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Covalent Bonds between Protein and DNA

FORMATION OF PHOSPHOTYROSINE LINKAGE BETWEEN CERTAIN DNA TOPOISOMERASES AND DNA*

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The cleavage of a DNA phosphodiester bond by *Escherichia coli* DNA topoisomerase I and the simultaneous covalent linkage of the enzyme to the 5′-phosphoryl group of the DNA at the cleavage site have been reported previously (Depew, R. E., Liu, L. F., and Wang, J. C. (1978) *J. Biol. Chem.* 253, 511–518; Liu, L. F., and Wang, J. C. (1979) *J. Biol. Chem.* 254, 11082–11088). With either *E. coli* or *Micrococcus luteus* DNA topoisomerase I, formation of the covalent complex with ^{32}P-labeled single-stranded DNA, followed by nucleolytic treatment of the complex, yields a ^{32}P-labeled protein. The stability of the linkage between the protein and the labeled phosphorus, in aqueous buffers ranging from 1 to 13 in pH and in 3.8 M hydroxyamine at pH 4.8, suggests that the linkage is unlikely to be phosphoserine, phosphothreonine, or a phosphorus-nitrogen bond. Analysis of a 5.6 M HCl hydrolysate of the labeled protein by paper electrophoresis and thin layer chromatography identifies *O*^{4}-phosphotyrosine as the labeled amino acid. Thus the protein-DNA covalent bond that can form between DNA and *E. coli* or *M. luteus* DNA topoisomerase I is most likely a phosphotyrosine linkage. The sites of cleavage by the topoisomerases in a number of single-stranded DNA restriction fragments have been determined at the nucleotide sequence level. There is no nucleotide specificity on either the 3′- or the 5′-side of the site of cleavage. The distribution of the cleavage sites is, however, nonrandom, and there appears to be considerable coincidence of cleavage sites for the two topoisomerases. The protein-DNA linkage formed upon cleavage of double-stranded DNA by *M. luteus* DNA gyrase has also been examined. It is found that the cleavage of the DNA by gyrase is accompanied by the covalent linking of subunit A, but not subunit B, of gyrase to the 5′-side of the DNA; the linkage is again a phosphotyrosine bond.

The existence of covalent bonds between certain proteins and DNA has been known for some time. Following the report in 1965 that there is a protein covalently linked to DNA extracted from phage P1-transducing particles (1), covalently linked proteins have been observed in *Escherichia coli* plasmid DNAs (2–10), *Bacillus subtilis* phage ϕ29 DNA (11–18), adenovirus DNA (19–23), simian virus 40 DNA (24–27), polyoma DNA (27, 28), and HeLa mitochondrial DNA (29).

For the examples cited above, little is known about the mechanisms of formation of the protein·DNA covalent complexes. It is also unknown whether these proteins, either before or after their covalent attachment to DNA, have any catalytic function. There are, however, a number of other protein·DNA covalent complexes for which the protein components themselves have been purified in catalytically active form, and, therefore, their covalent linking to DNA can be studied in some detail. These proteins are generally found to catalyze the breaking and rejoining of DNA backbone bonds.

In some cases, the breaking of a DNA internucleotide bond is followed in concert by the rejoining of the bond; thus the overall reaction usually manifests itself by the conversion of one DNA topological isomer (topoisomer) to another. The term DNA topoisomerases has been proposed for these enzymes (30). It was first postulated in 1971 that DNA topoisomerization catalyzed by such an enzyme involves the formation of a covalent link between the enzyme and DNA (31), and a number of covalent complexes between DNA and DNA topoisomerases have been detected in recent years (32–36). Usually these covalent intermediates appear to exist only transiently during the normal courses of reaction, and their isolation frequently involves treatments with protein denaturants.

