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Biochemical Characterization of the Herpes Simplex Virus-1 DNA Polymerase

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We have investigated the biochemical properties of the herpes simplex virus type 1 (HSV-1) DNA polymerase without the UL42 protein (Pol), purified from insect cells infected with a recombinant baculovirus containing the UL30 gene. BSA and DTT have inhibitory effects on dAMP incorporation. Pol showed a greater turnover rate of steady-state single nucleotide incorporation at 12 mM $MgCl_2$ than at 2 mM $MgCl_2$. However, it showed a greater processivity of DNA synthesis at lower $MgCl_2$ concentration (1 mM, 2 mM) than at a higher $MgCl_2$ concentration (12.5 mM). These results are consistent with a slow DNA dissociation at lower $MgCl_2$ concentrations. Pol does not incorporate a correct nucleotide into the primer with an incorrect nucleotide at the end; instead, it preferentially excises the incorrect nucleotide at the 3′ end of the primer. Pol has DNA polymerase activity at pHs 6.5 and 7.5 but little at pHs 5.5, 8.5, and 9.5. It has exonuclease activity at pHs 6.5, 7.5, and 8.5 but little at pHs 4.5, 5.5, and 9.5. The finding that Pol has exonuclease activity but not DNA polymerase at pH 8.5 suggests that DNA binds to Pol, but deoxynucleotide binding or incorporation does not occur at pH 8.5.

Keywords: Exonuclease, HSV-1 Pol, Polymerase activity.

Introduction

Herepes simplex virus type 1 (HSV-1) is a double-stranded DNA virus with a genome length of 153 kb (Roizman and Batterson, 1985; McGeoch *et al.*, 1988). The HSV-1 genome contains the seven open reading frames that are necessary and sufficient for the replication of plasmids containing a viral replication origin, either ori_L or ori_S (Wu *et al.*, 1988). The products of these seven genes are an origin binding protein, the product of the UL9 gene (Elias *et al.*, 1986; Olivo *et al.*, 1988), a two-subunit DNA polymerase composed of a catalytic core (UL30 product), and a processivity factor (UL42 product) (Crute and Lehman, 1989; Hernandez and Lehman, 1990; Purifoy *et al.*, 1977; Song and Lehman, 1998, a helicase-primase composed of UL5, UL8, and UL52 gene products (Crute *et al.*, 1988; 1989), and a single-stranded DNA binding protein (ICP8 or UL29 gene product) (Weller *et al.*, 1983). The UL30 gene product, a DNA polymerase, has a proofreading 3′–5′ exonuclease as well as 5′–3′ exonuclease that can function as RNase H (Crute and Lehman, 1989). The DNA polymerase interacts with the UL8 protein of the heterotrimeric helicase-primase (Mardsen *et al.*, 1997) . The mutations in the Exo III motif of the polymerase incorporated into the viral genome produced increased mutation frequencies (Hwang *et al.*, 1997). The UL42 protein has been shown to interact with the origin-binding protein (UL9 protein) (Monahan *et al.*, 1998) .

We report here the expression of HSV-1 DNA polymerase alone (Pol) in the baculovirus-Sf9 cell system and its characterization. For steady-state incorporation of a single nucleotide, Pol showed a greater activity at a higher $MgCl_2$ concentration, but in the presence of all four

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Abbreviations: Pol, HSV-1 DNA polymerase without the UL42 protein; HEPES, *N*-[2-hydroxyethyl]piperazine-N′-[ethanesulfonic acid]; Bis-Tris, bis[2-hydroxyethyl]iminotris-[hydroxymethyl]methane; CHES, 2-[*N*-cyclohexylamino]-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ACTD, activated calf thymus DNA; DTT, dithiothreitol; BSA, bovine seum albumin.

nucleotides, it showed a greater processivity of DNA synthesis at lower $MgCl_2$ concentration. The pH dependence showed that at pH 8.5 there is a considerable exonuclease, but little DNA polymerase, activity.

Materials and Methods

Enzyme The herpes simplex virus type 1 DNA polymerase without the UL42 protein (Pol) was purified from insect cells (Sf9) infected with recombinant baculovirus containing the UL30 gene as described (Hernandez and Lehman, 1990; Song and Lehman, 1998). The protein was >95% pure, as judged by a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel.

