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Author(s)	Kamiya, Hiroyuki; Suzuki, Akihiro; Yamaguchi, Yuki et al.
Citation	Free Radical Biology and Medicine, 46(12), 1703-1707 https://doi.org/10.1016/j.freeradbiomed.2009.04.005
Issue Date	2009-06-15
Doc URL	https://hdl.handle.net/2115/38630
Type	journal article
File Information	46-12_p1703-1707.pdf

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Incorporation of 8-hydroxyguanosine 5'-triphosphate (8-oxo-7,8-dihydroguanosine 5'-triphosphate) by bacterial and human RNA polymerases

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ABSTRACT

Oxidized RNA precursors formed in the nucleotide pool may be incorporated into RNA. In this study, the incorporation of 8-hydroxyguanosine 5'-triphosphate (8-OH-GTP, 8-oxo-7,8-dihydroguanosine 5'-triphosphate) into RNA by *Escherichia coli* RNA polymerase was examined *in vitro*, using a primer RNA and a template DNA with defined sequences. 8-OH-GTP was incorporated opposite C and A in the template DNA. Surprisingly, 8-OH-GTP was quite efficiently incorporated by the bacterial RNA polymerase, in contrast to the incorporation of the 2'-deoxyribo-counterpart by DNA polymerases, as indicated by the kinetic parameters. The primer was further extended by the addition of a ribonucleotide complementary to the nucleobase adjacent to C or A (the nucleobase opposite which 8-OH-GTP was inserted). Thus, the incorporation of 8-OH-GTP did not completely inhibit further RNA chain elongation. 8-OH-GTP was also incorporated opposite C and A by human RNA polymerase II. These results suggest that 8-OH-GTP in the nucleotide pool can cause the formation of oxidized RNA and disturb the transmittance of genetic information.

Keywords: Oxidized ribonucleotide; 8-Hydroxyguanosine 5'-triphosphate; RNA polymerase; Transcription

Reactive oxygen species are produced endogenously through normal cellular metabolism, and their formation is further enhanced by ionizing radiation and various chemicals. Reactive oxygen species attack nucleic acids and their related compounds, generating various modified nucleobases that seem to play pivotal roles in mutagenesis, carcinogenesis, neurodegeneration, and aging [1-4]. Among these modified nucleobases, 8-hydroxyguanine (8-OH-Gua; also known as 8-oxo-7,8-dihydroguanine) is one of the most abundant ([5] and references therein). The highly reactive hydroxy radical and guanine-selective singlet oxygen are thought to be involved in the formation of 8-OH-Gua [6]. 8-OH-Gua can pair with both cytosine and adenine [7-10]. The oxidized nucleobase in DNA seems to be an important source of mutations, and it induces G:C→T:A transversions in living cells [11-19]. The oxidation of dGTP in the nucleotide pool by reactive oxygen species is another significant source in the mutation process. 8-Hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP; 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate) is an oxidized form of dGTP, and it induces A:T→C:G transversions in living cells [20,21]. The presence of *Escherichia coli* MutT and its functional homologues, which catalyze the hydrolysis of 8-OH-dGTP to the monophosphate derivative, and the phenotypes of mutant organisms lacking the MutT-type enzymes indicate the importance of this oxidized

DNA precursor ([22,23] and references therein). Recently, Pursell et al. reported the presence of 8-OH-dGTP in the mitochondrial nucleotide pool in rat tissues [24]. They reported that amount of 8-OH-dGTP was ~10% of that of dGTP in mitochondria of rat liver.

Reactive oxygen species also oxidize RNA and its precursors. Oxidized RNA is reportedly implicated in several neurological diseases, including Alzheimer disease, Parkinson disease, and Down syndrome ([25] and references therein). Because the pool size of ribonucleotides is hundreds of times larger than that of 2'-deoxyribonucleotides [26,27], oxidized RNA precursors may be more abundant than oxidized DNA precursors. Previously, Taddei et al. reported that an oxidized form of GTP, 8-hydroxyguanosine 5'-triphosphate (8-OH-GTP; 8-oxo-7,8-dihydroguanosine 5'-triphosphate), was incorporated into RNA [28] and concluded that 8-OH-GTP induced translational errors. The findings that the *E. coli* MutT, and mammalian MTH1 (NUDT1) and NUDT5 proteins catalyze the hydrolysis of oxidized RNA precursors suggest the importance of the hydrolytic elimination of oxidized ribonucleotides [28-30]. Moreover, disruption of the transcription factor S-II, which enhances the excision of ribonucleotides misincorporated by RNA polymerase (pol) II *in vitro*, confers sensitivity to oxidants together with low transcriptional fidelity in yeast, and antioxidant treatment relieves

this low transcriptional fidelity [31]. Thus, the yeast S-II protein may maintain transcriptional fidelity by enhancing the cleavage of misincorporated, oxidized ribonucleotides from nascent mRNA. These findings suggest that the oxidation of RNA precursors in the nucleotide pool disturbs transcription and translation, and occasionally causes cell death (sensitivity to oxidants). The formation of oxidized ribonucleotides may be one of the causative factors of the neurodegenerative diseases described above ([25] and references therein). Therefore, it is of great interest to examine the incorporation of oxidized ribonucleotides by RNA pols in detail.

