Digestion of 5-Bromodeoxyuridine-substituted λ-DNA by Restriction Endonucleases

(Received for publication, May 11, 1978)

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5-Bromodeoxyuridine (BrdUrd) DNA from bacteriophage λ affects both the rates and sites of cleavage by Endo R•Eco RI, HindIII, and Sma I. Endonucleases Eco RI and HindIII, both of which recognize nucleotide sequences that contain 4 thymidine residues, cleaved fully substituted BrdUrd-DNA at the same sites as unsubstituted DNA. However, when treated with limiting amounts of either of the two endonucleases, BrdUrd-DNA was digested more slowly than was the unsubstituted DNA.

Endo R•Sma I, which recognizes a nucleotide sequence that does not contain thymidine, digested BrdUrd-DNA at approximately the same rate as unsubstituted DNA. Surprisingly, one of the three Sma I sites in λ-DNA (located at 0.656 on the genome's physical map) appeared to be highly resistant to cleavage by Sma I in substituted DNA. Hence, cleavage of BrdUrd-DNA by Sma I generated three restriction products instead of the expected four products derived from unsubstituted DNA. These BrdUrd-DNA products were identified as the characteristic Sma I-A and C fragments as well as an unusual, large product that contained the fused B and D fragments. Therefore, the Sma I cleavage site at the junction of the B-D fragments appears to differ from the other two sites by aspects of DNA structure determined outside of the canonical Sma I recognition sequence. This finding indicates that site-specific DNA enzymes can be influenced by DNA determinants that reside outside of the accepted recognition sequences of the enzymes.

In the past several years, we have learned a considerable amount about the organization of nucleic acid sequences in small, well defined viral and prokaryotic plasmid chromosomes. Our knowledge of these chromosomes results largely from use of sequence-specific restriction endonucleases to construct DNA physical maps (reviewed in Ref. 1). To interpret these maps and most experiments based on them requires the assumption that a particular endonuclease cleaves all sites in DNA that contain its canonical recognition sequence. This assumption can be verified in small DNA molecules in which site-specific cleavages alter easily tested molecular properties of the DNA. More recently, restriction endonucleases have been used to construct physical maps of unique genes that reside within complex pro- and eukaryotic DNAs (2-7). The molecular size, i.e. complexity, of DNAs involved in these experiments and many individual restriction endonuclease recognition sites contained in them preclude clear assessment of the limit digestion condition. Attempts to assess the extent of these digestions, therefore, usually have required inclusion of trace amounts of small, well defined DNAs whose cleavage was monitored by conventional means.

Analysis of the relative frequencies with which Eco RI cleaves the λ-genome at each of its five, well characterized sites has indicated that sites in the right end of the viral DNA physical map are cleaved more rapidly than sites in the center of the genome (8). Thus, it was of interest to determine whether rare or modified DNA bases, which may or may not be contained in endonuclease recognition sequences, affect cleavage of a defined DNA molecule. Modified bases occur in large amounts in DNAs purified from many prokaryotes, in particular from species that express DNA restriction-modification systems (9), and in small but significant amounts in a wide variety of other pro- and eukaryotic DNAs (10).

One way to isolate newly replicated DNA from the bulk of a genome involves incorporation of 5-bromodeoxyuridine into growing DNA and isolation of this DNA on the basis of its increased buoyant density (11-15). To generate physical maps of replicating DNA sequences, therefore, it would be useful to isolate the sequences as dense DNAs that contain BrdUrd. Therefore, we examined the effect of BrdUrd substitution on the cleavage of a well defined bacteriophage DNA by several restriction endonucleases. We selected this modified pyrimidine because we could generate fully substituted DNA molecules in vitro and because techniques were available to isolate BrdUrd-DNA and to assess the extent of its base substitution quantitatively. When BrdUrd replaces thymidine in DNA, many of the chemical, physical, and biological properties of the DNA are altered (11, 16-24). For example, Escherichia coli lactose repressor protein binds to BrdUrd-substituted lac operator DNA with much greater affinity than to thymidine-containing operator DNA (20). Perhaps the binding of restriction endonucleases to DNA also is affected by substitution of BrdUrd for dT.

We describe here our studies on the endonucleolytic activities of two restriction enzymes, Eco RI and HindIII, which recognize DNA sequences that contain multiple thymidine (BrdUrd) residues and of one endonuclease, Sma I, whose recognition sequence does not contain a thymidine. For each enzyme we compared the site specificities and rates of cleavage of the DNA. More recently, restriction endonucleases have been used to construct physical maps of unique genes that reside within complex pro- and eukaryotic DNAs (2-7). The molecular size, i.e. complexity, of DNAs involved in these experiments and many individual restriction endonuclease recognition sites contained in them preclude clear assessment of the limit digestion condition. Attempts to assess the extent of these digestions, therefore, usually have required inclusion of trace amounts of small, well defined DNAs whose cleavage was monitored by conventional means.

