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# Detecting the ability of viral, bacterial and eukaryotic replication proteins to track along DNA

**Rachel L.Tinker, George A.Kassavetis and E.Peter Geiduschek**

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0634, USA

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**The phage T4 gene 45 protein (gp45), *Escherichia coli* β and the eukaryotic proliferating cell nuclear antigen (PCNA) function in replication as processivity factors of their corresponding DNA polymerases. The T4 gp45 also functions as the transcriptional activator that connects expression of viral late genes to DNA replication. DNA tracking is an essential component of the replication and transcription regulatory functions of T4 gp45. The ability of gp45, β and PCNA to track along DNA has been analyzed by photocrosslinking. Each of these proteins must be loaded onto DNA by a species-specific assembly factor. For gp45 and β, the density of traffic along DNA is determined by a dynamic balance between continuous protein loading and unloading, and is also dependent on interaction with the conjugate single-stranded DNA binding protein.**

*Key words*: DNA tracking proteins/gene regulation/photocrosslinking/processivity factor/replication

## Introduction

Certain proteins that function in DNA recombination and replication assemble as multimeric complexes with central holes that accommodate one or even two DNA helices (Kong *et al.*, 1992; Stasiak *et al.*, 1994; West, 1994). The catenation of these proteins with DNA confers two properties that are important for their functions: (i) they retain 1-D mobility, i.e. they can track along DNA, and (ii) they constrain protein ligands to the vicinity of DNA. Three proteins of this class [the phage T4 gene 45 protein (gp45), a component of the T4 DNA polymerase holoenzyme, the β dimer of the *Escherichia coli* DNA polymerase III holoenzyme and the eukaryotic nuclear DNA polymerase δ-associated proliferating cell nuclear antigen (PCNA)] execute comparable functions in DNA replication. By tethering their respective DNA polymerases, these three proteins confer processivity on replicative DNA chain elongation (Huang *et al.*, 1981; O'Donnell and Studwell, 1990; Studwell and O'Donnell, 1990; Tsurimoto and Stillman, 1990; Kuriyan and O'Donnell, 1993). RuvB protein, which promotes the migration and eventual resolution of recombinational structures (Holliday junctions), also forms a catenane on DNA but has a distinctive structure (Stasiak *et al.*, 1994; West, 1994).

One of these proteins, the T4 gp45, also serves as a transcriptional activator, connecting expression of a large part of the viral genome to DNA replication. The atypical mechanism that is proposed for transcriptional activation by gp45 involves DNA 'entry' or 'loading' at enhancer-like distal sites, facilitated by an assembly factor complex composed of the T4 gene 44 and 62 proteins (gp44–62) and by ATP hydrolysis. Once gp45 has been loaded, it tracks along DNA, encounters RNA polymerase and eventually becomes stably associated with the upstream end of the enhanced promoter complex (Herendeen *et al.*, 1989, 1990, 1992; Tinker *et al.*, 1994).

The structural analysis on which the above picture of transcriptional activation by gp45 is based involves physical mapping of enhanced T4 late promoter complexes by site-specific protein–DNA photocrosslinking (Tinker *et al.*, 1994). These experiments also show a background of general RNA polymerase-independent gp45 photocrosslinking, regardless of the specific location of the photoactive nucleotide. The conditions for generating and eliminating this non-specific effect have led us to suppose that it might be due to crosslinking of gp45 as it tracks along DNA. The experiments that are presented below show that this is the case. We compare the DNA tracking properties of T4 gp45, *E.coli* $\beta_2$ and human PCNA. We show that these three proteins are only loaded onto DNA by their species-specific assembly factors, and also deduce the existence of certain specific interactions with single-stranded DNA binding proteins.

## Results

### Detecting DNA tracking by gp45

DNA for these photocrosslinking experiments was synthesized on single-stranded circular DNA templates by primer extension, essentially as described previously (Tinker *et al.*, 1994). The final products contain a single residue of the photoactive nucleotide 5-[*N'*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP ($N_3$RdUMP; Bartholomew *et al.*, 1990) adjacent to a radioactive nucleotide. Their single-stranded–double-stranded junctions, one of which is a primer–template junction, are the potential assembly sites for the T4 DNA polymerase accessory proteins gp45, 44 and 62. In probes A and B (Figure 1a) the $N_3$RdUMP residue is located 142 or 127 nucleotides, respectively, from the 5′ end of the synthesized DNA strand [except in the 5–10% of DNA molecules in which oligonucleotide 1 (Figure 1a) fails to ligate to the primer extension product of oligonucleotide 2; in these DNA molecules, the photoactive nucleotide is located 78 or 68 nucleotides, respectively, from a nick]. In assembling proteins on this DNA we always included the phage T4 single-stranded DNA binding protein, gp32, so as to favor assembly of the DNA polymerase accessory proteins at double-stranded–single-stranded DNA junctions (Munn and



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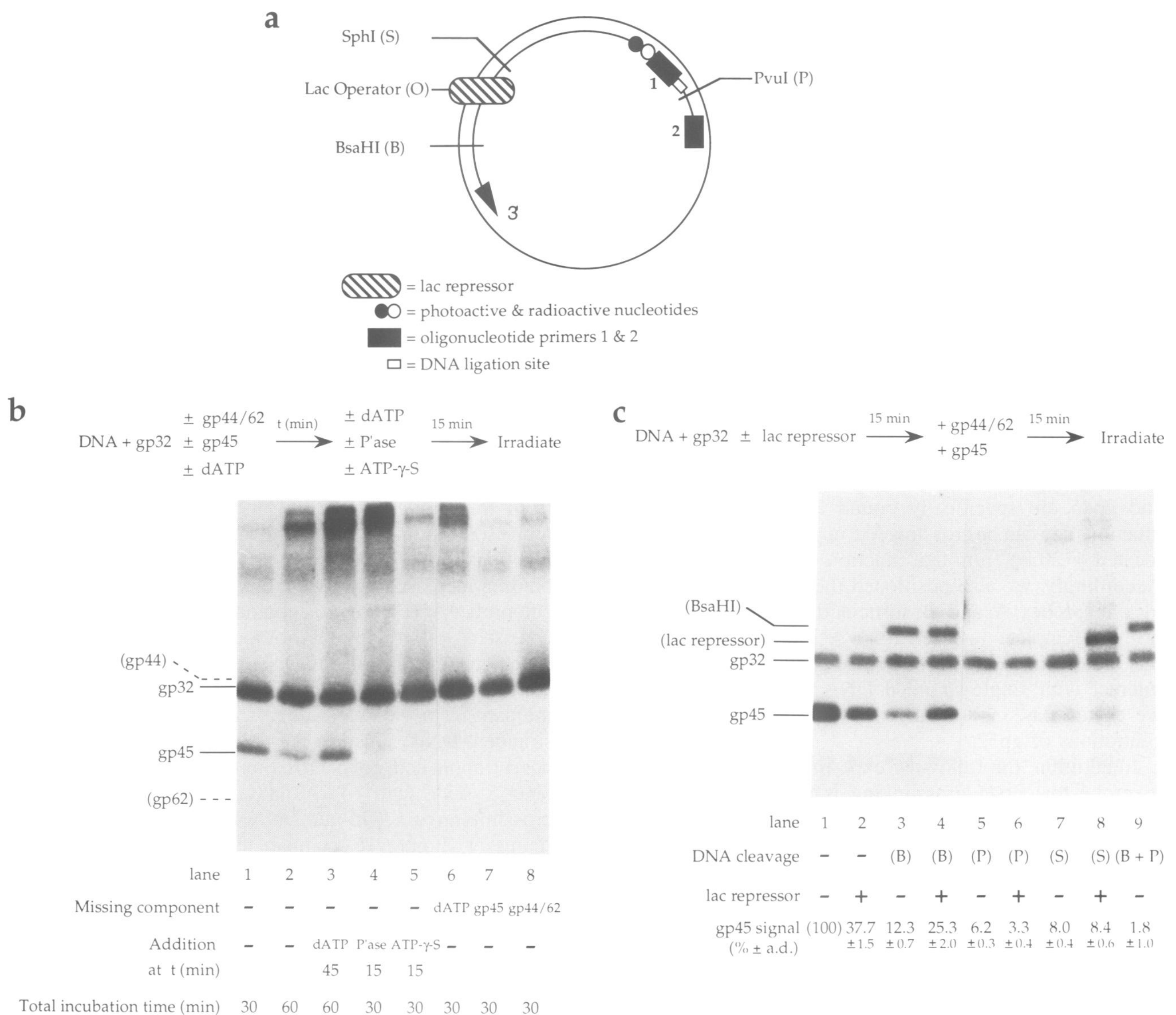


