Dynamics of DNA-tracking by two sliding-clamp proteins

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Bacteriophage T4 gene 45 protein (gp45) and *Escherichia coli* β are DNA-tracking sliding-clamp proteins that increase processivity by tethering their conjugate DNA polymerases to DNA. gp45 also activates T4 late transcription. DNA loading of gp45 and β requires ATP or dATP hydrolysis; efficient loading at primer–template junctions is assisted by single-stranded DNA-binding proteins. The kinetics of gp45 loading and tracking have been examined by DNase I footprinting of linear DNA with one blunt end, one primer–template junction, and binding sites for proteins that block gp45 tracking. DNA loading of gp45 can also be interrupted by adding the non-hydrolyzable ATP analog ATP-γ-S. At saturation, DNA is very closely packed with gp45 or β. When gp45 loading is interrupted, or when a segment of the track is blocked off, the gp45 footprint dissipates within seconds, but the DNA-tracking state of β is much more stable. The stability of the tracking state of gp45 is, however, increased by the macromolecular crowding agent polyethylene glycol. We suggest that labile gp45 catenation directly generates the coupling of late transcription to DNA replication during bacteriophage T4 multiplication.
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Introduction

Proliferating cell nuclear antigen (PCNA) from eukaryotic cells, the β subunit of *Escherichia coli* DNA polymerase III holoenzyme and bacteriophage T4 replication accessory protein gp45 are functional homologs, belonging to the family of 'sliding-clamp' proteins (reviewed by Kuriyan and O'Donnell, 1993; Kelman and O'Donnell, 1995b), which tether DNA polymerases onto DNA templates to increase the speed and processivity of DNA replication (reviewed by Alberts, 1987; Stillman, 1994; Kelman and O'Donnell, 1995a). The connection between the structures and functions of these sliding clamps is strikingly established for yeast PCNA and β. Each protein has a central channel that is large enough to accommodate a DNA duplex, with positive charges lining the inside of the channel and negative charges distributed along the outside (Kong *et al.*, 1992; Krishna *et al.*, 1994), clearly implying the ability of DNA to be topologically associated. Although

the three-dimensional structures of PCNA and β are very similar (Kong *et al.*, 1992; Krishna *et al.*, 1994), there is insufficient amino acid sequence identity between the two proteins for this close resemblance to have been independently discerned in advance of the determinations of structure (Kelman and O'Donnell, 1995b).

PCNA is a trimer and β is a dimer (Johanson and McHenry, 1980; Kong *et al.*, 1992; Krishna *et al.*, 1994). Interactions between the constituent monomers that might stabilize their ring structures include hydrogen bonds between β sheets, as well as hydrophobic and ion pair contributions (Kelman and O'Donnell, 1995b). The gp45 sliding clamp is a trimer (Jarvis *et al.*, 1989a) and is likely to be held together by similar interactions between its constituent monomers.

Loading each of these replication clamps onto DNA requires the intervention of a conjugate multisubunit DNA-dependent ATPase, serving as an ATP or dATP hydrolysis-driven assembly factor, and acting at an adequate DNA-loading site, such as a nick, gap or single-stranded tail. One can think of the assembly factors as protein catenases because they must open their respective clamps at a monomer–monomer interface and subsequently reclose them around a DNA duplex (Stillman, 1994). The specific loading factors of PCNA, β and gp45 are replication factor C (RF-C, also called Activator-1), γ complex and gp44/62, respectively.

gp45 also serves as a transcriptional activator of phage T4 late genes (Herendeen *et al.*, 1989, 1990, 1992). Most of the earlier analysis of the DNA-tracking property of gp45 focused on the processivity of DNA replication and the enhancement of T4 late gene transcription (Huang *et al.*, 1981; Herendeen *et al.*, 1992), but cryoelectron microscopic and photochemical cross-linking methods have also been applied to the analysis (Gogol *et al.*, 1992; Tinker *et al.*, 1994a,b). Cryoelectron microscopy of the association of T4 replication accessory proteins gp44, 62 and 45 with DNA revealed hash-mark clusters (Gogol *et al.*, 1992) that were eventually attributed to gp45 tracking along DNA (reviewed by Kuriyan and O'Donnell, 1993). A photochemical cross-linking analysis confirmed the DNA-tracking property of gp45 (Tinker *et al.*, 1994b), which was proposed originally to explain topological and physical constraints on enhancement of T4 late gene transcription (Herendeen *et al.*, 1992), and also demonstrated similarities between gp45, β and PCNA. These experiments further suggested that the DNA-tracking state of gp45 might be quite transient (Tinker *et al.*, 1994b) and this has been shown to be the case (Yao *et al.*, 1996).

