Interaction of recA protein with left-handed Z-DNA

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The ability of recA protein to interact with a Z-DNA polymer, Br-poly(dG-dC), or M13 bacteriophage single-stranded DNA was investigated. RecA protein binds more avidly to Z-DNA than to single-stranded DNA in the absence of a nucleotide cofactor. This binding pattern changes in the presence of adenosine 5'-(γ -thio)triphosphate (ATP[S]), however, such that the binding to Z-DNA decreases while binding to single-stranded DNA increases roughly 2-fold. When present together, the two forms of DNA compete with each other in the presence of ATP[S]. Experiments involving recA protein binding to recombinant plasmids showed neither a preferential binding of recA protein to the plasmid containing Z-DNA nor a similar effect of ATP[S] to that observed with the Z-DNA polymer. In contrast, maximal binding was obtained with a plasmid (linear or supercoiled) containing a polypurine-polypyrimidine insert, thus suggesting that recA protein displays sequence preferences in its interaction with DNA. The results of the present study provide no evidence that recA protein specifically interacts with or stabilizes the Z-DNA insert of a recombinant plasmid in the left-handed conformation.

INTRODUCTION

RecA protein of *Escherichia coli* is interesting in view of its manifold activities. It is essential for homologous genetic recombination and for the induction of the SOS repair system. The governing role of single-stranded DNA (ssDNA) in the pairing reaction of recA protein, involving linear duplex and circular ssDNAs, is well established *in vitro* (reviewed in ref. [1]). The binding of recA protein to ssDNA triggers its interaction with duplex DNA and results in the unwinding (untwisting of the double strands around the helical axis) of the latter [2,3]. This is not surprising in that, for strand exchange to occur, the recipient double helix must become unwound or partially denatured to accept the invading strand. RecA protein can also bind and unwind negatively supercoiled duplex DNA in the absence of ssDNA [3]. This binding has been shown to be somewhat dependent upon the superhelical density of the DNA molecule, which suggests that recA protein first binds to unwound regions and then polymerizes on the contiguous double-stranded lattice [4–6]. RecA protein also promotes pairing and strand exchange between two duplex DNA molecules, provided that one of the molecules contains a short ssDNA gap [7]. These studies support a recombination model where recA protein initiates binding at single-stranded regions in duplex DNA, such that the rate-limiting step of binding to the duplex is overcome; the resulting nucleoprotein filament, whether it contains ssDNA or double-stranded DNA (dsDNA), is then capable of pairing with homologous duplex DNA.

Recently, left-handed Z-DNA has been implicated as a participant in genetic recombination, largely on the basis of the observation that both recA protein [8] and rec1 protein of *Ustilago maydis* [9] bind preferentially to Z-DNA polymers over B-DNA. The role of Z-DNA in the process(es) of recombination, however, remains unclear. Kmiec & Holloman [10] have proposed a mechanism of strand exchange (similar to that described above) for the rec1 protein of *Ustilago*, i.e. rec1 protein might initiate pairing at the left-handed stretches contained within an otherwise B-DNA molecule, then move into the flanking

sequences and align homologous regions. Since rec1 protein also requires ssDNA in pairing reactions [11], the effect of unwound regions at B–Z junctions certainly cannot be ignored. It should also be taken into consideration that simple and uniform sequences such as dinucleotide repeats, (dG-dC)_n, used in the formation of Z-DNA *in vitro*, may facilitate the pairing reaction. Therefore propositions as above [10] should be viewed with caution unless the binding activities of rec1 or recA protein to different conformations and sequences of DNA have been worked out in detail and a relationship between DNA binding and a protein's enzymic function has been established. Kim *et al.* [12] have recently shown that the enhanced binding of recA protein to the Z form of poly(dG-m⁵dC) over the B form does not reflect a special affinity for the Z structure formed by this polymer but rather a kinetic effect.

This paper presents a comparison of the binding of recA protein to ssDNA with its binding to a Z-DNA polymer, Br-poly(dG-dC). The interaction of recA protein with plasmids containing or lacking segments of Z-helix is also studied, in an attempt to understand whether Z-DNA stretches contained within a large excess of B-DNA could provide a site of nucleation for the protein, as is known to be the case with ssDNA. The results indicate that, depending on the presence or absence of a nucleotide cofactor, recA protein preferentially binds either ssDNA or the Z-DNA polymer, Br-poly(dG-dC). However, the characteristics of recA binding to the chemically modified Z-DNA polymer are not reflected in its binding to a plasmid that contains a small chemically unmodified Z-DNA insert. Thus the present experiments do not provide evidence that recA protein preferentially binds to and stabilizes the left-handed helix in a plasmid.

MATERIALS AND METHODS

Nucleic acids

The synthesis of labelled and unlabelled poly(dG-dC), its chemical conversion by bromination into the Z-DNA [Br-

Abbreviations used: ATP[S], adenosine 5'-(γ -thio)triphosphate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; PEG, poly(ethylene glycol).

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poly(dG-dC)] and the verification of the latter structure were carried out as described previously [13].

M13 bacteriophage ssDNA was prepared by a modified method of Dente *et al.* [14], with the poly(ethylene glycol) (PEG) precipitation step of Carlson & Messing [15]. ³H-labelled M13 DNA was prepared by the addition of 2 mCi of [³H]thymidine to a 500 ml culture of bacteriophage-infected *E. coli* strain JM109.

