Complete Nucleotide Sequence of the Cohesive Ends of Bacteriophage P2 Deoxyribonucleic Acid*

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R. Padmanabhan,‡ Ray Wu,§ and Richard Calendar¶

From the Section of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, New York 14850

SUMMARY

At the ends of bacteriophage P2 DNA, the 5'-terminated strands are 19 nucleotides longer than the 3'-terminated strands. The complete nucleotide sequence of the two cohesive ends of P2 DNA has been determined. This was accomplished by repair synthesis, using Escherichia coli DNA polymerase and labeled deoxyribonucleoside triphosphates with or without a ribonucleoside triphosphate, followed by partial enzymatic digestion of the labeled segments and sequence analysis of the isolated oligonucleotides. Starting from the 5' end of one cohesive end, the 19 nucleotides are in the sequence dpGpTpGpCpTpTpTpCpCpCpCpGpCpCpT'pCpGpCpC. The sequence from the other cohesive end is exactly complementary to this one. The underlined bases differ from the sequence of the corresponding cohesive end of bacteriophage 186 DNA. Thus, DNA molecules from two related phages can form mixed dimers and help each other in infectivity assay even though the cohesive end sequences are not identical. The functional and thermodynamic significance of these findings is discussed.

We have previously reported the complete sequence of the left-hand cohesive end of bacteriophage 186 DNA (3). P2 DNA belongs to the same family as 186 DNA on the basis of (a) formation of mixed dimers between the 2 DNA molecules (4); (b) the ability of 1 DNA molecule to help in the infectivity assay by the other phage (4); and (c) the extensive hybridization homology between them (5). Furthermore, P2 and 186 have similar morphology (6, 7), and depend on the Escherichia coli rep gene product for DNA replication (8). In order to understand the morphogenesis and evolution of these phages, we have carried out sequence analysis of the P2 cohesive ends, using two independent methods. The first method is the same as published previously

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† Present address, Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Mo. 63110

§ To whom reprint requests should be made.

¶ Department of Molecular Biology, University of California, Berkeley, Calif. 94720.

(3, 9, 10). In this method, the DNA was labeled with one 32P-labeled and all four 3H-labeled deoxynucleoside triphosphates. The labeled DNA was then digested partially with micrococcal nuclease and the oligonucleotides were separated by two-dimensional electrophoresis (11) and sequenced. In the second method, we used the conditions for the incorporation of one ribonucleotide in the presence of two or three deoxyribonucleotides, DNA polymerase I and Mn++ (12, 13) for repairing the cohesive ends. The cohesive ends of the DNA were then cleaved with a specific ribonuclease. When rGTP, dCTP, and dTTP were used to repair the cohesive ends, ribonuclease T1 was used for the cleavage of the partially repaired cohesive ends. When rCTP, dGTP, and dATP were used to label the cohesive ends, pancreatic RNase was used for digestion. The sequence analysis of the cleavage products of the ribo-substituted DNA confirmed the sequence derived by the first method. In this communication, we wish to report the complete sequence of the cohesive ends of P2 DNA which is found to be homologous but not identical with 186 DNA. Partial sequences for the cohesive ends of P2 DNA have been reported by Murray and Murray (14) who used a terminal labeling method. These authors have determined the sequence of a 9-mer and a 12-mer from the two 5' ends, and they have deduced the complete sequence by assuming that the two cohesive ends are complementary and are 19 nucleotides in length, even though the actual length of the cohesive ends cannot be obtained with their method.

MATERIALS AND METHODS

DNA—Phage P2 was grown and purified according to previously published procedures (15, 16). DNA was extracted by phenol and further purified by sucrose gradient sedimentation as described elsewhere (17).

Enzymes—Human semen phosphatase was purified according to the unpublished procedure of Doctors M. Singer and L. Heppel (the details are given in Ref. 17). Purified spleen phosphodiesterase which is free of phosphatase activity was a gift of Dr. G. Bernardi, Institut de Biologie Moleculaire, Universite Paris VII. Ribonuclease T1 was from Calbiochem. Pancreatic ribonuclease, micrococcal nuclease, and bacterial alkaline phosphatase were purchased from Worthington Biochemical Co. Venom phosphodiesterase (Worthington) was further purified to remove 5'-nucleotidase (18).

† Where there is no ambiguity, the symbols A, G, T, and C are used throughout to stand for deoxynucleosides. The prefix d is used in some cases for emphasis. The prefix r is used for all ribonucleic acids. 3H-Labeled nucleotides are represented with a dot on top of the nucleoside, thus rG stands for [3H]guanosine; 32P-labeled nucleotides are represented with an asterisk on top of the phosphate, thus rG stands for [32P]dpG; P stands for 32P.
Labeled Deoxyribonucleoside Triphosphates—Tritiated dATP, dTTP, and dCTP were purchased from Schwarz BioResearch, Inc., and tritiated dGTP was from Amersham Searle Co. α-32P-Labeled deoxyribonucleoside triphosphates were prepared according to the procedure described by Symons (19) with a few modifications (3), or purchased from New England Nuclear for more recent experiments.

Repair Synthesis and Sequence Analysis—The first approach for the sequence determination of the cohesive ends of P2 DNA was identical with that employed for λ (10) and λ186 DNA (3). This involved DNA polymerase I-catalyzed repair synthesis of the cohesive ends, partial micrococcal nuclease digestion of the labeled DNA, and separation of the resulting oligonucleotides by twodimensional ionophoresis followed by sequence analysis of the oligonucleotides. The two-dimensional ionophoresis fractionating system (22, 23) was used for Experiment 1. The products of this experiment were separated by ionophoresis (11). The purified phosphorylated pyrimidine oligonucleotides were further analyzed on the dephosphorylated oligonucleotides by one-dimensional homochromatography (22, 23). From the mobility shifts of successively smaller oligonucleotides resulting from a partial enzymatic digestion of an oligonucleotide in this system, it is possible to determine the sequence of the parent oligonucleotide (25, 26, 27).

