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A fidelity assay using "dideoxy" DNA sequencing: A measurement of sequence dependence and frequency of forming 5-bromouracil·guanine base mispairs

(base analogue mutagenesis/replication errors)

ROGER S. LASKEN AND MYRON F. GOODMAN

Molecular Biology Section, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-1481

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ABSTRACT **DNA replication fidelity has been assayed by using a modified DNA sequencing reaction. In one experimental approach, dideoxycytidine 5′-triphosphate (ddCTP) was used as a chain terminator during replication of M13 phage DNA by the large fragment of DNA polymerase I. The deoxyribonucleotide analogue BrdUTP was used to compete against ddCTP-induced chain terminations as an assay for B·G base mispairing (B represents bromodeoxyuridine when the analogue is present as a base pair or base mispair). By comparing BrdUTP to dCTP for competition against ddCTP, an average misincorporation frequency for BrdUMP of 0.2% was found. A similar average misincorporation frequency has been measured previously for the incorporation of radioactively labeled BrdUMP and dCMP into the synthetic template-primer poly[d(G,T)]·oligo(dA). The advantage of the sequencing method is that an error frequency is determined for each template guanine in a defined DNA sequence, thus providing information on the effect of neighboring base sequences on fidelity. Misincorporation frequencies varied no more than 5-fold among 50 template guanines tested. The approach used here is not limited for use with nucleotide analogues but is generally applicable in determining misincorporation frequencies and sequence specificities for any deoxynucleoside triphosphate substrate. In a second experimental approach, base mispairing between bromouracil and guanine was demonstrated directly by using 5-bromodideoxyuridine 5′-triphosphate (BrddUTP). A comparison of chain terminations attributable to BrddUTP and to dideoxythymidine 5′-triphosphate (ddTTP) revealed that B·A and T·A base pairs formed at about the same rate, whereas B·G mispairs occurred 4–10 times more frequently than T·G. The elevation in the frequency of B·G over T·G mispairs is consistent with the mutagenic behavior of the base analogue.**

5′-Bromouracil-induced mutations are thought to result from the formation of B·G base mispairs (for a review, see ref. 1). This idea (2) originated from the finding that BrdUrd predominantly induces transition mutations in the bacteriophage T4 (3) and from a consideration of the structure of BrUra, which suggested that it would be more likely than thymine to exist in a tautomeric form capable of base-pairing with guanine (2). Incorporation of BrdUMP opposite template G has recently been inferred by using synthetic DNA *in vitro* (4). In the reciprocal experiment, template BrUra was found to stimulate misincorporation of dGMP; however, nearest neighbor analysis (5) and nucleotide competition experiments (4) suggested that BrdUMP is not incorporated solely opposite guanine. Uncertainties about the effects of homopolymer or copolymer DNA on fidelity have complicated this type of analysis.

In this paper, we describe a method to visualize the presence of B·G base mispairs in DNA directly, to measure their dependence on local template sequences, and to quantitate their frequency of occurrence in comparison with C·G base pairs and T·G mispairs. The method is not limited for use with nucleotide analogues but should be applicable equally well to replication fidelity measurements using the four common deoxyribonucleotides.

MATERIALS AND METHODS

Nucleotides. 5-Bromodideoxyuridine 5′-triphosphate (BrddUTP) was synthesized from dideoxycytidine 5′-triphosphate (ddCTP) (P-L Biochemicals) by bromination (6), purified with 20% packed volume Norite, deaminated in nitrous acid (7) purified with Sephadex A-25, and stored in the dark at −20°C. BrdUTP was purchased from Boehringer Mannheim and repurified by HPLC on a Waters Associates Radial-Pac μBondapak C_{18} cartridge in a mobile phase of 0.2 M NH_4PO_4 (pH 5), and then desalted with Norite. Dideoxythymidine 5′-triphosphate (ddTTP), dideoxyadenosine 5′-triphosphate (ddATP), ddCTP, dideoxyguanosine 5′-triphosphate (ddGTP), dTTP, dATP, dCTP, and dGTP were purchased from P-L Biochemicals.