In other cases, the breaking of a DNA internucleotide bond, accompanied by the formation of a protein-DNA covalent link, is well separated from the subsequent reactions. The gene A enzyme of phage ϕX174 is one of the well documented examples (37, 38). When negatively supercoiled ϕX DNA is incubated with gene A enzyme, especially when the enzyme is in molar excess (39), a phosphodiester bond at a unique position in the viral strand of the duplex DNA is cleaved, and the enzyme is linked to the 5′-phosphoryl end of the break. The gene A protein in this complex is presumably in its active form, since the complex supports replication of the DNA when incubated with uninfected *E. coli* extract, whereas the replication of negatively supercoiled ϕX DNA itself requires gene A protein in addition to extract of uninfected *E. coli* cells (39). The gene A enzyme is also likely to be directly involved in the reformation of a DNA phosphodiester bond after one round of replication (38, 40). The gene II product of phage fd appears to be similar to ϕX gene A enzyme in many respects (41). Other enzymes that probably fall in the ϕX gene A protein category are the phage P2 gene A product (42) and the phage ϕ29 protein mentioned earlier. All these proteins are involved in the initiation of DNA replication.

Because of the ubiquity of covalent protein·DNA complexes and the vital roles they play in the few well documented cases, we have initiated studies on the chemical nature of such covalent linkages. We report in this communication the identification of a phosphotyrosine linkage between DNA and several DNA topoisomerases, *E. coli* and *Micrococcus luteus* DNA topoisomerase I, and subunit A of *M. luteus* DNA gyrase.[1] Such a bond has previously been identified as the

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[1] We have previously denoted *M. luteus* gyrase subunit A the α subunit. Apparently, this subunit corresponds to *E. coli* gyrase sub-

linkage between protein VPg and poliovirus RNA (44, 45).

EXPERIMENTAL PROCEDURES

Materials—*E. coli* DNA topoisomerase I and *M. luteus* DNA topoisomerase I and DNA gyrase (topoisomerase II) were the preparations or similar preparations described previously (32, 34, 46, 47). *E. coli* DNA polymerase I, T4 polynucleotide kinase, and calf thymus alkaline phosphatase were purchased from Boehringer-Mannheim. Highly purified snake venom phosphodiesterase and *Pseudomonas* Bal 31 nuclease were generous gifts from Drs. M. Laskowski, Sr., Roswell Park Memorial Institute, and H. B. Gray, University of Houston, respectively. Staphylococcal nuclease and pancreatic DNase I were purchased from Worthington Biochemical Co. Pronase was purchased from Calbiochem. DNA restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Phage PM2 DNA and plasmid pBR322 DNA were prepared according to published procedures (48, 49). [α-^{32}P]dATP with a specific activity of about 400 Ci/mmol was purchased from New England Nuclear. *In vitro* ^{32}P-labeling of DNase I-nicked PM2 DNA was done by nick translation with *E. coli* DNA polymerase I in the presence of [α-^{32}P]dATP and the other three unlabeled deoxynucleoside triphosphates, as described (50). O^4-Phosphotyrosine was synthesized according to Rothberg *et al.* (44). The formation of covalent complexes between DNA and the topoisomerases is described in the appropriate figure legends. To form the covalent gyrase·DNA complex, the enzyme was first incubated with double-stranded PM2 DNA which had been ^{32}P-labeled by nick translation. The reaction mixture (300 μl) contained 50 mM Tris, pH 8, 0.1 mM EDTA, 20 mM KCl, 25 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/ml of bovine plasma albumin, 1 mM ATP, 40 μg/ml of oxolinic acid, 5 μg of DNA, and 10 μg each of *M. luteus* gyrase subunits A and B. After 1½ h at 37°C, KOH was added to a final concentration of 100 mM. The solution was neutralized after 1 min with a measured amount of HCl, and staphylococcal nuclease digestion and G-50 gel filtration were then carried out in the same way as described in the legend to Fig. 1. An aliquot of the gel-excluded radioactive material was mixed with 5 μg each of *M. luteus* subunits A and B, precipitated with 10% trichloroacetic acid, and loaded on a sodium dodecyl sulfate-5% polyacrylamide gel. Electrophoresis was carried out at about 5 V/cm for 6 h. Upon staining with Coomassie blue, the gel was photographed, and then dried and autoradiographed. The rest of the gel-excluded radioactive material was used in the identification of the covalent linkage as described in the legend to Fig. 2.

