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Edited* by Ed Harlow, Harvard Medical School, Boston, MA, and approved July 27, 2012 (received for review April 25, 2012)

Ribosomal frameshifting entails slippage of the translational machinery during elongation. Frameshifting permits expression of more than one polypeptide from an otherwise monocistronic mRNA, and can restore expression of polypeptides in the face of frameshift mutations. A common mutation conferring acyclovir resistance in patients with herpes simplex virus disease deletes one cytosine from a run of six cytosines (C-chord) in the viral *thymidine kinase* (*tk*) gene. However, this mutation does not abolish TK activity, which is important for pathogenicity. To investigate how this mutant retains TK activity, we engineered and analyzed viruses expressing epitope-tagged TK. We found that the mutant's TK activity can be accounted for by low levels of full-length TK polypeptide produced by net −1 frameshifting during translation. The efficiency of frameshifting was relatively high, 3–5%, as the polypeptide from the reading frame generated by the deletion, which lacks stop codons (nonstop), was poorly expressed mainly because of inefficient protein synthesis. Stop codons introduced into this reading frame greatly increased its expression, but greatly decreased the level of full-length TK, indicating that frameshifting is strongly stimulated by a new mechanism, nonstop mRNA, which we hypothesize involves stalling of ribosomes on the polyA tail. Mutational studies indicated that frameshifting occurs on or near the C-chord, a region lacking a canonical slippery sequence. Nonstop stimulation of frameshifting also occurred when the C-chord was replaced with a canonical slippery sequence from HIV. This mechanism thus permits biologically and clinically relevant TK synthesis, and may occur more generally.

Ribosomal frameshifting is a mechanism of translational recoding (1) in which ribosomes, which ordinarily maintain reading frame with extraordinary accuracy (2), switch to an alternate reading frame during translational elongation (reviewed in ref. 3). This mechanism thus permits the expression of two polypeptides from an otherwise monocistronic mRNA. Frameshifting can either be a programmed event that controls the expression of the polypeptides or it can compensate for single- or double-base insertion or deletion (frameshift) mutations (reviewed in ref. 4). A well-studied example of programmed frameshifting is used by many RNA and retroviruses, including HIV, to express their polymerases. In these systems, −1 frameshifting occurs on a "slippery sequence," which conforms to the motif X XXY YYZ and permits tandem slippage of peptidyl and aminoacyl tRNAs, where X can be any nucleotide, Y is A or U, and Z is not a G and is stimulated by an RNA secondary structure, such as a pseudoknot or stem-loop (reviewed in refs. 5 and 6).

An example of ribosomal frameshifting compensating for frameshift mutations comes from drug-resistant HSV mutants. Clinically relevant drug resistance implies that a mutant evades drug action while retaining pathogenicity. The first-line treatments for diseases caused by HSV are acyclovir (ACV) and related drugs that are more orally available. ACV resistance (ACV^r) is a serious problem for immunocompromised patients with HSV disease (7, 8). Ninety-five percent of ACV^r mutations in clinical isolates occur in the *thymidine kinase* (*tk*) gene; TK activates ACV (9). These mutations most commonly are insertions or deletions in homopolymeric stretches of guanines and cytosines that alter the TK reading frame. However, in many cases, these mutations, which should obliterate TK activity, do not. In the case of the most common mutation, G8, an insertion of a G into a run of seven Gs (G-string), net +1 ribosomal frameshifting results in low levels of TK (~0.1% those of WT virus) (10–12). These small amounts of TK or even lower amounts are nevertheless biologically relevant because they suffice to permit reactivation from latency in a mouse model but an isogenic TK-negative mutant does not (13, 14). [HSV expresses much more TK than is required for WT levels of replication and reactivation in mouse sensory ganglia (15).] Frameshifting on the G-string also suffices for expression of an epitope recognized by T cells (16).

The C5 mutation (also termed C6-1C), which is a deletion of one cytosine from a run of six cytosines referred to as the "C-chord" (nucleotides 548–553 in HSV-1 *tk*) (Fig. S1) is present in 5–10% of clinical ACV^r *tk* mutants (9, 17–19). Despite the deletion, TK activity comparable to that of G8 mutants can be detected by plaque autoradiography in infected cells (20). Introducing a stop codon into the TK reading frame ~30 bases downstream of the C-chord abolishes TK activity (20); thus, expression of the TK reading frame downstream of the deletion is essential for retaining enzyme activity. Studies using a dual-reporter assay system in rabbit reticulocyte lysates showed that sequences near the C-chord can serve as an internal ribosome entry site (IRES) (20). A mutation that decreases IRES activity in vitro also decreased TK activity in the virus, raising the possibility that a C-terminal fragment of TK could restore TK activity to the out-of-frame polypeptide (20). However, the TK polypeptides expressed in infected cells were not identified.

