Gauthier, F., Pagano, M., Esnard, F., Moury, H., & Engler, R. (1983) *Biochem. Biophys. Res. Commun. 110*, 449.
Hirado, M., Iwata, D., Niinobe, M., & Fujii, S. (1981) *Biochim. Biophys. Acta 669*, 21.
Järvinen, M. (1979) *FEBS Lett. 108*, 461.
Kato, H. (1980) *Seikagaku 52*, 1173.
Kato, H., Nagasawa, S., & Iwanaga, S. (1981) *Methods Enzymol. 80*, 172.
Katunuma, N., Wakamatsu, K., Takio, K., Titani, K., & Kominani, E. (1983) in *Proteinase Inhibitors* (Katunuma, N., Umezawa, H., & Holzer, H., Eds.) pp 135–145, Japan Scientific Society Press, Tokyo.
Kitamura, N., Takagaki, Y., Furuto, S., Tanaka, T., Nawa, H., & Nakanishi, S. (1983) *Nature (London) 305*, 545.
Lenney, J. F., Liao, J. R., Sagg, S. L., Gopalakrishnan, V., Wong, H. C. H., Ouge, K. H., & Chan, P. W. H. (1982) *Biochem. Biophys. Res. Commun. 108*, 1581.
Machleidt, W., Borchat, U., Fritz, H., Brzin, J., Ritonja, A., & Turk, V. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1481.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning, A Laboratory Manual*, pp 371–372, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mori, K., Sakamoto, W., & Nagasawa, S. (1981) *J. Biochem. (Tokyo) 90*, 503.
Muller-Estrel, M., Dittmann, B., Fritz, H., Lottspeich, F., & Henschen, A. (1983) *Adv. Exp. Med. Biol. 156A*, 157.
Nawa, H., Kitamura, N., Hirose, T., Asai, M., Inayama, S., & Nakanishi, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 90.
Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.
Ryley, M. C. (1979) *Biochem. Biophys. Res. Commun. 89*, 871.
Sakamoto, W., & Nishikaze, O. (1979) *J. Biochem. (Tokyo) 86*, 1549.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T., & Ito, N. (1977) *Biochem. Biophys. Res. Commun. 76*, 917.
Sasaki, M., Taniguchi, K., & Minakata, K. (1981) *J. Biochem. (Tokyo) 89*, 169.
Sasaki, M., Taniguchi, K., Suzuki, K., & Imahori, K. (1983) *Biochem. Biophys. Res. Commun. 110*, 256.
Staden, R. (1977) *Nucleic Acids Res. 4*, 4037.
Staden, R. (1978) *Nucleic Acids Res. 5*, 1013.
Takio, K., Kominani, E., Wakamatsu, N., Katunuma, N., & Titani, K. (1983) *Biochem. Biophys. Res. Commun. 115*, 902.
Takio, K., Kominami, E., Bando, Y., Katunuma, N., & Titani, K. (1984) *Biochem. Biophys. Res. Commun. 121*, 149.
Tarr, G. E. (1975) *Anal. Biochem. 63*, 361.
Turk, V., Brzin, J., Longer, M., Ritona, A., Eropkin, M., Borchart, U., & Machleidt, W. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1487.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259.
Wakamatsu, N., Kominami, E., & Katunuma, N. (1982) *J. Biol. Chem. 257*, 14653.
Young, R. A., & Davis, R. W. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194.
Young, R. A., & Davis, R. W. (1983b) *Science (Washington, D.C.) 222*, 778.

Articles

Effect of Polyamines and Basic Proteins on Cleavage of DNA by Restriction Endonucleases†

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ABSTRACT: We have investigated the effect of the polyamines spermine, spermidine, and putrescine and the prokaryotic histone-like proteins NS1 and NS2 on the restriction endonuclease *Eco*RI catalyzed cleavage of plasmid and bacteriophage DNAs. At low concentrations of spermine and spermidine, the rate of DNA cleavage by *Eco*RI is increased, while high concentrations of spermine as well as of spermidine are inhibitory. These phenomena are also observed with other restriction endonucleases. They are, therefore, probably due to the interaction of the polyamines with the DNA. Putrescine does not have such an effect within the concentration range investigated. Remarkably, low concentrations of spermine and spermidine very efficiently suppress *Eco*RI* activity. An inhibition of the *Eco*RI-catalyzed cleavage of DNA is also observed with NS1 and NS2, an effect that can be mimicked with other basic proteins that interact with DNA. The results are discussed in terms of the mechanism of restriction in vivo.

Class II restriction endonucleases cleave DNA with very high specificity at their respective recognition sites. Few enzyme activities have been described so far that discriminate as effectively between similar substrates as restriction endonucleases: it has been estimated for the *Eco*RI endonuclease that its reactivity toward its recognition site is at least 10^7 times higher than that at alternative sequences (Halford, 1980). The enormous specificity of restriction endonuclease is presumably due to several hydrogen bonds formed between the enzyme and the nucleotide bases of its substrate during the process of enzymatic catalysis (Seeman et al., 1976; Smith, 1979). A

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detailed analysis of the recognition mechanism of the *Eco*RI endonuclease indeed has shown that the experimental data are consistent with a recognition model that assumes that there are 12 specific hydrogen bonds, two per base pair of the hexanucleotide recognition site (Rosenberg & Greene, 1983). Any effect that leads to a distortion of the precise alignment of enzyme and substrate, therefore, is likely to affect the cleavage reaction. In accordance, it has been demonstrated for several restriction endonucleases that their activity and specificity is reduced by (I) covalent modification of the substrate or enzyme, (II) binding of ligands including carcinogens, drugs, dyes, and antibiotics to the substrate, and (III) addition to the assay buffer of organic solvents or heavy metal cations that may alter the conformation not only of the protein but also of the DNA [for a review, cf. Modrich & Roberts (1982) and Wells et al. (1981)].

