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Inhibition of the *Bam*HI Cleavage and Unwinding of pBR322 Deoxyribonucleic Acid by the Antitumor Drug *cis*-Dichlorodiammineplatinum(II)†

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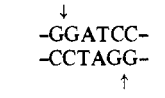
ABSTRACT: The antitumor drug *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) binds to pBR322 DNA and inhibits the cleavage of this circular DNA into a linear form by the restriction endonuclease *Bam*HI. The binding of platinum to DNA was monitored by agarose gel electrophoresis, and the amount of platinum bound per nucleotide (r_b) was measured by carbon rod atomic absorption spectroscopy. Electrophoretic mobility changes reflect a shortening and unwinding of the DNA duplex upon platinum binding as observed previously for the reaction of *cis*- and *trans*-DDP with pSM1 DNA [Cohen, G. L., Bauer, W. R., Barton, J. K., & Lippard, S. J. (1979) *Science (Washington, D.C.) 203*, 1014-1016]. The inhibition of *Bam*HI nuclease activity occurs at very low binding levels and is complete at r_b = 0.045. This value corresponds to the binding of one platinum atom within ±3 base pairs of the recognition sequence of the enzyme shown below. Treatment of the DNA with 0.2 M sodium cyanide after *Bam*HI cutting removes the platinum but does not alter the point at which *cis*-DDP inhibits the formation of the linear form III DNA. This result is in contrast with a previous report claiming that *Bam*HI could cut across a *cis*-DDP-induced GpG cross-link in DNA which could be subsequently revealed by cyanide reversal of platinum binding. When the platinum is removed by cyanide treatment, the drug-induced mobility changes are reversed and there is a pronounced sharpening of the bands in the gel. Quantitative study of the cyanide reversal shows the presence of a small amount of unremovable platinum tightly bound to the DNA at high ratios (~0.1) of bound platinum per nucleotide.



Although the site of cytotoxic action of the antitumor drug *cis*-DDP[1] (Rosenberg et al., 1969) is believed to be DNA (Roberts & Thomson, 1979), the exact nature of the platinum-induced lesion remains unknown. The antineoplastic activity of *cis*-DDP compared with the inactivity of the trans isomer suggests bifunctional coordination of the platinum drug to DNA utilizing its cis geometry: for example, intrastrand cross-linking of two adjacent guanine or cytosine bases (Roberts & Thomson, 1979; Kelman et al., 1977). DNA interstrand cross-links occur but at platinum binding levels greater than those required for cytotoxic action (Shooter et al., 1972; Munchausen, 1974).

We previously demonstrated (Cohen et al., 1979) that *cis*-DDP unwinds and shortens closed circular, supercoiled

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[1] Abbreviations used: DDP, dichlorodiammineplatinum(II); form I DNA, covalently closed circular duplex DNA; form II DNA, nicked circular duplex DNA; form III DNA, linear duplex DNA; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TBE, 90 mM Tris base, 90 mM boric acid, and 2.2 mM EDTA, pH 8.3; BSA, bovine serum albumin; P_0, concentration of nucleotide phosphate determined at 260 nm by using 6600 M^{-1} cm^{-1} as the extinction coefficient; C_0, the initial concentration of platinum species in the reaction; C_b, the concentration of platinum bound to nucleic acid; r_f, ratio of C_0 to P_0; r_b, ratio of C_b to P_0; AAS, atomic absorption spectroscopy; DTT, dithiothreitol; EtdBr, ethidium bromide; σ_0, the superhelix density.

