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Repair of DNA strand gaps and nicks containing 3′-phosphate and 5′-hydroxyl termini by purified mammalian enzymes

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ABSTRACT

A putative role for mammalian polynucleotide kinases that possess both 5′-phosphotransferase and 3′-phosphatase activity is the restoration of DNA strand breaks with 5′-hydroxyl termini or 3′-phosphate termini, or both, to a form that supports the subsequent action of DNA repair polymerases and DNA ligases, i.e. 5′-phosphate and 3′-hydroxyl termini. To further assess this possibility, we compared the activity of the 3′-phosphatase of purified calf thymus polynucleotide kinase towards a variety of substrates. The rate of removal of 3′-phosphate groups from nicked or short (1 nt) gapped sites in double-stranded DNA was observed to be similar to that of 3′-phosphate groups from single-stranded substrates. Thus this activity of polynucleotide kinase does not appear to be influenced by steric accessibility of the phosphate group. We subsequently demonstrated that the concerted reactions of polynucleotide kinase and purified human DNA ligase I could efficiently repair DNA nicks possessing 3′-phosphate and 5′-hydroxyl termini, and similarly the combination of these two enzymes together with purified rat DNA polymerase β could seal a strand break with a 1 nt gap. With a substrate containing a nick bounded by 3′- and 5′-OH termini, the rate of gap filling by polymerase β was significantly enhanced in the presence of polynucleotide kinase and ATP, indicating the positive influence of 5′-phosphorylation. The reaction was further enhanced by addition of DNA ligase I to the reaction mixture. This is due, at least in part, to an enhancement by DNA ligase I of the rate of 5′-phosphorylation catalyzed by polynucleotide kinase.

INTRODUCTION

Scission of the DNA sugar–phosphate backbone is a common occurrence that can arise from regular enzyme activity or as the result of damage and/or repair induced by a broad range of genotoxic agents. However, the term ‘strand break’ covers an array of diverse chemical structures. Aside from the simple biophysical differences of single- and double-strand breaks, there are many chemically distinct end groups found at strand-break termini. Nonetheless, all 3′-termini have to be restored to hydroxyl groups, and 5′-termini to phosphate groups, in order to allow DNA polymerases and ligases to catalyze repair synthesis and strand rejoining.

Among the more frequently encountered terminal modifications that would require processing prior to the actions of DNA polymerases and ligases are 3′-phosphate groups and 5′-hydroxyl groups. The former, together with 3′-phosphoglycolate groups, constitute the major strand-break modifications produced by ionizing radiation (1). Such strand breaks are usually accompanied by the loss of 1 nucleoside. Ionizing radiation and certain antineoplastic alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), also produce 5′-hydroxyl termini (2,3). Incision of DNA by DNase II, an enzyme recently implicated in apoptosis and lens cell differentiation (4–6), generates nicks with 3′-phosphate and 5′-hydroxyl termini (7). Similarly, eukaryotic topoisomerase I incision, in the presence of specific inhibitors like camptothecin and after enzymatic removal of the dead-end covalent complex, produces nicks with this configuration of termini (8).

It has been suggested several times, most recently by Yang *et al.* (8) and Nitiss and Wang, (9) that the process of restoring the integrity of the DNA at such sites would require the action of an enzyme or enzymes that phosphorylates 5′-hydroxyl termini and removes 3′-phosphate groups. One enzyme that has the capacity to phosphorylate 5′-termini is polynucleotide kinase (PNK). Several PNK enzymes have been purified from a variety of different mammalian tissues (10–17). Those that have an acidic pH optimum for 5′-phosphorylation, also appear to possess a 3′-phosphatase activity (17–19). Indeed when Pheiffer and Zimmerman (18) and Habraken and Verly (19) purified 3′-phosphatase activity from rat liver nuclei, the major enzyme activity copurified with polynucleotide kinase. Although the major mammalian apurinic/apyrimidinic endonuclease can also remove 3′-phosphate termini (20,21), this enzyme displays a markedly

