Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5769–5773, August 1986
Biochemistry

Mechanism of DNA polymerase I: Exonuclease/polymerase activity switch and DNA sequence dependence of pyrophosphorolysis and misincorporation reactions

(idling-turnover/misinsertion)

VALERIE MIZRAHI, PATRICIA BENKOVIC, AND STEPHEN J. BENKOVIC

Department of Chemistry, The Pennsylvania State University, University Park, PA 16802

Contributed by Stephen J. Benkovic, April 4, 1986

ABSTRACT **Mechanistic features of several processes involved in the idling-turnover reaction catalyzed by the large (Klenow) fragment of *Escherichia coli* DNA polymerase I have been established. The exonuclease → polymerase activity switch involved in the excision/incorporation mode of idling-turnover occurs without an intervening dissociation of the enzyme from its DNA substrate. Comparative studies on the pyrophosphorolysis kinetics of related DNA substrates indicate a significant dependence of the reaction rate upon the DNA sequence within the duplex region upstream of the primer–template junction. Finally, a gel electrophoretic analysis of the products of the idling-turnover reaction has provided direct evidence for an alternative DNA sequence-dependent misincorporation/excision pathway.**

DNA polymerase I (Pol I) of *Escherichia coli* is a multifunctional repair enzyme possessing a reversible polymerase activity and distinct 3′ → 5′ and 5′ → 3′ exonuclease activities (1). Extensive kinetic (2, 3), stereochemical (4, 5), and structural (6) studies of Pol I and its large proteolytic (Klenow) fragment have provided detailed mechanistic insight into the enzymology of DNA replication. Recent studies aimed at attempting to describe a unified mechanism of the interrelated activities of the Klenow fragment (KF) focused upon the nature of the dNTP → dNMP turnover reaction that is observed when the enzyme is constrained to idle at the 3′ terminus of the DNA template–primer (7). The idling-turnover reaction was found to involve an alternating excision/incorporation cycle in which the 3′-deoxynucleotide residue of the primer strand is partitioned via exonucleolytic and pyrophosphorolytic degradation into its 5′-mono- and 5′-triphosphate derivatives, respectively.

In this paper, we report further mechanistic studies concerning the nature of the exonuclease → polymerase activity switch and the subsequent pyrophosphorolysis reaction that constitute the excision/incorporation mode of idling-turnover. In addition, we present direct evidence implicating a DNA sequence-dependent misincorporation/excision cycle as a minor alternative pathway of the idling-turnover reaction.

MATERIALS AND METHODS

Materials. KF was purified from *E. coli* CJ155 according to Joyce and Grindley (8). The *E. coli* strain was provided by C. Joyce. *Bam*HI, *Pst* I, *Hae* III, *Taq* I, and *Hin*dIII restriction endonucleases were from New England Biolabs. *Eco*RI restriction endonuclease was kindly provided by P. Modrich. T4 polynucleotide kinase was from United States Biomedical. Plasmid pBR322 was isolated from transformed *E. coli* by a standard procedure (9). $p(dA)_{1000}$ and $(dT)_{16}$ were from P-L Biochemicals. The partially complementary oligonucleotides 5′ d(GATCCTCTACGCCGGACGC) 3′ (19-mer) and 5′ d(TGCGTCCGGCGTAGAG) 3′ (16-mer) were synthesized using an Applied Biosystems 380A DNA synthesizer.

[α-^{32}P]dATP, [α-^{32}P]dGTP, [α-^{32}P]dTTP, and [γ-^{32}P]ATP (>3000 Ci/mmol; 1 Ci = 37 GBq) were from New England Nuclear. dNTPs were from Sigma. DE-81 and GF/C filters (2.5 cm) were from Whatman. TLC was performed as described (7). Scintiverse II scintillation fluid was from Fisher. All other reagents were of the highest available reagent-grade quality.

Methods. *Restriction digestions.* Restriction digestion reactions were carried out by standard procedures (9).

Preparation of oligonucleotide duplex. A solution containing 1 μM of each template (19-mer) and primer (16-mer) oligonucleotide, 5 mM $MgCl_2$, and 50 mM NaCl in 15 mM Tris·HCl (pH 7.4) was hybridized by heating at 100°C for 2 min and then cooling to room temperature over *ca.* 2 hr in an oil bath initially set at 65°C. For gel electrophoretic polymerization assays, trace 5′-^{32}P-labeled primer was added to the mixture prior to hybridization, which was then carried out as above.

5′-end-labeling. A mixture (10 μl) containing 1 μM oligonucleotide (expressed as 5′ ends), 10 mM $MgCl_2$, 0.4 μM [γ-^{32}P]ATP (1000–3000 Ci/mmol), and 4 units of T4 polynucleotide kinase in 50 mM Tris·HCl (pH 7.4) was incubated at 37°C for 30 min. The reaction was terminated by heating at 65°C for 15 min.

