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Studies of the interaction of RecA protein with DNA

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ABSTRACT

Ethidium fluorescence assays were adapted for the rapid and sensitive detection of precA; in addition, fluorescence measurements on binding precA to linear, OC and CCC PM2 DNAs have enabled the stoichiometry of precA binding as well as the precA-induced unwinding angle of DNA to be determined. The stoichiometry of binding was independently confirmed by sedimentation analysis to be one precA molecule per 3 bp. The unwinding angle was also independently confirmed by measurements of fluorescence changes induced by the binding of precA to CCC DNA which was relaxed by topoisomerase to give a precA-induced unwinding angle of 51°. Electron microscopy of OC DNA molecules which bound non-saturating amounts of precA revealed that the length increase in DNA due to precA was approximately 55%. Finally, examination of negatively stained precA complexes with a variety of linear DNAs showed that the minor groove is the primary site of interaction for this protein.

INTRODUCTION

RecA protein (precA) has received a great deal of recent attention (for reviews see 1 and 2). It is a protein that has both catalytic (ATPase and protease) and structural functions (3). The clearest structural change to DNA on adding precA is a directed strand exchange, with unique polarity of single-stranded DNA, for the homologous strand in a DNA duplex (4). Quantitative studies on the stoichiometry of precA binding to DNA by electron microscopy (5) and by nuclease sensitivity or gel filtration (6) have been employed to calculate values of one precA molecule bound per 3 bp and 2 bp, respectively. Electron micrographs of platinum shadowed precA-DNA complexes show characteristic banding patterns (5) which appear to reflect the helical repeat of the DNA. This has been confirmed by an independent electron microscopic approach using DNAs of various superhelical densities

saturated with precA (7).

To evaluate these results by solution methods, we have used a rapid ethidium fluorescence assay for measuring protein unwinding angles. This assay relies on the fact that at a critical level of an unwinding agent added to negatively supercoiled DNA, the CCC DNA will be relaxed and topoisomerase added to such a complex will not change the linking number α (8). Using a modification of our previous ethidium fluorescence assays (reviewed in 9 and 10), we have also been able to rapidly and sensitively assay precA, and to determine the stoichiometry of binding by two independent methods. Our results are consistent with the interpretation of electron micrographs (7).

In initiating this work we were interested in the possibility that precA may induce the formation of tetrastranded DNA complexes (tetraplexes) during synapsis. McGavin has suggested such complexes may be intermediates in recombination (11,12). Howard-Flanders and his colleagues (13,14) also obtained suggestive evidence that such complexes may form. There are two major problems with the formation of a tetraplex: (i) the search for homology and (ii) the plectonemic problem of first having to untwist the DNA without disrupting base pairs for the base tetrads to form and then rewinding the 4 strands together. Since in the tetraplex the homologous DNAs recognize one another through their mutual major grooves, if precA has any role in the formation of such putative complexes one might expect it to bind in the minor groove of the DNA and also to unwind the primary helix. Our electron micrographs of precA bound to DNAs containing adducts in the major groove (i.e. T4 DNA which contains glucosylated hydroxymethylcytosine, and ØW-14 DNA which contains putrescinylthymine) showed exactly the same zig-zag structure with negative staining as did control calf thymus and phage PM2 DNAs. Thus large substituents in the major groove had no effect on the electron microscopic appearance of precA-DNA complexes. This is in contrast to the pattern obtained with calf thymus and PM2 DNAs which had been reacted with anthramycin, which binds covalently to the 2-amino group of guanosine (15) in the minor groove without disturbing the secondary structure of DNA.

MATERIALS AND METHODS

General procedures for using ethidium fluorescence to study DNA/RNA physical structures and DNA metabolizing enzymes have been described in two review articles (9,10). ØW-14 DNA was the gift of Dr. Tony Warren, University of British Columbia, and contains 5-(4-aminobutylaminomethyl)uracil, (i.e. putrescinyl-thymine) in place of the usual base thymine to the extent of about 50% (16). T4 DNA was purchased from Miles Biochemicals. The ATPγS was from Boehringer Mannheim Biochemicals. Anthramycin was the gift of Dr. J.W. Lown. The recA protein was purified by the procedure of Cox et al. (17), who kindly provided the precA over-producing strain, E. coli KM1842. The molecular weight of precA was taken as 38 Kd (18); the $E^{1\%}_{280}$, 5.16 (Weinstock et al.; 19), a value based on the amino acid composition (18).

