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Single-Strand Deoxyribonucleic Acid Binding Protein from Rat Liver Changes the Helical Structure of Deoxyribonucleic Acid†

Michel Duguet,* Catherine Bonne, and Anne-Marie de Recondo

ABSTRACT: Incubation of rat liver single-strand DNA binding protein S25 with covalently closed relaxed SV40 DNA in the presence of rat liver topoisomerase I induced a decrease in the linking number L_K of DNA, so that it appeared negatively supertwisted after removal of the protein. ΔL_K was found to be a linear function of protein to DNA ratio and reached a plateau corresponding to about 15 superhelical turns. The same result was obtained when S25 was incubated with form I or form Ir before addition of topoisomerase I or when SV40 was replaced by PM2 DNA. The observed reduction in the linking number of DNA when it is closed in the presence of rat liver protein S25 can be explained either by supercoiling of DNA induced by S25 or by detorsion or unwinding of DNA.

A number of single-strand DNA binding proteins (SSB)[1] isolated from eukaryotes and virus-infected eukaryotes as well as from prokaryotes or phage-infected prokaryotes have been studied today or are under investigation [for review, see Champoux (1978); Coleman & Oakley, 1980)]. For some of these proteins, their in vivo role in replication or in genetic recombination has been firmly established (Alberts, 1970; Sigal et al., 1972; Cavalieri et al., 1976; Meyer et al., 1979). For others it was only suggested (Banks & Spanos, 1975; Herrick et al., 1976). Some are melting proteins, i.e., are able to lower the melting point of DNA (Sigal et al., 1972; Kelly et al., 1976); some, in contrast, stabilize the DNA (Fowlkes et al., 1979); others have no reported effect on the melting point (Novak & Baril, 1978). Some bind cooperatively; others do not. The way by which each of these proteins binds to DNA and "shapes" it thus appears original. For instance, T_4 gene 32 protein binds cooperatively to single-stranded DNA and puts the strand in an extended and rigid conformation (Alberts, 1970), while gene 5 protein of fd binds by wrapping of single-stranded DNA around protein dimers in rodlike structures (Pratt et al., 1974). Little is known on the way by which eukaryotic proteins bind to DNA. Calf thymus single-strand binding proteins (or HDP) were reported to bind noncooperatively to ss-DNA and to bring it in an extended conformation (Herrick et al., 1976), while *Ustilago maydis* binding protein binds cooperatively to ss-DNA (Banks & Spanos, 1975).[2]

In previous works, we have isolated and studied some properties of a single-strand binding protein from rat liver. The protein isolated from regenerating liver, called HD25, was able both to lower the melting point of polyd(A-T) (Duguet & de Recondo, 1978) and to stimulate rat liver DNA polymerases (Duguet et al., 1977). These results supported the idea that it could be involved in DNA replication. In contrast, the protein from normal rat liver called S25, although it is a single-strand binding protein, was not found to be a melting protein and, in some conditions, inhibits homologous DNA polymerases (Bonne et al., 1979; de Recondo et al., 1980). At this time, the only difference found between these proteins is the ability of HD25 to oligomerize, a property that is not shared by S25. Otherwise, HD25 and S25 proteins are undistinguishable, even by the pattern of proteolytic degradation (Bonne et al., 1979; Bonne et al., 1980).

In search for a possible function of S25, we decided to look more carefully at the way it binds to DNA. Using competition experiments with ^{32}P-labeled M13 single-stranded DNA and various relaxed double-stranded DNAs, we have estimated that, at low ionic strength, S25 binds about 1000-fold better on single-stranded DNA (Bonne et al., 1980). We also know from previous studies that both HD25 and S25 bind to SV40 DNA; this was followed both by sedimentation analysis and by nitrocellulose filter assay. The use of a covalently closed circular DNA like SV40 or PM2 provides a very sensitive probe to test any change in the DNA helix parameters that is immediately reflected in the shape of the molecule and consequently in its electrophoretic mobility (Germond et al., 1975).

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[1] Abbreviations used: SSB, single-strand DNA binding protein; $NaDodSO_4$, sodium dodecyl sulfate; EtBr, ethidium bromide; HDP, helix-destabilizing protein; DTT, dithiothreitol; ss-DNA, single-stranded DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] Both are reported to be melting proteins. In contrast, the 72K protein from adenovirus, although it binds specifically to ss-DNA, is not a melting protein (Fowlkes et al., 1979).

