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Selective Protection of 5′ . . . GGCC . . . 3′ and 5′ . . . GCNGC . . . 3′ Sequences by the Hypermodified Oxopyrimidine in *Bacillus subtilis* Bacteriophage SP10 DNA

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The DNA of *Bacillus subtilis* bacteriophage SP10 is partially resistant to cleavage and methylation in vitro by restriction enzyme R · *Bsu*RI and its cognate methylase even though >20 copies of the target sequence, 5′. . . GGCC . . . 3′, are present on the phage genome. YThy, a hypermodified oxopyrimidine that replaces a fraction of the thymine residues in SP10 DNA, was responsible for this protection, since YThy-free DNA was no longer resistant. Sites that were normally resistant could nevertheless be cleaved or methylated in vitro if the salt concentration was reduced or dimethyl sulfoxide was added to the reaction buffer. Analysis of the termini produced by cleavage suggested that resistant sites occurred in the sequence 5′ . . . GGCC-YThy . . . 3′, whereas sensitive sites, of which there were only two per genome, occurred in the sequence 5′ . . . GGCCG . . . 3′. These in vitro results provide an explanation for the in vivo resistance of SP10 to restriction-modification by *B. subtilis* R. They also suggest ways in which the presence of the atypical base YThy in regions that flank the target might upset critical DNA-enzyme interactions necessary to locate and recognize the specific site of cleavage or methylation. YThy also strongly protected 5′ . . . GCNGC . . . 3′ (R · *Fnu*4HI) sequences on SP10 DNA, but the biological relevance of this protection is unclear.

Restriction endonucleases cut DNA at sites defined by a specific base sequence (31). The effective target for these nucleases includes not only the specific site of cleavage but also the nonspecific flanking regions into which the enzyme initially binds. After binding, the enzyme locates a cut site via a one-dimensional random walk; if no cut site is located within some finite period of time, the enzyme disengages from the DNA (19, 40). Therefore, conditions outside the site of cleavage strongly influence the extent of the random walk and, consequently, the probability that a given site will be located within a given DNA-binding event. A particularly striking example of this principle is the cleavage of coliphage lambda DNA by R · *Eco*RI (32). In this case, the rightward-most site is cut 10 to 12 times more rapidly than the leftward-most site, which in turn suggests that certain ensembles of the four conventional bases can render potential cut sites virtually inaccessible to the restriction endonuclease.

Atypical bases play an important role in protecting potential cut sites from attack. A completely nonspecific type of protection is manifested by DNAs containing atypical bases that are further modified through the addition of carbohydrate material. Such DNAs occur in the T-even coliphages (37) and *Bacillus subtilis* bacteriophage SP15 (2, 10). Basically, the high amount of carbohydrate constitutes a steric barrier that is impenetrable to practically all restriction endonucleases tested, regardless of sequence specificity (17, 20, 36). The occurrence of a protecting base inside the recognition sequence represents a more specific mechanism. Typically, the protecting base is N^6-methyladenine or 5-methylcytosine, generated by a methyltransferase with the same sequence specificity as the restriction endonuclease (31). However, other atypical bases occasionally serve in this capacity. For instance, when grown by lysogenic induction, coliphage Mu hypermodifies 20% of the adenines in its DNA (13). Hypermodification is limited to the general sequence 5′ . . . (G,C)A(G,C)N(T,C) . . . 3′ (13), which happens to overlap the recognition sequence of several restriction endonucleases (1).

Recently, evidence for yet a third protective mechanism has come to light (3). Here, protection of specific sites is effected by an atypical base residing outside the recognition sequence. The existence of such a mechanism attests to the importance of nonspecific enzyme-DNA interactions, inasmuch as bases residing outside the specific site of cleavage can protect it only by upsetting such interactions.

This communication deals with the latter mechanism as it applies to *B. subtilis* phage SP10 (33). The oxopyrimidine fraction of mature SP10 DNA has two components (37, 39). The major component, accounting for 85% of the total oxopyrimidine, is thymine, and the minor component is an incompletely characterized hypermodified analog, designated YThy. Acid digests of SP10 DNA contain thyminyl-*N*-pyroglutamate, but this is the breakdown product of a more complicated structure (M. Mandel, personal communication). Both oxopyrimidines in mature SP10 DNA are synthesized by postreplicational modification of 5-hydroxymethyluracil (HOMeUra) via its pyrophosphoric acid ester (39).

During the course of our investigations, we noticed that SP10 phage containing unmethylated DNA propagated normally in *B. subtilis* R, which contains a restriction-modification system specific for the sequence 5′ . . . GGCC . . . 3′ (4, 5, 12), even though preliminary data showed that these sequences do occur on phage DNA. It was decided to determine whether YThy protected 5′ . . . GGCC . . . 3′ sequences on SP10 DNA even though the atypical base cannot be part of the target sequence.

The results indicate that, indeed, YThy strongly protects such sequences both in vivo and in vitro. Protection is conditional in vitro and can be bypassed, to a limited degree,

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by reaction conditions similar to those that reduce the sequence specificity of at least some restriction endonucleases (11, 16, 28, 34). Of all other target sequences tested, only 5′ . . . GCNGC . . . 3′ sequences were strongly protected.

Phage and bacteria. *B. subtilis* RM125 (*nonA1 hsrM1 hsmM1 leuA8 argA15 att*$^{SP\beta}$) is a nonrestricting, nonmodifying derivative of *B. subtilis* Marburg 168 (35); it was obtained from T. Uozumi. *B. subtilis* R (*hsrR*$^+$ *hsmR*$^+$) was provided by T. Trautner, and its restriction-modification properties are well documented (3, 4, 12). SP10wt is the clear-plaque variant described in our earlier studies (summarized in reference 37). Two heat-sensitive mutants of phage S10 used in this study will be described below.