These are, however, nonphysiological conditions. The question arises, whether normal constituents of bacterial cells affect the cleavage of DNA by restriction endonucleases. We have been particularly interested in natural ligands of DNA, that occur intracellularly in high concentrations. Among those are the polyamines, in particular, spermidine and putrescine, and several basic proteins, notably the prokaryotic histone-like proteins NS1 and NS2.

Polyamines occur ubiquitously in living cells. They exert a profound effect on many metabolic pathways, in particular those involving nucleic acids [for a review, cf. Morris & Marton (1979) and Tabor & Tabor (1976)]. Polyamines bind to polynucleotides, preferentially to double-stranded ones, and thereby may influence the binding of other ligands to nucleic acids. If these other ligands are enzymes acting on nucleic acids, it is to be expected, therefore, that polyamines modulate enzymatic activities. Indeed, it was shown in in vitro experiments that polyamines speed up the conversion of single-stranded phage DNA to its replicative form (Shekman et al., 1972; Geider & Kornberg, 1974; Weiner et al., 1975), as well as transcription (Fuchs et al., 1967; So et al., 1967; Lee-Huang & Warner, 1969) and translation (Nathans & Lipmann, 1961; Pastuszyn & Loftfield, 1975; Lövgren et al., 1978; Jelenc & Kurland, 1979).

Polyamines not only enhance the rate of replication, transcription, and translation. They have also been shown to improve the accuracy of some of the reactions in which they are involved, viz., the aminoacylation reaction (Loftfield et al., 1981) and ribosomal protein synthesis (Jelenc & Kurland, 1979).

Our results show that polyamines and basic proteins affect the rate of cleavage of DNA by restriction endonucleases. At low concentration, spermine and spermidine have a stimulatory effect on the rate and accuracy of the cleavage reaction. At high concentration, these polyamines as well as NS1 and NS2 inhibit the cleavage.

Experimental Procedures

Materials

Bacteriophage λ DNA was obtained from Miles (Frankfurt). Bacteriophage φX 174 RF DNA was a kind gift of A. Rienitz (Medizinische Hochschule, Hannover, FRG). *Pst*I and *Bam*HI endonucleases were from New England Biolabs (Schwalbach, FRG). Spermine, spermidine, and putrescine were purchased from Serva (Heidelberg, FRG). Cytochrome *c* from horse heart was from Sigma (München, FRG). Bovine serum albumin was obtained as a 30% solution from Biotest-Serum-Institut (Frankfurt, FRG). All other chemicals were of pro analysis grade and obtained from Merck (Darmstadt, FRG).

EcoRI Endonuclease. *Eco*RI endonuclease was isolated from an *Eco*RI-overproducing strain (M. Zabeau, unpublished results). All buffers used throughout the isolation procedure contained 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM 1,4-dithioerythritol, and 0.01% (w/v) lubrol. All operations were carried out at 4 °C. Approximately 10 g of wet cells suspended in 50 mL of 1 M NaCl–0.03 M potassium phosphate buffer, pH 7.2, was homogenized in a MSE sonifier. Cell debris was removed by centrifugation at 30000*g* for 30 min. To the clear supernatant was added $(NH_4)_2SO_4$ to 70% saturation. The pH was adjusted to 7.5 with NH_4OH. After being stirred overnight, the precipitate was collected by centrifugation at 30000*g* for 30 min. The precipitate was suspended in 100 mL of 0.03 M potassium phosphate buffer, pH 7.2, and dialyzed overnight against 0.03 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl. The dialyzate was diluted 1:1 with water and loaded onto a phosphocellulose column (5 × 20 cm) equilibrated with 0.03 M potassium phosphate buffer, pH 7.2, and eluted with a linear gradient of 2 L each of 0.0–1 M NaCl. Enzymatically active peak fractions were pooled and dialyzed overnight against 0.03 M potassium phosphate buffer, pH 7.2, containing 0.1 M NaCl. The dialyzate was diluted 1:1 with water, loaded onto a DEAE-cellulose column (1.5 × 2.5 cm), and eluted with 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.2, 0.5 M NaCl, and 60% (v/v) glycerol. Enzymatically active peak fractions were pooled and stored at −40 °C. The yield of electrophoretically pure (95%) enzyme was 20 mg.

NS1 and NS2. NS1 and NS2 were prepared as described by Miano et al. (1982).

Plasmid DNA. The pEMBL8+ plasmid (Dente et al., 1983) was a gift of R. Cortese (EMBL Heidelberg). For large-scale preparations, HB 101 *Escherichia coli* cells containing the plasmid were grown in 8 L of L broth overnight. Cells were harvested by centrifugation and washed with 50 mM Tris-HCl, 0.1 M EDTA, and 10% sucrose, pH 8.0. The cells (ca. 20 g of wet paste) were resuspended in 150 mL of the same buffer and lysed 20 min by 0.3 mg/mL lysozyme. After addition of 30 mL of 0.5 M EDTA (pH 8.0) and 15 mL of 10% Triton X-100, cell debris and a large part of chromosomal DNA were removed by 30-min centrifugation at 35000 rpm in a Beckman Ti-45 rotor. The cleared lysate was mixed with an equal volume of 30% poly(ethylene glycol)–1.5 M NaCl and left at 4 °C for at least 1 h. The precipitate was redissolved in 20 mL of 50 mM Tris-HCl. After 1 g of CsCl was dissolved per mL of this solution, the proteins were removed by centrifugation for 60 min at 30000 rpm in a Beckman Ti-70 rotor. After the solid upper phase was removed, ethidium bromide (10 mg/mL) was added to a concentration of 0.5 mg/mL. The density gradient was formed by 18-h centrifugation at 45000 rpm in a Beckman VTi-50 vertical rotor. The heaviest band containing the superhelical DNA was collected and diluted with a 2.5-fold volume of water, and the DNA was precipitated for 1 h at −25 °C by addition of a 3-fold volume of ethanol. The precipitate was redissolved in 1 mL of 10 mM Tris-HCl–1 mM EDTA, pH 7.8, and extracted twice with phenol. The phenol was removed by ether extraction, and the DNA was precipitated for at least 15 min at 4 °C by addition of a 0.2-fold volume of 3 M sodium acetate, pH 6.0, and a 0.6-fold volume of 2-propanol. This procedure yielded ca. 6 mg of plasmid, which was more than 80% superhelical as judged from the agarose gel electropho-

