Determination of the Rate of Base-Pair Substitution and Insertion Mutations in Retrovirus Replication

JOSEPH P. DOUGHERTY[†] AND HOWARD M. TEMIN*

McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706

Received 24 March 1988/Accepted 26 April 1988

We recently described a protocol for determination of retrovirus mutation rates, that is, the mutation frequency in a single cycle of retrovirus replication (J. P. Dougherty and H. M. Temin, *Mol. Cell. Biol.* 6:4378-4395, 1987; J. P. Dougherty and H. M. Temin, p. 18-23, in J. H. Miller and M. P. Calos, ed., *Gene Transfer Vectors for Mammalian Cells*, 1987). We used this protocol to determine the mutation rates for defined mutations in a replicating retrovirus by using a spleen necrosis virus-based vector. We determined that the mutation rate for a single base pair substitution during replication of this avian retrovirus is 2×10^{-5} per base pair per replication cycle and the insertion rate is 10^{-7} per base pair per replication cycle. It will be possible to use this protocol to determine mutation rates for other retroviruses.

Retroviruses are RNA viruses that replicate through a DNA intermediate, the provirus. The provirus integrates into the target cell genome, where it is stably maintained. Spleen necrosis virus (SNV) is an avian retrovirus that can also infect some mammalian cells, such as rat and dog cells. Previously, we described a protocol that can be used to determine retrovirus mutation rates (3, 4). Using an SNV-based vector, we showed that the mutation rate leading to expression of a suppressed gene is 5×10^{-3} per base pair (bp) per replication cycle. However, the nature of the mutations leading to expression of the suppressed gene were not characterized. They could have been point mutations, deletions, insertions, or inversions (3, 4, 14).

In the present paper, we describe the determination of retrovirus mutation rates for base pair substitutions and for insertions. We found that the base pair substitution rate is 2×10^{-5} per bp per replication cycle and the insertion rate is 10^{-7} per bp per replication cycle. We also studied apparent gene conversion of a provirus and found that after 15 cell generations the apparent gene conversion frequency was 10^{-3} .

MATERIALS AND METHODS

Nomenclature. Mutation rate means the mutation frequency per single cycle of virus replication. *hygro* and *neo* refer to genes, while *neo*^r and *hygro*^r refer to resistance phenotypes. Plasmids have a small p before their names, while viruses derived from these plasmids do not.

Plasmid constructions. The construction of pJD216NeoHy was previously described (3, 4). pJD216Neo(Am)Hy was derived from pJD216NeoHy by substituting a *Pvu*II fragment containing the amber codon for the wild-type *Pvu*II *neo* fragment (6).

Cells. The D17 cell line is an osteosarcoma-derived dog cell line which is permissive for SNV infection. The C321 and .2G cell lines are helper cell lines derived from D17 cells. C321 cells have already been described (22). The only

difference between the C321 and .2G cell lines is that C321 cells contain wild-type copies of *neo* (in pSV2*neo*), which confers resistance to G418, whereas .2G helper cells contain copies of a mutant dihydrofolate reductase gene, which confers resistance to methotrexate (17). D17 and helper cells were grown as previously described (22). Selection for hygromycin-resistant cells was done in the presence of 50 to 100 µg of hygromycin per ml. Selection for G418-resistant cells was done in the presence of 400 µg of G418 per ml. Selection for methotrexate-resistant cells was done in the presence of 90 ng of methotrexate per ml.

Transfections and virus infections. Transfections were done by the calcium phosphate method (7, 23). Infections were done as previously described (3). Virus collected from helper cells was clarified by centrifugation followed by immediate use or storage at -70°C . Virus titers were determined by infecting 2×10^5 D17 cells in 60-mm-diameter petri dishes with 10-fold serial dilutions of virus from each helper cell clone in the presence of 100 µg of Polybrene per ml in 0.4 ml of medium, followed by selection for hygromycin resistance or G418 resistance. Titters are given in *hygro*^r or *neo*^r transforming units (TU) per 0.2 ml of virus stock. TU are the number of phenotypically transformed cell colonies formed after infection and selection, multiplied by the dilution.

