Effect of pH on the Base-Mispairing Properties of 5-Bromouracil during DNA Synthesis†

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ABSTRACT: We have utilized an electrophoretic assay of misincorporation to investigate the possibility that ionization of 5-bromouracil (BU) may play a role in its mispairing during DNA synthesis in vitro. We examined the effects of increasing pH on the relative rates of formation of BU·G and T·G mispairs during chain elongation catalyzed by various DNA polymerases. For the Klenow fragment of *Escherichia coli* DNA polymerase I, increasing pH facilitated BU·G mispair formation (relative to T·G mispairing) when BU was present in the template strand. This effect showed a strong dependence on sequence context. Increasing pH had little effect on the relative rate of misincorporation of BrdUMP versus dTMP (at template G) by the Klenow polymerase. Misincorporation opposite template BU residues catalyzed by Maloney murine leukemia virus DNA polymerase and DNA polymerase β (Novikoff hepatoma) also increased with pH, but for these two enzymes, there was no apparent dependence on sequence context. With T4 DNA polymerase and *E. coli* DNA polymerase III holoenzyme, a similar occurrence of BU·G and T·G mispairing during polymerization was observed, whether BU was present in the template or in the incoming nucleotide, and there was little effect of pH. The results reported here are consistent with a mispairing mechanism for template BU wherein the anionic form of the base mispairs with G.

The mutagenicity of 5-bromouracil (BU)[1] is generally believed to be due to its ability to occasionally mispair with G during DNA replication. According to this hypothesis, ambiguous base pairing of bromouracil should lead to transition mutations via two pathways. Misincorporation of BrdUMP opposite template G would lead to G·C → A·T transitions, while misincorporation of dGMP opposite template BU would lead to A·T → G·C transitions. The mutagenic intermediates in both pathways are BU·G mispairs. The most widely accepted explanation for the greater mispairing ability of BU compared to T stems from the proposal (Watson & Crick, 1953) that the rare tautomeric forms of the normal nucleic acid bases could occasionally mispair during DNA chain elongation. According to a model first forwarded by Freese (1959; attributed to Meselson), the presence of the Br atom at C5 of BU increases the proportion of the rare enol tautomer, which could mispair with G without distortion of Watson–Crick geometry. Katritzky and Waring (1962) measured the tautomeric equilibrium constant of BrUra in aqueous solution and found that compared to Ura, BrUra had approximately 10-fold more of the enol form.

A different mispairing mechanism has been suggested by Lawley and Brooks (1962). Using spectroscopic methods, these authors found the pK_a values for ionization of BrdUrd and Thd to be 8.1 and 9.8, respectively. At neutral pH, 7.4% of BrdUrd would be in the anionic form, whereas Thd would be only about 0.16% ionized. These authors proposed a model for BU·G mispairs in which the anionic form of BU forms two hydrogen bonds with G without distortion of Watson–Crick geometry.

In a third mispairing model, the BU·G mispair exists in a wobble conformation and is more stable than the T·G mispair because of strengthened base stacking conferred by the Br at C5 of the pyrimidine (Bugg et al., 1974). X-ray crystallography of d(BU-G-C-G-C-G) duplexes (Brown et al., 1986) has shown these mispairs to be in a wobble conformation, in agreement with the model.

Although there is some experimental basis for each of these proposals, none of them has been demonstrated to be actually involved in the process of BU-induced mutagenesis. In order to investigate the possibility that ionized species may play a role in mutation induction by BU, we have examined the effects of increasing pH on the relative rates of misincorporation opposite BU versus T in the template strand during chain elongation catalyzed by DNA polymerase I Klenow fragment of *Escherichia coli* (Kf pol), DNA polymerase of bacteriophage T4 (T4 pol), Maloney murine leukemia virus DNA polymerase (MMLV pol), DNA polymerase β from Novikoff hepatoma cells (pol β), and *E. coli* DNA polymerase III holoenzyme (pol III holoenzyme). We have also examined the effects of pH upon misincorporation of BrdUMP versus dTMP at template G residues.

Experimental Procedures

Enzymes and Chemicals. Deoxynucleoside 5′-triphosphates (dNTPs) were purchased from Pharmacia/P-L and extensively

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[1] Abbreviations: dNTP, 2′-deoxynucleoside 5′-triphosphate; dNMP, 2′-deoxynucleoside 5′-monophosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphate; BrUrd, 5-bromouridine; BU or BrUra, 5-bromouracil; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DTT, dithiothreitol; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TE buffer, 10 mM Tris-HCl (pH 7.5) and 1 mM Na_2EDTA; Kf pol, DNA polymerase I (Klenow fragment) of *Escherichia coli*; pol β, DNA polymerase β (Novikoff hepatoma); T4 pol, T4 DNA polymerase; pol III holoenzyme, DNA polymerase III holoenzyme of *E. coli*; MMLV pol, DNA polymerase of Maloney murine leukemia virus; BU-DNA, DNA template in which BU substitutes for T; T-DNA, unsubstituted DNA template; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin.

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purified by HPLC, as described previously (Revich et al., 1984). [γ-^{32}P]ATP was from Amersham or ICN Pharmaceuticals. 2′,3′-Dideoxynucleoside 5′-triphosphates (ddNTPs) were purchased from Pharmacia/P-L. DEAE-Sephacel and Sephadex G-200 were from Pharmacia. Kf pol either was purchased from Pharmacia or Bethesda Research Labs or was prepared in this laboratory by a published procedure (Joyce & Grindley, 1983), using the overproducing strain CJ155, which was kindly provided by Drs. Joyce and Grindley. Restriction endonucleases were purchased from New England Biolabs, Pharmacia, Bethesda Research Labs, or Boehringer Mannheim. T4 DNA polymerase was purchased from Pharmacia, and MMLV DNA polymerase was purchased from Bethesda Research Labs. DNA polymerase β from Novikoff hepatoma cells was a gift from Dr. Dale Mosbaugh. DNA polymerase III holoenzyme and single-stranded DNA binding protein of *E. coli* were kindly provided by Dr. Charles McHenry.