DNA and Enzymes. Single-stranded M13 DNA was prepared as described (8) from M13mp8 replicative form I (RFI) or M13mp9 RFI DNA obtained from New England Biolabs. Synthetic M13 primer (New England Biolabs, catalogue no. 403-2) was labeled at the 5′ terminus by using 5000 Ci of [γ-^{32}P]ATP (New England Nuclear or ICN) per mmol (1 Ci = 37 GBq) and T4 polynucleotide kinase (New England Biolabs) and in some cases purified on a Sephadex G-50 column. By labeling primers rather than using incorporation of [^{32}P]dAMP during the DNA synthesis reaction, autoradiograph band intensities are directly proportional to the extent of chain termination at any template guanine independent of the distance from the primer. The large fragment of DNA polymerase I (the Klenow fragment) was purchased from Bethesda Research Laboratories.

Fidelity Assays. Equimolar BrddUTP and ddTTP (300 μM) were compared as substrates for DNA synthesis in parallel reaction tubes. dATP and dGTP were 20 μM, dTTP was 1 μM, and dCTP was absent. The large fragment of *Escherichia coli* DNA polymerase was used to elongate ^{32}P-labeled primers by the procedure suggested by the supplier (8). Reactions (5.7 μl) were carried out at room temperature for 15–60 min followed by an additional 15 min in the presence of all four dNTPs (100 μM each) in order to extend all primers not

Abbreviations: B, BrdUrd when the nucleotide analogue is present as one component of a base pair—e.g., A·B base pair with adenine or B·G base mispair with guanine; ddNTP, dideoxynucleoside 5′-triphosphate; BrddUTP, 5-bromodideoxyuridine 5′-triphosphate; ddCTP, dideoxycytidine 5′-triphosphate; ddTTP, dideoxythymidine 5′-triphosphate; ddATP, dideoxyadenosine 5′-triphosphate; ddGTP, dideoxyguanosine 5′-triphosphate.

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specifically terminated by a dideoxynucleoside 5′-triphosphate (ddNTP). Competition assays were carried out the same way except that BrdUTP (or dTTP) ranging up to 1 mM in the presence of a fixed concentration of dCTP (0.1 μM) was compared to dCTP ranging from 0.1 μM to 1.0 μM in the absence of BrdUTP for competition against ddCTP. In all reactions ddCTP was 1.8 μM and dATP, dGTP, and dTTP were 20 μM. Reactions were incubated for 15 min at room temperature and then the four dNTPs were added at 100 μM for another 15 min.

Polymerase reaction products were analyzed by electrophoresis on an 8% polyacrylamide gel 40 cm long essentially as described (8), the gels were dried on filter paper, and autoradiographs were made with Kodak XRP-5 x-ray film and quantitated with a microdensitometer (Joyce, Loebl model MKIIIC). Peak areas were determined by paper cutting and weighing the densitometric tracings.

RESULTS

A method has been devised for determining error frequencies during replication of a natural DNA template *in vitro*. Dideoxyribonucleotides are employed as chain terminators during the replication of M13 single-stranded DNA by the large fragment of DNA polymerase I. Normal or base analogue deoxynucleoside triphosphates of interest are tested for their ability to compete against the dideoxynucleotide-induced terminations. The extent to which primer strands have been terminated is determined by electrophoretic sequencing (9).

As an example, the nucleotide analogue BrdUTP was tested for competition against chain-terminating incorporations of ddCMP opposite template guanine. BrUra primarily base pairs with adenine; however, it is believed that mispairing between BrUra and guanine occurs, resulting in the BrdUrd-induced transition mutations observed *in vivo* (2). In order to assay for the putative B·G mispairs, BrdUTP was allowed to compete against ddCTP. Incorrect incorporation of BrdUMP opposite template guanine should occur at the expense of the correct but chain-terminating incorporations of ddCMP. One millimolar BrdUTP was found to nearly eliminate ddCTP-induced terminations as determined by autoradiography, indicating that B·G base pairs were being formed to the exclusion of C·G base pairs (Fig. 1). In these experiments 100–150 nucleotides of the M13 template sequence could be clearly resolved by polyacrylamide gel electrophoresis. A representative sample of an autoradiograph spanning 38 nucleotides and its densitometric tracings are shown in Fig. 1. Note that the presence of BrdUTP (Fig. 1, lane 2, of both the autoradiograph and the tracing) has resulted in a large decrease in ddCTP-induced terminations at all template guanine sites compared to the reaction lacking BrdUTP (lane 1). In control reactions, 1 mM BrdUTP also was strongly competitive against ddTTP as expected since BrUra forms essentially correct base pairs with adenine, whereas 1 mM BrdUTP gave little or no competition against ddATP and ddGTP (data not shown).