Plasmids pDPL6, pDHg16 and pDHd16 were kindly provided by Dr. Pulleyblank. pDPL6 is a 2.2 kb derivative of pBR322 with a 36 bp polylinker region [16]. Plasmids pDHg16 and pDHd16 contain a 24 bp alternating (dG-dC)·(dG-dC) and a 44 bp alternating (dT-dC)·(dG-dA) insert respectively in the *sma*I site of the polylinker region of pDPL6. Plasmids were grown in *E. coli* HB101, amplified with chloramphenicol and isolated by the alkali-lysis procedure [17]. ³H-labelled plasmids were grown in medium containing 2 μ Ci of [³H]thymidine/ml. The specific radioactivities of ³H-labelled ssDNA and plasmid DNAs ranged from 0.7×10^4 to 1.3×10^4 c.p.m./ μ g. Poly(dA-Br⁵dU), dGTP, dCTP and ATP were obtained from Pharmacia, adenosine 5'-(γ -thio)triphosphate (ATP[S]) was from Boehringer Mannheim and [³H]thymidine was from Amersham.

DNA concentrations were calculated using $A_{260} = 1$ as equivalent to 50 μ g of dsDNA/ml, 36 μ g of ssDNA/ml, 55 μ g of poly(dG-dC)/ml, 69 μ g of Br-poly(dG-dC)/ml and 78 μ g of poly(dA-Br⁵dU)/ml.

Proteins

The recA protein was purified by the procedure of Cox *et al.* [18]. The molecular mass of recA was taken as 38 kDa [19]. The concentration of recA protein in stock solutions was determined by the absorbance at 280 nm by using $A_{280,1\text{cm}}^{0.1\%} = 0.59$ [20]. For confirmation of certain studies 200 μ g of purified recA protein was purchased from Pharmacia.

The polyclonal anti-(Z-DNA) antibody AZ-6 was produced by repeated injection of left-handed Br-poly(dG-dC) into rabbits and was kindly provided by T. Hutcheon, University of Calgary.

Filter binding assays

A typical binding reaction was performed in a total volume of 100 μ l containing 20 mM-Tris/HCl buffer, pH 7.5, 1.2 mM-MgCl₂, 1 mM-dithiothreitol and 1 μ g of [³H]ssDNA or 2 μ g of [³H]Z-DNA. Various amounts of recA protein were added and the reaction mixture was incubated for 30 min at 37 °C. ATP or ATP[S] (200 μ M) was present in the reaction where stated. Most of the reaction mixture (95 μ l) was then passed through a nitrocellulose filter (2.5 cm-diameter Millipore type Ha, 0.45 μ m pore size, prewashed in 0.3 M-NaOH). Filters were washed with 2 ml of the binding buffer and dried; retained radioactivity was measured by liquid-scintillation counting. The binding of recA protein to plasmid DNA was measured similarly. Plasmids were linearized by digestion with the restriction endonuclease *Pst*I. Maximal binding was not achieved in several binding experiments, most probably because of incomplete retention of the protein-DNA complexes.

Competitive filter binding assays were carried out in one of two ways. By the first method, reaction mixtures contained [³H]ssDNA and increasing amounts of equivalent Z-DNA as competitor DNA in binding buffer. In these studies 1 μ g of ssDNA has been considered to be equivalent to 2 μ g of ds-Z-DNA. RecA protein was then added to the reaction mixtures, which were incubated for 30 min at 37 °C before filtration. Control reaction mixtures contained all components with the exception of Z-DNA. Reciprocal inhibition studies were carried out by including [³H]Z-DNA and increasing amounts of equivalent ssDNA as competitor DNA in the reaction mixtures. The reaction mixtures were treated as above.

In another method, recA protein was preincubated with [³H]DNA probe in the binding buffer for 15 min at 37 °C. An equal volume of challenge solution containing increasing amounts of competitor DNA in the binding buffer was then added to the preincubation solution, and the total reaction mixture was incubated for 30 min at 37 °C before filtration. ATP[S] was included where stated.

DNA-unwinding assay

Unwinding of plasmid DNA was initiated by incubating 1 μ g of supercoiled plasmid DNA with increasing amounts of recA protein for 30 min at 37 °C, in a modified binding buffer (20 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-MgCl₂, 1 mM-dithiothreitol and 10 mM-KCl) containing 200 μ M-ATP[S]. The binding buffer was modified to ensure activity of calf thymus topoisomerase 1 (BRL), 10 units of which were added next, and the reactions were further incubated for 3–4 h at 37 °C. Each reaction mixture was extracted twice with phenol and twice with chloroform to ensure removal of the proteins, and was then directly loaded on a 1.5% agarose gel. Electrophoresis was carried out in Tris/borate/EDTA (TBE) buffer at 5 V/cm for 14 h. The gel was stained in 0.5 μ g of ethidium bromide/ml and then photographed. To see if the antibody AZ-6 inhibited the unwinding activity of recA, it was first incubated with plasmid DNA at 22 °C for 30 min followed by addition of recA protein and a second incubation of the total reaction for 30 min at 37 °C. Topoisomerase was then added and the reaction mixture was treated as described above. Dithiothreitol was omitted from the modified binding buffer when AZ-6 was used.

