4532–4536 *Nucleic Acids Research, 1997, Vol. 25, No. 22*

Higher fidelity of RNA-dependent DNA mispair extension by M184V drug-resistant than wild-type reverse transcriptase of human immunodeficiency virus type 1

Mayla Hsu[1,2], Phil Inouye[1,2], Lisa Rezende[3], Nathalie Richard[1,2], Zhuo Li[2], Vinayaka R. Prasad[3] and Mark A. Wainberg[1,2,*]

[1]Department of Microbiology and Immunology, McGill University, Montreal, Canada, [2]McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, 3755 Chemin de la Côte Ste. Catherine, Montreal H3T 1E2, Canada and [3]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA

Received August 6, 1997; Revised and Accepted October 1, 1997

ABSTRACT

Reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) has low fidelity compared with RTs of other retroviruses and cellular DNA polymerases. We and others have previously found that the fidelity of DNA-dependent DNA polymerization (DDDP) of M184V-mutated HIV-1 RT is significantly higher than that of wild-type RT. Viruses containing the M184V substitution are highly resistant to (–)-2′-dideoxy-3′-thiacytidine (3TC) *in vitro* and in patients treated with 3TC monotherapy. It was of interest to determine the fidelity of RNA-dependent DNA polymerization (RDDP) of M184V RT compared with wild-type because this step occurs first in reverse transcription; errors made during this step may be copied in subsequent polymerization steps. Using an *in vitro* mispaired primer extension assay, M184V-mutated RT exhibited 3–49-fold decreased frequency of mispair extension compared with wild-type RT. Fidelity differences between M184V and wild-type RT were most marked in extension of A:G (49-fold) and A:C (16-fold) mispairs, with only a marginal (3-fold) decrease in the extension of A:A mispairs. RT containing a methionine to isoleucine (M184I) mutation showed only slight increases in RDDP fidelity compared with wild-type, ranging from 1.5- to 6-fold increases. Of the three RTs tested, wild-type RT was the most error-prone, with mispair extension frequencies ranging from 6.674×10^{-1} to 7.454×10^{-2}.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) has a highly error-prone reverse transcriptase (RT) (1–3), a high rate of replication (4–6), and lacks 3′ to 5′ proofreading activity (2). These characteristics result in enormous genetic heterogeneity in HIV-infected individuals, which is thought to underlie both viral escape from immunological pressure as well as drug resistance. Therefore, studies of viral replication dynamics, fidelity, and evolution are relevant to considerations of pathogenesis and treatment.

Mutations in RT that encode drug resistance have been mapped to various domains of RT (7). Of particular interest is an M184V substitution, located in the polymerase active site of the enzyme, that confers extremely high-level resistance to the (–) enantiomer of 2′-deoxy-3′-thiacytidine (3TC) (8–10), as well as lower level resistance to both 2′, 3′-dideoxyinosine (ddI) and 2′, 3′-dideoxycytidine (ddC) (8,11). The M184V substitution is present in viruses isolated from patients receiving 3TC (12). Interestingly, treatment with 3TC monotherapy is associated with a reduction in viral load (13) despite the drug resistance conferred by the presence of the M184V mutation (12). This substitution also causes reversion of resistance to 3′-azidothymidine (AZT) (10), and is observed in patients on AZT–3TC combination therapy alongside decreased viral load (14). Although M184V-containing viruses may be marginally less infectious than wild-type viruses (14,15), their RTs do not differ significantly in enzymatic efficiency (16).

HIV-1 RT is a multifunctional enzyme with RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and RNase H activities, and is responsible for the conversion of viral genomic RNA into double-stranded preintegrative DNA (17). The error frequency of RDDP by wild-type RT has been determined *in vitro* to be on the order of 10^{-2} to 10^{-4} in primer extension assays (3,18), 10^{-4} when copying HIV-1 *env* RNA as a template (19), and 10^{-3} to 10^{-4} in a ϕXam16 reversion assay (20).

