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Strand breakage by the DNA untwisting enzyme results in covalent attachment of the enzyme to DNA

(superhelical simian virus 40 DNA/DNA strand breakage/DNA replication/DNA–protein complex/hydroxylamine)

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ABSTRACT **Strands of DNA that have been broken by the DNA untwisting enzyme exhibit a reduced buoyant density in alkaline CsCl due to bound protein. A covalent linkage between the DNA and the enzyme was indicated by the stability of the complex in alkali (pH > 12.7), in 7 M guanidine·HCl, and at 90° in 1% Sarkosyl for 5 min. The single-strand breaks generated by the enzyme are resistant to exonuclease III, indicating that the protein is attached to one of the ends of the broken strands. The free end of the broken strand bears a 5′-hydroxyl group, indicating attachment of the protein to the 3′-phosphoryl terminus. A nucleotide–peptide linkage involving a phosphoamide bond is unlikely since the complex is resistant to 3.5 M hydroxylamine at pH 4.75.**

The DNA untwisting enzyme and the similar enzyme from *Escherichia coli* (ω protein) were originally discovered by their ability to relax superhelical closed circular DNA molecules (1, 2). In order to explain the relaxation process and at the same time to account for the fact that the circular DNA remained closed, it was postulated that these enzymes must catalyze the formation of a transient single-strand break (nick) in duplex DNA. This mechanism has been confirmed by the recent finding of a nicked intermediate in the reaction catalyzed by the DNA untwisting enzyme (3).

If strand breakage involves hydrolysis of a phosphodiester bond, then resynthesis of the bond requires an input of >3 kcal/mole (12 kJ/mole) (4). Yet the DNA untwisting enzyme, unlike the polynucleotide ligase (5), is able to reseal the break in the absence of any energy-donating cofactors. In order to circumvent this problem, it was suggested that phosphodiester bond breakage involves not hydrolysis, but transfer of one end of the broken DNA strand to a site on the enzyme (1, 2). If the bond between the enzyme and the DNA has a free energy of hydrolysis comparable to that for the phosphodiester bond, then the resealing reaction is energetically possible. This hypothesis predicts that the enzyme should be covalently bound to the intermediate containing the single-strand break. In this paper evidence is presented for a covalent linkage between the enzyme and the nicked DNA. In addition, evidence is presented that the enzyme is attached to the DNA through a 3′-phosphate group.

MATERIALS AND METHODS

General. The sources of most of the reagents and materials have been given elsewhere (3). Guanidine·HCl was obtained from Matheson, Coleman & Bell and hydroxylamine hydrochloride from Sigma Chemical Co. Sarkosyl NL30 was purchased from ICN-K&K Laboratories, Inc. [γ-^{32}P]ATP (20 Ci/mmol) was purchased from New England Nuclear.

Enzymes. The DNA untwisting enzyme was purified from rat liver nuclei by a described procedure (6). Proteinase K was purchased from Beckman and stored in 50 mM Tris·HCl (pH 8.0)/0.1 mM EDTA/1 mM $CaCl_2$ at 0°. Pancreatic DNase and micrococcal nuclease were purchased from Worthington Biochemical Corp. *E. coli* exonuclease III and T4 polynucleotide kinase were from New England Biolabs.

Reaction Conditions for DNA Untwisting Enzyme. Each reaction mixture (final volume 15–60 μl) contained in addition to the stated amount of enzyme and simian virus 40 (SV40) DNA, 35 mM potassium phosphate buffer (pH 7.4), 0.25 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. The reaction mixtures were incubated at 24° for 10 min and stopped by adjusting the pH either to >12.5 or to 4.5. Both of these treatments prevented the resealing step of the reaction and thereby trapped the nicked intermediate. To stop the reaction with alkali, the mixture was rapidly mixed with 3 volumes of 0.20 M NaOH. For the low pH step the mixture was diluted with 3 volumes of 0.20 M sodium acetate buffer (pH 3.8) to give a final pH of 4.5.