We exploited epitope tags and immunoblots to detect the N- and C-terminal fragments of TK expressed in C5 mutant-infected cells. Our studies led us to discover a net −1 frameshifting event that is stimulated by the absence of stop codons in the primary ORF of an mRNA (nonstop).

Results

TK Expression in C5 Mutant-Infected Cells. To investigate how the C5 mutant expresses active TK in infected cells, we engineered a FLAG-tag at the C terminus of the TK ORF in a virus containing the C5 mutation (C5-FLAG) and its WT parental virus, HSV-1 strain KOS (WT-FLAG) (Fig. 1*A*). The tag did not affect the mutant's TK activity, as shown by plaque autoradiography (Fig. 1*B*). To quantify very low levels of TK protein in infected cells, we immunoprecipitated tagged protein using anti-FLAG antibody followed by Western blot using anti-FLAG antibody [IP-Western (FLAG)], which can detect as little as 0.01% of WT

Author contributions: D.P. and D.M.C. designed research; D.P. performed research; D.P. and D.M.C. analyzed data; and D.P. and D.M.C. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1206582109/-/DCSupplemental.

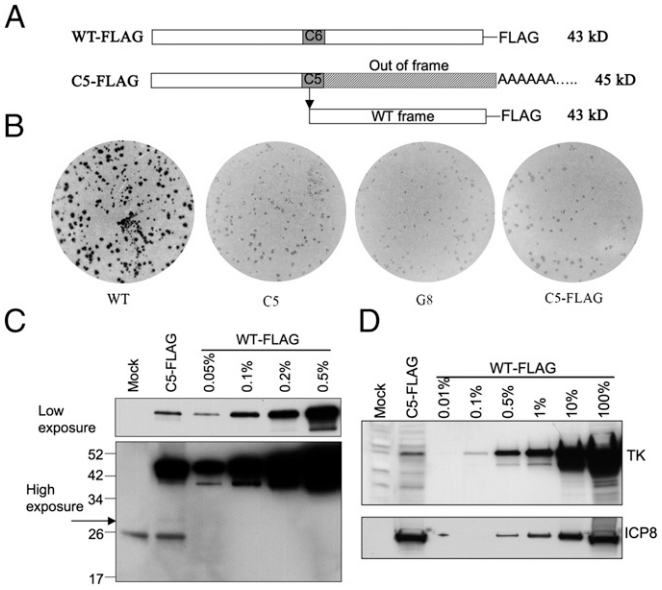

Fig. 1. TK expression and activity in infected cells. (*A*) Diagram of *tk* genes of tagged viruses. To the left are the names of viruses. The bars represent *tk* coding sequence, with the open segments representing the WT TK reading frame; the solid gray segments, the position of the C-chord; the striped segments, the reading frame generated by the one base deletion (out-of-frame). The arrow shows where a frameshifting event could occur to re-enter the WT TK reading frame. The positions of FLAG tags at the C termini are indicated. The molecular masses to the right of the bars were calculated using the on-line Protein Calculator (The Scripps Institute). For the out-of-frame polypeptide of C5-FLAG, the indicated molecular mass was derived from the prediction that translation terminates near the start of the poly(A) tail mapped by Cole and Stacy (21). (*B*) Plaque autoradiography (virus names below the autoradiographs). Black or gray dots represent plaques with TK activity. Diameters of images are 0.45× actual diameters. (*C*) Two exposures (low, *Upper*; high, *Lower*) of IP-Western (FLAG) analysis of full-length TK expression in mock-, mutant-, and WT-infected 143B cells compared with a dilution series of an IP from WT-infected cells (12 hpi) with the viruses and dilutions used indicated at the top of the panel. The arrow points to the faint band present in the C5-FLAG lane but absent in the mock-infected lane. The numbers to the left show the positions of molecular mass markers. (*D*) Western blot analysis of infected 143B cells [12 hours post infection (hpi)] using anti-TK antibody (*Upper*) or using anti-ICP8 antibody (*Lower*). The lysates and dilutions of lysates of WT-infected cells analyzed are indicated at the top of the panel.

