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cis Acting RNA Sequences Control the Gag-Pol Translation Readthrough in Murine Leukemia Virus

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Received January 10, 1991; accepted March 28, 1991

The *pol* gene of the Moloney murine leukemia virus (M-MuLV) is expressed as a Gag-Pol fusion protein through an in-frame suppression of the UAG termination codon located between the two genes. The role of nucleotide context in suppression was investigated, in a rabbit reticulocyte lysate translation system, using site-directed mutagenesis. The results indicate that the translational readthrough is mediated by at least 50 bases long RNA sequence located 3' to the *gag* UAG termination codon. Within this sequence a short purine-rich sequence adjacent to the amber codon, highly conserved among different retroviruses, appears essential for M-MuLV suppression. Two alternative putative stem and loop like RNA structures can be drawn at the *gag-pol* junction, one abutting the *gag* UAG codon, and the second downstream to it. None of these structures appears to be important to the suppression process. © 1991 Academic Press, Inc.

INTRODUCTION

All replication competent retroviruses possess three common genes, *gag*, *pol*, and *env*, which have been mapped as 5'-*gag-pol-env*-3' (Weiss *et al.*, 1984). The *gag* gene is translated as a single polypeptide, which is subsequently cleaved to produce the viral structural proteins (Jamjoom *et al.*, 1977). The initial translation product of the *pol* gene is a Gag-Pol fusion protein that is processed during virion assembly to yield the viral catalytic proteins (Jamjoom *et al.*, 1977; Murphy *et al.*, 1978; Schwartzberg *et al.*, 1984). Synthesis of the Gag-Pol polyprotein is dependent upon occasional readthrough of termination codon during translation (Jamjoom *et al.*, 1977; Murphy *et al.*, 1978; Philipson *et al.*, 1978). There are two general strategies by which readthrough is achieved, depending upon the relative organization of the *gag* and *pol* genes in the particular retrovirus: (i) Ribosomal frameshifting in viruses such as Rous sarcoma virus (RSV), in which the *gag* and *pol* open reading frames (ORFs) overlap out of frame (Jacks and Varmus, 1985; Varmus, 1988). (ii) In Moloney murine leukemia virus (M-MuLV) and the closely related AKV and feline leukemia virus (FeLV), translational readthrough involves suppression of the *gag* termination codon located in frame with *gag* and *pol* (Philipson *et al.*, 1978; Murphy *et al.*, 1980; Yoshinaka *et al.*, 1985b). The amount of Gag-Pol fusion protein produced in M-MuLV infected cells is about 1:10 to 1:50 of the Gag protein (Jamjoom *et al.*, 1977). The mainte-

nance of this ratio between the major structural Gag proteins and the catalytic *pol* gene products is essential for proper viral replication and infectivity (Felsenstein and Goff, 1988). Thus, suppression efficiency appears to be a key step in the replication cycle of M-MuLV.

Starvation of M-MuLV infected cells for glutamine diminished specifically *pol* gene expression (Gloger and Panet, 1986) suggesting glutamine suppression of the *gag* UAG termination codon. This assumption was shown to be correct by direct amino acid sequencing of the junction between Gag and Pol (Yoshinaka *et al.*, 1985b). As substitution of the amber codon at the end of *gag* to either opal or ochre codons did not significantly affect synthesis of Gag-Pol polyprotein (Feng *et al.*, 1989a), the suppression is probably not determined by a simple codon-anticodon interaction. The importance of nucleotide context in readthrough of termination codons was demonstrated in several retroviruses (Wilson *et al.*, 1988; Jacks *et al.*, 1988a,b). In RSV, which employ ribosomal frameshifting for translation of the *pol* gene, two distinct sequences appear to be important for the readthrough mechanism: a primary "slippery" sequence at the frameshift site, and a downstream RNA secondary structure (Jacks *et al.*, 1988b). In the light of the latter observation, it is notable that the M-MuLV *gag* amber termination codon is flanked by a potential stem and loop structure (Shinnick *et al.*, 1981). In this work we examined the involvement of the neighboring RNA sequences in suppression of the M-MuLV *gag* termination codon.

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MATERIALS AND METHODS

Materials

Restriction enzymes, T4 DNA ligase, *Escherichia coli* DNA polymerase I and its large fragment (Klenow), and T4 polynucleotide kinase, were purchased from New England Biolabs, Inc., and International Biotechnologies, Inc. (IBI). Radioactive materials were purchased either from Amersham Corp. or from New England Nuclear Corp.