The experiments that are described below pursue the analysis of DNA tracking by DNase I footprinting. We show that efficient loading of gp45 and β generates a dense occupancy of DNA by these tracking proteins. While gp45 readily falls off the track, we show that this
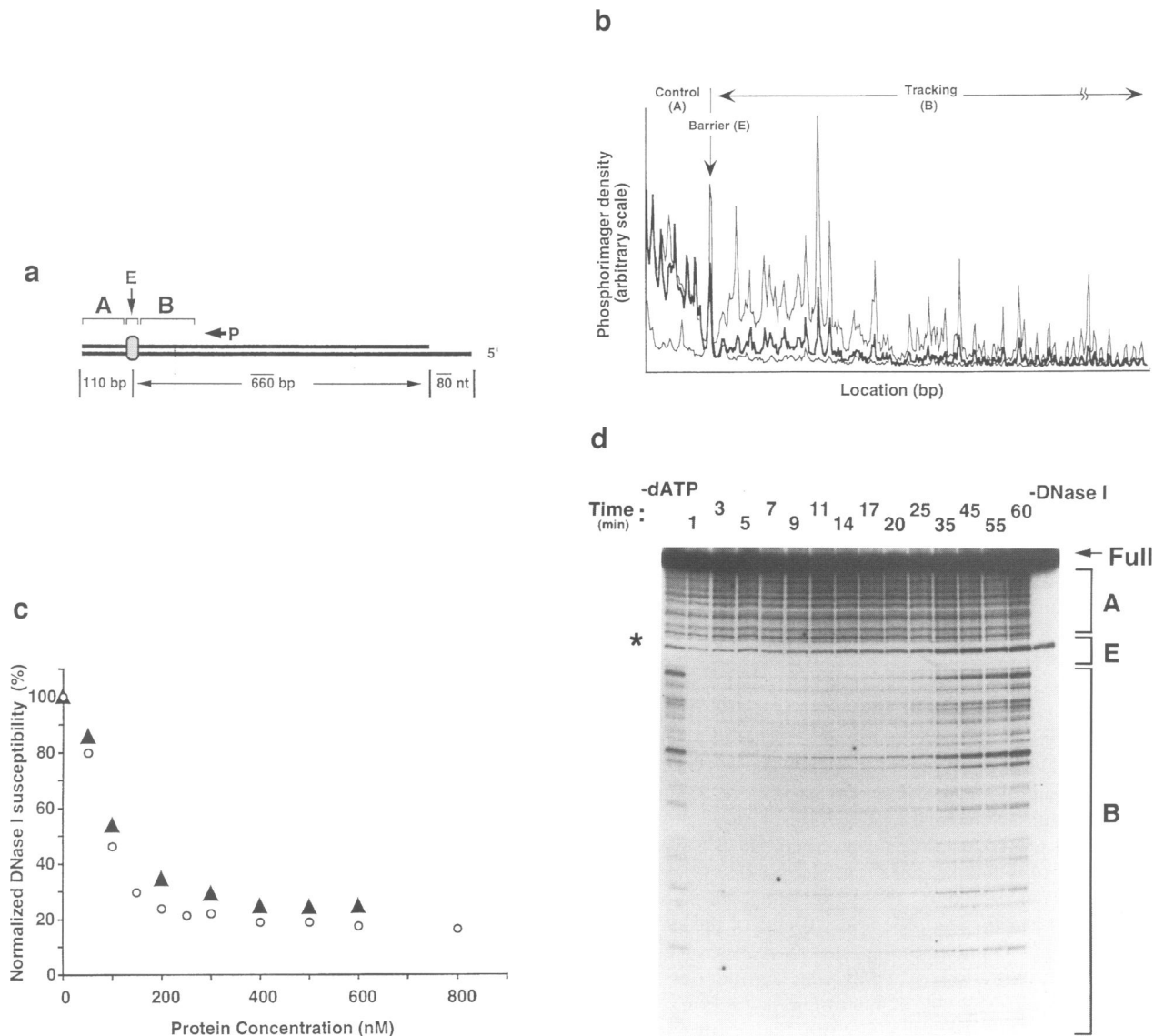
b

a

c

d

Fig. 1. Rapid loading and close packing of gp45 on DNA. (**a**) DNA T1. The filled oval indicates the position of the *Eco*RI site (E). DNA segments accessible (B) to gp45 tracking from the primer–template junction at the right, and blocked by *Eco*RI-Gln111 (A) are marked. The arrow indicates the location of the primer (P) (180 bp from the *Eco*RI binding site) that is used for developing the DNase I footprint. (**b**) Scan of a footprint of tracking gp45. The densitometric traces are, from the bottom up: background (mock digestion without DNase I; thin black line), footprint (heavy black line), no-tracking control (thin line). All samples contain *Eco*RI-Gln111, gp45, gp44/62 complex and gp32. The footprint sample contains, and the no-tracking control sample lacks, dATP. (**c**) Dependence of gp45 loading on the concentrations of gp44/62 complex and gp45. The gp44/62 titration (○) was done with gp45 fixed at 720 nM, and the gp45 titration (▲) was done with gp44/62 fixed at 300 nM. (**d**) Time course of gp45 loading. Samples were pre-incubated at 25°C for 5 min before adding dATP to a final concentration of 200 μM. Samples for DNase I footprinting were taken at the times indicated at the top of the figure. The band marked with an asterisk is due to a residual enzyme activity of *Eco*RI-Gln111. Samples lacking dATP and not treated with DNase I are shown in the left- and right-hand lanes, respectively, as indicated.

transience of its DNA-tracking state is mitigated by the macromolecular crowding agent polyethylene glycol (PEG). We also confirm that the DNA-tracking state of β is relatively more stable than that of gp45.

Results

Footprinting a DNA-tracking protein

The design of the DNA constructs for these experiments is based on the expectation that protection from DNase I afforded by a DNA-tracking protein would be continuous along the entire track, and that quantification of such a featureless 'footprint' would require an internal standard—

a DNA segment that is inaccessible to the tracking protein. DNA T1 (Figure 1a) provides a single efficient DNA-loading site for the gp45 sliding clamp, consisting of a primer–template junction (Mace and Alberts, 1984a; Tinker *et al.*, 1994b; Sanders *et al.*, 1995) with an 80 nt (on average) 5′ single-stranded tail at one end; the other end of DNA T1 is blunt to prevent loading. *Eco*RI-Gln111 binding to its site on this DNA separates two regions, one accessible and the other inaccessible to gp45 (B and A, respectively, in Figure 1a). When this DNA is loaded with gp45 by its cognate clamp loader, the gp44/62 complex, and subjected to DNase I digestion, a characteristic pattern is expected due to the presence of gp45 freely tracking

on DNA segment B only, with segment A, the no-gp45 side of the DNA probe, serving as the internal standard.

Figure 1b shows a scan of a DNase I footprint at near-saturating concentrations of gp45 and of gp44/62 complex. The entire segment B, which is accessible to gp45, is highly protected, while DNase I digestion of segment A is essentially unaffected by gp45 loading. Quantification (Figure 1b legend) yields an estimate of 80% protection, implying at least that degree of close packing of gp45 on DNA. (Since it is conceivable that completely close-packed gp45 would yield <100% protection from DNase I, the estimate of 80% close packing is a lower limit.) No protection is afforded when gp45 is omitted (data not shown).

