Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8636–8640, September 1992
Biochemistry

Ribosomal movement impeded at a pseudoknot required for frameshifting

(double-stranded RNA virus/*Saccharomyces cerevisiae* virus/translation/yeast)

CHIALING TU, TZY-HWA TZENG, AND JEREMY A. BRUENN*

Department of Biological Sciences, State University of New York, Buffalo, NY 14260

Communicated by Edwin D. Kilbourne, June 15, 1992 (received for review April 7, 1992)

ABSTRACT **Translational frameshifting sometimes occurs when ribosomes encounter a "shift" site preceding a region of unusual secondary structure, which in at least three cases is known to be a pseudoknot. We provide evidence that ribosomes have a decreased rate of movement through a pseudoknot required for frameshifting. These paused ribosomes are directly situated over the shift sequence. Ribosomal pausing appears to be necessary but not sufficient for frameshifting.**

Ribosomal frameshifting is the shifting of ribosomes from one reading frame to another at a specific sequence on a mRNA during translation. There are numerous examples of ribosomal frameshifting in prokaryotic and eukaryotic systems (1, 2). It is a commonly used mechanism by which RNA viruses translationally regulate the level of synthesis of proteins. The frequency with which frameshifting occurs may vary from a few percent to more than half of the time a ribosome traverses the critical sequence (3). Sequences that induce frameshifting often include heptanucleotide runs of purines and/or pyrimidines, a "slippery" or "shift" sequence (4–6), followed by unusual secondary and/or tertiary structure (5–7). Alternatively, rare codons in the original (zero) reading frame may cause frameshifting (8). It has been postulated that ribosomes slip backward or forward into a new reading frame on a shift sequence when they encounter a region that inhibits their progression (5). Some retrovirus frameshifting commonly occurs just prior to a region capable of forming required secondary structure (5) that may form a pseudoknot (9), although in human immunodeficiency virus type 1 no such downstream structure is necessary (6, 10). A pseudoknot just downstream of the shift site has been shown to be essential for frameshifting in the coronavirus infectious bronchitis virus (7), in the *Saccharomyces cerevisiae* virus (ScV) (11, 12), and in the retrovirus mouse mammary tumor virus (6).

In ScV, there are two large open reading frames on the plus strand of the double-stranded RNA genome (L1): *cap* and *pol*. The first, *cap*, encodes the major viral capsid polypeptide, and the second, *pol*, encodes the viral RNA-dependent RNA polymerase. The two reading frames overlap and a −1 frameshift is required for translation of *pol* (11–13). A region of 71 nucleotides, including a required (11) shift sequence (1958–1964, GGGUUUA) and a required (11, 12) pseudoknot (1969–2022), is sufficient for frameshifting in yeast *in vivo* and in wheat germ translation systems *in vitro* (12). Frameshift sequences behave similarly in homologous and heterologous systems, except that efficiencies are higher in some heterologous contexts in the homologous system (11, 12). The protein sequence of a ScV frameshift fusion protein demonstrates a ribosomal slippage similar to the −1 frameshift of some retroviruses (4, 5, 14, 15): in this case, a $tRNA^{Gly}$-$tRNA^{Leu}$ pair, in the P and A sites, respectively, slips backward one base to erroneously read the Gly-Phe codons (T.-H.T., unpublished data).

In this work, ribosomes are demonstrated to progress slowly through the pseudoknot, so that "paused" ribosomes accumulate, apparently flush up against its first stem. Mutations that eliminate either stem of the pseudoknot greatly reduce ribosomal frameshifting and ribosomal pausing at this site. Only the secondary structure of the RNA is important in this region, not its primary sequence.

MATERIALS AND METHODS

Vectors for Detection of Frameshifting. The pGEM7ZCTU family of vectors for detection of ScV frameshifting has been described, as have the point mutants and deletion mutants used to define the minimal frameshift region and to show that the pseudoknot is necessary (12). The locations of altered sequences in the mutants used are described in Table 1.

***In Vitro* Transcripts.** SP6 transcripts of pGEM7ZCTU and its derivatives (12) were made as described (12, 16, 17) in the presence of m^7GpppG to cause capping of the resultant RNA.