Buffers. Buffer D contained 10 mM Tris-hydrochloride (pH 7.9), 10 mM NaCl, and 0.1 mM disodium EDTA (pH 7.9). Buffer RO contained 10 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, and 100 μg of bovine serum albumin per ml. Buffer PB contained 10.0 mM Tris acetate (pH 7.9), 4 mM sodium acetate, 2 mM NaCl, 0.2 mM disodium EDTA (pH 7.9), 8% (wt/vol) sucrose, and 0.125% (wt/vol) bromphenol blue. Buffer E contained 50 mM Tris acetate (pH 7.9), 2 mM sodium acetate, 1 mM NaCl, and 0.1 mM disodium EDTA (pH 7.9).

Enzymes and fine chemicals. All radioactive chemicals were obtained from New England Nuclear Corp., Boston, Mass. Unlabeled materials were obtained from Sigma Chemical Co., St. Louis, Mo. Pancreatic DNase I, calf intestine phosphatase, and snake venom phosphodiesterase were the products of Boehringer Mannheim Biochemicals, Indianapolis, Ind. R · *Hae*III, R · *Fnu*4HI, and λ exonuclease were supplied by New England Biolabs, Beverly, Mass. Endonuclease R · *Bsu*RI (R · *Bsu*RI) was partially purified as previously described (5). R · *Bsp*I (22) was obtained from B. Nichols, University of Illinois at Chicago. DNA methyltransferase from *B. subtilis* R (M · *Bsu*R) was partially purified by the phosphocellulose chromatography method of Hattman et al. (14). M · *Bsu*R prepared in this manner methylated only the internal cytosine of 5′ . . . GGCC . . . 3′ sequences (not shown), which agrees with published results (12). Exonuclease I was purified from *Escherichia coli* as previously described (41). Di- and trideoxynucleotide markers were obtained from Collaborative Research, Inc., Waltham, Mass.

Isolation of phage DNA. Phage were propagated in cells grown in MS2 broth at 37°C (39). The viruses were concentrated by the salt-polyethylene glycol procedure (42), followed by banding in discontinuous CsCl gradients (6). Phage were dialyzed against buffer D to remove CsCl. Sarkosyl was added to the preparations to 1% (wt/vol), and the phage were incubated at 37°C for 2 h. Detergent was removed by overnight dialysis against buffer D. Pronase (100 μg/ml) was added, and the mixture was incubated at 37°C for 3 h. After three extractions with phenol-$CHCl_3$-isoamyl alcohol (25:24:1), the DNA was precipitated with ethanol, dissolved in buffer D (300 to 500 μg/ml), and dialyzed extensively against the same buffer. DNA was stored at −20°C.

Cleavage of DNA with restriction endonucleases. Reaction mixtures (60 μl) contained 2 μg of DNA and 4 U of enzyme in buffer RO plus the desired concentration of NaCl. The reaction mixtures were incubated at 37°C for the desired interval of time, and reactions were terminated by heating at 65°C for 5 min. After the addition of 20 μl of buffer PB, a 50-μl portion of each reaction mixture was electrophoresed through vertical slabs (20 by 20 cm) of 0.75% (wt/vol) agarose. Buffer E was the electrophoresis buffer.

DNA methyltransferase assay. The standard reaction contained (in 500 μl) 5 μmol of Tris-hydrochloride (pH 7.9), 5 μmol of NaCl, 0.1 μmol of dithiothreitol, 5 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (15 Ci/mmol), 25 μg of *B. subtilis* RM125 DNA, and 50 μg of protein. The reaction mixtures were incubated at 37°C for 1 h. The amount of label transferred to the DNA was determined (14). Unlabeled reaction mixtures contained 120 μM *S*-adenosylmethionine.

Labeling of 5′ termini with ^{32}P. DNAs were restricted as described above except that all quantities were increased 20-fold. After restriction, the DNA was purified and end labeled with T4 polynucleotide kinase as previously described (23). Protein was extracted with phenol, and unreacted ATP was removed by centrifugation through 0.9-ml Sephadex G50 columns (23). The fragments were eluted with 100 μl of 20 mM Tris-hydrocholoride (pH 7.5)–1 mM disodium EDTA (pH 7.5)–20 mM NaCl.

Digestion with pancreatic DNase I and *E. coli* exonuclease I. The Sephadex G50 column eluate was diluted threefold with buffer containing DNase I. The final conditions were 10 mM Tris-hydrochloride (7.5)–0.3 mM disodium EDTA (pH 7.5)–6.7 mM NaCl–10 mM $MgCl_2$–50 μg of DNase I per ml. The reaction mixtures were incubated at 30°C for 18 h; an additional 10 μg of DNase I was added, and incubation was continued for another 3 h (14, 15). The mixtures were heated at 65°C for 5 min and then chilled. Glycine buffer (pH 9.2) was added to 100 mM, and exonuclease I (41) was added. The reaction mixtures were incubated at 37°C for 3 h, neutralized with HCl, and frozen at −20°C until used.

Separation of oligodeoxynucleotides by chain length. Frozen digests were thawed and diluted fivefold with distilled water. The sample was applied to a 1- by 12-cm column of DEAE-cellulose (in water). The column was washed with 3 bed volumes of 10 mM lithium acetate (pH 5.3). The isostichs were fractionated by elution with a 250-ml linear gradient of LiCl, 0 to 300 mM in 10 mM lithium acetate (pH 7.3). All column elution buffers contained 7 M urea (7, 27). The di- and trideoxynucleotide fractions were pooled separately, desalted (14), and dried in vacuo. Exonuclease I digests DNA in the 3′ → 5′ direction and reportedly conserves the 5′-terminal dideoxynucleotide. Our digests, however, routinely contained roughly equal amounts of 5′-terminal di- and trideoxynucleotides (not shown). Others have reported the persistence of trideoxynucleotides in exonuclease I digests (14).

