nucleotides monitor the spacing of atoms during tRNA-mRNA interactions, and only cognate interactions are sterically favored (56). Miscoding, the insertion of the wrong amino acid at a codon, occurs very infrequently in normal eukaryotic translation (approximately 1 error per 10^4 to 10^5 elongation steps) (35). Termination of translation at a stop codon involves recognition of the codon by termination factors eRF1 and eRF3, leading ultimately to release of the polypeptide. The process of translational readthrough involves disruption of the normal functions of these factors and the misreading of the stop codon by a tRNA. Normally, suppression of termination also occurs at very low frequency. The MoMLV readthrough event at the *gag-pol* junction specified by the mRNA pseudoknot, occurring at very high frequency, must involve a profound manipulation of the ribosomal decoding center. The work presented here shows that the efficiency of readthrough, and thus the ratio of Gag to Gag-Pol, can be modulated in *trans* by the overexpression of a ribosomal protein, mRPL4. How the pseudoknot achieves this, and how mRPL4 might modulate this process, remains unclear.

Our fundamental observation is that the cotransfection of cells with an mRPL4 expression construct and the dual luciferase reporter leads to dramatically increased readthrough, but the mechanism by which this occurs is not clear. Our efforts to document the overexpression of mRPL4 protein itself were not successful. While we could see a clear increase in mRPL4 mRNA resulting from cotransfection, the levels of mRPL4 protein as detected by antibodies were not significantly increased over the basal levels. We suspect that the levels of mRPL4 protein may be controlled by posttranscriptional mechanisms and that transient increases are quickly restored to normal levels by inhibition of translation or protein turnover. In spite of our inability to detect increased mRPL4 levels, the increased readthrough seems to require the intact mRPL4 ORF and, therefore, presumably the mRPL4 protein *per se*.

The mRPL4 protein exhibiting the ability to modulate readthrough was first identified in a screen for pseudoknot-binding proteins as expressed in yeast. One possible mechanism of action is that mRPL4 alters the equilibrium between two interconverting conformations of the pseudoknot, selectively stabilizing the acidic conformation. Subsequent tests of the binding both in yeast and *in vitro*, however, did not reveal any strong specificity for the recognition of the pseudoknot structure over control RNAs (data not shown). It is thus not clear whether the enhancing activity depends on direct binding of the pseudoknot by mRPL4. The identity of the active protein as a component of the ribosome suggests, rather, that its activity may depend on an interaction with the ribosome, either with other ribosomal proteins or with the rRNA. Another possibility is that mRPL4 facilitates the binding of the pseudoknot to a specific site on the ribosome. Yet another possi-

bility is that mRPL4 affects ribosomes' fidelity or interaction with eRF1 or eRF3, or their efficiency of termination.

RPL4 is a highly conserved subunit of ribosomes among all kingdoms. In bacteria, RPL4 lines the exit tunnel of the ribosome and has extensions near the peptidyl transferase center, although there appears to be no direct contact between RPL4 and the decoding center (58). Recently determined crystal structures of 80S ribosomes reveal that eukaryotic RPL4 occupies a similar position, although there are numerous eukaryote-specific contacts and extensions of RPL4 in the 80S ribosome (7, 27). It is unlikely that RPL4 overexpression acts in the context of its normal position in the ribosome; overexpression would not be expected to lead to overexpression of all the other ribosomal subunits or RNA. Furthermore, RPL4 is an intrinsic subunit, deeply embedded in the core, and unlikely to be exchanged in or out of an assembled ribosome. We thus suppose that mRPL4 might act as an extraribosomal protein. mRPL4 has been shown to interact with helicase Gu alpha in the nucleolus and to have a role in the proper processing of rRNA (53). It has also been implicated in a regulatory role in the nucleus, increasing the transcription of Myb-regulated genes (11). Neither the location of RPL4 in the ribosome nor its other proposed activities shed light on its activity in enhancing readthrough.

