On the Molecular Basis of Transition Mutations

FREQUENCY OF FORMING 2-AMINOPURINE-CYTOSINE BASE MISPAIRS IN THE G-C → A-T MUTATIONAL PATHWAY BY T4 DNA POLYMERASE IN VITRO*

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An in vitro model system including wild-type T4 DNA polymerase, the mutagenic nucleotide analogue 2-aminopurine deoxyribonucleoside triphosphate, and poly(dA,C)·oligo(dT) poly(dC)·oligo(dG) template-primers is used to measure the frequency of 2-aminopurine-cytosine base mispairs formed in the G-C → A-T mutational pathway. Incorporation and turnover of the analogue into DNA is dependent on the presence of cytosine on the template strand and is reduced significantly in the presence of dGTP. 2-Aminopurine-cytosine mispairs are observed to occur at a 2-3 order of magnitude greater frequency than adenine-cytosine mispairs. The frequency of inserting 2-aminopurine deoxyribonucleoside monophosphate in place of dGMP opposite template cytosine sites is about 3-6% when either strong or weak base-stacking partners are present on the primer strand. However, enzymatic proofreading of the mispair strongly depends on base-stacking partners. Greater than 85% of misinserted 2-aminopurine deoxynucleotides are excised whenever the mispairs are formed next to 5'-primer thymine sites. A 5-fold reduction in proofreading frequency occurs when the mispair is formed with 2-aminopurine deoxyribonucleoside monophosphate stacked adjacent to a 5'-primer guanine. The frequency of 2-aminopurine-cytosine base mispair formation in the G-C → A-T pathway is similar to that found previously in the A-T → G-C pathway (Watanabe, S. M., and Goodman, M. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 78, 2864–2868). We propose a criterion for base selection by DNA polymerase to account for the unexpected similarity in base mispairing rates in the two transition pathways.

AmPur, a base analogue of adenine, has had a historically important role to play in mutagensis studies involving Escherichia coli and bacteriophage T4-infected E. coli (1-5). According to its hypothesized mode of mutagenesis (2), AmPur pairs with cytosine to form an AmPur-C base mispair in DNA. Following replication, the AmPur-C heteroduplex helix is resolved predominantly into A-T and G-C base pairs. 2-Aminopurine stimulates λ-T → G-C and G-C → A-T base substitution mutations bidirectionally. An AmPur-C base mispair can arise in either of two ways. First, in the A-T → G-C mutational pathway, 2-aminopurine deoxyribonucleoside triphosphate serving as a substrate for DNA polymerase can be inserted opposite thymine situated on the DNA template. In a subsequent round of replication, AmPur on the template pairs with an incoming dCTP substrate to form an AmPur-C base mispair. Alternatively, in the G-C → A-T pathway, the mispair is formed in a single step when dAmPurTP is inserted opposite a template cytosine.

We propose a criterion in modeling the AmPur-induced A-T → G-C transition in vitro is to demonstrate the extent to which AmPur-C forms in preference to A-C base mispairs. What effect does the replacement of A by AmPur on the template have on the probability of misincorporating dCMP at that site? We have shown that AmPur-C are favored over A-C mispairs by a factor of 230 when cytosine is stacked adjacent to a 5'-primer guanine, and by a factor of 35 when the base-stacking partner is thymine.

In this paper, we report the frequency of forming AmPur-C base mispairs in the G-C → A-T mutational pathway. Several questions will be considered. First, analogous to the A-T → G-C pathway, is dAmPurTP, as a substrate for DNA polymerase, inserted more readily opposite a template cytosine than dATP? Second, with what efficiency is dAmPurTP inserted opposite cytosine in competition with dGTP? Third, how thoroughly are AmPur-C base mispairs proofread by the active 3'-exonuclease of T4 DNA polymerase; this latter point was not investigated in the A-T → G-C studies (6, 7) which utilized α-DNA polymerase lacking 3'-exonuclease activity (8). Fourth, how do purine-purine as opposed to pyrimidine-purine nearest neighbor base-stacking partners influence the formation and editing of AmPur-C base mispairs?

EXPERIMENTAL PROCEDURES

Enzymes—Wild-type T4 DNA polymerase was purified according to the method of Morris et al. (9). Terminal deoxyribonucleotidyl transferase was purified from calf thymus glands (10, 11).