Fractionation of di- and trideoxynucleotides by base composition. Residues of the pooled DEAE-cellulose column fractions were dissolved into 50 μl of distilled water that contained authentic markers. Duplicate 20-μl portions were ionophoresed in one direction on DE81 paper at pH 2 (25). The position of the markers was determined by scanning under UV light. Fractions (1 by 1 cm) of each lane were cut out and counted for radioactivity. When necessary, oligonucleotides were eluted from the paper with 1 ml of 2 M triethylammonium bicarbonate (pH 9.5), dried in vacuo, and dissolved in 10 to 20 μl of distilled water.

Digestion with snake venom phosphodiesterase. A 10-μl portion of oligonucleotide eluted from DEAE-cellulose columns or DE81 paper was diluted into 90 μl of 50 mM Tris-hydrochloride (pH 7.9)–10 mM $MgCl_2$. For complete digestion, 20 μg of enzyme was present, and the reaction mixtures were incubated at 37°C for 3 h. For partial digestion, 2.0 μg of enzyme was present; samples (10 μl) were removed after 0, 1, 2, 5, 10, and 30 min of incubation. The samples were

subjected to either two-dimensional chromatography on thin layers of unmodified cellulose (9) or one-dimensional ionophoresis on DE81 paper.

Identification of 5'-terminal deoxynucleotide. Unrestricted and restricted DNAs were labeled at their 5' termini with ^{32}P. After purification (23), the DNAs were incubated in 50 μl of reaction mixture with λ exonuclease at 37°C for 30 min (29). After being diluted to 1 ml with water, the reaction mixtures were passed over a 1-cm column of Norit A. The column was washed with 5 ml of water, and nucleotydic material was eluted with 2 ml of water-ethanol-concentrated NH_4OH (30:67:3). The eluate was evaporated to dryness in vacuo, and the residue was redissolved in 10 μl of ethanol-ammonia containing marker nucleotides. Mononucleotides were fractionated by two-dimensional chromatography on thin layers of unmodified cellulose. Material corresponding to the markers was eluted from the chromatograms (6), and the radioactivity was counted. Controls indicated that ≥99% of the input ^{32}P cochromatographed with the optical markers.

Isolation of unhypermodified phage DNA. The heat-sensitive mutants of phage SP10 produce unhypermodified DNA at nonpermissive temperatures (manuscript in preparation). Briefly, the first round of replication produces hybrid duplexes comprising a parental hypermodified strand hydrogen bonded to a nascent unhypermodified strand. Thereafter, the phage continue to replicate their DNA, producing homoduplexes composed of two unhypermodified strands. Under nonpermissive conditions, DNA synthesis in the mutants proceeds at only 20% of the normal rate, but lysis is delayed by several hours; as a result, large quantities of the homoduplexes accumulate. Since virtually all cellular DNA is hydrolyzed to acid-soluble fragments (24), the homoduplexes can be isolated in pure form by straightforward procedures (6). One mutant produces homoduplexes containing HOMeUra as the sole oxopyrimidine, whereas the other mutant generates homoduplexes in which thymine is the sole oxopyrimidine; these are referred to as *h*-DNA and *t*-DNA, respectively.

RESULTS

Effect of conditions on in vitro cleavage of SP10wt · RM125 DNA by R · *Hae*III (R · *Bsp*I, R · *Bsu*RI). SP10wt · RM125 DNA used in this study had the following nucleotide composition: dGMP, 21 mol%; dCMP, 21 mol%; dAMP, 29 mol%; dTMP, 24 mol%; and YdTMP, 5 mol%. There was no detectable methyl dAMP, methyl dAMP, or methyl dCMP (not shown). The reported experiments were repeated with all three enzymes. Except where noted, the data obtained with R · *Hae*III are presented.

R · *Hae*III was routinely assayed in buffer containing 10 mM NaCl. At this NaCl concentration, the standard amount of enzyme (2 U/μg of DNA) produced 20 to 25 fragments (Fig. 1). When the NaCl concentration was raised to 20 mM, several lower-molecular-weight bands disappeared and were replaced by their presumably higher-molecular-weight precursors. No further changes in the pattern were evident until the NaCl concentration exceeded 50 mM. NaCl concentrations ≥100 mM evidently limited cleavage to only two sites.

With the standard amount of nuclease, the two-site cleavage observed at higher NaCl concentrations was complete within 45 s, and enzyme levels as high as 128 U/μg of DNA yielded only the two-site cleavage (not shown). Under conditions favoring more extensive cleavage, the data obtained with 10 mM NaCl were typical. Within 1 min, the standard nuclease concentration cut SP10wt DNA into the same three fragments as did NaCl concentrations ≥100 mM

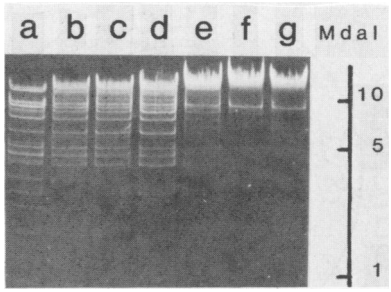

FIG. 1. Cleavage of SP10wt · RM125 DNA at various NaCl concentrations. DNA was cut by R · *Hae*III in standard reaction mixtures that contained different concentrations of NaCl. The tubes were incubated at 37°C for 2.5 h. Buffer RO was supplemented with (lane) 10 (a), 20 (b), 35 (c), 50 (d), 75 (e), 100 (f), or 125 (g) mM NaCl. Mass is indicated in megadaltons (Mdal).

(Fig. 1, lanes a through c; unpublished data). Between 1 and 15 min, no new fragments appeared (Fig. 2, lanes c and d). Thereafter, additional fragments became progressively more evident, and the reaction was complete by 90 min (Fig. 2, lanes e through j). Thus, complete fragmentation of SP10wt DNA at 10 mM NaCl was a complex reaction composed of two kinetically distinguishable sets of cleavages, i.e., a rapid two-site cleavage, equivalent to the only reaction observed at higher salt concentrations, and a more general but slower series of cuts unique to lower salt concentrations. Hereafter, the two sites that reacted at all NaCl concentrations are referred to as class I sites. Sites uniquely reactive at NaCl concentrations ≤50 mM are referred to as class II sites.

