
The effect of specific mutations at and around the *gag-pol* gene junction of Moloney murine leukaemia virus

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ABSTRACT

By carrying out oligonucleotide-directed mutagenesis, *in vitro*, on a 3.3 kb *XhoI-HindIII* fragment from Moloney murine leukaemia virus Mo-MuLV proviral DNA, inserted into the phagemid pTZ19R, nine separate fragments have been prepared in which mutations have been inserted at and around the *gag-pol* gene junction. Using these mutant fragments Mo-MuLV proviral DNA has been reassembled and cloned into pBR322. Examination of the mutant proviral DNAs in mouse culture cells indicates that a terminator codon at the *gag-pol* junction is essential for function, but any of the three chain terminator codons gives an active virus. Also the region of secondary structure surrounding the terminator codon must be preserved.

INTRODUCTION

The components which retroviruses have in common consist of the three genes *gag*, *pol* and *env*. The initial protein products of these genes are cleaved to give small functional proteins (See ref. 1 for a review). The *gag* and *pol* precursor proteins are translated from the same genomic mRNA. The *pol* precursor protein is synthesized only as a part of a larger product of the *gag* and *pol* genes (2,3), which requires a read-through mechanism of a chain terminator codon at the end of the *gag* gene (3,4). In some of the retroviruses, e.g. murine mammary tumour virus (MMTV) (5,6) and HIV (7), this involves a frameshifting mechanism, but for Moloney murine leukaemia virus (Mo-MuLV) it is accomplished by the insertion of a glutamine residue at the terminator codon, UAG, by a nonsense suppression mechanism (8). A UAG suppressor glutamine tRNA is coded for by mouse host cells and its amount is increased by infection with Mo-MuLV (9). This glutamine tRNA, in assays *in vitro*, is able to read through the leaky UAG terminator codon of TMV RNA (9).

Since in the retroviruses such as Mo-MuLV read-through of a terminator codon is necessary for expression of the *pol* gene, it is likely that this mechanism plays an essential role in the function of the virus. Also since it is likely that the suppressor glutamine tRNA is selectively utilized in reading particular terminator codon(s), such as that located at the junction of *gag-pol* genes of Mo-MuLV, the context of the terminator codon is of importance. Previously it has been suggested that the secondary structure may be an important factor in suppression efficiency (10,11).

In order to provide further insight into the importance of the terminator codon at the *gag-pol* junction, and the secondary structure which surrounds it we have prepared a series of mutations at and around the *gag-pol* gene junction of Mo-MuLV and examined the effects of these mutations on the infectivity and reproduction properties of the virus in NIH3T3 cells.

Near the completion of this work Felsenstein and Goff (12) reported results on the expression of a mutated Mo-MuLV in which the UAG terminator codon had been replaced by the normal glutamine codon, CAG. Our results with this mutant support their findings.

MATERIALS AND METHODS

Materials

Chemicals used were of the highest purity available. Radiochemicals were from Amersham. T4-DNA ligase, T4 polynucleotide kinase and DNA polymerase I—'Klenow' fragment were from Boehringer Mannheim. DNA polymerase I and restriction endonucleases were in the main from Toyobo Co. Ltd. The phagemid pTZ19R and the helper phage M13K07 for use with the phagemid were obtained from Pharmacia. For oligonucleotide-directed mutagenesis a kit available from Amersham was used, and DNA sequencing was, in the main, carried out using the 7-deazaguanosine kit from Takara Shuzo (13).

The proviral Mo-MuLV DNA used in this work originates from clone #48, which is a lambda clone containing an *EcoRI* DNA fragment from a Mo-MuLV infected mouse fibroblast line (14). This DNA fragment was subcloned into *EcoRI* cut pBR 322. This recombinant plasmid is infectious and the Mo-MuLV proviral DNA inserted into it was found to be different from the published sequence (4) by a single C to G base change in the sense strand at position 2255.

