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# The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase $\beta$ and increases its DNA substrate utilisation efficiency: implications for DNA repair

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## ABSTRACT

In eukaryotic cells, checkpoints are activated in response to DNA damage. This requires the action of DNA damage sensors such as the Rad family proteins. The three human proteins Rad9, Rad1 and Hus1 form a heterotrimeric complex (called the 9-1-1 complex) that is recruited onto DNA upon damage. DNA damage also triggers the recruitment of DNA repair proteins at the lesion, including specialized DNA polymerases. In this work, we showed that the 9-1-1 complex can physically interact with DNA polymerase  $\beta$  *in vitro*. Functional analysis revealed that the 9-1-1 complex had a stimulatory effect on DNA polymerase  $\beta$  activity. However, the presence of 9-1-1 complex neither affected DNA polymerase  $\lambda$ , another X family DNA polymerase, nor the two replicative DNA polymerases  $\alpha$  and  $\delta$ . DNA polymerase  $\beta$  stimulation resulted from an increase in its affinity for the primer–template and the interaction with the 9-1-1 complex stimulated deoxyribonucleotides misincorporation by DNA polymerase  $\beta$ . In addition, the 9-1-1 complex enhanced DNA strand displacement synthesis by DNA polymerase  $\beta$  on a 1 nt gap DNA substrate. Our data raise the possibility that the 9-1-1 complex might attract DNA polymerase  $\beta$  to DNA damage sites, thus connecting directly checkpoints and DNA repair.

## INTRODUCTION

Checkpoints are activated upon DNA damage in eukaryotic cells in order to stop cell cycle progression. Activation of these checkpoints requires the action of DNA damage sensors and transducers (1) such as ATM, ATR, ATRIP, Rad17, Rad9, Rad1 and Hus1. The three human proteins hRad9, hRad1

and hHus1 form a heterotrimeric complex (9-1-1 complex) which exhibits structural similarity with the homotrimeric clamp formed by proliferating cell nuclear antigen (PCNA) (2–5). In addition, hRad17 associates with the four small subunits of the heteropentameric replication factor C complex (RF-C) in a manner similar to the RF-C complex (2,4–7). Several studies showed that the 9-1-1 complex and Rad17-RF-C<sub>2–5</sub> function as a clamp/clamp–loader pair, similarly to PCNA and RF-C (8–11). Moreover, the 9-1-1 complex, Rad17-RF-C<sub>2–5</sub> and PCNA co-localize in foci formed upon DNA damage (12–14). These data suggested a mechanism in which Rad17-RF-C<sub>2–5</sub> would recognize DNA lesions, allowing the recruitment of the 9-1-1 complex to these sites. ATM and ATR kinases are recruited simultaneously at the same sites of DNA damage but in a 9-1-1 complex and Rad17-RF-C<sub>2–5</sub> independent manner (15–18). The proposed mechanism is that the 9-1-1 complex may serve as a recruiting platform for the checkpoint effector kinases such as Chk1 or Chk2 (19,20), that are subsequently phosphorylated by the ATR/ATM kinases (1). According to the currently accepted model, the 9-1-1 complex is present where the action of DNA repair has to take place, suggesting the possibility that it may play a direct role in subsequent stages of the DNA repair processes.

Following recognition by DNA damage sensors, the lesions are processed by multiprotein complexes specialized in different DNA repair pathways such as base excision repair (BER), nucleotide excision repair (NER), double-strand break repair (DSBR) or mismatch repair (MMR). An essential component of these machineries are DNA polymerases (pols) that are needed to synthesize DNA during the DNA repair processes. In human cells, pol  $\beta$ , which belongs to the pol X family, is the major repair pol, and it is essential for the BER pathway. Pol  $\beta$  preferentially uses shortly gapped DNA substrates (21), performs strand displacement synthesis (22) and also possesses a 5' dRP lyase activity which is essential for completion of BER (23–25). Moreover, pol  $\beta$  is able to bypass *in vitro* several types of DNA lesions like apurinic (AP)

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sites, cisplatin (cis-Pt) adducts or cyclobutane pyrimidine dimer (CPD) lesions, albeit with a relatively low efficiency (26–29).

The mechanisms by which DNA repair occurs are now beginning to be unraveled (30). Likewise, the way cells trigger the DNA damage checkpoints is also starting to be depicted (1). However, the link between checkpoint engagement and the recruitment to DNA lesions of pols involved in repair machineries is far from being understood. In view of its localization onto DNA lesions, the 9-1-1 checkpoint complex could be a potential candidate for the role of 'recruiting platform' for DNA repair effectors. In this context, recent studies have shown interaction or co-localization of the 9-1-1 complex with proteins involved in various DNA repair processes upon DNA damage (13,31,32). In order to address further the link between checkpoint proteins and DNA repair, we tested the possibility of a physical and functional interaction of the 9-1-1 complex with the main repair pol, pol  $\beta$ .

In this work we present evidence that the 9-1-1 complex directly interacts with and stimulates pol  $\beta$  activity, by increasing its affinity for the 3'-OH primer end, resulting in an increase of nucleotide misincorporation without affecting the efficiency of correct nucleotide incorporation. In addition, we show that the 9-1-1 complex enhances DNA strand displacement synthesis by pol  $\beta$  on a 1 nt gap DNA substrate. Our results are discussed in the view of a possible role of the 9-1-1 complex in recruiting pol  $\beta$  to DNA damage sites.

## MATERIALS AND METHODS

### Chemicals

[<sup>3</sup>H]dTTP (40 Ci/mmol) and [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) were from Amersham Biosciences; unlabeled dNTPs, poly(dA) and oligo(dT)<sub>18</sub> were from Roche Biochemicals. The oligonucleotides were from Microsynth (Switzerland). Whatman was the supplier of the GF/C filters. All other reagents were from Merck or Fluka.

### Antibodies

The hHus1 cDNA (amino acid 1–134) was cloned fused to the hexahistidine tag of the vector pQE-30 (Qiagen). The hRad9 cDNA was fused to the GST of the vector pGEX 4T-3 (Pharmacia). The hHus1 and hRad9 proteins were mainly insoluble. Proteins were purified according to the manufacturer's instructions. Antibodies against the recombinant proteins were raised in rabbit by standard procedures (33). The goat anti-Rad1 antibody (N-18) was from Santa Cruz biotechnology. The mouse monoclonal antibody against rat pol  $\beta$  was purchased from Neomarkers.