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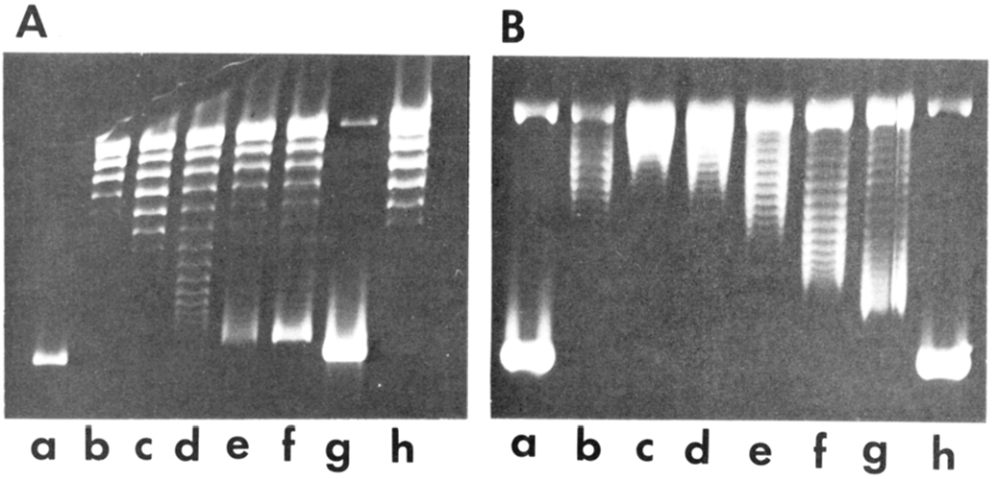

FIGURE 1: Generation of superhelical turns in closed circular DNA. DNA form Ir was incubated with increasing amounts of S25 at 30 mM NaCl in the presence of topoisomerase for 45 min at 37 °C and electrophoresed as described under Materials and Methods. (A) SV40 DNA. (Lane a) DNAI; (Lanes b–f) Complexes of relaxed DNA (Ir) with S25 at a protein to DNA ratio of (b) 0, (c) 0.22, (d), 0.88, (e), 1.76, and (f) 2.65. (g) Supercoiled DNA (I) incubated for 45 min with S25 at ratio 2.65 in the absence of topoisomerase. Lane h is as lane f except that topoisomerase was omitted. (B) PM2 DNA. (Lanes a–h) DNA I. (Lanes b–g) Complexes with S25 at a protein to DNA ratio of (b) 0, (c) 0.1, (d) 0.2, (e) 0.4, (f) 0.84, and (g) 1.24.

In this paper, we describe changes induced in such DNAs upon binding of rat liver protein S25.

Materials and Methods

Protein Purification. Rat liver topoisomerase I was purified according to Champoux & Mac Conaughy (1976) from nuclei through a phosphocellulose pool (fraction III). This fraction still contained a residual nuclease activity, but it is not detectable under the conditions used in the present study (i.e., in the absence of magnesium). Polynucleotide ligase from phase T_4 was obtained from Miles Research Laboratories, Inc.

Rat liver single-strand binding protein S25 was purified to homogeneity from normal rat liver as previously described (Bonne et al., 1979). It is free of nucleolytic activity.

T_4 gene 32 protein, purified according to Huberman et al. (1971), was a gift from M. Yaniv, Institut Pasteur. Single-strand DNA binding protein (72K) from adenovirus was purified by C. Cajean and M. Girard from this Institute.

DNA. Simian virus 40 DNA I, prepared according to the Hirt method (Hirt, 1967), was from J. Feunteun (this Institute). Phage PM2 DNA I was prepared by J. Laval (Institut G. Roussy). Relaxed, covalently closed SV40 and PM2 DNA (Ir) were prepared by 20-min incubation with topoisomerase under the conditions described by Champoux & Mac Conaughy (1976). After deproteinization, the DNA was recovered by ethanol precipitation. Values of 3.3×10^6 and 6.5×10^6 were used for the molecular weight of SV40 and PM2 DNA, respectively. DNA concentrations were determined by using $E^{260}_{mg/mL} = 20$. Nicked DNA (form II) was prepared according to Wang (1974).