Cleavage of unhypermodified SP10 by R · *Hae*III. At 10 and 150 mM NaCl, both unhypermodified varieties of SP10 DNA were cleaved identically, and at the standard nuclease concentration, fragmentation was complete within 20 min (Fig. 3). Therefore, the absence of the hypermodified base eliminated the salt effect and rendered the class II sites more reactive.

In vivo and in vitro methylation of SP10 DNA. SP10wt · R phage were prepared by single-cycle lysis of *B. subtilis* R. DNA extracted from these phage proved to be resistant to R · *Bsu*RI in 150 mM NaCl, but in 10 mM NaCl, this DNA was still cut many times (Fig. 4). By all the criteria described above, SP10wt · RM125 and SP10wt · R DNAs were equivalent substrates except that the latter failed to show any fragments characteristic of class I cut sites (Fig. 4; unpublished data), implying that protective methylation in vivo was limited to that class.

The same dichotomy was found to exist in vitro with respect to methylation by M · *Bsu*R. SP10wt · RM125 DNA was incubated with M · *Bsu*R and either 10 or 150 mM NaCl. The DNA was reisolated and tested for its sensitivity to R · *Bsu*RI under conditions that normally permit cleavage at both site classes (10 mM NaCl) and conditions that normally limit cleavage to class I sites (150 mM NaCl). DNA methylated at 10 mM NaCl was completely resistant to R · *Bsu*RI (Fig. 5, lanes a and b), but DNA methylated at 150 mM NaCl was resistant to nuclease only under conditions that limit cleavage to class I sites (Fig. 5, lanes c and d). In 10 mM NaCl, protective methylation of class I sites was complete within 10 min (not shown), whereas complete methylation of class II sites required 3 to 4 h. Again, differential methylation was a function of the atypical base since, regardless of the NaCl concentration, all cut sites on SP10 *h*-DNA and *t*-DNA

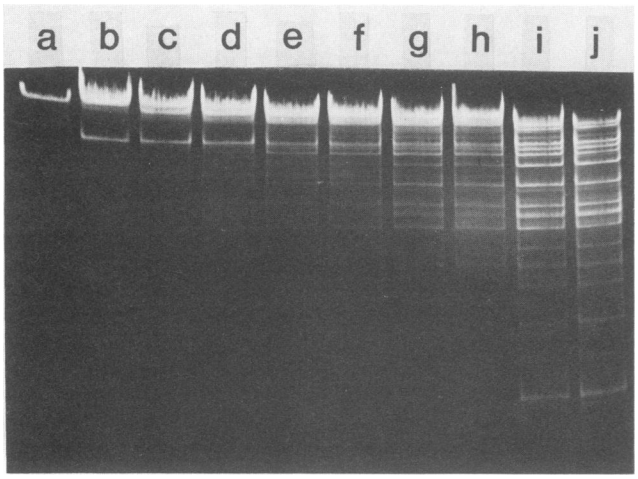

FIG. 2. Rate of cleavage of SP10wt · RM125 DNA in 10 mM NaCl. A scaled-up reaction mixture that contained the standard ratio of R · *Hae*III to DNA was set up and incubated at 37°C. Samples (60 μl) were removed at (lane) 0 (a), 0.5 (b), 1 (c), 15 (d), 30 (e), 45 (f), 75 (g), 90 (i), and 120 (j) min.

were protectively methylated within 25 min (Fig. 5, lanes e through h; unpublished data).

With radioactively labeled donor, SP10wt · R and SP10wt · RM125 DNAs accepted essentially equimolar amounts of methyl groups when incubated with M · *Bsu*R at 10 mM NaCl (not shown). Therefore, most M · *Bsu*R-specific sites on SP10wt · R DNA were evidently unmethylated.

Cleavage site of SP10 DNA by R · *Hae*III. The results presented above defined the conditions affecting the frequency with which an isoschizomeric series of endonucleases cleaved a particular DNA. Since a change in the frequency of cleavage could result from any of several causes (8, 11, 16, 28), it was deemed necessary to locate the site of cleavage on SP10 DNA.

DNA was cleaved with R · *Hae*III, and the 5′ termini were labeled with ^{32}P. A portion of the ^{32}P-oligonucleotides was digested with λ exonuclease to identify the 5′-terminal nucleotide. Under all conditions tested, dCMP was the only labeled nucleotide released from R · *Hae*III-created termini (not shown).

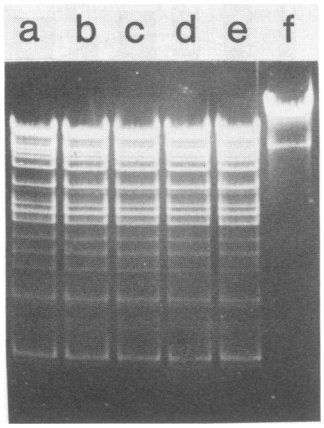

FIG. 3. Cleavage of unhypermodified SP10 DNAs by R · *Hae*III. All DNAs were cut in the standard mixture. Unhypermodified DNAs were incubated at 37°C for 20 min, whereas SP10wt · RM125 DNA was incubated for 2.5 h. Lanes: a, c, and e, 10 mM NaCl; b, d, and f, 150 mM NaCl; a and b, c and d, SP10 *t*-DNA; e and f, SP10wt · RM125 DNA.

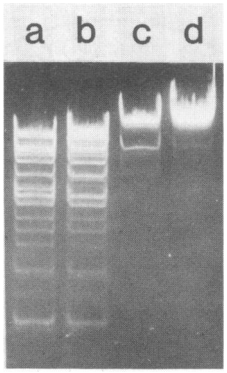

FIG. 4. Cleavage of SP10wt · R DNA by R · *Bsu*RI. DNA was incubated with enzyme under the standard conditions. Incubation was at 37°C for 2.5 h. Lanes: a and c, SP10wt · RM125 DNA at 10 (a) and 150 (c) mM NaCl; b and d, SP10wt · R DNA at 10 (b) and 150 (d) mM NaCl.