In this study, we analyzed the incorporation of 8-OH-GTP by the *E. coli* and human RNA pols *in vitro*. We found that the oxidized ribonucleotide was incorporated into RNA opposite C and A in the template DNA. This result suggests that 8-OH-GTP is incorporated into RNA, resulting in transcriptional and translational errors. In contrast, another oxidized ribonucleotide, 2-hydroxyadenosine 5'-triphosphate (2-OH-ATP) [29], was incorporated "correctly" opposite T.

Materials and methods

Materials

E. coli RNA polymerase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human RNA pol II was purified from HeLa cells expressing Flag- and His-tagged human Rpb3 (the third subunit of RNA pol II), as described previously [32]. Ribonuclease inhibitor was from Takara (Kusatsu, Japan). 8-OH-GTP and 2-OH-ATP were prepared by the oxidation of GTP and ATP, respectively, as described previously [29]. The purities of the 8-OH-GTP and 2-OH-ATP were both estimated to be more than 99%, as determined by an HPLC analysis (data not shown). The unmodified ribonucleoside 5'-triphosphates were from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). The Alexa Fluor 647 (Alexa 647)-modified oligoribonucleotide and the unmodified oligodeoxyribonucleotides (Table 1) were purchased from Japan Bio Services (Asaka, Japan) and Invitrogen Japan (Tokyo, Japan), respectively, in purified forms.

In vitro *RNA synthesis*

An RNA primer-extension assay, in which the elongation complexes were assembled on nucleic acid scaffolds [33-38], was used to monitor nucleotide incorporation. The reactions catalyzed by the *E. coli* RNA pol were performed in mixtures containing an oligodeoxyribonucleotide template annealed with the Alexa 647-modified

oligoribonucleotide primer (0.2 μM), 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 0.5 U/μl ribonuclease inhibitor, 0.1 μg/μl bovine serum albumin, various concentrations of NTP, and the enzyme at 37°C. Experiments with RNA pol II were conducted in a reaction mixture containing the primed template (0.2 μM), 10 mM Hepes-NaOH (pH 7.9), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 U/μl ribonuclease inhibitor, 500 μM NTP and the enzyme at 30°C. In both reactions, the RNA/DNA hybrid was incubated with RNA pol for 5 min to assemble the elongation complex (preincubation), and then the substrate ribonucleotide was added to start the reactions.

Reactions were stopped by the addition of a two-fold volume of termination solution (90% formamide, 50 mM EDTA). Samples were heated at 98°C for 5 min and chilled on ice, and were then applied to an 8 M urea, 20% polyacrylamide gel. Fluorograms were obtained with a Fujifilm FLA-2000 Fluorescent Image Analyzer (Fuji Photo Film, Tokyo, Japan; excitation 633 nm, emission 675 nm). The relative amounts of RNA were calculated based on the intensity of the experimental bands.

The Michaelis constant (K_m) and the maximum velocity of the reaction (V_{max}) values were obtained from Hanes-Woolf plots of the kinetic data. To obtain steady-state kinetic parameters, reactions catalyzed by the

E. coli RNA pol were carried out at 37°C for two to ten min.

Results

Incorporation of 8-OH-GTP by E. coli *RNA polymerase*

To monitor nucleotide incorporation, we utilized a primer-extension assay, in which the elongation complexes were assembled on nucleic acid scaffolds [33-38]. An Alexa 647-modified RNA primer (14mer) was annealed to a DNA template (30mer) that had A, G, C, or T just downstream of the primer (Table 1). Using DNA templates with defined sequences, we analyzed the incorporation mode of 8-OH-GTP during *in vitro* RNA synthesis catalyzed by *E. coli* RNA pol.

Fig. 1 shows the pattern of primer extension catalyzed by *E. coli* RNA pol on different DNA templates. The primer annealed to template-C was elongated by the RNA pol to yield a 15mer after the addition of 500 μM 8-OH-GTP (Fig. 1, lane 8). Moreover, the incorporation of 8-OH-GTP was observed when the primer was annealed to template-A (lane 6). These results indicate that *E. coli* RNA pol could incorporate 8-OH-GTP opposite C and A. The incorporation of 8-OH-GTP opposite A and that opposite C seemed to occur with similar efficiencies. This result is in agreement with

the biophysical observations that 8-OH-Gua can pair with C and A [7-10], and suggests that 8-OH-GTP is “mutagenic”.

