Template properties of mutagenic cytosine analogues in reverse transcription

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ABSTRACT
We have studied the mutagenic properties of ribonucleotide analogues by reverse transcription to understand their potential as antiretroviral agents by mutagenesis of the viral genome. The templating properties of nucleotide analogues including 6-(β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c](1,2)oxazin-7-one, N⁴-hydroxycytidine, N⁴-methoxycytidine, N⁴-methylcytidine and 4-semicarbazidocytidine, which have been reported to exhibit ambiguous base pairing properties, were examined. We have synthesized RNA templates using T3 RNA polymerase, and investigated the specificity of the incorporation of deoxyribonucleoside triphosphates opposite these cytidine analogues in RNA by HIV and AMV reverse transcriptases. Except for N⁴-methylcytidine, both enzymes incorporated both dAMP and dGMP opposite these analogues in RNA. This indicates that they would be highly mutagenic if present in viral RNA. To study the basis of the differences among the analogues in the incorporation ratios of dAMP to dGMP, we have carried out kinetic analysis of incorporation opposite the analogues at a defined position in RNA templates. In addition, we examined whether the triphosphates of these analogues were incorporated competitively into RNA by human RNA polymerase II. Our present data supports the view that these cytidine analogues are mutagenic when incorporated into RNA, and that they may therefore be considered as candidates for antiviral agents by causing mutations to the retroviral genome.

INTRODUCTION
Genetic diversities of retroviruses and RNA viruses caused by their higher mutation rates allow the viral population to escape host immune responses, resulting in rapid antiviral drug resistance (1,2). The higher mutation rates may result in many defective virions, because deleterious mutations in essential genes should also take place (2). Thus, it has been suggested that these viruses exist near their ‘error thresholds’, maximal mutation rates to sustain production of infectious progenies (3). Induction of mutations exceeding these thresholds could lead these viruses to extinction, which is termed error catastrophe or lethal mutagenesis (4–7). Among the various types of mutagen, nucleotide analogues, which do not incorporate into DNA, may be suitable for this purpose. Therefore, it is important to explore the use of such analogues for their potential to serve as novel antiviral agents. Indeed, it has been suggested that ribavirin, a broad-spectrum antiviral ribonucleoside, forces poliovirus and hantavirus into error catastrophe (4,8,9). A small increase in the mutation frequency by 5-azacytidine or 5-fluorouracil causes dramatic reduction in the survival levels of poliovirus, vesicular stomatitis virus, foot-and-mouth disease virus and lymphohytic choriomeningitis virus (10–13). In retroviruses, experiments with 5-hydroxy-2'-deoxycytidine (5-OH-dC) and 5-aza-5, 6-dihydro-2'-deoxycytidine (KP-1212) it has been demonstrated that it is possible to increase mutation rates and eradicate HIV by repeated passages in the presence of these deoxyribonucleoside analogues (6,14). However, deoxyribonucleoside analogues may also be incorporated into host genomic DNA, and mutations in the host genome may be induced. Unlike deoxynucleosides, ribonucleoside analogues are not directly incorporated into the host genomic DNA. Ribonucleoside analogues can be incorporated into cellular RNA resulting in altered proteins. However, it will be a transient aberration, because mRNAs have short half-lives and proteins tolerate a wide variety of mutations (15–19).

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Therefore, ribonucleosides might be more suitable for use in error catastrophe than deoxynucleosides (20). We have studied the mutagenic properties of several ribonucleotide triphosphate analogues using a reverse transcription assay to determine their mutagenic potential; mutagenic analogues can be considered as potential antiretroviral agents by inducing mutations to the viral genome, which in turn leads to lethal mutagenesis.

