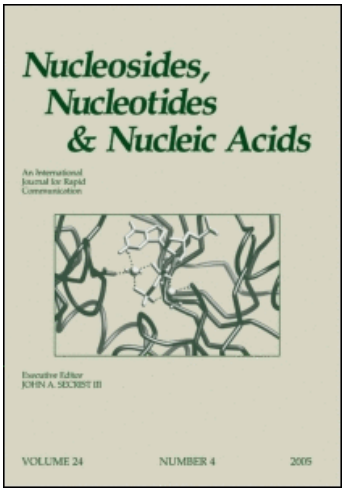

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

HIGHLY EFFICIENT RANDOM MUTAGENESIS IN TRANSCRIPTION-REVERSE-TRANSCRIPTION CYCLES BY A HYDROGEN BOND AMBIVALENT NUCLEOSIDE 5'-TRIPHOSPHATE ANALOGUE: POTENTIAL CANDIDATES FOR A SELECTIVE ANTI-RETROVIRAL THERAPY

K. Moriyama[a]; C. Otsuka[a]; D. Loakes[b]; K. Negishi[a]
[a] Gene Research Center, Okayama University, Tsushima, Okayama, Japan [b] Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK

Online publication date: 31 July 2001

To cite this Article Moriyama, K. , Otsuka, C. , Loakes, D. and Negishi, K.(2001) 'HIGHLY EFFICIENT RANDOM MUTAGENESIS IN TRANSCRIPTION-REVERSE-TRANSCRIPTION CYCLES BY A HYDROGEN BOND AMBIVALENT NUCLEOSIDE 5'-TRIPHOSPHATE ANALOGUE: POTENTIAL CANDIDATES FOR A SELECTIVE ANTI-RETROVIRAL THERAPY', Nucleosides, Nucleotides and Nucleic Acids, 20: 8, 1473 — 1483
To link to this Article: DOI: 10.1081/NCN-100105242
URL: http://dx.doi.org/10.1081/NCN-100105242

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(8), 1473–1483 (2001)

HIGHLY EFFICIENT RANDOM MUTAGENESIS IN TRANSCRIPTION-REVERSE-TRANSCRIPTION CYCLES BY A HYDROGEN BOND AMBIVALENT NUCLEOSIDE 5′-TRIPHOSPHATE ANALOGUE: POTENTIAL CANDIDATES FOR A SELECTIVE ANTI-RETROVIRAL THERAPY

K. Moriyama,[1] C. Otsuka,[1] D. Loakes,[2] and K. Negishi[1,*]

[1]Gene Research Center, Okayama University, Tsushima, Okayama 700-8530, Japan
[2]Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

ABSTRACT

The nucleoside P can base pair with both A and G. We evaluated the mutation frequency induced by the 5′-triphospbate of the ribonucleoside P (PTP) in an *in vitro* retroviral replication model. After 4 cycles of replication in the presence of PTP, the mutation frequency was raised to 3.8×10^{-2} per nucleotide and C-to-U and U-to-C mutations were dominantly observed. These results suggest that ambivalent NTP analogues, like PTP, could induce mutations beyond the error threshold of retroviruses.

*Corresponding author. Fax: +81-86-251-7264; E-mail: knegishi@cc.okayama-u.ac.jp

INTRODUCTION

The very high spontaneous mutation frequency and replication rate of RNA viruses make control of diseases caused by them difficult. In the case of human immunodeficiency virus (HIV), vaccine therapy has not yet become available, and many drug resistant strains have appeared due to mutations of the virus[1–3]. On the other hand, Domingo[4] suggests that there should be the minimum limit on the copying fidelity, what is called, "an error threshold" and that RNA viruses exist near the limit. It has been observed that the viral production and fitness of vesicular stomatitis virus (VSV)[5] and foot-and-mouth disease virus (PMDV)[6] were decreased in the presence of chemical mutagens as a result of violation of the error threshold toward extinction. These results indicate that selective and excess mutation induction of RNA viruses could be a new strategy for selective antiviral drug therapies.

