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Analysis of Moloney Murine Leukemia Virus Revertants Mutated at the *gag-pol* Junction

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Among Moloney murine leukemia viruses (Mo-MuLVs) having stop codons other than UAG at the *gag-pol* junction, Mo-MuLV with UAA, but not with UGA, had a replication disadvantage. Mo-MuLV with a glutamine codon (CAG) at the junction did not replicate. A revertant of this virus consisted of the original virus and a virus with a deletion of the *pol* region. Protease and Pr65^{gag} encoded by their respective genomes complemented each other.

In murine leukemia virus (MuLV), genome-sized mRNA encodes Pr65^{gag} and Pr200$^{gag\text{-}pol}$. Translation termination at the UAG codon at the *gag-pol* junction produces the former, while UAG nonsense suppression produces the latter (11, 22, 24). Feng et al. (5–7) found that other stop codons, i.e., UAA and UGA, could also be suppressed and that MuLV triplet was mutated to other stop codons or the CAG glutamine codon.

Plasmid pArMLV-D(UGA) or pArMLV-A(UAA) carries Moloney MuLV (Mo-MuLV) provirus with a UGA or a UAA at the *gag-pol* junction. NIH 3T3 cells transfected with pArMLV-D(UGA) were XC positive, while those transfected with pArMLV-A(UAA) were XC negative (12). The viruses recovered from the transfected NIH 3T3 cells were propagated once and used to infect NIH 3T3 cells; both viruses produced XC plaques. Extrachromosomal DNA (8) isolated 20 h after the infection contained 8.8-kb linear DNA and closed circular DNAs with one or two long terminal repeats (Fig. 1A). One hundred base pairs around the *gag-pol* junction was amplified by the polymerase chain reaction (PCR) and directly sequenced after in vitro transcription (20). The UAA in pArMLV-A(UAA) reverted to the wild-type UAG, while the UGA in pArMLV-D(UGA) remained unchanged (data not shown). Thus, the UGA at the

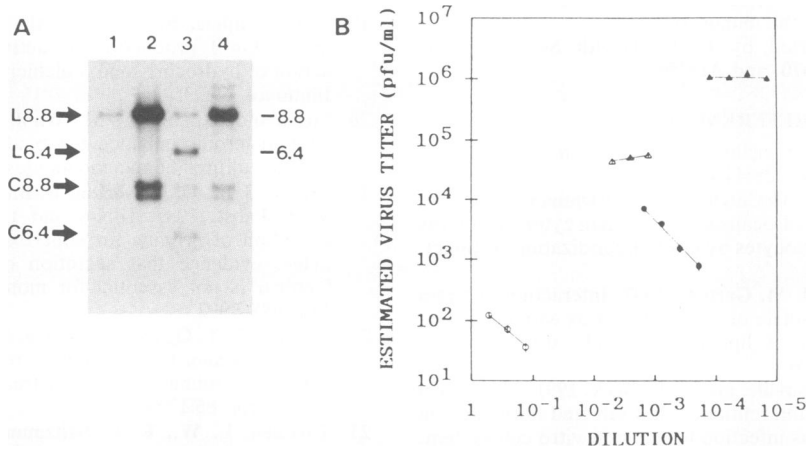

FIG. 1. Proviruses of revertants and titration pattern of MLV-B^{rev}. (A) Extrachromosomal DNAs of NIH 3T3 cells infected for 20 h with wild-type Mo-MuLV (lane 1) or viruses recovered from pArMLV-A(UAA) (lane 2), pArMLV-B(CAG) (lane 3), or pArMLV-D(UGA) (lane 4) transfectants were analyzed by Southern blotting with Mo-MuLV p8.2 (19) as a probe. L8.8, linear 8.8-kb DNA; C8.8, circular 8.8-kb DNA with two long terminal repeats; L6.4, linear 6.4-kb DNA; C6.4, circular 6.4-kb DNA with two long terminal repeats. (B) Titration of MLV-B^{rev} (○) and virus recovered from the pArMLV-B(CAG)–pGE$^{6.4}$ cotransfectant (●) on NIH 3T3 cells by the UV-XC assay (14). △ and ▲ represent wild-type Mo-MuLV. The estimated virus titer (mean ± range) is the product of the dilution factor and the number of XC plaques per plate.