Synthesis of Homopolymer and Copolymer Templates—Primer—Deoxyribonucleotide homopolymers consisting of either Ade or Cyt or mixed copolymers containing various proportions of Ade and Cyt were prepared using terminal deoxyribonucleotidyl transferase. Covalently attached "hooked" primers of either oligo(dT) or oligo(dG) were added as described previously (6, 12). Composition of each template-primer was determined by enzymatic digestion of the DNA polymers to mononucleotides (6) followed by analysis with high performance liquid chromatography on a Waters Associates Radial Pakmondapak C8 reverse-phase cartridge in a mobile phase of 0.2 M ammonium phosphate, pH 5.5, buffer.

Nucleotides and Chemicals—All nonradioactive deoxyribonucleoside triphosphates were purchased from P. L. Biochemicals. ATP and 2-amino-6-chloropurine (6-chloroguanine) were purchased from Sigma. [3H]2-Aminopurine was prepared by catalytic reduction of 6-chloroguanine by ICN Pharmaceuticals. [3H]2-Aminopurine deox-

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The abbreviations used are: AmPur, dAmPurMP, and dAmPurTP, the free base, deoxynucleoside monophosphate, and deoxyribonucleoside triphosphate of 2-aminopurine.
ribonucleoside triphosphate was synthesized from the tritiated free base (13). [3H]dATP, [3H]dTTP, and [32P]dGTP were purchased from New England Nuclear. All other chemicals were of the highest obtainable purity from commercial sources.

Assays—The T4 DNA polymerase and turnover assays were done essentially as previously described (13). Reaction mixtures consisted of the following components: 67 mM Tris-HCl, pH 8.8; 17 mM (NH4)2 SO4; 9.5 mM B-mercaptoethanol, 1.2 μg/ml of bovine serum albumin; 6.7 mM MgCl2, 0.63 mM ATP; 0.67 A260 units/ml of template-primer poly[d(A,C)]-oligo(dT), poly(dA)-oligo(dT), or 0.53 A260 units/ml of poly(dC)-oligo(dG); 43 units/ml of T4 DNA polymerase and deoxynucleoside triphosphate substrates in a total volume of 30 μl. One polymerase unit equals 10 pmol of dATP incorporated into DNA in 30 min at 30 °C. Substrate concentrations used in different experiments are described in the appropriate figure captions. Direct competition experiments were done in the same assay tube using [32P]dGTP and [3H]dAmPurTP. The specific activity of [32P]dGTP ranged from 6 × 106 cpm/μmol to 6.5 × 107 cpm/μmol. Specific activity of [3H]dAmPurTP was 2 × 105 cpm/μmol; that of [3H]dATP was 7 × 106 cpm/μmol, and [3H]dTTP was 9 × 106 cpm/μmol.

RESULTS

The rate-limiting step in the induction of G·C → A·T mutations by 2-aminopurine is believed to be the misincorporation of dAmPurMP in place of dGMP opposite cytosine (2). To model this step in vitro, we allowed [3H]dAmPurTP to compete against [32P]dGTP for insertion opposite C on synthetic DNA polymers using wild-type T4 DNA polymerase. Both incorrect (dAmPurMP) and correct (dGMP) incorporation and turnover (3'-exonucleolytic proofreading) were measured using two different nearest neighbor base-stacking configurations shown in Fig. 1.

The rate of dAmPurTP misincorporation is dependent on the amount of cytosine present on the template strand. This point is illustrated in Fig. 2a where incorporation of the nucleotide analog was measured at different time points using hooked copolymer template-primers, poly[d(A,C)]-oligo(dT), containing primarily dAMP with varying amounts of dCMP interspersed randomly along the template strand (Fig. 1a). Rapid dAmPurMP incorporation is observed on templates containing about 44% C, 56% A. A slower initial rate of dAmPurMP incorporation is observed on templates containing 20% C, 80% A. A much smaller but, nonetheless detectable level of dAmPurMP appears to be incorporated into poly(dA)·oligo(dT) when cytosine has been excluded from the template strand.

Removal of misinserted dAmPurMP by the active 3'-exonuclease activity of T4 DNA polymerase is shown in Fig. 2b. Similar turnover rates were observed for the two poly [d(A,C)]·oligo(dT) copolymer templates. For the data in Fig. 2, the competing substrate, dGTP, was not included in the reaction mixture. Incorporation of dAmPurMP opposite the first of a sequence of two or more consecutive C sites on the template acts as a block toward further elongation resulting in similar total AmPur incorporation (t > 30°) on the two copolymers (Fig. 1a).

