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Ribosomal Frameshifting Efficiency and *gag/gag-pol* Ratio Are Critical for Yeast M_1 Double-Stranded RNA Virus Propagation

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About 1.9% of ribosomes translating the *gag* open reading frame of the yeast L-A double-stranded RNA virus positive strand undergo a −1 frameshift and continue translating in the *pol* open reading frame to make a 170-kDa *gag-pol* fusion protein. The importance of frameshifting efficiency for viral propagation was tested in a system where the M_1 (killer toxin-encoding) satellite RNA is supported by a full-length L-A cDNA clone. Either increasing or decreasing the frameshift efficiency more than twofold by alterations in the slippery site disrupted viral propagation. A threefold increase caused by a chromosomal mutation, *hsh1* (high shifter), had the same effect. Substituting a +1 ribosomal frameshift site from Ty1 with the correct efficiency also allowed support of M_1 propagation. The normal −1 frameshift efficiency is similar to the observed molar ratio in viral particles of the 170-kDa *gag-pol* protein to the 70-kDa *gag* gene product, the major coat protein. The results are interpreted in terms of a packaging model for L-A.

Normal translation efficiently maintains a single reading frame that is fixed at the time of initiation. A now-substantial number of cases of frequent, purposeful shift of frame in which the product formed is a fusion protein encoded by two or, in a few cases, three open reading frames have been described (reviewed in references 1, 18, 22, and 35). These are usually −1 frameshifts, although cases of +1 frameshifting or more radical "hops" are also known. This phenomenon is known as ribosomal frameshifting, to distinguish it from frameshift events caused by mutant tRNAs that can produce a shift of frame in translation to compensate for a frameshift mutation. Most of the genes involved are from viruses, especially retroviruses, which form their *gag-pol* fusion protein by this means; no eukaryotic host gene is known to use this device for expression, making it an attractive target for antiretroviral drugs.

The mechanism of −1 ribosomal frameshifting is the subject of the Jacks and Varmus simultaneous slippage model (25), in which two features of the mRNA are critical: a slippery-site heptamer, which is the actual site of the frameshift, and, just 3′ to this site, a strong secondary structure, often an RNA pseudoknot (5). The mRNA slippery site is a sequence of the form X XXY YYZ, where the initial (*gag*) frame is indicated. This type of sequence allows frameshifting because the tRNAs initially sitting at the P site (initially paired to XXY) and at the A site (initially paired to YYZ) may shift back one nucleotide and re-pair to XXX and YYY, respectively, while maintaining correct pairing of their nonwobble bases. Extensive data support this aspect of the model. We have suggested that the frequency of unpairing at the A site is also important (9). This would explain the natural occurrence of only A and U for Y in the slippery site and the very inefficient shifting in artificial constructs when Y is C or G (9, 23).

The L-A double-stranded RNA (dsRNA) virus of *Saccharomyces cerevisiae* has two open reading frames (20). As in retroviruses, the 5′ open reading frame (*gag*) encodes the 76-kDa major coat protein and the 3′ open reading frame (*pol*), which is expressed only as a 170-kDa *gag-pol* fusion protein, encodes a single-stranded RNA-binding domain and has the consensus sequence patterns for RNA-dependent RNA polymerases of plus-strand RNA viruses and dsRNA viruses (16, 20). The 170-kDa protein is formed by −1 ribosomal frameshifting by a mechanism indistinguishable from that of retroviruses (9). The efficiency of the L-A frameshift is 1.9% (9), similar to the observed molar ratio in viral particles of the 170-kDa fusion protein to the major coat protein (16). To assess the effect on viral propagation of frameshift efficiency mutants, both viral and chromosomal, we used a system in which a vector expressing a full-length L-A cDNA supports replication of M_1, a satellite virus of L-A that encodes the killer toxin (36). In this work, we studied the role of the slippery site and pseudoknot structure in determining the efficiency of frameshifting and thus the ratio of *gag* protein to *gag-pol* fusion protein. Using these data, we show that frameshift efficiency is critical for virus propagation.

MATERIALS AND METHODS

Strains and media. The strains of *S. cerevisiae* used are listed in Table 1. YPAD, YPG, SD, complete synthetic medium (H-Trp), and 4.7MB plates for testing the killer phenotype were as previously reported (37).

Genetic methods. Transformation was accomplished by the lithium acetate method (21). The killer test was carried out by replica plating colonies to be tested to 4.7MB plates with a newly seeded lawn of strain 5X47 (0.5 ml of a suspension at 1 unit of optical density at 550 nm per ml per plate). After 2 days at 20°C, killer activity was observed as a clear zone around the killer colonies. Cytoduction (cytoplasmic mixing without nuclear fusion) was carried out as described by Ridley et al. (30). The donor *kar1* strain 2629, which was defective in nuclear fusion (karyogamy) and harbored the L-A and M_1 viruses, was mixed with the recipient strain 3063, which lacked both viruses and mitochondrial DNA. The mixture was incubated on a YPAD plate for 6 to 8 h at 30°C and plated on medium lacking both leucine (to select against the donor strain) and tryptophan (to select for retention of the L-A cDNA clone). Clones were

TABLE 1. Strains of *S. cerevisiae*[a]

Strain	Description
5X47	*MATa/MATα his1/+trp1/+ura3/+*K^- R^-
3063	*MATa ade1 his3 trp1 mak10* [*rho*^{0}]
2629	*MATα leu1 kar1-1* L-A-HNB M_1
2907	*MATa his3-d200 leu2 trp1-d901 ura3-52 ade2-10* K^-
EMS46	*MATa his3-d200 leu2 trp1-d901 ura3-52 ade2-10 hsh1* pF8 K^-
1995	*MATα spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1* K^+
JD17-2D	*MATa ura3-52 ade2,6 trp1-d901 hsh1* pF8 K^+
JD54	JD17-2D cured of pF8
2956	*MATα ade1 his3 trp1 mak10*
JD44-1A	*MATa ade2 his3 trp1 mak10*
JD44-1B	*MATa ade trp1d901 mak10 hsh1-1*
JD44-2B	*MATa ade2 his3 trp1 mak10*
JD44-2C	*MATa ade trp1 mak10 hsh1-1*

[a] JD44 strains are segregants of the cross JD54 × 2956. JD17-2D is a segregant of the cross EMS46 × 1995.

checked for growth on minimal medium (to screen out diploids) and on glycerol medium (to check for receipt of the donor cytoplasm, including the mitochondrial genome) and for the killer phenotype (as evidence of propagation of M_1).

