

The Effect of Neighboring Bases on Miscoding Properties of N²,3-Ethenoguanine

Małgorzata M. Mroczkowska and Jarosław T. Kuśmierek

Institute of Biochemistry and Biophysics, Polish Academy of Sciences,
36 Rakowiecka Str., 02532 Warsaw, Poland

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The miscoding potential of N²,3-ethenoguanine (εG), one of the carcinogen vinyl chloride adducts to DNA bases, has been examined by copying of poly (A, εG) templates with DNA-dependent RNA polymerase and reverse transcriptase. In contrast to the results previously obtained with poly (C, εG) templates where εG acts as G and A, in poly (A, εG) templates εG acts almost exclusively as A. These results suggest that mutagenic potential of εG *in vivo* can depend on the nature of neighboring bases.

Introduction

Vinyl chloride (VC), an industrial chemical, is a known carcinogen in both animals and man. This chemical and its metabolites, chloroethylene oxide (CEO) and chloroacetaldehyde (CAA) are mutagenic in various systems. N²,3-Ethenoguanine (εG), one of the VC adducts to nucleobases, appears to be responsible for the most frequent mutation G → A which is observed in CEO-treated *E. coli* (briefly reviewed in [1]).

The miscoding properties of εG in poly (C) templates were studied in AMV reverse transcriptase [2] and in *E. coli* DNA-dependent RNA polymerase [1] systems. In both cases it has been shown that εG can form pairs with C and T (or U). Additionally, the εG-A pairing was observed in RNA polymerase system. The studies on incorporation of εdGTP opposite defined template T or C residues by various DNA replicating enzymes again demonstrated the ability of εG to form pairs with T and C [3]. The formation of the εG-T pair leads to a G → A transition during DNA replication. This was recently confirmed in an experiment where εG was located in a preselected site in DNA of a bacteriophage vector which was transfected into *E. coli* [4].

In order to compare the influence of the nature of neighboring bases on the miscoding potential of εG, we now report studies on miscoding of εG

in poly (A) templates using AMV reverse transcriptase and *E. coli* DNA-dependent RNA polymerase.

Materials and Methods

Chemicals and enzymes

³H-Labeled ribonucleoside- and deoxyribonucleoside-5'-triphosphates (12–60 Ci/mmol) were Amersham products. Non-radioactive nucleotides, polynucleotide phosphorylase from *Micrococcus lysodeikticus*, *E. coli* K-12 RNA polymerase and bacterial alkaline phosphatase were products of Sigma. *Avian myeloblastosis virus* reverse transcriptase was from Life Sciences. Deoxyribonuclease I was from Cooper Biomedical whereas crude snake venom (*Russel Viper*) used as a source of phosphodiesterase and 5'-nucleotidase was a generous gift from Professor David Shugar. Polyribonucleotide templates containing εG were prepared as described elsewhere [1].

DNA-dependent RNA polymerase reaction

The copying of poly (A, εG) templates using RNA polymerase and analysis of the product of reaction followed the procedure for the copying of poly (C, εG) templates under non-competitive conditions in the presence of Mn²⁺ cation [1]. In order to obtain a satisfactory incorporation of radioactivity into the product it was necessary to increase the concentration of ribonucleoside-5'-phosphates and enzyme in the incubation mixture. Therefore during copying of poly (A) templates the concentration of each ribonucleoside-5'-tri-

Reprint requests to Dr. J. T. Kuśmierek.

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phosphate was 1.2 mM (total 2.4 mM) and the concentration of RNA polymerase was 10 units in the 400 µl standard incubation mixture.

Reverse transcriptase reaction

The 100 µl standard incubation mixture contained 0.1 A₂₆₀ of template (previously primed with 0.005 A₂₆₀ oligo (dT)₁₂), 0.8 mM ³H-dTTP (25 mCi/mmol), and 0.8 mM ³H-dCTP (125 mCi/mmol), 50 mM Tris-HCl pH 8.3, 10 mM MgCl₂, 40 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 2.5 units of reverse transcriptase. The mixture was incubated for 45 min at 37 °C.

The reaction was terminated by addition of 10 µl 3 M sodium acetate pH 5.2 and 100 µl of isopropanol. After 18 h at -70 °C the sample was centrifuged and the resulting pellet dissolved in 200 µl 0.5 M Tris-HCl pH 8.8. The polymer fraction was separated from deoxynucleoside-5'-triphosphates using a Sephadex G-50 column [1]. Fractions containing newly synthesized polymer were reduced to about 50 µl (with a Speedvac) and then precipitated by addition of 5 µl 3 M sodium acetate pH 5.2 and 150 µl of ethanol. After 18 h at -70 °C the sample was centrifuged and the pellet dried and subjected to enzymatic hydrolysis.

