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Novel properties of a restriction endonuclease isolated from *Haemophilus parahaemolyticus*

(DNA sequencing/phage lambda operators/RNA polymerase termination site/DNA alkylation/DNA-protein interaction)

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ABSTRACT **The sequences in λ DNA in and around six sites cut by *Hph*, a restriction enzyme isolated from *Haemophilus parahaemolyticus,* are compared. The enzyme produces a staggered cut around an AT or TA base pair, but the sequences immediately surrounding the cleavage sites bear no obvious relation to one another. Eight (in some cases nine) base pairs to one side of each cleavage site is the common sequence $\substack{\text{TCACC}\\\text{AGTGG}}$. Two lines of evidence indicate that these bases constitute part or all of the *Hph* recognition site. First, mutations in this sequence prevent *Hph* cutting. Second, dimethylsulfate-mediated methylation of Gs and As in this site prevent cutting, whereas methylation of purines in the region between this sequence and the cleavage sites has no such effect. There is discernible 2-fold rotational symmetry neither in the common sequence nor around the cleavage sites.**

An endonuclease activity of *Haemophilus parahaemolyticus* (*Hph*) cuts lambda DNA at more than 50 specific sites. The enzyme is similar to typical class II restriction endonucleases in that it requires Mg^{++} but neither ATP nor *S*-adenosylmethionine (Middleton, Edgell, Hutchison, and Roberts, personal communication). Nearly 20 examples of this class of restriction endonuclease have been reported to recognize and cut sequences that are partially or completely 2-fold rotationally symmetric (1). These symmetric sequences range from four to six base pairs, and in one case it has been shown explicitly that a symmetric oligonucleotide duplex eight base pairs in length is recognized and cut by a restriction enzyme (*Eco*RI) (2). In this paper we show that *Hph* has the following unique properties that distinguish it from restriction endonucleases described previously: (*i*) this enzyme apparently recognizes a specific but asymmetric sequence, and cuts at sites located eight (or nine) base pairs to one side of that sequence; (*ii*) the enzyme produces a staggered cleavage around an AT or TA base pair, but the sequences surrounding the cleavage sites bear no obvious relation to one another, nor are they symmetric. We refer to *Hph* as a restriction endonuclease although it (*Hph*) has not been shown to be involved in biological restriction.

MATERIALS AND METHODS

Enzymes. Enzymes were prepared as described (3), except for *Hph*. This endonuclease was purified from *H. parahaemolyticus* cells grown nearly to stationary phase in brain-heart infusion supplemented with 10 μg of hemin per ml and 2 μg of NAD per ml. Ten grams of cells were disrupted by sonication in 10 ml of 10 mM Tris·HCl pH 7.4, 10 mM 2-mercaptoethanol. The supernate fluid from a 100,000 × *g* centrifugation (1 hr) was made 1 M in NaCl and layered onto a Bio-Gel A 0.5 M column (200–400 mesh, 2.5 × 50 cm). Fractions were assayed as described in Sharp *et al.* (4) using 10 mM Tris·HCl, pH 7.4, 10 mM MgCl, 10 mM 2-mercaptoethanol, 6 mM KCl (*Hph* buffer). Active fractions were made to 50% saturation with ammonium sulfate at 0°, and centrifuged. The precipitated protein was resuspended and dialyzed against 10 mM K_2HPO_4, pH 7.5, 1 mM dithiothreitol, 100 mM KCl, then loaded on a (1 × 10 cm) phosphocellulose column. The activity was eluted with a 0.1 M to 1 M KCl gradient, made in the same buffer. Active fractions, eluting between 0.25 and 0.30 M KCl, were concentrated by dialysis against *Hph* buffer containing 50% glycerol and stored at −20°. Ten grams of cells yielded approximately 5–20 units of enzyme, where 1 unit is the amount of enzyme that cuts 1 mg of lambda DNA to a limit product in 1 hr at 37°.