Another portion of the ^{32}P-oligonucleotides was enzymatically degraded to a collection of mononucleotides (unlabeled), ^{32}P-dinucleotides, and ^{32}P-trinucleotides, which were then separated by DEAE-cellulose chromatography. The latter two isostichs were independently fractionated into their component isopliths by one-dimensional ionophoresis on DE81 paper. Material from the natural termini migrated more slowly than any optical marker employed, and since the absolute amount of label recovered from this material did not vary with the degree of fragmentation by R · *Hae*III (data not shown), it is ignored in all subsequent discussions.

For the dideoxynucleotide isostich, more than 95% of the label unique to R · *Hae*III-created termini was recovered in the pCpC isoplith (not shown). A more complex picture was

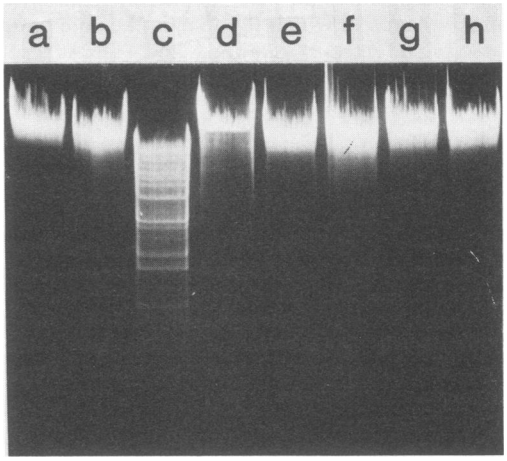

FIG. 5. Methylation of SP10 DNA in vitro by M · *Bsu*R. Parallel reaction mixtures containing 10 μg of DNA, 125 μM *S*-adenosylmethionine, and 20 U of M · *Bsu*R were set up. The NaCl concentration was either 10 or 150 mM. The reaction mixtures were incubated at 37°C for 5 h; prolonged incubation is necessary to ensure complete methylation at reduced NaCl concentrations (see text). Methylated DNA was reisolated and incubated with R · *Bsu*RI at either 10 or 150 mM NaCl. Lanes: a and b, SP10wt · RM125 DNA plus M · *Bsu*R in 10 mM NaCl then R · *Bsu*RI in either 10 (a) or 150 (b) mM NaCl; c and d, SP10wt · RM125 DNA plus M · *Bsu*R in 150 mM NaCl then R · *Bsu*RI in either 10 (c) or 150 (d) mM NaCl; e and f, SP10 *t*-DNA plus M · *Bsu*R in 10 mM NaCl then R · *Bsu*RI in either 10 (e) or 150 (f) mM NaCl; g and h, SP10 *t*-DNA plus M · *Bsu*R in 150 mM NaCl then R · *Bsu*RI in either 10 (g) or 150 (h) mM NaCl.

observed for the trinucleotide isostich (Fig. 6A and B). When SP10 *t*-DNA was cleaved with either 10 or 150 mM NaCl, virtually all of the label was recovered in the pCpCpT and pCpCpA isopliths. Since SP10 DNA contains neither R · *Stu*I nor R · *Bal*I target sequences (manuscript in preparation), these data suggest that the vast majority of the R · *Hae*III cut sites occur in a sequence equivalent to 5′ . . . TGGCCT . . . 3′/3′ . . . ACCGGA . . . 5′. When SP10wt DNA was cleaved at 10 mM NaCl, i.e., under conditions that allow cleavage at both class I and class II sites, approximately half the label was recovered in the pCpCpA isoplith, whereas most of the remaining label was recovered in a novel isoplith designated X (Fig. 6A). When X was subjected to limited digestion with snake venom phosphodiesterase, the initial product was indistinguishable from pCpC (not shown), implying that the unusual ionophoretic properties of X resided in its 3′-terminal nucleotide. When SP10 *h*-DNA was analyzed, an atypical isoplith, migrating between the pCpGpC and pCpTpC optical markers, was again observed (not shown). The simplest interpretation of these data is that the novel isopliths contain either YThy- or HOMeUra-based deoxynucleotides at their 3′ termini. When SP10wt DNA was cut at 150 mM NaCl, i.e., under conditions that limit cleavage to class I sites, label was recovered in the pCpCpA and pCpCpG isopliths (Fig. 6B). Overall, these data are consistent with the interpretation that, on SP10wt DNA, class I sites occur in the sequence 5′ . . . oxopyrimidine-GGCCG . . . 3′, whereas class II sites occur in the sequence 5′ . . . oxopyrimidine-GGCC-YThy . . . 3′. Since the 5′ proximal oxopyrimidine cannot be identified by the methods at hand, the presence of YThy at the 3′-proximal position is the minimum requirement for the protection of class II sites.

R · *Hae*III and its isoschizomers cut 5′ . . . GGCC . . . 3′ sequences at the axis of symmetry (4). The results presented above are consistent with the interpretation that these nucleases cut SP10 DNA at the normal target sequence and at the normal site of strand scission within that sequence.

Conditional cleavage of SP10wt · RM125 DNA by other restriction endonucleases. Of the nucleases thus far tested (manuscript in preparation), only one other, R · *Fnu*4HI, displayed conditional cleavage (Fig. 7). This nuclease cleaves at 5′ . . . GC ↓ NGC . . . 3′ sequences (31). End group analysis of SP10wt DNA cut with R · *Fnu*4HI at 10 mM NaCl gave the following distribution of nucleotides at the 5′ terminus: dGMP, 17.3%; dCMP, 18.9%; dAMP, 33.1%; dTMP, 30.2%; and hypermodified dTMP (YdTMP), 0.5%. Therefore, of the 40 or so R · *Fnu*4HI cut sites on SP10wt DNA, few, if any, contained YdTMP within the target sequence. Since both unhypermodified varieties of SP10 DNA were cleaved constitutively (not shown), the conditional response observed with SP10wt DNA must stem from YdTMP residues present outside the canonical target sequence.