**Fig. 1.** Protein tracking along DNA identified by photocrosslinking. (**a**) Construction of DNA probes (see Materials and methods). Two oligonucleotide primers are used to define the structural parameters of the *in vitro* synthesized DNA. Primer 1 determines the site of insertion of the photoactive and radioactive nucleotide by partial primer extension. Primer 2 determines the location of the 5′ end of the synthesized DNA strand. Symbols are defined in the figure. The following sites are defined in probe A by their distance in bp from the 5′ end of oligonucleotide 2: *Pvu*I (P), 27; ligation site, 64; photoactive nucleotide ($N_3$RdUMP), 141; radioactive nucleotide ([$\alpha$-$^{32}$P]dGMP), 142; *Sph*I(S), 622; *lac* operator (AATTGTGAGCGGATAACAATT), 684; and *Bsa*HI (B), 857. Probe B differs from probe A only in the choice of the oligonucleotide directing the insertion of $N_3$RdUMP and [$\alpha$-$^{32}$P]dGMP (oligonucleotide 1 in a), generating concomitantly a change in the location of the ligation site, 59; $N_3$RdUMP, 127; and [$\alpha$-$^{32}$P]dGMP, 126. (**b**) Photocrosslinking reactions were performed with probe B in the presence of gp32, with or without gp44–62, gp45 and dATP, as indicated below each lane and as described in Materials and methods. Reaction mixtures were incubated at 37°C for 15 (lanes 1 and 4–8) or 45 min (lanes 2 and 3). dATP, shrimp alkaline phosphatase (P′ase), ATPγS or mock reaction buffer was subsequently added, as specified, and reaction mixtures were incubated for an additional 15 min at 37°C, followed by irradiation. $^{32}$P-labeled crosslinked proteins are identified at the side. (**c**) Probe A was linearized with *Bsa*HI (B), *Pvu*I (P), *Sph*I (S) or both *Bsa*HI and *Pvu*I (B + P), as described in Materials and methods. Probe A, either circular or linear, was incubated with gp32 and with or without *lac* repressor for 15 min at 37°C. gp45 and gp44–62 were subsequently added, and incubation continued for 15 min at 37°C, followed by irradiation. Experimental details are specified in Materials and methods.

Alberts, 1991), and to coat the single-stranded DNA so as to diminish the non-specific binding of other proteins. gp32 also generated a standardizing photocrosslinking signal, presumably by non-specifically and transiently binding to DNA in the vicinity of the photoactive nucleotide due to template breathing. When the complete set of components (gp32, gp44–62, gp45 and dATP) was incubated with probe B for 30 min and then irradiated, gp45 was crosslinked to DNA, but gp44 and gp62 were not (Figure 1b, lane 1, and data not shown). Photocrosslinking of gp45 required the presence of gp44–62 complex and dATP (Figure 1b, compare lane 1 with lanes 6–8). The efficiency of gp45 crosslinking remained constant for 30 min (data not shown), subsequently decreased (Figure 1b, compare lane 1 with lane 2), but was restored by adding more dATP (lane 3). This is the result that would be expected if dATP hydrolysis were continuously required to maintain gp45 on DNA and if the DNA-dependent

ATPase activity of gp44–62 were capable of depleting dATP below the required level. Indeed, incubation with alkaline phosphatase abolished crosslinking within 15 min (lane 4), as did addition of ATPγS (lane 5) which competitively inhibits the ATPase activity of gp44–62 (Piperno *et al.*, 1978).

Activation by the DNA polymerase accessory proteins of transcription initiating at T4 late promoters is known to require tracking of protein along DNA (Herendeen *et al.*, 1992). Does the photocrosslinking in Figure 1b detect tracking by gp45, or is it generated by statically bound gp45 that becomes photocrosslinked to a distant nucleotide 'through space' as a result of DNA looping? Two observations argue against the latter possibility: (i) ATPγS, which stabilizes the gp44–62–45 complex with a primer–template junction (Munn and Alberts, 1991), blocked gp45 crosslinking (lane 5); and (ii) only gp45 was crosslinked in these experiments, but gp44 and gp62 were not (Figure 1b and data not shown). When gp44–62 and gp45 are specifically bound at a primer–template junction they can be crosslinked to a photoactive nucleotide located 4–20 bp from that junction (Capson *et al.*, 1991). Accordingly, we also positioned the photoactive nucleotides in probes A and B sufficiently far away from the primer–template junction (and residual nicks) to avoid this background (Figure 1a); since gp45 and gp44–62 also interact with single-stranded DNA (Jarvis *et al.*, 1989), we coated the single-stranded DNA with saturating concentrations of gp32.