Bacterial strains

E. coli strain DR100 (Mador *et al.*, 1989) was used for plasmid manipulation and preparation. *E. coli* strain JM107 was used for M13 bacteriophage sequencing experiments.

Plasmid constructions and site-directed mutagenesis

An M-MuLV DNA fragment extending from nucleotide 567 to nucleotide 4894 (Shinnick *et al.*, 1981) was isolated from the infectious clone pMOV9 and inserted in a polylinker site of plasmid vector pSP64 (Melton *et al.*, 1984) downstream to the SP6 promoter (plasmid pSPgp). Oligonucleotide-directed mutagenesis was performed according to Morinaga *et al.* (1984), using chemically synthesized oligonucleotides (see below) supplied by Biotechnology General Inc. (Rehovot, Israel). To simplify isolation of the mutants all the oligonucleotides (except No. 1) were designed to create a new site for a restriction endonuclease (underlined). The mutations are indicated by dots.

(1) 5' CACTATCGCTAGTTGCTCC 3'	pSPg
(2) 5' GATGACTAG <u>ACGCGT</u> CAGGGTCAGG 3'	pSPgp1
(3) 5' CCCAGACCTCCCTCCT <u>AAAT</u> TAGATGACTAGGG 3'	pSPgpA
(4) 5' TAGGGAGGTCA <u>TCA</u> TGAGGACCCCCCTGAACC 3'	pSPgpB
(5) 5' CCCCCCT <u>CA</u> TG <u>AT</u> AGGATAACCCTCAA 3'	pSPgpC

In vitro transcription and translation

Plasmids were cleaved with *Bam*HI endonuclease and the linear DNA templates were transcribed *in vitro* using SP6 RNA polymerase. The run-off transcripts were translated in nuclease-treated rabbit reticulocyte lysates in presence of [³⁵S]methionine (Amersham Corp.). Translation products were immune precipitated (Jacks and Varmus, 1985) with anti-M-MuLV p30 Gag rabbit serum and protein A-Sepharose. The protein products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8%). Equal amounts of radioactive translation products, determined after precipitation with trichloroacetic acid, were loaded on the gels. Quantitation of the translation products was carried out by scanning of the autoradiograms by a computerized photoscanner (620 Video Densitometer, Bio-Rad Laboratories). For details, see Mador *et al.* (1989).

DNA sequencing

The M-MuLV DNA fragment located between restriction sites of endonucleases *Nru*I-nucleotide 2121 and *Sac*I-nucleotide 2558, (Shinnick *et al.*, 1981) in the mutated pSPgp clones were subcloned into M13mp18 vector plasmid (New England Bio-labs). Single-strand DNA was purified and the nucleotide sequence was

determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

RESULTS

To examine the role of RNA sequences surrounding the M-MuLV *gag* amber codon in suppression, we set up an *in vitro* translation system for analysis of the products of *gag* and *pol* genes. An M-MuLV DNA fragment containing the corresponding sequences [nucleotides 567 to 4894 (according to the sequence of Shinnick *et al.*, 1981)] was inserted in the polylinker site of pSP64, downstream to the SP6 promoter. The clone, designated pSPgp, served as a template in the SP6 RNA polymerase transcription system (Melton *et al.*, 1984). The DNA templates were linearized at the *Bam*HI restriction site (nucleotide 3229) within the *pol* gene, and transcribed. The run-off BAM-RNA (2662 bases long) was translated, using the rabbit reticulocyte lysate translation system. ³⁵S-labeled translation products were immunoprecipitated with anti-p30 rabbit sera and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). The clearly discernible 65-kDa protein band represents the predicted product of the *gag* gene (G) (Fig. 1A, lane 1). In addition, a 94-kDa protein band was observed when BAM-RNA was translated. This translation product