RESULTS

Partial Incorporation of Nucleotides into Cohesive Ends of P2 DNA—When only dATP and dGTP were present for the E. coli DNA polymerase I-catalyzed repair synthesis, no pA but 3 pG residues were incorporated. An analysis revealed that the ratio of the labeled nucleoside to nucleotide was 2:1. This could be interpreted to mean that 2 pG residues were added to one cohesive end and the 3rd pG was added to the other cohesive end (Table I, Experiment 1a). In analogy to 186 DNA, the cohesive end of P2 to which 2 pG residues were added will be arbitrarily referred to as the right-hand cohesive end, and the end to which 1 pG was added as the left-hand cohesive end. When dGTP was used for incorporation (Experiment 1b) one pA was transferred from pG to pA which is one of the natural 3′ ends of P2 DNA, and two pG were transferred to pG. This would be consistent if 2 pG residues were added to the right hand end and 1 pG to the left-hand end. When dCTP and dGTP were present, 4 pG residues were incorporated and nearest neighbor analysis showed the presence of one ApG, two GpG, and one CpG sequence (Experiment 2). When dGTP and dTTP were present 1 pT and 4 pG residues were incorporated. Nearest neighbor analysis indicated the presence of one ApG, two GpG, and one TpG sequence (Experiment 3). Again, in analogy to 186 DNA it was assumed that the sequence of nucleotides added to the right-hand end was G-G-C-G-OH and the sequence at the left-hand end was G-T-G-OH (Fig. 1). Although the possibility existed that the sequence at the right-hand end could be G-G-T-G and at the left-hand end be G-C-G, it was ruled out by the complete sequence determination of the cohesive ends of P2 DNA. When dGTP, dATP, and dTTP were present (Table I, Experiment 4), no incorporation of pA residue occurred and 3′ end group analysis showed a 1:1 ratio of pG:pC, supporting the sequence shown for Experiment 2. When dGTP, dCTP, and dATP were present (Experiment 5), 4 pC, 5 pA, and 11 pG residues were incorporated. As will be shown later, the entire length of the right-hand cohesive end of P2 DNA was repaired in this experiment. Since the second nucleotide to be added at the left-hand end (pT) was not present, repair synthesis presumably did not take place beyond the addition of a pG residue at this end, and 19 nucleotides were added to the right-hand end.

3′ End Group and Nearest Neighbor Analyses of P1 DNA Completely Labeled at Cohesive Ends—By using one labeled and three nonradioactive deoxynucleoside triphosphates, it was found that 14 pG, 14 pC, 5 pT, and 5 pA residues were incorporated into both cohesive ends of P2 DNA to give the total number of 38. Thus, each cohesive end is 19 nucleotides long, similar to the cohesive end of 186 DNA.

In order to determine the 3′ ends of the completely labeled cohesive ends, all four tritium-labeled deoxynucleoside triphosphates were used for incorporation. Analysis for its 3′ end showed that P2 DNA has only C at its 3′ ends after repair and the ratio of pG:pC was 6:1. Since there were 14 pC residues incorporated into the cohesive ends, this could only be explained if there were 2 C residues at the two 3′ ends and 12 pC residues at the internal regions of completely repaired cohesive ends. This was confirmed by the determination of the 5′ ends of native

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TABLE I
Incorporation of deoxynucleotides into P2 DNA

| Expt. | Nucleoside Residues incorporated† | Products found after enzyme digestion‡ | Sequence deduced |
|-------|----------------------------------|----------------------------------------|------------------|
|       |                                  |                                        |                  |
| 1 a   | dCTP and dATP                     | 2.8 pG                                  | 0.9 pG           |
|       |                                  |                                        | 2 G              |
| 1 b   | dCTP                             | 3.3 pG                                  | 1 Ap, 1.8 pG     |                  |
|       |                                  |                                        |                  |
| 2     | dCTP and dCTP                    | 3.7 pG                                  | 1 Ap             |
|       |                                  |                                        | 2.3 C            |
|       |                                  |                                        | 0.9 C            |
| 3     | dCTP and dCTP                    | 1.2 pT                                  | 1.2 Ap           |
|       |                                  |                                        | 1.8 C            |
|       |                                  |                                        | 0.8 Tp           |
| 4     | dCTP, dATP, and dCTP             | -                                       | 1 Ap, 1.8 pG     | 2 G              |
| 5     | dCTP, dCTP, and dCTP             | 3.7 pC                                  | 1 Ap             |
|       |                                  |                                        | 2.3 pG           |
| 6     |                                  | 11 pG                                   | 3 Cp, 10.2 pG    |                  |

† The number of residues incorporated was obtained after the plateau was reached under standard conditions of incubation as described previously (9).
‡ Labeled DNA was digested with micrococcal nuclease and spleen phosphodiesterase to 3'-nucleotides and nucleosides followed by separation by two-dimensional paper chromatography as described previously (9). Nucleotides within brackets represent the original 3' end nucleotide present in the native molecule of P2 DNA before repair synthesis. Symbol * over p residue denotes 32P label and the symbol ‡ over the nucleoside moiety denotes 3H label.

Fig. 1. Sequence determined by partial incorporation into the cohesive ends of P2 DNA.

P2 DNA using the procedures as described previously (28). It was found that both 5' ends of P2 DNA were pG. The method used for the determination of 16 possible dinucleotide sequences at the repaired cohesive ends of P2 DNA was the same as described by Wu and Taylor (10). The results summarized in Table II suggest that the nucleotide sequences at the cohesive ends of P2 DNA are complementary and of opposite polarity. For example, there were two GpA to match two TpC sequences and one GpT to match one ApC. There were three ApG but only two CpT sequences which can be explained as follows. It has been established (see Table I, Experiment 1b) that pG was the first nucleotide to be added to both ends and one of them was next to the pA at the natural 3' end, thus giving one extra ApG sequence. For the same reason, there was one extra GpG over CpC sequence. There could be either 1 or 2 molecules of TpG per molecule of λDNA on the basis of the experimental value of 1.4. However, since only 1 molecule of CpA was found, there must be only 1 TpG.