DNA Sequencing. The methods used to determine the DNA sequences shown below have been described previously (3). The conditions used for 5′-hydroxyl ^{32}P labeling of restriction fragments with T_4 polynucleotide kinase were optimized according to ref. 5. The nucleotide at the 5′ end of each restriction fragment was determined by digesting the 5′-^{32}P-labeled fragment to mononucleotides with snake venom phosphodiesterase and pancreatic DNase and examining the products by paper electrophoresis as described (6). The operator mutations not described previously, for example, *v*003, were isolated by Stuart Flashman; sequences were determined as described in ref. 3 (unpublished).

DNA Methylation. Partial methylation of 5′-hydroxyl labeled restriction fragments using dimethylsulfate was according to Gilbert *et al.* (7).

RESULTS

Sequences surrounding six *Hph* cleavage sites

Fig. 1 shows the sequences in and around six sites on lambda DNA cut by *Hph*. Sequences *a* through *d* are located in the lambda operators (see Fig. 2) (3, 6, 8–11). Sequence *e* is from a portion of the *N* gene (9). Sequence *f* is from λ DNA and includes the 25 base pairs coding for the 3′ end of the so-called "OOP" RNA and the 62 base pairs distal to the site of termination of the RNA (12–14). The figure also shows that the *Hph* site 14 base pairs from the "OOP" terminator is bracketed closely by two other restriction endonuclease cleavage sites, a fact which proved useful in experiments described below. These various sequences were determined as described in the figure legend.

Examination of the sequences of Fig. 1 reveals the following: (*i*) the cleavage is staggered, producing a protruding 3′ end; (*ii*) the cleavage sites bear no obvious similarities to each other; (*iii*) with the exception of site *e*, the bases immediately surrounding the cleavage sites do not display any obvious symmetries; (*iv*) the sequence $\substack{\text{TCACC}\\\text{AGTGG}}$ is located eight (or nine) base pairs to one side of each cleavage site. The latter sequence is not symmetric and does not lie in some region of larger symmetry, and we shall refer to it as the "common sequence." We have not discerned any other similarities in the

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(a)  5' CGTCCTGCTGATGTGCTCAGTATCACCGCCAGTG
     3' GCAGGACGACTACACGAGTCATAGTGGCGGTCAC

(b)     CAACACCGCCAGAGATAATTTATCACCGC
        GTTGTGGCGGTCTCTATTAAATAGTGGCG

(c)     TCCTTAGTACATGCAACCATTATCACCGCCAGAG
        AGGAATCATGTACGTTGGTAATAGTGGCGGTCTC

(d)     TTGCTCATAAGTG-TAAATCTATCACCGCAAGGG
        AACGAGTATTCAC-ATTTAGATAGTGGCGTTCCC

(e)     CAGGGCTTAATTTTTAAGAGCGTCACCTTCATGG
        GTCCCGAATTAAAAATTCTCGCAGTGGAAGTACC
                                                    ...."OOP" RNA .....
(f)                                              AUUUUUUGCGGGCCGCCGUUGGCUC
TGGGGGGTCGTTGACGACGACATGGTCCGATTGGCGCGACAAGTTGCTGCGATTCTCACCAATAAAAAACGCCCGGCGGCAACCGAG
ACCCCCCAGCAACTGCTGCTGTACCAGGCTAACCGCGCTGTTCAACGACGCTAAGAGTGGTTATTTTTTGCGGGCCGCCGTTGGCTC

