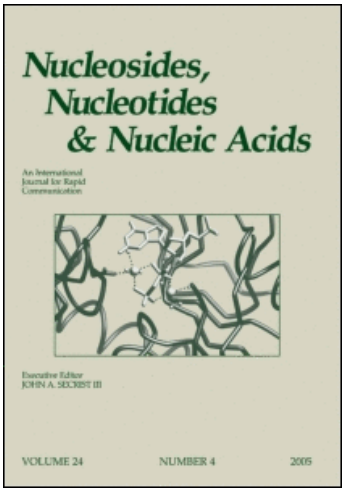

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SENSITIVITY OF BACTERIOPHAGE RB69 DNA POLYMERASE TO N^2-(*P*-N-BUTYLPHENYL)-2'-DEOXYGUANOSINE NUCLEOTIDES.

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Abstract: The response of bacteriophage RB69 DNA polymerase to N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP), related nucleotides and non-nucleoside inhibitors was measured and compared to values obtained for the closely related DNA polymerase from bacteriophage T4. Both enzymes showed similar responses to inhibitors in terms of K_i values and the ability to utilize BuPdGTP as a substrate. These results provide the relevance of using the recent crystal structure of RB69 DNA polymerase for analysis of BuPdGTP/B family DNA polymerase interactions.

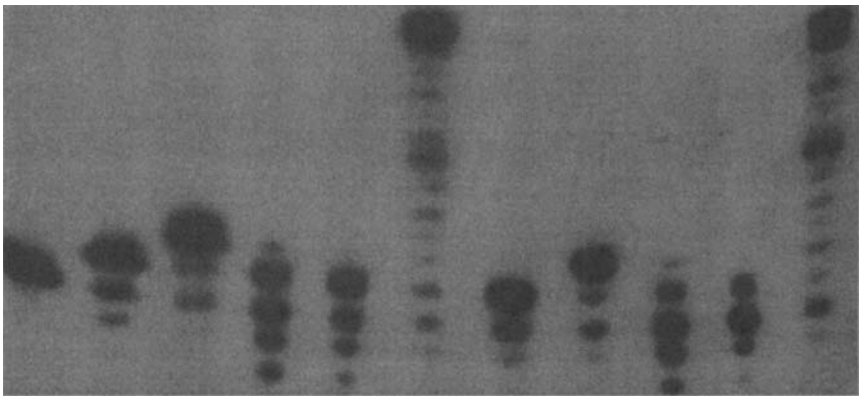
DNA-dependent DNA polymerases are complex enzymes that bind multiple substrates, and some of them may possess, in addition to polymerase (pol) activity, 3'-5' exonuclease (exo) and 5'-3' exo activities [1]. The largest family of DNA polymerases, the B family, consists of animal cell DNA polymerases α, δ and ε, the corresponding enzymes from yeast, herpes virus DNA polymerases, such as HSV1 DNA polymerase (HSV1 polymerase), T-even bacteriophage DNA polymerases, such as T4 DNA polymerase (T4 polymerase), and other small phage DNA polymerases such as ϕ29 DNA polymerase (ϕ29 polymerase) [2]. B family polymerases contain up to seven regions of significant sequence homology in the C-terminal region suggested to be in the pol domain, and three regions of homology in the N-terminal region which comprise the exo domain [2].

Elucidation of structural features of B family pol active sites has been accomplished, in part, by use of specific inhibitors that have unique action against B family polymerases. Of particular interest are the varied responses of members of this family to N^2-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP).

BuPdGTP was initially reported to be a potent and selective inhibitor of HeLa pol α, with a competitive K_i = 1-5 nM [3,4]. Subsequent analysis showed that immunopurified calf thymus pol α, although highly sensitive to the inhibitor, did not utilize BuPdGTP as a substrate [5]. BuPdGTP has subsequently been found to inhibit T4 polymerase [6], ϕ29 polymerase [7], and HSV1 polymerase [C. Knopf, personal communication] with competitive K_is = 0.8 μM, 20 μM and 100 μM, respectively. All three enzymes incorporate BuPdGTP into DNA, in contrast to results seen for immunopurified calf thymus pol α.

Isolation of bacteriophage RB69 DNA polymerase (RB69 pol), a new member of the B family of DNA polymerases, has been reported recently [8]. RB69 pol is closely related to T4 pol (76% amino acid homology), and the cloned wild-type polymerase gene of phage RB69 can substitute for T4 pol activity, and vice versa, in phage complementation experiments [8]. Furthermore, the X-ray crystal structure of RB69 pol has now been solved, representing the first B family DNA polymerase to have its three dimensional structure mapped [9]. In addition to the comparison with active site structures of other pol families, this structure can now be used to help find the region of the pol site responsible for the B family's sensitivity to inhibitors, especially BuPdGTP. As a means of evaluating the relevance of the RB69 pol structure to determine the conserved residues that interact with the butylphenyl group, we have studied the response of RB69 pol to "butylphenyl" nucleotides, and here compared the results to those observed for T4 pol. Our goal is to ensure the relevance of using the RB69 pol structure for analysis of BuPdGTP/DNA polymerase interactions.