A comparison of the effectiveness of BrdUTP, dTTP, and dCTP in competing with ddCTP allows the determination of misincorporation frequencies for BrdUMP and dTMP compared to the correct incorporation of dCMP. In these experiments, control reactions containing 0.1 μM dCTP and 1.8 μM ddCTP were compared to parallel reactions in which the competing nucleotide BrdUTP, dTTP, or additional dCTP was also added. It was found that doubling the dCTP concentration from the control level of 0.1 μM up to 0.2 μM resulted in an average reduction in terminations of about 50% ± 5% as determined by densitometry. In contrast, about 50 μM BrdUTP or 1 mM dTTP was required to cause a similar 50% ± 15% reduction in terminations.

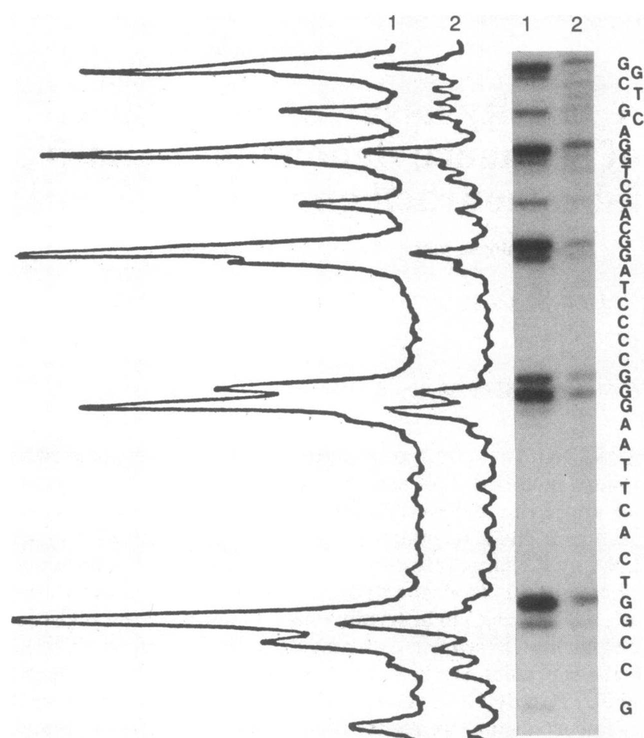

FIG. 1. Competition between BrdUTP and ddCTP. In reaction 1 (labeled above both the autoradiograph and its densitometric tracing) is seen the pattern of chain terminations generated by ddCTP in the absence of BrdUTP. In reaction 2, the presence of 1 mM BrdUTP has greatly reduced the level of chain terminations. The template sequence is shown with the G at the bottom of the autoradiograph being nearest the primer. The representative portion of the autoradiograph shown spans template nucleotide positions 11–48 downstream from the 3′-primer terminus. The baseline densities of the two densitometric tracings were equal but have been offset here for clarity.

In Table 1, the results of competition between BrdUTP and ddCTP are shown for 8 template guanines situated between 8 and 30 nucleotides downstream from the 3′-primer terminus. Peak areas (expressed in milligrams of paper weight) are shown for reactions in which BrdUTP was absent or present at 50 μM. Also shown is the percent peak area remaining in the presence of BrdUTP. Note that although the peak areas vary over a 20-fold range, the level of competition is fairly similar between different template guanine sites. By dividing the concentration of dCTP (0.1 μM) by the concentration of BrdUTP (50 μM) or dTTP (1 mM) required to produce the same average level of competition, misincorporation frequencies are obtained of about 2×10^{-3} for BrdUMP and 1×10^{-4} for dTMP.

Note that incorporation frequencies are being determined for nucleotides with normal deoxyribose groups. The dideoxynucleotide ddCTP present in this experiment serves only to indicate the relative level of incorporation of the deoxynucleotides BrdUMP, dTMP, and dCMP. It is possible for many template guanines as far as 50–100 nucleotides removed from the primer to be directly compared to guanine more proximal to the primer because the infrequent terminations due to ddCMP do not cause a rapid attenuation of primer elongation. The misincorporation frequency for about 50 template guanines tested has been found to vary by no more than an estimated 5-fold.

