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Partial Purification and Cleavage Specificity of a Site-Specific Endonuclease, *Sci*NI, Isolated from *Spiroplasma citri*

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A site-specific endonuclease, *Sci*NI, has been partially purified from the plant pathogen *Spiroplasma citri*. The enzyme recognizes the sequence 5′-G-C-G-C-3′ and cleaves between the first G and C. 3′-C-G-C-G-5′ *Sci*NI is an isoschizomer of *Hha*I, but generates DNA fragments with 5′ rather than 3′ single-stranded protrusions.

The use of sequence-specific type II endonucleases has greatly facilitated the structural analysis of DNA molecules. Although type II endonucleases have been isolated from more than 90 different procaryotic species (9), none have previously been isolated from bacteria of the class *Mollicutes*. A site-specific endonuclease, *Tha*I, has been isolated from *Thermoplasma acidophilum* (8)–an organism originally believed to belong to the class *Mollicutes*—although recent studies have suggested that *T. acidophilum* is a member of the Kingdom Archaebacteria (5). The observation that certain strains of the plant pathogen, *Spiroplasma citri* (class *Mollicutes*, order *Mycoplasmatales*), were capable of restricting viruses (M. A. Stephens, 1979, *In* the Sixty-Ninth Annual Report of the John Innes Institute, Norwich, England, p. 88–89, 1978) suggested that site-specific endonucleases may exist in spiroplasmas. We report here the isolation from *S. citri* of a type II site-specific endonuclease, *Sci*NI, which recognizes and cleaves within the tetranucleotide sequence 5′-GCGC-3′.

MATERIALS AND METHODS

Growth of organisms. Cells of *S. citri* strain ASP2 (supplied by R. Townsend) were grown in SMC broth supplemented with 10% (vol/vol) fetal calf serum (16). The serum was filter sterilized (Millipore Corp., pore size, 0.22 μm) and heat inactivated (55°C for 45 min) before being added to the growth medium.

Purification of DNA. pBR322 DNA was purified by a method adapted from the cleared lysis procedure of Clewell and Helinski (3). Chromosomal DNA from strain ASP2 was purified from exponentially growing cells. Nucleic acids were released from the resuspended cell pellet by the cell lysis procedure of Scott and Ingle (11), and after phenol extraction the DNA was further purified by isopycnic centrifugation in cesium chloride in the presence of ethidium bromide (M. A. Stephens, Ph.D. thesis, University of East Anglia, England, 1980). Fragments of pBR322 DNA were eluted from agarose gels either by adsorbing the DNA to glass beads in the presence of NaI (17) or by adsorbing the DNA to hydroxyapitite (12). DNA was eluted from polyacrylamide gels by the procedure of Maxam and Gilbert (7).

Preparation of *Sci*NI active fraction. A culture of *S. citri* strain ASP2 was grown to the late exponential phase (8×10^8 colony-forming units per ml) in 2 liters of SMC broth. Cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. All further purification procedures were carried out at either 0 or 4°C. The cell pellet was resuspended in resuspension buffer (100 mM Tris-hydrochloride [pH 7.5], 7 mM β-mercaptoethanol, 7% [wt/vol] sorbitol, 10 mM $MgCl_2$) and the cells were disrupted by sonicating for six 10-s pulses. Cell debris was removed by centrifugation at 60,000 × *g* for 2 h, and NaCl was added to the resulting supernatant to a final concentration of 0.1 M. Nucleic acids were removed by precipitation with polyethyleneimine, and the proteins were concentrated by ammonium sulfate precipitation essentially as described by Bickle et al. (1). The ammonium sulfate pellet that resulted was suspended in 1 ml of column buffer (20 mM Tris-hydrochloride [pH 7.5], 0.5 mM EDTA, 7 mM β-mercaptoethanol), and after dialysis against 2,000 volumes of column buffer for 2 h and centrifugation at 8,000 × *g* for 15 min, the soluble extract was applied to a 2.5-ml column of heparin–Bio-Gel (1). After washing with column buffer, the material bound to the column was eluted with 50 ml of a linear 0 to 1 M NaCl gradient. Fractions (approximately 1 ml) were collected and assayed for a nuclease activity that cleaved pBR322 DNA. Active fractions were concentrated by dialysis against 200 volumes of storage buffer (20 mM Tris-hydrochloride [pH 7.5], 0.5 mM EDTA, 7 mM β-mercaptoethanol, 50 mM NaCl, 50% [vol/vol] glycerol) for 6 h at 0°C. Before storage at −20°C, bovine serum albumin was added to a final concentration of 100 μg/ml.