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on bacteriophage \( \lambda \)-DNA and \( \lambda \)-DNA that was nearly fully substituted with BrdUrd.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases HindIII (Control No. 1077401) and Sma I (Control No. 1097201) were purchased from Boehringer Mannheim; Eco RI (Lot 8O) was obtained from Miles Laboratories, Inc. \( {\text{H}}[^{32}\text{P}]\text{PO}_4 \) and Liquifluor scintillation mixture were products of New England Nuclear Corp. \([H]^{-}\) and \([C]^{-}\)Thymidine, as well as optical grade CaCl\(_2\), were products of Sigma-Mann. Thymidine, 5-bromodeoxyuridine, and ethidium bromide were purchased from St. Louis Chemical Co. Pancreatic RNase (CalBiochem) was heat-inactivated at 90°C for 10 min to destroy contaminating DNase activities. Electrophoretically pure DNase I and pronase were purchased from Worthington Biochemical Co. Pronase was autodigested for 60 min at 37°C prior to use. Agarose was purchased from Seakem. We purchased \([3H]^{-}\)thymidine-labeled E. coli K-12 DNA and \([3H]^{-}\)Thymidine-labeled unifilar Chinese hamster cell BrdUrd-DNA by purchased from New England Nuclear Corp. \([{\text{H}}]^{-}\) and \([\%]^{-}\)Thymidine, 1077401) and Sma I (Control No. 1097201) were purchased from 90°C Endo R Cleavage of BrdUrd-DNA

90°F Endo R Cleavage of BrdUrd-DNA was performed in reduced light. DNA was analyzed by CsCl gradient centrifugation (14). A stock to achieve a refractive index of 1.4017 (\( \rho = 1.725 \)).

**Growth of Bacteriophage X and Purification of Its DNA—**RW682 (\( \lambda \)-\( C \)-\( S \)) was supplied by R. Weisberg (National Institutes of Health). We verified that both bacterial strains contained lysogenic \( \lambda \) with a thermolabile c1 gene product as well as the amber mutant yielding X phage upon induction (<10\(^{-8} \) plaque-forming units/ml) in the absence of exogenous thymidine, thymine, or BrdUrd.

**RESULTS**

Substitution of BrdUrd for Thymidine in h-DNA—To evaluate the effect of BrdUrd substitution on digestion of DNA with restriction endonucleases, it was necessary to obtain a well-characterized DNA substrate in which BrdUrd replaced thymidine. We defined 1 unit of restriction endonuclease as the amount of activity required to cleave 1 \( \mu \)g of \( \lambda \)-DNA in 1 h. Digestion with 0.1 unit of endonuclease for 10 h in all cases was equivalent to 1-h digestion with 1.0 unit of enzyme. Thus, in all reactions, the extents of digestion were directly proportional to the amounts of endonuclease added. In addition to their activities on \( \lambda \)-DNA, all three restriction endonucleases were assayed for their site specificities and nuclease and exonuclease activities using SV40 DNA I. As expected for preparations free of exonuclease activities, Eco RI and HindIII activities on full-length linear SV40 DNA, HindIII yielded only the six well characterized SV40 DNA fragments, and Sma I did not cleave the SV40 DNA I, even when reactions contained as much as 10 units of endonuclease per \( \mu \)g of SV40 DNA for 24 h (data not shown). Eco RI was stored at \(-96^\circ C \) in 10 mm KPO\(_4\), 200 mm NaCl, 7 mm 2-mercaptoethanol, 1 mm EDTA, and 50% (w/v) glycerol, pH 7.0. Sma I was stored in the same buffer, but containing 5 mm dithiothreitol and 300 mm NaCl. HindIII storage buffer was 10 mm Tris-HCl, 250 mm NaCl, 1 mm EDTA, 1 mm dithiothreitol, 50% (w/v) glycerol, and 5 mg/ml of bovine serum albumin, pH 7.4.

