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

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The pal 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-pal 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.

INTRODUCTION

All replication competent retroviruses possess three common genes, gag, pal, and env, which have been mapped as 5'-gag–pal–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 pal 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 codons 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 pal genes in the particular retrovirus: (i) Ribosomal frameshifting in viruses such as Rous sarcoma virus (RSV), in which the gag and pal 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 pal (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 maintenance of this ratio between the major structural Gag proteins and the catalytic pal gene products is essential for proper viral replication and infectivity (Felsenstein and Griff, 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 pal 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 pal 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 (Shinick 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' CACTATCGCTAG-TTGCTCC 3' pSPgp
(2) 5' GATGACTAGACGCGTCAGGGTCAGG 3' pSPgp1
(3) 5' CCCCAGACCTCCCTCCTATTATTTAGATGACTAGGG 3' pSPgpA
(4) 5' TAGGGAGGTCATCATGAGGACCCCCCCCTGAACC 3' pSPgpB
(5) 5' CCCCCCCCTCATGATAGGATAACCCTCAAA 3' pSPgpC

In vitro transcription and translation

Plasmids were cleaved with *Bam*III 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 (Jack 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 (Laemmli, 1970). The clearly discernible 65-kDa protein band represents the predicted product of the gag gene (Fig. 1A, lane 1). In addition, a 94-kDa protein band was observed when BAM-KNA was translated. This translation product

DNA sequencing

The M-MuLV DNA fragment located between restriction sites of endonucleases *Nru*I–nucleotide 2121 and *Sal*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).
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 with BamHI or SalI endonucleases for transcription in the GPG 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 BamHI, 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).

Fig. 1. Identification of in vitro M-MuLV Gag and Gag–Pol translation products. (A) Plasmid pSPgp was linearized with BamHI or SalI and endonucleases for transcription in the GPG 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 BamHI, 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).

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 (Ganger 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
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 quantify 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-
niscent 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 (pSPgpB, 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 (Tem 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 (pSPgpB). The mutations at the 5' arm (pSPgpB) are presented in Fig. 2A. pSPgpB (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.](image-url)
volved 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.

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