Suppression of UAA and UGA Termination Codons in Mutant Murine Leukemia Viruses

YA-XIONG FENG,¹ JUDITH G. LEVIN,¹ DOLPH L. HATFIELD,² TIMOTHY S. SCHAEFER,^{3†}
ROBERT J. GORELICK,³ AND ALAN REIN^{3*}

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development,¹ and Laboratory of Experimental Carcinogenesis, National Cancer Institute,² Bethesda, Maryland 20892, and Laboratory of Molecular Virology and Carcinogenesis, Bionetics Research, Inc., Basic Research Program, National Cancer Institute—Frederick Cancer Research Facility, P.O. Box B, Frederick, Maryland 21701³

Received 29 December 1988/Accepted 1 March 1989

Genomes of mammalian type C retroviruses contain a UAG termination codon between the *gag* and *pol* coding regions. The *pol* region is expressed in the form of a *gag-pol* fusion protein following readthrough suppression of the UAG codon. We have used oligonucleotide-directed mutagenesis to change the UAG in Moloney murine leukemia virus to UAA or UGA. These alternate termination codons were also suppressed, both in infected cells and in reticulocyte lysates. Thus, the signal or context inducing suppression of UAG in wild-type Moloney murine leukemia virus is also effective with UAA and UGA. Further, mammalian cells and cell extracts contain tRNAs capable of translating UAA and UGA as amino acids. To our knowledge, this is the first example of natural suppression of UAA in higher eucaryotes.

The universal genetic code contains three triplets, UAA, UAG, and UGA, which function as termination signals for protein synthesis. However, in some cases a termination signal is suppressed, i.e., inefficiently translated as an amino acid, in normal cells (9, 23). One of these situations involves the mammalian type C retroviruses. The genomes of these viruses contain a UAG codon between the *gag* and *pol* coding regions; ~95% of the translation products of this mRNA end here, but in ~5% of the polypeptide chains, the UAG is suppressed by insertion of a glutamine residue, allowing synthesis of a large *gag-pol* fusion protein (5, 15, 18, 27).

The molecular mechanisms underlying this suppression phenomenon are unknown, but they may involve codon context or secondary structure in the viral RNA near the termination codon (e.g., references 17 and 23). It is not known whether these mechanisms are unique to UAG. We have used oligonucleotide-directed mutagenesis to change the *gag* UAG termination codon in Moloney murine leukemia virus (Mo-MuLV) to UGA or UAA. We find that these termination codons are, like UAG, suppressed by mammalian cells and cell extracts. Thus, the signals which cause suppression in Mo-MuLV are not specific to UAG, and tRNAs capable of suppressing UGA and UAA are present in mammalian cells. While suppression of UGA has previously been observed in mammalian cells (3, 7, 9, 10, 23), this is, to our knowledge, the first example of natural suppression of UAA in higher eucaryotes.

Mutants were generated as follows. A *PvuI-SacI* fragment (nucleotides 2117 to 2554 [22]) of an infectious clone of Mo-MuLV (20) was cloned into M13mp18 (26). Oligonucleotide-directed mutagenesis was performed as described elsewhere (28) to change the UAG termination codon at the *gag-pol* junction (nucleotides 2235 to 2237) (22) to UGA or UAA. Mutant bacteriophage clones were identified by sequencing (21) after initial screening by plaque hybridization

as described elsewhere (28). The entire *PvuI-SacI* fragments were sequenced to establish that they were wild type except for the desired change.

We initially tested for suppression in the mutant Mo-MuLVs containing UGA (Mo-UGA) or UAA (Mo-UAA) termination codons in an in vitro assay system (6). The mutant fragments, in combination with appropriate wild-type fragments, were used in the construction of clones (termed pMXH-UGA and pMXH-UAA) in which Mo-MuLV sequences from *XbaI* (nucleotide -151) to *HindIII* (nucleotide 4894) (22) were placed downstream of the SP6 promoter in pGEM4Z (Promega Biotec). The presence of the mutations in these clones was confirmed by colony hybridization by using the respective mutagenic oligonucleotides as probes. Templates were linearized with *SacI* or *HindIII*, which cut the DNA 1.5 kilobases or 2.6 kilobases, respectively, beyond the termination codon. They were then transcribed with SP6 RNA polymerase, and the RNA was translated by using a rabbit reticulocyte lysate supplemented with 50 µg of calf liver tRNA per ml (Promega Biotec), as described by Feng et al. (6). [³⁵S]methionine-labeled proteins were precipitated with anti-p30^{gag} as described elsewhere (11) and were analyzed by electrophoresis in an 8% sodium dodecyl sulfate-polyacrylamide gel followed by fluorography.