        Hind II                              Hph                         Hpa II
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FIG. 1. Sequences at six *Hph* cleavage sites in λ DNA. The *Hph* cleavage sites, indicated with arrows, and the "common sequences" by dashed arrows, are aligned. (*a*) Sequence in and around the *Hph* site adjacent to the λ repressor bindings site O_L1 (see Fig. 2). The sequence was reported in refs. 6, 8, 9, and 10, and the 5′-terminal nucleotides at the cleavage site were determined by partial exonuclease digestion of 5′ end-labeled DNA as described in ref. 3 (unpublished). The identity of the 5′-terminal nucleotide after *Hph* digestion was confirmed by complete nuclease digestion followed by paper electrophoresis (unpublished). (*b*) Sequences in and around the *Hph* site in O_L2 (see Fig. 2). The sequence was reported in ref. 10, and the cleavage site was located as in *a* (unpublished). (*c*) Sequences in and around the *Hph* site in O_R1 (see Fig. 2). Sequence was reported in refs. 3 and 11, and confirmed by partial exonuclease digestion (unpublished). The cleavage site was located as in *a*, and found to be one nucleotide to the right of that previously reported (3). (*d*) Sequence in and around the *Hph* site in O_R3..The sequence was determined in part in refs. 3 and 11 and by partial exonuclease digestion [unpublished and unconfirmed by pyrimidine tract analysis (3)]. Cleavage site was located as in *a*, and found to differ slightly from that previously reported (3). (*e*) Sequence in and around the *Hph* site in gene *N*. RNA sequence reported in ref. 9, and cleavage site located as in *a*. (*f*) Sequence in and around the "OOP" RNA termination site. [The "OOP" RNA is a 4S RNA molecule that maps near the origin of replication of phage λ (12).] The determination of this sequence and arguments concerning its relevance to the termination event will be presented elsewhere [D. Kleid and M. Ptashne, manuscript in preparation; and Rosenberg *et al.* (13)]. The sequence contains an *Hph* cleavage site, as well as the cleavage sites of the enzymes *Hin*dII and *Hpa* II. Also shown are the terminal 25 bases of the "OOP" RNA. The three bases underlined in the "OOP" RNA sequence are absent from the sequence reported in ref. 14. The DNA sequence at this site was determined as in *a*.

sequences surrounding the *Hph* sites. The experiments presented below provide additional evidence that the integrity of the "common sequence" is required for *Hph* action.

Operator mutants and *Hph* cutting

Fig. 2 shows the sequences of the lambda operators and the first few bases distal to the startpoint of transcription of the adjacent genes *N* and *tof*. These operators contain multiple repressor binding sites (15), and it has been argued elsewhere that the sequences recognized by the repressor are those set off in brackets (10). The figure also shows four *Hph* "common sequences," corresponding to the four *Hph* cleavage sites in the operators, as well as the sequence change caused by 11 mutations in various parts of the operators (ref. 10 and unpublished). Two, and only two, of these mutations lie in a *Hph* "common sequence," and we have found that of the mutations shown in the figure, these and only these block *Hph* action. Fig. 3 shows that *v*2 and *v*003, which replace the same GC with an AT and a TA, respectively, block *Hph* cutting at the adjacent *Hph* site. A restriction endonu-

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  Hph            OR3                     OR2      Hind II            OR1           Hph   tof
5' G-TAAATCTATCACCGCAAGGGATAAATATCTAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCATGTA
3' C-ATTTAGATAGTGGCGTTCCCTATTTATAGATTGTGGCACGCACAACTGATAAAATGGAGACCGCCACTATTACCAACGTACAT
   cI                                 T   GA     T               G    A   A
                                      A   CT     A               C    T   T
                                   Prm116

          OL3              Hph    OL2      Hind II           OL1              Hph   N
  --CC--CTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACATCAG
  --GG--GACGCCACTATTTAATAGAGACCGCCACAACTGTATTTATGGTGACCGCCACTATGACTCGTGTAGTC
                                           T         G       TA
                                           A         C       AT
                                          Sex1
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FIG. 2. The operators of phage λ. The leftward operator is reversed from its usual orientation on the lambda map. The sequences believed to be recognized by repressor (10) are set off in brackets. The start points of transcription of the adjacent genes *N* and *tof* are indicated by horizontal arrows. The sites cut by the restriction enzymes *Hin*dII and *Hph* are indicated by vertical arrows. The *Hph* "common sequences" are indicated by dashed arrows. The base changes caused by 11 mutations are indicated. Nine of these decrease repressor affinity, and the other two, *Prm*116 and *Sex*1, are promoter mutations that decrease transcription of genes *c*I and *N*, respectively.

clease fragment, 320 base pairs long (13), produced by the action of *Hin*dII on λ DNA, contains part of gene *N* and O_L; this fragment also contains *Hph* sites 30 and 75 base pairs from one end (see Fig. 3). The figure shows that the mutant DNAs are not cut at the site nearest the right end of the *Hin*dII 320 fragment, but the site 75 base pairs from the end is cut. The effect on *Hph* action is not due to nonisogenicity of strains, because, as shown in the figure, the parental strain from which *v*003 was derived, as well as the presumed parent of *v*2, both have intact *Hph* cleavage sites. Moreover, another operator mutation located in the base pair immediately adjacent to a "common sequence" (see Fig. 2) does not affect *Hph* action. We have found a mutation (*vs*387) that abolishes *Hph* cutting at the site adjacent to O_R1 (not shown), and we predict this mutation lies in the common sequence in O_R1.