Enzyme assay and electrophoresis conditions. *Sci*NI activity was detected in fractions collected from the heparin–Bio-Gel column by incubating a 3-μl sample from every other fraction with 0.3 to 0.5 μg of pBR322 DNA in a final volume of 25 μl containing 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl (*Sci*NI buffer) for 5 to 11 h at 37°C. The reactions

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were heated to 65°C for 10 min, and after the addition of 1/10 volume of dye mixture (20% [wt/vol] Ficoll, 0.15% [wt/vol] bromophenol blue) the samples were electrophoresed into a 1% agarose gel. One unit of *Sci*NI activity was defined as that amount of enzyme required to digest 1 μg of pBR322 DNA to completion in 1 h at 37°C. Digestions with other restriction endonucleases were carried out under the conditions suggested by the respective manufacturer.

Agarose gels (20 by 20 by 0.3 cm), formed on glass plates, contained 1% (wt/vol) agarose dissolved in running buffer (40 mM Tris-acetate [pH 8.3], 20 mM sodium acetate, 1 mM tetrasodium EDTA) and were run at constant voltage (see legends to figures for details). Polyacrylamide gels, containing either 5 or 10% acrylamide (0.17 or 0.33% [wt/vol] bisacrylamide) in TBE buffer (90 mM Tris-borate [pH 8.3], 2.5 mM EDTA), were polymerized in vertical gel molds (20 by 18 by 0.4 cm). The running buffer was TBE buffer, and electrophoresis was at 12.5 V · cm^{-1} for 2 to 3 h. Polyacrylamide gels used in the sequencing of the *Sci*NI cleavage site were prepared and run as described by Maxam and Gilbert (7).

Gels were stained in a solution of ethidium bromide (1 μg · ml^{-1}), visualized under 365-nm UV light by using a "Blakray" transilluminator (Ultra-Violet Products, Inc.), and photographed onto Polaroid 665 positive-negative film. Radioactively labeled DNA fragments were visualized by autoradiography as described by Maxam and Gilbert (7).

Nucleotide sequencing and determination of cleavage site. The unique 375-base-pair (bp) *Eco*RI-*Bam*HI fragment from pBR322 was obtained by cleaving pBR322 simultaneously with *Eco*RI and *Bam*HI and purifying the fragment from an agarose gel. The fragment was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase essentially as described by Maxam and Gilbert (7). After cleavage with *Hae*III and electrophoresis into a polyacrylamide gel, the 174-bp *Eco*RI-*Hae*III fragment, labeled only at the *Eco*RI end, was purified and subjected to the base-specific chemical reactions of Maxam and Gilbert (7). The guanosine (G), guanosine plus adenosine (G+A), cytosine (C), and cytosine plus thymidine (C+T) reactions were used. The products were analyzed on a 0.5-mm-thick, 8% polyacrylamide gel. Determination of the site of cleavage was carried out as described by Brown and Smith (2).

Enzymes and chemicals. *Bam*HI, *Hae*III, *Hha*I, and *Taq*I were obtained from New England Biolabs; *Eco*RI and *Hin*dIII were from Miles Laboratories; T4 polynucleotide kinase was from Boehringer-Mannheim Biochemicals; T4 DNA polymerase and nuclease-free bovine serum albumin were from Bethesda Research Laboratories; and [γ-^{32}P] ATP was from New England Nuclear Corp.

RESULTS

Isolation of *Sci*NI active fraction. An extract from strain ASP2 was fractionated on a Heparin–Bio-Gel column and assayed for nuclease activity by using pBR322 DNA as substrate as described above. Electrophoretic analysis (Fig. 1) showed that intact pBR322 DNA was present in fractions 10 to 22 and 40 to 48. The multiple DNA bands from these fractions represent conformational isomers of the plasmid DNA. A nonspecific nuclease activity—which degraded the DNA without producing distinct bands—was detected in fractions 28 to 30, whereas a site-specific endonuclease, which cleaved the pBR322 DNA to produce a large number of relatively low-molecular-weight fragments migrating to the bottom of the gel, was detected in fractions 32 to 36 (eluting at 0.5 to 0.6 M NaCl). This site-specific endonuclease activity was given the name *Sci*NI, following the nomenclature suggested by Smith and Nathans (13).