**Restriction endonucleases were diluted in reaction buffer containing bovine serum albumin (1 mg/ml) immediately before their addition to digestion mixtures. Reaction buffer for Eco RI and Eco R- Sma I contained 10 mm Tris-HCl, 50 mm NaCl, and 10 mm MgCl\(_2\), pH 7.5. Reaction buffer for Eco R- HindIII contained 10 mm Tris-HCl, 50 mm NaCl, 10 mm MgCl\(_2\), and 14 mm dithiothreitol, pH 7.6. Digests with Eco RI and HindIII were carried out in 0.05 ml at 37°C, and digests using Sma I were performed at 35°C. All reactions were terminated by the addition of 10 \( \mu \)l of 5% (w/v) sodium dodecyl sulfate, 20 mm NaEDTA, 50% (w/v) glycerol, 0.1% (w/v) bromophenol blue. The samples then were stored at \(-20^\circ C \) until electrophoresis.

**Garagose Gel Electrophoresis—**The DNA products of endonuclease digestion were analyzed on horizontal slab gels (14 \( \times \) 18 \( \times \) 0.3 cm) of agarose formed and electrophoresed in 40 mm Tris-HCl, 5 mm sodium acetate, 1 mm Na\(_2\)EDTA, pH 7.8, as described by Ketner and Kelly (5). Immediately before electrophoresis, samples of DNA were heated to 70°C for 5 min to dissociate complementary fragment ends. Unless specified otherwise, electrophoresis was carried out at 54 V (100 mA) for 15 h in 1.4% (w/v) agarose gels. The gels were stained with ethidium bromide (10.8 \( \mu \)g/ml) in distilled water for 30 min at room temperature. DNA fragments in the gels were visualized with a Transilluminator (Ultraviolets Products) and were photographed through a type 23A red filter.

**RESULTS**

**Substitution of BrdUrd for Thymidine in \( \lambda \)-DNA—**To evaluate the effect of BrdUrd substitution on digestion of DNA with restriction endonucleases, it was necessary to obtain a well-characterized DNA substrate in which BrdUrd replaced virtually all thymidine residues. We accomplished this by inducing \( \lambda \) prophage growth in \( E \) coli RW682, a thymidine-auxotroph lysogenic for X (~1857). Several types of analysis indicated the near-quantitative replacement of BrdUrd for thymidine in the DNA of the progeny phage.

First, RW682 absolutely requires thymidine or an analog of thymidine to produce progeny phage after induction. In the absence of thymidine or BrdUrd fewer than 10\(^{-7} \) plaque-forming units/ml of phage were obtained after induction. In the presence of 10\(^{-6} \) mm thymidine or of 10\(^{-7} \) mm BrdUrd, phage yields were 0.5 to 1.0 \( \times \) 10\(^{11} \) and 0.5 to 1.0 \( \times \) 10\(^{10} \) plaque-forming units/ml of lysisate, respectively. The specific infectivity of bacteriophage \( \lambda \) grown in the presence of BrdUrd was 1.25 \( \times \) 10\(^{10} \) plaque-forming units/\( \mu \)g of phage, whereas the infectivity of thymidine-containing phage preparations were approximately 25-fold higher. These data agree well with optical cross-sections reported by others (22-24).

Second, a culture of RW682 was grown for seven generations in J broth that contained 10\(^{-5} \) mm \([H]\)dT (0.1 \( \mu \)Ci/ml), and
and low M BrdUrd. The prophage was induced and progeny phage were purified as described in the text.

Based on the content of aDNA nucleotides, we calculated that a molecule of X-DNA in the preparation contained approximately 500 residues of [3H]thymidine, and was, therefore, at least 98% substituted with BrdUrd. The latter calculation is subject to the errors implicit in measurement of very low absorbance.

Isopycnic CsCl gradient centrifugation of the 32P-labeled, BrdUrd-substituted DNA described in Table I indicated a buoyant density of 1.80 g/cm³ (Fig. 1). We determined this density from the refractive indices of gradient fractions, as well as from the positions of two DNA standards of known density, E. coli [3H]DNA, \( \rho = 1.7035 \) g/cm³ (A), and unifilar BrdUrd-[14C]DNA from Chinese hamster cells, \( \rho = 1.750 \) g/cm³ (C), were included in the gradient as internal density standards.

We confirmed these results by a third experimental method. Isopycnic CsCl gradient centrifugation of the 32P-labeled, BrdUrd-substituted DNA described in Table I indicated a buoyant density of 1.80 g/cm³ (Fig. 1). We determined this value from the refractive indices of gradient fractions, as well as from the positions of two DNA standards of known density. The data suggest complete substitution of BrdUrd in our preparations of λ-DNA (15). The three experiments described above indicate that the λ-DNA we used is nearly quantitatively substituted (98 to 99.5%) with bromodeoxyuridine.

Sensitivity of BrdUrd-DNA to Restriction Endonucleases—We examined two classes of restriction endonuclease for their abilities to digest BrdUrd-substituted λ-DNA described above. The first class of endonuclease included two enzymes, Eco RI and HindIII, both of which recognize DNA sequences that contain no thymidine residues (28). Reaction products were analyzed by agarose gel electrophoresis. The Eco RI cleavage map of λ-DNA and molecular weights of individual restriction fragments also are shown (8).