Its concentration was determined by active site titration as previously described (Song and Lehman, 1998), with saturating DNA substrate (30/50 mer) using a rapid quench-flow apparatus (KinTek Instruments). The reaction was initiated by mixing Pol [~ 5 nM estimated by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard] with a solution of 50 nM 5′-^{32}P labeled 30/50 mer and 100 μM Mg^{2+}•dATP. The reaction was stopped with 0.5 M EDTA, pH 8.0, at variable times (10 ms–60 s). The burst size was taken as the enzyme concentration.

DNA substrate The oligonucleotides shown in Table 1 were purchased from Oligos Etc. (Wilsonville, USA) and prepared as previously described (Song and Lehman, 1998).

Methods Unless otherwise indicated, experiments were performed with the use of a rapid-quench flow apparatus, RQF3 (KinTek Instruments) in 40 mM HEPES (pH 7.5) containing 150 mM KCl, 12 mM $MgCl_2$, and 2.5% glycerol at 23°C. The reaction was stopped with 0.5 M EDTA, pH 8.0. The reaction products were electrophoresed through 15% polyacrylamide containing 7 M urea and the gel was either exposed to X-ray film (X-OMAT AR 5; Kodak) or analyzed with use of a PhosphorImager (Molecular Dynamics).

Factors affecting dAMP incorporation The effects of buffer, EDTA, DTT, BSA, salt, and concentration of $MgCl_2$ were tested under the following condition. Reaction mixture (20 μl) containing 0.35 μM Pol, 1.8 μM 5′-^{32}P labeled 30/50 mer, and 200 μM Mg^{2+}•dATP in 1× buffer of each indicated in Fig. 1 was incubated for 2 min at 23°C. The reaction was stopped with 20 μl of 0.5 M EDTA, pH 8.0. The quenched reaction mixture was electrophoresed through 15% polyacrylamide gel containing 7 M urea. Following electrophoresis, the gel was dried on DE81 paper (Whatman) and exposed to X-ray film.

Table 1. The sequence of oligonucleotide substrates.

30/50mer	5′-GCCTCGCAGCCGTCCAACCAACTCTACCCT-3′ 3′-CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC-5′
29A/50mer	5′-GCCTCGCAGCCGTCCAACCAACTCTACCC^{A}-3′ 3′-CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC-5′
29C/50mer	5′-GCCTCGCAGCCGTCCAACCAACTCTACCC^{C}-3′ 3′-CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC-5′
29G/50mer	5′-GCCTCGCAGCCGTCCAACCAACTCTACCC^{G}-3′ 3′-CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC-5′

Dependence of DNA synthesis on the number of nucleotides and $MgCl_2$ The reaction was initiated by mixing a solution of Pol (5 nM) and 5′-^{32}P labeled 30/50 mer (1 nM) with a solution of 0.26 mg/ml ACTD, 12 mM $MgCl_2$, dNTP (100 μM each, added nucleotide indicated in Fig. 2A), and stopped with EDTA at variable times (5 ms–2 s). For $MgCl_2$ dependence, the reaction was initiated by mixing a solution of Pol (5 nM) and 5′-^{32}P labeled 30/50 mer (1 nM) with a solution of 0.26 mg/ml ACTD, $MgCl_2$ (concentrations indicated in Fig. 2B), dNTP (100 μM each) and stopped with EDTA at variable times (5 ms–2 s). ACTD was added to prevent elongation of free 5′-^{32}P labeled 30/50 mer and intermediate products. Following electrophoresis, the gel was exposed to X-ray film.