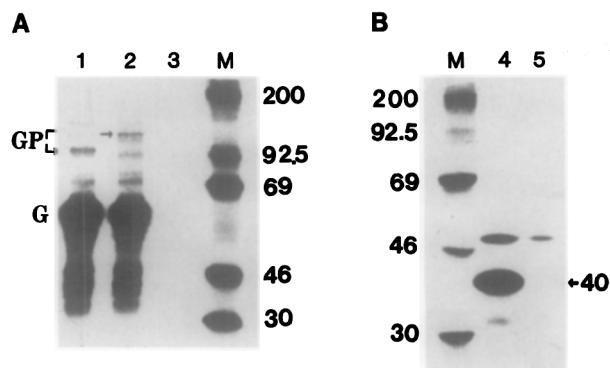


Fig. 1. Identification of *in vitro* M-MuLV Gag and Gag-Pol translation products. (A) Plasmid pSPgp was linearized with *Bam*HI or *Sal*I endonucleases for transcription in the SP6 RNA polymerase transcription system. The resulting RNAs were translated *in vitro* and protein products Gag (G) and Gag-Pol (GP) were separated by polyacrylamide gel electrophoresis, following immune precipitation with anti P30 rabbit antibodies. BAM-RNA, (lane 1), SAL-RNA, (lane 2), translation without exogenous RNA (lane 3), molecular mass markers were applied in lane M. Protein molecular weights are indicated in kilodaltons. (B) *In vitro* translation of M-MuLV RNA harboring an amber mutation in the *gag* gene. The glutamine codon CAG in *gag* at positions 1623–1625, was converted to UAG, and the resulting plasmid pSPg was cleaved with restriction enzyme *Bam*HI, prior to transcription of g-RNA. The translation mixtures were applied to the SDS-PAGE without prior immune precipitation. The truncated 40-kDa Gag polypeptide product of g-RNA is indicated by an arrow (lane 4). Translation mixture without exogenous RNA (lane 5).

corresponded in size to the expected runoff Gag-Pol fusion protein, as calculated from the M-MuLV DNA sequence (Shinnick *et al.*, 1981). To verify the identity of the runoff protein product, the DNA template pSPgp was linearized at the *Sal*I restriction site (nucleotide 3705), within the *pol* gene and transcribed into SAL-RNA of 3138 bases long. The translation products of this RNA (Fig. 1A, lane 2) corresponds to the 65-kDa Gag protein and to the readthrough product of 110 kDa. The SAL-RNA readthrough product is larger than the BAM-RNA product by 16 kDa as expected from the difference in the size of the two RNAs. Thus, both the immunoprecipitation reaction and the translations of two related RNAs indicated that the runoff product represents the Gag-Pol polyprotein. In repeated experiments BAM-RNA appeared to serve as a more efficient template for synthesis of the Gag-Pol runoff fusion protein compared to longer RNAs with 3' ends located further downstream in the *pol* gene. The Gag-Pol translation products of the later RNAs were distributed between the runoff proteins and a shorter 92-kDa protein which probably represent a premature termination product. We, therefore, used the BAM-RNA in all subsequent experiments. In a translation mixture without exogenous RNA none of the M-MuLV translation products was immunoprecipitated, indicating the specific-

ity of the antiserum for M-MuLV p30 (Fig. 1A, lane 3). We conclude that authentic suppression of the UAG termination codon occurs in the *in vitro* translation system, giving rise to Gag-Pol and Gag polypeptides, with molar ratio of 1:20–1:50, as determined by densitometry measurements, in the range observed previously *in vitro* and *in vivo* (Jamjoom *et al.*, 1977; Murphy *et al.*, 1978). The pattern of low molecular weight proteins observed in Fig. 1A, lane 1, is similar to that obtained prior to immunoprecipitation (data not shown). These Gag containing polyproteins could result either from internal translation initiation of the Gag protein or premature termination and they are typical by-products of *in vitro* translation systems (Philipson *et al.*, 1978; Feng *et al.*, 1989a).

To determine whether the level of translation level through the amber codon located between the *gag* and *pol* genes is unique, or may represent a general infidelity of the reticulocyte translation system, we converted, by site-directed mutagenesis (Morinaga *et al.*, 1984), a glutamine codon (CAG) within the *gag* gene (nucleotides 1623–1625) to a UAG codon (pSPg). Screening of the mutants was carried out by differential colony hybridization, and the mutations were verified by DNA sequencing (Sanger *et al.*, 1977). Translation of the RNA transcribed from pSPg (g-RNA) was expected to terminate at the UAG codon and yield a 40-kDa truncated Gag product. Suppression of the inserted amber codon would have produced, in addition, a full-size 65-kDa Gag protein. Only the 40-kDa viral specific protein was observed, even after long exposure periods of the autoradiograms (Fig. 1B, lane 4). The additional 47-kDa translation product represents an endogenous reticulocyte protein as it also appears in the control without viral RNA (lane 5). This result indicates that unique signals which control the amber codon suppression may be present at the *gag-pol* junction.

