Effect of Nucleotide Cofactor Structure on RecA Protein-Promoted DNA Pairing. 2. DNA Renaturation Reaction†

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Received December 17, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: We have examined the effects of the structurally related nucleoside triphosphates, adenosine triphosphate (ATP), purine riboside triphosphate (PTP), inosine triphosphate (ITP), and guanosine triphosphate (GTP), on the recA protein-promoted DNA renaturation reaction (φX DNA). In the absence of nucleotide cofactor, the recA protein first converts the complementary single strands into unit-length duplex DNA and other relatively small paired DNA species; these initial products are then slowly converted into more complex multipaired network DNA products. ATP and PTP stimulate the conversion of initial product DNA into network DNA, whereas ITP and GTP completely suppress network DNA formation. The formation of network DNA is also inhibited by all four of the corresponding nucleoside diphosphates, ADP, PDP, IDP, and GDP. Those nucleotides which stimulate the formation of network DNA are found to enhance the formation of large recA–ssDNA aggregates, whereas those which inhibit network DNA formation cause the dissociation of these nucleoprotein aggregates. These results not only implicate the nucleoprotein aggregates as intermediates in the formation of network DNA, but also establish the functional equivalency of ITP and GTP with the nucleoside diphosphates. Additional experiments indicate that the net effect of ITP and GTP on the DNA renaturation reaction is dominated by the corresponding nucleoside diphosphates, IDP and GDP, that are generated by the NTP hydrolysis activity of the recA protein.

The recA protein of *Escherichia coli* is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The purified recA protein is a DNA-dependent ATPase and will promote a variety of ATP-dependent DNA pairing reactions that reflect in vivo functions (Radding, 1982; Griffith & Harris, 1988; Roca & Cox, 1990; Kowalczykowski, 1991). In the preceding paper (Menge & Bryant, 1992), we showed that the structurally related nucleoside triphosphates, adenosine triphosphate (ATP), purine riboside triphosphate (PTP), inosine triphosphate (ITP), and guanosine triphosphate (GTP), are all hydrolyzed by the recA protein with the same turnover number, but that only ATP and PTP are able to function as cofactors for the recA protein-promoted three-strand exchange reaction. Our results suggested that ITP and GTP are inactive in strand exchange because the recA protein–ssDNA[1] presynaptic complex is destabilized by the corresponding nucleoside diphosphates, IDP and GDP, that are generated by the NTP hydrolysis activity of the recA protein (Menge & Bryant, 1992).

In addition to the complex three-strand exchange reaction discussed in the preceding paper, the recA protein will also promote the simple renaturation of complementary single-stranded DNA molecules to form duplex DNA products (Weinstock et al., 1979). Our initial mechanistic analysis of the recA protein-promoted renaturation of the linear complementary (+) and (−) strands of φX DNA at 25 °C established the following: (1) optimal renaturation occurs at a ratio of 1 recA monomer/30 nucleotides of ssDNA, a level of recA protein sufficient to cover about 15% of the ssDNA; (2) although recA protein protein-promoted renaturation is stimulated by ATP, a significant reaction occurs in the absence of a nucleotide cofactor; (3) the ATP-stimulated reaction follows apparent first-order kinetics, indicating that the reaction mechanism involves the formation of intermediate recA–ssDNA complexes prior to renaturation of the complementary strands (Bryant & Lehman, 1985).

In a subsequent study employing a quantitative agarose gel assay, we found that the recA protein-promoted renaturation reaction proceeds in two kinetically-distinct stages. In the first stage, the complementary single strands are converted into unit-length duplex DNA (FIII DNA) as well as a distribution of other products that migrate above and below FIII DNA on an agarose gel ("initial product DNA"). In the second stage, initial product DNA is converted into complex multipaired DNA structures that do not migrate from the gel well ("network DNA"). The time-dependent formation of initial product DNA and network DNA from complementary single strands for both the cofactor-independent and the ATP-stimulated reactions could be modeled by a simple two-step sequential kinetic scheme (Figure 1). This analysis indicated that the primary stimulatory effect of ATP in the recA protein-promoted renaturation reaction is not on the initial pairing step (which leads to the formation of initial product DNA), but rather is to increase the rate of subsequent DNA pairing events (Bryant et al., 1989).

In order to extend the studies that are described in the preceding paper (Menge & Bryant, 1992), and to more precisely define the molecular basis for the mechanistic deficiency associated with ITP and GTP, we have examined the DNA renaturation activity and the corresponding ssDNA aggregation properties of the recA protein in the presence of these

† This work was supported by Grant GM 36516 from the National Institutes of Health.

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; φX, bacteriophage φX174; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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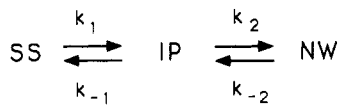

FIGURE 1: Two-step sequential kinetic scheme for the recA protein-promoted renaturation reaction. In this scheme, SS represents the complementary ϕX single strands, IP represents initial product DNA, and NW represents network DNA (as defined for the agarose gel electrophoresis system described in the text). RecA protein has been deliberately omitted from the kinetic formulation; since the renaturation data points were obtained by first quenching aliquots of the reaction mixtures with EDTA, it is possible that the renaturation products that are observed in the agarose gel assay are not formed as such until after the recA protein is removed (Bryant et al., 1989).

alternate nucleotide cofactors. The results of these studies are described in this report.

Experimental Procedures

Materials

E. coli recA protein was purified as previously described (Cotterill et al., 1982). Adenosine, inosine, and guanosine diphosphates and triphosphates were from Sigma; purine riboside diphosphate and triphosphate were synthesized as described (Menge & Bryant, 1992). ATPγS and GTPγS were from Boehringer-Mennheim. [^{3}H]ATP and [^{3}H]GTP were from ICN, and [γ-^{32}P]ATP was from Amersham.

