Journal of Virology, Sept. 1995, p. 5878–5882
0022-538X/95/$04.00+0
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Vol. 69, No. 9

Modification of Retroviral RNA by Double-Stranded RNA Adenosine Deaminase

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Received 10 March 1995/Accepted 30 May 1995

In this report, we describe a recombinant provirus generated during in vitro passage that contains a short region of adenosine-to-guanosine hypermutation. The hypermutated region is restricted to complementary sequences present in the recombinant provirus. We propose that a duplex was formed in the recombinant RNA prior to reverse transcription. This duplex was a substrate for double-stranded RNA adenosine deaminase, an activity found in all cells examined that deaminates A in double-stranded RNA, converting it to inosine, which is further converted to a guanosine by reverse transcription. It appears that *cis* viral sequences facilitated the A→G transitions.

A double-stranded RNA (dsRNA) unwinding and modifying activity was originally found in *Xenopus* oocytes that had been injected with antisense RNA (1, 26). This activity, later called dsRNA adenosine deaminase (dsRAD), hydrolytically deaminates adenosine in dsRNA, converting it to inosine, thereby disrupting the duplex (2, 25, 34). dsRAD is localized to the nuclei of all somatic cells examined (35). dsRAD acts efficiently on duplexes of 100 bp or longer, modifying about 50% of the adenosines (19). Both inter- and intramolecular duplexes have been shown to be modified by dsRAD in vitro, and even short duplexes (20 bp) could be modified, although inefficiently (19). A→G modification of the human immunodeficiency virus type 1 (HIV-1) *trans*-activation response element, which forms a stable stem-loop structure, has been demonstrated by injecting this RNA into *Xenopus* oocytes (28). Preferential deamination of certain A's has been demonstrated in vitro, with a 5′ neighbor preference of A = U > C > G (24). dsRAD has also been hypothesized to selectively modify a single A in the mRNA of an L-glutamate-activated cation channel (GluR-B), converting a glutamine codon (CAG) into an arginine codon (CGG) (10, 29). A duplex between sequences present in the exon and intron is hypothesized to have been the substrate for dsRAD. Additionally, dsRAD is thought to be involved in biased hypermutation in some RNA viruses (3). A→G hypermutation in vesicular stomatitis virus, a rhabdovirus, was first described in 1984, prior to the description of dsRAD (21). Biased hypermutation has been detected in measles virus isolated from patients with subacute sclerosing panencephalitis or measles inclusion body encephalitis (6, 36). The measles matrix gene contained U→C mutations which were hypothesized to be the result of dsRAD activity (3, 36), possibly by acting on RNA duplexes that may form during virus transcription or replication. However, such duplexes have not been directly demonstrated in measles virus-infected cells. A→G hypermutations were also found in two escape mutants of the human respiratory syncytial virus, another paramyxovirus (27).

It is not known if dsRAD plays a role in retroviral variation. In one published clone, A→G hypermutations were observed in the U3 region of a c-*mil*/c-*raf* transducing avian lymphomatosis retrovirus, but there was no obvious dsRNA template for the modified region since retroviruses, unlike other RNA viruses, do not have a minus-sense RNA intermediate (7). Retroviruses undergo genetic variation at a very high rate, estimated to be a millionfold higher than the rate in eukaryotic genomes (4, 5). Such variation includes base substitutions, insertions, and deletions. Retroviruses contain two copies of their RNA genome in a single viral particle and are thus diploid. Information from both RNA strands can be reverse transcribed into a single viral DNA which is then integrated into the host cell genome, although a single RNA is probably sufficient to generate a provirus (11). Two strand transfers are required to generate a provirus. It has been proposed that the high mutation rate observed in retroviruses is due to reverse transcriptase (RT) and is the result of the ability of RT to jump between strands (31). RT has a DNA polymerase domain that lacks proofreading function. By using an in vitro DNA strand transfer system, it has been shown that HIV RT is capable of synthesizing DNA past a mismatched base pair (22, 23). In these studies, RT was found to add a nontemplated nucleotide prior to strand transfer. Upon strand transfer, however, RT was capable of extension to the end of the second template without single-base insertions at the site of strand transfer (23). dATP was the most commonly misincorporated nucleotide in the in vitro system (22, 23). Analysis of the base compositions of various retroviruses has revealed base preference variations. For example, HIV is rich in adenosine (consistent with HIV RT's preferential misincorporation of A at strand transfers), whereas the human T-cell leukemia virus is rich in cytosine (5). Analysis of HIV-1 variation during passage in tissue culture revealed G→A hypermutation, especially at GpA dinucleotides, and a model of dislocation mutagenesis was proposed (32). It is also hypothesized that an intracellular depletion of dCTP could lead to the misincorporation of dTTP, with the resultant G→A mutation in the provirus (17, 32, 33).

