Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4594–4598, August 1982
Biochemistry

Site-directed psoralen crosslinking of DNA

(mercurated pyrimidines/restriction nucleases/DNA damage/photochemistry)

WILMA A. SAFFRAN, MERRILL GOLDENBERG, AND CHARLES R. CANTOR

Department of Human Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Communicated by Gilbert Stork, May 3, 1982

ABSTRACT **A technique has been developed for placing site-specific crosslinks into DNA by using a psoralen derivative containing a sulfhydryl group. Plasmid pBR322 was nicked at the unique *Bam*HI restriction site, and mercurated pyrimidines were introduced by nick-translation. Then the psoralen was specifically directed to this site in the dark through a Hg-S linkage prior to irradiation to form a crosslink. The location of interstrand crosslinks was examined by electron microscopy of *Pst* I-cut molecules and found to be at or near the *Bam*HI site.**

Interstrand crosslinks in nucleic acids are readily formed by reaction with psoralen or its derivatives (1, 2). Psoralens bind to double-stranded DNA or RNA noncovalently in the dark, by intercalation. Irradiation of this complex with 365-nm light results in the formation of a cycloadduct between a pyrimidine residue on one strand and the psoralen. This may then react photochemically with a second pyrimidine on the opposite strand to form a crosslink. Although the relative rates of reaction with various pyrimidines differ, there is little indication of any more general sequence specificity.

In addition to its medical application in the treatment of psoriasis, the psoralen photoreaction has been used in studies of nucleic acid secondary structure, synthesis, mutagenesis, and DNA repair and recombination (3, 4). Although some of these investigations have taken advantage of the double-strand specificity of the crosslinking photoreaction to map secondary structure in nucleic acids or to probe the organization of chromatin, the molecules under study reacted randomly with psoralen, and a heterogeneous mixture of products was produced.

We describe here a method of directing psoralen crosslinking to a specific location in a DNA molecule by using a derivative of psoralen containing a thiol group. We have prepared plasmid pBR322 DNA molecules with interstrand crosslinks placed near the unique *Bam*HI restriction site by enzymatically incorporating mercurated nucleotides at this site and then directing psoralen to the modified bases through a Hg-S linkage.

MATERIALS AND METHODS

Synthesis of Site-Specific Psoralen. The sequence is shown in Fig. 1.

1,2-Bis(2-methylaminoethoxy)ethane (I). Twenty grams of 1,2-bis(2-chloroethoxy)ethane (Eastman) and 165 g of methylamine (40% in water) (Matheson, Coleman, and Bell) were placed in a pressure bottle and heated at 85°C for 3 days. The solution was made basic with sodium hydroxide and the product was extracted into chloroform, and dried over anhydrous sodium sulfate. After rotoevaporation of the solvent, the product (76% yield) was purified by vacuum distillation: bp at 12 mm Hg 112–114°C; NMR(C^2HCl_3, Varian T60) δ 3.6 (8H, s, superimposed on t, J = 5 Hz, $-CH_2-O-$), 2.75 (4H, t, J = 8 Hz, $-CH_2-N$), 2.4 (6H, s, CH_3-N-), 2.5-2.2 (2H, s, H-N); mass spectrum (chemical ionization in methane) 177 (M + 1), 205 (M + 29), 217 (M + 41).

Chloromethyltrimethylpsoralen (II). This compound was made (with minor modifications) according to the literature (5): yield (not including mother liquor) 32%; mp 212–215°C (lit. 215–217°C); NMR (Varian T60) δ 7.5 (1H, s, phenyl), 6.17 (1H, s, lactone), 4.7 (2H, s, $Cl-CH_2-Ar$), 2.5 (9H, m, methyls).

Methylaminodiethoxyethanemethylaminomethyltrimethylpsoralen (III). A mixture of 176 mg of II and 1.69 g of I was heated with stirring in 20 ml of dry toluene overnight. The reaction was monitored by TLC on silica gel with benzene/methanol, or 1:1 (vol/vol) 95% ethanol/concentrated aqueous ammonia, 4:1, as eluants. The product (140 mg) was eluted from a silica gel (Baker, 60–200 mesh) flash column with 95% ethanol/concentrated aqueous ammonia, 4:1, as a pale yellow oil: NMR (80 MHz) δ 7.68 (1H, s, phenyl), 6.2 (1H, s, lactone), 3.9-3.4 (10H, m, $-CH_2-O-$, $-N-CH_2-Ar$), 3-2.2 (19H, m, $-CH_3$ and $-CH_2-N-$); mass spectrum (chemical ionization in methane) 417 (M + 1), 445 (M + 29), 457 (M + 41).

