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Mutational Analysis of the *gag-pol* Junction of Moloney Murine Leukemia Virus: Requirements for Expression of the *gag-pol* Fusion Protein

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The *gag-pol* polyprotein of the murine and feline leukemia viruses is expressed by translational readthrough of a UAG terminator codon at the 3′ end of the *gag* gene. To explore the *cis*-acting sequence requirements for the readthrough event in vivo, we generated a library of mutants of the Moloney murine leukemia virus with point mutations near the terminator codon and tested the mutant viral DNAs for the ability to direct synthesis of the *gag-pol* fusion protein and formation of infectious virus. The analysis showed that sequences 3′ to the terminator are necessary and sufficient for the process. The results do not support a role for one proposed stem-loop structure that includes the terminator but are consistent with the involvement of another stem-loop 3′ to the terminator. One mutant, containing two compensatory changes in this stem structure, was temperature sensitive for replication and for formation of the *gag-pol* protein. The results suggest that RNA sequence and structure are critical determinants of translational readthrough in vivo.

The genomes of all replication-competent retroviruses contain three major genes termed *gag* (for group-specific antigen), *pol* (for polymerase), and *env* (for envelope glycoprotein). The initial protein products of these genes are polyprotein precursors that are posttranslationally cleaved into mature proteins with distinctive biochemical properties and replication functions (see reference 32 for a review). The *gag* polyprotein, responsible for assembly of the virion core, is translated from a large mRNA identical to the genomic RNA present in the virion particle. The *pol* gene, which encodes several replication enzymes, is expressed only as a *gag-pol* fusion protein that is translated from the same mRNA (21). Formation of the *gag-pol* polyprotein requires that translation bypass the terminator at the 3′ end of the *gag* gene and continue downstream into the *pol* open reading frame. Different retroviruses utilize either of two different mechanisms to achieve controlled synthesis of the *gag-pol* protein (14, 30; for a recent review, see reference 11). In some viruses, a translational frameshifting event occurs at a specific site 5′ to the *gag* terminator codon at a controlled rate, allowing ribosomes to continue synthesis past the terminator into the *pol* gene in the −1 frame relative to *gag*. In the murine and feline leukemia viruses, a translational readthrough event occurs at a defined frequency, allowing translation to continue through a single UAG terminator into the downstream *pol* sequences in the same reading frame (10, 22, 24, 37). We are interested in understanding the mechanism of this suppression event.

Amino acid sequence analysis of the *gag-pol* fusion protein of Moloney murine leukemia virus (M-MuLV) showed the presence of a glutamine at the position specified by the UAG terminator codon, suggesting that a glutamine tRNA can recognize the codon (37). There seems to be no absolute requirement, however, for a specific tRNA in the process. Mutant viruses containing UAA or UGA codons in place of the normal terminator are viable and mediate nearly normal levels of readthrough (7), and in the case of the UGA codon, other amino acids appear in the protein (5). Translation of the viral RNA in reticulocyte extracts in vitro recapitulates the readthrough event, suggesting that normal cellular tRNAs and normal ribosomes are capable of mediating the process (21, 24). Furthermore, RNAs transcribed from artificial constructs that contain only a small region from the *gag-pol* junction region can also mediate readthrough (23). These results suggest that the context of the terminator is important; that is, *cis*-acting sequences near the terminator somehow specify a high frequency of suppression. In contrast to one report (17), there seems to be no need for viral proteins, since extracts from uninfected and infected cells show similar abilities to suppress (6).

Several fusion constructs that contain the terminator region have been generated in various laboratories, and a number of mutations have been introduced into these constructs to define the sequence requirements for readthrough (8, 13, 15, 23, 34). The results suggest that a very limited sequence near the terminator is required. Several RNA structures that might form have been proposed by computer-assisting modelling of these sequences (30). By analyzing mutants and their effects on predicted RNA secondary structures, several of these candidate RNA structures have been eliminated (8, 13, 34). By analyzing double mutants with compensatory changes that should restore structure and function, one particular structure has been confirmed as a likely feature required for the event (8, 34). This structure is a pseudoknot, a folded structure containing two paired sequence blocks separated by two loops. How the presence of this structure promotes readthrough remains unclear.

To define more precisely the RNA structures needed for translational suppression and to determine whether the requirements for suppression in vivo are similar to those in vitro, we generated a collection of mutant proviruses of M-MuLV with point mutations in the *gag-pol* junction region. The mutants were tested for viability and for formation of the *gag-pol* fusion protein in mammalian cells. The

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results support the idea that efficient suppression is essential for replication of the virus. The results also suggest that there is a critical region downstream of the terminator codon and that the required sequences are similar, although perhaps not identical, to those needed in vitro. One double mutant, which contains compensatory changes in a proposed RNA stem, yielded a temperature-sensitive phenotype. We suggest that the resulting altered RNA structure, or its interaction with the translational machinery, is temperature sensitive in this mutant.