Effect of DMSO on conditional cleavage. Dimethyl sulfoxide (DMSO), a helix destabilizer, affects the cleavage of DNA by several restriction endonucleases (8, 11, 16, 28, 34). The lower salt concentrations at which conditional sites become reactive are helix-destabilizing conditions as well. Therefore, it was of interest to see whether DMSO partially removed (alleviated) protection of these sites at 150 mM NaCl. For the trials shown here, cleavage was estimated from the amount of ^{32}P transferable to R · *Bsu*RI-treated DNAs. With SP10wt DNA, the cleavage frequency increased stepwise (Fig. 8A). The first wave represented protection alleviation, as evidenced by the facts that (i) the

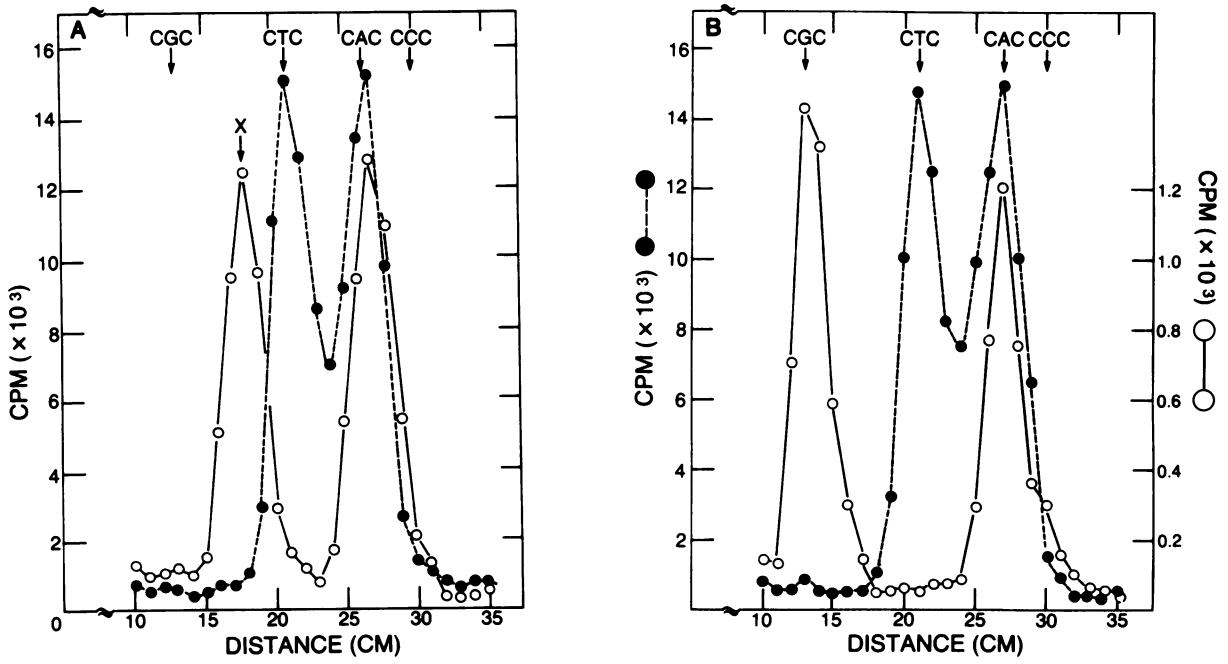

FIG. 6. Ionophoresis of 5′-^{32}P-labeled trideoxynucleotides derived from R · *Hae*III-created termini. Duplicate (20-μl) samples of each restricted DNA were spotted onto sheets of DE81 paper. The ionophoretograms were run in one direction at pH 1.9, basically as described by Murray (25). Fractions (1 cm^2) of each panel were cut out, and the amount of label was determined. The markers were pCpCpC, pCpGpC, pCpApC, and pCpTpC. Note that the latter three markers are positional isomers of the actual R · *Hae*III-specific 5′-terminal sequences; the analytical systems used should not separate positional isomers (25). Shown are trideoxynucleotides from DNA incubated with R · *Hae*III in 10 (A) or 150 (B) mM NaCl. Note the different scales on the ordinates. Symbols: ○, SP10wt · RM125 DNA; ●, SP10 *t*-DNA.

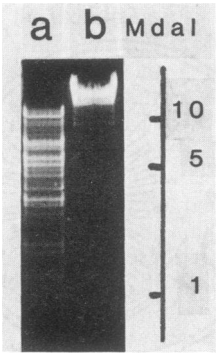

FIG. 7. Cleavage of SP10wt · RM125 DNA by R · *Fnu*4HI. DNA was restricted under standard conditions in 10 (a) or 150 (b) mM NaCl. Mdal, Megadaltons.

plateau value was identical to that observed with 10 mM NaCl alone (see below) and (ii) the nuclease-specific 5′-dideoxynucleotide was exclusively pCpC (Fig. 8B). The second wave represented specificity reduction, i.e., a situation in which cleavage can now occur at 5′ . . . NGCN . . . 3′ sequences in addition to the normal target sequence (16), since the pCpC isoplith now constituted progressively fewer of the total nuclease-created 5′ termini (Fig. 8B). Unhypermodified varieties of SP10 DNA showed only the specificity reduction characteristic of higher DMSO concentrations (Fig. 8A and B). When the experiment was repeated at 10 mM NaCl, only specificity reduction was observed, and this occurred even at low DMSO concentrations (Fig. 8C and D). For the sake of brevity, only the relative abundance of the pCpC isoplith is shown. Under conditions of specificity reduction, R · *Bsu*RI-created 5′ termini contained all possible pCpN isopliths for any given DNA. The distribution of label in these latter isopliths varied approximately with the relative abundance of the 3′-terminal nucleotide in the DNA (data not shown). Therefore, under conditions of specificity reduction, at least a portion of the pCpC isopliths must have originated from these atypical cleavages. Overall, these data suggest that protection alleviation and specificity reduction, although discrete events, are nevertheless manifestations of the same underlying phenomenon.