2-Pyridyldithioethylmethylamidodiethoxyethanemethylaminomethyltrimethylpsoralen (IV) [site-specific psoralen (SSP)]. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (6) (SPDP; 29 mg; Pharmacia) was dissolved in 200 μl of dry methylene chloride. Then 35 mg of III dissolved in 100 μl of dry methylene chloride was added and the reaction mixture was stirred in the dark at room temperature. The disappearance of III and SPDP and the appearance of the product and *N*-hydroxysuccinimide were monitored by TLC (silica gel with methanol eluant). The reaction was complete within 15 min. The product was eluted with benzene/methanol, 1:1, on a reversed-phase (silica gel 60, Merck) flash column. The fractions were analyzed on a linear KC18 plate. (On this TLC system, the R_Fs of the product, SPDP, *N*-hydroxysuccinimide, and III, were 0.24, 0.7, 0.46, and 0.04, respectively.) On all TLC plates the product spot absorbs 260 nm light, fluoresces pale blue on irradiation with 370 nm light and is stained by iodine. Yield was 41 mg. The product was further characterized by UV spectroscopy. With a molar extinction coefficient of 6,884 (at 280 nm) for psoralen (5), 5,100 (at 280 nm) for 2-pyridyl disulfide, and (after reduction of the disulfide with dithiothreitol) 8,080 (at 345 nm) for 2-thiopyridone (7), it was determined that the molar ratio of 2-pyridyldisulfide to psoralen was 1:1: mass spectrum (chemical ionization in methane) 614 (M + 1), 628 (M + 15), 642 (M + 29).

Preparation of SSP Crosslinks in Plasmid DNA. *Nick-translation with mercurated nucleotides.* Plasmid pBR322 was isolated by the method of Bolivar and Boekman (8). *Bam*HI was prepared by the method of Wilson and Young (9). Plasmid was nicked with *Bam*HI in the presence of ethidium bromide under the following conditions: 100 μg of DNA per ml, 150 mM NaCl, 6 mM Tris·HCl (pH 8.0), 6 mM $MgCl_2$, 0.5 mg of bovine serum

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Abbreviations: SSP, site-specific psoralen; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate.

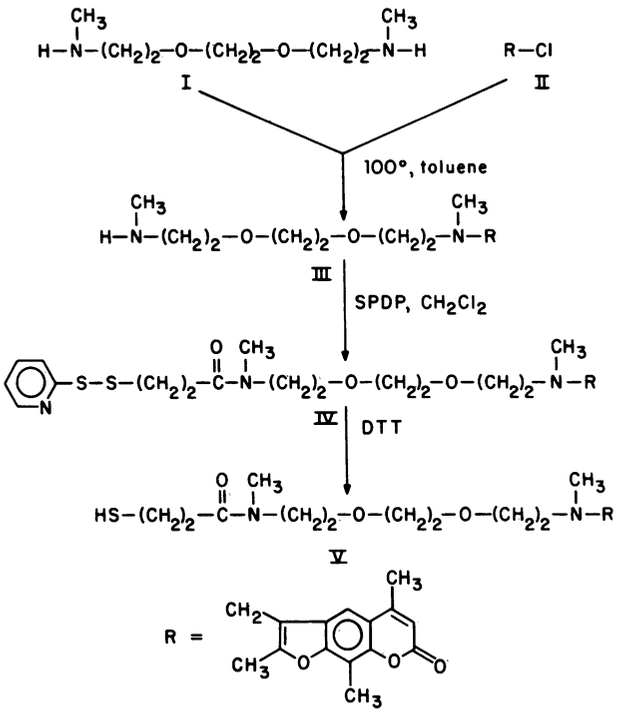

FIG. 1. Synthesis of site-specific psoralen (SSP).

albumin per ml, 25 μg of ethidium bromide per ml, and 100 units of *Bam*HI per ml. After incubation for 1 hr at 37°C, the reaction mixture was extracted with 1 vol of phenol and then ethanol precipitated. Up to this step, all procedures were carried out under red light to minimize random nicking by ethidium bromide. The pellet was washed once with 80% ethanol and resuspended in 10 mM Tris (pH 8.0) at a concentration of 1 mg/ml.