Polymerase chain amplification and DNA sequencing. Polymerase chain amplification reactions were performed with 1.5 µg of genomic DNA in 75-µl reactions containing 67 mM Tris hydrochloride (pH 8.8), 6.7 mM MgCl_2 , 16.6 mM ammonium sulfate, 10 mM β -mercaptoethanol, 6.7 mM EDTA, 0.4 mM deoxynucleoside triphosphates, 4.5 µg of each primer, 4.5 U of *Taq* polymerase, and 10% dimethyl sulfoxide. The first step was to heat the polymerase chain reaction (PCR) samples to 90°C for 2 min, followed by 30 cycles of incubation at 70°C for 5 min, followed by incubation at 90°C for 1 min. The samples were then electrophoresed in an 8% acrylamide gel and stained (11). After electrophoresis, amplified DNA was electroeluted from the acrylamide gel, followed by ^{32}P end labeling with T4 polynucleotide kinase and [γ - ^{32}P]ATP, followed by digestion with *Bgl*II, electrophoresis in an 8% acrylamide gel, and electroelution of the larger end-labeled fragment. The purified end-labeled fragment was then sequenced by the Maxam and Gilbert protocol (12).

* Corresponding author.

[†] Present address: Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854.

FIG. 1. Single cycle of retrovirus replication and vectors. Single round of retrovirus replication. The open rectangles represent long terminal repeats, the horizontal lines represent viral sequences, the open and filled boxes represent inserted genes, and the jagged lines represent chromosomal sequences. (B) Vectors used. ss, Splice site. The solid boxes represent *hygro* gene coding sequences, and the open boxes represent *neo* gene coding sequences. The asterisk represents an amber codon introduced into the *neo* gene (6). The amber codon creates a *DdeI* restriction site (CTTAG). The inverted triangle and sequence above the *neo* gene of pJD216NeoHy represents the wild-type *neo* sequence where the point mutation was introduced to create an amber codon and a *DdeI* restriction enzyme cleavage site. The inverted triangle over the *neo* sequence of pJD216Neo(Am)Hy shows the change that was made.

RESULTS

Single round of retrovirus replication. Retrovirus helper cells provide *trans*-acting functions required for retrovirus vector replication. Superinfection of such helper cells is blocked by a factor of over 100 as a result of superinfection interference (data not shown), and vector virus cannot spread in the target cells because it is defective and there is no helper virus to supply viral proteins. Therefore, going from a defective retrovirus vector provirus in a helper cell to a defective vector provirus in a target cell is a single cycle of replication. Such a single cycle involves one round of RNA transcription and one round of reverse transcription (Fig. 1A). Growth of a clone of cells then involves multiple rounds of cell replication.

JD216NeoHy expresses both the *neo* and *hygro* genes. For our studies of retrovirus mutation rates, we used splicing vectors JD216NeoHy and JD216Neo(Am)Hy (Fig. 1B). A splicing vector is a vector in which two genes can be expressed from a single long terminal repeat promoter, one from unspliced viral RNA and the other from spliced viral RNA. JD216NeoHy contains both the *neo* gene, which

TABLE 1. *neo* and *hygro* titers produced by JD216NeoHy proviruses in .2G or C321 helper cells^a

Clone	Titer (TU) of ^b :	
	<i>neo</i>	<i>hygro</i>
.2G clones		
1	110×10^2	130×10^2
2	51×10^2	47×10^2
3	34×10^2	39×10^2
4	31×10^2	27×10^2
5	17×10^2	18×10^2
6	47×10^1	83×10^1
7	75×10^1	61×10^1
8	65×10^1	56×10^1
9	51×10^1	55×10^1
10	36×10^1	43×10^1
C321 clones		
1-1	190×10^2	180×10^2
1-2	60×10^2	60×10^2
1-3	14×10^2	17×10^2

^a To establish .2G and C321 helper cells with a single JD216NeoHy provirus, C321 cells were transfected with pJD216NeoHy, transfected cells were selected for hygromycin resistance, and virus was harvested from *hygro*^r C321 cells and used to infect either .2G or C321 helper cells at a low multiplicity of infection. Infected .2G or C321 cells were selected for hygromycin resistance, and individual cell clones were picked and grown. Virus titers for each helper cell clone harboring a JD216NeoHy provirus were determined as described in Materials and Methods after freezing and thawing at least once before use.