Steady state kinetics

We then measured the kinetic parameters during *in vitro* RNA synthesis catalyzed by *E. coli* RNA pol. The incorporation of 8-OH-GTP opposite C and A was examined. As a control, the incorporation of unmodified GTP opposite C was also analyzed. The results of the kinetic studies are shown in Table 2.

With template-C, the K_m value of GTP was ~100 μM. Unexpectedly, the K_m value of 8-OH-GTP (18 μM) was 5.8 times lower than that of GTP. The relative V_{max} value of the incorporation of 8-OH-GTP was 3.3 times lower than that of GTP. The relative V_{max} / K_m value of 8-OH-GTP was 1.7, which means that the insertion of 8-OH-GTP opposite C was favored over that of GTP.

With template-A, the K_m value of 8-OH-GTP was 27 μM, which is 3.9 times lower than that of GTP opposite C. The relative V_{max} value of the incorporation of 8-OH-GTP was 2.3 times lower than that of GTP. The relative V_{max} / K_m value of 8-OH-GTP was 1.7, which means that the insertion of 8-OH-GTP opposite A occurred more frequently, as compared to that of GTP opposite C. A comparison of the incorporations of

8-OH-GTP opposite C and A revealed that the K_m value and the V_{max} value were 1.5 times and 1.4 times lower, respectively, in the case of C•8-OH-GTP pair formation (Table 2). The insertions of 8-OH-GTP opposite C and A were similar, based on the kinetic parameters obtained.

Extension from 8-OH-Gua at the 3'-end of the primer

We next analyzed the extension from the 8-OH-Gua residue after incorporation. We added both 8-OH-GTP and ATP to the reaction mixture containing template-C or template-A. ATP was used because the 5'-flanking position of the nucleobase opposite which 8-OH-GTP was inserted was T (Table 1). As shown in Fig. 2, part of the primer was extended to 16mer and 17mer by the addition of 8-OH-GTP and ATP (lanes 7 and 10). This result is explained by the incorporation of two ATP molecules opposite the T bases at positions 16 and 17 from the 3'-end of the template. When unmodified GTP instead of 8-OH-GTP was used in the reaction, a 17mer RNA was obtained (lane 4). The elongation seemed to occur more efficiently with GTP than with 8-OH-GTP, although the results could not be quantitatively interpreted. These results suggest that the incorporation of 8-OH-GTP did not cause complete inhibition of further RNA chain elongation.

Incorporation of 8-OH-GTP by human RNA polymerase II

We then examined the incorporation pattern of 8-OH-GTP during *in vitro* RNA synthesis catalyzed by human RNA pol II, which produces mRNA. As in the case of *E. coli* RNA pol, we utilized nucleic acid scaffolds containing the Alexa 647-modified RNA primer annealed to a DNA template (Table 1).

Fig. 3 shows the pattern of primer extension catalyzed by human RNA pol II on different DNA templates. The primer was elongated by the addition of 500 μM 8-OH-GTP with template-C and template-A to similar degrees, as with *E. coli* RNA pol. This result indicates that 8-OH-GTP is "mutagenic" in the reaction catalyzed by this human transcription enzyme.

Interestingly, a noteworthy amount of unmodified GTP was incorporated opposite T (Fig. 3, lane 4).

Incorporation of 2-OH-ATP by RNA polymerases

Last, we examined the incorporation of another oxidized ribonucleotide, 2-OH-ATP [29]. 2-OH-ATP (500 μM) was added to the reaction mixture containing the primed template and RNA pol. The 14mer primer was extended when annealed with template-T, indicating the incorporation of 2-OH-ATP opposite T by the *E. coli* and human RNA pols (Fig. 4, lanes 8 and 16). In contrast to the 2'-deoxyribo-derivative [39,40],

no apparent misincorporation was observed. Therefore, this oxidized RNA precursor seems to be “nonmutagenic” during transcription in bacterial and human cells.

Discussion

The objective of this study was to examine the incorporation of oxidized GTP, 8-OH-GTP, by RNA pols. Because 8-OH-dGTP concentrations in mitochondrial 2'-deoxyribonucleotide pool in rat tissues are ~1 μM [24], the pool size of GTP is ~100 times larger than that of dGTP [41], and the hydrolysis of 8-OH-GTP by *E. coli* MutT and human MTH1 is less efficient than that of 8-OH-dGTP [29, 42], significant amounts of 8-OH-GTP could be present in cells. The incorporation of 8-OH-GTP by *E. coli* RNA pol was efficient and error-prone (Fig. 1). In addition, the incorporation of the following nucleotide opposite the next nucleobase was observed (Fig. 2), indicating that the extension from the incorporated 8-OH-Gua residue occurs. Thus, 8-OH-Gua could be formed in cellular RNA via the incorporation of 8-OH-GTP. Because this oxidized ribonucleotide has been suggested to be involved in transcriptional and translational errors [28], the oxidation of GTP would perturb the accurate expression of genetic information.

