Effect of Base-Pair Stability of Nearest-Neighbor Nucleotides on the Fidelity of Deoxyribonucleic Acid Synthesis†

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ABSTRACT: The influence of the stability of base pairs formed by nearest-neighbor nucleotides on misincorporation frequency has been studied with the large fragment of DNA polymerase I, the alternating DNA copolymers, poly(dI-dC) and poly(dG-dC), as template-primers, and dGTP, dITP, and dCTP as substrates. We have utilized the difference in thermodynamic stability between the doubly H-bonded I·C base pair and triply H-bonded G·C base pair to examine the effects of base-pair stability of both the "preceding" and the "following" nucleotides on the frequency of insertion of a mismatched nucleotide, as well as on its stable incorporation into polynucleotide. The present studies demonstrate that the stability of the base pairs formed by nearest-neighbor nucleotides affects the frequency of incorporation of noncomplementary nucleotides. Misincorporation frequency is increased when the nearest-neighbor nucleotides form more stable base pairs with the corresponding nucleotides in the template and is decreased when they form less stable base pairs. The stability of the base pair formed by a nucleotide either preceding (5′ to) or following (3′ to) a misincorporated nucleotide influences misincorporation frequency, but by different mechanisms. The stability of base pairs formed by preceding nucleotides affects the rate of insertion of mismatched nucleotide but does not protect the mismatched nucleotide from removal by the 3′ to 5′ exonuclease activity. In contrast, the stability of a base pair formed by a following nucleotide determines whether a misincorporated nucleotide is extended or excised by affecting the ability of the enzyme to edit errors of incorporation.

It has long been postulated that mutation frequency at a particular site in a genome is in some way a function of the base sequence surrounding that site. The idea that neighboring DNA sequence influences mutation rate was first suggested by Benzer (1960) as an explanation for a much higher frequency of spontaneous mutation at certain loci (hot spots) in the rII region of bacteriophage T_4. Benzer suggested that less stable regions of the DNA might be better able to accommodate a mismatch than more stable regions. However, Koch (1971), in his studies of 2-aminopurine-induced substitution mutation in the rII region of T_4, demonstrated that chainging an A·T base pair to a G·C base pair at a neighboring site resulted in a 10-fold increase in mutation frequency. Furthermore, it has been suggested (Bessman & Reha-Krantz, 1977; Clayton et al., 1979) that more stable regions of the DNA would be more rather than less mutable, because they would be less susceptible to the proofreading 3′ to 5′ exonuclease activity of DNA polymerase. In support of this hypothesis, Bessman & Reha-Krantz (1977) found that both in vitro incorporation of 2-aminopurine nucleotides into DNA by T_4 DNA polymerase and the 2-aminopurine-induced mutation rates at several sites in the rII region of T_4 were markedly decreased at elevated temperatures. In more recent studies from Bessman's laboratory (Pless et al., 1981) the nearest-neighbor distribution frequencies for 2-aminopurine nucleotides were compared with those for adenine nucleotides following their incorporation into DNA by several DNA polymerases with varying levels of 3′ to 5′ exonuclease activity. The results of these experiments were consistent with the hypothesis that stabilization of a primer terminus increases the frequency of misincorporation with DNA polymerases which have an active 3′ to 5′ exonuclease; i.e., there was a bias for incorporation of 2-aminopurine after guanine and against incorporation of 2-aminopurine after adenine or thymine.

Other studies have suggested that the "following" nucleotide, i.e., the nucleotide incorporated immediately following a mismatched nucleotide, is also important in influencing misincorporation frequency. In studies in which the reversion frequency of amber mutants of ϕX174, replicated in vitro, was used to measure fidelity of DNA synthesis, Fersht & Knill-Jones (1981), using *E. coli* DNA polymerase III holoenzyme, have found that misincorporation frequency is proportional to following nucleotide concentration when the following nucleotide is dGMP and independent of following nucleotide concentration when the following nucleotide is dAMP. On the other hand, Kunkel et al. (1981), using *E. coli* DNA polymerase I, have reported concentration-dependent following nucleotide effects on misincorporation when the following nucleotide is dAMP.

In the present studies we have examined the effects of the stability of the base pairs formed by nearest-neighbor nucleotides on the frequency of misincorporation by the large fragment of DNA polymerase I. We have utilized the difference in thermodynamic stability between the doubly H-bonded I·C base pair and the triply H-bonded G·C base pair to examine the effects of base-pair stability both on the insertion of a mismatched nucleotide and on its stable incorporation into polynucleotide, to distinguish between "preceding" nucleotide effects and "following" nucleotide effects. We have chosen this system because both I and G are complementary to C and the geometric spacing of the I·C and G·C base pairs is very similar. Furthermore, the H-bonding strength of the I·C base pair is similar to that of the A·T base pair; the T_m value for poly(dA-dT) and poly(dI-dC) at 0.1 M NaCl, pH 7.5, is 39 °C whereas the T_m value for poly(dG-dC) is 95 °C (Wells et al., 1970). The experimental system is depicted schematically in Figure 1.