Nick-translation was carried out in 50 mM Tris·HCl, pH 7.6/6.7 mM $MgCl_2$/6 mM mercaptoethanol/10 μM dATP/100 μM dGTP with 100 μg of nicked plasmid per ml. In addition, the samples without mercury contained 100 μM dCTP and 100 μM dTTP; the samples with mercury contained 100 μM HgdCTP and 100 μM HgdUTP (Boehringer Mannheim). Samples were allowed to react for 5–10 min at 16°C with *Escherichia coli* DNA polymerase I (50 units/ml; New England BioLabs) and then were extracted with 1 vol of phenol. They were dialyzed twice against 10 mM Tris·HCl, pH 8.0/10 mM EDTA/100 mM NaCl and once against 10 mM Tris·HCl, pH 8.0.

^{32}P-Labeled DNA was nick-translated by the same procedure except that the reaction mixture included 10–50 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{32}P]dATP (2,000 Ci/mmol). Aliquots were assayed after dialysis, to determine [^{32}P]dATP incorporation. The incorporation was 8–12 mol of [^{32}P]dATP per mol of pBR322 for samples without mercury and 6–8 for samples containing mercury.

Crosslinking. Nick-translated DNA, at 100 μg/ml, was incubated overnight at room temperature with 0.25 mM SSP in the presence of 0.1 mM mercaptoethanol to reduce the disulfide, forming reduced SSP (V). The DNA was extracted with 1 vol of phenol to remove nonspecifically bound SSP and then was ethanol precipitated; the pellet was washed once with 80% ethanol. The sample was resuspended in 10 mM Tris, pH 8.0/100 mM NaCl and degassed for 5 min with N_2 gas. All steps up to this point were carried out under red light and samples were protected from light to prevent random crosslinking by nonspecifically bound SSP. Crosslinks were produced by irradiating the samples with the apparatus described by Isaacs *et al.* (5). Irradiation time was generally 10 min.

Crosslinks were detected by electrophoresis of native and denatured DNA on 1.2% agarose in Tris·acetate/EDTA pH 7.6 buffer (10). Equal aliquots of sample, one native and one denatured in 0.2 M NaOH, were run in adjacent lanes. Denatured, noncrosslinked DNA ran as single strands, with higher mobility than the double-stranded species; the rapidly renaturing crosslinked molecules had the same mobility as the native DNA. The amount of crosslinked DNA in ^{32}P-labeled samples was measured by cutting out the ethidium bromide-stained gel bands, dissolving them in 1 ml of formamide for 1 hr at 50°C, and then assaying them in 10 ml of scintillation fluid. DNA was denatured and spread by the method of Cech and Pardue (11).

RESULTS

Synthesis and Properties of SSP. This psoralen derivative was constructed to optimize the chances of site-specific reaction near mercurated bases in DNA. It has the following properties: (*i*) a disulfide group that is readily reduced to a thiol [this moiety binds specifically and tightly ($K_d = 10^{-16}$ M) to mercury in the dark (12)]; (*ii*) is soluble in water; (*iii*) is positively charged, which increases the affinity for DNA; and (*iv*) has a long and flexible linker between the furocoumarin and the thiol, which enables the psoralen moiety to intercalate into DNA while attached to a mercurated base through the sulfur at its other end. This linker is 20 Å long, allowing intercalation three to four bases from the site of attachment. Cystaminylmethyltrimethylpsoralen, another thiol-containing psoralen derivative with a shorter (5 Å) linker (13), failed to produce any specific interstrand crosslinks.

Preparation of pBR322 Crosslinked near the *Bam*HI Site. To prepare pBR322 DNA containing mercury near or at a specific site, intact plasmid was first nicked with *Bam*HI in the presence of ethidium bromide (Fig. 2*A*). Ethidium bromide inhibits restriction endonucleases, and this treatment results in the production of single-strand cuts rather than the normal staggered double-strand cuts (14). At the optimal ethidium bromide concentration, about half of the supercoiled substrate was nicked (converting it to the relaxed circle) and half was cut,