TK expression (12). Using this assay, the predominant product detected in C5-FLAG–infected cells comigrated with full-length TK. Based on comparisons to a dilution series from lysates of WT-FLAG–infected cells, this product was present at ∼0.1% of the level of TK found in WT-infected cells (Fig. 1*C*). Similar results were obtained in two additional independent experiments. At high exposures of the blot (Fig. 1*C*, *Lower*), we could also detect a faint band migrating with an apparent molecular mass of 28 KDa in the C5-FLAG–infected sample that was not in the mock-infected sample, as well as a band migrating with an apparent molecular mass of 25 KDa in both the C5-infected and mock-infected samples. One or both of these species in the C5-infected sample may contain the C-terminal TK fragment proposed to arise from utilization of an IRES (20). However, these products were much less abundant than full-length TK.

To confirm expression of full-length TK by the C5 mutant, we performed Western blot analysis of cell lysates using a polyclonal anti-TK antibody. This approach also detected full-length TK from C5-FLAG (Fig. 1*D*). Based on comparisons to a dilution series and normalizing to another viral protein (ICP8), again we found that C5 expressed full-length TK at ∼0.1% of the level in WT-infected cells. This amount is similar to that found in G8-infected cells (12), which correlates with C5 and G8 expressing similar amounts of active TK in plaque autoradiography assays (Fig. 1*B*) (20). Thus, synthesis of full-length TK accounts for the TK activity of C5.

Full-Length TK Expressed by C5 Is Produced by Frameshifting During Translation. We next investigated whether full-length TK was generated by reversion, transcriptional errors, or frameshifting during translation in C5-infected cells. Arguing against reversion, several plaque autoradiography experiments that together assayed about 2,000 plaques did not detect any plaque exhibiting high levels of TK activity (e.g., Fig. 1*B*) (20). To consider the possibilities of low levels of reversion and transcription errors further, we analyzed *tk* transcripts from mutant-infected cells in duplicate by reverse transcription followed by deep sequencing. We found that only ∼0.02% of the *tk* transcripts had an addition of one C (C6; i.e., WT *tk* sequence) (Fig. 2, Y intercept), ∼fivefold less than the relative level of full-length TK in C5-infected cells. This assay likely overestimated the frequency of C6 transcripts in C5-infected cells, because errors could be generated during reverse transcription, PCR amplification, and sequencing. To address the sensitivity of the assay, mRNA from WT-infected cells was spiked into the assay at ratios of 0.05%, 0.1%, and 0.2% of the mutant mRNA sample. The ratio of C6 to C5 sequences as a function of the percent of WT mRNA spiked into the C5 mRNA samples displayed a straight line with a slope close to 1 (Fig. 2), indicating that the method was more than sensitive enough to detect C6 transcripts that could account for the amount of full-length TK found in C5-infected cells. Therefore, neither reversion nor transcription errors likely account for the level of full-length TK expressed by the mutant. We conclude that expression of full-length TK is mainly a result of net −1 frameshifting during translation.

Where Does Frameshifting Occur? To address the location of frameshifting, we engineered a mutant virus (C5stop1) with a stop codon just upstream of the C-chord in the −1 reading frame relative to that of TK, without changing the amino acids coded for by the TK reading frame (Fig. 3*A*). If frameshifting occurred before this stop codon, then no full-length TK should be expressed. We also engineered a second mutant (C5stop2) with a stop codon in the TK reading frame at the end of the C-chord, so that if frameshifting occurred before the stop codon, then no TK would be made (Fig. 3*A*). Both mutant viruses also encode a FLAG tag at the C terminus of the TK frame, and an HA tag near the N terminus (in a location that does not perturb the *UL24* gene that overlaps *tk*). These two tags enabled detection of

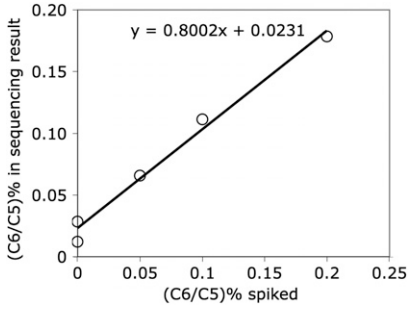

Fig. 2. Deep sequencing of *tk* transcripts in infected 143B cells. The figure displays the percentages of reads of transcripts containing six Cs (C6, open circles) relative to those containing five Cs (C5) as a function of the percentages of the amount of WT (C6) mRNA spiked into the *C5* mRNA. The points for C6/C5 were fit to a straight line, with the fitting function shown above the line.

