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Dedicated to Professor David Shugar on the occasion of his 80th birthday

Miscoding properties of isoguanine (2-oxoadenine) studied in an AMV reverse transcriptase *in vitro* system*

Agnieszka M. Bukowska and Jarosław T. Kuśmierek**

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5A, 02–106 Warsaw, Poland

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We have found that isoguanine (iG) can pair with thymine (iG·T) and the non-natural base, 5-methylisocytosine (iG·iCM) during template directed synthesis catalyzed by AMV reverse transcriptase. The ratio of these pairings is about 1:10, irrespectively which of the templates, poly(C,iG) or poly(I,iG) is used. This ratio corresponds to the ratio of 2-OH and 2-keto tautomers in monomer in aqueous solution and apparently it is not influenced by the template context. Our results indicate also that formation of the reverse transcriptase catalyzed base pairs between iG and A, G or C can occur only at a low frequency, comparable to the frequency, of mismatches of

Isoguanosine (crotonoside) was discovered over 60 years ago as a constituent of the croton bean, *Croton tiglium* L. [1]. More recently isoguanosine, its 1-methyl- (doridosine) and O^2-methylderivative (spongosine) have been found to be widespread amongst marine organisms and they are being extensively studied because of their pharmacological properties [2, 3]. Although isoguanine (as a ribonucleoside) occurs in Nature, this base has not been established to represent a natural constituent of nucleic acids. However, isoguanine (iG, 1,2-dihydro-2-oxoadenine, 2-hydroxyadenine) can be formed in DNA in the reaction of oxygen radicals with adenine. This damaged adenine has been found to be present among the other oxidized bases in DNA of normal and cancerous human tissues [4].

Oxygen radicals are produced through normal cellular metabolism, and formation of such radicals is further enhanced by ionising radiation and various chemicals. The reaction of oxygen radicals with DNA bases produces a variety of lesions of which 8-oxoguanine (7,8-dihydro-8-oxoguanine, 8-hydroxyguanine) has been studied most extensively. It was shown that this oxidized base could miscode either when present in DNA template or in dNTP pool [5, 6]. We have undertaken our studies to elucidate the possible miscoding of another oxidized base, isoguanine.

In solution, isoguanine exists as a mixture of tautomeric forms and the tautomeric equilibrium strongly depends on polarity of a solvent. In water, the 2-keto form (Fig. 1A) is predominant (about 90%) whereas the 2-enol form (Fig. 1B) is favoured by a decrease in solvent polarity [7]. In their studies on template-directed base-pair formation catalyzed by various polymerases Benner and coworkers

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**To whom correspondence should be addressed.

Abbreviations: AMV, avian myeloblastosis virus; diCM, 2'-deoxy-5-methylisocytidine; iG, isoguanine.