In each of our studies we examined the DNA fragments generated from unsubstituted and BrdUrd-substituted λ-DNA after digestion with various amounts of restriction endonuclease by agarose gel electrophoresis. The amounts of endonuclease activity used in each study ranged from an activity that completely cleaved the unsubstituted DNA to an activity far in excess of that which completely cleaved the unsubstituted DNA. In contrast, Sma I recognizes a DNA sequence that contains no thymidine residues (30), and therefore the three sites in λ-DNA cleaved by Sma I (31) should not contain BrdUrd in the fully substituted DNA.

Incorporation of [3H]dT and [32P]Pi into λ-DNA after heat induction of RW682

RW682 was grown for seven generations in medium supplemented with [3H]dT at a specific activity of \( 1.3 \times 10^5 \) cpm/nmol. At a density of \( A_{So} = 0.5 \), the culture was harvested, and then was resuspended in medium that contained [32P]Pi (specific activity of \( 6.6 \times 10^5 \) cpm/nmol) and \( 1 \times 10^{-4} \) M BrdUrd. The prophage was induced and progeny phage were purified as described in the text.

Based on the molecular weight of λ-DNA, 3.1 \times 10^7 (26), λ DNA is 25.8% thymine (27). Thus, each genome (9.4 \times 10^4 nucleotides) contains approximately 24.2 \times 10^3 residues of dT.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Content within preparation of λ-DNA</th>
<th>[3H]dT</th>
<th>A_{So}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.26 \times 10^5 cpm</td>
<td>1.35 \times 10^5 cpm</td>
<td>0.0575 A_{So}</td>
</tr>
<tr>
<td>2</td>
<td>1.89</td>
<td>1.04 \times 10^{-2}</td>
<td>8.74</td>
</tr>
<tr>
<td>3</td>
<td>5.50 \times 10^{-3}</td>
<td>1.19 \times 10^{-3}</td>
<td>112</td>
</tr>
<tr>
<td>4</td>
<td>517</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.0218</td>
<td>0.0046</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>97.82%</td>
<td>99.54%</td>
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The electropherograms illustrated in Fig. 2 summarize our studies with Eco RI. This enzyme's recognition sequence, C[3H]ATTG written as duplex DNA is expected to cleave only some of the potential recognition sites within the unsubstituted λ-genome to an activity far in excess of that which completely cleaved the unsubstituted DNA.
to contain 4 BrdUrd residues in the fully substituted DNA substrate, C\textsubscript{G}AA\textsubscript{B}B\textsubscript{C}. Cleavage at these sites in the DNA occurs in \textit{trans} to 1 residue of BrdUrd. The six expected \textit{Eco RI} fragments of \textlambda-DNA (A to F), their positions in the physical map of viral DNA, and their molecular weights, all established by Thomas and Davis (8), are also indicated in the figure. Lanes e and f depict the patterns of DNA cleaved from the \textlambda-genome when we used a 10-fold excess of \textit{Eco RI} (10 unit h/\mu g of DNA). Both the substituted and unsubstituted \textlambda-DNAs were cleaved into fragments of apparently identical molecular weights. From these data we conclude that \textit{Eco RI} cleaves fully substituted BrdUrd-DNA from \textlambda at all five sites characteristic of the unsubstituted viral DNA.

To examine possible differences in the rates of cleavage of the substituted and unsubstituted DNAs, we digested \textlambda-DNA with lesser quantities of \textit{Eco RI}. Lanes a to d (Fig. 2) illustrate the products of these reactions. At levels of 0.5 and 1.0 unit h of enzyme activity per \mu g of DNA substrate, \textit{BrdUrd-DNA} (Lanes a and c, respectively) was cleaved by \textit{Eco RI} more slowly than was unsubstituted DNA (Lanes b and d). Comparison of the \textit{BrdUrd-DNA} products analyzed in Lane c (1.0 unit h/\mu g of DNA) with the unsubstituted DNA products in Lane b (0.5 unit h/\mu g of DNA) indicates that \textit{Eco RI} cleaved the \textit{BrdUrd-DNA} at approximately half the rate that it cleaved unsubstituted DNA. As reported by others (8), we also observed that cleavages between the \textit{Eco-C-D}, \textit{D-E}, and \textit{E-F} fragments occurred more rapidly than cleavages in the center of the physical map (A-B and B-C) in unsubstituted DNA. Note the early occurrence of the complete D and E fragments (Lane b) and the F fragment, which was more easily seen on the stained gel than in the photograph. Similarly, in \textit{BrdUrd-DNA} (Lane c), the D, E, and F fragments were the first to be resolved as the amount of endonuclease was increased. Thus, substitution of \textlambda-DNA with \textit{BrdUrd} only modestly affected the rate of cleavage of the DNA by \textit{Eco RI}, and did not alter the preference of the enzyme for the right-most cleavage sites within the \textlambda-genome, i.e., the C-D, D-E, and E-F junctions.