The elongation block at consecutive C sites, which requires the insertion of successive AmPur nucleotides to extend the primer, can be alleviated by addition of dGTP to the reaction. For the template primer poly[d(As4, C6)]-oligo(dT), dAmPurMP incorporation is about 1.7 pmol after 30 min in the presence of 0.2 mM dGTP (Fig. 3), while in the absence of dGTP, dAmPurMP incorporation is about 3-fold lower (Fig. 2a). The data in Fig. 3 illustrate the important point that AmPur is most likely forming base mispairs at template C sites because dAmPurMP incorporation can be competed out by the addition of increasing concentrations of dGTP. As expected, the addition of excess dATP has no discernible effect on dAmPurMP incorporation.

A time course for incorporation and turnover of dAmPurMP into poly(dC)·oligo(dC) template-primer DNA is shown in Fig. 4. The presence of dGTP is required to achieve net DNA synthesis and net incorporation of dAmPurMP; indeed the incorporation of dAmPurMP and dGMP roughly parallel one another (Fig. 4a). Here one may be observing an example of the "next nucleotide" effect (13, 14) which occurs whenever an incorporated dAmPurMP exhibits reduced susceptibility to exonucleolytic proofreading by the presence of high concentrations of the next nucleotide to be added, in this case dGTP, in a direction 3' to the AmPur residue.

A direct demonstration that increased levels of dGTP cause a reduction in the probability of dAmPurMP excision is contained in Fig. 4b which shows that turnover of the deoxynucleotide analogue is reduced significantly as dGTP concentration is increased from 0.013 to 13.3 mM. At the high concentration of dGTP, significant turnover of dAmPurMP is observed only at the 30-min incubation point when DNA syntheses, as measured by dGMP incorporation, has begun to level off, see Fig. 4a. When nucleotide incorporation is no longer increasing with time, newly inserted deoxynucleotides are known to exhibit greatly increased susceptibility to excision by the 3'-exonuclease of T4 DNA polymerase (15–17).

Spontaneous deamination of cytosine to uracil in DNA is known to occur in vivo; the analogous deamination of 5-methyluracil to thymine leads to mutational hot spots in the lacI gene of *E. coli* (18). Since 2-aminopurine can form two stable hydrogen bonds with uracil (19), it is important to determine to what possible extent the incorporation of dAmPurMP into poly[d(A,C)]-oligo(dT) and poly(dC)-oligo(dG) might be caused by the presence of a uracil contaminant in the template strand. A precise determination of template-primer composition was carried out by making a limit enzymatic digest of synthetic DNA polymers to deoxyribonucleoside monophosphates followed by analysis using high performance liquid chromatography. No dUMP was detected in the DNA digests (data not shown). An upper bound estimate for the presence of uracil in the synthetic DNA polymers is approximately 0.001%. Thus, the high-performance liquid chromatography data taken in conjunction with the observations that dAmPurMP incorporation can be competed out by the addition of dGTP (Fig. 3) and that no measurable dAMP was incorporated in any of the templates studied argues strongly that incorporation of the nucleotide
AmPur-induced G C→A·T Transitions

FIG. 2. Time course for incorporation (a) and turnover (b) of dAmPurMP opposite C on poly-[d(A,C)]·oligo(dT) templates by T4 DNA polymerase. Incorporation and turnover assays were performed as described under "Experimental Procedures" in the presence of 13.3 μM [3H]dAmPurTP (2 × 10^6 cpm/μmol) and 66.7 μM unlabeled dTTP. Template-primers are: poly[d(A,C)]·oligo(dT) (x), poly[d(A,C)]·oligo(dT) ( ), opposite poly[d(A,C)]·oligo(dT) ( ).

FIG. 3. Incorporation of dAmPurMP on poly[d(A,C)]·oligo(dT) template as a function of increasing concentrations of either dGTP ( ) or dATP ( ). The reactions were incubated for 30 min in the presence of 66.7 μM unlabeled dTTP, 13.3 μM [3H]dAmPurTP (2 × 10^6 cpm/μmol) and 2 × 10^4 to 13.3 μM [32P]dGTP (1 × 10^6 to 6.8 × 10^5 cpm/μmol). The concentration of dATP ranged from 0 to 13.3 μM.

The dAmPurMP/dGMP misinsertion and misincorporation ratios are the most relevant parameters which relate these in vitro results to the mutagenic potential of 2-aminopurine. These ratios have been normalized by multiplying by the initial ratio of the two competing nucleotide substrates, dGTP/dAmPurTP. We find that a misinsertion ratio of between 3 and 6% is observed for each of the template-primers studied (Table I). The misincorporation ratio, which includes the effect of proofreading, is about a factor of 6-12 less than the misinsertion ratio on the mixed d(A,C) copolymer templates (Table I). In contrast to the mixed copolymer templates, we attribute a diminished proofreading of the homopolymer poly(dC)·oligo(dG) template-primer to strong base-stacking stabilization of a misinserted dAmPurMP by 5' nearest neighbor guanine residues (20), and to a reduced susceptibility of misinserted dAmPurMP residues to proofreading because the error becomes "locked in" by the subsequent incorporation of dGMP (13, 14).