The fractions containing the peak of *Sci*NI activity were pooled and concentrated, and a sample of this stock solution was incubated for various lengths of time with a fixed quantity of pBR322 DNA in either the presence or absence of Mg^{2+} ions. The reactions were incubated at 70°C for 10 min before electrophoretic analysis on a polyacrylamide gel (Fig. 2). More than 30 individual bands could be seen when the DNA was incubated for more than 30 min at 37°C in the presence of 10 mM Mg^{2+} ions. These bands were considered to be the result of a complete digestion of the pBR322 DNA since no additional bands appeared upon incubation for a longer time (Fig. 2) and with increasing amounts of enzyme (data not shown). Additional fragments of DNA of higher molecular weight, observed as bands of slower mobility, were visible when the reactions were heated before 30 min. These additional fragments apparently represented the products of partial cleavage. Their existence showed that the *Sci*NI cleavage activity was inactivated at 70°C. *Sci*NI was almost completely inactive in the absence of magnesium ions, a common characteristic of type II endonucleases.

Since there was complete digestion after 30 min in the presence of magnesium ions, the activity of the enzyme preparation was at least 4,000 U/ml. The presence of considerable nonspecific nuclease activity in the crude cell lysate precluded an estimation of the efficiency of extraction of the enzyme. More than 200 U of *Sci*NI, however, was isolated from liter of culture.

Determination of the *Sci*NI recognition site. Comparison of the sizes of the fragments produced by complete cleavage of pBR322 DNA with *Sci*NI with the sizes of fragments that would be produced by type II endonucleases of known specificities (14) suggested that *Sci*NI probably recognized the same nucleotide sequence as *Hha*I, i.e. 5′-GCGC-3′. Digests of pBR322 DNA with either *Sci*NI (Fig. 3, lane D) or *Hha*I (Fig. 3, lane C) produced identical digest patterns, and simultaneous cleavage of pBR322 with *Hha*I and *Sci*NI (lane B) did not produce any additional bands. Thus, *Sci*NI and

