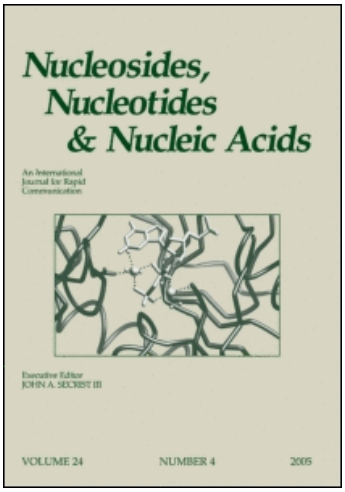
This article was downloaded by:
On: *26 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Inhibition of Restriction Enzyme Ksp 632-I Via Triple Helix Formation by Phosphorothioate Oligonucleotides

Satoru Tsukahara[a]; Hidefumi Yamakawa[a]; Kazuyuki Takai[a]; Hiroshi Takaku[a]
[a] Department of Industrial Chemistry, Chiba Institute of Technology, Chiba, Japan

To cite this Article Tsukahara, Satoru , Yamakawa, Hidefumi , Takai, Kazuyuki and Takaku, Hiroshi(1994) 'Inhibition of Restriction Enzyme Ksp 632-I Via Triple Helix Formation by Phosphorothioate Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 13: 6, 1617 — 1626
To link to this Article: DOI: 10.1080/15257779408012176
URL: http://dx.doi.org/10.1080/15257779408012176

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

NUCLEOSIDES & NUCLEOTIDES, 13(6&7), 1617-1626 (1994)

INHIBITION OF RESTRICTION ENZYME Ksp 632-I VIA TRIPLE HELIX FORMATION BY PHOSPHOROTHIOATE OLIGONUCLEOTIDES#

Satoru Tsukahara, Hidefumi Yamakawa, Kazuyuki Takai and Hiroshi Takaku*

Department of Industrial Chemistry, Chiba Institute of Technology, Tsudanuma, Narashino, Chiba 275, Japan

Downloaded At: 18:04 26 January 2011

Abstract: The ability of pyrimidine-rich oligonucleotide phosphorothioate to form stable triple helical structures with the sequence containing the recognition site for the class II-S restriction enzyme *Ksp* 632-I was examined. First, we prepared double strand oligonucleotides corresponding to the major groove of SV40 DNA at 17 base pair homopurine-homopyrimidine sequences, and studied their interaction with homopyrimidine oligodeoxyribonucleotides including replacement of the PS group in the second nucleotide position from the 5'-terminus (SO-ODNs) and of the PS group at both the 3'- and 5'-ends (S_2O-ODNs). The resulting perfect DNA triplexes were detected by the gel-mobility shift. The phosphorothioate oligonucleotide analogues (SO-ODNs) and (S_2O-ODNs) were shown to inhibit enzymatic cleavage under conditions that allow for triple helix formation. Inhibition is sequence-specific and occurs in the micromolar concentration range. Of particular interest is the Sp-phosphorothioate analogue (Sp-SO-ODNs) which inhibited endonuclease more than the other phosphorothioate oligonucleotide analogues (Rp-SO-ODNs or S_2O-ODNs).

INTRODUCTION

The sequence specific recognition of double-helical DNA is an essential biological process responsible for the regulation of cellular functions including transcription, replication, and cell division. In most cases, regulation of gene expression in living organisms is achieved by specific nucleic acid binding proteins. Any method which can

---

#This article is dedicated to Emeritus Professor Morio Ikehara on the occasion of his seventieth birthday.

Copyright © 1994 by Marcel Dekker, Inc.