^b For .2G clones, the overall *neo* and *hygro* titers were 27×10^3 and 29×10^3 TU, respectively, and for C321 clones the corresponding values were both 26×10^3 .

confers resistance to G418 (8), and the *hygro* gene, which confers resistance to hygromycin B (9). We used two D17 (dog cell line)-based SNV helper cell lines, C321 and .2G. C321 has already been described (22). The only difference between the C321 helper line and .2G helper line is that .2G contains a mutant dihydrofolate reductase gene, which confers resistance to methotrexate (17), while C321 contains wild-type copies of the *neo* gene. As a positive control, we established 10 .2G helper lines and 3 C321 helper cell lines, each containing a JD216NeoHy provirus. We harvested virus from each cell line, infected fresh D17 cells, selected for G418-resistant (*neo*^r) or hygromycin-resistant (*hygro*^r) cell colonies, and obtained *neo* and *hygro* titers expressed as TU (Table 1). JD216NeoHy was able to form equal numbers of *neo*^r and *hygro*^r cell clones.

Rate of base pair substitutions. To determine the rate of base pair substitutions, we constructed pJD216Neo(Am)Hy, which differs from pJD216NeoHy by a single base pair change resulting in the introduction of an amber codon into the 5' coding region of *neo* (Fig. 1B) (6). We established by infection 43 .2G helper cell lines, each containing a JD216Neo(Am)Hy provirus. We harvested virus from each cell line, infected fresh D17 cells, selected for *neo*^r or *hygro*^r cell colonies, and obtained *neo* and *hygro* TU titers. The *neo*^r/*hygro*^r ratio obtained with the 10 clones giving the highest overall titers was 2×10^{-5} (Table 2). The sum of *neo*^r/*hygro*^r colonies obtained for all 43 clones was 129/5,916,000 or 2.2×10^{-5} . The *neo*^r colonies represent the number of vector mutants that express the *neo* gene, while the *hygro*^r colonies represent the overall vector titer. Therefore, the *neo*^r/*hygro*^r ratio represents the minimal mutation frequency (see below). The mutation rate is the mutation frequency per replication cycle. Since only one round of virus replication occurred, 2×10^{-5} is the mutation rate per replication cycle.

TABLE 2. Rate of base pair substitutions for virus from .2G cells^a

Clone	Titer (TU) of ^b :	
	<i>neo</i>	<i>hygro</i> (10 ⁴)
1	9	68
2	17	67
3	10	44
4	4	43
5	1	23
6	4	22
7	7	19
8	6	15
9	2	15
10	3	14

^a .2G cell clones containing a JD216Neo(Am)Hy provirus were established in the same manner as the cell lines described in Table 1, except that the original transfections were done with pJD216Neo(Am)Hy. Virus titers were determined as described in Table 1, footnote *a*, except that the virus stocks from the cell clones were not frozen and thawed but only clarified by centrifugation. The titers in this experiment are higher than those in Table 1 because this virus was not frozen and thawed before assay.

^b The overall *neo* and *hygro* titers were 63 and 330 × 10⁴, respectively, and the mutation rate was determined as follows: *neo* NU/*hygro* NU = 63/330 × 10⁴ = 2 × 10⁻⁵.

To test whether the fluctuations in the *neo*^r/*hygro*^r ratios obtained with individual cell clones were clonally distributed, we applied the chi-square goodness-of-fit test to the results obtained with 31 of these clones. We found that the differences were not significant, indicating that the proportions were equal and the distribution was not clonal.

To prevent any possible virus spread during growth of the cell clones, we performed the same experiment with 12 .2G helper cell lines, each harboring a JD216Neo(Am)Hy provirus, except that we grew the cell clones in the presence of a neutralizing antibody to SNV proteins (3). The antibody was removed from the cell cultures 24 h before harvest of the virus. The number of *neo*^r/*hygro*^r colonies obtained was 28/1146 × 10³ or 2.4 × 10⁻⁵ and was the same as that obtained when the .2G clones were grown in the absence of neutralizing antibody.