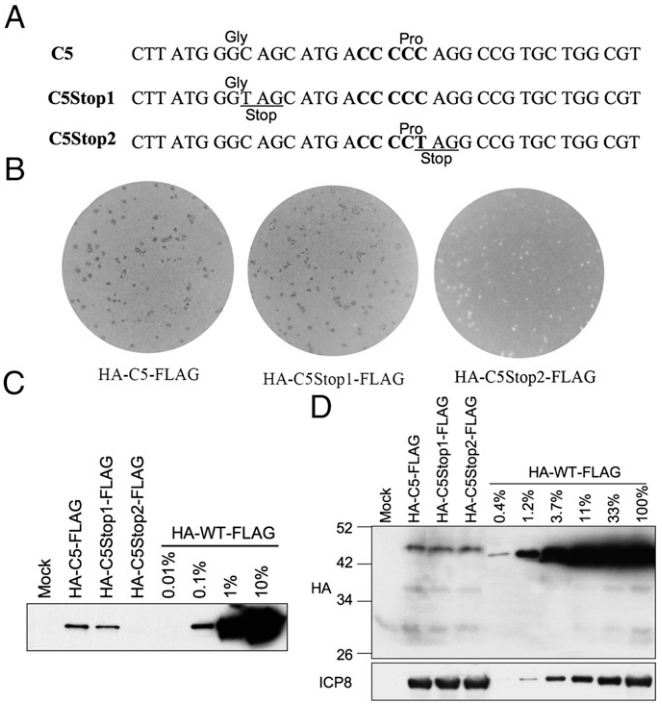

Fig. 3. Effects of stop codons in the TK reading frame. (*A*) Sequences surrounding the C-chord (boldface) of C5 and two stop codon mutants (names to the left) are presented in the primary reading frame (the one created by the one base deletion). Underlined are the positions of the introduced stop codons. Above the sequences are the amino acids (unaltered by the mutations) encoded by the mutated codons in the primary reading frame. (*B*) Plaque autoradiography of mutant viruses (names below the autoradiographs). White dots or absence of dots indicate undetectable TK activity. Diameters of images are 0.53× actual diameters. (*C*) IP-Western (FLAG) analysis of full-length TK expression in mutant infected 143B cells (10 hpi). (*D*) Western blot analysis using anti-HA antibody (*Upper*) or anti-ICP8 antibody (*Lower*) (10 hpi). Molecular mass markers are shown to the left of the panel. For *C* and *D*, mock infection (mock), the viruses, and dilutions used are indicated at the top of the panel.

full-length TK and all polypeptides that include the N-terminal portion of TK, respectively. WT virus and a C5 mutant with both tags (HA-WT-FLAG and HA-C5-FLAG) were also engineered for comparison. The tags did not affect TK activity (12) (Fig. 3*B*), and like C5-FLAG, the doubly tagged C5 mutant expressed full-length TK at a level about 0.1% of HA-WT-FLAG (Fig. 3*C*). The C5stop1 mutation (HA-C5Stop1-FLAG) exerted no effect on full-length TK expression relative to HA-C5-FLAG, as shown by IP-Western (FLAG), or TK activity, as shown by plaque autoradiography, but the C5stop2 mutation (HA-C5Stop2-FLAG) eliminated both TK expression and TK activity (Fig. 3 *B* and *C*). The C5stop mutant viruses and HA-C5-FLAG control virus expressed similar levels of polypeptides that reacted with anti-HA antisera and migrated somewhat more slowly than full-length TK (Fig. 3*D*), with an apparent molecular mass that matches the prediction for translation termination near the start of the poly (A) tail (Fig. 1*A*). These results are consistent with frameshifting occurring between the two engineered stop codons, and with the primary reading frame of C5 *tk* mRNA (i.e., the reading frame created by the deletion mutation) being nonstop.