Plasmids. pF8 and pF'8 have *lacZ* in the −1 frame relative to the initiation codon and L-A sequences from the *gag-pol* overlap region in between (9). pF'8 differs from pF8 only in having the f1 origin. pJD12, 13, 16, 24, 26, 27, 28, 31, and 32 are derived from pF'8 and were previously described (9). pTI25 is the same as pF8 except that there are no L-A sequences and *lacZ* is in the 0 frame (9). The L-A cDNA expression vector pI2L2 has a *PGK1* promoter, the entire L-A sequence except for 8 bases at the 3' end, the yeast *TRP1* gene, the 2μm DNA origin of replication, pBR322 sequences, and the phage f1 origin of replication (36). pJD35, 43, 44, 45, 46, 48, 49, 55, 57, 58, 68, 69, 70, 78, 79, and 80 were derived by modification of pF'8 with synthetic oligonucleotides. pJD45 and 46 were modified by synthetic oligonucleotides to create pJD47, 51, and 52. pJD47 was further modified to create pJD53. Plasmid pI2L2 was modified by synthetic oligonucleotides to create pJD34, 38, 39, 40, 41, 42, 50, 56, 59, 60, 71, 72, and 73. All mutations were confirmed by sequence analysis. pJD54 was constructed by insertion of a 200-bp *Sau*3AI fragment from pMM54 (kindly provided by Peter Leeds) containing SUF1 (27) into the *Bam*HI site of YEp13.

Isolation of chromosomal mutations affecting ribosomal frameshifting. Strain 2907 carrying pF8 was mutagenized with ethyl methanesulfonate (26), and 2×10^4 CFU were plated on H-Trp containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (pH 6.8) per ml. Ninety-six dark and light blue colonies were initially assayed (see below), and 12 were assayed again. Of these, five were cured of plasmid pF8 by streaking onto YPAD and scored by replicating to H-Trp. These were transformed with pF8 and pTI125; upon reassessment, four were found to be true chromosomal mutants comprising three complementation groups. These will be described more fully in a later publication. One of the mutants, EMS46, gave a high-frameshifting (*hsh1*) phenotype, and derivatives of this were used in experiments to determine (i) that the phenotype was due to the effect of a single gene (cross JD17) and (ii) the effect of the *hsh1* mutation on the support of M_1 by the L-A cDNA expression plasmid pI2L2 in a *mak10* background (cross JD44). Cells of the JD44 cross were made [*rho*^{0}] by streaking for single colonies on YPAD plates containing 1 μg of ethidium bromide per ml. These cells were then transformed with pI2L2 and used as acceptor strains in cytoductions with strain 2629. Cytoductants were streaked for single colonies on H-Trp-Leu plates and scored on YPG, SD, and killer indicator plates.

β-Galactosidase assays. Assays of permeabilized yeast cells were as described previously (17). Cells were grown in H-Trp or H-Trp-Leu medium to the mid-logarithmic phase, and assays were normalized with respect to the optical density at 595 nm of the culture and to the assay time. Three independently derived mutants of each mutagenesis reaction were assayed in triplicate against three wild-type plasmids that had gone through the same mutagenesis reaction.

Preparation of dsRNA. dsRNA was prepared as described previously (14). Cells were grown to the mid-logarithmic phase, and the optical density at 550 nm was determined. Equal amounts of cells were washed with 50 mM disodium EDTA (pH 7.0), incubated for 15 min in 50 mM Tris–H_2SO_4 (pH 9.3) containing 2.5% 2-mercaptoethanol, and then stirred for 1 h at room temperature with 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM disodium EDTA, 0.2% sodium dodecyl sulfate (SDS), and an equal volume of redistilled phenol. Nucleic acid in the aqueous phase was recovered by ethanol precipitation. Nucleic acids were resolved on 1.5% Tris-acetate-EDTA agarose gels containing 0.5 μg of ethidium bromide per ml.

Immunodetection methods. Mid-logarithmic-phase yeast cells were normalized with respect to optical density at 550 nm, and equal amounts of cells were suspended in 120 μl of 4 mM KH_2PO_4–16 mM Na_2HPO_4 (pH 7.2)–115 mM NaCl (PBS). Cells were lysed by agitation with acid-washed glass beads. Then 40 μl of 4× reducing buffer (5% 2-mercaptoethanol, 13.2% SDS, 20% glycerol, 25 mM Tris base, 0.005% bromophenol blue) was added, and the whole-cell lysates were incubated at 100°C for 10 min. Cell debris was removed by low-speed centrifugation, and the supernatants were analyzed by SDS–7.5% polyacrylamide gel electrophoresis. The gels were equilibrated in Tris-glycine buffer for 20 min, and proteins were electrotransferred to nitrocellulose. The nitrocellulose was rinsed with PBS–0.05% Tween 20 and blocked at room temperature with PBS-Tween 20 containing 1% nonfat dry milk (Carnation, St. Louis, Mo.). The nitrocellulose sheets were cut transversely. The upper half was incubated with rabbit anti-170-kDa protein antibody (16) at a dilution of 1:1,000, and the lower half was incubated with an affinity-purified rabbit anti-major coat protein antibody. Affinity purification was as described by Beall and Mitchell (2). Briefly, a preparative gel of an extract of L-A-containing cells was transferred to nitrocellulose, blocked as described above, and incubated with the rabbit anti-76 kDa antiserum (16) at a dilution of 1:100 overnight at 4°C. Vertical strips were cut from the nitrocellulose sheet on either side and in the middle. The strips were washed with PBS-Tween 20 and incubated with alkaline phosphatase-conjugated protein G (Gammabind-G AP; Genex, Gaithersburg, Md.). The remainder of the sheet was stored at 4°C. After the immunoreactive bands were washed with PBS-Tween 20, they were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$ as recommended by the manufacturer (Promega). The immunostained strips were aligned with the unreacted sheets, and horizontal strips corresponding to the region of the nitrocellulose containing the 76-kDa band were cut out with a razor blade. These strips were washed for 1 h