In addition, the following experiments were performed to prove that gp45 crosslinking is a consequence of its tracking along DNA. The experimental design follows previous work on transcriptional activation by the T4 DNA polymerase accessory proteins which has shown that: (i) transcriptional activation requires tracking of protein along DNA; (ii) DNA nicks serve as assembly and entry sites for gp45 in a gp44–62-dependent and dATP (or ATP) hydrolysis-dependent process (Herendeen *et al.*, 1989, 1992); and (iii) blunt or short (two or four nucleotides) overhanging DNA ends serve as exit sites, allowing tracking proteins to fall off DNA (Stukenberg *et al.*, 1991), and are not efficient for transcriptional activation (Herendeen *et al.*, 1989). DNA tracking can be blocked by tightly and stably bound protein (Stukenberg *et al.*, 1991; Herendeen *et al.*, 1992). We verified that photocrosslinking of gp45 requires tracking along DNA by examining the effects of cutting probe A with different restriction endonucleases, and of binding *lac* repressor to its operator on photocrosslinking. (The corresponding sites on the DNA are shown in Figure 1a.) If distantly bound gp45 could be crosslinked as a consequence of DNA looping, DNA cut at a single restriction site would still have gp45 statically bound in *cis* to the photoactive nucleotide; only doubly cutting DNA (to sever the connection to both single-stranded–double-stranded DNA junctions) should dramatically diminish crosslinking. On the other hand, if gp45 crosslinking was due to tracking on DNA, linearization should diminish gp45 loading (since one of the two gp45 loading sites is separated from the site of crosslinking by a long stretch of gp32-bound single-stranded DNA) and moreover allow gp45 to slide off the cut DNA ends, and should consequently diminish photocrosslinking of gp45. gp45 crosslinking was, in fact, reduced on all linearized DNA templates (Figure 1c, compare lanes 3, 5 and 7 with lane 1). The quantitatively different effects of cutting with *Bsa*HI and *Pvu*I on photocrosslinking of gp45 may reflect different efficiencies of loading or unloading gp45 at the two double-stranded–single-stranded DNA junctions, as well as effects of the newly created DNA ends on unloading gp45. These results are consistent with photocrosslinking of gp45 as it tracks along DNA and are not readily rationalized in terms of a DNA looping mechanism. Nevertheless, it remained remotely conceivable that conversion of circular to linear DNA might reduce DNA looping sufficiently to account for the observed decrease in photocrosslinking. The experiment that follows, in which the *lac* repressor was used to obstruct tracking along DNA, eliminates this possibility.

*lac* repressor binding to intact probe A reduced the efficiency of gp45 crosslinking by ~60% (Figure 1c, compare lane 1 with lane 2), implying that gp45 tracking from the primer–template junction is blocked by *lac* repressor, but that both single-strand–double-strand DNA junctions serve as loading sites for gp45. *lac* repressor binding increased gp45 crosslinking efficiency on *Bsa*HI-cut probe A (Figure 1c, compare lane 3 with lane 4), consistent with the repressor blocking the path of gp45 to its exit at the *Bsa*HI-cut DNA end. In contrast, placing *lac* repressor on the *Pvu*I-cut probe, where it would block the path from the entry site at a single-stranded–double-stranded DNA junction to the photoactive nucleotide, nearly abolished gp45 crosslinking (Figure 1c, compare lane 5 with lane 6). As a control, we showed that crosslinking on *Sph*I-cut DNA was not reduced by *lac* repressor (Figure 1c, compare lane 7 with lane 8), suggesting that the latter does not intrinsically and non-specifically interfere with gp45 tracking or photocrosslinking. Since probes A and B efficiently tagged gp45, we concluded that crosslinking was not sequence-specific but was instead random, supporting the argument that gp45 is accessible to photocrosslinking as it tracks along DNA. (Crosslinking of *lac* repressor and *Bsa*HI, which is readily noted in Figure 1c, was not analyzed further, although it might have interesting implications.)

In summary, these experiments provide strong evidence that: (i) tracking of gp45 along DNA is detected by photocrosslinking; (ii) loading of this protein onto DNA requires dATP hydrolysis and gp44–62; and (iii) gp45 is continuously loaded onto, and unloaded from, DNA under the conditions tested.

### β and PCNA

The β-dimer sub-assembly of the *E.coli* DNA polymerase III holoenzyme and the PCNA cofactor of the nuclear DNA polymerase δ are bacterial and eukaryotic homologs of gp45; the multiprotein *E.coli* DNA polymerase III holoenzyme γ complex (subunits γ, δ, δ′, χ and ψ) and replication factor RF-C are the functional bacterial and eukaryotic homologs of gp44–62. $\beta_2$ is known to be a DNA tracking protein (Stukenberg *et al.*, 1991) and evidence for DNA tracking by PCNA has also been presented (Burgers and Yoder, 1993). We were interested in extending the analysis of photocrosslinking to $\beta_2$ and PCNA, and are grateful to M.O'Donnell, D.R.Herendeen, G.Brush, T.J.Kelly and M.S.Wold for generous gifts of