DdeI site lost in most of the cases tested. Introduction of the amber codon into pJD216Neo(Am)Hy also resulted in creation of a *DdeI* restriction site CTNAG (Fig. 1B). A base change in position 2 or 3 of this amber codon results in loss of the *DdeI* restriction site (Fig. 1B). If the *neo* revertants obtained in the experiment described in the legend to Table 2 were the result of base pair substitutions at position 2 or 3 of this amber codon, then the *neo* revertants should contain proviruses that lost the *DdeI* restriction site. To test this hypothesis, we grew 17 *neo*^r D17 cell clones, obtained as described in Table 2, footnote *a*, isolated genomic DNA from these clones, digested the genomic DNA with *DdeI*, electrophoresed the DNA in a 1.2% agarose gel, blotted the DNA to nitrocellulose, and hybridized it with a *neo*-specific probe, followed by autoradiography (Fig. 2). In the three cases shown, the *DdeI* site was lost. In all, we found that 15 of 17 clones had lost the *DdeI* site.

Reversion frequency with C321 helper cells. We also performed an experiment with the protocol described in Table 2, footnote *a*, with C321 helper cells, which contain multiple wild-type copies of *neo* in pSV2*neo* (17). That is, we established 10 C321 helper cell lines, each containing a JD216Neo(Am)Hy provirus. We harvested virus from these cell lines, infected fresh D17 cells, selected for G418 or hygromycin resistance, and obtained *neo* and *hygro* TU

FIG. 2. Analysis of JD216Neo(Am)Hy proviruses in *neo*^r cells. Three individual *neo*^r D17 cell clones described in Table 2 were grown. Genomic DNA was isolated, and 10 µg was digested with *DdeI*, followed by electrophoresis in a 1.2% agarose gel and blotting of the gel to nitrocellulose. The blot was then hybridized with a ³²P-labeled *neo*-specific probe, followed by autoradiography (19). Genomic DNAs from the clones were run in lanes 1 to 3. Lane M is a control of plasmid DNA in which the *DdeI* site in *neo* is present. DNAs from such a clone and from one without the *DdeI* site were run in all gels. At the bottom is a diagram of JD216Neo(Am)Hy DNA with the *DdeI* restriction cleavage sites in the *neo* gene shown (arrowheads). ss, Splice site. The asterisk indicates an amber codon introduced into the *neo* gene (6).

titers. The reversion frequency we obtained was 10⁻³ (Table 3), which is 50 times higher than that obtained with .2G helper cells, which do not contain endogenous copies of wild-type *neo*.

We also tested whether the proviruses in the *neo* revertants obtained in the experiment described in Table 3, footnote *a*, had lost the *DdeI* restriction site. In all of the 19 cases tested, the *DdeI* restriction site was lost. We suggest

TABLE 3. Reversion frequency of virus grown in C321 helper cells^a

Clone	Titer (TU) ^b of:	
	<i>neo</i>	<i>hygro</i> (10 ³)
1	36	28.0
2	13	23.0
3	13	20.0
4	11	12.0
5	19	11.0
6	5	10.0
7	7	5.0
8	1	0.7
9	0	0.5
10	1	0.5

^a C321 cell clones containing a JD216Neo(Am)Hy provirus were established in the same manner as the cell lines described in Table 1, footnote *a*, except that the original transfections were done with pJD216Neo(Am)Hy. C321 helper cell clones were grown for approximately 15 cell doublings before virus was harvested. Virus titers were determined as described in Table 2, footnote *a*.

^b The overall *neo* and *hygro* titers were 106 and 110.7 × 10³, respectively, and the reversion frequency was determined as follows: *neo*/*hygro* = 106/110.7 × 10³ = 1 × 10⁻³.

FIG. 3. Genomic sequencing of *neo* revertants. (A) Acrylamide gel of amplified DNA obtained with three separate *neo*⁺ D17 cell clones infected with virus from .2G helper cell clones. M refers to molecular weight markers (*Msp*I-digested pBR322). (B) Autoradiograms obtained from DNA sequencing of two clones. The bracket labeled *Dde*I indicates the position of the *Dde*I site in the original JD216Neo(Am)Hy vector. In I there was a 4-bp insertion, CCGA, indicated at the left.

that the higher reversion frequency obtained with the C321 helper cells is the result of gene conversion (see Discussion).