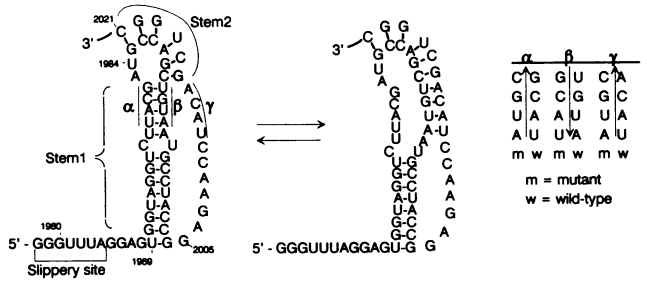

FIG. 1. The region of the L-A plus strand responsible for ribosomal frameshifting has the potential to form different pseudoknot structures, of which the two extremes are shown. Stem 1 and stem 2 make up the pseudoknot. Strand β could pair with either strand α, elongating stem 1, or strand γ, elongating stem 2. Nucleotides are numbered from the 5′ end of the L-A plus strand. Specific sequence changes made in the experiments of Fig. 2 are indicated on the right: 5′-1979, UACG→AUGC; 5′-1991, UGUA→GCAU; and 5′-2012, UACA→AUGC.

in three changes of 10 mM Tris-HCl (pH 8.0)–0.15 M NaCl, 0.05% Tween 20, for 30 min in 0.1 M boric acid–0.5 M NaCl (pH 8.0), and for 30 min in PBS. Bound antibody was eluted with 2 ml of 0.1 M glycine–0.15 M NaCl (pH 2.6) for 2 min, and the pH of the eluate was immediately adjusted to 8.0 with 2 M Tris-HCl (pH 8.0). Bovine serum albumin was added to the eluate to 20%, and aliquots were frozen at −80°C. This antibody preparation was used at a dilution of 1:100 for the detection of the 76-kDa protein. After incubation with primary antibody preparations overnight at 4°C, all blots were washed with three changes of PBS-Tween 20 and incubated with Gammabind-G, and immunoreactive bands were visualized as described above.

RESULTS

A breathing pseudoknot and frameshift efficiency. The −1 ribosomal frameshift observed in the translation of the L-A plus strand requires the slippery site GGGUUUA, followed closely by an RNA pseudoknot (9) (Fig. 1). Several possible pseudoknot structures can be drawn, depending on the relative lengths of stems 1 and 2; two possible models are shown in Fig. 1. A complete series of mutants was constructed by altering the sequences of strands α, β, and γ (Fig. 1) such that pairing of this region could occur only in stem 1, only in stem 2, or in neither (Fig. 2). Although these constructs change the coding sequence, they do not introduce in-frame termination codons, and when all three mutations are made, the constructs leave intact the abilities of their respective regions to base pair. Figure 2 shows that stem 1 pairing rather than stem 2 pairing is important for frameshifting efficiency. In all cases that allowed for maximum base pairing in stem 1, frameshifting was at or near wild-type levels (pJD47, pJD48, pJD53), whereas frameshifting in mutants in which base pairing of this region was only possible in stem 2 (pJD45, pJD52) was comparable to that in mutants in which base pairing was disrupted entirely (pJD46, pJD51). The rate of frameshifting in pJD47, in which a G · C pair replaces a G · U pair at the top of stem 1, was 156% of the wild-type rate, as previously observed with a similar construct (pJD20 [9]). Both changes strengthen stem 1 pairing and increase frameshift efficiency, as also noted by Brierley et al. (6) in infectious bronchitis virus.

Unpairing of the A-site aminoacyl tRNA from the 0-frame (*gag*) codon can limit frameshifting. The Jacks et al. simultaneous slippage model (23) properly emphasizes the importance of re-pairing of tRNAs with mRNA in the −1 frame as a dominant factor determining the occurrence of ribosomal frameshifting. But in our previous report we found evidence