Chemical cross-linking of protein-DNA complexes

Plasmid DNA (1 μ g) was incubated for 30 min at 37 °C with the antibody AZ-6 in 10 mM-triethanolamine/HCl buffer, pH 8.0, containing 1 mM-EDTA and 20 mM-NaCl, or with recA protein in 10 mM-sodium phosphate buffer, pH 7.5, containing 1.2 mM-MgCl₂, 0.1 mM-dithiothreitol and 200 μ M-ATP[S]. The protein-DNA complexes were cross-linked sequentially in 1% formaldehyde and 0.6% glutaraldehyde on ice for 15 min [21]. The reaction mixtures were immediately placed in a microdialysis apparatus and dialysed overnight at 4 °C against 1 mM-Tris/HCl buffer, pH 8.0. The volume of the dialysed reactions was reduced to 15 μ l and then adjusted to 20 μ l in low-salt buffer. Cross-linked complexes were digested with *Hpa*II at 37 °C for 4–5 h. The restriction fragments generated were labelled by the fill-in reaction with the Klenow fragment of DNA polymerase I by addition of 10 μ Ci of [α -³²P]d(CTP) and 2 units of Klenow enzyme. The reaction was allowed to proceed at 22 °C for 30 min and was then terminated by incubation at 70 °C for 5 min to inactivate the enzyme. Samples were loaded on a 8% polyacrylamide gel and electrophoresed in TBE buffer at 15 V/cm for 4–5 h. After electrophoresis, the gel was covered with Saran wrap and exposed directly to the X-ray film (Kodak XAR).

RESULTS

Binding of recA protein to ssDNA and Z-DNA

Molecular aspects of the interaction of recA protein with ssDNA have been investigated in detail [1]. At neutral pH, recA protein polymerizes on to ssDNA in a highly co-operative manner, coating the DNA completely and extending it significantly. Recently it has been reported that recA protein binds preferentially to Z-DNA polymers compared with B-DNA polymers and that the binding to Z-DNA requires ATP or its non-hydrolysable analogue ATP[S] [8]. Assuming that ssDNA and Z-DNA regions may occur as unusual conformations *in vivo*,

Fig. 1. Binding of recA protein to Z-DNA and to ssDNA

Binding of recA protein to [3 H]Z-DNA (2 μ g) (■, ▲ and ●) and [3 H]ssDNA (1 μ g) (□, △ and ○) was measured as a function of protein concentration in the absence of a nucleotide cofactor (■ and □), in the presence of 200 μ M-ATP[S] (▲ and △) and in the presence of 200 μ M-ATP (● and ○).

we have attempted to compare recA protein binding to ssDNA with its binding to Z-DNA. Although the binding of recA protein to ssDNA and Z-DNA is greater in the presence of 10 mM-MgCl₂, we nevertheless used the low MgCl₂ concentration throughout our studies to avoid aggregation of Z-DNA.

Titration experiments carried out with a nitrocellulose filter binding assay to measure the amount of DNA bound indicate that recA protein binds to left-handed Br-poly(dG-dC) in the absence of a nucleotide cofactor and also exhibits higher binding to the Z-DNA polymer than to ssDNA. Fig. 1 shows that whereas 40 % of the ssDNA probe was retained in the absence of a nucleotide cofactor with high concentrations (0.2 mg/ml) of recA protein, approx. 80 % of the Z-DNA probe was retained under similar conditions and at the same concentration of the protein. Since the Z-DNA probe (average size 700 bp) was smaller in length than the ssDNA probe, size would not be a factor in the higher retention of Z-DNA, which may be due, for example, to recA protein binding to DNA ends. The observation that a nucleotide cofactor is not required in the interaction of recA protein with left-handed Br-poly(dG-dC), at either 1.2 mM- or 10 mM-MgCl₂ concentration, differs from the result obtained by Blaho & Wells [8]. Also, the extent of Z-DNA probe retained in the presence of 200 μ M-ATP[S] was found to decrease (Fig. 1) as compared with that retained in the absence of a nucleotide cofactor. Under these conditions a maximum of 60 % of the Z-DNA probe was bound to the filter. In a similar experiment conducted in the presence of ATP[S], the binding to ssDNA was consistent with previously reported behaviour [22], and increased roughly 2-fold, resulting in 100 % retention of the ssDNA probe on the filter (Fig. 1). The stoichiometry of recA protein binding to ssDNA in the presence of ATP[S] was calculated, from the 50 % retention point for ssDNA probe, as approximately eight

nucleotide residues per molecule of recA protein. This value is in good agreement with the reported values [1,22] and verifies the activity of our recA protein.

To confirm the unusual behaviour of recA protein towards Z-DNA in the presence of ATP[S], another preparation of recA protein (Pharmacia) was used to bind ssDNA and Z-DNA in the presence or in the absence of ATP[S]. Similarly to the behaviour shown by the previous recA protein, whereas binding to ssDNA increased in the presence of ATP[S], the binding to Z-DNA again decreased under identical conditions (results not shown).