MATERIALS AND METHODS

Construction of mutants. Plasmid pNCA, which contains a complete infectious proviral DNA copy of the M-MuLV genome (2), was used as the parent for generation of mutants. To create point mutations, a 2.2-kb fragment containing the *gag-pol* junction was excised by cleavage with *Pvu*II plus *Sal*I and subcloned into bacteriophage vector M13mp18 (36). Uracil-containing single-stranded DNA was prepared and used as the template for DNA synthesis primed with various synthetic oligonucleotides (18). The oligonucleotides (Genosys Biotechnologies, Inc., Houston, Tex.) were all 25 to 30 nucleotides long, with the mispaired bases centrally located. Whenever possible, the mutations were designed to minimize changes in the amino acids encoded by the *gag* and *pol* genes. The mutant DNAs were recovered after transformation of strain BD1528 (*ung* mutant). The structures of all of the mutations were confirmed by DNA sequence analysis (26); the analysis was extended approximately 100 nucleotides on each side of the oligonucleotide to ensure that no unexpected mutations had been formed. To transfer the mutations into a complete viral genome, a 2.1-kb *Xho*I-*Sal*I fragment was excised from the M13 replicative-form DNA and used to replace the corresponding wild-type fragment in pNCA. All cloning methods used were based on standard procedures (19).

Transfection of mammalian cells and tests for virus replication. NIH 3T3 and Rat2 cells were maintained in Dulbecco's modified Eagle medium containing 10% calf serum. Mutant DNAs were tested for biological activity by measuring virus production after transient transfection of NIH 3T3 and Rat2 cells, using the DEAE-dextran procedure (20), applying 0.1 μg of viral DNA per 2×10^5 cells in 6-cm-diameter dishes. Cells were allowed to reach confluence (3 to 5 days), transferred at a 1:10 dilution to 10-cm-diameter dishes, and allowed to reach confluence again. Cultures were tested for the appearance of replicating virus by the XC plaque assay (25) and by enzymatic assays for reverse transcriptase (RT) in the culture medium (9). Virus-negative cultures were maintained with passage for a total of 4 weeks and reassayed at that time to test for the appearance of partially defective virus. No slowly replicating viruses were identified.

Establishment of producer cells and analysis of viral proteins. To examine the viral protein products synthesized by defective mutants, producer cell lines were generated by stably introducing the proviral DNAs into NIH 3T3 cells. Cultures were transformed by a mixture of the viral DNA (1 μg) and pSV2neo DNA (0.1 μg) (28) in calcium phosphate (33), and recipient cells were recovered by selection with G418 (400 μg/ml; GIBCO) for 2 weeks. Colonies were pooled and grown into large cultures, and single-cell clones were isolated by limiting dilution in 96-well cloning trays. To analyze the intracellular viral proteins, cell lines were labelled with [^{35}S]methionine (150 μCi/10-cm dish, 45 min), and the proteins were extracted, immunoprecipitated, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography (29). Specific polyclonal goat antisera, obtained from the National Cancer Institute, were reactive against total MuLV virion proteins (81S-107) and p30^{gag} (77S-158). To analyze the virion proteins released from the lines, cultures were labelled with [^{35}S]methionine (100 μCi per dish, 16 to 20 h), and the culture medium was collected. Virion proteins were pelleted and analyzed by immunoprecipitation and electrophoresis as described before (29).

RESULTS

Construction of mutant M-MuLV proviruses. To generate mutations near the *gag-pol* boundary, a DNA fragment of a wild-type clone of M-MuLV proviral DNA was subcloned into an M13 vector (see Materials and Methods). A collection of oligonucleotides was used to create the mutations, and the sequences of the clones recovered were confirmed by DNA sequence analysis. In some cases, mutant clones were used as substrates for a second round of mutagenesis with a second oligonucleotide to generate double mutants. DNA fragments containing the mutations were then used to replace the corresponding fragment in plasmid pNCA, which contains a complete infectious provirus (2).

Each proviral DNA was tested first for the ability to generate replication-competent virus after introduction of the DNA into NIH 3T3 cells. The appearance of virus was monitored by both XC plaque assay and RT assays. In these assays, the wild-type virus generated 500 to 1,000 plaques per 100 ng of DNA and generated high levels of RT 5 to 8 days posttransfection. In almost all cases, the mutants either gave fully wild-type levels or no detectable virus at all. Each DNA that induced the appearance of virus in NIH 3T3 cells was subsequently tested in Rat2 cells to eliminate the possibility that the virus arose through recombination with endogenous viral sequences. In all cases, the mutants that were viable in NIH 3T3 cells were also viable in Rat2 cells. To analyze the defective mutants further, stable producer cell lines were established by cotransformation of NIH 3T3 cells with a selectable marker and the mutant proteins were analyzed after immunoprecipitation of extracts of labelled cells. We will discuss the mutants in groups based on position relative to the terminator codon.

Mutations flanking the UAG terminator: a candidate stem-loop structure is not required. Examination of the nucleotide sequence of M-MuLV and the related virus AKV has led several investigators to note the possibility that the viral RNA forms a stable stem-loop structure, with the UAG terminator codon located in the loop (12, 23, 27; Fig. 1). The stem would contain a "bulged" A nucleotide, similar to that seen at a site of suppression in phage R17 (35). To test the importance of this stem-loop structure, we generated nine mutations that would disrupt the base pairing of the stem, with changes lying on either the 5′ or the 3′ side of the stem (Fig. 1 and 2). Each mutant DNA was introduced into permissive cells by transfection, and the cultures were tested for appearance of replicating virus. All of the mutants with alterations on the 5′ side of the terminator codon (sub1 through sub5) were fully viable in both NIH 3T3 and Rat2 cells, yielding wild-type levels of XC plaques and generating RT with kinetics indistinguishable from that of the wild-type. The time of appearance of the virus makes it unlikely that reversion of the mutations was required for replication, and the recovery of virus from Rat2 cells makes it unlikely that recombination with endogenous retroviral sequences was