Single-Stranded DNA. The 396-base-pair (bp) L1 cDNA sequence (bases 1783–2179) in pGEM7ZCTU (12) was first subcloned to the pGEM7Zf(+) vector (Promega) to create plasmid pGEM7ZCTU. Isolation of single-stranded DNA from pGEM7ZCTU for heelprinting experiments (see below) was performed as described (18). The L1 minus strand cDNA is packaged in this construct.

Heelprinting. Heelprinting was as described (19) with the following modifications. A 25-μl translation reaction mixture contained 3 μg of capped RNA, 1 unit of RNase inhibitor per μl (RNasin; Promega), 20 nM amino acid mix, and 17.5 μl of rabbit reticulocyte lysate (Promega). Following a 25-min incubation at 26°C and addition of cycloheximide to a final concentration of 1 mM, micrococcal nuclease (final concentration, 16 units/μl; Boehringer Mannheim) was added to digest RNA. The volume of the reaction mixture was adjusted to 200 μl with buffer T (19), and ribosomes were collected by pelleting through a 120-μl cushion of 0.25 M sucrose in buffer T at 53,000 rpm, 5°C for 2.5 hr in a TLS55 rotor in a Beckman TL100 ultracentrifuge. The top 240 μl was removed after sedimentation; then proteinase K (200 μg/ml) was added. Ribosome-protected RNA fragments were purified and redissolved in 10 μl of distilled water. One microliter of ribosome-protected fragments and 0.12 ng of a 5′-labeled oligonucleotide primer with the L1 sequence of bases 1892–1907 were annealed to an L1 minus strand cDNA complementary to bases 1783–2179. The annealing reaction mixture was heated to 65°C for 5 min and then placed at 37°C for 1 hr. Following annealing, 2.5 units of T4 DNA polymerase and dNTPs (final concentration, 0.334 mM) were added to the annealing reaction mixture (the polymerase accessory proteins gp44/62 and gp45 were omitted from the primer extension reaction because we found them unnecessary). After a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: ScV, *Saccharomyces cerevisiae* virus.

*To whom reprint requests should be addressed.

Table 1. Correlation between frameshifting and ribosomal pausing

Mutant	Bases altered	Stem altered	FS*, % WT	Heelprint
m1	1969–1973	1	10–15	−
m2	1984–1991	2	10–15	−
m3	2000–2004	1	10–15	−
m4	2015–2022	2	10–15	−
m5†	1969–1973			
	2000–2004	1	40	+
m6†	1984–1991			
	2015–2022	2	150	+
d3	2021–2022			
	deleted	2	ND	+
d9	2013–2022			
	deleted	2	ND	−

The presence (+) or absence (−) of the normal heelprint (ribosomal pausing) is indicated for each of the mutants tested, along with a brief description of each and the extent of frameshifting *in vitro* with each. ND, not detectable.
*Percent of wild-type frameshifting.
†Mutant m5 has the changes of m1 and m3; mutant m6 has the changes of m2 and m4.

15-min incubation at 37°C, the primer extension products were purified, precipitated, and redissolved in 10 μl of sequencing sample buffer (20). Five microliters of extension products was denatured and fractionated on each lane of an 8% polyacrylamide sequencing gel.

RESULTS

Strategy Used for Detecting Frameshifting. We have constructed a number of vectors in which the ScV frameshift sequence can direct frameshifting in heterologous contexts, *in vitro* and *in vivo* (12). We examined ribosomal pausing in pGEM7ZCTU, containing a 396-bp fragment (bases 1783–2179) including the ScV shift site (1958–1964) and pseudoknot (1969–2022). In this construct (WT in Fig. 1), 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; the second reading frame (*pol*) is in frame with the α peptide of β-galactosidase of pGEM-7Zf(+). *In vitro* transcription with SP6 RNA polymerase results in transcripts capable of *in vitro* translation in wheat germ (12) or rabbit reticulocyte (21) lysates, in which a non-frameshifted (termination) product of 12 kDa and a frameshift fusion product of 27 kDa result. The frameshifting efficiency measured in this manner is about 3% in wheat germ extracts (12) and 2.5–5% in the rabbit reticulocyte lysate system used for the current experiments (21).