Unlabeled and [^{3}H]-labeled circular ϕX ssDNA and circular ϕX dsDNA were prepared as described (Cox & Lehman, 1981). Linear ϕX dsDNA (Form III DNA) was prepared by *Xho*I (Bethesda Research Laboratories) cleavage of circular ϕX dsDNA, using the conditions recommended by the manufacturer. Linear ϕX dsDNA was [^{32}P]-labeled at the 5′-termini using calf intestinal phosphatase (Boehringer-Mannheim), T4 polynucleotide kinase (New England Biolabs), and [γ-^{32}P]ATP as described (Maniatas, 1982); the [^{32}P]-labeled DNA was purified by agarose gel electrophoresis and electoeluted from the gel using an Elutrap device (Schleicher & Schuell). Linear ϕX dsDNA was denatured to the complementary single strands as previously described (Bryant et al., 1989). DNA concentrations were calculated by using A_{260} of 1 as equivalent to 36 μg/mL ssDNA or 50 μg/mL dsDNA and are expressed as total nucleotides.

Methods

DNA Renaturation Reactions. DNA renaturation reaction mixtures contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 30 μM denatured [^{32}P]ϕX DNA [15 μM each of the (+) and (−) strands], 1 μM recA, and 1 mM NTP or NDP where indicated. After incubation of the reaction buffer for 8 min at 37 °C, denatured ϕX DNA was added and the incubation continued for an additional 2 min. Reactions were then initiated by the addition of recA protein and were carried out at 37 °C. Aliquots were quenched at various times with EDTA (15 mM) and then analyzed by electrophoresis on 0.8% agarose gels (containing 5 μg/mL ethidium bromide) using a Tris–acetate–EDTA buffer system (Maniatis et al., 1982). The gels were photographed under UV irradiation with Polaroid Type 57 film. We have obtained identical results in our gel assays when EDTA alone or SDS/EDTA was used as the quenching solution; thus the products that are visualized on the gels are not due to residual protein. For the kinetic analyses, the agarose gels were dried down onto DEAE paper (Whatman) and then exposed overnight to Kodak XAR-5 film. Using the autoradiograph as a template, each lane of the dry gel was cut into 0.6-cm^2 squares, beginning at the position of the gel well; each square was assigned to either unreacted ssDNA (identified from standards), network DNA (defined as all DNA species appearing in gel wells), or initial product DNA (defined as all DNA species migrating between ssDNA and network DNA, including FIII DNA product). The radioactivity in each square was determined by liquid scintillation counting, and the relative amounts of ssDNA, initial product DNA, and network DNA were calculated as a percent of the total radioactivity in that lane.

RecA Protein-Dependent Aggregation of ssDNA. The aggregation of ϕX ssDNA by recA protein was measured as described by Tsang et al. (1985). Reactions mixtures contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 30 μM [^{3}H]ϕX ssDNA [(+) strand], 1 μM recA protein, and 1 mM nucleotide where indicated. Reactions in the absence of nucleotide were initiated by addition of recA protein after incubation of all other components for 10 min at 37 °C; the reactions in the presence of NTP or NDP were initiated by addition of nucleotide after incubation of all other components (including recA protein) for 10 min at 37 °C. All incubations were then continued at 37 °C. Aliquots (48 μL) were removed at various times and centrifuged at maximum speed in a benchtop microfuge for 2 min. Three successive aliquots (12 μL) of the supernatant were added to 100 μL of TE [10 mM Tris-HCl, 1 mM EDTA (pH 7.5)], and the radioactivity of each was determined by liquid scintillation counting. The pellet and remaining supernatant were resuspended in 100 μL of TE, and the radioactivity was determined by liquid scintillation counting. The distribution of radioactivity between the supernatant and pellet fractions was corrected for the remaining supernatant (12 μL) in the pellet. The radioactivity present in the pellet fraction corresponded to [^{3}H]ϕX ssDNA that was in the form of large recA–ssDNA aggregates.

Results

Analysis of the RecA Protein-Promoted DNA Renaturation Reaction at 37 °C. Our earlier studies on the recA protein-promoted DNA renaturation reaction were carried out at 25 °C (Bryant et al., 1989). The reactions described in this paper, however, were carried out at 37 °C so that the results could be compared directly with the NTP hydrolysis and three-strand exchange reactions that are described in the preceding paper (Menge & Bryant, 1992). The reaction solutions contained 30 μM ϕX ssDNA [15 μM each of the (+) and (−) linear strands] and 1 μM recA protein, conditions previously determined to be optimal for the cofactor-independent and ATP-stimulated renaturation reactions promoted by the recA protein at 25 °C. Under these conditions, no significant renaturation of complementary ssDNA occurs in the absence of recA protein (data not shown). The renaturation reactions were monitored by agarose gel electrophoresis.

The time course for the renaturation reaction promoted by the recA protein in the absence of a nucleotide cofactor is shown in Figure 2A. The (+) and (−) linear strands were well separated on the gel and decreased concurrently throughout the reaction time course. Initially, unit-length linear duplex DNA (FIII DNA) was formed, as well as a distribution of other renaturation products that migrated above and below the FIII DNA band; these products will be referred to collectively as "initial product DNA". The initial product DNA was then slowly converted into a form that did not migrate from the gel well; these final products correspond to complex networks of multipaired DNA molecules (Bryant & Lehman, 1985) and will be referred to as "network DNA". The relative amounts of single-stranded DNA, initial product DNA, and network DNA were measured as described in