We have also varied the ratio of polymerase to 3′ to 5′ exonuclease activity in this system by partially inhibiting the exonuclease activity with nucleoside 5′-monophosphates (5′-NMP) (Que et al., 1978) to evaluate the relative contribution

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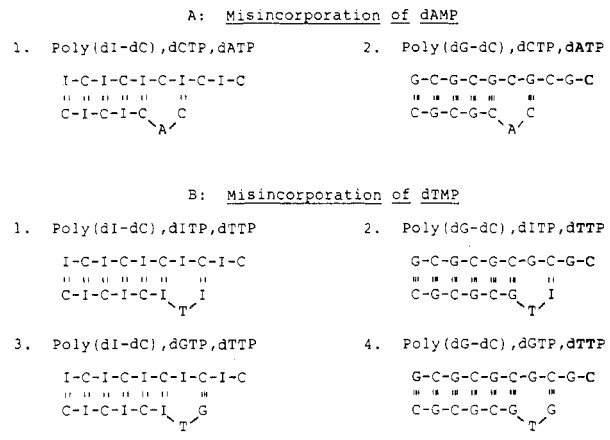

FIGURE 1: Schematic representation of the base pairs formed by the nearest-neighbor nucleotides to a misincorporated nucleotide. (A) Misincorporation of dAMP into either (1) poly(dI-dC) or (2) poly(dG-dC) with dCTP as alternating substrate. (B) Misincorporation of dTMP into either (1) poly(dI-dC) or (2) poly(dG-dC) with dITP as alternating substrate; misincorporation of dTMP into either (3) poly(dI-dC) or (4) poly(dG-dC) with dGTP as alternating substrate.

of the proofreading exonuclease activity to replication fidelity.

Materials and Methods

^{3}H-Labeled and ^{32}P-labeled deoxynucleoside triphosphates (dNTP) were purchased from New England Nuclear or ICN Pharmaceuticals, Inc.; unlabeled dNTPs and deoxynucleoside monophosphates (5′-NMP) were from P-L Biochemicals, Inc. The purity of dNTP solutions was determined by poly(ethylenimine) (PEI)–cellulose thin-layer chromatography using solvent systems described by Randerath & Randerath (1964) and Cashel et al. (1969). Radioactively labeled dNTPs were contaminated by less than 1% of nucleoside mono- or diphosphates. The large fragment of DNA polymerase I was obtained from New England Enzymes. Micrococcal nuclease, bovine spleen phosphodiesterase, and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp. Poly(dG-dC) and poly(dI-dC) were from P-L Biochemicals, Inc.

Misincorporation Assays. Determination of the rate of incorporation of a noncomplementary purine nucleotide, [^{3}H]dATP (9 μM, 11 Ci/mmol), into either poly(dG-dC) or poly(dI-dC) was carried out in the absence of a competing complementary purine nucleotide (dGTP or dITP) and in the presence of saturating concentrations of the complementary pyrimidine nucleotide (50 μM dCTP). Misincorporation frequency was determined in a parallel reaction by measuring the rate of incorporation of [^{3}H]dGTP (9 μM, 11 Ci/mmol) in the presence of 50 μM dCTP.

Determination of the rate of incorporation of a noncomplementary pyrimidine nucleotide, [^{3}H]dTTP (5 μM, 71 Ci/mmol), was carried out in the absence of a complementary pyrimidine nucleotide (dCTP) and in the presence of saturating concentrations of a complementary purine nucleotide, either dGTP (20 μM) or dITP (60 μM). Misincorporation frequency was determined in a parallel reaction by measuring the rate of incorporation of [^{3}H]dCTP (5 μM, 22 Ci/mmol) in the presence of either 20 μM dGTP or 60 μM dITP.

Rates were determined under conditions of enzyme concentration and incubation time where incorporation was linear with both parameters, and the observed values were normalized to allow comparisons.

The reaction mixture also contained, in a final volume of 0.1 mL, 50 mM HEPES[1] buffer, pH 7.8, 5 mM $MgCl_2$, 0.05 A_{260} unit of poly(dI-dC) or 0.125 A_{260} unit of poly(dG-dC), and 0.03–0.15 unit of the large fragment of DNA polymerase I. After incubation at 37 °C, the reaction was stopped by the addition of cold 5% trichloroacetic acid containing 0.02 mM sodium pyrophosphate, and unlabeled dNTP was added to a final concentration of 2 mM. The radioactive precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976).