### DNA substrates

The d60mer and the d73mer, as well as the corresponding primers, were chemically synthesized and purified on denaturing polyacrylamide gels. The sequences are the following: d73mer, 5'-GATCGGGAGGGTAGGAATATTGAGGATGAAAGGGT-TGAGTTGAGTGGAGATAGTGGAGGGTAGTATGGTGG-ATA-3'; d60mer<sub>a</sub>, 5'-GCTGATTGGTGGCTATCCCTTGG-TTCTTTTCGGCACTGGCCGTCGTATGCTCTTGTTG-TA-3'; d60mer<sub>b</sub>, 5'-CAATGATCTCGTCAGCATCTGTGTG-AATTCGGCACTGGCCGTCGTATGCTCTTGTTGTA-3'

(see Figure 5A). The sequences complementary to the corresponding primers are underlined. For the d60mer<sub>a</sub>, primers from 14 to 17 nt complementary to the 3' end of the template were used (see Figure 4), the sequence underlined corresponds to the d17mer. For the 60mer<sub>b</sub> template, (1 nt gap substrate) the underlined sequences correspond respectively to the 20mer primer and to the 20mer downstream oligonucleotide.

### Enzymes and proteins

The 9-1-1 complex was isolated by coexpressing in Sf9 cells the three baculovirus encoding the recombinant hRad1, hRad9 and hHus1 (provided by T. Tsurimoto, Japan). The complex was subsequently purified according to Shiomi *et al.* (4). Single infected Sf9 cell extracts were obtained by expression of each of the recombinant proteins followed by extraction according to Shiomi *et al.* (4). GST-tagged pol  $\beta$  and GST were expressed in TG1 *Escherichia coli* strain and purified by binding on glutathione-sepharose beads as described by Hasan *et al.* (34). Natural pol  $\alpha$  from mammalian cells, recombinant human pol  $\delta$  and pol  $\beta$  were purified as previously described (35–37). Human recombinant PCNA was purified as previously described (38). Human pol  $\lambda$  was purified according to Garcia-Diaz *et al.* (39).

### Enzyme assays

**DNA polymerase assay.** Pol  $\beta$  activity on poly(dA)-oligo(dT)<sub>10:1</sub> was assayed in a final volume of 25  $\mu$ l containing 50 mM Tris-HCl (pH 7.6), 0.25 mg/ml bovine serum albumin, 1 mM DTT, 0.8 mM MnCl<sub>2</sub> and 50  $\mu$ M [<sup>3</sup>H]dTTP (5 Ci/mmol) unless otherwise indicated in the figure legends. The reactions were incubated for 15 min at 37°C unless otherwise stated, and the DNA was precipitated with 10% trichloroacetic acid containing 100 mM Na pyrophosphate. Insoluble material was determined by scintillation counting as described (40). DNA polymerase product analysis, following oligonucleotide substrates replication, was performed by using sequencing gels. The reaction mixture included in a final volume of 10  $\mu$ l: 50 mM Tris-HCl (pH 7.6), 0.25 mg/ml bovine serum albumin, 1 mM DTT, 0.8 mM MnCl<sub>2</sub> and 100  $\mu$ M of each unlabeled dNTPs, [<sup>32</sup>P]-5' end-labeled primer annealed to the template and as indicated in the figure legends. The processivity assays were performed in the presence of 100-fold molar excess over the labeled template of cold poly(dA)-oligo(dT) as a trap. Reactions were incubated for 15 min at 37°C unless otherwise stated, stopped by addition of sequencing gel loading buffer [95% (v/v) formamide, 20 mM EDTA, pH 8.0] and heated for 5 min at 95°C. Reaction products were resolved on a 10% polyacrylamide, 7 M urea gel. The gels were dried and exposed to X-ray film.

**Misincorporation assay.** Assay conditions are the same as those described above except that the pol activity was tested in the presence or in the absence of 100  $\mu$ M of either dGTP, dATP, dTTP or dCTP. Reactions were incubated for 30 min at 37°C. The products were analyzed as described above.

### Pulldown assay

GST-tagged pol  $\beta$  or GST alone bound to glutathione-sepharose beads were incubated with cell extracts or purified proteins, as indicated in the figure legends, for 2 h at 4°C in

50 mM Tris-HCl pH 8.0, 90 mM NaCl, 0.05% (v/v) NP-40, 1 mM  $\beta$ -mercaptoethanol, 1 mM PMSF. After washing four times in the same buffer, the beads were heated for 5 min at 95°C in Laemmli buffer and the coprecipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods (41,42).

### Immunoprecipitations

Sf9 cell extracts were incubated with purified His-tagged pol  $\beta$  in IP buffer [50 mM Tris pH 8, 80 mM NaCl, 1 mM DTT, 0.05% (v/v) NP-40, 1 mM  $\text{Na}_3\text{VO}_4$ , 10 mM glycerophosphate, 2  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  pepstatin, 1  $\mu\text{g}/\text{ml}$  bestatin] for 2 h at 4°C. 5  $\mu\text{l}$  anti-Rad9 antiserum or control serum from unimmunized rabbits were added and incubated for 2 h at 4°C. 25  $\mu\text{l}$  equilibrated protein A sepharose were added and the samples were incubated for 1 h at 4°C. After washing three times in IP buffer the beads were heated 5 min at 95°C in Laemmli buffer and the coprecipitated proteins analyzed by western blot using the corresponding antibodies according to established methods (41,42).

### Gel filtration analysis

His-tagged pol  $\beta$  alone, the 9-1-1 complex alone or both proteins together were incubated for 2 h at 4°C in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (v/v) NP40, 1 mM  $\beta$ -mercaptoethanol, 1 mM PMSF. The proteins were subsequently separated on a sephacryl S-200 column (3.5 ml) in the same buffer but lacking NP-40. The eluted fractions were analyzed by DNA polymerase assay as described above and by western blot using the corresponding antibodies according to established procedures (41,42).