The genetic information of retroviruses is propagated to the next generation in three stages, transcription, cDNA synthesis and the amplification of integrated DNA[7]. By comparing this propagation pathway with that of host cells, transcription is involved only in the case of retroviruses, since the genetic information of the host cell is propagated by DNA-dependent DNA replication. For this reason, errors in transcription should cause specific retroviral mutagenesis. Therefore, if we can induce frequent mutations *via* transcriptional errors, this may be a new strategy to attack retroviruses selectively. This is in contrast to the anti-retroviral effect of many nucleoside analogues, which are, in effect, chain terminators[8–11].

The base analogue, 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazin-7-one (P), has a tautomeric constant (K_T) of the order $10-30$[12]. It therefore forms stable base-pairs with both A and G (Fig. 1)[13–15]. Biological properties of the 2′-deoxyribonudeoside of P (dP) and oligodeoxyribonucleotides contaning dP, and its applications have been investigated in depth[12,16–18]. The nucleoside dP induces G:C-to-A:T and A:T-to-G:C transition mutations

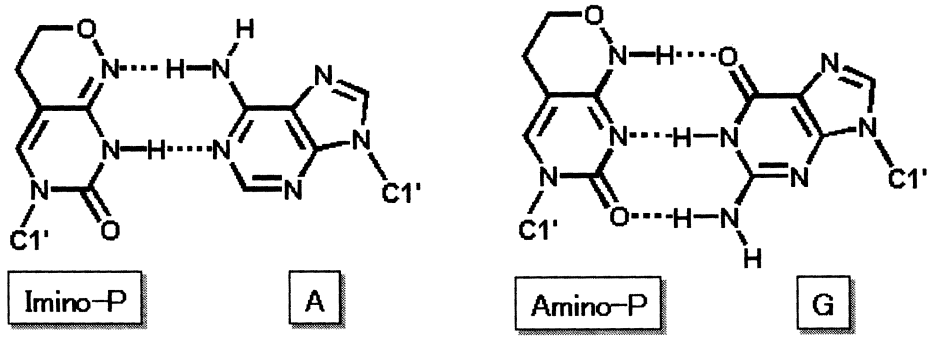

Figure 1. Structures of base-pairing of P with A and G.

potently in *Escherichia coli*[19]. This mutagenesis may be caused by ambiguous incorporation of the 5′-triphosphate of dP (dPTP), which is a metabolite of dP in the cell, opposite both A an G by a DNA polymerase. The subsequent replication of dP in the template fixes the mutation (Fig. 2*A*). Zaccolo *et al.*[20] showed that PCR in the presence of dPTP, and the normal dNTPs, could lead to very high frequency random transition mutagenesis. This was much more efficient than 8-oxo-dGTP-induced PCR transversion mutagenesis used in the same strategy.

Recently, the ribonucleoside 5′-triphosphate of P (PTP) was synthesized and this was found to be a good substrate for RNA polymerases[21]. Various phage RNA polymerases[21] and *E. coli* RNA polymerase (Moriyama, *et al.*, unpublished data) incorporated PTP in place of both UTP and CTP. We considered that by a mechanism similar to the dPTP-induced mutagenesis, introduction of such ambiguous ribonuoleoside 5′-triphosphate (NTP) analogues into RNA transcripts might randomly induce mutations in retroviruses specifically (Fig. 2B).

To investigate the PTP-induced mutagenesis, we have constructed a retroviral replication model *in vitro*. The ability of PTP to induce mutations in this system was investigated by a *Xho*I digestion assay, as well as transcription and cDNA sequencing after cloning.

MATERIALS AND METHODS

***In vitro* Transcription.** PTP was prepared as described previously[21]. One μg of purified plasmid DNA, 500 μM each of four NTPs (ATP, CTP, GTP and UTP) with or without PTP, 100 U recombinant RNasin (Promega), 5 U T7 RNA polymerase (Promega), 40 mM Tris-HCl (pH 7.9), 10 mM NaCl,