Efficiency of Frameshifting. The efficiency of frameshifting is the synthesis of the frameshifted polypeptide divided by the synthesis of the product of the primary reading frame. Accordingly, we measured the levels of expression of the out-of-frame TK polypeptides generated by the one-base deletion in the C-chord in HA-C5-FLAG and its mutants by comparing them to a dilution series from lysates of HA-WT-FLAG–infected cells (Fig. 3*D*). These out-of-frame polypeptides were expressed at low levels, 0.5–1% of HA-WT-FLAG TK (Fig. 3*D*), as expected for the products of nonstop mRNAs (22, 23). As observed previously with viruses expressing untagged TK (20), the *tk* mRNA level in C5-FLAG–infected cells was similar to that in WT-FLAG–infected cells, as shown by Northern blot (Fig. S2*A*). To assess whether protein degradation could account for the low levels of the out-of-frame TK polypeptides, we first analyzed the time course of polypeptide expression by Western blot, which showed that although the levels of full-length TK and ICP8 increased until about 10 h postinfection (hpi) and then remained constant in HA-WT-FLAG–infected cells, the levels of the out-of-frame TK polypeptide in HA-C5-FLAG–infected cells decreased slightly from 10 to 18 hpi (Fig. S3). We further analyzed protein stability by examining protein levels by Western blot at different times after adding cycloheximide (CHX) at 6 hpi (Fig. 4*A*). Band intensities were compared with a dilution series of the sample harvested at the moment of CHX addition. Whereas the levels of full-length TK and ICP8 were constant during the 6 h after CHX addition in HA-WT-FLAG–infected cells, those of the out-of-frame product were reduced by four- to eightfold in HA-C5-FLAG–infected cells, suggesting that the out-of-frame product had a half life of 2–3 h. However, at 10 hpi, when the 100- to 200-fold reduction in the level of the out-of-frame product from C5 mutants relative to full-length TK from by WT viruses was measured (Fig. 3*D*), most TK polypeptides had been present in the cell for less than 4 h, as seen from the time course of protein expression (Fig. S3), so protein degradation contributed less than fourfold to the reduction in the level of the out-of-frame product. As a third approach, we found that MG132, a proteasome inhibitor, increased the level of the out-of-frame product by ~threefold in mutant-infected cells but did not affect TK or ICP8 protein levels in HA-WT-FLAG–infected cells (Fig. 4*B*). Taken together, these results lead us to estimate that translation of the out-of-frame product in C5 mutant-infected cells is 2–3% that of full-length TK in WT-infected cells. Given that full-length TK is synthesized in C5-infected cells at about 0.1% that in WT-infected cells (Fig. 1 *C* and *D*), we calculate that the frameshifting efficiency is 3–5%.

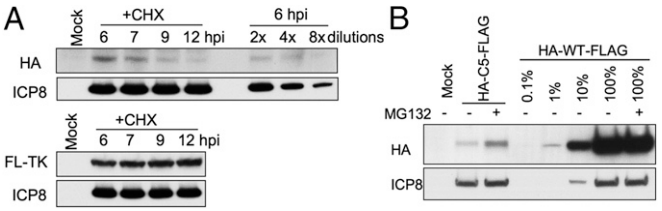

Fig. 4. Analysis of stability of the out-of-frame polypeptide in C5 mutant infected 143B cells. (*A*) Stability assay. Cells were infected with HA-C5-FLAG (*Upper*) or HA-WT-FLAG (*Lower*): 100 μg/mL CHX was added at 6 hpi. At the times postinfection indicated at the top of each panel, cells were analyzed by Western blot using anti-HA antibody (*Upper* image of each panel) and ICP8 antibody (*Lower* image of each panel). (*Upper*) Out-of-frame polypeptide produced by HA-C5-FLAG. A dilution series (indicated at top) prepared from the sample obtained at the 6 h time point is resolved on the right side of the gel; (*Lower*) full-length TK (FL-TK) from HA-WT-FLAG. Mock-infected sample, Mock. (*B*) Effect of MG132. Cells were infected with HA-C5-FLAG or HA-WT-FLAG. MG132 (10 μM; +) or DMSO (–) was added at 3 hpi. At 10 hpi, cells were analyzed by Western blot using anti-HA and anti-ICP8 antibodies. Mock-infection (Mock), the viruses assayed and the dilutions made from HA-WT-FLAG lysate obtained in the absence of MG132 are indicated at the top.

Stop Codons Introduced into the Reading Frame Created by the C5 Mutation Drastically Decrease Frameshifting Efficiency. In the course of these studies we engineered a version of the FLAG-tagged C5 mutant using a bacterial artificial chromosome (BAC). Unlike the other C5 mutants, this virus (C5-FLAG-BAC) contains a loxP site in the *tk* gene downstream of the C-chord, and that site includes a nonsense codon in the reading frame created by the C5 single-base deletion (Fig. 5*A*). C5-FLAG-BAC exhibited undetectable TK activity (Fig. S4), a much lower level of full-length TK (Fig. 5*B*), and readily detectable out-of-frame polypeptide (Fig. S5*A*). Viruses derived using this BAC ordinarily exhibit the same levels of TK activity and full-length TK as those derived without using a BAC (12) (Fig. S5*B*). This finding suggested that converting the nonstop mRNA to one with a stop codon in that frame might affect the efficiency of ribosomal frameshifting. We therefore engineered another mutant virus, HA-C5Stop3-FLAG, with such a stop codon, the C5 mutation, and HA and FLAG tags at the N- and C-termini of the TK reading frame (Fig. 5*A*) without using a BAC. This mutant, and the other viruses just discussed, expressed similar levels of *tk* mRNA (Fig. S2*B*). Full-length TK and out-of-frame polypeptides were detected by IP-Western (FLAG) (Fig. 5*C*) and Western blot (HA) (Fig. 5*D*), respectively. HA-C5Stop3-FLAG expressed >10-fold more out-of-frame product (Fig. 5*D*) but >10-fold less full-length TK compared with HA-C5-FLAG (Fig. 5*C*), and showed undetectable TK activity by plaque autoradiography (Fig. S4). Accordingly, the stop codon reduced frameshifting efficiency by >100-fold. This result strongly suggests that nonstop mRNA greatly stimulates frameshifting on C5 *tk* mRNA.