In this report, we describe an avian retroviral recombinant that was generated in our studies of nonhomologous recombination. This recombinant provirus contains a 150-nucleotide (nt) inverted repeat that contains A→G hypermutations. We propose that this hypermutation is the result of the activity of dsRAD.

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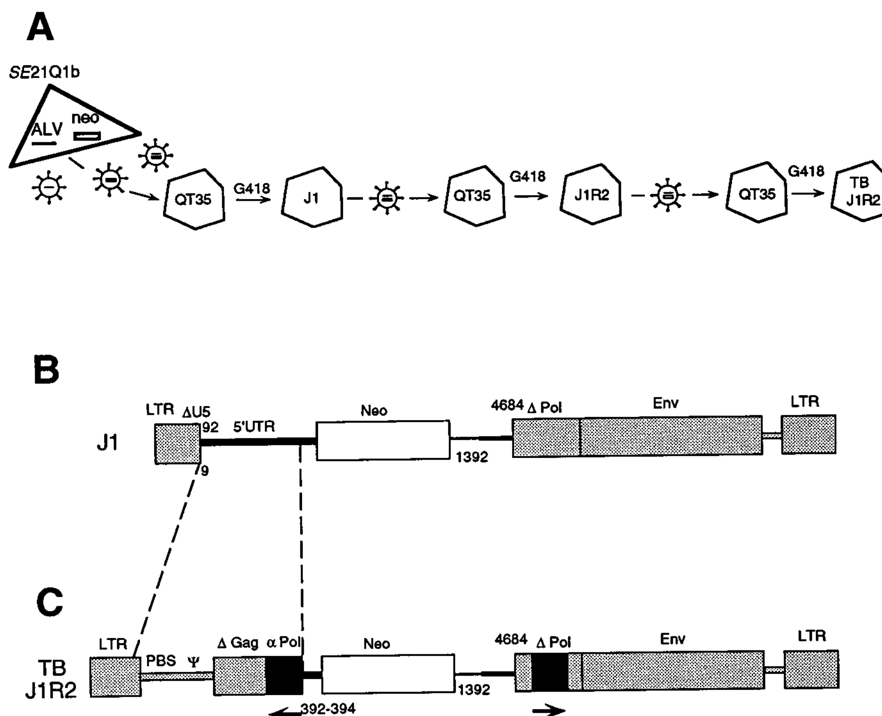

FIG. 1. Genetic organization of recombinant provirus TBJ1R2 and its parental provirus J1. (A) Derivation of TBJ1R2. *SE*21Q1b cells transfected with pCMVneo and infected with ALV were used as a source of virus to infect QT35 cells, which were then selected in G418. A single G418-resistant clone, J1, was selected and expanded. Virus harvested from J1 cells was used to infect QT35 cells, producing clone J1R2. Virus from this culture was used to obtain G418-resistant clone TBJ1R2. (B) Structure of the integrated J1 provirus. Numbers above the provirus correspond to the sequence of ALV (GenBank accession number M37980), while those below correspond to plasmid pCMVneo (14). Viral sequences are denoted by stippling. The white box denotes *neo* coding sequences, and the thick black line represents the 5′ UTR from the *neo* plasmid. ΔU5 indicates that a portion of the 5′ long terminal repeat (LTR) U5 region is deleted (at nt 92). (C) Structure of the integrated TBJ1R2 provirus derived from J1 as indicated in panel A. The dashed lines connecting panels B and C indicate the region of further recombination between J1 and ALV. The 5′ UTR-*neo* sequences have been replaced by the ALV leader (stippled line) including the plus-strand primer binding site (PBS) and the major packaging sequences (Ψ). The black boxes and arrows indicate the inverted repeat generated during recombination that is the region of A→G hypermutation. Recombination between antisense *pol* sequences and *neo* occurred at nt 392 to 394 in the *neo* RNA at a stretch of 3-nt identity. The region 3′ of *neo* has not been altered relative to J1 except for the A→G hypermutated black box.