\textit{HindIII} was the second restriction endonuclease we examined for activity on \textit{BrdUrd-DNA}. Our studies with this enzyme are summarized in Fig. 3. The figure indicates the positions and sizes of the \textit{HindIII} fragments cleaved from the \textlambda-genome (32). \textit{HindIII} recognizes a duplex DNA sequence which, like the sequences recognized by \textit{Eco RI}, should contain 4 residues of \textit{BrdUrd} in the substituted DNA, A\textsubscript{G}AC\textsubscript{B}C \textsubscript{B}CG\textsubscript{A}A\textsubscript{B} (29). In this case, however, the duplex should be cleaved at these sequences in \textit{trans} to 2 residues of \textit{BrdUrd}. In the electropherograms shown in Fig. 3, the \textit{HindIII-G} fragment (\textit{M} = 0.5 \times 10^6) was allowed to run off of the gels to better resolve the larger DNA fragments. At high levels of enzyme activity (200 unit h/\mu g of DNA, Lanes g and h), the \textit{BrdUrd} substrate (Lane g) was cleaved nearly to completion. The six authentic \textit{λ-DNA} digestion products, fragments A to F, were observed in this analysis of the \textit{BrdUrd-DNA}, indicating that \textit{HindIII} cleaved all of the recognition sites within the substituted genome. The amount of \textit{HindIII} activity required to cleave the \textit{BrdUrd-substrate} nearly to completion was between 16 and 200 times the amount required to similarly digest unsubstituted viral DNA (1.0 unit h/\mu g of DNA, Lane b). This large rate difference perhaps reflects the fact that all sites at which \textit{HindIII} cleaves substituted \textlambda-DNA are \textit{trans} to 2 residues of \textit{BrdUrd}.

When we digested a mixture of unsubstituted and \textit{BrdUrd-DNAs} from phage \textlambda with \textit{HindIII}, the \textit{BrdUrd-DNA} did not inhibit the activity of \textit{HindIII} on the thymidine-containing substrate. Similarly, when both \textit{HindIII} and \textit{Eco RI} were added to a digestion mixture that contained \textit{BrdUrd-DNA}, the activity of \textit{Eco RI} (Fig. 2) was not inhibited by \textit{HindIII} (data not shown). These two control experiments demonstrated that neither our preparations of \textit{BrdUrd-DNA} nor of \textit{HindIII} contained inhibitory substances. Berkner and Folk have observed only a small reduction (3-fold) in the activity of \textit{HindIII} on \textit{BrdUrd-DNA} from phage \textlambda when compared to unsubstituted DNA. Despite qualitative agreement of their observation and ours, there remains a quantitative difference between our findings that might reflect the lesser extent of \textit{BrdUrd} substitution (83%) in their preparations of \textlambda-DNA.

As was the case with \textit{Eco RI}, \textit{HindIII} appeared to cleave the six recognition sites within \textlambda-DNA at unequal rates. Similar to \textit{Eco RI}, the recognition sites in the right-most region of the viral physical map were cleaved more rapidly than were the sites nearer to the center. This was observed most easily when cleavage occurred at a reduced rate in reactions that contained \textit{BrdUrd-DNA} (Lanes c and e, Fig. 3), where the \textit{HindIII-C} and D fragments were the first to be resolved in the gels.

As a control that involved a restriction endonuclease whose sites of cleavage within \textlambda-DNA were expected not to contain \textit{BrdUrd}, we examined the ability of \textit{Endo R-Sma I} to cleave \textit{BrdUrd-DNA}. We thus hoped to distinguish effects of \textit{BrdUrd} within an enzyme's recognition sequence (which for \textit{Eco RI} and \textit{HindIII} resulted in reductions in the rates of digestion) from effects that \textit{BrdUrd} might have when external to the sites of cleavage. Our observations on the digestion of \textit{BrdUrd-DNA} from \textlambda with \textit{Sma I} are summarized in Figs. 4 to 6.