### Steady-state kinetics data analysis

For kinetic data analysis, time-dependent nucleotide incorporation was measured in the presence of increasing concentrations of DNA or nucleotide substrate. The slopes of the curves obtained by linear interpolation of the data points were taken as the initial velocities of the reaction at any given substrate concentration. The  $K_m$  and  $V_{max}$  values were calculated by plotting the initial velocities in dependence of the substrate concentrations and fitting the data according to the Michaelis-Menten equation in the following form,

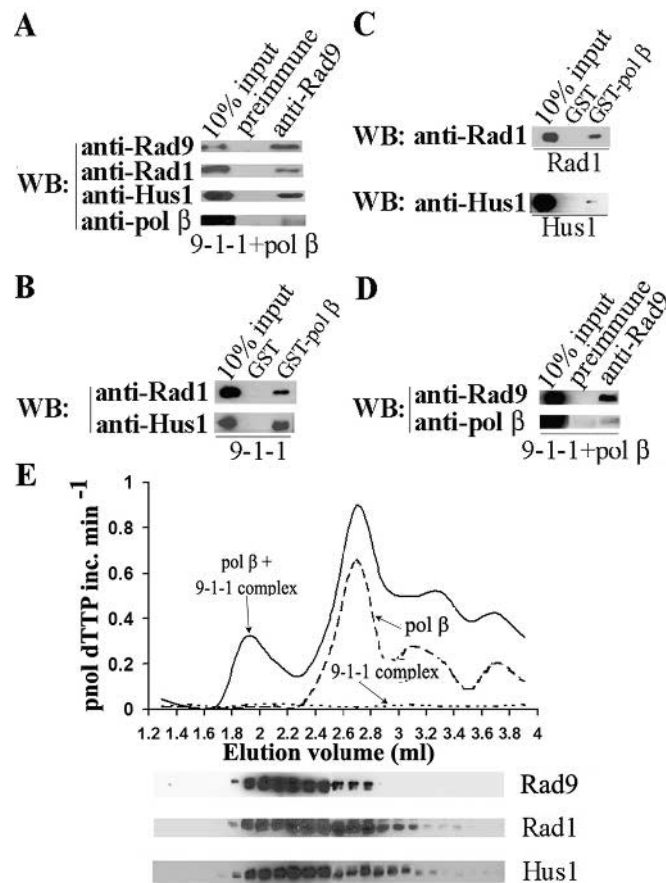
$$v = (k_{cat} \cdot E_0) / (1 + K_m/[S])$$

where  $k_{cat} \cdot E_0 = V_{max}$ .

## RESULTS

### DNA polymerase $\beta$ physically interacts with the 9-1-1 complex

The initial aim was to determine whether pol  $\beta$ , the major DNA repair pol, was able to interact with the 9-1-1 checkpoint complex. To address this question, we first incubated purified His-pol  $\beta$  with Sf9 cell extracts expressing the 9-1-1 complex. From these extracts, anti-Rad9 immunoprecipitation by using a polyclonal anti-Rad9 antibody was carried out. Western blot analysis showed that Rad9 was immunoprecipitated, and that Rad1, Hus1 and pol  $\beta$  were all co-immunoprecipitated with Rad9 (Figure 1A). Second, a GST-pulldown experiment was performed after incubation of the purified 9-1-1 complex with



**Figure 1.** DNA polymerase  $\beta$  physically interacts with the 9-1-1 complex. (A) Sf9 cell extracts (45  $\mu\text{g}$ ) expressing the 9-1-1 complex were incubated with His-tagged pol  $\beta$  (3  $\mu\text{g}$ ). From these extracts, anti-Rad9 immunoprecipitation was performed by using a polyclonal anti-Rad9 antibody. (B) GST-pulldown experiments were performed in the presence of either GST or GST-pol  $\beta$  (5  $\mu\text{g}$ ) and purified 9-1-1 complex (1.8  $\mu\text{g}$ ). (C) GST-pulldown experiments were performed in the presence of either GST or GST-pol  $\beta$  (5  $\mu\text{g}$ ) and Sf9 cell lysates expressing either Rad1 or Hus1 separately (15  $\mu\text{g}$ ). (D) Sf9 cell extracts (15  $\mu\text{g}$ ) expressing only Rad9 were incubated with purified His-tagged pol  $\beta$  (3  $\mu\text{g}$ ). Anti-Rad9 immunoprecipitation was performed by using a polyclonal anti-Rad9 antibody. The presence of coprecipitated proteins was determined by SDS-PAGE followed by western blot analysis. (E) Purified His-tagged pol  $\beta$  (2  $\mu\text{g}$ ) and 9-1-1 complex (6  $\mu\text{g}$ ) were incubated together and the proteins were subsequently separated on a Sephacryl S-200 column as described in Materials and Methods. The presence of pol  $\beta$  in the eluted fractions was detected by determining DNA polymerase activity as described in Material and Methods. The presence of each of the 9-1-1 complex proteins in the corresponding fractions was determined by SDS-PAGE followed by western blot analysis. His-tagged pol  $\beta$  (2  $\mu\text{g}$ ) and the 9-1-1 complex (6  $\mu\text{g}$ ) alone were analyzed as controls separately on a Sephacryl S-200 column. The incorporations of [ $^3\text{H}$ ]dTTP into acid-precipitable material is represented as broken line (for free pol  $\beta$ ) and dotted line (for free 9-1-1 to exclude DNA polymerase contamination).