Derivation of TBJ1R2. We have been studying recombinant proviruses that were generated following copackaging of a *neo*-containing RNA derived from plasmid pCMVneo (14), devoid of any viral sequences, with avian leukosis virus (ALV) genomic RNA (9). Such virions were produced by the avian packaging cell line *SE*21Q1b, which is capable of packaging RNAs lacking Ψ, the viral packaging sequence normally required for RNA encapsidation (15, 16). Recombinant proviruses are thought to be formed during reverse transcription of the copackaged RNAs, with RT jumping onto the *neo* RNA after initiating at the normal primer binding site in the viral genome (9, 37). Our experiments were done with *SE*21Q1b cells infected with replication-competent ALV because the *SE*21Q1b provirus lacks a primer binding site and thus cannot normally initiate reverse transcription. Virions produced from ALV-infected *SE*21Q1b cells containing pCMVneo were used to infect QT35 cells (18), a chemically transformed quail cell line that is devoid of ALV-like endogenous sequences. Single-cell clones were isolated following G418 selection. One of these clones, J1, was used in the studies described here (Fig. 1A).

The 5′ long terminal repeat-Neo recombination junction of J1 has already been reported (9). The 3′ Neo-long terminal repeat junction was obtained following nested PCR amplification of 1 μg of cell DNA isolated from J1 cells. Oligonucleotide primers used in the first reaction were Rous sarcoma virus αs (antisense)-U3/*Eco*RI (5′-GCGGAATTCAGTGGTTCGTCC-3′) and CMVneo 1719 (5′-CTGGATTCATCGACTGTGGCC-3′). For the nested reactions, Rous sarcoma virus αs-U3/*Pvu*I (5′-GCACGATCGTACCACCTTACT-3′) and Neo *Rsr*II (5′-GGTGTGGCGGACCGCTATCAG-3′) were used. Reaction conditions consisted of 0.5 μg of each primer, 200 μM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 0.01% (wt/vol) gelatin, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus). A denaturing step of 95°C for 5 min was followed by 25 cycles (for the first reaction) or 35 cycles (for the nested reaction) of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min (first reaction) or 4 min (nested reaction). Amplified products were purified from a 0.8% agarose gel by using a QIAEX kit (Qiagen) and ligated into the pCRII vector by using a TA cloning kit (Invitrogen). A 3.2-kbp amplified product was obtained for J1, and a schematic representation of the J1 provirus is shown in Fig. 1B.

In an attempt to generate new recombinant proviruses, virus from J1 cells, which contain replication-competent ALV in addition to the J1 recombinant provirus, was used to infect fresh QT35 cells. Virus from a single cell clone obtained following G418 selection (J1R2) was used in another round of infection and selection, and the G418-resistant cell clone TBJ1R2 was isolated (Fig. 1A). The sequence of the 5′ recombination junction was determined following PCR amplification of cell DNA with a U3 and antisense *neo* primer as previously described (9) except that the amplified products were directly

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ALV Pol DNA 5'-  GATGGAATGTGCTGGTCTGGGGACGAGGTTATGCAGCTGT 1
TBJ1R2 [ sense                                 G          2
       [ αsense                             GG G          3

                 GAAAAACAGGGACACTGATAAGGTTATTTGGGTACCCTCT 1
                                    GG     G       G      2
                                G  G     G  G           G 3

                 CGGAAAGTTAAACCGGATGTCACCCAAAAGGATGAGGTGA 1
                      G   G                GGGG     G     2
                        GG        G G             G    G  3