Nucleotide Turnover Assays. This assay measures the template-dependent conversion of dNTP to dNMP by sequential action of the polymerase and 3′- to 5′-exonuclease activities of the large fragment of DNA polymerase I. Reaction mixtures were as described for the misincorporation assay. After incubation at 37 °C, aliquots were applied either to DE-81 filter paper disks to determine incorporation or to the origin of a PEI–cellulose thin-layer plate, to which unlabeled markers had been previously applied, to determine turnover. The DE-81 disks were washed batchwise with 0.3 M ammonium formate, pH 7.8, and ethanol as previously described (Que et al., 1978). The PEI–cellulose plates were developed with 1.0 M acetic acid when turnover of dATP was studied and with either 0.5 M sodium formate, pH 3.5, or 0.5 M LiCl when turnover of dTTP was studied. After development of the chromatogram, markers were visualized by UV absorption, and radioactivity was quantitated by cutting the thin-layer plate into sections, extracting with 0.6 mL of 2 M ammonium hydroxide for 30 min, and counting in 10 mL of Biofluor scintillation cocktail.

Nearest-Neighbor Analysis. The reaction mixture was as described for the misincorporation assay except for the use of [α-^{32}P]dNTPs as substrates. Reactions were carried out at 23 °C for 60 min and stopped by the addition of EDTA to a final concentration of 1 mM. DNA was precipitated by the addition of 2.5 volumes of cold ethanol and allowed to stand at −20 °C for 6 h. The DNA precipitate was collected by centrifugation at 10000g for 15 min and resuspended in 0.1 mL of buffer containing 10 mM Tris-HCl, pH 7.5, 60 mM KCl, and 1 mM unlabeled dNTP. Precipitation and resuspension were repeated 3 times, and the final precipitate was dried under vacuum. A small aliquot of the resuspended DNA was directly subjected to thin-layer chromatography to determine the extent of contamination by free nucleotides. Another aliquot was digested to nucleoside 5′-monophosphates by incubation with 0.05 mg of snake venom phosphodiesterase for 12 h at 37 °C, to determine whether all of the ^{32}P-labeled nucleotide that was incorporated was due to misincorporation. A third aliquot was subjected to nearest-neighbor analysis by the procedure of Agarwal et al. (1978) using 150 units of micrococcal nuclease and 0.11 unit of spleen phosphodiesterase for 6 h at 37 °C. Under these conditions all of the ^{32}P-labeled nucleotide that had been incorporated into polynucleotide was rendered acid soluble. Nucleoside 3′-monophosphates were separated by thin-layer chromatography, eluted, and counted as described above.

Results

Effects of Base-Pair Stability of Nearest-Neighbor Nucleotides on Misincorporation of dAMP. The effect of the stability of the base pairs formed by nearest-neighbor nucleotides on the misincorporation of dAMP opposite dCMP in the template is shown in Table I, in which the misincorporation of dAMP into either poly(dI-dC) or poly(dG-dC) is

[1] Abbreviations: HEPES, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Table I: Effect of Base-Pair Stability of Nearest-Neighbor Nucleotides on the Frequency of Misincorporation of dAMP[a]

substrates	incorporation (pmol/30 min) −5′-AMP	+5′-AMP	misincorporation frequency −5′-AMP	+5′-AMP
poly(dI-dC)				
[^{3}H]dATP, dCTP	<0.02	0.70	<1/12 800	1/457
[^{3}H]dGTP, dCTP	256	320		
poly(dG-dC)				
[^{3}H]dATP, dCTP	0.89	1.50	1/226	1/160
[^{3}H]dGTP, dCTP	201	240		

[a] Misincorporation assays were carried out as described under Materials and Methods. 5′-AMP, when present, was 1.4 mM. The concentrations of [^{3}H]dATP and [^{3}H]dGTP were each 9 μM, and dCTP was 50 μM.