Alkaline CsCl Equilibrium Centrifugation. Alkaline CsCl gradients were run by the procedure of Blair and Helinski (7). The polyallomer tubes were soaked in bovine serum albumin (5 mg/ml) before use. Each centrifuge tube contained the sample DNA diluted to 2.1 ml with 10 mM Tris·HCl (pH 7.5)/1 mM EDTA plus 0.13 ml of 0.10 M EDTA, 1.0 ml of 0.5 M Na_3PO_4, 0.03 ml of 2.5% Sarkosyl, 0.017 ml of bovine serum albumin (5 mg/ml), and 3.5 g of CsCl. The samples were centrifuged for 60 hr in the SW56 rotor at 35,000 rpm and 20°. The gradients were dripped into 45 fractions and the radioactivity was determined as described (8). The CsCl density was calculated from the refractive index after correcting for the contribution due to the other components of the mixture. Since SV40 DNA has a GC content of about 41% (9), the buoyant density of the native form of the DNA in neutral CsCl is 1.700 g/cm^3 (10). The buoyant densities of the closed and open forms of SV40 DNA in alkaline CsCl were calculated to be 1.780 and 1.760 g/cm^3, respectively, using the increments observed for polyoma DNA by Vinograd *et al.* (11).

Preparation of Nicked DNAs. The procedure for nicking SV40 DNA with pancreatic DNase and the purification of the nicked DNA through a CsCl/ethidium bromide gradient has been given (12). The reaction conditions for nicking SV40 DNA with micrococcal nuclease were 25 mM glycine buffer (pH 9.0), 5 mM $CaCl_2$, 34 μg of DNA per ml, and 0.16 ng of enzyme per ml. The mixture was incubated for 5 min at 37° and the reaction was stopped by the addition of ethylene glycol-bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid (EGTA). The nicked DNA was purified as described for the pancreatic DNase nicked DNA. The average number of single-strand breaks per molecule

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Abbreviations: SV40, simian virus 40; nick, single-strand break.

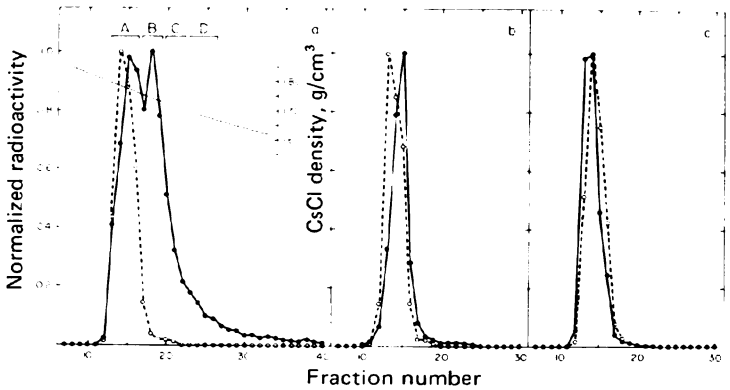

FIG. 1. Alkaline CsCl equilibrium centrifugation of DNA–protein complexes. SV40 DNA (19 μg/ml) was reacted with the purified DNA untwisting enzyme (30 μg/ml) for 10 min at 24°. An aliquot of the reaction mixture was removed and added to a tube containing KCl to bring the final salt concentration up to 0.50 M. The remainder of the reaction was stopped with alkali. (*a*) An aliquot of the alkali-stopped reaction was centrifuged to equilibrium in alkaline CsCl. The indicated fractions were combined to yield pools A, B, C, and D, which comprise 42, 36, 15, and 7% of the total [^{3}H]DNA, respectively. The peak fraction for the substrate [^{3}H]DNA (●) contained 605 cpm; the peak fraction for the marker [^{14}C]DNA (○) contained 314 cpm. (*b*) A second aliquot of the alkali-stopped reaction was neutralized and treated with proteinase K (100 μg/ml) before analysis in alkaline CsCl. The peak fraction for the [^{3}H]DNA contained 1432 cpm; the peak fraction for the [^{14}C]DNA contained 449 cpm. (*c*) The aliquot that had been diluted into 0.5 M KCl was incubated for another 10 min at 24°, stopped with alkali, and centrifuged to equilibrium in alkaline CsCl. The peak fraction for the [^{3}H]DNA contained 1939 cpm; the peak fraction for the [^{14}C]DNA contained 608 cpm. The fractions not plotted contained no radioactivity.