Scanning the sequences of different retroviruses at the *gag-pol* or *gag-pro* junctions revealed a common five nucleotide long purine-rich stretch (mostly G's) adjacent to the *gag* termination codon (Weiss *et al.*, 1984). To test the function of this consensus sequence in translation readthrough, we substituted the nucleotide sequence GGAGG following the amber codon with ACGCG, giving rise to plasmid pSPgp1 (Fig. 2A). Translation readthrough of the *gag* termination codon was completely abolished when RNA transcribed off pSPgp1 (gp1-RNA) served as a template (Fig. 2B, lane 2), demonstrating the importance of this nucleotide sequence in M-MuLV suppression.

Ribosomal frameshifting mechanism in several retroviruses appears to involve an RNA stem and loop

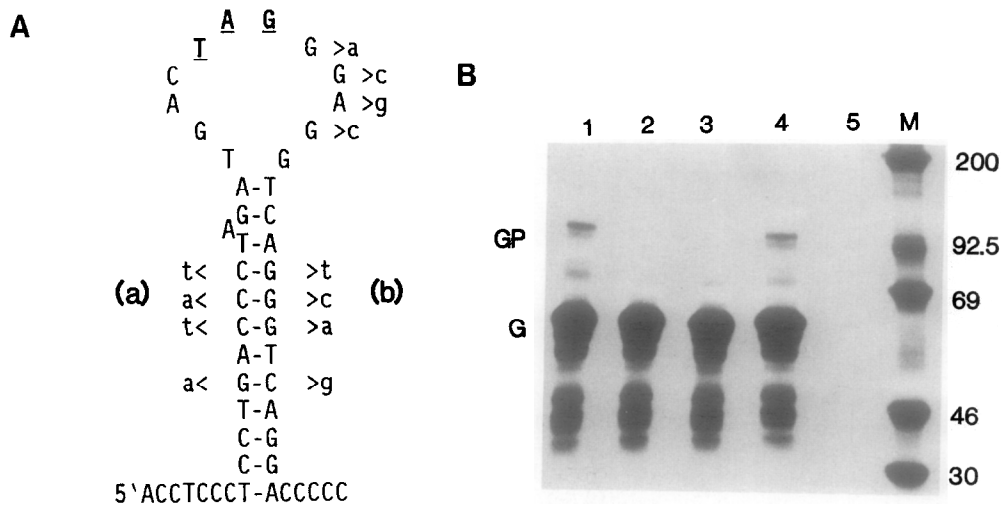


FIG. 2. Mutational analysis of the putative secondary structure abutting the *gag* termination codon. (A) Schematic illustration of the putative RNA stem (a)–(b) and loop structure flanking the amber codon (underlined bold letters) between the *gag* and *pol* genes of M-MuLV. The base changes generating the mutations at the loop (pSPgp1), at the 5' arm of the stem (pSPgpA), and at the 3' arm of the stem (pSPgpB) are indicated. (B) Reticulocyte lysate translation products of RNA transcribed from pSPgp and its mutated derivatives: lane 1, translation products derived from gp-RNA; lane 2, from gp1-RNA; lane 3, from gpB-RNA; lane 4, from gpA-RNA; and lane 5, without exogenous RNA. The positions of Gag and Gag–Pol proteins and the molecular mass markers (M) are indicated. Translation products were immunoprecipitated prior to electrophoresis.

structure downstream to the frameshift site, in addition to a primary sequence signal (Jacks *et al.*, 1988a,b). DNA sequence analysis of M-MuLV and the related AKV and FeLV revealed a putative secondary structure flanking the *gag-pol* amber codon (Shinnick *et al.*, 1981; Herr, 1984; Laprevotte *et al.*, 1984; Fig. 2A). To investigate the role of this structure in suppression we destabilized the stem by creating four-nucleotide changes within the sequence indicated as (a) region or the (b) region (pSPgpA and pSPgpB; Fig. 2A). As shown in Fig. 2B, suppression efficiency directed by gpA-RNA (lane 4) was equal to wild-type levels (lane 1), indicating that this putative stem and loop RNA structure does not play an important role in the suppression process. However, suppression was abolished in pSPgpB (Fig. 2B, lane 3). In light of this observation, we considered the following possibilities: either the mutated sequence (b) which is adjacent to the GGAGG sequence represents an extended primary signal essential for suppression, or an additional interaction may occur between the mutated nucleotides in the (b) region and nucleotides downstream in the mRNA to create an alternative secondary structure to that discussed above. Sequences within 100 nucleotides of the suppression site were analyzed, and a potential stem and loop structure downstream to the amber codon was identified (Fig. 3A). Furthermore, close examination of the sequence revealed a stretch of 6 guanosine residues, 16 nucleotides downstream to the predicted stem and loop, which could potentially base-

