Ribosomal Frameshifting Requires a Pseudoknot in the *Saccharomyces cerevisiae* Double-Stranded RNA Virus

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The large double-stranded RNA of the *Saccharomyces cerevisiae* (yeast) virus has two large overlapping open reading frames on the plus strand, one of which is translated via a -1 ribosomal frameshift. Sequences including the overlapping region, placed in novel contexts, can direct ribosomes to make a -1 frameshift in wheat germ extract, *Escherichia coli* and *S. cerevisiae*. This sequence includes a consensus slippery sequence, GGGUUUA, and has the potential to form a pseudoknot 3' to the putative frameshift site. Based on deletion analysis, a region of 71 nucleotides including the potential pseudoknot and the putative slippery sequence is sufficient for frameshifting. Site-directed mutagenesis demonstrates that the pseudoknot is essential for frameshifting.

The *Saccharomyces cerevisiae* viruses (ScV) are noninfectious double-stranded RNA viruses with separately encapsidated segments present in the cytoplasm of most laboratory strains. The large viral double-stranded RNA from ScV subtype 1, L1, has two large open reading frames (ORFs) overlapping by 129 bases on the plus strand (7). The first large ORF (*cap*) encodes the major capsid polypeptide P1 (7, 8, 12), the predicted size of which is 76 kDa. The second ORF (*pol*) encodes a polypeptide containing an RNA binding domain (10), a predicted RNA-dependent RNA polymerase domain (4, 7, 24), and a nucleotide phosphohydrolase domain (9a). Our previous results suggest that the second ORF is read via a -1 ribosomal frameshifting event within the overlapping region (7). The predicted size of the resulting *cap-pol* fusion protein is about 171 kDa. Antibody against a synthetic peptide derived from the predicted RNA-dependent RNA polymerase sequence has been shown to recognize a viral protein of this size by immunoblotting of viral proteins (7) and immunoprecipitation of in vitro translation products of heat-denatured L1 dsRNA (9b). Two sequences in the overlapping region, UUUA and UUUUA, were suggested as possible slippery sites (7). A stem-loop structure with a continuous 11-bp stem downstream from the UUUA was predicted (7). A potential pseudoknot was also suggested to be present 3' from the UUUA (31).

Ribosomal frameshifting has been demonstrated in retroviruses (13–16, 23), the coronavirus infectious bronchitis virus (IBV) (3), the yeast retrotransposon Ty (5, 20), and a number of prokaryotic systems (6). A translational frameshift has also been proposed to be involved in expression of the polymerase in barley yellow dwarf virus (22). While retroviruses have a -1 frameshift responsible for the synthesis of *gag-pol* or *gag-pro-pol* fusion protein (13–16, 23, 37) and the coronavirus IBV has a -1 frameshift responsible for the synthesis of the F1-F2 fusion protein (3), the TYA-TYB fusion polypeptide of Ty elements is synthesized by a $+1$ frameshift (2, 5, 20). The slippery site of retroviruses and IBV has a characteristic heptanucleotide RNA sequence, starting with two homopolymeric triplets (3, 13), which is not present in the frameshift site of Ty elements (2).

Sequences required for frameshifting, in addition to the slippery site, have been extensively studied by using deletion analysis and site-directed mutagenesis. In some cases, RNA secondary structure, which might slow down the passage of ribosomes, is required for efficient frameshifting (3, 13). A stem-loop structure downstream of the slippery site has been shown to be essential for frameshifting in vitro for Rous sarcoma virus (13), and a pseudoknot structure (25, 26) may be required (13). The presence of a pseudoknot following the coronavirus IBV frameshift site is required for efficient frameshifting (3). Secondary structure has also been suggested to be important for frameshifting in mouse mammary tumor virus (15), and many other retroviruses (19, 31). In contrast, human immunodeficiency virus type 1 (HIV-1) does not depend on the predicted stem-loop structure following the slippery site to accomplish frameshifting in either the mammalian or yeast system, but requires only 16 nucleotides (nt) surrounding the slippery site (37). Similarly, only 7 nt are required for frameshifting in Ty elements (2). In this case, ribosomal pausing at the frameshift site may be caused by the presence of a rare codon in the original reading frame (2). Some aspects of frameshifting must be similar in eukaryotes and prokaryotes, since frameshift signals found in retroviruses accurately direct *Escherichia coli* ribosomes to shift frame in a similar manner (36).