The duplex DNA sequence cleaved by \textit{Sma I} is CCC\textsuperscript{GGG} \textsuperscript{GGG} \textsuperscript{CCC} (30); it differs from the sequences cleaved by \textit{Eco RI} and \textit{HindIII} in two respects. First, it contains no thymidine, or in substituted DNA, no \textit{BrdUrd}. Second, in contrast to the staggered cleavages of the two strands in duplex DNA by \textit{Eco RI}...
RI and HindIII, Sma I cleaves the duplex at sites immediately across from one another and results in “blunt-ended” DNA fragments. As above, first we examined the extent and the relative rates with which Sma I cleaved the two λ-DNA substrates (Fig. 4). As before, Lanes a, c, e, g, and i contained fragments generated from BrdUrd-DNA, and Lanes b, d, f, h, and j, fragments of unsubstituted DNA. Enzyme activities were varied from 0 (a and b) to 10 unit h/pg of DNA (i and j). Sma I cleaved BrdUrd-DNA at approximately the same rate as unsubstituted DNA. Surprisingly, little if any BrdUrd-DNA cleavage products of BrdUrd-DNA, and fragments of unsubstituted X-DNA. Enzyme activities were cleaved at approximately the same rate as unsubstituted DNA. Subjects of Smu I -B and Sma I -D fragments were produced, even when large amounts of endonuclease were added to digestion mixtures (Fig. 4, Lanes g and i; Fig. 5, Lane c). These data indicate that the Sma I cleavage site which separates the B and D fragments, located at 0.656 on the physical map of λ-DNA (31), was largely resistant to cleavage in BrdUrd-DNA. In contrast, the two other Sma I sites, located at 0.406 (A-B) and 0.825 (D-C) (31) were cleaved at approximately normal rates in λ-DNA fully substituted with BrdUrd.

If inhibition of Sma I is specific for the site in BrdUrd-substituted λ-DNA at 0.656, then the Sma I-B-D fragment should have been present among the large DNA products of digestions that contained excess endonuclease. Note the large intermediate cleavage product that migrated somewhat slower than the Sma I-A fragment in Lanes g and i (Fig. 4). The persistence of this large fragment is consistent with the failure of the enzyme to cleave the B-D junction in fully substituted λ-DNA. To confirm the identity of the putative B-D intermediate fragment observed in Fig. 4 (g and i), we altered the electrophoretic conditions to better resolve the larger DNA fragments and analyzed the products of a digestion mixture that contained 14 unit h of Sma I/μg of BrdUrd-DNA (Fig. 5, Lane c). In this analysis, we also included standards λ-DNA fragments of well characterized molecular weights. Lane a contains intact λ-DNA (31 × 10^6 daltons) as well as the HindIII-A, B, and C fragments (see Fig. 3). Lane b contains both the larger Eco RI and HindIII digestion products (see Figs. 2 and 3); and Lane d contains Sma I λ fragments A, B, C, and D. Again, note the absence of the Sma I-B and D fragments in the BrdUrd-DNA digestion products (Lane c). Also note in Lane c the fragment that is clearly smaller than the HindIII-A and Eco RI-A fragments, but is larger than the Sma I-A fragment. This fragment has an estimated molecular weight of 13.12 × 10^6 (Fig. 6), and corresponds closely to the sum of the molecular weights reported for the Sma I-B and D fragments (31).

Failure of Sma I to cleave the recognition site located at 0.656 fractional λ-genome lengths is the only interpretation of the data in Figs. 4 to 6 that explains both the gel patterns and the molecular weight calculated for the large, persistent par-

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**Fig. 4.** Sensitivity of unsubstituted and BrdUrd-DNA to Endo R Sma I. 0.5 μg of BrdUrd-DNA (Lanes a, c, e, g, and i) or unsubstituted DNA (Lanes b, d, f, h, and j) were digested with various amounts of Sma I. Endonuclease activities used in each reaction were: 0 (a, b); 0.125 (c, d); 0.5 (e, f); 1.0 (g, h); and 10 (i, j) unit h/μg of DNA. Products were analyzed by electrophoresis as described in Fig. 1. The Sma I cleavage map of λ and molecular weights of restriction fragments also are illustrated (31).

**Fig. 5.** Analysis of Sma I cleavage products of BrdUrd-DNA purified from bacteriophage λ. BrdUrd-DNA was digested with Sma I (14 unit h/μg of DNA), and products were analyzed by electrophoresis for 15 h at 54 V in a 0.7% agarose gel. Standard fragments derived from unsubstituted λ-DNA were analyzed in the adjacent lanes. Lane a contains untreated DNA and HindIII-A, B, and C fragments (see Fig. 3). Lane b contains these same HindIII fragments and the Eco RI-A, D, E, and C fragments (see Fig. 2). Lane c contains the Sma I digestion products of BrdUrd-DNA, and Lane d, the four Sma I fragments of unsubstituted λ-DNA.