                 CTAAGAAAGATGAGGCGAGCCCTCTTTT-3'          1
                   GG                                     2
                  G        G                G             3
```

FIG. 2. Nucleotide sequence of the 3′ inverted repeat in TBJ1R2, corresponding to the sense ALV *pol* sequences. Line 1 indicates the DNA sequence of the *pol* region duplicated in TBJ1R2. The sense sequence (line 2) indicates the A→G changes detected in the *pol* region 3′ of *neo* in TBJ1R2. The antisense (αsense) sequence (line 3) indicates A→G changes found in the antisense *pol* sequences 5′ of *neo* in TBJ1R2.

cloned into pCRII (TA cloning kit), without digestion with restriction endonucleases. The 3′ junction was cloned into pCRII following a single round of amplification using the Neo *Rsr*II and αs-U3/*Eco*RI primers. Reaction conditions were as previously described, and amplification conditions consisted of a 5-min 95°C denaturing step, then 30 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 4 min, and finally a 10-min 72°C extension.

TBJ1R2 sequence. A schematic representation of the TBJ1R2 provirus is shown in Fig. 1C. TBJ1R2 has undergone further recombination, compared with the J1 provirus, having acquired all of U5, the primer binding site, the 5′ leader region containing the packaging region Ψ (16), and part of *gag*. Most of the 5′ untranslated region (UTR) of *neo* that was present in J1 has been deleted in TBJ1R2. Additionally, 150 nt of antisense *pol* sequences (nt 4918 to 5064 of ALV-RSA) are now present between the *gag* and *neo* sequences. These sequences are also located (in the sense orientation) at the 3′ end of J1. Fifty percent (17 of 34) of the A's were found to be mutated to G in the antisense sequences of TBJ1R2 (Fig. 2, line 3). The 3′ junction of TBJ1R2 is identical to that in J1 except that the sense *pol* sequences in the potential duplex region also contain A→G transitions (14/43 = 32%; Fig. 2, line 2). It is possible that in the generation of TBJ1R2, an intermediate recombinant was formed that contained the unmodified complementary *pol* sequences, which then formed a duplex with the sense *pol* sequences that could act as a substrate for dsRAD to deaminate A into I. Upon infection of a new cell, reverse transcription of I in the modified RNA would result in a C in the minus strand, which would then be converted to a G in the provirus, leading to the observed A→G transitions. dsRAD is the only cellular activity described to date that could result in A→G hypermutations in dsRNA. There are no cells available that are devoid of dsRAD activity in which to definitively test its role in modification of duplex RNA; however, full-length human and rat dsRAD cDNAs were recently cloned, and such experiments may be forthcoming (12, 20).

The role of replication-competent virus and retroviral *cis* sequences in A-to-G transitions. Since the hypermutated sequences were found in cells in which there was replication-competent ALV, we next tested the role of replication competent virus in A→G transitions. To this end, a proviral construct, pLAGαsPE, similar to TBJ1R2 but with a different duplex region, was created (Fig. 3A). The cloning strategy involved multiple steps, and the details are available upon request. The duplex region is 150 nt long (ALV-RSA nt 5101 to 5249) and is 48% G+C, with 1.5 kb of sequences between the inverted repeats. pCN-180αs, a construct devoid of retroviral *cis* sequences, was also constructed (Fig. 3B). This plasmid is a derivative of pCMVneoAva^{D} (13). The RNA encoded by pCMVneo contains a 430-nt 5′ UTR. The 180-nt fragment *Afl*III-*Nar*I from this 5′ UTR was cloned in the opposite orientation at the *Sma*I site, located 3′ of the Neo open reading frame. This inverted repeat is 180 nt long, 55% G+C, and separated from the next inverted repeat by 1.1 kb. Each construct was transfected into QT35 cells as previously described (9), and mass cultures and single-cell clones were isolated following G418 selection.

To determine whether A→I changes had occurred in the encoded *neo* RNA, we isolated total cell RNA by using Tri-Reagent or Tri-Reagent LS (Molecular Research Center, Inc.). RNA was treated with RQ1 DNase (Promega) and reverse transcribed by using a Superscript RNAseH−kit (Bethesda Research Laboratories) and random hexamers as primers. The cDNA, in which inosine would have been converted to guanosine, was then PCR amplified with specific primers to amplify one of the inverted repeats. For pLAGαsPE, MAgag1 (5′-ATGGAAGCCGTCATTAAGGT-3′) and 5′ αs-neo 1140 (5′-GGCCGGAGAACCTGC-3′) were used, whereas for pCN-180αs, Neo 781 (5′-GGCGCAAGGGCTGCTAAAGG-3′) and 5′ αs-neo 1140 were used (arrowheads in Fig. 3). The amplified