GST-tagged pol  $\beta$  or with GST alone (Figure 1B). Western blot analysis against Rad1 and Hus1 showed a specific coprecipitation of Rad1 and Hus1 with GST-pol  $\beta$  but not with GST. Third, we tested which of the monomers were directly involved in this interaction. We performed a GST-pulldown experiment after incubation of GST-tagged pol  $\beta$  or GST alone with Sf9 cell extracts overexpressing either Rad1 or Hus1 separately. Western blot analysis revealed a specific direct interaction of Rad1 and Hus1 with GST-pol  $\beta$  (Figure 1C). These results were further confirmed when a GST pulldown of

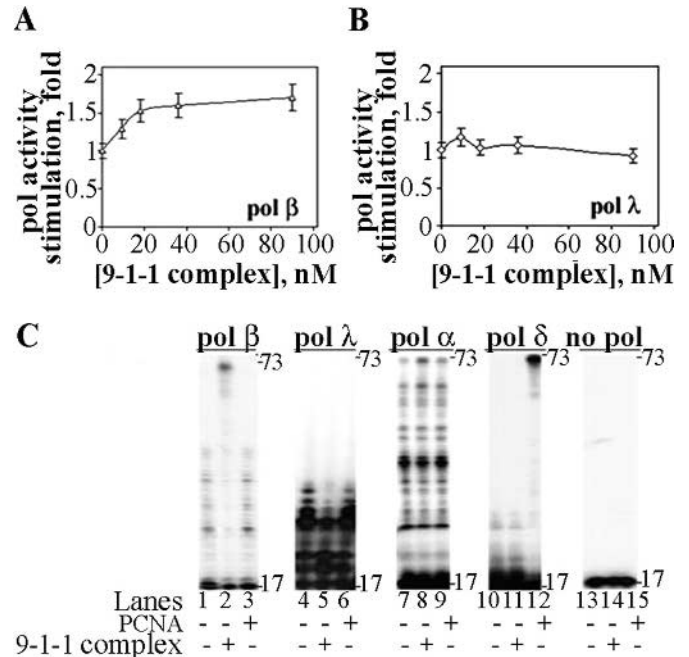
GSTRad1 and GSTHus1 with His-tagged pol  $\beta$  was performed (data not shown). Fourth, we performed an anti-Rad9 immunoprecipitation after incubation of His-tagged pol  $\beta$  with Sf9 cell extracts overexpressing only Rad9. Coprecipitation of pol  $\beta$  with Rad9 showed that these two proteins also interact (Figure 1D). Finally, in view of our data that pol  $\beta$  could independently interact with the three 9-1-1 complex monomers, a gel filtration analysis was performed with pol  $\beta$  and the 9-1-1 complex. As shown on Figure 1E, an additional activity peak eluting with the 9-1-1 complex ahead of pol  $\beta$  alone could be detected. A control elution of the 9-1-1 complex alone showed that the purified 9-1-1 complex does not contain any contaminating polymerase activity that may account for this activity peak. Western blot analysis against Rad9, Rad1 and Hus1 confirmed that the additional pol  $\beta$  peak co-eluted in the earliest fractions containing the three Rad proteins. This suggested that a high molecular weight complex containing pol  $\beta$  and the 9-1-1 complex was formed. In summary, these data suggested that pol  $\beta$  can form a complex with the 9-1-1 complex, and that this interaction appears to be mediated by each of the three monomers.

### The 9-1-1 complex specifically stimulates DNA polymerase $\beta$ activity

In order to check whether the physical interaction of the 9-1-1 complex with pol  $\beta$  had a functional relevance, we next analyzed the effect of the 9-1-1 complex on pol  $\beta$  activity in an *in vitro* DNA polymerization assay. As shown in Figure 2, the 9-1-1 complex was able to stimulate pol  $\beta$  activity in an acid precipitable assay (Figure 2A) whereas it did not affect nucleotide incorporation by pol  $\lambda$ , another X family pol (Figure 2B). Next, to confirm this finding, the elongation products synthesized by these two pols were visualized on polyacrylamide denaturing gel. For this, elongation of a d17:d73mer primer–template by pol  $\beta$  and pol  $\lambda$  was carried out in the absence or in the presence of either the 9-1-1 complex or PCNA (Figure 2C, lanes 1 to 6). A distinct increase in pol- $\beta$ -mediated elongation of the primer was observed in the presence of 9-1-1 complex (lane 2), whereas PCNA did not affect pol  $\beta$  DNA synthesis (lane 3), as expected from the data in literature (22). On the other hand, pol  $\lambda$  activity was not affected by the presence of the 9-1-1 complex and even a slight decrease in the products length was observed (lane 5). PCNA did not stimulate pol  $\lambda$  activity under distributive conditions (lane 6) as previously reported (43). In order to investigate further the specificity of the 9-1-1 complex effect on pol  $\beta$  DNA synthesis, we next analyzed the ability of 9-1-1 to stimulate replicative pols. As shown in Figure 2C, the 9-1-1 complex did not affect either pol  $\alpha$  or pol  $\delta$  elongation patterns under the tested conditions (lanes 8 and 11). As expected, PCNA had no effect on pol  $\alpha$  activity but allowed pol  $\delta$  to synthesize full-length products (lanes 9 and 12). Lanes 13 to 15 show that neither the 9-1-1 complex nor PCNA contained intrinsic pol activity. Taken together these data show a specific stimulation of pol  $\beta$  by the 9-1-1 complex.

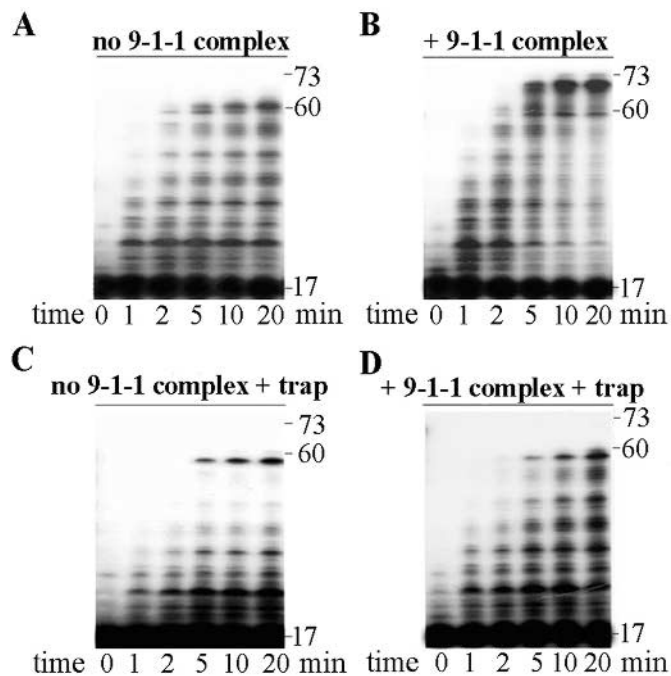
### The 9-1-1 complex does not influence the processivity of DNA polymerase $\beta$