General Methods

Plasmid/phagemid preparations. Rapid plasmid preparations were carried out by the alkaline lysis method (Ref. 15, p368–9). Purified plasmid DNA samples were prepared from 50 ml cell cultures, grown overnight at 37°, by scaling up the method. After the phenol:CHCl₃ step the DNA was precipitated with 0.6 Vol. isopropanol and washed with 70% ethanol. This crude DNA was dissolved in 400 µl of 10 mM Tris-HCl pH8.0, 1 mM EDTA (TE) containing DNase-free RNase (20 µg/ml) and incubated at 37° for 1–2 h. After extraction with phenol: CHCl₃, the DNA was precipitated with 2 Vol. cold ethanol, and washed with 70% ethanol. The pellet was dissolved in 4 ml 50 mM Tris-HCl, pH 8.0, 1 mM EDTA to which 4 g CsCl and 0.2 ml ethidium bromide (10 mg/ml) were added. The solution was centrifuged at 45,000 rpm in a vertical rotar (Beckman, VTi65-2) for between 10 h and 16h. The lower band (visualized using 360 nm light) was collected and the ethidium bromide removed by several extractions with isopropanol saturated with a saturated CsCl solution. To each portion of 400 µl solution, 500 µl H₂O, 100 µl 3M sodium acetate, pH 5.2 and 530 µl isopropanol was added. After mixing and keeping at room temperature for 5 min. the precipitated DNA was collected by centrifugation. The combined pellets were dissolved in 100 µl 0.1 × TE, pH 8.0, and the plasmid DNA was reprecipitated with cold ethanol and washed with 70% ethanol. Depending on the plasmid or phagemid used, between 30 µg and 60 µg of purified plasmid DNA was obtained.

Sequencing of DNA. Sequencing was routinely carried out by the dideoxy-method of Sanger (16) using α-³²P-dCTP (400 Ci/mole) as the label and the '7-deazaguanosine' protocol and kit provided by Takara Shuzo (13). In earlier work single stranded DNA was used as the template but in later work it was found that sufficient sequence information could be obtained using double stranded DNA. For sequencing DNA obtained from rapid plasmid preparations, the DNA was firstly partially purified by precipitation from a solution containing 0.5 M NaCl and 6.0 % polyethylene glycol. The double stranded DNA was denatured at room temperature in 2N NaOH: 2 mM EDTA prior to annealing the primer.