Ethidium fluorescence binding studies with precA: DNAs were incubated with precA at 37°C in 20 mM sodium cacodylate buffer (pH 6.5), 10 mM $MgCl_2$, 1 mM ATPγS, precA as indicated and DNA at about 1.0 A_{260}. In calculations of stoichiometry 1 A_{260} is taken as 50 μg/mL of DNA. The kinetics of binding of precA to DNA under these conditions were followed by monitoring the decrease in fluorescence due to precA blocking ethidium binding sites. This effect levelled off after an incubation time of about one hour. If the incubation was allowed to extend overnight, however, the results were not reproducible because of extensive aggregation of the precA-DNA complexes. Samples from the DNA-precA incubation mixture of 10 μL were added to 2 mL of ethidium assay solution which contained 10 mM sodium cacodylate (pH 6.5) and 0.5 μg/mL ethidium bromide. The fluorescence readings were taken immediately since the complex dissociates easily.

Sucrose density gradient of precA-DNA complexes: A 10-20% linear sucrose gradient, 4.1 mL, was made up containing 20 mM cacodylate (pH 6.5), 2 mM $MgCl_2$ and 0.6 mM ATPγS. On the top of the gradient was layered 0.3 mL of a solution containing 21.4 μg of linear PM2 DNA, 0.206 mg of precA, 20 mM cacodylate, 6 mM $MgCl_2$ and 0.6 mM ATPγS (preincubated at 37°C for 30 min). The gradient was centrifuged in an SW60 Ti rotor for one hour at 40,000 rpm at 4°C, then analysed for DNA and protein by the "pH 12" ethidium fluorescence assay (9) and by the Bio-Rad

Coomassie Blue procedure respectively. In brief the "pH 12" assay measures only duplex DNA concentrations by the fluorescence of intercalated ethidium. precA is not bound at this pH.

Anthramycin-DNA adduct formation: Anthramycin (0.5 mg/mL in dimethylsulfoxide) was reacted with calf thymus DNA at room temperature such that the final concentrations (per mL) in the reaction were: 45 mM cacodylate (pH 6.5), 4.7 A_{260} calf thymus DNA, 0.12 mg anthramycin and 25% DMSO. Samples (3 µl) were added to the pH 8 ethidium fluorescence assay solution (12). The initial fluorescence reading of 82 dropped to 33 after about 32 hours. PM2 DNA was reacted with anthramycin as for calf thymus DNA, but at 38°C. The drop in fluorescence was essentially the same as for calf thymus DNA and took place within one day. DNAs are isolated by exclusion on Biogel A50m (100-200 mesh).

RESULTS

(a) Fluorescence DNA renaturation assay for precA. We decided to first apply our ethidium fluorescence assays (9,10) to study the renaturation of denatured DNA catalyzed by precA. Nuclease S1-treated PM2 DNA (a mixture of open circular and linear molecules) was heat-denatured at 98°C for 10 min in 10 mM Tris (pH 8), 0.1 mM EDTA. Using the reaction conditions of Weinstock *et al*. (20) it was found that at 22°C virtually no renaturation of denatured PM2 DNA occurred. In contrast, at 37°C appreciable rates of renaturation were obtained in the absence of precA. As illustrated in Figure 1, catalytic quantities of precA promoted the renaturation of PM2 DNA, and this reaction was completely dependent on the addition of ATP. ATPγS did not substitute for ATP. As a further control, heat-denatured (60°C for 20 min) precA was found to have no effect. Since the "pH 12" ethidium assay conditions were used to measure the formation of duplex DNA precA was expected to be entirely dissociated from the DNA. This was confirmed by showing that addition of the highest level of precA which was used (13.6 µg/mL), to the pH 12 assay mixture had no effect on the fluorescence. Of interest, however, was the observation that the DNA never completely renatured, even after overnight incubation when all samples showed about 80% of the fluorescence expected for complete renaturation. The product

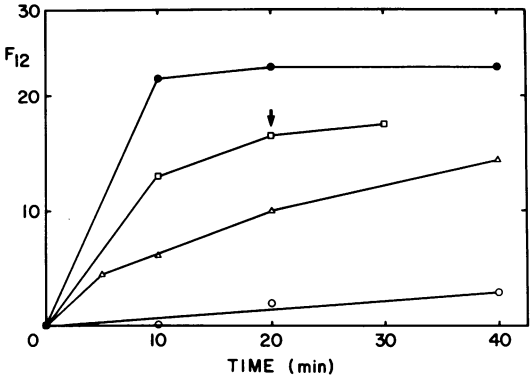

Fig. 1 Renaturation rates of heat denatured PM2 DNA catalyzed by precA. The reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 μg BSA/ml, 10 mM KCl, 5% (v/v) glycerol, heat-denatured PM2 DNA (S1 nicked and 0.4 A_{260} before denaturation), 0.8 mM ATP and precA as indicated. Incubation was at 22°C and 20 μL samples were added to "pH 12" ethidium assay solution for fluorescence readings. These are expressed in arbitrary units (F_{12}) and shown on the ordinate. In the absence of ATP or with heat-denatured precA, the renaturation was the same as when no precA was added. x-x, no precA or 13.6 μg/mL of precA previously heat-denatured; Δ-Δ, precA 3.4 μg/mL; □-□, 6.8 μg/mL; ●-●, 13.6 μg/mL. The arrow indicates a second addition of ATP.

of precA renaturation is a large complex which does not enter a 1% agarose gel on electrophoresis.