Use of a chemical probe to identify the *Hph* recognition site

Gilbert *et al.* (7) have described the use of the alkylating agent dimethylsulfate for identification of bases required for specific DNA-protein interaction. Under the conditions used, dimethylsulfate methylates the N^7 of guanine and N^3 of adenine, but does not react with pyrimidines. We have used dimethylsulfate to identify the *Hph* recognition site as follows. We isolated the 63 base pair *Hin*dII/*Hpa* II restriction fragment of *f* of Fig. 1, labeled with ^{32}P at the *Hpa* II end in one case and at the *Hin*dII end in the other, as described in the legend to Fig. 4. These fragments were treated separately with dimethylsulfate to the extent that about 40% of the fragments became resistant to *Hph* digestion. We estimate that each of these fragments contains on the average approximately two added methyl groups. These molecules were then digested with *Hph*, and the products separated by gel electrophoresis. In each case two labeled fragments were seen on the gels. One was the uncut parental 63 base pair fragment rendered resistant to *Hph* by methylation. The other was one of the two cleavage products: with molecules labeled at the *Hpa* II end (left, Fig. 4) we recovered the right part of the molecule, 27 base pairs long; with molecules labeled at the *Hin*dII end (right, Fig. 4) we recovered the left part of the molecule, 36 base pairs long (see Fig. 1f). These labeled molecules were extracted from the gel, and treated as described in ref. 7 to depurinate and break each chain at the site of methylation. The products were then examined by gel electrophoresis and autoradiography (Fig. 4). The figure shows that the molecules resistant to *Hph* cutting were preferentially methylated in the purines of the sequence AGTGG, and only at this sequence (see columns 2 and 5). In contrast, in the molecules successfully cut by *Hph*, this sequence and only this sequence is *deficient* in methylation (columns 1 and 4). Remarkably, methylation of the purines between this sequence and the cleavage site has no apparent effect on *Hph* action. It is difficult to assess the effect of methylation of the central AT (see column 2). The figure also shows as a control (columns 3 and 6) the results of methylating the 63 base pair fragment and analyzing without *Hph* treatment; in this case the various purines are labeled uniformly, Gs being about 7-fold more reactive than As, as previously observed (7).

We have used the dimethylsulfate technique to confirm that, in contrast to *Hph*, *Hin*dII requires integrity only of the six base pairs surrounding its cleavage site. In this experiment, we used the 75 base pair fragment produced by *Hph* digestion that contains most of O_R (3) (see Fig. 2); this fragment also contains a *Hin*dII cleavage site. This fragment, labeled at both ends with ^{32}P, was methylated and cut with *Hin*dII, and the resistant and cleaved products isolated on gels. In this case the resistant fragment was preferentially methylated at the *Hin*dII recognition (cleavage) site, $\genfrac{}{}{0pt}{}{\text{GTCAAC}}{\text{CAGTTG}}$ (17) and only at this site, whereas the cleaved pieces were deficient in methylation at this site and only at this site (not shown). These results are consistent with the conclusion that in contrast to *Hph*, *Hin*dII recognizes only the six base pairs surrounding its cleavage site.