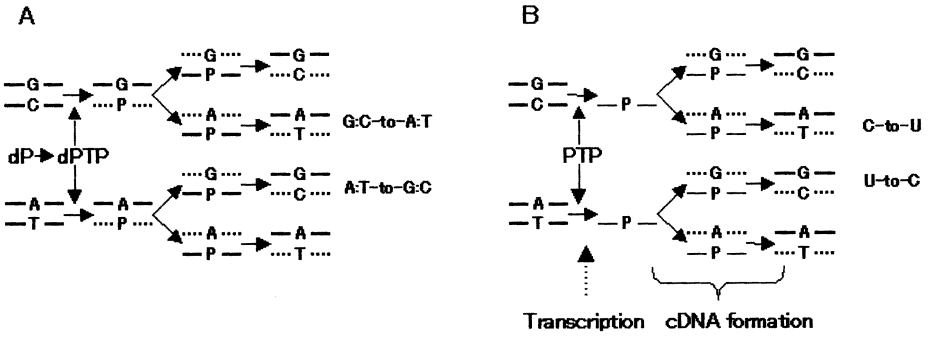

Figure 2. Possible pathways of dP-lnduced mutagenesis based on DNA-dependent-DNA-replication machinery (A) and PTP-induced mutagenesis based on retrovirus replication machinery (B). Dotted lines indicate newly synthesized DNA strand. Thick lines and thin lines indicate DNA and RNA, respectively.

6 mM $MgCl_2$, 2 mM spermidine in 20 μl were incubated at 37 °C for 1 hour. Plasmid DNA was then digested by the treatment with 2 U RQ1 RNase-free DNase (Promega) for 30 min.

RT-PCR. Portions (2 μl) of the above transcripts were mixed with 200 μM each of four dNTPs (dATP, dCTP, dGTP and TTP), 50 pmol "*Sac*" primer (5′-AAAGCTGGAGCTCCACCGCG-3′), 50 pmol "*Kpn*" primer (5′-CGAATTGGGTACCGGGCCCC-3′), 5 U AMV reverse transcriptase (Promega), and 5 U *Tfl* DNA polymerase (Promega) in 1× AMV/*Tfl* reaction buffer (Promega) in the total volume of 50 μl. These two primers are complementary to the *Sac*I and *Kpn*I sites of pBluescriptII SK(+), respectively. The mixture was incubated at 48 °C for 45 min. After heat inactivation (94 °C, 2 min), the mixture was cycled (94 °C 30 sec, 60 °C 1 min, 68 °C 2 min, ×10 cycles).

Plasmid Reconstruction and Amplification in *E. coli*. One μg of pBluescriptII SK(+) was digested with 10 U *Sac*I and 10 U *Kpn*I at 37 °C for 3 hours and purified by 1% agarose gel electrophoresis. The linearized DNA extracted from the gel was treated with 0.05 U alkaline phosphatase. A portion of RT-PCR solution containing 1 μg of the amplified fragment was also treated with *Sac*I and *Kpn*I under the same conditions. A mixture of 40 ng of the digested RT-PCR fragment and 100 ng of the vector DNA were incubated for ligation with 1.5 U T4 DNA ligase at 1.6 °C for 12 hours. The ligated DNA was introduced into competent *E. coli* DH5α cells by electroporation under standard conditions (1.5 kV, 25 μF, and 800 ohm), and 800 μl of SOC medium was added immediately. After incubation at 37 °C for 1 hour, 100 μl aliquot of the culture, which contained about 350 independent transformants, was inoculated into 10 ml LB medium containing 50 μg/ml ampicillin and incubated for 18 hours. Plasmid DNA was then purified from the culture.

XhoI Digestion Assay. 0.5 μg of purified plasmids were incubated with 10 U *Xho*I at 37 °C for 3 hours. A portion (0.2 μg DNA) of the solution was loaded onto 1% agarose gel to separate electrophoretically undigested (circular) plasmid from digested (linear) plasmid. DNA was visualized by ethidium bromide staining.

Cloning and DNA Sequencing. *E. coli* DH5α cells were transformed by electroporation with 0.1 ng of purified plasmid in the same condition as described above. After incubation for 1 hour in SOC medium, 100 μl of the culture was plated onto LB plate containing 50 μg/ml ampicillin and incubated overnight at 37 °C. Ten well separated colonies were picked and inoculated into 3 ml LB medium containing 50 μg/ml ampicillin. Cloned plasmids were

purified from the overnight culture. Purified plasmids were then applied to the cycle sequencing reaction (95 °C 30 sec, 60 °C 30 sec, 72 °C 1.5 min, ×25 cycles) using ThermoSequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) with T7 sequencing primer (5′-(Cy5)-AGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGG-3′). DNA sequencing was carried out using an ALF*express* DNA sequencer (Amersham Pharmacia Biotech).