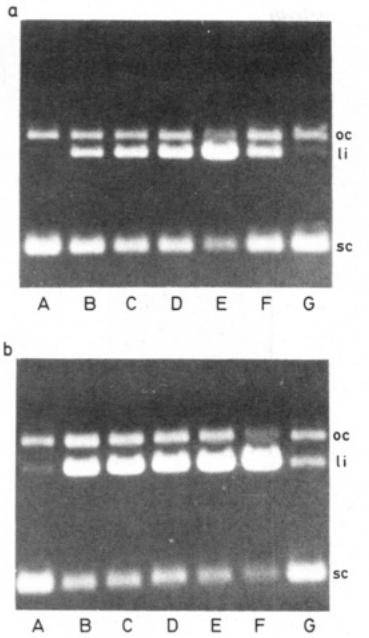

FIGURE 1: pEMBL DNA (27.5 μg/mL) was incubated with 3.7 nM *Eco*RI for 60 min at 37 °C in the presence of 1 mM $MgCl_2$ (a) or 10 mM $MgCl_2$ (b) and increasing amounts of spermine (lanes B–G): (lane B) 0, (lane C) 0.04, (lane D) 0.08, (lane E) 0.17, (lane F) 0.33, and (lane G) 0.67 mM spermine. Lane A is the reference: DNA in the absence of *Eco*RI. The lower band represents supercoiled pEMBL (form I) DNA, the upper band nicked pEMBL (form II), and the middle band linear pEMBL (form III).

resis. Some of the experiments (see Figures 2 and 5) reported here were carried out with samples that had accumulated open circular DNA upon prolonged storage.

Methods

DNA Cleavage. Plasmid pEMBL, bacteriophage λ DNA and φX 174 DNA were incubated with *Eco*RI, *Pst*I, or *Bam*HI, respectively, in 0.02 M Tris-HCl, pH 7.2, 0.05 M NaCl, and $MgCl_2$ or $MnCl_2$ as indicated at 37 °C. The reaction was stopped by adding 5 μL of a solution containing 0.25 M EDTA, 0.2% sodium dodecyl sulfate (SDS), 25% sucrose, and 0.1% bromphenol blue to a 15-μL aliquot. Reaction products were analyzed by electrophoresis on 1% agarose gels. Gels were stained with ethidium bromide and photographed with a Chema 4 UV illuminator supplied by Vetter (Wiesloch, FRG). For quantitative evaluation, the negatives were scanned with a gel scanner (Zeiss PMQ II/ZK 4). The scans were digitized and numerically integrated. The relative amount of the cleavage products in each sample was normalized to the total DNA concentration.

Circular Dichroism. Circular dichroism spectra of pEMBL and λ DNA were recorded on a Jobin-Yvon Dichrographe III at ambient temperature in cylindrical cuvettes of 1-cm path length.

Results

Effect of Polyamines on Rate of Cleavage of DNA by Restriction Endonucleases. We have studied the effect of polyamines on the cleavage of DNA by restriction endonucleases. Figure 1 shows the product analysis of the digestion of pEMBL, a supercoiled plasmid DNA with one *Eco*RI site, by the *Eco*RI endonuclease in the presence of varying amounts of spermine. At low concentrations of spermine, the rate of DNA cleavage is enhanced, while high concentrations of spermine are inhibitory. The spermine concentration range in which stimulation or inhibition is observed depends on the

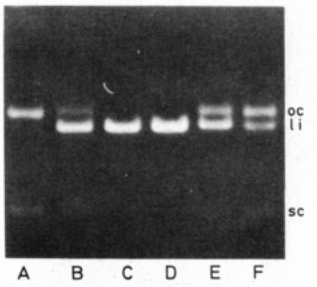

FIGURE 2: pEMBL DNA (27.5 μg/mL) was incubated with 167 units/mL *Pst*I for 5 min at 37 °C in the presence of 1 mM $MgCl_2$ and increasing amounts of spermine (lanes B–F): (lane B) 0, (lane C) 0.04, (lane D) 0.08, (lane E) 0.17, and (lane F) 0.33 mM spermine. Lane A is the reference: DNA in the absence of *Pst*I.

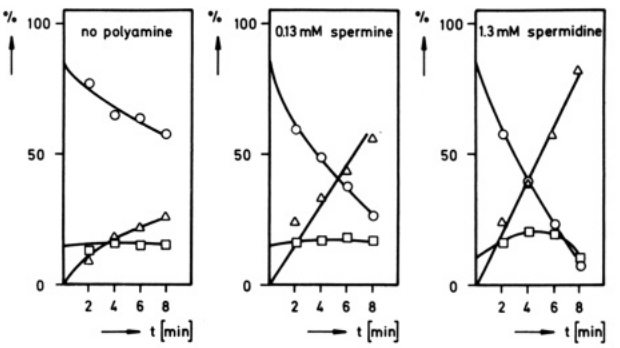

FIGURE 3: Quantitative evaluation of the kinetics of cleavage of 28.1 μg/mL pEMBL DNA by 121 nM *Eco*RI in the presence of 1 mM $MgCl_2$ and in the absence of polyamines (left), in the presence of 0.13 mM spermine (middle), and in the presence of 1.3 mM spermidine (right): (○) supercoiled substrate; (□) relaxed circular intermediate; (△) linearized product.

concentration of Mg^{2+} ions. At 1 mM $MgCl_2$, for example, the stimulating or inhibitory effect of spermine is seen at lower concentrations of the polyamine than at 10 mM $MgCl_2$.