Genomic sequence analysis of *neo* revertants by PCR amplification. The exact nature of the changes giving the *neo*⁺ phenotype was analyzed by direct sequencing of PCR-amplified genomic DNA from 13 revertants (13, 16, 25). Two 20-nucleotide oligomers were synthesized and used as primers to amplify a 264-bp region that spans the amber codon. Thirty sequential cycles of primer annealing, DNA polymerase extension (with DNA polymerase from *Thermus aquaticus*), and denaturation were performed. Amplified DNA was purified in an 8% polyacrylamide gel (Fig. 3A) and sequenced by the Maxam and Gilbert protocol (Fig. 3B) (12). Figure 3B shows amplified genomic DNA from three *neo*⁺ D17 cell clones originally infected with JD216Neo(Am)Hy virus harvested from .2G helper cell clones. Typically, we obtained 1 to 5 μ g of amplified DNA from 1.5 μ g of genomic DNA, indicating approximately 10^8 -fold amplification.

We performed this amplification on DNAs from seven *neo*⁺ D17 cell clones originally infected with JD216Neo(Am)Hy virus from .2G helper cell clones and on six DNAs from cells originally infected with JD216Neo(Am)Hy virus from C321 helper cell clones (containing endogenous *neo* genes), all of which had lost the *Dde*I site (Fig. 2). In all cases in which the *Dde*I site was lost, base pair 4 of the *Dde*I

site (corresponding to base 2 of the amber codon) was converted from A-T to G-C (Fig. 3B, II).

Two *neo*⁺ D17 cell clones originally infected with JD216Neo(Am)Hy virus from .2G helper cell clones did not lose their *Dde*I sites. DNAs from these two clones were used as templates for amplification and sequencing. The sequence obtained for one clone is shown in Fig. 3B, I. As expected, the *Dde*I site was maintained. There was no change from T to C. However, there was an insertion of four bases (CCGA) seven bases downstream from the amber codon. The insertion is a duplication of the adjacent CCGA. Thomas and Capecchi have shown that +1, +4, +7, etc., frameshift mutations just downstream of this amber codon can restore *neo* function (see Discussion) (21). Sequencing of the other *neo* revertant that did not lose the *Dde*I site confirmed that the *Dde*I site was maintained without change, but we were able to find no compensating mutations within 40 bases surrounding the amber codon.

DISCUSSION

In this paper we describe the direct measurement of mutation rates during retrovirus replication for both a single base pair substitution at a defined locus and insertions in a defined area.

Base pair substitution mutation rate. The base pair substitution rate that we obtained was 2×10^{-5} per bp per replication cycle. Initially we thought that mutations would occur at any of the three bases of the amber codon (TAG) unless they produced another stop codon. However, genomic sequencing of PCR-amplified DNA showed that substitutions were limited to a single change at the second base of the amber codon, that being a transition from A-T to G-C which restores the original wild-type sequence. The wild-type codon is TGG, which is the tryptophan codon. All other base substitutions at the amber codon would result in changes to codons for different amino acids. It seems possible that we were limited to this change because when the 5' end of the wild-type *neo* gene product is present, there may be an absolute requirement for tryptophan at the locus we were studying. Thus, the value we obtained for the rate of base pair substitution is a minimal one, because there may have been selection against other mutations. Experiments with other mutations (in progress) will test this hypothesis.

The base pair substitution rate is similar to that determined with cell-free systems (10). Furthermore, the mutation rate we observe is similar to that calculated for two other RNA viruses, poliovirus type 1 (less than 10^{-5} for the VP1 gene) and influenza virus (8×10^{-5} for the NS segment), and higher than that for a third, Sindbis virus (2×10^{-7}) (5, 15, 18). These other RNA viruses utilize different types of polymerases than do retroviruses.

Mutations in our system could have occurred at either the RNA transcription step or the reverse transcription step. We are not sure at which step the mutations occurred. It is possible to separate these two steps physically, which may allow determination of the mutation rate at each step.