RESULTS

PTP-Mediated Induction of Mutations and its Accumulation during the Model Replication Cycles

One cycle of the retroviral replication model is shown in Fig. 3. This model consists of three stages, transcription, cDNA formation, and insertion into the carrier DNA, mimicking the retroviral life cycle. The model "genome" is an 82 nucleotide long region encompassing the *Kpn*I and *Sac*I sites of pBluescriptII SK(+). This region is transcribed from the T7 promoter adjacent to it.

The plasmid was transcribed in the absence or presence of PTP, together with the four normal NTPs, with T7 RNA polymerase. The RNA transcripts were reverse transcribed using AMV reverse transcriptase, and then the area

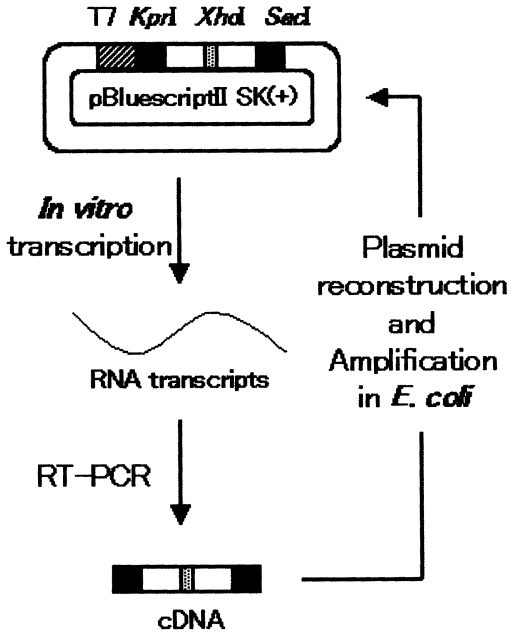

Figure 3. One cycle of retrovirus replication model *in vitro*.

between *Sac* and *Kpn* primer was converted to double strand DNA by PCR using *Tfl* DNA polymerase. This sequence (82 nt.) between the two primer sites was the target for mutagenesis induced by PTP. The RT-PCR product was then ligated into pBluscriptII SK(+) vector DNA and this reconstructed plasmid was amplified in *E. coli* to obtain purified plasmid used for the next cycle. This represents one cycle of our retroviral replication model.

To measure mutation induction by PTP in this model, one cycle of replication in the presence of PTP at various ratios of PTP and normal NTP ([PTP]/[NTP] ratio) was carried out. Mutations were detected by *Xho*I digestion of the purified plasmid, because mutations in this restriction site (CTCGAG) would confer the resistance to *Xho*I digestion and prevent plasmid linearization. Fig. 4 shows that the ratio of circular ("C" in Fig. 4) to linear form increased with the concentration of PTP, indicating that PTP induced mutations at the *Xho*I site in a concentration-dependent manner. It should be noted that only very efficient mutagenesis can give positive results in this assay, since it represents mutations at only 3 (CTC in the restriction site) out of the 82 nucleotides of the target sequence. This mutation induction is considered to be by the mechanism shown in Fig. 2*B*. As PTP is not likely to be incorporated into DNA by either reverse transcriptase or DNA-dependent-DNA-polymerases (*Tfl* DNA polymerase in this system), mutations due to PTP are not likely to arise during PCR reactions. Thus, PTP has mutation-inducing ability in this *in vitro* model during transcription and reverse transcription.

Three further cycles of replication were carried out in the absence or presence of PTP at [PTP]/[NTP] ratio of 1.0. The result of agarose gel electrophoresis after *Xho*I digestion is shown in Fig. 5. While plasmid digested completely by *Xho*I was obtained after four cycles in the absence of PTP, the ratio of circular form increased during successive cycles in the presence of PTP. This indicates that mutations increasingly accumulated at the *Xho*I site by PTP during the four cycles of the replication.