In a second type of experiment, the formation of B·G base pairs was directly visualized by using BrddUTP as a chain terminator. Under normal DNA-sequencing conditions (9), terminations would not have occurred frequently enough to detect; about one BrddUMP misincorporation per 10^6 dCMP

Table 1. BrdUTP competition against ddCTP-induced terminations

Nucleotide position of template guanine	Peak area, mg		% peak area remaining
	0.1 μM dCTP	0.1 μM dCTP, 50 μM BrdUTP	
8	3.4	1.4	41
11	5.6	2.1	38
14	8.0	3.0	38
15	32.0	18.9	59
20, 21	67.6	46.7	69
25	4.2	1.7	40
30	11.0	3.4	31

3′ A-C-A-T-T-T-T-G(8)-C-T-G(11)-C-C-G(14)-G(15)-T-C-A-C-G(20)-G(21)-T-T-C-G(25)-A-A-C-C-G(30)-A

Dideoxy sequencing reactions included 0.1 μM dCTP and 1.8 μM ddCTP. BrdUTP was either absent or present at 50 μM. Peak area from densitometry is expressed in mg of paper weight. Results are shown for template guanine occurring in the first 30 nucleotides following the 3′-primer terminus. The template sequence is included and the position of guanine relative to the primer is indicated. The % peak area remaining when BrdUTP was present is calculated by dividing the peak area for a guanine position in column 3 by the peak area in column 2. The peak at position 20 occurred as a small shoulder in the position 21 peak, so one area is given for the two peaks combined.

incorporations was expected since the frequency of B·G base-pairing compared to C·G is about 10^{-3} and in general ddNTPs are incorporated about 10^{-3} as frequently as dNTPs. To overcome this problem, dCTP was omitted from reactions so that BrddUTP would be free from competition from dCTP and also because the polymerase would pause at template guanine, allowing long reaction times. BrddUTP was found to cause terminations at template adenine, which is expected to "correctly" base-pair with BrUra, and at template guanine, which is predicted to "incorrectly" base-pair with BrUra (Fig. 2, lane 1). In addition, bands appear corresponding to all template nucleotides preceding a guanine. These reflect sets of primer strands prevented from elongating due to pausing of the polymerase at template guanine in the absence of dCTP. When a saturating concentration of dCTP is subsequently added to the "minus" dCTP reaction, the pause bands fade while specific terminations at template guanine and adenine due to the incorporation of BrddUMP persist (Fig. 2, lane 2). An exception is seen at the template guanine 30 nucleotides downstream from the 3′-primer terminus (Fig. 2, 30-G); addition of dCTP resulted in a large decrease in the band intensity opposite the guanine probably because pausing due to a downstream template guanine (33-G not seen in Fig. 2) has contributed largely to the band at 30-G. This kind of effect is seen most clearly at the template guanine sites most distal to the primer at which elongation has been largely attenuated due to the absence of dCTP.

When minus dCTP reactions are carried out in the absence of any ddNTP, followed by addition of dCTP, no bands appear for template adenine and guanine (Fig. 2, lane 3), demonstrating that BrddUMP is required for the chain terminations and that the initial minus dCTP condition does not contribute to these bands.

It is seen that, in spite of the minus dCTP condition, BrddUMP incorporations were not limited only to the first template guanine situated 8 nucleotides downstream from the primer (Fig. 2, 8-G). It is likely, therefore, that one of the normal deoxynucleotides present in the reaction—for example, dTTP—was inserted opposite guanine (e.g., see refs. 10 and 11), allowing extension of the primer up to subsequent template guanines.