Characterization of RB69 pol. Biochemical properties and inhibitor sensitivities of RB69 pol were determined and compared with those of T4 pol (Table 1). As anticipated, the two enzymes have similar K_m values for nucleotides and DNA under our assay conditions. RB69 pol also showed similar sensitivity to phosphonoacetic acid (PAA) and enhanced sensitivity to aphidicolin, both of which are non-nucleotide inhibitors of B family DNA polymerases [1]. The ability of BuPdGTP and related derivatives to inhibit RB69 polymerase activity was measured. RB69 pol exhibited reduced sensitivity to "butylphenyl" nucleotide analogs in comparison to T4 pol (Table 1), and showed a different pattern of inhibition than seen for both T4 pol and recombinant human pol α [11]. For T4 pol (Table 1) and pol α [11], BuPdGMPCH$_2$PP was about 5-fold less potent and BuPdGMPNHPP 8-fold more potent than the parent compound, BuPdGTP. RB69 pol showed reduced sensitivity to both derivatives in comparison to BuPdGTP (Table 1). Variable substrate analysis of the effect of BuPdGTP showed competitive inhibition of RB69 pol with respect to dGTP (data not shown). T4 pol showed mixed inhibition in response to BuPdGTP, a result interpreted as a result of incorporation of the inhibitor into



lane: 1 2 3 4 5 6 7 8 9 10 11

FIG. 1. 12% PAGE analysis of nucleotide incorporation by T4 pol and RB69 pol into 17:29-mer primer:template (p:t) system. Reactions were performed in the presence of 6 mM GMP as described in EXPERIMENTAL PROCEDURES. Lanes 2-6: T4 polymerase; lanes 7-11: RB69 polymerase. Contents: lane 1: p:t alone; lanes 2,7: 25 μM dGTP; 3,8: 25 μM dGTP/dTTP; 4,9: 10 μM BuPdGTP; 5,10: 10 μM BuPdGTP/25 μM dTTP; 6,11: 25 μM of 4 dNTPs.

primer:template [6], implying that RB69 pol either does not incorporate BuPdGTP at all, or does so at a rate sufficiently low that the kinetic mechanism is not affected.

Primer extension assays. Because of the difference between RB69 pol and T4 pol in response to BuPdGTP in the variable substrate assay, we measured the ability of RB69 pol to utilize BuPdGTP as a substrate using a synthetic primer:template (17:29-mer, sequence in EXPERIMENTAL PROCEDURES). The 17:29-mer, for which dGTP is the first required nucleotide, was previously used to establish that BuPdGTP is a terminating substrate for T4 pol [6,11]. These reactions contained 6 mM GMP to inhibit 3'-5'-exo activity [12,13]; even so, significant degradation of the 17-mer by both enzymes was observed, especially in the presence of BuPdGTP (Figure 1, lanes 4 and 9). Extension of the 17-mer by both enzymes was observed in the presence of dGTP alone (lanes 2 and 7) and in the presence of dGTP and dTTP (lanes 3 and 8). RB69 pol did incorporate BuPdGTP (lane 9), although the extent of incorporation appeared to be lower than that seen for T4 pol under identical conditions (lane 4). The rate of BuPdGTP incorporation for both enzymes appeared to be much lower than the incorporation rate of dGTP (compare lanes 2 and 4 and lanes 7 and 9), suggesting that the presence of the butylphenyl group interferes with a critical step in the pol reaction in both cases. This is further supported, indirectly, by the presence of significant 3'-5'-exo degradation in

TABLE 1. Biochemical properties and inhibitor sensitivities of T4 DNA polymerase (T4 pol) and RB69 DNA polymerase (RB69 pol). K_m assays were performed by varying the substrate concentration with enzyme and remaining substrate concentrations remaining constant. Inhibition assays utilized activated calf thymus DNA under "truncated" conditions as described in EXPERIMENTAL PROCEDURES [1]. Assays with aphidicolin lacked dCTP, all other inhibitor assays lacked dGTP. Assays with phosphonoacetic acid (PAA) were performed under "full" conditions, containing all 4 dNTPs.

K_m	T4 pol	RB69 pol
dGTP:	0.80 ± 0.11 μM	0.48 ± 0.05 μM
dCTP:	1.20 ± 0.17 μM	0.87 ± 0.20 μM
Activated DNA:	0.30 ± 0.07 mg/mL	0.57 ± 0.11 mg/mL
Apparent K_i		
BuPdGTP:	0.020 ± 0.012 μM	0.192 ± 0.055 μM
Aphidicolin:	10 ± 2 μM	1.7 ± 0.7 μM
PAA:	63 ± 34 μM	241 ± 92 μM
BuPdGMPCH$_2$PP:	0.120 ± 0.050 μM	0.760 ± 0.112 μM
BuPdGMPNHPP:	0.011 ± 0.003 μM	0.480 ± 0.124 μM

reactions containing BuPdGTP, implying that since BuPdGTP does not function well as a substrate, the enzymes switch to 3'-5'-exo function more readily. No extension of the BuPdG-modified 18-mer by dTTP was observed (lane 10), consistent with reported results of incorporation studies with 3'-BuPdG modified primers and the previous finding that BuPdGTP is a terminating substrate for DNA polymerases [5,6,11].