Methods—DNA sequencing was done according to the procedures of Maxam and Gilbert (51). High voltage paper electrophoresis was performed on Whatman No. 3MM paper with a Savant water-cooled apparatus. Electrophoresis at pH 3.5 was carried out in 5% acetic acid adjusted to the pH with pyridine for a duration of 2 h at 3000 V (30 V/cm). Electrophoresis at pH 1.8 was carried out in 2.5% formic acid plus 7.8% acetic acid for 4 h at the same field strength. The paper was dried and then autoradiographed on Kodak XR-5 film with a Dupont "lightning-plus" intensifying screen, as described by Laskey and Mills (52). Orthophosphate was made visible with Hanes-Isherwood reagent (53), and ninhydrin was used to stain unlabeled phosphotyrosine marker. Unlabeled dAMP was visualized under uv light. Thin layer chromatography was performed on silica gel plates (Macherey-Nagel Polygram SilG). The two solvent systems used were ethanol/*n*-butyl alcohol/ammonia/H_2O (volume ratios 4/1/2/3) and chloroform/methanol/ammonia (volume ratios 2/2/1). After drying the plates, autoradiography and visualization of the unlabeled spots were done as described above.

RESULTS

Covalent Complex Formation and the Transfer of DNA ^{32}P-label to M. luteus and E. coli DNA Topoisomerase I—We have previously shown that in the covalent complex between DNA and *E. coli* or *M. luteus* DNA topoisomerase I, the protein is most likely linked to a DNA 5′-phosphoryl group (32, 34). This immediately suggests that if ^{32}P-labeled DNA is used, complex formation followed by appropriate hydrolysis of the DNA should lead to the transfer of a radioactive DNA phosphorus label to the protein. The chemical nature of the original protein-DNA linkage can then be determined by identifying, upon hydrolysis of the protein, the labeled phosphorylated amino acid.

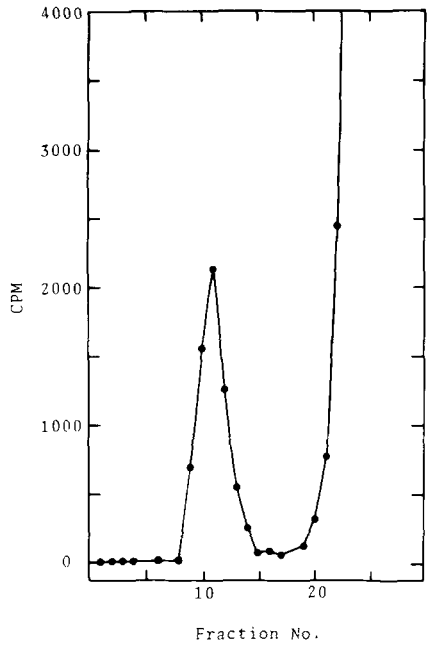

FIG. 1. **Formation of the covalent complex between denatured ^{32}P-labeled PM2 DNA and *M. luteus* DNA topoisomerase I.** Nicked PM2 DNA in 10 mM Tris·HCl, pH 8, 0.1 mM Na_3EDTA, which had been ^{32}P-labeled by nick translation, was denatured by adding KOH to 50 mM. After 10 min at 37°C, the DNA was neutralized with 0.1 volume of an equal volume mixture of 1 M HCl and 1 M Tris·HCl, pH 8. A reaction mixture (900 μl total volume) containing 10 mM Tris·HCl, pH 8, 20 mM NaCl, 50 μg/ml of bovine plasma albumin, 12 μg/ml of the denatured DNA, and 16 μg/ml of *M. luteus* DNA topoisomerase I was immediately prepared and then incubated at 37°C for 30 min. KOH was added to a final concentration of 50 mM, and the mixture was neutralized as before after 1 min at 37°C. $CaCl_2$ and staphylococcal nuclease were added to 1 mM and 30 units/ml, respectively. The mixture was incubated overnight at 37°C and concentrated approximately 10-fold by lyophilization. A Sephadex G-50 column (9 cm × 1.1 cm^2) was equilibrated with 20 mM potassium phosphate, pH 7.4, and 3 mg of bovine plasma albumin were loaded. After washing off the albumin with the phosphate buffer, the concentrated reaction mixture was loaded and eluted with the same phosphate buffer. Fractions of approximately 150 μl each were collected, and 10 μl of each fraction were counted after mixing with a scintillation counting cocktail (ScintiVerse, New England Nuclear).