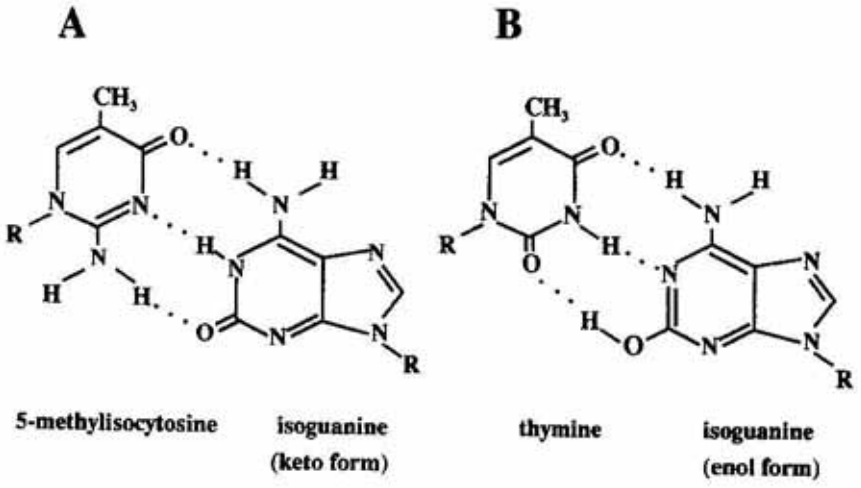

Fig. 1. Proposed base-pairing of isoguanine (iG).
A, iG in its keto form (1,2-dihydro-2-oxoadenine) can pair with 5-methylisocytosine (iCM); B, iG in its enol form (2-hydroxyadenine) can pair with thymine (T). iCM and T can be replaced by isocytosine and uracil, respectively, without changing the pattern of hydrogen bonds.

[8] found that iG can form a pair with another odd base, isocytosine, and also with thymine. They postulated that isocytosine is paired with iG in its keto form, whereas thymine is paired with iG in its enol form (Fig. 1). Since keto-enol equilibrium of isoguanosine is solvent-dependent, one can expect that the nature of neighbouring bases in the template would also influence this equilibrium and could result in changing the coding properties of iG. The examination of the possible influence of neighbours in the template on coding properties of iG was another purpose of our work.

MATERIALS AND METHODS

Chemical syntheses. Isoguanosine was prepared according to [9]. 2'-Deoxy-5-methylisocytidine (diCM) was prepared from thymidine based on methods described in [10, 11]. Thymidine was tosylated and 5'-tosylthymidine was reacted with methanolic ammonia (70°C, 15 h) to give diCM as a main product. After purification on Dowex 1 × 4 (carbonate form) which does not retain diCM, and then on silica gel plates, diCM was obtained in 25–30% yield. diCM is about 2–3 times more resistant than 2'-deoxyisocytidine to acid depyrimidination (pH 4.5) and to alkaline deamination (1 M KOH). Neither of the compounds shows any degradation after one week incubation in water at 37°C. Radioactive [^{3}H]diCM (5 mCi/mmol) was obtained by the same method using 1 mCi [^{3}H]thymidine and 0.2 millimole of non-radioactive thymidine.

Isoguanosine 5'-monophosphate (5'-iGMP) was obtained by enzymatic phosphorylation of isoguanosine according to [12]. This was converted to 5'-iGDP by the method of Moffatt & Khorana [13]. 5'-Triphosphate of diCM (diCMTP) was synthesized from diCM using the method described for triphosphorylation of 2'-deoxyisocytidine [8].

Reverse transcriptase reaction. The templates used for copying were prepared by copolymerization of appropriate ribonucleoside-5'-diphosphates with the aid of polynucleotide phosphorylase. The methods of preparation and analysis of templates are described in [14].

The templates primed with complementary oligodeoxynucleotides were copied by avian myeloblastosis virus (AMV) reverse transcriptase in the presence of appropriate non-radioactive and ^{3}H-labelled deoxynucleoside-5'-triphosphates (see Figure legends). The time course of the reaction was followed by spotting of aliquots of reaction mixture on DEAE paper disks at various times of incubation. Disks were washed with 6% Na_2HPO_4 and radioactivity on disks was measured. In order to determine the proportion of incorporated radioactive dNTPs, the newly synthesized polymer was separated from non-incorporated radioactive dNTPs using Sephadex G-50 column. After enzymatic digestion of the polymer, the resulting deoxynucleosides were separated by paper chromatography and the radioactivity in each

deoxynucleoside area was measured. The experimental details are described in [14, 15].

RESULTS AND DISCUSSION

In order to study the miscoding potential of iG we have employed an *in vitro* system, the copying of randomly composed polyribonucleotide templates with AMV reverse transcriptase. Reverse transcriptase can use for DNA synthesis either a ribo- or a deoxyribo-template in the presence of a complementary oligodeoxynucleotide primer [16]. The possibility of use in experiments of ribotemplates can be advantageous since polyribonucleotides containing randomly placed modified bases are easily synthesized under mild conditions by copolymerization of appropriate nucleoside-5'-diphosphates with the aid of polynucleotide phosphorylase. In the case of labile adducts the use of enzymatically prepared ribotemplates could be the only choice [17].