6-((β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido[4,5-c] (1,2) oxazin-7-one triphosphate (rPTP) has been shown to induce U-to-C and C-to-U transition mutations in an in vitro retroviral replication model using phage T3 RNA polymerase and AMV reverse transcriptase (21). It was reported that the triphosphates of N4-hydroxycytidine (ho4C), N4-methoxycytidine (mo4C) and N4-methylcytidine (m4C) exhibited ambiguous properties in polymerization (22–24), and N4-amino deoxycytidine triphosphate can be incorporated in place of both dCTP and dTTP (25,26). We have found that 4-semicarbazidocytidine triphosphate, a N4-aminocytidine derivative, can be incorporated as UTP by phage RNA polymerase (27) (Figure 1). These N4-cytidine analogues except m4C show ambiguous base pairing properties by amino-imino tautomerism; the presence of the electronegative element on the N4-amino group reduces their tautomeric constants close to unity (28,29). In the present study, we have investigated the specificity of incorporation of deoxyribonucleoside triphosphates opposite these cytidine analogues in RNA by HIV and AMV reverse transcriptases. Except m4C, HIV and AMV reverse transcriptases incorporated both dAMP and dGMP opposite these analogues when present in RNA. This indicates that these substrates would be highly mutagenic if present in the viral RNA.

### MATERIALS AND METHODS

**Materials**

Recombinant HIV reverse transcriptase was expressed in *Escherichia coli* by using plasmid p6HRT-PROT kindly provided by Dr S. F. Le Grice of National Institutes of Health (30), and purified by Ni-NTA agarose (QIAGEN). AMV reverse transcriptase, Tfl DNA polymerase, T3 RNA polymerase, HeLa nuclear extract and calf intestinal alkaline phosphatase were purchased from Promega (Madison, WI).

**Synthesis of ribonucleoside analogues**

**N4-Hydroxycytidine 5’-triphosphate**

N4-Hydroxycytidine 5’-triphosphate was prepared as described previously (31) with a slight modification. Briefly, CTP (30 mg) was treated in 0.2 ml of 2 M hydroxylamine hydrochloride (pH 5.0) at 55°C for 5 h. The samples of ho4CTP were purified by reverse phase high-performance liquid chromatography (HPLC) using an ODS-120A (TOSOH) column eluted with 3% methanol-10 mM triethylammonium bicarbonate (pH 6.8), at 0.7 ml/min. The samples were further purified by anion exchange HPLC using a DEAE-2sw (TOSOH) column (0.06 M disodium phosphate).

**N4-Methoxycytidine 5’-triphosphate**

N4-Methoxycytidine 5’-triphosphate was similarly prepared by treating CTP with 2 M hydroxylamine hydrochloride (pH 5.0) at 55°C for 5 h.

**N4-Methylcytidine 5’-triphosphate**

N4-Methylcytidine 5’-triphosphate was similarly prepared by treating CTP with 2 M hydroxylamine hydrochloride (pH 5.0) at 55°C for 5 h.

**4-Semicarbazidocytidine 5’-triphosphate**

4-Semicarbazidocytidine 5’-triphosphate was similarly prepared by treating CTP with 2 M hydroxylamine hydrochloride (pH 5.0) at 55°C for 5 h.

**Figure 1.** Structure of N4-modified cytidine analogues used in this study.
hydrogenphosphate-20% acetonitrile (pH 6.6) and 0.7 ml/min] and then purified by reverse phase HPLC again as described above.

$N^4$-Methoxycytidine 5'-triphosphate

$N^4$-Methoxycytidine 5'-triphosphate was prepared as described previously (23), and purified by three-step HPLC as ho$^4$CTP.

$N^4$-Methylcytidine 5'-triphosphate

$N^4$-Methylcytidine 5'-triphosphate was prepared by bisulfite-catalyzed transamination of CTP (32). CTP (15 mg) was reacted in a solution (0.1 ml) containing 1 M semicarbazide hydrochloride and 0.5 M sodium bisulfite (pH 5.4) at room temperature for 4 h. To this solution was added 4 ml of 0.5 M sodium phosphate (pH 6.5) and 1.8 ml of water and the reaction kept at 37°C for 2 h. The crude sc$^4$CTP sample was diluted by addition of 80 ml water, loaded on to a DE52 column (2 x 9 cm), which was washed with 50 ml of 25 mM sodium phosphate (pH 7) and 50 ml of 25 mM sodium phosphate (pH 7) supplemented with 7 M urea. The triphosphate was eluted in a gradient from 25 mM sodium phosphate (pH 7) supplemented with 7 M urea and then purified by reverse phase HPLC as described for ho$^4$CTP.