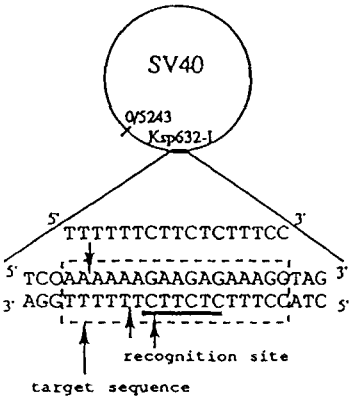
alter selectively the interaction of a protein at a certain site on DNA, without affecting that similar interaction elsewhere on the chromosome, offers the opportunity to modulate biological functions. The triplex formation may serve this function. An intermolecular triple helix can be formed by interaction of thymine with A-T, where thymine forms Hoogsteen hydrogen bonds with Watson-Crick A-T base pairs and protonated cytosine with G-C base pairs.[1-3] The demonstration that homopyrimidine oligodeoxynucleotides can bind specifically to homopurine-homopyrimidine tracts of duplex DNA via intermolecular triplex formation has opened a new field on the inhibition of enzymatic cleavage.[4-7]

Recently, we reported[8] that oligonucleotides having phosphorothioate internucleotidic bonds as a mixture of diastereoisomers could not form triple helix structures, whereas one of the two diastereoisomers (Rp or Sp) could form triple helix structures with double strands. To examine the possibility of inhibiting sequence-specific DNA binding proteins by phosphorothioate oligonucleotides, we have tested the ability of a homopyrimidine phosphorothioate oligonucleotide to inhibit sequence-specific cleavage in SV40 DNA by the class II-S restriction endonuclease *Ksp* 632-I.[9] The *Ksp* 632-I enzyme recognizes a 6 base pairs homopurine-homopyrimidine sequence. We have synthesized 17mer homopyrimidine phosphorothioate oligonucleotide analogues (Rp-SO-ODNs, Sp-SO-ODNs, RpRp-S_2O-ODNs, SpSp-S_2O-ODNs, and SpRp-RpSp-S_2O-ODNs) designed to bind to the major groove of this 17 base pair sequence according to the Hoogsteen base pairing (Fig. 1).[8] We found that these oligonucleotides selectively inhibit the cleavage of supercoiled SV40 DNA by the restriction enzyme *Ksp* 632-I under reaction conditions that allow the formation of triple helices.

Downloaded At: 18:04 26 January 2011

RESULTS AND DISCUSSION

In order to provide evidence of the influence of homopyrimidine phosphorothioate oligonucleotides on triplex formation, we synthesized the 17 nucleotide duplexes, 5'AAAAAAGAAGAGAAAGG3'/3'TTTTTTCTTCTCTTTCC5' (SV40 target sequence) and studied their interactions with the homopyrimidine phosphorothioate oligonucleotide analogues (Fig. 1). The electrophoretic profiles show that the oligonucleotide mixtures formed triple-helical structures, as indicated by the appearance of bands with mobilities lower than that of the reference target duplex (Fig. 2).[8] It is worth noting that the band corresponding to a weaker mode of binding of the pyrimidine



ODNs	5'-TTTTTTCTTCTCTTTCC-3'
Rp-SO-ODNs	5'-TTsTTTTCTTCTCTTTCC-3'
Sp-SO-ODNs	5'-TTsTTTTCTTCTCTTTCC-3'
RpRp-S_2O-ODNs	5'-TsTTTTTCTTCTCTTTCsT-3'
SpSp-S_2O-ODNs	5'-TsTTTTTCTTCTCTTTCsT-3'
SpRp-RpSp-S_2O-ODNs	5'-TsTTTTTCTTCTCTTTCsT-3'
Target Duplex	5'-AAAAAAGAAGAGAAAGG-3'
	3'-TTTTTTCTTCTCTTTCC-5'

Fig. 1. Schematic representation of SV40 DNA showing the recognition site for *Ksp* 632-I enzyme and the target sequence for the homopyrimidine phosphorothioate oligonucleotide analogues whose sequence is shown above the boxed target sequence.Short arrows indicate the cleavage sites for the restriction endonuclease.