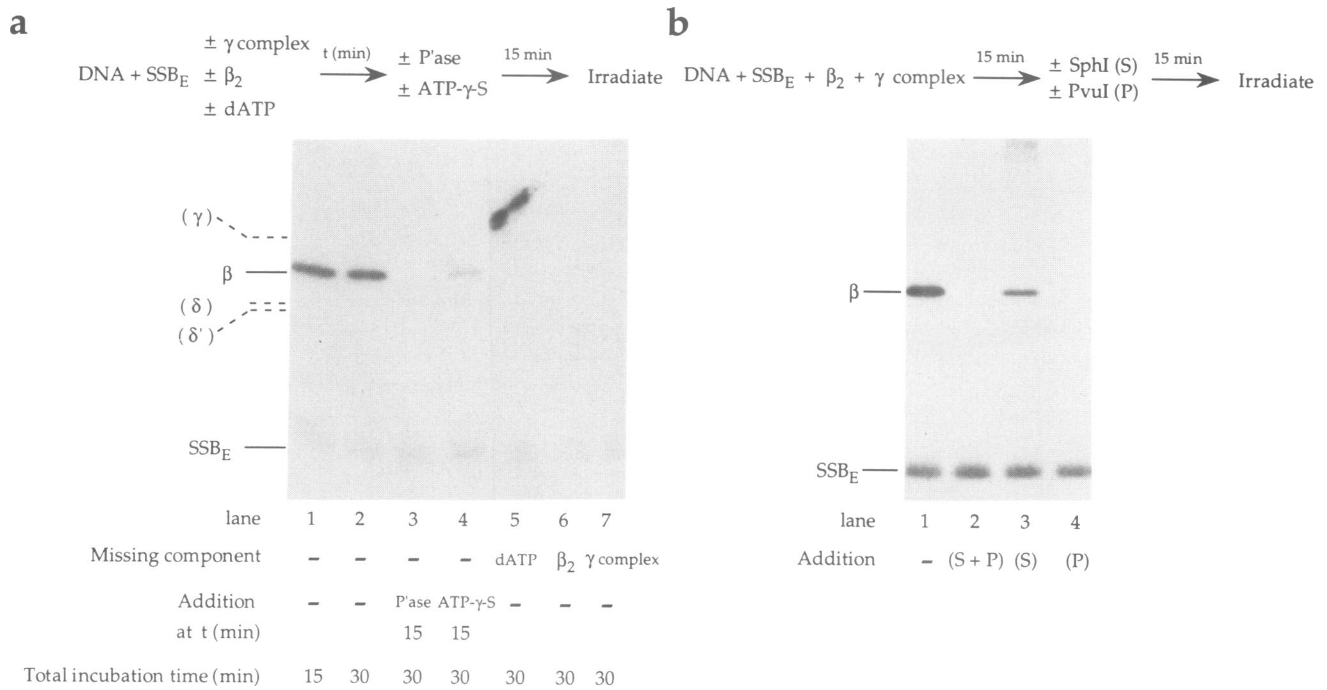


**Fig. 2.** Detection of tracking by *E.coli* $\beta_2$ by photocrosslinking. (**a**) Protein complexes were formed on probe B in the presence of *E.coli* SSB ($SSB_E$), with or with out $\beta_2$, γ complex and dATP, as noted below each lane, for 15 min at 37°C. The reaction mixture in lane 1 was subsequently irradiated. As specified, phosphatase, ATPγS or mock buffer was added to the remaining samples and mixtures were incubated for an additional 15 min at 37°C before irradiation. (**b**) Probe B was incubated with $SSB_E$, $\beta_2$ and the γ complex for 15 min at 37°C and then linearized by the addition of *Sph*I, *Pvu*I or both *Sph*I and *Pvu*I, as noted below each lane, for 15 min at the same temperature. Other experimental details are specified in Materials and methods.

materials for the experiments that are described below. When $\beta_2$, γ complex, *E.coli* single-stranded DNA binding protein ($SSB_E$) and dATP were incubated with probe B for 15 or 30 min and then irradiated, β was photocrosslinked but the components of the γ complex were not (Figure 2a, lanes 1 and 2). Photocrosslinking of β depended on the presence of γ complex and dATP (lanes 5–7), and was also sensitive to the addition of phosphatase and ATPγS (Figure 2a, compare lanes 1 and 2 with lanes 3 and 4, respectively). In contrast to what was found with gp45, subsequently added ATPγS reduced, but did not eliminate, β crosslinking (lane 4). However, when ATPγS was substituted for dATP in the initial reaction mixture, there was no crosslinking of β (data not shown), consistent with a hydrolyzable form of ATP being required to load $\beta_2$ onto DNA (Fradkin and Kornberg, 1992). When DNA was preloaded with $\beta_2$ for 15 min and then cut with *Sph*I, *Pvu*I or both restriction endonucleases for 15 min before irradiation, β photocrosslinking diminished (Figure 2b). These findings are consistent with the detection of $\beta_2$ tracking along DNA by photocrosslinking. Although the quantitative differences between Figures 1 and 2 suggest differences between the properties of gp45 and $\beta_2$ tracking assemblies on DNA, these differences were less extreme than we had anticipated on the basis of prior work (Stukenberg *et al.*, 1991). Under reaction conditions very similar to ours, $\beta_2$ remains bound to DNA after separation from γ complex and ATP, incubation and subsequent gel filtration (altogether requiring >20 min) at 5°C (Stukenberg *et al.*, 1991), but dissociates from DNA more rapidly at 37°C, consistent with the results in Figure 2a (M.O'Donnell, personal communication). Moreover, the continued presence of the γ complex in our experiments (Figure 2b) and its removal by gel filtration in prior work could generate different rates of $\beta_2$ unloading.

When *E.coli* $\beta_2$ was found to have the form of a donut with a central hole large enough to accommodate a DNA duplex, it was proposed that its functional homologs, the PCNA trimer and the gp45 trimer, would turn out to have generally similar structures (Kong *et al.*, 1992). This has now been shown for PCNA (T.S.R.Krishna and J.Kuriyan, personal communication). To examine by photocrosslinking whether PCNA tracks on DNA, we assembled it on DNA probe A in the presence of RF-C, dATP and single-stranded DNA binding protein. The *E.coli* $SSB_E$ has been successfully substituted for the three-subunit human SSB, RPA, in SV40 DNA-leading strand replication (Kenny *et al.*, 1989), and we found that both $SSB_E$ (Figure 3a) and RPA (Figure 3b) were compatible with photocrosslinking of PCNA. Irradiation after a 15 or 30 min incubation photocrosslinked PCNA (Figure 3a, lanes 1 and 2). Crosslinking of PCNA was dependent on RF-C and dATP (Figure 3a, lanes 7–9). In contrast to what was seen with gp45 and $\beta_2$, crosslinking of PCNA was not eliminated by the subsequent addition of phosphatase or ATPγS (Figure 3a, lanes 3 and 4). When ATPγS was added first and followed by dATP (Figure 3a, lane 6), it did inhibit PCNA crosslinking, albeit weakly. In fact, RF-C even generated relatively inefficient PCNA crosslinking with ATPγS alone (Figure 3a, lane 5). It may be that ATPγS stabilizes the RF-C–PCNA complex at a primer–template junction (Tsurimoto and Stillman, 1991a), and allows some release of PCNA for DNA tracking without the need for ATP hydrolysis. We also noted an additional protein,