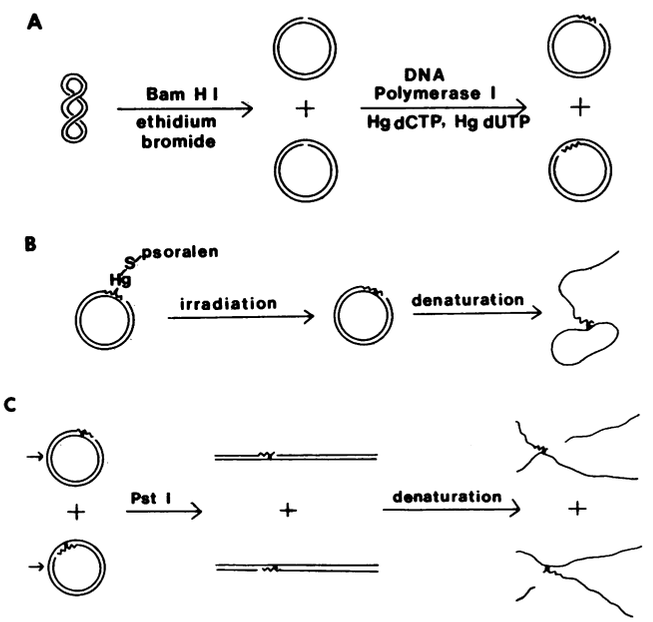

FIG. 2. Preparation of SSP crosslinks. (*A*) Mercuration at the *Bam*HI site. (*B*) Crosslinking with SSP. (*C*) Cutting with *Pst* I.

yielding linear DNA. The two strands of the plasmid are nicked with approximately equal frequency.

Next, a short stretch of mercurated nucleotides was inserted near the *Bam*HI site by nick-translation with *E. coli* DNA polymerase I (Fig. 2*A*). The mercury-substituted pyrimidine nucleotides 5-mercurideoxycytidine triphosphate (HgdCTP) and 5-mercurideoxyuridine triphosphate (HgdUTP) are good substrates for DNA polymerase I and were incorporated nearly as well as the unmodified nucleotides (15). The modified nicked circular DNA molecules produced had a short patch of mercurated bases starting at the site of the original nick and extending 20–40 bases in the 3′ direction along that strand. Any single molecule had mercury on only one strand, but the population of nicked circles had mercury on both strands, lying on both sides of the *Bam*HI site. Because the double-strand cuts were staggered, the linear products of *Bam*HI cutting had cohesive ends with 5′ overhangs which served as templates for nucleotide addition by DNA polymerase I. The 3′ ends were filled in, producing blunt-ended linear molecules.

The modified DNA was then incubated with SSP. The psoralen moiety bound readily to double-stranded DNA by intercalation. In addition SSP bound tightly and specifically to the mercurated bases through a Hg-S bond. The noncovalently bound psoralen was completely removed by phenol extraction. The plasmid was then irradiated with near-UV light to produce crosslinks at the site of modification (Fig. 2*B*).

Measurement of Crosslinking. Psoralen crosslinking was detected by denaturing the DNA with alkali and analyzing it on a nondenaturing agarose gel. Molecules with crosslinks rapidly renatured to double strands, whereas noncrosslinked molecules remained single-stranded after denaturation and ran with higher mobility on the gel. Interstrand crosslinking was specific to mercurated plasmid. All the DNA nick-translated with unmodified nucleotides remained single-stranded after alkali denaturation (Fig. 3). In contrast, a portion of the sample nick-translated with mercurated nucleotides snapped back to a double-strand form. Crosslinks were seen in the nicked circular mercurated DNA, but the linear DNA, which contained little mercury, was almost completely uncrosslinked. The double-stranded DNA seen after alkali denaturation was due to psoralen crosslinking rather than to interstrand Hg-S bonds between mercurated nucleotides and monoadducted SSP, because incubation with 50 mM dithiothreitol failed to reduce the intensity of this band (data not shown).

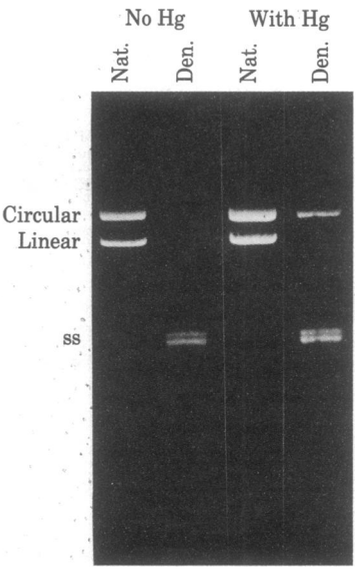

FIG. 3. Electrophoretic analysis of crosslinking. pBR322 nick-translated with dCTP and dTTP (no Hg) or HgdCTP and HgdUTP (with Hg) was treated with SSP plus light. Half of each sample was run without further treatment [native (Nat.)] and the other was alkali denatured before electrophoresis [denatured (Den.)]. Crosslinked molecules are those running with the same mobility with and without alkali treatment. ss, Single-stranded.