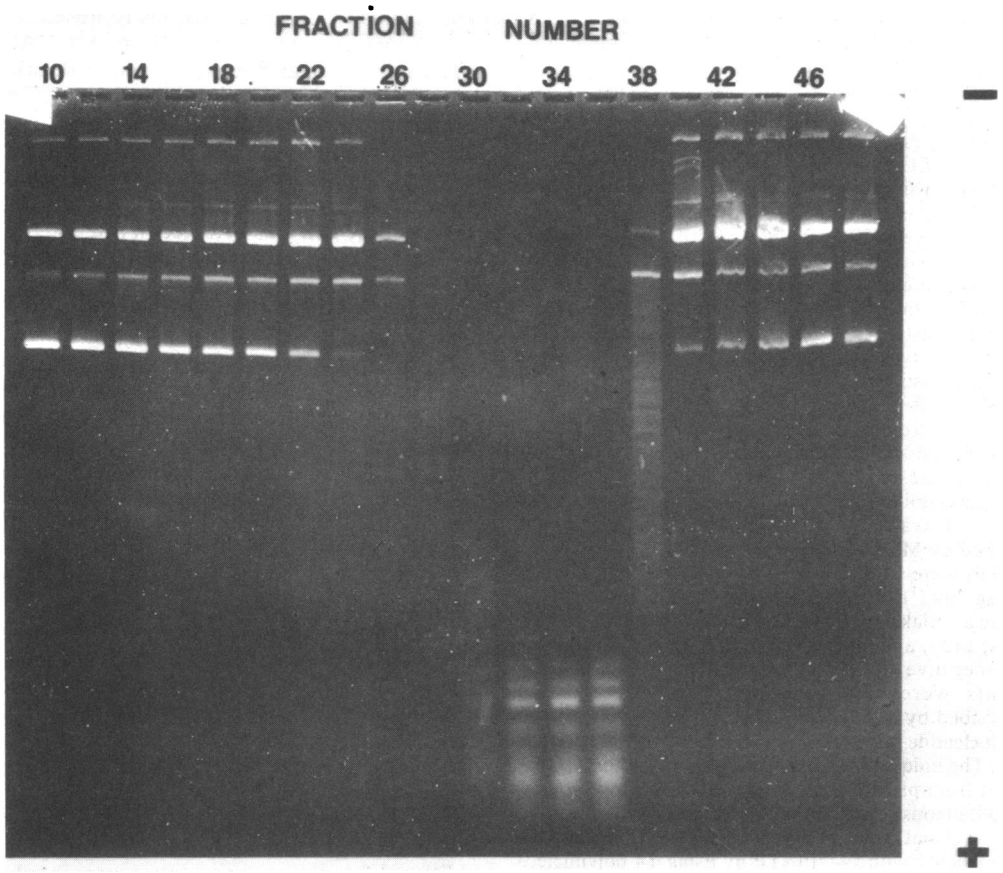

FIG. 1. Detection of *Sci*NI activity eluted from heparin–Bio-Gel column. *Sci*NI activity was detected by incubating the fractions eluted from the Heparin–Bio-Gel column with pBR322 DNA followed by electrophoretic analysis as described in the text.

*Hha*I were isoschizomers; i.e., they recognized the same nucleotide sequence. The appearance of distinct bands on the gels after the DNA samples had been extensively incubated with *Sci*NI suggested that there was very little nonspecific deoxyribonuclease contamination in the *Sci*NI enzyme preparation. Incubation of phage lambda DNA with *Sci*NI also produced the same pattern as that produced by *Hha*I cleavage (data not shown).

To confirm that *Sci*NI cleaves close to, or within, the sequence 5′-GCGC-3′, the enzyme was incubated with a specific, purified fragment of pBR322, the complete nucleotide sequence of which has previously been determined (15). *Sci*NI cleaved the 346-bp *Hin*dIII-*Bam*HI fragment from pBR322 to produce four smaller fragments approximately 132, 113, 73, and 28 bp in length (Fig. 4). Bands were also observed which corresponded to the intact 346-bp fragment and a 161-bp fragment produced as a result of partial cleavage at one of the *Sci*NI sites. The sizes of the fragments were those expected if *Sci*NI cleaved close to, or within, the sequence 5′-GCGC-3′, which occurs at three positions in the 346-bp *Hin*dIII-*Bam*HI fragment of pBR322.

Determination of the cleavage specificity of *Sci*NI. The exact position of cleavage of *Sci*NI at one of the sites on the 346-bp pBR322 fragment was determined by using the procedure of Brown and Smith (2). The 174-bp *Eco*RI-*Hae*III fragment from pBR322, labeled only at the *Eco*RI end, was partially sequenced (Fig. 5, lanes 1 through 4). A sample of this fragment, not subjected to the chemical sequencing reactions, was cleaved with *Sci*NI, and the products were electrophoresed alongside the sequencing reaction products (lane 5). A strand which appears as a result of chemical cleavage at a particular base has lost that base and ends with the nucleotide to the 5′ side of that base. The *Sci*NI-cleaved product had nearly the same mobility as a strand chemically cleaved to the 5′ side of a cytosine base (C) which lies in the sequence 5′-GCGC-3′. The *Sci*NI-cleaved fragment therefore ended in a G, showing that *Sci*NI