Unexpectedly, the kinetic parameters obtained in this study indicated that 8-OH-GTP was efficiently incorporated by the bacterial RNA pol. The K_m value for the incorporation of 8-OH-GTP opposite C was 5.8 times lower than that for the incorporation of GTP opposite C (Table 2). This result indicates that *E. coli* RNA pol did not discriminate against C•8-OH-GTP and C•GTP at the level of the K_m. This enzyme discriminated against C•8-OH-GTP and C•GTP, as revealed by the lower V_{max} value (Table 2). Likewise, the K_m and V_{max} values for A•8-OH-GTP were 3.9 times and 2.3 times, respectively, lower than those for C•GTP. Thus, 8-OH-GTP was erroneously incorporated into RNA quite efficiently.

Similar kinetic studies using replicative DNA pols have been reported for the 2'-deoxyribo-counterpart, 8-OH-dGTP. *E. coli* DNA pol III (the α subunit) incorporates 8-OH-dGTP opposite C and A, with 5 to 10 times higher K_m values and 2 to 7 times lower V_{max} values than those for the Watson-Crick pairing [43]. Thus, the incorporation of 8-OH-dGTP by this prokaryotic DNA pol is 30 times less efficient than the normal pairing. Calf thymus DNA pol α-primase complex exhibits 90-fold higher K_m and >1,000-fold lower V_{max} values for C•8-OH-dGTP than for C•dGTP, resulting in a 130,000-fold bias against this damaged 2'-deoxyribonucleotide [44]. The K_m values for the incorporation of 8-OH-dGTP opposite C and A by calf thymus DNA pol δ in the presence

of proliferating cell nuclear antigen are 9,700 to 190,000 times, respectively, larger than that for dCTP incorporation opposite G [45]. On the other hand, the V_{max} values for 8-OH-dGTP are similar to that for the normal pairing. Thus, the discrimination of 8-OH-GTP by *E. coli* RNA pol was completely different from that of 8-OH-dGTP by these replicative DNA pols.

This study focused on the incorporation of 8-OH-GTP by RNA pols. On the other hand, the effects of 8-OH-Gua in template DNA on *in vitro* transcription by RNA pols were previously reported [46-51]. In general, this oxidized nucleobase in the transcribed strand can potentially block transcription and induce the misincorporation of ATP [46-51]. Thus, in cells, the oxidation of G bases, in DNA and as the ribonucleotide, seems to affect transcription quantitatively and qualitatively.

In contrast to 8-OH-GTP, another type of oxidized RNA precursor, 2-OH-ATP, was incorporated correctly by the RNA pols examined (Fig. 4), although the 2'-deoxyribo-counterpart, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate, was shown to be incorporated erroneously by the replicative DNA pols [39,40]. It is interesting that the incorporation of the 8-OH-Gua nucleotides was more erroneous in transcription than in replication, while that of the 2-hydroxyadenine nucleotides had the opposite rend.

Hayakawa *et al*. reported that the *E. coli* and human polynucleotide phosphorylase proteins and the human Y box-binding protein 1 (YB-1) bound specifically to RNA containing 8-OH-Gua [52-54]. They proposed that these proteins may recognize and discriminate the RNA molecule containing 8-OH-Gua from undamaged ones, thus contributing to the fidelity of translation in cells by inhibiting the translation of the damaged RNA. *E. coli* cells lacking the polynucleotide phosphorylase protein are reportedly hyperresistant to an oxidative stress-inducing agent [52]. In contrast, human cells with a knocked-down polynucleotide phosphorylase protein are sensitive to H_2O_2 [55]. These results suggest the roles of the polynucleotide phosphorylase proteins in cells under oxidative stress, although the reduction in the levels of the proteins gave different effects.

8-OH-GTP was previously used in *in vitro* transcription reactions conducted by RNA pols from phage T7, *E. coli*, and calf thymus [28,56, 57]. Poly(dA-dT), *E. coli* and calf thymus DNAs, and linearized plasmid DNA were employed as templates in the previous studies, and thus a detailed analysis of 8-OH-GTP incorporation was difficult. In this study, we used the primed oligoribonucleotide template with defined sequences and directly demonstrated that 8-OH-GTP was efficiently incorporated opposite C and A by RNA pols. The results obtained in this study suggest that the formation of 8-OH-GTP in cells affects transcription and

translation. Further studies are necessary to reveal the effects of 8-OH-GTP on transcription and translation in living cells.