Assay for the Generation of Superhelical Turns. The standard incubation mixture contained, in a total volume of 30 μL, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 30 mM NaCl, 1.2 μg of DNA Ir, 1 μL of topoisomerase (fraction III), and various amounts of protein S25. After a time of incubation indicated in the legends to the figures, the reaction was stopped by 1% $NaDodSO_4$ (final concentration) for 5 min at 37 °C, and the DNA was analyzed by agarose gel electrophoresis. Modifications in the standard procedure are indicated in the legends to the figures.

Incubations of nicked SV40 DNA (form II) with T_4 ligase were performed in 30 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.3 mM ATP, and varying amounts of protein for 10 min at 30 °C. The reaction was stopped by 1% $NaDodSO_4$ (Depew & Wang, 1975).

Electrophoresis. Electrophoreses were performed in 1% agarose (0.7% in the case of PM2 DNA) by using horizontal slab gels prepared and run in 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA, pH 7.8. Gels were run at 2 V/cm for 26 h at room temperature and stained with ethidium bromide. Photographs of the gels were taken under short-wavelength UV light with Illford HP4 film. Densitometric traces were made by using a Joyce Loebl microdensitometer.

Electron Microscopy. Samples of DNA or protein–DNA complexes were fixed for 10 min at 37 °C with 10 mM glutaraldehyde, diluted 10-fold in distilled water, and absorbed on grids according to Dubochet et al. (1971). Preparations were observed with Siemens Elmiskop A electron microscope.

Results

Rat Liver Single-Strand Binding Protein S25 Generates Superhelical Turns in Relaxed SV40 and PM2 DNA after Incubation with Topoisomerase. Covalently closed circular relaxed SV40 DNA (form Ir) was incubated with increasing amounts of S25 in the presence of rat liver topoisomerase I (fraction III; see Materials and Methods) for 45 min at 37 °C. After deproteinization of the complex, the DNA was analyzed by agarose gel electrophoresis (Materials and Methods). As shown in Figure 1A, a population of topoisomers with different electrophoretic mobilities was revealed in each lane, produced by thermal fluctuations within the helix at the time of the closure by topoisomerase (Pulleyblank et al., 1975). It is now well established that the DNA in each band differs from its neighbors by one unit in its linking number and consequently in the number of its superhelical turns (Depew & Wang, 1975). This number increased with protein S25 added (lanes b–f) to reach a plateau of 14–15 superhelical turns for protein to DNA ratios of 1.7–2.6 (w/w) (lanes e and f). When S25 or topoisomerase was omitted, no change in the electrophoretic pattern of form Ir occurred (lanes b and h, respectively). The same results were obtained when S25 was preincubated with DNA I or DNA Ir prior to addition of topoisomerase.[3] The result is also unchanged with a 10-fold increase in the amount of topoisomerase I.

[3] In most of the experiments, DNA Ir was preferred to DNA I as a substrate for S25 to rule out the possibility that the appearance of superhelical turns with increasing S25 was only due to the protection of DNA by increasing S25 against the activity of topoisomerase I.