DNA Substrates. Preparation of bacteriophage template DNAs and construction of template-primers were described previously (Hillebrand et al., 1984). Synthetic oligonucleotide primers were prepared in our laboratory by the phosphoramidite procedure (Beaucage & Caruthers, 1981) on an Applied Biosystems 380A DNA synthesizer or on a Cruachem PS200 DNA synthesizer. Previous papers described the electrophoretic assay of misincorporation, including HPLC purification of dNTPs, 5′-end-labeling of oligonucleotide primers, and conditions for DNA synthesis and electrophoresis (Hillebrand et al., 1984; Revich et al., 1984).

Preparation of Nascent Strand Templates. T-DNA (unsubstituted) and BU-DNA (BU substituted for T) templates were prepared as outlined in Figure 1. An oligonucleotide primer (usually 30–50 pmol, annealed to bacteriophage M13mp9 template DNA) was elongated by Klenow polymerase in the presence of 100 μM dNTPs and up to 200 units of a suitable restriction endonuclease. The corresponding BU-DNA templates were prepared by substituting BrdUTP for dTTP in the elongation/cleavage reaction. Nascent strands were isolated by electrophoresis of the DNA in a 5% polyacrylamide–7 M urea gel, following cleavage by a restriction enzyme chosen to generate an end-labeled product of 200–400 bases in length. Positions of products in the gel were revealed by autoradiography, and the bands were excised and placed in sterile Eppendorf tubes. DNA was eluted from gel slices by soaking overnight in 1 mL of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM Na_2EDTA). Low molecular weight contaminants were then removed by gel filtration on Sephadex G-200 equilibrated with TE buffer. Reverse primers were 5′-^{32}P-labeled and then purified by anion-exchange chromatography on DEAE-Sephacel, followed by preparative electrophoresis in a 10% polyacrylamide–7 M urea gel. Primer bands were visualized by autoradiography, excised, and eluted in TE buffer. The eluate was passed through a Poly-Prep chromatography column (Bethesda Research Labs), and the purified primers were ethanol-precipitated and then dissolved in a small volume of TE buffer. Annealing of reverse primers to nascent strand templates was carried out at 55 °C for 30 min in 0.45 M NaCl, with primer present in 5-fold excess over template. Annealed template-primers were separated from unannealed primers by gel filtration on Sephadex G-200.

Reaction Conditions. Reactions with Kf pol contained 50 mM Tris-HCl, 6.5 mM $MgCl_2$, and 10 μM dNTPs. Reactions with pol III holoenzyme contained 25 mM Tris-HCl, 6% glycerol, 1.25 mg/mL BSA, 12.5 mM DTT, 5 mM $MgCl_2$, 5 mM spermidine hydrochloride, 62.5 μM dNTPs, 6 μM

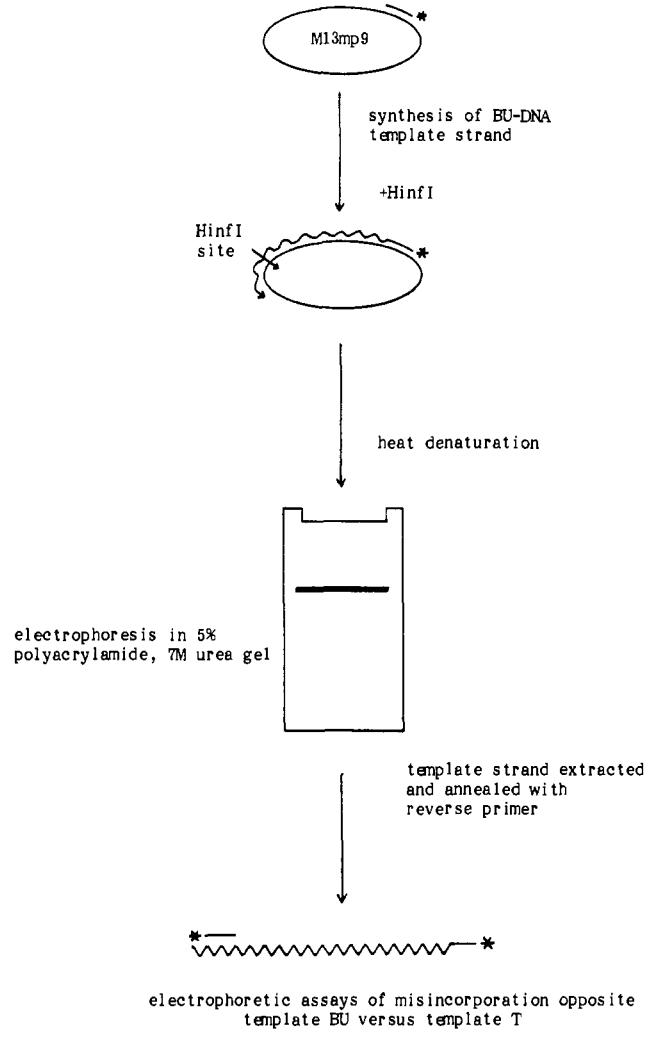

FIGURE 1: Preparation of nascent strand templates. See text for details.

twice-HPLC-purified ATP, and *E. coli* single-stranded DNA binding protein, sufficient to give a ratio of one protein molecule per five template nucleotide residues. DNA polymerase β reactions contained 50 mM Tris-HCl, 15% glycerol, 0.5 mM Na_2EDTA, 3.75 mM $MgCl_2$, and 20 μM dNTPs. Reactions with T4 DNA polymerase contained 50 mM Tris-HCl, 6.4 mM $MgCl_2$, 5 mM DTT, and 30 μM dNTPs. MMLV DNA polymerase reactions contained 50 mM Tris-HCl, 1 mM DTT, 3.2 mM $MgCl_2$, and 250 μM dNTPs. All polymerase reactions were carried out at 30 °C.