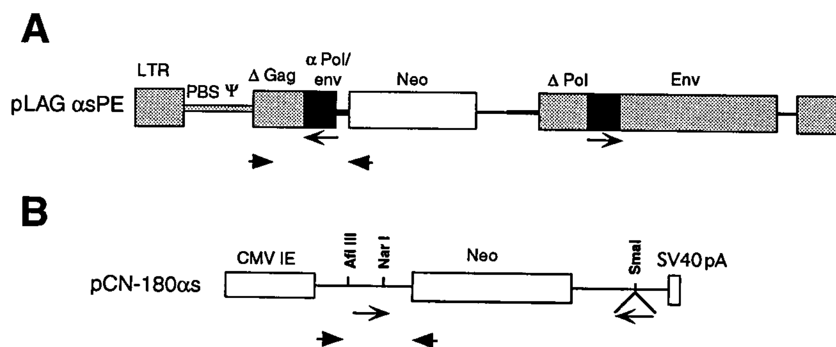

FIG. 3. Schematic representation of the pLAGαsPE and pCN-180αs constructs. (A) pLAGαsPE. A derivative of an ALV plasmid containing the selectable *neo* gene (white box) in place of most of the *gag* and *pol* genes was constructed. Viral sequences are denoted by stippling. A region corresponding to nt 5101 to 5249 from ALV was placed in reverse orientation between Δ*gag* and *neo*, leading to an inverted repeat of 150 bp. LTR, long terminal repeat; PBS, primer binding site; α, antisense. (B) pCN-180αs. This construct is a derivative of pCMVneoAva^{D} (13) in which 180 bp from the 5′ UTR (between *Afl*III and *Nar*I) were inserted in antisense orientation at the *Sma*I site in the 3′ UTR, upstream of the simian virus 40 polyadenylation (SV40pA) sequences, leading to an inverted repeat of 180 bp. Arrows indicate inverted repeats, and arrowheads indicate locations of PCR primers used in RT-PCR. CMV IE, cytomegalovirus immediate-early region.

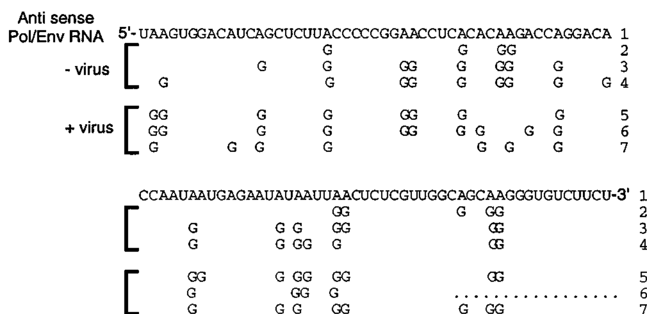

FIG. 4. Sequence of the *pol/env* region duplicated in pLAGαsPE. Line 1 indicates the antisense *pol/env* RNA sequence 5′ of *neo* in pLAGαsPE (black box in Fig. 3A). Lines 2 to 4 indicate the nucleotide changes (A→G) in the absence of replication-competent virus; lines 5 to 7 indicate A→G transitions in the presence of helper virus. Each line is derived from sequencing a separate RT-PCR clone. Dots indicate a region that was not sequenced.

products were cloned into pCRII by the TA cloning kit and sequenced with a Sequenase kit (U.S. Biochemical). Several PCR clones from each amplification reaction were sequenced.

Five of six single-cell clones containing pLAGαsPE were found to have modifications in their cDNAs. A→G changes were present even in the absence of replication-competent virus (Fig. 4, lines 2 to 4). When modifications were present, 30 to 50% of the A's were mutated to G in a single PCR clone. No difference in the pattern of modifications was noted in the presence or absence of replication-competent ALV, and the six clones depicted in Fig. 4 are representative of the sequences that we obtained. The remaining clones that we obtained were only partially sequenced. Eighty percent (29 of 36) of A's with a 5′ U neighbor were modified, whereas only 14% (5 of 36) of A's with a 5′ G neighbor were modified. When C was the 5′ neighbor, 54% (28 of 52) of the A's were modified, and when A was the 5′ neighbor, 45% (24 of 53) of the A's were modified. The pattern of modifications described for dsRAD is a 5′ neighbor preference of A = U > C > G. This pattern was detected by using an in vitro system with a 36-nt duplex as a substrate for dsRAD (24). In these experiments, only a few (20%) of the A's in the RNA were modified to I, but these were at reproducible sites. A longer template (48-mer) was more highly modified (50% of A's), although less selectively. It is possible that the difference that we observed in our in vivo 5′ neighbor preference of U > C = A >> G relative to the in vitro system is due to the lengths of the duplexes examined. Both systems, however, detected a strong preference for a 5′ U and a strong inhibition by a 5′ G. In terms of 3′ neighbor preferences, only 25% (9 of 36) of A's with 3′ U were modified, whereas no strong selection or inhibition was seen with 3′ A (31/53 = 58%), 3′ C (23/54 = 42%), or 3′ G (23/40 = 58%). The in vitro system did not reveal a 3′ neighbor preference (24). The in vitro system also did not detect any modifications within the first 3 nt at the 5′ end of the templates, whereas we found that 50% (6 of 12) of the A's were mutated in this region. However, the 5′ end of the duplex region in our system is not the 5′ end of the RNA, potentially making the end nucleotides more accessible to dsRAD.

The sixth clone, which did not have A→G changes, appeared to have a deletion in pLAGαsPE, as judged from Southern blots of cell DNA isolated from this clone (data not shown). When pLAGαsPE was cotransfected with an ALV-encoding construct (p882-29 [30]), RNA from mass cultures or single-cell clones also revealed modifications (Fig. 4, lines 5 to 7). Surprisingly, examination of two single-cell clones containing pCN-180αs DNA did not show any modifications in RT-PCR-derived clones, even after infection of these clones with replication-competent virus. pCN-180αs mass cultures also did not show modifications.