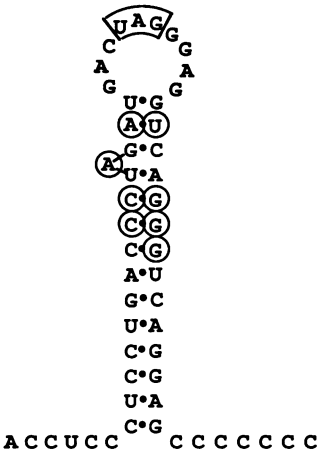

FIG. 1. One proposed secondary structure (12, 23, 27) surrounding the UAG terminator at the 3′ end of the *gag* gene. The UAG terminator is boxed. Bases altered in this study to address the potential involvement of such a structure in translational readthrough are circled. The relevant mutants are sub1 through sub9 (see Fig. 2).

involved. In contrast, all of the mutants with alterations on the 3′ side of the terminator (sub6 through sub9) were replication defective. These results suggest that the proposed stem-loop structure is not formed or is not important for virus replication, in agreement with other reports (13, 34). To probe the possibility of the importance of the structure further, two double mutants were generated, containing compensatory changes on the 5′ and 3′ sides of the stem and restoring the predicted base pairing (mutants sub2+7 and sub1+8). These mutants were defective, like the single mutants with changes on the 3′ side of the stem (Fig. 2). These results suggest that the mutations on the 3′ side of the stem were involved with a different structure and are probably not paired with the 5′ side of the stem.

To analyze the proteins encoded by the defective mutants, producer cells were established for all of the mutants and labelled with [^{35}S]methionine, and the viral proteins were examined by immunoprecipitation and electrophoresis (Fig.

Virus	Pro	Gln	Thr	Ser	Leu	Leu	Thr	Leu	Asp	Asp	***	Gly	Gly	Gln	Gly	Gln	Glu	Pro	Pro	Viability
Wild type	CCC	CAG	ACC	UCC	CUC	CUG	ACC	CUA	GAU	GAC	UAG	GGA	GGU	CAG	GGU	CAG	GAG	CCC	CCC	+
sub1	===	===	===	===	===	===	===	==G	===	===	===	===	===	===	===	===	===	===	===	+
sub2	===	===	===	===	===	===	===	G==	===	===	===	===	===	===	===	===	===	===	===	+
sub3	===	===	===	===	===	===	===	==C	===	===	===	===	===	===	===	===	===	===	===	+
sub4	===	===	===	===	===	===	===	==G	A==	===	===	===	===	===	===	===	===	===	===	+
sub5	===	===	===	===	===	===	===	===	=G=	===	===	===	===	===	===	===	===	===	===	+
sub6	===	===	===	===	===	===	===	===	===	===	===	===	==A	===	===	===	===	===	===	−
sub7	===	===	===	===	===	===	===	===	===	===	===	===	==C	===	===	===	===	===	===	−
sub8	===	===	===	===	===	===	===	===	===	===	===	===	===	C==	===	===	===	===	===	−
sub9	===	===	===	===	===	===	===	===	===	===	===	===	===	=C=	===	===	===	===	===	−
sub2+7	===	===	===	===	===	===	===	G==	===	===	===	===	==C	===	===	===	===	===	===	−
sub1+8	===	===	===	===	===	===	===	==G	===	===	===	===	===	C==	===	===	===	===	===	−
sub10	===	U==	===	===	===	===	===	===	===	===	===	C==	===	===	===	===	===	===	===	−
sub11	===	===	===	===	===	===	===	===	===	===	===	C==	U==	===	===	===	===	===	===	−
sub12	===	===	===	===	===	===	===	===	===	===	===	==A	===	===	===	===	===	===	===	+
sub13	===	===	===	===	===	===	===	===	===	===	=GA	===	===	===	===	===	===	===	===	+
sub14	===	===	===	===	===	===	===	===	===	===	==-	A==	===	===	===	===	===	===	===	+
sub15	===	===	===	===	===	===	===	===	===	===	===	UC=	===	===	===	===	===	===	===	+
sub16	===	===	===	===	===	===	===	===	===	===	===	=C=	===	===	===	===	===	===	===	+-
sub17	===	===	===	===	===	===	===	===	===	===	==C	===	===	===	===	===	===	===	===	+
sub18	===	===	===	===	===	===	===	===	===	===	=C=	===	===	===	===	===	===	===	===	−
sub19	===	===	===	===	===	===	===	===	===	===	C==	===	===	===	===	===	===	===	===	−

FIG. 2. Mutants generated in this study with alterations flanking the UAG terminator at the 3′ end of the *gag* gene. The wild-type sequence and the amino acids encoded by that sequence are given at the top (from references 15 and 27). The new bases in each mutant are indicated below. The viability of each mutant after transformation of mammalian cells is indicated at the right. +, equivalent to the wild type by XC and RT assays; −, completely negative for virus.