The 50 µl mixture contained 20 mM Tris-HCl pH 6.8, 10 mM MgCl₂ and 20 units of deoxyribonuclease I and was incubated for 16–24 h at 37 °C. Then concentrated Tris-HCl, MgCl₂ and crude Russell Viper venom was added and 175 µl mixture contained 0.2 M Tris-HCl pH 9.0, 40 mM MgCl₂ and 250 µg of snake venom. After 16–24 h

incubation at 37 °C, another portion of 250 µg of snake venom was added together with 1 µg of bacterial alkaline phosphatase and incubation continued for another 16–24 h at 37 °C.

Following digestion, deoxynucleoside markers were added and the mixture was subjected to descending paper chromatography with water-saturated *n*-butanol, which separates all four deoxynucleosides. The separation was carried out twice for 24 h in the same direction. Chromatograms were cut into 1 cm strips and radioactivity counted using toluene scintillation fluid.

Results

Table I presents the detailed analysis of products of copying of poly (A), poly (A, ϵ G) and poly (A, G) templates by RNA polymerase and reverse transcriptase under non-competitive conditions where UTP and CTP (or dTTP and dCTP, respectively) were present in the incubation mixtures in a ratio 1:1. The incorporation of C nucleotides by either of enzyme caused by ϵ G was only slightly higher than the background incorporation measured on unmodified poly (A) template. The similar results were obtained with reverse transcriptase under competitive conditions, where all four dNTPs were present at concentration 0.4 mM (not shown). The competitive experiment with RNA polymerase was not possible to perform, since in this case transcription of poly (A) templates in the presence of both UTP and ATP results in formation of additional poly (A) strand

Table I. A single typical analysis of incorporation of radioactive nucleoside-5'-triphosphates during the copying of poly (A) templates containing ϵ G and G by *E. coli* DNA-dependent RNA polymerase and by AMV reverse transcriptase^a.

Poly (A) template containing	RNA polymerase ^b Incorporation of			Reverse transcriptase ^b Incorporation of		
	UTP [cpm]	CTP [cpm]	CTP [%] ^c	dTTP [cpm]	dCTP [cpm]	dCTP [%] ^c
–	7618	166	0.4	77,294	314	0.1
5% ϵ G	3427	118	0.7	35,257	489	0.3
14% ϵ G	917	66	1.4	19,024	453	0.5
10% G	521	270	9.4	4,626	2278	9.0

^a The concentration of each nucleoside-5'-triphosphate in the incubation mixtures was 1.2 mM for RNA polymerase and 0.8 mM for AMV reverse transcriptase reaction, ratio of C nucleotides to U (or T) nucleotides was 1:1. See Table II for higher ratios.

^b See text for details of RNA polymerase and reverse transcriptase reactions.

^c Note that specific activity of [³H]CTP (or [³H]dCTP) was five times higher than specific activity of [³H]UTP (or [³H]dTTP).

which is synthesized on a newly generated poly (U) strand.

The ϵ G-provoked incorporation of G was not possible to measure using poly (C, ϵ G) templates [1, 2]. Using poly (A, ϵ G) templates we have found that incorporation of dGTP by reverse transcriptase as well as incorporation of GTP by RNA polymerase was however negligible, alike incorporation of C nucleotides in both enzymatic assays (data not shown).

Table II presents the influence of the ratio of C:T (or C:U) nucleotides in the incubation mixtures on C nucleotides incorporation. The increase of the ratio of concentration of dCTP over dTTP (up to 10-fold) does not change the level of incorporation of dCTP in the reverse transcriptase reaction. However, the incorporation of CTP can be achieved under forced conditions in the RNA polymerase reaction. At the same time, unmodified G in poly (A) template directs incorporation of dCTP (or CTP) in accordance with the expectation.

Discussion

There is a striking difference of apparent miscoding potential of ϵ G tested in poly (C, ϵ G)

templates *versus* poly (A, ϵ G) templates. In poly (C, ϵ G) templates ϵ G acts as G and A in the ratio about 4:1 in reverse transcriptase system [2] and in the ratio about 1:1 in RNA polymerase system [1]. In contrast, in poly (A, ϵ G) templates ϵ G acts almost exclusively as A.

The presence of ϵ G in poly (A) templates diminishes the efficiency of synthesis of the new strand measured as incorporation of UTP by RNA polymerase or TTP by reverse transcriptase (Table I). This would suggest that the copying enzyme does not pass the modified base what results in incorporation of UTP or TTP only, but not other nucleotides which are potentially complementary to ϵ G. However, it appears unlikely that ϵ G is a completely blocking lesion since in poly (C) templates where the presence of ϵ G also diminishes the synthesis of the new strand, a substantial ϵ G-provoked incorporation of nucleotides by both enzymes is observed [1, 2]. Therefore, we consider this change of miscoding potential as the result of change of neighboring bases rather than the result of blocking action of ϵ G.

The influence of neighboring base sequence on replication errors occurring at unmodified or modified bases, both *in vivo* and *in vitro*, has been evidenced since many years. However there is not

Table II. The influence of the ratio of C:T (or C:U) nucleotides in the incubation mixtures on C nucleotides incorporation.