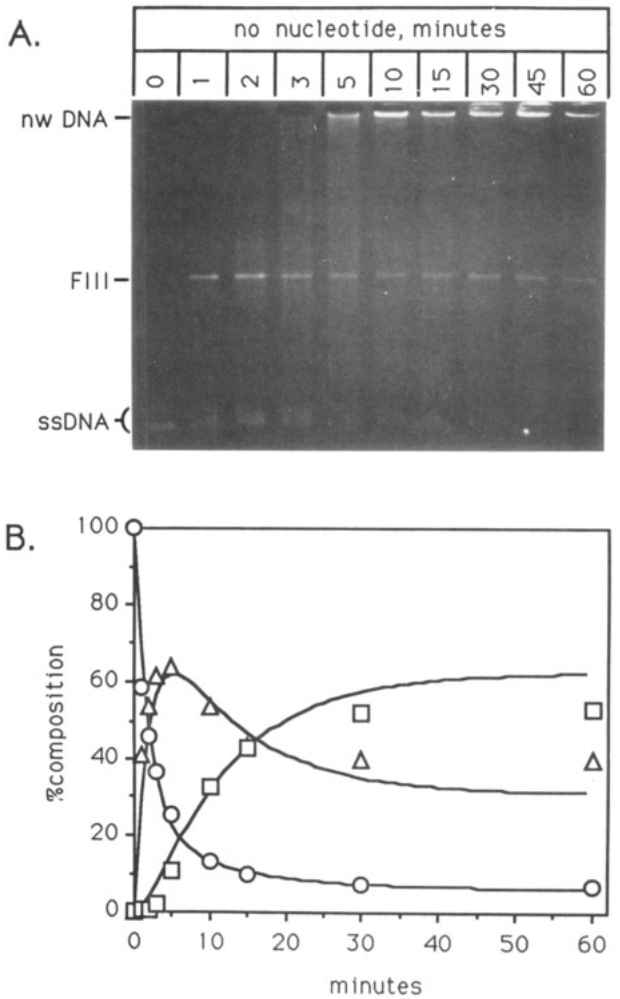

FIGURE 2: RecA protein-promoted renaturation reaction in the absence of nucleotide cofactor. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands] and 1 μM recA protein. The reactions were initiated by the addition of recA protein. Aliquots were quenched with EDTA at the indicated times and then subjected to agarose gel electrophoresis. (A) Agarose gel showing representative reaction time points. Markers: ssDNA, single-stranded DNA; FIII, linear duplex DNA; nw DNA, network DNA. (B) Kinetic time course. The relative amounts of ssDNA (circles), initial product DNA (triangles), and network DNA (squares) were determined as described in Experimental Procedures and are plotted as a function of reaction time; each point represents the average of 2–3 individual determinations [note: the particular gel pictured in (A) was not used for the kinetic analysis]. The simulated curves (solid lines) are based on the kinetic mechanism in Figure 1 and were calculated using the general equations for the two-step sequential mechanism (Moore & Pearson, 1981) and the parameters given in Table I.

Experimental Procedures and are plotted as a function of reaction time in Figure 2B. The kinetics of the appearance and disappearance of the FIII DNA paralleled that of the other initial products (when analyzed individually), indicating that the FIII DNA and other initial products are formed in a common step and that the entire population behaves as a single kinetic species in the subsequent conversion into network DNA (data not shown). The overall reaction time course can be modeled with the two-step sequential mechanism shown in Figure 1 and the parameters $k_1 = 0.4$ min^{-1}, $k_{-1} = 0.08$ min^{-1}, $k_2 = 0.07$ min^{-1}, and $k_{-2} = 0.035$ min^{-1} (Figure 2B, Table I). The significance of these parameters in terms of the physical mechanism of the renaturation reaction will be considered in the Discussion.

The time course for the renaturation reaction promoted by the recA protein in the presence of ATP (1 mM) is shown in

Table I: Kinetic Parameters for the RecA Protein-Promoted Renaturation Reaction at 37 °C[a]

nucleotide	k_1	k_{-1}	k_2	k_{-2}
none	0.4	0.08	0.07	0.035
ATP	1.6	≤0.08	1.0	0.04
PTP	1.6	≤0.08	0.80	0.08
ITP	0.12	0.008		
GTP	0.12	0.008		
ADP	0.12	0.024		
PDP	0.12	0.024		
IDP	0.12	0.024		
GDP	0.12	0.024		

[a] These parameters were determined by fitting the experimental data in Figures 1–5 to the mechanism shown in Figure 1, using the general equations for the two-step sequential mechanism (Moore & Pearson, 1981). The rate constants are expressed in units of min^{-1}.

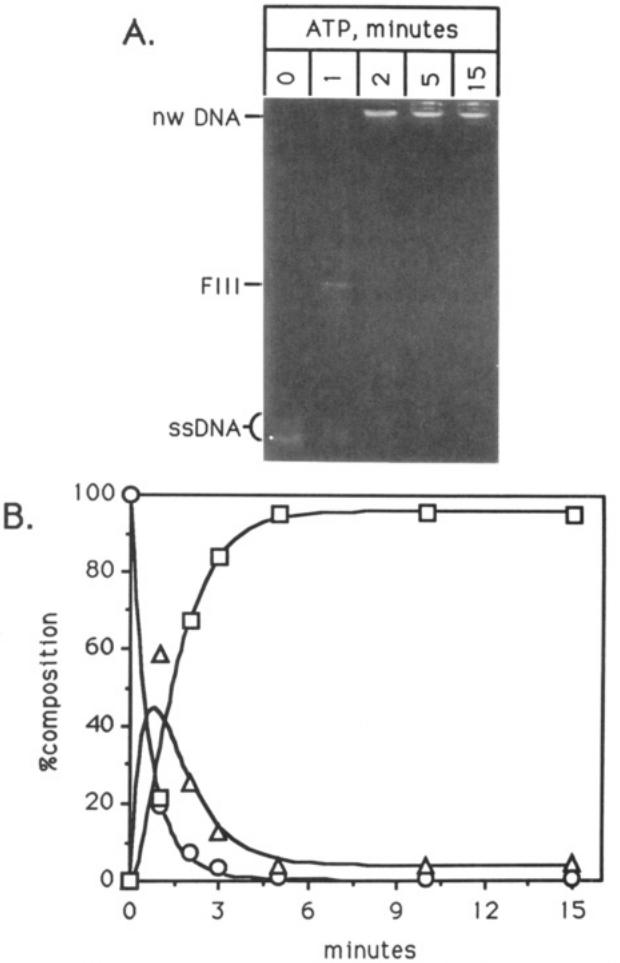

FIGURE 3: RecA protein-promoted renaturation in the presence of ATP. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 1 mM ATP. The reactions were analyzed as described in the legend to Figure 2. (A) Agarose gel showing representative reaction time points. Markers: ssDNA, single-stranded DNA; FIII, linear duplex DNA; nw DNA, network DNA. (B) Kinetic time course. The relative amounts of ssDNA (circles, initial product DNA (triangles), and network DNA (squares) are plotted as a function of reaction time. The simulated curves (solid lines) were calculated using the parameters given in Table I.