4-Semicarbazidocytidine 5'-triphosphate

4-Semicarbazidocytidine 5'-triphosphate was prepared according to the method described for 4-semicarbazidocytidine (33) with a slight modification. CTP (15 mg) was reacted in a solution (0.1 ml) containing 1 M semicarbazide hydrochloride and 0.5 M sodium bisulfite (pH 5.4) at 37°C for 2 days. The samples of m$^4$CTP were purified by HPLC as described for ho$^4$CTP.

Synthesis of the nucleosides and nucleotides, and confirmation of the modified triphosphates

Modified ribonucleosides, ho$^6$C, mo$^6$C, sc$^6$C and m$^6$C, and their 2'/3'-monophosphates were prepared with cytidine and 2'/3'-CMP, respectively, in an analogous reaction as used for the preparation of triphosphates, ho$^6$C, mo$^6$C, sc$^6$C and sc$^6$C were shown to be >98% pure in two HPLC systems, and verified by HPLC analysis. The nucleosides were shown to be co-eluted in two HPLC systems with the nucleosides obtained by digestion of triphosphates. For the preparation of its 2'/3'-monophosphate, RNA containing rP synthesized as below was digested with RNase T2, and purified by HPLC. All the triphosphates and monophosphates showed the same ultraviolet (UV) absorbance as the nucleosides at acidic, neutral and basic pHs.

6-(β-D-Ribofuranosyl)-3,4-di hydro-8H-pyrimido [4,5-c](1,2)oxazin-7-one 5'-triphosphate (rPTP)

6-(β-D-Ribofuranosyl)-3,4-di hydro-8H-pyrimido[4,5-c](1,2)oxazin-7-one, and its 5'-triphosphate were prepared as described previously (34).

Transcription by T3 RNA polymerase

The DNA fragment of pBluescript II SK (−) digested by BssHII was purified on agarose gel as a template to produce 155 nt runoff transcripts from T3 promoter. Standard transcription reaction mixtures (20 μl) contained 40 mM Tris–HCl (pH 7.4), 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM rNTPs, 50 ng DNA template, 20 U RNase inhibitor (Wako, Osaka, Japan), and 10 U T3 RNA polymerase. The mixtures were incubated at 37°C for 1 h. For the incorporation of the cytidine analogues, they were added in place of one of four normal nucleotides in an equal or 10-fold excess concentration of the normal nucleotides. After the transcription, the DNA template was degraded by incubation at 37°C for 30 min with 2 U DNase I. The reactions were monitored by PAGE. The reactions were terminated with 1/10 volume of 20 mM EGTA, deproteinized with phenol/chloroform/isoamyl alcohol (25:24:1) and desalted by Centri-Sep spin column. These RNA transcripts were used as templates for reverse transcription (Figure 2).

Analysis of base composition

The HPLC analysis of RNA transcripts was performed as described previously (35) with slight modification. The dried transcripts were dissolved in digestion mixtures (30 μl) containing 50 mM Tris–HCl (pH 7.4), 10 mM MgCl2 and 0.4 U snake venom phosphodiesterase I. The mixtures were incubated at 37°C for 90 min. Subsequently, 0.2 U snake venom phosphodiesterase I and 2 U calf intestinal alkaline phosphatase were added to the reactions, and then incubation was continued at 37°C for 2 h. After the digestion, non-digested materials were removed by ethanol precipitation. The supernatants were dried by a SpeedVac, and then dissolved in 20 μl buffer A [100 mM potassium phosphate (pH 7.0)]. The nucleosides were separated by reverse phase HPLC with ODS column (ultrasphere ODS, BECKMAN) eluting at a flow rate of 0.7 ml/min using sequential gradients of buffers A and B: 0–5 min 0% B. 5–20 min 0–15% B. 20–35 min 15–45% B. 35–60 min

5'-GGAAACAAAGCGUGAGACTCACCACCCAUGGGCGGCAGCUAG
DNA primer 2

AACUGUGGAUCCCCCCGGCUAGGAUUGAUUCAGUAAGCUA
<= Ho-primer

GAUACCCUGCACCUGAGGGGCGGGCCACCACAAUGGUAG
<= DNA primer 1

UAGUGACUGAUUAGGCGGGGAG

Figure 2. Sequence of RNA template and primer binding site. RNA templates contain ho$^6$C, mo$^6$C, rP or sc$^6$C in place of U, and m$^6$C in place of C.
45–100% B, 60–75 min 100–100% B [buffer B: 1 mM potassium phosphate (pH 7.0) and 90% methanol]. The concentration of each nucleoside was determined by monitoring A260 and integration using D-2500 Chromato-Integrator (Hitachi).