Poly (A) template containing	Enzyme	Incorporation [%] of C nucleotides ^a Ratio of C:T (or C:U) nucleotides ^b		
		1:1	4:1	10:1
5% ϵ G	RT ^c	0.2, 0.0	0.0, 0.2	0.1, 0.1
	RP ^c	0.3, 0.2	0.7, 0.2 ^d	9.3, 5.9 ^d
14% ϵ G	RT	0.4, 0.2	0.0, 0.4	0.3, 0.9
	RP	1.0, 0.9	1.1, 5.4 ^d	10.3, 13.4 ^d
10% G	RT	8.9, 11.2	13.1, 14.4	9.8, 9.8
	RP	9.0, 7.0	8.0, 8.2	NT ^e

^a The background incorporations obtained for poly (A) were subtracted. The values obtained from two independent experiments instead of one average value are presented in the Table. Such way of presentation was chosen due to large scattering of data in some cases. See also footnote (d).

^b Total concentration of dCTP and dTTP was always 1.6 mM in reverse transcriptase reactions whereas total concentration of CTP and UTP was 2.4 mM in each RNA polymerase reaction.

^c RT, reverse transcriptase; RP, RNA polymerase.

^d Because of the low extent of reaction, the radioactivity measured in C areas on chromatograms did not exceed the triple value of background (40 cpm).

^e NT, not tested.

simple and general explanation for these phenomena.

One of the possible explanation of the observed phenomenon could be that ϵ G is looped out of poly (A, ϵ G) template and the only A residues are copied. This would result in incorporation of T (or U), but not C. The misalignment of template leads to minus-one-base-frame-shift errors during DNA replication by a number polymerases. Such situation is preferred when template contains runs reiterated bases, especially pyrimidines [5]. Since stacking interactions between purines are stronger than between pyrimidines, the pyrimidine base can be easier looped out than purine base. However, when we consider the possible stacking interactions in poly (C, ϵ G) and poly (A, ϵ G) templates it seems that ϵ G can be easier looped out from the pyrimidine than from the purine template. The stacking of ϵ G is similar to that of A [6], so one can expect stronger interaction between ϵ G and A than between ϵ G and C.

The tautomeric structure of ϵ G has not been studied extensively, nevertheless the ¹⁵N NMR spectra show the presence of N₁-H tautomer (Fig. 1; ϵ G in *a* and *b* pairs) in non-aqueous solutions [6]. This indicates that the basic tautomeric structure of ϵ G is similar to the structure of unmodified G, however the existence of others minor tautomers can not be excluded. In Fig. 1 we propose possible structures of base pairs explaining miscoding properties of ϵ G. The ϵ G-C pair *a* can be formed like the normal G-C pair. For ϵ G-T pair we postulate structures *b*, *c* and *d*. Structure *b* is analogous to the G-T wobble pair and it can be formed with a change of conformation of template, whereas structures *c* and *d* involve minor tautomers ϵ G* and ϵ G** (N²-H and O⁶-H, respectively).

Since the physical nature of pairing between ϵ G and complementary bases has not been studied yet, the influence of neighboring bases on miscoding properties of this adduct can be speculated only. In addition to the discussed above looping out of ϵ G we would like to point out two other

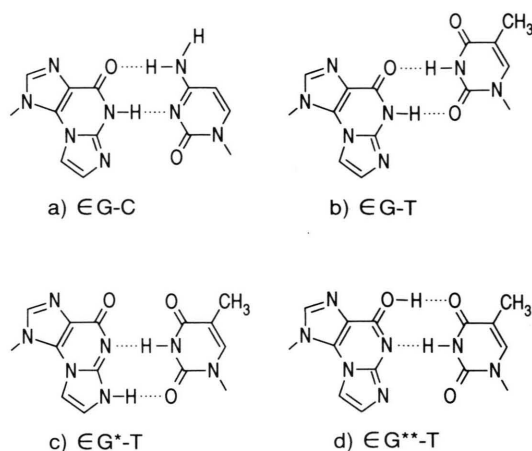


Fig. 1. Possible structure of ϵ G-C and ϵ G-T pairs involving different tautomeric forms of ϵ G.

possibilities. The first is that the conformation of poly (A, ϵ G) template can facilitate the formation of the ϵ G-T pair (Fig. 1, pair *b*) to a greater extent than conformation of poly (C, ϵ G) template. The second is that in the neighborhood of A residues the tautomeric equilibrium of ϵ G can be stronger shifted toward minor tautomer(s) than in the neighborhood of C residues. This would result in formation of ϵ G*-T or ϵ G**-T pair (Fig. 1, pair *c* or *d*) rather than of ϵ G-C pair (Fig. 1, pair *a*). The influence of neighboring bases on tautomeric equilibrium of mismatched bases was postulated by Fresco and co-workers [7].

We realize that any firm conclusion about physical nature of the observed phenomenon would be rather premature due to lack of experimental evidence. Nevertheless, irrespective of the detailed mechanism, we postulate that this phenomenon can occur during DNA replication *in vivo*.

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