Figure 3A. As in the cofactor-independent reaction, the complementary single strands were first converted into FIII DNA and a distribution of other species that migrated above and below the FIII DNA band (initial product DNA). By 5 min, however, the initial product DNA was completely converted into network DNA. This reaction time course can

Table II: Comparison of Kinetic Parameters for the RecA Protein-Promoted Renaturation Reaction at 37 and 25 °C[a]

nucleotide	k_1(rel)	k_{-1}(rel)	k_2(rel)
none	2	0.8	14
ATP	4	0.8	3.3

[a] The value k(rel) = k(37 °C)/k(25 °C), and represents the ratio of the individual rate constants measured at 37 °C (this paper) to those previously measured at 25 °C (Bryant et al., 1989).

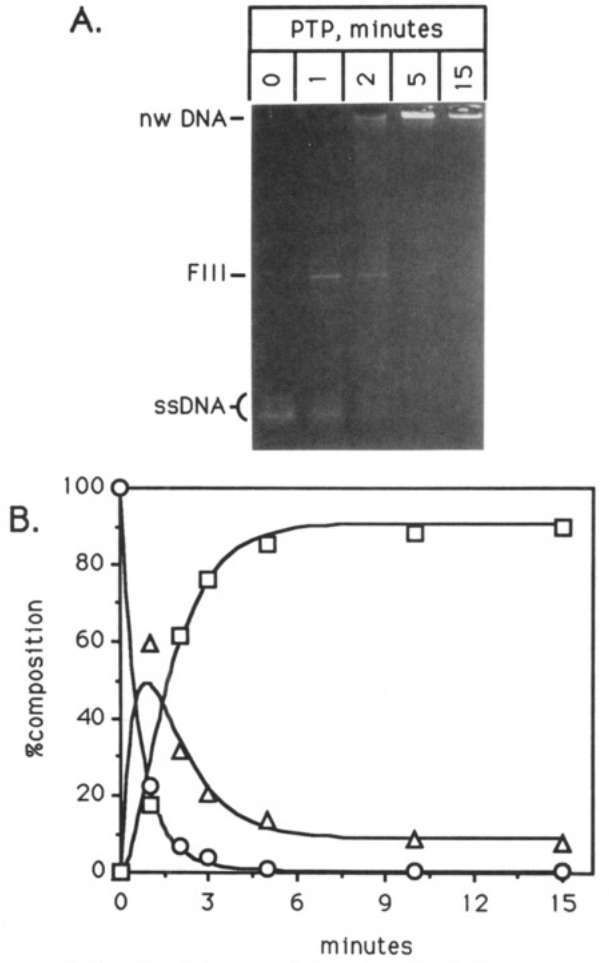

FIGURE 4: RecA protein-promoted renaturation in the presence of PTP. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 1 mM PTP. The reactions were analyzed as described in the legend to Figure 2. (A) Agarose gel showing representative reaction time points. Markers: ssDNA, single-stranded DNA; FIII, linear duplex DNA; nw DNA, network DNA. (B) Kinetic time course. The relative amounts of ssDNA (circles), initial product DNA (triangles), and network DNA (squares) are plotted as a function of reaction time. The simulated curves (solid lines) were calculated using the parameters are given in Table I.

be modeled with the kinetic mechanism shown in Figure 1 and the parameters k_1 = 1.6 min^{-1}, k_{-1} = 0.08 min^{-1}, k_2 = 1.0 min^{-1}, and k_{-2} = 0.04 min^{-1} (Figure 3B, Table I). The relative values of k_1 for the cofactor-independent and ATP-stimulated renaturation reactions indicate that the formation of initial product DNA is about 4-fold faster in the presence of ATP than in its absence. The relative values for k_2, on the other hand, indicate that the conversion of initial product DNA to network DNA is 14-fold faster in the presence of ATP than in its absence.

In Table II, the kinetic parameters for the cofactor-independent and ATP-stimulated renaturation reactions determined here at 37 °C are compared with those previously measured at 25 °C (Bryant et al., 1989). Although these

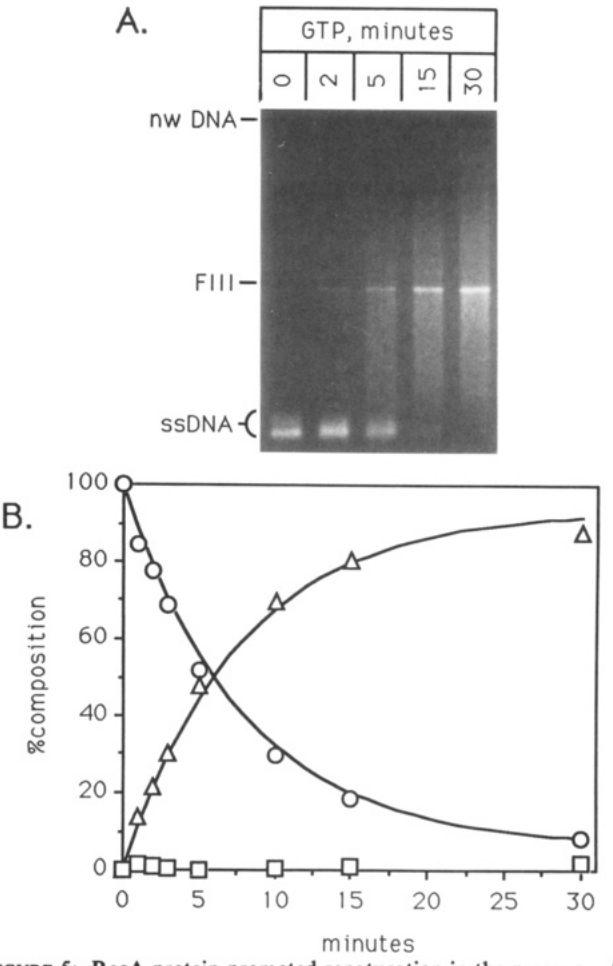

FIGURE 5: RecA protein-promoted renaturation in the presence of GTP. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 1 mM GTP. The reactions were analyzed as described in the legend to Figure 2. (A) Agarose gel showing representative reaction time points. Markers: ssDNA, single-stranded DNA; FIII, linear duplex DNA; nw DNA, network DNA. (B) Kinetic time course. The relative amounts of ssDNA (circles), initial product DNA (triangles), and network DNA (squares) are plotted as a function of reaction time. The simulated curves (solid lines) were calculated using the parameters given in Table I.

reactions are somewhat faster at 37 °C than at 25 °C, the same general kinetic mechanism appears to be followed at the two temperatures.