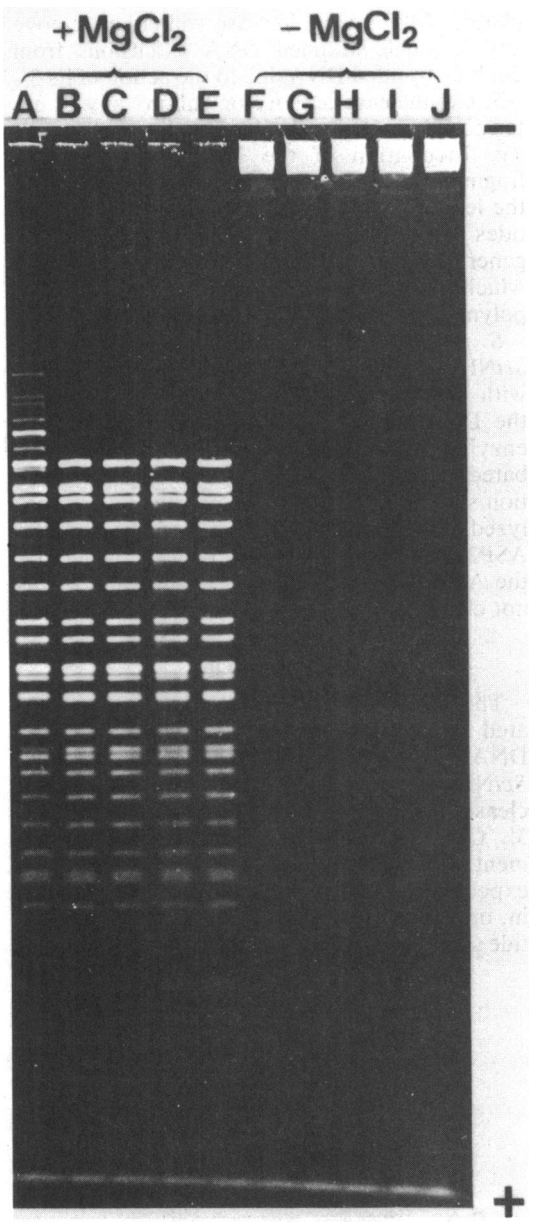

FIG. 2. Assay of *Sci*NI activity. Samples of pBR322 DNA were incubated with *Sci*NI (0.5 μl of *Sci*NI per μg of DNA) in the presence or absence of $MgCl_2$ (10 mM). At various times during the incubation (see below) samples were removed, heated to 70°C, and electrophoresed into a 5% polyacrylamide gel. Lanes: A and F, 15 min; B and G, 30 min; C and H, 60 min; D and I, 90 min; E and J, 20 min.

endonuclease cleaved the DNA between the first G and C. The *Sci*NI-cleaved strand migrated slightly slower than the corresponding chemical product, probably because the enzymatically cleaved fragment lacked the 3′ terminal phosphate which usually remains after chemical cleavage (2, 8).

Observations by autoradiography that there was very little radioactive label released from the end-labeled fragment when incubated with *Sci*NI suggested that there was very little contaminating phosphatase or 5′-exonuclease activity in the *Sci*NI preparation. It was not determined whether there was any 3′ exonuclease in the preparation.

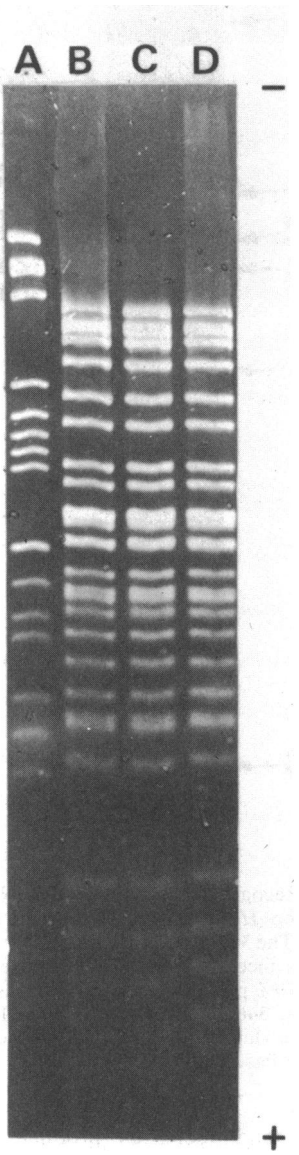

FIG. 3. Recognition specificity of *Sci*NI: single and double *Sci*NI and *Hha*I digests. Samples of pBR322 DNA were incubated with the following endonucleases and electrophoresed into a 10% polyacrylamide gel. Lanes: A, *Hae*III (size standards, see Fig. 4 for sizes); B, *Hha*I and *Sci*NI; C, *Hha*I; D, *Sci*NI.