Recently, studies of M184V RT fidelity have demonstrated less error-prone DDDP than wild-type (16,21). Using primer extension of homopolymeric templates, it was shown that M184V had 25–45-fold increased k_{cat}/K_m of selectivity for correct versus incorrect nucleotides, compared with wild-type

*To whom correspondence should be addressed at McGill University AIDS Centre. Tel: +1 514 340 8260; Fax: +1 514 340 7537; Email: mdwa@musica.mcgill.ca

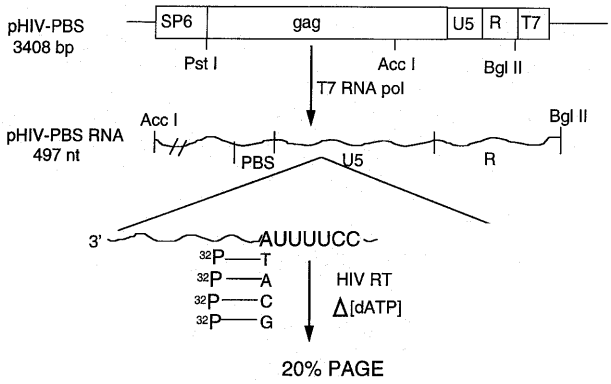

Figure 1. Schematic representation of pHIV-PBS and *in vitro* transcribed RNA template (497 nt) used in RDDP reactions (11). The position of matched and mismatched DNA primers is indicated.

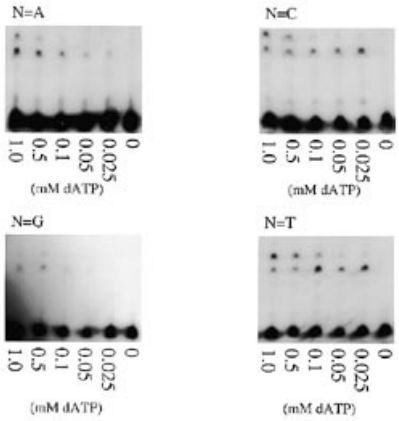

Figure 2. Primer extension reactions of wild-type RT with mismatched primers (N = A, C, G) and matched primer (N = T). The baseline band indicates the unextended 16 nt primer. Similar autoradiographs were obtained for primer extension by M184I and M184V RT (not shown).

(16). This was corroborated by primer extension assays, which demonstrated 1.37–16.97-fold decreases of misinsertion frequency for M184V compared with wild-type (21). In this study, we determined the RDDP fidelity of M184V, M184I, and wild-type RTs, using a mismatched primer extension assay. By calculating the kinetics of extension of mispairs, we observed that both mutated RTs had increased fidelity compared with wild-type, i.e. up to 48.6-fold increased mispair extension fidelity for M184V RT, depending on the mispair tested. M184I RT was between 1.5 and 6.12 times less likely to extend mispairs than wild-type RT. The largest differences in fidelity between mutated and wild-type RT were observed with extensions of a A:G mispair.

MATERIALS AND METHODS

RNA synthesis and *in vitro* RDDP assays

A plasmid encoding the gag-U5-primer binding site (PBS) of HIV-1 HXB2D RNA (pHIV-PBS) was linearized with *Acc*I (Gibco-BRL) and transcribed overnight following manufacturer's instructions (Ambion, Austin, TX) to yield a 497 nucleotide (nt) RNA template (22). DNA oligonucleotide primers of 16 nt (General Synthesis Diagnostics, Inc., Toronto, Canada) were designed to anneal to the RNA template at nucleotide positions 611–626 slightly upstream of the PBS. Primer sequences were 5′-ATTTTCCATTCTGACN-3′, (N = A, C, G, or T) with deliberate mismatches at the 3′ terminal positions, such that primer elongation represented mispair extension as a measure of RT RDDP fidelity (Fig. 1).

Reverse transcriptase

Enzymes were histidine-tagged and purified by Ni^{2+}-NTA affinity chromatography (Qiagen) (23). Activity was defined as the amount of enzyme required to incorporate one pmol of ^{3}H-dTTP into trichloroacetic acid (TCA)-precipitable material at 37°C in 30 min, with poly(rA).oligo(dT)$_{(12–18)}$ (Pharmacia) as a template:primer. The quantities of RT in each reaction were standardized such that polymerization activity was constant and optimal for primer extension and ranged between 21 and 48 nM (not shown).

Primer extension reactions

Primers were 5′-[γ-^{32}P] end-labelled with T4 polynucleotide kinase as described (Gibco-BRL) (24). Primers were annealed to template at a template:primer molar ratio of 1.3:1, corresponding to 820 ng RNA template and 12 ng labelled primer per reaction. Reactions were performed in 20 µl and included 10 mM dithiothreitol, 50 mM Tris–HCl (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, and 0.025–1 mM of dATP (Pharmacia). Reactions were boiled for 2 min, incubated at 55°C for 8 min, and transferred to a 37°C water bath for 10 min. The addition of dATP before or after heating resulted in no difference in extension of primers (not shown). RT was added and polymerization carried out at 37°C for 10 min, followed by phenol-chloroform extraction and ethanol precipitation.