The binding of recA protein to ssDNA and Z-DNA was also evaluated in the presence of ATP. It is known that recA protein possesses DNA-dependent ATPase activity and that hydrolysis of ATP to ADP causes dissociation of recA-DNA complexes [23,24]. In the presence of ATP, recA binding to both ssDNA and Z-DNA decreased (Fig. 1) as compared with the binding obtained in the absence of any nucleotide cofactor. Data presented in Fig. 1 show that Z-DNA is retained more than ssDNA in the presence of ATP.

Competition studies

The ability of recA protein to bind Z-DNA in the presence of ssDNA and vice versa was tested in binding competition experiments. These experiments were performed in two different ways as described in the Materials and methods section. In the first case [3 H]ssDNA and competing Z-DNA (unlabelled) were present together in the binding reaction. RecA protein was added to the reaction mixture, which was then incubated for 30 min and filtered. Results presented in Fig. 2(a) show that Z-DNA is a good competitor of ssDNA even in the presence of ATP[S]. An equivalent amount (see the Materials and methods section for definition) of competing Z-DNA caused 45 % inhibition of ssDNA binding. In a reciprocal competition experiment, in which [3 H]Z-DNA and competing ssDNA (unlabelled) were present together, an equivalent amount of ssDNA caused a 60 % inhibition of Z-DNA binding to recA protein (Fig. 2b). Thus recA protein appears to have a slightly higher affinity for ssDNA in the presence of ATP[S]. However, the competition curve presented in Fig. 2(b) is not linearly dependent on the concentration of the competing ssDNA; about 30 % of the Z-DNA probe remained bound to the protein even in the presence of a 5-fold excess of ssDNA. This indicates the presence of multiple binding sites on the Z-DNA polymer that have unequal affinities for the recA protein, or that at high concentrations of ssDNA a ternary complex forms between recA protein and the two different types of DNA.

Additional competition experiments with a 5-fold excess of poly(dG-dC) or poly(dA-Br⁵dU) (B-DNAs) as competitor resulted in approx. 20 % inhibition of Z-DNA binding. As for ssDNA binding, it is already known that large excesses of dsDNA as competitor cause little, if any, inhibition at pH 8.0 [25].

In another set of experiments, dissociation of preformed recA protein-DNA complexes with increasing concentrations of competing DNA was studied. It is important to note that these experiments determine the transfer of recA protein from one DNA molecule to another based on the concentration of the challenger DNA. Time was not varied in these experiments [22]. RecA protein was first incubated with [3 H]ssDNA for 15 min, and transfer was then initiated by the addition of increasing concentrations of unlabelled Z-DNA and 200 μ M-ATP[S] (Fig. 2a). Results of a reciprocal dissociation experiment are shown in Fig. 2(b). These results demonstrate that in the presence of ATP[S] ssDNA can dissociate preformed recA protein-Z-DNA complexes and Z-DNA can dissociate preformed recA protein-ssDNA complexes. However, the addition of ATP[S] at

Fig. 2. Competition of (a) ssDNA binding to recA protein by the addition of Z-DNA and (b) Z-DNA binding to recA protein by the addition of ssDNA

(a) Reaction mixtures containing 1 μg of [^3H]ssDNA, 200 μM -ATP[S], indicated amounts of unlabelled Z-DNA as competitor and 4 μg of recA protein were incubated for 30 min at 37 $^{\circ}\text{C}$ before filtration (■). Reaction mixtures containing 1 μg of [^3H]ssDNA and 4 μg of recA protein were first incubated for 15 min at 37 $^{\circ}\text{C}$. Equal volumes of challenge solution containing increasing amounts of unlabelled Z-DNA and 200 μM -ATP[S] were added and the total reaction mixtures were incubated for 30 min at 37 $^{\circ}\text{C}$ before filtration (□). (b) Reaction mixtures containing 1 μg of [^3H]Z-DNA, 200 μM -ATP[S], indicated amounts of unlabelled ssDNA as competitor and 4 μg of recA protein were incubated for 30 min at 37 $^{\circ}\text{C}$ before filtration (■). Reaction mixtures containing 1 μg of [^3H]Z-DNA and 4 μg of recA protein were first incubated for 15 min at 37 $^{\circ}\text{C}$. Equal volumes of challenge solution containing increasing amounts of unlabelled ssDNA and 200 μM -ATP[S] were added and the total reaction mixtures were incubated for 30 min at 37 $^{\circ}\text{C}$ before filtration (□).

the time of addition of unlabelled competing DNA increases the stability of recA protein–ssDNA complexes by preventing dissociation, which can be seen as the difference in the curves presented in Figs. 2(a) and 3(a). By and large, it can be inferred that ssDNA and Z-DNA compete with each other for binding to recA protein; the sites for binding to ssDNA and Z-DNA are presumably identical or overlapping.