By using standard protocols (14), it was established that, under all conditions, the methylated cytosine (C*) produced by M · *Bsu*R was always present in the sequence 5′ . . . pCpC*pC . . . 3′ (not shown). Therefore, unlike its cognate endonuclease, R · *Bsu*R does not undergo specificity reduction. By the same token, R · *Hae*III, R · *Bsp*I, and R · *Fnu*4HI also failed to display specificity reduction (not shown).

DISCUSSION

SP10wt DNA contains 20 to 25 target sequences recognized by the *B. subtilis* R restriction-modification system. Of these, only two (designated class I) are efficiently restricted and methylated in vitro under physiologically significant conditions, whereas the remainder (designated class II) are protected, seemingly, by the immediate proximity of at least one YThy (Fig. 1 through 6 and 8).

B. subtilis phage H1 DNA, in which HOMeUra replaces thymine, has four R · *Bsu*RI target sites, all of which are protected under physiological conditions by the atypical base (3). One of these sites is present on a cloned fragment, and on H1 DNA, this site would occur in the sequence 5′ . . . CAHAACHHHGGCCHAG . . . 3′, where H is HOMeUra. Protection of this and the other sites on H1 DNA presumably stems from the high local density of HOMeUra (3). Interestingly, none of the R · *Bsu*RI targets on SP10 *h*-DNA were protected (Fig. 3, 5, and 8), even though HOMeUra is the sole oxopyrimidine (manuscript in preparation), and therefore most targets on *h*-DNA would be in the sequence 5′ . . . HGGCCH . . . 3′ (see below and Fig. 6). If HOMeUra protects R · *Bsu*RI targets only when it occurs at a density comparable to that observed for the cloned H1 site, the corresponding targets on SP10 DNA may not be surrounded by the same high density of oxopyrimidines, and as a result, none of the targets on SP10 *h*-DNA would be surrounded by a protective density of HOMeUra.

A lower density of oxopyrimidines in the vicinity of R · *Bsu*RI targets on SP10 DNA could mean that YThy is a more effective protector than HOMeUra and that correspondingly reduced levels of the hypermodified base suffice to protect these targets on SP10wt DNA. This helps explain why protected sites occur five to six times more frequently on SP10wt than on H1 DNA. Thus, if YThy protects at lower local densities than HOMeUra does, the probability that a

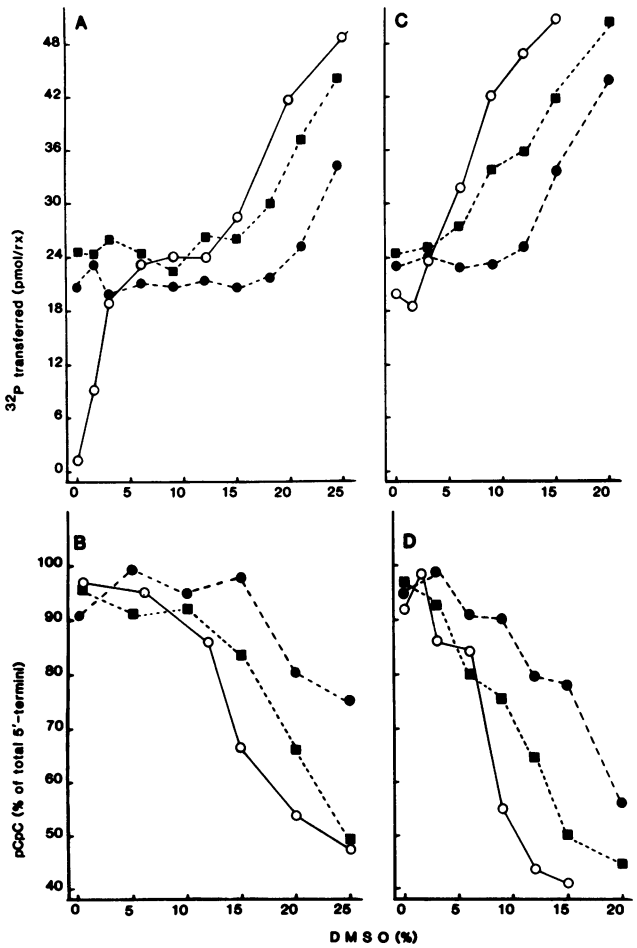

FIG. 8. Effect of DMSO on cleavage of DNA by R · *Bsu*RI. Standard reaction mixtures contained either 150 (A and B) or 10 (C and D) mM NaCl and the indicated concentration of DMSO. The mixtures were incubated at 37°C for 3 h. The amount of ^{32}P transferable to the polymer was determined, as well as the distribution of label among the 5′-terminal dideoxynucleotides. Symbols: ○, SP10wt DNA; ●, *t*-DNA; ■, *h*-DNA.

given target will be surrounded with an effective level of YThy is correspondingly increased, and consequently, more sites have been conserved on SP10wt DNA.

The overall rate at which a target site is cleaved depends largely on conditions prevailing outside the canonical recognition sequence (19, 28, 32, 40). Therefore it is highly significant that potentially restrictable sites on two unrelated phage DNAs are efficiently protected by atypical bases residing outside the recognition sequence. Atypical bases, with their known and putative effects on the conformational and electrostatic properties of DNA (37, 38), present in the flanking regions could upset critical DNA-nuclease interactions required to locate or recognize the target sequence, thereby protecting the site. Inasmuchas HOMeUra on H1 DNA (3) and YThy on SP10wt DNA (Fig. 6 through 8) protect 5′ . . . GGCC . . . 3′ sequences from methylation, it follows that these considerations hold equally well for M · *Bsu*R and, perhaps, other type II methylases.