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pSM1 DNA. More recently (Cohen et al., 1980), we used a restriction enzyme to show that *cis*-DDP binds selectively and rapidly to a $(dG)_4 \cdot (dC)_4$ sequence in pSM1 DNA. An assay with exonuclease III also has revealed a selective binding mode of *cis*-DDP to $(dG)_n \cdot (dC)_n$ sequences in a restriction fragment of pBR322 (T. D. Tullius and S. J. Lippard, unpublished experiments). In this paper we present the results of our studies on the binding of *cis*-DDP to plasmid pBR322 (Bolivar et al., 1977) DNA. To probe the binding of the platinum drug, we used the restriction enzyme *Bam*HI which makes a double-stranded cut at a single, unique

$$\begin{array}{l} \downarrow \\ -GGATCC- \\ -CCTAGG- \\ \uparrow \end{array}$$

sequence in pBR322. The enzyme converts plasmid forms I and II to the linear form III DNA. These three forms of the plasmid are readily distinguishable by agarose gel electrophoresis.

Materials and Methods

pBR322 DNA, cis-DDP, and Enzymes. Plasmid pBR322 DNA was isolated from *Escherichia coli* strain MC1009 containing the plasmid by using a modification of a published procedure (Bolivar & Backman, 1979). Specifically, the Bio-Gel A50 column step was eliminated and a second RNase treatment was performed after clearing the lysate. After isolation of form I plasmid DNA from the lower band of a CsCl–propidium diiodide gradient, the DNA was placed onto a Sephadex G-50 column and eluted with 50 mM Tris-HCl, 0.5 M NaCl, and 1 mM EDTA, pH 7.8, buffer in order to remove residual RNA contamination.

cis-Dichlorodiammineplatinum(II) was prepared by a literature method (Dhara, 1970) and purified by recrystallization from 0.1 N HCl.

The restriction enzymes *Bam*HI and *Hinc*II and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories and used as directed. [γ-^{32}P]ATP was obtained as a 10 mCi/mL aqueous solution from Amersham. Its specific activity was 3000 Ci/mmol.

Binding Experiments. In a typical binding experiment the reaction between *cis*-DDP and pBR322 DNA was initiated by adding a sufficient volume of a freshly prepared 1 mg/mL (3.33×10^{-3} M) solution of the platinum complex in 1 mM sodium phosphate and 3 mM sodium chloride, pH 7.4, buffer to 1.5–1.8 mL of form I pBR322 DNA in the same buffer and incubating at 37 °C. The concentrations of platinum complex and pBR322 DNA in the reaction mixture were around 2.25×10^{-5} and 3.0×10^{-4} M (phosphates), respectively, giving an r_f value (C_0/P_0) of around 0.075. At various times aliquots were removed, the platinum reaction was quenched (Cohen et al., 1979) by raising the chloride concentration to 0.2 M with 4.0 M NaCl, and the samples were frozen for storage. After the samples were thawed, unbound platinum and the salt used to quench the reaction were removed by spin dialysis (Neal & Florini, 1973) through Sephadex G-25, leaving the samples ready for analysis by electrophoresis, restriction enzyme digestion, and atomic absorption spectroscopy (AAS). AAS was performed on a Varian AA-375 atomic absorption spectrometer equipped with a flameless carbon rod atomizer. By use of the spin-dialyzed DNA, a direct measurement of C_b, the amount of platinum bound per nucleotide, was obtained.

Cyanide Reversal of Platinum Binding. Removal of bound platinum by the addition of cyanide to form the very stable ($K_f \sim 10^{41}$) $[Pt(CN)_4]^{2-}$ complex (Bauer et al., 1978; Lippard & Hoeschele, 1979) was accomplished by adding a sufficient volume of 1 M NaCN as to bring the cyanide concentration in the reaction tube to 0.2 M and incubating at 37 °C for 3 h. Usually, 12.5 μL of 1 M NaCN was added to 50 μL of the DNA–Pt containing solution. Sodium cyanide hydrolyzes, giving solutions of pH ~11. For this reason, the cyanide salt was dissolved in 0.1 M Tris-HCl, pH 8.0, and the pH readjusted to 8.5 with concentrated HCl. This procedure was carried out in an efficient fume hood since gaseous HCN is liberated. Previous results (Bauer et al., 1978) have shown that the presence of cyanide and $[Pt(CN)_4]^{2-}$ ions does not affect the mobilities of DNA on gels. Consequently, samples were not redialyzed prior to electrophoresis. Cyanide-reversed samples were spin dialyzed again prior to AAS quantitation of platinum binding and before enzyme digestion, however.