Fig. 1 shows the transfer of DNA ^{32}P-label to *M. luteus* DNA topoisomerase I. Phage PM2 DNA was first labeled to a high specific activity by the nick translation action of *E. coli* DNA polymerase I in the presence of [α-^{32}P]dATP and three other unlabeled deoxyribonucleoside triphosphates. The labeled DNA was denatured, incubated with *M. luteus* DNA topoisomerase I, and then treated with KOH to form the covalent complex (32). The mixture was digested extensively with staphylococcal nuclease, and the labeled protein was separated from the bulk of the radioactivity, which was in the form of nuclease-degraded DNA, by gel filtration through Sephadex G-50. The elution profile clearly shows a radioactive peak around Fraction 11 at the exclusion limit of the gel. Several control experiments were carried out to show that the ^{32}P-labeled protein was derived from the covalent protein·DNA complex. Omission of the topoisomerase in the incubation mixture, substitution of ^{32}P-labeled DNA with [α-^{32}P]dATP, or the replacement of the KOH treatment step by the addition of an equivalent amount of water all lead to the abolishment of the gel-excluded ^{32}P-labeled peak. Sodium

unit A (43), and it is redesignated subunit A to conform to the nomenclature for the *E. coli* enzyme. *M. luteus* gyrase subunit β is now redesignated subunit B.

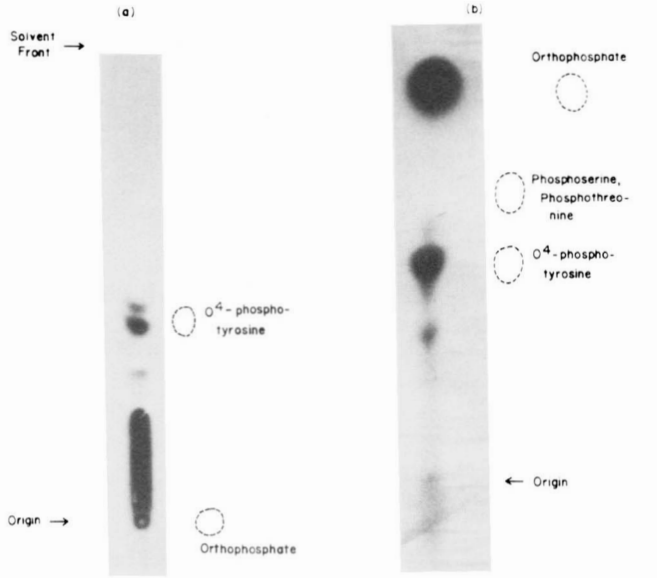

FIG. 2. **Identification of phosphotyrosine in the acid hydrolysate of the protein-DNA covalent complex.** *a*, Twenty microliters of the gel-excluded fraction shown in Fig. 1, containing approximately 2000 cpm of ^{32}P, were dried *in vacuo* in a 1.5-ml polyethylene centrifuge tube. Acid hydrolysis was then carried out by the addition of 30 μl of constant boiling (5.6 M) HCl (obtained from Pierce Chemical Co.) and incubation at 100°C for 30 min. The acid was removed by drying *in vacuo*, and the residue was dissolved in 10 μl of water and spotted on a silica gel thin layer plate. Orthophosphate and phosphotyrosine markers were spotted on a parallel lane on the same plate. *b*, the gel-excluded material was first digested overnight at 37°C with 10 mg/ml of pronase, which had been autodigested for 30 min at 37°C prior to use. Acid hydrolysis was carried out as described in *a*, and the final solution was spotted on Whatman No. 3MM paper and analyzed by electrophoresis at pH 1.8 as described under "Experimental Procedures."

dodecyl sulfate-polyacrylamide gel electrophoresis of the gel-excluded material gives a ^{32}P-labeled band with an electrophoretic mobility indistinguishable from that of *M. luteus* DNA topoisomerase I. Essentially identical results are obtained with *E. coli* DNA topoisomerase I.