Table II: Incorporation, Turnover, and Insertion of dAMP into Poly(dI-dC) and Poly(dG-dC)[a]

substrates	5′-AMP	rate (pmol/30 min) incorporation	turnover	insertion
poly(dI-dC)				
[^{3}H]dATP, dCTP	−	<0.02	0.70	0.70
[^{3}H]dATP, dCTP	+	0.53	0.77	1.30
poly(dG-dC)				
[^{3}H]dATP, dCTP	−	1.10	3.32	4.42
[^{3}H]dATP, dCTP	+	2.15	1.55	3.70

[a] Incorporation and turnover assays were carried out as described under Materials and Methods. Insertion was derived by summing incorporation and turnover. 5′-AMP, when present, was at 1.4 mM.

compared with the incorporation of dGMP. With poly(dI-dC) as template-primer, a misincorporated dAMP is both preceded and followed by an I·C base pair [see Figure 1A(1)], whereas when poly(dG-dC) is the template-primer, a misincorporated dAMP is both preceded and followed by a G·C base pair [see Figure 1A(2)]. It is evident that when the 3′- to 5′-exonuclease activity was fully active, the presence of the stable G·C base pairs as nearest neighbors increased the frequency of misincorporation of dAMP by at least 45-fold (<0.02 pmol vs. 0.89 pmol). Inhibition of the 3′ to 5′ exonuclease by 5′-AMP increased the frequency of misincorporation into both poly(dI-dC) and poly(dG-dC), although the effect was more marked with poly(dI-dC) (>35-fold) than with poly(dG-dC) (1.7-fold). However, even in the presence of an exonuclease inhibitor, the frequency of misincorporation of dAMP into poly(dG-dC) was 3-fold greater than into poly(dI-dC). Under the experimental conditions used for dAMP misincorporation, i.e., in the absence of the complementary purine nucleotide dGTP, dAMP misincorporation occurred exclusively opposite dCMP in the template, as determined by nearest-neighbor analysis using [α-^{32}P]dATP (data not shown).

To gain further insight into the effect of nearest-neighbor nucleotides on misincorporation frequency, we measured the insertion of dAMP into either poly(dI-dC) or poly(dG-dC) by measuring both the rate of stable incorporation of dAMP into polymer and the rate of template-dependent conversion of dATP to dAMP (turnover). As seen in Table II, when the 3′ to 5′ exonuclease was fully active, all of the dAMP incorporated into poly(dI-dC) was hydrolyzed, and no stable incorporation of dAMP into poly(dI-dC) was detectable. With poly(dG-dC) as template-primer, although measurable levels of dAMP were stably incorporated, 75% of the dAMP that was incorporated was hydrolyzed and released as free dAMP. The effects of inhibition of 3′ to 5′ exonuclease activity by 5′-NMP (5′-AMP) were different for the two template-primers. With poly(dG-dC) as template-primer, inhibition of exonuclease activity had relatively little effect on the rate of insertion of dAMP; however, it did result in a 2-fold decrease in the rate of turnover of dAMP and in a 2-fold increase in stable incorporation of dAMP into the polymer. In contrast, when poly(dI-dC) was the template-primer, addition of 5′-NMP resulted in a significant increase in stable incorporation and a 2-fold increase in the rate of insertion of dAMP; however, it had little or no effect on the rate of turnover of dAMP. It is likely that the increase in the rate of insertion of dAMP into poly(dI-dC) by 5′-NMP was the result of stabilization of the primer terminus at the polymerase active site, since 5′-NMP is a competitive inhibitor of the binding of the primer terminus at the exonuclease active site (Que et al., 1978).

Table III: Effect of Base-Pair Stability of Nearest-Neighbor Nucleotides on the Frequency of Misincorporation of dTMP[a]

substrates	incorporation (pmol/30 min) −5′-AMP	+5′-AMP	misincorporation frequency −5′-AMP	+5′-AMP
poly(dI-dC)				
[^{3}H]dTTP, dITP	<0.02	0.56	<1/27 800	1/2100
[^{3}H]dCTP, dITP	556	1173		
[^{3}H]dTTP, dGTP	0.72	3.35	1/1600	1/420
[^{3}H]dCTP, dGTP	1153	1420		
poly(dG-dC)				
[^{3}H]dTTP, dITP	<0.02	0.15	<1/12 700	1/3580
[^{3}H]dCTP, dITP	254	537		
[^{3}H]dTTP, dGTP	0.61	0.72	1/870	1/900
[^{3}H]dCTP, dGTP	530	651		

[a] Misincorporation assays were carried out as described under Materials and Methods. 5′-AMP, when present, was 1.4 mM. [^{3}H]dTTP and [^{3}H]dCTP were 5 μM, dITP was 60 μM, and dGTP was 20 μM.

Comparison of the rates of insertion and incorporation of dAMP into poly(dG-dC) and poly(dI-dC) in the absence of an inhibitor of exonuclease activity shows that the insertion of dAMP after a G·C pair was 6 times more frequent than after an I·C pair, suggesting that the stability of the base pair at the primer terminus is important in promoting misinsertion. However, stable misincorporation of dAMP was >55 times more frequent with poly(dG-dC) than with poly(dI-dC), suggesting that the stability of the base pair formed by the nucleotide incorporated following a mismatched nucleotide may be even more important in promoting stable misincorporation.