Acknowledgement

This work was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science and the Research Foundation for Pharmaceutical Sciences.

Abbreviations: 8-OH-Gua, 8-hydroxyguanine; 8-OH-dGTP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate; 8-OH-GTP, 8-hydroxyguanosine 5'-triphosphate; pol, polymerase; 2-OH-ATP, 2-hydroxyadenosine 5'-triphosphate; Alexa 647, Alexa Fluor® 647; K_m, Michaelis constant; V_{max}, maximum velocity of the reaction.

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FIGURE LEGENDS

Figure 1 Incorporation of 8-OH-GTP by *E. coli* RNA pol. The DNA templates were annealed to the labeled RNA primer. The template-primer complex (0.2 μM) was treated with the RNA pol (0.66 units) in the presence of 500 μM GTP (lanes 2-5) and 8-OH-GTP (lanes 6-9) under the conditions described under Materials and methods. The reaction mixtures were incubated at 37°C for 10 min and were processed as described under Materials and methods. Lane 1, untreated primer; lanes 2 and 6, template-A; lanes 3 and 7, template-G; lanes 4 and 8, template-C; lanes 5 and 9, template-T.

Figure 2 Extension from the incorporated 8-OH-Gua residue by *E. coli* RNA pol. The template-primer complex (0.2 μM) was treated with the RNA pol (0.063 units) in the presence of ribonucleotide(s) (500 μM each). The reaction mixtures were incubated at 37°C for 10 min and were processed as described under Materials and methods. Lanes 2-7, template-C; lanes 8-10, template-A. Lane 1, untreated primer; lane 2, GTP; lanes 3, 6, and 9, ATP; lane 4, GTP and ATP; lanes 5 and 8, 8-OH-GTP; lanes 7 and 10, 8-OH-GTP and ATP.

Figure 3 Incorporation of 8-OH-GTP by human RNA pol II. The template-primer complex (0.2 μM) was treated with the RNA pol (1 pmol) in the presence of 500 μM GTP (lanes 1-4) and 8-OH-GTP (lanes 5-8). The reaction mixtures were incubated at 30°C for 60 min and were processed as described under Materials and methods. Lanes 1 and 5, template-A; lanes 2 and 6, template-G; lanes 3 and 7, template-C; lanes 4 and 8, template-T.

Figure 4 Incorporation of 2-OH-ATP by RNA pols. The template-primer complex (0.2 μM) was treated with *E. coli* RNA pol (0.66 units, lanes 1-8) or human RNA pol II (1 pmol, lanes 9-16) in the presence of 500 μM ATP (lanes 1-4 and 9-12) and 2-OH-ATP (lanes 5-8 and 13-16) under the conditions described under Materials and methods. The reaction mixtures were incubated at 37°C for 10 min (*E. coli* RNA pol) or at 30°C for 30 min (RNA pol II) and were processed as described under Materials and methods. Lanes 1, 5, 9, and 13, template-A; lanes 2, 6, 10, and 14, template-G; lanes 3, 7, 11, and 15, template-C; lanes 4, 8, 12, and 16, template-T.