The effect of 3′-end mismatch on correct nucleotide incorporation The correct nucleotide at the 3′-end of the primer is dT for 30/50 mer and the next correct nucleotide to be incorporated is dA. Correct nucleotide incorporation followed an incorrect nucleotide at the 3′-end of the primers, dA (29A/50 mer), dC (29C/50 mer), or dG (29G/50 mer). The reaction was initiated by mixing a solution of 4 nM Pol and 1 nM 5′-^{32}P labeled DNA substrate with 200 μM Mg^{2+}•dATP, and stopped with EDTA at variable times (5 ms–480 ms).

pH dependence of dAMP incorporation Incorporation of dAMP was tested at pH 5.5 (potassium acetate), 6.5 (Bis-Tris, Sigma), 7.5 (HEPES), 8.5 (Tris), and 9.5 (CHES, Sigma). The reaction was initiated by mixing a solution of 5 nM Pol and 1 nM 5′-^{32}P labeled 30/50 mer with a solution of 0.5 μM Mg^{2+}•dATP, and stopped at variable times (5 ms–2 s) with EDTA. After gel electrophoresis through 15% polyacrylamide with 7 M urea, products were quantitated with use of a Phosphorimager. The kinetic constants were obtained by nonlinear squares fits to the data (KaleidaGraph, version 3.0.1; Synergy Software).

pH dependence of exonuclease The pH dependence of exonuclease was tested with a DNA substrate (29C/50 mer) containing an incorrect nucleotide (dC) at the 3′-end of the primer. The reaction was initiated by mixing a solution (20 μl) of 1 μM Pol and 0:8 μM 5′-^{32}P labeled DNA substrate with 12 mM $MgCl_2$ (20 μl). The buffers used were sodium acetate (pH 4.5 and 5.4), PIPES (pH 6.5), HEPES (pH7.5), Tris (pH 8.8), and CHES (pH 9.5).

Formaldehyde with electrophoresis dyes (60 μl) was added to stop the reaction at 0.5 and 5 min after mixing, and the mixture was vortexed. After electrophoresis, products were analyzed with use of a PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

Result

Factors affecting dAMP incorporation The effects of DTT, BSA, salt, salt concentration, and $MgCl_2$ concentration on single nucleotide incorporation are summarized in Fig. 1. DTT and BSA showed inhibitory effects at 12.5 mM $MgCl_2$ (lanes 1, 2, 3), but at low $MgCl_2$

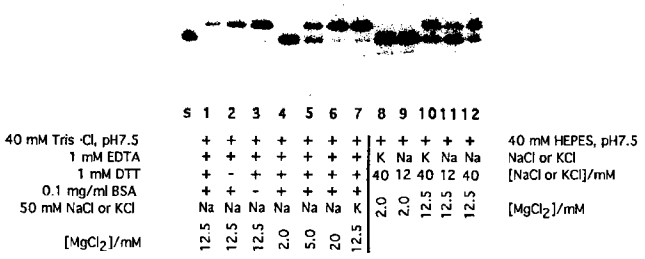

Fig. 1. Condition dependence of single nucleotide incorporation. The reaction mixture (20 μl) containing 0.35 μM Pol, 1.8 μM 5′-labeled 30/50 mer, and 200 μM Mg^{2+}•dATP was incubated for 2 min at 23°C. The reaction was stopped with EDTA. The products were electrophoresed through a 15% denaturing polyacrylamide gel. The s represents substrate (30 mer).

concentration their effects were not detected due to a low incorporation activity (lanes 4, 8, 9). Incorporation activity increased with increasing $MgCl_2$ concentration from 2 to 12.5 mM but activity at 20 mM $MgCl_2$ was similar to that at 12.5 mM $MgCl_2$ (lanes 4–7). Activity at 40 mM NaCl was greater than that at 12 mM NaCl (lanes 11, 12). Salt species showed little effect (lanes 1, 7, 10, 12).

Dependence of processive DNA synthesis on the number of nucleotide species and $MgCl_2$ Since the sequence of DNA template (30/50 mer) is defined, extension of the primer can be controlled by the number of nucleotides, as shown in Fig 2A. In the presence of dATP and dCTP, the primer was extended to 33/50 mer though the expected extension product based on the sequence being 32/50 mer. In the presence of dATP, dCTP, and dTTP, the expected 47/50 mer was synthesized (see the arrow in Fig. 2A).

The effect of $MgCl_2$ on processive DNA synthesis in the presence of four dNTPs is shown in Fig. 2B. The products were formed from single binding since multiple initiation was prevented by added ACTD. The larger amount of full-length product was observed at lower $MgCl_2$ concentrations (1, 2 mM). Strong stop sites were observed at the 6th and 17th nucleotide positions.