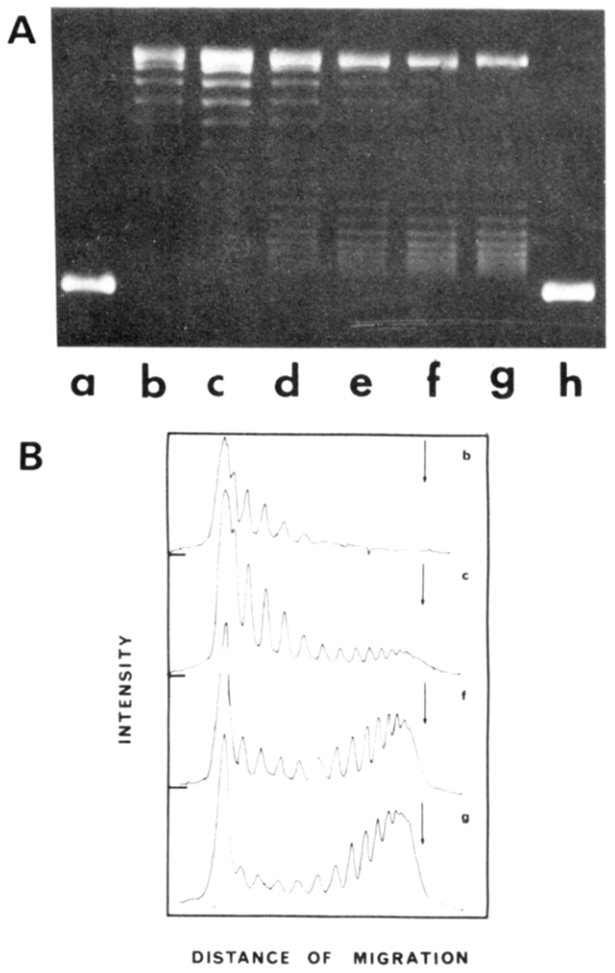

FIGURE 2: Time course of the generation of superhelical turns. (A) DNA Ir was incubated with S25 at a protein to DNA ratio of 1.3 at 37 °C for (c) 5, (d) 20, (e) 40, (f) 60, and (g) 90 min. Lanes a and h are DNA I control. Lane b is form Ir. (B) Densitometric traces of lanes b, c, f, and g. Migration is from left (–) to right (+). The arrow indicates the migration of form I. The band remaining at the position of relaxed DNA after 90-min incubation is nicked DNA (form II).

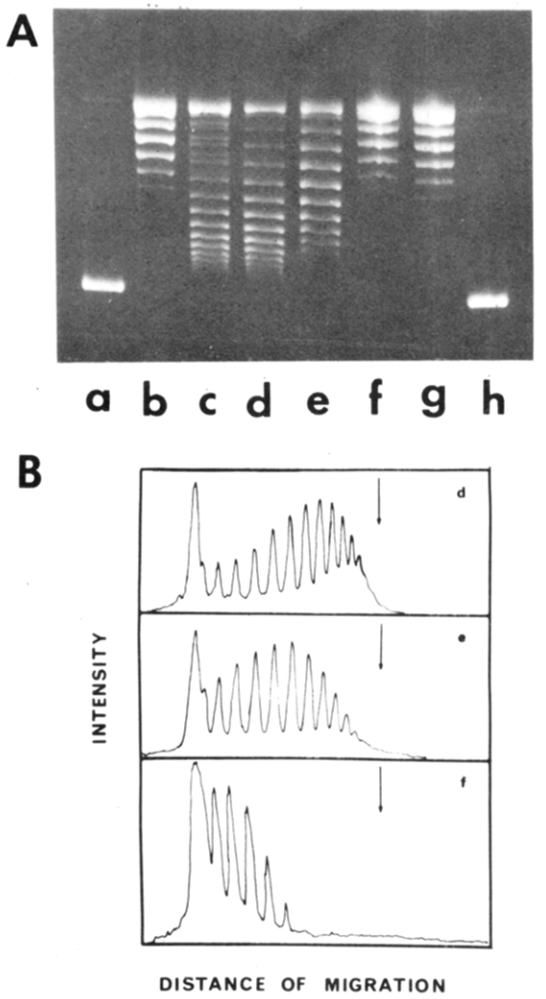

FIGURE 3: Effect of ionic strength of the formation of superhelical turns. (A) DNA Ir was incubated with S25 at protein to DNA ratio of 1.0 for 60 min and 37 °C at 33 (c), 66 (d), 100 (e), and 300 mM salt (f). Lanes a and h are DNA I, and lanes b and g are DNA Ir. (B) Densitometric traces of lanes d, e, and f. Migration is from left to right. The arrow indicates the migration of form I.

In other experiments performed with PM2 DNA, incubation with S25 again generated superhelical turns (Figure 1B). Due to the larger size of the genome (9850 bp), species of different electrophoretic mobilities were closer. Besides, in the experiment illustrated in Figure 1B, the relaxed PM2 DNA (lane b) appeared more twisted than it was after incubation with two increasing concentrations of S25 (lanes c and d); in the conditions of electrophoresis, the "relaxed" PM2 DNA was positively twisted so that incubation with S25 generated first relaxed DNA and then negative twists (this is discussed in more detail in a following section).