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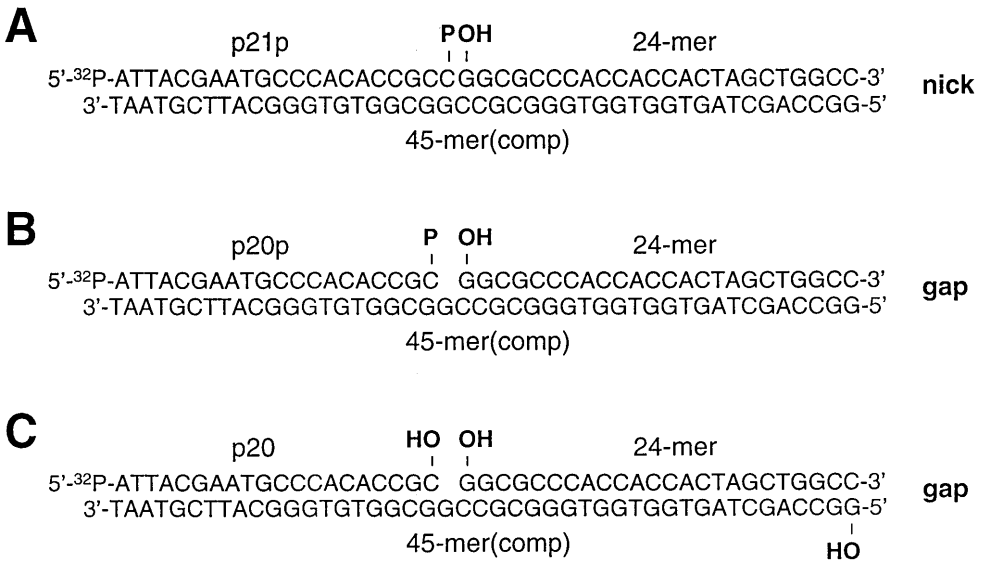

Figure 1. Composition of oligonucleotides and model substrates. Model duplexes were prepared as described in Materials and Methods by annealing three oligonucleotides to generate either a nick (duplex A) or a single-nucleotide gap (duplexes B and C). The pertinent termini for this study are indicated above or below their respective oligonucleotides. Here and in the text p20p and p21p refer to the 20mer and 21mer oligonucleotides with 3′- and 5′-phosphate termini. The 5′-phosphate is radiolabelled. 45mer(comp) refers to the complementary 45mer as opposed to p45 used in the text and other figures to indicate the product of ligation of the 21mer and 24mer oligonucleotides.

higher activity towards internal abasic sites than towards terminal groups (20,22). There is thus an additional incentive to examine the activities of alternative 3′-phosphatases.

We have recently shown that polynucleotide kinase, isolated from calf thymus, is capable of efficiently phosphorylating sterically hindered 5′-OH termini at single-strand breaks in DNA duplexes (17). Here we report an extension of these studies to examine the 3′-phosphatase activity of PNK and the use of this enzyme in conjunction with purified mammalian DNA polymerase β and DNA ligase I to repair nicks and gaps in duplex DNA with 5′-OH and 3′-phosphate termini.

MATERIALS AND METHODS

Enzymes

Polynucleotide kinase was isolated from calf thymus as previously described (17). One unit of PNK is the amount of enzyme required to incorporate 1 nmol of phosphate from ATP into micrococcal nuclease treated DNA in 30 min at 37°C under standard assay conditions (23). Recombinant human DNA ligase I was purified as described previously (24). Recombinant rat DNA polymerase β (25) was generously provided by Dr S. H. Wilson (NIEHS, Research Triangle Park, NC). T4 polynucleotide kinase (3′-phosphatase-free) was purchased from Boehringer Mannheim (Laval, PQ).

Preparation of substrates

Oligonucleotides (Fig. 1), including those with a 3′-phosphate, were chemically synthesized by the DNA Synthesis Service of the Microbiology Department, University of Alberta. Oligonucleotides 20p and 21p were radiolabelled by incubation of 24 pmol of oligonucleotide with 24 pmol of [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham, Oakville, ON) and phosphatase-free T4 polynucleotide kinase in 30 µl of buffer containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol and 0.1 mM spermidine (pH 8.2) for 30 min at 37°C, followed by incubation with 100 pmol of unlabelled ATP for 5 min. The labelled oligonucleotides were purified by passage through Seppak cartridges (Waters Ltd, Mississauga, ON) (26). The dried labelled oligonucleotides were resuspended in 30 µl of water. For annealed substrates, labelled oligonucleotide (4 pmol) was mixed in a total volume of 25 µl with a slight excess of the 24mer and complementary 45mer in either pH 6.4 or 7.6 buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol), heated at 70°C for 5 min and allowed to cool to room temperature over 2 h. Small aliquots were examined by non-denaturing polyacrylamide gel electrophoresis to ensure that all the labelled oligonucleotide was annealed with the complementary strand.