Effect of Base-Pair Stability of Nearest-Neighbor Nucleotides on dTMP Misincorporation. To further distinguish between preceding and following base-pair effects, the misincorporation of dTMP into either poly(dG-dC) or poly(dI-dC) was investigated with either dITP or dGTP as alternating substrate. With poly(dI-dC) as template-primer and dITP as alternating substrate, the misincorporated dTMP would be both preceded and followed by an I·C base pair [see Figure 1B(1)], whereas with the same template-primer and dGTP as alternating substrate, dTMP would be preceded by an I·C base pair and followed by a G·C base pair [see Figure 1B(3)]. Conversely, with poly(dG-dC) as template-primer and dGTP as alternating substrate, misincorporated dTMP would be preceded and followed by a G·C base pair [see Figure 1B(4)], and with the same template-primer and dITP as alternating substrate, dTMP would be preceded by a G·C base pair and followed by an I·C base pair [see Figure 1B(2)].

The frequency of dTMP misincorporation with the four possible nearest neighbors is shown in Table III. In the

absence of an exonuclease inhibitor, the misincorporation of dTMP into either poly(dI-dC) or poly(dG-dC) with dITP as alternating substrate was not detectable. In contrast, when dGTP was the alternating substrate, misincorporation of dTMP was measurable and about the same with either poly(dG-dC) or poly(dI-dC) as template-primer. These results suggest that a stable following base pair is essential to fix a misincorporation in place. The lack of detectable misincorporation of dTMP into poly(dG-dC) with dITP as alternating substrate further suggests that the stability of the preceding base pair is relatively less important than that of the following base pair in promoting stable misincorporation. Inhibition of the 3′ to 5′ exonuclease activity by 5′-NMP resulted in measurable misincorporation of dTMP in all four cases. As was the case with dAMP misincorporation, inhibition of exonuclease activity had a much greater effect when the preceding and following nucleotides formed weaker base pairs with the template.

The effect of base-pair stability of nearest-neighbor nucleotides on misinsertion of dTMP was also determined by measuring both stable incorporation of dTMP into polymer and turnover of dTTP to dTMP. These results are shown in Table IV. With poly(dG-dC) as template-primer, i.e., with a stable primer terminus, the insertion (incorporation plus turnover) of dTMP was relatively unaffected either by inhibiting exonuclease activity or by the base-pairing capacity of the alternating substrate. However, there was a marked effect of these factors on the stable misincorporation of dTMP into poly(dG-dC). Inhibition of exonuclease activity increased dTMP misincorporation 7.5-fold when dITP was the alternating substrate (<0.02 pmol vs. 0.15 pmol), and changing the alternating substrate to dGTP in the absence of inhibitor resulted in a >30-fold stimulation.

When the primer terminus was less stable, as with poly(dI-dC) as template-primer, addition of 5′-NMP resulted in a marked stimulation of insertion of dTMP into polymer (5-fold), as well as a >28-fold stimulation of stable misincorporation of dTMP. Furthermore, the turnover of dTTP to dTMP was also stimulated 4-fold. These results suggest that binding of 5′-NMP at the active site of the 3′ to 5′ exonuclease stabilizes the weak I·C base pair at the polymerase active site, thereby increasing the rate of insertion of a mismatched nucleotide. It is obvious that the exonuclease activity is only partially inhibited by 5′-NMP, since 85% of the dTMP that was inserted into poly(dI-dC) in the presence of 5′-NMP with dITP as alternating substrate was hydrolyzed and released as free dTMP. The marked increase in the rate of misinsertion of dTMP into poly(dI-dC) that was seen when the alternating substrate was changed from dITP to dGTP may also be due to stabilization of the primer terminus, i.e., to replacement of dIMP at the primer terminus by dGMP.

To examine this possibility, the misincorporation of dTMP into poly(dI-dC) with dITP and/or dGTP as alternating substrates was followed by nearest-neighbor analysis using [α-^{32}P]dTTP. In these experiments misincorporation of [^{32}P]dTMP was increased by extending the time of incubation to 60 min and lowering the temperature to 23 °C. The results (Table V) showed that when dGTP was the alternating substrate, 95% of the dTMP was incorporated after dGMP, being incorporated either following chain elongation or following replacement of dIMP by dGMP at the primer terminus of poly(dI-dC). In the presence of both dITP and dGTP, dTMP misincorporation after a dIMP primer terminus occurred 13% of the time in the absence and 22% in the presence of 5′-NMP, again suggesting that stabilization of a weakly base-paired primer terminus by 5′-NMP increases the rate of misinsertion of dTMP. However, even when the exonuclease activity was inhibited, misincorporation was still favored after the more stable G·C pair.