The other notable feature of the Figure 1b footprint is a band (under the vertical arrow) in the center of the site that is occupied by *Eco*RI-Gln111. The intensity of this band increases with time of incubation and is due to an unanticipated residual enzymatic activity of the *Eco*RI-Gln111 protein. Under the conditions of these experiments, the fraction of DNA chains that is cleaved at this site is always <3%. We have not considered it necessary to eliminate this minor side-product or analyze it further. At worst (i.e. if the action is hydrolytic, if *Eco*RI-Gln111 detaches from hydrolyzed DNA and if gp45 can then track across all hydrolytic DNA sites, thereby eliminating the normalization property of segment A for those particular DNA molecules), this residual enzymatic activity of *Eco*RI-Gln111 would generate a mere ~3% underestimation of protection from DNase I cleavage over segment B.

The density of occupancy of DNA by gp45, analyzed by DNase I protection as just described, was examined as a function of the concentrations of gp45 and gp44/62 complex. The results of these titrations are shown in Figure 1c. gp44/62 complex was titrated in the presence of 720 nM gp45; maximum protection requires ~400 nM gp44/62. The titration of gp45 was carried out in the presence of 300 nM gp44/62; maximum protection requires ~400 nM gp45. Slightly suboptimal concentrations of gp45 (300 nM) and gp44/62 complex (300 nM) were used in the experiments that follow. (For gp45, concentrations are specified as the trimer, and for gp44/62 as the complex with 4:1 subunit stoichiometry.)

A time course DNase I footprinting series reveals the quick pace of gp45 loading and packing on DNA (Figure 1d): ~80% of the maximum protection is reached within 1 min. Eventually, starting ~25 min later, protection diminishes. The DNA loading of gp45 is driven by ATP or dATP hydrolysis, and the loss of DNase I protection at later times is due to exhaustion of the ATP supply (cf. Tinker *et al.*, 1994b). Both ATP and dATP have been used in this experiment with very similar results (data not shown), in agreement with the observation that gp44/62 complex has the same rates of hydrolysis for ATP and dATP (Mace and Alberts, 1984b).

The transience of gp45 tracking

ATP-γ-S, a non-hydrolyzable ATP analog, blocks the stimulation of DNA polymerase activity by the accessory proteins (Huang *et al.*, 1981). ATP-γ-S appears to 'freeze' the accessory protein complex at a primer–template junction (Munn and Alberts, 1991), presumably because release of gp45 from that complex for tracking along DNA
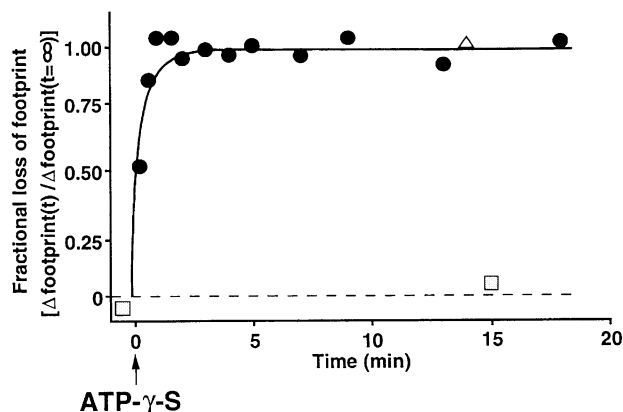

Fig. 2. Unloading gp45 from DNA. gp45 was loaded onto DNA T1 at 25°C for 5 min in the presence of 200 μM ATP. A large sample was added to ATP-γ-S (final concentration 500 μM) and aliquots were taken for DNase I digestion at the subsequent times indicated on the abscissa (●). Samples to which ATP-γ-S was not added (□) and a sample without ATP (△) were taken for DNase I digestion at the indicated times. The ordinate reports the fractional loss of the DNase I footprint (0 = full footprint due to DNA-tracking gp45; 1.0 = full sensitivity to DNase I).

requires ATP hydrolysis. Fixing the gene 44/62 and 45 proteins at the primer–template junction generates favorable conditions for DNase I and chemical footprinting (Munn and Alberts, 1991). For the experiment that is shown in Figure 2, we have used ATP-γ-S to examine the persistence of the DNA-tracking state of gp45. DNA has been loaded with gp45 for 5 min before adding ATP-γ-S to block further loading (Tinker *et al.*, 1994a). Time-course DNase I footprinting allows one to follow the disappearance of gp45. The persistence of the DNA-tracking state of gp45 is so brief that the apparent half-life of gp45 trimer on DNA in the absence of continuous ATP-dependent loading can only be roughly estimated as <30 s (~15 s plus one-half of the DNase I reaction time of 15 s). A similar experiment, with hexokinase and glucose added in place of ATP-γ-S [i.e. with very rapid conversion of ATP to ADP at time zero (<0.2 s)], had the same outcome.

The preceding experiment fails to specify the mode of gp45 unloading, in particular whether it takes place only at the loading end or everywhere along the DNA. In order to distinguish among these possibilities, we constructed DNA template T2, with one *lac* operator (O_l) and one *Eco*RI binding site separated by 260 bp, as well as a 180 nt 5' single-stranded tail (Figure 3a). In experiments with DNA T2, Lac repressor serves as a first road block to gp45 tracking, confines this protein to segment B of DNA T2, and excludes it from segment A (Figure 3a). Applying *Eco*RI-Gln111 as the second road block isolates segment B_1 from the primer–template junction loading site for gp45, so that the fate of gp45 remaining in region B_1 can be examined independently from events at the DNA ends.