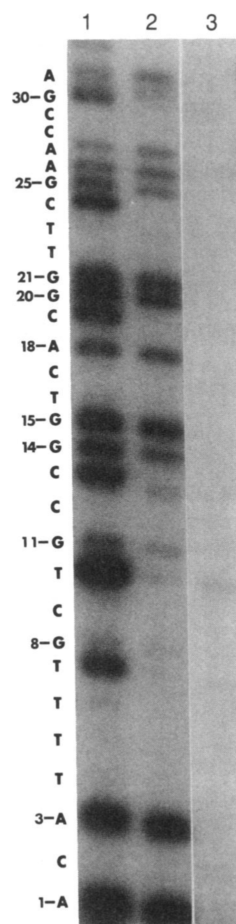

FIG. 2. DNA sequencing using BrddUTP as a chain terminator. BrddUTP was used in polymerase reactions, and the terminated primer strands were sequenced by electrophoresis and autoradiographed. Lane 1, dCTP was omitted from the reaction in order to enhance misincorporations opposite template guanine. The 60-min room temperature incubation resulted in terminations due to BrddUMP misincorporations opposite template guanine. Terminations are also seen opposite template adenine, which forms "correct" base pairs with BrUra. The bands at all template sites preceding a guanine result from pausing of the polymerase in the absence of dCTP. Lane 2, the reaction was also carried out for 60 min in the absence of dCTP; however, excess dCTP was then added to the reaction for an additional 15-min incubation in order to extend all primer strands not specifically terminated by BrddUMP. The pause bands have faded while the specific terminations persist. Lane 3, control reaction in which the 60-min, minus dCTP reaction followed by the 15-min, plus dCTP reaction was carried out in the absence of BrddUTP. The template sequence is shown to the left. Numbers indicate the nucleotide position relative to the primer, with "1-A" being the first site for incorporation following the 3′-primer terminus. The sequence of the DNA template used here (M13mp8) diverges from the template sequence seen in Fig. 1 (M13mp9) from nucleotide position 20 on.

The base analogue BrUra can substitute for the parent compound thymine in the DNA of both prokaryotes and eukaryotes. The mutagenic effect of BrUra is thought to result from a greater tendency than thymine to mispair with guanine, resulting in both G·C → A·T and A·T → G·C transition mutations (2). We have again compared BrUra to thymine, but now as the dideoxynucleotides BrddUTP and ddTTP (Fig. 3, lanes 1 and 2, respectively). Terminations at guanine occur about 4–10 times as frequently from BrddUTP than from an equal concentration of ddTTP. However, at one site (Fig. 3, 25-G), BrddUMP is misincorporated while ddTMP

misincorporation is almost absent. In contrast, terminations at template adenine are about equal. In fact, ddTTP causes slightly more terminations than BrddUTP at the adenine in the 18th nucleotide position (Fig. 3, 18-A), possibly because more primers have been allowed to be extended beyond preceding template guanine in the case of ddTTP. Again, the progressive attenuation of primer extension complicates any attempt to identify relative "hot spots" for BrddUMP incorporation in a minus dCTP reaction. Note that BrddUMP confers a slightly greater mobility than ddTMP to the shorter primer strands. This might be expected since >60% of the BrUra will be ionized under the conditions of electrophoresis (pH 8.3).

"Pausing" bands preceding template guanine are also seen (Fig. 3, lanes 1 and 2). Originally, it was assumed that these represented residual pause bands created by the minus dCTP reaction that had survived the subsequent plus dCTP reaction; however, in reactions carried out in their entirety in the presence of all four dNTPs, these bands are also seen (Fig. 3, lane 3).

In addition to the apparent hot spot for BrddUMP misincorporation compared to ddTMP misincorporation (Fig. 3, 25-G), two successive template guanine sites appear to be hot spots for misincorporation of both BrddUMP and ddTMP compared to single template guanine sites (Fig. 3, lanes 1 and 2, 14,15-G and 20,21-G). Note, however, that misincorporation frequencies as indicated by the competition experiments shown in Table 1 differ very little between single or double template guanines. The large differences in BrUra misincorporation seen in Fig. 3 apparently result from the minus dCTP condition that creates a "polymerase trap" at successive guanine sites; the polymerase can only advance past the first guanine if one of the normal deoxynucleotides present (e.g., dTTP) is misinserted. However, such a misinsertion will be quickly removed by the 3′ exonuclease proofreading activity (12, 13) because the polymerase is forced to pause at the second guanine. An excess of BrddUMP terminations will occur at the first guanine because ddNMPs are resistant to the 3′ exonuclease (14). The polymerase can only escape the trap if two successive misinsertions of dNMPs occur. A requirement to have two successive misinsertions will result in an extremely inefficient primer and, thus, it is likely that frequent insertions of BrddUMP or nonspecific stalling of the polymerase at the second guanine will occur. Template guanines occurring singly appear to be cold spots for BrUra misincorporation when compared to guanine doublets. We also find that the absolute level of misincorporation varies between different single template guanine sites. For example, in Fig. 3, severalfold more terminations occurred at 11-G than at 8-G. However, competition studies (Table 1) indicate that the misincorporation frequencies for 11-G and 8-G are virtually identical. In fact, the misincorporation frequencies at all four single guanine sites are similar (Table 1). Again, the effect of leaving out dCTP on proofreading is thought to be involved as discussed below.