The self-complementary sequences ApT, TpA, GpC, and CpG were either even numbered or zero as would be expected of completely complementary cohesive ends. Therefore, the data presented in Tables I and II suggest that the two cohesive ends are each 19 nucleotides long and they are complementary.

Isolation and Characterization of Oligonucleotides from Cohesive Ends—In order to determine the complete sequence of the cohesive ends of P2 DNA, the DNA was labeled with all four deoxyribonucleotides. In general, one 32P-labeled and all four tritium-labeled deoxynucleotides were incorporated into the cohesive ends of 1 molecule of DNA. The values are the average of three sets of experiments. For the explanation of symbol *, see Table I.

Table II
Nearest neighbor analysis of P2 DNA completely labeled at cohesive ends

The results are expressed as the number of indicated dinucleotide sequences in the repaired ends of one P2 DNA molecule. The calculation was based on a knowledge of the total number of the particular 32P-labeled nucleotide residues incorporated into the cohesive ends of 1 molecule of DNA. The values are the average of three sets of experiments. For the explanation of symbol *, see Table I.

| Isolated 3'-deoxyribonucleotide | Reaction 1: \( \text{pppA} \) | Reaction 2: \( \text{pppG} \) | Reaction 3: \( \text{pppT} \) | Reaction 4: \( \text{pppC} \) |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Sequence | No. | Sequence | No. | Sequence | No. | Sequence | No. |
| Ap      | 2.2 | ApG     | 3.0 | ApT      | 0   | ApC      | 1.1 |
| Gp      | 2.0 | GpG     | 5.7 | GpT      | 1.0 | GpC      | 5.8 |
| Tp      | 0   | TpG     | 1.4 | TpT      | 2.2 | TpC      | 2.1 |
| Cp      | 0.8 | CpG     | 3.9 | CpT      | 1.9 | CpC      | 5.0 |
| Sum     | 5.0 | 14.0    | 5.1 | 14.0     |     |           |     |
TABLE III

Isolation and sequence analysis of oligonucleotides incorporated into cohesive ends of P2 DNA

All of the oligonucleotides that were analyzed were products of partial micrococcal nuclease digestion of labeled P2 DNA. The products were first separated by two-dimensional isophoresis (11) as shown in Figs. 2 and 3. Oligonucleotides 1, 2, 7, 10, 12, 13, 14 came from Fig. 2 (DNA labeled with $pC, pG, pA, pT$) or related experiments (not shown here) and oligonucleotides 4 and 15 came from Fig. 3 (DNA labeled with $pG, pC, pA, pT$). The numbers of nucleotides and nucleosides were calculated from the ratios of specific activities of tritium as well as $32P$-labeled deoxynucleoside triphosphates used for incorporation.

| Oligonucleotide | Analysis | Nucleoside | Nucleotides | Sequence present | Sequence deduced $^5$ |
|----------------|----------|------------|-------------|-----------------|----------------------|
| 1              | S$_0$    | 1 C        | 1 $pC$, 1 $pG$, 1 $Ap$ | C as 3' end | ApGpCpA-C-OH |
|                | V        | 1 A        | 1.1 $pA$, 2.1 $pC$, 1 $pG$ | A as 5' end | R(15 to 19) |
| 2              | S$_0$    | 1 C        | 1.2 $pC$, 1 $Ap$ | C as 3' end | ApApGpCpA-C-OH |
|                | V        | 1 C        | 2.3 $pC$, 1 $pG$ | C as 5' end | R(14 to 19) |
| 7              | S$_0$    | 1.1 C      | 1.2 $pC$, 0.8 $pG$, 1 $pC$ | C as 3' end | CpGpCpA-C-OH |
|                | V        | 1 C        | 2.3 $pC$, 1 $pG$ | C as 5' end | L(16 to 19) |
| 10             | S$_0$    | 1 T        | 4 $pC$ | T as 5' end | L(7 to 11) |
|                | V        | 1 T        | 4 $pC$ | T as 5' end | L(7 to 14) |
| 12             | S$_0$    | 1 C        | 1.2 $pG$, 1.2 $Tp$ | 1 $pG$, 1 $Tp$ | L(2 to 4) |
|                | S$_1$    | 1.2 C      | 0.9 $pG$, 1.2 $Tp$ | 1 $Tp$, 3 $pC$ | L(1 to 4) |
|                | V        | 0.8 T      | 5.3 $pC$, 1 $pG$ | T as 5' end | L(7 to 14) |
| 13             | S$_0$    | 1 C        | 1.3 $pG$, 0.9 $Tp$ | 1 $pG$ | TpGpCp |
|                | V        | 1 T        | 1 $pG$, 0.8 $pC$ | T as 5' end | L(2 to 4) |
| 14             | S$_0$    | 1.1 $pC$, 0.9 $pG$, 1 $Tp$ | 1 $pC$ | GpTpApGp |
|                | V        | 1 A        | 1.1 $pC$, 0.8 $pG$, 1 $Ap$ | 1 $ApG$ | ApGpGp |
| 15             | S$_0$    | 1 C        | 1 $pC$, 1 $Tp$, 1 $pG$ | 1 $Tp$ | TpGpC |
|                | S$_1$    | 1 C        | 1 $pG$, 1 $Tp$ | 1 $Tp$ | TpGpC-OH |