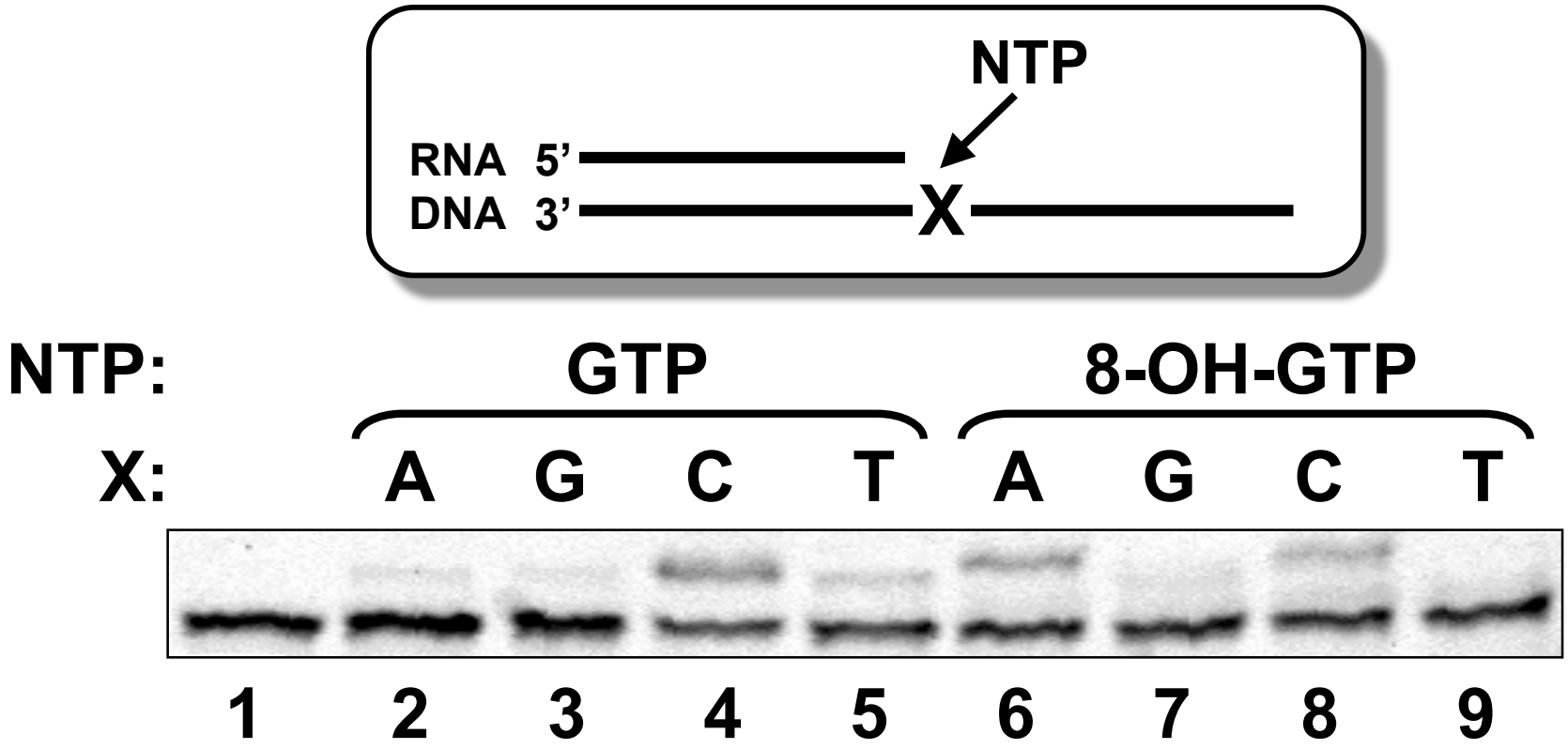
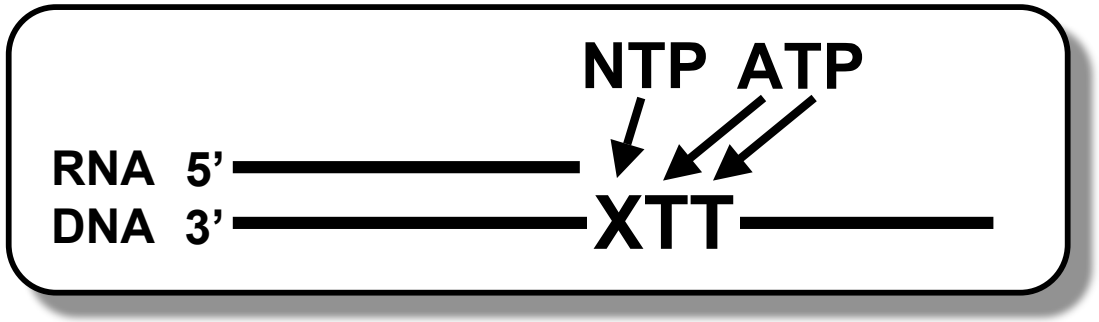
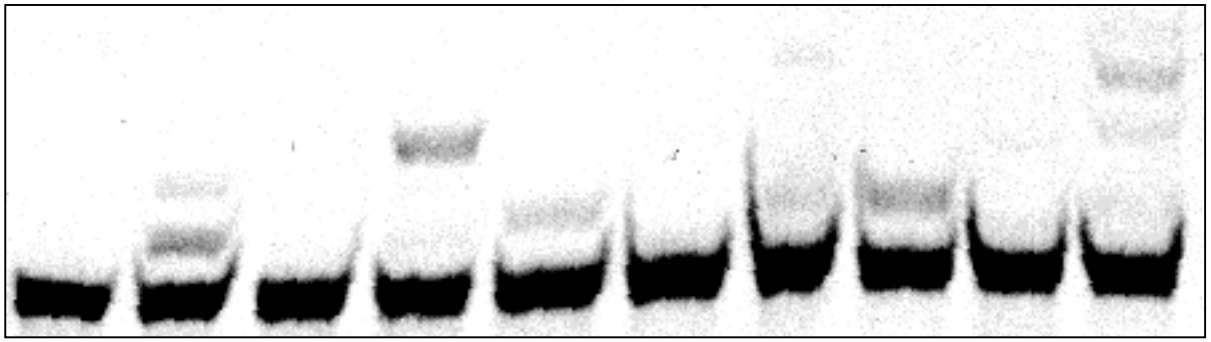