This renaturation assay is rapid, reproducible and sensitive such that each sample in Figure 1 (for the lowest precA concentration used) contained 0.05 μg of precA.

(b) The stoichiometry of binding of precA to DNA. Various DNAs were incubated as described in Materials and Methods with precA of various concentrations. This should allow the stoichiometry of precA bounding to be determined as has been done for other DNA binding proteins (10). The main problem encountered was the instability of the DNA-precA complex, even in the presence of ATPγS, under the "pH 8" ethidium assay conditions. Fortunately, at pH 6.5 the complex dissociates relatively slowly. In Figure 2 are shown the results of such titrations of precA against three forms of PM2 DNA: (i) HpaII restricted PM2 DNA; (ii) CCC PM2 with a natural superhelix density taken as -0.15 (see discussion); and

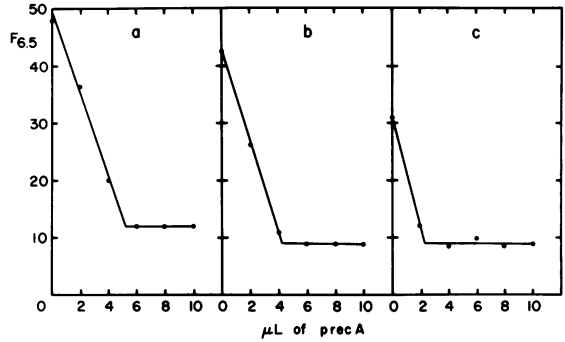

Fig. 2 Ethidium fluorescence titration of linear, and supercoiled and topoisomerase-relaxed PM2 DNAs. The assay conditions were as described in Materials and Methods except that 20 μL samples were taken for c. The ordinate is in arbitrary fluorescence units at pH 6.5 ($F_{6.5}$) and the abscissae in μL of precA (4.8 μg/μL) in a total volume of 30 μL for a and b and 40 μL for c. The concentrations of DNA were a, 42.9 μg/mL of linear PM2 DNA; b, 49 μg/mL of supercoiled CCC PM2 DNA; and c, 30 μg/mL of topoisomerase-relaxed PM2 DNA. At the titration end points the ratio of precA molecules per nucleotide (P) were in a, b and c, 1:6.1, 1:8.6 and 1:12.5 respectively. The CCC DNA molecules were contaminated with OC DNA. In b the fluorescence readings before and after heating in pH 12 ethidium assay solution were 41 and 24, indicating 65% of the DNA was supercoiled CCC PM2 DNA (12). Assuming there are x nucleotides bound per precA for pure CCC DNA, $.65/x + .35/6.1 = 1/8.6$, or $x = 11$. In c the fluorescence reading before and after heating in pH 12 ethidium assay solution were 35 and 21, indicating 75% of the DNA was relaxed CCC DNA (9). By a similar calculation, the corrected value for precA bound per nucleotide is 19 for pure relaxed CCC PM2 DNA.

(iii) PM2 DNA relaxed with calf thymus topoisomerase (13). As would be expected for a protein that unwound DNA, the saturation level of precA bound per nucleotide decreased from a ratio of 1:6.1 to 1:8.6 to 1:12.5. However, in cases (ii) and (iii) corrections had to be made for the amount of nicked DNA contaminating the topologically closed DNA. This could be readily quantitated by our fluorescence assay (9), and the corrected ratios for pure CCC DNAs of $\sigma = -0.15$ and 0 are 1:11 and 1:19.

From these data it is possible to determine the unwinding angle for precA, making the same assumptions as Stasiak and DiCapua (7); namely that precA binds the DNA cooperatively until that portion of the DNA free of precA reaches a critical positive superhelix density beyond which the cost in free energy of

further supercoiling is too high to allow further binding of precA. It is also assumed that precA unwinds the DNA a constant amount regardless of the topological stresses in the remainder of the DNA molecule, and that the portion of DNA bound by precA itself shows no significant supercoiling. This last point is evident from electron micrographs which show that precA-covered DNA can form two thick filaments lying side by side (7). The occasional supertwist of the thick filaments will have no significant affect on the calculations.

