hydroxylamine, which did not have much structure similarity, formed PLP oxime without any intermediate.

Registry No. SHMT, 9029-83-8; OADS, 20311-84-6; AAA, 645-88-5; DCS, 68-41-7; PLP–AAA oxime, 17780-81-3; PLP–canaline oxime, 77111-63-8; PLP oxime, 634-25-3; PLP, 54-47-7; NH_2OH, 7803-49-8; L-canaline, 496-93-5.

References

Aniento, F., Garcia-Espana, A., Portoles, M., Alonso, E., & Cabo, J. R. (1988) *Mol. Cell. Biochem. 79*, 107.

Baskaran, N., Prakash, V., Appu Rao, A. G., Radhakrishnan, A. N., Savithri, H. S., & Appaji Rao, N. (1989) *Biochemistry* (preceding paper in this issue).

Baxter, C. F., & Roberts, E. (1961) *J. Biol. Chem. 236*, 3287.

Beeler, T., & Churchich, J. E. (1976) *J. Biol. Chem. 251*, 5267.

Braunstein, A. E. (1973) *Enzymes (3rd Ed.) 9*, 379.

Braunstein, A. E., Azarkh, R. M., & Ching-Hsieh, H. (1961) *Biokhimiya (Moscow) 26*, 882.

Brown, F. C., Hudgins, W. R., & Roszell, J. A. (1969) *J. Biol. Chem. 244*, 2809.

Churchich, J. E., & Bieler, J. (1971) *Biochim. Biophys. Acta 229*, 813.

Hotta, S. S. (1968) *Arch. Biochem. Biophys. 127*, 132.

Hyvonen, T., Alakuijala, L., Anderson, L., Khomutov, R. M., Khomutov, R. A., & Eloranta, T. (1988) *J. Biol. Chem. 263*, 11138.

Jencks, W. P. (1964) *Prog. Phys. Org. Chem. 2*, 63.

Jencks, W. P., & Cordes, E. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P., Braunstein, A., & Rossi-Fanelli, A., Eds.) p 57, Pergamon Press, New York.

John, R. A., Charteris, A., & Fowler, L. J. (1978) *Biochem. J. 171*, 771.

Kito, K., Sanada, Y., & Katunuma, N. (1978) *J. Biochem. 83*, 201.

Klosterman, H. J. (1986) in *Coenzymes and Cofactors. Vitamin B_6: Pyridoxal Phosphate Chemical, Biochemical and Medical Aspects* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Vol. I, Part A, p 391, Wiley-Interscience, New York.

Korpela, T., Lundell, J., & Makinen, E. (1979) *Org. Magn. Reson. 12*, 376.

Manohar, R., Ramesh, K. S., & Appaji Rao, N. (1982) *J. Biosci. 4*, 31.

Manohar, R., Appu Rao, A. G., & Appaji Rao, N. (1984) *Biochemistry 23*, 4116.

Raunio, R. P., Lindberg, R., & Jenkins, W. T. (1984) *Arch. Biochem. Biophys. 233*, 43.

Rognstad, R., & Clark, D. G. (1974) *Arch. Biochem. Biophys. 161*, 638.

Rosenthal, G. A. (1981) *Eur. J. Biochem. 114*, 301.

Roze, U., & Strominger, J. L. (1966) *Mol. Pharmacol. 2*, 92.

Schirch, L. (1975) *J. Biol. Chem. 250*, 1939.

Stammer, C. H. (1962) *J. Org. Chem. 27*, 2957.

Wang, E., & Walsh, C. (1978) *Biochemistry 17*, 1313.

Inhibition of Restriction Endonuclease Cleavage via Triple Helix Formation by Homopyrimidine Oligonucleotides†

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ABSTRACT: A 17-mer homopyrimidine oligonucleotide was designed to bind to the major groove of SV40 DNA at a 17 base pair homopurine·homopyrimidine sequence via Hoogsteen base pairing. This sequence contains the recognition site for the class II-S restriction enzyme *Ksp* 632-I. The oligonucleotide was shown to inhibit enzymatic cleavage under conditions that allow for triple helix formation. Inhibition is sequence-specific and occurs in the micromolar concentration range. Triple helix formation by oligonucleotides opens new possibilities for sequence-specific regulation of gene expression.