In this report, a cDNA fragment comprising the putative ScV-L1 frameshift signal is shown to direct frameshifting in wheat germ extract, *E. coli*, and *S. cerevisiae*. Deletion analysis and site-directed mutagenesis show that a sequence of 71 nt is sufficient for frameshifting and that the potential pseudoknot structure following the putative slippery sequence GGGUUUA is required. To our knowledge, this is only the second example in which a 3' pseudoknot has been shown to be essential for frameshifting.

MATERIALS AND METHODS

Construction of plasmids for frameshift analysis. Plasmids pGEM7ZCTU and pFS1 (Fig. 1) were constructed for frameshift analysis in wheat germ extract and in *E. coli*, respectively. These were constructed by inserting an L1 cDNA fragment of 396 bp (1783 to 2179), which contains the putative frameshift signal, into pGEM-7Zf(+) (Promega). The fragment was ligated to *Hind*III-*Csp*45I-digested pGEM-

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FIG. 1. Plasmids used for frameshift analysis in wheat germ extract (pGEM7ZCTU), *E. coli* (pFS1), and *S. cerevisiae* (p3pFS). The first reading frame (*cap*) is in frame with the first AUG in the multiple cloning site 3' to the start point for the SP6 promoter in pGEM7ZCTU and in frame with the AUG under the control of the

7Zf(+) to create pGEM7ZCTU and *Csp45I*-digested pGEM-7Zf(+) to create pFS1; both insert and vectors were blunt ended with *E. coli* DNA polymerase Klenow fragment (Promega) prior to ligation. The first reading frame (*cap*) is in frame with the first AUG in the multiple cloning site 3' to the start point for the SP6 promoter in pGEM7ZCTU and in frame with the AUG under the control of the *lacZ* promoter in pFS1; the second reading frame (*pol*) is in frame with the α peptide of β-galactosidase of pGEM-7Zf(+) in both constructs. A third plasmid, p3pFS (Fig. 1), derived from p3p (3), was constructed for frameshift analysis in yeast cells. The yeast expression vector p3p was digested with *Bam*HI and *Kpn*I, blunt ended, and ligated with a blunt-ended *Hind*III-*Sma*I fragment (bp 1952 to 2179 of L1) to create the frameshift plasmid p3pFS, in which the initiation codon of *his4A* is in frame with the *cap* ORF and *lacZ* is in frame with the *pol* ORF.

Plasmids were maintained in *E. coli* DH5α (Bethesda Research Laboratories) or JM109. Yeast expression experiments were performed in strain CGY150 (*MATα ura3-52 leu2-3 GAL⁺*) (11). All plasmid constructs were verified by dideoxy sequencing (28).

Construction of deletion mutants. Constructions of 5' and 3' deletion mutations for frameshift analysis both in vitro and in *E. coli* were done by inserting different lengths of L1 cDNA fragments encompassing different ranges of the *cap/pol* overlap sequence into the pGEM-7Zf(+) vector. In the pFS series, consisting of deletion mutations for analysis in *E. coli*, both the *cap* and *pol* reading frames are in frame with the α-peptide β-galactosidase sequence in pGEM-7Zf(+). In the pGEM7ZCTU series, consisting of deletion mutations for analysis in wheat germ extract, the *cap* reading frame is in frame with the first AUG following the transcription start point of the SP6 promoter (in the multiple cloning site) and the *pol* reading frame is in frame with the α-peptide sequence of the vector. In both cases, a -1 frameshift event is required for the production of the β-galactosidase α-peptide fusion protein.

The boundaries of the frameshift signal were first defined by ligating different lengths of restriction fragments derived from pFS1 with pGEM-7Zf(+) digested with different restriction enzymes. Figure 5A indicates the region of the L1 cDNA in these constructs. In all of the constructs, both the inserts and vectors were blunt ended prior to ligation. A *Hind*III-*Dde*I fragment and a *Cla*I-*Dde*I fragment of pFS1 were inserted into *Hind*III-*Csp*45I-digested and *Hind*III-digested pGEM-7Zf(+), respectively, to make pGEM7ZCTUd1 and pFSd1. pFSd2 was constructed by inserting a *Cla*I-*Pvu*II fragment into *Hind*III-digested pGEM-7Zf(+). pGEM7ZCTUd2 was made by modifying pFSd2; pFSd2 was digested with *Bam*HI and the ends filled before self-ligation. pGEM7ZCTUd3 and pFSd3 were constructed by inserting a *Hind*III-*Hae*II fragment and a *Cla*I-*Hae*II fragment, respectively, into *Bam*HI-digested and *Hind*III-digested pGEM-7Zf(+). A *Hind*III-*Ava*II fragment and a *Cla*I-*Ava*II fragment were cloned into *Hind*III-*Sma*I-digested and *Hind*III-digested pGEM-7Zf(+) to construct pGEM7ZCTUd4 and pFSd4, respectively. To construct pFSd5 and pGEM7ZCTUd5, pFS1 and pGEM7ZCTU were