3′-end-labeling. *Bam*HI- or *Eco*RI-digested pBR322 was 3′-end-labeled as described (7).

Polymerase assays. Polymerization reactions were followed either by the DE-81 filter paper assay (2) or by gel electrophoresis. In the gel electrophoretic assay, final reaction solutions contained *ca.* 3×10^4 cpm/μl as 5′-^{32}P-labeled primer. Aliquots (2 μl) were quenched directly into 10 μl of the gel loading buffer (80% formamide and 0.1% each of bromophenol blue and xylene cyanol FF in electrophoresis buffer). Samples were heated at 100°C for 1 min and chilled on ice, and 1-μl aliquots then were loaded on a 10% polyacrylamide gel (30 cm × 38 cm × 0.4 mm) containing 8 M urea and TBE buffer (90 mM Tris borate/2.5 mM Na_2EDTA, pH 8.3). Electrophoresis was carried out at 1200–1400 V for 2½–3 hr. Gels were autoradiographed using Kodak XAR-5 film with a single intensifier screen at −70°C for 16 hr.

Exonuclease → polymerase switch experiments. All incubations were conducted at 21°C. A reaction mixture (28 μl) containing 25 nM poly(dA), 250 nM $(dT)_{16}$, 140 nM KF, and 5.5 mM $MgCl_2$ in 50 mM Tris·HCl (pH 7.4) was incubated for 30 min prior to the addition of a mixture (10.8 μl) containing

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase(s); KF, Klenow fragment; bp, base pair(s).

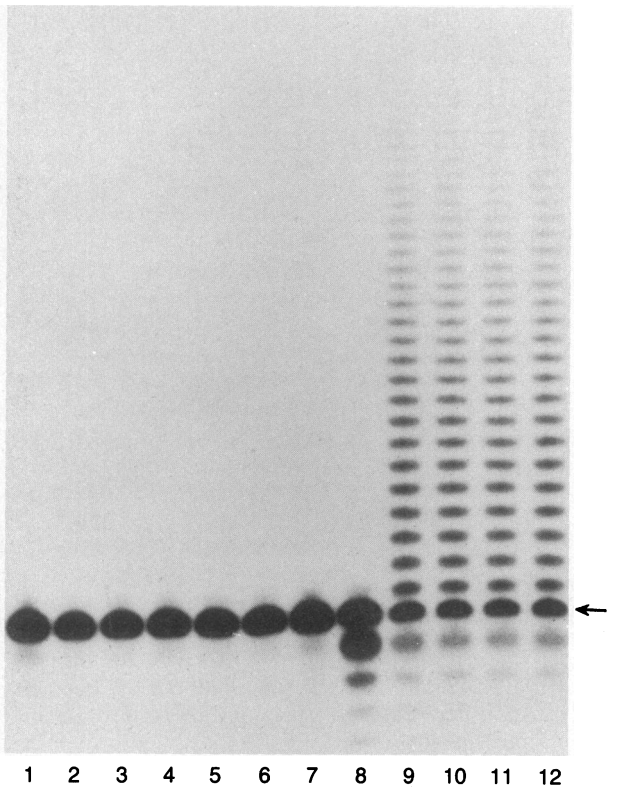


FIG. 1. Gel electrophoretic analysis of reaction products of exonuclease → polymerase switch experiments. Lanes 1–6 are the products of a reaction in which a mixture of KF, poly(dA)·oligo(dT), challenger DNA, and $MgCl_2$ were preincubated for 0 and 30 min (lanes 1 and 2) and then treated with dTTP for a further 15, 30, 45, and 60 sec (lanes 3–6, respectively). Lanes 7–12 illustrate the products of a reaction containing KF, poly(dA)·oligo(dT), and $MgCl_2$ at 0 and 30 min (lanes 7 and 8), to which was added a mixture of challenger DNA and dTTP; the mixture was then allowed to react for a further 15, 30, 45, and 60 sec (lanes 9–12, respectively). Final solutions contained 18 nM poly(dA), 180 nM oligo(dT), 28 μM [α-^{32}P]dTTP, 4 mM $MgCl_2$, 100 nM KF, and 556 μg of challenger DNA per ml in 36 mM Tris·HCl (pH 7.4). Each lane contained *ca.* 5000 cpm of ^{32}P introduced as 5′-^{32}P-labeled dT_{16}. The arrow indicates the migration position of $p(dT)_{16}$ in this system. Gel electrophoresis was carried out in a 10% polyacrylamide/8 M urea gel, followed by autoradiography.