Incorporation into the DNA template with a mismatch at the 3′-end In order to test the possibility that Pol incorporates a correct nucleotide into a DNA template with a mismatch at the 3′-end of the primer, the nucleotide (dT) at the 3′-end of the 30/50 mer primer was replaced with dA, dC, or dG, with the mutated templates being 29A/50 mer, 29C/50 mer, and 29G/50 mer, respectively. Pol incorporated little dAMP into the 29A/50 mer, 29C/50 mer or 29G/50 mer products whereas it incorpoated dAMP into the standard 30/50 mer substrate (Fig. 3). There was a small increase in 29 mer for the mutated substrates that could be due to excision of the mismatch at the 3′-end of the primer by exonuclease.

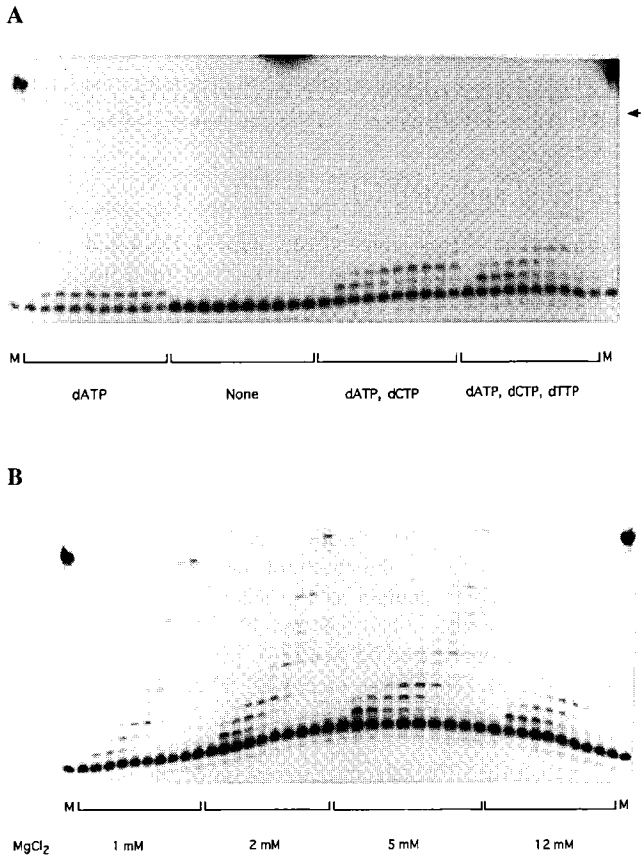

Fig. 2. The effect of number of nucleotide species and $MgCl_2$ on DNA synthesis. The reaction was initiated by mixing a solution of 5 nM Pol and 1 nM 5′-labeled 30/50 mer with a solution of ACTD, 100 μM of each dNTP and $MgCl_2$, and was stopped with EDTA. The products were electrophoresed through a 15% denaturing polyacrylamide gel. Each set of ten lanes represents different time points, 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 s. M, markers of 30 mer and 50 mer. (**A**) $MgCl_2$, 12 mM; (**B**) $MgCl_2$, 1, 2, 5, 12 mM.

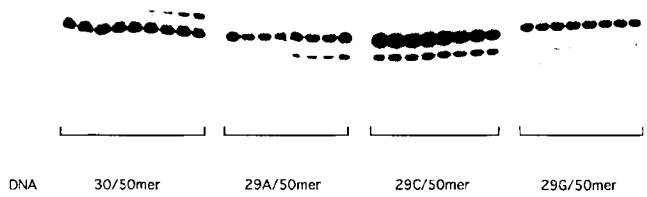

Fig. 3. The effect of 3′-end mismatch on incorporation. DNA substrates with a correct nucleotide, dT (30/50 mer) or an incorrect nucleotide, dA (29A/50 mer), dC (29C/50 mer), dG (29G/50 mer) were examined for incorporation of correct nucleotide (dAMP). The reaction was initiated by mixing a solution of 4 nM Pol and 1 nM 5′-^{32}P labeled DNA substrate with 200 μM Mg^{2+}•dATP, and stopped with EDTA at variable times (0, 5, 20, 60, 120, 180, 240, 480 ms).