An experiment showing the time course of DNase I footprinting after the addition of 2.5 nM *Eco*RI-Gln111 is presented in Figure 3b. It is clear that the footprint over segment B_1 disappears with time, indicating gp45 detachment from the isolated, central stretch of DNA while the protection of segment B_2 from DNase I is
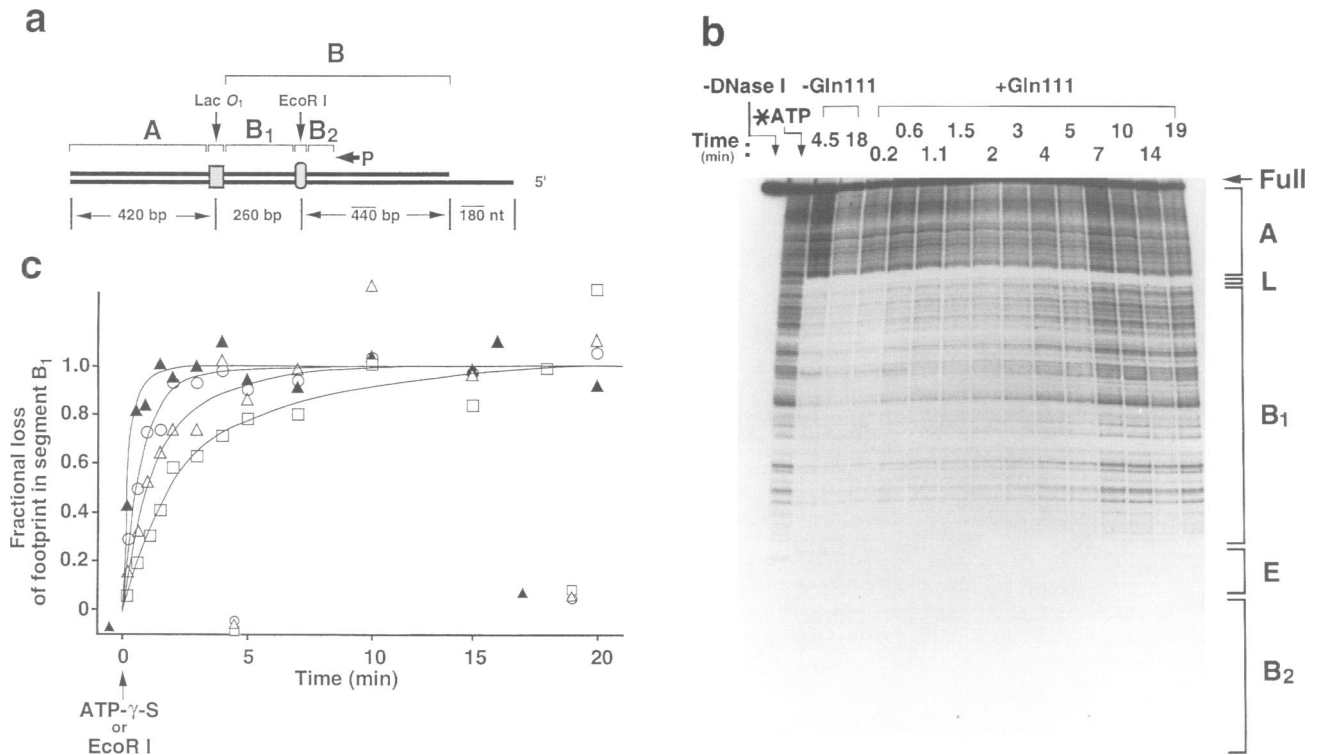
Fig. 3. gp45 unloading from internal locations. (**a**) DNA T2. The filled rectangle and oval indicate the positions of a *lac* operator and *Eco*RI binding site, respectively. The arrow shows the location of primer (P) (170 bp from the *Eco*RI site). gp45 loaded at the primer–template junction at the right tracks over segment B, but is excluded from A by lac repressor bound at O_1. Segment B_1 becomes isolated from the gp45-loading site upon addition of *Eco*RI-Gln111. (**b**) A time course. A mixture containing 1 nM DNA template, 300 nM gp44/62, 100 μg/ml gp32, 300 nM gp45, 200 μM ATP and 4 nM Lac repressor was incubated for 5 min at 25°C. Aliquots were taken for DNase I digestion at the times after the addition of Gln111 protein (final concentration 2.5 nM) that are noted at the top. A separate sample was retained without *Eco*RI-Gln111 and aliquots were analyzed at the times shown. Control samples respectively delaying the addition of ATP, by adding *Eco*RI-Gln111 first (*ATP), and not digested with DNase I are shown in two left-hand lanes. (**c**) gp45 unloading at internal sites follows rapidly upon isolation of a DNA segment from a loading site. The experiment shown in (b) (□) was repeated with 7.5 (△) and 22.5 (○) nM *Eco*RI-Gln111 and analyzed as described in the legend to Figure 2. A control experiment with ATP-γ-S added instead of *Eco*RI-Gln111 (▲) (a repetition of the Figure 2 experiment, but with DNA T2) is also shown. Control samples respectively lacking *Eco*RI and without ATP-γ-S, and therefore fully loaded with gp45, were analyzed in parallel at the indicated times (corresponding small symbols, □, △, ○ and ▲).

retained, presumably because the track between the gp45 loading site and DNA segment B_2 remains open. The rate of detachment of gp45 from segment B_1 (Figure 3c, open squares) is considerably slower than indicated in the comparison experiment in Figure 2. On the other hand, a closer examination of the footprint over segment E in Figure 3b indicates that occupancy of the *Eco*RI binding site by its ligand is not instantaneous.

That the disappearance of gp45 from DNA segment B_1 of template T2 follows closely upon its isolation by binding of protein to the *Eco*RI site is shown by repeating the Figure 3b experiment at increasing concentrations of *Eco*RI-Gln111 (Figure 3c). At the highest concentration shown, at which the occupation of site E by *Eco*RI-Gln111 is too fast to measure accurately, the rate of disappearance of gp45 tracking in DNA segment B_1 is also too fast to measure accurately (half-life ~0.5 min). We conclude that gp45 can get off the track anywhere, and not just at the DNA ends, that it does so rapidly under these conditions, and that the rates of decay of the DNA-tracking state of gp45, as measured by the two methods shown in Figures 2 and 3, are indistinguishable.