$^5$ The enzyme used for Analyses $S_0$ and $S_1$ was spleen phosphodiesterase. Analysis $S_0$, spleen phosphodiesterase digestion of the original oligonucleotide. Analysis $S_1$, digestion after treatment with semen phosphatase. Analysis $V$, venom phosphodiesterase digestion. Semen phosphatase treatment preceded the venom phosphodiesterase digestion if the original oligomer was found to have a phosphate at its 3' end. The condition for semen phosphatase treatment is given under "Materials and Methods." $^\S$ L and R refer to the arbitrary assignment of left-hand and right-hand cohesive ends of P2 DNA (see Fig. 9), respectively. All nucleotides contain tritium; the dots on the nucleosides are omitted in the structure given here; the symbol * is as defined in Table I.
FIG. 2 (left). Two-dimensional ionophoresis of partial micrococcal nuclease digest of P2 DNA (6 pmole, 135 μg) labeled with pA, pC, pG, and pT. Dimension I, electrophoresis on cellulose acetate strip at pH 3.5 was carried out at 3500 volts. Dimension II, electrophoresis on DEAE-cellulose paper at pH 1.9 for 20 hours at 2200 volts. O, origin of electrophoresis; Y, position of nucleotide 4 as ApGpCp which is included in the sequence of oligonucleotides 1 to 2.

Oligonucleotide 7 was a tetramer with a C at the 3' end, and a C at the 5' end. In addition, there were one GpC and one CpC sequences and hence the sequence deduced was CpGpCp-OH. The alternative structure CpCpGpCp-OH was ruled out by the structure analysis of a related oligonucleotide (not shown) TpCpGpCp-OH. Occasionally we have observed that the repair synthesis stopped in some DNA molecules (30% of all molecules in this experiment) at the nucleotide penultimate to the 3' end of the completely repaired cohesive ends as in the case of TpCpGpCp-OH. Oligonucleotide 13 had one more Gp than the oligomer 13 giving rise to two possible structures. The structure GpTpGpCp is the more likely than TpCpGpCp in view of the fact that it overlaps with the sequence of G-T-G established by partial incorporation experiments at the left-hand cohesive end of P2 DNA. The structure of oligonucleotide 15 which is identical with oligonucleotide 13, but obtained from a DNA digest labeled with pA, pG, pC, and pT, was shown to be TpGpCp. This also rules out the alternative possibility of TpCpGpCp for oligonucleotide 14 because the 3'-PO₄ group in TpGpCp was not labeled with ³²P in oligonucleotide 15.

Sequence Analysis of Pyrimidine Oligonucleotides from Labeled Cohesive Ends of P2 DNA—Fig. 4a shows the two-dimensional fractionation of the phosphorylated pyrimidine oligonucleotides obtained as described under "Materials and Methods." Nearest neighbor, 3' and 5' end group analyses of these oligonucleotides (after dephosphorylation) are given in Table IV. The analysis of Spot 2 shows the presence of one CpT, the presence of a C at both the 3' end and 5' end and 1 additional Cp residue; the sequence C(CpT)C-OH can be deduced. The alternative sequence C(TpC)C-OH could not be ruled out from this data. This was ruled out, as will be discussed later, by an independent observation using a synthetic octanucleotide as primer for the repair synthesis of the cohesive end of P2 DNA. The analysis of Spot 3 (Table IV, Experiments a and b) showed the presence of a total of 5 C and 3 T residues. Both 3' and 5' ends were
For labeling the pyrimidine cluster, 4 pmoles of P2 DNA were used. The labeled DNA was denatured as described elsewhere (3, 20) and the products were subjected to two-dimensional ionophoresis (11). FIG. 4 (left). a, Fingerprint of the phosphorylated pyrimidine cluster (labeled with \( P_2 \) DNA) from the labeled cohesive ends of P2 DNA by two-dimensional electrophoresis. Dimension I, electrophoresis on cellulose acetate strip at pH 3.5 was carried out for 1.5 hours at 3500 volts. Dimension II, electrophoresis on DEAE-paper at pH 1.9 was carried out for 20 hours at 2200 volts. 0, origin of electrophoresis; Y, position of yellow dye marker; B, position of blue dye marker. b, A spot similar to Spot 3 from a, but from a DNA labeled with \( \text{p'T, p'C} \), was dephosphorylated using bacterial alkaline phosphatase-F (3) and purified by one-dimensional ionophoresis (pH 1.9) at 2200 volts for 14 hours.

FIG. 5 (right). One-dimensional homochromatographic separation of ribonuclease digestion products from the ribonucleotide substituted repaired cohesive ends of P2 DNA. Conditions for the repair synthesis in the presence of one ribonucleoside triphosphate and two deoxynucleoside triphosphates are as described under "Materials and Methods" and elsewhere (12, 13). P2 DNA was substituted with \( \text{pG, p'C, and p'T} \) before digestion with ribonuclease \( T_1 \) and fractionation (a). In b the DNA was labeled with \( \text{pG, p'A, and p'C} \), digested with ribonuclease A and fractionated by one-dimensional homochromatography. Homomix VI was used for fractionation in a and homomix III (23) was used in b.

found to be C and there were one CpT and two TpT sequences. The analysis of Spot 4 which was identical with Spot 3 but obtained from a DNA labeled with \( \text{p'C and p'T} \) showed the presence of three CpC and one TpC sequences; the sequence C(T-T-T, C-C-C)-OH could be deduced for Spot 3 (and 4).

The exact structure of Spot 3 (and hence 4) was established by isolation of the degradation products after partial spleen phosphodiesterase (Table V, Experiment a) and partial venom phosphodiesterase (Experiment b). Partial spleen phosphodiesterase digestion of the pyrimidine cluster showed a successive removal of \( \text{Cp, Tp, and Tp} \) residues from its 5' end. This established the location of the two TpT sequences. This information together with the 5'-nucleotide analysis of oligonucleotides 2, 3, and 4 established the sequence of the octanucleotide as C-T-T-T-C-C-C-OH. This sequence was confirmed in Experiment b.