To further investigate the mechanism of pol  $\beta$  stimulation by the 9-1-1 complex, we analyzed its effect on the time course



**Figure 2.** The 9-1-1 complex stimulates DNA polymerase but not DNA polymerase  $\alpha$ ,  $\delta$  and  $\lambda$ . (A) The 9-1-1 complex was titrated into a pol  $\beta$  assay by measuring the incorporation of [ $^3$ H]dTTP in the presence of 20 nM pol  $\beta$  and 70 nM of poly(dA)-oligo(dT) (in terms of 3'-OH ends). (B) The 9-1-1 complex was titrated into a pol  $\lambda$  assay by measuring the incorporation of [ $^3$ H]dTTP in the presence of 120 nM pol  $\lambda$  and 70 nM of poly(dA)-oligo(dT) (in terms of 3'-OH ends). (C) Product analysis of DNA synthesis by different pols on a d17:d73 DNA substrate (1 nM) was carried out in the presence of 4 nM pol  $\beta$  (lanes 1–3), 50 nM pol  $\lambda$  (lanes 4–6), 0.1 units pol  $\alpha$  (lanes 7–9), 15 nM pol  $\delta$  (lanes 10–12), or in the absence of any pol (lanes 13–15). The reactions were carried out in the absence (lanes 1, 4, 7, 10 and 13) or in the presence of 70 nM 9-1-1 complex (lanes 2, 5, 8, 11 and 14) or 70 nM PCNA (lanes 3, 6, 9, 12, 15). Positions of the 17mer primer and 73mer oligonucleotide are indicated on the right side of the figures.

incorporation of pol  $\beta$  on the d17:d73 primer–template under distributive and processive conditions (Figure 3). Under distributive conditions, pol  $\beta$  alone was not able to synthesize full length products even after 20 min incubation, and the majority of the products stopped at a strong termination site around position 60, where a G-rich sequence is present (Figure 3A). In the presence of the 9-1-1 complex, however, the termination site was overcome and full length products accumulated within the first 5 min (Figure 3B). In agreement with the data shown in Figure 2C, the DNA synthesis rate appeared to be enhanced by the 9-1-1 complex (compare Figure 3A and B). Next, the same analysis was performed under processive conditions [presence of a 100-fold molar excess of unlabeled poly(dA)-oligo(dT) as a trap]. Under these conditions, all the pol  $\beta$  products terminated at the strong pausing site around position 60 both in the absence (Figure 3C) and in the presence of the 9-1-1 complex (Figure 2D). While at early time points (1 and 2 min), the length of the products was higher in the presence of 9-1-1 complex, at later time points the maximal length of products in the presence of 9-1-1 complex was similar to the maximal length observed in its absence. However, when the 9-1-1 complex was present, intermediate products accumulated, suggesting that pol  $\beta$  can reinitiate synthesis more efficiently under these conditions. These

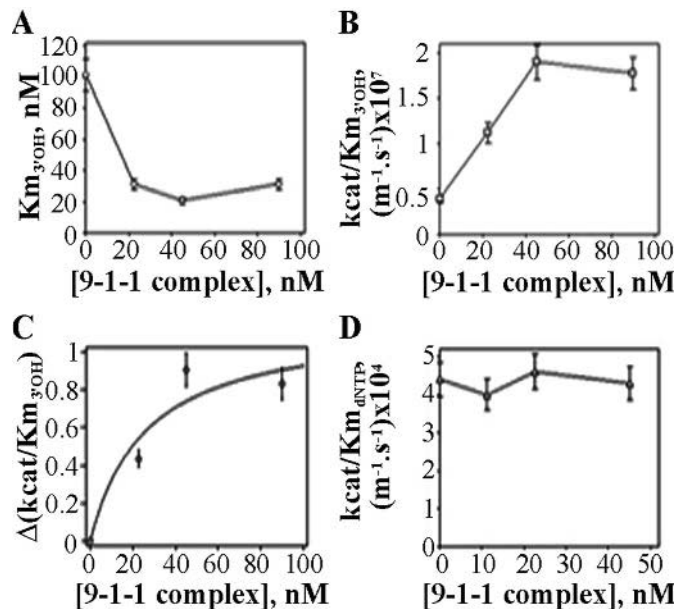


**Figure 3.** The 9-1-1 complex does not stimulate the processivity of DNA polymerase  $\beta$ . (A and B) Time-dependent elongations (0, 1, 2, 5, 10 and 20 min) of a 5' end-labelled DNA primer (d17:d73 DNA substrate, 1 nM) under distributive conditions were performed as described under Materials and Methods in the presence of pol  $\beta$  (4 nM). (A) Pol  $\beta$  alone. (B) Pol  $\beta$  in the presence of the 9-1-1 complex (70 nM). (C and D) Time-dependent elongations (0, 1, 2, 5, 10 and 20 min) of a 5' end-labelled DNA primer d17:d73 (1 nM) under processive conditions were performed as described under Materials and Methods in the presence of pol  $\beta$  (4 nM) and a 100-fold excess of unlabeled poly(dA)-oligo(dT) (100 nM) as a trap. (C) Pol  $\beta$  alone. (D) Pol  $\beta$  in the presence of the 9-1-1 complex (70 nM). Positions of the 17mer primer, and 73mer and 60mer oligonucleotides are indicated on the right side of the figures.

data indicate that the stimulation of pol  $\beta$  by the 9-1-1 complex is not achieved via a substantial increase of its processivity.