pair with a stretch of cytosine residues in the single-strand loop region of the second hairpin, to create a pseudoknot. Based on this observation, we sought a direct evidence for interaction between the (b)–(c) sequences in the proposed stem and loop, by introducing destabilizing mutagenic changes within the (c) region (Fig. 3A). A significantly reduced suppression efficiency was observed with gPC RNA (Fig. 3B, lane 4). Since residual readthrough was so low with this RNA, densitometric analysis could not be applied to quantitate the Gag–Pol product. Finally, to examine the importance of the proposed secondary RNA structure (b)–(c) in the readthrough process, complementary mutagenic changes were made both in (b) and (c) sequences of the same plasmid, restoring the potential for stem and loop formation by a different sequence (pSPgpBC). Suppression efficiency was not restored in the double-mutant gpBC-RNA (lane 3) compared with gpB-RNA (lane 2). Thus, mutations at sequences extending at least 50 bases 3' to the amber codon affect the translational readthrough. Sequences immediately distal to the UAG codon (mutations pSPgp1, pSPgpB) are of major importance for the event whereas further downstream sequences (mutation pSPgpC) may have a somewhat smaller effect on the readthrough frequency.

DISCUSSION

Our findings that sequences downstream to the termination codon are crucial for the suppression is remi-

niscient of the suppression in prokaryotic systems. In *E. coli*, suppression efficiency has been shown to depend upon the downstream adjacent nucleotides (Miller and Albertini, 1983; Bossi, 1983; Engelberg-Kulka, 1981). A comparative analysis of sequences at the vicinity of the *gag* termination codon of different retroviruses revealed a conserved purine rich five-nucleotide sequence, resembling the prokaryotic Shine–Dalgarno sequence. The latter sequence has been shown to be involved in translation initiation and in ribosomal frameshift mechanisms in *E. coli* (Weiss *et al.*, 1988). Disruption of this sequence and the adjacent downstream sequences (pSPgp1, pSPgpB) appears most detrimental to the translation readthrough in M-MuLV. Base changes further downstream of this sequence were also inhibitory but enabled residual readthrough of the termination codon (pSPgpC). It is not clear at this point how all of these control sequences interact with the suppressing glutamine tRNA (Yoshinaka *et al.*, 1985a,b; Kuchino *et al.*, 1987) and/or with the ribosome complex to facilitate the readthrough event.

Sequence analysis has revealed two alternative putative stem and loop structures adjacent to the UAG codon. Our analysis indicates that the first secondary structure (a)–(b) is dispensible for the suppression. Jones *et al.* (1989), have introduced single-base mutations in sequences flanking the M-MuLV *gag* termination codon and analyzed the effect on virus infectivity. Point mutations 5' to the UAG codon had no effect on virus infectivity while some mutations 3' to the UAG codon inactivated the virus. Our molecular analysis is

in agreement with this observation. In one experiment (Jones *et al.*, 1989), a single complimentary base change 5' to the UAG in a defective virus due to a mutation 3' to the UAG, partially restored viral infectivity. However, since no analysis of the viral Gag and Pol protein synthesis is described in this work, the relevance of the later observation to the suppression mechanism is not clear.

The second stem and loop like structure [(b)–(c), Fig. 3] resembles the RNA pseudoknot required for efficient ribosomal frameshifting in coronaviruses (Brierly *et al.*, 1989). The sequences downstream of the *gag*–*pol* junction are relatively conserved in feline leukemia virus, Baboon endogenous virus and murine leukemia virus. Furthermore, computer-aided analysis has predicted a similar secondary structure in this region (Ten Dam *et al.*, 1990). Moreover, free energy calculations predicted that the pseudoknot structure is more stable than the first stem and loop structure (a)–(b). Mutations of this structure in M-MuLV RNA which destabilized the stem structure, reduced suppression efficiency (pSPgpB, lane 2, and pSPgpC, lane 4), however, restoration of the stem with a different sequence (pSPgpBC, complementary mutations, lane 3) did not restore the suppression. This would suggest either that the primary sequence is of major importance or that the complementary mutations did not restore the proper mRNA folding to enable high level of suppression. However, since all of the mutations introduced downstream of the UAG codon reduced readthrough efficiency we favor the first possibility. Analysis of genetic signals in-