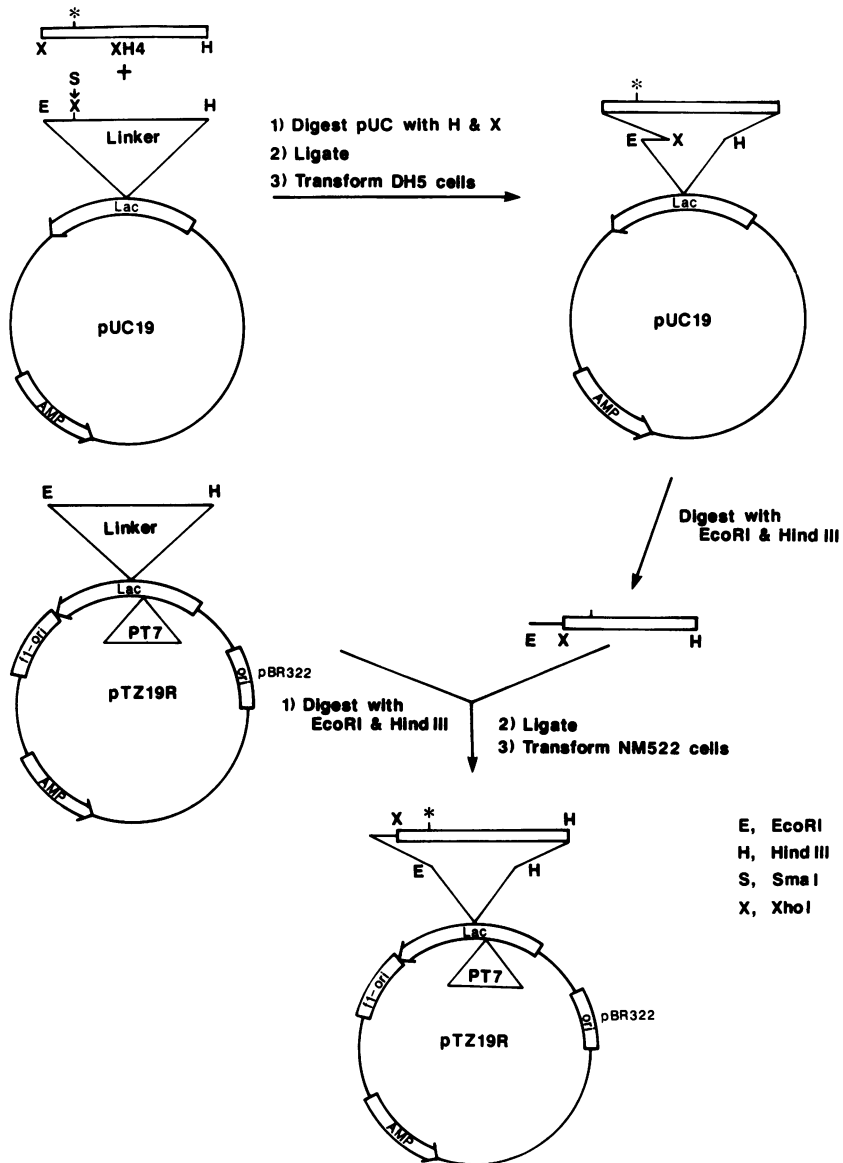


Fig. 1. Construction of pTZ19 recombinant. * indicates the position of the gag terminator codon in XH4. The recombinant is designated pTZ/EH1.

Construction of mutant proviral DNA-containing plasmids.

Preparation of the recombinant phagemid, pTZ/EH1. Initial attempts to obtain an XhoI/KpnI fragment of about 1.1 kb containing the gag-pol junction of proviral Mo-MuLV were

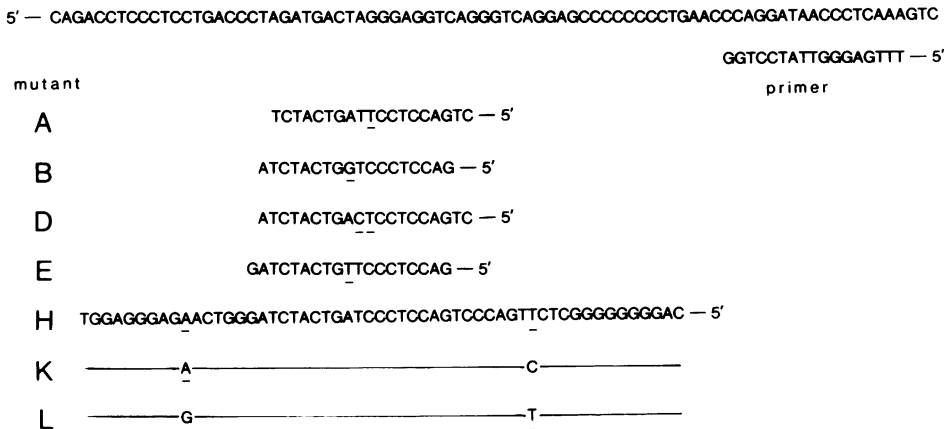


Fig. 2. Sequences of synthetic oligonucleotides. The top line shows part of the sequence of the template single stranded pTZ/EH1 DNA. A–L are the synthetic oligonucleotides used for mutagenesis. Mismatched bases are shown underlined. The synthetic primer used for sequencing the mutant DNAs is also shown.

unsuccessful owing to the resistance of the proviral DNA to digestion at the *KpnI* site downstream from the junction. It was decided, therefore, to clone a larger fragment into the phagemid pTZ19R (17) for oligonucleotide-directed mutagenesis rather than into M13 since, during replication, M13 recombinant DNA containing an insert of greater than 2 kb tends to be unstable. Fig. 1 outlines the construction of PTZ/EH1. Recombinant phagemid containing colonies were obtained by selecting white colonies from plates containing X-gal, IPTG and ampicillin. The recombinant phagemid DNA (pTZ/EH1) was isolated from these colonies by the method described above and identified by restriction enzyme analysis. *Preparation of single stranded pTZ/EH1 DNA.* The procedure described by the suppliers of pTZ19R (Pharmacia) using the helper phage M13107 was followed. 10 ml overnight cultures yielded of the order of 100 µg of DNA.