RESULTS

In order to examine the effect of pH upon the rate of primer elongation catalyzed by Klenow polymerase, we carried out "complete" reactions (containing all four dNTPs) at pH values ranging from 5.5 to 10.0 (Figure 2). Four buffer systems were used: 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.5, 6.0, and 6.5; 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 6.5, 7.0, and 7.5; tris(hydroxymethyl)aminomethane (Tris) at pH 7.5, 8.0, 8.5, and 9.0; 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES) at pH 9.0, 9.5, and 10.0. Kf pol exhibited a broad peak of activity between pH 7.0 in MOPS (Figure 2, lane 5) and pH 9.0 in CHES (lane 11), with maximal elongation activity at pH 7.5 in Tris (lane 7). The efficiency of primer utilization appeared to increase with pH. Slight degradation of primer by 3′-exonucleolytic activity appeared at pH 8.5 (lane 9) and increased with pH. Sequence-dependent variation in the rate of chain elongation, reflected by nonuniform banding patterns as reported previ-

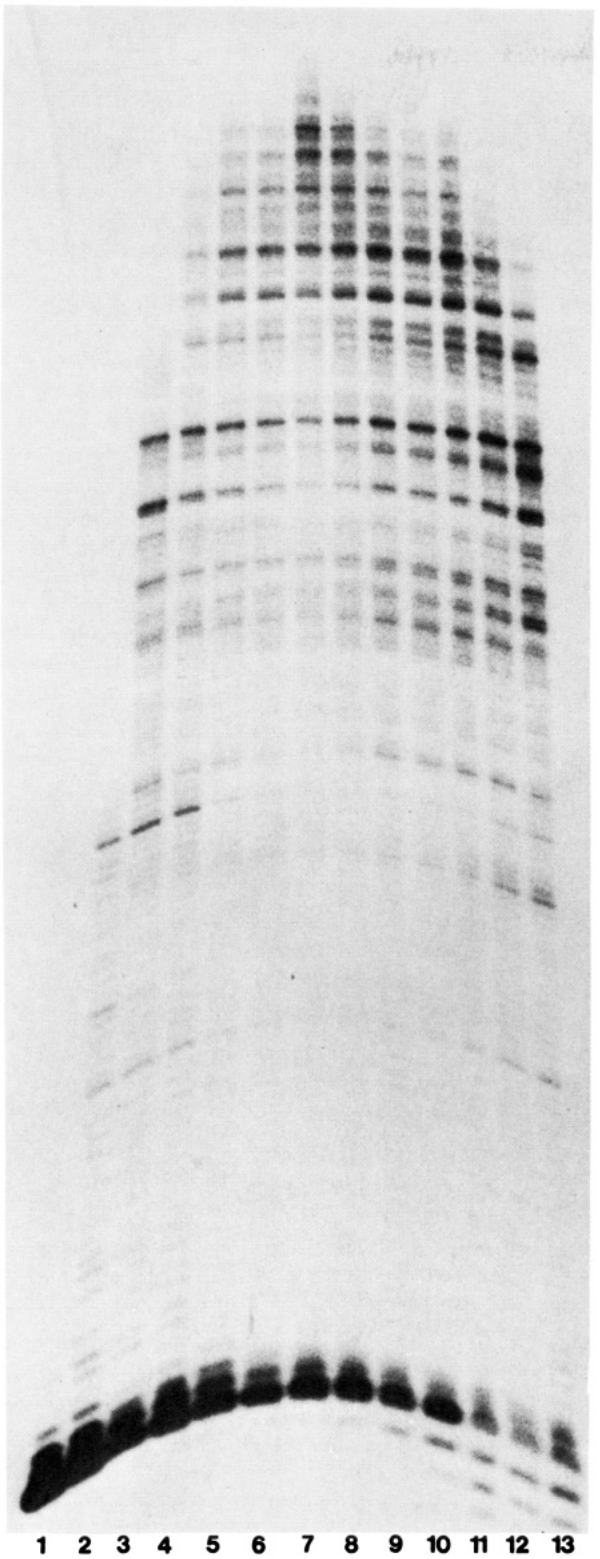

FIGURE 2: Effect of pH on primer elongation in the "complete" reaction catalyzed by DNA polymerase I (Klenow fragment). Reactions (50 μL total volume) contained 0.5 nM (3′-OH termini) template-primer, 20 μM each of the four dNTPs, and 6.5 mM $MgCl_2$. Reactions were buffered with 50 mM MES (lanes 1–3), MOPS (lanes 4–6), Tris (lanes 7–10), or CHES (lanes 11–13). The pH values were as follows: lane 1, pH 5.5; lane 2, pH 6.0; lane 3, pH 6.5; lane 4, pH 6.5; lane 5, pH 7.0; lane 6, pH 7.5; lane 7, pH 7.5; lane 8, pH 8.0; lane 9, pH 8.5; lane 10, pH 9.0; lane 11, pH 9.0; lane 12, pH 9.5; and lane 13, pH 10.0. Incubation was at 30 °C for 45 s. Reactions were initiated by addition of 1 unit of enzyme and terminated by addition to 5 μL of 250 mM Na_2EDTA. DNA products were displayed by electrophoresis through a 10% polyacrylamide–7 M urea gel, followed by autoradiography.