was calculated from the proportion of unnicked molecules using the Poisson distribution.

Digestion with Exonuclease III. The reaction mixture (0.10 ml) contained 66 mM Tris·HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, the indicated amount of DNA (expressed as fmol of single-strand breaks per reaction), and 4.5 units (13) of exonuclease III. After incubation for 10 min at 37° the reaction was stopped by dilution into 0.5 ml of 10 mM EDTA containing 0.5 mg of salmon sperm carrier DNA per ml. The DNA was precipitated with 5% trichloroacetic acid and the precipitate was removed by centrifugation. The radioactivity in the supernatant was determined in toluene scintillation fluid containing Triton X-100. Under these conditions the release of acid-soluble nucleotides was directly proportional to the concentration of susceptible single-strand breaks (see *Results*).

Other Methods. The procedure for alkaline sucrose gradient sedimentation to separate closed circular SV40 DNA from nicked circles has been described (3). In order to resolve the single-stranded circular and linear forms of SV40 DNA, the same procedure was used except the time of centrifugation was extended from 80 min to 4 hr. The DNA was treated with polynucleotide kinase under the conditions described by Richardson (14) except that the final ATP concentration was 3.5 μM and the reaction was at 0° in order to minimize the exchange reaction (15). The DNA–protein complexes were treated with 3.5 M hydroxylamine at pH 4.75 by the procedure of Gumport and Lehman (16).

RESULTS

A DNA–Protein Complex. Single-strand breaks, introduced into duplex DNA by high concentrations of the DNA untwisting enzyme, were previously detected by stopping the enzyme reaction with alkali (pH > 12.5) (3). The level of nicked intermediate present in the reaction was assayed by sedimenting the products through an alkaline sucrose gradient. While this assay discriminated between nicked and closed circular DNA, it did not reveal whether the more slowly sedimenting broken DNA strands contained any bound protein.

A complex containing both protein and DNA will exhibit a lower buoyant density in CsCl than free DNA. If the DNA strands that are broken by the untwisting enzyme contain protein bound through an alkali-stable linkage, the existence of such a complex should be detectable by equilibrium centrifugation in alkaline CsCl. SV40 DNA was reacted with purified DNA untwisting enzyme from rat liver under the conditions previously used for detection of the nicked intermediate. The reaction was stopped with alkali and analyzed by equilibrium centrifugation in alkaline CsCl. Fig. 1*a* shows the results of an experiment in which the substrate DNA was 91% nicked at the moment the reaction was stopped. A large proportion of the ^{3}H-labeled substrate DNA was less dense than the ^{14}C-labeled marker DNA. The marker used in these experiments contained approximately equal proportions of closed and nicked SV40 DNA molecules which band in alkaline CsCl at approximately 1.780 and 1.760 g/cm^3, respectively.

When the reaction products were treated with proteinase K (17) prior to analysis in alkaline CsCl (Fig. 1*b*), essentially all of the ^{3}H-labeled DNA banded at the position expected for single-strand circular and linear pieces of SV40 DNA. This result shows that protein is responsible for the reduced density of the DNA in Fig. 1*a*.