Strategy Used for Detecting "Pausing." We used the "heelprinting" technique (19, 22) to locate regions protected from nuclease digestion by ribosomes translating the frameshift region. This technique locates the 5′ extent of regions protected from micrococcal nuclease digestion by steric hindrance of the nuclease by proteins bound to RNA (in this case, ribosomes). SP6 transcripts were incubated in rabbit reticulocyte lysates and then treated with micrococcal nuclease. The protected RNA fragments were isolated as complexes with ribosomes by centrifugation through a sucrose cushion followed by phenol extraction and ethanol precipitation. The resultant protected fragments were hybridized to single-stranded DNA with the sequence of the minus strand from the appropriate region of L1 (complementary to bases 1783–2179). Primer extension was accomplished by T4 DNA polymerase primed with a 5′-end-labeled oligonucleotide with the sequence of bases 1892–1907 of the L1 plus strand. The resultant labeled extension products were separated on an 8% polyacrylamide sequencing gel in parallel with the Sanger sequencing reactions generated with the same primer. Because the RNA·DNA hybrid halts the progression of the T4 DNA polymerase, which lacks a 5′ exonuclease activity (23), the length of the extension product reflects the position of the 5′ end of the protected fragment. This then represents the position of the "heel" of the ribosome protecting the RNA from digestion by micrococcal nuclease. The result is shown as Fig. 1. The protected fragments cause two strong T4 DNA polymerase terminations at bases 1946 and 1949, just prior to the slippery site, beginning at base 1958. We will refer to this as the normal heelprint.

Ribosomal Pausing Is Correlated with Frameshifting. To determine if the strong polymerase termination products were correlated with the presence of the pseudoknot, we tried the same experiment with RNA transcripts from four substitution mutants in which frameshifting is greatly reduced, *in vivo* and *in vitro*, by disruption of the pseudoknot. These were m1, m2, m3, and m4, mutants derived by site-directed mutagenesis, in which the first stem (m1 and m3) or the second stem (m2 and m4) of the pseudoknot is abolished. Mutant m6, which shows normal frameshifting (12), has a restored pseudoknot in which 8 bp of the second

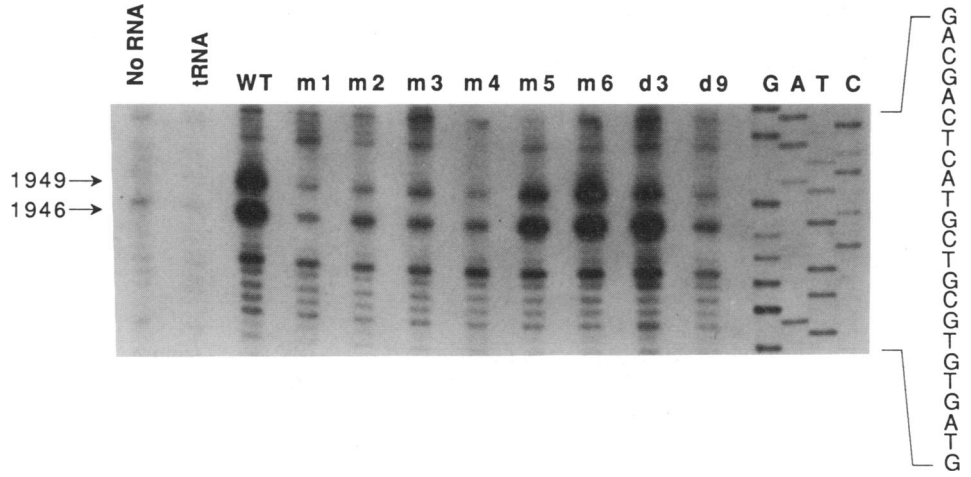

FIG. 1. Heelprinting of ribosomes paused at the frameshift site. After purification, *in vitro* transcripts were incubated in rabbit reticulocyte lysates, followed by micrococcal nuclease digestion, isolation of protected fragments, hybridization of protected fragments to L1 minus strand cDNA, and primer extension inhibition assays using T4 DNA polymerase with a 5′-end-labeled oligonucleotide primer (see text). The products were analyzed by 8% polyacrylamide sequencing gels. WT is the wild-type sequence in a pGEM7ZCTU vector (see text). Mutants m1–m6, d3, and d9 are as described (12) and are explained in the text and Table 1. The sequencing ladder was generated with the same primer and DNA as the heelprints.