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed *in vitro* mutagenesis was performed based on the method of Eckstein and his co-workers (18) using a commercially available kit from Amersham. The restriction endonuclease *NciI* was used to nick the non-mutant strand, since a suitable site is located in the 3.3 kb fragment approx. 300 bp from the *gag-pol* junction. Fig. 2 shows the sequence of the template DNA around the *gag-pol* junction and the sequences of the synthetic oligonucleotides used for the mutagenesis. Mismatched base pairs are shown underlined. oligonucleotides A and B were synthesized chemically using a DNA synthesizer (Applied Biosystems 380A). Oligonucleotides D, E, H, K and L were purchased from Operon Technologies, Inc., San Pablo, Ca. USA and were obtained after purification by HPLC. After the mutagenesis reactions, *E. coli* cells strain TGI (K12, $\Delta(lac-pro)$, *supE*, *thi*, *hsd D5/F'* *tra D36*, *proA+B+*, *lacI^q*, *lacZ* Δ M15) were transformed with the d.s. pTZ/EH1 mutant DNAs and plated onto ampicillin containing plates.

Reassembly of proviral Mo-MuLV DNAs incorporating the mutations. Fig. 3 outlines the reassembly of proviral Mo-MuLV mutants constructed in a truncated pBR322 vector. pArMLV was derived from the original proviral Mo-MuLV, clone #48, subcloned into pBR322. This recombinant plasmid was digested with *EcoRI* and partially digested with

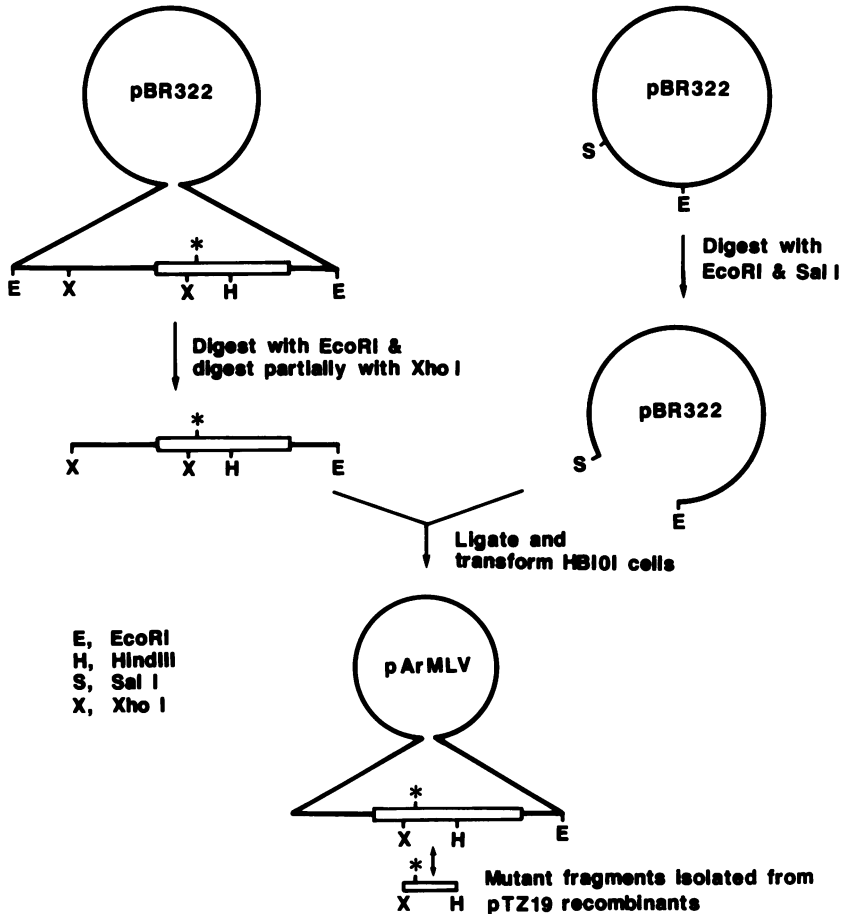


Fig. 3. Reconstruction of mutant pMo-MuLVs. *indicates the position of the *gag* terminator codon.