To determine whether dTMP residues stably incorporated into poly(dG-dC) and poly(dI-dC) are present in internucleotide linkage or whether they are present at termini, nearest-neighbor analysis was carried out with [α-^{32}P]dGTP (Table VI). With poly(dI-dC) as template-primer, 86% of the dGMP residues were incorporated following dTMP, and 14% were incorporated following dCMP. Similar results were obtained with poly(dG-dC) as template-primer. Since dCTP was not present in the reaction mixture, the transfer of ^{32}P to dCMP suggests that the dCMP was originally present at primer termini. The ratio of ^{32}P transferred to dTMP relative to that to dCMP was only 6 to 1, suggesting that primers containing mismatched nucleotides at 3′-termini are not efficiently extended. These results demonstrate that dTMP residues are incorporated into internucleotide linkage and, together with the data in Table V, indicate that misincorporation of dTMP occurs primarily by purine·pyrimidine mispairing under these assay conditions.

Discussion

The results of the present studies demonstrate that the stability of the base pairs formed by nearest-neighbor nucleotides affects the frequency of incorporation of noncomplementary nucleotides. Misincorporation frequency is increased when the nearest-neighbor nucleotides form more stable base pairs with the corresponding nucleotides in the template and decreased when they form less stable base pairs. The stability of the base pairs both preceding (5′ to) and following (3′ to) a misincorporated nucleotide affects misincorporation frequency, but by different mechanisms.

The base-pairing capacity of preceding nucleotides directly affects misinsertion frequency. Since phosphodiester bond formation between a primer terminus and a mismatched deoxynucleoside triphosphate requires that both substrates are bound at the polymerase active site, and since binding at the polymerase active site is favored for termini that form base pairs with the corresponding nucleotides in the template, a more stable base pair at the primer terminus increases the rate of phosphodiester bond formation (Que et al., 1979). Thus, misinsertion is more frequent after a G·C base pair than after an I·C base pair. This is consistent with the work of Bessman and his colleagues, who found that the base analogue 2-aminopurine was inserted more frequently after a guanine than after an adenine or thymine residue with DNA polymerases capable of proofreading (Pless et al., 1981).

The stimulatory effect of partial inhibition of the 3′ to 5′ exonuclease activity of DNA polymerase I on misinsertion with a weakly base-paired primer terminus suggests that the exonuclease inhibitor 5′-NMP stabilizes the primer terminus in the polymerase active site by blocking its entry into the exonuclease active site, thereby increasing the frequency of phosphodiester bond formation.

The base-pairing capacity of the following nucleotide affects the frequency of stable misincorporation by affecting the proofreading capacity of the enzyme. Since the direction of exonucleolytic hydrolysis is 3′ to 5′, extension of a mismatched nucleotide by a following nucleotide that forms a stable base pair with the template would protect the misincorporated nucleotide from "peelback" hydrolysis. In the present studies the ability of a following nucleotide to form a stable base pair with the template appears to be of primary importance in generating stable misincorporation; i.e., regardless of the

Table IV: Incorporation, Turnover, and Insertion of dTMP into Poly(dI-dC) and Poly(dG-dC)[a]

substrates	5'-AMP	rate (pmol/30 min) incorporation	turnover	insertion
poly(dI-dC)				
[^{3}H]dTTP, dITP	–	<0.02	0.75	0.75
[^{3}H]dTTP, dITP	+	0.56	3.11	3.67
[^{3}H]dTTP, dGTP	–	0.72	3.33	4.05
[^{3}H]dTTP, dGTP	+	3.35	1.22	4.57
poly(dG-dC)				
[^{3}H]dTTP, dITP	–	<0.02	1.37	1.37
[^{3}H]dTTP, dITP	+	0.15	0.84	0.99
[^{3}H]dTTP, dGTP	–	0.61	1.61	2.22
[^{3}H]dTTP, dGTP	+	0.72	0.69	1.41

[a] Incorporation and turnover assays were carried out as described under Materials and Methods. Insertion was derived by summing incorporation and turnover. 5'-AMP, when present, was 1.4 mM.

Table V: Nearest-Neighbor Analysis of [^{32}P]dTMP Misincorporated into Poly(dI-dC)[a]

substrates	5'-AMP	GpT (%)	IpT (%)	incorporation (pmol/60 min)
[^{32}P]dTTP, dITP	–	<0.2	99.8	0.12
[^{32}P]dTTP, dITP	+	<0.2	99.8	0.36
[^{32}P]dTTP, dITP, dGTP	–	87	13	0.99
[^{32}P]dTTP, dITP, dGTP	+	78	22	1.34
[^{32}P]dTTP, dGTP	–	95	5	1.59
[^{32}P]dTTP, dGTP	+	95	5	1.77

[a] Nearest-neighbor analysis was carried out as described under Materials and Methods. 5'-AMP, when present, was at 1.4 mM. Incubation was at 23 °C for 60 min. The concentration of [α-^{32}P]dTTP was 5 μM, dGTP was 20 μM, and dITP was 60 μM.