This finding was consistent with the fact that a synthetic octanucleotide with the sequence, C-T-T-T-C-C-C-OH could serve as primer for the DNA polymerase I-catalyzed repair synthesis. The sequence beyond the 3' end of this octanucleotide primer was established as G-C-C-T-C-OH. As mentioned above, this ruled out an alternative possibility of sequence for Spot 2 in Table IV and established its sequence as C-C-T-C.

Sequence Analysis of Ribonucleases T, and A Digestion Products from Ribonucleotide-substituted Cohesive Ends of P2 DNA—In order to isolate more fragments from the cohesive ends of P2 DNA for a better overlap with the previously determined sequences, the original observation of Berg et al. was used (12). The cohesive ends of P2 DNA were repaired in the presence of one ribonucleoside triphosphate, two deoxyribonucleoside triphosphates, DNA polymerase, and Mn\(^{2+}\) ions. The conditions used for incorporation were essentially the same as described previously (24) and are given under "Materials and Methods." The reason for using only two deoxynucleoside triphosphates instead of three, in the presence of one ribonucleoside triphosphate, is the following. The incorporation data shown in Table I, Experiment 5 when only dGTP, dCTP, and dATP were present (or when dGTP, dCTP, and dTTP were present) only one of the
The pyrimidine oligonucleotides were separated by ionophoresis as described under "Materials and Methods" from P2 DNA labeled with \( \hat{p}T \) and \( pC \) (Spots 1 to 3 in Fig. 4a) or \( \hat{p}C \) and \( pT \) (Spot 4 in Fig. 4b) in the presence of nonradioactive \( pA \) and \( pG \). Spots 1, 2, and 3 from Fig. 4a were dephosphorylated with semen phosphodiesterase as described under "Materials and Methods" before they were analyzed in Experiment a or b. Spot 4 was identical with dephosphorylated Spot 2 except that the DNA used to isolate it was labeled with \( pC \) and \( \hat{p}T \). Results after complete spleen phosphodiesterase digestion for 3' end and nearest neighbor analysis are shown in Experiment a. Results after complete venom phosphodiesterase digestion for 5' end and composition analyses are shown in Experiment b. All nucleosides are tritium-labeled and dots on nucleosides are omitted here for simplicity, only the location of \( ^{32}P \) is shown by an asterisk.

| Experiment | Spot No. | Products after enzymatic digestion | Sequence present |
|------------|----------|------------------------------------|-----------------|
| \( a \)    | 1        | T                                  | (Pu) \( \hat{p}T \) (Pu) |
|            | 2        | 1 C                                | C as 3' end      |
|            | 3        | 1 C                                | C as 3' end      |
|            | 4        | 1.1 C                             | C as 3' end      |
|             |          |                                    |                 |
| \( b \)    | 1        | T                                  | (Pu) \( \hat{p}C \) (Pu) |
|            | 2        | 1 C                                | C as 5' end      |
|            | 3        | 1 C                                | C as 5' end      |
|            | 4        | 1 C                                | C as 5' end      |

The pyrimidine oligonucleotides were separated by ionophoresis as described under "Materials and Methods" from P2 DNA labeled with \( \hat{p}T \) and \( pC \) (Spots 1 to 3 in Fig. 4a) or \( \hat{p}C \) and \( pT \) (Spot 4 in Fig. 4b) in the presence of nonradioactive \( pA \) and \( pG \). Spots 1, 2, and 3 from Fig. 4a were dephosphorylated with semen phosphodiesterase as described under "Materials and Methods" before they were analyzed in Experiment a or b. Spot 4 was identical with dephosphorylated Spot 2 except that the DNA used to isolate it was labeled with \( pC \) and \( \hat{p}T \). Results after complete spleen phosphodiesterase digestion for 3' end and nearest neighbor analysis are shown in Experiment a. Results after complete venom phosphodiesterase digestion for 5' end and composition analyses are shown in Experiment b. All nucleosides are tritium-labeled and dots on nucleosides are omitted here for simplicity, only the location of \( ^{32}P \) is shown by an asterisk.

| Experiment | Spot No. | Products after enzymatic digestion | Sequence present |
|------------|----------|------------------------------------|-----------------|
| \( a \)    | 1        | T                                  | (Pu) \( \hat{p}T \) (Pu) |
|            | 2        | 1 C                                | C as 3' end      |
|            | 3        | 1 C                                | C as 3' end      |
|            | 4        | 1.1 C                             | C as 3' end      |
|             |          |                                    |                 |
| \( b \)    | 1        | T                                  | (Pu) \( \hat{p}C \) (Pu) |
|            | 2        | 1 C                                | C as 5' end      |
|            | 3        | 1 C                                | C as 5' end      |
|            | 4        | 1 C                                | C as 5' end      |

The analysis of oligomer 2 in Fig. 5a showed the presence of 1 T, 3 C, and 1 rG residues. Oligomer 2 also contained one TpC, one CpC, and one rGpC sequences. It had a C as the 5' end, thus the sequence C(pTpCpCrGp) could be deduced. The two possible structures C(pTpCpCrGp) and C(pTpCpCrGp) could not be distinguished by these analyses alone. But by using a synthetic octanucleotide \( pCpTpTpCpCpCrGp \) as primer for DNA polymerase-catalyzed repair synthesis, a pentanucleotide sequence GpCpGpTpC was determined beyond the 3' end of the primer. It also established that oligomer 1 with the sequence GpCpCpTpCpCrGp(C) was at the 5' end of oligomer 2 with the sequence GpCpCpTpCpCrGp(C). By overlapping rGpC, oligomer 1 and 2 can be joined to give a 16 mer, rGpCpTpCpCpCrGpCrGpCrGpCpTpCp.