BamHI Restriction Enzyme Digestion. *Bam*HI was stored in and diluted with buffer containing 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 500 μg/mL BSA, and 50% glycerol. Enzyme digestions were carried out in 20 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, and 100 mM NaCl on 2 μg of platinated DNA for 8 h at 37 °C. The amount of enzyme used was that necessary to cut 2 μg of unplatinated DNA in 1 h at 37 °C. Since platinum has a high affinity for sulfur-containing compounds such as DTT, a control experiment was performed in which a series of DNA samples with varying amounts of bound platinum ($0.004 \leq r_b \leq 0.121$) were incubated with DTT at a concentration of 9.8×10^{-5} M for 7 h at 37 °C. These conditions are comparable to those used in the enzyme digests. The samples were spin dialyzed to remove any platinum–DDT complexes formed and the bound platinum was measured by AAS. The r_b values recorded before and after incubation with DTT agreed within experimental error, indicating that this concentration of the thiol does not remove bound platinum.

Digestions were quenched by the addition of a $^1/_4$ digest volume of 0.125 M EDTA, 1% $NaDodSO_4$, 0.05% bromophenol blue, 0.05% xylene cyanol, and 10% Ficoll 400 (Pharmacia). Alternatively, the reactions were quenched by the addition of a $^1/_4$ volume of 0.250 M EDTA, followed by chilling in ice.

Gel Electrophoresis. Agarose gel electrophoresis was carried out in vertical slab gels (Studier, 1973). Good resolution between pBR322 forms I, II, and III was obtained with 1.5% (w/v) gels which were prepared with and run in 90 mM Tris base, 90 mM boric acid, and 2.2 mM EDTA, pH 8.3 (TBE), buffer. Gels were run at 100 V (7.4 V/cm) at room temperature for ~10 h. The DNA was visualized by staining the gels for 0.5 h in 0.5 mg/L ethidium bromide and illuminating from below with ultraviolet light. The gels were photographed with a Polaroid MP-4 camera by using a red filter and Polaroid 107 and 665 films.

Results and Discussion

Platinum Binding to DNA. Figure 1 is a representative plot of the kinetics of binding *cis*-DDP to pBR322 DNA. Platinum binding increases with time, gradually leveling off after 24 h at which time r_b is ~80% of r_f.

Following reaction with *cis*-DDP and spin dialysis, ~0.25 μg of DNA was subjected to electrophoresis as described under Materials and Methods. The results are shown in Figure 2. As platinum binding increases along the time coordinate, the band due to form I DNA broadens and becomes markedly retarded in its mobility. Its mobility decreases until a point at which it joins the form II DNA. The mobility of the form II band increases slightly with time prior to coalescence (4 h) and more rapidly thereafter. After 4 h a substantial amount

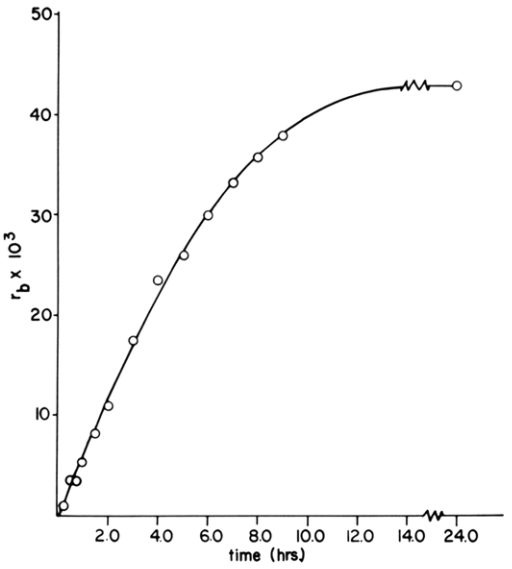

FIGURE 1: The kinetics of binding of *cis*-DDP to pBR322 DNA at 37 °C in 3 mM chloride and 1 mM phosphate, pH 7.4. $C_0 = 1.39 \times 10^{-5}$ M, $P_0 = 2.52 \times 10^{-4}$ M, and $r_f = 0.055$.