If x is the number of base pairs per turn of DNA helix covered by precA, and σ is the superhelix density of the DNA uncomplexed to precA under saturating conditions of precA, then two equations can be obtained using the knowledge that the topological linking numbers (α) of the two forms of DNA are unaffected by precA binding. (Note also that the fluorescence assays showed that none of the DNA had been nicked during these binding studies.) Thus $\tau + \beta$, (the number of supercoils + the number of turns in the primary helix, which equals the topological linking number) is constant. The fraction of the DNA covered by precA is simply obtained from the precA to nucleotide ratio at saturation for CCC PM2 DNA (σ = -0.15 and 0), compared with the case where there is no topological restriction. Thus for PM2 DNA, σ = -0.15, the fraction of DNA covered by precA is 6.1/11 = 0.55 and for σ = 0, 6.1/19 = 0.32 (see legend to Figure 2). Finally we take the average number of base pairs per turn of helix for natural B DNA to be 10.5 (21,22). Then the two equations for the two CCC DNAs are as follows (the number of base pairs for PM2 DNA should appear in each term but can be omitted since we are dealing with intensive functions):

	no precA		with precA		
	τ	β	τ	$+\ \beta$	
Superhelical DNA	$\frac{-0.15}{10.5} + \frac{1}{10.5}$	=	$\frac{0.45\sigma}{10.5}$	$+ \frac{0.45}{10.5} + \frac{0.55}{x}$	
Relaxed DNA	$\frac{1}{10.5}$	=	$\frac{0.68\sigma}{10.5}$	$+ \frac{0.68}{10.5} + \frac{0.32}{x}$	

Solving for x and σ, x = 18.8 bp of DNA per turn of helix when covered with precA and σ = + 0.2. Stasiak and DiCapua obtained a value of 18.6 for x, in unexpectedly good agreement, considering

the difference in techniques. In another titration, the ratios of precA to nucleotide at saturation for DNAs (i), (ii) and (iii) were changed from 1:6.1, 1:11 and 1:19 to 1:7, 1:11.5 and 1:20.5, respectively. These ratios give a value for x of 17; however the end points were not as well defined as for those of Figure 2. The major problem we encountered in the fluorescence assays could be ascribed to aggregation of the precA-DNA complexes. For example, when the solutions were left overnight, although those points where precA was limiting were usually the same, where precA was more than saturating a good end point could not be obtained. We also found it advantageous to use as low a DNA concentration as possible. The stoichiometry of precA interaction with DNA is important particularly since there is considerable disagreement in the literature. Volodin et al. (6) obtained a value of 2 bp per precA whereas DiCapua et al. (5) found 3, as we do. We therefore determined the stoichiometry of binding and the unwinding of DNA by two further different approaches.

(c) The stoichiometry of the precA-DNA complex by sedimentation analysis. Initial attempts to quantitate the amount of precA bound to DNA under saturating conditions of precA in the presence of ATPγS by gel filtration (to separate the precA-DNA complex from free precA) only worked with CCC DNA. With linear DNAs large side-by-side complexes formed (see Figure 6) which would not enter the gel, even Biogel A50m. Therefore we resorted to 10-20% sucrose gradients, which were run as described in Materials and Methods. The DNA-precA complex sedimented very rapidly to form a pellet at the bottom of the gradient while free precA remained at the top. The pellet was dissolved in 20 mM Tris pH 8, 1 mM mercaptoethanol, 10% glycerol and 30 mM EDTA, in order to dissociate the precA from the DNA. A clear solution was obtained after leaving the mixture at 37° for about one hour. That no protein remained bound to the DNA was confirmed by adding 5 µL samples to 2 mL of ethidium assay solutions at pH 6.5 (Materials and Methods), pH 8 and pH 12 (9). The ratio of the fluorescence at these three pH's was the same as that for purified DNA. If any precA had been bound to the DNA, there should have been a considerable relative decrease in the pH 6.5 ethidium fluorescence. The concentration of precA was then determined

against a standard plot for BSA using the Bio-Rad Coomassie Brilliant Blue assay. 1 μg of precA gives the same reading as 0.63 μg of BSA at 595 nm taking into account their different molar extinction coefficients at 280 nm. In two samplings of the DNA, the ratios of nucleotides bound per precA monomer were calculated to be 5.9 and 5.6, in reasonably good agreement with the value of 6 in section b, and of DiCapua et al. (5).