### The 9-1-1 complex stimulates DNA polymerase $\beta$ activity by increasing its primer utilization efficiency, but does not affect its nucleotide incorporation efficiency ( $k_{\text{cat}}/K_m$ )

The stimulatory effect of the 9-1-1 complex on pol  $\beta$  might be due either to an increased nucleotide incorporation, or to an increased affinity of the pol for the DNA primer template. To address this question, we next measured the initial velocities of the reaction catalyzed by pol  $\beta$ , using poly(dA)-oligo(dT) as a primer/template, in the absence or the presence of increasing amounts of the 9-1-1 complex. This allowed the determination of the apparent affinity ( $K_m$ ) and the catalytic rate ( $k_{\text{cat}}$ ) values for the 3'-OH primer end at each concentration of the 9-1-1 complex. As shown in Figure 4A, the apparent affinity of pol  $\beta$  for the 3'-OH primer end was increased in the presence of 9-1-1 complex, as indicated by the decrease of the  $K_m$  value. As shown in Figure 4B, the 9-1-1 complex also induced a dose-dependent increase of the efficiency of primer utilization ( $k_{\text{cat}}/K_m$  ratio) of pol  $\beta$ . In Figure 3C the variation of  $k_{\text{cat}}/K_m$  [ $\Delta(k_{\text{cat}}/K_m)$ ] was plotted as a function of the 9-1-1 complex



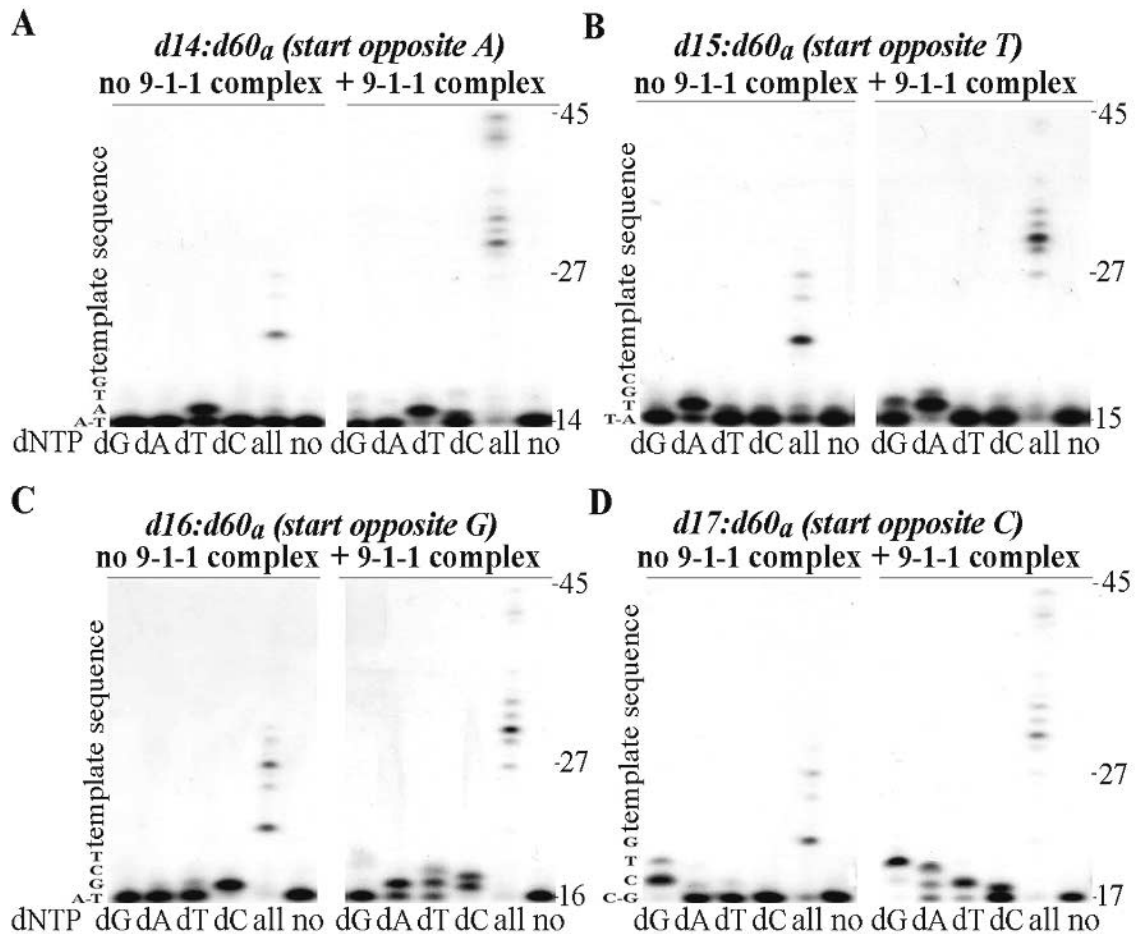
**Figure 4.** The 9-1-1 complex increases the primer utilization efficiency but not the nucleotide incorporation efficiency by DNA polymerase  $\beta$ . (A) Dependence of the apparent  $K_m$  value for the 3'-OH primer end from the 9-1-1 complex concentration.  $K_m$  were determined in the presence of 10 nM pol  $\beta$  and 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTTP, by using poly(dA)-oligo(dT)<sub>10:1</sub> as a primer-template. (B) dependence of the catalytic efficiency for the 3'-OH primer end ( $k_{\text{cat}}/K_m$ ) from the 9-1-1 complex concentration.  $K_m$  and  $k_{\text{cat}}$  values were determined in the presence of 10 nM pol  $\beta$  and 50  $\mu\text{M}$  [ $^3\text{H}$ ]dTTP, by using poly(dA)-oligo(dT)<sub>10:1</sub> as a primer-template. (C) dependence of the increase of  $k_{\text{cat}}/K_m$  for the 3'-OH primer end from the 9-1-1 complex concentration. Data were fitted to the equation,  $\Delta(k_{\text{cat}}/K_m) = \Delta_{\text{max}}/(1 + (K_d/[9-1-1 \text{ complex}]))$ . (D)  $K_m$  and  $k_{\text{cat}}$  values for the dNTPs were determined in the presence of 10 nM pol  $\beta$  and 70 nM poly(dA)-oligo(dT)<sub>10:1</sub> (in terms of 3'-OH ends). The variation of  $k_{\text{cat}}/K_m$  ( $\Delta(k_{\text{cat}}/K_m)$ ) was plotted as a function of the 9-1-1 complex concentration.

concentration. The curve was fitted to a hyperbolic equation that allowed estimating an apparent dissociation constant ( $K_d$ ) for the pol  $\beta$ /9-1-1 complex interaction of 26 nM.