The extent of crosslinking was quantitated by incorporating ^{32}P-labeled nucleotides along with the mercurated nucleotides during nick-translation and measuring the radioactivity in the gel bands. As expected, the nicked circles had a much higher specific activity than the linear molecules. The fraction of crosslinked molecules was calculated as the ratio of counts in the double-stranded nicked circular band with and without alkali denaturation. SSP, irradiation, and mercuration all were required for crosslinking. In the absence of any one of these factors there was no crosslinking (Table 1); with all three, >40% of the mercurated DNA became crosslinked. SSP plus light alone efficiently crosslinked either mercurated or unmercurated DNA if the step of extracting nonspecifically bound SSP was omitted. In this case, all three forms of DNA were crosslinked, rather than the nicked circles alone.

Crosslink Location. Crosslinked plasmid molecules were examined by electron microscopy. Denaturation of nicked circular DNA with one or more crosslinks located near the nick should result in molecules shaped as diagrammed in Fig. 2*B*. This species was seen only in samples that contained crosslinks, as detected on agarose gels. Electron micrographs of such molecules are presented in Fig. 4*A*. The contour lengths of the linear and circular portions were found to correspond to the full length of unmodified denatured pBR322.

The crosslinks were located close to the site of the nick; the two parts of the figure were joined at the very end of the linear part. Extending the patch of modified bases by increasing the duration of the nick-translation reaction resulted in the appearance of molecules with crosslinks some distance in from the end of the linear portion.

The site of crosslinking was confirmed by cutting the SSP-treated DNA at a second unique restriction site with *Pst* I. The linearized molecules should then appear Y-shaped after denaturation (Fig. 2*C*), and they did in electron micrographs (Fig. 4*B*). These molecules had either two short or two long arms, depending on which strand was initially nicked by *Bam*HI. We saw equal numbers of these two species (within statistical error), indicating that *Bam*HI nicking occurs with equal frequency on the two strands.

The *Bam*HI and *Pst* I sites are 1,125 bases apart on the 4,362-base-pair plasmid (16), or 0.258 times the unit length of the molecule. The contour lengths of the arms of the Y-shaped molecules were measured and the site of crosslinking was expressed as the ratio of the length of the short arm to the total length of the molecule. The mean (± SD) distance of the in-

Table 1. Effect of treatment steps on crosslinking

Treatments			Crosslinks, %*
Hg	Light	SSP	
−	−	−	3.3
−	−	+	2.3
−	+	+	2.4
+	−	+	2.2
+	+	+	43.8

* Double-stranded relaxed circular bands were cut from agarose gels and assayed; % crosslinking = 100 × [^{32}P cpm (denatured)/^{32}P cpm (native)].