stem have been inverted. Mutant m5 also shows almost normal frameshifting (12) and has a restored pseudoknot in which 5 bp of the first stem have been inverted. A schematic diagram showing the stems of the pseudoknot and the locations of mutations is part of Fig. 2, and the mutants are further described in Table 1. Mutants m1–m4 show about 10% of the normal level of frameshifting *in vivo* or *in vitro* (12) and a commensurate reduction in the strong T4 DNA polymerase termination products in the heelprinting experiment (Fig. 1). Mutant m6 shows normal frameshifting and a normal heelprint, whereas m5 has an almost wild-type level of frameshifting and a nearly normal heelprint (Fig. 1). Controls with tRNA or no RNA (Fig. 1) do not give any heelprint.

Two deletion mutants lacking essential parts of the pseudoknot and in which frameshifting is undetectable (12) were also tested (d9 and d3). Mutant d9 lacks 10 bases of the pseudoknot and also shows a reduced heelprint. In mutant d3, two bases at the 3′ end of the second stem of the pseudoknot are replaced by the same bases present in the formerly complementary region of stem 2, so that the stem is shortened by 2 bp. This mutant, in which frameshifting is undetectable (12), still shows the normal heelprint (Fig. 1). Apparently, the decreased mobility of ribosomes through the frameshift region in d3 is insufficient to cause detectable frameshifting. A summary of the results with the eight mutants tested is shown in Table 1.

Pausing Is Not an Artifact Due to Secondary Structure of the RNA. We next performed experiments to eliminate the possibility that the heelprint characteristic of wild-type L1 mRNA incubated with ribosomes is due to protection of some regions of the RNA from nuclease digestion by secondary and tertiary structure of the RNA rather than its protection by ribosomes. Controls with the wild-type RNA but with excess m^7GDP, which inhibits translational initiation (Fig. 3, lane 1), or with L1 RNA without ribosomes (Fig. 3, lane 3) show no heelprint. Ribosome protection experiments with ribonuclease V_1, a double-stranded RNA-specific ribonuclease, in addition to micrococcal nuclease, give identical heelprints as the experiments with micrococcal nuclease alone (data not shown). This is significant, since micrococcal nuclease alone does not succeed in digesting all unprotected RNA (see Fig. 4). These three control experiments with naked RNA, with excess m^7GDP, and with ribonuclease V_1 verify that secondary structure of the RNA alone is not responsible for the nuclease protected fragments.

Sizes of the RNA Fragments Protected by Ribosomes. The sizes of the protected fragments were estimated by preparing transcripts labeled with [α-^{32}P]UTP, isolating ribosome-protected fragments, and sizing the protected fragments on an 8% polyacrylamide sequencing gel. The result (Fig. 4, lane 2) demonstrates that the region protected by ribosomes is 30 or 31 nucleotides in extent, which is confirmed by characterization of RNA fragments protected by ribosomes from digestion with ribonuclease V_1 in addition to micrococcal nuclease (Fig. 4, lane 4). This region was not protected from nuclease digestion in naked RNA (Fig. 4, lanes 3 and 5) or when incubations with rabbit reticulocyte lysates took place in the presence of excess m^7GDP (Fig. 4, lanes 1 and 6). Together with the heelprint, this places the maximum protected region within bases 1946–1979. The shift site, bases 1958–1964, falls in the middle of the protected region (Fig. 2).