carrying either codon replicated well. MuLV with UAA, however, had an anomaly in the induction of XC cell fusion (12). When UAG was replaced by a glutamine codon, CAG, cells carrying the mutant provirus produced no processed *gag*, despite Pr200$^{gag\text{-}pol}$ expression, and hence no infectious virions (4, 12). In this paper, we characterize viruses recovered from cells transfected with proviruses whose junctional

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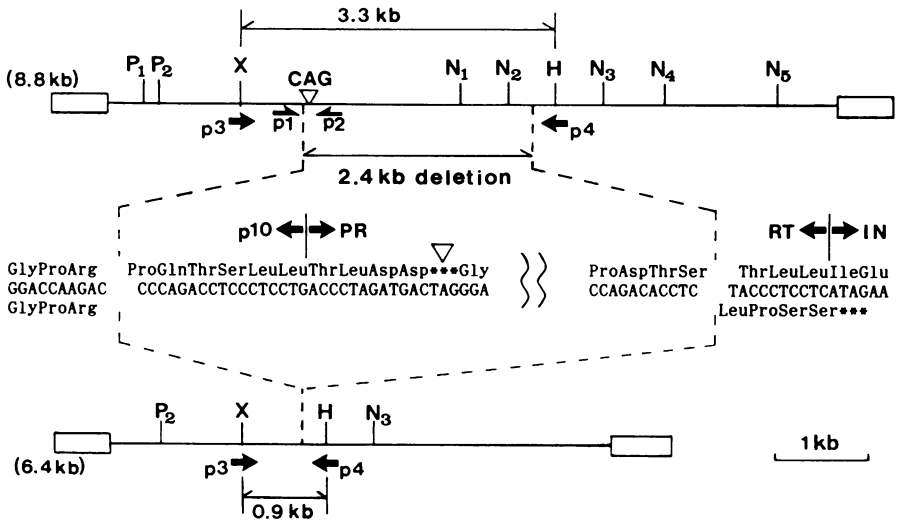

FIG. 2. Restriction map of MLV-B^{rev}. X, *Xho*I; H, *Hin*dIII; P_1 and P_2, *Pst*I; N_1 to N_5, *Nco*I. The triangle indicates the *gag-pol* junction. Primers for 100-base amplification around the *gag-pol* junction are p1 and p2, and those for 900-base amplification, including the deleted site, are p3 and p4. PCR products obtained with primers p1 and p2 were in vitro transcribed with SP6 or T7 RNA polymerase and sequenced by means of reverse transcription. Those obtained with primers p3 and p4 were doubly digested with *Xho*I and *Hin*dIII, subcloned into pIBI30, and sequenced. The nucleotide sequence around the deletion in the 6.4-kb genome and predicted amino acid sequence are shown. p1, 5′-GGGTACCTAATACGACTCACTATAGGGAGAGATTGTCCCAAGAAACCACG-3′ (the T7 promoter connected to nucleotide positions 2166 to 2185 of p10 to transcribe from left to right); p2, 5′-GGGTACCACGATTTAGGTGACACTATAGAATACGTGACGGGTTGC CCCCCGAC-3′ (the SP6 promoter connected to nucleotide positions 2305 to 2286 of *pol* to transcribe from right to left); p3, 5′-CCCCTCGAGCGCCCAGACTGGG-3′ (nucleotides 1557 to 1578); p4, 5′-GCACAAGCTTTGCAGGTCTC-3′ (nucleotides 4903 to 4884); PR, protease; RT, reverse transcriptase; IN, integration protein.

gag-pol junction caused no replication disadvantage, but the UAA did so. The suppression of UAG and UGA, but not that of UAA, has been observed in natural viral infections in eukaryotic cells (13, 21). The apparent discrepancy between the conclusions of Feng et al. (5–7) and our conclusions may be caused by the different assay systems.