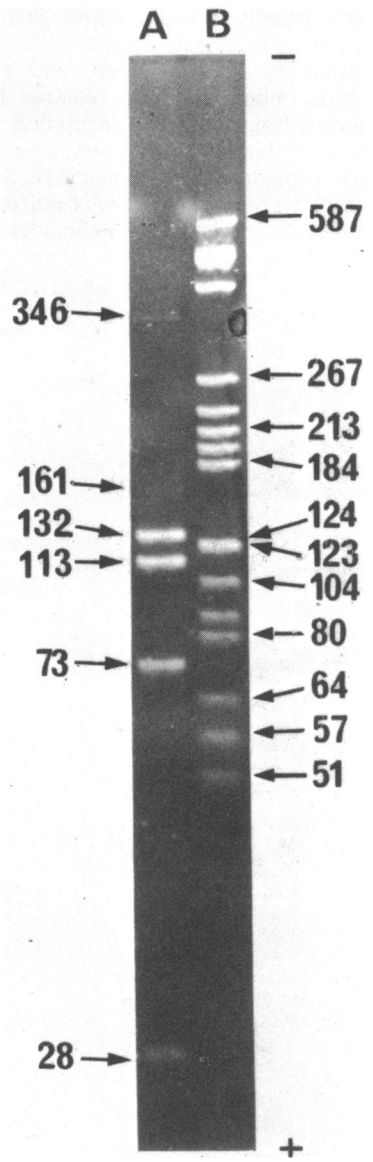

FIG. 4. Recognition specificity of *Sci*NI: cleavage of the 346-bp *Hin*dIII-*Bam*HI fragment of pBR322 with *Sci*NI. The 346-bp *Hin*dIII-*Bam*HI fragment from pBR322 was incubated with *Sci*NI and electrophoresed into a 10% polyacrylamide gel. Lanes: A, *Sci*NI digest of the 346-bp fragment; B, *Hae*III digest of pBR322 (size standards). The sizes of the fragments are shown in base pairs.

Type II endonucleases produce fragments with 5′ or 3′ single-strand extensions or flush-ended fragments. The cleavage position on the unlabeled strand was determined to establish the type of end produced by *Sci*NI. Under the appropriate incubation conditions, when provided with all four deoxyribonucleotide triphosphates, T4 DNA polymerase will either remove any 3′ single-stranded DNA extensions from double-stranded DNA due to the action of its 3′->5′ exonuclease activity or "fill-in" any 5′ extensions by utilizing its 5′->3′ polymerase activity. Incubation of the 102-bp *Eco*RI-*Sci*NI fragment with T4 DNA polymerase increased the length of the labeled strand by two nucleotides (Fig. 5, lane 6), showing that the *Sci*NI-generated end possessed a two-base 5′ extension which could act as a template for the 5′->3′ polymerase activity of T4 polymerase.

***S. citri* DNA is protected against cleavage by *Sci*NI.** A sample of ASP2 DNA was incubated with *Sci*NI endonuclease to determine whether the DNA was susceptible to cleavage by this enzyme. As a control, the DNA was also incubated separately with *Hha*I and *Taq*I (recognition site, 5′-TCGA-3′). The products were analyzed electrophoretically (Fig. 6). Although the ASP2 DNA was cleaved by *Taq*I, showing that the ASP2 DNA could act as a substrate, it was not cleaved by either *Sci*NI or *Hha*I.

DISCUSSION

The number and sizes of the fragments generated when pBR322 DNA and lambda phage DNA were cleaved by *Sci*NI suggested that *Sci*NI was an isoschizomer of *Hha*I, an endonuclease which recognizes the sequence 5′-GCGC-3′. Cleaving the 346-bp *Hin*dIII-*Bam*HI fragment from pBR322 generated the fragments expected if *Sci*NI recognized and cleaved within, or very close to, this sequence. The nucleotide sequencing of one of these sites showed that

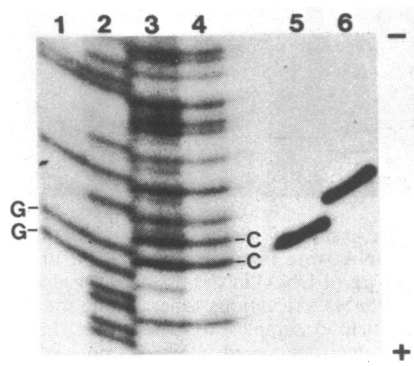

FIG. 5. Determination of the cleavage specificity of *Sci*NI. The 5′ ^{32}P-labeled *Eco*RI-*Hae*III fragment described in the text was subjected to the chemical sequencing reactions and electrophoresed into an 8% polyacrylamide gel. Lanes: 1, cleavage at G; 2, cleavage at G+A; 3, cleavage at C+T; 4, cleavage at C; 5, *Eco*RI-*Hae*III fragment cleaved with *Sci*NI; 6, *Eco*RI-*Hae*III fragment cleaved with *Sci*NI and incubated with T4 DNA polymerase.