lacZ promoter in pFS1; the second reading frame (*pol*) is in frame with the α peptide of β-galactosidase of pGEM-7Zf(+) in both constructs. In p3pFS, the initiation codon of *his4* is in frame with the *cap* ORF and *lacZ* is in frame with the *pol* ORF.

digested with *Pst*I and *Xba*I and blunt ended before self-ligation. To make pGEM7ZCTUd6, a *Hae*II-*Eco*RI fragment was inserted into *Bam*HI-digested pGEM-7Zf(+). pFSd6 was made by modifying pGEM7ZCTUd6; pGEM7ZCTUd6 was digested with *Sac*I and then blunt ended by T4 DNA polymerase before self-ligation. pGEM7ZCTUd7 and pFSd7 were constructed by inserting a *Dde*I-*Eco*RI fragment and a *Dde*I-*Sma*I fragment into *Hind*III-digested and *Hind*III-*Sma*I-digested pGEM-7Zf(+), respectively. pFSd8 was constructed by inserting a *Sau*3AI-*Sma*I fragment into *Csp*45I-*Sma*I-digested pGEM-7Zf(+). pGEM7ZCTUd8 was made by modifying pFSd8; pFSd8 was digested with *Bam*HI and then end filled before self-ligation.

To further define the 3' limit of the frameshifting signal, deletion mutants d9 to d11 were made by *Bal* 31 digestion downstream of the slippery sequence. pGEM7ZCTU was linearized with *Eco*RI and treated with nuclease *Bal* 31 as described previously (27). *Bal* 31-treated pGEM7ZCTU was digested with *Bam*HI, and resulting fragments were separated from the vector by electrophoresis. Fragments of desired size were isolated from agarose gels and ligated to *Bam*HI-*Sma*I-digested pGEM-7Zf(+) for in vitro assay. For assays in *E. coli*, the same fragments were ligated to *Bam*HI-*Sma*I-digested pGEM-7Zf(+) that had previously been modified by *Sac*I digestion followed by flush ending with T4 DNA polymerase and religation.

The minimum sequence required for frameshifting was then inserted into both pGEM-7Zf(+) and the yeast expression vector p3p for frameshift analysis in both homologous and heterologous systems. Deletion mutants, pGEM7ZCTUd12 and pFSd12, containing the minimum sequence required for frameshifting, were constructed by *Bal* 31 digestion starting from pFSd7, using a procedure similar to that described above. The L1 sequence in the deletion mutant d12 was then subcloned into the yeast expression vector, p3p, to create p3pFSd, in which the *cap* ORF was in frame with the initiation codon of *his4A* and the *pol* ORF was in frame with *lacZ*.

All of the deletion clones were maintained in *E. coli* DH5 α or JM109. Yeast expression experiments were performed in strain CGY150 (11).

Site-directed mutagenesis. The significance of the pseudoknot was tested by creating complementary changes within the pairing region of the pseudoknot. Site-specific mutations within the pairing region were prepared by a modification of the method of Kunkel (18). Plasmids subjected to mutagenesis were transformed into *E. coli* CJ236 (*dut ung*) (IBI), and uracil-containing single-stranded DNA templates were isolated following superinfection with helper phage M13K07 (32). Mutagenic oligonucleotides were synthesized by the State University of New York at Buffalo microinstrumentation center. All of the mutants were identified by restriction digestion and confirmed by DNA sequencing. The mutagenesis efficiency was between 40 and 100%. Mutants were constructed as follows: m1, with a synthetic oligonucleotide of sequence GCATCGTAAGACCATGGCCTCTAAACC CTG to disrupt stem S1; m2, with CCGGTAGGCATTAC TCGACCGCTCGTAAGACCTACC to disrupt stem S2; m3, with GCTGTAGGTTCTCGCCATGGATTACAGCTGG to disrupt stem S1; and m4, with TCTTCGTGGCAGGCCG GTTCGAGTAGGTTCTCCGG to disrupt stem S2 (see Fig. 4). Altered bases are indicated by underlining in the sequences of the oligonucleotides. Stem-disrupting mutants m1 to m4 were constructed from pFS1. Mutant m5 combined m1 with m3 and mutant m6 combined m2 with m4, restoring base pairing in both putative stems of the pseudoknot in each

case but altering the sequence of one stem (S1) by an inversion of 5 bp and the sequence of the second (S2) by an inversion of 8 bp in the resultant cDNAs.