**Fig. 6.** A curve of electrophoretic mobility versus fragment molecular weight was constructed based on the migration of the standard DNA fragments analyzed in the gel displayed in Fig. 5. From this curve, the Sma I-B-D fragment is estimated to be 13.12 × 10^6.
trated in Figs. 2 to 4 were analyzed containing X-DNA. Photographic negatives of weights These areas were normalized by comparison to the peaks...generated in limit digests, which were taken to be 100%. In limit digests, the intensities of the ethidium bromide-stained bands were directly proportional to the molecular weights of the fragments. For example, densitometer tracings indicated a molar ratio of 0.880:0.93: 1.10:1.06:1.00 for the Eco RI-A, B, C + E, D, and F fragments, respectively. As described in the text, for each enzyme the liberation of the right-most λ-DNA fragments from dT-DNA (O) and from BrdUrd-DNA (O) were analyzed.

As described above, Eco RI and Smal I appeared to digest λ-DNA substituted with BrdUrd-DNA, then among the partial digestion products of substituted DNA we should not have observed the Smal I-D-C fragment. This fragment has a molecular weight of 10.75 × 10^6. The D-C fragment is the only Smal I partial digestion product of λ-DNA that migrates between the Smal I-A and B fragments, as was observed after digestion of unsubstituted λ-DNA (Fig. 4, Lanes d and f). It was not seen after digestion of BrdUrd-substituted DNA (Fig. 4, Lanes e, g, and i).

As described above, Eco RI and Smal I appeared to digest λ-DNA substituted with BrdUrd-DNA only slightly slower than DNA containing thymidine. In contrast, HindIII cleaved dT-DNA much more rapidly than BrdUrd-DNA. To quantitate the rates of these enzymes on BrdUrd- and dT-DNAs better, the stained gels illustrated in Figs. 2 to 4 were analyzed using a densitometer (Fig. 7). Because: 1) Eco RI and HindIII cleaved specific sites in λ-DNA with different rates; 2) production of all DNA fragments except those derived from the termini of the linear genome require two endonucleolytic cleavages; and 3) the DNA fragments produced by all three restriction endonucleases. These are the Eco RI-F, HindIII-D, and Smal I-C-DNA fragments.

The data in Fig. 7 confirmed our qualitative assessment of the agarose gel patterns. Smal I digested BrdUrd- and dT-DNAs with approximately equal rates. Eco RI digested BrdUrd-DNA 10-fold slower than dT-DNA. The initial rate of the HindIII reaction on the BrdUrd substrate was approximately 20-fold lower than on the dT substrate as judged by cleavage at the λ HindIII-C-D junction. However, limit digestion at this site required between 20- and 200-fold more enzyme for BrdUrd-DNA than for dT-DNA.

**DISCUSSION**

We used phage λ-DNA that was almost fully substituted (98 to 99.5%) with 5-bromodeoxyuridine to investigate the effect of a halogenated pyrimidine on cleavage of DNA by three restriction endonucleases. Eco RI and HindIII exhibited reduced rates of cleavage when tested with fully substituted BrdUrd-DNA; HindIII was affected more (approximately a 20-fold reduction in rate) and Eco RI was affected less (approximately 10-fold reduction in rate). The rate of digestion by Smal I was inhibited less than 2-fold by incorporation of BrdUrd into the λ-DNA. We have obtained these results with four independent preparations of BrdUrd-DNA whose extents of BrdUrd substitution were approximately the same as described in this report.

While our studies were in progress, Berkner and Folk reported their investigation of other modifications in position 5 of DNA pyrimidine nucleotides and the effects of these nucleotides on the endonucleolytic and methylating activities of Eco RI (21). They assessed relative rates of endonuclease activity by measuring Eco RI-generated 5'-phosphoryl termini within the DNA substrates, and reported that viral DNAs containing glucosylated 5-hydroxymethylcytosine and 5-hydroxymethyluridine are poor substrates for Eco RI endonuclease. Furthermore, Berkner and Folk discussed the effects of modified pyrimidine DNA bases on restriction endonuclease activity in terms of the structure of the canonical Eco RI recognition sequence itself. They considered both steric effects of the position 5 substituents as well as the relative electro-negatives of the substituents, and concluded that these features of the substituents might account for the differences in enzyme activity observed. The effects of BrdUrd substitution upon cleavage of λ-DNA by Eco RI and HindIII which we have observed are consistent with their interpretation.