2 mg of challenger DNA per ml (heat-denatured calf-thymus DNA) and 100 μM [α-^{32}P]dTTP (1.5×10^4 cpm/pmol). Aliquots (9 μl) were withdrawn after 15, 30, 45, and 60 sec and were quenched with EDTA to a concentration of 50 mM. The incorporation of radioactivity into DNA was measured by the DE-81 filter assay. In the first control experiment, $MgCl_2$ was omitted from the first solution (28 μl) and included instead in the second solution (10.8 μl) at a concentration of 14.4 mM. Aliquots (9 μl) were withdrawn and processed as above. In the second control, the first solution (36 μl) contained 19.4 nM poly(dA), 194 nM $(dT)_{16}$, 0.6 mg of challenger DNA per ml, 108 nM KF, and 4.3 mM $MgCl_2$ in 39 mM Tris·HCl (pH 7.4). Polymerization was initiated with a second solution (2.8 μl) containing 390 μM [α-^{32}P]dTTP. Aliquots were withdrawn and processed as above. For the gel electrophoretic analysis of the products of these reactions, 5′-^{32}P-labeled $(dT)_{16}$ was included in the appropriate solutions, and the [α-^{32}P]dTTP was replaced by unlabeled dTTP. Final solutions contained *ca.* 3×10^4 cpm/μl. Aliquots (2 μl) were quenched directly into 8 μl of gel loading buffer. Electrophoresis and autoradiography were carried out as described above.

Sequence dependence of pyrophosphorolysis. Reaction mixtures (30 μl) containing 143 nM 3′-end-labeled *Bam*HI-digested pBR322, 10 nM KF, 6.6 mM $MgCl_2$, 50 μM dGTP, 0.2 mM PP_i, and 2 mM dithiothreitol in 50 mM Tris·HCl (pH 7.4) were incubated at 21°C. Aliquots (5 μl) were quenched after 0, 1, 2, 10, and 20 min by the addition of 2 vol of a PhOH/$CHCl_3$ (1:1) mixture followed by rapid Vortex mixing. Aliquots were supplemented with NaCl to a concentration of 50 mM prior to restriction digestion with *Pst* I. The digests were loaded on a 1% agarose gel and electrophoresed in Tris acetate buffer containing 0.5 μg of ethidium bromide per ml (50 V, 4 hr). Following UV visualization of the restriction fragments, the gel lanes were cut into blocks of equal size, which were then placed in scintillation vials and assayed for radioactivity following the addition of 5 ml of Scintiverse II.

Gel electrophoretic analysis of misincorporation. Solutions (30 μl) containing 200–250 nM 3′ ends (*Bam*HI- or *Eco*RI-digested pBR322), 14 nM KF, 5 mM $MgCl_2$, and 10 μM of the appropriate dNTP ([α-^{32}P]dGTP at 2.5×10^4 cpm/pmol for *Bam*HI-digested pBR322; [α-^{32}P]dATP at 1.2×10^4 cpm/pmol for *Eco*RI-digested pBR322) in 50 mM Tris·HCl (pH 7.4) were incubated at 21°C. At various times, aliquots (5 μl) were withdrawn and quenched by PhOH/$CHCl_3$ extraction, as described above. Restriction digestion (*Hae* III or *Taq* I for *Bam*HI-digested pBR322; *Hae* III, *Taq* I, or *Hin*dIII for *Eco*RI-digested pBR322) was carried out as above. Aliquots (1 μl) of the digestion reaction (10 μl) were quenched into 9 μl of the gel loading buffer. Samples were heated at 100°C for 2 min, and 1-μl aliquots were then immediately loaded on a 10% polyacrylamide gel containing 8 M urea and TBE buffer. Electrophoresis was carried out at 1700–1900 V maintaining a gel temperature of 42–45°C. Misincorporation at the 3′ primer terminus of the 19/16-mer