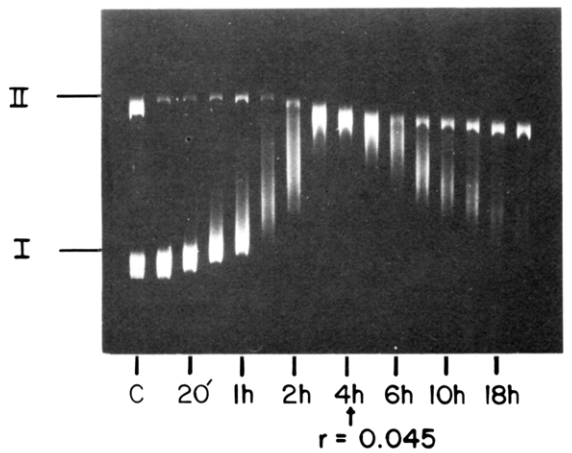

FIGURE 2: Electrophoresis in a 1.5% agarose gel of ~0.5 μg of pBR322 DNAs I and II following reaction with *cis*-DDP. Channels correspond to DNA samples incubated for 0 (control), 5 min, 20 min, 40 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h, 18 h and 24 h at 37 °C in 3 mM chloride and 1 mM phosphate, pH 7.4, buffer. $C_0 = 2.06 \times 10^{-5}$ M, $P_0 = 2.71 \times 10^{-4}$ M, and $r_f = 0.076$.

of the DNA travels with the nicked, relaxed circles in a band that increases slightly in mobility with time. The rest of the DNA increases in mobility at a faster rate as it migrates in very smeared bands. The coalescence of forms I and II DNA was determined by AAS to occur when 45 platinum atoms are bound per 1000 nucleotides ($r_b = 0.045$).

The changes in the electrophoretic mobility of form I pBR322 DNA upon platinum binding are analogous to what is seen in progressive intercalation of a reagent such as ethidium bromide (Bauer & Vinograd, 1968). It has been shown (Howe-Grant et al., 1976; Wakelin, 1974), however, that *cis*-DDP does not bind intercalatively to DNA. The results seen here with pBR322 agree closely with those of Cohen et al. (1979), who studied *cis*-DDP binding to pSM1 DNA and concluded that *cis*-DDP and *trans*-DDP unwind and shorten closed circular DNA. A significant difference between the two studies, however, is the amount of bound *cis*-DDP necessary to unwind form I DNA to the point where it has the same mobility as form II, $r_b = 0.045$ for pBR322 DNA vs. 0.10 for PSM1 DNA. This difference may reflect differences in the superhelix densities of the two plasmids, a point

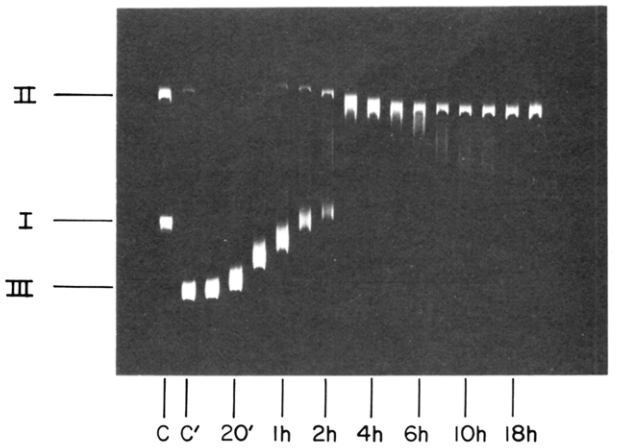

FIGURE 3: Electrophoresis in a 1.5% agarose gel following digestion of platinated pBR322 (see Figure 2) with *Bam*HI. Control channels correspond to 0-time incubation with *cis*-DDP before (C) and after (C′) treatment with the restriction endonuclease.