The UAG codon at the *gag-pol* junction was mutated to a CAG glutamine codon. NIH 3T3 cells transfected with pArMLV-B(CAG) carrying such a provirus were XC negative (12). After serial passages for nearly 3 months, cells positive for the UV-XC assay appeared and took over the culture. The XC-positive virus, MLV-B^{rev}, contained parental 8.8-kb and short 6.4-kb proviruses (Fig. 1A, lane 3). The restriction map of the 6.4-kb provirus deduced from various combinations of *Pst*I, *Xho*I, *Hin*dIII, and *Nco*I digestions is shown in Fig. 2. Titration of MLV-B^{rev} in NIH 3T3 cells followed two-hit kinetics (Fig. 1B), indicating that infection with two virions was necessary for plaque formation.

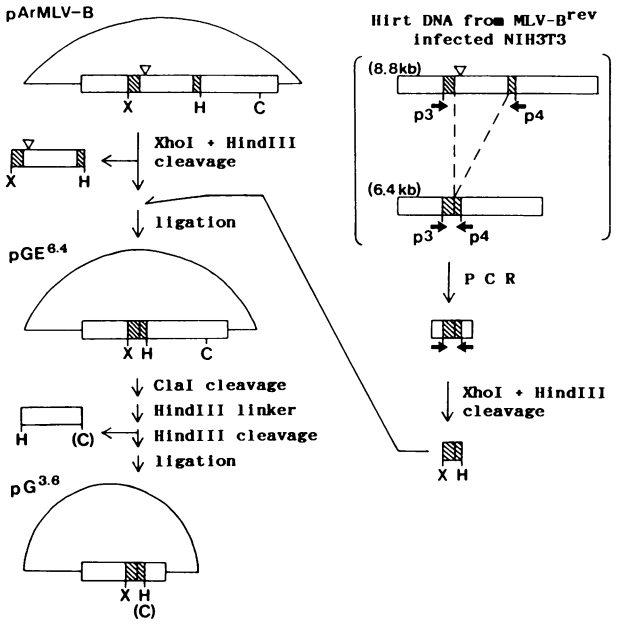

FIG. 3. Construction of plasmids pGE$^{6.4}$ and pG$^{3.6}$. Plasmid pGE$^{6.4}$ was constructed by replacing the *Xho*I-*Hin*dIII fragment of pArMLV-B(CAG) with that of the 6.4-kb virus amplified by PCR. Plasmid pG$^{3.6}$ was constructed by removing the *Hin*dIII-*Cla*I fragment from pGE$^{6.4}$. The *gag-pol* junction is indicated by the triangles. X, *Xho*I; H, *Hin*dIII; C, *Cla*I; p3 and p4, PCR primers.

TABLE 1. XC plaque formation by proviral DNAs[a]

Expt	Plasmid(s) transfected	No. of XC plaques/culture on the indicated day after transfection:		
		6	9	12
1	pArMLV-B(CAG)	0	0	0
	pGE$^{6.4}$	73	67	ND
	pArMLV-B(CAG) + pGE$^{6.4}$	Confluent	Confluent	Confluent
2	pArMLV-B(CAG)	0	0	0
	pGE$^{6.4}$	67	7	5
	pGE$^{3.6}$	0	0	0
	pArMLV-B(CAG) + pGE$^{6.4}$	Confluent	Confluent	Confluent
	pArMLV-B(CAG) + pG$^{3.6}$	0	0	4

[a] NIH 3T3 cells were transfected with 15 μg (experiment 1) or 20 μg (experiment 2) of each plasmid. Cultures were split into two (experiment 1) or three (experiment 2) parts on the next day and subsequently every 3 days. ND, not determined.