In vitro transcription and in vitro translation. For transcription, plasmids were linearized by digestion with *Ssp*I. In vitro transcription was done according to Krieg and Melton (17).

Translation in wheat germ extracts was carried out according to Anderson et al. (1). Translation products were analyzed by 17.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The termination and frameshift products were quantified by measuring the radioactivity of excised gel slices and by densitometry of autoradiographs; these two methods gave similar results. The numbers were corrected for methionine content of each product. The frameshift efficiency is expressed as the ratio of frameshift product to frameshift product plus termination product.

β -Galactosidase activity measurements. β -Galactosidase activity in yeast cells was measured by the whole-cell assay of Miller (21), and the yeast cells were permeabilized according to Belcourt and Farabaugh (2).

Immunoblotting. Frameshift products were detected by immunoblotting of total bacterial or yeast proteins with anti- β -galactosidase antibody (Sigma). Termination products in *E. coli* were detected with anticapsid antibody (7). The *E. coli* cells were induced with isopropylthiogalactopyranoside (IPTG) and lysed in SDS gel-loading buffer (27). Yeast cell lysates were prepared from CGY150 carrying p3p-derived plasmids as described previously (27). Total cell lysates were subjected to 17.5% SDS-PAGE (*E. coli*) or 7.5% SDS-PAGE (*S. cerevisiae*), transferred to nitrocellulose paper by electrophoresis, and reacted with the indicated antibody as described previously (7). Anti- β -galactosidase antibody was detected by an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma); anticapsid antibody was detected by alkaline phosphatase-conjugated protein A (Cappel).

RESULTS

Frameshifting assays in vitro, in *E. coli*, and in *S. cerevisiae*.

Our previous work (7) suggests that the 171-kDa polypeptide in the ScV viral particle is a fusion protein translated via a -1 ribosomal frameshift within the overlapping region between *cap* and *pol*. A cDNA fragment encompassing the overlapping region was cloned into pGEM-7Zf(+) and p3p vectors and tested for its ability to direct frameshifting in a heterologous genetic context. The results indicate that the L1 frameshift signal functions in both homologous and heterologous eukaryotic systems as well as in prokaryotes.

Two plasmids, pGEM7ZCTU and pFS1, were constructed for assay by translation in wheat germ extract and *E. coli*, respectively (Fig. 1). In both cases, frameshift products were synthesized as fusion proteins in which the α peptide of β -galactosidase in pGEM-7Zf(+) was fused in frame with the *pol* reading frame. The predicted sizes of the termination products of pGEM7ZCTU and pFS1 are 12 and 14 kDa, and the frameshift products are 27 and 29 kDa, respectively. We used two methods to measure frameshifting: in vitro synthesis of fusion proteins in wheat germ extract and immunoblots of *E. coli* proteins in which fusion proteins were detected with anti- β -galactosidase antibody.

Frameshifting was detected in both the wheat germ extract and *E. coli* (Fig. 2). Two polypeptides were detected after translation of SP6 transcripts derived from *Ssp*I-digested

FIG. 2. Frameshifting analysis in vitro (A) and in *E. coli* (B). (A) Autoradiogram of a 17.5% SDS-PAGE containing ^{35}S -labeled proteins produced from wheat germ extract translation directed by SP6 transcripts from *Ssp*I-digested pGEM7ZCTU and pGEM7ZCTUR. (B) Immunoblot of a 17.5% SDS-PAGE of bacterial lysates from two independent transformants with plasmids pFS1 (Fig. 1), pFSRO (contains the same cDNA responsible for frameshifting in the reverse orientation), pGEM7ZCTU (Fig. 1), or pGEM-7Zf(+) induced with IPTG. Primary antibody is a polyclonal anti- β -galactosidase antibody. FP, the frameshift product, a *cap-pol*- β -galactosidase α -peptide fusion; TP, the termination product; IC, a nonspecifically staining band in all strains (including complete *lac* deletions; not shown).