In additional experiments using the 17:29-mer assay system, RB69 pol did not incorporate BuPdGMPCH$_2$PP or BuPdGMPNHPP (data not shown) under conditions which supported dGTP and BuPdGTP incorporation. Thus, RB69 pol behaved like T4 pol and human pol α in their inability to incorporate α,β-phosphate-modified BuPdGTP derivatives [10,11].

The recently solved RB69 DNA polymerase crystal structure will provide a means to analyze the positions of substrates and inhibitors within the B family pol active site [8]. Overall, RB69 pol is similar to T4 pol in response to B family DNA polymerase inhibitors, as expected. The affinities of RB69 pol for BuPdGTP, aphidicolin and PAA were generally similar to those found for T4 pol. The α,β-phosphate-modified derivatives of BuPdGTP were potent active site inhibitors of RB69 pol, although with slightly lower affinities than seen for T4 pol and human pol α [11] and with a different response pattern. For T4 pol (Table 1) and pol α [11], BuPdGMPNHPP was more potent than BuPdGTP, while BuPdGMPCH$_2$PP was less potent. This inhibitory pattern may be

attributed, in part, to differences in pK values for protons and divalent cations of the triphosphate group with substitutions of the α,β-anhydride oxygen [10,11].

Structures of members of the A and D polymerase families have been solved in the presence of primer:template and nucleotide [14-16], and these structures have been extremely useful in identifying the residues critical for substrate interaction. Since the RB69 polymerase structure was solved in the absence of primer:template and nucleotide substrate [9], the α,β-substituted BuPdGTP derivatives could be used in co-crystallization trials to provide a stable, catalytically relevant structure of polymerase, primer:template and non-incorporable substrate analog. From such a structure, residues that interact directly with a substrate and with the butylphenyl group can be determined.

EXPERIMENTAL PROCEDURES

Chemicals. BuPdGTP and related inhibitors were synthesized as described [3,10,11]. Aphidicolin was a gift from the National Cancer Institute. dNTPs were from New England Biolabs. Polyacrylamide and dithiothreitol (DTT) were from Bio-Rad. Calf thymus DNA was from Worthington. Buffers and other reagents were from Sigma.

Enzymes. Dr. Linda Reha-Krantz, University of Alberta, provided wild type T4 DNA polymerase. Dr. William Konigsberg, Yale University, provided wild type RB69 DNA polymerase. T4 polynucleotide kinase (T4 PNK) was obtained from New England Biolabs.

Polymerase assays. Assays of DNA polymerase activity on activated DNA were carried out as described [6,11] in 25 μL volumes containing 30 mM Tris-Cl (pH 7.5), 20% glycerol, 4 mM DTT, 10 mM $MgCl_2$, 0.4 mg/mL activated calf thymus DNA, 25 μM dNTPs and 10 μM [^{3}H]-dTTP (New England Nuclear) at 1250 cpm/pmol. Reactions were initiated by the addition of polymerase (1-5 units) and incubated for 10 min. at 30°C. Reaction mixtures were quenched by addition of 1 mL 10% trichloroacetic acid/100 mM sodium pyrophosphate. Acid-insoluble product was collected on Whatman GF/A filters, washed three times with 0.1 M HCl/100 mM $NaPP_i$. Radioactivity was quantified in 1 mL Optifluor.

Inhibitor assays. Compounds were tested in triplicate in the presence of ten-fold serial dilutions of inhibitors stored in 50 mM Tris-Cl (pH 7.5). For determinations of apparent IC_{50} values, 6 concentrations of inhibitor were used; the results of three independent experiments are plotted as % inhibition vs. log inhibitor concentration. For apparent K_i determinations, 6 concentrations of dGTP were used at a constant inhibitor concentration; results were plotted as 1/pmol dGMP incorporated vs. 1/dGTP concentration. Regression lines were obtained using the Macintosh (HyperCard) program Enzyme Kinetics (*D.G.* Gilbert, Indiana University).

Primer extension assays. A synthetic 17-mer primer (3'-GTAAAACGACGGCCAGT-5') was radiolabeled using [γ-^{32}P]ATP and T4 PNK and annealed to a template 29-mer (5'-CATTTTGCTGCCGGTCACATGCCGATCCC-3') as described previously [6,11]. Primer extension assays were performed in 10 µL volumes containing 30 mM Tris-Cl (pH 7.5), 5% glycerol, 4 mM DTT, 10 mM $MgCl_2$, 1.1 pmol [^{32}P]-17:29-mer and ten-fold serial dilutions of nucleotides. These reactions also contained 6 mM GMP to inhibit 3'-5'-exonuclease activity [12,13]. Reactions were initiated by the addition of enzyme (0.5-2 units) and quenched after 10 min. by addition of 3 µL 90% formamide/0.01% bromophenol blue. Samples were loaded onto a 12% polyacrylamide gel and electrophoresed at 1250 v. for approximately 3 hr. Radioactivity was measured using a Molecular Dynamics phosphorimager.

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