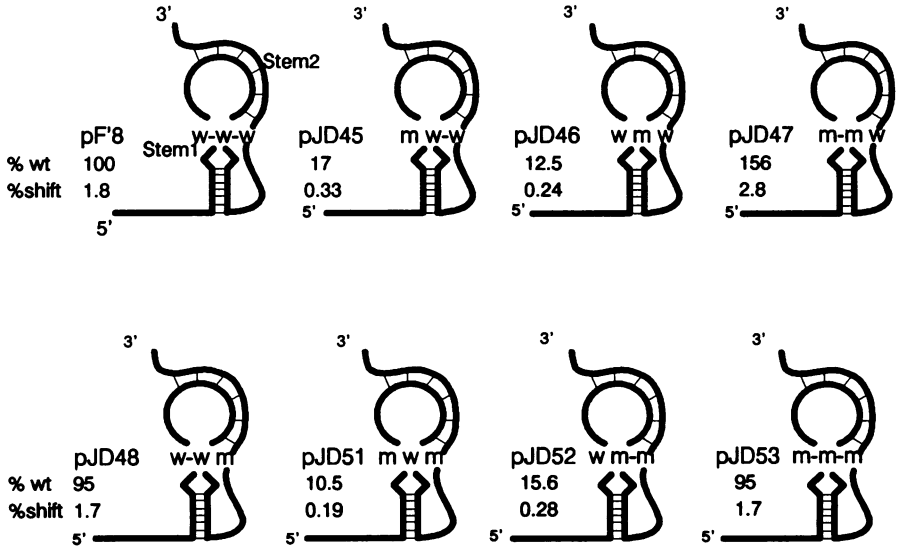

FIG. 2. Pairing in stem 1 is more important for frameshifting than is pairing in stem 2. The specific changes made are shown in Fig. 1. All combinations of wild-type (w) and mutant (m) sequences were constructed by starting with pF′8 carrying the wild-type frameshifting sequence. As can be seen from Fig. 1, pairing (shown by hyphens) is possible between two wild-type sequences or between two mutant sequences but not between wild-type and mutant sequences. The efficiency of frameshifting was measured as β-galactosidase activity and compared with the value for pTI25, the 0-frame plasmid (% shift) and with pF′8 (% wt). Only when pairing in stem 1 is possible, in the cases pF′8 (w-w-w), pJD47 (m-m w), pJD48 (w-w m), and pJD53 (m-m-m), is frameshifting normal. When no pairing of the sequences affected by the mutations is possible (pJD46 [w m w] or pJD51 [m w m]) or when only pairing in stem 2 is possible (pJD45 [m w-w] or pJD52 [w m-m]), frameshifting is substantially reduced.

```
        |<---------L-A sequences---------->|
                  slippery site
     ATG----------GGGUUUAGGA--5'Psi----3'Psi--LacZ
       ---ORF1--->|_|_|
                  |_|_|  ----------ORF2---------->
```

PLASMIDS (from pF'8)	0 frame	ACTIVITY %of pF'8
pF'8 (wt)---------GGGUUUAGGA----------	1.9	100
pJD13-------------AAA----------	2.2	120
pJD31-------------CCC----------	4.7	261
pJD32-------------UUU----------	12.0	667
pJD24----------------AAA----------	5.0	270
pJD16----------------CCC----------	0.17	9
pJD26----------------CCCC----------	0.41	20
pJD27----------------AAAC----------	3.45	150
pJD28-------------UUUGGGC----------	0.11	6
pJD49-------------UUUCCCC----------	0.34	17
pJD43-------------UUUUUCA----------	1.57	88
pJD78-------------UUUGAAA----------	0.37	23
pJD12-------------GAAUUUA----------	0.4	21
pJD79-------------GGGUUUC----------	0.52	36
pJD80-------------UUUUUUC----------	2.3	126
pJD35--------------------GGU----------	0.41	23
pJD44--------------------AGG----------	1.3	66
pF'8 (wt)---------GGGUUUAGGA-- + SUF1 plasmid---	1.9	100
pJD32-------------UUUUUUAGGA-- + SUF1 plasmid---	3.0	158

FIG. 3. Slippery site alterations and frameshift efficiency. The indicated changes were made in the slippery site region of pF'8, the altered plasmids were introduced into strain 2907, and the β-galactosidase activity of mid-log-phase cells was measured. The *SUF1* gene encodes Gly-tRNA$_{UCC}$, which recognizes the GGA codon in the 0 frame. The *SUF1* gene was introduced on YEp13, a high-copy-number plasmid. Data from Dinman et al. (9) are shown in the top part of the figure for comparison with new constructs.

that only A's (GGGAAAA, 5.0% frameshifting; GGGA AAC, 3.45% frameshifting) and U's (GGGUUUA, 1.9% frameshifting) in the second triplet of the slippery site promoted efficient frameshifting, whereas C's (GGGCCCA, 0.17% frameshifting; GGGCCCC, 0.41% frameshifting) and G's (UUUGGGC, 0.11% frameshifting) did not (Fig. 3). Here we show that, although a triplet of U's in the first position increased frameshifting rates in cases where efficient frameshifting could occur (UUUUUUA, 12.0% frameshifting), substituting U's for G's in the first triplet did not affect the rate of frameshifting when the A-site aminoacyl tRNA involves G · C base pairs (UUUCCCC, 0.34% frameshifting) (Fig. 3). In pJD43 (UUUUUCA, 1.57% frameshifting) re-pairing after the shift would not be as good as for UUUCCCC, and yet frameshifting is much better. This may be because the A-site 0-frame UCA codon involves only a single G · C base pair, so unpairing should be easier in this case than for UUUCCCC.

As suggested by Hatfield and Oroszlan (18), it is possible that certain tRNAs are particularly able to shift while others are less so. If the tRNAs recognizing UUC and UUG were uniquely unshiftable, one could explain the low shifting efficiencies of UUUCCCC (0.34% frameshifting), UUUC CCA (0.17% frameshifting), UUUGGGC (0.11% frameshifting), and GGGUUUG (0.33% frameshifting). However, we find that UUUGAAA (0.37% frameshifting) is comparable to GAAUUUA (0.4% frameshifting), which has the same requirement for a G · U base pair after a −1 frameshift but no UUG codon. Although the shifting efficiency of the UUUUUUC heptad (2.3% frameshifting) is lower than that of UUUUUUA (12% frameshifting), it is comparable to that of UUUUUCA (1.57% frameshifting), which also has one G · C pair at the A site. The reduced shifting of GGGUUUC (0.52% frameshifting) compared with that of the wild-type could be explained by the unshiftable tRNA hypothesis, the additional G · C base pair in the A site, or a combination of these mechanisms.