Effect of PTP on the RecA Protein-Promoted Renaturation Reaction. The time course for the renaturation reaction promoted by recA protein in the presence for PTP (1 mM) is shown in Figure 4A. This reaction time course is virtually identical to that obtained in the presence of ATP and can be modeled with the sequential mechanism using the parameters k_1 = 1.6 min^{-1}, k_{-1} = 0.08 min^{-1}, k_2 = 0.8 min^{-1}, and k_{-2} = 0.08 min^{-1} (Figure 4B, Table I). Thus PTP, like ATP, stimulates the conversion of initial product DNA into network DNA.

Effect of GTP and ITP on the RecA Protein-Promoted Renaturation Reaction. The time course for the renaturation reaction promoted by recA protein in the presence of GTP (1 mM) is shown in Figure 5A. This reaction time course is dramatically different from those obtained in the presence of ATP and PTP. In the presence of GTP, the complementary single strands were converted into initial product DNA, but the initial product DNA was not converted into network DNA. The time course for the GTP reaction can be modeled with the kinetic mechanism in Figure 1 by omitting the second step

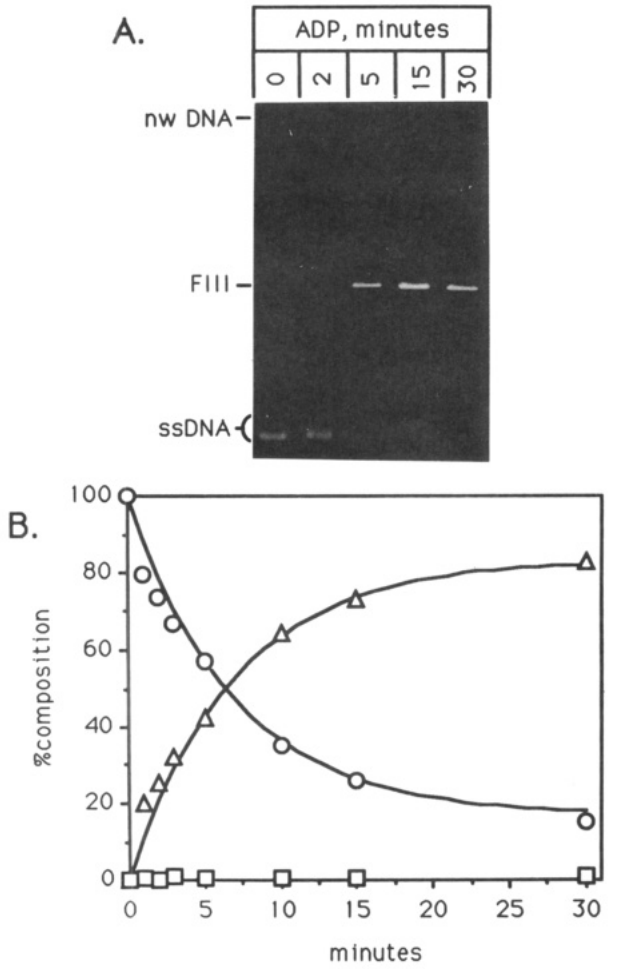

FIGURE 6: RecA protein-promoted renaturation in the presence of ADP. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 1 mM ADP. The reactions were analyzed as described in the legend to Figure 2. (A) Agarose gel showing representative reaction time points. Markers: ssDNA, single-stranded DNA; FIII, linear duplex DNA; nw DNA, network DNA. (B) Kinetic time course. The relative amounts of ssDNA (circles), initial product DNA (triangles), and network DNA (squares) are plotted as a function of reaction time. The simulated curves (solid lines) were calculated using the parameters are given in Table I.

($k_2 = 0$) and using the parameters $k_1 = 0.12$ min^{-1} and $k_{-1} = 0.008$ min^{-1} (Figure 5B, Table I). The time course for the renaturation reaction in the presence of ITP was virtually identical to that obtained in the presence of GTP (gel not shown) and can be modeled using the parameters given in Table I. The values for k_1 indicate that the conversion of initial product DNA is about 3-fold slower in the presence of GTP or ITP than in the absence of cofactor. GTP and ITP, however, completely suppress the formation of network DNA.

Effect of Nucleoside Diphosphates on the RecA Protein-Promoted Renaturation Reaction. The time course for the renaturation reaction promoted by the recA protein in the presence of ADP (1 mM) is shown in Figure 6A. This reaction resembles the GTP and ITP reactions in that initial product DNA is formed, but is not converted into network DNA. Although the fraction of form III DNA in the initial product DNA appears to be somewhat higher in the ADP reaction than in the GTP and ITP reactions, the overall time course for the ADP reaction can be modeled with the kinetic mechanism in Figure 1 using parameters similar to those used for GTP and ITP (Figure 6B, Table I). The time courses for renaturation reactions promoted by the recA protein in the presence of PDP, IDP, and GDP (1 mM) were identical to

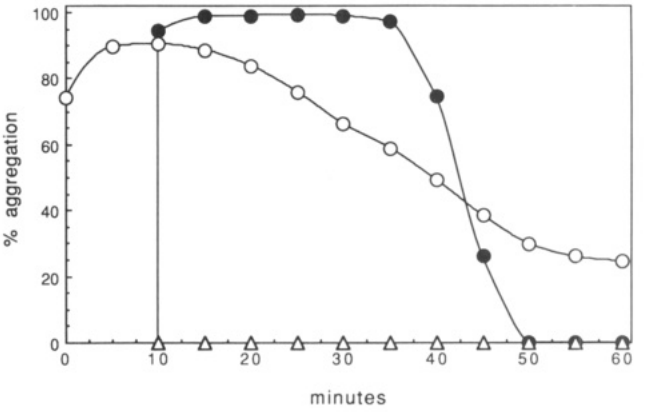

FIGURE 7: Effect of various nucleotides on the aggregation of ssDNA by recA protein. Reactions were carried out as described in Experimental Procedures. Reaction solutions contained 30 μM [^{3}H]ϕx ssDNA [(+) strand], 1 μM recA protein, and either no nucleotide (open circles), 1 mM ATP (closed circles; identical results were obtained with 1 mM PTP), or 1 mM ITP (triangles; identical results were obtained with 1 mM GTP, ADP, PDP, IDP, or GDP). The plots represent the amount of ssDNA found in an aggregated form as a function of incubation time.

that obtained with ADP (Table I, gels not shown). Thus, all four nucleoside diphosphates have only a minor inhibitory effect on the formation of initial product DNA, but completely suppress the formation of network DNA.