The analysis of Spots 1 and 3 from Fig. 5b are given in Table VII. Spot 1 was a nonanucleotide containing 5 G, 3 A, and 1 rC residues. The 5' end of this oligomer was an A and it contained one GpA, two ApA, and one rCp sequences, thus the sequence Gp(AapAapA) could be the 5' end of this oligomer. The 3' end of this oligomer was the product of ribonuclease A digestion. The 3' terminus was \( ^{32}P \)-labeled as shown by the presence of one rCpA sequence. The complete sequence of this oligomer was established by partial digestion by spleen phosphodiesterase followed by fractionation by two-dimensional (ionophoresis-homochromatography) system as shown in Fig. 7. From the characteristic mobility shifts of the oligonucleotides, the presence of 4 G and 1 A from the 5' end of this oligomer was established. Thus the sequence GpGpGpGpGpApGpApGpGrCp could be established unambiguously for this oligomer, and the presence of the sequence rCpGpCpGpCpCrGpApGpCrGpCrA in one of the cohesive ends was indicated. The rCp at the 5' end is expected since the nonamer was released after ribonuclease A digestion of the ribo-C-substituted P2 DNA. The pA at the 3' end came from nearest neighbor transfer of \( ^{32}P \) from \( pA \) to \( rC \).

The analysis of Spot 3 from Fig. 5b showed the presence of 3 pG residues and one GpA sequence (Table VII). A rCp residue must be at the 3' end of this oligomer for the reason stated for Spot 1 but this was not 3' terminally labeled since nonradioactive rCTP was used for incorporation and there was no rCpA sequence present. The 5' end of this oligomer was a G and thus, the sequence GpApGpGpGpCrGpCr could be deduced. The complete sequence of this oligonucleotide was obtained by partial digestion with spleen phosphodiesterase followed by the two-dimensional fingerprinting method (22, 27) as shown in Fig. 8. From the characteristic mobility shifts of the pG- and pA-labeled oligonucleotides, the sequence GpApGpGpCrGpCr could be deduced for Spot 3 in Fig. 5b, and the presence of the sequence rCpGpApGpGpCrGpCr in one of the cohesive ends was indicated.

Alignment of Oligonucleotides to Give Complete Sequence—The sequences of the radioactive oligonucleotides, which are complementary to the cohesive ends of P2 DNA, are derived from three types of experiments: (a) partial incorporation, (b) complete re-
The pyrimidine cluster used in this experiment, identical with Spot 4 mentioned under Table IV, was isolated from P2 DNA labeled with pC and pT in the presence of nonradioactive pG. Spots 1 to 4 under Experiment a were obtained by partial spleen phosphodiesterase digestion and Spots 1 and 2 under Experiment b were obtained by partial venom phosphodiesterase digestion of the pyrimidine cluster. For partial spleen phosphodiesterase digestion, the material was divided into two parts and digested with two levels of the enzyme (0.04 μg or 0.02 unit, and 0.08 μg or 0.04 unit) in an incubation mixture containing 25 mM KPi, 2 mM EDTA, and 0.01% Tween-80. Incubation was carried out at 37° for 30 min. The products were separated by one-dimensional ionophoresis at pH 3.5 (2200 volts for 8.5 hours). The composition of the partial degradation products and its 5' end were determined by complete venom phosphodiesterase digestion. The incubation mixture contained 20 mM Tris-acetate (pH 8.5) and 2 mM magnesium acetate and 0.3 μg of purified venom phosphodiesterase. The incubation was for 17 hours. For partial venom phosphodiesterase digestion (Experiment b), 120 nmoles of the pyrimidine cluster with 7,500 cpm of 32P and 22,700 cpm of 3H were used. The material was divided into two parts and digested with two levels (0.08 and 0.16 μg) of the enzyme for 30 min at 37°. The incubation mixture contained 40 mM Tris-acetate (pH 8.4) and 4 mM magnesium acetate. Nearest neighbor and 3' end group analyses of these products were carried out by spleen phosphodiesterase digestion. The incubation mixture contained 20 mM KPi, 2 mM EDTA, 0.01% Tween-80, and 0.4 mM in each of the four 3'-deoxyribonucleotides and four deoxyribonucleosides added as carriers. The incubation was for 17 hours at 37°. The nucleotides and nucleosides were separated and analyzed as described earlier (9). All nucleotides are tritium-labeled, but the dots on nucelosides are omitted here for simplicity. Only the location of 32P is shown by an asterisk.

Table V

| Expt. | Spot # | Mobility from origin (cm) | M. value | Products after enzyme digestion | Sequence present | Sequence deduced |
|-------|--------|--------------------------|----------|-------------------------------|-----------------|------------------|
|       |        |                          |          | Nucleoside  | Nucleotide      |                  |
| a     | 1      | 4                        |          | 1 C       | 4.1 pC          | CpTpTpTpCpCpCpCpCpOH |
|       |        |                          |          | (5'end)    | 2.9 pT          |                  |
|       | 2      | 6.5                      | 0.62     | 1 T       | 1.9 pT          | TpTpTpTpCpCpCpCupoH |
|       |        |                          |          | (5'end)    | 4.4 pC          |                  |
|       | 3      | 12.5                     | 0.92     | 1 T       | 0.7 pT          | TpTpTpTpCpCpCupoH |
|       |        |                          |          | (5'end)    | 4.3 pC          |                  |
|       | 4      | 23.5                     | 0.88     | 1 T       | 4 pC            | TpTpTpTpCpCupoH   |
|       |        |                          |          | (5'end)    |                |                  |
| b     | 1      | 5.5                      |          | 1 C       | 1.1 Cp, 3.1 Cp  | TpC            |
|       |        |                          |          | (3'end)    | 2.3 Tp, 1.3 Tp  | Cp               |
|       | 2      | 3.5                      | 0.54     | 1 C       | 0.7 Cp, 2 Cp    | TpC            |
|       |        |                          |          | (3'end)    | 2.1 Tp, 0.9 Tp  | Cp               |

Pair with deoxynucleotides followed by partial digestion with micrococcal nuclease, and (c) ribo-substitution experiment (repair with ribo and deoxyribonucleotides) followed by digestion with ribo-nuclease. Of these experiments, the third type produced the largest amount of sequence information. Therefore, ribo-substitution experiments should be carried out whenever possible.