(d) The unwinding angle of DNA by precA using a topoisomerase assay. We have described a rapid assay for measuring unwinding angles of ligands bound to supercoiled DNA (8). In Figure 3 is shown the result of binding increasing amounts of precA to supercoiled CCC PM2 DNA either in the presence or absence of topoisomerase. In the absence of topoisomerase there is no nicking by precA, as evidenced by the horizontal lines in the fluorescence assay before and after heating. The difference in fluorescence between the two lines is due to contaminating OC PM2 DNA (9). In the absence of any nicking, the samples treated with topoisomerase in addition to precA should show the same crossover point both before and after heating (18). Since this was not observed there was evidently a small amount of nicking by the topoisomerase preparation. Normally this is not a problem as the assay is done in the presence of EDTA, but the DNA-precA complex requires Mg^{++} for stability. Fortunately, since the slopes of the lines in the presence of topoisomerase both before and after heating are the same, the amount of nicking is independent of precA concentration. The before and after heating fluorescence difference for PM2 DNA in the absence of topoisomerase is 16 units, but in the presence of topoisomerase it is 22 units. The difference of 6 fluorescence units is due to nicking of CCC PM2 DNA (9). That the DNA had been totally relaxed is demonstrated by the after heating fluorescence readings with and without topoisomerase. The fluorescence due to CCC PM2 DNA decreases by 33% when completely relaxed with topoisomerase (10), and from Figure 3 the decrease is from 40 fluorescence units to 27, corresponding to a 32.5% decrease. When relaxed CCC DNA is nicked, its fluorescence increases by 100% and there is a 100% loss in fluorescence after heating (10). Therefore, the 6 fluorescence unit difference referred to above means that line a in Figure 2 should be lowered

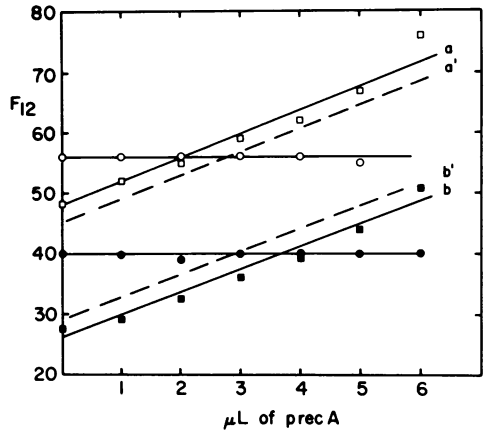

Fig. 3 The unwinding angle of precA bound to PM2 DNA as determined by topoisomerase relaxation. The reaction mixtures were all identical except for increasing amounts of precA along the abscissa, and were either with (squares) or without (circles) topoisomerase. Open symbols indicate the F_{12} readings before heating and filled symbols after heating. The dashed lines indicate the corrections made for nicking by the topoisomerase (see text, section d). The reaction mixtures contained 20 mM cacodylate (pH 6.5), 0.8 mM ATPγS, 10 mM $MgCl_2$, CCC PM2 DNA at 61 μg/mL and, as indicated on the abscissa, various volume additions of precA (4.8 μg/mL). The reaction mixture (40 μL) was incubated for one hour at 37°C to allow precA to bind, then 5 μL of 1 M NaCl and, where indicated (squares), 1 μL of partly purified calf thymus topoisomerase (10) were added. Incubation was continued for a further 2 hours and 20 μL samples were then added to pH 12 ethidium solution for the fluorescence reading (F_{12}).

by 3 fluorescence units to line a′, and line b should be raised by 3 fluorescence units to line b′. This corrects for the effects of nicking. The cross-over points should now, in principle, be identical. They are to within 3% (the positions where lines a′ and b′ cross the horizontal lines). From these data it can be calculated that one precA molecule binds 20 nucleotides, given the reasonable assumption that all the precA is bound. Electrophoresis in 1% agarose gels showed that there was not a bimodal distribution of DNA molecules from the topoisomerase assay, and in Figure 4 is shown an electron micrograph of OC PM2 DNA with limiting amounts of precA, which confirms that under our conditions, patches of DNA remained free of precA. Thus the topoisomerase assay does measure the average amount of precA bound at

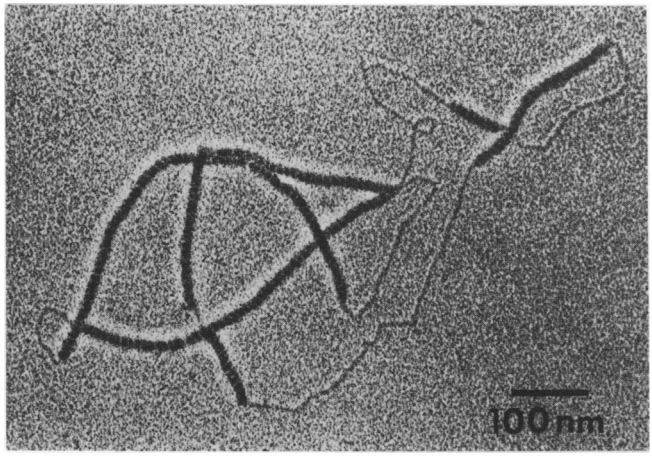

Fig. 4 An open-circular PM2 DNA molecule partially covered by precA. The components for incubation were 20 mM cacodylate (pH 6.5), 0.6 mM ATPγS, 10 mM $Mg(OAc)_2$, HpaII digested PM2 DNA (2.4 μg/mL; a mixture of circular and linear forms) and precA (23 μg/mL). DNA was therefore in excess, with 12 nucleotides per precA molecule. For electron microscopic observation, the precA-DNA complexes were mounted on carbon films by the poly-lysine method of Williams (28), stained with aqueous uranyl acetate and rotary-shadowed with 2.0 nm of 95% Pt-5%C.

the equivalence point of relaxation of supercoiled PM2 DNA.