The results of this experiment are shown in Fig. 1. It can be seen that the major precipitated translation product from each construct was a 65-kilodalton (kDa) protein, the product of the *gag* gene (5). A 75-kDa protein, presumably the species which is glycosylated in vivo (5), was also observed. In addition, a band of higher molecular weight was seen with wild-type pMXH and pMXH-UGA; the size of this product was ~115 kDa if the template was linearized with *SacI* but ~157 kDa if *HindIII* was used. These are the sizes expected for translation extending through the termination codon at the 3' end of the *gag* gene. In the case of pMXH-UAA, these bands were very faint (Fig. 1, lanes 3 and 7) unless the reticulocyte lysates were supplemented with mouse cell tRNAs (lanes 4 and 8). We conclude that the context or signal causing suppression of UAG in Mo-MuLV (17) is also

* Corresponding author.

† Present address: Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331.

FIG. 1. Suppression of UAA and UGA in reticulocyte lysates. Templates were linearized with *Sall* (lanes 1 through 4) or *HindIII* (lanes 5 through 8) and analyzed as described in the text. Lanes: 1 and 5, wild-type control pMXH (6); 2 and 6, pMXH-UGA; and 3, 4, 7, and 8, pMXH-UAA. Reactions shown in lanes 4 and 8 were additionally supplemented with 40 µg of NIH 3T3- cell tRNA per ml (6). The molecular size markers shown on the left are myosin (200K), phosphorylase B (97.4K), and bovine serum albumin (69K).

effective for UGA and UAA. The results also show that the reticulocyte lysate (supplemented with calf liver tRNA) contains an isoacceptor capable of efficiently suppressing the UGA in pMXH-UGA but very little tRNA which can suppress the UAA in pMXH-UAA; further, NIH 3T3 cells do contain a tRNA which can suppress UAA in pMXH-UAA. The presence of a UAA suppressor tRNA is not unique to mouse cells, however, since addition of rabbit liver tRNA to the system also stimulates *gag-pol* readthrough in pMXH-UAA (data not shown).

We also investigated the possibility that UGA and UAA could be suppressed, allowing translation of the *pol* gene, in intact viral genomes in vivo. The *pol* portion of the *gag-pol* fusion polyprotein is ultimately processed to the viral protease, reverse transcriptase, and integrase proteins, each of which plays a critical role in virus replication (4). We placed intact viral genomes in a pSV2Neo-derived vector (8), transfected them into CHO hamster cells, and tested the cells for the production of replication-competent MuLV by using the S^+L^- focus assay (1). Transient expression assays, in which cells were assayed for infectious-center formation the day after transfection, showed that Mo-UGA and Mo-UAA plasmid DNAs generated infectious MuLV with approximately the same efficiency as the wild-type Mo-MuLV plasmid (data not shown).

We also selected stable transfectants using G-418 and tested the resulting cultures for synthesis of viral proteins and production of infectious MuLV. The three viral genomes gave rise to virus particles with similar levels of reverse transcriptase activity and similar infectivity titers (Table 1). For additional confirmation that the *pol* genes of Mo-UGA and Mo-UAA are expressed in the stable transformants, we labeled the cells with [35 S]methionine, harvested the virions produced by the labeled cells, and precipitated the lysed virions with antisera against viral proteins. The virions produced by these cells contain p30^{gag} (Fig. 2, lanes 1 through 4), which is released from Pr65^{gag} by the action of the viral protease (4, 5). Precipitation with antiserum raised against bacterially expressed *pol* gene products (11) showed that the virions also contain the other *pol* proteins, reverse transcriptase (80 kDa) and integrase (46 kDa) (lanes 5 through 8). p30^{gag} was also quantitated by immunoblotting, and the levels were also similar for all three cultures (Table 1). Taken together, these results show that the three viral genomes give rise to comparable levels of virus particles which have comparable specific infectivities. We conclude that the level of suppression of UAA and UGA in both hamster cells and the mouse S^+L^- indicator cells is ade-