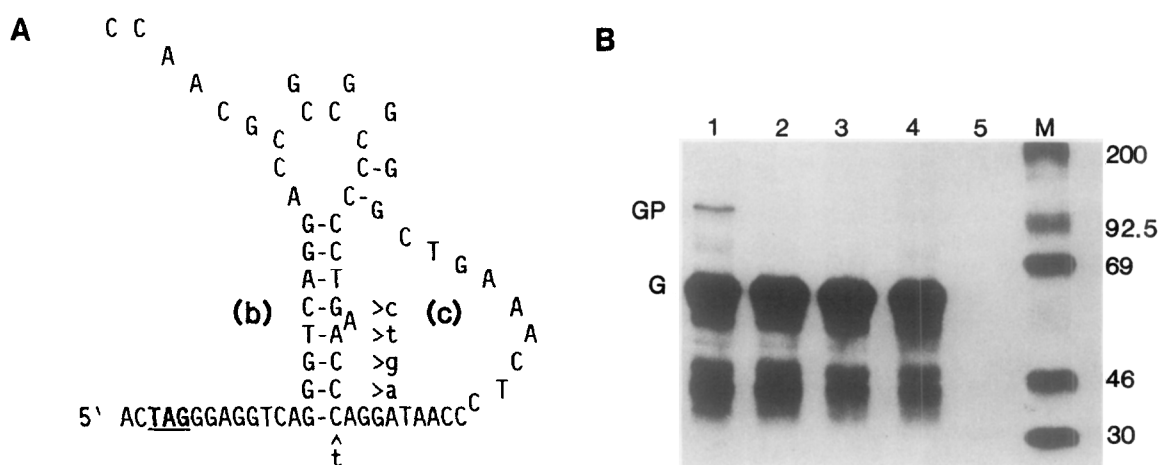


Fig. 3. Mutational analysis of the putative secondary structure downstream to the *gag* termination codon. (A) A schematic illustration of the potential RNA pseudoknot structure downstream to the *gag* amber codon. The positions of the stem destabilizing mutations at the 3' arm are indicated (pSPgpC). The mutations at the 5' arm (pSPgpB) are presented in Fig. 2A. pSPgpBC (not shown) carries both complementary mutations present in pSPgpB and in pSPgpC and thus may regenerate the putative stem and loop structure. (B) Translation products Gag (G) and Gag–Pol (GP) of RNA transcribed from pSPgp or the mutant templates described in (A): lane 1, gp-RNA; lane 2, gpB-RNA; lane 3, gpBC-RNA; lane 4, gpC-RNA. Translation products were immunoprecipitated prior to electrophoresis.

involved in translational frameshift between the *gag* and *pol* of HIV (Wilson *et al.*, 1988) did not reveal a requirement for an RNA secondary structure in the readthrough event. On the other hand, the consensus sequences required for Gag-Pol frameshift in Rous sarcoma virus (RSV) include a stem and loop structure downstream to the termination codons (Jacks *et al.*, 1988a). It seems that the involvement of a secondary structure in translation readthrough by frameshift or by suppression is not a general requirement but rather specific for some viruses (Ten Dam *et al.*, 1990).

The finding that suppression of *gag* translation termination codon occurs *in vitro* and *in vivo* in various cell lines, supports the notion that a cellular glutamine tRNA is responsible for the readthrough of the nonsense codon UAG (Kuchino *et al.*, 1987; Feng *et al.*, 1989b; Panganiban, 1988). Replacement of the UAG *gag* termination codon by UAA or UGA triplets did not affect suppression, suggesting that the UAG termination codon is not a part of the readthrough signal (Feng *et al.*, 1989a; Jones *et al.*, 1989). Thus, our study indicates that the main determinant which facilitates *pol* synthesis during M-MuLV replication, appears to be the *cis* RNA sequences downstream of the *gag* termination codon. This unique genetic system mediates the controlled expression of two genes from one mRNA molecule.

ACKNOWLEDGMENTS

We are grateful to Drs. S. Israel, A. Bar-shira, and C. Kronman for continued interest and helpful advice; Drs. S. Goff, A. Rein, and J. Levin for the communication of results prior to publication; and to Drs. B. Amit and A. Worcel, Biotechnology General Inc., for synthesis of oligonucleotides.

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