Identification of a Phosphotyrosine Linkage—With either *E. coli* or *M. luteus* DNA topoisomerase I, less than 10% of the ^{32}P-counts are rendered acid-soluble when the labeled protein is incubated at 37°C for 1 h in dilute aqueous buffers ranging from 1 to 13 in pH. Incubation in 3.8 M hydroxyamine at pH 4.8 also results in little release of acid-soluble counts. Since phosphoserine and phosphothreonine bonds are rather labile at alkaline pH and P-N bonds are generally sensitive to hydroxyaminolysis and acid hydrolysis (54), these results suggest that it is unlikely that the ^{32}P-label is linked to the protein via phosphoserine, phosphothreonine, or a P-N bond.

Acid hydrolysis of the labeled protein at 100°C in constant boiling HCl for 30 min yields orthophosphate as the major labeled species, as shown by thin layer chromatographic analysis of the products. A significant fraction of the radioactivity, however, co-chromatographs with chemically synthesized phosphotyrosine on thin layer silica gel in two different solvent systems (Fig. 2*a*). The yield of this species is improved if the labeled protein is digested with pronase prior to acid hydrolysis (Fig. 2*b*). The identity of this species as phosphotyrosine is further confirmed by paper electrophoresis. At pH 1.8 or 3.5, the species co-migrates with the chemically synthesized phosphotyrosine marker.

The experiments described above employed KOH treatment in the formation of the covalent link (32). We have also carried out parallel experiments in which the alkali treatment was substituted by sodium dodecyl sulfate treatment (32, 34). The covalent link so formed has been again identified as a phosphotyrosine bond. It should also be noted that the DNA was labeled with [α-^{32}P]dATP. The selection of a purine rather than a pyrimidine nucleoside triphosphate in the labeling is to ensure the cleavage of the phosphodiester bond

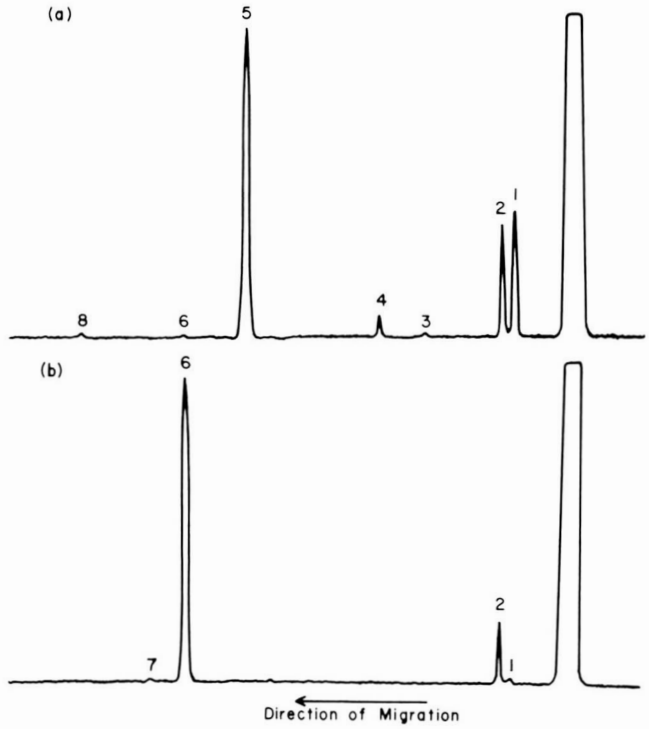

FIG. 3. **The patterns of cleavage of an end-labeled single strand DNA fragment upon incubation with *M. luteus* DNA topoisomerase I (scan *a*) and *E. coli* DNA topoisomerase I (scan *b*) and subsequent treatment with alkali.** A single-stranded DNA fragment ^{32}P-labeled at its 5′-end was kindly provided to us by Lawrence Peck. Each reaction mixture, which contained the labeled DNA and excess (1 μg) unlabeled sonicated calf thymus DNA as a carrier, was made 50 mM in KOH and incubated for 5 min at 37°C to denature the carrier DNA. Each mixture was neutralized and adjusted to a total volume of 200 μl containing 12.5 mM Tris, pH 7.6, 12.5 mM KCl, 75 μg/ml of bovine plasma albumin, and 1.8 μg of *E. coli* or *M. luteus* DNA topoisomerase I by the addition of 25 μl of 0.1 M Tris·HCl, pH 2.8, other stock solutions, and water. Following incubation of the reaction mixtures at 37°C for 45 min, 10 μl of 1 M KOH was added to each, and incubation was continued for 1 min. Neutralization of the mixtures and ethanol precipitation of the DNA were then performed, and the precipitate of each mixture was redissolved in 10 μl of a gel electrophoresis loading buffer containing 95% formamide and tracking dyes. The same labeled DNA was also subjected to DNA-sequencing reactions as described by Maxam and Gilbert, and all samples were loaded in parallel lanes of a sequencing gel. Electrophoresis and autoradiography of the gel were done as described (51), and the autoradiogram was scanned with a Helena densitometer. The rightmost band in each tracing, which ran off scale, corresponds to the uncleaved DNA. The horizontal scale of the tracings is compressed to depict the complete patterns. The nucleotide sequence read off the autoradiogram is shown in *C*; the sites of cleavage yielding the enumerated bands shown in the tracings are marked by *arrows* enumerated accordingly.