Effect of Various Nucleotides on RecA Protein-Dependent Aggregation of ssDNA. It has been shown that the binding of subsaturating levels of recA protein to ssDNA results in formation of large *complementarity-independent*, nucleoprotein aggregates that sediment at greater than 10 000 S. The formation of these nucleoprotein aggregates is strongly inhibited by saturating levels of recA protein, indicating that aggregation is due to ssDNA–recA–ssDNA interactions (Tsang et al., 1985). It has been proposed that these nucleoprotein aggregates are intermediates on the recA protein-promoted renaturation pathway (Tsang et al., 1985; Bryant et al., 1989). In order to determine the basis for the effects of the various nucleoside diphosphates and triphosphates on the renaturation reaction, we investigated the aggregation of ssDNA by recA protein in the presence of each nucleotide. The reaction solutions contained 30 μM ϕX ssDNA, 1 μM recA protein, and 1 mM nucleotide where indicated. These conditions are identical to those for the renaturation reactions except that only (+) strand ϕX ssDNA was used; this prevents DNA renaturation from occurring and thus allows the formation of the nucleoprotein aggregates to be distinguished from the formation of network DNA renaturation products (which also sediment upon centrifugation). The time courses for the recA protein-dependent ssDNA aggregation reactions are shown in Figure 7.

In the absence of nucleotide factor, maximal aggregation was observed after 5–10 min, when 90% of the ssDNA was in an aggregated state. The aggregation level then decreased steadily until, after 50 min, only 25% of the DNA was in the form of aggregates (Figure 7). This time-dependent decrease in aggregation was not due to an inactivation of the recA protein; the protein remained fully active throughout the entire incubation period when assayed for ATPase activity (data not shown). Instead, the recA protein appears to undergo a conversion in the absence of nucleotide cofactor from a form with full aggregation activity to a form that is ineffective in aggregation.

As shown in Figure 7, the addition of any of the four nucleoside diphosphates to an aggregation reaction mixture re-

sulted in the immediate and complete dissociation of the nucleoprotein aggregates. Addition of either GTP or ITP also resulted in the dissociation of the aggregates (Figure 7). However, addition of the nohydrolyzable GTP analog, GTPγS, resulted in the immediate incorporation of ssDNA into aggregates that were stable for at least 60 min (data not shown). This suggests that the dissociation of nucleoprotein aggregates that was observed in the presence of ITP and GTP was actually caused by the nucleoside diphosphates that were generated by the NTP hydrolysis activity of the recA protein. Since the total amount of ITP or GTP hydrolysis that occurs on the time scale of the aggregation experiments is small (2% hydrolysis in 2 min; Menge & Bryant, 1992), the aggregate dissociation was probably caused by the nucleoside diphosphates as they were formed in the NTPase active site, rather than by diphosphates that had accumulated in solution. The aggregate dissociation that was observed with GTP, ITP, and the nucleoside diphosphates was not due to a lack of interaction between recA protein and ssDNA in the presence of these nucleotides, since the recA protein exhibits substantial DNA renaturation activity (Figures 5 and 6) and full ssDNA-dependent ITPase and GTPase activity under these conditions [see Menge and Bryant (1992)]. However, it is possible that the binding of recA protein to ssDNA is weakened in the presence of these nucleotides, relative to that in the absence of nucleotide cofactor, and that this weakened binding results in the destabilization of the large nucleoprotein aggregates.

In contrast to the results obtained with ITP and GTP, the addition of ATP or PTP resulted in the incorporation of all of the ssDNA into nucleoprotein aggregates (Figure 7). Furthermore, the disaggregated ssDNA that was formed after prolonged incubation in the absence of nucleotide cofactor could be immediately reincorporated into aggregates upon addition of ATP (data not shown). However, after 25 min, the aggregates that formed in the presence of ATP and PTP suddenly dissociated, most likely due to accumulation of ADP or PDP from the ssDNA-dependent NTPase reaction. Consistent with this suggestion, the addition of the nonhydrolyzable ATP analog, ATPγS, to an aggregation reaction mixture resulted in the immediate incorporation of ssDNA into aggregates that were stable for at least 60 min (data not shown).

The results in this section demonstrate that those nucleotides which stimulate the formation of network DNA (ATP, PTP) also stimulate the formation of recA–ssDNA aggregates, whereas those nucleotides which suppress the formation of network DNA (ITP, GTP, and all nucleoside diphosphates) disrupt the nucleoprotein aggregates.

Effect of ATPγS and GTPγS on RecA Protein-Dependent Renaturation. The time courses for the renaturation reactions promoted by the recA protein in the presence of ATPγS and GTPγS (1 mM) are shown in Figure 8. With both analogs, the complementary single strands were converted into initial product DNA at rates comparable to those of the other nucleotide-mediated reactions. However, in both cases, the reaction stopped after 15 min, when about 60% of the ssDNA had been converted into renaturation product, and very little network DNA was formed. The premature cessation of the reaction was likely due to the irreversible binding to the recA protein to ssDNA that occurs in the presence of ATPγS and GTPγS under these conditions (see Discussion).