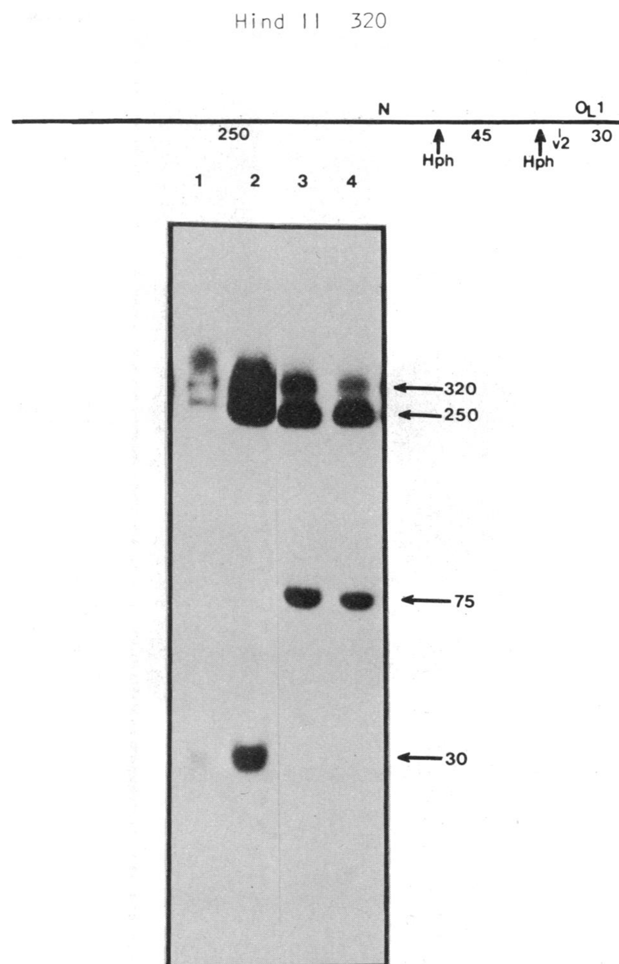

FIG. 3. *Hph* digestion of wild-type *Hin*dII 320 and of this fragment bearing the mutations *v*003 and *v*2. *Hin*dII 320, labeled with ^{32}P at each end, was digested with *Hph* and electrophoresed on a 15% polyacrylamide gel (bottom). The digested molecules carried either the mutation *v*2 (column 3) or *v*003 (column 4), or were derived from the DNA of the wild-type parents of these mutants (columns 1 and 2). The length in base pairs of the fragments produced by *Hph* digestion are indicated. Also shown (top) is a map of *Hind* 320 showing the location of 2 *Hph* cleavage sites, distances along the molecule in base pairs, and approximate location of *v*2.

Sequences closely related to the *Hph* "common sequence"

Examination of sequences found in the *lac* operon (18, 19) and in λ DNA reveals 10 of the possible 15 sequences that differ from the *Hph* "common sequence" by one base pair (Fig. 5). We have found that none of these sequences is recognized by *Hph* (not shown). The sequences in *lac* DNA were tested by incubating with *Hph* a 200 base pair fragment produced by digestion of λp*lac* DNA with *Hae* III;