One might be concerned that the highest concentrations of *Eco*RI-Gln111 used in this experiment permit binding to variant (i.e. 'star') *Eco*RI sites. There is no indication,

however, that the track between the gp45 loading site and segment B_2 is blocked by non-specifically or subspecifically bound protein.

We examined whether the dynamics of DNA tracking can be slowed down sufficiently at 0–4°C to allow more accurate measurements, but found that gp45 does not afford significant DNase I protection at low temperature. Apparently, the balance between gp45 loading, tracking and falling off DNA is biased against analysis of the dynamics of the process at low temperature (data not shown).

A comparison with β

Extensive analysis of the dynamics of DNA tracking (Stukenberg *et al.*, 1991, 1994; Naktinis *et al.*, 1996; Yao *et al.*, 1996), primarily by gel filtration, shows that the topological linkage of *E.coli* β to DNA is relatively stable, and that the γ complex acts as a catalyst for decatenation as well as topological linkage of β to nicked circular DNA. Loading of this DNA-tracking protein requires ATP hydrolysis; requirements of the unloading reaction are the subject of ongoing study.

The experiment presented in Figure 4 shows an analysis, by DNase I footprinting, of β loading onto, and subsequent unloading from, DNA T2 (Figure 3a). The γ complex
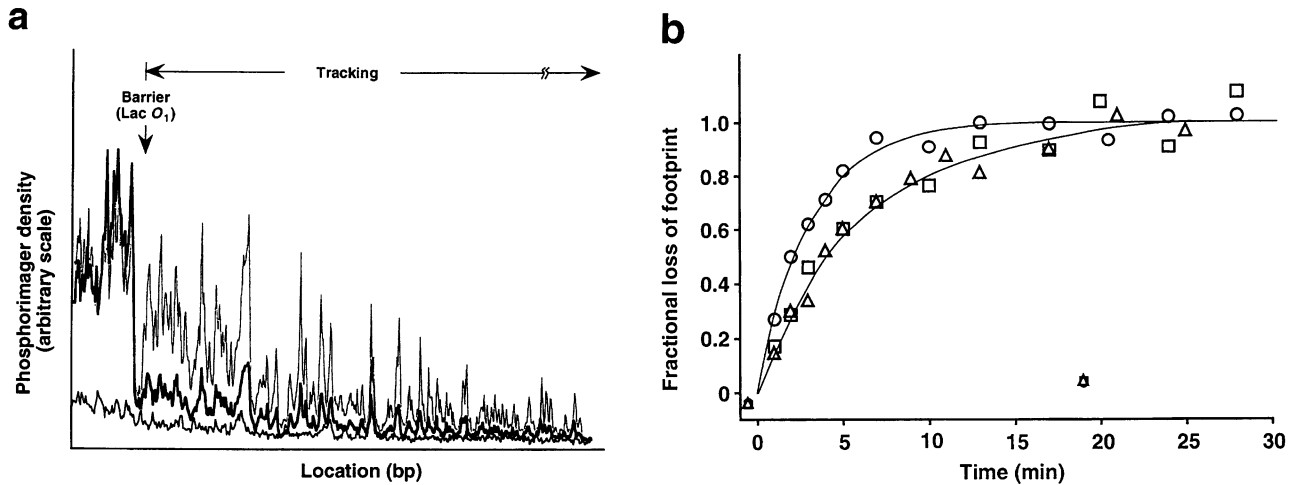
a

b

Fig. 4. Loading and unloading β. (**a**) Scan of a footprint of tracking β on DNA T2. The densitometric traces are, from the bottom up: background (mock digestion without DNase I; thin black line), the footprint (β sliding along segments B_1 and B_2 of DNA T2, but excluded from segment A; heavy black line) and the no-tracking control (ATP omitted, thin gray line). Each sample contains Lac repressor, β, γ complex and *E.coli* single-stranded DNA-binding protein. (**b**) Rate of disappearance of β from segment B_1. The following components are added at $t = 0$: (○) hexokinase and glucose; (□) *Eco*RI-Gln111 to 22.5 nM final concentration; (△) hexokinase, glucose and *Eco*RI-Gln111. Each time course is accompanied by two control samples to which no additions have been made, taken at the indicated times (corresponding small symbols, superimposing).

(optimal at 80–100 nM) is more efficient than the gp44/62 complex at loading the conjugate sliding clamp. Figure 4a shows a footprint generated by 400 nM β with 80 nM γ complex, showing 73% protection over an ~320 bp stretch of DNA next to the *lac* operator. Unloading of β from this stretch of DNA is examined in Figure 4b under several conditions: after ATP is rapidly hydrolyzed (within <0.2 s) by adding hexokinase and glucose; after the path from the loading site to segment B_2 is blocked by DNA-bound *Eco*RI-Gln111 (Figure 3a); or by adding hexokinase and glucose along with *Eco*RI-Gln111. Comparison with Figure 3c shows that the DNA-tracking state of β is much more stable than that of gp45. Unloading of β does take place preferentially at the primer–template junction, since blocking off both ends of segment B_1 with *lac* repressor and *Eco*RI considerably diminishes the rate of disappearance of the footprint after destruction of ATP (Figure 3b; compare circles and triangles). However, the rate at which β falls off this isolated DNA fragment is essentially the same whether ATP or ADP is present (compare squares and triangles).