Our preliminary experiments have shown that in the presence of complementary oligodeoxynucleotide primers AMV reverse transcriptase can copy efficiently poly(C), poly(A) and poly(I) but not poly(U) templates. This is somewhat inconsistent with the results of Battula & Loeb [16] who found that poly(C) and poly(A) are active whereas poly(I) and poly(U) are nonactive templates. The presence of other unmodified or modified bases in the homopolymer template diminishes the efficiency of synthesis of the new strand, nevertheless the conformity is observed between the proportion of minor base in the template and incorporation of its complementary base to the new strand. This is in agreement with observations of other authors and also with our previous findings [15, 17].

Isoguanine can pair with thymine and 5-methylisocytosine

Since iG can form a pair with another odd base isocytosine, in addition to thymine ([8] Fig. 1), we have undertaken studies on the relative ability of base-pair formation by both counterparts with iG. We used in our studies 5-methylisocytosine (iCM), a close analogue of isocytosine. The reason was that [^{3}H]-5-methyldeoxyisocytidine-5'-triphosphate ([^{3}H]diCMTP), a substrate in the reverse transcriptase reaction, can be synthesized from relatively inexpensive [^{3}H]thymidine (see Materials and Methods). We expected that the 5-methyl substituent would not change the base-pair formation ability of isocytosine, by analogy to thymine *vs* uracil.

The relative incorporation of [^{3}H]diCMTP *vs* [^{3}H]dTTP was studied by copying of poly(C,iG) and poly(I,iG) templates. The radiochromatogram of enzymatic digest of the product of copying poly(C,15%iG) is shown in Fig. 2 as an example. The legend to this Figure comprises the details of experiment. The results are summarized in Table 1.

The template-directed iG·iCM pair formation catalyzed by AMV reverse transcriptase is about 10 times more efficient than formation of the iG·T pair. The small differences among templates tested can be ascribed to experimental errors. The ratio 1:10 corresponds to the ratio of 2-OH to 2-keto tautomers of isoguanosine in aqueous solution [7]. This supports the base pairing scheme presented in Fig. 1. According to this scheme iCM pairs with keto form of iG (Fig. 1A) whereas T pairs with enol form of iG (Fig. 1B). The change of template context, at least C- *vs* I-neighbourhood, does not change the coding properties of iG to an unquestionably measurable extent. It seems that in polymer, the ratio of tautomeric forms of iG is very similar to the ratio in monomer and that stacking interactions do not exert any apparent influence on tautomeric equilibrium of iG. A similar conformity between the ratio of the amino- and imino tautomers of N^4-methoxycytosine and base pair formation with G and A was observed during the copying of templates containing the above analogue by RNA polymerase [18].

The total incorporation of iCM+T is lower than iG content in template. In poly(C,iG) templates about 2/3 of iG was copied. A similar extent of copying of another modified base, N^2,3-ethenoguanine and unmodified G, A and U, was observed in analogous experiments [17]. The very low efficiency (about 1/50) of copying of iG present in poly(I,iG) needs a separate comment. The simplest explanation of this phenomenon could be that iG residues are easily looped out of the poly(I) template. Another possibility is that iG causes efficient incorporation of dCTP which is also complementary to the dominating base in the template and this strongly biases the incorporation of dTTP +