A non-dissociable complex is reported to form between recA protein and ssDNA in the presence of ATP[S] [22,25]. To assess whether increasing amounts of Z-DNA had any effect on such complexes, a competition titration was performed in which [^3H]ssDNA was incubated with recA protein in the presence of ATP[S] for 15 min at 37 $^{\circ}\text{C}$. To these reaction mixtures increasing amounts of unlabelled Z-DNA were added as challenge DNA. An insignificant dissociation of 10 % of the recA protein–ssDNA complexes was seen at the highest concentration of Z-DNA added (Fig. 3a). When recA protein–Z-DNA complexes formed in the presence of ATP[S] were challenged with unlabelled ssDNA, a maximum dissociation of 25 % was observed (Fig. 3b). Similar dissociation experiments were also performed in the absence of a nucleotide cofactor. An intriguing finding is that recA protein–ssDNA complexes formed in the absence of a nucleotide cofactor are very efficiently dissociated by Z-DNA (Fig. 3a). A 5-fold excess of Z-DNA caused 83 % dissociation of the protein–ssDNA complexes. In comparison, a 5-fold excess of unlabelled ssDNA caused a dissociation of the same complex by only 18 % (Fig. 3a). In a reciprocal experiment ssDNA caused a maximum dissociation of 22 % of recA protein–Z-DNA complexes formed

in the absence of a nucleotide cofactor (Fig. 3b). In contrast, 50 % dissociation was observed with a 5-fold excess of unlabelled Z-DNA (Fig. 3b). From these results it is clear that Z-DNA is capable of dissociating recA protein–ssDNA complexes or recA protein–Z-DNA complexes, formed in the absence of ATP[S], much more efficiently than does ssDNA. However, in the presence of 10 mM-MgCl₂, Z-DNA was an inefficient competitor for recA protein binding to ssDNA (results not shown). This may be due to the fact that recA protein binding to ssDNA is optimal at the high Mg²⁺ concentration.

Binding of recA protein to plasmids

Evidence suggests that the binding of recA protein to supercoiled dsDNA is dependent upon the superhelical density of the DNA molecule [4,6,26]. It is also well documented that negative supercoiling of DNA strongly stabilizes Z-DNA (for a review see ref. [27]). If recA protein binding to Z-DNA is a functional activity of this protein, then the binding to supercoiled plasmids that contain Z-segments should also be detectable. Four different ^3H -labelled plasmids were used in this study: pDPL6, pDHg16, pDhd16 and pBR322 (see the Materials and methods section). All plasmids were used at their natural superhelical densities found *in vitro* to bind recA protein, and the binding was detected by the nitrocellulose filter binding assay. The presence of Z-DNA in these plasmids was determined by binding to anti-(Z-DNA) antibody (AZ-6). Plasmid pDHg16 showed maximum binding to AZ-6 and pDhd16 showed the minimum (results not shown). This mode of binding behaviour would be expected since (dG-

Fig. 3. Dissociation of (a) preformed recA protein-ssDNA complexes (b) preformed recA protein-Z-DNA complexes

(a) Reaction mixtures containing 1 μ g of [3 H]ssDNA and 4 μ g of recA protein were preincubated for 15 min at 37 °C either in the absence (● and ■) or in the presence (□) of ATP[S]. Equal volumes of challenge solution containing increasing amounts of unlabelled ssDNA (●) or Z-DNA (■ and □) were added and the total reaction mixtures were incubated for 30 min at 37 °C before filtration. (b) Reaction mixtures containing 1 μ g of [3 H]Z-DNA and 4 μ g of recA protein were incubated for 15 min at 37 °C in either the absence (■ and ●) or the presence (○) of ATP[S]. Equal volumes of challenge solution containing increasing amounts of unlabelled Z-DNA (■) or ssDNA (● and ○) were added and the total reaction mixtures were incubated for 30 min at 37 °C before filtration.

dC)_n tracts are known to assume a left-handed conformation at natural superhelical densities [16] whereas (dT-dC)_n tracts do not adopt a Z-conformation but are characterized to form an unusual structure at low pH and on negative supercoiling of the plasmid [28,29]. In contrast with the results obtained with AZ-6, recA protein bound preferentially to pDHd16 and showed almost no difference in its binding to pDHg16 and pDPL6. pBR322 was bound the least (Fig. 4a). The result of equal binding of recA protein to plasmids with or without Z-DNA helices is in agreement with the previously reported results obtained by Blaho & Wells [8], although different plasmids at superhelical densities of -0.06 were used by these authors. In the absence of ATP[S], as opposed to the results obtained with the Z-DNA polymer, an increase in binding was not observed with the plasmid pDHg16 (results not shown). This indicates that recA protein binding to the chemically modified Z-DNA polymer is a separate activity and is not reflected in its binding to plasmids.

When linear plasmids pDHg16, pDHd16 and pDPL6 were used to bind recA protein, linear pDHd16 was retained maximally (Fig. 4b). The binding to linear pDHd16 and pDHg16 was somewhat similar to that obtained with the respective supercoiled molecules. The binding to pDPL6 was slightly diminished when the plasmid was in the linear state. A likely interpretation of these results is that recA protein has sequence preferences for dinucleotide repeats, in this case more for the (dT-dC)_n tracts of pDHd16 than for the (dG-dC)_n tracts of pDHg16.