An aliquot of the reaction mixture analyzed in Fig. 1*a* was diluted into 0.50 M KCl and the incubation continued for another 5 min before it was stopped with alkali. We have previously shown that under these conditions all of the nicked DNA is resealed and the untwisting reaction ceases (3). When this sample was analyzed in alkaline CsCl (Fig. 1*c*), none of the low density material remained and nearly all of the DNA banded at the expected position for denatured closed circular DNA. This result suggests that the untwisting enzyme and not a trace contaminant in the purified preparation was responsible for the buoyant density shift of the DNA.

Relationship between Strand Breakage and Formation of DNA–Protein Complex. The fractions indicated by the brackets in Fig. 1*a* were pooled, neutralized, treated with proteinase K, and dialyzed to remove the high concentration of salt. An aliquot of each pool was sedimented for a short time in an alkaline sucrose gradient to determine the proportion of closed circular DNA molecules. Pool A contained 20% closed circles, while pools B, C, and D contained no detectable closed circles (gradients not shown). A second aliquot of each pool was analyzed by a longer sedimentation in alkaline sucrose in order to resolve single-stranded circles from unit-length linear molecules and smaller fragments (Fig. 2). Single-stranded circles were present only in pool A. These results show that treatment with the DNA untwisting enzyme did not affect the buoyant density of the DNA strands that remained unbroken.

Conversely, the DNA strands that exhibited a lower buoyant density due to bound protein are broken strands of less than or equal to unit length (Fig. 2). Moreover, the length of the DNA strands decreases as the buoyant density of the complexes decreases. In addition, the extent of the buoyant shift increases as the number of breaks per molecule increases [compare Figs. 1*a* (91%) and 3*a* (98%)]. These results suggest that the complexes may be composed of one enzyme molecule attached to DNA fragments of various lengths.

This hypothesis can be tested since the buoyant density of a DNA–protein complex depends on the relative size of each of

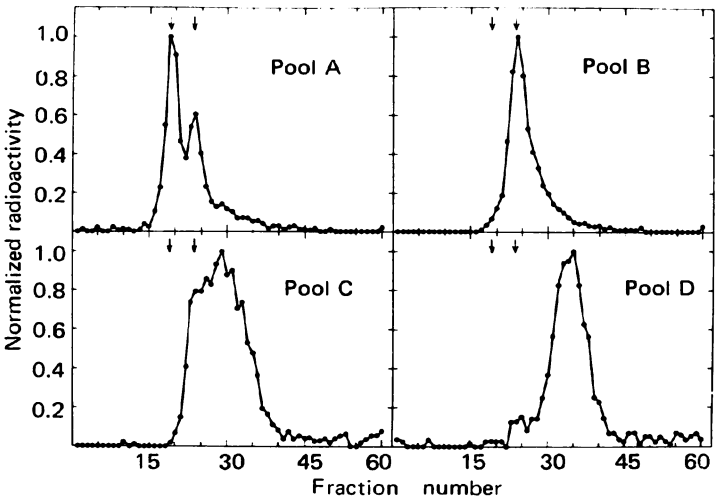

FIG. 2. Alkaline sucrose gradient analysis of the [^{3}H]DNA in pools A, B, C, and D from Fig. 1*a*. Sedimentation is from right to left. The arrows indicate the positions of the 18S and 16S single-stranded circular and linear strands of SV40 DNA, respectively. The peak fractions contained: pool A, 229 cpm; pool B, 457 cpm; pool C, 107 cpm; pool D, 83 cpm.

its constituents. The enzyme is composed of a single polypeptide chain with a molecular weight of 66,000 (6). For each set of pooled fractions shown in Fig. 1*a*, the molecular weight range of the DNA strands can be determined from their sedimentation rates in alkali (Fig. 2) (18). From the weight fraction of protein (f_P) and DNA (f_D) in a composite structure, one can calculate the buoyant density of the complex (ρ_C) according to the following equation:

$$\rho_C = \rho_P\rho_D/(f_P\rho_D + f_D\rho_P)$$

where ρ_D and ρ_P represent the buoyant densities of the pure DNA strands and pure protein, respectively. In alkaline CsCl, SV40 single-stranded linear molecules have a buoyant density of about 1.760 g/cm^3, while fragments of SV40 DNA will have a buoyant density that depends on their GC content. In neutral CsCl the buoyant density of protein has been taken to be 1.29 g/cm^3 (19). We have used this value to approximate the buoyant density of protein in alkaline CsCl.