Downloaded At: 18:04 26 January 2011

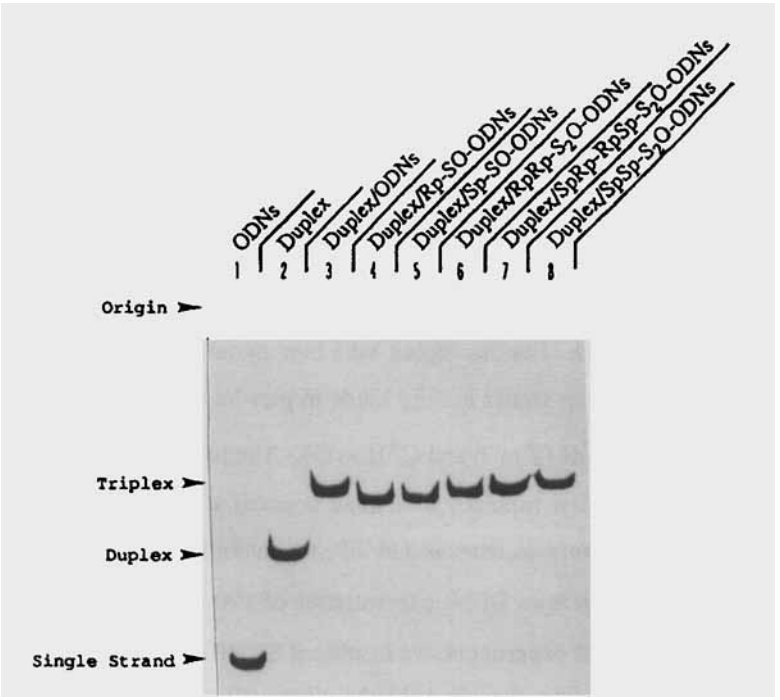


Figure 2. 15% Non-denaturing polyacrylamide gel electrophoresis in 90 mM Tris-borate/5 mM $MgCl_2$ (pH 8.3).

oligonucleotides to the imperfect C-G sites in all oligonucleotide analogues was not observed.

The restriction enzyme *Ksp* 632-I recognizes the sequence 5'CTCTTC3'/ 3'GAGAAG5' and cleaves the two strands in an asymmetric way outside the recognition sequence (Fig. 1). We have synthesized the homopyrimidine phosphorothioate oligonucleotide analogue, 17 bases in length, which has a parallel orientation as compared to the homopurine sequence of the 17 bp SV40 sequence.[10] In previous studies, this oligonucleotide could bind to the major groove of duplex DNA at the homopurine sequence.[4,11,12] Concerning the stability of the phosphorothioate oligonucleotides, we have recently shown that the oligonucleotides with replacement of the PS group in the second nucleotide position from the 5'-terminus and of the PS group at both 3'- and 5'-ends (S2O-ODNs) were strongly resistant to exonucleases in the presence of nucleases.[13]

Downloaded At: 18:04 26 January 2011

To test the ability of the 17mer homopyrimidine phosphorothioate oligonucleotide analogues to recognize selectively the *Ksp* 632-I site within the SV40 DNA, the experiments were carried out at 37°C in a pH 6.9 buffer in the presence of 10 mM $MgCl_2$ and 1 mM spermine, conditions under which the enzyme makes a single double-strand cut in supercoiled SV40 DNA. The oligonucleotides were added to the SV40 DNA mixture before addition of the restriction enzyme. Fig. 3a shows the digestion of SV40 DNA by *Ksp* 632-I in the presence of unmodified oligomer (ODNs) at 20-100 μM. Densitometric analysis of the gels indicated that the concentration required to inhibit 50% of the reaction was 59 μM. The third strand is generally pyrimidine rich, but in some cases may be purine rich. For the triplex with two pyrimidine strands and one purine strand, the extra pyrimidine strand usually binds in parallel to the purine strand through Hoogsteen hydrogen bonds (T to A and C^+H to G). The loss of cytosine protonation in the third strand causes these triplexes to be more unstable at basic pH values. When the pH of the cleavage reaction was increased to 7.9, the inhibition of the restriction enzyme activity was similar to that at an ODNs concentration of 100 μM (compare lanes 5 and 6, Fig. 3a). In a subsequent experiment, we incubated SV 40 DNA with *Ksp* 632-I in the presence of homopyrimidine phosphorothioate oligonucleotides (Rp-SO-ODNs or Sp-SO-ODNs). The analysis of the gels for Rp-SO-ODNs and Sp-SO-ODNs indicated that the concentration required to inhibit 50% of the reaction was 70 μM (Rp-SO-ODNs) and