TABLE 1. Production of virus by cells containing Mo-UAA and Mo-UGA

Plasmid ^a	Infectivity (FIU/ml) ^b	RT activity (pmol incorporated/ml) ^c	p30 ^{gag} (arbitrary units) ^d
Mo-UAA	3×10^4	15.0	7
Mo-UGA	4×10^4	8.5	4
Mo-MuLV	5×10^4	24.7	12
pGCCos3Neo	$<2 \times 10^0$	0	0

^a Intact, nonpermuted MuLV genomes were reconstructed in the selectable vector pGCCos3Neo as described elsewhere (8) by using appropriate mutant *PvuII-SacI* fragments. The presence of the mutations in these clones was confirmed by colony hybridization. The clones were transfected into CHO hamster cells, and stable transfectants were selected with G-418 (GIBCO Laboratories) as described elsewhere (20). Culture fluids were harvested from these cells and assayed for virus production as indicated.

^b Infectivity was assayed by the S^+L^- focus assay (1). FIU, Focus-inducing units.

^c Reverse transcriptase (RT) activity in response to poly(rA)-oligo(dT) was determined as described (13).

^d Relative amounts of p30^{gag} were determined by protein immunoblotting as described elsewhere (8).

quate for efficient replication of the MuLVs with these termination codons.

It might be argued that the suppression documented above represents expression of contaminating or revertant wild-type Mo-MuLV genomes in our plasmid preparations or cell cultures. However, CHO cells have no cell surface receptors for Mo-MuLV. Therefore, if a wild-type genome were present or arose in a few cells, it would be unable to spread to other cells in the culture and thus could not result in the wild-type levels of infectious virus production which we observed (Table 1; Fig. 2). The suppression seen in vitro

FIG. 2. Presence of *pol* proteins in virions produced by cells containing Mo-UAA or Mo-UGA. Stable G-418-resistant transformants were labeled with 100 µCi of [35 S]methionine per ml for ~8 h in methionine-free medium. The labeling medium was replaced with nonradioactive complete medium, and virus was collected the following day by ultracentrifugation through a 20% sucrose cushion. The virus pellet was disrupted with lysing buffer (16), and equal portions were precipitated with anti-p30^{gag} (24) (lanes 1 through 4) or anti-reverse transcriptase-integrase. (anti-pRT24 [11]) (lanes 5 through 8). The precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Cells were transfected with Mo-UAA (lanes 1 and 5), Mo-UGA (lanes 2 and 6), wild-type Mo-MuLV (lanes 3 and 7), or pGCCos3Neo (8) (lanes 4 and 8). Lanes 1 through 4 were exposed for 43 h, while lanes 5 through 8 were exposed for 25 days.

(Fig. 1) also cannot be explained by contamination of pMXH-UGA or pMXH-UAA with wild-type pMXH: the reticulocyte lysates require supplementation with exogenous tRNA for efficient readthrough of UAA but not of UAG or UGA (Fig. 1). We also found that readthrough of UGA, but not UAG, is stimulated by supplementing a tRNA-dependent lysate (6) with yeast opal suppressor tRNA (data not shown), while yeast amber suppressor tRNA specifically enhances UAG readthrough (6, 15, 18).

Translation is normally terminated when a protein, the release factor, recognizes a termination codon; in eucaryotes a single factor mediates termination in response to all three termination triplets (2). The results presented in this report indicate that the signals which cause suppression in Mo-MuLV are effective with all three termination codons. Perhaps secondary structure in the viral RNA retards or impedes the action of the release factor, allowing time for a charged tRNA to interact with the termination codon. The identities of these tRNAs are not known. They might be tRNAs which normally function to read amino acid code words but can also misread termination codons. Thus, in yeast cells, glutamine isoacceptors which normally read CAA (19) or CAG (14, 25) can also misread UAA or UAG, respectively, by "first-position wobble." Alternatively, they might be authentic suppressor tRNAs whose only function is to translate a termination codon; such a tRNA has previously been described for UGA in mammalian cells (3, 9, 10). However, we are not aware of any precedent for translation of UAA in higher eucaryotes. Indeed, UAA has been described as an "absolute" termination signal for higher eucaryotes (7, 23), unlike UAG and UGA, which are known to be suppressible under certain conditions. Experiments to identify the amino acids inserted in Mo-MuLV proteins in response to UGA and UAA codons are now in progress.