Pool B is composed mostly of unit length linear strands (Fig. 2). The expected buoyant density of a 1:1 complex between unit length linear molecules and the enzyme is 1.737 g/cm^3, while a complex containing two enzymes per DNA molecule should have a density of 1.716 g/cm^3. The observed value of 1.742 g/cm^3 for the average density of the fractions in pool B is in good agreement with the predicted value for a 1:1 complex. The calculated buoyant densities for 1:1 complexes between the enzyme and the DNA strands in pool C (assuming the same GC content as unit linear molecules) range from 1.709 to 1.737 g/cm^3. The average densities observed for the fractions in pool C extend from 1.706 to 1.724 g/cm^3. The corresponding predicted densities for pool D range from 1.689 to 1.719 g/cm^3, while the observed values extend from 1.672 through 1.695 g/cm^3. For pools C and D there appears to be a bias towards higher predicted buoyant densities as compared with the observed values. This effect is likely due to the presence of overlapping Gaussian bands in the original CsCl gradient (Fig. 1*a*), which occur preferentially on the dense side of each pool. Since the denser DNA fragments are longer, the DNA size distribution is weighted to somewhat higher values which leads to an overestimate of the buoyant density of the complex. The agreement between the predicted and the observed densities supports the hypothesis that each complex is the result of the attachment of one enzyme molecule to a broken DNA strand.

Table 1. Susceptibility of single-strand breaks to exonuclease III

Source of single-strand breaks	DNA molecules, fmol/reaction	Single-strand breaks, fmol/reaction	Nucleotides acid solubilized, pmol/reaction
DNA untwisting enzyme (76% nicked)*	25	35	2.8
Mock treated DNA (8% nicked)†	31	2.5	3.4
Pancreatic DNase (5′-*P*, 3′-OH)	18	22	9.2
Pancreatic DNase	36	45	24
Micrococcal nuclease (5′-OH, 3′-*P*)	17	32	17
Micrococcal nuclease	41	76	49
DNA untwisting enzyme	25	35	24
+ pancreatic DNase	36	45	
DNA untwisting enzyme	25	35	50
+ micrococcal nuclease	41	76	

* SV40 DNA (84 μg/ml) was reacted with the purified DNA untwisting enzyme (68 μg/ml) for 8 min at 24°. The reaction was stopped by adjusting the pH to 4.5. An aliquot from this mixture was tested directly for its susceptibility to exonuclease III. The average number of nicks per DNA molecule was calculated from the proportion of unnicked molecules [determined as described (3)] using the Poisson distribution.

† SV40 DNA was treated exactly as described above except the DNA untwisting enzyme was omitted from the initial reaction mixture. The single-strand breaks in this DNA are due, at least in part, to ^{3}H decay.

What proportion of the broken strands contain bound protein? From Fig. 2 it can be seen that pool A contains only a few linear fragments shorter than unit length. Thus, virtually all of the small fragments generated by the enzyme exhibited a lower buoyant density than free DNA and must have bound protein. An extension of this analysis to the longer strands is complicated by the fact that the DNA preparation used in this experiment initially contained 13% nicked molecules, which should yield single strands lacking bound protein (see below).

Susceptibility of Broken Strands to Exonuclease III and Polynucleotide Kinase. In order to determine directly whether the enzyme is attached to the DNA at the site of the nick, I examined the sensitivity of the single-strand breaks to digestion by exonuclease III. SV40 DNA was treated with the DNA untwisting enzyme as described above and the reaction was stopped by adjusting the pH of the mixture to 4.5. Control experiments showed that, like alkaline denaturation, this procedure trapped the nicked intermediate (see Fig. 4). After neutralization the single-strand breaks were not resealed, indicating that the low pH block is essentially irreversible.