Reaction product visualization and quantitation

Reaction products were resuspended in formamide gel-loading buffer (24), and were boiled for 2 min, iced for 10 min, and run on 20% polyacrylamide urea sequencing gels. Gels were dried and subjected to molecular imaging analysis (Bio-Rad) in order to calculate primer extension as a fraction of total primers per lane. Percent of primer extension/min was plotted against dATP concentration, and K_m and v_{max} were determined as described (GraphPad Prism 2.0).

RESULTS

Figure 1 shows the position of 16 nt long annealed primers relative to the HIV-1 R-U5-gag RNA template. The next four incoming nucleotides to be added onto the extending primer are dATPs. Thus, the kinetics of primer extension were determined by varying the concentration of this substrate. For mispaired primers ending in A, C, or G, the incorporation of dATP represented mispair extension, and was a measure of the infidelity of the enzyme when compared with the correctly paired primer ending in T. Molecular image analysis allowed quantitation of primer extension as a fraction of total primers present in the reaction (Fig. 2). The percentage of primer extension as a function of substrate concentration was characteristic of Michaelis–Menten kinetics of single-substrate binding (Fig. 3). K_m and v_{max} were

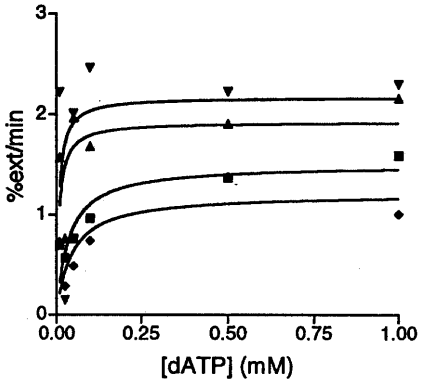

Figure 3. Mispair extension by wild-type RT, using primers ending in A (■), C (▲), T (▼) or G (◆). Curves for M184I and M184V RT were similarly calculated (not shown).

determined for extension of each template:primer pair. The following equation was used to determine the frequency of mispair extension, F_{ext}:

$$F_{ext} = \frac{(v_{max}/K_m)^{mismatch}}{(v_{max}/K_m)^{match}}$$

Extensions of matched primers (N = T) and mismatched primers (N = A, C, G) by wild-type RT are shown in Figure 2. With an increase in dATP concentration, there was an increase in the accumulation of extended primers varying in length between 17 nt (addition of 1 nt) and 21 nt (addition of 5 nt). The 21 nt product represents the incorporation of four incoming dATP, followed by one subsequent dATP which is a misincorporated nucleotide. For wild-type RT, the extension of primers A and C appeared similar to extension by the primer N = T, indicating a lack of discrimination between matched and mismatched primers by wild-type RT. There appeared to be a preponderance of 17 nt and 20 nt product, corresponding to addition of 1 and 4 nt, respectively, rather than accumulation of primer extension products of intermediate length. Similar results were obtained in regard to primer extension by the M184I and M184V mutant RTs (results not shown). In contrast to wild-type RT, the mutant RTs were less able to extend mismatched primers, particularly those ending in G (Table 1).

The frequencies of mispair extension (F_{ext}) were calculated for each template:primer pair for each RT and are shown in Table 1. The lower the F_{ext}, the lesser the probability of the primer being extended and the greater the fidelity. In this system, wild-type RT was shown to have a very high frequency of mispair extension, ranging from 6.674×10^{-1} for extension of the A:C mispair to 7.454×10^{-2} for extension of the A:G mispair, relative to extension of the correctly paired primer. For mutant RTs, the F_{ext} values were lower than those of wild-type RT, indicating increased discrimination between extension of mismatched and matched primers and, thus, increased fidelity. For all enzymes, the mispairs most likely to be extended were the A:C mispairs.

The fold decreases in F_{ext} between mutant and wild-type RT, representing increases in fidelity, were calculated for each primer:template mispair (Table 1). The largest increases in accuracy between mutant and wild-type RT were seen in the extension of the A:G mispair, of which M184V showed the largest increase in fidelity (48.6-fold). In contrast, M184I RT showed only a modest increase (4.2-fold) in extension of A:G and of A:C (6.1-fold). Generally, differences in fidelity, in comparison with wild-type, were greater with M184V than with M184I RT.