XhoI, and the 15.5 kb/*XhoI-EcoRI* fragment was isolated. This was then ligated into *SalI/EcoRI* digested pBR322 to yield pArMLV. This now has as single *XhoI* and *HindIII* site. In order to obtain the mutant pArMLV plasmids, pArMLV and the pTZ/EH1 mutants were each digested with *XhoI* and *HindIII* and the mutant 3.3 kb fragments isolated from the pTZ/EH1 phagemids were ligated into the 15 kb fragment isolated from pArMLV. The resulting mutant plasmids (pArMLV-A, -B, -D, -E, -H, -K, -L, -M, -N) were cloned in *E. coli* cells, strain SCS1 (F⁻, *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, (*rk*⁻, *mk*⁺), *supE44*, *relA1*, λ⁻). The reassembly was also carried out using the 3.3 kb fragment isolated from non-mutant pTZ/EH1. This is designated pArMLV-48.

Biological assays of wild-type and mutant pArMLV plasmids. Semiconfluent cultures of NIH3T3 cells (10⁵ cells per 60 mm Petri dish) were co-transfected with 10 μg pArMLV and 2 μg pSV2neo using the standard calcium precipitation method. The selection in G418 (200 μg/ml) was started 36 h after transfection. Two week later about three hundred G418

Table I. Replication and Interfering Capacity of Mutated Moloney Murine Leukemia Virus Genomes

Genome	Number of XC plaques per 10 ⁵ neo ^r transfected cells	XC plaque titer in the culture medium (pfu/ml)	Formation of XC cell syncytia in the Mo-MuLV superinfected culture
pArMLV-48	Confluent	1.6 × 10 ⁴	nd
-A	Confluent ^a	1.8 × 10 ²	—
-B	0 ^b	0 ^b	+
-D	Confluent	1.3 × 10 ⁴	nd
-E	34	11	+
-H	Confluent	1.3 × 10 ²	nd
-K	Confluent	1.5 × 10 ⁴	nd
-L	8	0	+
-M	8	0	+
-N	Confluent	1.1 × 10 ²	nd

a: Atypical fusion pattern was detected in XC assay.

b: Serial passages for approximately 3 months produce XC positive populations.

nd: not detected.

resistant colonies appeared on each transfected plate. These were pooled and, after removal of an aliquot, the remainder of the cells were cultured in a corning 25cm² flask for harvesting of the virus one week later. The aliquot (10⁵ cells) was plated onto a 60 mm petri dish and this culture was submitted to XC assay after three days. The XC assay (19) was performed as follows: the infected cells were UV-irradiated at a dose of 500 erg/mm² and overlaid with XC cells (10⁶ cells per 60 mm dish). The medium was changed on the following day and the cultures were fixed two to three days later.

RESULTS

Oligonucleotide-directed mutagenesis. After transformation of TG1 cells a few colonies were selected randomly, and from these the recombinant phagemid DNA was isolated and examined by restriction enzyme analysis and DNA sequencing. It was found that 50–75% of the DNA samples examined after each mutagenesis contained the expected base change(s). The primer used for sequencing is shown in Fig. 2. In one exceptional case, using oligonucleotide L, unexpectedly, additional base changes were found in the mutant recombinant phagemid. This is dealt with in more detail in the Discussion Section.

Analysis of mutant pArMLV plasmids. Because of the large size of the Mo-MuLV fragment used in the mutagenesis experiments it was impractical to carry out complete nucleotide sequence analysis on the mutant fragments. However, using the sequencing primer (Fig. 2), approx. 200 nucleotide residues of the mutated and reconstructed wild-type pArMLV plasmids were sequenced at and around the region bounded by the synthetic oligonucleotides. Also for mutants A and B, the 3.3 kb fragments were digested with a series of restriction endonucleases (*AluI*, *AvaII*, *BamHI*, *HaeIII*, *HinfI*, *HpaII*, *Sau3A*) and their patterns of fragments on agarose gel electrophoresis were compared with the patterns obtained after digestion of the wild-type 3.3 kb fragment. The mutants and the wild -type gave identical patterns of fragments. This indicates that no unexpected gross random mutations had taken place in regions distant from the *gag-pol* junction. This along with the fact that the expected sequences were obtained in the region of mutagenesis provides strong evidence that only the site-specific mutations were present.