The nicked DNA was treated directly with exonuclease III. From the results presented in Table 1 it can be seen that the sensitivity of the enzyme-nicked DNA to hydrolysis by exonuclease III was no greater than what one would expect based on the level of nicks preexisting in the DNA preparation. As expected (13), exonuclease III can degrade DNA from a nick regardless of whether it contains a 3′-phosphate or a 3′-hydroxyl group. Mixing experiments (last two lines, Table 1) show that

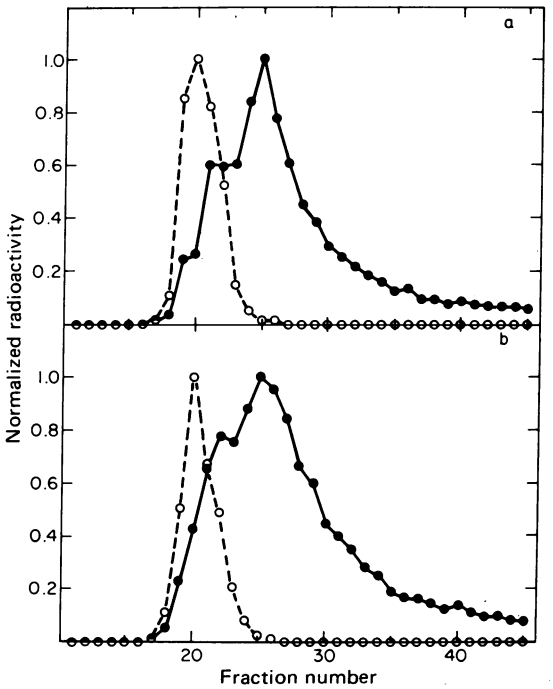

FIG. 3. Effect of guanidine·HCl on the DNA–protein complex. SV40 DNA (11 μg/ml) was reacted with DNA untwisting enzyme (34 μg/ml) as described for Fig. 1. The reaction was stopped with alkali and one aliquot (*a*) was banded directly in alkaline CsCl. A second aliquot (*b*) was diluted into 7 M guanidine·HCl and incubated for 30 min at 37° followed by centrifugation in alkaline CsCl. At the time the reaction was stopped the substrate DNA was 98% nicked. The peak fractions of ^{3}H-labeled DNA (●) and ^{14}C-labeled marker DNA (○) contained (*a*) 583 and 357 cpm, respectively, and (*b*) 423 and 421 cpm, respectively.

the resistance of the enzyme-induced nicks to exonuclease III is not due to the presence of an inhibitor for exonuclease III in the preparation of nicked DNA. These results suggest an association of the untwisting enzyme with the end of one of the broken strands, which blocks the action of exonuclease III (see *Discussion*). Since there is no detectable increase in the susceptibility to exonuclease III, it appears that all of the breaks caused by the untwisting enzyme are blocked.

In order to determine which end of the broken strand contained bound enzyme, the susceptibility to phosphorylation by polynucleotide kinase was examined. A reaction mixture similar to the one analyzed in Fig. 1*a* was stopped with alkali as described above. The sample was neutralized and treated with polynucleotide kinase in the presence of [γ-^{32}P]ATP. After equilibrium centrifugation in alkaline CsCl, the ^{32}P label was found associated with all of the broken strands containing bound protein. The fraction in the gradient containing complexes between the protein and unit length linear strands (corresponding to fraction 18, Fig. 1*a*) contained 9.5 fmol of single strands (assuming a single-strand molecular weight of 1.75×10^{6}). The same fraction contained 7.6 fmol of ^{32}P, indicating that 80% of the strands had become phosphorylated. Therefore, the DNA strands containing bound protein also contain a free 5′-hydroxyl. The details of this analysis will be published elsewhere.