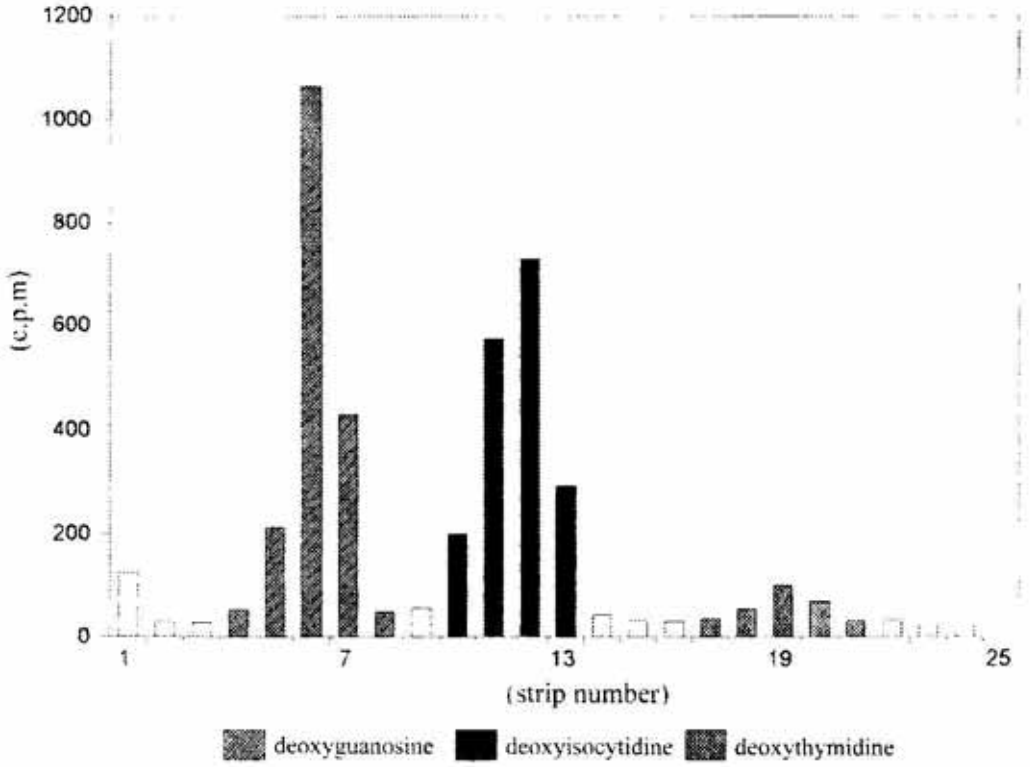

Fig. 2. Chromatogram of enzymatic digest of the product of copying of poly(C,15% iG) template by AMV reverse transcriptase.

The 5 ml-reaction mixture contained 10 absorbance units of template primed with 0.5 absorbance unit of dG_6, 2 mM [^{3}H]dGTP (0.5 mCi/mmol) 0.1 mM [^{3}H]dTTP (5 mCi/mmol), 0.1 mM [^{3}H]diCMTP (5 mCi/mmol) and 100 units of enzyme in: 50 mM Tris/HCl, pH 8.3, 10 mM $MgCl_2$, 40 mM KCl, 1 mM EDTA and 1 mM DTT. After 1 h incubation at 37°C the reaction was stopped by ethanol precipitation. The polymeric material was separated from unreacted dNTPs by chromatography on Sephadex G-50 column and then hydrolyzed to deoxynucleosides by combined action of micrococcal nuclease, P1 nuclease, deoxyribonuclease I, phosphodiesterase I and bacterial alkaline phosphatase. The deoxynucleoside markers were added and the entire sample was applied to Whatman 3MM paper for descending chromatography. Chromatography was performed with the solvent system: water-saturated *n*-butanol. The separation was carried out twice in the same direction for 18–20 h at room temperature. The chromatogram was cut into 2 cm strips and radioactivity on each strip was determined using a liquid scintillation counter. The UV-marker areas are indicated by different shadowing of appropriate radioactivity bars.

diCMTP. However, this is rather unlikely, since iG present in poly(C) and in poly(A) templates does not provoke any measurable incorporation of dCTP (see below).