pGEM7ZCTU (Fig. 2A). The sizes of the two products, 14 and 26 kDa, were approximately as expected for the termination and frameshift products, respectively. As a negative control, the same cDNA was placed in reverse orientation to create pGEM7ZCTUR, and a small translation product (around 7 kDa) was detected. The small size of this translation product reflects the position of the nonsense codon in the insert. The frameshift efficiency in the wheat germ extract varied from 3 to 9% in three independent experiments. The synthesis of an α -peptide fusion protein in *E. coli* was demonstrated by immunoblotting with polyclonal anti- β -galactosidase antibody (Fig. 2B). A protein with the expected size was detected only in bacterial lysates from two independent transformants containing pFS1, not in those containing control plasmids in which the cDNA was placed in reverse orientation (pFSRO) or in which the *E. coli* translation initiation site was not in frame (pGEM7ZCTU). A polypeptide (IC; Fig. 2B) recognized by our anti- β -galactosidase antibody in all strains is probably not related to β -galactosidase because it was also present in an *E. coli* strain with a complete *lac* deletion (data not shown).

Frameshifting was also demonstrated in a homologous yeast system by inserting a fragment of 228 bp encompassing the frameshift region (1952 to 2179) into the *his4-lacZ* fusion in the p3p vector (2) so that the N-terminal codons of *his4* were in frame with the *cap* ORF and the truncated *lacZ* was in frame with the *pol* ORF (p3pFS). A frameshift fusion product of approximately the predicted size (125.3 kDa), somewhat larger than the in-frame fusion β -galactosidase of the control plasmid p3pF (117.5 kDa), is produced (Fig. 3). Negative controls, in which the frameshift region was inserted in the wrong orientation (p3pFSR), produce no β -galactosidase. The frameshift efficiency in yeast cells was relatively high; the β -galactosidase activity in p3pFS was about 20% of that in p3pF, while p3pFSR had less than 0.01% of the level in p3pF.

FIG. 3. Frameshifting in yeast cells. Synthesis of the *cap-pol*- β -galactosidase frameshift products was detected by immunoblotting of total proteins of yeast transformants after 7.5% SDS-PAGE using anti- β -galactosidase antibody. The control plasmids with the fragments inserted in the reverse orientation (p3pFSR and p3pFSRd) produced no β -galactosidase, while the positive control (p3pF) produces a His4- β -galactosidase fusion product of 116 kDa (theoretical) and the frameshift plasmids (p3pFS and p3pFSd) produce His4-*cap-pol*- β -galactosidase fusion products of 125 and 120 kDa (theoretical). Sizes estimated from the migration of markers were consistent with these values. FP, the frameshift product, a *cap-pol*- β -galactosidase α -peptide fusion.

Defining the boundaries of the frameshifting signal. Retroviral frameshifting appears to involve two components: a slippery sequence and a 3' stem-loop or pseudoknot structure. Only in IBV RNA has the formation of a pseudoknot tertiary structure been shown to be essential for efficient frameshifting. Inspection of the ScV-L1 RNA *cap-pol* overlap reveals similar sequence motifs: a putative slippery sequence motif (GGGUUUA) (1958 to 1964) 5 nt upstream of a potential stem (S1) and loop, some bases of which can form a stem (S2) with a downstream sequence, resulting in a pseudoknot (1969 to 2022) (Fig. 4). To investigate the role of these sequences and the other possible slippery sequence UUUUA (2059 to 2063), we created a series of in-frame deletions by using either restriction enzymes or Bal 31 exonuclease.

Deletions upstream of the slippery site (pGEM7ZCTUd7

FIG. 4. One possible structure of the proposed pseudoknot. The pseudoknot was drawn such that two stems could stack coaxially to form a quasi-continuous helix according to the principle described by Pleij et al. (25). S1 is stem 1 (1969 to 1979 and 1994 to 2004), S2 is stem 2 (1984 to 1993 and 2013 to 2022), L1 is loop 1, and L2 is loop 2. Nomenclature is as in reference 31. Site-directed mutagenesis (see Materials and Methods) was used to alter 5 nt in one stem (m1 and m3) and 8 nt in the other (m2 and m4) in the putative pseudoknot (Materials and Methods). Those bases which were altered in the mutants have asterisks.