Table VI: Nearest-Neighbor Analysis of [^{32}P]dGMP Incorporated into Poly(dI-dC) or Poly(G-dC)[a]

substrates	5'-AMP	TpG (%)	CpG (%)	incorporation (pmol/60 min)
poly(dI-dC)				
[^{32}P]dGTP, dTTP	+	86	14	1.59
[^{3}H]dCTP, dGTP	+			229
poly(dG-dC)				
[^{32}P]dGTP, dTTP	+	78	22	1.04
[^{3}H]dCTP, dGTP	+			105

[a] Nearest-neighbor analysis was performed as described under Materials and Methods. The concentration of either [α-^{32}P]dGTP or unlabeled dGTP was 18 μM, and the concentration of either [^{3}H]dCTP or unlabeled dTTP was 100 μM. The concentration of 5'-AMP was 1.4 mM. Incubation was at 23 °C for 60 min.

stability of the preceding base pair, no stable misincorporation was detected when the following base pair was I·C.

The effects of base-pair stability of nearest-neighbor nucleotides on misincorporation frequency reported here were determined in the presence of saturating concentrations of the alternating complementary dNTP. However, one might expect that these sequence-dependent following nucleotide effects might be manipulated by varying the concentration of the following nucleotide. Misincorporation frequency dependence on following nucleotide concentration has been reported in two studies. Fersht & Knill-Jones (1981) have reported that with ϕX174am16 DNA, replicated in vitro with DNA polymerase III holoenzyme, reversion frequency is linearly dependent on following nucleotide concentration when dGMP is the following nucleotide and independent of following nucleotide concentration when dAMP is the following nucleotide. Thus, the effect of the stability of the following G·C base pair was kinetically enhanced by increasing the rate of formation of the phosphodiester bond between the misincorporated nucleotide and the following dGMP. Since in this system the $n + 2$ nucleotide is also dGMP (Sanger et al., 1977), it is likely that the base-pair stability of the $n + 2$ nucleotide also contributed to misincorporation frequency by further protecting the mismatched nucleotide from hydrolysis.

In contrast to the results of Fersht and Knill-Jones, Kunkel et al. (1981) observed a dependence of misincorporation frequency on following nucleotide concentration when the following nucleotide was dAMP with ϕX174am3 DNA; i.e., kinetic enhancement of misincorporation frequency was apparently not dependent on the ability of the following nucleotide to form a strong base pair with the template. However, in this study, the dependence of misincorporation frequency on dATP concentration was only seen when the concentration of the misincorporated nucleotide, dCTP, was extremely high. Since, in this system, both the $n - 2$ and $n - 1$ nucleotides as well as the $n + 2$ nucleotide are also dCMP (Sanger et al., 1977), it is likely that the stability of the base pairs formed by both preceding and $n + 2$ nucleotides may have influenced misincorporation frequency. Clearly the effects of DNA sequence on misincorporation frequency may not be limited to immediately adjacent nucleotides.

The importance of the 3' to 5' exonuclease activity of DNA polymerase I in maintaining the fidelity of DNA synthesis appears to be influenced by DNA sequence. Partial inhibition of exonuclease activity can increase misincorporation frequency nearly 30-fold when the misincorporated nucleotide is both preceded and followed by a weak base pair. In contrast, when the misincorporated nucleotide is both preceded and followed by a strong base pair, partial inhibition of the exonuclease activity has little effect on misincorporation frequency.

The present studies would suggest that substitution mutation "hot spots" should occur within clusters of G·C base pairs in the DNA and would predict that nearest-neighbor effects on mutation frequency should follow the trend G·C(preceding), G·C(following) > A·T(preceding), G·C(following) > G·C(preceding), A·T(following) > A·T(preceding), A·T(following).

Registry No. Poly(dI-dC), 34639-43-5; poly(dG-dC), 36786-90-0; dAMP, 653-63-4; dGMP, 902-04-5; dTMP, 365-07-1; dATP, 1927-31-7; dCTP, 2056-98-6; dGTP, 2564-35-4; dTTP, 365-08-2; dITP, 16595-02-1; DNA polymerase, 9012-90-2; 3'–5'-exonuclease, 79393-91-2.