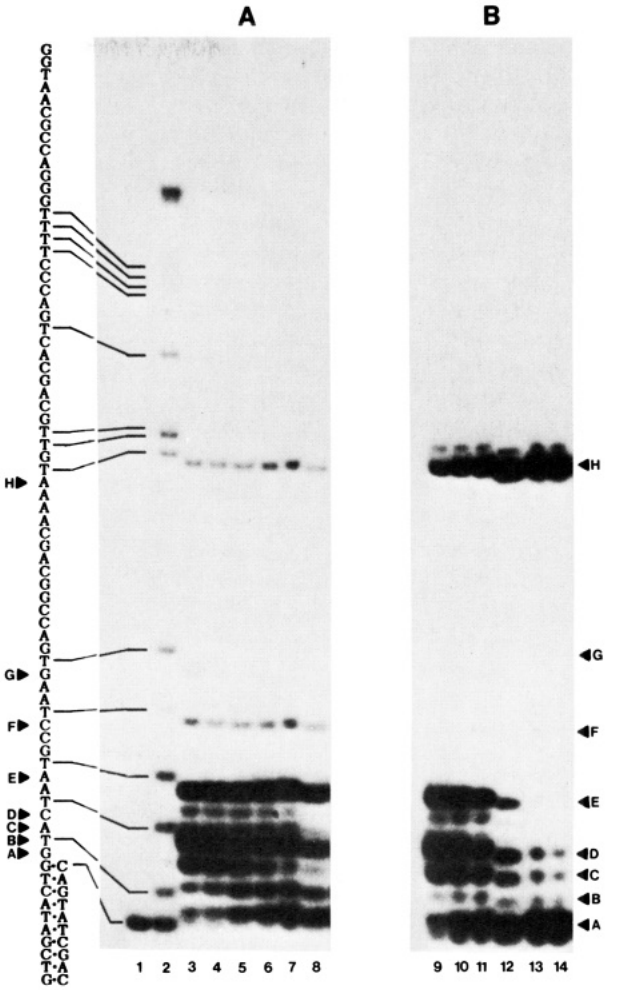

FIGURE 3: Effect of pH on the relative rates of misincorporation opposite template BU versus T by DNA polymerase I (Klenow fragment). Reaction mixtures (50 μL total volume) contained 0.5 nM (3′-OH termini) template-primer, 20 μM dGTP, dCTP, and dTTP, 6.5 mM $MgCl_2$, and 50 mM Tris-HCl. Reactions were performed at pH values of 7.0 (lanes 3 and 9), 7.3 (lanes 4 and 10), 7.6 (lanes 5 and 11), 8.0 (lanes 6 and 12), 8.2 (lanes 7 and 13), and 8.5 (lanes 8 and 14). Incubation was at 30 °C for 30 min after addition of 1 unit of enzyme. Reactions were terminated by addition of 2 μL of 250 mM Na_2EDTA. DNA was processed and electrophoresed through a 10% polyacrylamide–7 M urea gel. The nucleotide sequence of the template downstream from the 3′-OH terminus of the primer is shown on the left, with specific residues aligned with their corresponding "ddA" bands produced in lane 2. Reactions of lanes 3–8 were performed with T-DNA template-primer, whereas those of lanes 9–14 were performed with BU-DNA template-primer.

ously (Hillebrand & Beattie, 1985), was essentially unaffected by the different buffers and pH values.

In the experiment shown in Figure 3, we examined the effect of increasing pH upon the relative rates of misincorporation by Kf pol opposite BU versus T in the template strand. In the electrophoretic assay carried out for this purpose, elongation of 5′-^{32}P primer is conducted in the absence of dATP. The degree of "pausing" at successive BU (or T) residues in the template is a measure of the relative fidelity of the polymerase at these sites. "−A" reactions were carried out at pH 7.0, 7.3, 7.6, 8.0, 8.2, and 8.5 in Tris buffer. This range of pH values spans the broad peak of maximal activity for Kf pol (lanes 6–9 of Figure 2). The position marked "G" in the template sequence (Figure 3) immediately preceded a misincorporation hotspot at all pH values in both T and BU templates (as seen by the lack of premisincorporation pausing at this site), whereas the position marked "F" preceded a BU-specific misincorporation hotspot at all pH values [pausing

occurred at template T (panel A) not BU (panel B)]. At the second and third misincorporation sites (preceded by pause sites D and E), rates of misincorporation opposite BU increased with pH (as seen by progressive decrease in band intensity in lanes 9 → 14), whereas misincorporation rates opposite T at these sites were unaffected by pH (constant band intensities, lanes 3 → 8).

A sequence-dependent phenomenon known as "postmisincorporation pausing" (Hillebrand et al., 1984) is seen in Figure 3, wherein pausing occurs *after* misincorporation at certain sites, resulting in primers of electrophoretic mobility identical with that of the corresponding "dideoxy" sequencing bands. This phenomenon presumably results from decreased efficiency of utilization of a primer strand containing a 3′-terminal mismatch (for both incorporation and 3′-exonuclease action). For example, postmisincorporation pausing occurred at the first misincorporation site (position B) and was more evident on the T-DNA template (lanes 3 → 8) than on the BU-DNA template (lanes 9 → 14). A similar effect was seen at the second misincorporation site (preceded by pause site D).