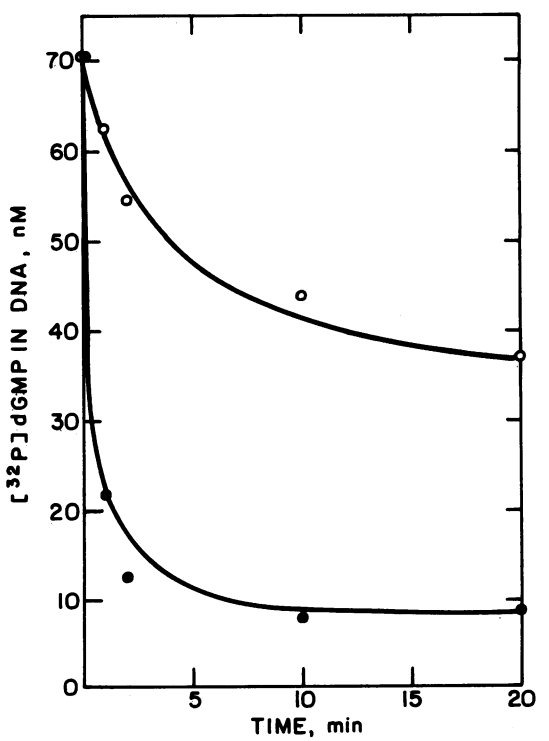


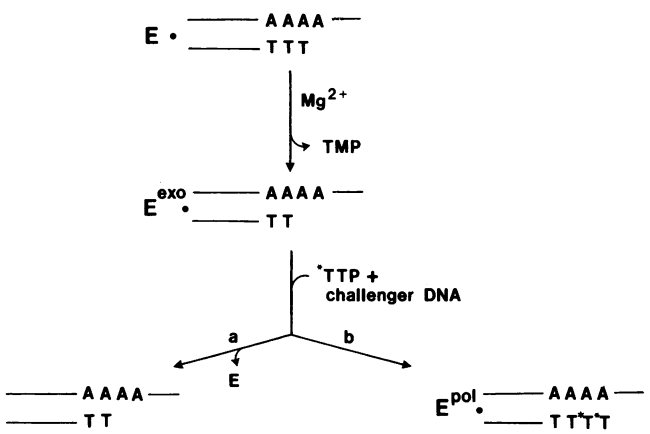
FIG. 2. Resolved pyrophosphorolysis time courses for KF on 3′-end-labeled *Bam*HI-digested pBR322. A 30-μl reaction mixture containing 143 nM 3′ ends, 10 nM KF, 6.6 mM $MgCl_2$, 2 mM dithiothreitol, 50 μM dGTP, and 0.2 mM PP_i in 50 mM Tris·HCl (pH 7.4) was incubated at 21°C. Aliquots (6 μl) were withdrawn at various times and were quenched, *Pst* I-digested, and electrophoresed. ○, ^{32}P label remaining in the 1.1-kilobase (kb) *Pst* I fragment; ●, ^{32}P label remaining in the 3.2-kb *Pst* I fragment. The nonexcisable label remaining in the 3.2-kb fragment (≈10%) is probably due to misincorporation during end-labeling, which renders a fraction of the DNA resistant to pyrophosphorolysis (*Discussion*).

oligonucleotide duplex under the above conditions was monitored by including 5′-^{32}P-labeled 16-mer in the reaction and using unlabeled dNTPs. The gel electrophoretic analysis was conducted as described above for a normal polymerization assay.

RESULTS

Exonuclease → Polymerase Activity Switch. The first step in excision/incorporation idling-turnover involves a 3′ → 5′ exonucleolytic removal of the terminal residue, followed by a rapid incorporation step to reinsert the correct nucleotide from the available dNTP pool (7). The conversion of the enzyme from an exonuclease to a polymerase mode, when presented with a complementary dNTP pool, suppresses further exonuclease activity (10). We have investigated whether an intervening dissociation of KF from its duplex DNA substrate is involved in such an exonuclease → polymerase activity switch.

The strategy designed to address this question is outlined in Scheme I.



The enzyme is initially bound to poly(dA)·(dT)$_{16}$ in a mode conducive to exonuclease activity. The simultaneous addition of [α-^{32}P]dTTP and excess challenger DNA to this system promotes conversion of the E·DNA complex to a binding mode supporting polymerization on the partially degraded template–primer. If the conversion is preceded by an obligatory dissociation of KF from the DNA, the enzyme would be quantitatively trapped by the challenger substrate and thus prevented from incorporating [^{32}P]dTMP residues into the homopolymer substrate (pathway a). If, however, the activity switch can proceed without an intervening E·DNA dissociation step, the enzyme would remain inaccessible to the challenger DNA trap and could thus catalyze polymerization on poly(dA)·oligo(dT) for the duration of a single processive cycle (pathway b).

This experiment was conducted in conjunction with two controls. In the first control, KF was incubated with poly(dA)·(dT)$_{16}$ in the absence of Mg^{2+} to form the E·DNA complex. Reaction with a mixture of Mg^{2+}·[α-^{32}P]dTTP and challenger DNA allows a single processive cycle of polymerization to occur to an extent determined by the concentration of E·poly(dA)·(dT)$_{16}$ (2, 3). The apparent processivity of polymerization thus measured was identical to that measured in single turnover experiments conducted at excess levels of poly(dA)·(dT)$_{16}$ relative to KF (11, 12), thereby demonstrating the effectiveness of the challenger DNA trap. In the second control, KF was incubated with a mixture of poly(dA)·(dT)$_{16}$ and challenger DNA in the presence of Mg^{2+}. The level of incorporation following addition of [α-^{32}P]dTTP represents the background polymerization on the mixture of DNAs. In the actual experiment, KF was incubated with