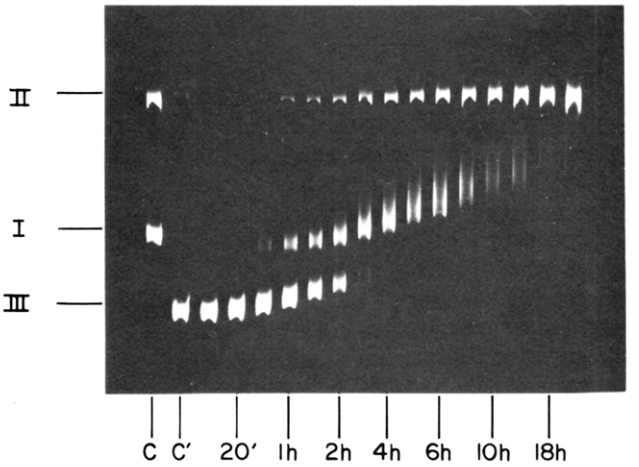

FIGURE 4: Electrophoresis in a 1.5% agarose gel following cyanide treatment of *Bam*HI-digested platinated DNA. Channel assignments are as in Figure 3.

that is currently being investigated.

Inhibition of BamHI Cleavage. The results of electrophoresis after *Bam*HI digestion of platinated pBR322 DNA are presented in Figure 3. At very low platinum binding levels there is complete cutting with all DNA migrating in the form III band. As the amount of platinum on the DNA increases, there is a gradual inhibition of cutting as evidenced by the growing presence of form I and form II DNA. At the 4-h time point with an r_b of 0.045, inhibition of cutting is complete with no form III DNA in evidence. This result appears more clearly in Figure 4 which shows the data for *Bam*HI-digested DNA from which platinum has subsequently been removed with cyanide. The removal of platinum largely, but not completely, arrests the mobility changes and sharpens the bands. The absence of any form III DNA at the 4-h time point is clearly evident. As is seen in Figures 2 and 3, the point of total inhibition of *Bam*HI cutting corresponds to the point at which forms I and II pBR322 DNA comigrate in the gel.

The mobility of the *Bam*HI-produced form III DNA decreases with increasing platinum binding (Figure 3). This effect is more pronounced in borate- than in acetate-containing buffers. That this DNA is actually linear was confirmed by an ethidium bromide containing "dye gel" (Cohen et al., 1979; Espejo & Lebowitz, 1976). The mobility changes of the form III band were unaffected by addition of a saturating amount of EtdBr. Studies with linear 146 base-pair nucleosome core

particle DNA have shown a retardation and smearing in gels upon *cis*-DDP binding (Lippard & Hoeschele, 1979) similar to that observed here for form III pBR322 DNA.

When platinated DNA was treated with excess cyanide and spin dialyzed to remove $[Pt(CN)_4]^{2-}$ and CN^- ions *prior* to *Bam*HI digestion, only linear DNA was produced by action of the enzyme. This result shows that the platinum lesion is reversible. As is evident in Figure 4, there is some residual smearing in the form I bands after cyanide reversal. The inability of excess cyanide to remove all the bound platinum at high r_b values has been observed previously (Bauer et al., 1978). In one case (r_b = 0.09), ~8% (r_b = 0.007) of the platinum was measured by AAS to have remained bound at the 24-h time point. The inability of the cyanide to remove this platinum may be due to the formation of very strong Pt–DNA interactions as would be expected if three or four bonds formed between DNA and a given platinum atom. The implications of this finding have been discussed previously (Bauer et al., 1978; Barton & Lippard, 1978, 1980).