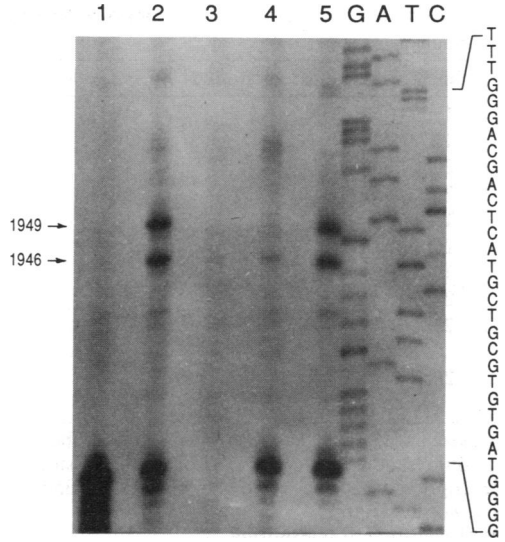

FIG. 3. Heelprinting of ribosomes at the frameshift site: controls. The experiment of Fig. 1 with wild-type RNA was repeated with excess m^7GDP (100 mM, lane 1) to inhibit translational initiation (22), with RNA not incubated with ribosomes (naked RNA, lane 3), and with wild-type (lane 2), m1 (lane 4), and m6 (lane 5) RNAs as in Fig. 1. The DNA sequence generated with the same primer and DNA used for lanes 1–5 is shown on the right. The heelprinting experiments have been repeated three times with identical results.

DISCUSSION

The heelprinting experiments are consistent with a ribosomal pause at the pseudoknot; it is not clear if the first stem of the pseudoknot is unwound by the paused ribosome. This places the paused ribosome exactly over the shift site, as shown in

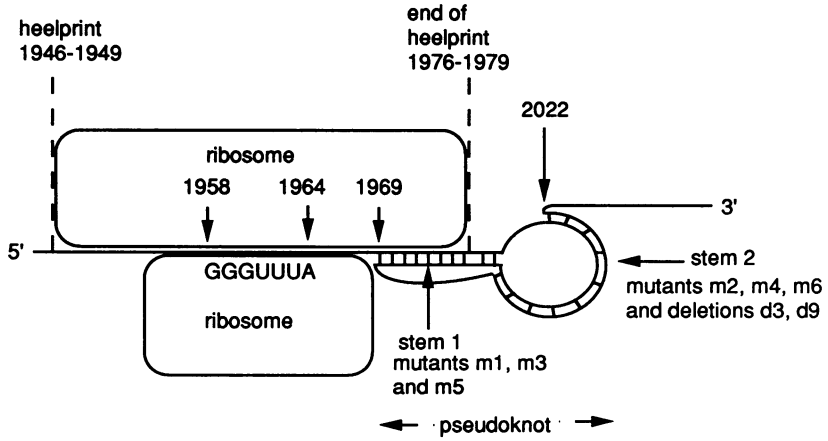

FIG. 2. Model for ribosomal pausing at the pseudoknot. Base numbers on the L1 plus strand are given starting from the 5′ end of the RNA. The positions of base pairs in the two stems of the pseudoknot are shown with cross bars. The locations of mutations in stem 1 or stem 2 are shown.