At position H (Figure 3), misincorporation seemed relatively insensitive to increasing pH on both templates, although the sensitivity of the assay was low, due to insufficient elongation past this point. Occasionally, it is impossible to distinguish whether a band (e.g., at position C) is due to postmisincorporation pausing or to 3′-exonucleolytic degradation of primers which were elongated to the next premisincorporation pause site. Despite these complexities, the data of Figure 3, along with results obtained with a variety of other template sequences (data not shown), clearly indicate that at some template BU residues misincorporation by Kf pol is facilitated by increased pH, but not at template T residues.

In the experiment shown in Figure 4, we examined the effect of increasing pH upon misincorporation opposite template T and BU for MMLV pol and pol β. A shift in pH from 7.0 (lane 3) to 9.0 (lane 4) resulted in a small increase in misincorporation opposite template T by MMLV pol. The pH increase gave a much greater increase in misincorporation at template BU residues by this enzyme, as indicated by the greatly reduced band intensity at positions A, B, and C in lane 6 (pH 9), compared with lane 5 (pH 7). Misincorporation by pol β opposite template T also increased with pH (lanes 7 and 8) but not nearly as dramatically as at template BU (lanes 9 and 10). The three bands at the top of lane 10 (marked "E") did not migrate to the expected positions of misincorporation products. We have repeatedly observed this banding pattern, but only in the "–A" reactions catalyzed by pol β and only on this particular template-primer. We concluded that this banding pattern was probably not attributable to secondary structure in the template strand but rather to a unique interaction between pol β and this particular template-primer that causes chain termination.

Since we have found that the rate of misincorporation opposite BU residues in the template strand by three different DNA polymerases can be stimulated by increasing pH, we investigated whether misincorporation of BrdUMP (or dTMP) at template G is also facilitated by increasing pH. In the experiment shown in Figure 5, we examined the pH dependence of the relative rates of misincorporation of BrdUMP versus TMP in "–C" reactions (i.e., opposite template G residues) with Kf pol. As also noted previously with this template sequence (Driggers & Beattie, 1988a), misincorporation at positions A and C (Figure 5) is faster in the presence of BrdUTP (lanes 6–8) than in the presence of dTTP (lanes 3–5), whereas position B is bypassed faster in the presence of dTTP than in the presence of BrdUTP. Increasing pH had little effect overall in either reaction. The lack of stimulation of BrdUMP misincorporation by increased pH was also observed with a variety of other template-primers, in reactions catalyzed by Kf pol, T4 pol, and MMLV pol (data not shown).

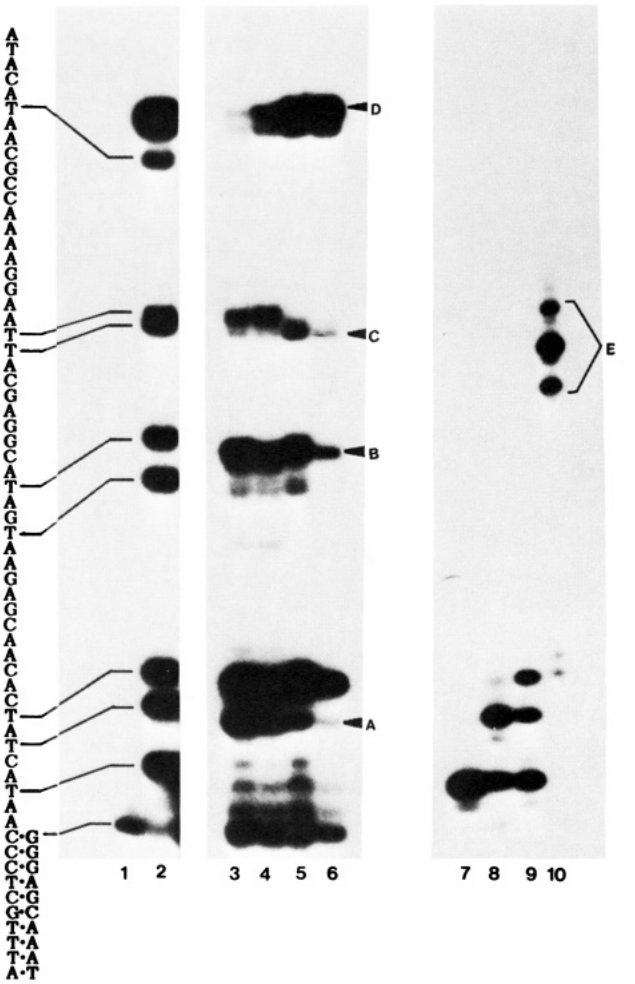

FIGURE 4: Effect of pH on misincorporation opposite template T and BU during primer elongation catalyzed by DNA polymerase of Maloney murine leukemia virus and DNA polymerase β (Novikoff hepatoma). Reactions with MMLV pol (lanes 3–6) contained (in 50 μL total volume) 0.5 nM (3′-OH termini) template-primer, 50 mM Tris-HCl, 1 mM DTT, 3.2 mM $MgCl_2$, and 250 μM dNTPs. Reactions with pol β (lanes 7–10) contained (in 50 μL total volume) 0.5 nM (3′-OH termini) template-primer, 50 mM Tris-HCl, 15% glycerol, 0.5 mM Na_2EDTA, 3.75 mM $MgCl_2$, and 20 μM dNTPs. The template sequence near the 3′ end of the primer is displayed along the left edge, with lines connecting T (or BU) residues in the template with corresponding "ddA" bands in lane 2. Reactions were initiated by the addition of 1 unit of enzyme and incubated at 30 °C for 30 min. Reactions were terminated by addition of 2 μL of 250 mM Na_2EDTA. DNA products were displayed by electrophoresis through a 10% polyacrylamide–7 M urea gel, followed by autoradiography. Reactions were performed at pH 7.0 (lanes 3, 5, 7, and 9) or at pH 9.0 (lanes 4, 6, 8, and 10). Polymerization was carried out on T-DNA template (lanes 3, 4, 7, and 8) or on BU-DNA template (lanes 5, 6, 9, and 10).