The time course of the reaction with SV40 is illustrated in Figure 2. The DNA was incubated with S25 at a protein to DNA ratio of 1.3 for 5–90 min at 37 °C in the presence of topoisomerase. Bands with maximum number of superhelical turns (about 13 in this experiment) appeared very early and increased in intensity without important changes in the position of the maximum (Figure 2A). Densitometric tracings of the photograph (Figure 2B) revealed two Gaussian distributions of topoisomers, one centered close to the position of nicked DNA and a second centered in a position near 12 superhelical turns. The latter distribution increased in intensity with incubation time at the expense of the former (lanes c and g), but the reaction is not completed, even after 90-min incubation. This result was also apparent in Figure 1A where a distribution of topoisomers near the position of nicked DNA remained after 45-min incubation. Since the kinetics of relaxation by topoisomerase I are very fast at 37 °C, the rate of the above reaction is probably limited by the rate of formation of the nucleoproteic complex between S25 and DNA rather than by the relaxation process. The kinetics of the generation of superhelical turns were found to be faster when S25 was incubated with form I rather than with form Ir before relaxation, and this is consistent with the higher affinity of S25 for superhelical DNA (Bonne et al., 1980).

The generation of superhelical turns by S25 is sensitive to ionic strength. As shown in Figure 3, the reaction performed at S25 to DNA ratio of 1.0 is slightly inhibited at 100 mM salt and completely at 300 mM salt. The center of the distribution is shifted toward the position of nicked DNA as the ionic strength increased (Figure 3B). Since topoisomerase I is fully efficient at 200 mM salt (Champoux & Mac Conaughy, 1976), the observed inhibition presumably reflected an inhibition in the formation of the nucleoproteic complex with S25. This is consistent with the salt sensitivity observed when formation of the complex is followed by filter binding or by sedimentation analysis (not shown).

We have tested the ability of various proteins to generate superhelical turns after incubation with SV40 DNA in the conditions described above. These proteins include basic proteins such as lysozyme and cytochrome *c* able to contract

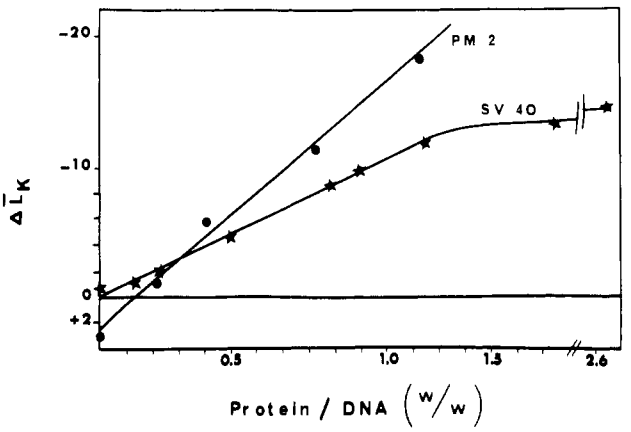

FIGURE 4: Variation in the linking number of SV40 and PM2 DNA as a function of protein S25 to DNA ratio. ΔL_K is deduced from electrophoretic data in measuring the position of the center of the Gaussian distribution of bands generated by incubation with increasing amounts of S25.

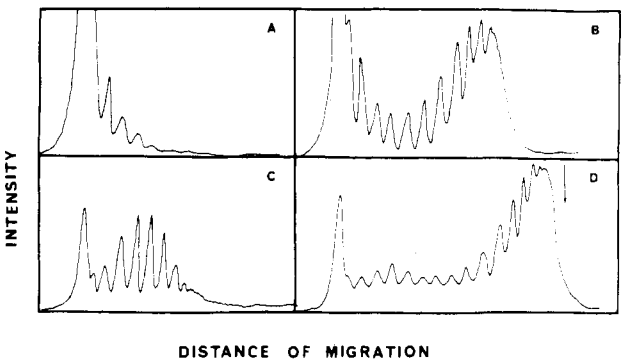

FIGURE 5: Effect of the temperature of electrophoresis on the position of Gaussian distribution of DNA bands. DNA Ir was incubated with S25 at a protein to DNA ratio of 1.3 for 60 min at 37 °C. Ir alone incubated at 37 °C and electrophoresed at 33 °C (A) or 11 °C (C). Ir incubated at 37 °C with S25 and electrophoresed at 33 °C (B) or 11 °C (D). Migration is from left to right. The arrow indicates the migration of form I. The band remaining at the position of relaxed DNA in trace C is nicked DNA.