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Inhibition of DNA Replication Coordinately Reduces Cellular Levels of Core and H1 Histone mRNAs: Requirement for Protein Synthesis†

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ABSTRACT: Cellular levels of H1 and core histone mRNAs have been examined in exponentially growing HeLa S3 cells as a function of DNA synthesis inhibition under varying concentrations of three DNA synthesis inhibitors. Total cellular histone mRNAs were analyzed by Northern blot hybridization, and their relative abundance was shown to be stoichiometrically and temporally coupled to the rate of DNA synthesis. In the presence of cytosine arabinoside, hydroxyurea, or aphidicolin, a rapid, proportionate decrease of histone mRNA levels resulted in an apparent mRNA half-life of less than 10 min. Using inhibitors of transcription and translation, we show that transcription is not necessary for the coordinate decrease of histone mRNA levels that occurs when DNA synthesis is inhibited. When protein synthesis is inhibited by addition of cycloheximide, core and H1 histone mRNAs do not decrease in parallel with reduced rates of DNA synthesis but instead are stabilized and accumulate with time, thus uncoupling histone mRNA levels and DNA replication. These last observations suggest that protein synthesis, either of histones or of some unidentified regulatory molecules, is required for the stoichiometric turnover of H1 and core histone mRNAs coordinate with reduced rates of DNA synthesis.

Histone genes comprise a family of moderately reiterated sequences (Wilson & Melli, 1977; Kedes, 1979) whose products, histone proteins, play key roles in the structural and transcriptional properties of the eukaryotic genome (Isenberg, 1979; McGhee & Felsenfeld, 1980; Felsenfeld & McGhee, 1982; Weisbrod, 1982). It has been well established that histone proteins are required for packaging eukaryotic DNA into chromatin; hence, it is not surprising that histone genes are expressed predominantly during the S phase of the cell cycle. A temporal and functional coupling of histone gene expression and DNA replication in most eukaryotic cells is suggested by the synthesis of histone proteins (Spalding et al., 1966; Robbins & Borun, 1967; Stein & Borun, 1972; Wu & Bonner, 1981; Delegane & Lee, 1972; Marashi et al., 1982) and histone mRNAs (Borun et al., 1967, 1975; Jacobs-Lorena et al., 1973; G. Stein et al., 1975; J. Stein et al., 1975; Tarnowka et al., 1978; Parker & Fitschen, 1980; Hereford et al., 1981, 1982; Heintz et al., 1983; Plumb et al., 1983b; Sittman et al., 1983) in conjunction with DNA synthesis. Although evidence points to control of this relationship by both transcriptional (Borun et al., 1967, 1975; G. Stein et al., 1975; Hereford et al., 1981, 1982; Heintz et al., 1983; Plumb et al., 1983a; Sittman et al., 1983) and posttranscriptional (Butler & Mueller, 1973; Borun et al., 1975; Heintz et al., 1983; Plumb et al., 1983a; Plumb et al., 1983b; Sittman et al., 1983) processes, the mechanisms regulating coordinate DNA replication and histone gene expression remain largely unresolved.

One approach to studying the relationship between histone gene expression and DNA replication has involved the use of metabolic inhibitors to examine the requirements for specific biochemical events. Early studies, which used high concentrations of DNA synthesis inhibitors such as Ara-C,[1] HU, or aminopterin, showed that DNA synthesis, histone protein synthesis (Spalding et al., 1966; Robbins & Borun, 1967; Stein & Borun, 1972; Borun et al., 1975; Marashi et al., 1982), histone mRNA levels (Breindl & Gallwitz, 1973, 1974a,b; Borun et al., 1975; Gallwitz, 1975; G. Stein et al., 1975; Stahl & Gallwitz, 1977; Stein et al., 1977a; Shephard et al., 1982), and radiolabeled precursor incorporation into histone mRNA (Spalding et al., 1966; Borun et al., 1967, 1975; Craig et al., 1971; Adesnik & Darnell, 1972; Schochetman & Perry, 1972; Butler & Mueller, 1973; Jacobs-Lorena et al., 1973; Perry & Kelley, 1973) were all diminished. However, these early studies lacked a direct assay for cellular levels of specific histone mRNAs and did not address the stoichiometry of the relationship between DNA replication and histone gene expression. The availability of cloned human histone genes (Heintz et al., 1981; Sierra et al., 1982) and the development of sensitive methods for detection of mRNAs have permitted us to examine systematically the nature of this relationship.

In this study, we have used a wide range of concentrations of three DNA synthesis inhibitors, Ara-C, HU, or aphidicolin, to examine the relationship between DNA replication and histone gene expression in HeLa S3 cells. Regardless of the

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[1] Abbreviations: Ara-C, cytosine arabinoside; HU, hydroxyurea; Act-D, actinomycin D; TCA, trichloroacetic acid.

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