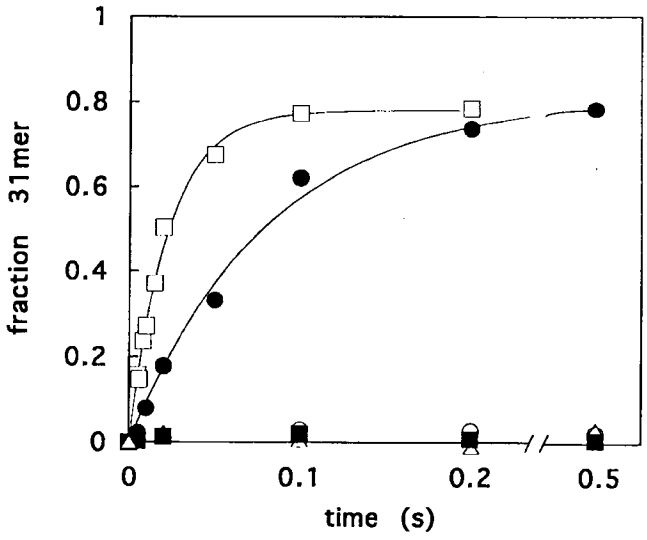

Fig. 4. pH dependence of dAMP incorporation. The reaction was initiated by mixing a solution of 5 nM Pol and 1 nM 5′-^{32}P labeled 30/50 mer with 0.5 μM Mg^{2+}•dATP, and stopped with EDTA at variable times (5 ms–0.5 s). The observed rate constants are 44 s^{-1} at pH 7.5 (□), 12 s^{-1} at pH6.5 (●), and 0 s^{-1} at pHs 5.5 (○), 8.5 (■) and 9.5 (△).

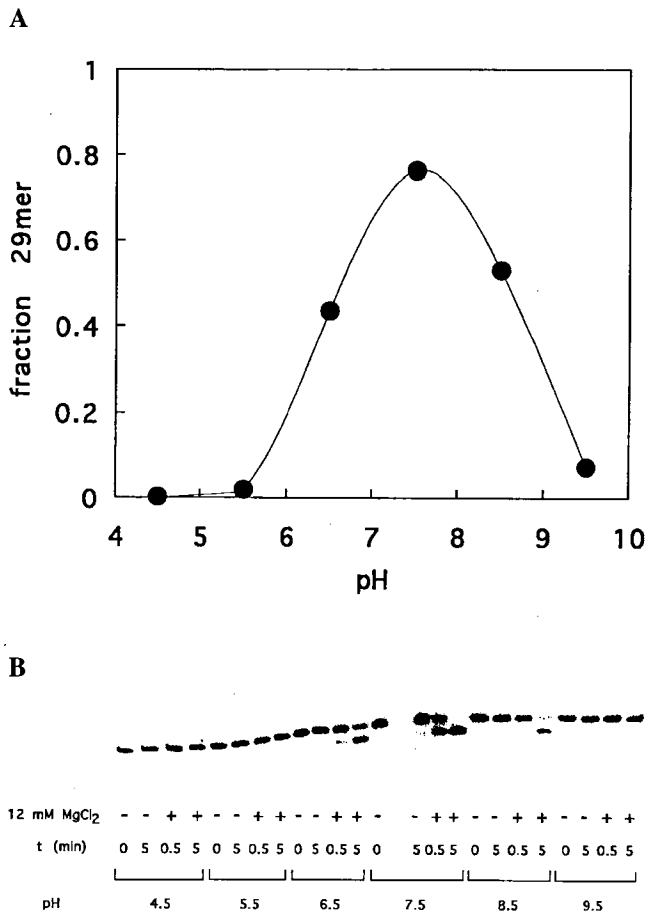

Fig. 5. pH dependence of exonuclease. DNA substrate with a incorrect nucleotide at the 3′-end of the primer (29C/50 mer) was used. The reaction was initiated by mixing a solution (20 μl) of 1 μM Pol and 0.8 μM 5′-^{32}P labeled 29C/50 mer with 12 mM $MgCl_2$ at indicated pHs, and stopped at 0.5 and 5 min with formaldehyde-containing dyes (60 μl). The reaction products were electrophoresed through a 15% denaturing polyacrylamide gel, and the gel was dried and autoradiographed (**B**). The fraction of product (29 mer) at 5 min was plotted against pH (**A**).

pH dependence of dAMP incorporation and exonuclease The pH dependence of dAMP incorporation was examined in the presence of subsaturating dATP concentration (0.5 μM) at pHs 5.5, 6.5, 7.5, 8.5, and 9.5 (Fig. 4). No incorporation was detected at pHs 5.5, 8.5, and 9.5, while at pHs 6.5 and 7.5 dAMP was incorporated at rates of 12 s^{-1} and 44 s^{-1}, respectively.