Nonstop mRNA Stimulates Frameshifting on a Canonical Slippery Sequence. We next asked if the nonstop mechanism could stimulate other frameshifting signals. For this experiment, we transfected 293T cells with plasmids expressing TK tagged with HA and FLAG tags at the N and C termini, respectively, followed by infection with virus expressing untagged TK to stimulate TK expression from the plasmid, in an approach similar to one used to study *tk* promoter mutants (24). The plasmid-expressed TK

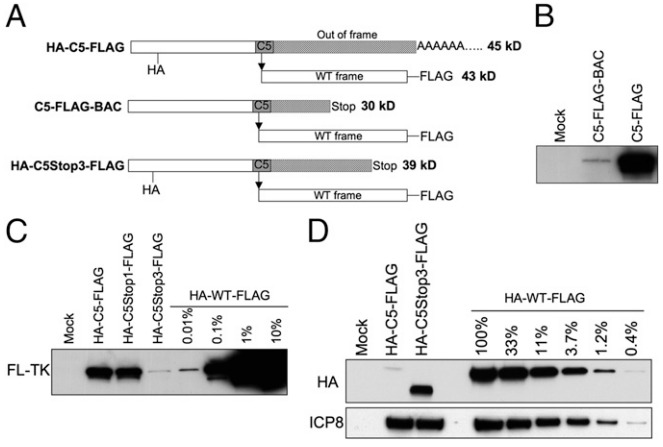

Fig. 5. Effects of stop codons in the primary reading frame. (*A*) Diagram of *tk* genes of tagged viruses presented the same way as in Fig. 1*A*. The positions of HA tags at the N terminus and the introduced stop codons are indicated. (*B*) IP-Western (FLAG) analysis of full-length TK expression from C5-FLAG-BAC or C5-FLAG (12 hpi). (*C*) IP-Western (FLAG) analysis of full-length TK expressed by mutant viruses compared with a dilution series derived from IP of HA-WT-FLAG. Full-length TK, FL-TK. (*D*) Western blot analysis of the out-of-frame polypeptides expressed by HA-C5-FLAG and HA-C5stop3-FLAG viruses and dilution series of the HA-WT-FLAG lysate using anti-HA (*Upper*) and anti-ICP8 (*Lower*) antibodies. Mock-infection (Mock), the viruses assayed and the dilutions are indicated at the top of each panel.

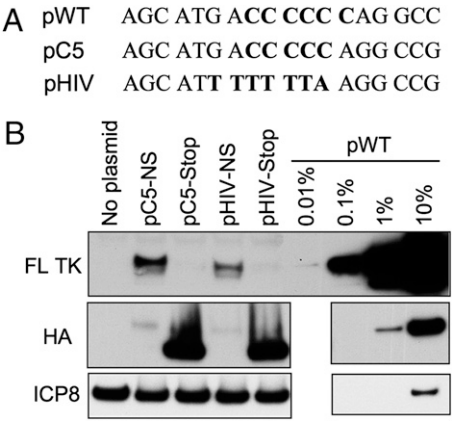

Fig. 6. Effects of stop codons on TK expression from transfected *tk* genes. (*A*) Sequences surrounding the C-chord of plasmids (names to the left) are presented in the primary reading frame. The C-chord or the sequence replacing it is in boldface. (*B*) Plasmids expressing tagged TK (or no plasmid) as indicated at the top of the panel were transfected into 293T cells. After 16 h, the cells were infected with untagged WT KOS for 10 h before being lysed and analyzed by IP-Western (FLAG) for full-length TK (FL TK, *Top*), or Western blot using anti-HA antibody (*Middle*), or using anti-ICP8 antibody (*Bottom*). Dilutions derived from the lysate with the pWT plasmid are resolved on the right side of the blots.