FIG. 5. (A) Deletion mutants. Deletion for analysis of the minimal frameshift sequence were constructed as described in Materials and Methods. WT indicates the wild-type construction (pFS1 or pGEM7ZCTU), and d1 through d12 are the deletions. The frameshift efficiency determined in vitro is the ratio of frameshift product to frameshift product plus termination product, as determined by densitometry of autoradiographs and by measuring the radioactivity of the gel slices. Correction has been made for different molarities of methionine in each case. The relative frameshift efficiency determined in *E. coli* is the ratio of frameshift product to termination product, as determined by scanning the immunoblots and Coomassie brilliant blue-stained gels, normalized to 100% for the wild type. (B) Wheat germ extract translation products synthesized in response to mRNA from pGEM7ZCTU and deletion mutants (d6 to d11), which were used to determine the minimum sequence required for frameshifting. (C) Detection of *cap-pol*- β -galactosidase α -peptide fusions synthesized in *E. coli* transformed with pFS1 and deletion mutants (d6 to d11), which were used to determine the minimum sequence required for frameshifting. FS% is the frameshift efficiency; ND indicates that the frameshift efficiency is less than 0.4% in the wheat germ extract system and the relative frameshift efficiency is less than 10%. FP indicates the frameshift product, a *cap-pol*- β -galactosidase α -peptide fusion; TP indicates the termination product.

and -d8) or downstream of the potential pseudoknot structure (pGEM7ZCTUd4 and -d5) had no effect on efficient frameshifting in wheat germ extract (Fig. 5A and B). As we predicted, deletion mutant pGEM7ZCTUd2, in which one side of the predicted stem-loop was removed, displayed no frameshifting. Even a 2-bp deletion (pGEM7ZCTUd3) in the 3'-terminal portion of the second stem of the pseudoknot sequence reduced the in vitro frameshifting efficiency to an undetectable level. In pGEM7ZCTUd6, the putative frameshift region (1958 to 1964) and the predicted pseudoknot

were deleted. No detectable frameshifting occurred in this mutant, although this result is ambiguous because of the inability to detect the termination product in reasonable quantity (Fig. 5B). This may be due to degradation of the very short termination products (see below). The sizes of frameshift and termination products in all other deletion clones were similar to what we expected. These results show that the sequence essential for efficient frameshifting is located on the L1 plus-strand sequence between nt 1952 and 2096.

Deletion analysis of frameshifting in *E. coli* was consistent with in vitro results (Fig. 5). All of the mutants that frameshifted in vitro also frameshifted in *E. coli*, and all of the mutants that did not frameshift in vitro also do not frameshift in *E. coli*. Again, all termination and frameshift products had sizes close to those predicted. The frameshift products were detected in immunoblots probed with a polyclonal anti- β -galactosidase antibody. The termination products were readily visible on Coomassie brilliant blue-stained gels because they were the most abundant proteins on the gels, and their identity was confirmed by immunoblots probed with anticapsid antibody. Since most of the epitopes recognized by anticapsid antibody appear to be located downstream to the frameshift site, neither frameshift products nor truncated termination products missing the region 3' to the slippery site could be recognized by the antibody. Because different antibodies were used to recognize the frameshift and termination products, only a relative frameshift efficiency was determined. The amounts of termination products were quantified by scanning Coomassie brilliant blue-stained gels, and the amounts of frameshift products were quantified by scanning immunoblots with a densitometer. Equal amounts of total protein were analyzed from all strains. The relative frameshift efficiency determined in *E. coli* is the ratio of frameshift product to termination product, as determined by scanning the immunoblots and Coomassie brilliant blue-stained gels, normalized to 100% for the wild type. The relative frameshift efficiencies of the deletion mutants that did exhibit frameshifting (except d7) were similar to that of the wild type in each case.

The high ratio of frameshift product to termination product in pFSd7 might be the result of faster degradation of the termination product in *E. coli*. Consistent with this interpretation, the even shorter termination product of d6 is not detectable either in vivo in *E. coli* or in vitro in wheat germ extracts. In addition, a point mutant (m5a) with an altered pseudoknot sequence similar to that of m5 (see below) but with a termination codon in the *cap* reading frame just after the putative slippery site makes normal amounts of the expected frameshift product but no detectable termination product in vitro, although the termination product is detectable in *E. coli* (data not shown). The calculated sizes of the termination products that were not detectable or reduced in quantity in one or more systems were 2.9 (d6), 5.7 (d7), and 9.8 (m5a) kDa. All of the termination products that were detectable in all systems were 10.7 kDa or larger.