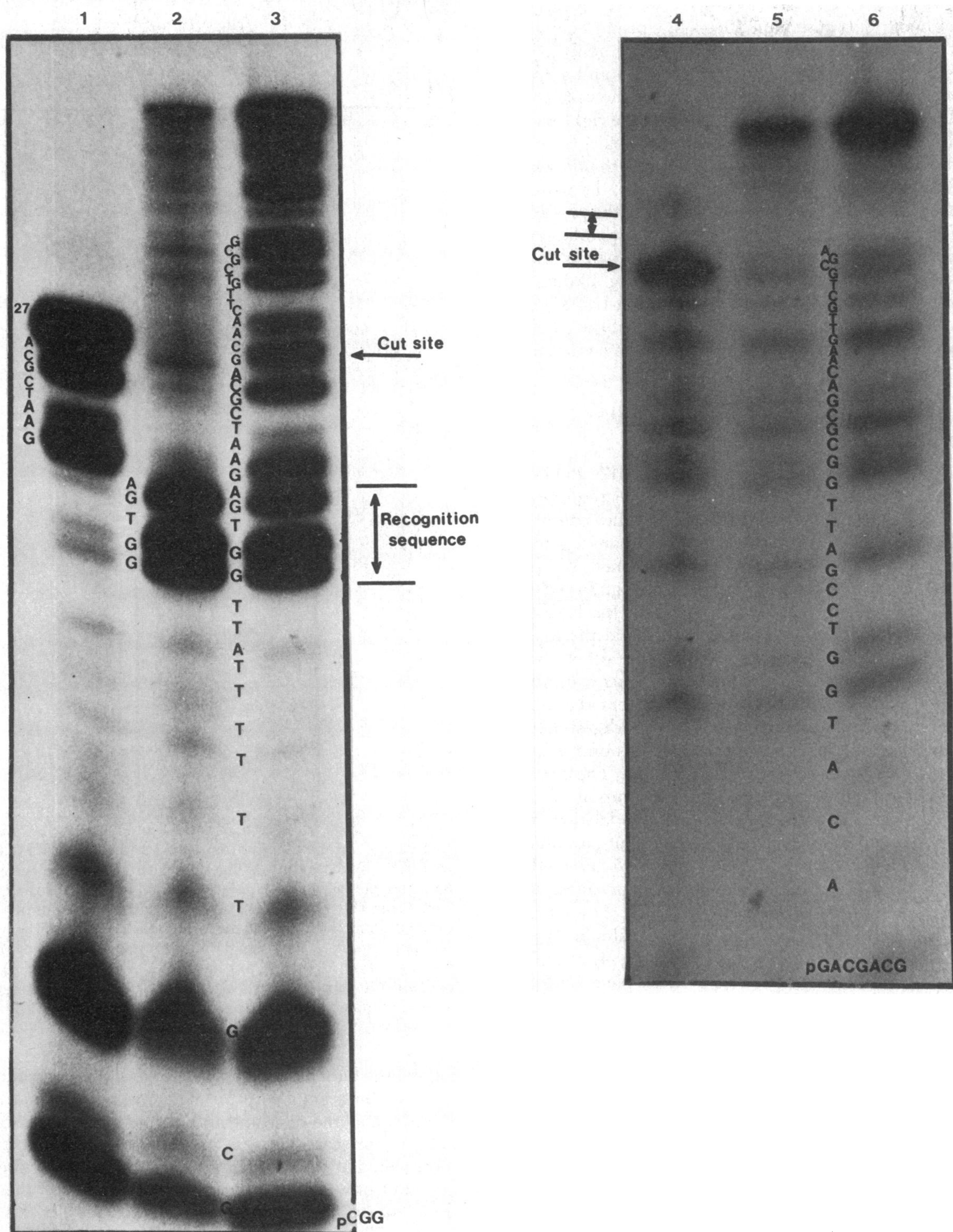

FIG. 4. Bases recognized by *Hph* as determined with dimethylsulfate. The 63 base pair *Hpa* II/*Hin*dII fragment was contained within a larger 1190 base pair long fragment produced by the action of *Hae* III on λ DNA (16). Independent labeling of the *Hpa* II end (columns 1, 2, and 3) was accomplished by treating the 1190 fragment successively with *Hpa* II, bacterial alkaline phosphatase, T_4 polynucleotide kinase and [γ-^{32}P]ATP, and *Hin*dII. The *Hin*dII end (columns 4, 5, and 6) was labeled similarly by reversing the order of endonucleolytic cleavages. Fragments were methylated at 0° for 16 hr in 12 mM dimethylsulfate in 250 μl of buffer containing 50 mM sodium cacodylate, pH 8, 10 mM $MgCl_2$, and 20 μg of tRNA. The mixture was then made 0.5 M in Tris·HCl and 25 mM in 2-mercaptoethanol and the DNA was precipitated with ethanol. The precipitates were resuspended in *Hph* buffer, and aliquots were digested with *Hph*. The molecules were then electrophoresed through a 15% polyacrylamide gel, and two fragments end-labeled with ^{32}P were visualized by autoradiography and eluted as described (3). [Fragments 63 and 27 base pairs long were recovered from the molecules labeled at the *Hpa* II end and fragments 63 and 36 base pairs long from the molecules labeled at the *Hin*dII end (see text).] One fragment (63 base pairs long) was recovered from each sample not treated with *Hph*. Methylated bases were released from the DNA by 5-min incubation at 95° in buffer containing 20 mM phosphate, pH 7, and 5 mM EDTA. The DNA was then cleaved at the sites of depurination by incubation at 95° for 10 min in 0.1 M NaOH. The fragments were fi-