Low-abundance tRNAs and frameshifting efficiency. Belcourt and Farabaugh (3) showed that the +1 ribosomal frameshift that generates the Ty1 *gag-pol* fusion protein is due to a +1 slip by a unique tRNA^{Leu} whose anticodon is UAG and that can pair, in the P site, with either the 0-frame codon CUU or the overlapping +1-frame codon UUA. This slip is stimulated by the next 0-frame codon being AGG, specifying a rare tRNA^{Arg}. Supplying this tRNA^{Arg} gene on a high-copy-number vector suppressed the frameshifting, as did changing the codon to one that was not so rare. In L-A the next codon after the slippery site in the 0 frame is GGA, which is a Gly codon rarely used in highly expressed genes of *S. cerevisiae* and is assumed, therefore, to be low in abundance (31). The tRNA cognate to GGA is encoded by three genes, whereas that for GGU is represented by about 15 genes (27). When we changed this codon to the frequently used Gly codon GGU (pJD35), we observed a fivefold decrease of frameshifting (Fig. 3). To determine whether this effect was due to the difference in tRNA abundance, we introduced pJD54, a high-copy-number plasmid carrying the *SUF1* gene encoding a copy of the tRNA^{Gly} that reads GGA (27), into a strain carrying pF'8 (the wild-type slippery site) or pJD32 (slippery site U UUU UUA, 12% frameshifting). The efficiency of frameshifting by the wild-type site was not affected, but the very shifty pJD32 was reduced in efficiency to 3.0% (Fig. 3). In the work of Belcourt and Farabaugh, the AGG codon was the only one tested that gave efficient frameshifting, so we introduced AGG in place of the normal GGA (pJD44), expecting to see an increase in frameshifting efficiency. Instead, we observed a slight decrease of frameshift efficiency to 1.3%. Taken together, these results suggest that the GGA codon in the L-A slippery region does not play the same role in frameshift efficiency as the AGG codon plays in Ty1.

Rates of frameshifting affect virus viability. The L-A virion has approximately 120 major coat protein molecules per viral particle (7, 12). The 1.9% frequency of frameshifting suggests that there are two fusion proteins per virion, and this is approximately the amount found in isolated particles. An L-A cDNA expression plasmid has been shown to be able to support M_1 in a *mak10* host in the absence of the L-A virus. The M_1 RNA is encapsidated in viral particles and is replicated and transcribed (36). The L-A virus is not supported in these circumstances, allowing us to manipulate the *gag/gag-pol* ratio by altering the L-A expression plasmid. We constructed a series of mutants of this plasmid containing slippery sites with known frequencies of frameshifting (Fig. 4, Table 2). The M_1 satellite virus was cytoduced into cells containing these mutants. We observed that M_1 could only be maintained and propagated in a narrow window of frameshifting efficiency. A greater-than-twofold increase in frameshifting completely eradicated the ability of the plasmid to maintain M_1, as did a sixfold decrease. Changes in the rate of frameshifting that allowed for maintenance of M_1 affected the observed killer phenotype. The wild-type slippery site sequence gave a strong killer phenotype. The zones and intensities of killing decreased as frameshifting deviated further from the wild-type rate (Fig. 4C). This phenotypic change was reflected in the M_1 dsRNA copy number (Fig. 4B). Immunoblotting demonstrated (i) that all of the strains contained plasmids that made both *gag* and *gag-pol* fusion proteins and (ii) that, within the limits of quantitation imposed by the technique, the observed relative amounts of *gag-pol* fusion proteins are generally consistent with the frequencies of frameshifting as measured more accurately by the β-galactosidase assay (Fig. 4A).

Host mutations affecting ribosomal frameshifting efficiency. We isolated a number of host chromosomal mutants that

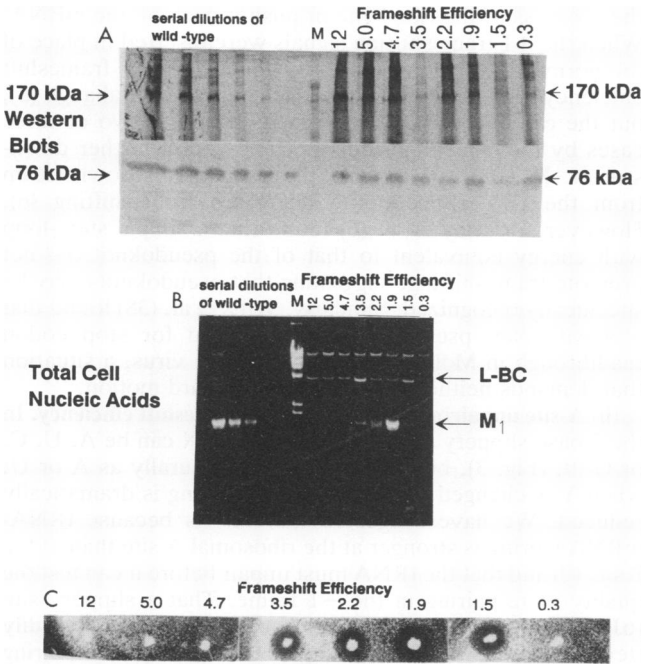

FIG. 4. Effect of altered frameshift efficiency on the ability of the L-A cDNA expression plasmid to support the propagation of M_1 dsRNA. Variants of the L-A cDNA expression plasmid pI2L2 with various slippery site sequences were constructed. The efficiency of frameshifting given is that measured for the same slippery site in the β-galactosidase vector pF'8 (Table 2). L-A and M_1 were introduced into strain 3063 carrying each variant L-A expression plasmid by cytoduction from strain 2629. Because strain 3063 has the *mak10-1* mutation, L-A is lost but M_1 propagation is stably supported by the normal L-A expression plasmid (37). (A) Extracts of cytoductant strains carrying the variant L-A expression plasmids were analyzed by Western immunoblots. The upper portion of the blot was probed with anti-170-kDa *gag-pol* fusion protein antiserum. The lower portion of the blot was separately probed with an affinity-purified antibody to *gag*. Twofold serial dilutions of extracts of a strain carrying the wild-type plasmid pI2L2 are shown for reference. M, molecular weight markers. (B) Total nucleic acids extracted from the same strains, analyzed on a 1.5% agarose gel, and stained with ethidium bromide. L-BC is a dsRNA virus unrelated to L-A and M. (C) Killer test of the same cytoductant strains.