If binding of a protein to a covalently closed circular DNA molecule is associated with an unwinding of the DNA helix, then unwinding can be measured by relaxing DNA with a topoisomerase, removing the protein and then analysing the

DNA molecular on a gel. RecA binding to supercoiled plasmids is accompanied by extensive unwinding in the presence of ATP or ATP[S] at low Mg²⁺ conditions [3,30]. We wished to determine whether this mode of binding is similar with the three plasmids pDPL6, pDHg16 and pDHd16. The plasmids were treated with increasing concentrations of recA protein in the presence of ATP[S]. The results presented in Fig. 5(a) show that both pDPL6 and pDHg16 were unwound and that the unwound species increased with increasing concentrations of recA protein. Similar results were obtained with pDHd16 (results not shown). Thus no unusual mode of interaction was observed with pDHg16. Further, no intermediate topoisomers between the relaxed and supercoiled forms were seen, which would be expected if recA protein were to bind exclusively and stabilize the 24 bp insert in the Z-form, as is the case with the antibody AZ-6 (Fig. 5b, lane 7). These results indicate that pDHg16 is co-operatively bound and unwound by recA protein, presumably with destabilization of the Z-helix. Although pDHg16 is unwound by recA protein as is any other plasmid, does the Z stretch in pDHg16 serve as a nucleation point in the binding of recA protein and in its initiation of unwinding? This problem was addressed by testing how well AZ-6, which binds and stabilizes the Z-DNA region in pDHg16, could block the unwinding of the plasmid by recA protein. In this experiment the incubation of plasmids with AZ-6 preceded the addition of recA protein and was followed by treatment with topoisomerase I. The Z-DNA antibody (used at a concentration that bound 100 % of the supercoiled fraction of pDHg16 as judged by a gel retardation assay) partially inhibited the formation of the unwound species of both pDPL6 and pDHg16, suggesting thereby that some interference occurred

Fig. 4. Retention of radioactive plasmids on nitrocellulose filters as a function of increasing amounts of recA protein

(a) The binding of recA protein to supercoiled plasmids (1 μg each) pDPL6 (▲), pDHg16 (□), pDHd16 (■) and pBR322 (○) was measured in the presence of 200 μM-ATP[S]. (b) The binding of recA protein to linearized plasmids (1 μg each) pDPL6 (▲), pDHg16 (□) and pDHd16 (■) was measured in the presence of 200 μM-ATP[S].

other than at the protein–DNA level (Fig. 5b, lanes 4 and 9). However, the observation that pDHg16 could be unwound in the presence of high concentrations of AZ-6 suggests that the Z-helix is not exclusive for the initiation of unwinding, and that once unwinding occurs the antibody may no longer remain bound to that fraction of the plasmid. On the other hand, the centre of the topoisomer distribution of the rest of the supercoiled fraction of pDHg16 (not bound by recA protein) is seen to be shifted from the fully relaxed distribution (lane 9), indicating that the (dG-dC)_n tract is maintained in the Z-form by the Z-DNA antibody.

Protein–DNA cross-linking studies

In order to map specific interactions of recA protein with pDHg16 and pDPL6, the recA protein–DNA complexes were first chemically fixed [21] and then subjected to restriction-enzyme analysis. Fragment A' of pDHg16 represents the sum of fragments A and a of pDPL6 and a (dG-dC)₁₂ insert (Fig. 6a). When plasmids pDHg16 and pDPL6 were allowed to react and cross-linked with AZ-6, fragment A' of pDHg16 was specifically retarded to the origin of the gel whereas no such shift in migration was obtained with the analogous fragments A and a of pDPL6. This indicates a specific interaction of AZ-6 with the Z-DNA stretch contained within the fragment A' of pDHg16.

The interaction of recA protein with duplex DNA is co-operative, and, once initiated, the extension of the recA filament and unwinding of the DNA are not rate-limiting [5]. If this were true for recA protein binding to the plasmid containing the Z insert, then at high concentrations of recA protein it would be impossible to observe any specific interaction of this protein with regions of DNA. For this reason the experiment was carried out at non-saturating concentrations of recA protein. Increasing concentrations of recA were incubated with plasmid DNA in order to observe a gradual protein-dependent effect. No dis-

placement was seen of any one of the labelled fragments of either pDPL6 or pDHg16 at any protein concentration (results not shown). Fig. 6(b) presents the results of an experiment carried out at the highest concentration of recA protein (13 μg of recA/μg of plasmid DNA) employed in these experiments. A portion of the labelled DNA was retained at the origin of the gel for both pDPL6 and pDHg16, and this effect was more pronounced for the supercoiled plasmid DNA as compared with the relaxed DNA, indicating that recA protein interacts more favourably with supercoiled DNA than with relaxed DNA. However, as judged from the intensity of the labelled bands, the A' fragment derived from supercoiled pDHg16 showed no specific retardation relative to the other bands of the same lane or those of the appropriate control lanes. These results indicate that, unlike antibody binding, once the recA protein binding is initiated it occurs simultaneously throughout the DNA molecule. This observation is substantiated by the results of the unwinding experiments reported here (Fig. 5) and by the results obtained by Pugh & Cox [5], where less unwound intermediates were not detected either under conditions of low protein concentrations (see Fig. 5) or within short time periods [5] respectively.

Thus the experiments described here do not indicate that recA binding to a plasmid containing a Z-DNA stretch is in any way unusual as compared with the control vector plasmid.