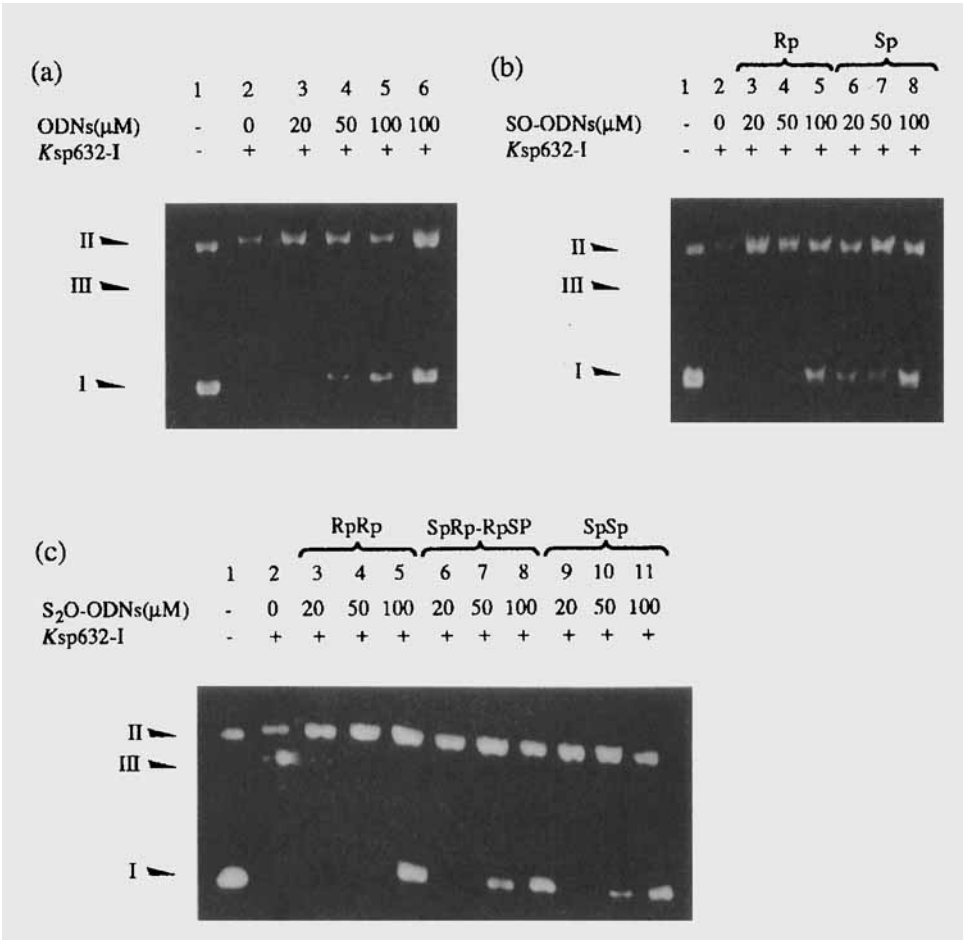


Figure 3. Specific inhibition of restriction enzyme *Ksp* 632-I by the phosphorothioate oligonucleotide analogues. Enzymatic assays were performed by incubating SV40 DNA (6 nM) at 37°C for 1 h with *Ksp* 632-I (20 units/μL) and several concentrations (0, 20, 50, or 100 μM) of oligonucleotide in a buffer containing 33 mM Tris-acetate, 66 mM potassium acetate (pH 6.9 or 7.9), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM spermine. Form I: supercoiled DNA; form II: open circular DNA; form III: linear DNA. a) The enzymatic assay was carried out in the presence of the unmodified oligonucleotide (ODNs). Lane 6 was the same as lane 5 except for higher pH (7.9 instead of 6.9). Lane 1 represents SV40 DNA incubated without the restriction enzyme and oligonucleotides. b) The enzymatic assay was carried out in the presence of the modified oligonucleotides with the PS group in the second nucleotide position (from 5'-terminus) (Rp-SO-ODNs or Sp-SO-ODNs). Lane 1 represents SV40 DNA incubated without the restriction enzyme and oligonucleotides. c) The enzymatic assay was carried out in the presence of the end capped oligonucleotide with PS group at both 3'- and 5'-ends (RpRp-S_2O-ODNs, SpSp-S_2O-ODNs, and SpRp-RpSp-S_2O-ODNs). Lane 1 represents SV40 DNA incubated without restriction enzyme and oligonucleotides.