If each precA molecule unwinds the DNA by y turns of helix, then knowing that one precA molecule is bound per 20 nucleotides (=10 base pairs) to just relax PM2 DNA with a superhelix density of -0.15, DNA unwound by precA equals the number of superhelical turns; that is

$$\frac{10,000}{10} \times y = \frac{10,000}{10.5} \times -0.15$$

Solving the equation, y =0.143 turns (or 51°) unwinding of the duplex per precA bound. Now if Z is the number of base pairs per turn of helix in DNA covered by precA, if one precA molecule binds 3 base pairs of DNA (6 nucleotides) at saturation we have:

$$\frac{Z}{10.5} - \frac{Z}{3} \times 0.143 = 1$$

Therefore, Z =21 base pairs.

This value is again close to that of DiCapua et al. (5) who obtained 18.6. In the experiment of Figure 3, a rather low DNA

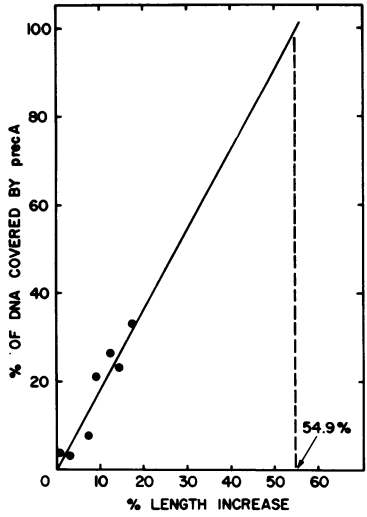

Fig. 5 Plot of the percentage of PM2 DNA covered by precA vs the percentage length increase. The data were obtained from measurements of molecules such as that shown in Figure 4. Naked PM2 DNA (i.e. with no precA bound), when measured on photographic prints at a final magnification of 185,000 with a Hewlett-Packard Digitizer coupled to a Tektronix Graphics Computer, had a length of 20,430 ± 230 arbitrary units (6 molecules). This was taken as the zero reference point, and measurements of PM2 DNA molecules with precA bound were calculated as percentages of this value. From the least squares straight line plot of the data, the increase in length for a PM2 DNA molecule completely covered by precA is 54.9 ± 3.4% (standard deviation).

concentration had to be used to prevent large aggregates of the DNA-precA complex. Thus within experimental error our data agree with those obtained from electron microscopy (5).

(e) Electron microscopy of precA with various DNAs. Our principle aim here was to determine if precA gave identical complexes with various DNAs having substituents in their major or minor grooves. In Figure 4 is shown an electron micrograph of precA bound to nicked PM2 DNA under limiting precA concentrations. Although highly cooperative in its binding, there are still random patches on each DNA molecule without precA. The total length of precA-free patches was measured for a series of molecules, as was the increase in contour length compared to "naked" PM2 DNA circles (as described in the legend to Figure 5). The percentage of DNA covered by precA was then plotted against

the percent length increase. Extrapolation to DNA 100% covered by precA predicted an increase in the contour length of 55%; this value is comparable to that of Stasiak et al. (23) and Dunn et al. (24) of 50%. As suggested by Stasiak et al. the most obvious explanation for the DNA length increase mediated by precA is an intercalative mode of binding, with nearest neighbor exclusion. We checked for the possibility of intercalation of tryptophan in precA using tryptophan fluorescence as a probe, but found no effect on adding DNA to precA.

Figure 6 is a composite of electron micrographs of precA bound to different DNAs and made visible by uranyl acetate negative staining. As controls, panels a and c show excess precA bound to calf thymus and PM2 DNAs. The zig-zag pattern corresponds in pitch to the cross-striations seen by platinum shadowing, and this has been interpreted (5) as being due to the helical periodicity of the precA-DNA complex and the periodicity of the partly unwound DNA duplex. In panels e and f are shown the complexes obtained on adding precA to T4 DNA and ØW-14 DNA. These DNAs contain, respectively, glucosylated hydroxymethyl groups on the 5 positions of cytosine and putrescinyl groups on 50% of the 5-methyl residues of thymine. The same zig-zag pattern is obtained as for calf thymus and PM2 DNAs. There was an interesting tendency for the precA-T4 DNA complex to form extensive lateral aggregates, producing the herringbone appearance seen in e. These micrographs demonstrate that modifications of residues on either C or T in the major groove of the DNA helix have no significant affect on complex formation. The ethidium fluorescence assay also showed that precA binding to T4 and ØW-14 DNAs was unimpeded.