The platinated DNAs were carefully spin dialyzed to remove unbound platinum prior to enzyme digestion. Moreover, the rate of spontaneous loss of *cis*-DDP bound to DNA under our conditions is negligible (Johnson et al., 1980). The *Bam*HI inhibition is therefore the result of *cis*-DDP binding to the DNA and is not caused by platination of the enzyme. This conclusion was verified by a control experiment in which the enzyme was found to cut unplatinated pBR322 DNA in the presence of additional DNA sufficiently platinated (r_b = 0.06) to inhibit all enzyme cutting in the earlier experiment. This result also shows that the inhibition of *Bam*HI is not due to a platinum-induced DNA–enzyme cross-link.

An experiment was performed to ensure that, under the conditions of *cis*-DDP binding to pBR322 DNA, *Bam*HI maintains its site specificity. *cis*-DDP was bound to pBR322 DNA at approximate r_b values of 0.003 and 0.02, corresponding to modest and substantial inhibition of *Bam*HI, respectively. These samples were digested with the enzyme, treated with cyanide to remove platinum, and then 5′ labeled with [γ-^{32}P]ATP by using the polynucleotide kinase exchange reaction (Berkner & Folk, 1977). This material was next digested with *Hinc*II, which cuts at positions 652 and 3907 in the pBR322 sequence (Sutcliffe, 1979). If *Bam*HI had cut at position 375, its normal recognition sequence, the *Hinc*II digest would yield labeled fragments 830 and 277 base pairs long. The existence of these pieces was confirmed by agarose and polyacrylamide gel electrophoresis. Guanine-specific sequencing reactions (Maxam & Gilbert, 1980) were also carried out on these two labeled fragments. The results after autoradiography of a 20% sequencing gel revealed that the sequences of control samples matched the sequences of the platinated DNA, confirming that the site specificity of *Bam*HI is maintained.

The results presented in Figure 4 demonstrate that removal of *cis*-DDP with cyanide ion after *Bam*HI digestion does not restore form III pBR322 to the gels past the 4-h time point. This finding contrasts with previously reported data for λ DNA (Kelman & Buchbinder, 1978). These workers stated that, in the presence of bound dichloroethylenediamineplatinum(II), an active antitumor agent with functionality and reactivity similar to *cis*-DDP, *Bam*HI still cut at its five recognition sites in λ DNA, even though the bands due to the restriction fragments were missing from the resulting, very smeared gels. Upon reversal of the platinum binding with cyanide, these workers observed the appearance of sharp bands at the positions expected for the *Bam*HI digest. On the basis of this evidence, it was concluded that the restriction enzyme maintains its ability to cut in the presence of platinum but the fragments are not seen due to intrastrand cross-linking by *cis*-DDP of adjacent guanines in the recognition site. It was further proposed that cyanide reversal removes these platinum atoms, which serve to link the restriction fragments, and allows the DNA to separate into its various pieces. Analysis of the present data for pBR322 DNA strongly suggests that the band sharpening observed for λ DNA was due to the removal of platinum atoms from short DNA fragments that were retarded in mobility and smeared because of the effect of duplex unwinding (Lippard, 1980); there is no reason to believe that the postulated intrastrand GpG cross-link occurred.

Total inhibition of restriction enzyme activity occurs at an r_b of 0.045, or 1 bound platinum atom per 11 base pairs. Since the recognition sequence is six base pairs in length, a platinum atom will be within ±3 base pairs of the

sequence, assuming random binding. The binding of *cis*-DDP within or close to the unique recognition sequence would unwind the duplex (Cohen et al., 1979) and probably result in disruption of the 2-fold site symmetry required for cutting (Smith, 1979).