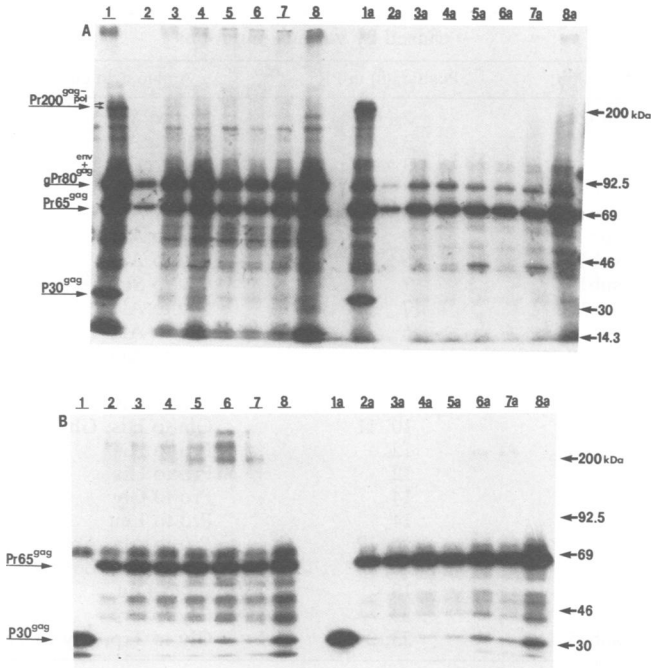

FIG. 3. SDS-gel electrophoresis of viral proteins encoded by various mutants. (A) Intracellular viral proteins synthesized in cell lines that express various mutant proviral DNAs. Cell lines were labelled with [^{35}S]methionine for 1 h and lysed, and proteins were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. (B) Virion proteins pelleted from culture supernatants collected after overnight labelling. Lanes: 1 to 8, immunoprecipitation with total anti-M-MuLV serum; 1a to 8a, immunoprecipitation with anti-p30 (CA) serum; 1, wild-type M-MuLV; 2, mutant sub20; 3, mutant sub9; 4, mutant sub21; 5, mutant sub24; 6, mutant sub29; 7, mutant sub32; 8, mutant sub34. The positions of the major viral proteins are indicated at the left. The positions of migration of marker proteins of various sizes are indicated at the right.

3). Intracellular Pr65^{gag} and gPr80^{env} were readily detected after brief labelling in all cases, although no cleavage of the *gag* precursor to the mature p30 CA protein was detected (only a few examples are shown in Fig. 3A; the results for the remaining mutants were identical). Importantly, no Pr200$^{gag\text{-}pol}$ was detected in any of the cell lines that carried the mutants, suggesting that readthrough itself was directly blocked by the mutations. Parallel experiments with protease-deficient mutants (3) and with a constitutive readthrough mutant (4) showed that the Pr200$^{gag\text{-}pol}$ protein could readily have been detected (data not shown). Uninfectious virion particles were formed and released from the cells; *gag* and *env* virion proteins were detected in the extracellular medium after appropriate labelling periods (examples are shown in Fig. 3B). Unlike the wild-type virus, virtually all of the *gag* protein was in the form of the uncleaved Pr65^{gag} precursor. No *pol* products were visible in the mutant particles. This phenotype is exactly as expected for mutants that express no *gag-pol* protein: the absence of the viral PR protease encoded by the *pol* gene is known to block cleavage of the *gag* protein without blocking release of the immature virion cores (3, 16). It should be noted that three of the five mutations on the 5′ side of the stem resulted in changes in amino acids (Table 1). These changes were apparently all phenotypically silent and were

TABLE 1. Amino acid substitutions in the protease gene product caused by various mutations

Mutation	Position(s) in PR[a]	Amino acid change(s)
sub2	2	Leu to Val
sub4	3	Asp to Asn
sub5	3	Asp to Gly
sub7	8	Gln to His
sub8	9	Gly to Arg
sub9	9	Gly to Ala
sub14	6	Gly to Arg
sub15	7	Gly to Ser
sub16	7	Gly to Ala
sub18	6	Gly to Ala
sub19	6	Gly to Arg
sub20	9	Gly to Ala
sub22	11	Glu to Gln
sub23	10, 11	Gln to His, Glu to Gln
sub24	12	Pro to His
sub26	12	Pro to His
sub28	14	Pro to Gly
sub29	14	Pro to Leu
sub30	16, 17	Pro to Ala, Arg to Ser
sub31	16, 18	Pro to Ala, Ile to Leu
sub32	18	Ile to Leu
sub34	23	Gly to Trp
sub35	23, 24	Gly to Trp, Gly to Trp

[a] Amino acid position relative to the N-terminal PR residue formed after cleavage of PR from the *gag-pol* precursor.

tolerated by these viable viruses. These changes affect the very N terminus of the PR protease, which is partially encoded by sequence upstream of the terminator (37). Three of the four mutations on the 3′ side also resulted in changes in PR. Thus, the defect in these mutants could in principle be due to the changes in either the amino acid sequence of PR or the RNA structure. The absence of detectable $Pr200^{gag\text{-}pol}$ protein in a short labelling period, however, argues for a block in translational readthrough. The remaining mutation, sub6, must block readthrough, since it causes no amino acid substitutions.

Mutants with ectopic terminator codons: position, not identity, of the terminator is important. Two mutants were generated to test whether terminator codons at other positions near the normal site of the wild-type UAG could be utilized for readthrough. The normal terminator was replaced with a CAG codon, which specifies glutamine (4), and new termination codons were created elsewhere. In one case (sub10), a new UAG codon was created to replace a CAG codon for glutamine 9 codons 5′ of the normal terminator; in the other (sub11), a UGA codon was created to replace a GGA codon for glycine 1 codon 3′ of the normal terminator. Both mutants were defective (Fig. 2). Analysis of stable producer cell lines revealed that both mutants made normal levels of *gag* and *env* proteins but no detectable *gag-pol* protein. The *gag* protein detected in mutant sub10, as predicted by the structure of the mutation, was shorter than the wild type (data not shown). For both mutants, virions were released but the *gag* precursors were not processed, suggesting that no *pol* products were formed. The result suggests that the position of the terminator in the *gag-pol* region is critical for proper readthrough.