Kuchino et al. (12) have reported that Mo-MuLV raises the level of a minor glutamine isoacceptor in infected cells; it was suggested that this alteration is required for efficient suppression of the viral UAG codon. However, we have shown here that suppression in Mo-MuLV is essentially independent of the identity of the termination codon. It seems difficult to reconcile this finding with the hypothesis that the virus must affect the populations of cellular tRNAs in order to reproduce itself. Indeed, our results, in apparent contrast to those of Kuchino et al. (12), show that tRNAs isolated from infected and control cells are equally capable of in vitro suppression of UAG in Mo-MuLV and that the glutamine tRNAs of infected and uninfected cells are qualitatively and quantitatively indistinguishable (6). We also find that tRNAs from Mo-MuLV-infected and uninfected NIH 3T3 cells are equivalent in their abilities to suppress UAA and UGA in mutant Mo-MuLVs (data not shown). In addition, it is pertinent that Panganiban placed a 300-base-pair restriction fragment containing the MuLV *gag-pol* junction upstream of the *lacZ* gene; suppression of UAG in this construct was equally efficient in control and MuLV-infected cells (17). Taken together, these results strongly suggest that suppression in MuLV is mediated by tRNAs normally present in mammalian cells.

We thank Janet Hanser, Emily Keene, Sandra Ernst, and Hue Nguyen for technical assistance; Ramesh Kumar for critical contributions; Alan Schultz and Tom Wood for help in protein analysis and plasmid constructions, respectively; Marilyn Powers for oligonucleotides; Ray Gesteland for yeast amber suppressor tRNA; Stephen Oroszlan and Dave Ott for many helpful comments on the manuscript; and Carol Shawver for typing.

This research was sponsored by the National Cancer Institute,

Department of Health and Human Services, under contract NO1-CO-74101 with Bionetics Research, Inc.

ADDENDUM

After this work was submitted for publication, a report (G. Li and C. M. Rice, *J. Virol.* **63**:1326–1337, 1989) appeared describing experiments in which a suppressible UGA codon in Sindbis virus was changed to UAA or UAG by oligonucleotide-directed mutagenesis. As in the case of Mo-MuLV, Li and Rice found that all three termination codons could be suppressed in Sindbis virus in vivo.