Table 1

The template-directed relative iG·iCM and iG·T base-pair formation catalyzed by AMV reverse transcriptase

Template	iCM + T (% of total incorporation)	iCM:T ratio
poly(C,6%iG)	4.0	9
poly(C,15%iG)	9.4	12
poly(I,12%iG)	0.25	8

Isoguanine does not pair with DNA bases other than thymine

Isoguanine (2-oxoadenine) is one of the products of the reaction of mutagenic oxygen radicals with adenine in DNA [4]. In the view of the foregoing, it is important to evaluate the miscoding potential of this lesion, i.e. the ability of iG to provoke the incorporation of each particular dNTP to the primer strand during template-directed synthesis catalyzed by a DNA polymerase. The iG provoked incorporation of dTTP (see above) can not lead to mutation and this indicates only that iG retains to some extent the coding properties of the parent base, adenine. On the other hand, the fact that iG provoked incorporation of diCMTP (or deoxyisocytidine-5′-triphosphate [8]) is not reic-

vant to mutagenesis because iCM (or isocytosine) is not a DNA base.

Poly(A), poly(I) and poly(C) templates containing iG in various proportions were copied in the presence of all four 0.4 mM [^{3}H]dNTPs and the composition of the newly synthesized strands was analyzed (the idea of these experiments is presented in the legend to Fig. 2). In all cases the incorporations of A, G and C caused by the presence of iG in templates were below or did not exceed significantly the background values (not shown).

Since the miscoding of iG can not be detected under competitive conditions, i.e. where all four dNTPs are present at equal concentration, in the next experiments we have used the "forced" conditions. In general, in these experiments we studied time course of incorporation of [^{3}H]dNTP, potentially complementary to iG, in the presence of non-radioactive dNTP complementary to the dominant base in template. Figure 3 ([^{3}H]dTTP incorporation) and Fig. 4 ([^{3}H]dATP incorporation) display the time course of copying of poly(A,12%iG) and poly(A,10%G) templates in the presence of 0.1 mM dATP and 0.1 mM dTTP.

The incorporation of [^{3}H]dTTP (Fig. 3), which is complementary to the dominant base in templates, exhibits a characteristic plateau, usually observed under such conditions. In contrast, the incorporation of [^{3}H]dATP (Fig. 4), which is tested for complementarity to iG and G bases and which is complementary to the new T-dominated strand shows first some lag phase and/or a plateau, and then an accelerating course. The incorporation of [^{3}H]dATP tested in the presence of poly(A) template is marginal and does not exceed significantly background values up to 60 min of incubation.

The foregoing observations lead to the conclusion that iG·A and G·A mismatches are formed during the copying of poly(A,iG) and poly (A,G) by AMV reverse transcriptase. These mismatches create structural irregularities which can originate the utilization of the new strand as an alternative template. As it can be evaluated on the basis of the actual amount of radioactivity in aliquots of reaction mixtures corresponding to the time points (see Figure legends), this utilization can account for about 1% of the total synthesis after 60 min of incubation.

The new strand cannot serve as the alternative template when mismatches are excluded as it is the case when unmodified poly(A) is copied in the presence of [^{3}H]dATP and non-radioactive dTTP (Fig. 4). Also the copying of poly-(C,10%iG) in the presence of [^{3}H]dCTP and