Assays

Conditions for each reaction are given in the respective figure legends. The reaction products were separated on 8% polyacrylamide–7 M urea sequencing gels, which were scanned by PhosphorImager (GS-250 Molecular Imager™ System, BioRad) and quantified with the Molecular Analyst™ software (BioRad).

RESULTS

3′-Phosphatase activity

The influence of DNA structure on PNK-catalyzed 3′-dephosphorylation was examined using oligonucleotides p21p and p20p (Fig. 1) either as single-stranded species or in the fully annealed double-stranded form. The results show very similar enzyme concentration dependence regardless of the structure of the substrate (Fig. 2). In each case, ~50% reaction was observed after 5 min using 0.03 U of PNK. [The units are defined for the kinase

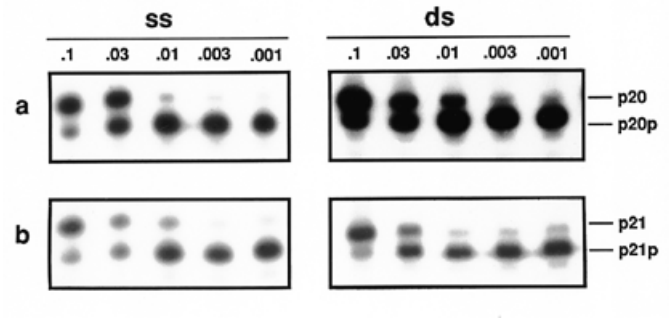

Figure 2. 3′-Phosphatase activity of PNK. 3′-Dephosphorylation of (**a**) p20p and (**b**) p21p either as single-stranded oligonucleotides (ss) or when annealed in a duplex (ds). In each reaction, 2.4 pmol of labelled oligonucleotide was incubated at 37°C for 5 min in 15 µl of buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0) with PNK varying from 0.1 to 0.001 U (indicated at the top of each lane).

activity, based on the substrate and conditions originally used by Richardson (23)].

Repair of nicks and 1 nt strand gaps with 3′-phosphate and 5′-OH termini

Having previously established that PNK can efficiently phosphorylate 5′-OH termini at nicks and short strand gaps (17) and having now determined a similar capacity of PNK to dephosphorylate 3′-phosphate groups at such sites, we examined the possibility of using purified enzymes in a single reaction mixture to repair strand breaks containing 3′-phosphate and 5′-OH termini. Figure 3a shows the results obtained with duplex A, which contains a nick. As expected human DNA ligase I alone had no effect (lane 3), while PNK alone removed the 3′-phosphate group from p21p (lane 4). However, the combination of the two enzymes (lane 5) sealed the nick to produce the labelled 45mer (p45). The results of enzyme treatment of the duplex containing a nucleotide gap (duplex B) are shown in Figure 3b. Incubation with PNK alone dephosphorylated p20p to give p20 (lane 3). Lane 4 shows the result of incubation with rat DNA polymerase β and human DNA ligase I. Surprisingly a small quantity of p21 was detected. This is due to a weak 3′-phosphatase activity in the polymerase, converting p20p to p20, and the subsequent addition of a nucleotide. (This appears to be a genuine phosphatase activity displayed by polymerase β and not a contaminant in the enzyme preparation). By comparison, lane 6 reflects the influence of PNK in generating p21 in high yield because of PNK-mediated removal of the 3′-phosphate from p20p. Lane 7 shows that the combination of all three enzymes effectively filled in and sealed the nucleotide gap to produce p45.