increase the rate of −1 ribosomal frameshifting (*hsh* = high frameshifting). One of these, *hsh1*, which had a frequency of frameshifting that was approximately threefold higher than normal (Table 3), was examined for effects on the support of M_1 propagation by the L-A cDNA expression vector in a *mak10* background. Cytoductants of *hsh1* mutants did not have the killer phenotype (Fig. 5A), the M dsRNA genome was lost (Fig. 5B), and *hsh1* cells containing the plasmid produced more *gag-pol* fusion protein than did their *HSH1* counterparts (Fig. 5C).

A Ty1 +1 frameshift signal can substitute for the wild-type L-A frameshift signal. Belcourt and Farabaugh (3) defined a native 7-nucleotide sequence and mutations thereof that effect +1 ribosomal frameshifting in the yeast retrotransposon Ty1. The wild-type CTTAGGC sequence was reported to yield a 40% rate of +1 frameshifting, whereas mutants CTTCGGC, CTTTGGC, and CTTAGTC gave rates of approximately 1.8, 2.1, and 3.9%, respectively. We replaced the bases GGTTTA of the slippery site with these +1 slippery sites in both the β-galactosidase-containing vector

TABLE 2. Effect of slippery site on frameshift efficiency and propagation of M_1 dsRNA[a]

Plasmid[b]	Slip site	% Shift	Killer
pJD32/pJD34	TTTTTTA	12.0	−
pJD24/pJD40	GGGAAAA	5.0	−
pJD31/pJD39	CCCTTTA	4.7	−
pJD27/pJD41	GGGAAAC	3.5	+
pJD13/pJD38	AAATTTA	2.2	+
pF'8/pI2L2 (wild type)	GGGTTTA	1.9	+
pJD43/pJD42	TTTTTCA	1.5	+
pJD49/pJD50	TTTCCCC	0.3	−

[a] The percent shift was measured by measuring β-galactosidase activity in strain 2907 carrying pF'8 modified to have the slippery site shown and dividing by the activity produced in the same strain by pTI125, the in-frame plasmid. Killer activity is the result of placing the same slippery site into the L-A cDNA expression plasmid pI2L2 and determining whether M_1 was supported by this plasmid in the *mak10* host, strain 3063. The killer assay, dsRNA analysis, and fusion protein synthesis of cells carrying these plasmids are shown in Fig. 4.

[b] The first plasmid in each pair is the β-galactosidase expression plasmid with the indicated slippery site. The second plasmid is the L-A cDNA expression plasmid.

pF'8 and in the L-A cDNA expression vector pI2L2 (Fig. 6). These constructs were such that a +1 frameshift would place the frame in the L-A *pol* open reading frame. Each of these plasmids showed rates of +1 frameshifting that were substantially lower than that reported by Belcourt and Farabaugh for the same sequences in the Ty1 context, and none allowed support of M_1 by the L-A cDNA expression plasmid (Fig. 6). However, introduction of an additional codon (a frequently used Arg^{AGA}) between the +1 slippery site and the pseudoknot substantially increased the rate of shifting of two of the three constructs. That with the highest shifting efficiency (1.3%) allowed stable propagation of M_1 from the L-A cDNA plasmid, whereas those with efficiencies in the 1.0-to-1.2% range gradually lost the killer phenotype. This shows that a +1 shift is as good as a −1 shift, as long as the ratio of *gag* to *gag-pol* is correct. The increase of +1 shifting when the slippery site was moved back from the pseudoknot supports the notion that the pseudoknot, whose proximity increases −1 shifts (6), is really holding back the progress of the ribosomes, preventing the +1 shift.

DISCUSSION

Factors affecting frameshift efficiency. (i) The breathing pseudoknot. The importance of the pseudoknot after the slippery site of the coronavirus infectious bronchitis virus has been described by Brierley and coworkers (4–6). They showed that the distance of the pseudoknot from the slippery site is critical (6). We found that L-A also has a pseudoknot that is necessary for efficient frameshifting (9). The L-A

TABLE 3. Chromosomal high-shift (*hsh*) mutants increase L-A ribosomal frameshift[a]

Strain	Genotype	% Shift	*hsh1*/*HSH1* shift ratio
JD44-1A	*HSH1*	2.7	
JD44-1B	*hsh1*	10.6	3.9
JD44-2B	*HSH1*	3.2	
JD44-2C	*hsh1*	8.5	2.7

[a] The percent shift was measured by assaying β-galactosidase activity in each strain carrying pF'8 and dividing by the activity produced in the same strain from pTI125, the in-frame plasmid.