To confirm the analogue peak, the digested products and each nucleoside analogue were co-eluted by HPLC.

Reverse transcription with HIV RT

Reverse transcription reaction by HIV reverse transcriptase was performed using RNA templates containing ho4C, mo4C, rP or sc 4C in place of U, and m 4C in place of T. One base insertion reaction by HIV reverse transcriptase was initiated by addition of 2 µl of 5× dNTP, and terminated by addition of 10 µl of loading buffer [90% formamide, 0.05% BPP and 50 mM EDTA (pH 8.0)]. The reaction products were analyzed by 7 M urea 20% denaturing PAGE. The gels were exposed to Imaging Plate and analyzed with Imaging Analyzer BAS-1800II (Fujifilm) to quantitate each band. The velocity of reaction was determined using the following equation: \( v_{rel} = 100 I_0 / (I_0 + 0.5 I_t) \), where \( I_0 \) is intensity of the unextended band, \( I_t \) is intensity of the extended band and \( t \) is reaction time (36). The reaction time was chosen to be in the linear range. Graphs of r versus dNTP concentration were analyzed using non-linear regression in KaleidaGraph 4.0 for the determination of Michaelis constant (Km) and relative maximum velocity (Vmax, rel). The frequency values of insertion (fins) and extension (fext) were defined by a ratio of Vmax, rel/Km for each base pair to Vmax, rel/Km for dA:U pair.

Transcription by human RNA polymerase II and the nearest-neighbor analysis

RNA transcription by human RNA polymerase II was performed using HeLa Nuclear Extract (Promega). A PCR product (1140 bp) containing CMV immediate early promoter and supF gene amplified from pCMV-supF plasmid was used as a template for runoff transcription. A standard reaction mixture (25 µl) contained 20 mM HEPEs (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 3 mM MgCl2, 0.4 mM ATP, CTP and UTP, 0.016 mM GTP, 450 ng DNA template, 40 U RNase inhibitor, 10 µCi [α-32P]GTP (3000 Ci/mmol) and 8 U HeLa nuclear extract. To study incorporation of the ribonucleoside triphosphate analogues, they were added in place of CTP or UTP. The mixtures were incubated at 30°C for 1 h. The transcripts were deproteinized with phenol/chloroform/isooamyl alcohol (25:24:1) extraction and purified with Centri-Sep spin column. The transcripts were separated by 7 M urea 5% denaturing polyacrylamide gel, and analyzed with Imaging Analyzer BAS-1800II (Fujifilm).

For the nearest-neighbor analysis, the RNA transcripts were digested in 10 µl of 15 mM sodium acetate (pH 4.5), 1.5% glycerol and 1 U RNase T2 at 37°C for 14 h. To the dried reaction mixtures, 0.25 A260 U of non-isotopic 2',3'-NMPs were added as standards, and the digests were analyzed by two-dimensional cellulose TLC (Funacel SF 10 × 10 cm) with the use of isobutyric acid/ammonia/water (66:1:33, v/v/v) for the first dimension, and ammonium sulfate/0.1 M sodium phosphate (pH 6.8)/n-propanol (60:100:2, wt/v/v) for the second dimension. The TLC products were deproteinized with phenol/chloroform/isooamyl alcohol (25:24:1) extraction and purified with Centri-Sep spin column. The transcripts were separated by 7 M urea 5% denaturing polyacrylamide gel, and analyzed with Imaging Analyzer BAS-1800II (Fujifilm).
plates were analyzed with Imaging Analyzer BAS-1800II (Fujifilm).