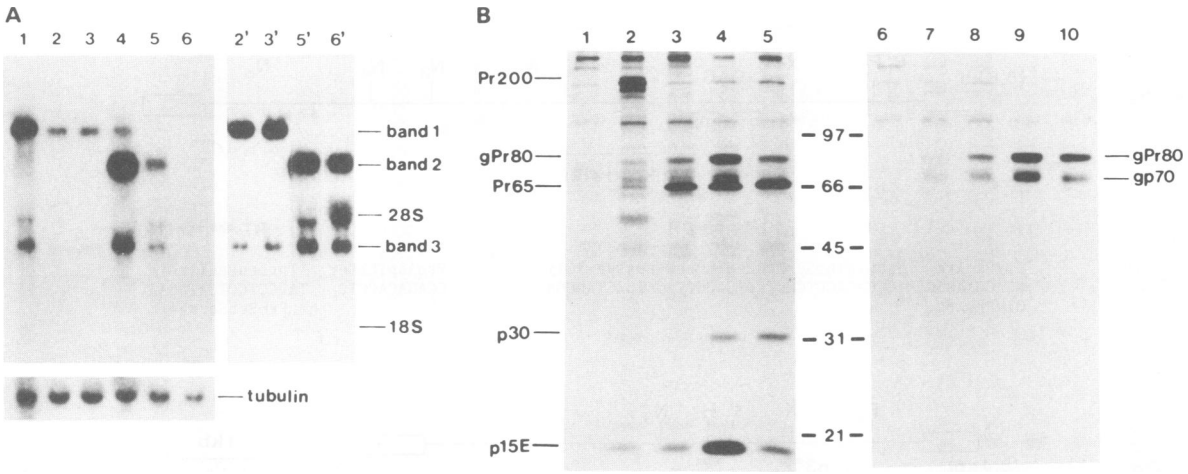

FIG. 4. Viral transcripts and translation products. (A) Northern blot analysis with Mo-MuLV p8.2 as a probe. Total cellular RNA (10 μg) was loaded in each lane. Lanes: 1, NIH 3T3 cells infected with wild-type Mo-MuLV; 2, pArMLV-B(CAG) transfectant, B10 cells; 3, pArMLV-B(CAG) transfectant, B2 cells; 4, cell clone coinfected with MLV-B(CAG) and $GE^{6.4}$; 5, $pGE^{6.4}$ transfectant, GE5 cells; 6, $pGE^{6.4}$ transfectant, GE18 cells. Lanes 2′, 3′, 5′, and 6′ represent longer exposures of lanes 2, 3, 5, and 6, respectively. Bands: 1, unspliced 8.8-kb RNA; 2, unspliced 6.4-kb RNA; 3, spliced *env* mRNA. The total amount of RNA loaded was monitored by hybridization with an α-tubulin probe. (B) Viral proteins in transfected cells. Cells were labeled with 25 μCi of L-[^{35}S]methionine per ml for 3.5 h. Cell lysates with the same radioactivity were immunoprecipitated with anti-ecotropic virus serum (lanes 1 to 5) or anti-gp70 antibody (lanes 6 to 10) and separated by sodium dodecyl sulfate-PAGE. Lanes: 1 and 6, NIH 3T3 cells; 2 and 7, pArMLV-B(CAG) transfectant; 3 and 8, $pGE^{6.4}$ transfectant; 4 and 9, NIH 3T3 cells coinfected with MLV-B(CAG) and $GE^{6.4}$; 5 and 10, NIH 3T3 cells infected with wild-type Mo-MuLV. Molecular mass markers (in kilodaltons) are shown between lanes 5 and 6.

A 0.9-kb sequence around the deletion was amplified by PCR (15) and sequenced. The deletion started at base 2206 and ended at base 4600 (numbering of Shinnick et al. [18]), encompassing 2,395 bases. Six C-terminal amino acids (ProGlnThrSerLeuLeu) in p10 were replaced by four unrelated ones (LeuProSerSer) derived from *pol* (Fig. 2). Thus, the 6.4-kb virus encodes $Pr65^{gag}$ modified at the C terminus and intact *env*. A 100-base sequence around the *gag-pol* junction of the 8.8-kb genome was amplified and sequenced. The mutated base, CAG, was retained (data not shown).

To exclude the possibility that a mutation(s) other than the 2.4-kb deletion was responsible for complementation, plasmid $pGE^{6.4}$ was constructed by replacing the *Xho*I-*Hin*dIII fragment of pArMLV-B(CAG) with that of the 6.4-kb virus (Fig. 3). NIH 3T3 cells cotransfected with $pGE^{6.4}$ and pArMLV-B(CAG) became confluently XC positive by day 6, and the culture fluid contained XC-positive virus (Table 1). Titration of the virus followed two-hit kinetics (Fig. 1B). NIH 3T3 cells transfected with $pGE^{6.4}$ alone were positive in the UV-XC assay shortly after transfection but did not produce any infectious virions. They lost the capacity to induce typical XC cell fusion during passage.