proximal to the 5′-carbon during acid hydrolysis of the protein·DNA covalent complex (55).

It has been shown in a number of cases that a phosphotyrosine linkage can be cleaved by snake venom phosphodiesterase (44, 45, 56, 57). We have, therefore, carried out similar experiments with the covalent protein·DNA linkage. When the gel-excluded material shown in Fig. 1 is treated with the phosphodiesterase, radioactive orthophosphate and dAMP are released. The result of this experiment lends further support to the idea that the covalent linkage between DNA and *E. coli* or *M. luteus* DNA topoisomerase I is a phosphodiester bond. If the staphylococcal nuclease digestion step employed in the preparation of the labeled topoisomerase I is replaced by digestion with *Pseudomonas* Bal 31 nuclease (58, 59), subsequent treatment with snake venom phosphodiesterase gives mainly dAMP. This difference can be attributed to the difference in specificity of the nucleases; the staphylococcal enzyme cleaves DNA phosphodiester bonds proximal to the 5′-carbon and the Bal 31 enzyme cleaves DNA phosphodiester bonds proximal to the 3′-carbon (58–60).

The DNA Sequences at the Sites of Cleavage of E. coli and M. luteus DNA Topoisomerase I—We have previously noted that the cleavage of DNA and the simultaneous formation of covalent protein·DNA bond by *E. coli* DNA topoisomerase I is not stringently dependent on DNA nucleotide sequence. Negatively supercoiled and single-stranded DNAs from natural sources, as well as poly(dT), poly(dC), and a random copolymer poly(dC,dT) are all capable of forming the covalent complex with the enzyme (32). DNA cleavage and covalent linking are not random with respect to sequence, however, as illustrated by the experiment below.

A 119-base pairs long restriction fragment was labeled with ^{32}P at the 5′-ends, and the complementary strands were separated by polyacrylamide gel electrophoresis after denaturation. Aliquots containing one of the strands recovered from the gel were incubated separately with *E. coli* and *M. luteus* DNA topoisomerase I, and NaOH was added to each to effect the cleavage of the DNA chain by the topoisomerase (32). We have shown previously that DNA chain cleavage by either the *E. coli* or the *M. luteus* enzyme generates a 3′-hydroxyl group on one side of the scission, and the protein is linked to the other side (32, 34). Since the original DNA strand is labeled at the 5′-end, a cleavage event gives a shortened, 5′-labeled fragment ending with a 3′-hydroxyl group, and the length of this fragment immediately gives the position of cleavage. If the experiment is carried out with a topoisomerase/DNA ratio such that there is less than one scission per DNA, the size distribution of the labeled bands and their relative intensities, which can be determined by gel electrophoresis and autoradiography, give directly the distribution of the cleavage sites along the DNA and their relative susceptibilities to cleavage by the topoisomerase. When aliquots of the original 5′-end labeled fragment treated according to the Maxam-Gilbert sequencing reactions are run as length markers, the cleavage sites can be precisely located at the sequence level (61). The patterns of cleavage of this fragment by *E. coli* and *M. luteus* DNA topoisomerase I are depicted in Fig. 3, *a* and *b*, respectively, and the nucleotide sequence of the fragment and the positions of the cleavage sites are shown in Fig. 3*C*. A summary of the results obtained with other single-stranded restriction fragments is tabulated in Table I.