Downloaded At: 18:04 26 January 2011

14 μM (Sp-SO-ODNs) (Fig. 3b). This observation is somewhat different from that we reported previously.[8] However, perfect DNA triplexes were detected on the gel-mobility shift (Fig. 2). On the other hand, in the case of the end capped oligonucleotide with PS groups at both 3'- and 5'-ends, RpRp-S2O-ODNs, SpSp-S2O-ODNs, and SpRp-RpSp-S2O-ODNs, the cleavage reaction was inhibited 50% at 70 μM, 30 μM, and 37 μM, respectively (Fig. 3c). These findings suggest that the homopyrimidine phosphorothioate oligonucleotide analogues having Sp specifically inhibit the DNA-protein interaction via triplex formation to a greater extent than the analogues having Rp or the unmodified oligonucleotide (ODNs). Of particular interest is the Sp-phosphorothioate analogue (Sp-SO-ODNs) which showed higher endonuclease inhibition than the other phosphorothioate oligonucleotide analogues (S2O-ODNs or Rp-S-ODNs). This effect has been attributed to the inhibition of molecular interactions by the oligonucleotide with a single PS group at the *Ksp* 632-I recognition site.

Downloaded At: 18:04 26 January 2011

Furthermore, in order to show that the inhibition was due to the interaction of the oligonucleotide with the target DNA rather than with the restriction enzyme, the experiments were carried out with the plasmid pBR 328[14], which contains one restriction site for *Ksp* 632-I but no sequence fully complementary to that of 17mer homopyrimidine (Fig 4). The inhibitory effect of the cleavage of pBR 328 was less than that of the SV 40 DNA at a concentration of 100 μM oligonucleotides (Fig. 5). These findings showed that the inhibition was not due to the oligonucleotide binding to the enzyme but to triple helix formation at the binding site to the restriction enzyme.

Here we have shown that the recognition of the DNA sequence by a restriction endonuclease can be specifically inhibited by homopyrimidine phosphorothioate oligonucleotide analogues that allow recognition of the target site by triple helix formation. We have studied previously the influence of the oligonucleotide phosphorothioate pyrimidine strands on triplex formation.[8] We found that the Rp-phosphorothioate oligonucleotide ($dT_{15}S_1$ (R)) had a sightly higher stability for the triplex structure than did the Sp-phosphorothioate oligonucleotide ($dT_{15}S_1$ (S)), but in contrast to the present findings the Sp-phosphorothioate oligonucleotide analogues showed greater endonuclease inhibition than the Rp-phosphorothioate oligonucleotide analogues. The homopyrimidine phosphorothioate oligonucleotide having Sp at the *Ksp* 632-I recognition site inhibited the DNA-protein interactions more than the

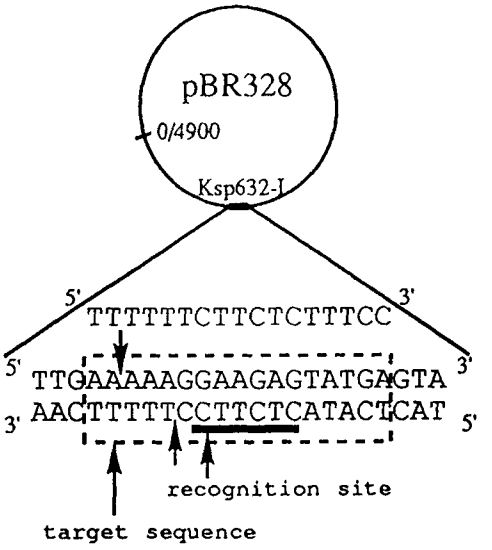


Figure 4. Schematic representation of the plasmid pBR 328 showing the recognition site for *Ksp* 632-I enzyme and the target sequence for the homopyrimidine phosphorothioate oligonucleotide analogues the sequence of which is shown above the boxed target sequence.Short arrows indicate the cleavage sites for the restriction endonuclease.