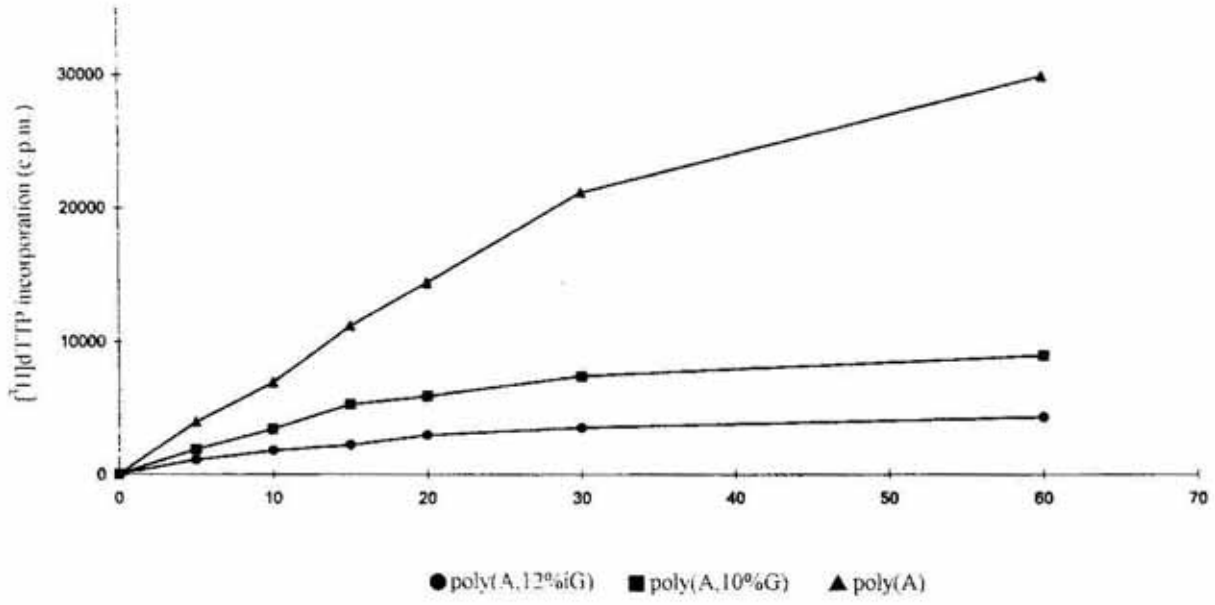

Fig. 3. Time course of copying of poly(A,12%iG), poly(A,10%G) and poly(A) templates in the presence of 0.1 mM dATP and 0.1 mM [^{3}H]dTTP (0.2 Ci/mmole).

Each 100 µl reaction mixture contained 0.2 absorbance unit template primed with 0.05 absorbance unit of dT_{15} and 2 units of reverse transcriptase. Other conditions were as in Fig. 2. Aliquots of 12 µl were spotted on DEAE paper disks at indicated times.