The stimulating as well as the inhibitory effect of spermine on the restriction endonuclease catalyzed cleavage of DNA is unique neither to the *Eco*RI endonuclease nor to the supercoiled plasmid DNA pEMBL; it is also seen with other restriction endonucleases, e.g., *Pst*I (Figure 2) and *Bam*HI (not shown), and with other DNAs, e.g., linear bacteriophage λ DNA (not shown). Analogous results were obtained with spermidine at higher concentration levels (not shown). This finding seems not to be related to the observation that spermidine enhances the rate of cleavage of DNA in the presence of dextran sulfate (Bouche, 1981). The kinetics of the activation of the cleavage reaction in the presence of spermine or spermidine is shown in Figure 3 for the cleavage of pEMBL DNA by *Eco*RI. From the quantitative evaluation it is apparent that 0.13 mM spermine and 1.3 mM spermidine respectively enhance the rate of cleavage under the conditions given approximately 2–3-fold and 3–4-fold. With putrescine, no stimulating or inhibitory effect was seen up to a concentration of 13.3 mM (not shown). It should be mentioned that the optimal concentrations of spermine and spermidine differ slightly for different restriction enzymes (compare Figures 1 and 2).

Influence of Polyamines on Accuracy of EcoRI Endonuclease Catalyzed Cleavage of DNA. Since under normal conditions the specificity of restriction endonucleases is very high, we chose to investigate the influence of polyamines on the accuracy of restriction endonucleases under conditions of relaxed specificity. Many restriction endonucleases lose their normally high accuracy when exposed to organic solvents, low

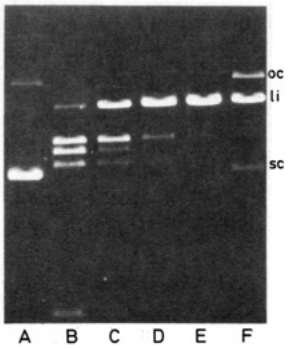

FIGURE 4: pEMBL DNA (28.1 μg/mL) was incubated with 1.2 μM *Eco*RI for 15 min at 37 °C in the presence of 1 mM $MgCl_2$ and increasing amounts of spermine (lanes B–F): (lane B) 0, (lane C) 0.04, (lane D) 0.08, (lane E) 0.17, and (lane F) 0.33 mM spermine. Lane A is the reference: DNA in the absence of *Eco*RI. The extra bands in lane B, which are not as pronounced in lanes C and D, represent *Eco*RI* products.

ionic strength, elevated pH, or Mn^{2+} or at high enzyme to DNA ratios and recognize some nucleotide sequences that are similar to their canonical recognition site [for a review, cf. Fuchs & Blakesley (1983)]. Under conditions of reduced specificity the *Eco*RI endonuclease recognizes sites that differ in a single position from the canonical *Eco*RI sequence at any one of the six positions in the recognition site, with the exception of A → T or T → A changes within the central tetramer (Gardner et al., 1982). The hydrolysis rates at these *Eco*RI* sites are rather different: some sites are very labile; others are refractory to cleavage. We have investigated the products of an *Eco*RI digest of DNA under *Eco*RI* conditions in the presence of increasing concentrations of polyamines in order to find out whether they influence the cleavage of DNA by the *Eco*RI endonuclease at sites other than the canonical recognition site. Figure 4 shows that low concentrations of spermine suppress the *Eco*RI* activity with pEMBL DNA as substrate. Similar results were obtained with other DNAs, e.g., λ DNA and φX 174 RF DNA (not shown), and with other restriction enzymes, e.g., *Pst*I and *Bam*HI (not shown). The suppression of cleavage at noncanonical sites occurs within the range of concentration of spermine or spermidine that has been found to enhance the rate of cleavage at canonical sites. Spermidine at millimolar concentrations has a similar effect as spermine, while putrescine has no effect (not shown).

Influence of Basic Protein on Cleavage of DNA by EcoRI Endonuclease. We have noticed recently that excess *Eco*RI endonuclease leads to the inhibition of the *Eco*RI-catalyzed cleavage of DNA (Alves et al., 1982). We have interpreted this self-inhibition as nonspecific binding of excess enzyme molecules leading to a distortion of the structure of the substrate, which then can no longer be cleaved. If this interpretation is correct, then this effect can be produced by other proteins as well that interact with DNA in a nonspecific manner. We have chosen for this investigation the prokaryotic DNA binding proteins NS1 and NS2, also known as HU proteins, which are associated with the bacterial nucleoid and presumably are mainly responsible for the packaging of DNA [for a review, cf. Rouvière-Yaniv (1977)].