RESULTS
Incorporation of ribonucleotide analogues in transcription by T3 RNA polymerase
Incorporation of the 5'-triphosphates of the N4-modified cytidine analogues, ho4C, mo4C, m4C, rP and sc4C, into RNA by T3 RNA polymerase was examined. Transcription was performed using reaction mixtures where one of the four triphosphates was replaced by an analogue. RNA transcripts were produced when ho4CTP and mo4CTP were used in place of UTP, and when m4CTP was used in place of CTP. Other combinations, e.g. ho4CTP used in place of CTP, GTP or ATP, produced no RNA transcripts even if 10-fold excess concentration was added (data not shown). rPTP and sc4CTP were incorporated into the transcripts as a substitute of UTP as well as CTP (Supplementary Figure S2). HPLC analysis demonstrated that almost all uridine or cytidine in RNA was replaced by the analogues in transcription by T3 RNA polymerase as shown in Supplementary Table S1 and Supplementary Figure S3. These transcripts were then used as templates for reverse transcription. In addition to these cytidine analogues, the incorporation of the 5'-triphosphates of 3',N6-ethenocytidine, 9-(β-D-ribofuranosyl)-N6-methoxy-2,6-diaminopurine, 2-hydroxyadenosine, inosine and 8-bromoadenosine were studied, but these were not incorporated in place of any normal nucleotides in this assay (data not shown).

Templating properties of mutagenic cytidine analogues by reverse transcription
The RNA templates containing the N4-modified cytidine analogues were reverse-transcribed with HIV and AMV reverse transcriptases to produce cDNAs, which were amplified by PCR. The incorporation of native dNTPs opposite the analogues was examined by direct sequencing of the amplified cDNAs. In the direct sequencing electropherograms of the cDNA produced with HIV reverse transcriptase, every peak consists of a single line when the RNA template containing only natural nucleotides was used. However, C peaks accompany T peaks when the RNA templates contained ho4C, mo4C, rP or sc4C in place of U (Supplementary Figure S4a). From the RNA containing the various substrates, the average peak height ratios of C to T were 0.60 ± 0.06, 0.48 ± 0.03, 0.21 ± 0.02 and 7.2 ± 0.4 for cDNAs from ho4C-RNA, mo4C-RNA, rP-RNA and sc4C-RNA, respectively. AMV reverse transcriptase gave similar results (Supplementary Figure S4b). The average ratios of C peak height to T peak height were 0.75 ± 0.04, 0.43 ± 0.03, 0.27 ± 0.04 and 4.0 ± 0.5 for cDNAs from ho4C-RNA, mo4C-RNA, rP-RNA and sc4C-RNA, respectively. These ratios correspond to those of incorporated nucleotides opposite the analogues (data not shown). The mixed patterns in the electropherograms indicated that these analogues in RNA are mutagenic, causing ambiguous misincorporation by HIV and AMV reverse transcriptases. The RNA template containing m4C exhibited no such ambiguity.

Sequencing analyses after cloning (Figure 3 and Supplementary Table S2) gave similar results. The ratio of incorporated dGMP to dAMP opposite ho4C and rP was 0.57 and 0.15 for HIV reverse transcriptase and 1.4 and 0.17 for AMV reverse transcriptase, respectively. The G/A ratio for mo4C was approximately 0.35 for both reverse transcriptases. The G/A ratio for sc4C was 10.7 and 4.8 for HIV and AMV reverse transcriptase, respectively. Therefore, these analogues in RNA have the potential for inducing mutations at different rates. In contrast, dGMP was preferentially incorporated opposite m4C by both reverse transcriptases consistent with the results of the sequencing analyses on the cDNA without cloning.

Steady-state kinetics of insertion opposite ribonucleotide analogues by HIV RT
Steady-state kinetics of insertion opposite ribonucleotide analogues by HIV RT
To study the ambiguous incorporation opposite the analogues by HIV and AMV reverse transcriptases, the same template was annealed to a primer to initiate incorporation of dNTPs opposite the analogues (Figure 2 and Supplementary Figure S1). Figure 4 shows typical patterns of the primer extension. The steady-state Vmax and Km for the insertion of all four natural dNTPs opposite the N4-modified cytidine analogues in RNA template were determined. As a control reaction, the Vmax and Km for the RNA template containing uridine at the same position were measured. The observed values of kinetic data for each reaction are shown in Table 1. The Km for the incorporation of dA opposite U is about the same as a reported one (37). Among them, ho4C, mo4C and rP are shown to be highly ambiguous in their base pairing properties, consistent with the aforementioned sequencing analyses. However, ho4C and mo4C are different from rP in the mechanism that makes them mutagenic because ho4C and mo4C can adopt either syn or anti conformers, whilst
rP is constrained in the anti conformation. In the syn conformation, the hydroxyl or methoxyl group protrudes into the hydrogen bonding face.