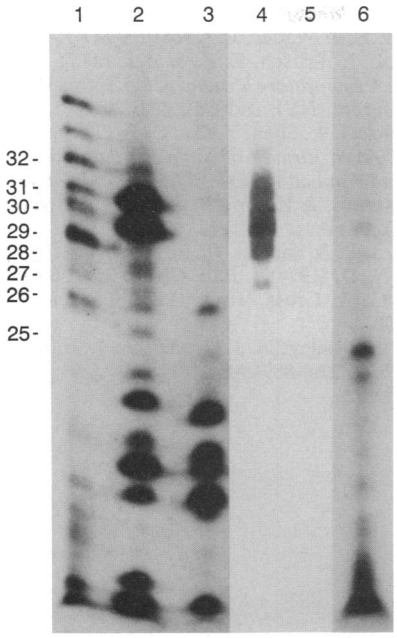

FIG. 4. Sizing of ribosome-protected RNA fragments. RNA fragments protected from micrococcal nuclease digestion (16 units/μl) or from combined micrococcal nuclease digestion (16 units/μl) or from combined micrococcal nuclease (16 units/μl) and ribonuclease V_1 (0.01 unit/μl; Pharmacia) digestion (22) were sized on a sequencing gel with DNA markers. The SP6 transcripts were prepared as in Fig. 1, but the concentration of nonradioactive UTP was decreased to 0.1 mM, and [α-^{32}P]UTP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq) was added to a final concentration of 1 μM. Lane 1, fragments protected from micrococcal nuclease digestion when incubations with ribosomes were in the presence of excess m^{7}GDP; lane 2, fragments protected from micrococcal nuclease digestion by ribosomes; lane 3, fragments protected from micrococcal nuclease in the absence of ribosomes; lane 4, fragments protected by ribosomes from combined micrococcal and ribonuclease V_1 digestion; lane 5, fragments protected from micrococcal and ribonuclease V_1 digestion in the absence of ribosomes; and lane 6, fragments protected from micrococcal and ribonuclease V_1 digestion when incubations took place in the presence of excess m^{7}GDP. This is a composite figure from lanes run on the same gel. Size markers (not shown, but indicated in nucleotides) were end-labeled DNA oligonucleotides.

the two-dimensional schematic of Fig. 2. The P and A sites would be just about in the middle of the ribosome if they were occupied with the tRNAs reading the shift sequence. The total extent of the protected fragments in the heelprinting (30 or 31 bases) is consistent with a single paused ribosome, which, in other heelprinting experiments, protects 29–32 bases (19), as diagramed in Fig. 2.

The two distinct positions of the paused ribosome might reflect ribosomes in the zero (before frameshifting) and −1 (after frameshifting) reading frames. Ribosomes paused in the −1 frame would be expected to be located a single nucleotide 5′ of ribosomes in the zero frame, rather than the 3 nucleotides 5′ that is measured (at bases 1946 and 1949). However, micrococcal nuclease has a distinct preference for cleavage prior to A and U (24), and the two U residues at 1946 and 1949 are separated by a C and G, where cleavage would be less likely.

There are two possible interpretations of heelprinting experiments: paused ribosomes may be in the process of translation or they may be "dead" ribosomes that have terminated but not disengaged from the mRNA. We think that the first explanation for our results is correct for two reasons. First, we calculate that the amount of RNA in this region protected from nuclease digestion can only be accounted for by the presence of a single ribosome in this region in a fraction of about 40% of the mRNA. Hence, at least 40% of translations would have to result in premature termination at this point (some 100 nucleotides upstream of the *cap* termination codon), and a premature termination product of this size (8 kDa) should be obvious in *in vitro* translation. No such product is detectable (ref. 12; C.T., unpublished data).

Second, it is clear from the sizes of the protected fragments and the heelprint that ribosomes are not commonly stacked up against the pseudoknot, as would be expected behind ribosomes that pause for an extended period, but that a single ribosome is predominant at this position. The sensitivity of the heelprinting would be adequate to detect stacked ribosomes if they constituted more than about 5% of the population of paused ribosomes. Stacked ribosomes are commonly present at pause sites in other mRNAs in rabbit reticulocyte lysates *in vitro* and contribute a large percentage to the heelprint (19, 22).

We conclude that ribosomes detected at the L1 frameshift site *in vitro* are paused ribosomes that will continue to translate and that some of them will frameshift before continuing to translate (or have already frameshifted).

Although these experiments were performed in a heterologous *in vitro* system, the accurate recognition of frameshift signals (3), including the ScV frameshift sequence (12), in all translation systems tested indicates that ribosomal movement is probably impeded at this frameshift site *in vivo* in *Saccharomyces cerevisiae*.

Our results show that in at least one frameshift sequence a 3′ pseudoknot is capable of causing ribosomes to linger directly over the shift site. Note that this pause takes place over bases 1946–1979, nearly 100 bases upstream of the *cap* termination codon (2070–2072). Analysis of six frameshift defective and two frameshift nondefective mutants shows that this ribosomal pause is highly correlated with the ability to cause frameshifting (Table 1). However, in one mutant with a normal shift site but a pseudoknot with a slightly shortened second stem (d3), frameshifting is abolished even though ribosomal pausing is detectable. We conclude that ribosomal pausing at a pseudoknot may be necessary, but it is not sufficient for frameshifting at a shift site.