Insertion mutation rate. Two *neo* revertant D17 cell clones originally infected with JD216Neo(Am)Hy virus from .2G helper cells did not lose the *Dde*I site. Genomic sequencing with PCR-amplified DNA revealed that one clone retained the *Dde*I site and the amber codon (Fig. 3B, I), but there was a 4-bp insertion 7 bp downstream from the amber codon. Thomas and Capecchi have found that +1 frameshift mutations within an area of 11 bp downstream of the amber codon can compensate for the stop codon (21). Translation can be

initiated at an AUG in the -1 reading frame upstream of the amber codon, allowing readthrough of the amber codon which is in the 0 reading frame. The $+1$ frame shift then allows the ribosome to regain the proper phase. The target size for selectable insertions is about 11 bp downstream from the amber codon (21). Since the only insertions that score as revertants are $+1$ frameshift mutations, our effective target is the equivalent of a single codon. The insertion mutation rate per codon is $1/17$ times the base substitution mutation rate. Therefore, the insertion mutation rate at this site is about 10^{-7} per bp per replication cycle.

The other clone that retained the *DdeI* site also retained the amber codon. We discovered no compensating mutations (20 bases $5'$ or 20 bases $3'$ to the amber codon) to account for its neo^r phenotype. It is possible that there is a compensating mutation outside of the area we sequenced or that there is a cellular mechanism by which this cell clone can suppress the amber codon. (We have not been able to recover neo^r -transforming virus by superinfection of cells carrying this provirus, even though the superinfecting virus replicated well [unpublished data]).

If the retroviral mutation rates we measured are true for the entire genome, we can roughly estimate how many replication cycles different retrovirus isolates have undergone since divergence from a cell or other virus isolate. Reticuloendotheliosis virus strain T (REV-T) contains the oncogene *v-rel*. Comparing *v-rel* from REV-T coordinates 4290 to 4675 with its proto-oncogene, *c-rel*, we found that *v-rel* and *c-rel* differ by 6 bp and an insertion of 6 bp in *v-rel* (24). The changes in this area of *v-rel* are not important for the transforming ability of *v-rel* (20), and so we consider them silent. Therefore, from the time the *c-rel* sequence was transduced into REV-T until the REV-T provirus was cloned (2), REV-T underwent approximately 800 replication cycles (number of replication cycles = $6 \text{ bp substitutions} / 2 \times 10^{-5} \text{ substitutions per bp per replication cycle} \times 385 \text{ bp}$). A similar calculation can be made for other retroviruses, if we assume that the mutation rates we measured also apply to them and the observed differences in nucleotide sequence are primarily silent. Thus, we can calculate that the observed substitution rate of lentiviruses (visna and human immunodeficiency viruses) of approximately 10^{-3} per nucleotide per year (1, 26) indicates approximately 50 replication cycles per year since the divergence of different isolates.

Gene conversion. We also used the procedure described in Fig. 1A with C321 helper cells, which contain multiple wild-type copies of *neo* in pSV2*neo*. The reversion frequency that we obtained with C321 cells was 10^{-3} , which is 50 times higher than that obtained with .2G helper cells, which do not contain endogenous copies of *neo*. Since there is no other difference between C321 and .2G cells, we suggest that the higher reversion frequency is the result of gene conversion which occurred either during growth of the C321 helper cells containing the JD216Neo(Am)Hy proviruses or during retrovirus replication or both. It is also possible that this high reversion frequency was a result of recombination. However, because the wild-type *neo* RNA contains no retrovirus sequences, it would only be packaged at a very low frequency, and a double crossover would be necessary to recover a wild-type *neo* virus. As expected, all revertants obtained with virus from C321 cells contained the wild-type sequence.

ACKNOWLEDGMENTS

We thank S. Coe, J. Couch, S. Hinz, J. Rein, and R. Wisniewski for technical assistance; K. Thomas and M. Capecchi for a gift of

pRH4-14/TK; H. S. Kim and O. Smithies for help with PCR amplification; Tom Leonard for help with the chi-square goodness-of-fit test; and Ralph Dornburg, Celine Gelinas, Wei-Shau Hu, Mark Hannink, Antonio Panganiban, and Bill Sugden for helpful comments on the manuscript.

This research was supported by Public Health Service research grants CA-22443 and CA-07175 from the National Institutes of Health. J.P.D. was supported by Public Health Service research award CA-09075 from the National Institutes of Health. H.M.T. is an American Cancer Society research professor.