Figure 5a,b shows that NS1 and NS2 at high concentration inhibit the cleavage of pEMBL DNA. The inhibition is more pronounced with supercoiled DNA than with open circular DNA, in agreement with the stronger binding of NS1 and NS2 to superhelical DNA relative to relaxed DNA. According to Rouvière-Yaniv (1977), double-stranded DNA is saturated at a weight ratio of 2:1 for NS and DNA. The onset of inhibition is observed at concentrations of the NS proteins

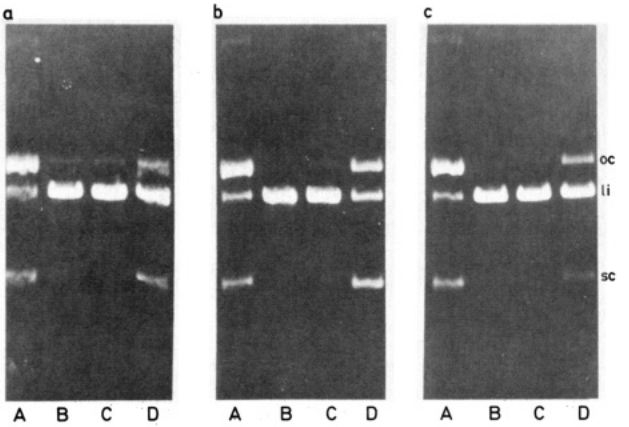

FIGURE 5: pEMBL DNA (27.8 μg/mL) was incubated with 37.3 nM *Eco*RI in the presence of 1 mM $MgCl_2$ for 10 min at 37 °C. The incubation was carried out in the absence (lane B) and in the presence of 33 (lane C) and 133 μg/mL (lane D) NS1 protein (a), NS2 protein (b), and cytochrome *c* (c). Lane A is the reference: pEMBL DNA in the absence of *Eco*RI.

sufficient to saturate the DNA present. The effect of NS1 and NS2 on the *Eco*RI-catalyzed cleavage of DNA can be mimicked by cytochrome *c*, the classical agent to complex DNA for electron microscopy (Kleinschmidt & Zahn, 1959). Figure 5c shows that cytochrome *c* inhibits the cleavage of DNA by the *Eco*RI endonuclease, although not quite as effectively as NS1 or NS2. With bovine serum albumin, on the other hand, in concentrations up to 5 mg/mL, no inhibition of the cleavage reaction could be detected (not shown), demonstrating that the inhibition is not due to proteins per se but proteins interacting strongly with DNA. The inhibitory effect of NS1, NS2, and cytochrome *c* is very similar to the one of the peptide antibiotics distamycin A and netropsin, as well as the intercalating drugs proflavin, ethidium bromide, and actinomycin D, which all inhibit the *Eco*RI action on DNA by binding at or near the *Eco*RI recognition site (Goppelt et al., 1981).

Correlation with Structural Data of Inhibitory Effect of Spermine and NS1 on EcoRI Endonuclease Catalyzed Cleavage of DNA. Polyamines bind preferentially to double-stranded DNA and stabilize the DNA to thermal melting. They compete with other cations for binding to DNA. The apparent equilibrium constant for the binding of polyamines to DNA, therefore, is dependent on the concentration of mono- and divalent cations present (Shapiro et al., 1969). In 20 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 1 mM $MgCl_2$, K^{assoc} for spermine binding to DNA can be estimated to be of the order of 10^3 M^{-1}.

The binding of polyamines to DNA leads to the condensation of DNA [for a review, cf. Morris & Marton (1979)]. At a critical concentration of the polyamine that depends on the kind of DNA, on its concentration, and on the presence of other cations, precipitation occurs (Osland & Kleppe 1977).

There is little information on the structure of polyamine–DNA complexes. The X-ray fiber diffraction pattern of the complex of calf thymus and salmon sperm DNA with spermine, however, indicates that at 92% relative humidity the complex has the same helical parameters as B DNA (Suwalsky et al., 1969; Huse et al., 1978).

We have been interested to find out whether the effects that polyamines and basic proteins have on the cleavage of DNA by restriction endonucleases can be correlated with structural alterations of the DNA. For this purpose, circular dichroism spectra of pEMBL DNA were recorded at several spermine and NS1 concentrations. Figure 6a shows that spermine has