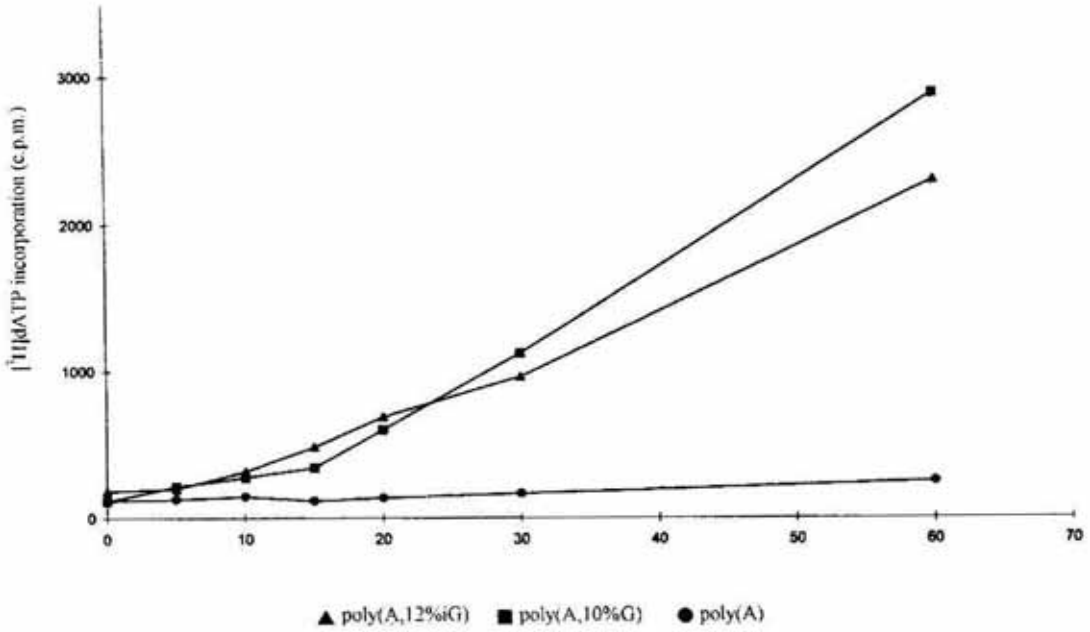

Fig. 4. Time course of copying of poly(A,12%iG), poly(A,10%G) and poly(A) templates in the presence of 0.1 mM [^{3}H]dATP (1.0 Ci/mmol) and 0.1 mM dTTP.
The conditions were as in Fig. 3 except that total volume of each incubation mixture was 350 µl and 48 µl-aliquots were spotted on disks.

non-radioactive dGTP does not lead to incorporation of radioactivity into the polymer (not shown). This would indicate that iG·C or iG·G mismatches possibly formed at low frequency can not disrupt the strong secondary structure of G·C-dominated duplex, in contrast to mismatches in the less stable A·T-dominated duplex.

An increasing concentration of non-radioactive diCMTP (0.1 mM–2 mM) causes a gradual decrease of incorporation of [^{3}H]dATP during the copying of poly(A,12%iG) template (Fig. 5). The similar effect is exerted by increasing concentration of dTTP (not shown). This indicates that iCM and T which are complementary to iG, compete with A in base pair formation with iG.

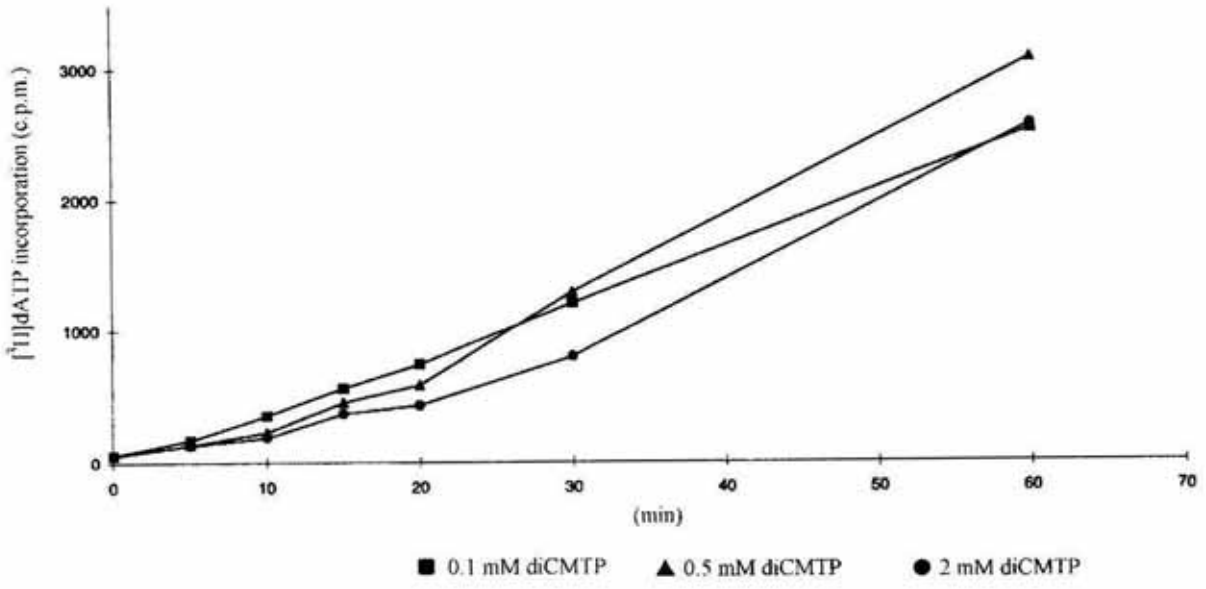

Fig. 5. The influence of increasing concentration of diCMTP on [^{3}H]dATP incorporation during the copying of poly(A,12%iG).
All conditions were identical to those in Fig. 4.