LITERATURE CITED

1. Bassin, R. H., N. Tuttle, and P. J. Fischinger. 1971. Rapid cell culture assay for murine leukaemia virus. *Nature (London)* **229**:564–566.
2. Caskey, C. T. 1980. Peptide chain termination. *Trends Biochem. Sci.* **5**:234–237.
3. Diamond, A., B. Dudock, and D. Hatfield. 1981. Structure and properties of a bovine liver UGA suppressor serine tRNA with tryptophan anticodon. *Cell* **25**:497–506.
4. Dickson, C., R. Eisenman, and H. Fan. 1985. Protein synthesis and assembly, p. 135–148. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich. 1982. Protein biosynthesis and assembly, p. 513–648. *In* R. A. Weiss, N. M. Teich, H. E. Varmus, and J. M. Coffin (ed.), *Molecular biology of tumor viruses: RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. Feng, Y.-X., D. L. Hatfield, A. Rein, and J. G. Levin. 1989. Translational readthrough of the murine leukemia virus *gag* gene amber codon does not require virus-induced alteration of tRNA. *J. Virol.* **63**:2405–2410.
7. Geller, A. I., and A. Rich. 1980. A UGA termination suppression tRNA^{Trp} active in rabbit reticulocytes. *Nature (London)* **283**:41–46.
8. Gorelick, R. J., L. E. Henderson, J. P. Hanser, and A. Rein. 1988. Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence. *Proc. Natl. Acad. Sci. USA* **85**:8420–8424.
9. Hatfield, D. 1985. Suppression of termination codons in higher eucaryotes. *Trends Biochem. Sci.* **10**:201–204.
10. Hatfield, D., A. Diamond, and B. Dudock. 1982. Opal suppressor serine tRNAs from bovine liver form phosphoserine-tRNA. *Proc. Natl. Acad. Sci. USA* **79**:6215–6219.
11. Hu, S. C., D. L. Court, M. Zweig, and J. G. Levin. 1986. Murine leukemia virus *pol* gene products: analysis with antisera generated against reverse transcriptase and endonuclease fusion proteins expressed in *Escherichia coli*. *J. Virol.* **60**:267–274.
12. Kuchino, Y., H. Beier, N. Akita, and S. Nishimura. 1987. Natural UAG suppressor glutamine tRNA is elevated in mouse cells infected with Moloney murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **84**:2668–2672.
13. Levin, J. G., R. J. Crouch, K. Post, S. C. Hu, D. McKelvin, M. Zweig, D. L. Court, and B. I. Gerwin. 1988. Functional organization of the murine leukemia virus reverse transcriptase: characterization of a bacterially expressed AKR DNA polymerase deficient in RNase H activity. *J. Virol.* **62**:4376–4380.
14. Lin, J. P., M. Aker, K. C. Sitney, and R. K. Mortimer. 1986. First position wobble in codon-anticodon pairing: amber suppression by a yeast glutamine tRNA. *Gene* **49**:383–388.
15. Murphy, E. C., Jr., N. Wills, and R. B. Arlinghaus. 1980. Suppression of murine retrovirus polypeptide termination: effect of amber suppressor tRNA on the cell-free translation of Rauscher murine leukemia virus, Moloney murine leukemia virus, and Moloney murine sarcoma virus 124 RNA. *J. Virol.* **34**:464–473.
16. Oroszlan, S., C. W. Long, and R. V. Gilden. 1976. Isolation of a murine type C virus p30 precursor protein by DNA-cellulose

- chromatography. *Virology* **72**:523–526.
17. **Panganiban, A. T.** 1988. Retroviral *gag* gene amber codon suppression is caused by an intrinsic *cis*-acting component of the viral mRNA. *J. Virol.* **62**:3574–3580.
 18. **Philipson, L., P. Andersson, U. Olshevsky, R. Weinberg, D. Baltimore, and R. Gesteland.** 1978. Translation of MuLV and MSV RNAs in nuclease-treated reticulocyte extracts: enhancement of the *gag-pol* polypeptide with yeast suppressor tRNA. *Cell* **13**:189–199.
 19. **Pure, G. A., G. W. Robinson, L. Naumovski, and E. C. Friedberg.** 1985. Partial suppression of an ochre mutation in *Saccharomyces cerevisiae* by multicopy plasmids containing a normal yeast tRNA^{Gln} gene. *J. Mol. Biol.* **183**:31–42.
 20. **Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz.** 1986. Myristylation site in Pr65^{gag} is essential for virus particle formation by Moloney murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **83**:7246–7250.
 21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 22. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. *Nature* (London) **293**:543–548.
 23. **Valle, R. P. C., and M.-D. Morsch.** 1988. Stop making sense or regulation at the level of termination in eukaryotic protein synthesis. *FEBS Lett.* **235**:1–15.
 24. **Versteegen, R. J., and S. Oroszlan.** 1980. Effect of chemical modification and fragmentation on antigenic determinants of internal protein p30 and surface glycoprotein gp70 of type C retroviruses. *J. Virol.* **33**:983–992.
 25. **Weiss, W. A., and E. C. Friedberg.** 1986. Normal yeast tRNA^{Gln}_{CAG} can suppress amber codons and is encoded by an essential gene. *J. Mol. Biol.* **192**:725–735.
 26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
 27. **Yoshinaka, Y., I. Katoh, T. D. Copeland, and S. Oroszlan.** 1985. Murine leukemia virus protease is encoded by the *gag-pol* gene and is synthesized through suppression of an amber termination codon. *Proc. Natl. Acad. Sci. USA* **82**:1618–1622.
 28. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA* **3**:479–488.