Table 1. Fold decrease in RDDP mispair extension of mutant relative to wild-type HIV-1 RTs

T:P[a]	K_m (μM)[a]	v_{max} (%ext/min)[b]	F_{ext}	Fold decrease[c]
Wild-type				
A:A	40.78 ± 8.29	1.522 ± 0.075	7.673×10^{-2}	–
A:C	6.09 ± 2.036	1.977 ± 0.002	6.674×10^{-1}	–
A:T	4.48 ± 2.594	2.179 ± 0.145	1	–
A:G	33.98 ± 10.429	1.232 ± 0.101	7.454×10^{-2}	–
M184I				
A:A	66.963 ± 15.168	3.2273 ± 0.174	4.96×10^{-2}	1.5
A:C	29.037 ± 6.697	3.0645 ± 0.155	1.09×10^{-1}	6.12
A:T	2.664 ± 1.684	2.587 ± 0.078	1	–
A:G	102.21 ± 19.59	1.755 ± 0.010	1.768×10^{-2}	4.2
M184V				
A:A	12.49 ± 4.892	1.1965 ± 0.072	2.254×10^{-2}	3.4
A:C	11.72 ± 5.622	2.067 ± 0.141	4.15×10^{-2}	16.1
A:T	0.6716 ± 1.98	2.854 ± 0.125	1	–
A:G	207.6 ± 47.818	1.354 ± 0.114	1.535×10^{-3}	48.6

[a]T:P denotes template: primer pair.
[b]Values are given as means of three experiments and standard error of the mean.
[c]Relative to corresponding template: primer pair for wild-type.

DISCUSSION

The methods used were adapted from Bakhanashvili and Hizi (18,25). Briefly, the ability of wild-type and mutant RTs to extend primers mispaired at the 3′ end was used as a quantitative measure of RDDP error frequency. The template used was an *in vitro* transcribed RNA corresponding to the PBS-U5 region of the HIV-1 RNA genome, which was used because of its similarity to the *in vivo* template for RDDP. Reverse transcription involves the generation of a double-stranded pre-integrative DNA from a single strand of viral genomic RNA. Initially, RT catalyzes the synthesis of a minus-strand DNA (RDDP) followed by transcription of the complementary positive strand DNA (DDDP) (26). The relative contribution of errors during RDDP to overall *in vivo* mutation frequency is unknown. However, a series of frameshift mutations in a spleen necrosis virus-derived vector increased significantly in homopolymeric runs in a single replication cycle. Most of these were shown to have occurred during RDDP (27).

The *in vivo* relevance of RDDP in generation of errors could be greater than that of DDDP; errors in the initial step of reverse transcription should be found in both strands of the double-stranded DNA product, whereas errors generated during DDDP might ultimately be present on only one strand. Studies of RDDP of HIV-1 RT have revealed either increased or decreased error frequency, or no significant difference relative to DDDP (18–20,28). For example, the RDDP of *lacZ*α-encoding RNA had a mutation frequency of 91–210 $\times 10^{-4}$, compared with $410 \pm 87 \times 10^{-4}$ for DDDP of a DNA template of *lacZ*α (28). Conversely, the use of 16S ribosomal RNA of *Escherichia coli* or ϕX174am DNA for DDDP revealed higher mispair extension frequencies for RDDP than DDDP (18,25).

Figure 2 shows the extremely high frequency of extension of terminal mispairs by wild-type RT, which was reflected in the calculated F_{ext} for this enzyme (Table 1). Wild-type RT has an extremely high frequency of misincorporation mutations (3), particularly during homopolymeric runs (29,30), that are susceptible to RT pausing (31). Misincorporations are then maintained by continued polymerization of the elongating primer. For wild-type RT, the accumulation of the 20 nt product, or primer plus 4 nt, may represent the processive addition of four correct nucleotides, followed by a pause. Longer primer extension products result from subsequent addition of misincorporated dATP.

The 17 nt product, corresponding to the addition of a single nucleotide, was observed in mispair extension reactions with wild-type as well as mutant RTs, as shown in a representative autoradiograph of wild-type RT (Fig. 2). The abundance of this oligonucleotide, relative to 18 and 19 nt products suggests decreased processivity, either pausing or dissociation of RT after addition of 1 nt. This is consistent with the observation that RT preferentially terminates chain elongation after addition of 1 nt (32).