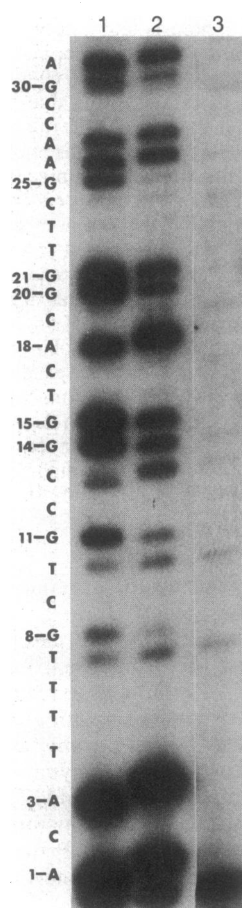

FIG. 3. Comparison of BrddUTP- and ddTTP-induced terminations. BrddUTP (lane 1) and ddTTP (lane 2) were used in minus dCTP (60-min incubation) and subsequent plus dCTP (15-min incubation) reactions. In a control reaction (lane 3) all four normal dNTPs were present throughout the reaction and no ddNTP was present.

DISCUSSION

A method has been developed for determining the fidelity of DNA replication *in vitro*. By employing a DNA sequencing procedure, base-pairing accuracy is determined for each nucleotide of a known sequence. In one approach, deoxynucleotides of interest are compared for their ability to compete against termination of elongation by dideoxynucleotides. As an example, incorrect base pairing of BrdUMP or dTMP with template guanine was detected as a decrease in ddCTP-induced terminations (Fig. 1 and Table 1). In comparison to the correct incorporation of dCMP, misincorporation frequencies were about 2×10^{-3} for BrdUMP and 1×10^{-4} for dTMP.

It is assumed that deoxy- and dideoxynucleotides affect each other's incorporation in no other way than as simple, mass action competitors. The validity of the misincorporation measurement reported here is independently verified by a previous study in which the misincorporation of BrdUMP compared to dCMP was also found to be about 0.2% (4); this result was obtained from a direct measurement of [^{32}P]-BrdUMP misincorporation into a synthetic DNA template copolymer of thymine and guanine. It is also assumed that relative nucleotide incorporation frequencies are proportional to substrate concentrations over a wide range of concentrations. We have determined that misincorporation of [^{32}P]-BrdUMP is linearly related to substrate concentration over at least a 5×10^5-fold range from 5 nM to 2.5 mM (unpublished data).

The experimental approach described here should be applicable to a broad range of problems concerning DNA synthesis for the following reasons:

(*i*) Data can be rapidly obtained for a large sample of template sequences, allowing analysis of local sequence effects under a variety of experimental conditions.

(*ii*) Virtually any nucleotide, present either as a substrate triphosphate or situated on the template, can be tested for its ability to base mispair. Error frequencies as low as 10^{-5} can be detected. The high sensitivity to base-pairing errors derives from the fact that incorporation of a dideoxynucleotide is itself an unfavorable reaction; in the example reported here, rare misincorporations of BrdUMP (composed of a base analogue and normal sugar group) were competitive against similarly rare incorporations of ddCMP (a normal base and analogue sugar). The high sensitivity of electrophoretic sequencing has allowed a quantitative measurement of terminations accurate to within 20% as a conservative estimate.

(*iii*) The natural DNA template used here is more readily applicable for modeling *in vivo* mutagenesis than synthetic templates, which are generally limited to one or two bases and possibly introduce special influences upon fidelity (15). Genetic analysis of natural DNA templates replicated *in vitro* (16–18) will be complemented by the dideoxy sequencing method, which can be used to rapidly screen a large sample of base sequence permutations free from genetic restraints upon the detection of errors or the possibility of unspecified alterations within a host cell such as heteroduplex repair.