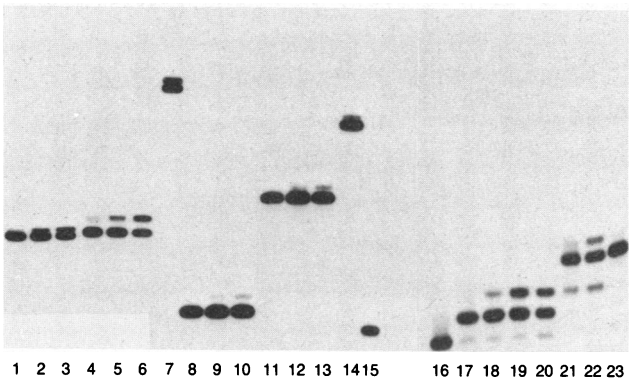


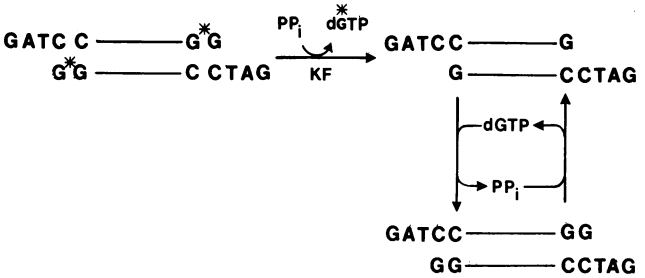
FIG. 3. Electrophoretic analysis of misincorporation. Lanes 1–7, polymerization products of a reaction containing 200 nM *Bam*HI-digested pBR322, 14 nM KF, 5 mM $MgCl_2$, and 10 μM [α-^{32}P]dGTP in 50 mM Tris·HCl (pH 7.4). Reactions were quenched after 2 (lanes 1 and 4), 10 (lanes 2 and 5), and 30 min (lanes 3, 6, and 7) and restricted with *Hae* III (lanes 1–6) or *Taq* I (lane 7). Lanes 8–14, polymerization products of a reaction containing 200 nM *Eco*RI-digested pBR322, 14 nM KF, 5 mM $MgCl_2$, and 10 μM [α-^{32}P]dATP in 50 mM Tris·HCl (pH 7.4). Reactions were quenched after 2 (lanes 8 and 11), 10 (lanes 9 and 12), and 30 min (lanes 10, 13, and 14) and restricted with *Hae* III (lanes 8–10), *Taq* I (lanes 11–13), or *Hin*dIII (lane 14). Lane 15, 5′-^{32}P-labeled p(dT)$_{16}$ standard. Products were applied to a 10% polyacrylamide/8 M urea gel and electrophoresed for 6 (lanes 1–3) or 3 hr (lanes 4–15). Lanes 16–19, polymerization products of a reaction containing 200 nM 19/16-mer duplex, 14 nM KF, 5 mM $MgCl_2$, 25 mM NaCl, and 10 μM dGTP in 50 mM Tris·HCl (pH 7.4). Reactions were quenched after 0 (lane 16), 2 (lane 17), 10 (lane 18), and 30 min (lane 19). Lanes 20–22, products after treatment of reaction mixture of lane 19 with either 0.5 mM PP_i for 10 min (lane 20), 10 μM dNTPs (N = A, G, T, C) for 90 min (lane 21), or 10 μM dNTPs (N = G, T, C) and 0.4 mM dATP for 90 min (lane 22). Lane 23, product of a reaction containing 200 nM 19/16-mer duplex, 14 nM KF, 5 mM $MgCl_2$, 25 mM NaCl, and 10 μM dNTPs (N = A, G, C, T) in 50 mM Tris·HCl (pH 7.4) quenched after 30 min. Lanes 16–23 contained *ca.* 5000 cpm of ^{32}P introduced as 5′-^{32}P-labeled 16-mer.

poly(dA)·(dT)$_{16}$ in the presence of Mg^{2+} to initiate the exonuclease activity followed by addition of [α-^{32}P]dTTP and challenger DNA to favor polymerization. At 30–45 sec, 7.1 pmol of dTTP was incorporated into the template–primer versus 6.0 pmol in the first control (both corrected for background). The results of these experiments suggest that, under the given reaction conditions, KF is capable of quantitative conversion from an exonuclease to a polymerase mode of action on a poly(dA)·oligo(dT) template–primer.† The reaction products were further analyzed by denaturing gel electrophoresis (Fig. 1). The effectiveness of the challenger DNA as an enzyme trap is demonstrated in lanes 1–6: neither hydrolysis (lane 2) nor polymerization products (lanes 3–6) of the p(dT)$_{16}$ primer were observed over the time course of the experiment. The ability of the enzyme to switch activity modes is illustrated in lanes 7–12: simultaneous addition of dTTP and challenger DNA to an exonuclease reaction mixture (lane 8) gave rise to a ladder of polymerization products characteristic of processive synthesis (lanes 9–12).