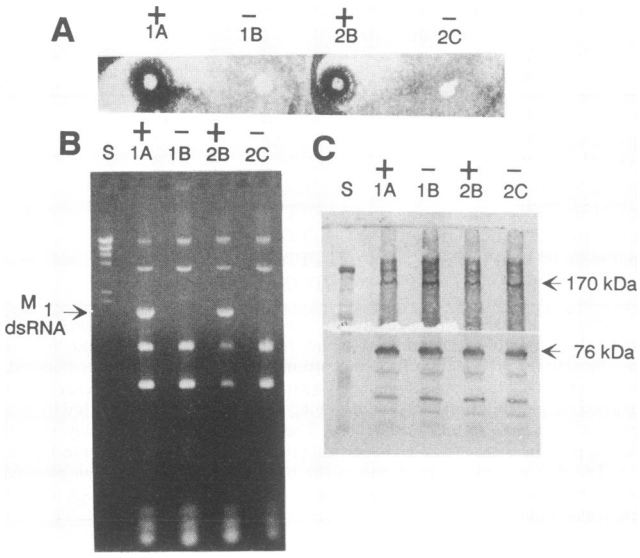

FIG. 5. L-A cDNA expression plasmid cannot maintain M_1 in *hsh1* (high shifter) chromosomal mutants. JD44 segregants 1A, 1B, 2B, and 2C that were *trp1 mak10* [*rho*^{0}] were transformed with the L-A cDNA expression vector pI2L2, and then M_1 was introduced by cytoduction from strain 2629. +, wild type; −, *hsh1* mutant. (A) Killer assay of cytoductants. (B) Agarose gel electrophoresis of nucleic acids from cytoductants. (C) Immunoblot of extracts of JD44-1A, -1B, -2B, and -2C carrying pI2L2 (before cytoduction) probed with rabbit anti-170 kDa *gag-pol* fusion protein antiserum (top) or with affinity-purified rabbit anti-76 kDa *gag* protein antibody (bottom). Each lane represents 6×10^6 cells.

pseudoknot has the potential to breathe; the length of stem 2 increases by 4 bp at the expense of stem 1 (Fig. 1). The same breathing, though only by 2 bp, is also possible in IBV (5). We show here that the breathing is not necessary for promoting frameshifting and that the pairing in stem 1 is more important than the pairing in stem 2.

Our work also provides support for the notion that the role of the pseudoknot in −1 frameshifting is to physically block

Plasmid	%Shift in Ty	%Shift in pF'8	%wild type	Killer	Site
pF'8/pI2L2	-	1.8	100	+	1956-CAG GGUUUAGG AG^U
pJD55/56	1.8	0.9	50	-	CAG CUUCGGC AG^U
pJD57/59	2.1	0.10	4.2	-	CAG CUUUGGC AG^U
pJD58/60	3.9	0.15	7.4	-	CAG CUUAGUC AG^U
pJD68/71	1.8	1.1	62	-	CAG CUUCGGC AGA AG^U
pJD69/72	2.1	1.2	67	w	CAG CUUUGGC AGA AG^U
pJD70/73	3.9	1.3	73	+	CAG CUUAGUC AGA AG^U
					0 frame ->
					+1 frame

FIG. 6. M_1 propagation supported by an L-A cDNA clone making *gag-pol* by a +1 frameshift. Sequences that should produce a +1 ribosomal frameshift at some frequency (%Shift in Ty) (3) were introduced into either pF'8 to measure the frequency in the context of the L-A sequence (%Shift in pF'8 and %wild type) or into pI2L2 to determine whether the sequence would allow the support of M_1 propagation (Killer). Each altered pI2L2 was transformed into strain 3063, and M_1 was introduced by cytoduction from strain 2629. The plasmid numbers refer to the variants of pF'8 and pI2L2, respectively. %Shift in Ty, value reported by Belcourt and Farabaugh (3) for the indicated shifty site in the Ty1 context. %wild-type, frameshift efficiency as a percentage of that of pF'8, which has the normal L-A shift site. Killer, phenotype of the cytoductants (w, weak killer). ^, position at which the pseudoknot structure begins. The last three constructs are the same as the preceding three, except that the pseudoknot has been moved one codon away from the slippery site.

the progress of the ribosome or push it back on the mRNA. When the +1 frameshifting signals were inserted in place of the normal L-A slippery site, we found a lower frameshift efficiency than that reported by Belcourt and Farabaugh (3), but the efficiencies were increased 10-fold in two of three cases by moving the pseudoknot one codon further downstream. In contrast, moving the pseudoknot downstream from the IBV slippery site decreases frameshifting (6). However, Brierley et al. (6) found that a simple stem-loop with energy equivalent to that of the pseudoknot did not promote frameshifting, suggesting that pseudoknots may be specifically recognized. Similarly, Wills et al. (38) found that a downstream pseudoknot was required for stop codon readthrough in Moloney murine leukemia virus, a situation that demands neither forward nor backward motion.

(ii) A-site unpairing is important for frameshift efficiency. In the 7-base slippery site ..X XXY YYZ.., X can be A, U, C, or G (9) (Fig. 3), but Y is only found naturally as A or U; when Y is changed to C or G, frameshifting is dramatically reduced. We have suggested that this is because tRNA-mRNA pairing is stronger at the ribosomal A site than at the P site (9) and that the tRNA must unpair before it can test the quality of re-pairing in the −1 frame. That a slippery site (pJD43) with an A-site triplet that unpairs more readily (fewer G · C pairs) but forms a poorer match upon re-pairing frameshifts more efficiently than pJD49, in which the conditions are reversed, supports this notion. However, it is also possible that there are specific tRNAs that, as suggested by Hatfield and Oroszlan (18), are particularly good at shifting and others that are reluctant to do so.

Effect of frameshift efficiency on virus propagation. Viral ribosomal frameshifting occurs in many retroviruses, including Ty1 of *S. cerevisiae*, in the coronaviruses, in the L-A dsRNA virus, in several plus-strand single-stranded RNA plant viruses, and in bacteriophages T7 and lambda (4–6, 9, 11, 19, 20, 23–25, 28, 32, 33, 39; and Levin, Hendrix, and Casjens [cited in reference 35]). Neither the *gag* protein alone nor the *gag-pol* fusion protein alone will support replication (13, 29, 36); Moloney murine leukemia virus *gag* and *gag-pol*, when expressed from separate vectors, form virus poorly (13). In none of these viral cases, however, has the physiological significance of the efficiency of frameshifting been critically tested until the present study.