It is interesting that we only observe a significant base-stacking effect on proofreading but not on dAmPurMP misinsertion frequencies in agreement with the observations of Pless et al. (20, 21). A similar observation has been made for the case of AmPur C base mispairs formed in the A·T→G·C mutational pathway where AmPur is a template base and dCMP is misinserted opposite AmPur in competition with dTMP (6). A failure of base-stacking forces to influence AmPur·C misinsertion frequencies may relate to AmPur's ability to have two hydrogen bonds with cytosine when stabilized by surrounding proper Watson-Crick base pairs (Ref. 22; see "Discussion").

DISCUSSION

The classic model proposed to account for 2-aminopurine-induced mutagenesis (2) invokes the formation of a DNA intermediate having 2-aminopurine mispaired opposite cytosine. We have shown (22) that AmPur and Cyt are joined by two hydrogen bonds when the mispair is located in the vicinity of stable Watson-Crick base pairs.

In the A·T→G·C direction, the formation of AmPur·C mispairs occur with AmPur situated on the DNA template with dCTP and dTTP competing for insertion opposite AmPur. Neither of the two competing base pairs, AmPur·T nor AmPur·C, form ideal Watson-Crick structures. It has been observed that AmPur·T base pairs are less stable than A·T base pairs by about 1-1.3 kcal/mol (13). We have also

analogue must be occurring predominantly opposite template C sites.

The high-performance liquid chromatography analysis also enabled us to make an accurate measurement of the relative amounts of A and C in the templates. We verified that the biological activity of the templates using the common deoxyribonucleoside triphosphates as substrates for DNA polymerase is consistent with C/A ratios determined by high-performance liquid chromatography (data not shown); this is an important consideration since the distribution of bases on DNA synthesized by terminal deoxyribonucleotidyl transferase is not entirely random (see, e.g. Ref. 11).

We have also observed the incorporation of dAmPurMP at template C by electrophoretic analysis of defined sequence M13 DNA fragments (R. L. Lasken and M. F. Goodman, manuscript in preparation).
FIG. 4. Time course for incorporation of dAmPurMP and dGMP (a) and turnover of dAmPurMP (b) on poly(dC)-oligo(dG) template by T4 DNA polymerase. The incorporation and turnover assays were performed as described under "Experimental Procedures" using [3H]dAmPurTP/[32P]dGTP pool (a) or [3H]dGTP (b) for incorporation and turnover, respectively. The number of experiments carried out is given in parentheses.

TABLE I

<table>
<thead>
<tr>
<th>Template-primer</th>
<th>Misinsertion ratio</th>
<th>Misincorporation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly[d(A,C)]-oligo(dT)</td>
<td>3.1 ± 1.1 (6)</td>
<td>0.4 ± 0.3 (16)</td>
</tr>
<tr>
<td>poly[d(A,C)]-oligo(dG)</td>
<td>5.9 ± 2.3 (3)</td>
<td>0.2 ± 0.1 (4)</td>
</tr>
<tr>
<td>poly(dC)-oligo(dG)</td>
<td>3.7 ± 1.2 (4)</td>
<td>2.7 ± 0.7 (8)</td>
</tr>
</tbody>
</table>

shown that AmPur.C base pairs formed in the A-T → G-C pathway are less stable than AmPur.T base pairs by about 1.8 kcal/mol (6, 7). In other words, when AmPur is situated on the DNA template strand, dTMP/dCMP insertions opposite AmPur occur in a ratio of about 20:1.

In the G-C → A-T mutational pathway, the nucleotide analogue is present as a substrate for the DNA polymerase; dAmPurTP competes with dGTP for insertion opposite template cytosine. The incorporation and turnover of dAmPurMP is dependent on the presence of cytosine on the template strand (Fig. 2). Incorporation and turnover of the analogue is reduced significantly either when excess dGTP is present to compete with dAmPurTP (Fig. 3) or when cytosine is absent from the template (Fig. 2). Hence, these data imply that AmPur.C base mispairs are being formed in the G-C → A-T pathway in vitro.