Since the 9-1-1 complex appears to stimulate pol  $\beta$  synthesis by increasing its affinity for the 3'-OH primer end, we next wanted to determine if it also affects its nucleotide incorporation efficiency. For this purpose, we performed experiments similar to the ones described above, but by varying the dTTP concentrations. The  $K_m$  and  $k_{\text{cat}}$  values of pol  $\beta$  for dTTP were determined in the absence or in the presence of increasing concentrations of 9-1-1 complex. As shown in Figure 4D, the efficiency of nucleotide incorporation ( $k_{\text{cat}}/K_m$ ) was not affected by the presence of 9-1-1 complex. Taken together, these results suggested that the 9-1-1 complex stimulates pol  $\beta$  activity by increasing its primer-template utilization efficiency.

### The 9-1-1 complex increases misincorporation by DNA polymerase $\beta$ in different sequence contexts

Next, we determined whether the presence of the 9-1-1 complex has an effect on the misincorporation ability of pol  $\beta$ . For this, we used a 60mer template annealed to different primers allowing DNA incorporation to start by a T (d14:d60<sub>a</sub>,



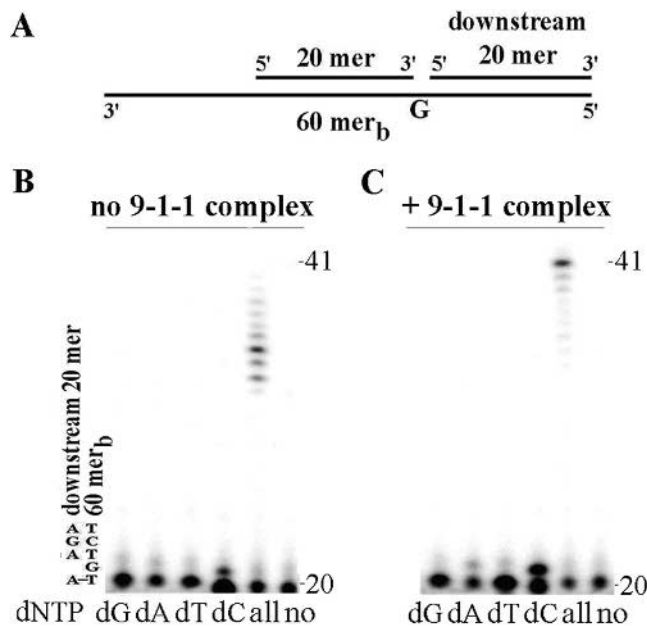
**Figure 5.** The 9-1-1 complex enhances misincorporation by DNA polymerase  $\beta$  in dependence of the sequence context. DNA synthesis by pol  $\beta$  was analyzed in the presence of 4 nM pol  $\beta$  and 1 nM DNA substrate (panel A, d14:d60<sub>a</sub>, panel B, d15:d60<sub>a</sub>, panel C, d16:d60<sub>a</sub>, panel D, d17:d60<sub>a</sub>) in the absence or in the presence of each of the four dNTPs alone (100  $\mu$ M) or of a mixture of all four dNTPs (100  $\mu$ M each). Experiments were carried out in the absence (left panel) or in the presence (right panel) of the 9-1-1 complex (70 nM). Positions of the primers and 27mer and 45mer oligonucleotides are indicated on the right side of the figures.

Figure 5A), an A (d15:d60<sub>a</sub>, Figure 5B), a C (d16:d60<sub>a</sub>, Figure 5C) or a G (d17:d60<sub>a</sub>, Figure 5D). The left panels of Figure 5A–D show that pol  $\beta$  alone was capable of limited misincorporation depending on the sequence context. Misincorporation could be observed when DNA synthesis started opposite C (Figure 5D, left panel) and when incorporation started opposite G (Figure 5C, left panel). No misincorporation was seen when DNA synthesis started opposite A or T (Figure 5A and B, left panels). As expected, elongation products up to 27–30 nt length were observed in the presence of all four dNTPs with all the primer–template pairs tested (Figure 5A–D, left panels). In the presence of the 9-1-1 complex an increase in misincorporation by pol  $\beta$  could be observed, with all four primer–template pairs (Figure 5A–D, right panels), but mainly opposite G and C (Figure 5C and D, right panels). As it can be seen, the addition of 9-1-1 complex caused an increase of the products length to about 45 nt in the presence of the four dNTPs with all the four tested primers (Figure 5A–D, right panels). Using longer exposure times, products corresponding to full-length synthesis were also detected (data not shown). According to these results, the 9-1-1 complex, under conditions allowing incorporation of only one of the four desoxynucleotides, apparently led to increased misincorporation by pol  $\beta$ . In view of the fact that the 9-1-1 complex did not affect the nucleotide

incorporation efficiency by pol  $\beta$ , this misincorporation effect is likely to be due to an increased stability of the pol  $\beta$ /primer complex in the presence of the 9-1-1 complex.

### The 9-1-1 complex stimulates the strand displacement synthesis by DNA polymerase $\beta$ on a 1 nt gap DNA template

Subsequently we tested the effect of the 9-1-1 complex using a 1 nt gap template as a substrate for pol  $\beta$  (represented on Figure 6A). Such a template mimics BER intermediates that are substrates for pol  $\beta$  gap filling *in vivo*. Figure 6B shows the products synthesized by pol  $\beta$  on this BER substrate. Each of the four dNTPs was tested separately or all together. Pol  $\beta$  was able to incorporate the correct nucleotide, dCTP, opposite to the free nucleotide position. Furthermore, in the presence of the four dNTPs pol  $\beta$  showed strand displacement DNA synthesis (22), and replicated the template up to position +19. In the presence of the 9-1-1 complex (70 nM), however, an increase in incorporation of dCTP at position +1 was observed (Figure 6C). A slight misincorporation of dATP was also detected, which corresponded to the main misincorporation observed opposite a G when a classical primer–template



**Figure 6.** The 9-1-1 complex stimulates strand displacement synthesis by DNA polymerase  $\beta$  on a 1 nt gap DNA template. (A) Schematic representation of the 1 nt gap substrate used in the strand displacement synthesis experiment. (B and C) DNA synthesis by pol  $\beta$  was analyzed in the presence of 10 nM pol  $\beta$  and 1 nM of the 1 nt gap DNA substrate (shown on panel A) in the absence or in the presence of each of the four dNTPs alone (100  $\mu$ M) or a mixture of all four dNTPs (100  $\mu$ M each). (B) Pol  $\beta$  alone. (C) Pol  $\beta$  in the presence of the 9-1-1 complex (70 nM). Positions of the 20mer primer and 41mer oligonucleotide are indicated on the right side of the figures.

oligonucleotide was used (see Figure 5C, right panel). When all four dNTPs were present, the 9-1-1 complex enhanced strand displacement DNA synthesis by pol  $\beta$  and mainly full length products were observed. These results suggest that the 9-1-1 complex can enhance strand displacement DNA synthesis by pol  $\beta$  on a 1 nt gap DNA.