To confirm the findings of other laboratories that the two other terminator codons can mediate readthrough and are compatible with viral replication, we introduced mutations to form UAA and UGA triplets in place of the wild-type UAG (mutants sub12 and sub13; Fig. 2). As previously reported, these mutants were fully replication competent when assayed in NIH 3T3 and Rat2 cells. This result reinforces the conclusion that the position of the terminator in its flanking sequences is critical for the process, as in other systems.

Mutations immediately 3′ to the terminator codon: a limited role in readthrough. In prokaryotic systems, the level of suppression of terminator codons can be significantly affected by the sequence immediately following the terminator (1). The five nucleotides 3′ to the UAG terminator of a variety of murine and feline leukemia viruses are relatively well conserved and are generally purines (11). The two nucleotides immediately 3′ to the UAG terminator have been proposed to interact with nucleotides U and Cm in the tRNA to promote use of the glutamine tRNA (23), similar to proposed pairings in readthrough in bacteriophage Qβ (31). To test the importance of these bases in vivo, we generated several substitution mutations and tested the mutants for viability after transfection (sub14 through sub19; Fig. 2). Surprisingly, many of these mutants were replication competent. Changing the first G residue to A had no effect, and changing the central A or the last two G's was also tolerated. These results differ from those obtained through analysis of readthrough on mutant constructs in vitro (8); in these studies, changing the first G to A or the last G to C abolished readthrough. Thus, the requirement for these G residues is apparently less stringent in vivo than in vitro. Of the mutations studied in vivo here, only changes of either of the first two G's to C's were able to abolish replication of the viruses. We thus conclude that these two G residues are important for function. The viability of the substitution of the first G with A and not with C is consistent with a need for pairing of this base to the U base of the tRNA. It is also possible that these G residues are involved in other aspects of RNA structure or interactions with ribosomal factors and that there is only a requirement for purines at these positions.

To confirm that the defects in the two defective mutants (sub18 and sub19) were due to a failure in readthrough rather than to changes in the PR protein sequence, cell lines were established and the viral proteins were tested for formation of $Pr200^{gag\text{-}pol}$. As for the earlier mutants, no $Pr200^{gag\text{-}pol}$ was formed, confirming that readthrough was indeed affected.

Mutations far downstream of the terminator: a complex structure. The most detailed mutational analyses of the *gag-pol* region for translational readthrough in vitro have led to the proposal of a pseudoknot structure formed from sequences on the 3′ side of the terminator (8, 34). To probe the nature of the requirements in this area in vivo, we made a number of single and double mutations (Fig. 4). The positions of the bases altered in these mutants on the proposed pseudoknot structure are indicated in Fig. 5. The mutant proviruses were introduced into NIH 3T3 cells as before, and the effects of the mutations on virus replication were tested. Nearly all of these mutations blocked replication of the virus completely, suggesting that an extended portion of the region is critically involved in readthrough. Many of the mutations in this area (11 of 18) resulted in changes in amino acids in the encoded PR protein (Table 1). To determine whether formation of the *gag-pol* protein or only the function of the PR protein was blocked, producer cell lines were generated for the defective mutants and the viral proteins were analyzed as before. In all cases, the *gag-pol* protein was not detected, strongly suggesting that

Virus																			Viability
Wild	***	Gly	Gly	Gln	Gly	Gln	Glu	Pro	Pro	Pro	Glu	Pro	Arg	Ile	Thr	Leu	Lys	Val	Gly	Gly	
type	UAG	GGA	GGU	CAG	GGU	CAG	GAG	CCC	CCC	CCU	GAA	CCC	AGG	AUA	ACC	CUC	AAA	GUC	GGG	GGG	+
sub6			=A																		−
sub9			=C																		−
sub20			=A		=C																−
sub21					=A																−
sub22						C=															−
sub23					=C	C=															−
sub24							=A=														−
sub25								=A													−
sub26							=A=	=A													+
sub27								=A	=A												+
sub28									GG=												−
sub29									=U=												−
sub30										G=	=U										−
sub31										G=		U=									−
sub32												U=									−
sub33																		=A	=A	=A	−
sub34																		U=			−
sub35																		U=	U=		−

FIG. 4. Mutants generated in this study with alterations far downstream from the UAG terminator at the 3′ end of the *gag* gene. The wild-type sequence and the amino acids encoded by that sequence are given at the top. The new bases in each mutant are indicated below. The viability of each mutant in mammalian cells is indicated at the right. +, equivalent to the wild type by XC and RT assays. −, completely negative for virus.

Virus																			Viability
Wild	***	Gly	Gly	Gln	Gly	Gln	Glu	Pro	Pro	Pro	Glu	Pro	Arg	Ile	Thr	Leu	Lys	Val	Gly	Gly	
type	UAG	GGA	GGU	CAG	GGU	CAG	GAG	CCC	CCC	CCU	GAA	CCC	AGG	AUA	ACC	CUC	AAA	GUC	GGG	GGG	+
sub23+28						=C	C=			GG=											−
sub20+30			=A		=C							G=	=U								−
sub20+31			=A		=C							G=		U=							−
sub21+29					=A				=U												+/− (TS)
sub27+35										=A	=A									U=	U=	−
sub6+32			=A														U=				−
sub25+34										=A										U=		−

FIG. 6. Double mutants generated in this study with (potentially) compensatory alterations far downstream from the UAG terminator at the 3′ end of the *gag* gene. The wild-type sequence and the amino acids encoded by that sequence are given at the top. The new bases in each mutant are indicated below. The viability of each mutant in mammalian cells is indicated at the right. −, completely negative for virus; +/−, conditionally positive by XC and RT assays. Cultures were positive at 32°C and negative at 39°C. TS, temperature sensitive. ***, terminator.

the effect of all these mutations was, at least temporally first, to block readthrough.