Using a macromolecular crowding agent to stabilize the DNA-tracking state of gp45

The preceding experiments imply that if the DNA-tracking state of gp45 is due to the assembly of a ring-form trimer around DNA (as one fully expects; cf. Tinker *et al.*, 1994b), then the transience of the tracking state that is demonstrated by the preceding experiments is due to the dynamics of spontaneous ring opening. Macromolecular crowding reagents, such as very hydrophilic polymers, are known to generate conditions in solution that are favorable to intermacromolecule association (Zimmerman and Minton, 1993; Reddy *et al.*, 1995). PEG increases the ability of gp45 to stimulate T4 DNA polymerase processivity independently of gp44/62 complex and ATP hydrolysis (Reddy *et al.*, 1993). PEG also makes the enhancement of T4 late transcription by gp45 independent of the gp44/62 and ATP hydrolysis-requiring DNA-loading
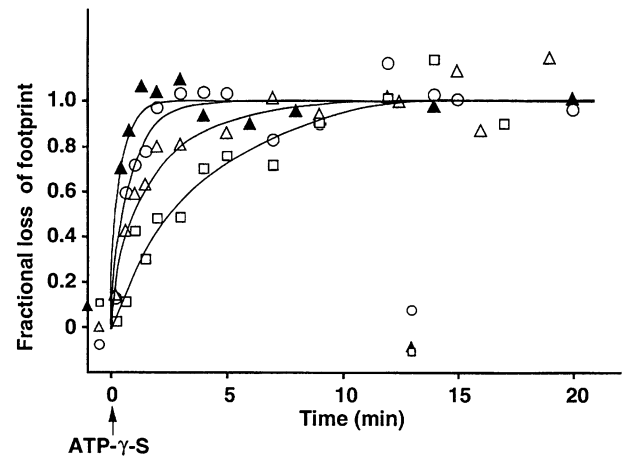

Fig. 5. Stabilizing gp45 on DNA with PEG. Reaction mixtures containing 0–7.5% PEG 8K were pre-incubated at 25°C for 5 min. The concentration of gp45 was 720 nM. The experiment shown in Figure 2 (▲), using DNA T1, was repeated with 5 (○), 6 (△) and 7.5 (□) % PEG 8K, and analyzed as described in the legend to Figure 2. Control samples lacking ATP-γ-S and, therefore, fully loaded with gp45, were analyzed in parallel at the indicated times (corresponding small symbols).

process (Sanders *et al.*, 1994). We have therefore examined whether PEG can stabilize gp45 on DNA by repeating the Figure 2 experiment, on the dissipation of a gp45 footprint after addition of ATP-γ-S, in the presence of different concentrations of PEG (Figure 5). PEG does indeed stabilize gp45 tracking on DNA: the apparent half-life of the tracking state after cessation of loading is <0.5, ~0.5, 1 and 2.5 min in the presence of 0, 5, 6 and 7.5% PEG 8K, respectively. (We have noted, however, that protection of DNA from DNase I is nevertheless consistently lower in the presence of PEG, suggesting that the efficiency of operation of the clamp loader may actually be diminished by macromolecular crowding.)

Discussion

A rapidly loaded and densely packed DNA-tracking state of gp45

The gp45 DNA-tracking protein can be so closely packed on DNA at 25°C that it reduces access of DNase I by ~80% (Figure 1b). This high occupancy must be established against a continuous and rapid loss of gp45 (Figures 2 and 3); the steady state of high occupancy is reached rapidly (Figure 1d), consistent with rapid turnover of DNA-tracking gp45. Loading of gp45 takes place at a DNA end with a primer–template double-stranded–single-stranded DNA junction, but gp45 also falls off the DNA at internal locations (Figure 3).

Assuming that the gp45 sliding clamp has similar structure and dimensions to PCNA, 80–100% of close packing would allow 53–66 gp45 trimers to be loaded onto the 660 bp (on average) of DNA lying between the loading site and track-blocking EcoRI-Gln111. This is ~15% of the total amount of gp45 trimer; considering the presence in the reaction mixture of a second DNA fragment with one gp45-loading end, one blunt DNA end and 1800 bp of double-stranded DNA in between, it is clear that, under the conditions of these experiments, a substantial fraction of the gp45 in the reaction mixture is loaded onto DNA at steady state. DNA that is so tightly packed with a tracking protein can be thought of as a dense one-dimensional fluid. We hoped to detect internal structure in this fluid, particularly in the vicinity of the EcoRI 'wall', but have found no indications (such as uneven DNase I protection) of structure thus far (Figure 1b).

In a cryoelectron microscopic analysis of the interactions of T4 DNA polymerase accessory proteins with DNA, Gogol and co-workers (1992) saw 'hash marks' along the DNA. The identification of these images posed a substantial technical problem, but they were ultimately interpreted as most consistent with gp44/62 complex or, alternatively, gp45 trimer. Subsequent determination of the structures of the β and PCNA sliding clamps (Kong et al., 1992; Krishna et al., 1994), and analysis of DNA tracking by β, PCNA and gp45 (Stukenberg et al., 1991; Burgers and Yoder, 1993; Tinker et al., 1994b; Sanders et al., 1995; Naktinis et al., 1996; Yao et al., 1996), make it overwhelmingly likely that the hash marks observed by Gogol and co-workers are representations of tracking gp45.

Gogol and co-workers found these cryomicroscopic hash marks distributed along DNA in a highly irregular manner, suggesting some cooperativity. Since the relatively wide spacing of hash marks militates against direct protein–protein interactions on DNA, it was suggested that clustering might be generated by the DNA-loading process. However, the large stoichiometric excess of gp44/62 complex over DNA-loading sites that is required for near-optimal loading of gp45 (Figure 1c) indicates a relatively loose association of gp44/62 complex with the gp45-loading site and does not favor highly cooperative or processive loading. The difficulty of footprinting the gp44/62/45 complex at a primer–template junction in the presence of ATP (Munn and Alberts, 1991) also suggests a loose, non-persistent association, as does the large stoichiometric excess of gp44/62 complex that is required for optimal DNA-dependent ATPase activity (Jarvis et al., 1989b). It therefore seems more probable that the observ-

ations of large and small clusters, as well as isolated hash marks, reflect some non-uniform interaction of gp45-loaded DNA with electron microscope grids and irregular loss of gp45 during sample preparation.