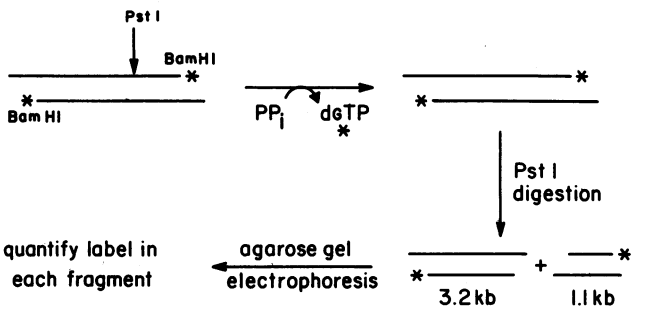
Sequence Dependence of Pyrophosphorolysis. Previous studies demonstrated that the accumulation of PP_i during idling-turnover results in a partitioning of the excision pathway into hydrolysis and pyrophosphorolysis of the 3′-

†Direct comparison of the level of incorporation observed in the switch experiment with that observed in the first control assumes that the concentration of E·poly(dA)·(dT)$_{16}$ available for processive polymerization is not significantly dependent upon Mg^{2+}, since the binary complex is formed in the presence of Mg^{2+} in only the former case. The marginally higher level of incorporation observed in the switch experiment (*ca.* 15%) may indeed reflect a slight Mg^{2+}-dependent lowering of the E·poly(dA)·(dT)$_{16}$ dissociation constant.

terminal residue of the primer strand (7). As part of an effort to define the kinetics of the idling-turnover process, a study of the rate of KF-catalyzed pyrophosphorolysis on 3′-end-labeled *Bam*HI-digested pBR322 was undertaken. The rate of pyrophosphorolysis was measured by monitoring the accumulation of [α-^{32}P]dGTP in an unlabeled dGTP pool, as outlined in Scheme II (7).



The reaction time course was found to be biphasic, with the rapid initial phase resulting in the loss of approximately one-half of the total end-label from the DNA. In order to establish whether the observed biphasicity is a consequence of differential pyrophosphorolysis kinetics at the two 3′ ends of the linear DNA substrate, the reactions at each end were independently monitored according to the protocol of Scheme III.



The resolved time courses thus obtained are illustrated in Fig. 2 and clearly demonstrate that 3′-end-labeled *Bam*HI-digested pBR322 behaves as a mixture of two kinetically distinct substrates in the pyrophosphorolysis reaction.

Evidence for Misincorporation/Excision During Idling-Turnover. The terminal residue of 3′-end-labeled *Eco*RI- or *Bam*HI-digested pBR322 is not protected from excision during idling-turnover, suggesting that the reaction involves an excision/incorporation cycle rather than one of misincorporation/excision (7). In order to directly investigate the possible involvement of the latter pathway, the products of the reactions of *Bam*HI- and *Eco*RI-digested pBR322 with [α-^{32}P]dGTP and [α-^{32}P]dATP, respectively, were subjected to a gel electrophoretic analysis. To allow the single-base resolution that is required to monitor misincorporation (13), the reaction products were first restriction-digested, and the 3′-end-labeled fragments thus formed were resolved by standard strand-separating gel electrophoretic methodology (9). The results of these analyses are summarized in the autoradiograph shown in Fig. 3. The two labeled 3′ ends formed by the reaction of *Bam*HI-digested pBR322 with [α-^{32}P]dGTP under idling-turnover conditions were analyzed following *Hae* III or *Taq* I digestion (lanes 1–7). Normal single end-labeling at each 3′ end generates labeled fragments of 79 and 23 base pairs (bp) (*Hae* III) or 37 and 274 bp (*Taq* I) (9). A significant degree of misincorporation is clearly illustrated by the time-dependent appearance of labeled oligonucleotides of length 80 (lanes 1–3), 24 (lanes 4–6), and 38 (lane 7). The two labeled 3′ ends formed by the reaction of *Eco*RI-digested pBR322 with [α-^{32}P]dATP under idling-turnover conditions were analogously analyzed following *Hae* III, *Taq* I, or *Hin*dIII digestion. Normal double end-labeling at each terminus generates fragments of size 18 and 174 (*Hae* III), 344 and 26 (*Taq* I), or 4333 and 33 bp (*Hin*dIII) (9). As illustrated in lanes 8–14, misincorporation at the *Eco*RI-derived sequence occurs at a much lower frequency than at the *Bam*HI-derived sequence.