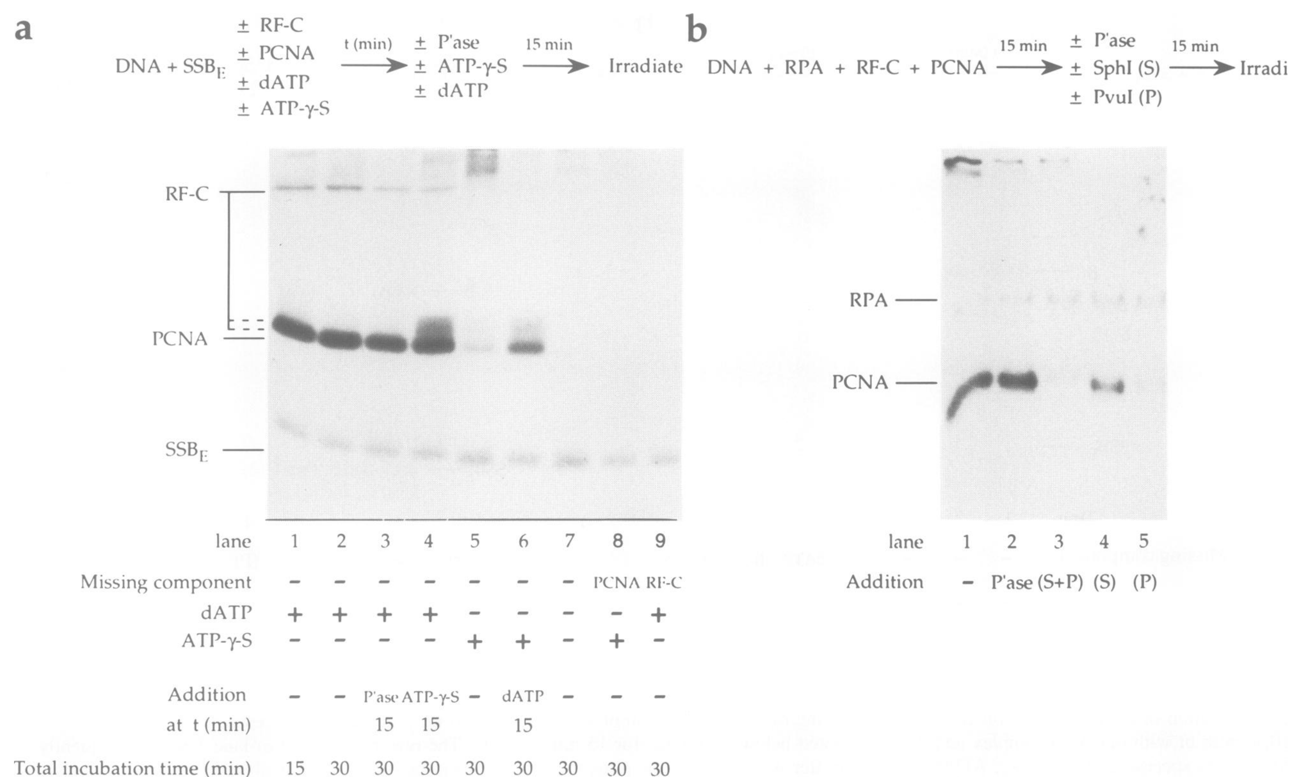


**Fig. 3.** Photocrosslinking detects PCNA tracking along DNA. (**a**) Photocrosslinking reaction mixtures containing probe A and $SSB_E$ were incubate with or without RF-C and PCNA, and in the presence or absence of dATP or ATPγS, for 15 min at 37°C. The sample in lane 1 was subsequently irradiated; the remaining samples were treated with either phosphatase, ATPγS, dATP or reaction buffer, further incubated for 15 min at 37°C and then irradiated. (**b**) Probe A was incubated with RPA, RF-C and PCNA for 15 min at 37°C. Mock reaction buffer, phosphatase, *Sph*I, *Pvu*I or both *Sph*I and *Pvu*I, was added to the reaction mixture, as indicated below each lane, and the reaction was allowed to proceed for 15 min at 37°C, followed by irradiation. Other experimental details are specified in Materials and methods.

slightly larger than PCNA, crosslinking to DNA. This is probably one of the smaller (36.5–41.0 kDa) subunits of RF-C; crosslinking only occurred in the presence of PCNA and ATPγS (Figure 3a, compare lanes 4–6 with lanes 1 and 8). RF-C is known to bind strongly to single-stranded DNA in the presence of PCNA and ATPγS (Tsurimoto and Stillman, 1991a,b); the formation of such a complex in the vicinity of the photoactive nucleotide might be facilitated by DNA template breathing that is, in turn, favored by PCNA (Figure 3a, compare lanes 4 and 6 with lane 5).

We further examined the retention of PCNA on DNA once it has been loaded. When probe A was preloaded with PCNA and subsequently linearized with *Sph*I, *Pvu*I or both endonucleases, PCNA crosslinking diminished (Figure 3b, compare lane 1 with lanes 3–5). Yet, as already stated, PCNA was retained on DNA after phosphatase treatment (in the presence of RPA or $SSB_E$; Figure 3b, compare lane 1 with lane 2, and Figure 3a, compare lane 1 with lane 3). N.Yao, Z.Kelman, Z.Dong and M.O'Donnell have also detected DNA tracking by PCNA, using a gel filtration method applied originally to the analysis of DNA tracking by $\beta_2$ (Stukenberg *et al.*, 1991; M.O'Donnell, personal communication).

Each of the three DNA tracking proteins (gp45, $\beta_2$ and PCNA) rigorously required the cognate assembly factor, gp44–62, γ complex and RF-C, respectively, for loading onto DNA (Table I). This is consistent with, and provides an explanation for, previous reports on the specificity of function of these components in SV40 DNA replicati (Tsurimoto *et al.*, 1990). It was more surprising to fi (Figure 4) that loading of gp45 (lanes 1–4) and β (lan 5–8), but not PCNA (lanes 9–12), also required th cognate single-stranded DNA binding protein. When RF and PCNA were used at limiting concentrations, RPA d not dramatically increase the efficiency of PCNA loadi over the other single-stranded DNA binding proteins (da not shown). No photocrosslinking of any DNA tracki protein was seen in the absence of single-stranded DN binding protein (lanes 1, 5 and 9), probably due

**Table I.** Loading of the DNA tracking protein requires the homologous assembly factor

| Assembly factor | DNA tracking protein | | |
|---|---|---|---|
| | gp45 | $\beta_2$ | PCNA |
| gp44–62 | + | – | – |
| γ complex | – | + | – |
| RF-C | – | – | + |

Photocrosslinking assays were performed as described in Materials a methods. For experiments with gp44–62 and the γ complex, the sing stranded DNA binding protein was gp32 and $SSB_E$, respectively. Fo experiments with RF-C, each of the SSBs (gp32, $SSB_E$ or RPA) yielded qualitatively the same outcome. When gp44–62 and $SSB_E$, c the γ complex and gp32, were incubated with gp45, $\beta_2$ or PCNA, nc of the tracking proteins were loaded (Figure 4 and data not shown). (+) the DNA tracking protein was crosslinked; (–) no crosslinking the DNA tracking protein was detected.