electrostatic interactions with DNA and single-strand binding proteins such as T_4 gene 32 protein and adenovirus 72K protein. None of these were able to change the distribution pattern of relaxed SV40 DNA. In contrast, preliminary results indicate that the protein from regenerating liver (HD25) generates superhelical turns as S25 does.

Finally, the same distribution of topoisomers was obtained when S25 was incubated with nicked SV40 DNA in the presence of T_4 ligase in the conditions described under Materials and Methods (not shown).

Change in Linking Number of SV40 or PM2 DNA Induced by S25 Is a Linear Function of Protein to DNA Ratio. As pointed out by several authors (Pulleyblank et al., 1975; Depew & Wang, 1975), the position of the center of each Gaussian distribution obtained in the above experiments provides a measure of the average linking number $\bar{L}_K$ of the DNA. When the change in $\bar{L}_K$ is plotted against S25 protein to DNA ratio, a linear relation is found, as illustrated in Figure 4. This result is in favor of a noncooperative binding of S25 to SV40 DNA. Furthermore, if we make the hypothesis that all the protein molecules are bound to DNA and contribute to change the parameters of the helix, we can calculate the change in rotation angle per protein molecule, independently of any hypothesis on the mechanism implied. Values between 24 and 27° were calculated both for SV40 and for PM2 DNA (when reduced to a given number of nucleotides, the slopes of Figure 4 for SV40 and PM2 are almost identical). These values are similar to those obtained for HMG proteins (Javaherian et al., 1979) and can be compared to the value of 240° reported by Wang et al. (1977) for the effect of RNA polymerase on fd RF DNA in similar ionic environment.

Polarity of the Superhelical Turns Generated by S25 Is Negative. The change in the twist of DNA illustrated in the above sections can be interpreted as positive or as negative superhelical turns. This question can be solved by using a very simple experiment. It has been shown by Depew & Wang (1975) and by Pulleyblank et al. (1975) that the helix rotation angle in DNA (measured by the number of helical turns, T_w or β) is temperature dependent and increases about 0.012° per °C and base pair. When DNA is closed by topoisomerase I at a given temperature, the value of its linking number $\bar{L}_K$ (Anderson & Bauer, 1978) depends on temperature and ionic environment at the time of the closure and cannot be further changed without covalent breakage. This implies that any change in T_w imposed by temperature during electrophoresis leads to a change in the number of superhelical turns ($\bar{W}_r$ or τ) in the DNA,[4] revealed by a shift in the Gaussian distribution of species observed. In the experiment illustrated in Figure 5, SV40 DNA was incubated alone or in the presence of protein S25 with topoisomerase I for 60 min at 37 °C. After deproteinization, half of the DNA was run at 11 °C in the standard electrophoresis buffer, and the second part was run at 33 °C. As shown in Figure 5A,C, the Gaussian distribution for DNA alone was shifted from about four negative superhelical turns at low temperature, as expected from the above data. For the sample incubated in the presence of S25 (Figure 5B,D), the DNA appeared again with about four more superhelical turns at low temperature. This result shows unambiguously that S25 generates negative superhelical turns in DNA; in other words, S25 seems to decrease the twist of DNA or to unwind it.

This result is consistent with the experiment shown in Figure 1B where PM2 DNA with positive superhelical turns appears first relaxed and then negatively twisted (after incubation with increasing amounts of S25 in the presence of topoisomerase and deproteinization).