Table I and Fig. 1 show that the sequence of nucleotides incorporated into one cohesive end of F2 DNA during partial incorporation is G-G-C-G and the sequence at the other end is G-T-G (possibility I). An alternative possibility (II) that the terminal oligonucleotides 1 and IVw+ which was catalyzed by DNA polymerase I should also ruled out from the experimental evidence discussed below.

The repair synthesis in the presence of dATP, dGTP, rCTP, and Mn++ which was catalyzed by DNA polymerase I should also take place essentially at one cohesive end. Therefore, the two fragments rC-G-G-G-A-A-A-G-rCpA and rC-G-A-G-G-rCpG-G-G-G-A-A-A-G-C-A-C-OH could be deduced from the data given in Table II. On the other hand, if the repair synthesis starts with G-T-G (possibility I) as the starting point of repair synthesis, the sequence rC-G-G-G-G-A-A-A-G-C-A-C-OH could be deduced to be present at the R end (see Fig. 9). The sequence rC-G-C-G-A-A-A-G-Cp could be joined by overlapping four nucleotides with the sequence of 3'-terminal oligonucleotide 2 in Table III, A-A-G-C-A-C-OH; thus the sequence rC-G-G-G-G-A-A-A-G-C-A-C-OH could be deduced to be present at the R end (see Fig. 9). The sequence rC-G-A-G-G-rCp which is also present at this end could only be at the 5' end of the above dodecanucleotide sequence; thus a 17-nucleotide sequence rC-G-A-G-G-rCpG-G-G-G-A-A-A-G-C-A-C-OH can be derived by overlapping a rCp. The sequence established by partial incorporation from the starting point of repair synthesis, namely G-G-C-G (possibility I) or G-C-G (possibility II) should also come from this end. Only possibility I, G-G-C-G, can give rise to the correct length of 19 nucleotides for this end. In fact,
TABLE VI
Nearest neighbor analysis of products obtained by ribonuclease T1 digestion of rpG-substituted repaired cohesive ends of P2 DNA

Spots 1 and 2 (Experiment a) were from Fig. 5a. Spot 1 (Experiment b) was from a similar experiment as described under Fig. 5a except that the DNA was labeled with $T, pC in the presence of rpG. Experiments a and b gave results after spleen phosphodiesterase digestion, and Experiment c gave results after venom phosphodiesterase digestion of Spots 1 and 2. The conditions for the enzymatic digestion have been described elsewhere (27). All nucleoside moieties contain tritium except rG, and dots are omitted for simplicity.

| Expt. | Spot # | Products after enzyme digestion | Sequence present |
|-------|--------|---------------------------------|-----------------|
| a     | 1      | 1 T; 1.9 Tp                      | 1 Tp(C)         |
|       | 2      | 3 C; 2 cp                        | 3 C;            |
|       |        | 0.8 rG;                          | 1 rG(C)         |
| b     | 1      | 1 Tp, 3.7 Cp                      | 1 Tp(C)         |
|       |        | 0.9 C;                           | 1 C;            |
|       |        | 0.9 rG;                          | 1 rG(C)         |
| c     | 1      | 1 C; 3.9 Cp                       | 1 C;            |
|       | 2      | 1.1 Tp                           | 2 Tp            |
|       |        | 1.8 Tp                           | 1.8 Tp          |

Only the sequence G-G-C-G for the R end is consistent with all the data.

Once the sequence determined by partial incorporation techniques at the R end is established to be G-G-C-G, the sequence at the L end must be G-T-G (see Figs. 1 and 9). This is also consistent with the occurrence of fragment G-T-G-C (oligonucleotide 14 in Table III) in the micrococcal nuclease digest of labeled P2 DNA.

TABLE VII
Nearest neighbor analysis of products obtained by ribonuclease A digestion of rpC-substituted repaired cohesive ends of P2 DNA

Spots 1 and 3 were from Fig. 5b. Experiment a was due to spleen phosphodiesterase digestion and Experiment b was due to venom phosphodiesterase digestion. The conditions for digestions have been described elsewhere (27). Only the deoxyguanosine moiety was tritium-labeled.

| Expt. | Spot No. | Products after enzyme digestion | Nucleotide | Sequence present |
|-------|-----------|---------------------------------|------------|-----------------|
| a     | 1         | 4.8 $Gp$, 1.2 $Gp^*$             | 1 $Gp^*$   |
|       | 3         | 2.3 $Ap$, 0.8 $Cp$               | 2 $Ap$, 1 $Cp^*$ |
| b     | 1         | 1 $Gp$, 1.8 $Gp$                 | 1 $Gp$     |
|       | 3         | 4.1 $pG$, 3.2 $pA$               | 5 au 5’end  |

FIG. 6 (left). Fingerprint of a partial spleen phosphodiesterase digest of Spot 1 from Fig. 5a. An aliquot (5-ul) of Spot 1 from the experiment was described under Fig. 5a was digested with 0.05 unit of spleen phosphodiesterase (0.13 pg) in a total volume of 10 ul. The conditions for partial digestions and the fractionation by two-dimensional ionophoresis-homochromatography have been described elsewhere (23).

FIG. 7 (center). Fingerprint of a partial spleen phosphodiesterase digest for Spot 1 from Fig. 5b. An aliquot of Spot 1 from the experiment described under Fig. 5b was digested as described under Fig. 6. The conditions for digestion and for the fractionation of the digest have been described elsewhere (23).