Discussion

We have examined the effects of the structurally related nucleoside triphosphates, ATP, PTP, ITP, and GTP, on the recA protein-promoted DNA renaturation reaction. In the absence of nucleotide cofactor, the recA protein converts the

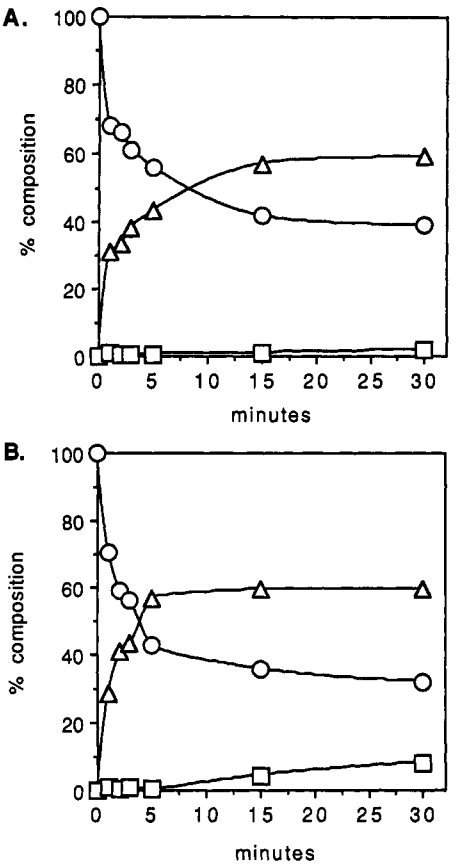

FIGURE 8: RecA protein-promoted renaturation in the presence of ATPγS and GTPγS. Reaction solutions contained 30 μM denatured φX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 1 mM ATPγS (panel A) or 1 mM GTPγS (panel B). The relative amounts of ssDNA (circles), initial product DNA (triangles), and network DNA (squares) were determined using the agarose gel assay (gels not shown) and are plotted as a function of reaction time.

complementary single-stranded DNA molecules into unit-length duplex DNA (FIII DNA) and other relatively small paired DNA species; these initial products are then slowly converted into more complex network DNA products. The four nucleoside triphosphates have only a minor effect on the formation of initial product DNA. However, ATP and PTP stimulate the conversion of initial product DNA into network DNA, whereas ITP and GTP completely suppress network DNA formation. The formation of network DNA is also inhibited by all four of the corresponding nucleoside diphosphates, ADP, PDP, IDP, and GDP.

ATP and PTP were also found to enhance the formation of large, complementarity-independent recA–ssDNA aggregates, whereas ITP, GTP, and all four nucleoside diphosphates were found to cause the dissociation of these nucleoprotein aggregates. In contrast to the opposing effects of ATP and GTP, the nonhydrolyzable analogs ATPγS and GTPγS both stimulate the formation of nucleoprotein aggregates. These results indicate that the net effect of ITP and GTP on aggregate formation is actually due to the corresponding nucleoside diphosphates, IDP and GDP, that are generated in the NTPase active site by the ssDNA-dependent NTP hydrolysis activity of the recA protein.

In addition to establishing the functional equivalency of ITP and GTP with the nucleoside diphosphates, the correlation that we observe between nucleoprotein aggregate formation and network DNA formation indicates that the large nucleoprotein aggregates, though not needed to form initial product DNA, are required for the formation of network DNA. This finding

allows us to expand our mechanistic model for the recA protein-promoted renaturation of complementary DNA strands (Bryant et al., 1989). In our current model, recA protein first binds to ssDNA to form (nonaggregated) recA–ssDNA complexes. The binding is cooperative, and the reactive species in the renaturation reaction are ssDNA molecules that are only partially covered with clusters of recA protein (Bryant & Lehman, 1985). Next, the recA–ssDNA complexes may interact with additional DNA strands through a second DNA binding site on the recA protein. These transient multistrand complexes bring some fraction of complementary DNA sequences into homologous proximity, leading to the formation of FIII DNA and other initial product DNA species in a step that is largely nucleotide cofactor-independent. This initial pairing step is described in the kinetic mechanism shown in Figure 1 by the rate constants k_1 and k_{-1}. In this formulation, the relative values of k_1 and k_{-1} probably represent the renaturation efficiency of the single strands held within the initial nucleoprotein complex, rather than an actual equilibrium between paired and unpaired strands [see Bryant et al. (1989)].

The second step of the renaturation reaction, which involves conversion of initial product DNA into network DNA, requires the formation of large recA–ssDNA nucleoprotein aggregates and is therefore sensitive to nucleotide cofactors: those nucleotides which enhance aggregation stimulate the formation of network DNA, and those nucleotides which disrupt aggregation suppress network DNA formation. An apparent exception to this rule occurs with ATPγS and GTPγS; although these analogs greatly stabilize nucleoprotein aggregates, they do not stimulate the formation of network DNA. In the presence of ATPγS and GTPγS, however, the recA protein binds irreversibly to ssDNA, and as a consequence, transfer of recA protein between strands is prevented (Bryant et al., 1985; Menge and Bryant, unpublished observations). Thus, these results suggest that the formation of network DNA may require the movement of recA protein within the large nucleoprotein aggregate.

Following this interpretation, the increased rate of network DNA formation in the presence of ATP and PTP, relative to that in the absence of nucleotide, may be due to the increased rate of transfer of recA protein between DNA strands that occurs in the presence of these nucleotides (Bryant et al., 1985). Faster transfer of recA protein within the nucleoprotein aggregates may increase the rate at which complementary sequences are brought within pairing proximity and thereby increase the rate of network DNA formation. Although the rate of transfer is even faster in the presence of ADP (Bryant et al., 1985), this nucleotide disrupts aggregation (possibly as a direct result of the high rate of transfer) and therefore inhibits network DNA formation.

The second step of the renaturation reaction is depicted in the kinetic mechanism shown in Figure 1 as an equilibrium between initial product DNA and network DNA, described by the rate constants k_2 and k_{-2}. However, the only reaction that absolutely requires a k_{-2} step in order to be modeled satisfactorily (i.e., where the value of k_{-2} is greater than $0.1k_2$) is the cofactor-independent reaction; in the reaction time course shown in Figure 2B, an equilibrium between initial product DNA and network DNA appears to be established after approximately 20–30 min. As shown in Figure 7, however, the recA protein undergoes a time-dependent change (in the absence of nucleotide cofactor) to a form that has reduced aggregation activity, and the time frame of this change corresponds to the apparent approach to equilibrium between initial product DNA and network DNA in the cofactor-independent renaturation reaction. Furthermore, the addition of ADP at the end of the cofactor-independent renaturation reaction does not lead to the conversion of network DNA back into initial product DNA as might be expected if the second step were an equilibrium process (Menge and Bryant, unpublished observation). Thus, the relative values of k_2 and k_{-2} in the cofactor-independent reaction most likely reflect the distribution of initial product and network DNA that existed at the time of aggregate dissociation. Similarly, the small values of k_{-2} (relative to k_2) that are required to optimally model the ATP- and PTP-stimulated renaturation reactions probably reflect the dissociation of nucleoprotein aggregates that occurs after 20–30 min as a result of the accumulation of ADP and PDP from the concurrent NTP hydrolysis reactions.