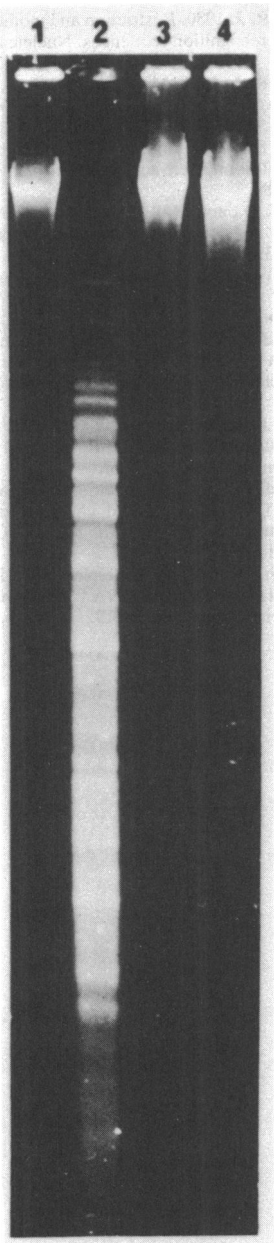

FIG. 6. ASP2 DNA is protected against cleavage by *Sci*NI. Samples of ASP2 DNA (1 μg) were incubated (lanes: 1, without endonuclease; 2, with *Taq*I endonuclease; 3, with *Sci*NI endonuclease; 4, with *Hha*I endonuclease) and electrophoresed into a 1% agarose gel.

*Sci*NI cleaved the tetranucleotide sequence between the first G and C and produced a fragment which ended with a 5′ single-strand extension two nucleotides in length. In contrast with *Sci*NI, *Hha*I endonuclease (as well as another isoschizomer of *Hha*I, endonuclease *Fnu*DIII) has been shown to cleave the recognition sequence 5′-GCGC-3′ between the second G and C, thereby generating fragments ending with 3′ single-strand extensions two nucleotides in length (6, 10). The cleavage specificities of two other isoschizomers of *Hha*I, namely, *Mnn*IV and *Cfo*I (9), have not been reported. *Sci*NI therefore appears to have a cleavage specificity which is unique among those thus far characterized.

*Sci*NI did not cleave DNA from strain ASP2, even though the sequence 5′-GCGC-3′ should occur (on a random basis) approximately 300 to 400 times in a genome of 10^9 daltons with a guanine plus cytosine content of 25% (Stephens, Ph.D. thesis). It is likely, therefore, that the *Sci*NI sites are protected by base modification. Although protection against cleavage due to a general, nonspecific modification of the DNA cannot be ruled out, it is likely that a corresponding *Sci*NI modification enzyme exists. It is interesting that *Hha*I also does not cleave ASP2 DNA, suggesting that the *Sci*NI modification activity also protects against cleavage by *Hha*I. In the reciprocal experiment *Sci*NI endonuclease did not cleave *Haemophilus haemolyticus* DNA (R. J. Roberts, personal communication).

Spiroplasmas have been shown to be susceptible to infection by virus particles and naked viral DNA (4; A. Liss and R. M. Cole, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, G22, p. 86). Although classical restriction and modification of viruses have been observed in some strains of *S. citri*, no correlation of the *Sci*NI activity with any restriction and modification system has been established (Stephens, Ph.D. thesis). It is likely, therefore, that as well as *Sci*NI, there are other site-specific endonucleases present in *S. citri* strains and in the variety of spiroplasma species which have recently been isolated.

In summary, *Sci*NI is the first site-specific endonuclease to be isolated from a member of the class *Mollicutes*. The endonuclease is easily purified to a stage relatively free of nonspecific nucleases and appears to recognize all of the sites that are recognized by *Hha*I, but complements *Hha*I in that it produces fragments with 5′ extensions. The enzyme is likely to be a useful alternative to *Hha*I for the structural analysis of DNA.

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