FIG. 8 (right). Fingerprint of a partial spleen phosphodiesterase digest of Spot 3 in Fig. 5b. Spot 3 corresponding to the one in Fig. 5b but obtained from the DNA labeled with rpC, $pG$, and $pA$ was digested under Fig. 6. The conditions for digestion and for fractionation have been described elsewhere (23).
The sequence C-G-C-OH for oligonucleotide 7 in Table III come from the 3' terminus of the cohesive end L. By overlapping three nucleotides with the 16-mer for the L end mentioned earlier, the 17-mer sequence G-C-T-T-C-C-C-G-C-G-C-T-G-C-OH can be established. This could be joined to G-T-G-C by overlapping two nucleotides to give G-T-G-C-T-T-T-C-C-C-C-G-C-C-T-C-G-C-OH which is the complete sequence for the cohesive end L. This sequence is also consistent with that of all the fragments analyzed in Table III. The correctness of the sequences for the two cohesive ends is supported by the total number of nucleotides incorporated at the two cohesive ends, and also their nearest neighbor analysis shown in Table III. Also, the sequences at the two cohesive ends are exactly complementary in agreement with the data in Table II.

\[ \text{FIG. 9. Nucleotides incorporated into the cohesive ends of P2 DNA.} \]

\[ \text{FIG. 10. Comparison of the sequences at one of the cohesive ends of P2 and 186 DNA.} \]

\[
\begin{align*}
\text{DISCUSSION} \\
\text{Differences and Similarities between Cohesive End Sequences—} \\
\text{The studies reported here were started as a part of our investigation of the role of cohesive ends in the formation of mixed dimers between 2 DNA molecules and in the helper-mediated infectivity assay. Two members of the same group of bacteriophages, such as } & \lambda \text{ and } \phi 80 \text{ or P2 and 186 are able to form mixed dimers with each other and function as helpers. But the members of different groups, such as } & \lambda \text{ and 186 are unable to perform these two functions (6, 29). Sequences at the cohesive ends of } & \lambda \text{ and 186 have been shown to be completely different (3, 10). The studies on the sequence determination of } & \phi 80 \text{ and P2 started 2 years ago in our laboratory to find out whether their sequences are identical or similar to } & \lambda \text{ and 186, respectively. This would define also the criterion for the formation of mixed dimers between 2 DNA molecules, and for one phage to serve as a helper in the infectivity assay by the other. Previous studies established that the sequences at the cohesive ends of } & \lambda \text{ and } \phi 80 \text{ DNA are exactly identical (14, 30). Results from this report showed that, out of the 19 nucleotides at each cohesive end of P2 and 186 DNA, two nucleotides are different. In addition, our results point out the exact location of the differences between 186 and P2 DNA as shown in Fig. 10. It also gives the exact length of the homology between the 2 DNA molecules as 13 nucleotides. Our previous studies (31, 32) on the hybridization of oligonucleotides to native DNA indicated that a stable duplex can be formed between a DNA and a nine-nucleotide long oligomer. Therefore, a 13-nucleotide homology between P2 and 186 should be more than sufficient to form a stable duplex. This is indeed the case from the ability of the 2 DNA molecules to form mixed dimers with each other. These studies also show that identical sequences at the cohesive ends of 2 DNA molecules are not required to be able}
\end{align*}
\]
to form mixed dimers and to function in helper-dependent infectivity of DNA molecules.

Thermodynamic Considerations—The thermal stability of end-to-end DNA aggregates has been studied for P2-related phages P2, 186, and 290 (4, 33). The first results indicated that the base sequences at the cohesive ends of P2 and 186 are similar but not identical. An expanded study by Wang et al. (34) gave a more precise conclusion. These authors proposed that 186 cohesive ends have at least one internal AT pair, replacing a GC pair in the P2 cohesive ends. The possibility of another mismatched base pair was not eliminated. A second mismatch between 186 and 290 implies that 186 cohesive ends have purines at both 3' and 5' ends (1, 27, 28) which must be thermodynamically stable to form mixed dimers and to function in helper-dependent infectivity of DNA molecules.

Functional Significance—Before sequence work on cohesive ends was begun, it was attractive to think that a sequence with 2-fold rotational symmetry would be found. Such a sequence would allow a single enzyme to cleave both phosphodiesters bonds in the formation of cohesive ends from a double-stranded precursor molecule. One interesting observation from the sequence studies of λ, 290, and P2 is that all these DNA molecules have purines at both 3' and 5' ends (1, 27, 28) which must be generated by endonucleolytic attacks of purine-purine bonds (25). This supports the notion that there might be a common recognition sequence in these molecules for the enzyme and points out the necessity for the determination of sequence near the 3' termini of these molecules. Such studies have already been reported for λ and 290 (25, 35). Alternatively, the cohesive end sequence might provide a recognition site for the phage tail attachment. Evidence for this proposal has been presented for phage λ (36–39), as well as for P2, 186, and satellite phage 1'2 (39). This supports the notion that there might be a common recognition sequence in these molecules for the enzyme and points out the necessity for the determination of sequence near the 3' termini of these molecules. Such studies have already been reported for λ and 290 (25, 35). Alternatively, the cohesive end sequence might provide a recognition site for the phage tail attachment. Evidence for this proposal has been presented for phage λ (36–39), as well as for P2, 186, and satellite phage 1'2 (39).

Possibility of Modified Bases—A special nucleotide sequence, such as the cohesive end sequence of phage DNAs, might be suspected to carry modified bases. Our results shed little light on this possibility, since we are determining complementary sequences, using normal nucleoside triphosphate precursors. However, our studies show that the complements at each cohesive end are self-complementary, and thus any potential modified base is not disturbing the normal base pairing and repair replication. Furthermore, the terminal label method of Murray and Murray (14), which analyzes sequence directly, has not detected modified bases. Our results shed little light on this possibility, since we are determining complementary sequences, using normal nucleoside triphosphate precursors. However, our studies show that the complements at each cohesive end are self-complementary, and thus any potential modified base is not disturbing the normal base pairing and repair replication. Furthermore, the terminal label method of Murray and Murray (14), which analyzes sequence directly, has not detected modified bases.

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