Several features of the cleavage of the single-stranded DNA by the topoisomerases can be seen from these results. Firstly, there is no specificity in terms of the nucleotide on either the 3′- or the 5′-side of the site of cleavage. Secondly, it is apparent that the cleavage sites are not randomly distributed. We do not know, however, whether this nonrandomness in distribution is due to the sequence specificity of the enzymes *per se*, or is due to the secondary or higher structural features of the single-stranded DNA. Furthermore, although the intensity patterns, which reflect the relative susceptibilities of the sites to cleavage as discussed in the paragraph above, are different for the *E. coli* and the *M. luteus* enzymes, there is considerable coincidence of cleavage sites for the two enzymes. Finally, it might be significant that the fourth nucleotide on the 3′-side of the cleavage site is a C in 90% of the sequences examined.

Cleavage of DNA by Gyrase and the Covalent Linking of

TABLE I

Relative cleavage frequencies at different sites by E. coli and M. luteus DNA topoisomerase I

No entry signifies a relative cleavage frequency of less than 1. The relative frequencies of cleavage by a given enzyme are comparable only within each of the three sets of sites measured with three different restriction fragments. The sequences of cleavage sites were determined as described in the legend to Fig. 3. In all cases the results agree with the published pBR322 sequence of Sutcliffe (62).

Region of pBR322 examined[a]	Position of cleavage[b]	Relative frequency of cleavage[c]	
		E. coli enzyme	*M. luteus* enzyme
Bottom strand, 4170–4340	4174	2	30
	4177		3
	4195	19	
	4207	33	5
	4218		3
	4245		25
	4246	3	45
	4247		37
	4248		17
	4253	55	41
	4255		26
	4267		20
	4272	65	18
	4274	67	35
	4281	47	15
	4286	47	24
	4290	29	23
	4291	44	15
	4327	7	19
	4328	100	100
	4329	13	28
	4330	11	3
Top strand, 0–60	20	11	
	25	5	
	28	7	
	35	15	
	38	29	33
	43	100	100
	54	18	
	56	25	
Bottom strand, 290–370	297	8	
	305	11	
	310	7	
	319	13	
	326	71	
	327	36	100
	329	27	
	338	100	
	339	15	
	342	26	
	353	4	10

[a] The designation bottom strand, 4170–4340, for example, refers to the region from nucleotide 4170 to nucleotide 4340 on the bottom strand of the pBR322 DNA sequence of Sutcliffe (62).

[b] The position of cleavage refers to the nucleotide (numbered according to Sutcliffe (62)) on the 3′-side of the cleavage site.

[c] In all cases the average number of cleavages per labeled fragment is lower than 1, so that the relative frequencies of cleavage are proportional to the intensities of the bands on the autoradiogram, as described under "Results" and in the legend to Fig. 3.

the gyr A Subunit to DNA—It has been reported that incubation of double-stranded DNA and *E. coli* DNA gyrase in the presence of the inhibitor oxolinic acid, followed by the addition of dodecyl sulfate, results in the cleavage of the DNA duplex and the linking of the enzyme to the 5′-ends generated (35, 36, 63). With *M. luteus* DNA gyrase, the oxolinic acid-dodecyl sulfate procedure leads to little cleavage of DNA. Incubation of duplex DNA and gyrase followed by the addition of alkali results in some DNA cleavage and covalent protein·DNA complex formation, though the yield is much lower than cleavage with *M. luteus* DNA topoisomerase I at the same molar ratio of protein to DNA.

The following experiment shows that *M. luteus* gyrase subunit A, and not subunit B, becomes covalently linked to DNA in the cleavage reaction. ^{32}P-Labeled double-stranded DNA was incubated with *M. luteus* DNA gyrase, and alkali was then added. Upon neutralization and digestion with staphylococcal nuclease, the mixture was treated with dodecyl sulfate and analyzed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel. Autoradiography of the Coomassie blue-stained gel shows that radioactivity is present only in the A subunit band.

DNA·Gyrase Subunit A Covalent Linkage is Also a Phosphotyrosine Bond—Experiments identical with those with the covalent complexes between DNA and *E. coli* and *M. luteus* DNA topoisomerase I were carried out with the covalent complex between DNA and *M. luteus* gyrase subunit A. The covalent linkage is again identified as a phosphotyrosine bond.