Covalent Linkage between DNA and Enzyme. The assay for DNA-bound protein described here relies on the fact that the linkage is stable in alkali (pH > 12.7) for at least the 60 hr required to reach equilibrium in CsCl centrifugation. The nature of the linkage was further examined by testing its stability in guanidine·HCl. Fig. 3 shows the results of incubating

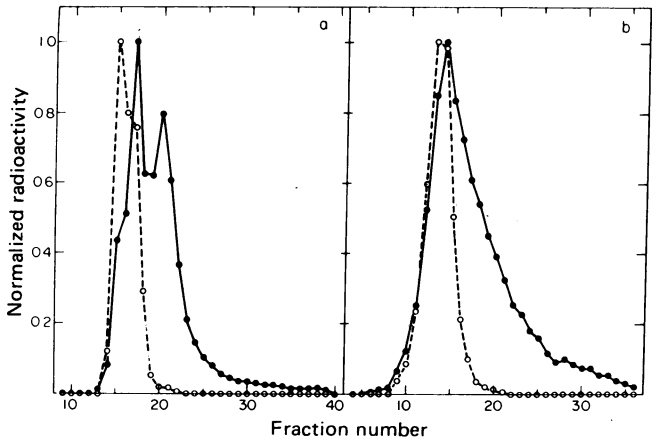

FIG. 4. Effect of hydroxylamine on the linkage between the DNA and the enzyme. SV40 DNA (14 μg/ml) was reacted with the DNA untwisting enzyme (37 μg/ml) as described for Fig. 1. The reaction was stopped by adjusting the pH to 4.5. The reaction mixture was divided into two equal aliquots. (*a*) One aliquot was banded directly in alkaline CsCl. The peak fraction for the ^{3}H-labeled substrate DNA (●) contained 755 cpm; the peak fraction for the ^{14}C-labeled marker DNA (○) contained 424 cpm. (*b*) The second aliquot was treated with 3.5 M hydroxylamine (pH 4.75) for 30 min at 37° followed by alkaline CsCl centrifugation. The ^{3}H peak contained 466 cpm; the ^{14}C peak contained 373 cpm. The recovery of ^{3}H-labeled DNA was 87% relative to the untreated control.

the complexes (reaction stopped with alkali) for 30 min at 37° in 7 M guanidine·HCl (pH 7) prior to analysis in alkaline CsCl. The linkage is not destroyed by this treatment. In other experiments (data not shown) it was found that the linkage was stable to incubation for 5 min at 90° in 1% Sarkosyl. Taken together these results indicate that the enzyme is most probably attached to the 3′-phosphoryl terminus through a covalent bond.

The stability of the linkage in alkali suggested that the protein might be bound to the DNA through a phosphoamide bond (20). Most phosphoamide bonds are cleaved by treatment with hydroxylamine at pH 4.75 (20). Since hydroxylamine reacts more readily with single-stranded as compared with double-stranded DNA (21), the intermediate was trapped using the low pH method rather than alkaline denaturation. Fig. 4*a* shows the results of stopping a reaction by rapidly adjusting the pH of the reaction mixture to 4.5. As before, analysis of the products in alkaline CsCl revealed the presence of a complex with a decreased buoyant density. When the reaction products were treated with 3.5 M hydroxylamine (pH 4.75) prior to equilibrium centrifugation in alkaline CsCl (Fig. 4*b*), most of the low density material was still present, although the band of radioactivity appeared considerably broader than in the untreated sample. Control experiments (not shown) demonstrated that treatment of native DNA at pH 4.75 with 3.5 M hydroxylamine resulted in breakage of approximately 15% of the DNA strands (probably the result of depurination), but otherwise had no effect on the average buoyant density of the DNA in alkaline CsCl. However, the presence of hydroxylamine in the alkaline CsCl gradient (at ~0.15 M) did cause a broadening of the bands for the uncomplexed DNA as well as for the DNA–protein complexes. Attempts to remove the hydroxylamine by dialysis prior to analysis in alkaline CsCl resulted in preferential binding of the complexes to the dialysis membrane. However, sufficient material with a reduced buoyant density was recovered in these experiments to confirm the resistance of the complex to acidic hydroxylamine.