The effect of pH on misincorporation opposite template T and BU residues catalyzed by pol III holoenzyme is shown in Figure 6, panel A. Comparison of lanes 3–6 shows that misincorporation on neither template was affected by the pH increase. Furthermore, pol III holoenzyme exhibited no greater misincorporation opposite template BU than it did opposite template T, at any pH. Finally, we examined the effect of pH on the relative rates of misincorporation of dTMP or BrdUMP in the "–C" reaction catalyzed by pol III holo-

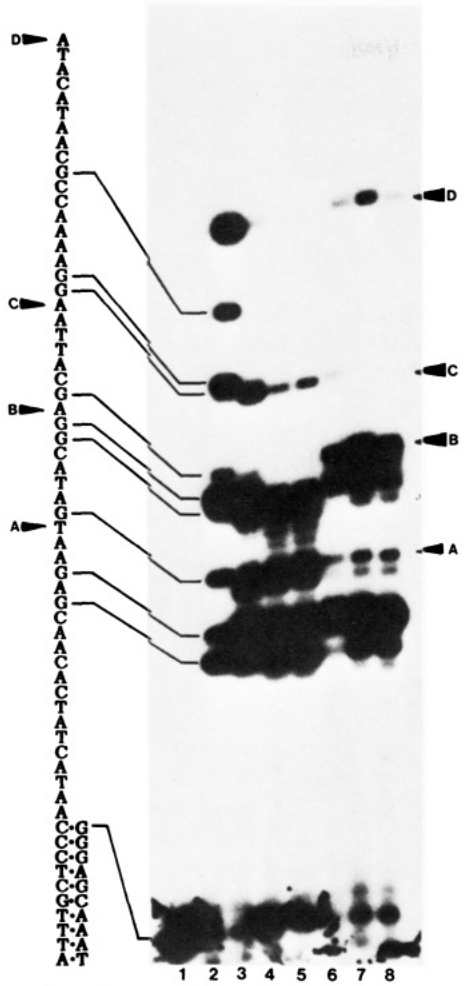

FIGURE 5: Effect of pH on misincorporation of BrdUMP or dTMP in "–C" reactions during primer elongation catalyzed by DNA polymerase I (Klenow fragment). Reactions (50 μL total volume) contained 50 mM Tris-HCl, 6.5 mM $MgCl_2$, 20 μM dNTPs, and 0.5 nM (3′-OH termini) template-primer. "–C" reactions contained dATP, dGTP, and either dTTP (lanes 3–5) or BrdUTP (lanes 6–8). Reactions were performed at pH values of 7.0 (lanes 3 and 6), 8.0 (lanes 4 and 7), or 9.0 (lanes 5 and 8). After initiation by addition of 1 unit of enzyme, reactions were incubated at 30 °C for 30 min. Reactions were terminated by addition of 2 μL of 250 mM Na_2EDTA. DNA products were displayed by electrophoresis through a 10% polyacrylamide–7 M urea gel, followed by autoradiography. The nucleotide sequence of the template downstream from the 3′-OH terminus of the primer is shown on the left, with specific residues aligned with their corresponding bands produced in the dideoxy C sequencing reaction in lane 2. Arrows on the right side of the diagram point to the expected positions of migration of bands produced by pausing of the enzyme prior to misincorporation opposite template G residues. Arrows on the left side of the figure point to positions of premisincorporation pausing in the "–C" reaction.