The mechanistic basis for the time-dependent change in the DNA aggregation properties of the recA protein in the absence of nucleotide cofactor is not clear. However, the recA monomers in a recA protein–ssDNA filament may exist in a distribution of conformational states, and this distribution may somehow change with time from a population that, on average, favors aggregation to one that is ineffective in aggregation. ATP and PTP may preferentially stabilize the aggregation-proficient conformational state of the recA protein, whereas GTP, ITP, and all nucleoside diphosphates may stabilize the aggregation-inactive state.

The results described in this paper complement the results that are described in the preceding paper regarding the effects of the various nucleoside triphosphates on the recA protein-promoted three-strand exchange reaction. In that paper, we show that ATP and PTP support isomerization of the recA protein to the strand exchange-active conformational state, whereas ITP and GTP lead to a state that is functionally equivalent to the strand exchange-inactive conformational state, a state also induced by nucleoside diphosphates. These results are consistent with our proposal that the net effect of ITP and GTP on recA protein-promoted DNA pairing activities is dominated by the corresponding nucleoside diphosphates, IDP and GDP, that are generated by the ssDNA-dependent NTPase reaction. As discussed in the preceding paper, the higher $S_{0.5}$ values of ITP and GTP may reflect altered rates of NTP association/dissociation, which disrupt the balance between conformational states and leave the recA–ssDNA filament susceptible to destabilization by NDPs as they are generated in the NTPase active sites (Menge & Bryant, 1992). In order to test this hypothesis, we are currently evaluating the elementary rate constants for the binding of various NTPs to the recA protein.

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Solution Studies on the Structure of Bent DNA in the cAMP Receptor Protein–*lac* DNA Complex†

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Received December 20, 1991; Revised Manuscript Received March 4, 1992

ABSTRACT: Cyclic AMP receptor protein is involved in the regulation of more than 20 genes. A step in the mechanism of activation of transcription is to induce a significant bending of the DNA upon complex formation between specific DNA and the protein. The induced DNA bending and a structure of the protein–DNA complex were studied by fluorescence energy transfer in 50 mM Tris, 1 mM EDTA, and 50 mM KCl at pH 7.8 and 20 °C. The symmetry of the DNA bend was estimated by measuring the efficiency of transfer between the protein and a label on either the upstream or the downstream end of a *lac* DNA fragment. The results show that the bend, despite the asymmetry in the DNA sequence, is symmetrical, for the fragments which length ranges from 26 to 40 bp. Using fluorescence energy transfer, the extent of DNA bending was estimated by measuring the end-to-end distance of the DNA fragment which was labeled with a donor–acceptor pair on two opposite ends. Both steady-state and time-resolved measurements showed that in a 26 bp *lac* DNA fragment complexed with cyclic AMP receptor protein, the end-to-end distance is about 77 Å which corresponds to a bending angle of 80° or 100°, depending on the actual contour length between the fluorophores in the free DNA fragment. The results using longer DNA fragments show no measurable amount of energy transfer; thus, it is very unlikely that the DNA completely wraps around the CRP molecule. This study shows that the approach of fluorescence energy transfer has proven to be a versatile technique to provide useful structural information on the DNA–protein complex in potentially any solution conditions.

The formation of a complex between DNA and DNA-binding protein frequently results in a change in DNA structure. These changes include DNA unwinding (Siebenlist et al., 1980), DNA looping (Schleif, 1988), and DNA bending (Wu & Crothers, 1984). Protein-induced or -mediated changes in DNA structure most likely play an important role in the regulatory mechanism of gene expression (Wu & Crothers, 1984; Ptashne, 1986; Gralla, 1989); thus, in studying any system which involves the DNA–protein complex, it is important to consider the structure of the DNA in such a complex.

cAMP receptor protein (CRP)[1] from *Escherichia coli* is known to be involved in the regulation of transcription of more than 20 genes (de Crombrugghe et al., 1984). The mechanism of activation of transcription by CRP is still unclear. There is clear evidence that CRP binding results in significant bending of the DNA (Wu & Crothers, 1984; Schultz et al., 1991). The size of the bending domain and the bending angle were measured (Liu-Johnson et al., 1986), and sequence determinants of the bending were established (Gartenberg & Crothers, 1988). Almost all of the studies by Crothers and co-workers are based on results using gel electrophoresis. Recently, the crystal structure of a CRP–DNA complex became available, and it shows that the DNA is sharply bent (Schultz et al., 1991). Thus, DNA bending is an integral step of the mechanism of transcriptional regulation by CRP.

Recent experiments show that the role of bending can be quite complex. It was observed that the CRP-binding site can be replaced with naturally bent DNA sequences. This results in a mimicking of the CRP effect; i.e., a pronounced activation of transcription was observed (Bracco et al., 1989). A detailed study of this effect showed that the induced activation seems to be coupled with supercoiling, i.e., transduction of the superhelical stress to the promoter, thus facilitating the unwinding of the promoter during its conversion from a closed to an open complex (Zinkel & Crothers, 1991; Gartenberg & Crothers, 1991). Interestingly, synergistic effects of CRP and supercoiling were indeed observed (Meiklejohn & Gralla, 1989).

It was also suggested that the energy stored in bent DNA can be used to help melt promoter DNA and therefore promote

† This work was supported by U.S. Public Service Grants DK-21489 and GM 45579 and by The Robert A. Welch Foundation.
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[1] Abbreviations: CRP, cAMP receptor protein; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; FM, fluorescein maleimide; ssDNA and dsDNA, single-stranded and double-stranded DNA, respectively; DMF, *N*,*N*'-dimethylformamide.

0006-2960/92/0431-5165$03.00/0