mRPL4 may act in collaboration with other proteins to modulate readthrough. We found its activity to be cell line dependent. This suggests that other cellular factors with different expression profiles in these cell lines may influence readthrough and so may be able to block or compensate for mRPL4 induced modulations of readthrough. The cell lines responsive to mRPL4 did not suggest any simple characteristic—neither species of origin nor cell type—that accounted for their response. We also tested a panel of other ribosomal proteins to rule out the possibility that overexpression of any such protein could cause a general disruption in the stoichiometry of ribosomal proteins, i.e., that the increase in readthrough was a nonspecific effect of a ribosomal protein imbalance. Although the proteins mRPL7 and mRPS20 showed a modest increase in MoMLV readthrough over basal levels, all other proteins tested had no effect whatsoever on readthrough. Some aspect of mRPL4 provides for an unusually, and perhaps uniquely, potent enhancement of recoding.

mRPL4 overexpression did not grossly increase the frequency of all termination events, and the mRPL4 effect on readthrough is not a result of general disturbance of the translation machinery. A control reporter plasmid with a stop codon followed by a scrambled pseudoknot sequence showed no increase in readthrough in response to mRPL4 overexpression. mRPL4 did, however, exhibit activity in several other settings. An HIV-1 frameshift reporter and a Sindbis readthrough reporter both showed enhancement of recoding by mRPL4. Because the HIV-1 RNA contains a hairpin rather than a pseudoknot, and the Sindbis recoding signal is merely a leaky stop codon without secondary structure, we conclude that the mRPL4 enhancement of MoMLV readthrough is not dependent specifically on the MoMLV *gag-pol* junction sequences, or even on pseudoknot structure generally. Rather, mRPL4 seems to enhance the propensity for recoding to occur in the presence of any of several distinct recoding signals. Perhaps mRPL4 can increase the time during which recoding decisions are made, or can shift the equilibrium away from normal decoding to abnormal decoding, but only when the ribosome has been primed by these RNA signals to allow recoding. Correspondingly, mRPL4 is also able to enhance readthrough of all three stop codons. The UAA stop codon is reportedly resistant to readthrough by aminoglycoside antibiotics (12); however, the UAA stop codon demonstrates mRPL4-increased readthrough in the context of the MoMLV pseudoknot.

The normal ratio of Gag to Gag-Pol expression is important for virus replication, and disruption of the correct Gag:Gag-Pol stoichiometry is likely to interfere most directly with virion assembly. Virus particles are assembled under the plasma membrane from the pool of available Gag and Gag-Pol precursors, and the abundance of Gag-Pol in the virion closely matches the level in the cytoplasm. Furthermore, the Gag domains that are required for the incorporation of Gag-Pol into the virion match closely with the domains that are required for Gag-mediated assembly itself (2, 3, 9, 45). Thus, Gag-Pol is likely incorporated as a Gag with an extended C-terminal Pol domain (although portions of the Pol protein may also play some role in its incorporation) and in proportion to its abundance in the cytoplasm. During and after assembly, in a poorly understood way, dimerization of the Gag-Pol protein results in activation of the embedded viral protease, leading to its excision from the precursor and ultimately cleavage of virtually all of the Gag and Gag-Pol precursors. The timing of the activation of the protease is a critical aspect of virus assembly, and either premature activation of the protease or insufficient activation should be deleterious to the efficient production of mature infectious virus. High levels of Gag-Pol could lead to aberrant particle formation due to steric constraints, and premature activation of the protease before virion completion could lead to disruption of the structure and viral protein turnover (26). Our results suggest that increased readthrough induced by mRPL4 overexpression can indeed limit the ability of the virus to assemble. Overexpression of mRPL4 led to a dose-dependent and profound decrease in virion yield, with no decrease in the intracellular levels of the Gag precursor. The similar overexpression of a control ribosomal protein, mRPS3a, showed no such inhibition of virion production. These results are consistent with a block to virion assembly in the face of abnormal precursor levels. The sensitivity to modest changes in readthrough highlights the importance of the correct ratio for the virus and accounts for the complex machinery that has been developed to provide for the fine tuning of the readthrough frequency.

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