Two mutants with changes in this region were viable. These mutants (sub26 and sub27) each contain two substitutions of A residues within a long stretch of eight contiguous C residues in the wild-type sequence. These mutants did not yield a completely normal number of XC plaques after transformation of NIH 3T3 cells but rather gave about 10-fold fewer plaques. This is a subtle defect, however, and the kinetics of appearance of RT after transient transfection was not significantly delayed compared with the wild type. The substitutions in these mutants should disrupt the pairing of the stretch of C's with a downstream stretch of G's that has been predicted to occur in the most likely pseudoknot structure (Fig. 4), replacing C-G pairs with A-G mismatches. This result suggests that these stretches do not in fact pair, that pairing is not essential for readthrough, or that some pairing of the regions can still occur in vivo, even with two disruptive mismatches. Results of studies in vitro have similarly shown that changes in this stretch of C residues cause only modest reductions in readthrough (8).

Analysis of double mutants with compensatory changes to restore base pairing. A very large number of alternate RNA structures can be generated by computer-assisted modelling, and the effects of the mutations tested above are consistent with many such structures. To obtain further evidence for or against various structures, we combined pairs of the mutations to generate double mutants (Fig. 6). The double mutants were designed such that base pairing would be restored if the two altered sequences were indeed paired with each other (Fig. 7). Each mutant DNA was tested as before for biological activity after transient transfection of NIH 3T3 cells at 37°C. All seven mutants were defective by this criterion, yielding no replication-competent virus. Cell lines that stably express all of these mutants were established, and analysis of the viral proteins at 37°C gave results identical to those obtained for the previous defective mutants: intracellular *gag* and *env* proteins were formed, but no *gag-pol* precursor was detected. The virion particles showed *gag* and *env* proteins, and the *gag* precursor remained uncleaved. There was no detectable RT activity in the medium. All of these results are consistent with continued lack of expression of the viral *gag-pol* protein. These results suggest that the various possible RNA pairings are not formed or, alternatively, that the pairings form but the sequences of the paired regions themselves are also critical.

It seemed possible to us that compensatory changes in paired stem regions might restore formation of the stem but that the presence of a base pair in the stem different from that in the wild-type stem might have subtle effects on the structure of the stem or on its relationship with other parts of

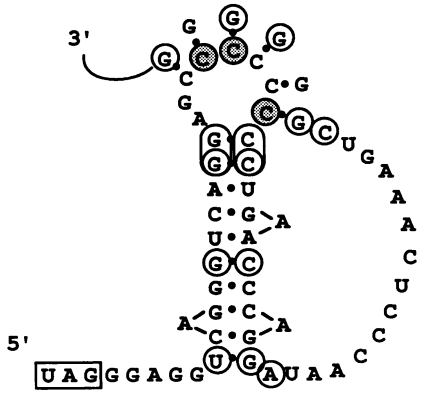

FIG. 5. Proposed secondary structure (8, 34) formed downstream of the *gag* gene. The UAG terminator is boxed. Bases altered in this study to address the potential involvement of the structure are circled. Shaded bases indicate changes that, in some combinations, permitted replication of the virus (Fig. 4).

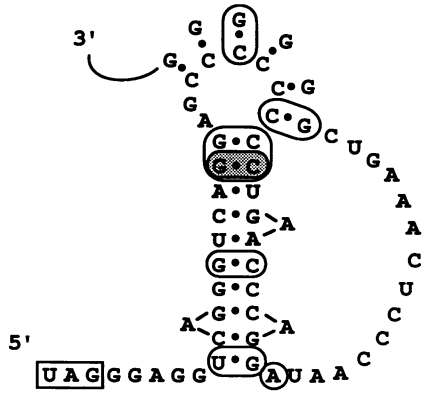

FIG. 7. Proposed secondary structure formed downstream of the *gag* gene. Pairs of bases altered in this study to address the potential involvement of the structure are circled. The shaded bases indicate the pair change that permitted conditional replication of the virus (Fig. 5).

the RNA structure. Such subtle changes might produce weak, partial, or conditional phenotypes. To probe for such phenotypes, we tested clonal NIH 3T3 cell lines that produce all of the mutants generated in this study for virus release as measured by RT at 32, 37, and 39°C. Six of the seven double mutants were still unable to induce release of RT at any temperature. One mutant, however, sub21+29, which contains two single substitutions, released considerable levels of RT at 32°C and none at all at 37 or 39°C. This result suggested that mutant sub21+29 encodes an RNA which could be translated to yield *pol* proteins at the lower temperature.

To confirm and extend these results, we generated new producer cell lines by cotransformation of Rat2 cells with mutant and pSV2neo DNAs. The cells were transformed, cloned, and expanded at 37°C, and the cultures were then shifted to various temperatures before collection of virus supernatants. Analysis of the release of virus from cells that harbored mutant sub21+29 showed behavior consistent with the results obtained after transient transfection: the cells released substantial levels of RT activity at 32°C and none detectable above the background at 37 or 39°C (data not shown). Virus was collected from these cells at 32°C and then used to infect fresh Rat2 cells at various temperatures, and the recipient cells were assayed for release of RT activity into the medium. Only cultures infected and maintained at 32°C were able to produce virus and active enzyme.