In order to carry out a more detailed analysis of these reactions, we first determined enzyme conditions under which the action(s) of PNK was rate limiting. Because of the acidic pH optimum (pH 5.5–6) of the kinase activity of PNK (11,17), reactions were performed at two pHs to determine to what extent pH influences the overall reaction. [The preceding reactions were carried out at pH 7.0, the pH optimum of the phosphatase activity of PNK (17)]. Results of 5 min reactions using increasing concentrations of PNK with constant levels of polymerase and ligase indicated that the rate of formation of p45 was comparable at both pHs and that

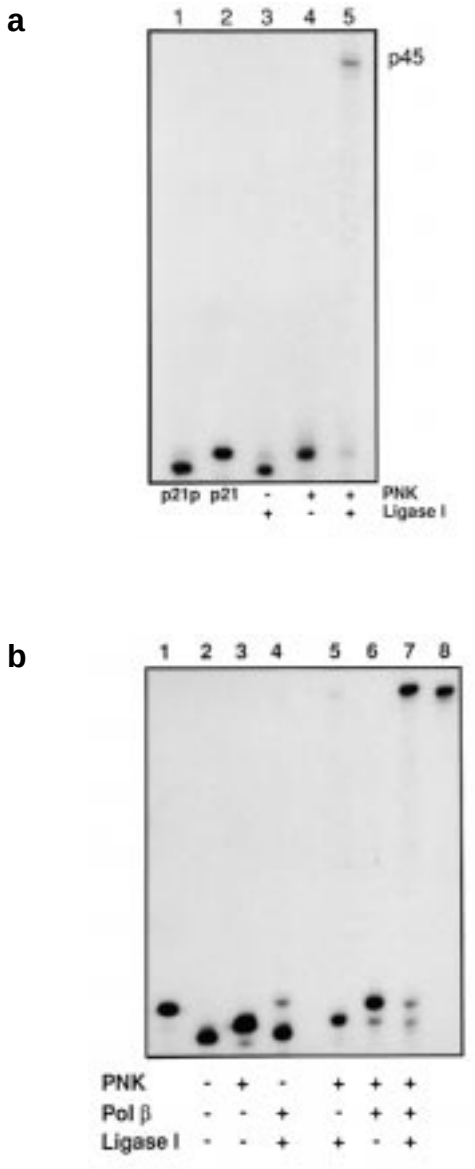

Figure 3. Repair of strand breaks with 3′-phosphate and 5′-hydroxyl termini. Autoradiograms showing the results of incubating (**a**) duplex A with PNK and DNA ligase I and (**b**) duplex B with PNK, DNA polymerase β and DNA ligase I. Duplex A (containing 0.8 pmol of each oligonucleotide) was incubated at 37°C for 1 h with 20 µM ATP, 0.1 U PNK or 1 µg DNA ligase I, or both, in 10 µl buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0). Duplex B was incubated under similar conditions with the inclusion of dCTP (0.25 µM) and DNA polymerase β (1.7 µg). In (a), lanes 1 and 2 show markers for p21p and p21, and in (b), lanes 1 and 8 show markers for p21 and p45, respectively.

0.005 U of PNK would be a suitable concentration to monitor the reactions over time.

An example of an autoradiogram following the time course over 40 min is shown in Figure 4a for the reaction of duplex B with PNK, polymerase β and DNA ligase at pH 6.4. Plots of the reactions are displayed in Figure 4b (B–E). For the reactions with duplex B, very little, if any, p21 was seen in the autoradiograms, therefore, the plotted products are p20p, p20 and p45. This would