(Legend continued at top of next page)

(Legend to Fig. 4 continued)
nally electrophoresed through an 18% polyacrylamide gel containing 7 M urea, 45 mM Tris-borate, pH 8.3, and 1.25 mM EDTA. The bands shown in the autoradiograph were assigned to the known sequence (see Fig. 1), according to the rules described by Gilbert *et al.* (7). That is, the dark bands correspond to methylation of Gs, lighter bands to As, and spaces to pyrimidines. Slight irregularities are expected; for example, not all the bands due to methylation of the As are of equal intensity (7). Columns 1, 2, and 3: molecules labeled at *Hpa* II end. Column 1, 27 base pair fragment generated by *Hph* cleavage; column 2, 63 base pair fragment rendered resistant to *Hph* cutting by methylation; column 3, 63 base pair fragment methylated and not treated with *Hph*. Columns 4, 5, and 6: molecules labeled at *Hin*II end. Column 4, 36 base pair fragment generated by *Hph* cleavage; column 5, 63 base pair fragment rendered resistant to cutting by methylation; column 6, 63 base pair fragment methylated and not treated with *Hph*.

Hph did not cleave this fragment, but did cleave a control fragment included in the reaction mixture (not shown). The sequences in λ DNA were tested by incubating with *Hph* DNA fragments that contained one or more bonafide *Hph* cleavage sites and one or more of the test sequences on the same fragment. Only the bonafide *Hph* cleavage sites were recognized.

DISCUSSION

Hph differs from previously described restriction endonucleases. It resembles enzymes of class II in its cofactor requirements and in that it cuts at specific sites. It differs from enzymes of that class, however, in that bases required for recognition are located eight or nine base pairs to one side of the cut site. Moreover, there is no apparent symmetry in or around these bases, nor in the bases immediately surrounding the cut site. We have reported here three lines of evidence that suggest that the *Hph* enzyme recognizes the "common sequence" $\frac{\text{TCACC}}{\text{AGTGG}}$: (*i*) this sequence is found eight or nine base pairs to one side of six *Hph* cleavage sites; (*ii*) methylation of purines within this sequence prevents *Hph* cutting; and (*iii*) mutations within this sequence prevent *Hph* cutting.

Methylation experiments performed *in vitro* suggest that the "common sequence" may constitute the entire *Hph* recognition site. Methylation of any one G or A in the sequence lowers *Hph* action nearly two orders of magnitude. Remarkably, methylation of the bases between this sequence and that of the bases immediately surrounding the cleavage site do not prevent cleavage, suggesting that the enzyme may not have any sequence requirement between the "common sequence" and the cleavage site. This idea is supported by the fact that there are no obvious similarities among the sequences between the various cleavage sites and their neighboring "common sequence." The cleavage sites so far observed are staggered about either an AT or a TA base pair, and we do not know whether these base pairs are required. The fact that a change of the fourth position of the "common sequence" by mutation abolishes *Hph* action proves that that base pair is required. The fact that the sequence we identify as the *Hph* recognition sequence is not symmetric is consistent with the fact that the enzyme cuts only to one side of that sequence.

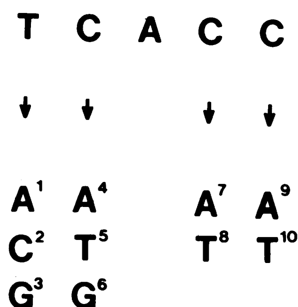

FIG. 5. The *Hph* recognition sequence, and various sequences that differ from it in one base pair. Only one strand of the recognition sequence is shown. The base change in 10 related sequences is shown. The location of these sequences is as follows: 1 is in wild-type O_L and O_R; 5 is in wild-type O_R; 7 and 8 are the mutant *v*2 and *v*003 in O_L; 2 and 4 are in the *N* gene (9); 3, 6, 9, and 10 are in a 200 base pair fragment isolated from λ*plac* DNA containing the sequenced *lac* operator (19, 20).

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