Acknowledgments

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Preparation of Milligram Amounts of 21 Deoxyribonucleic Acid Restriction Fragments†

Wolfgang Hillen,‡ Ronald D. Klein, and Robert D. Wells*

ABSTRACT: Twenty-one DNA restriction fragments ranging in size from 12 to 880 base pairs (bp) were purified to homogeneity in milligram amounts. The developments which facilitated this work were (a) procedures for the rapid preparation of gram quantities of pure recombinant plasmid DNAs, (b) selective poly(ethylene glycol) (PEG) precipitation of DNAs according to broad classes of lengths, and (c) large-scale high-pressure liquid chromatography on RPC-5 for the purification of fragments to homogeneity. The 95- and 301-bp sequences from the lactose control region of *Escherichia coli* were cloned into the single *Eco*RI site of pVH51 in up to four copies per plasmid. These tandem inserts are separated by *Eco*RI sites and have a head to tail orientation in all cases. A total of 50 and 90 mg of the 95- and 301-bp fragments, respectively, were prepared from 300-L fermentations of *E. coli* cells transformed with these plasmids. A rapid and improved method, which can easily be scaled up, for the purification of plasmids and DNA restriction fragments was developed. Also, the linear pVH51 vector DNA was digested with *Hae*III to yield fragments ranging in size from 12 to 880 bp. The five smaller fragments (from 12 to 180 bp) were purified quantitatively by a selective PEG precipitation enrichment step followed by RPC-5 column fractionation. The larger fragments (245-880 bp) were prepared in milligram amounts. Ten subfragments from the 301-bp *lac* fragment were prepared by *Hpa*II, *Hin*fI, or *Hae*III/*Alu*I digestions followed by separation of the reaction products on RPC-5.

The role of the physical properties of DNA in biological regulation has been studied principally with large chromosomal DNAs (or heterogeneous populations of broken chromosomal DNAs) or with biosynthetic DNA polymers containing defined and repeated nucleotide sequences (reviewed in Wells et al., 1977, 1980b; von Hippel, 1979). The existence of sequence-dependent structural differences was demonstrated by a variety of physical and spectroscopic studies on the DNA polymers (Wells et al., 1970, 1977, 1980b). Extension of this approach to natural DNA molecules requires the development of methodology for the isolation of milligram quantities of small (10-200 base pairs) and homogeneous DNA sequences. Four developments in the past several years have laid the groundwork for this methodology: (i) the characterization of a large number of restriction endonucleases with precise specificities, (ii) the development of gene cloning with multicopy plasmid vectors, (iii) the utilization of rapid and reliable sequencing methods for characterizing fragments, and (iv) high-pressure liquid chromatography (HPLC)[1] on RPC-5 for the fractionation of milligram amounts of fragments.

An alternate approach is the chemical synthesis of oligonucleotides which correspond to a DNA sequence of interest (reviewed in Wu et al., 1978). Whereas this approach is useful for relatively small molecules (less than 20 bp in length), it is much more difficult to synthesize milligram amounts of homogeneous duplex DNAs containing 40-100 bp.

Recombinant DNA methodology has been utilized previously for the preparation of homogeneous DNA sequences. Plasmids were constructed containing the desired sequences which can be easily excised with restriction endonucleases and then purified from the vector DNA. Most of the plasmids studied to date contain fragments derived from the lactose control region of *Escherichia coli* (Hardies et al., 1979a; Hardies & Wells, 1979; Sadler et al., 1979; Kallai et al., 1980). The amount of fragments prepared by standard procedures (Hardies & Wells, 1979) was sufficient for optical studies (i.e., DNA melting) but was inadequate for other types of physical

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[1] Abbreviations used: bp, base pairs; PEG, poly(ethylene glycol); DOC, deoxycholate; UV, ultraviolet; HPLC, high-pressure liquid chromatography; *Hae*III/*Alu*I digest, the slash indicates a double digest of the DNA with the two endonucleases indicated; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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