The $f_{\text{ins}}$ values of dGTP opposite ho$^5$C and mo$^5$C were about the same as insertion of dGTP opposite U. However, the values of dATP insertion opposite ho$^5$C and mo$^5$C were 20 and 42-fold smaller than opposite U, respectively. Thus, the insertion ratio of dGTP to dATP opposite ho$^5$C and mo$^5$C were about 50-fold higher than opposite U. In contrast, the $f_{\text{ins}}$ value of dGTP opposite rP was 18-fold higher than opposite U, but the value of dATP was only 2-fold higher than opposite U. Therefore, the insertion ratio of dGTP to dATP opposite rP was 10-fold higher than opposite U.

Steady-state kinetics of extension of ribonucleotide analogues pair by HIV RT

The other important factor to affect the yields of the mutated cDNA is the efficiency of extension from the nucleotides incorporated opposite the analogues. For this purpose,
Table 1. Steady-state kinetics data for insertion opposite cytidine analogues, hoC, moC, rP and scC in RNA templates by HIV RT

| T:P       | $K_m$ (µM) | $V_{max,rel}$ (%/s) | $V_{max,rel}/K_m$ (× 10^−2) | $f_{ext}$ |
|-----------|------------|----------------------|-----------------------------|------------|
| U:dA      | 2.5 ± 0.5  | 15 ± 2               | 6.3(± 1.4) × 10^−2          | 1          |
| U:dG      | 59 ± 17    | 3.1 ± 0.3            | 5.4(± 1.0) × 10^−4          | 8.8(± 1.6) × 10^−3 |
| U:dC      | 2038 ± 575 | 6.0 ± 1.3            | 3.1(± 1.0) × 10^−5          | 5.0(± 1.3) × 10^−4 |
| U:dT      | 2680 ± 381 | 1.8 ± 0.4            | 6.8(± 0.7) × 10^−6          | 1.1(± 0.2) × 10^−3 |
| hoC:dA    | 71 ± 13    | 2.2 ± 1              | 3.2(± 0.6) × 10^−3          | 5.1(± 0.4) × 10^−2 |
| hoC:CdA   | 52 ± 4     | 1.1 ± 2              | 4.7(± 0.4) × 10^−1          | 2.8(± 0.5) × 10^−2 |
| hoC:CdG   | 1963 ± 1086| 7.8 ± 1.7            | 4.5(± 1.4) × 10^−2          | 7.5(± 3.0) × 10^−4 |
| hoC:CT    | 2239 ± 807 | 11 ± 1               | 5.5(± 2.9) × 10^−3          | 8.7(± 3.9) × 10^−4 |
| moC:dA    | 131 ± 15   | 20 ± 2               | 1.0(± 0.2) × 10^−3          | 2.6(± 1.0) × 10^−2 |
| moC:dG    | 158 ± 75   | 9.8 ± 1.7            | 6.9(± 2.5) × 10^−5          | 1.1(± 0.3) × 10^−2 |
| moC:dc    | 2742 ± 962 | 3.7 ± 0.8            | 1.5(± 0.8) × 10^−3          | 2.3(± 0.9) × 10^−4 |
| moC:DT    | 3486 ± 477 | 5.8 ± 0.3            | 1.7(± 0.3) × 10^−5          | 2.8(± 0.6) × 10^−4 |
| rPa:dA    | 2.4 ± 0.2  | 26 ± 7               | 1.1(± 0.3) × 10^−1          | 1.9 ± 0.7  |
| rPa:dG    | 15 ± 2     | 14 ± 2               | 3.2(± 1.0) × 10^−4          | 1.6(± 0.6) × 10^−1 |
| rPa:dc    | 1870 ± 296 | 7.6 ± 0.9            | 4.2(± 1.2) × 10^−2          | 7.0(± 2.5) × 10^−4 |
| rPa:DT    | 1730 ± 457 | 3.7 ± 0.3            | 2.3(± 0.8) × 10^−3          | 3.7(± 1.2) × 10^−5 |
| scC:dA    | 234 ± 21   | 5.3 ± 1.3            | 2.3(± 0.7) × 10^−3          | 3.9(± 2.0) × 10^−4 |
| scC:CdA   | 5.0 ± 0.7  | 3.1 ± 0.0            | 6.3(± 0.8) × 10^−3          | 1.0(± 0.3) × 10^−1 |
| scC:cd    | 1.7 ± 0.9  | 6.0 ± 0.8            | 3.8(± 1.2) × 10^−2          |             |
| CdG       |            |                      |                             |             |

No extended products from scC:dc and scC:ct.