The recognition of DNA sequences by regulatory proteins is central to the control of cellular processes at the level of gene expression. Techniques for selectively inhibiting protein–nucleic acid recognition would provide means for controlling gene expression at the level of DNA replication and transcription. Homopyrimidine oligonucleotides were recently shown to bind to the major groove of double-stranded DNA at homopurine·homopyrimidine sequences (Le Doan et al., 1987; Moser & Dervan, 1987; Praseuth et al., 1988; Lyamichev et al., 1988; François et al., 1988, 1989). A triple helix is locally formed where the homopyrimidine oligonucleotide is oriented parallel to the homopurine-containing strand of DNA. Thymine forms two hydrogen bonds with adenine in a Watson–Crick A·T base pair. The formation of two hydrogen bonds between cytosine and a Watson–Crick G·C base pair requires protonation of cytosine.

Hoogsteen base pairing of thymine and protonated cytosine to a homopurine sequence of duplex DNA might not be the only molecular code for recognition of Watson–Crick base pairs by oligonucleotides. A purine-rich oligonucleotide, 27 nucleotides in length, was recently shown to bind duplex DNA and to reduce the transcription of the human c-myc gene in

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vitro (Cooney et al., 1988). The possibility might exist for recognition of A·T and G·C base pairs by A and G, respectively (Letai et al., 1988).

In order to provide evidence for the possibility of inhibiting sequence-specific DNA-binding proteins, we have tested the ability of a homopyrimidine oligonucleotide to inhibit sequence-specific cleavage by a restriction endonuclease. The class II-S restriction endonuclease *Ksp* 632-I recognizes a 6 bp homopurine·homopyrimidine sequence (Bolton et al., 1988). A single recognition site exists in SV40 DNA. It is part of a homopurine·homopyrimidine sequence, 17 base pairs in length, located in the T antigen gene (Tooze, 1981). We have synthesized a 17-mer homopyrimidine oligonucleotide designed to bind to the major groove of this 17 bp sequence according to the Hoogsteen base pairing scheme described above. We show that this oligonucleotide selectively inhibits the cleavage of supercoiled SV40 DNA by the restriction enzyme *Ksp* 632-I under reaction conditions that allow the formation of triple helices.

Materials and Methods

The restriction enzyme *Ksp* 632-I purified from *Kluyvera* sp. strain 632 (Bolton et al., 1988) was a gift from Dr. G. Schmitz, Boehringer-Mannheim. Enzymatic assays were performed in a buffer containing 33 mM Tris–acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mg/mL tRNA. The pH and temperature of the incubation medium were changed as indicated in the text and the legends of the figures. The concentration of target DNA (SV40 or pBR328) was usually 6 nM; 0.08 unit/μL restriction enzyme was used in each assay. In some experiments 0.4 mM spermine was added to the reaction mixture. Even though organic solvents were previously shown to stabilize triple helices (Moser & Dervan, 1987; François et al., 1989), no organic solvent was added to the enzymatic reaction mixture to avoid any unexpected effect on *Ksp* 632-I enzyme such as the relaxation of base-sequence specificity which has been previously described for other restriction endonucleases (Modrich & Roberts, 1982).

After incubation, enzymatic reactions were stopped by adding EDTA (10 mM). Samples were then analyzed by gel electrophoresis with horizontal slab gels (0.8% agarose). Densitometric analysis of gels stained with ethidium bromide was performed on a LKB laser densitometer.

The oligonucleotides d-$^{5'}$TTTTTTCTTCTCTTTCC$^{3'}$ and d-$^{5'}$TTTTCTTTTCCCCCCT$^{3'}$ were synthesized on a Pharmacia automatic synthesizer. They were purified by HPLC and their purity controlled by polyacrylamide gel electrophoresis.