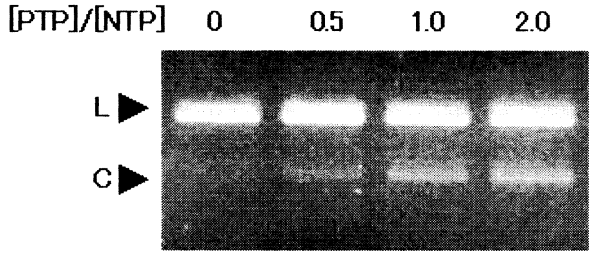

Figure 4. Analysis of mutation induction on *Xho*I site of purified plasmid after one cycle of retrovirus replication model *in vitro* in the presence of PTP at [PTP]/[NTP] ratio of 0–2.0 by 1% agarose ge1 electrophoresis. "L" and "C" indicate linear form and circular form of plasmid, respectively.

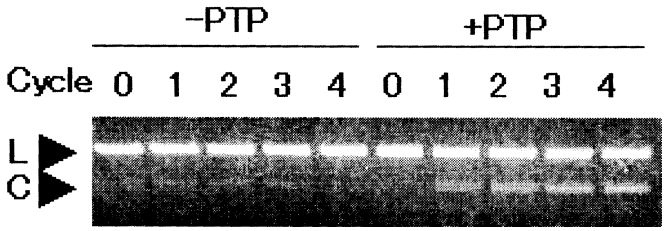

Figure 5. Analysis of mutation accumulation on *Xho*I site of purified plasmid after 0, 1, 2, 3 and 4 cycle(s) of retrovirus replication model *in vitro* in the absence and presence of PTP at [PTP]/[NTP] ratio of 1.0 by 1% agarose gel electrophoresis. "L" and "C" indicate linear form and circular form of plasmid, respectively.

Spectrum of PTP-induced Mutagenesis

Plasmids were cloned after each of the 4 cycles in the presence and absence of PTP. Ten plasmids per cycle were sequenced to evaluate the mutation frequency. In the absence of PTP as control, only three mutations were induced in the 82 nt. target sequence (Table), and this is assumed to be due to polymerase error during one of the three copying events. Fig. 6 shows the mutations in the target area occurring during each cycle in the presence of PTP. More than 90% of the detected mutations were either C-to-T or T-to-C transitions. Four A-to-G transitions and one G-to-T transversions were also detected. As the latter cannot readily be accounted for by PTP, it presumably arose due to error in the incorporation of normal dNTPs. Mutations detected are listed in the Table. Mutation frequencies at each cycle were calculated by dividing the number of point mutations by total nucleotides sequenced. Like

Table. Spectrum of Mutation Induced on RNA Transcripts by PTP in Each Cycle

		−PTP				+PTP				
	0	1	2	3	4	1	2	3	4	(cycle)
Base substitution										
Transition										
C → U	0	0	0	0	0	2	3	7	18	
U → C	0	0	0	0	0	2	3	2	11	
A → G	0	1	0	0	0	0	1	2	1	
Transversion										
C → A	0	0	0	0	2	0	0	0	0	
G → U	0	0	0	0	0	0	0	1	0	
Deletion	0	0	0	0	0	0	0	0	1	(△30 nt.)
Total	0	1	0	0	2	4	7	12	31	
Sequenced (nucleotides)	820	820	820	820	820	820	820	820	790	

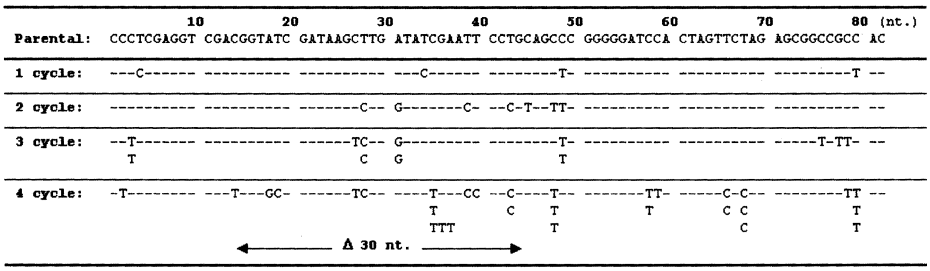

Figure 6. Mutation spectrum at the target site of ten cloned plasmids after 1, 2, 3 and 4 cycle of retrovirus replication model *in vitro* in the presence of PTP. Mutated bases are listed below the parental DNA sequence of the target site.

the *Xho*I digestion assay shown in Fig. 5, the mutation frequency increased during each of the 4 cycles and reached 3.8×10^{-2} per nucleotide after 4 cycles (Fig. 7). C-to-U mutation (2.3×10^{-2} per nucleotide was more frequent than U-to-C (1.4×10^{-2} per nucleotide) after the fourth cycle.