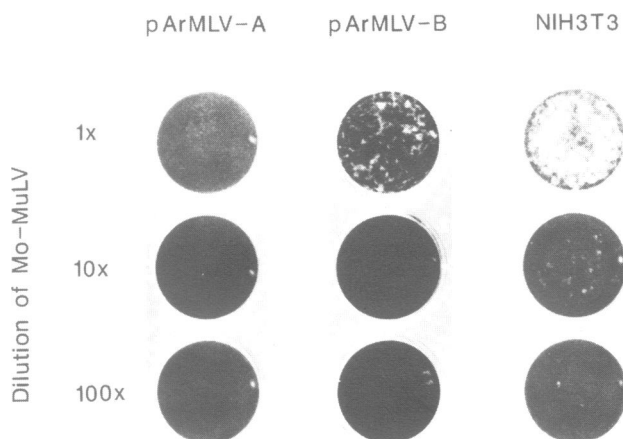


Fig. 5. Interference test. pArMLV-A, or pArMLV-B transfected NIH3T3 cell, or control NIH3T3 cells were infected with Mo-MuLV (wild-type) serially diluted 10-fold. The XC plaque assay was performed four days past infection. The cultures were fixed with formalin and stained with crystal violet.

Note. The light mottled effects seen in some areas of the wells (e.g. pArMLV-A, 100× NIH3T3) are not plaques but are an artifact of the photography.

Table 1. The cells transfected with pArMLV-B, -L and -M produced an undetectable amount of MLV, and those transfected with pArMLV-E produced MLV of a very low titer. pArMLV-48, -A, -D, -H, -K, and -N-transfected cells gave relatively high XC titers. As pArMLV-A-transfected cells were XC-negative, and as their supernatant contained XC-positive MLV, they must have produced XC-negative virions together with XC-positive virions. As for pArMLV-B, the above data do not indicate any sign of MLV replication in the transfected cells. However, prolonged culture (for as long as two months) resulted in an appearance of XC positive cells. This means that the pArMLV-B transfected cells were actually harbouring the viral genome but failed to produce virions which are detectable by the interference or the XC test. The XC positive cells which appeared two months later are considered to harbour revertant viruses.

In summary, pArMLV-B, -E, -L, and -M had a defect in replication, while pArMLV-A, though capable of replication, probably had a defect in some step of viral replication, very probably in *env* gene expression. The reversion frequency was relatively high for pArMLV-E, -L, -M and -A, while it was low for pArMLV-B. This difference in reversion rates was reproducible, but its cause is not known.

DISCUSSION

Mutations at the *gag-pol* gene junction of Mo-MuLV were obtained using the oligonucleotide-directed mutagenesis method usually performed with M13 clones. Because of the large size of the proviral Mo-MuLV DNA fragment being used in this study it was inserted into the phagemid pTZ19R (see Fig. 1). Preparation of single stranded DNA from the recombinant phagemid pTZ/EH1 using the helper phage M13K07 proved to be straight forward and good yields of s.s. DNA (sense strand of Mo-MuLV proviral DNA) were obtained.

Table 2. Point Mutations in the Stem-loop Structure near the gag-pol junction of Mo-MuLV

<u>Genome</u>	<u>Mutations and amino acid changes</u>				<u>Virus production</u>			
pArMLV-48	TCC CTC CTG ACC CTA GAT GAC TAG GGA GGT CAG GGT CAG GAG CCC	Gly	Gly	Gln	Glu	Pro	+	
	Ser Leu Thr Leu Asp Asp	Asp	Asp	Gln	Gln	Glu	Pro	
-A	-----	TAA	-----	-----	-----	-----	-----	±
-B	-----	CAG	-----	-----	-----	-----	-----	-
	-----	Gln	-----	-----	-----	-----	-----	
-D	-----	TGA	-----	-----	-----	-----	-----	+
-E	-----	AAG	-----	-----	-----	-----	-----	-
	-----	Lys	-----	-----	-----	-----	-----	
-H	-----	T	-----	-----	A	-----	-----	+
-K	-----	T	-----	-----	G	-----	-----	+
-L	-----	C	-----	-----	A	-----	-----	-
-M	-----	C-C	-----	-----	A	-----	-----	-
	-----	Pro	-----	-----	-----	-----	-----	
-N	-----	C	-----	-----	A	G	-----	+
	-----	Ala	-----	-----	-----	-----	-----	