To determine whether 2-aminopurine exhibits "mutagenic" potential in vitro commensurate with its strong mutagenic behavior in vivo, it is particularly relevant to determine whether base mispairs involving AmPur.C greatly exceed those involving A.C. The data clearly illustrate that mispairs involving AmPur.C occur at a frequency of perhaps 3 orders of magnitude higher than those involving A.C catalyzed by wild-type T4 polymerase. It is difficult to give a precise estimate for the AmPur.C/A.C mispair ratio because the incorporation of dAMP opposite C, either in the presence or absence of dGTP, is only marginally detectable. Our best estimate for the ratio of dAmPurMP/dAMP incorporation is made using a poly(dC)-oligo(dG) template-primer molecule; here the AmPur.C/A.C mispair ratio is about 1500/1.

A comparison between misinsertion and misincorporation ratios (Table I) illustrates the importance of nearest neighbor base-stacking and hydrogen-bonding interactions during DNA replication. Base context in the immediate vicinity of the AmPur.C mispair appears to influence AmPur proofreading primarily, while exhibiting no discernible effect on AmPur misinsertion. Thus, the frequency of misinserting dAmPurMP (3-6%) is similar on the two template-primers, poly[d(A,C)]-oligo(dT) and poly(dC)-oligo(dG), and does not appear to depend upon whether dAmPurMP is inserted next to a presumably strong base-stacking partner, G, or weaker base-stacking partner, T. The absence of base-stacking effects on deoxynucleotide insertion frequencies has been observed to occur whenever hydrogen bonds are present to override or mask base-stacking forces (6, 23). Since base-stacking free energies can be several times larger than H-bonding free energies, it is possible that the active site of DNA polymerase orients incoming dNTP substrates to optimize H-bonding at the expense of base-stacking interactions.

In contrast to the lack of base context influence on the insertion of an AmPur deoxynucleotide, exonucleolytic proofreading of dAmPurMP is dependent on nearest neighbor base-stacking and hydrogen-bonding interactions. Greater than 85% of all misinserted AmPur deoxynucleotides are removed whenever AmPur.C base mispairs are formed with dAmPurMP inserted next to a primer thymine. However, when dAmPurMP is inserted adjacent to guanine on the primer strand, the dAmPurMP removal frequency is less than 17%.

The 5-fold reduction in proofreading when a newly inserted AmPur is located next to guanine on the primer strand may be caused, in part, by G-AmPur base-stacking and by the presence of nearest neighbor G.C base pairs; each may aid in joining AmPur to C by two hydrogen bonds (22). The stabilizing influence of a G.C base pair following AmPur.C may be as important as the influence of a preceding G.C pair (24) since the addition of dGMP to the 3' terminus of dAmPurMP reduces the probability for exonuclease protection (13, 14). An AmPur.C mispair is more likely to be melted out and hence more easily excised when surrounded by A.T base pairs (24).

An interesting question is how can dAmPurMP compete so successfully with dGTP for insertion at template C sites. The AmPur.C/G.C insertion ratio is roughly 3-6% on both
poly(dC)-oligo(dG) and poly[d(A,C)]-oligo(dT) template-primer complexes (Table I). We were surprised that the rate of forming AmPur-C base mispairs in competition with G-C base pairs opposite template AmPur sites, although somewhat lesser, were in fact quite close to the rate of forming AmPur-C versus AmPur-T mispairs opposite template AmPur sites (6). We would like to speculate on a criterion for base selection by DNA polymerase which may provide a qualitative explanation for the unexpected similarities in the two AmPur-C formation rates.

Supposing that the polymerase active site were structured so that the presence of just two hydrogen bonds were sufficient to stabilize the [polymerase-template-dNTP substrate] complex long enough to ensure that a phosphodiester bond is likely to be formed. Any additional increase in the lifetime of the complex, as might be provided by the presence of a third hydrogen bond in a G-C base pair, would not significantly increase the deoxynucleotide insertion probability. Experimental evidence consistent with this idea is that Michaelis constants for the insertion of dATP opposite T (template), dGTP or dCTP were to form a longer-lived complex with the polymerase and template than dATP or dTTP, then the free energy difference between G and C base pairs do not exhibit significantly longer lifetimes than those involving A-T base pairs. If either dGTP or dCTP were to form a longer-lived complex with the polymerase and template than dATP or dTTP, then the free energy difference between G-C and A-T base pairs of about 1 kcal/mol emanating from one additional hydrogen bond should have resulted in a 7-fold larger value in the insertion $K_m$ for A-T base pairs. It is possible, therefore, that two rather than three hydrogen bonds govern deoxynucleotide insertion rates. Excision rates, however, are strongly dependent on the extent of single-stranded character exhibited by a terminal base pair, and hence proofreading rates differ according to the number of H bonds present (24).

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REFERENCES