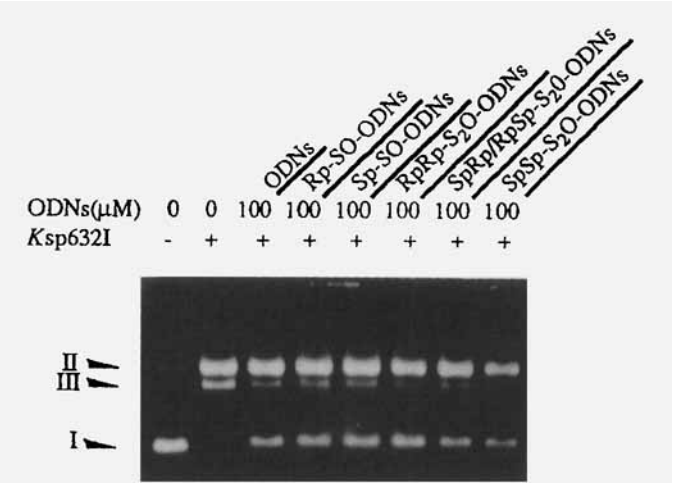


Figure 5. Inhibition of restriction enzyme *Ksp* 632-I by the phosphorothioate oligonucleotide analogues. Enzymatic assays were performed by incubating the plasmid pBR 328 (6 nM) at 37°C for 1 h with *Ksp* 632-I (20 units/μL) in the presence of 100 μM oligonucleotides in a buffer containing 33 mM Tris-acetate (pH 6.9), 66 mM potassium acetate, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM spermine.

Downloaded At: 18:04 26 January 2011

homopyrimidine phosphorothioate oligonucleotide having Rp. Selective binding of homopyrimidine phosphorothioate oligonucleotide analogues might modulate transcription either by interfering with the binding of regulatory protein factors or by preventing the elongation of RNA. DNA replication might be similarly inhibited. Furthermore, the end capped homopyrimidine oligonucleotide with PS groups at both 3'- and 5'-ends are more resistant to exonucleases than the unmodified oligonucleotide. This stabilization should help us to design much more efficient transcription or replication inhibitors which could be used as tools in cellular biology.

EXPERIMENTAL SECTION

Materials. The restriction enzyme *Ksp* 632-I and the plasmid pBR 328 were purchased from Boehringer-Mannheim. SV 40 DNA was purchased from GIBCO.

Oligonucleotide Synthesis. The homopyrimidine phosphorothioate oligonucleotide analogues (Rp-SO-ODNs, Sp-SO-ODNs, RpRp-S2O-ODNs, SpSp-S2O-ODNs, and SpRp-RpSp-S2O-ODNs) were synthesized with an Applied Biosystems DNA synthesizer using our new phosphite approach.[15] The oligonucleotide diastereoisomers were separated by reverse phase HPLC. The characterization of P-S bonds was shown using ^{31}P-NMR [(85% H_3PO_4 in D_2O as an external reference) Rp-SO-ODNs: 54.65, -1.17 ppm; Sp-SO-ODNs: 53.64, -1.75 ppm; RpRp-S2O-ODNs, SpSp-S2O-ODNs, and SpRp-RpSp-S2O-ODNs: 54.85 to 54.59, -1.56 to -1.75 ppm] spectroscopy.

Gel Electrophoresis. The duplex DNA was made by combining designated amounts (200 μM) of oligonucleotides in a 40 mM Tris-acetate (pH 7.0) buffer/100 mM NaCl/10 mM $MgCl_2$, keeping the mixture at 90°C for 10 min, and slowly cooling it to room temperature. The triplex DNA was made by the addition of an equimolar amount of the third strand to the duplex followed by incubation at 4°C for overnight. The concentration of each strand was 1.23 X 10^{-3} mM in a total vol. of 10 mL. Electrophoresis experiments were conducted using gels containing 15% polyacrylamide (acrylamide/bisacrylamide, 19:1) prepared in a Bio-Rad Protean II gel apparatus with 20 X 22 X 0.75 cm glass slabs. 90 mM Tris-borate//5 mM $MgCl_2$ (pH 8.3) was used in the electrophoresis reservoirs. Experiments were conducted at a constant temperature (4°C) and 200V for 16h. The gel was stained using methylene blue.