DISCUSSION

DNA strands that have been broken by the DNA untwisting enzyme exhibit a lower buoyant density in alkaline CsCl than free single-stranded DNA. From the magnitude of the buoyant shift for unit length linear strands, we conclude that each DNA molecule contains one bound enzyme molecule. The single-stranded interruptions introduced into the DNA by the untwisting enzyme are resistant to the action of exonuclease III. The strands containing bound protein can be phosphorylated by polynucleotide kinase, indicating a free 5′-hydroxyl terminus. Taken together, these results show that the enzyme is attached to the broken strand at the 3′-phosphate end. It seems probable that this attachment of the enzyme to the DNA strand serves to conserve the energy required for resealing the single-strand break.

Pool A in Fig. 1*a* accounts for 42% of the DNA in the gradient, of which 20% are closed circles. Of the remaining molecules, 60% are single-stranded circles (Fig. 2, pool A). Using these values one can calculate that 29% of the DNA strands remained unbroken at the time the untwisting reaction was stopped. In other experiments, at much higher ratios of enzyme to DNA, virtually every strand was nicked one or more times. The fact that greater than 50% of the DNA strands can be broken at least once by the DNA untwisting enzyme means that nicking by the enzyme is not strand specific. In addition, the fact that the fragments resulting from alkaline denaturation of the nicked DNA can be less than 25% the length of SV40 DNA (Fig. 2) indicates that one strand of SV40 DNA can be cut by the DNA untwisting enzyme at multiple sites. The question of sequence specificity of the enzyme is being investigated further.

One of the intermediates in the reaction catalyzed by polynucleotide ligase involves covalent attachment of an AMP group to the enzyme through a phosphoamide bond (5). By analogy with the ligase-catalyzed reaction, one could hypothesize that a similar linkage might be involved in the attachment of the nicked intermediate to the DNA untwisting enzyme. The ligase adenylate is relatively stable in alkali, but is almost completely cleaved by hydroxylamine at pH 4.75 (16). When tested under exactly the same conditions, the bond between the untwisting enzyme and the DNA appears to be resistant to cleavage by acidic hydroxylamine. Although quantitation in this experiment was difficult, these results suggest that a linkage other than a phosphoamide bond may be responsible for the attachment of the protein to the DNA.

Several examples of complexes between nucleic acid and protein are known. Nicking of the colicin plasmid (ColE1 and ColE2) relaxation complexes results in covalent attachment of a protein to the 5′ end of the DNA strand that is broken (7, 22). The gene A protein of ϕX174 may also be covalently linked to the DNA strand at the site of breakage (23). These activities differ from that exhibited by the DNA untwisting enzyme in that they are specific with regard to the strand and the sequence at which they introduce a nick and they are attached to the 5′-phosphoryl rather than the 3′-phosphoryl terminus. The ColE1 reaction has not yet been shown to be reversible, whereas the A protein can restore continuity to the strand it breaks (A. Kornberg, personal communication).

A situation similar to the colicin plasmid relaxation complex may exist for SV40. DNA extracted from SV40 virions with sodium dodecyl sulfate in the presence of dithiothreitol has been reported to contain protein linked to nicked molecules (24). Recent evidence indicates that a small protein is covalently attached to the RNA extracted from polio virions (25, 26). Finally, the ends of adenovirus DNA appear to be complexed with protein (27–30). The nature of the nucleic acid–protein linkage and its significance remain to be elucidated in all of these examples.

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