Fig. 1



ATP:	–	+	+	–	+	+	–	+	+
8-OH-GTP:	–	–	–	+	–	+	+	–	+
GTP:	+	–	+	–	–	–	–	–	–
X:	C	C	C	C	C	C	A	A	A



1 2 3 4 5 6 7 8 9 10

Fig. 2

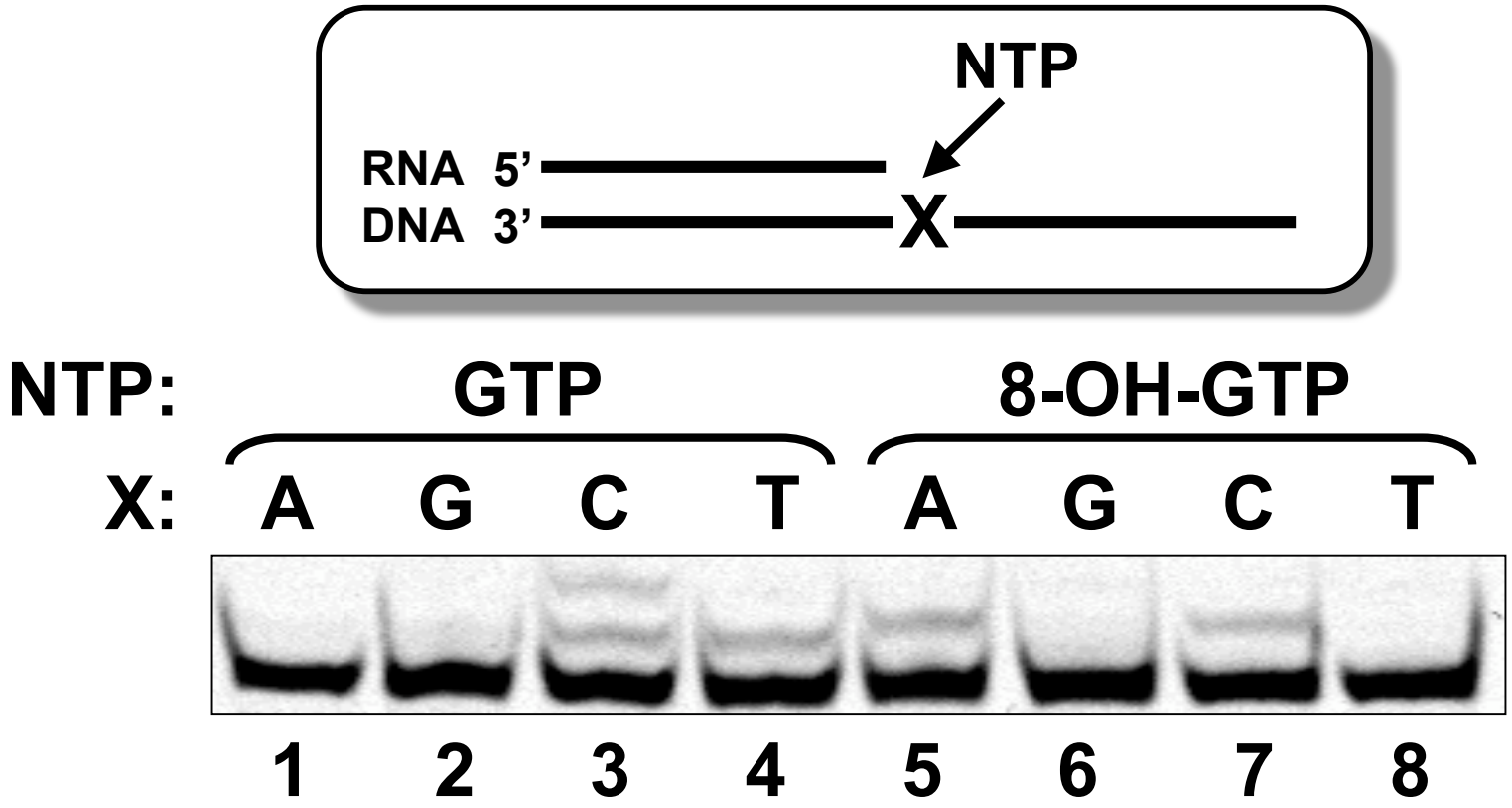


Fig. 3

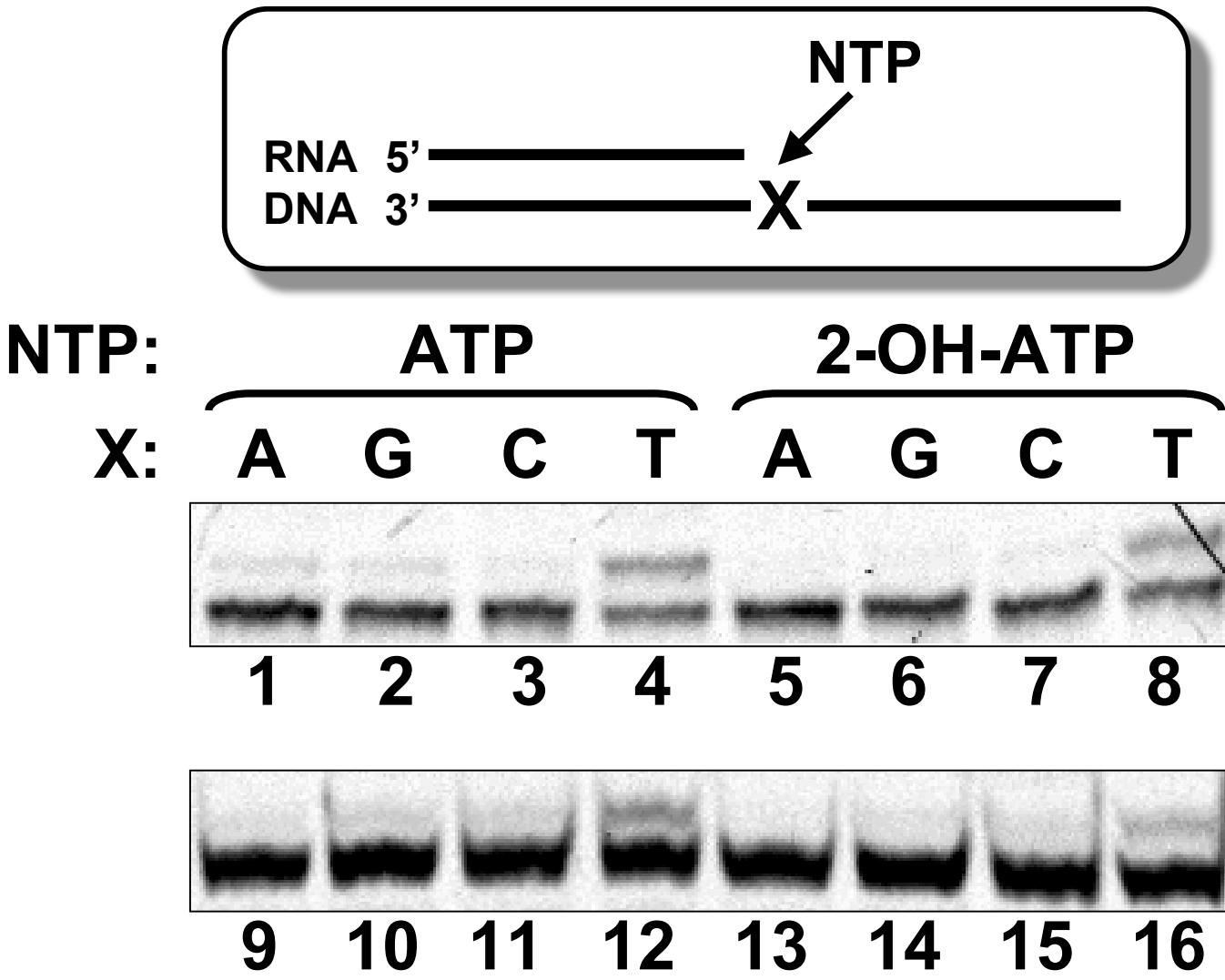

NTP
RNA 5'
DNA 3'
X
NTP:
ATP
2-OH-ATP
X:
A G C T A G C T
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16

Fig. 4

Table 1

Table 1 Oligonucleotides used in this study

oligonucleotide	sequence
RNA	
RNA primer	5'-UUGUCCGAGCGCGG
DNA	
template-A	3'-AACAGGCTCGCGCC**A**TTAAGGCTAAGTCTG
template-G	3'-AACAGGCTCGCGCC**G**TTAAGGCTAAGTCTG
template-C	3'-AACAGGCTCGCGCC**C**TTAAGGCTAAGTCTG
template-T	3'-AACAGGCTCGCGCC**T**ATAAGGCTAAGTCTG

Table 2

Table 2. Kinetic parameters of nucleotide insertion reactions by *E. coli* RNA polymerase

template	nucleotide	K_m[a] (μM)	V_{max}[a] (X 10^{-3} %/sec)	V_{max} / K_m (X 10^{-3} %/sec/μM)
template-C	GTP	104	30	0.29
template-C	8-OH-GTP	18	9	0.50
template-A	8-OH-GTP	27	13	0.48

[a]Average of four independent experiments.