DISCUSSION

In this study, we have investigated the templating properties of ribonucleotide analogues, hoC, moC, mC, scC and rP in reverse transcription for an assessment of their mutagenic potentials for the development of antiviral drugs. Ribonucleotides might be more suitable for this purpose than deoxyribonucleotides because they are less likely to disturb the host genetic machinery. In the present study, three N4-oxo, one N4-amino and one N4-alkylcytidine derivatives were examined. N4-modified cytidine analogues contain the most potent nucleoside analogue mutants (27,38). Results of the sequencing analysis of cDNA products indicated that substitution of U by hoC, moC, rP and scC directs the incorporation of both DA and DG, suggesting their ability to induce U-to-C mutations.

N4-oxocytidines exist preferentially as the imino tautomer (ratio 9:1) (22–24,28,29,39–42), which results in base pairing with each analogue. We found that dAMP insertion opposite hoC and moC was less efficient than opposite U, while dGMP was incorporated in a similar efficiency opposite hoC, moC and U. Thus, the incorporation of dGMP/dAMP opposite the analogues increased. In contrast, an increase in the insertion of dGMP with unchanged incorporation of dAMP caused the increase in the dGMP/dAMP opposite rP. The decrease in the insertion of dATP opposite hoC and moC can be explained by the fact that the N2-hydroxyl or N3-methoxy group prefers the syn conformation, which interferes with hydrogen bonding in a Watson–Crick base pair (40–42). On the other hand, the insertion of dATP opposite rP and U was of the same order and the by HIV RT. It is important to know whether they can be incorporated into RNA by human RNA polymerase II. This was examined by nearest-neighbor analysis of the products of transcription reaction using HeLa nuclear extract in the presence of [α-32P]GTP and the N4-cytidine analogues. Following transcription, the transcripts were digested to nucleoside 3’-monophosphates, which were separated by two-dimensional TLC. Radioactive spots corresponding to the analogues were observed when hoCCTP, moCCTP and rPCTP were used in place of UTP and scCCTP in place of CTP (Figure 6). From the intensities of the spots, the amount of incorporated hoC, moC, rP and scC were calculated to be 3.6, 3.6, 12 and 12% of total nucleotides in synthesized transcripts, respectively (Table 3). Up and Cp derived from UTP and CTP, which were not added to the reaction, were also detected possibly due to presence of small amounts of the triphosphates in the extract. When UTP and CTP were added at 20% of the concentration of rPCTP and scCCTP, the spots of the rPp and scCp were detected, and expected products were formed (Supplementary Figure S5).

Incorporation of ribonucleotide analogues in transcription by human RNA polymerase II

In this study, we have analyzed the templating properties of N4-cytidine analogues in RNA during reverse transcription.
ratio of dATP and dGTP inserted opposite rP was similar to the ratio of its imino and amino tautomers (29). This suggests that the imino and amino tautomers of rP behave like natural U and C, because the N4-hydroxyl group is constrained in an anti-form away from the hydrogen bonding face enabling hydrogen bonding by Watson–Crick base pairing (43,44).

The extension frequencies from dA and dG paired with N4-oxy-cytidine analogues also show an interesting feature. The extension frequency from dA:rP pair was 10-fold higher than those from dA:ho4C pair and dA:mo4C pair, while the frequencies from dG were identical. This is clearly due to the preference for the syn-configuration in the imino-forms of ho4C and mo4C. It interferes with Watson–Crick pairing with dA, but can pair with dG through the wobble-type conformation (45), which possibly changes to Watson–Crick base pairing with a transition to the amino-form (46) after its incorporation. From the latter base pair the chain could extend efficiently. It should also be noted that the G/A ratio of incorporation opposite analogues should depend upon the
dGTP/dATP ratio, but the extension from G-analogue pair should depend solely upon the concentration of dGTP, which will be incorporated opposite C next to the analogue. The concentrations of all the dNTPs used in our experiments of cDNA synthesis were higher than the $K_m$ for dG paired with the analogues, but lower than the $K_m$ for dG mismatched with uridine. Therefore, the dNTP concentration would be sufficient to extend from dG paired with the analogues, but not uridine. Thus this selection step may efficiently complement the low fidelity insertion step to avoid the errors in cDNA synthesis from normal RNA, but it may not in the synthesis from RNA containing analogues.