The likelihood of wild-type and mutant RT extension of mispairs was A:C > A:A > A:G. This is consistent with observations of the RTs of HIV-1, HIV-2, and murine leukemia virus (MLV) RT (18). It is interesting that the greatest difference in fidelity between M184V and wild-type enzymes occurred with the A:G mispair (48.6-fold), since the latter is the least likely to be erroneously extended. This suggests that extension of the A:G mispair is particularly discriminated against by all RTs, but more so by higher-fidelity enzymes. The increase in fidelity is largely attributable to a decrease in K_m for primers ending in T, rather than a significant increase in v_{max} (16). This phenomenon may be characteristic of demonstrable increases in fidelity (18).

The M184V mutation is of interest because it confers high-level resistance to 3TC (8–10), and low-level resistance to ddI and ddC (11). As shown in Table 1, the M184V variant of RT has a 6.7-fold lower K_m (0.6716 μM) for incoming dNTPs in the extension reaction with the A:T matched template pair than wild-type enzyme (4.48 μM). However, M184V RT also had a 6.1-fold higher K_m than wild-type RT in this same reaction with the A:G mismatched primer pair, i.e., a difference for M184V of 48.6-fold, depending on whether the A:T matched primer or A:G mismatched primer was used. This suggests that the ability of M184V to efficiently extend from the matched primer A:T may have been increased alongside a corresponding decrease in regard to the efficiency of extension from the mismatched primer A:G. The contribution of differences in K_{cat} between wild-type and M184V RTs to these results may be minor.

The M184V mutation is selected rapidly *in vitro* by 3TC (10) and is also observed in patients treated with this drug (12,14,33). The methionine at position 184 within the conserved YMDD polymerase active site is a critical determinant of RT catalytic activity (15,16). This amino acid may also play a role in determining enzyme fidelity. An M184I substitution has also been observed during 3TC therapy, but may rapidly be outgrown by the more fit M184V variant (14,33,34). It may be important to quantitate whether M184I RT has diminished processivity, as has been demonstrated for M184V RT, a finding thought to underscore the diminished growth of M184V virus in comparison with wild-type in primary cells (34).

Note added in proof

Since submission of this paper, a manuscript also showing the increased RDDP fidelity of M184V and M184I RT, on the basis of both mispair extension and mispair insertion assays, has appeared in the literature (35). Interestingly, the latter paper also reported that these RT variants had diminished processivity.

ACKNOWLEDGEMENTS

This work was performed by M.H. in partial fulfillment of the Ph.D. degree, Faculty of Graduate Studies and Research, McGill University, Montréal, Canada. M.H. was supported by a scholarship from the National Health Research Development Program, Health Canada, that also funded this research. M.A.W. is a National AIDS Scientist of Health Canada.

REFERENCES

1 Preston, B.D., Poiesz, B.J. and Loeb, L.A. (1988) *Science*, **242**, 1168–1171.
2 Roberts, J.D., Bebenek, K. and Kunkel. T.A. (1988) *Science*, **242**, 1171–1173.
3 Yu, H. and Goodman. M.F. (1992) *J. Biol. Chem*., **267**, 10888–10896.
4 Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M. and Markowitz. M. (1995) *Nature*, **373**, 123–126.
5 Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M. and Ho. D.D. (1996) *Science*, **271**, 1582–1586.
6 Wei, W., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H., Saag, M.S. and Shaw. G.M. (1995) *Nature*, **373**, 117–122.
7 Arts, E.J. and Wainberg. M.A. (1996) *Antimicrob. Agents Chemother*., **40**, 527–540.