To further define the 3' limit of the frameshifting signal, we created a series of deletions downstream of the slippery sequence by using Bal 31 exonuclease (Fig. 5A). Several of these (d9 to d11) were analyzed in both wheat germ extract and *E. coli*. As shown in Fig. 5, the region sufficient for frameshifting includes nt 1952 to 2022. Hence, the region essential for frameshifting is 71 nt in length, encompassing the putative slippery sequence and the proposed pseudoknot. The lack of requirement for other 5' and 3' sequences was confirmed by frameshifting directed by this 71 nt in both

FIG. 6. Analysis of pseudoknot mutants. Mutants m1 to m6 were analyzed for synthesis of *cap-pol*- β -galactosidase α -peptide fusions in the wheat germ extract system (A) and in *E. coli* (B) as described in the legend to Fig. 2. Only the two double mutants, m5 (m1 + m3) and m6 (m2 + m4) have frameshift efficiencies comparable to that of the wild type. FP, the frameshift product, a *cap-pol*- β -galactosidase α -peptide fusion; TP, the termination product.

wheat germ extract (pGEM7ZCTUd12) (Fig. 5A) and yeast cells (p3pFSd) (Fig. 3). The frameshift efficiency in p3pFSd is around 29% as determined by a β -galactosidase activity assay. This 71 nt, however, failed to direct *E. coli* ribosomes to frameshift efficiently (Fig. 5), although a comparable frameshift efficiency (102%) was detected when a slightly longer sequence (1952 to 2054) was used (data not shown).

Requirement for pseudoknot. The sequence downstream of the GGGUUUA site is predicted to form a pseudoknot. One possible secondary structure in this region is illustrated in Fig. 4. The significance of the two predicted stems was tested by creating complementary changes within both pairing regions (Fig. 4). In m1 and m3, we replaced five consecutive nucleotides to disrupt the first stem (S1); in m2 and m4, we replaced eight consecutive nucleotides to disrupt the second stem (S2). To restore the base pairing, both mutagenic changes in m1 and m3 were made in m5 and both mutagenic changes in m2 and m4 were made in m6. The frameshift efficiencies of the six mutants were measured and compared with that of wild type both in vitro and in *E. coli*.

As shown in Fig. 6, frameshifting activity requires stems S1 and S2 that constitute the predicted pseudoknot both in vitro and in *E. coli*. The frameshift products were present in very low amounts in the four single mutants, but they were synthesized at near-wild-type level in the two double mutants. The frameshift efficiencies in the four single mutants were only 0.4 to 0.5%, while the efficiency in the wild type was 3.5% in the wheat germ extract in vitro. The frameshift product was synthesized in normal amount in the m6 double mutant (5.5%), although the m5 double mutant had lower frameshift efficiency (1.4%). Similar results were obtained in *E. coli*. The relative frameshift efficiencies were only 13% in m4 and less than 10% in m1 to m3, whereas the relative frameshift efficiencies of m5 and m6 were 96 and 129%, respectively. Since the two double mutants, m5 and m6, have different sequences within the two stems than the wild type, we conclude that secondary and tertiary structure rather than primary sequence is important.

DISCUSSION

The L1 double-stranded RNA virus of *S. cerevisiae*, like the coronavirus IBV and many retroviruses, uses a -1 frameshifting event to join two ORFs. We have defined a stretch of 71 nt within the overlapping region which contains the necessary information for directing frameshifting in a heterologous context. This region includes a consensus slippery site, GGGUUUA, and has the potential to form a pseudoknot structure 3' to the putative frameshift site. The pseudoknot has been shown to be essential.

The sequence GGGUUUA appears to be the slippery site for the -1 frameshifting in ScV. This sequence, a heptanucleotide RNA sequence starting with two homopolymeric triplets, is similar to the slippery sites for the -1 frameshifting in the coronavirus IBV and retroviruses (3, 13). Deletion clones (pFSd6 and pGEM7ZCTUd6), in which this sequence was removed, displayed no frameshifting, even though the second possible slippery sequence (UUUUA) and its 3' region were intact. This hypothesis is further supported by direct protein sequencing of the frameshift product purified from yeast cells transformed with p3pFS (31a).