During PCR amplification, which is DNA-dependent-DNA-replication like genomic replication in the cell, dPTP induced G:C-to-A:T and A:T-to-G:C transition mutations frequently[20]. The mutation frequency of dPTP-induced PCR mutagenesis was $\sim 10 \times 10^{-2}$ per nucleotide over 30 cycles, and is higher than any other dNTP analogue-induced PCR mutagenesis[22].

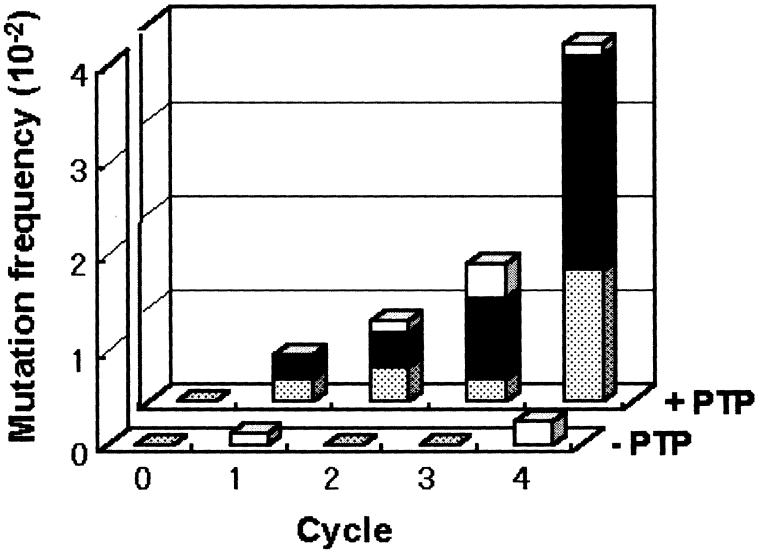

Figure 7. Frequency of point mutation on RNA transcripts in each cycle of retrovirus replication model *in vitro* in the absence and presence of PTP. C-to-U, U-to-C and the other point mutation are indicated by filled box, dotted box and open box, respectively.

Taking these results together, we conclude that PTP should be a potent random mutation inducer in retroviral replication. Since frequent mutation induction during transcription may be selective for the retrovirus as discussed earlier, the mutation induction by PTP is clearly a candidate for this novel anti-retroviral strategy.

DISCUSSION

In this work, we have investigated a new possible anti-retroviral strategy excess mutation induction specifically to the retrovirus. Loeb *et al.*[23] reported a similar trial using the mutagenic deoxyriboside analogue, 5-hydroxydeoxycytidine (5-OH-dC). 5-OH-dC was metabolized in cells to the corresponding triphosphate, 5-OH-dCTP, which can be incorporated into DNA by reverse transcriptase. As 5-OH-dC can base pair with A as well as G, mutations will be fixed by cDNA synthesis through 5-OH-dC. Although 5-OH-dCTP may also be incorporated into the genomic DNA of the host cells, this might be removed by repair systems in the cell. In the presence of 5-OH-dC, HIV lost viability during the sequential passage in human CEM cells[23].

Loeb and Mullins[24] have pointed out how a mutagenic ribonucleoside is to be considered as a potentially anti-retroviral agent in terms similar to those described above with kinase steps to generate the analogue triphosphate. We previously suggested that ambiguous ribonucleoside triphosphates may be used against retroviruses[21, 25]. In this proposal, the nucleoside *via* its 5′-triphospbate as a substrate for RNA polymerase is introduced into the viral genome leading, in subsequent rounds of replication, to an overburdening mutation rate. While such a residue could be introduced into host mRNAs, their rapid turnover[26] would only lead to short term damage. The efficient DNA repair system of the host should diminish any effect of conversion of the analogue ribonucleotide to the deoxy triphosphate analogue. The earlier work on PTP as a substrate of RNA polymerases[21] together with the present work shows that the essential features of the proposed anti-retroviral strategy are fulfilled.