It is possible that a duplex RNA could not form in the pCN-180αs RNA, even though this construct encoded an RNA with a longer potential duplex, having a higher G+C content and a shorter intervening sequence but lacking viral sequences compared with pLAGαsPE. However, it is also possible that the *cis* viral sequences present in pLAGαsPE direct the RNA into a subnuclear compartment where dsRAD is found. In the case of the glutamate-gated cation channel protein GluR-B, it appears that dsRAD modifies the RNA prior to splicing, since the potential dsRNA template is composed of exon and intron sequences (10). It is possible that dsRAD is associated with the spliceosome, since dsRAD appears to be associated with ribonucleoprotein (RNP) complexes in brain cells (35a). It is interesting that while pCN-180αs does not contain any splicing signals, pLAGαsPE has both a 5′ splice site (SS; at the 5′ end of *gag*) and a 3′ SS (just upstream of *env*) that are normally used by the retrovirus to generate an *env* RNA. *neo*, in this construct, is in the intron, but since we are selecting for G418 resistance, we are selecting for nonspliced RNA. In normal retroviral replication, only a subset of the RNA is spliced. A negative regulator of splicing (NRS) has been found in *gag*, to which additional small nuclear RNP (snRNP) complexes (namely, U11/U12) can bind, thereby inhibiting splicing (8). Mature spliceosomes did not form on NRS-containing RNA even though the spliceosomal snRNP complexes were associated with the RNA (8). The NRS has been deleted in pLAGαsPE, but it is possible that other signals are inhibiting splicing. In any case, it is possible that the spliceosomal snRNP complexes are associating with the 5′ and 3′ SS in pLAGαsPE RNA and that dsRAD is associating with these snRNP complexes and is thus able to modify the RNA. Since pCN-180αs lacks SS, the spliceosomal snRNP and dsRAD would not associate with the RNA, and hence no modifications are observed in the RNA. Alternatively, retroviral sequences could have an effect in a splice-independent manner.

In conclusion, we have described a viral recombinant that showed A→G hypermutation in an inverted repeat that was most likely due to the dsRAD activity that has been found in all eukaryotic cells examined. It appears that viral *cis* sequences may have enhanced the detection of these modifications, since RNA from pCN-180αs, which lacks *cis* viral sequences, did not show any modifications, whereas RNA from pLAGαsPE, which encodes retroviral sequences, was modified. It is possible that in the rare cases in which antisense retroviral RNAs are transcribed in infected cells, modification of the resultant duplexes that form with genomic RNA by dsRAD could occur prior to packaging of the genomic RNA, and A→G transitions could then be observed in the provirus in the next round of infection. Hypermutation would likely render the virus replication defective. However, dsRAD has been shown to act on short duplexes, modifying a few select A's, and in such cases, a resultant virus could still be infectious. It will be interesting to examine the role of splicing in dsRAD activity. Deletion of the 5′ and 3′ SS in pLAGαsPE should inhibit spliceosomal snRNP complexes from associating with the RNA, and it will be interesting to determine whether A's are still modified.

We thank Lamia Sharmeen and Andrew Polson for helpful discussions and suggestions. We are very grateful to Timothy Wong for sharing results from his laboratory with us and pointing out a potential splicing link. We extend many thanks to all members of the Linial

laboratory, especially Andrei Tikhonenko, Shuyuarn Yu, Kate Levine, Ashly Yeo, Richard Lum, and Tae Kang, for insightful comments and debate, and we thank Julie Overbaugh, Andrew Kirsh, A. Tikhonenko, and T. Wong for helpful comments on the manuscript.

This work was supported by NCI grant CA18282 to M.L.L. A.M.H. is supported by NIH research scientist development award RR00079. The Biocomputing Resource Center (for sequence homology searches) is supported by NCI grant P30CA15704.

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