was either unmutated (pWT), the C5 mutant (pC5-NS), or a mutant in which the C-chord was replaced by a canonical slippery sequence from HIV in a manner that shifts the reading frame to that found in the C5 mutant (pHIV-NS) (Fig. 6*A*). The two mutants would thus produce nonstop mRNAs. We also generated versions with the nonsense codon found in the C5Stop3 virus (pC5-Stop and pHIV-Stop, respectively). Following transfection and infection, IP-Western (FLAG) and Western blots using anti-HA antibody were performed to detect full-length TK and out-of-frame TK polypeptides, respectively (Fig. 6*B*). Western blots were also probed with anti-ICP8 antibody, which showed that cells were infected at similar efficiencies (Fig. 6*B*, *Bottom*). In both pC5-NS– and pHIV-NS–transfected cells, full-length TK was readily detected, and as expected, only low levels of out-of-frame products were observed (Fig. 6*B*). In contrast, the pC5-stop and pHIV-stop plasmids expressed much less full-length TK and much more out-of-frame polypeptide (Fig. 6*B*). Thus, the stop codons greatly reduced the frameshifting efficiency in both cases. These results suggest that nonstop mRNAs generally promote frameshifting.

Discussion

In this study, we found that an HSV-1 mutant with a one-base deletion in its *tk* gene expresses low levels of full-length TK that could account for the mutant's TK activity, and that this expression is due to net −1 frameshifting during translation. Despite the low expression of TK, the efficiency of frameshifting is relatively high (3–5%), similar to that seen for retroviral −1 ribosomal frameshifting (25). Additionally, frameshifting is stimulated >100-fold by a new mechanism, nonstop mRNA, which can also stimulate canonical −1 ribosomal frameshifting. We discuss these findings in terms of how the translation machinery might shift frame on the *tk* sequence, how nonstop results in decreased synthesis of the out-of-frame polypeptide and how it might stimulate frameshifting, and the biological and clinical relevance of the frameshifting mechanism that we have uncovered.

How Does the Translation Machinery Shift Frame on or near the C-Chord? The simplest interpretation of our results, particularly the effects of the C5stop1 and C5stop2 mutations, is that

ribosomes shift reading frame somewhere between a site just upstream of the C-chord and the last C of the C-chord. A more complicated interpretation is that the C-to-T mutation in C5stop2 affects the frameshifting signal rather than acting as a stop codon. Regardless, a stop codon ~30 bases downstream of the C-chord in the TK reading frame also eliminates TK activity (20). Thus, a conservative interpretation would be that frameshifting occurs between the locations of the C5stop1 mutation and this latter stop codon. Even within that longer segment, there is no sequence that corresponds to the canonical X XXY YYZ slippery sequence that promotes tandem −1 slippage of peptidyl and aminoacyl tRNAs. We emphasize that we do not know whether this *tk* frameshifting event is −1 or +2. Regardless, we speculate that the event may entail a −1 shift of a single tRNA rather than a tandem shift of both tRNAs (reviewed in ref. 4, but see also ref. 26). In that case, the C-chord itself appears to be a possible site for slippage. In this scenario, a proline tRNA that base pairs with CCC (Fig. 3) could slip back one base and thus cause a shift in reading frame.

Although this scenario is simple, other nearby sequences may play a role in frameshifting. In particular, a one-base substitution 11 nucleotides downstream of the C-chord resulted in a ~40% reduction in TK activity (20). This effect was previously thought likely to be because of an effect on an IRES, but now seems more likely to be an effect on frameshifting. More work is needed to elucidate the sequence dependence and molecular mechanisms of frameshifting in or near the C-chord.

How Does Nonstop mRNA Decrease Synthesis of the Out-of-Frame Polypeptide and Stimulate Frameshifting? Nonstop mRNAs usually arise as errors during DNA replication or transcription and are targeted and processed as aberrant molecules by different mechanisms in different organisms (reviewed in ref. 27). In eukaryotes, nonstop mRNAs are poorly translated, although in yeast and in human mitochondria, they are also rapidly degraded (22, 23, 28–34). Our finding of poor translation without RNA degradation is consistent with those in other mammalian systems (22, 30, 31). However, unlike Akimitsu et al. (22), who described a smear of products from translation of a nonstop mRNA in rabbit reticulocyte lysates with sizes consistent with multiple premature dissociation events from the body of the mRNA, we found a tight distribution of product sizes expressed from C5 mRNA in infected cells, consistent with translation of the out-of-frame polypeptide terminating on or just upstream of the poly(A) tail as has been found in yeast for translation of nonstop mRNAs (23). The MG132-sensitive degradation of the out-of-frame TK polypeptide may be related to findings in yeast that polypeptide products of nonstop mRNAs are assigned for proteasomal degradation by ubiquitylation triggered by stalling of ribosomes during translation on the poly(A) tail (32). Such stalling on the poly(A) tail could be because of poly(A)-encoding polylysine, which may interact with the negatively charged ribosome exit tunnel (35, 36). The presence of poly(A)-binding protein on the poly(A) tail might further inhibit movement of ribosomes on the poly(A) tail, as suggested (37).