An analogous gel electrophoretic analysis of misincorporation during idling-turnover was carried out by using a synthetic DNA template–primer (*Materials*), the sequence of which corresponds to that of one of the termini of *Bam*HI-digested pBR322. The products of the reaction of this substrate with dGTP under standard idling-turnover conditions are illustrated in Fig. 3. The extensive time-dependent misincorporation that is observed (lanes 17–19) is in qualitative agreement with that observed at the same terminal sequence of the *Bam*HI-digested pBR322 substrate (lanes 4–6). The gel electrophoretic assay was further used to demonstrate that a mismatched 3′ primer terminus generated during idling-turnover is not subject to rapid excision by pyrophosphorolysis in the presence of 0.5 mM PP_i (lane 20). Furthermore, negligible polymerization beyond the mismatched terminus is observed upon addition of an equimolar mixture of all four dNTPs (10 μM each, lane 21). However, the mismatched terminus can support polymerization provided a large molar excess of the following correct dNTP is available, as illustrated by the appearance of two extended primers in lane 22. Finally, the extensive misincorporation that occurs under the constraints of the idling-turnover reaction (e.g., lanes 17–19) is totally suppressed by the inclusion of the following correct dNTP(s) that are required for normal polymerization (lane 23).

DISCUSSION

The present study was undertaken with the aim of elucidating mechanistic details of the KF-catalyzed idling-turnover reaction. Our earlier studies suggested that the idling-turnover reaction on DNA substrates such as *Eco*RI- or *Bam*HI-digested pBR322 involves an alternating cycle of excision/incorporation (7). An initial exonuclease/polymerase cycle results in the accumulation of PP_i and the subsequent participation of a parallel pyrophosphorolysis/incorporation cycle. In view of the uncertainty in the location of the polymerase and exonuclease active sites with respect to one another,‡ isotope-trapping experiments were carried out to investigate whether KF is capable of converting from an exonuclease to a polymerase mode of action within a single binding event to its DNA substrate. That switch is achieved by T5 DNA polymerase (11). The results clearly demonstrate that such an activity conversion can indeed occur while the enzyme remains bound to a poly(dA)·oligo(dT) template–primer.§ This observation thus suggests that the first two steps of the idling-turnover reaction—namely, hydrolysis followed by polymerization—may occur within a single E·DNA association event. Consequently, the DNA within the E·DNA complex appears to equilibrate between sites responsible for exonuclease and polymerase activity, if indeed the sites prove to be separate.

‡Kornberg (1) has proposed a model in which the active sites are coincident, whereas the structural data of Steitz and co-workers (6) have been tentatively interpreted in terms of distinct exonuclease and polymerase active sites.

§Relatively high background incorporation problems precluded the use of either *Eco*RI- or *Bam*HI-digested pBR322 in the exonuclease → polymerase switch experiments.

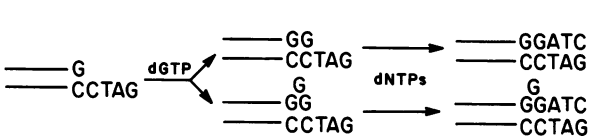
Kinetic analysis of the pyrophosphorolysis/incorporation cycle of the idling-turnover reaction on 3'-end-labeled *Bam*HI-digested pBR322 revealed that the two 3' ends of this DNA behave as kinetically distinct substrates for pyrophosphorolysis. The 3' termini and the corresponding upstream sequences of the kinetically slow and fast substrates are illustrated below (**1** and **2**, respectively):

```
5' ... CACACCCGTCCTGTG*G 3'   5' ... GCGTCCGGCGTAGAG*G 3'
   ... GTGTGGGCAGGACAC CTAG      ... CGCAGGCCGCATCTC CTAG
```

1 **2**

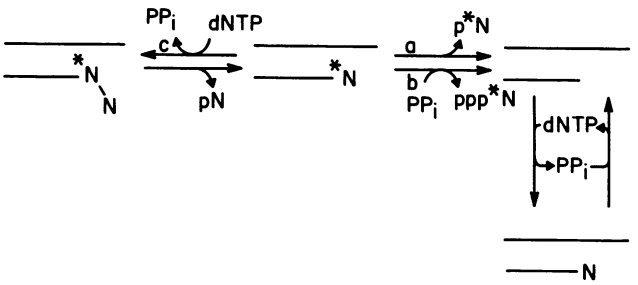
This result thus offers direct evidence for a dependence of binding and/or catalysis upon the DNA sequence upstream of the 3' terminus. Studies aimed at separating the sequence dependencies of binding and catalysis are necessary.