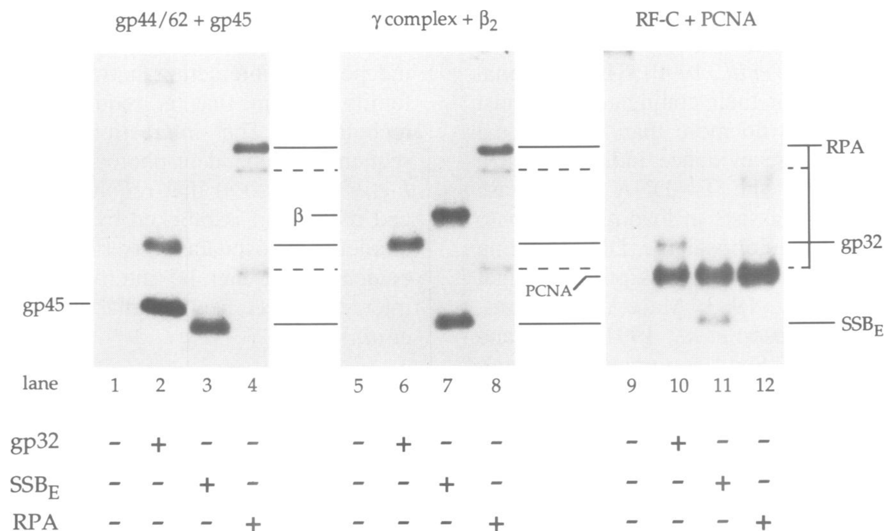


**Fig. 4.** Species-specific effects of single-stranded DNA binding proteins on the loading/retention of tracking proteins. Protein mixtures containing either gp44–62 and gp45, γ complex and $\beta_2$, or RF-C and PCNA, were assembled on probe B that was preincubated on ice with either gp32, $SSB_E$ or RPA (bringing the final volume to 15 μl). After 15 min at 37°C, samples were irradiated and processed. Crosslinked protein–DNA complexes were resolved on a Tricine–SDS–9.8% polyacrylamide (32:1 acrylamide:bisacrylamide) gel with a Tricine–SDS–3.8% polyacrylamide (32:1 acrylamide:bisacrylamide) stacking gel overlay (Schagger and von Jagow, 1987). Crosslinked proteins are identified at the sides. Experimental details are specified in Materials and methods.

sequestration by single-stranded DNA to which all of these DNA polymerase accessory proteins are known to bind (Jarvis *et al.*, 1989; Tsurimoto and Stillman, 1991b; Fradkin and Kornberg, 1992). We suggest that the ability of the homologous single-stranded DNA binding protein to selectively stimulate DNA loading of gp45 and β, and/or diminish unloading, reflects specific protein–protein interactions at the single-stranded–double-stranded DNA junctions at which gp45 and β are assembled onto DNA by their respective assembly factors.

## Discussion

The gene 45 protein is accessible to photocrosslinking by $N_3$RdUMP as it tracks along DNA. The presence of the arylazido side chain at an interior site of the photoprobe does not interfere with DNA tracking-dependent transcriptional activation (Tinker *et al.*, 1994) and does not prevent detection of DNA tracking by crosslinking. We have not examined whether $N_3$RdUMP perturbs DNA tracking traffic; it is even conceivable that such perturbations would generate a greater efficiency of crosslinking than might otherwise exist. The prokaryotic and eukaryotic cellular homologs of gp45 (the dimeric *E.coli* β and the trimeric human PCNA) can also be detected in this way. Each of these three proteins requires its conjugate assembly factor and dATP (functioning as a hydrolyzable analog of ATP). Complexes of T4 gp45, 44 and 62 with primer–template junctions are stabilized by the non-hydrolyzable ATPγS (Munn and Alberts, 1991). Thus, ATP hydrolysis must be required for release of gp45 from the gp44–62 complex in a tracking-competent state. Neither gp44 nor gp62 is efficiently crosslinked to interior-located $N_3$RdUMP. The tracking-competent state of gp45, presumably as a catenated protein ring on circular, partly double-stranded and partly single-stranded DNA, is not stable over the time scale of our experiments, and so ATP hydrolysis is continuously required to retain the balance between gp45 loading and unloading (Figure 1b). This lability distinguishes DNA-tracking gp45 from enhanced T4 late promoter complex-associated gp45 (Tinker *et al.*, 1994). The presumptively catenated gp45 tracking complex with double-stranded DNA is also not stable to gel filtration at 5°C and in the presence of relatively high electrolyte concentrations, whereas the promoter complex-associated gp45 is sufficiently stable to be detected (Tinker *et al.*, 1994). Thus, the T4 late gene-transcribing RNA polymerase holoenzyme and the sliding clamp of DNA replication mutually stabilize their associations with DNA.

Gogol *et al.* (1992) have used cryoelectron microscopy to visualize complexes formed between gp44–62, gp45 and DNA. Structures, termed hash marks, were seen distributed along DNA when gp44–62, gp45 and ATP were incubated with nicked or gapped DNA. The number of these hash marks increased in the presence of gp32, and they disappeared within minutes if ATP was removed, indicating that they had a relatively short life-time. The conditions for the formation of these structures, their estimated molecular size and the data that are presented here are consistent with the suggestion that these hash marks are indeed gp45 trimers tracking along DNA.

*lac* repressor only incompletely blocks photocrosslinking (Figure 1c), suggesting that gp45 loading is not confined to primer–template junctions but also occurs at the other single-stranded–double-stranded DNA junctions of these constructs. Blunt or short (2 bp) 3′ or 5′ overhanging DNA ends do not allow efficient loading of gp45 (Figure 1c, lane 9), consistent with their failure to allow transcriptional enhancement (Herendeen *et al.*, 1989, 1992). Short overhanging DNA ends also facilitate gp45 unloading (Figure 1c, compare lanes 3, 5 and 7 with lane 1) under our experimental conditions.

The requirement of each of these three DNA tracking replication proteins for its conjugate assembly factor

indicates specificities of protein–protein interaction, and is sufficient to account for non-interchangeability in DNA replication *in vitro* (Tsurimoto *et al.*, 1990). The additional specificity of gp45 and $\beta_2$ for their conjugate SSB must mean that the latter proteins do more than merely coat single-stranded DNA. Diverse evidence indicates that a specific interaction between the T4 DNA polymerase accessory proteins and gp32 assists in loading gp45 onto DNA, and that the general, non-specific DNA binding activity of the SSB is insufficient for this process (Sigal *et al.*, 1972; Formosa *et al.*, 1983; Mace and Alberts, 1984; Jarvis *et al.*, 1989; Capson *et al.*, 1991; Munn and Alberts, 1991; S.Moody and G.Sanders, unpublished data). Thus, these SSB may be able to facilitate loading of the conjugate tracking protein onto DNA, interfere with unloading, or both. [The specific requirement for participation of $SSB_E$ in DNA loading of $\beta_2$ by the $\gamma$ complex is not absolute. Photocrosslinking of $\beta$ in the presence of gp32 or in the entire absence of any single-stranded DNA binding protein has been detected at ~20-fold higher than standard concentrations of $\beta_2$ and $\gamma$ complex (data not shown).] It is also known that RPA is specifically required for lagging strand DNA synthesis by polymerase $\alpha$ in the SV40 replication system and cannot be substituted by $SSB_E$ or T4 gp32 (Kenny *et al.*, 1989).

PCNA is distinctive in the substantial persistence of its crosslinking to DNA after depletion of dATP with phosphatase (Figure 3a, lane 3, and b, lane 2), but the substantial elimination of crosslinking upon cutting with *Pvu*I, or *Pvu*I and *Sph*I together (Figure 3b), implies that PCNA does not unload quickly at double-stranded–single-stranded DNA junctions that are 'sealed' with $SSB_E$ or RPA. PCNA is also functionally distinctive in that it participates in actively blocking the progression of the initiator DNA-generating DNA polymerase $\alpha$ (Waga and Stillman, 1994) and is required in DNA excision repair (Shivji *et al.*, 1992).