Complexes of S25 with SV40 Appear as Beaded Structures That Resemble Minichromosomes. Formation of nucleoproteic complexes between S25 and supercoiled SV40 DNA can be followed by nitrocellulose filter binding assay or be sedimentation in sucrose gradients (Bonne et al., 1980). A complex sedmenting at about 30 S is thus revealed. As shown in Figure 6, complexes fixed with glutaraldehyde appeared as beaded structures that resemble those obtained by assembly of SV40 DNA I with the four core histones (Germond et al., 1975) or with the *Escherichia coli* HU protein (Rouvière-Yaniv et al., 1979). The average number of beads at protein saturation was 14–16 per genome, a good correlation with the number of superhelical turns generated after deproteinization. Moreover, at intermediate protein to DNA ratios, we have observed an intermediate number of beads correlated with an intermediate number of superhelical turns observed in electrophoresis. But in contrast with histones or *E. coli* HU–SV40 complexes, we failed to observe a significant change in the contour length of the DNA complexed with S25. Values of 1.61 ± 0.01 and 1.57 ± 0.03 μm were obtained for naked SV40 DNA and complexes with S25, respectively. The result suggests that those structures are presumably not formed by wrapping of the DNA around a protein core.

[4] For a covalently closed circular DNA, $\bar{L}_K$ can be written as the sum of the twist $\bar{T}_w$ and the writhing number $\bar{W}_r$ or $\bar{L}_K = \bar{T}_w + \bar{W}_r$ (Crick, 1976).

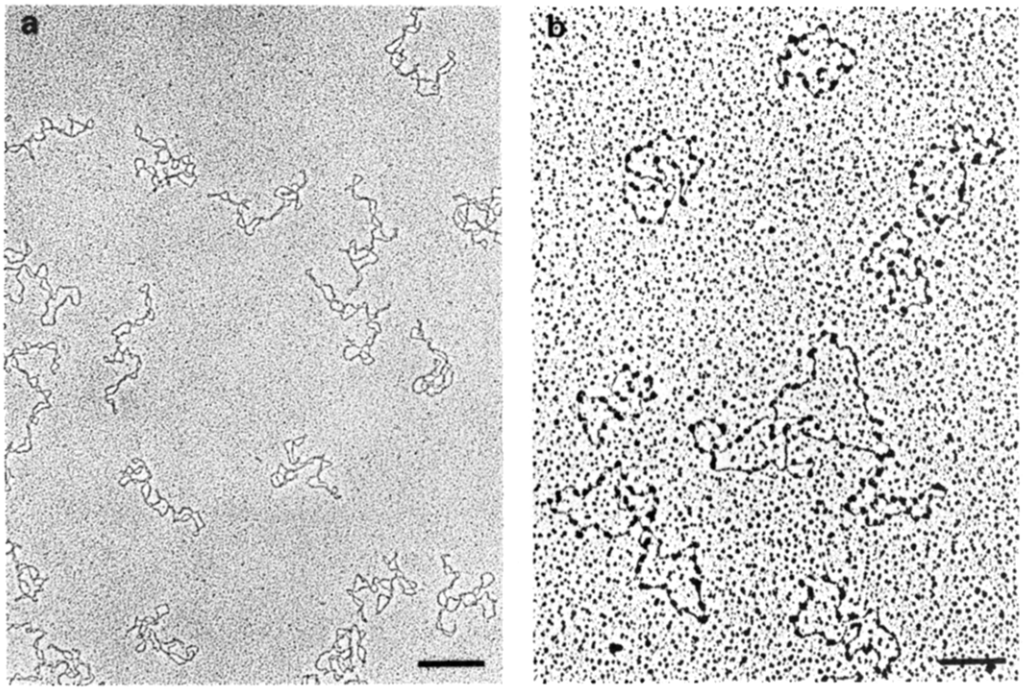

FIGURE 6: Electron microscopy of SV40–S25 complexes. SV40 DNA I and S25 were incubated in 10 mM potassium phosphate, pH 7.0, for 20 min at 37 °C and processed as described under Materials and Methods. (a) SV40 DNA I control. (b) SV40–S25 complexes at protein saturation. The bar represents 0.2 μm.

Discussion

The experiments described in this paper show that normal rat liver single-strand binding protein S25 changes the conformation of DNA so that its linking number is changed when a transient covalent break made by topoisomerase allows DNA in the complex to reach an energetically favored conformation (i.e., to relax). A linear relation was found between the change in linking number of DNA and the amount of protein present, indicating that each active protein molecule equally contributes to this change. In other words, the effect of S25 on DNA was found to be noncooperative, as was the binding of S25 to SV40 DNA I when followed by sedimentation analysis (Bonne et al., 1980).