Northern (RNA) blot analysis (Fig. 4A) showed that all the pArMLV-B(CAG)-transfected or $pGE^{6.4}$-transfected clones expressed unspliced genomic and spliced *env* mRNAs. However, the expression of *env* in pArMLV-B(CAG)-transfected cells was low. Lysates of cells labeled with L-[^{35}S]methionine for 3.5 h were immunoprecipitated with anti-Rauscher MuLV or with anti-gp70 antibodies (10, 23) and analyzed by polyacrylamide gel electrophoresis (PAGE) (Fig. 4B). pArMLV-B(CAG)-transfected cells synthesized unprocessed $Pr200^{gag\text{-}pol}$, as reported previously (4), and a 55-kDa protein as well. On the basis of its molecular size, the 55-kDa protein was probably a product of $Pr200^{gag\text{-}pol}$ cleaved at the p30-p10 junction (3) but was not processed further. No p30 was detected. In the $pGE^{6.4}$ transfectant, the truncated *gag* polyprotein $Pr65^{gag}$ was present but was not processed. In cells doubly infected with viruses encoded by pArMLV-B(CAG) and $pGE^{6.4}$ [MLV-B(CAG) and $GE^{6.4}$, respectively], mature *gag* proteins were produced, as in the wild type. The above-described experiment showed that $GE^{6.4}$ and MLV-B(CAG) complemented each other functionally. The complementation must have been brought about by the cleavage of $GE^{6.4}$-encoded $Pr65^{gag}$ by MLV-B(CAG)-encoded protease. Felsenstein and Goff (4) reached the same conclusion by showing that transfection of a MLV-B(CAG)-type provirus to *gag* precursor-expressing M23 cells (17) resulted in efficient cleavage of the *gag* precursor. However, the interpretation of the experiment was compromised by the facts that untransfected M23 cells exhibited a low level of *gag* precursor cleavage (16) and that the wild-type virus was quickly formed by recombination.

If the complementation between MLV-B(CAG) and $GE^{6.4}$ is at the level of $Pr65^{gag}$ processing, it could be expected that MuLV encoding only the *gag* gene would complement MLV-B(CAG). To test this idea, we constructed $pG^{3.6}$ by removing the *Hin*dIII-*Cla*I fragment from $pGE^{6.4}$ (Fig. 3). When cells were cotransfected with $pG^{3.6}$ and pArMLV-B(CAG), XC-positive cells appeared as late as 10 to 15 days later [in contrast to the less than 5- to 6-day lag in the case of cotransfection with pArMLV-B(CAG) and $pGE^{6.4}$] (Table 1). When a pArMLV-B(CAG) transfectant was supertransfected with $pG^{3.6}$ or, inversely, a $pG^{3.6}$ transfectant was supertransfected with pArMLV-B(CAG), the emergence of XC-positive cells was again slow (data not shown). No simple complementation between $G^{3.6}$ (the virus encoded by $pG^{3.6}$) and MLV-B(CAG) existed. A recombination event was required for the appearance of XC-positive cells, because the XC-positive virus recovered from such cotransfected cells was a mixture of 8.8- and 6.4-kb viruses and not of 8.8- and 3.6-kb viruses (data not shown). This result indicates that the *env* portion deleted from $GE^{6.4}$ was

necessary for complementation, through efficient transcription, transcript stability, or efficient translation.

In pArMLV-B(CAG)-transfected cells, the level of the *env* message (Fig. 4A) and consequently the translation of this message (Fig. 4B) were very low. This result is surprising in view of the fact that MLV-B(CAG) has intact splice donor and acceptor sites for *env* mRNA. In murine retroviruses, some deletions in the *gag-pol* region have been shown to decrease splicing efficiency (1, 2, 9). Whether a single point mutation at the *gag-pol* junction can exert such an effect remains to be tested.

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