8 Gao, Q., Gu, Z., Parniak, M.A., Cameron, J., Cammack, N., Boucher, C. and Wainberg. M.A. (1993) *Antimicrob. Agents Chemother.*, **37**, 1390–1392.
9 Schinazi, R.F., Lloyd, Jr., R.M., Nguyen, M.-H., Cannon, D.L., McMillan, A., Ilksoy, N., Chu, C.K., Liotta, D.C., Bazmi, H.Z. and Mellors. J.W. (1993) *Antimicrob. Agents Chemother.*, **37**, 875–881.
10 Tisdale, M., Kemp, S.D., Parry, N.G. and Larder. B.A. (1993) *Proc. Natl. Acad. Sci. USA.*, **90**, 5653–5656.
11 Gu, Z., Gao, Q., Li, X., Parniak, M.A. and Wainberg. M.A. (1992) *J. Virol*., **66**, 7128–7135.
12 Schuurman, R., Nijhuis, M., Van Leeuwen, R., Schipper, P., de Jong, D., Collis, P., Danner, S.A., Mulder, J., Loveday, C., Christopherson, C., Kwok, S., Sninsky, J. and Boucher. C.A.B. (1995) *J. Infect. Dis*., **171**, 1411–1419.
13 Ingrand, D., Weber, J., Boucher, C.A.B., Loveday, C., Robert, C., Hill, A., Cammack, N. and The Lamivudine European HIV Working Group. (1995) *AIDS*, **9**, 1323–1329.
14 Larder, B.A., Kemp, S.D. and Harrigan. P.R. (1995) *Science*, **269**, 696–699.
15 Wakefield, J.K., Jablonski, S.A. and Morrow. C.D. (1992) *J. Virol*., **66**, 6806–6812.
16 Pandey, V.N., Kaushik, N., Rege, N., Sarafianos, S.G., Yadav, P.N.S. and Modak. M.J. (1996) *Biochemistry*, **35**, 2168–2179.
17 LeGrice, S.F.J. (1993) Skalka, A.M. and Goff. S.P. (ed.), *Reverse Transcriptase*, Cold Spring Harbor Laboratory Press, Plainview, NY, pp.163–191.
18 Bakhanashvili, M. and Hizi. A. (1992) *Biochemistry*, **31**, 9393–9398.
19 Ji, J. and Loeb. L.A. (1992) *Biochemistry*, **31**, 954–958.
20 Hübner, A., Kruhoffer, M., Grosse, F. and Krauss. G. (1992) *J. Mol. Biol*., **223**, 595–600.
21 Wainberg, M.A., Drosopoulos, W.C., Salomon, H., Hsu, M., Borkow, G., Parniak, M.A., Gu, Z., Song, Q., Manne, J., Islam, S., Castriota, G. and Prasad. V.R. (1996) *Science*, **271**, 1282–1285.
22 Gu, Z., Arts, E.J., Parniak, M.A. and Wainberg. M.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 2760–2764.
23 LeGrice, S.F.J. and Grüninger-Leitch. F. (1990) *Eur. J. Biochem*., **187**, 307–314.
24 Sambrook, J., Fritsch, E.F. and Maniatis. T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25 Bakhanashvili, M. and Hizi. A. (1992) *FEBS Lett.*, **306**, 151–156.
26 Telesnitsky, A. and Goff. S.P. (1993) In Skalka, A.M. and Goff. S.P., eds, *Reverse Transcriptase*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 49–83.
27 Burns, D.P.W. and Temin. H.M. (1994) *J. Virol*., **68**, 4196–4203.
28 Boyer, J.C., Bebenek, K. and Kunkel. T.A. (1992) *Proc. Natl. Acad. Sci. USA.*, **89**, 6919–6923.
29 Bebenek, K., Abbotts, J., Roberts, J.D., Wilson, S.H. and Kunkel. T.A. (1989) *J. Biol. Chem.*, **264**, 16948–16956.
30 Bebenek, K., Abbotts, J., Wilson, S.H. and Kunkel. T.A. (1993) *J. Biol. Chem*., **268**, 10324–10334.
31 Klarmann, G.J., Schauber, C.A. and Preston. B.D. (1993) *J. Biol. Chem*., **268**, 9793–9802.
32 Majumdar, C., Abbotts, J., Broder, S. and Wilson. S.H. (1988) *J. Biol. Chem.*, **263**, 15657–15665.
33 Kavlick, M.F., Shirasaka, T., Kojima, E., Pluda, J.M., Hui, Jr., F., Yarchoan, R. and Mitsuya. H. (1995) *Antiviral Res*., **28**, 133–146.
34 Back, N.K.T., Nijhuis, M., Keulen, W., Boucher, C.A.B., Oude Essink, B.B., van Kuilenburg, A.B.P., van Gennip, A.H. and Berkhout, B. (1996) *EMBO J*., **15**, 4040–4049.
35 Oude Essink, B.B., Back, N.K.T. and Berkhout, B. (1997) *Nucleic Acids Res.*, **25**, 3212–3217.