Results and Discussion

The restriction enzyme *Ksp* 632-I, purified from *Kluyvera* sp. strain 632, is a class II-S enzyme which recognizes the sequence $^{5'}$CTCTTC$^{3'}$/$^{3'}$GAGAAG$^{5'}$ and cleaves the two strands in an asymmetric way outside the recognition sequence (Figure 1) (Bolton et al., 1988). This enzyme has a single recognition site in SV40 which is part of a 17 bp homopurine·homopyrimidine sequence (Figure 1). We have synthesized a homopyrimidine oligonucleotide, 17 bases in length, which has a parallel orientation as compared to the homopurine sequence of the 17 bp SV40 sequence. According to previous works (Moser & Dervan, 1987; Praseuth et al., 1988) this oligonucleotide should bind to the major groove of duplex DNA in a parallel orientation with respect to the homopurine sequence. Recognition involves Hoogsteen base pairing of thymine and protonated cytosine with Watson–Crick A·T and G·C base pairs, respectively.

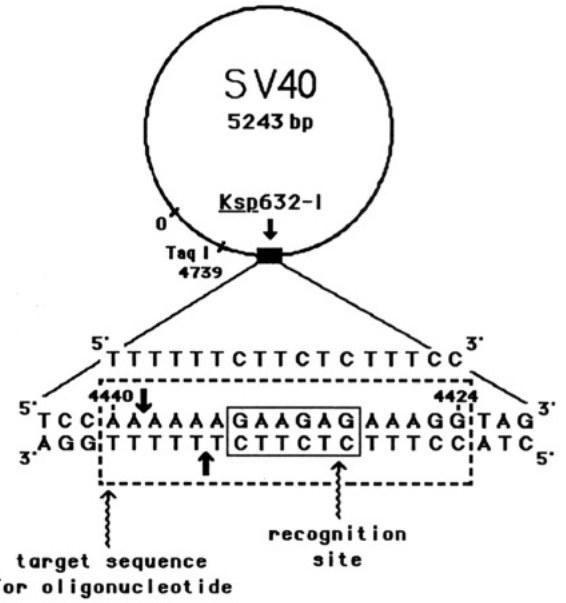

FIGURE 1: Schematic representation of SV40 DNA showing the recognition site for *Ksp* 632-I enzyme and the target sequence for the homopyrimidine oligonucleotide whose sequence is shown above the boxed target sequence. Short arrows indicate the cleavage sites for the restriction endonuclease. Numbers refer to position on the SV40 genome according to Tooze (1981).

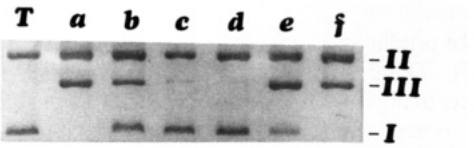

FIGURE 2: Specific inhibition of restriction enzyme *Ksp* 632-I by oligonucleotide d-$^{5'}$TTTTTTCTTCTCTTTCC$^{3'}$. Enzymatic assays were performed by incubating SV40 DNA (6 nM) at 37 °C for 20 min with *Ksp* 632-I (0.08 units/μL) and varying concentration of oligonucleotide in a buffer containing 33 mM Tris–acetate, pH 6.5, 66 mM potassium acetate, 0.5 mM DTT, 10 mM $MgCl_2$, and 1 mg/mL tRNA. In lanes a–d, increasing amounts of oligonucleotide (0, 10, 50, and 100 μM) were added to the reaction mixture. Lane e was the same as lane d except for higher pH (7.9 instead of 6.5). A 16-mer oligonucleotide whose sequence is d-$^{5'}$TTTTCTTTTCCCCCCT$^{3'}$ was unable to inhibit the restriction enzyme at a concentration of 100 μM at pH 6.5 (lane f). Lane T represents SV40 DNA incubated without restriction enzyme and oligonucleotides.