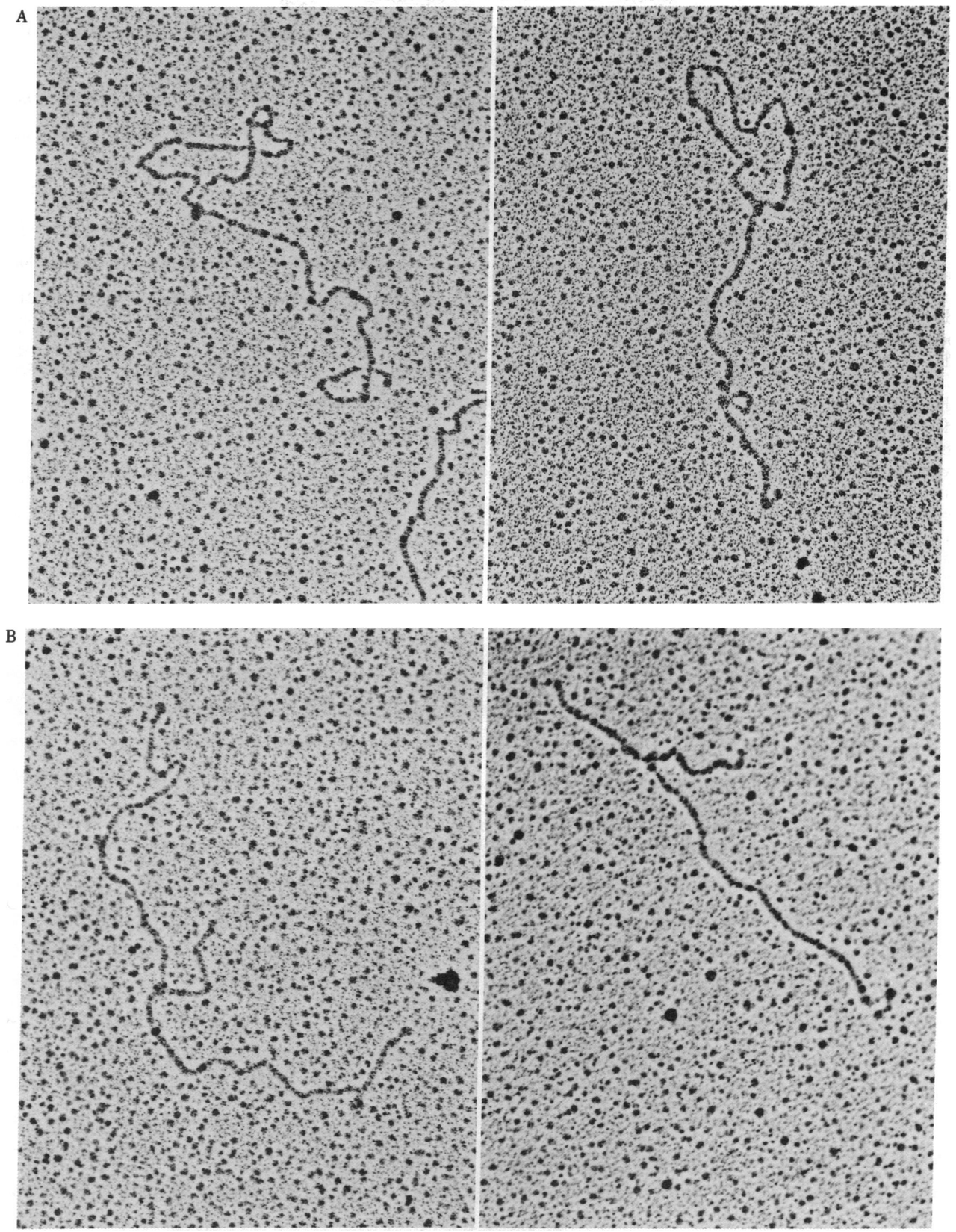

FIG. 4. Electron micrographs of denatured crosslinked pBR322. (*A*) Uncut. (*B*) Cut with *Pst* I. (Bar = 0.5 μm.)

tersection from the *Pst* I site was found to be 0.25 ± 0.04 (n = 47), in good agreement with the predicted value. We conclude that, within the resolution of the electron microscope, the crosslinks are located near the *Bam*HI site.

DISCUSSION

We have placed SSP crosslinks at a chosen location in the plasmid pBR322 in a two-step reaction. The nick-translation pro-

cedure used directs SSP to a small segment of the DNA molecule rather than to a specific base. The limited resolution of electron microscopic analysis indicates that the crosslinks are located within 150 bases of the *Bam*HI site, although the actual locations of the crosslinks may be considerably closer to the restriction endonuclease site. Preliminary results indicate that 20–25 nucleotides are introduced by nick-translation on each side of the restriction site. The length of the mercurated patch can be controlled to some extent by the conditions of the nick-translation reaction, although control is limited by the processivity of DNA polymerase I. SSP crosslinking should prove to be useful in a number of areas, including DNA structure, repair, recombination, and replication. SSP may be introduced at the site of any specific nick. The crosslinking reaction also can be used to identify any sites containing mercurated bases inserted by other techniques.

The reactions of this psoralen derivative are extremely flexible. SSP is a trifunctional reagent, able to react with DNA at its thiol group as well as at the 3,4 and 4′,5′ double bonds of the furocoumarin. The mercury-specific binding can be easily reversed by adding an excess of reducing agent, whereas the cycloaddition to pyrimidines can be photoreversed by short wavelength UV light (17). The timing and extent of the photoreactions can be finely controlled; irradiation at 390 nm will result in monoaddition, and crosslinks can be formed by subsequent irradiation at 360 nm (18). Other thiol-containing reagents, such as spectroscopic probes, could be attached to DNA at known sites by the same scheme as the one outlined here.

This work was supported by National Institutes of Health Grant GM14825 and by a National Institutes of Health Postdoctoral Fellowship to W.A.S.

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