We therefore hypothesize that the stalled ribosomes on the poly(A) tail of C5 *tk* mRNA slow down the movement of upstream ribosomes, resulting in (*i*) greatly reduced synthesis of the out-of-frame polypeptide, and (*ii*) pausing of ribosomes at the frameshift signal, which would stimulate net −1 slippage back into the TK reading frame. Other known stimulators of ribosomal frameshifting, such as stem-loops or pseudoknots, can cause pausing, which is generally thought to be responsible for stimulation of frameshifting (5, 6). Thus, the same mechanism that greatly reduces synthesis of the out-of-frame polypeptide would greatly increase synthesis of full-length TK. However, as piled-up ribosomes generally inhibit translation (38), a more complicated mechanism may be involved.

The strong stimulation of frameshifting on C5 mutant *tk* mRNA by the nonstop mechanism provides an explanation for why frameshifting was not detected on the C-chord and surrounding sequences using a dual-reporter gene assay in rabbit reticulocyte lysates, as the dual-reporter constructs contained stop codons in all three reading frames (20). This then permitted detection of an IRES in *tk* mRNA that can drive expression of the downstream reporter in rabbit reticulocyte lysates. If the IRES is active in HSV-infected cells, it is much less so than was observed in reticulocyte lysates.

Biological and Clinical Relevance. Although the efficiency of ribosomal frameshifting that we observed is relatively high, the C5 mutant only expresses TK at ~0.1% that of a WT virus. Nevertheless, that level of TK expression is biologically relevant. In particular, the level is sufficient to permit some reactivation from latency in mouse ganglia, while an isogenic truly TK^- mutant does not reactivate (13, 14, 39–41). The C5 mutation is one of the more common ACV^r mutations found in patients. It seems reasonable to suggest that the low level of TK expressed by this mutant contributes to its ability to cause disease, but is insufficient to activate ACV and thus confer drug resistance. The low level of TK expression might combine with other mechanisms by which HSV *tk* mutations can confer drug resistance yet still retain pathogenicity, such as reversion to create mixtures of TK^+ and mutant virus, and alleles in other loci that compensate for the loss of TK (39, 42).

Although all of our experiments were performed with HSV-infected cells, our results raise the possibility that ribosomal frameshifting stimulated by nonstop mRNA may occur more generally. Typically, reading frames that do not end in a stop codon are ignored in genome analyses. It seems quite plausible that at least some transcripts thought to be noncoding or monocistronic might express low, but biologically relevant levels of novel polypeptides because of nonstop stimulation of translational recoding.

Materials and Methods

Cells, Plasmids, and Viruses. Vero and TK^- 143B cells were maintained as previously described (12). 293T cells (a kind gift of Bryan Cullen, Duke University, Durham, NC) were maintained as for 143B cells. *Tk* mutant plasmids and viruses were constructed as previously described (39), except for C5-FLAG-BAC, which was engineered from a BAC, as previously described (12). Primer sequences are provided in Tables S1 and S2. All virus infections, except for plaque autoradiography, were at a multiplicity of 10 at 37 °C. Plasmid transfections of 293T cells are detailed in *SI Materials and Methods*.

Assays of TK Activity and TK Expression. Plaque autoradiography, immunoprecipitation, Western blot analyses including densitometric comparison with a dilution series, and Northern blot hybridization were performed as previously described (12).

Deep Sequencing. RNAs purified from infected cells were reverse transcribed, the products were amplified by PCR using primers spanning the C-chord, and subjected to Illumina sequencing. The numbers of reads of sequences containing 5Cs and 6 Cs in the C-chord and otherwise matching *tk* sequence were recorded. Details are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Bill Summers and David Knipe for generously providing antibodies, Bryan Cullen for kindly providing 293T cells, and Anthony Griffiths for helpful discussions. This work was supported by National Institutes of Health Grant R01 AI026126.

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