In an attempt to see if the minor groove was an important determinant in precA binding to DNA, the drug anthramycin seemed a good choice (15). It not only binds covalently to DNA through the 2-amino of guanine via an aminal linkage which is stable at neutral and alkaline pH's, but it does not perturb the structure of DNA as shown by the lack of any effect on the superhelical density of SV40 DNA (15). CPK models indicate the whole of the drug binds neatly, without protusion, into the minor groove of DNA and forms specific hydrogen-bonds. Unlike other drugs which

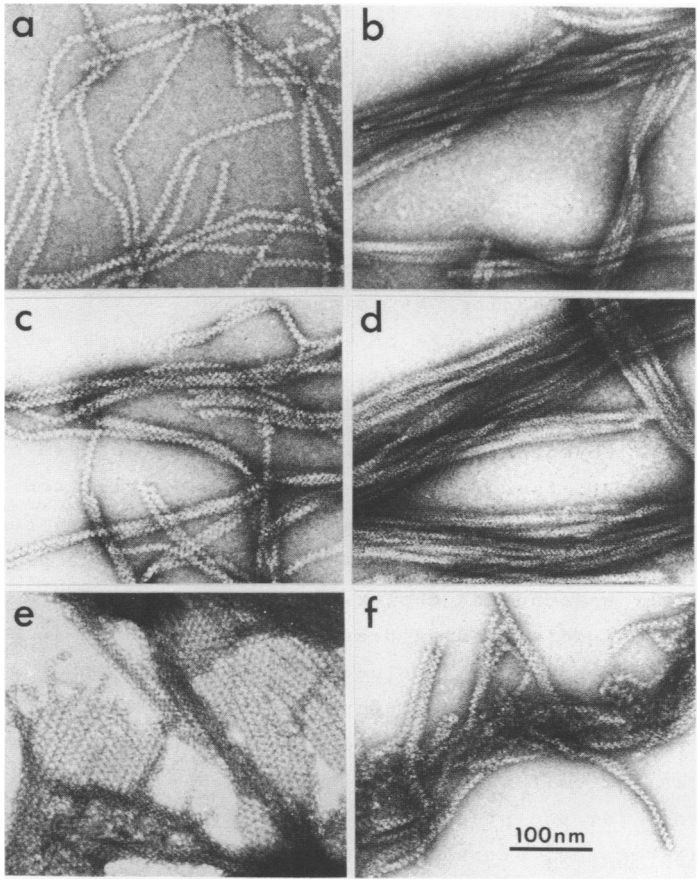

Fig. 6 Electron micrographs of precA-DNA complexes. precA (23 μg/mL) was incubated with DNA (2.5 μg/mL) in a buffer consisting of 10 mM $Mg(OAc)_2$, 0.6 mM ATPγS and 20 mM sodium cacodylate (pH 6.5) for 4 hrs at 22°C. Samples were adsorbed to glow-discharge-treated carbon films, washed with 5 mM $Mg(OAc)_2$ and stained with 2% uranyl acetate. precA plus (a) calf thymus DNA; (b) calf thymus DNA pre-treated with anthramycin (Materials and Methods); (c) linear PM2 DNA; (d) linear PM2 DNA pre-treated with anthramycin (see b); (e) T4 DNA; (f) DNA from the Pseudomonas acidivorans phage, ØW-14 (29).

bind to DNA and cause local distortion of structure (such as 2-acetylaminofluorene), and which are rapidly recognized and excised by repair enzymes, anthramycin-treated DNA is very slowly repaired. Various DNAs were treated with excess anthramycin and the drop in ethidium fluorescence in the pH 8 assay was measured

as an indication of the extent of reaction, and to determine when the reaction had reached completion. Under the conditions described in Materials and Methods, equilibrium appeared to have been reached after 2 days at 22° with calf thymus DNA, with 37% of the "pH 8" ethidium fluorescence remaining. It was shown that the complex was stable for at least 2 days in ice as shown by no increase in fluorescence due to reversal of the reaction [which is quite rapid with denatured DNA or at lower pH's (15)]. PM2 DNA gave almost exactly the same kinetics of interaction with anthramycin.

The most striking effect, as seen in panels b and d of Figure 6, is that the anthramycin-DNA adducts of calf thymus and PM2 DNAs when reacted with precA have a quite different appearance to the zig-zag pattern seen with the other DNAs. Evidently precA still binds to the anthramycin-DNA adduct (as was also confirmed by the fluorescence assays) but there is no longer a well-defined regular pattern. The tendency for lateral aggregation does, however, persist.