It is difficult to gauge a priori what properties of HOMeUra and YThy are most important in the observed protection of 5′ . . . GGCC . . . 3′ sequences. Assuming that protected sequences are located in spans of DNA containing a high local density of an atypical oxopyrimidine, two not necessarily exclusive possibilities can be considered. (i) Both atypical bases project groups into the major groove of DNA that are larger and more hydrophilic than the methyl group of thymine (37; M. Mandel, personal communication). Relative to the normal substituent, the $-CH_2OH$ group is twice as large (3), and the known portion of the hypermodification in YThy is nearly six times as large. Due to their greater hydrophilicity, both atypical substituents would organize more water into the major groove. Larger substituent size coupled to increased hydration might reduce the extent to which those portions of the enzyme molecule responsible for sequence recognition can occupy the major groove. Accordingly, even if a target sequence is encountered, the enzyme may not be able to establish contacts that are sufficiently strong or numerous to effect strand cleavage or methylation under physiological conditions (ii) HOMeUra and, especially, YThy are helix destabilizers (37; unpublished data). Such destabilizing perturbations can be translated to adjoining regions to a degree dictated by the magnitude of the perturbation and neighboring base sequence, as well as by environmental factors such as ionic strength and the presence of exogenous helix destabilizers (37, 38). Consequently, protected regions should be underwound relative to unprotected regions. The resulting conformational irregularity could bias DNA-enzyme interactions as outlined above.

Studies with R · *Bsu*RI and SP10wt DNA suggest that protection alleviation and specificity reduction are related phenomena (Fig. 8 and attendant discussion). In specificity reduction, the requirement for the two end-proximal base pairs relaxes sufficiently to permit limited cleavage at 5′ . . . NGCN . . . 3′ sequences (16) (Fig. 8B and D). It is interesting to speculate that protection alleviation stems from a lower degree of specificity relaxation in which contact with the end-proximal base pairs is still mandatory but the contacts need not be as firmly established as under physiological conditions. If these suppositions are true in substance, all of the enzymes studied here can undergo the degree of specificity relaxation required for protection alleviation (Fig. 1, 5, and 8; unpublished data), but only R · *Bsu*RI, for whatever reasons, can undergo the additional relaxation necessary to achieve actual specificity reduction (cf. reference 16).

A key element in specificity relaxation seems to be the degree of helix unwinding at or near the site of reaction. At 150 mM NaCl, for instance, the amount of DMSO necessary to bring about protection alleviation was lower than the amount required to effect specificity reduction (3) (Fig. 8). Offhand, this and the fact that protection alleviation can be observed independently of specificity reduction imply that the former requires less unwinding than the latter. Although this is probably true, the differences may not be as great as the data would seem to indicate. Due to their destabilizing base content, regions adjacent to protected R · *Bsu*RI targets are especially sensitive to helix-destabilizing conditions (DMSO, reduced NaCl concentration). As a result, such regions would reach the requisite level of local unwinding under milder conditions than would regions with a more random distribution of bases, i.e., regions in which most of the abbreviated sequences probably reside. Exactly how partial unwinding of bihelical DNA can promote specificity relaxation remains to be seen.

Class I R · *Bsu*RI sites on SP10wt DNA were unprotected by YThy in vitro (Fig. 1, 2, 3, and 5) and in vivo (Fig. 4). Seeing that two presumably accessible sites exist on SP10wt DNA, how do intact phage escape restriction? When cut with R · *Hae*III in vitro at 150 mM NaCl, SP10wt · RM125 DNA rapidly lost its transfectability, implying that one or both scissions are lethal; moreover, mock-infected and SP10wt-infected cultures of strain R yielded similar levels of R · *Bsu*RI and M · *Bsu*R, so it is unlikely that this phage codes for an inhibitor (unpublished data). It has been suggested that DNA with a low incidence of cut sites tend to be resistant to restriction by strain R (26), and in fact the DNAs of many *B. subtilis* phage are characterized by an exceptionally low incidence of such sequences (18, 21, 30). In SP10wt, a low incidence of accessible sites seemingly favors protective methylation over restriction.

YThy also protects most, if not all, R · *Fnu*4HI recognition sequences on SP10wt DNA (Fig. 7 and attendant discussion). It is significant that, of enzymes representing more than 60 separate sequence specificities (manuscript in preparation), YThy simultaneously protects R · *Fnu*4HI and R · *Bsu*RI sequences, because *B. subtilis* phage in the SPβ serogroup automethylate their DNAs at these two sequences (26). Although both kinds of protection may serve to facilitate phage survival in certain restricting strains of bacteria, to date no potential host has been identified for these phage that has a system conspecific to R · *Fnu*4HI. Nevertheless, the fact that unrelated phages have evolved vastly different mechanisms to achieve the same end suggests that the simultaneous protection of both sequences is an important function.

Newly replicated phage SP10 DNA contains HOMeUra that is subsequently either reductively modified to thymine or hypermodified to YThy (39). Little is known about the relative distribution of thymine and YThy. The results presented here indicate that the distribution is nonrandom. Were the two oxopyrimidines randomly distributed, each oxopyrimidine site should contain thymine and YThy in the same relative proportions as global DNA does. End group analysis of R · *Fnu*4HI-created fragments showed that the central position of this sequence can contain thymine but rarely, if ever, YThy (Fig. 7 and attendant discussion); therefore, reductive modification occurs at these sites to the exclusion of hypermodification. The converse is true with class II R · *Bsu*RI cut sites in that most, if not all, of these sites occur in the sequence 5′ . . . oxopyrimidine-GGCC-YThy . . . 3′ (Fig. 6). Therefore, hypermodification of

SP10wt DNA, like the hypermodification of Mu DNA (13), may be a sequence-specific event.

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