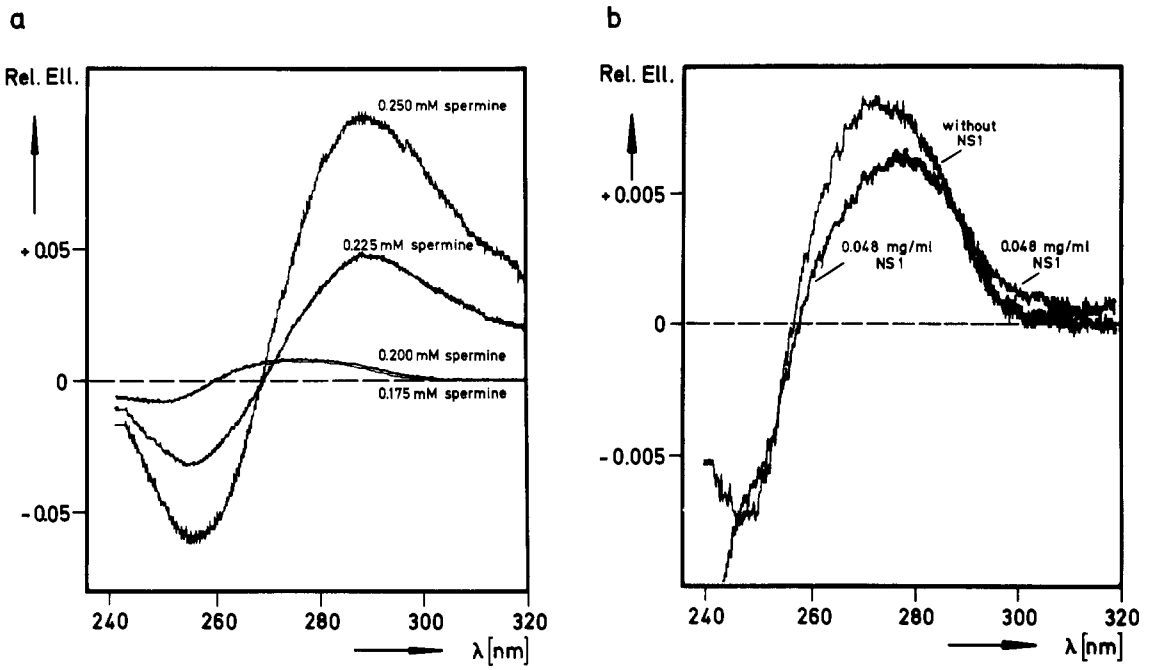

FIGURE 6: Circular dichroism spectra of 26.6 μg/mL pEMBL DNA in 0.02 M Tris-HCl, pH 7.2, 0.05 M NaCl, and 1 mM $MgCl_2$ in the presence of 0.175, 0.2, 0.225, and 0.25 mM spermine (a) and in the absence and presence of 48 μg/mL NS1 protein (b). Intensities are given as θ values by using a mean residue weight of 650, corresponding to the average molecular weight of a base pair. Identical spectra were obtained after incubation for 2 h at 25 °C.

a profound effect on the circular dichroism of pEMBL DNA. With the same DNA concentration as used in the cleavage assay, spermine induces a dramatic increase in the apparent circular dichroism intensities of pEMBL. This change is observed at the same concentration of spermine that has been found to inhibit the cleavage reaction. At this concentration of spermine, the DNA remains in solution.

However, as can be judged from light scattering measurements, a highly cooperative condensation occurs between 0.2 and 0.25 mM spermine; under the experimental conditions given, precipitation is observed only at concentrations above 0.3 mM spermine. At a concentration lower than 0.2 mM, spermine does not significantly change the circular dichroism spectrum of DNA. The effect of spermine on the structure of DNA is also apparent with other DNAs. The magnitude of the effect, however, depends on the kind of DNA and its conformation. With pEMBL in a supercoiled structure, a significantly different effect is seen as compared with, e.g., λ DNA or linearized pEMBL (not shown). Nonconservative circular dichroism spectra of DNA in the presence of basic polypeptides and proteins have been observed; evidence is accumulating that these spectra are not so much the result of changes in the secondary structure of DNA but are due to asymmetric structures with long-range periodicities within DNA aggregates (Brunner & Maestre, 1974; Shin & Eichhorn, 1984). It cannot be decided, nevertheless, whether the inhibitory effect of spermine and, by analogy, of spermidine on the restriction endonuclease catalyzed cleavage of DNA is caused by a possibly small conformational change of the substrate or by a decreased accessibility of the substrate for the enzyme due to the condensation of the DNA.

NS1 also changes the circular dichroism spectrum of supercoiled pEMBL DNA (Figure 6b). The effect is not as dramatic as the one observed with spermine. Furthermore, no NS1-induced aggregation or precipitation of the DNA can be detected. Again, it is not clear whether the inhibition by NS1 and, by analogy, by NS2 of the *Eco*RI-catalyzed cleavage of DNA is primarily due to the binding of NS1 and NS2 to the DNA at or near the cleavage site or due to a subsequent conformational change of the substrate.

Discussion

We have tried to analyze in this investigation what the effect of naturally occurring ligands of DNA on the cleavage of DNA by restriction endonucleases is like. The rational of this investigation was the following: DNA within *E. coli* is not just DNA with Na^+ and Mg^{2+} as counterions to neutralize the negative charge of DNA, but it is also complexed with polyamines, in particular spermidine and putrescine, as well as basic proteins such as the prokaryotic histone-like proteins NS1 and NS2. In *E. coli*, the total intracellular concentrations are 13.1 μmol/g of wet wt for putrescine and 4.7 μmol/g of wet wt for spermidine (Tabor & Tabor, 1976) [0 μmol/g of wet wt for spermine, when cells are grown in purified media; spermine, however, can be efficiently accumulated from the medium (Tabor & Tabor, 1966)]. The concentrations of NS1 and NS2 were estimated to be 60 000/*E. coli* cell (Rouvière-Yaniv, 1977), i.e., approximately 100 μM. The physiological Mg^{2+} ion concentration on the other hand is considered to be approximately 1 mM (Lövgren et al., 1978). The action of restriction endonucleases presumably was optimized during evolution for their substrate complexed in part with these ligands. Consequently, both optimum rate and specificity (accuracy) may depend on the presence of these "natural ingredients". Our results demonstrate that both polyamines and the histone-like proteins NS1 and NS2 have profound effects on the cleavage of DNA by restriction endonucleases.

At low concentrations, spermine and spermidine enhance the rate of cleavage in the presence of physiological concentrations of Mg^{2+} ions. This enhancement is accompanied by an increase in specificity, since *Eco*RI* activity induced by Mn^{2+}, glycerol, or high concentrations of enzyme is very effectively suppressed. Since we have observed the suppression of "star" activity with several restriction enzymes, we suppose that the reduction of specificity could result from slight conformational changes in the DNA, which are enhanced by changing pH, ionic strength, or divalent metal ion or organic

solvent concentration and which may give rise to "star" products. Spermine and spermidine may stabilize the structure of DNA and thus will prevent conformational changes that would make the interaction between the protein and the DNA less exact. Our results, however, do not rule out the possibility that the relaxation of specificity is due at least in part to conformational changes of the enzyme that can be prevented or counteracted by polyamines.