Using the *in vitro* mutagenesis kit provided by Amersham and following, in the main, their protocol, the mutagenesis proceeded smoothly, on the whole, when carried out using single stranded pTZ/EH1 DNA. For reasons we cannot explain it was found that the annealing reaction was more efficient (as judged by the clarity of the sequencing gels when the oligonucleotides containing the mismatched base(s) were used as primers in sequencing reactions) if, after annealing at 60–65° and allowing to cool slowly to room temperature, the mixtures were stored at –20° for several hours (usually overnight).

When oligonucleotide L was used for mutagenesis, as well as obtaining a phagemid containing the expected G to A base change, two additional mutants were isolated and characterized by sequencing. As well as containing the G to A base change, one, M, contained an A to C base change upstream from the *gag-pol* junction and the other, N, contained a C to G base change downstream from the designed G to A base change (see Table 2). Sequence analysis of the region (greater than 200 nucleotides) showed no other unexpected base changes. It is difficult to be sure what might have caused these additional base changes, but the most likely cause would be in the synthesis of the oligonucleotides. In both cases the 'wrongly' inserted nucleotide follows a run of G residues and it is either the same residue as the one succeeding it (M) or the one preceding it (N). Therefore L may have been a mixture of three oligonucleotides of the same length but differing in one residue which did not separate during purification by HPLC. This mixture then gave rise to the three mutants L, M and N.

The series of mutants which have been designed involve changes at the terminator codon and in the proposed stem region surrounding it. The proposed (20) stem-loop structure of this region of the genomic mRNA along with the mutations which we have introduced are shown in Fig. 4. It will be seen that four separate mutations have been made to the UAG terminator codon. Two of these, UAG → UAA (A) and UAG → UGA (D) have converted the amber terminator codon into the ochre and opal terminator codons respectively. The two other mutations UAG → CAG (B) and UAG → AAG (E) have converted the terminator codon into sense codons. The CAG mutant (previously reported by Felsenstein and Goff, 12) codes for glutamine, the amino acid found in the read-through protease (8) and the AAG mutant codes for lysine. This latter mutant was introduced as a control in order to determine whether the insertion of any amino acid at this site is permissible.

The other mutations are to the stem structure, and were designed to weaken and then to restore base-pairing. The changes made at positions 2220 (C to U) and 2252 (G to A) do not alter the amino acid sequence. The single mutations C to U (K) and G to A (L) each destroy the G-C base pair, although the C to U change would introduce the weaker G-U base pair (21). The double mutant (H) C to U plus G to A restores the base pairing but replaces the original C-G base pair with a U-A base pair. Mutants M and N are derived from L (see above) and therefore have the G to A base change at position 2252. In addition mutant M has an A to C base change at position 2223. This removes an A-U base pair and therefore two base pairs are absent from the stem of mutant M. The A₂₂₂₃ to C mutation also alters the coding at this position from threonine to proline (See Table 2). Mutant N has an additional C to G base change at position 2256. This introduces an additional C-G base pair at the foot of the stem. So, although, a base pair is lost in the G to A base change at position 2252 this is compensated for by the second mutation. The C to G base change at position 2256 also introduces a coding change from proline to alanine (see Table 2).

Reassembly of the complete Mo-MuLV proviral DNA was carried out using the wild-type 3.3 kb fragment and each of the mutant fragments. This reconstructed wild type Mo-MuLV (pArMLV-48) was used as a control for the biological testing.

The G418 selection was started earlier than usual. This is because revertants with high infectivity, if they appear, will propagate rapidly among the cells and become dominant. As the G418 selection inhibits replication of the host cells, the secondary infection will be reduced to the minimum with this procedure. With this precaution, we could distinguish the mutants pArMLV-D, -H, -K, -N and the mutants pArMLV-E, -L, -M, and judge that the latter mutants were originally replication incompetent.