The restriction enzyme *Ksp* 632-I linearizes supercoiled SV40 at 37 °C in a wide range of pH values from 6.0 to 8.0 (see Figure 2). It reacts rapidly with supercoiled DNA but much more slowly with relaxed circular DNA and linear DNA (unpublished data). At 37 °C relaxed SV40 DNA is only slightly affected under conditions where 100% of supercoiled DNA is cleaved. At temperatures lower than 30 °C the enzyme makes both single-strand and double-strand cuts (see below, Figure 3A). Single-strand cuts lead to the accumulation of relaxed DNA which is no longer a good substrate for the enzyme. The ratio of single-strand to double-strand cleavage increases when the temperature decreases.

In order to test whether the 17-mer oligonucleotide could inhibit sequence-specific cleavage, a series of experiments was carried out at 37 °C in a pH 6.5 buffer in the presence of 10 mM $MgCl_2$, conditions under which the enzyme makes a single double-strand cut in supercoiled SV40 DNA (Figure 2). Increasing amounts of the 17-mer oligonucleotide were added to SV40 DNA before addition of the restriction enzyme. As shown in Figure 2, cleavage was inhibited in a dose-dependent manner. Densitometric analysis of the gels indicated that the concentration required to inhibit 50% of the reaction was 20

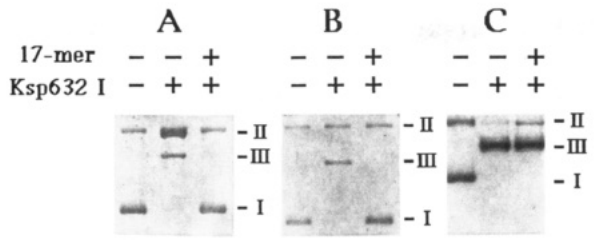

FIGURE 3: Inhibition of SV40 DNA cleavage by *Ksp* 632-I under conditions that favor triple helix formation. In part A, the enzymatic assay was carried out in the presence of 1 μM 17-mer oligonucleotide d-$^{5'}$TTTTTTCTTCTCTTTCC$^{3'}$ in the conditions described in Figure 2 (lanes a–d) except for temperature, which was decreased from 37 to 20 °C. In part B, the incubation temperature was raised to 37 °C and spermine (0.4 mM) was added to the reaction mixture. In part C, the plasmid pBR328 was incubated with *Ksp* 632-I restriction enzyme in the presence of 100 μM 17-mer oligonucleotide in the same conditions as in Figure 2 (lanes a–d).

μM. In order to show that inhibition was due to the interaction of the oligonucleotide with the target DNA rather than with the restriction enzyme, parallel experiments were carried out with the plasmid pBR328 (Soberon et al., 1980), which contains one restriction site for *Ksp* 632-I but no sequence fully complementary to that of the 17-mer homopyrimidine (Bolton et al., 1988). Over the same range of oligonucleotide concentration no inhibition of cleavage of pBR328 was observed (Figure 3C). Similarly, replacing the inhibitory 17-mer oligonucleotide by a homopyrimidine 16-mer of different sequence produced no inhibition of SV40 cleavage (Figure 2, lane f). These experiments demonstrated that inhibition was not due to oligonucleotide binding to the enzyme but to triple helix formation at the binding site of the enzyme.

Further evidence for the involvement of oligonucleotide binding to duplex DNA was provided by the use of conditions that are known to destabilize or stabilize triple-helical structures. Triple helix formation by a homopyrimidine oligonucleotide involves binding of thymine and protonated cytosine to Watson–Crick A·T and G·C base pairs, respectively. An increase in pH destabilizes the triple helix due to the loss of cytosine protonation which is required to form a C·G·C base triplet. When the pH of the cleavage reaction mixture was increased to 7.9, only a weak inhibition of the restriction enzyme was observed at a concentration of the 17-mer oligonucleotide (100 μM) that gave 100% inhibition at pH 6.5 (compare lanes d and e in Figure 2). Decreasing the incubation temperature should stabilize triple helices. At 20 °C 1 μM oligonucleotide was sufficient to produce 100% inhibition (Figure 3A), while no inhibition was observed at 37 °C. Triple helix stability is also increased upon addition of spermine (Moser & Dervan, 1987). When *Ksp* 632-I was used to cleave SV40 DNA at pH 6.5 in the presence of 1 μM oligonucleotide, no inhibition was observed at 37 °C. However, the addition of 0.4 mM spermine under the same reaction conditions resulted in 100% inhibition (Figure 3B). The enzymatic reaction in the absence of oligonucleotide was not affected by the addition of 0.4 mM spermine except for a slight increase in the rate of reaction (data not shown).