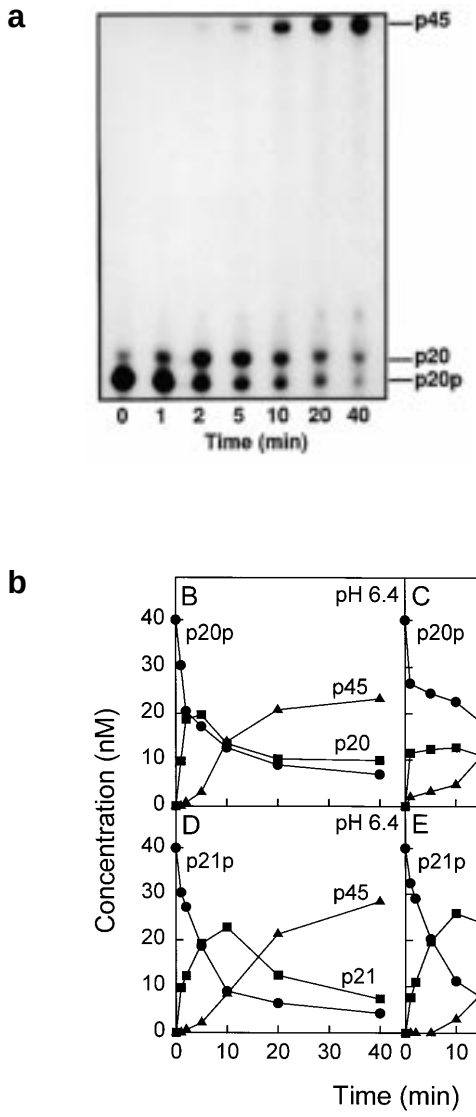

Figure 4. Time course of repair of nicked and gapped duplexes. (**a**) Autoradiogram showing the reaction products over the course of the 40 min reaction (at pH 6.4) to fill in and seal the gap in duplex B. (**b**) Plots of the complete repair of a 1 nt gap at pH 6.4 (**B**) and 7.6 (**C**). Duplex B (containing 0.8 pmol of each oligonucleotide) was incubated at 37°C for 40 min with 20 µM ATP, 0.25 µM dCTP, 0.005 U of PNK, 170 ng of DNA polymerase β and 1 µg DNA ligase I, in 20 µl buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 6.4 or 7.6). At the times indicated, 2 µl aliquots were withdrawn and the reaction in the aliquot stopped by addition of formamide loading buffer (25) and held at 4°C until loaded on the gel. (**D** and **E**) Plots of the nick-sealing reactions at pH 6.4 and 7.6, respectively. Reaction conditions are as described above except that duplex A was used in place of duplex B, and dCTP and DNA polymerase β were omitted from the reaction mixture.

suggest that the p21 produced by polymerase β was rapidly acted upon by DNA ligase. This may not be surprising given that the two enzymes can form a complex (27). As is evident from Figure 4, the pH of the reaction did not have a dramatic influence on the rates of reaction, although the overall rate appears to be a little slower at the higher pH.

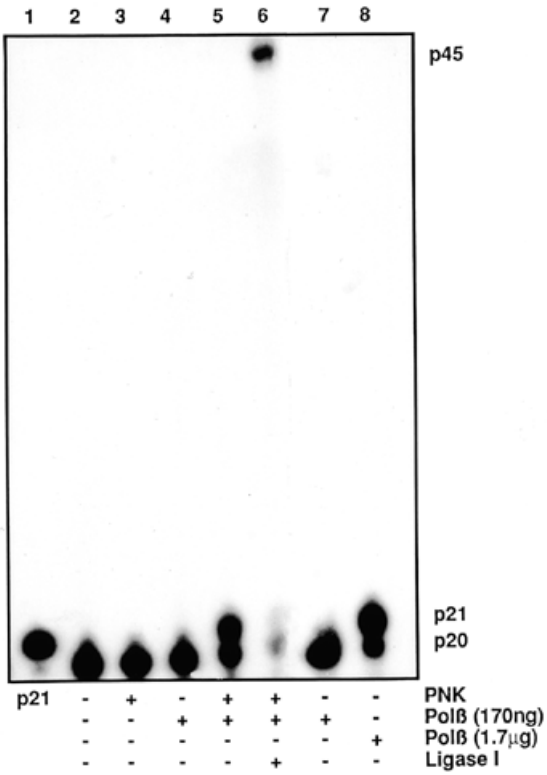

Figure 5. Repair of a 1 nt gap containing 3′- and 5′-hydroxyl termini. Autoradiogram showing the results of incubating duplex C (Fig. 1) with PNK, DNA polymerase β and DNA ligase I. Duplex C (containing 0.8 pmol of each oligonucleotide) was incubated in 10 µl of buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0) at 37°C for 1 h with 20 µM ATP, 0.25 µM dCTP and 0.005 U of PNK, 170 ng or 1.7 µg polymerase β and 1 µg DNA ligase I, as indicated. Lane 1 shows a marker for p21.