Dynamics of DNA tracking and cycling between free and DNA-linked states

The stability of gp45 on DNA is so low ($t_{1/2} < 30$ s), and its packing density so great, that all the gp45 in a reaction mixture containing 300 nM of the trimer (Figure 1b) must cycle through the DNA probe (at 1 nM) approximately every 3–5 min. Since untracking takes place everywhere along the DNA, and not merely at the primer–template junction (Figure 3), and since DNA-internal unloading is ATP hydrolysis independent (data not shown), it is not likely to be assisted by gp44/62 complex. In view of this lability, it is not surprising that tracking of gp45 on nicked circular DNA could not be detected by gel filtration (Tinker et al., 1994a; Yao et al., 1996). In contrast, gp45 is sufficiently stably bound to persist on DNA in the absence of continuous reloading when it is associated with RNA polymerase at enhanced T4 late promoter complexes (Tinker et al., 1994a), or with T4 DNA polymerase at primer–template junctions (Hacker and Alberts, 1994b; Yao et al., 1996).

The contrast with β, which can also be packed onto DNA to high density (Figure 4), is striking. (i) We observe 3- to 4-fold more efficient loading of β by γ complex than of gp45 by gp44/62. We note, parenthetically, that γ complex is being used in these experiments to generate a density of packing of β on DNA that is at least an order of magnitude greater than that previously analyzed by other means (Stukenberg et al., 1991; Yao et al., 1996). (ii) The persistence time on DNA upon hydrolysis of ATP is much greater for β than for gp45 under comparable conditions. (iii) The persistence of β within a stretch of duplex DNA that has been isolated at each end by a protein block is even greater, and independent of whether ATP or ADP is present.

The side-by-side analysis of β and gp45 by DNase I footprinting raises some interesting questions. The half-life for detachment of β from DNA in the absence of γ complex has been measured to be ~70 min (Yao et al., 1996), but we find β clamps removed with a half-life of <5 min from duplex DNA isolated at each end by a protein block. The reduced stability of β on DNA in this study, relative to previous work, may be due to the use of different conditions (100 mM NaCl instead of 200 mM KAc, 37°C instead of 25°C, and 120–190 nM γ complex instead of 80–100 nM γ complex); in the experiments reported here, the packing density of β is also very great compared with what has been done previously. We cannot confidently specify whether dilutely dispersed β clamps have the same properties as nearly close-packed β. At close packing, the 260 bp region B1 of DNA T2 (Figure 3a) should have 20–25 β rings loaded initially, and the DNase I footprinting analysis is insufficiently sensitive to specify the dynamic properties of the last one or two β clamps to remain on that DNA segment.

Alternatively, the dissociation of β from DNA may be assisted by the γ complex. It has been shown that addition of γ complex and ATP to β clamps on DNA results in rapid release ($t_{1/2} \sim 1$ min), and that this unloading action

of γ complex does not take place in the absence of ATP (Naktinis *et al.*, 1996). In the experiments that are reported here, dissociation of β clamps from an isolated internal section of DNA (segment B_1 of Figure 3a) is found to be as rapid in the presence of ATP as with ADP (produced from ATP with hexokinase and glucose). The mechanism of γ complex-catalyzed unloading of β, and whether ADP will also support this process, is still an open question and will be the subject of future analysis. Further, if γ complex unloads β clamps from an isolated segment of duplex DNA, then either a 3' primer terminus is not required for unloading activity (e.g. γ complex acts in solution or on duplex DNA), or γ complex bound to a distant 3' end can unload β clamps across a protein block (e.g. by looping out the intervening DNA, in the manner of transcriptional and recombinational enhancers).

Molecular crowding and the stability of the DNA-tracking state of gp45

The cytoplasm of *E.coli* is a macromolecularly crowded medium (Cayley *et al.*, 1991) and eukaryotic nuclei must also be macromolecularly crowded (Zimmerman and Minton, 1993). Macromolecular crowding favors protein–protein associations holding together multimeric protein complexes. It has been shown that the association of gp45 with the gp44/62 complex in the absence of DNA is strongly elevated by 7.5% PEG 12K (Jarvis *et al.*, 1990). The more modestly crowding conditions of these experiments (with PEG 8K) greatly stabilize the DNA-tracking state of gp45 (Figure 5), while retaining ATP hydrolysis-dependent loading. Even an average DNA residence time of only ~4 min (2.5 min half-life) of the sliding clamp would, of course, ensure a very high degree of processivity of leading strand chain elongation proceeding at 400 nt/s. Nevertheless, a further stabilization is afforded at the primer–template junction by association with the T4 DNA polymerase (Hacker and Alberts, 1994b; Kaboord and Benkovic, 1995; Yao *et al.*, 1996). In a sense, both the T4 sliding clamp and the T4 DNA polymerase serve as their partner's processivity factor.

The significance of the ephemeral DNA-tracking state of gp45

As also pointed out elsewhere, the sliding clamps of *E.coli* DNA polymerase III and of T4 DNA polymerase represent alternative design principles (Yao *et al.*, 1996). The more stably associated of these sliding clamps, β, requires the conjugate γ complex to serve as a loading and unloading factor. Competition between the DNA polymerase and the loading factor for the sliding clamp, and the responsiveness of the polymerase–clamp interaction to DNA structure, with dissociation taking place at the completion of an Okazaki fragment (Hacker and Alberts, 1994a; Naktinis *et al.*, 1996), are the other essential components of the efficient functioning of this sliding clamp.

gp45, the less stable of the clamps, challenges one's intuitive notions of a processivity factor, because its topological linkage to DNA is so labile. gp45 acquires additional stability for this linkage through diverse ligands: RNA polymerase at the late promoter transcription initiation complex (Tinker *et al.*, 1994a), DNA polymerase at a primer–template junction with exposed single-stranded DNA, as just mentioned, and gp55 during DNA tracking

(R.L.Tinker-Kulberg *et al.*, 1996). The association with T4 DNA polymerase is transient, with dissociation accompanying completion of DNA chain elongation (Hacker and Alberts, 1994a,b; Yao *et al.*, 1996); the association with RNA polymerase is also likely to be broken once RNA chain elongation gets under way. When that happens, gp45 can recycle, either by spontaneous dissociation and reloading, or by rapidly acquiring new partners as it continues to track along DNA.