At higher concentrations of spermine and spermidine, an inhibition of the restriction endonuclease cleavage of DNA is observed. With spermidine, this effect occurs within the physiological concentration range. This inhibition is correlated with a condensation of the DNA, as detected by turbidity and circular dichroism measurements. It is not clear at present whether the inhibition that we observe is due to a change in secondary structure of the DNA or the low accessibility of the cleavage sites in the condensed state. The lack of an effect of putrescine is most likely due to the relatively low affinity of this polyamine to DNA.

The prokaroytic histone-like proteins NS1 and NS2 also have an inhibitory effect on the cleavage of DNA by restriction endonucleases. A similar although not quite as effective inhibition is seen with other basic proteins, e.g., cytochrome *c*. The inhibition is probably due to a competition for binding sites on the DNA (Goppelt et al., 1981), although again it cannot be ruled out that conformational changes of the substrate upon binding of the basic proteins to the substrate are responsible for the inhibition, as is the case for the intercalating drugs proflavin, ethidium bromide, and actinomycin D, as well as for the antibiotics netropsin and distamycin A, which bind to the minor groove of the DNA and distort the DNA (Dattagupta et al., 1980).

It is hard to tell what the significance of our findings is for restriction in vivo. Although we know approximately the total intracellular concentrations of the polyamines and the NS proteins, it is impossible at present to give a reliable estimate of their free concentrations. The following arguments, therefore, are presented with due reservation: Fast-growing (2.4 doublings/h) *E. coli* cells contain an average of 3.4 chromosomes (Donachie et al., 1976; Pierucci, 1978), corresponding to 13.2×10^6 base pairs. The cellular content of NS1 and NS2 is probably half of the amount required to condense DNA to give rise to the observed repeating unit in the *E. coli* chromosome (Griffith, 1976). Accordingly, an essential function in the condensation of DNA was also attributed to polyamines (Rouvière-Yaniv, 1977). We suppose that not only is the chromosomal DNA complexed with NS1 and NS2 and polyamines but that also DNA of foreign origin that has got into the bacterial cell will eventually be interacting with NS1 and NS2 as well as polyamines. Foreign DNA when fully complexed will then be largely protected from cleavage. Restriction of this foreign DNA will, therefore, depend on the kinetics of the various processes involved, on one hand association of the DNA with polyamines and NS1 or NS2 and on the other hand cleavage by restriction endonucleases. In this context, the finding that *Eco*RI is localized in the periplasmic space (G. W. Kohring, personal communication) or associated with the *E. coli* membrane (H. Mayer, personal communication) is of particular interest, because it gives this enzyme a chance to cleave foreign DNA during or shortly after infection or transformation and before it is fully complexed with NS1, NS2, and polyamines. A partial complexation with polyamines may then even enhance the rate of cleavage and improve its accuracy. The protective complexation of the chromosomal DNA with NS1, NS2, and polyamines may explain the finding of Gingeras & Brooks (1983) that an active *Pae*R7 restriction enzyme gene can be present in an *E. coli* cell without an accompanying methylase gene.

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Registry No. *Eco*RI endonuclease, 80498-17-5; *Pst*I endonuclease, 81295-32-1; *Bam*HI endonuclease, 81295-09-2; spermine, 71-44-3; spermidine, 124-20-9; putrescine, 110-60-1.

References

Alves, J., Pingoud, A., Langowski, J., Urbanke, C., & Maass, G. (1982) *Eur. J. Biochem. 124*, 139–142.

Bouché, J. P. (1981) *Anal. Biochem. 115*, 42–45.

Brunner, W. C., & Maestre, M. F. (1974) *Biopolymers 13*, 345–357.

Dattagupta, N., Hogan, M., & Crothers, D. M. (1980) *Biochemistry 19*, 5998–6005.

Dente, L., Cesarini, G., & Cortese, R. (1983) *Nucleic Acids Res. 11*, 1645–1655.

Donachie, W. D., Begg, K. J., & Vicente, M. (1976) *Nature (London) 264*, 328–333.

Fuchs, R., & Blakesley, R. (1983) *Methods Enzymol. 65*, 3–37.

Fuchs, E., Milette, R. L., Zillig, W., & Walter, G. (1967) *Eur. J. Biochem. 3*, 183–193.

Gardner, R. C., Howarth, A. J., Messing, J., & Shepherd, R. J. (1982) *DNA 1*, 109–115.

Geider, K., & Kornberg, A. (1974) *J. Biol. Chem. 249*, 3999–4005.

Gingeras, T. B., & Brooks, J. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 402–406.

Goppelt, M., Langowski, J., Pingoud, A., Haupt, W., Urbanke, C., Mayer, H., & Maass, G. (1981) *Nucleic Acids Res. 9*, 6115–6127.

Griffith, J. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 563–568.

Halford, S. E. (1980) *Biochem. Soc. Trans. 8*, 399–400.

Huse, Y., Mitsui, Y., Iitaka, Y., & Miyaki, K. (1978) *J. Mol. Biol. 122*, 43–53.

Jelenc, P. C., & Kurland, C. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3174–3178.

Kleinschmidt, A., & Zahn, R. K. (1959) *Z. Naturforsch. 146*, 770–779.

Lee-Huang, S., & Warner, R. C. (1969) *J. Biol. Chem. 244*, 3793–3802.

Loftfield, R. B., Eigner, E. A., & Pastuszyn, A. (1981) *J. Biol. Chem. 256*, 6729–6735.

Lövgren, T. N. E., Petterson, A., & Loftfield, R. B. (1978) *J. Biol. Chem. 253*, 6702–6710.

Miano, A., Losso, M. A., Gianfranceschi, G. L., & Gualerzi, C. O. (1982) *Biochem. Int. 5*, 415–422.

Modrich, P., & Roberts, R. J. (1982) in *Nucleases* (Linn, S. M., & Roberts, R. J., Eds.) pp 109–154, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morris, O. R., & Marton, L. J., Eds. (1979) *Polyamines in Biology and Medicine*, Marcel Dekker, New York.