Inhibition of Restriction Enzyme Ksp 632-I by Phosphorothioate Oligonucleotides. Enzymatic assays were performed in a buffer containing 33 mM

Downloaded At: 18:04 26 January 2011

Tris-acetate (pH 6.9 or 7.9), 66 mM potassium acetate, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM spermine[11,16] at 37°C. The concentration of SV40 DNA and the plasmid pBR328 was usually 6 nM, and 20 units/μL restriction enzyme was used in each assay. After incubation, enzymatic reactions were stopped by the addition of EDTA (10 mM). Specimens were then analyzed by gel electrophoresis with slab gels (0.8% agarose). Densitometric analysis of gels stained with ethidium bromide was performed on a Milipore Bio Image 60S.

ACKNOWLEDGEMENT: This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas No. 03242104 and No. 05265220 from Ministry of Education, Science and Culture Japan and a Research Grant from the Human Research Sciences.

Downloaded At: 18:04 26 January 2011

REFERENCES

1. Rajagopal,P. and Feigon,J. (1989) *Biochemistry*, **28**, 7859-7870.
2. Santos,C.D.L., Rosen,M. and Patel,D. (1989) *Biochemistry*, **28**, 7282-7289.
3. Pilch,D.S., Brousseau,R. and Shafer,R.H. (1990) *Nucleic Acids Res.*, **18**, 5743-5750.
4. François,J.C., Saison-Behmoaras,T., Thoug,N.T. and Hélène (1989) *Biochemistry*, **28**, 9617-9619.
5. Maher,L.J., Wold,B. and Dervan,P.B. (1989) *Sicence*, **245**, 725-730.
6. Hanvey,J.C., Shimizu,M. and Wells,R.D. (1990) *Nucleic Acids Res.*, **18**, 157-161.
7. Xodo,L.E., Alunni-Fabbroni,M., Manzini,G. and Quadrifoglio,F. (1993) *Eur.J.Biochem.*,**212**, 395-401.
8. Kim,S.-G., Tsukahara,S., Yokoyama,S. and Takaku,H. (1992) *FEBS Letters*, **314**, 29-32.
9. Bolton,B.J., Schmitz,G.G., Jarsch,M., Conner,M.J. and Kessler,C. (1988) *Gene*, **66**, 31-43.
10. Reddy,V.B., Thimmappaya,B., Dhar,R., Subramanian,K.N., Zain,B.S., Pan,J., Ghosh,P.K., Celma,M.L. and Weissman,S.M. (1978) *Sicence*, **200**, 404-502.
11. Le Doan,T., Perrouault, L.Praseuth,D., Habhoub,N., Decout,J.L., Thuong,N.T., Lhomme,J. and Hélène,C. (1987) *Nucleic Acids Res.*, **15**, 7749-7760.
12. Praseuth,D., Perrouault,L., Le Doan,T., Chassignol,M., Thoug,N.T. and Hélène,C. (1988) *Proc.Natl.Acad.Sci.U.S.A.*, **85**, 1349-1353.

13. Kim,S.-G., Suzuki,Y., Nakashima,H., Yamamoto,N. and Takaku,H. (1991) *Biochem. Biophys.Res.Commun.*, **179**, 1614-1619.

14. Bolton,B.J., Schmitz,G.G., Jarsch,M., Conner,M.J. and Kessler,C. (1988) *Gene*, **66**, 31-34.

15. Hosaka,H., Suzuki,Y., Sato,H., Kim,S.-G. and Takaku,H. (1991) *Nucleic Acids Res.*, **19**, 2935-2940.

16. François,J.C., Saison-Behmoaras,T., Chassignol,M., Thoug,N.T. and Hélène,C. (1989) *J.Biol.Chem.*, **264**, 5891-5898.

Received 1/5/94
Accepted 2/7/94

Downloaded At: 18:04 26 January 2011