A salient characteristic of T4 late transcription is its dependence on, and continuous coupling to, DNA replication (Brody *et al.*, 1995). We propose that the limited stability of the gp45 clamp, even under conditions of macromolecular crowding, generates the coupling of late transcription to concurrent replication by making late promoter activation dependent on continuous loading of gp45 onto DNA at sites that are also unstable and must be continuously generated by recombination-coupled DNA synthesis.

Materials and methods

Proteins, DNA and chemicals

DNA exonuclease III, Vent (exo⁻) DNA polymerase, T4 polynucleotide kinase, restriction enzymes, DNase I, proteinase K, nucleotides and PEG 8K were purchased. The purification of T4 gp32, gp45 and gp44/62 complex from overproducing bacteria was described and referenced previously (Herendeen *et al.*, 1992; Sanders *et al.*, 1994). The purification of *E.coli* β and γ complex has been described elsewhere (Kong *et al.*, 1992; Onrust *et al.*, 1995). We are also grateful to M.Ouhammouch for a gift of β. Samples of the mutant *Eco*RI protein, *Eco*RI-Gln111 (Wright *et al.*, 1989) were generously provided by Drs P.Modrich, J.Taylor and J.Kadonaga.

DNA and chemicals. Plasmid pTE114 (Elliott and Geiduschek, 1984), a 2982 bp derivative of pUC8, and pLac-Eco, a 3867 bp derivative of pBR322, have one *Eco*RI binding site; pLac-Eco has, in addition, a single *lac* operator (O_l). DNA for analysis of DNA tracking was prepared as described (Sanders *et al.*, 1995): plasmid DNA was linearized with a restriction enzyme to generate 5' overhanging ends that were enlarged to 80 or 180 nt, on average, with exonuclease III, then cut with a blunt end-generating restriction enzyme to produce two DNA fragments, each with one blunt end and one long 5'-overhanging single-stranded end. These DNA fragments were not further separated, although only one of them features in the subsequent analysis.

DNase I footprinting of gp45

Unless otherwise noted, each sample contained 1 nM DNA (concentration referring to the DNA fragment that was analyzed by primer extension), 5 nM *Eco*RI-Gln111 protein, 100 μg/ml gp32, 300 nM gp44/62, 300 nM gp45 (trimer), and 200 μM ATP or dATP in 20 μl of reaction buffer containing 33 mM Tris–acetate (pH 7.5), 200 mM potassium acetate, 10 mM magnesium acetate, 150 μg/ml bovine serum albumin (BSA), 0.1 mM Na₃EDTA, 1 mM dithiothreitol (DTT) and 1% (w/v) glycerol at 25°C. Five microliters of 5 ng/μl DNase I in the same buffer were added and digestion was terminated 15 s later by adding 150 μl of stop solution [40 mM Na₃EDTA, 0.4% (w/v) SDS, 40 mM Tris–HCl, pH 8.0]. Five microliters of 20 mg/ml proteinase K were added and the mixture incubated at 65°C for 1 h, then extracted with phenol and precipitated with ethanol. Suitable DNase I concentrations for experiments with different concentrations of PEG were individually determined by titration. In order to retain DNase I digestion within the single-hit range, a minimum of 37% of uncut DNA strands was maintained.

DNase I footprints were developed by primer extension. DNA primers were 5' end-labeled and purified by electrophoresis in polyacrylamide gel. A 20 μl volume containing DNA template and a 5-fold excess of labeled primer in 1× Vent DNA polymerase buffer [10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% (w/v) Triton X-100, 20 mM Tris–HCl, pH 8.8] was first heated to 90°C for 1 min, quickly chilled on ice, then transferred to 55°C for 5 min. Thirty microliters of 167 μM dNTPs (334 μM dNTPs used in primer extension of DNA T2) and 5 μl of 0.8 U/μl of Vent (exo⁻) DNA polymerase were added. Samples were

incubated at 55°C for 4 min, then transferred to 65°C for another 6 min. Stop solution [150 µl of 40 mM Na$_3$EDTA, 0.4% (w/v) SDS, 40 mM Tris–HCl, pH 8.0] was added to terminate reactions, the mixture was extracted with phenol and DNA was precipitated with ethanol. Samples were loaded onto 8–12% TBE–polyacrylamide (19:1 acylamide:bisacrylamide) denaturing gels containing 8.3 M urea.

Quantification of footprints was carried out on a phosphorimager. Phosphorimager intensity was integrated in each lane of a gel for fixed DNA segments (i and j) within regions B and A (Figure 1a and b), i.e. on each side of the *Eco*RI barrier, E (Figure 1a). The ratio of these integrated densities (b_i/a_j) depends on the lengths (i, j) of the chosen DNA segments and on the extent of protection from DNase I in segment B (i.e. the footprint). Under single-hit conditions of nuclease digestion, b_i/a_j does not depend on the degree of digestion by DNase I and serves as a measure of protection by the DNA-tracking protein that is independent of the chosen intervals i and j. We verified that integrating phosphorimager density over different lengths, j, of segment B did not change the outcome of analysis.

DNase I footprinting of β

Unless otherwise noted, each sample contained 1 nM DNA, 33 nM *E.coli* SSB (tetramer), 80 nM γ complex, 300 nM β (dimer) and 200 µM ATP in the above-specified reaction buffer. All other steps were as described for DNase I footprinting of gp45.

Acknowledgements

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