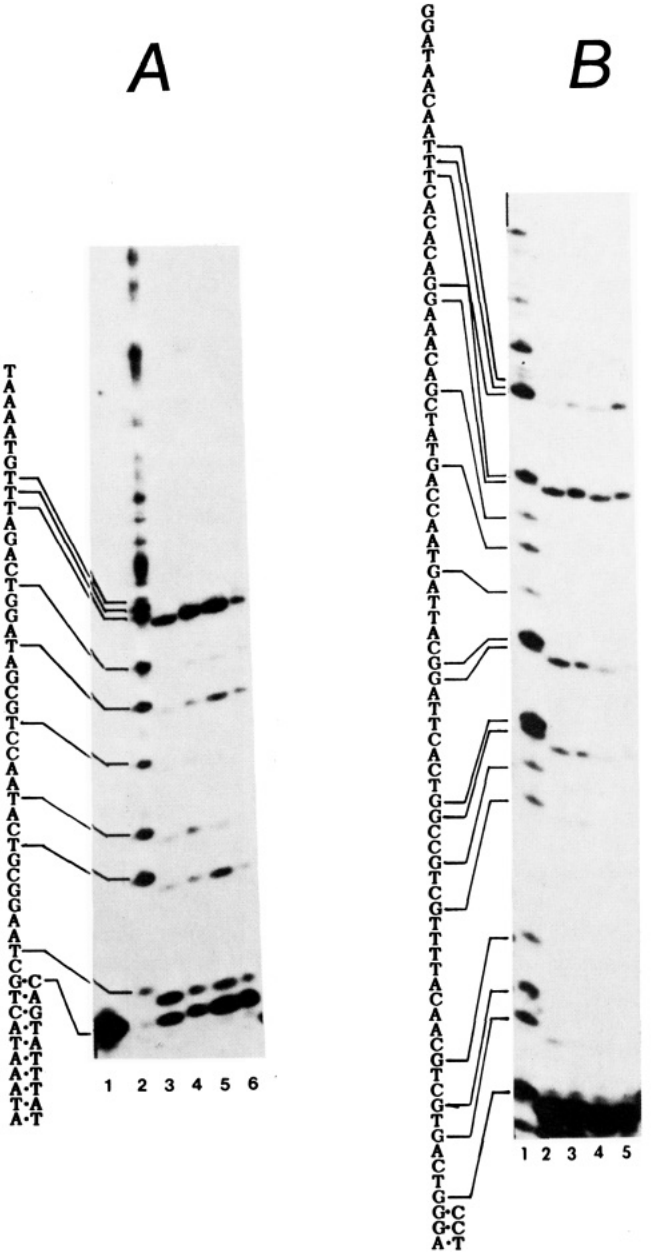

FIGURE 6: Effect of pH on mispairing of BU during primer elongation catalyzed by *E. coli* DNA polymerase III holoenzyme. Reaction mixtures (20 μL total volume) contained 0.5 nM (3′-OH termini) template-primer, 25 mM Tris-HCl, 6% glycerol, 1.25 mg/mL BSA, 12.5 mM DTT, 5 mM $MgCl_2$, 5 mM spermidine hydrochloride, 62.5 μM dNTPs, 6 μM twice-HPLC-purified ATP, and *E. coli* single-stranded DNA binding protein, sufficient to give a ratio of one protein molecule per five template nucleotide residues. Reactions were initiated by addition of enzyme and incubated at 30 °C for 30 min. After termination of polymerization by addition of 2 μL of 250 mM Na_2EDTA, DNA products were displayed by electrophoresis through a 10% polyacrylamide–7 M urea gel, followed by autoradiography. The "–A" reactions (panel A) were carried out on T-DNA template-primers (lanes 3 and 4) or on BU-DNA template-primers (lanes 5 and 6), in the presence of 62.5 μM dGTP, dCTP, and dTTP. In panel A, lane 1 represents unelongated primer, and lane 2 represents the "dideoxy-A" sequencing reaction; the "–A" reactions were carried out at pH 7.0 (lanes 3 and 5) or pH 9.0 (lanes 4 and 6). The "–C" reactions (panel B) were carried out on T-DNA template-primers, in the presence of 62.5 μM each of dATP and dGTP plus 62.5 μM dTTP (lanes 2 and 3) or BrdUTP (lanes 4 and 5). The "–C" reactions were performed at pH 7.0 (lanes 2 and 4) or pH 9.0 (lanes 3 and 5). Lane 1 in panel B represents the "dideoxy-C" sequencing reaction.

enzyme (Figure 6, panel B). An increase in the pH from 7.0 to 9.0 produced no significant differences in these reactions. Thus, we have been unable to detect a significant difference in the occurrence of BU·G mispairing versus T·G mispairing during DNA synthesis catalyzed by *E. coli* pol III holoenzyme, and we detected little if any stimulation of these mispairings by elevated pH.

Discussion

The most widely accepted explanation for the mechanism of mutation induction by BU involves the formation of G·BU mispairs as mutagenic intermediates. Of the three mispairing models proposed to account for greater stability of G·BU mispairs compared with G·T mispairs (tautomerization, ionization, and wobbling), none has ever been implicated in mispairing of BU during DNA synthesis. We have investigated the possibility that ionized species could be involved in

formation of BU·G mispairs during DNA synthesis. The pK_a values of BrdUrd and Thd in aqueous solution are 8.1 and 9.8, respectively (Lawley & Brookes, 1962). Thus, as the pH is raised from 7.0 to 9.0, there should be greater ionization of template BU than of template T, and a correspondingly greater existence of the potentially mispairing anionic base. Therefore, we began by examining the effect of increasing pH upon the rate of misincorporation opposite template BU versus T during primer elongation catalyzed by Kf pol. We found that increasing pH resulted in a variety of effects on the relative rates of misincorporation opposite BU and T in the template. At most of the sites which we have examined, increasing pH facilitated misincorporation opposite template BU but not template T. As seen in Figure 2, 3′-exonucleolytic activity increased slightly when the pH was raised to 8.5 or higher. This effect would tend to counteract the increased mispairing of BU at high pH. This effect, if sequence-dependent, could account for the lack of stimulation of misincorporation by Kf pol by elevated pH at certain template positions. Nevertheless, the data for Kf pol indicated that increasing pH facilitates misincorporation opposite BU at many template positions but not opposite T in the template strand.

We also examined the effect of pH upon misincorporation opposite template T or BU for four other DNA polymerases. For MMLV pol and pol β, we found that a shift in pH from 7.0 to 9.0 dramatically increased the frequency of misincorporation, apparently opposite every BU residue in the template. Increased pH also slightly facilitated misincorporation opposite template T with these polymerases. Since these two polymerases reportedly lack 3′-exonucleolytic activity, the primer elongation observed in "−A" reactions should directly reflect the ability of the enzymes to catalyze misinsertion opposite BU and T in the template. For pol III holoenzyme and T4 pol, which possess 3′-exonucleolytic activity, increasing pH did not affect the relative rates of misincorporation opposite T and BU in the template strand (which were similar to begin with).

Surprisingly, increasing pH had little effect on rates of misincorporation of dTMP and BrdUMP by any of the DNA polymerases tested. Thus, our data provide no evidence for the involvement of ionized incoming dNTPs in mispairing mechanisms, in contrast to the case of misincorporation opposite template BU. This lack of reciprocity is not predicted by a simple mispairing model, which does not distinguish between template and incoming nucleotide bases, and the same mispair geometry should be theoretically possible in either case. We expect stacking interactions to contribute to the stabilization of mispairs, and better stacking interactions with neighboring bases may improve the stability of BU·G mispairs relative to T·G mispairs. Stabilization by stacking would be less for an incoming BU residue than for a template BU, since in the former case the stacking interaction occurs on only one side of the BU residue. Additionally, it is possible that ionization could produce conformational changes in BrdUTP in solution (Uhl et al., 1983) or electrostatic repulsion, thereby affecting the binding of dNTP at the active site of the polymerase.