Conclusion

We have shown here that the recognition of a DNA sequence by a specific protein (a restriction endonuclease) can be selectively inhibited by a homopyrimidine oligonucleotide under conditions that allow recognition of the target site by triple helix formation. Homopurine·homopyrimidine sequences are often present within the coding or regulatory regions of genes, as exemplified by SV40 DNA [see Tooze (1981) for SV40 sequence]. Selective binding of homopyrimidine oligonucleotides to such regions might modulate transcription either by interfering with the binding of regulatory protein factors or by preventing the elongation of RNA chains. DNA replication might be similarly inhibited. After submission of this paper, Maher et al. (1989) described the inhibition of *Taq*I endonuclease and methylase by an oligonucleotide forming a triple helix. The substrate was a synthetic homopurine·homopyrimidine sequence overlapping *Taq*I binding site by only 2 base pairs. In our study a natural homopurine·homopyrimidine sequence of SV40 DNA was chosen as a target, and the oligonucleotide completely covered the restriction endonuclease binding site (see Figure 1). In both cases inhibition required concentrations of the competing oligonucleotide in the micromolar range. We have recently shown that attachment of an intercalating agent at the 5′-end of a homopyrimidine oligonucleotide strongly stabilizes the triple-stranded structure by intercalation at the triplex–duplex junction (Sun et al., 1989). This stabilization should allow us to design much more efficient transcription or replication inhibitors which could be used as tools in molecular and cellular biology and provide a rational basis for the development of potential therapeutical applications.

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References

Bolton, B. J., Schmitz, G. G., Jarsch, M., Conner, M. J., & Kessler, C. (1988) *Gene 66*, 31–43.

Cooney, M., Czernuszewicz, G., Postel, E. H., Flint, S. J., & Hogan, M. E. (1988) *Science 241*, 456–459.

François, J.-C., Saison-Behmoaras, T., & Hélène, C. (1988) *Nucleic Acids Res. 16*, 11431–11440.

François, J.-C., Saison-Behmoaras, T., Chassignol, M., Thuong, N. T., & Hélène, C. (1989) *J. Biol. Chem. 264*, 5891–5898.

Le Doan, T., Perrouault, L., Praseuth, D., Habhoub, N., Decout, J. L., Thuong, N. T., Lhomme, J., & Hélène, C. (1987) *Nucleic Acids Res. 15*, 7749–7760.

Letai, A. G., Palladino, M. A., Fromm, E., Rizzo, V., & Fresco, J. R. (1988) *Biochemistry 27*, 9108–9112.

Lyamichev, V. I., Mirkin, S. M., Frank-Kamenetskii, M. D., & Cantor, C. R. (1988) *Nucleic Acids Res. 16*, 2165–2178.

Maher, J. L., Wold, B., & Dervan, P. B. (1989) *Science 245*, 725–730.

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.

Moser, H. E., & Dervan, P. B. (1987) *Science 238*, 645–650.

Praseuth, D., Perrouault, L., Le Doan, T., Chassignol, M., Thuong, N. T., & Hélène, C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1349–1353.

Soberon, X., Covarrubias, L., & Bolivar, F. (1980) *Gene 9*, 287–305.

Sun, J. S., François, J.-C., Montenay-Garestier, T., Saison-Behmoaras, T., Roig, V., Thuong, N. T., & Hélène, C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Tooze, J. (1981) *DNA Tumor Viruses*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.