The pH dependence of exonuclease showed a bell-shaped curve with a maximum activity at pH 7.5 (Fig 5A). Little exonuclease activity was observed at pHs 4.5 and 5.5 and there was a small activity at pH 9.5. There was a large difference between incorporation and exonuclease activities at pH 8.5. While considerable exonuclease activity was observed, little DNA polymerase activity was detected at this pH (Fig. 4). After one nucleotide excision, little further excision products were detected (Fig 5B). Small amounts of exonuclease activity was detected in the absence of $MgCl_2$.

Discussion

We have investigated the factors affecting DNA polymerase activity, the effect of an incorrect 3′-end nucleotide on incorporation, and the pH dependence of DNA polymerase and exonuclease activities on HSV-1 DNA polymerase without the UL42 protein.

For single nucleotide incorporation, DNA polymerase activity was greater at 12.5 or 20 mM $MgCl_2$ than at a lower concentration of $MgCl_2$ (2 mM, Fig. 1). Since this was done under a steady-state condition, the amount of the product is controlled by the rate-limiting step of the reaction. One possibility is that the incorporation is the rate-limiting step and DNA polymerase has a greater intrinsic incorporation activity at lower $MgCl_2$ concentrations. Another possibility is that DNA dissociation is the rate-limiting step and DNA dissociation from the enzyme is faster at higher $MgCl_2$ concentrations. At lower $MgCl_2$ concentrations, the incorporation product (31/50 mer) dissociated slowly, such that it took more time for the enzyme to bind new DNA substrate. That is, a low $MgCl_2$ concentration reduces the turnover rate of steady-state single nucleotide incorporation by slowing down the rate of DNA dissociation. At high $MgCl_2$ concentrations, the rate-limiting DNA dissociation is faster and the turnover rate is larger. This is consistent with the result of $MgCl_2$ concentration effect on the processivity of DNA synthesis.

In the presence of four deoxynucleotides, Pol showed greater processivity at lower concentrations of $MgCl_2$ (1 mM, 2 mM) than at a higher concentration (12.5 mM), suggesting that more nucleotides are incorporated at lower $MgCl_2$ concentrations in the presence of all four nucleotides (Fig. 2). At lower $MgCl_2$ concentrations, the template is probably limited. Greater processivity at a low $MgCl_2$ concentration was observed for DNA polymerases α and δ from calf thymus (Hohn and Grosse, 1987; Sabatino and others, 1988). This result is consistent with slower DNA dissociation at the low $MgCl_2$ concentrations discussed in the previous section. The enzyme stays longer at low $MgCl_2$ concentrations and has more time to incorporate nucleotides. Direct measurement of the rate of DNA dissociation at various $MgCl_2$ concentrations is currently underway.

HSV-1 DNA polymerase does not incorporate a correct nucleotide after an incorrect nucleotide at the 3′ terminus of the primer. Exonuclease preferentially excises the incorrect nucleotide at the 3′-end, showing little activity towards the correct nucleotide at the 3′-end of the primer, because cleavage stopped after excision of the incorrect nucleotide (Fig. 5B).

HSV-1 DNA polymerase had incorporation activity at pHs 6.5 and 7.5 but showed little at other pHs tested (pHs 5.5, 8.5, 9.5). However, exonuclease showed a moderate activity at pH 8.5 as well as pHs 6.5 and 7.5. The difference between the two activities at pH 8.5 suggests that DNA binds to the enzyme at this pH, but there is no incorporation. The absence of DNA polymerase activity at pH 8.5 could be due to reasons other than DNA binding, for example, a lack of deoxynucleotide binding or a lack of incorporation activity at pH 8.5.

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