The analysis of the mutation spectrum demonstrated the selective induction of U-to-C and C-to-U mutations by PTP (Table and Fig. 7). As described schematically in Fig. 2*B*, this result is consistent with our earlier observation that T7 RNA polymerase incorporates PTP in place of both UTP and CTP[21]. From the ambiguous base pairing ability of P[13–15, 18], it is also expected that both dATP and dGTP are incorporated opposite rP in RNA transcripts by a reverse transcriptase, thereby causing additional mutagenic errors. Future work is required to evaluate the contribution of this step.

Although P-riboside may be taken up into infected cells and converted to PTP, phosphorylated P could be directly introduced to minimize the mutation induction to host DNA or inhibition of kinase steps of normal

nucleosides. Monnard *et al.*[27] and Szebeni *et al.*[28] have studied the entrapment of small nucleic acid monomer like ATP in liposomes. Furthermore, drug delivery systems into infected cells by liposomes in order to introduce phosphorylated species have been developing[29–32]. On the other hand, to bypass the first kinase step, S-Acyl-2-thioethyl (SATE) modified AZT 5′-monophosphate and its derivatives have been synthesized and the effect of these compounds on HIV-infected cells has been reported[33–36]. Using these techniques, we could induce mutations selectively in retroviruses with minimal effects to host cells. Together with the suggestion by Domingo *et al.* that excess mutation induction of RNA viruses, which exist near an error threshold, would result in their extinction[4–6], ambiguous ribonucleoside derivatives may be new candidates for a selective antiretroviral therapy.

ACKNOWLEDGMENTS

We thank the Japan Society for the Promotion of Science Grants-in-aid for JSPS fellows (no. 7807) (to KM) and Nycomed Amersham plc (to DL) for financial assistance, Dr. Dan Brown for his advice and helpful discussions and Prof. Hikoya Hayatsu for kind support.

REFERENCES

1. Shirasaka, T.; Yarchoan, R.; O'Brien, M.C.; Husson, R.N.; Anderson, B.D.; Kojima, E.; Shimada, T.; Broder, S.; Mitsuya, H. Proc. Natl. Acad. Sci. USA **1993**, *90*, 562–566.
2. Shirasaka, T.; Kavlick, M.F.; Ueno, T.; Gao, W.Y.; Kojima, E.; Alcaide, M.L.; Chokekijchai, S.; Roy, B.M.; Arnold, E.; Yarchoan, R.; Mitsuya, H. Proc. Natl. Acad. Sci. USA **1995**, *92*, 2398–2402.
3. Ueno, T.; Mitsuya, H. Biochemistry **1997**, *36*, 1092–1099.
4. Domingo, E. Virology **2000**, *270*, 251–253.
5. Lee, C.H.; Gilbertson, D.L.; Novella, I.S.; Huerta, R.; Domingo, E.; Holland, J.J. J. Virol. **1997**, *71*, 3636–3640.
6. Sierra, S.; Davila, M.; Lowenstein, P.R.; Domingo, E. J. Virol. **2000**, *74*, 8316–8323.
7. Coffin, J.M.; Hughes, S.H.; Varmus, H.E. (ed.) *Retroviruses*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor NY, 1997.
8. Langtry, H.D.; Campoli-Richards, D.M. Drugs **1989**, *37*, 408–450.
9. Clumeck, N.J. Antimicrob. Chemother. **1993**, *32*, 133–138.
10. Adkins, J.C.; Peters, D.H.; Faulds, D. Drugs **1997**, *53*, 1054–1080.
11. Perry, C.M.; Noble, S. Drugs **1999**, *58*, 1099–1135.
12. Hill, F.; Loakes, D.; Brown, D.M. Proc. Natl. Acad. Sci. USA **1998**, *95*, 4258–4263.
13. Kong Thoo Lin, P.; Brown, D.M. Nucleic Acids Res. **1989**, *17*, 10373–10383.
14. Stone, M.J.; Nedderman, A.N.R.; Williams, D.H.; Kong Thoo Lin, P.; Brown, D.M. J. Mol. Biol. **1991**, *222*, 711–723.