In closing, we point out that the intrinsic properties of the protein catenanes that are loaded onto DNA concomitantly with replication, recombination and perhaps also repair (Kong *et al.*, 1992; Shivji *et al.*, 1992; Grossman and Thiagalingam, 1993; Stasiak *et al.*, 1994; West, 1994), confer interesting potentialities for gene regulation that have only been explored in connection with phage T4 development, but should be expected to operate more broadly. The photocrosslinking analysis presented here provides a very sensitive and relatively straightforward method for analyzing and following the DNA tracking properties of these proteins, even for DNA tracking that may be too ephemeral to detect by other means. For example, attempts in our laboratory (Tinker *et al.*, 1994) and by others (M.O'Donnell, personal communication) to detect gp45 tracking by gel filtration have been unsuccessful. Through their interactions with protein ligands, the protein catenanes are able to recruit a variety of potential effectors to the vicinity of, and to traffic along, DNA. For example, PCNA is known to interact not only with the core eukaryotic replication apparatus, but also with D-type cyclins, with a cyclin-dependent protein kinase (CDK) and with the p21 CDK-regulator protein (Xiong *et al.*, 1992; Matsuoka *et al.*, 1994; Waga and Stillman, 1994). DNA photocrosslinking analysis can be used to demonstrate specific interactions between protein catenanes and such protein ligands. In experiments along these lines we have, for example, detected a direct RNA polymerase-independent interaction between gp45 and the gp55 $\sigma$-family protein that is required for T4 late promoter recognition. The interaction is manifested as DNA sequence-independent photocrosslinking of gp55 (but not *E.coli* $\sigma^{70}$ or $\sigma^{32}$) that requires gp45, gp44–62 complex and dATP, and is blocked by subsequently added ATP$\gamma$S or alkaline phosphatase (see Figure 1b). Suppressor genetic evidence that can be interpreted in terms of such an interaction has been available for many years (Coppo *et al.*, 1975).

## Materials and methods

### *Proteins and DNA*

The purification of gp45 and gp44–62 has been described previously (Morris *et al.*, 1979). We are grateful to M.O'Donnell (Cornell University School of Medicine) for $\beta_2$ and $\gamma$ complex, prepared as described previously (Johnson *et al.*, 1986; Maki and Kornberg, 1988), to T.Kelly, G.Brush and D.Herendeen (John Hopkins Medical School) for RF-C and PCNA, prepared as described previously (Prelich *et al.*, 1987; Tsurimoto and Stillman, 1989) and to M.Wold (University of Iowa) for RPA (Henricksen *et al.*, 1994). T4 gene 32 protein (gp32; BMB), single-stranded DNA binding protein (*E.coli* $SSB_E$; Stratagene), shrimp alkaline phosphatase (US Biochemical), *Sph*I, *Pvu*I and *Bsa*HI (Promega) were purchased. The construction of photocrosslinking probe A, previously designated as the '−39' probe, has been described (Tinker *et al.*, 1994). Briefly, the appropriate oligonucleotide 1 (Figure 1a) was hybridized to single-stranded pDH2 DNA, initially extended with 5-[*N'*-(*p*-azidobenzoyl)-3-aminoallyl]-dUTP ($N_3$RdUTP) and [$\alpha$-$^{32}$P]dGTP by exonuclease-free Klenow fragment DNA polymerase I (USB) in the presence of *E.coli* SSB, and subsequently chased with four dNTPs, as described previously (Tinker *et al.*, 1994). The reaction was stopped and inactivated protein and deoxynucleotides were removed from the sample by gel filtration on a Sephacryl S-400 (Pharmacia) spun column, as described (Tinker *et al.*, 1994). Oligonucleotide 2 was annealed to its upstream site (Figure 1a) in pDH2 and was extended and ligated to oligonucleotide 1 by adding ATP, all four dNTPs, T4 DNA polymerase (Gibco) and T4 DNA ligase (USB), as described (Tinker *et al.*, 1994). The reaction was stopped and the final DNA product was purified by two passages through Sephacryl S-400. The efficiency of ligation of probes varied between 90 and 95%. The final product (Figure 1a) was fully replicated past the *Bsa*HI site, but no completely replicated, double-stranded (i.e. ligatable) product was detected.

### *Standard photocrosslinking experiment*

Proteins were assembled on 4 fmol of photoactive DNA probe A or probe B (Figure 1a) for the indicated times at 37°C in 13 μl of reaction buffer containing 200 mM K acetate, 33 mM Tris acetate, pH 8, 10 mM Mg acetate, 0.8% (v/v) glycerol, 100 μg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.25 mM dATP (unless omitted or substituted by ATP$\gamma$S, as noted). When subsequent additions are specified, 2 μl of either mock reaction buffer, dATP (2.1 mM), shrimp alkaline phosphatase (P'ase) (0.2 U/μl), ATP$\gamma$S (3.75 mM), *Sph*I (0.5 U/μl), *Pvu*I (0.5 U/μl) or both *Sph*I and *Pvu*I (0.5 U/μl of each) were added, bringing the volume to 15 μl; the reaction mixture was incubated for an additional 15 min at 37°C. (DNA cleavage was 90–95% complete.) Samples were then irradiated at 254 nm for 4 min by setting the 1.5 ml polypropylene microcentrifuge tubes with their tops open at a distance of 60 cm from three 15 W germicidal lamps. The light intensity at this distance from the light source was previously measured to be 380 μW/cm$^2$ with an IL 1500 research radiometer (Bartholomew *et al.*, 1991). The samples were then processed by digestion with DNase I and S1 nuclease, as described previously (Tinker *et al.*, 1994). Crosslinked protein–DNA complexes were resolved by electrophoresis in SDS–12% polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels (unless otherwise indicated), silver stained, dried and subjected to autoradiography using intensifying screens.

The following quantities of proteins were used, as appropriate: 1.8 pmol of gp45 (24.8 kDa), 3.3 pmol of gp44–62 complex (subunits 35.7 and 21.3 kDa, respectively), 23 pmol of gp32 (33 kDa), 200 fmol

of β (37 kDa), 8 fmol of γ complex [subunits γ (52 kDa), δ (35 kDa), δ′ (33 kDa), χ (15 kDa) and ψ (12 kDa)], 40 pmol of *E.coli* SSB ($SSB_E$; 18.5 kDa), 17 fmol of human RF-C (subunits 140, 41, 38, 37 and 36.5 kDa; Pan and Hurwitz, 1993), 218 fmol of human PCNA (28.7 kDa) and 2.6 pmol of human RPA (subunits 70, 32 and 14 kDa). For the purpose of expressing protein quantity, gp45 and PCNA are taken to be trimeric (Jarvis *et al.*, 1989; Bauer and Burgers, 1990; Kong *et al.*, 1992), each molecule of gp44–62 complex to contain four monomers of gp44 and one of gp62 (Jarvis *et al.*, 1989), and the β subunit to be dimeric ($\beta_2$).