DISCUSSION

The ability of recA protein to interact with a Z-DNA polymer and M13 ssDNA was compared as a first step towards understanding what might be a preferred nucleation site for this protein. The results show that recA protein binding to a chemically modified Z-DNA polymer, Br-poly(dG-dC), is different from its B-DNA and ssDNA binding activity. RecA protein binds more

Fig. 5. Unwinding of supercoiled plasmid DNA by recA protein

(a) Plasmid DNA (1 μ g) was incubated with increasing amounts of recA protein in the presence of 200 μ M-ATP[S] for 30 min at 37 $^{\circ}$ C. Topoisomerase was then added and the reaction mixtures were incubated for 3–4 h. After removal of proteins, samples were analysed by agarose-gel electrophoresis. Lanes 1 and 6 represent supercoiled (s) plasmids pDPL6 and pDHg16 respectively. Lanes 2 and 7 represent the same plasmids treated with topoisomerase (r). Lanes 3–5 and 8–10 represent the same plasmids treated with 2.5, 5 and 30 μ g of recA protein and subsequently treated with topoisomerase. (b) Plasmid DNA (1 μ g) was first incubated with AZ-6 (18 μ g of protein) at 22 $^{\circ}$ C for 30 min. Subsequently 7 μ g of recA protein was added and the reaction mixture was further incubated for 30 min at 37 $^{\circ}$ C. Topoisomerase was then added and the reaction mixtures were treated as described above. Lanes 1 and 5 represent plasmids pDPL6 and pDHg16 respectively. Lanes 2 and 6 represent the same plasmids treated with topoisomerase. Lanes 3 and 8 represent the same plasmids treated sequentially with recA protein and topoisomerase. Lanes 4 and 9 represent the same plasmids treated sequentially with AZ-6, recA protein and topoisomerase. Lane 7 represents pDHg16 treated sequentially with AZ-6 and topoisomerase.

avidly to synthetic Z-DNA than to ssDNA in the absence of a nucleotide cofactor. However, in the presence of ATP[S], the binding to ssDNA increases approx. 2-fold, whereas binding to the Z-DNA probe decreases relative to the binding obtained when no cofactor was present. This effect of ATP[S] on recA binding to Z-DNA appears to be in contrast with its effect on recA binding to both ssDNA and B-DNA [1], in which case the increase in affinity of recA protein for the DNAs in the presence of ATP[S] is accompanied by higher retention of the DNAs. Although the exact reason for the decrease in Z-DNA binding in the presence of ATP[S] is currently not understood, it is possible that recA protein binds Z-DNA at random sites in the absence of ATP[S] and that the binding character changes for a co-operative assembly of recA monomers on DNA in the presence of ATP[S]. The affinity of recA protein for Z-DNA could still be higher in the presence of ATP[S]. Studies have shown that the structure of the bound DNA within the recA protein–DNA complexes can also be affected by nucleotide binding [24,31]. It remains to be seen if the structure of this particular Z-DNA polymer within a protein–Z-DNA complex is modulated by the presence of ATP[S].

The presence of ATP decreased the binding of recA protein to both Z-DNA and ssDNA. It has previously been suggested that

the inhibition is due to the decrease in affinity for DNA caused by increased ADP concentration arising from ATP hydrolysis [24]. The fact that recA protein binds to Z-DNA to a greater extent than to ssDNA under these conditions may indicate that recA protein–Z-DNA complexes are more stable in the presence of ADP and therefore possess a lower turnover rate. This could also possibly be due to a difference in the ATPase activities of recA protein that arise from its stimulation to various degrees by either ssDNA or Z-DNA. The binding of recA protein to Z-DNA could not be evaluated at steady-state concentrations of ATP, as inclusion of an ATP-regenerating system caused over 80% of the Z-DNA probe to be retained on the filter in the absence of recA protein.

The competition results show that the interaction of recA protein with one substrate is affected by the presence of the other substrate. At equivalent ratios, recA protein appears to have a slightly higher affinity for ssDNA over Z-DNA in the presence of ATP[S]. For higher concentrations of competing DNA, a clear interpretation of the results is presently unavailable. Nevertheless, it can be said that ssDNA and Z-DNA, in the presence of ATP[S], compete with each other for binding to recA protein. Because of the limited understanding of recA protein interactions with Z-DNA and because of the method of analysis, quantitative

Fig. 6. Comparison of the restriction endonuclease patterns obtained with plasmid DNA versus cross-linked plasmid DNA-protein complexes

(a) 1 μ g of supercoiled plasmid DNA was incubated with AZ-6 (18 μ g of protein) and the complexes were chemically fixed as described in the Materials and methods section. Untreated plasmid DNA and the cross-linked DNA-IgG complexes were then digested with restriction endonuclease *Hpa*II and subsequently labelled. The samples were analysed on an 8% polyacrylamide gel. The autoradiogram shows restriction fragment patterns for pDPL6 and pDHg16, as indicated either with (+) or without (–) cross-linked AZ-6. The origin of the gel and the restriction fragments of interest are indicated. (b) A 1 μ g portion of supercoiled (s) or relaxed (r) plasmid DNA was incubated with 13 μ g of recA protein and the complexes were chemically fixed, restricted, labelled and analysed on a gel as described above. The autoradiogram shows the restriction fragment patterns for pDPL6 and pDHg16, as indicated either with (+) or without (–) cross-linked recA protein. The origin of the gel and the restriction fragments of interest are indicated.

interpretations of the data obtained in binding experiments would be meaningless at this point in time. A complete analysis of recA protein binding to polynucleotides requires detailed knowledge of the physical properties of its single components and their interactions. For example, information on different protein species that are present in solution, and on different sites on a DNA molecule that may lead to the formation of different protein-DNA complexes, is required to obtain a quantitative picture of recA binding to Z-DNA. It is conceivable that Br-poly(dG-dC) offers more than one binding site to the protein because of different degrees of chemical modification and/or the presence of single-stranded regions. Two binding sites on this polymer have been reported to be recognized by one of the proteins isolated from bull testis [32].