Influence of PNK and ligase I on gap filling by polymerase β

In the preceding experiments, which show that a combination of the three enzymes can repair the gapped substrate, both functions of PNK were required. To look for a more subtle influence of PNK on the action of DNA polymerase β, other than the prerequisite of removing the 3′-phosphate group, we employed a gapped substrate, in which the gap was bounded by 3′- and 5′-OH termini (duplex C, Fig. 1). Lanes 4 and 5 in Figure 5 indicate that with the same quantity of DNA polymerase β as used in the previous gap filling reaction, i.e. 170 ng, no elongation of p20 occurred unless PNK was present; the combination of the two enzymes giving ~50% chain elongation in 1 h. This stimulation by PNK required ATP (data not shown). However, increasing the concentration of the polymerase 10-fold did result in gap-filling (lanes 7 and 8) in the absence of PNK. Interestingly, when DNA ligase I was included in the reaction mixture, gap-filling and ligation went almost to completion, implying that DNA ligase I also stimulated the steps prior to strand rejoining.

Influence of ligase I on 5′-phosphorylation by PNK

To test the possibility that the presence of DNA ligase I enhanced the kinase activity of PNK, a similar set of reactions were carried out

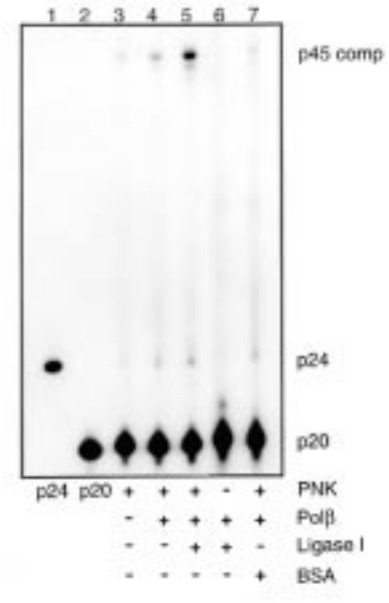

Figure 6. Influence of DNA ligase I on phosphorylation by PNK. Duplex C was incubated as described in Figure 5, but the ATP included in the reaction was radiolabeled (30 μCi at a specific activity of ~140 Ci/mmol), and the dCTP was omitted. It was thus possible to monitor the phosphorylation of the 24mer and the complementary 45mer (p45 comp). The radioactivity in p20 in lanes 3–7 indicated that there was no artefact due to unequal sample loading onto the gel.

as described above but dCTP was omitted to prevent gap-filling and ligation of the two shorter oligonucleotides. In addition the ATP in the reaction mixture was spiked with 30 μCi of [γ-^{32}P]ATP (the final specific activity of the ATP was ~140 Ci/mmol) in order to be able to monitor phosphorylation of the two available 5′-OH termini, i.e. the 5′-end of the 24mer and the complementary 45mer. PhosphorImager analysis of the gel shown in Figure 6 indicated a 1.5-fold elevation of labelling of the 24mer by PNK in the presence of DNA polymerase β (lane 4), and a 2.5-fold elevation in the presence of the polymerase plus DNA ligase I (lane 5). A similar level of enhancement, i.e. ~2.5-fold, was observed with PNK plus DNA ligase I (data not shown). Even more marked enhancement of phosphorylation was observed with the complementary 45mer oligonucleotide (p45 comp). The control, in which PNK was omitted (lane 6), confirmed that the other two enzyme preparations were free of kinase activity. A second control (lane 7), in which BSA was substituted for DNA ligase I, indicated that the effect of ligase was unlikely to be due to non-specific protein stabilization of PNK.