LITERATURE CITED

1. Braun, M. J., J. E. Clements, and M. A. Gonda. 1987. The visna virus genome: evidence for a hypervariable site in the *env* gene and sequence homology among lentivirus envelope proteins. *J. Virol.* 61:4046-4054.
2. Chen, I. S. Y., T. W. Mak, J. J. O'Rear, and H. M. Temin. 1981. Characterization of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. *J. Virol.* 40:800-811.
3. Dougherty, J. P., and H. M. Temin. 1987. High mutation rate of a spleen necrosis virus-based retrovirus vector. *Mol. Cell. Biol.* 6:4387-4395.
4. Dougherty, J. P., and H. M. Temin. 1987. Determination of retroviral vector mutation rates and a promoterless retroviral vector, p. 18-23. In J. H. Miller and M. P. Calos (ed.), *Gene transfer vectors for mammalian cells*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. Durbin, R. K., and V. Stollar. 1986. Sequence analysis of the E2 gene of a hyperglycosylated, host restricted mutant of Sindbis virus and estimation of mutation rate from frequency of revertants. *Virology* 154:135-143.
6. Folger, K. R., K. Thomas, and M. R. Capecchi. 1985. Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. *Mol. Cell. Biol.* 5:59-69.
7. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
8. Gritz, L., and J. Davies. 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression. *Gene* 25:179-188.
9. Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* 177:65-72.
10. Kunkel, T. A., F. Eckstein, A. S. Mildvan, R. M. Kopplitz, and L. A. Loeb. 1981. Deoxynucleoside [1-thio]triphosphates prevent proofreading during *in vitro* DNA synthesis. *Proc. Natl. Acad. Sci. USA* 78:6734-6738.
11. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, N.Y.
12. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
13. McMahon, G., E. Davis, and G. N. Wogan. 1987. Characterization of c-Ki-ras oncogene alleles by direct sequencing of enzymatically amplified DNA from carcinogen-induced tumors. *Proc. Natl. Acad. Sci. USA* 84:4974-4978.
14. Miller, C. K., J. E. Embretson, and H. M. Temin. 1988. Transforming viruses spontaneously arise from nontransforming reticuloendotheliosis virus strain T-derived viruses as a result of increased accumulation of spliced viral RNA. *J. Virol.* 62:1219-1226.
15. Parvin, J. D., A. Moscona, W. T. Pan, J. M. Leider, and P. Palese. 1986. Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. *J. Virol.* 59:377-383.
16. Saiki, R. K., S. J. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. Erlich, and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350-1354.
17. Simonsen, C. C., and A. D. Levinson. 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA.

- Proc. Natl. Acad. Sci. USA **80**:2495–2499.
18. **Smith, D. B., and S. C. Inglis.** 1987. The mutation rate and variability of eukaryotic viruses: an analytical review. *J. Gen. Virol.* **68**:2729–2740.
 19. **Southern P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**:327–341.
 20. **Sylla, B. S., and H. M. Temin.** 1986. Activation of oncogenicity of the *c-rel* proto-oncogene. *Mol. Cell. Biol.* **6**:4709–4716.
 21. **Thomas, K. R., and M. R. Capecchi.** 1986. Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. *Nature (London)* **324**:34–38.
 22. **Watanabe, S., and H. M. Temin.** 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. *Mol. Cell. Biol.* **3**:2241–2249.
 23. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes to eucaryotes. *Cell* **16**:777–785.
 24. **Wilhelmsen, K. C., K. Eggleton, and H. M. Temin.** 1984. Nucleic acid sequences of the oncogene *v-rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene *c-rel*. *J. Virol.* **52**:172–182.
 25. **Wong, C., C. E. Dowling, R. K. Saiki, R. G. Higuchi, H. A. Erlich, and H. H. Kazazian.** 1987. Characterization of beta-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. *Nature (London)* **330**:384–386.
 26. **Yokoyama, S., and T. J. Gojobori.** 1987. Molecular evolution and phylogeny of the human AIDS viruses LAV, HTLV-III, and ARV. *J. Mol. Evol.* **24**:330–336.