To determine whether RT production could be reversed by a temperature shift, the producer cell line was shifted from 32 to 39°C and grown for 48 h and the medium was collected and assayed. No activity above the background was detected, suggesting that the shutoff of virus release was relatively rapid.

To analyze these cells for the viral proteins synthesized at low and high temperatures, cultures were grown and labelled with [^{35}S]methionine at 32 or 39°C as before. Examination of the intracellular proteins encoded by the wild-type virus showed the presence of both the *gag* and *gag-pol* precursors at both temperatures, with a small amount of processed p30 CA protein visible (Fig. 8A). The mutant showed somewhat less viral protein synthesis overall, but the *gag* precursor was readily detected at both temperatures. At 32°C, low levels of the Pr200$^{gag\text{-}pol}$ precursor were formed, and substantial processing of the *gag* precursor to form p30 CA occurred, demonstrating that readthrough could proceed to form functional *pol* products. At 39°C, no *gag-pol* protein was detected and no processing of the *gag* precursor could be seen, even on long autoradiographic exposures. Examination of the virion-associated *gag* proteins provided still more evidence for temperature-sensitive formation of the *pol* products (Fig. 8B). The *gag* protein in the wild-type virus was essentially fully cleaved to form p30 CA at either 32 or 39°C. For mutant sub21+29, about half of the *gag* protein was processed to form a mixture of Pr65^{gag} and cleavage products at 32°C, only a little was processed to form a trace of CA at 37°C, and there was no detectable processing at 39°C. The data show that the level of *pol*-encoded PR activity was highly temperature sensitive in the mutant.

Taken together, these results indicate that mutant sub21+29 is temperature sensitive for formation of the *gag-pol* precursor and for virus replication. The two single mutations in mutant sub21+29, when present separately in the virus, did not permit temperature-sensitive growth but completely abolished replication and did not allow formation of the *gag-pol* protein (Fig. 4). Analysis of cell lines that

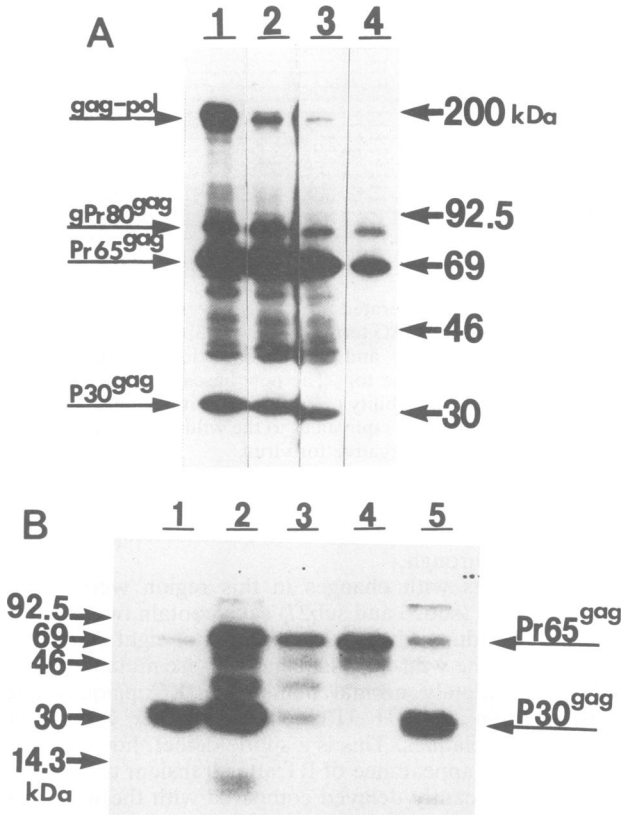

FIG. 8. SDS-gel electrophoresis of viral proteins encoded by double mutant sub21+29 at various temperatures. (A) Intracellular viral proteins detected after labelling with [^{35}S]methionine for 1 h, immunoprecipitated with anti-p30 (CA) antiserum. Lanes: 1, wild-type virus labelled at 32°C; 2, wild-type virus labelled at 39°C; 3, mutant sub21+29 labelled at 32°C; 4, mutant sub21+29 labelled at 39°C. (B) Virion proteins pelleted from the supernatant culture medium after overnight labelling and immunoprecipitated with anti-p30 (CA) serum. Lanes: 1, wild-type virus labelled at 32°C; 2, mutant sub21+29 labelled at 32°C; 3, mutant sub21+29 labelled at 37°C; 4, mutant sub21+29 labelled at 39°C; 5, wild-type virus labelled at 39°C. The positions of migration of marker proteins and major viral proteins are indicated.

produce these single mutant viruses showed that they did not produce *pol* proteins and did not induce release of RT activity at any temperature. Mutation sub21+29 causes a single amino acid substitution in the *pol* protein, which could in principle be responsible for the observed phenotype. This same substitution, however, is caused by the single mutation sub29, which conferred an unconditionally defective phenotype. The conditional viability of sub21+29 proves that the amino acid substitution is not responsible for the lethality of sub29 and supports the contention that the effect is on the RNA. The two mutations in mutant sub21+29 would restore base pairing across the top of a stem predicted to form as part of the pseudoknot favored by earlier analyses (Fig. 7). Thus, mutant sub21+29 supports the pairing of the two complementary stretches in the RNA that was previously proposed (8, 34) and the notion that this pairing is essential for readthrough in vivo. We cannot completely rule out the possibility that the double mutant restores function without pairing of these two bases, but this possibility seems remote.