Our previous studies had failed to provide evidence for an alternative misincorporation/excision pathway (7). In an attempt to directly monitor the occurrence of misincorporation events during idling-turnover, a gel electrophoretic analysis of the reaction products was carried out. Using this technique, the formation of misincorporation products during idling-turnover was directly observed. Comparative studies using two DNA substrates of differing 3'-terminal sequence suggest that the degree of misincorporation is dependent upon the specific nature of the mismatch. The observation of a much lower frequency of misincorporation at the 3' end of *Eco*RI-digested pBR322 (A-A mismatch) than at the 3' end of *Bam*HI-digested pBR322 (G-T mismatch) is consistent with the lower frequency of purine–purine than purine–pyrimidine mismatching (14, 15). This observation also rules against attributing the high G-T misinsertion frequency to the short length of the template overhang. However, by analogy to the recent proposal of Kunkel and Alexander (16), based on comparative fidelity studies of the eukaryotic polymerases α, β, and γ, the overall misincorporation frequency may depend upon a distinct site substitution preference of the polymerase itself, in addition to the intrinsic DNA structural properties. The observed inability of KF to excise 3'-terminal misincorporated nucleotide residues by pyrophosphorolytic cleavage is consistent with the strict base-pairing specificity of this reaction (17) and hence argues against the involvement of pyrophosporolysis in an error–correction proofreading capacity. Mismatches generated under the constraints of idling-turnover [i.e., in the absence of the following correct dNTP(s)] may become stably incorporated into the DNA upon addition of a dNTP pool that is heavily biased in favor of the following correct nucleotide. This reaction provides a possible mechanism for the formation of a frameshift mutation, as illustrated in Scheme **IV**.



It should be emphasized that the illustrated sequence is assumed from the conditions necessary for incorporation.

In light of the results of this study, an extended mechanistic scheme for the idling-turnover reaction is presented (Scheme **V**).



Reaction via pathway a involves consecutive hydrolysis and polymerization steps that may occur within a single association event between the enzyme and the DNA substrate. Competitive with this cycle is a misincorporation/hydrolysis cycle (pathway c) in which alternating incorporation and hydrolysis of an incorrectly base-paired residue occurs at the 3' primer terminus. The mismatched 3' end is a very poor substrate for further polymerization. The mismatch is not readily excisable by pyrophosphorolysis and accumulates despite the 3' → 5' exonuclease activity. The extent of reaction via pathway c is determined by the 3'-terminal sequence and the frequency of the particular misinsertion. The accumulation of PP_i formed via pathways a and c provides an alternative excision/incorporation route (pathway b) involving alternating pyrophosphorolysis and polymerization steps. The kinetics of the pyrophosphorolysis reaction are strongly influenced by the sequence of the duplex region of the DNA upstream of the 3' end.

The assistance of Dwayne Allen is most gratefully acknowledged. This work was supported by the National Institutes of Health Grant 2 R01 GM13306-21.

1. Kornberg, A. (1980) *DNA Replication* (Freeman, San Francisco).
2. Bryant, F. R., Johnson, K. A. & Benkovic, S. J. (1983) *Biochemistry* **22**, 3537–3546.
3. Mizrahi, V., Henrie, R. N., Marlier, J., Johnson, K. A. & Benkovic, S. J. (1985) *Biochemistry* **24**, 4010–4018.
4. Burgers, P. M. J. & Eckstein, F. (1979) *J. Biol. Chem.* **254**, 6889–6893.
5. Gupta, A. F. & Benkovic, S. J. (1985) *Biochemistry* **23**, 5874–5881.
6. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G. & Steitz, T. A. (1985) *Nature (London)* **313**, 762–766.
7. Mizrahi, V., Benkovic, P. A. & Benkovic, S. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 231–235.
8. Joyce, C. M. & Grindley, N. D. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1830–1834.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Brutlag, D. & Kornberg, A. (1972) *J. Biol. Chem.* **247**, 241–248.
11. Das, S. K. & Fujimura, R. K. (1980) *J. Biol. Chem.* **255**, 7149–7154.
12. Newport, J. W., Kowalczykowski, S. C., Lonberg, N., Paul, L. S. & von Hippel, P. H. (1980) *Mechanistic Studies of DNA Replication and Genetic Recombination* (Academic, New York), pp. 485–505.
13. Hillebrand, G. G., McCluskey, A. H., Abbott, K. A., Revich, G. G. & Beattie, K. L. (1984) *Nucleic Acids Res.* **12**, 3155–3171.
14. Saenger, W. (1984) *Principles of Nucleic Acid Structure* (Springer, New York).
15. Fersht, A. R., Shi, J.-P. & Tsui, W.-C. (1983) *J. Mol. Biol.* **165**, 655–667.
16. Kunkel, T. A. & Alexander, P. S. (1986) *J. Biol. Chem.* **261**, 160–166.
17. Deutscher, M. P. & Kornberg, A. (1969) *J. Biol. Chem.* **244**, 3019–3028.