Nathans, D., & Lipmann, F. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 497–504.

Osland, A., & Kleppe, K. (1977) *Nucleic Acids Res. 4*, 685–695.

Pastuszyn, A., & Loftfield, R. B. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 501.
Pierucci, O. (1978) *J. Bacteriol. 135*, 559–574.
Rosenberg, J. M., & Greene, P. (1982) *DNA 1*, 117–124.
Rouvière-Yaniv, J. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 439–447.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804–808.
Shapiro, J. T., Leng, M., & Felsenfeld, G. (1969) *Biochemistry 10*, 1675–1683.
Shekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L., & Kornberg, A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2691–2695.
Shin, Y. A., & Eichhorn, G. L. (1984) *Biopolymers 23*, 325–335.
Smith, H. O. (1979) *Science (Washington, D.C.) 205*, 455–462.
So, A. G., Davie, E. W., Epstein, R., & Tissieres, A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1739–1746.
Suwalsky, M., Traub, M., Shmueli, U., & Subirana, J. (1969) *J. Mol. Biol. 42*, 363–373.
Tabor, C. W., & Tabor, H. (1966) *J. Biol. Chem. 241*, 3714–3723.
Tabor, H., & Tabor, C. W. (1976) *Annu. Rev. Biochem. 45*, 285–306.
Weiner, J., Bertsch, L., & Kornberg, A. (1975) *J. Biol. Chem. 250*, 1972–1980.
Wells, R. D., Klein, R. D., & Singleton, C. K. (1981) *Enzymes, 3rd Ed. 14*, 157–191.

Left-Handed Helical Structure of Poly[d(A-C)]·Poly[d(G-T)] Studied by Infrared Spectroscopy†

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ABSTRACT: Infrared spectroscopic studies demonstrate the ability of poly[d(A-C)]·poly[d(G-T)] to adopt a Z-type conformation. The Z form of the unmodified polynucleotide is induced by Ni^{2+} counterions and not by Na^+. The B → Z equilibrium is shifted at room temperature, in the presence of 1 Ni^{2+}/nucleotide, by an increase in the concentration of poly[d(A-C)]·poly[d(G-T)]. The importance of specific binding of Ni^{2+} ions on the N7 site of purines in the stabilization of the Z form is also discussed.

Left-handed Z DNA is favored in crystals of perfectly alternating $[d(G-C)]_n$ sequences (Wang et al., 1979; Crawford et al., 1980; Drew et al., 1980; Fujii et al., 1982). However, it is predicted from steric considerations that any purine-pyrimidine sequence should be able to adopt a left-handed structure. The $[d(A-C)]_n$·$[d(G-T)]_n$ sequence often occurs in eukaryotic cells (Hamada & Kakunaga, 1982), and possible roles for Z elements in transcriptional activation have been suggested (Rich, 1982). The stabilization of the Z conformation of $[d(A-C)]_n$·$[d(G-T)]_n$ by negative supercoiling has been presented (Nordheim, 1983; Haniford, 1983). It has also been reported by X-ray fiber diffraction that poly(dA-dC)·poly(dG-dT) may occasionally adopt a left-handed helical structure at low humidity (Arnott et al., 1980). However, no UV or CD (circular dichroic) spectra characteristic of the Z conformation have yet been obtained with dilute solutions of the unmodified polymer, even in the presence of very high concentration of various metal ions or with alcoholic solutions (Vorlickova et al., 1982; Zimmer et al., 1982): the negative band at 295 nm of the canonical Z form of poly[d(G-C)]·poly[d(G-C)] has not been observed for poly[d(A-C)]·poly[d(G-T)]. In solution, to promote the Z conformation of poly[d(A-C)]·poly[d(G-T)], in addition to high salt content, covalent modification by (acetylamino)fluorene or high temperature and methylation on the C5 position of cytosine were required (Wells et al., 1982; McIntosh et al., 1983). By using infrared spectroscopy, we show that the unmodified poly[d(A-C)]·poly[d(G-T)] polymer in hydrated films can adopt A, B, or Z conformations depending on the type of counterions and on the degree of hydration. We have found that Ni^{2+} is able to induce in a condensed phase of poly[d(A-C)]·poly[d(G-T)], a Z form similar to that induced in poly[d(G-C)]·poly[d(G-C)], but Na^+ fails to convert poly[d(A-C)]·poly[d(G-T)] into the Z form. An increase in DNA concentration and an increase in cationic binding sites are the two factors that favor the Z conformation.

Materials and Methods

Poly[d(A-C)]·poly[d(G-T)] (lot 719-97) was purchased from P-L Biochemicals. Samples were deposited on ZnSe windows and gently dried so as to give homogeneous films. The desired amount of metal ions is obtained by diffusion of a droplet of the $NiCl_2$ solution followed by slow evaporation. Films are placed in cells with controlled relative humidity (H_2O or D_2O). Hydration of the complexes is determined directly from the IR spectra of the samples. The Perkin-Elmer 180 double-beam spectrophotometer is coupled to a Hewlett-Packard 9825 A calculator, allowing systematic data treatment such as base-line and water-contribution corrections and scaled spectrum subtraction.

Results

Three different infrared spectra have been obtained with poly[d(A-C)]·poly[d(G-T)] films depending on the counterion and the water content. In presence of Ni^{2+} (1Ni^{2+}/nucleotide), a Z-type spectrum is observed. In the case of Na^+ (1 Na^+ in excess/nucleotide), B- or A-type spectra are recorded, depending on the relative humidity (B form above 86% RH; A form between 71 and 58% RH). The Z conformation has never been observed in the case of Na^+ counterion, and no A

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