$N^4$-aminocytidine derivatives are also highly mutagenic analogues with tautomeric ambiguity in their structures (25,47). $N^4$-aminocytidine derivatives exist preferentially in the amino tautomeric form (28,48), and our results are consistent with this. We examined here $sc^4C$, an $N^4$-aminocytidine derivative, which directs the incorporation of both dGMP and dAMP but with a preference for dGMP. $N^4$-alkylated cytidine, $N^4$-methylcytidine behaves as cytidine only. Although we need to examine other conditions, $N^4$-modification without tautomerism does not seem to affect incorporation. These facts suggested that a cytidine analogue that has equal amino and imino tautomers and prefers the anti conformation, were such an analogue available, would be more mutagenic and a useful nucleotide as an antiviral drug as we have described.

| T:P       | $K_m$ (µM) | $V_{max,rel}$ (%) | $V_{max,rel}/K_m$ | $f_{ext}$ |
|-----------|------------|-------------------|-------------------|-----------|
| U:dA      | 0.17 ± 0.04 | 4.3 ± 0.2         | 2.6(± 0.4) × 10⁻¹ | 1         |
| $ho^4C:dA$| 2.6 ± 0.2   | 8.5 ± 0.1         | 3.3(± 0.2) × 10⁻² | 1.3(± 0.3) × 10⁻¹ |
| $mo^4C:dA$| 2.9 ± 1.1   | 10 ± 0.2          | 3.7(± 0.8) × 10⁻² | 1.5(± 0.5) × 10⁻¹ |
| rP:A      | 0.18 ± 0.04 | 6.7 ± 0.1         | 3.8(± 0.7) × 10⁻¹ | 1.5 ± 0.5 |
| $sc^4C:dA$| 4.6 ± 1.8   | 15 ± 0.2          | 3.5(± 1.2) × 10⁻³ | 1.3(± 0.3) × 10⁻² |
| U:dG      | 1600 ± 494  | 14 ± 3            | 9.2(± 1.9) × 10⁻⁵ | 3.6(± 0.9) × 10⁻⁴ |
| $ho^4C:dG$| 166 ± 7     | 17 ± 1            | 1.0(± 0.1) × 10⁻³ | 3.8(± 0.8) × 10⁻³ |
| $mo^4C:dG$| 162 ± 29    | 18 ± 1            | 1.1(± 0.1) × 10⁻³ | 4.3(± 0.8) × 10⁻³ |
| rP:G      | 149 ± 6     | 15 ± 1            | 1.0(± 0.1) × 10⁻³ | 3.9(± 1.0) × 10⁻³ |
| $sc^4C:dG$| 48 ± 15     | 2.6 ± 0.04        | 5.9(± 2.1) × 10⁻⁴ | 2.4(± 1.3) × 10⁻³ |

Figure 6. Incorporation of cytidine analogues with HeLa nuclear extract. The transcripts were analyzed by denaturing 5% polyacrylamide gel electrophoresis. Above each lane, rNTP added to the extract are recorded. Molar ratios of ATP:(32P)-GTP:CTP:UTP:C*TP is 1:0.04:1:1:1 if present (A). Two-dimensional cellulose TLC for nearest-neighbor analyses of the transcripts (B). In the transcription, the CTP analogues, $ho^4CTP$, $mo^4CTP$ and rP*TP, respectively, were added in place of UTP, while $sc^4CTP$ was added in place of CTP.
the latter are implicated in important cellular processes, such as metabolic process and signaling besides DNA and RNA synthesis. Thus, use of mutagenic pyrimidine analogues might be more effective, and result in less adverse cellular effects. Additionally, it will be possible to improve the therapeutic efficacy by combinations of mutagenic nucleoside and antiviral inhibitor (54–57). Further work would be required to determine the antiviral efficacy of these analogues, but we have provided evidence to support the fact that if incorporated into viral RNA the analogues could lead to lethal mutagenesis.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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