The time of incubation in the presence of S25 influences the amount of DNA made superhelical but does not seem to affect appreciably the position of the Gaussian distribution, i.e., the average value of the linking number. Since this value depends on the number of protein molecules bound, one can estimate that, at intermediate times, a fraction of DNA is complexed with high protein ratio while another is free. But, when equilibrium is reached (for long incubation times), all DNA molecules seem to be equally covered. A redistribution of protein molecules on DNA is not excluded since competition experiments suggest a reversible binding of S25 to SV40 DNA (Bonne et al., 1980).

In contrast, an increase of the ionic strength influences the average value of the linking number at equilibrium, presumably by decreasing the maximum number of S25 molecules that can be bound to each SV40 DNA molecule.

Comparable effects on the linking number of covalently closed circular DNA were reported in the case of *E. coli* RNA polymerase[5] (Wang et al., 1977), of *E. coli* HU protein (Rouvière-Yaniv et al., 1979), of histones (Germond et al., 1975), and of high mobility group (HMG) proteins 1 and 2 from calf thymus (Javaherian et al., 1979). In all cases, generation of negative turns was reported, but the mechanism proposed to explain the effect of these proteins on DNA was different in each case. In contrast, single-strand DNA binding proteins such as T_4 gene 32 protein or 72K protein of adenovirus appeared "inactive" toward the above reaction. This put into question the mechanism by which rat liver S25 changes the linking number of closed circular DNA. It was shown that, in any case, this change reflects the initial constraints imposed on DNA before relaxation by topoisomerase. According to the equation $\bar{L}_K = \bar{T}_w + \bar{W}_r$ of Fuller (1971) and Crick (1976) [a reexamination of Vinograd's relation $\alpha = \beta + \tau$ (Bauer & Vinograd, 1974)], any change in $\bar{T}_w$ or $\bar{W}_r$ or both will change $\bar{L}_K$ and vice versa. In other words, two general contributions to the variations in $\bar{L}_K$ are possible; one is a change in the twist (helical repeat) of DNA ($\bar{T}_w$) and the second is a change in the shape of DNA axis in the space ($\bar{W}_r$), or roughly superhelicity. This provides two general mechanisms for the generation of negative superhelical turns observed in a closed circular DNA.

The change in the writhing number $\bar{W}_r$ is illustrated by the in vitro assembly of the four core histones on SV40 DNA, catalyzed by topoisomerase I (Germond et al., 1979), where the mechanism probably involved is the supercoiling of the DNA around a protein core (formation of nucleosomes). In this case, each histone octamer stabilizes approximately one superhelical turn so that the number of nucleosomes roughly corresponds to the number of negative superhelical turns generated after deproteinization (Germond et al., 1979). The same mechanism was proposed for the effect of *E. coli* HU protein on SV40 DNA (Rouvière-Yaniv et al., 1979). In both cases, the consequence of the formation of such structures is a compaction of the DNA. Although the nucleoproteic complexes made by S25 on SV40 DNA resemble those made by HU, no reduction in the contour length of the DNA was found, unfavoring this mechanism in the case of rat liver protein.

The change in the twist $\bar{T}_w$ can occur by continuous change in the helical repeat of DNA (as for EtBr binding) or by discontinuous changes (local separation of strands). Since rat

[5] In this case, a rotation angle of 240° per holoenzyme molecule was reported, but, when calculated for a given protein to DNA ratio, this value was comparable to that found for the other proteins cited.

liver binding protein S25 has a high affinity for single-stranded DNA, it is tempting to think that this protein, like RNA polymerase and possibly HMG proteins, acts by local separation of the two strands. However, no denaturation loop appeared in electron microscopy on SV40 DNA (in contrast with gene 32 protein–SV40 complexes), providing that, if rat liver protein promotes strand separation in SV40 DNA, this would occur very locally, possibly within the beads. Nevertheless, a continuous change in the helix rotation angle induced by protein binding is not presently excluded. As pointed out by Benham (1979), any torsional stress imposed to DNA (for instance by protein binding) can be transmitted at a distance and can favor the starting of specific events such as transcription or replication, and this provides other in vivo possible functions for rat liver single-strand binding proteins.

Acknowledgments

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