It is rather difficult to evaluate the absolute frequency of iG·A mismatch formation on the basis of data presented here. Nevertheless, the time course curves of incorporation of [^{3}H]dATP in the presence of poly(A,12%iG) and poly(A,10%G) templates are very similar (Fig. 4): this suggests that the frequencies of iG·A and G·A mismatches are of the same order of magnitude.

To establish whether an iG·G mismatch can be formed in the AMV reverse transcriptase reaction, we studied the copying of poly(I, 10%iG) and poly(I,10%A) templates in the presence of [^{3}H]dGTP and non-radioactive dCTP (not shown). The results were analogous to these of the foregoing experiments where the formation of iG·A mismatch was studied. We conclude that the iG·G mismatch is formed with a frequency similar to the frequency of A·G mismatch formation.

In addition to the experiments described above we have tested for complementarity to iG the [^{3}H]dNTPs which were not complementary to the dominating base in the template or in the new strand. They are listed below:

poly(I,10%iG),	[^{3}H]dATP and dCTP
poly(C,6%iG),	[^{3}H]dATP and dGTP
poly(A,12%iG),	[^{3}H]dGTP and dTTP
poly(A,12%iG),	[^{3}H]dCTP and dTTP

In each case the incorporation of radioactivity was within the range of the background values (not shown). The results of all experiments presented here indicate that formation of template-directed base pairs catalyzed by AMV reverse transcriptase between iG and A, G and C can occur only at a low frequency, comparable to that of natural mismatch formation.

Comments to the recent relevant papers

Two papers relevant to the issue studied appeared during completion of this study. Kamiya *et al.* [19] studied the insertion of dNTPs opposite iG placed at a preselected site of synthetic oligodeoxynucleotide template, using the primer extension kinetic assay. The authors found some incorporation of dAMP and dGMP opposite to iG by the 3′,5′-exonuclease-deficient Klenow fragment of *E. coli* DNA polymerase I (KF exo$^-$) and dAMP by calf thymus DNA polymerase α (pol α). The frequency of these incorporations were within the range 10^{-5}–10^{-3} of the frequency of incorporation of dTMP opposite A, taken as an arbitrary unit (1.0). In the reaction of recombinant rat DNA polymerase β (pol β) the frequency of incorporation of dAMP opposite iG amounted to 0.14. On the basis of these results the authors state that iG formation in DNA will induce A→T and A→C transversions in cells.

However, a closer examination of the presented data shows that natural mismatches A·A and A·G are formed by KF exo$^-$ 3 and 9 times more efficiently than iG·A and iG·G, respectively, whereas with pol β the formation of iG·A is only 5 times more efficient than that of A·A. The high frequency of iG·A formation by pol β (0.14) can not be compared with the frequency of A·A because of lack of data. On the other hand, pol β is known as an extremely error prone polymerase with a strong preference to incorporate dAMP opposite to any base [20].

Bearing in mind that all these results were obtained under non-competitive conditions (under conditions of the assay only one dNTP can be tested at a time) and that none of the polymerases tested does possess any correcting activity, we think that the authors' statement is rather premature. In our opinion, the answer to the question whether the formation of iG in DNA shall induce A→T and A→C transversions *in vivo*, still requires more extensive studies.

In the other paper, Horn *et al.* [21] compared the stability of iG·iCM pair *vs* other pairs in oligodeoxynucleotide duplexes. The duplex containing iG·iCM pair was slightly more stable than the analogous duplex containing G·C pair, whereas the replacement of iCM by T, C, A or G led to substantial decrease of stability. This indicates that only iCM is the appropriate counterpart of iG.

In conclusion, thymine is the only natural base which can pair with iG in its minor 2-OH tautomeric form, and formation of iG in DNA does not seem to lead to mutations by the simple mispairing mechanism.

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