DISCUSSION

As we have summarized in the introduction, in cases where the protein components of protein·DNA covalent complexes are known to have catalytic functions, they are found to catalyze the breaking and rejoining of DNA backbone bonds. It appears plausible that most of the proteins in all known protein·polynucleotide covalent complexes serve the role of breaking and rejoining internucleotide bonds.

Among enzymes that are known to form covalent DNA·protein complexes, the modes of breaking and rejoining DNA backbone bonds vary considerably. Enzymes of the *E. coli* DNA topoisomerase I type appear to catalyze the breaking and rejoining of DNA backbone bonds in a concerted fashion. For these enzymes, intermediates in which the protein is linked covalently to the DNA are usually not detectable without the addition of a protein denaturant. Attempts to detect the generation of an exposed 3′-hydroxyl group by *E. coli* DNA topoisomerase I under normal reaction conditions, by the use of *E. coli* exonuclease III or *E. coli* DNA polymerase I in the presence and absence of deoxynucleoside triphosphates, have been unsuccessful.[2] Presumably, under normal conditions the covalent linking of such proteins to DNA occurs only transiently. In other cases, species in which the protein in its native form is covalently attached to DNA appear to have much longer lifetimes. It is well established that the gene A enzyme of phage ϕX174 can cleave negatively supercoiled ϕX DNA and link covalently to the 5′-phosphoryl end generated (37–40). The DNA becomes relaxed and the existence of a 3′-hydroxyl group has been demonstrated by its accessibility to *E. coli* exonuclease III (39).

The chemical nature of the protein·DNA linkage also appears to vary considerably. The gene II enzyme of phage fd, which in many ways resembles the gene A enzyme of ϕX174, converts negatively supercoiled fd DNA to either a covalently closed but relaxed form, or to a nicked form with a 3′-hydroxyl and a 5′-phosphoryl group straddling the single chain scission (41). The nicked form can be converted to the covalently closed form by DNA ligase in the presence of an appropriate ligase cofactor, but not by the gene II enzyme (41). Thus it appears that the gene II enzyme can break and rejoin a DNA phosphodiester bond, presumably via a readily hydrolyzable protein·DNA covalent link. A DNA topoisomerase from rat liver has been reported to form a covalent complex with DNA in which the protein is linked to a 3′-phosphoryl group (33). The same conclusion is reached for a similar enzyme from calf thymus.[3]

The role of tyrosine in protein-nucleic acid covalent bond formation has already been discussed in a number of cases. Adenylation of *E. coli* glutamine synthetase involves the formation of a phosphotyrosine bond (56). The regulatory protein PII of the same enzyme also reacts with UTP to give 5′-UMP covalently bound to the protein by a phosphotyrosine linkage (57). The covalent linkage in a poliovirus RNA·protein complex has also been shown to be a phosphotyrosine bond (44, 45). The present finding that under certain conditions a phosphotyrosine bond is formed between DNA and a number of DNA topoisomerases strengthens further the notion that certain tyrosine residues might play crucial roles in enzymes that form transient or stable covalent bonds to nucleic acids. In view of the apparent mechanistic diversity of such enzymes as discussed in the paragraphs above, however, it is most likely that other types of protein-DNA covalent bonds are also of common occurrence. Our preliminary studies on the gene A enzyme of ϕX174 indicate that it does not link to DNA via a phosphotyrosine bond,[4] but we have yet to identify the linkage.

It should be pointed out that we have not established rigorously that the covalent complexes we reported in this communication truly represent the covalent intermediates during DNA topoisomerization. Attempts to reform DNA phosphodiester bonds from the covalent protein·DNA complexes have been unsuccessful. Nor can we rule out the possibility that bond migration had occurred during the isolation of the phosphorylated amino acid, and, therefore, phosphotyrosine might represent a consequence of the chemical or enzymatic manipulations, or both. We note, however, that with *E. coli* and *M. luteus* DNA topoisomerase I, different treatments, using alkali, dodecyl sulfate, or pronase for the formation of the covalent bond all give phosphotyrosine after acid hydrolysis.

Acknowledgment—Some of the preliminary experiments on the covalent linkage between DNA and *M. luteus* DNA topoisomerase I were carried out by Viola T. Kung.

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