We find that, when M_1 dsRNA replication is supported by the L-A cDNA expression vector, the ratio of *gag* protein to *gag-pol* fusion protein reflects the efficiency of frameshifting as measured by β-galactosidase assay and that this ratio is critical for the efficient propagation of the M_1 satellite virus. This result was observed whether the alteration in −1 ribosomal frameshifting was due to changes introduced in the slippery site or due to a chromosomal mutation (*hsh*). The result cannot be due simply to the altered amino acid or two encoded by the altered slippery site; several changes that altered the encoded amino acids were compatible with propagation of M_1, and the *hsh* mutants, in which the coding of this site is not altered, were unable to maintain M_1. Although the efficiency of frameshifting by L-A appears to be critical, there is no evidence that it is regulated like the +1 frameshifting in the RF2 gene of *Escherichia coli* (8, 34).

When we substituted a +1 slippery site for the −1 slippery site, making synthesis of *gag-pol* dependent on +1 frameshifting, we found that M_1 could be supported but that, again, the ratio of *gag* to *gag-pol* was critical. This result shows that there is no specific requirement for a −1 frameshift. It also sets a more precise lower limit on the acceptable efficiency at about 1.3% (73% of the wild-type rate).

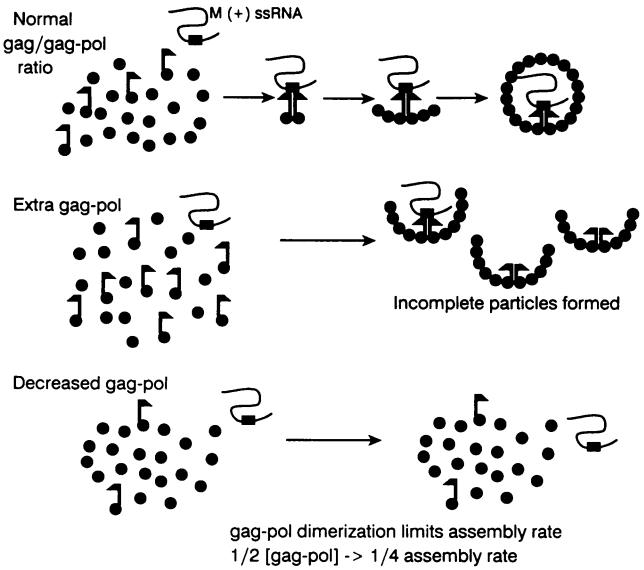

FIG. 7. Importance of ribosomal frameshift efficiency interpreted in terms of a model of assembly for L-A (16). The model proposes that a *gag-pol* fusion protein dimer binds the L-A plus strands with the demonstrated single-stranded RNA (ssRNA) binding activity of its *pol* domain and primes polymerization of *gag* monomers with its *gag* domain (top). An excess of *gag-pol* fusion protein might then result in many incomplete particles if binding of plus strands were not prerequisite to priming capsid polymerization. A relative excess of *gag* protein might lead to *gag* polymerization and closure of particles before the *gag-pol* fusion protein could find a plus strand, or, if dimerization of the *gag-pol* fusion protein is rate limiting, a modest decrease of fusion protein concentration might produce a dramatic decrease of the dimer and failure of assembly.

By altering frameshifting efficiency by any of three methods, we found the same clear result: the efficiency was critical for M_1 propagation with the proteins from the cDNA clone. To carry out the experiments described in this report, it was necessary to set up a rather special and carefully controlled situation. To control the ratio of *gag* to *gag-pol*, the source of L-A-encoded proteins had to be the L-A cDNA expression plasmid or variants thereof. We can introduce the L-A and M_1 viruses together by cytoduction, but we must get rid of the L-A virus so that L-A proteins are coming only from the plasmid. This is done by using a *mak10-1* host (36). We have, however, evidence that the *hsh1* mutants, like other chromosomal genes controlling M_1 propagation, are affected by other factors (10). Some *hsh1 MAK10* strains carrying a different L-A natural variant virus than that used to make the cDNA clone are able to maintain M_1. Since the *mak10-1* mutant allele, the particular L-A, and the viral proteins coming from a cDNA plasmid instead of a virus are all factors known to critically influence the replication of M_1, further work is needed to discern the origin of this effect.

The explanation for the importance of the ratio of *gag* protein to *gag-pol* protein must involve the process of viral particle assembly. It has been suggested that the *gag-pol* fusion protein is the key to assembly, its *pol* domain binding to the packaging site on viral plus strands with its known single-stranded RNA binding activity and priming assembly of *gag* monomers with its N-terminal *gag* domain (Fig. 7) (15, 16). In accord with this model, an excess of *gag-pol* fusion protein might be expected to result in many partially complete particles but few complete ones (Fig. 7). Similarly, if the binding of viral plus strands is not tightly coordinated with priming of *gag* polymerization, a decrease of *gag-pol* fusion protein might result in the particles being often completely closed before the *pol* domain has found a viral plus strand to bind, resulting in gradually decreasing M_1 levels and the eventual loss of M_1. The normal frameshift rate of 1.9% and the presence of 120 *gag* molecules per particle indicates that there are two *gag-pol* molecules per particle. Since there is direct evidence that the particles package only one viral plus strand per particle (15), this suggests that it is a *gag-pol* dimer that binds viral plus strands. In that case, decreasing the production of *gag-pol* might result in a more dramatic decrease of the *gag-pol* dimer and failure of packaging (Fig. 7).

The critical nature of the efficiency of ribosomal frameshifting for yeast virus propagation and the similarity of this system to retrovirus gene expression suggest that drugs affecting ribosomal frameshifting might be useful as antiretroviral agents. As yet, no examples are known of eukaryotic cellular genes whose proper expression requires ribosomal frameshifting. The fact that the *hsh* mutants that increase frameshifting efficiency do not noticeably affect yeast cell growth (10) suggests that drugs specifically affecting frameshifting might be well tolerated.

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