#### *Photocrosslinking with tracking along DNA blocked by lac repressor*

Photocrosslinking templates were linearized by treating 100 fmol of DNA probe A with 25 U of either *Bsa*HI, *Pvu*I, *Sph*I or with a combination of these restriction enzymes, for 1 h at 37°C in the appropriate buffer. Reaction mixtures were heated at 60°C for 15 min and stored at −20°C for later use. The efficiency of cutting was ~95%. In the photocrosslinking experiment, DNA was preincubated with gp32, with or without 7.5 ng of *lac* repressor (Stratagene; 48 fmol of tetramer) for 15 min at 37°C in standard reaction conditions. gp45 and gp44–62 were then added (bringing the final volume to 15 μl) and incubation continued for 15 min at the same temperature. The radioactivity in crosslinked gp45 was quantified by PhosphorImager analysis (software from Scanalytics) and values were normalized to the radioactivity in crosslinked gp32 in the same sample. The normalized efficiency of gp45 crosslinking is presented as the average of three experimental determinations (± the average deviation).

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## References

Bartholomew,B., Kassavetis,G.A., Braun,B.R. and Geiduschek,E.P. (1990) *EMBO J.*, **9**, 2197–2205.

Bartholomew,B., Kassavetis,G.A. and Geiduschek,E.P. (1991) *Mol. Cell. Biol.*, **11**, 5181–5189.

Bauer,G.A. and Burgers,P.M. (1990) *Nucleic Acids Res.*, **18**, 261–265.

Burgers,P.M. and Yoder,B.L. (1993) *J. Biol. Chem.*, **268**, 19923–19926.

Capson,T.L., Benkovic,S.J. and Nossal,N.G. (1991) *Cell*, **65**, 249–258.

Coppo,A., Manzi,A. and Pulitzer,J.F. (1975) *J. Mol. Biol.*, **96**, 601–624.

Formosa,T., Burke,R.L. and Alberts,B.M. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 2442–2446.

Fradkin,L. and Kornberg,A. (1992) *J. Biol. Chem.*, **267**, 10318–10322.

Gogol,E.P., Young,M.C., Kubasek,W.L., Jarvis,T.C. and von Hippel,P.H. (1992) *J. Mol. Biol.*, **224**, 395–412.

Grossman,L. and Thiagalingam,S. (1993) *J. Biol. Chem.*, **268**, 16871–16874.

Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) *J. Biol. Chem.*, **269**, 11121–11132.

Herendeen,D.R., Kassavetis,G.A., Barry,J., Alberts,B.M. and Geiduschek,E.P. (1989) *Science*, **245**, 952–958.

Herendeen,D.R., Williams,K.P., Kassavetis,G.A. and Geiduschek,E.P. (1990) *Science*, **248**, 573–578.

Herendeen,D.R., Kassavetis,G.A. and Geiduschek,E.P. (1992) *Science*, **256**, 1298–1303.

Huang,C.C., Hearst,J.G. and Alberts,B.M. (1981) *J. Biol. Chem.*, **254**, 4087–4094.

Jarvis,T.C., Paul,L.S., Hockensmith,J.W. and von Hippel,P.H. (1989) *J. Biol. Chem.*, **264**, 12717–12729.

Johnson,K.O., Haynes,T.E. and McHenry,C.S. (1986) *J. Biol. Chem.*, **261**, 11460–11465.

Kenny,M., Lee,S. and Hurwitz,J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 9757–9761.

Kong,X.P., Onrust,R., O'Donnell,M. and Kuriyan,J. (1992) *Cell*, **69**, 425–437.

Kuriyan,J. and O'Donnell,M. (1993) *J. Mol. Biol.*, **234**, 915–925.

Mace,D. and Alberts,B.M. (1984) *J. Mol. Biol.*, **177**, 279–293.

Maki,S. and Kornberg,A. (1988) *J. Biol. Chem.*, **263**, 6555–6560.

Matsuoka,S., Yamaguchi,M. and Matsukage,A. (1994) *J. Biol. Chem.*, **269**, 11030–11036.

Morris,C.F., Hama-Inaba,H., Mace,D., Sinha,N.K. and Alberts,B.M. (1979) *J. Biol. Chem.*, **254**, 6787–6796.

Munn,M.M. and Alberts,B.M. (1991) *J. Biol. Chem.*, **266**, 20024–20033.

O'Donnell,M. and Studwell,P. (1990) *J. Biol. Chem.*, **265**, 1179–1187.

Pan,Z.Q. and Hurwitz,J. (1993) *J. Biol. Chem.*, **268**, 20433–20442.

Piperno,J.R., Kallen,R.G. and Alberts,B.M. (1978) *J. Biol. Chem.*, **253**, 5180–5185.

Prelich,G., Kostura,M., Marshak,D.R., Mathews,M.B. and Stillman,B. (1987) *Nature*, **326**, 471–475.

Schagger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.

Shivji,K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.

Sigal,N., Delius,H., Kornberg,T., Gefter,M. and Alberts,B.M. (1972) *Proc. Natl Acad. Sci. USA*, **69**, 3537–3541.

Stasiak,A., Tsaneva,I., West,S., Benson,C., Yu,X. and Egelman,E. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7618–7622.

Studwell,P. and O'Donnell,M. (1990) *J. Biol. Chem.*, **265**, 1171–1178.

Stukenberg,P.T., Studwell,V.P. and O'Donnell,M. (1991) *J. Biol. Chem.*, **266**, 11328–11334.

Tinker,R.L., Williams,K.P., Kassavetis,G.A. and Geiduschek,E.P. (1994) *Cell*, **77**, 225–237.

Tsurimoto,T. and Stillman,B. (1989) *Mol. Cell. Biol.*, **9**, 609–619.

Tsurimoto,T. and Stillman,B. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 1023–1027.

Tsurimoto,T. and Stillman,B. (1991a) *J. Biol. Chem.*, **266**, 1950–1960.

Tsurimoto,T. and Stillman,B. (1991b) *J. Biol. Chem.*, **266**, 1961–1968.

Tsurimoto,T., Melendy,T. and Stillman,B. (1990) *Nature*, **346**, 534–539.

Waga,S. and Stillman,B. (1994) *Nature*, **369**, 207–212.

West,S. (1994) *Cell*, **76**, 9–15.

Xiong,Y., Zhang,H. and Beach,D. (1992) *Cell*, **71**, 505–514.

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