DISCUSSION

By two completely different methods (fluorescence titration, and sedimentation analysis of the DNA-precA complex) we have reached the same conclusion as DiCapua *et al*. (5), namely that the stoichiometry of binding of precA to DNA involves one molecule of precA per 3 bp. It must be remembered that this stoichiometry for our solution studies is under the set of conditions where the pH is relatively low (6.5) and ATPγS is present. Depending on the conditions, quite different precA-DNA complexes can be seen in the electron microscope (24, and Th. Koller, E. DiCapua and A. Stasiak, private communication). DiCapua *et al*. (5) obtained their value for the stoichiometry by quantitation of the elastically scattered electrons using STEM. The above value for the stoichiometry contrasts with the value of one precA molecule per 2.0 ± 0.4 bp of DNA obtained by Volodin *et al*. (6). The difference in extinction coefficients used for precA, $E^{1\%}_{280}$ = 5.16 (19) which we used, and of 5.9 used by Volodin *et al*. (6) means that if one takes their upper limit of 2.4 for the molar ratio of precA to DNA bp, and uses the extinction coefficient of

5.16 instead of 5.9, the molar ratio now becomes 2.75, i.e. quite close to 3. The data in Figures 2 and 3 of Volodin et al. (6), which relate to micrococcal nuclease sensitivity of precA covered DNA and the inability of the complex to enter a G75 Sephadex gel, both show a great deal of variability. We have also shown that denaturation of precA gives rise to a hyperchromicity of at least 5%, therefore the value of $E^{1\%}_{280} = 5.16$ which is calculated from the amino acid composition should, if anything, be lower than the actual value due to the hypochromicity of aromatic amino acids in precA stacking on one another. This would bias the value obtained by Volodin et al. (6) still further in the direction of 3 bp bound per precA. The one conceptual problem with the latter stoichiometry is that the increase in length of DNA bound by precA is approximately 50% (23); we obtained a value of $\sim$ 55% from electron microscopy (see Figure 5). This increase in length strongly suggests an intercalation model with excluded neighboring sites. We cannot conceive of a regular structure of precA covering 3 bp which would accommodate such a model. It is possible that alternating precA's would provide one and two intercalating groups, respectively. Another possibility is that the precA molecule actually spans 4 base pairs, and that each molecule overlaps with its neighbor in the terminal base pair. However such models still cannot give rise to identical nearest neighbor exclusion intercalation sites. Tryptophan fluorescence studies indicate that this aromatic residue is not involved in intercalation. Perhaps only X-ray diffraction will solve the mystery.

Using solutions of precA-DNA, the helical repeat as determined by both the binding assays to three PM2 DNAs, one topologically open and two topologically closed but with different linking numbers (Figure 2), and by the topoisomerase assay (Figure 3), is between 18 and 21 bp of DNA. This is in comforting agreement with that of 18.6 bp obtained by electron microscopy (5), since the length of the repeat unit seen by electron microscopy cannot unequivocally be correlated with the DNA helical repeat. There are good arguments for this being the case using a topological method but this again requires electron microscopy (7). We chose $\sigma = -0.15$ for the superhelical density of PM2 DNA, a value which corresponds to an unwinding angle by ethidium of

33°. The commonly used value of 26° (25) is probably too low since titration of PM2 DNA with the water soluble carbodiimide showed a minimum sedimentation rate with about 15% of the T's and G's derivatized (i.e. $\sigma = -0.15$). More recently Ciarrocchi and Pedrini (27) determined the unwinding of DNA caused by pyrimidine dimer formation and were able to calculate the ethidium unwinding angle more accurately as $31.4^{\circ} \pm 2.9^{\circ}$. We have taken the value as 33° since it is within their experimental error and equals the value we originally obtained (26). If the value is indeed lower, then for 26° instead of 21 bp for the helical repeat in section d it would be 16.5 bp.

The electron micrographs of precA bound to various DNAs (Figure 6) would support the notion that precA does not interact significantly with the major groove of the DNA helix, since it binds with the same apparent geometry to "normal" DNAs and to T4 and ØW-14 phage DNAs. However, blockage of the minor groove with anthramycin adducts at G residues, while not eliminating binding of precA, completely changes the staining pattern. It would therefore seem that the minor groove is important for precA binding to result in the helical pitch of about 10 nm and the associated unwinding of the duplex DNA. This mode of binding is at least consistent with models where synapsis promoted by precA involves a search for homology via the tetrastranded structures described by McGavin (11,12).

ABBREVIATIONS

bp, base pairs; CCC DNA, covalently-closed circular DNA; OC DNA, open circular DNA; ATPγS, adenosine 5'-(γ-thio)triphosphate; BSA, bovine serum albumin; STEM, scanning transmission electron microscopy.

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