DISCUSSION

The results presented here suggest that an extended sequence downstream of the *gag* gene's terminator of M-MuLV is essential for translational readthrough of the terminator to form the *gag-pol* protein. The mutants provide a map of the sites required for readthrough and for viability of the virus. Sequences upstream of the terminator do not seem to be important. There are two G nucleotides immediately downstream of the terminator that are needed; readthrough occurs when these bases are purines but not when they are converted to C residues. The next few bases after the two G's seem unimportant in vivo, perhaps serving only as spacers, and the following 50 or so bases seem extremely sensitive to alteration. The full extent of the sequence requirements in vivo remains uncertain, and more distant parts of the genome might be involved. The level of the effect of mutations in this downstream region on viral replication is striking: single base changes, even those that cause no changes in the encoded protein, can completely abolish translational readthrough and kill the virus. It seems likely that a complicated interplay of requirements for RNA and protein structure was involved in the evolution of this sequence stretch.

Some of the mutations generated in this study alter encoded amino acids and so could, in principle, affect either the function of the RNA in forming the *pol* proteins or the function of the PR protein. Analysis of cell lines that express all of these defective mutants showed that the $Pr200^{gag\text{-}pol}$ protein was not detectable after 45 min of labelling with [^{35}S]methionine. We argue on the basis of this result that the mutations almost certainly affect the readthrough event directly. Although it seems unlikely, we cannot absolutely rule out the possibility that some of the mutants encode an altered $Pr200^{gag\text{-}pol}$ protein that is so unstable that it is quickly degraded and cannot be detected. In these cases, translational readthrough might occur more efficiently than is suggested by the block to replication. In all cases, it is possible that the PR protein that would be encoded by the mutants is inactive; since all of the defective mutants fail to make detectable *gag-pol* protein, there is no PR formed to test. For mutants with no alterations in the encoded amino acids, the defect in replication must be due only to effects on the RNA and translational readthrough.

The role of the two bases immediately downstream of the terminator remains uncertain. These bases, normally G residues, could not be changed to C residues without blocking readthrough. This result is consistent with the possibility that these bases normally must interact with the U and Cm residues adjacent to the anticodon in the tRNA. It is also possible, of course, that these bases directly interact with another RNA, or with a host protein, in a very specific way during the process. It is interesting that the sequence requirements immediately downstream of the terminator for readthrough in vivo are distinct, and apparently less stringent, than in vitro; although the mutations of sub14 and sub16 are compatible with virus replication, the same changes have been shown to block readthrough in vitro (8). Presumably, some aspect of translation in vivo is different from that in vitro.

The mutagenesis results are consistent the notion that a complex RNA secondary structure, formed from the downstream region, is required for readthrough. The results are not consistent with the early proposal that a hairpin formed by sequences flanking the terminator could be important; this sequence has similarly been shown to be nonessential for readthrough in vitro (8, 13). The structure required for readthrough in vivo seems to lie exclusively downstream of the terminator codon, covering at least 50 nucleotides of the sequence. Another structure, proposed previously on the basis of analysis by translation in vitro (8, 34), does cover the region within which altered bases abolished readthrough (Fig. 5 and 7). But several double mutants that contain compensatory changes predicted to restore pairing in this structure did not restore readthrough (Fig. 7). Thus, the data do not allow us to lend support to the overall pseudoknot structure as being required in vivo. Perhaps the demands in the precise level of readthrough to permit virus replication are very strict and these compensatory changes do not restore an acceptable level of readthrough. It may be, for example, that a highly dynamic process of rearrangement occurs during readthrough and that the relative thermodynamic stability of the paired regions is critical. The behavior of the single conditionally viable double mutant (sub21+29), however, is supportive of a part of this structure and of the pairing of these two particular nucleotides.

The pairing of bases indicated by mutant sub21+29 might or might not be part of a simple pseudoknot in vivo. The second paired region in this proposed pseudoknot consists of pairing of a stretch of C's to a stretch of G's (Fig. 7), and some mutations in these sequences did block readthrough. Two mutants (sub26 and sub27) with two alterations each in the block of C's, however, were viable both in NIH 3T3 cells and in Rat2 cells. These results suggest that this part of the structure is not formed or is not strongly required in vivo. Curiously, mutants with only one (sub24) or the other (sub25) of the two changes in one of these mutants were not viable. It is very hard to understand how substitution of two C residues for A residues is tolerated better than one such substitution, if these C's are merely paired with the block of G's. Efforts to make compensatory changes in this region did not restore functional readthrough, in agreement with previous results (13). Possibly, the true structure required in vivo is more complex and involves other parts of the viral sequence, even regions not close to the terminator. Similar results were obtained in analysis of mutations in this stretch of C residues in vitro (8, 34).

How sequence or structure in the RNA perturbs the ability of the ribosome to recognize the UAG codon and terminate properly is still unclear. It is noteworthy that the position of the sequence relative to the terminator is similar to the position of the structures required for translational frameshifting relative to the site of frameshifting. Perhaps the structure impedes the progression of the ribosome, stalling elongation at the time of competition between the glutamine tRNA and protein release factors. Presumably, stalling alone is insufficient to specify readthrough; it seems likely that the relative efficiency of elongation versus termination should have to be altered. There might well be specific interactions between the viral RNA and rRNAs that promote stalling of the ribosome or that even alter the structure of the ribosome so as to alter its efficiency of termination. We suspect that a combination of genetic approaches, like that taken here, and biochemical approaches to ribosome structure and function is required to elucidate the detailed mechanism of readthrough.

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