The results of the biological testing show some extremely interesting results (See Table 1 and 2). Firstly, supporting the previously findings of Felsenstein and Goff (12), it is clear that chain termination at the *gag-pol* junction is essential for viral reproduction, since proviral DNA with the sense codon for glutamine (pArMLV-B) at this position fails to induce XC plagues. In the light of this result the similar negative result with the other sense mutant coding for lysine (pArMLV-E) is to be expected.

Interestingly, for viral reproduction, it would appear that any of the three terminator codons, UAA, UAG or UGA is acceptable. The results with the mutant proviral DNA (pArMLV-A) containing the UAA chain terminator are not so clear since the XC plague titer is two orders of magnitude less than with the wild-type and the fusion pattern is somewhat atypical. In molecular terms, however, the activity present in this mutant is not surprising since it might be expected that the suppressor glutamine tRNA (anticodon UmUG) would be able to read UAA as well as UAG by 3rd base (codon) wobble (22). For reading UAG and UAA 1st base (codon) G-U base pairing is also required. It has been reported previously that such base-pairing occurs with yeast glutamine tRNA (23, 24).

Much more surprising is the result with the UGA mutant (pArMLV-D). This mutant proviral DNA is very nearly as active as the wild-type, which presumably means that chain termination and read-through take place with efficiencies similar to the wild-type. There are no reports of a suppressor tRNA reading both a UAG and a UGA codon, therefore, it is highly unlikely that the suppressor glutamine tRNA reads through the UGA terminator in this mutant. It is possible, therefore, that a suppressor tRNA, other than the already identified glutamine tRNA, is present or is induced in the host cell, the most likely candidate being a UGA suppressor serine tRNA (25). Work is currently in progress to search for such a tRNA and to determine whether a different amino acid is inserted in the protease and the read-through Pr180^{*gag-pol*} precursor in response to UGA. It will be interesting to determine whether the presence of UGA specifically induces the synthesis of a 'new' suppressor tRNA or brings about a specific modification to an 'existing' tRNA providing it with suppressor activity.

The fact that the secondary structure of the mRNA at the *gag-pol* junction is important is supported by the results with mutants pArMLV-H, -K, -L, -M, and -N. In mutant pArMLV-L a G to A change at position 2252, which removes a single base pair in the stem structure leads to a total loss of viral reproduction. The additional change of an A to C at position 2223 (pArMLV-M) removes a second base pair and this double mutant has also lost all activity. However, the additional C to G change at position 2256 (pArMLV-N) introduces an extra base pair at the foot of the stem and this base pair partially compensates for the loss of the C₂₂₂₀-G₂₂₅₂ base pair, since viral reproduction is restored, albeit at a lower level. Also here the substitution of alanine for proline is likely to be an acceptable amino acid change for the protease activity. Perhaps at this point it is worthwhile

pointing out that the original Mo-MuLV proviral DNA that was sequenced (4) is inactive. This has a C at position 2255 which removes a base pair from the foot of the stem.

The C to U base change at position 2220(pArMLV-K) also amends the C₂₂₂₀-G₂₂₅₂ base pair but the replacement of C by U would still allow a base pair to be formed at this position but of somewhat lower strength (21). This mutant and the double mutant (pArMLV-H) in which the C₂₂₂₀-G₂₂₅₂ base pair is replaced with a U-A base-pair are both positive in the XC assay but the latter has lower activity.

The mutants we have generated at and around the *gag-pol* gene junction of Mo-MuLV clearly show that the terminator codon at this site and its context within the structure of the RNA are important for virus activity. Using these mutants in *in vitro* expression systems should help us to clarify the mechanism of read through and identify any additional factors which may be involved.

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ADDENDUM

After submission of this manuscript, Y.-X. Feng *et al.* (*J. Virol.* **63**, 2870–2873, 1989) reported that mutants of Mo-MuLV containing UAA and UGA at the *gag* terminus were suppressed both in infected cell and in reticulocyte lysates.

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