## DISCUSSION

The 9-1-1 complex has been shown to be recruited onto DNA following DNA damage as an early step of the checkpoint cascades. Its structural similarity to PCNA suggested that it might act as an alternative sliding clamp for certain pols, such as repair pols.

In this paper, we showed that the 9-1-1 complex and pol  $\beta$  can directly interact and form a complex *in vitro* (Figure 1A–C), and that all three monomers were able to directly interact with pol  $\beta$  (Figure 1C and D). The 9-1-1 complex specifically stimulated pol  $\beta$  (Figure 2) whereas it did neither stimulate pol  $\lambda$ , nor the replicative pol  $\alpha$  and  $\delta$ . The 9-1-1 complex did not act as a processivity factor for pol  $\beta$  (Figure 3) but significantly increased its efficiency of primer utilization (Figure 4A–C). This mechanism looks rather similar to the one observed for PCNA mediated pol  $\epsilon$  stimulation (44). An estimation of the apparent dissociation constant ( $K_d$ ) for the pol  $\beta$ /9-1-1 complex interaction gave a value of 26 nM, consistent with the affinities determined for PCNA and pol  $\delta$  or  $\epsilon$  (45), thus providing additional evidence for the physiological relevance of this effect. Addition of the 9-1-1 complex

also resulted in an increase of single nucleotide misincorporation by pol  $\beta$  (Figure 5), which could be a consequence of the observed polymerase stabilization at the 3'-OH end by the complex. Subsequently, using a substrate mimicking a BER intermediate (1 nt gap primer–template), a stimulation of pol  $\beta$  strand displacement synthesis in the presence of the 9-1-1 complex was observed (Figure 6B). This substrate not only mimics one of the repair intermediates occurring during BER, but gapped double-stranded DNA has also been shown to be the preferred substrate for loading of the 9-1-1 complex by Rad17-RFC<sub>2-5</sub> (8–10). We also found that PCNA was unable to stimulate pol  $\beta$  in its elongation of a primer template (Figure 2C, lane 6) and it had no effect on misincorporation by pol  $\beta$  or its strand displacement synthesis on a 1 nt gap template (data not shown). Hence, in spite of recent evidence that PCNA interacts with pol  $\beta$  (46), the increase in pol  $\beta$  affinity for the primer appears to be specific to the 9-1-1 checkpoint complex, rather than clamp-structure-dependent.

From the data presented here, we propose that the 9-1-1 checkpoint complex might have a direct implication in BER. In fact, the 9-1-1 complex increased the affinity of pol  $\beta$  for the 3'-OH end of the primer. This suggests that, once loaded onto sites of DNA damage, the 9-1-1 complex may attract and stabilize pol  $\beta$  to those sites where BER proceeds. Our data are in good agreement with a model very recently proposed by two different groups (9,11) where the 9-1-1 complex and the Rad17-RFC<sub>2-5</sub> clamp loader stabilize stalled replication forks. We propose an analogous mechanism where the 9-1-1 complex could exert a stabilization effect in BER.

Furthermore, the specificity of the functional interaction between the clamp and the pol suggests that it may act as a selection mechanism, recruiting and stimulating a particular pol where its activity is needed. Finally, strand displacement synthesis stimulation by the 9-1-1 complex could increase the efficiency of pol  $\beta$  in long patch BER, where pol  $\beta$  strand displacement synthesis is effective (47).

It remains to be assessed whether the Rad17-RFC<sub>2-5</sub> clamp loader would also have an influence on the observed pol  $\beta$ /9-1-1 interaction. However, analogy between the two clamp/clamp-loader pairs 9-1-1/Rad17-RFC<sub>2-5</sub> and PCNA/RF-C has been demonstrated by several groups, and the 9-1-1 complex is able to slide freely on DNA similarly to PCNA (8,10,11). In the case of the PCNA/RF-C pair, it has been shown that the main function of RF-C is to load PCNA onto DNA, without influencing PCNA affinity for the cognate pol  $\delta$  and  $\epsilon$ . Indeed, in the case of PCNA, the determination of its mechanism of stimulation of pol  $\delta$  and  $\epsilon$  were performed mainly in the absence of RF-C, in order to avoid interference of RF-C binding to single-strand DNA or to the primer terminus, in competition with the pol (44,48–51).

Finally, we would like to stress that our data, given the simplicity of the experimental system used, do not pretend to reflect the complexity of the *in vivo* situation, but rather aimed to highlight what appears to be an intrinsic property of the 9-1-1 complex, namely its ability to interact with and stimulate pol  $\beta$ .

In summary we propose that, *in vivo*, the 9-1-1 complex located at DNA lesions may participate to the recruitment not only of checkpoint effectors for ATM/ATR kinases phosphorylation (1,16–18) but also of DNA repair factors, such as pol  $\beta$ . However, our first attempts to co-immunoprecipitate pol  $\beta$

together with the 9-1-1 complex from cells under normal growth conditions have been unsuccessful. Nevertheless, the 9-1-1 complex has been shown to be recruited onto chromatin only under genotoxic stress conditions. According to this observation, it is conceivable that the interaction between the 9-1-1 complex and pol  $\beta$  may occur in response to DNA damage events, which may explain the absence of a 9-1-1/pol  $\beta$  complex in untreated cells. Consequently, it will be crucial to identify the genotoxic stress conditions, which induce this interaction in cells, and to elucidate the role of the 9-1-1 complex in BER.

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