A striking observation of the dissociation experiments is the ability of Z-DNA effectively and rapidly to dissociate recA protein-ssDNA complexes preformed in the absence of a nucleotide cofactor. Previous observations with ssDNA [22] suggest that recA protein released from the preformed complexes equilibrates with challenge DNA, in this case Z-DNA. This indicates

a high affinity of recA protein for Z-DNA in the absence of a nucleotide cofactor, and further consolidates the results of our direct binding experiments. RecA protein can also efficiently migrate from a recA protein-Z-DNA complex to free molecules of challenge Z-DNA. This migration is much more efficient than that from a recA protein-ssDNA complex to free ssDNA molecules, a behaviour that argues against Z-DNA being the active species in the search for homology. One would envisage a more stable recA protein-Z-DNA complex if it were involved in the pairing reaction.

The recA protein binding to recombinant plasmids showed no preferential binding to the plasmid-containing sequences that adopt a Z-conformation. It has previously been noted for Z-DNA-binding proteins that relative affinities of these proteins for plasmids with Z-forming inserts, as compared with their parent vector, are much smaller than the relative affinities determined for the Z-polymer versus B-DNA [33]. The evaluation with recA protein is even more complicated because of its ability to bind rapidly and stably to denatured, underwound or unwindable regions in a plasmid DNA [6], and further because of its co-operative mode of binding. Contrary to the binding of recA to Br-poly(dG-dC), the binding to the supercoiled form of plasmid pDHg16 did not increase when ATP[S] was omitted from the buffer. Previously Blaho & Wells [8] did not observe a correlation between the Z-DNA-stimulated ATPase activity of recA (characterized for Z-DNA polymers) and the interaction of the protein with plasmids containing Z-DNA tracts. These results together strongly suggest that binding mechanisms of recA protein to Z-DNA polymers are not reflected in its interaction with plasmids such as pDHg16, but they do not rule out the possibility of recA binding to (dG-dC)_n stretches in a plasmid. The observation that recA protein preferentially binds to supercoiled or linear pDHd16 over other plasmids suggests that recA protein exhibits sequence preferences in plasmid binding. Further justification of this concept comes from evidence described in an earlier report that recA protein displays significant sequence preferences in the process of homologous pairing [34].

If the recA protein-Z-DNA complex is an active species in the initiation of strand exchange as proposed by Kmiec & Holloman [10], then stabilization of Z-DNA by recA protein would be a natural assumption. Since the stabilization of one turn of Z-DNA results in a net unwinding of approximately two superhelical turns, the centre of the topoisomer distribution of the supercoiled fraction should have been shifted by four to five turns from the fully relaxed distribution in pDHg16. A pattern of topoisomer distribution similar to that obtained with AZ-6 in the unwinding assay was not observed, nor did we see any major differences in the topoisomer distribution of different plasmids upon interaction with recA protein. Therefore we conclude that pDHg16, like the other plasmids, underwent co-operative binding and unwinding in the presence of recA protein and ATP[S]. Extensive unwinding caused by recA protein results in the loss of negative supercoils. In this event the Z-conformation in a plasmid is likely to be destabilized unless otherwise acted on by the protein. Recently it has been demonstrated that recA protein does not stabilize the Z-conformation of the polynucleotide poly(dG-m⁵dC) [12,35], but that binding to the Z form of this polymer is faster than binding to B-DNA [12]. The kinetics of binding to Z-DNA remains controversial [12,35]. The results presented in Fig. 5(b) argue against Z-DNA being the initiation site on plasmid pDHg16 for recA binding. Alternatively (and more likely) unwinding, which is known to be initiated at denatured regions [5,6], could be initiated at sites like the B-Z junctions and then propagate to the rest of the molecule.

The results presented here, and those of Takahashi *et al.* [35], emphasize that it is premature to conclude that the binding of

recA protein to Z-DNA polymers is indicative of functional significance. The characteristics of Z-DNA polymers, including sequence, length, chemical modifications and the conditions used in their stabilization, do not necessarily reflect Z-DNA as it presumably would occur *in vivo*. It is well documented that recA protein binds with a much higher affinity to certain chemically modified ssDNA and dsDNA as compared with the unmodified DNA [24,35]. Therefore studies involving comparison of recA protein binding to a synthetic Z-DNA polymer versus chemically unmodified B-DNA or plasmid DNA could be misleading. The use of different Z-DNA polymers for recA protein binding *in vitro* has indeed led to differing results ([8,12,35]; the present study), which are most probably due to variations in polymer structure and differences in the experimental conditions. We have previously shown that even strong and preferential interactions of proteins with Z-DNA polymers are not functionally relevant [13,36].

In summary, these results do not indicate an association of recA protein with plasmid-localized Z-DNA tracts as such. However, a positive influence of unwound regions present at the B-Z junctions remains a possibility. The mere binding of recA protein to Z-DNA polymers [8,9,12] cannot be taken as sufficient proof of an important role for Z-DNA in recombination.

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