15. Nedderman, A.N.R.; Stone, M.J.; Williams, D.H.; Kong Thoo Lin, P.; Brown, D.M. J. Mol. Biol. **1993**, *230*, 1068–1076.
16. Kong Thoo Lin, P.; Brown, D.M. Nucleic Acids Res. **1992**, *20*, 5149–5152.
17. Loakes, D.; Brown, D.M.; Mahmood, N.; Balzarni, J.; De Clercq, E. Antiviral Chem. Chemother. **1995**, *6*, 371–378.
18. Moriyama, K.; Williams, D.M.; Loakes, D.; Brown, D.M.; Hayatsu, H.; Negishi, K. Nucleic Acids Symp. Ser. **1998**, *39*, 245–246.
19. Negishi, K.; Williams, D.M.; Inoue, Y.; Moriyama, K.; Brown, D.M.; Hayatsu, H. Nucleic Acids Res. **1997**, *25*, 1548–1552.
20. Zaccolo, M.; Williams, D.M.; Brown, D.M.; Gherardi, E. J. Mol. Biol. **1996**, *255*, 589–603.
21. Moriyama, K.; Negishi, K.; Briggs, M.S.J.; Smith, C.L.; Hill, F.; Churcher, M.J.; Brown, D.M.; Loakes, D. Nucleic Acids Res. **1998**, *26*, 2105–2111.
22. Hill, F.; Williams, D.M.; Loakes, D.; Brown, D.M. Nucleic Acids Res. **1998**, *26*, 1144–1149.
23. Loeb, L.A.; Essigmann, J.M.; Kazazi, F.; Zhang, J.; Rose, K.D.; Mullins, J.I. Proc. Natl. Acad. Sci. USA **1999**, *96*, 1492–1497.
24. Loeb, L.A.; Mullins, J.I. AIDS Res. Hum. Retroviruses **2000**, *16*, 1–3.
25. Moriyama, K.; Kikkawa, Y.; Loakes, D.; Negishi, K. Nucleic Acids Symp. Ser. **1999**, *42*, 131–132.
26. Ross, J. Trends Genet. **1996**, *12*, 171–175.
27. Monnard, P.-A.; Oberholzer, T.; Luisi, P. Biochim. Biophys. Acta **1997**, *1329*, 39–50.
28. Szebeni, J.; Wahl, S.M.; Betageri, G.V.; Wahl, L.M.; Gartner, S.; Popovic, M.; Parker, R.J.; Black, C.D.V.; Weinstein, J.N. AIDS Res. Hum. Retrovirus **1990**, *6*, 691–702.
29. Betageri, G.V.; Black, C.D.V.; Szebeni, J.; Wahl, L.M.; Weinstein, J.N. J. Pharm. Pharmacol. **1993**, *45*, 48–53.
30. Zelphati, O.; Degols, G.; Loughrey, H.; Leserman, L.; Pompon, A.; Puech, F.; Maggio, A.-F.; Imabach, J.-L.; Gosselin, G. Antiviral Res. **1993**, *21*, 181–195.
31. Desormeaux, A.; Bergeron, M.G. J. Drug Target. **1998**, *6*, 1–15.
32. Oussoren, C.; Magnani, M.; Fraternale, A.; Casabianca, A.; Chiarantini, L.; Ingebrigsten, B.; Underberg, W.J.M.; Storm, G. Int. J. Pharm. **1999**, *180*, 261–270.
33. Beltran, T.; Egron, D.; Lefebvre, I.; Perigaud, C.; Pompon, A.; Gosselin, G.; Aubertin, A.M.; Imbach, J.L. Nucleosides Nucleotides **1999**, *18*, 973–975.
34. Egron, D.; Arzumanov, A.A.; Dyatkina, N.B.; Krayevsky, A.; Imbach, J.L.; Aubertin, A.M.; Gosselin, G.; Perigaud, C. Nucleosides Nucleotides **1999**, *18*, 983–984.
35. Egron, D.; Perigaud, C.; Gosselin, G.; Aubertin, A.M.; Imbach, J.L. Nucleosides Nucleotides **1999**, *18*, 981–982.
36. Parang, K.; Wiebe, L.I.; Knaus, E.E. Curr. Med. Chem. **2000**, *7*, 995–1039.

Received: November 15, 2000
Accepted: December 27, 2000