We can consider two possible explanations for increased misincorporation opposite BU versus T in the template, in which the effect is mediated through the enzyme rather than through ionization of BU. In one model, increasing pH decreases the intrinsic fidelity of the polymerase. This explanation would seem to inadequately account for our results with Kf pol, MMLV pol, and pol β, since for all three of these enzymes the effect of pH on misincorporation was observed specifically at template BU and not template T, C, A, or G. It is difficult to imagine how the accuracy of the enzymes would be affected by pH exclusively at template BU residues. Additionally, the sequence dependence of the pH effect seen at template BU with Kf pol is not readily accounted for by this model. In another model, increasing pH affects the ionization state of a group within the enzyme, which affects its interaction with template BU but not T, so that BU·G mispairs are preferentially stabilized in comparison with T·G mispairs. Since such preferential stabilization would not necessarily be seen for every DNA polymerase, this could explain why some DNA polymerases showed no pH effect.

A more straightforward explanation for the effect of pH on misincorporation is that mispairing of template BU occurs more readily when the template BU is in its anionic form than when it is neutral. The model for ionized BU·G mispairing is in accord with the observed faster misinsertion of G opposite template BU (compared with template T), as we have observed with Kf pol, MMLV pol, and pol β. In accordance with this model, we might also expect to observe a small increase in the rates of misincorporation opposite T in the template, since the anionic form of this base also increases with pH and might form a similar mispair with G. This is consistent with our observation of a small increase in misincorporation by MMLV pol and pol β opposite template T when the pH was shifted from 7 to 9. There are precedents for the involvement of ionized bases in mispairs within duplex DNA. Ionized mispairs have recently been demonstrated for A·C mismatches in solution (Sowers et al., 1986a) and in oligonucleotide duplexes (Hunter et al., 1986), and for the 2-aminopurine·C mismatch in an oligonucleotide duplex (Sowers et al., 1986b).

At present, the ionization model appears to best fit our data. We may speculate that misinsertion opposite ionized BU residues is actually relatively fast for all of the polymerases examined. Both MMLV pol and pol β reportedly lack 3′-exonuclease activity, so that misinsertion and misincorporation should be the same. For the procaryotic polymerases, misincorporation is the net result of misinsertion and removal by 3′-exonucleolytic proofreading. For these polymerases, the relative rates of misincorporation at different template positions might be largely determined by the specificity of the 3′-exonuclease activity at different sites along the template. Thus, the sequence dependence of mispairing by Kf pol might be mediated through effects of sequence on the specificity of proofreading during polymerization. Furthermore, the lack of observed BU·G mispairing for pol III holoenzyme and T4 polymerase might reflect a highly active 3′-exonuclease activity that eliminates any difference in the occurrence of BU·G versus T·G mispairing during DNA synthesis.

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Registry No. BU, 51-20-7; G, 73-40-5; T, 65-71-4; DNA polymerase, 9012-90-2; DNA polymerase III, 37217-33-7.

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Refinement of the Solution Structure of the Ribonucleotide 5′r(GCAUGC)$_2$: Combined Use of Nuclear Magnetic Resonance and Restrained Molecular Dynamics†

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ABSTRACT: The solution structure of the self-complementary hexamer 5′r(GCAUGC)$_2$ is investigated by means of nuclear magnetic resonance spectroscopy and restrained molecular dynamics. The proton resonances are assigned in a sequential manner, and a set of 110 approximate interproton distance restraints are derived from the two-dimensional nuclear Overhauser enhancement spectra. These distances are used as the basis of a structure refinement by restrained molecular dynamics in which the experimental restraints are incorporated into the total energy function of the system in the form of effective potentials. Eight restrained molecular dynamics simulations are carried out, four starting from a structure with regular A-type geometry and four from one with regular B-type geometry. The atomic root mean square (rms) difference between the initial structures is 3.2 Å. In the case of all eight simulations, convergence is achieved both globally and locally to a set of very similar A-type structures with an average atomic rms difference between them of 0.8 ± 0.2 Å. Further, the atomic rms differences between the restrained dynamics structures obtained by starting out from the same initial structures but with different random number seeds for the assignment of the initial velocities are the same as those between the restrained dynamics structures starting out from the two different initial structures. These results suggest that the restrained dynamics structures represent good approximations of the solution structure. The converged structures exhibit clear sequence-dependent variation in some of the helical parameters, in particular helix twist, roll, slide, and propellor twist. The variation in roll follows that predicted by Dickerson [Dickerson, R. E. (1983) *J. Mol. Biol. 166*, 419–441], whereas those for helix twist and propellor twist follow the opposite trend to the predicted one.

As part of a study on the conformations of nucleic acids in solution we present a combined nuclear magnetic resonance (NMR)[1] and restrained molecular dynamics study on the self-complementary RNA hexamer 5′r(GCAUGC)$_2$. This particular sequence was chosen to enable a direct structural comparison with the analogous DNA oligonucleotide 5′d-(GCATGC)$_2$, whose three-dimensional structure had previously been determined by the same methods (Nilges et al., 1987a). We first assign the resonances of the RNA hexamer in a sequential manner using a combination of HOHAHA and NOESY spectroscopy; a set of approximate interproton distance restraints is then derived from the NOESY cross-peak

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; rms, root mean square; RD, restrained dynamics.

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