Additional interpretation of our data can be based upon the effect of BrdUrd on the structure of DNA both inside and outside of the canonical recognition sequences of the endonucleases. As we observed for Eco RI (Fig. 2) and for HindIII (Fig. 3), and as Thomas and Davis reported earlier for Eco RI (8), endonuclease recognition sites that reside toward the right end of the viral DNA physical map are cleaved more rapidly than are sites in the center of the genome. If each DNA site cleaved by these enzymes contains the canonical nucleotide recognition sequence, then structural features of the DNA in addition to these sequences must affect the rates of cleavage of individual sites. Such other features might involve nucleotide sequences adjacent to or in the proximity of the cleavage sites or effects of BrdUrd on DNA duplex structure and on intrastrand looping. In this regard, it is informative to consider our studies on Endo R-Smal I.

Sma I cleaves three sites in the λ-genome located at 0.406, 0.656, and 0.825 (31). In λ-DNA fully substituted with BrdUrd, however, the site at 0.656 was not cleaved by Sma I, or was cleaved at a much slower rate than the other two sites (Figs. 4 to 6). Assuming that the sites cleaved by Sma I all contain the sequence CCC|GGG|GGG|CCC, the site-specific inhibition of cleavage at 0.656 is particularly surprising. Clearly, a residue of BrdUrd can reside no closer to the phosphodiester bonds cleaved by Sma I than four nucleotides. Therefore, BrdUrd must affect cleavage of the site at 0.656 from outside the canonical recognition sequence. Since BrdUrd substitution increases stability of duplex DNA to thermal denaturation (16-18) and decreases its resistance to denaturation by alkali (19), alterations in the duplex structure itself might account for a generalized effect of BrdUrd substitution on cleavage by Sma I. To explain the site-specific inhibition on this basis, it is necessary to assume that the Sma I site at 0.656 in the λ-genome is surrounded by an A-T (or A-BU)-rich region of duplex DNA. However, Inman and Schnoss (19) have shown that the regions in which all three Sma I sites map in λ-DNA are resistant to partial denaturation by alkali, and thus likely reside within G-C-rich sequences of DNA. In particular, two
small regions of $\lambda$-DNA are most resistant to alkali (i.e., of highest G-C content). One of these maps very close to or includes the Smal I site at 0.656, i.e., within the $\lambda$ gene B. Thus, while denaturation maps of $\lambda$-DNA preclude a high concentration of BrdUrd at or near the Smal I site of 0.656, they do not preclude the presence of short, BrdUrd-containing sequences that protect this site from cleavage.

The nucleotide sequences of $\lambda$-DNA thus far determined (33, 34), suggest that much of the viral genome is compatible with DNA structures that involve extensive intrastand loops. Meselson pointed out that short loops can be formed within palindromic restriction enzyme recognition sequences (35) and has speculated that these small loops might signal sites for restriction and modification. The Smal I site at 0.656 lies very close to an Eco RI site at 0.656 to 0.659 (8, 31), i.e. within 150 base pairs. Structural features of BrdUrd-DNA, such as the presence or absence of particular intrastand loops, that may have inhibited cleavage of the Smal I site did not also inhibit cleavage by Eco RI (Fig. 2). Also, since the Smal I-B-D fragment persisted in digestion mixtures long after the sites at 0.402 and 0.825 were cleaved (Figs. 4 and 5), structural features of the BrdUrd-DNA that inhibited the site’s cleavage resided totally within the B-D fragment itself (a central 45% portion of the genome).

As we were completing this manuscript, we learned that BrdUrd substitution of DNA from bacteriophage P-22 produces a similar differential inhibition of cleavage at one site by Endo R-HindIII. This observation is quite similar to the site-specific inhibition of Endo R-Smal I at 0.656 on the $\lambda$ genome.

Occurrence of modified bases within a DNA therefore can alter the selectivity of particular restriction endonucleases. A few DNAs naturally contain large amounts of modified bases. DNAs from many sources contain small amounts of these bases (10, 36-38). Numerous experiments now indicate that discontinuous replication of both pro- and eukaryotic DNAs might involve the transient insertion of nucleic acid bases not generally found in mature DNAs (39-42). Recently, Bird and Southern (37, 38) observed that Xenopus rDNA sequences purified from somatic cells contained much higher contents of cytosine or 5-methylcytosine. oocyte rDNA sequences normally cleaved by several restric-
tion enzymes (13), and they found that sites within amplified oocytes. In addition, they found that sites within amplified DNA sequences that contained cytosine or 5-methylcytosine.

The observations we have described, as well as recent reports from a number of laboratories, prompt caution in experiments that are predicted on the stringent specificities with which restriction endonucleases cleave very large DNAs or DNAs that are in the process of replication.

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