In a second experimental approach, BrUra was present as the dideoxynucleotide BrddUTP in order to directly visualize the formation of B·G heteroduplexes. In this experiment, DNA polymerization was largely blocked at template guanine by the absence of dCTP in order to allow mispairing of BrUra for long reaction periods during which the polymerase acted on a limited number of template guanine sites. The ability of BrUra to mispair with guanine was demonstrated (Fig. 2), in agreement with previous results of an electrophoretic analysis in which the presence of BrdUTP relieved pausing of the polymerase at template guanine in the absence of dCTP (11) and studies with synthetic polymers in which template BrUra, but not thymine, allowed dGMP misincorporation (4, 5) and template guanine allowed misincorporation of BrdUMP (4).

BrddUMP was misincorporated about 4- to 10-fold more frequently than ddTMP opposite template guanine (Fig. 3). We assume that this accurately reflects the misincorporation frequencies of the deoxynucleotides BrdUMP and dTMP in spite of the 1000-fold higher K_m for dideoxy- versus deoxynucleotides. This assumption implies that for both dideoxy- and deoxynucleotides, hydrogen bonding involving B·G or T·G mispairs is the primary determinant of misincorporation frequencies. The 4- to 10-fold difference between misincorporation of the dideoxynucleotides BrddUMP and ddTMP is in good agreement with results found by using deoxynucleotides; here we observe a 20-fold increase in the misincorporation frequency by BrdUMP (2×10^{-3}) over dTMP (1×10^{-4}) as measured by direct competition of each deoxynucleotide separately versus ddCMP incorporation. Furthermore, a 10-fold difference was also found for mispairing of radioactively labeled BrdUMP and dTMP using synthetic templates (4).

The greater misincorporation frequency of BrdUMP compared to dTMP is a critical test of a model of BrdUrd-induced transition mutation pathways proposed by Freese (2), in which BrUra primarily base pairs like thymine but also mimics cytosine in base pairing with guanine. Our results show that this base-pairing ambiguity occurs and could underlie G·C → A·T transitions where substrate BrdUTP substitutes for dCTP.

The intensity of bands produced by BrddUMP mispairing varies among different template guanine sites (Fig. 3); however, the minus dCTP condition of these reactions greatly complicates any attempt to correlate results between different sites. Two consecutive template guanines are expected to enhance the 3′-exonucleolytic proofreading selection that favors incorporation of dideoxy- over deoxynucleotides. However, band intensities at the guanine occurring singly also vary greatly from each other (e.g., Fig. 3, 8-G and 11-G). The variability in band intensities may reflect differences in the effect of local sequences on proofreading activity.

A recent analysis of 2-aminopurine misincorporation (19) using a modified Maxam–Gilbert (19, 20) sequencing method strongly implied a role for proofreading in determining the sequence specificity of fidelity; the degree of sequence variation in misincorporation correlated with the level of 3′-exonuclease activity of three polymerases, antimutator and wild-type T4 DNA polymerase and *E. coli* polymerase I. The *ts*L141 antimutator DNA polymerase, with its very high level of 3′-exonuclease activity, generated the greatest variation in misincorporation frequency between different template sites. It is possible that our minus dCTP reaction simulates the effect of an antimutator polymerase by slowing the rate of DNA synthesis and thus increasing the exonuclease-to-polymerase ratio.

It has been observed that the rate of DNA synthesis appears to vary in response to DNA sequence in a characteristic way for different polymerases even in the presence of saturating concentrations of all four dNTPs (21). An example of this can be seen in Fig. 3, lane 3, in which pausing bands occurred in the presence of the four dNTPs at 100 μM. Pausing of the polymerase could be a basis for differential proofreading *in vivo* independently of dNTP pool biases. Many of the most intense pause bands appear to occur at nucleotides immediately preceding a template guanine in Fig. 3, lane 3, and in results obtained with other templates (data not shown).

No attempt is made to relate hot spots for BrddUMP misincorporation seen in the minus dCTP reaction (Fig. 3) to hot spots for BrUra-induced mutagenesis observed in genetic studies (22). Misincorporation frequencies derived from incorrect to correct base pairing provide a more direct test of mutagenic potential. We have compared B·G to C·G base pairing by measuring competition against ddCTP-induced terminations under conditions in which the dCTP concentration is sufficient to allow continuous polymerization. Misincorporation frequencies varied by no more than 5-fold among the 50 template guanine sites tested. Thus, for the limited number of guanine sites reported, we do not observe bromouracil misincorporation hot spots corresponding to the 100- to 1000-fold effects found *in vivo* (22).

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