It is possible that the lack of frameshifting in d3 reflects the inability of the altered pseudoknot to undergo a conformational change necessary for frameshifting. This result complements other work showing that the stability of the secondary structure of the pseudoknot does not account for its role in frameshifting (25). We have not yet examined mutants of the shift site defective in frameshifting. We would expect that these would continue to exhibit the ribosomal pause.

We thank Sandra Wolin for advice and guidance, Chuck Samuel for advice and communication of results prior to publication, R. F. Gesteland, J. Berry, S. Free, and M. Hollingsworth for critical reading of the manuscript, and the Public Health Service (Grant GM22200 from the National Institutes of Health) and the National Science Foundation (Grants DMB9106818 and INT-5020780) for support.

1. Craigen, W. J. & Caskey, C. T. (1987) *Cell* **50**, 1–2.
2. Atkins, J. F., Weiss, R. & Gesteland, R. F. (1990) *Cell* **62**, 413–423.
3. Weiss, R. B., Dunn, D. M., Shuh, M., Atkins, J. F. & Gesteland, R. F. (1989) *New Biol.* **1**, 159–169.
4. Jacks, T., Power, M. D., Maslarz, F. R., Luciw, P. A., Barr, P. J. & Varmus, H. E. (1988) *Nature (London)* **331**, 280–283.
5. Jacks, T., Madhani, H. D., Masiarz, F. R. & Varmus, H. E. (1988) *Cell* **55**, 447–458.
6. Chamorro, M., Parkin, N. & Varmus, H. E. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 713–717.
7. Brierley, I., Digard, P. & Inglis, S. C. (1989) *Cell* **57**, 537–547.
8. Belcourt, M. F. & Farabaugh, P. J. (1990) *Cell* **62**, 339–352.
9. ten Dam, E. B., Pleij, C. W. A. & Bosch, L. (1990) *Virus Genes* **4**, 121–136.

10. Wilson, W., Braddock, M., Adams, S. E., Rathjen, P. D., Kingsman, A. J. & Kingsman, S. M. (1988) *Cell* **55**, 1159–1169.
11. Dinman, J. D., Icho, T. & Wickner, R. B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 174–178.
12. Tzeng, T.-H., Tu, C. & Bruenn, J. A. (1992) *J. Virol.* **66**, 999–1006.
13. Diamond, M. E., Dowhanick, J. J., Nemeroff, M. E., Pietras, D. F., Tu, C.-L. & Bruenn, J. A. (1989) *J. Virol.* **63**, 3983–3990.
14. Jacks, T. & Varmus, H. E. (1985) *Science* **230**, 1237–1242.
15. Jacks, T., Townsley, K., Varmus, H. E. & Majors, J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4298–4302.
16. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
17. Krieg, P. A. & Melton, D. A. (1988) *Methods Enzymol.* **155F**, 397–415.
18. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
19. Wolin, S. L. & Walter, P. (1988) *EMBO J.* **7**, 3559–3569.
20. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed., p. B.25.
21. Dowhanick, J. J., Shen, Y., Tu, C.-L., Tzeng, T.-H. & Bruenn, J. A. (1992) in *Viruses of Simple Eukaryotes: Molecular Genetics and Applications to Biotechnology and Medicine*, eds. Leibowtitz, M. & Koltin, Y. (Univ. of Delaware Press, Newark, DE), in press.
22. Doohan, J. P. & Samuel, C. E. (1992) *Virology* **186**, 409–425.
23. Nossal, N. G. (1974) *J. Biol. Chem.* **249**, 5668–5676.
24. Krupp, G. & Gross, H. J. (1979) *Nucleic Acids Res.* **11**, 3481–3490.
25. Brierley, I., Rolley, N. J., Jenner, A. J. & Inglis, S. C. (1991) *J. Mol. Biol.* **220**, 889–902.