DISCUSSION

A role for the enzyme in DNA repair has been espoused ever since the existence of PNK in mammalian cells was first reported (28,29). The subsequent observation that the enzyme possessed a 3′-phosphatase activity, as well as its kinase activity, provided additional support for this hypothesis. Most recently, the discovery of a eukaryotic enzyme activity that can remove blocked topoisomerase I from 3′-termini, thus rendering strand breaks with 3′-phosphate and 5′-OH termini, has highlighted the need for an enzyme with the properties of PNK to complete a repair pathway that may be responsible for cellular resistance to topoisomerase I inhibitors (8). Clearly, as a prerequisite for such an enzyme to be involved in repair of single-strand breaks induced by topoisomerase or ionizing radiation it would have to act at the relatively sterically-hindered termini of nicks and 1 nt gaps, as opposed to more accessible ends of single-stranded DNA or of double-strand breaks. In contrast to the mammalian PNK, the polynucleotide kinase from phage T4, which also possesses 5′-kinase and 3′-phosphatase activities, phosphorylates exposed 5′-OH termini far more efficiently than hindered termini (30). This enzyme acts to restore tRNA, cleaved by a phage anticodon nuclease, to a ligatable form (31), and is not considered to be a DNA repair enzyme since PNK mutants of phage T4 display no phenotypic defect in repair (32–34).

Several substrates have been used in earlier examinations of the 3′-phosphatase activity of mammalian PNKs, including single-stranded oligonucleotides and micrococcal nuclease-nicked DNA. However, the latter is a relatively ill-defined substrate because such treatment can generate gaps as well as nicks (11). Habraken and Verly (35) generated a more defined poly(dA)/oligo(dT) substrate to test the potential direct transfer by PNK of a phosphate from a 3′- to a 5′-terminus. But even this substrate has the potential for gap formation by slippage of the oligo(dT) on the complementary strand. For this reason we employed oligonucleotide models with a well-defined nick or 1 nt gap. Figure 2 clearly indicates that calf thymus PNK can hydrolyse the phosphate group at the 3′-terminus of the single-stranded oligonucleotides and the more sterically hindered phosphate groups within the duplexes with similar efficiency. These data together with our earlier observation that PNK can efficiently phosphorylate 5′-OH termini at nicks and gaps (17) are strongly suggestive of a role for PNK in repair.

The study was then extended to look at the concerted action of PNK, DNA ligase I and DNA polymerase β in the repair of nicked and gapped sites with 5′-hydroxyl and 3′-phosphate termini. The latter two enzymes were chosen because they are believed to form a complex in cells which is involved in one of the two known pathways for base excision repair (27). Figure 3 establishes that the combination of these three mammalian enzymes rapidly restored the damaged sites. The repair of the substrates required both functions of PNK. The kinase activity of calf thymus PNK has a fairly sharp pH optimum at pH 6.0, while the phosphatase has an optimum at pH 7.0 (17). Thus, it would be anticipated that if the kinase activity was rate-determining, there should be substantial difference between the overall rates of reaction at pH 6.4 and 7.6. This was not observed (Fig. 4), suggesting that under the conditions used the phosphatase reaction was rate-determining.

Further analysis of the potential interactions between the enzymes showed that the presence of PNK had a pronounced effect on the activity of DNA polymerase β (Fig. 5). Since this also required ATP, it is highly likely that the enhancement of the DNA polymerase β reaction was due to phosphorylation of the 5′-OH terminus, and would thus strongly support the very recent report that the presence of a 5′-phosphate group markedly increases the efficiency of DNA polymerase β-catalyzed gap filling of a 1 nt gap (36). Although Prasad *et al*. (37) had earlier observed that 5′-phosphorylation is not required by polymerase β to fill in a 1 nt gap, our data would suggest that this is dependent on the concentration of the polymerase used to catalyze the reaction.

Equally intriguing was the observation that DNA ligase I appears to enhance the rate of phosphorylation by PNK (Fig. 6). This raises the possibilities that either the enzymes may form a

complex, or that ligase I renders DNA termini more susceptible to the action of PNK, or both. A potential interaction between PNK and DNA ligase I is also of interest in light of the recent observations that Okazaki fragments may have 5′-OH termini requiring a DNA kinase before ligation can occur (38), and DNA ligase I is the ligase that joins Okazaki fragments (39,40). It is reasonable to ask what the purpose might be of initially producing Okazaki fragments with non-ligatable termini. Since DNA nicks can direct mismatch repair (41), one possibility is that such non-ligatable strand breaks would not only allow the DNA mismatch repair system to distinguish between nascent and parental strands, but also provide the time to carry out the repair before phosphorylation of the 5′-termini and strand ligation.

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