Translational frameshifting appears to occur by a number of mechanisms in both prokaryotes and eukaryotes. In prokaryotes, translational frameshifting of homologous genes may occur by shifty stops, in which the ribosome, stopped at a termination codon in the initial reading frame, slips on a string of purine nucleotides (34, 35). The frequency of frameshifting in *E. coli* peptide chain release factor 2 mRNA is increased by a 3' stop codon and by a 5' sequence complementary to the 3' end of the 16S rRNA (34, 35). Frameshifting may also occur at positions in which rare ("hungry") codons are present in the original reading frame (33). In eukaryotes, there also appears to be a multiplicity of mechanisms by which frameshifting may occur. Most retroviral frameshift signals are slippery sequences followed by a secondary (13, 15, 19, 23, 31) and probably tertiary (pseudoknot) structure (13, 31). The coronavirus IBV requires a 3' pseudoknot for frameshifting (3). In contrast, the +1 ribosomal frameshift in the yeast retrotransposon Ty requires only a short slippery sequence with a rare codon in the original reading frame (2), similar to frameshifting at hungry codons in *E. coli* (33). Frameshifting in HIV requires only the short slippery sequence but not the 3' sequence with its predicted stem-loop structure (37).

The generation of the ScV *cap-pol* fusion protein is the second example of a frameshifting event that requires an mRNA pseudoknot 3' to a slippery sequence. Our deletion analysis indicates that the sequence downstream of the putative slippery site GGGUUUA is necessary for frameshifting. The requirement of a possible secondary or tertiary structure for the frameshift in ScV was explored by site-directed mutagenesis. The requirement for the two proposed long stems (Fig. 4 and 6) for frameshifting demonstrates that the ScV pseudoknot exists at least frequently enough to cause frameshifting for some of the ribosomes that traverse it. Given the sizes of the loops, this structure is more stable than either stem and loop separately (38). Clearly, the significance of RNA tertiary structure of this kind in all aspects of RNA functions is only beginning to be appreciated (3, 29–31). One possible explanation for the dependence on downstream secondary or tertiary structure is ribosomal pausing at or near the slippery site, which might be caused by delayed unwinding of the secondary structure or tertiary structure (3, 13). This might allow the tRNA slippage at the slippery site. Our preliminary data indicate that ribosomes

do pause at the ScV-L1 slippery site longer or more frequently in the wild type and two double mutants which have restored the pseudoknot structure than in the mutants that do not frameshift (31a).

It has been shown that ribosomal frameshift signals direct frameshifting in heterologous systems (16, 36, 37). The frameshift signal of HIV has been shown to direct yeast ribosomes to shift with efficiency comparable to that in the mammalian system, and the same sequence requirements were demonstrated in both systems (37). Ribosomal frameshifting occurred on an Rous sarcoma virus RNA in an *E. coli* cell-free translation system, although the efficiency was 10-fold less than that in rabbit reticulocyte lysates (16). Ribosomal slippage directed by retroviral frameshift signals also occurs at precisely the same places in both *E. coli* and mammalian systems (36). Where secondary structure appears to be important in frameshifting in eukaryotes, the same secondary structure plays a role in *E. coli* (36). Our results show that a yeast frameshifting signal can direct a -1 frameshifting event in heterologous systems, in wheat germ extract in vitro translation system, in rabbit reticulocyte lysate in vitro translation (not shown), and in *E. coli* in vivo, although the minimum sequence failed to function in *E. coli*. Therefore, the ribosomes from prokaryotic and several different eukaryotic cells appear to respond to the ScV frameshift signal in a similar way. The failure of the 71 nt to direct frameshifting efficiently in *E. coli* may be due to the inhibitory effect of surrounding vector sequences, which may interfere with the formation of the tertiary structure required for efficient frameshifting. Frameshifting can be inhibited by placing frameshift signals in proximity to initiation codons located in yeast expression vectors (2).

The levels of frameshifting directed by the same frameshift signal were different in yeast in vivo and wheat germ extract in vitro translation systems. The frameshift efficiency in the wheat germ system averaged about 3.5%, which is close to the ratio of the 171-kDa *cap-pol* fusion protein to P1 in the viral particle (3.6%) and produced by in vitro translation of denatured L1 dsRNA (5.9%) (7). A higher frameshift efficiency, however, was detected in the yeast system; the frameshift efficiency was around 20% in p3pFS and 29% in p3pFSd as determined by a β -galactosidase activity assay. This may be due to the yeast vector used for the frameshift analysis. The level of the frameshifting directed by the same sequence may vary dramatically if different vectors are used, even when the same assay is used for each (2).

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ADDENDUM

While this report was in preparation, another report establishing the requirement for an RNA pseudoknot in the ScV-L1 frameshift appeared (9).

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