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Location of the 5-Methylcytosine Group on the Bacteriophage φX174 Genome

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Bacteriophage φX174 DNA was labeled in vivo with [*methyl*-^{3}H]methionine. The methyl-labeled progeny DNA was extracted from purified bacteriophage φX174 particles and was used as template for in vitro synthesis of the complementary strand in the presence of the nucleoside triphosphates and *Escherichia coli* polymerase I. The resultant replicative form DNA was then cleaved, in separate experiments, with restriction endonucleases from *Haemophilus influenzae* and *H. aegyptius*. The DNA fragments were analyzed by polyacrylamide gel electrophoresis. It is concluded that the single methylcytosine in the viral DNA is located in a specific region of the φX174 genome, very likely in gene *H*.

Methylation of DNA occurs in viruses, bacteria, and higher organisms and has been the topic of intensive study. A possible role of DNA methylation in cell differentiation has been discussed (15). Other evidence suggests that methylation is involved in DNA modification and restriction (1). Experiments carried out in bacteria have demonstrated that methylation of DNA is related to DNA replication, and that this process normally occurs in the nascent DNA strand close to or at the replication point (3, 10, 11).

The small circular DNA of bacteriophage φX174 provides a convenient system for the study of DNA methylation. Experiments previously reported from this laboratory showed that the only methylated base present in φX174 DNA is 5-methylcytosine, occurring at a frequency of one per DNA molecule. In addition, this minor base was found exclusively in the dinucleotide fraction of the pyrimidine isopliths, suggesting that it is present in a nonrandom distribution in the φX genome (13). Further studies on the process of in vivo DNA methylation during the infection of *Escherichia coli* C with φX174 revealed that methylation of φX occurs on the nascent DNA strand of the replicating intermediates involved in the synthesis of progeny single-stranded DNA (14).

The use of restriction enzymes to cleave φX174 DNA into specific fragments has been previously described (5, 7, 12), and a cleavage map of the φX174 genome by these enzymes has been prepared (11b). In this paper, we report the results of restriction enzyme digestions of the methyl-labeled viral DNA, after its conversion to the replicative form by in vitro synthesis in the presence of nucleoside triphosphates and *E. coli* polymerase I, and the location of the methyl group in the genome.

MATERIALS AND METHODS

E. coli strain. *E. coli* C416 (*pur*$^-$, *met*$^-$, *arg*$^-$, *try*$^-$) was used as the host strain.

Phage strain. A lysis-defective mutant of phage φX174, *am3*, was used.

Medium. The growth medium used was the Tris-pyruvate-glucose medium of Sinsheimer et al. (16) supplemented with (per milliliter): 50 μg of 18 amino acids, 4 μg each of L-methionine and L-cysteine, 4 μg of thymine, and 10 μg of adenosine.

Reagents. [*methyl*-^{3}H]methionine (specific activity 2.6 Ci/mmol) was purchased from Schwartz Bio Research, Inc. (Orangeburg, N.Y.).

[α-^{32}P]dXTP (specific activity ~100 Ci/mmol) was a product of New England Nuclear (Boston, Mass.).

Oligonucleotide $(_pA_pG)_4$ was purchased from Collaborative Research Inc. (Waltham, Mass.).

Enzymes. *E. coli* DNA polymerase I was the generous gift of Arthur Kornberg. *Haemophilus influenzae* and *H. aegyptius* restriction enzymes were prepared by modifications (7) of the Smith and Wilcox method (17). The bulk of the enzyme activity from *H. aegyptius* was eluted at 0.7 M KCl from the phosphocellulose column. The phosphocellulose-column fractions were used without further purification and were stored at 4 C in 10% glycerol.

Preparation of [methyl-^{3}H]-viral DNA. [*methyl*-^{3}H]-labeled viral DNA was prepared under two different conditions with the same results.

In the first method, *E. coli* C416 cells were grown to 5×10^8 cells/ml in a 500-ml culture in the growth medium described above. The cells were washed once with growth medium (without methionine) and resuspended in the original volume of growth medium including all supplements, except the methionine concentration was adjusted to 2.8 μg/ml and the thymine concentration was increased to 8 μg/ml. After

20 min of aeration at 37 C, the cells were infected with *am3* (multiplicity of infection = 10). After infection (5 min), 10 mCi of [*methyl*-^{3}H]methionine (2.6 Ci/mmol) was added to the culture. The final concentration of L-methionine was 4 μg/ml, and the cells were collected by centrifugation after 2 h.

Alternately, a 100-ml culture of *E. coli* C416 was grown up to 5 × 10^8 cells per ml. The cells were washed free of methionine and resuspended in the same volume of growth medium, including all supplements except methionine.

After 15 min of aeration at 37 C, 10 mCi of [*methyl*-^{3}H]methionine (2.6 Ci/mmol) was added. The final concentration of methionine was 5 μg/ml (carrier free). The cells were infected with *am3* (multiplicity of infection = 10) after 10 min. Incubation was at 37 C for 2 h with aeration.

The DNA of the progeny virus was prepared as described by Razin et al. (13). After alcohol precipitation, the viral DNA was suspended in a 70-μliter solution of 0.01 M Tris (pH 8) and 0.001 M EDTA, and was stored at −10 C.

In vitro synthesis of the complementary strand of methyl-labeled DNA. The reactions were carried out in 130 mM Tris (pH 7.4), 13 mM KCl, and 5 mM $MgCl_2$. The reaction mixture (300 μliters) contained 0.25 μmol each of dCTP, dGTP, dTTP, and dATP, and 0.16 nmol of [α-^{32}P] dXTP (108 Ci/mmol), 0.04 μmol (in nucleotides) of methyl-labeled DNA, 0.003 μmol of $(_pA_pG)_4$ as primer (K. Grohmann, personal communication), and 15 U of DNA polymerase I. The progress of synthesis was followed by measuring the accumulation of acid-insoluble radioactivity with time. The mixture was incubated at 15 C for 9 h, at which time repair synthesis was complete (4). Reaction was stopped by the addition of EDTA to 10 mM.

Polyacrylamide gel electrophoresis. Before electrophoresis, the digestion mixture was adjusted to 1% sodium dodecyl sulfate and 15% sucrose. The mixture was placed on the sample well (11a) of a cylindrical polyacrylamide gel. The electrophoresis buffer consisted of 0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (pH 7.8). Conditions of electrophoresis are described in the figure legends. To quantitate the radioactivity, gels were frozen and cut into 1-mm segments by using a Mickle gel slicer (Brinkman Instruments). Gel slices were solubilized with toluene-liquifluor containing 9% NCS (Amersham-Searle) and 1% water, and the radioactivity was measured in a Beckman liquid scintillation counter.

RESULTS

The discovery of a unique methylcytosine group in the progeny phage φX DNA by Razin et al. (14) promoted us to locate it in restriction enzyme fragments and thereby on the φX genetic map.

Sedimentation profile of the methyl-labeled DNA. To verify the purity of the methyl-labeled DNA prepared by the above methods, viral DNA samples were sedimented through an alkaline cesium chloride gradient as shown in Fig. 1. The sedimentation profiles of viral DNA prepared by either labeling condition were identical and cosedimented in a single sharp band with the ^{14}C-single-stranded phage DNA marker. About 5% of the ^{3}H-radioactivity was found at the top of the gradient, probably indicative of the presence of a small amount of low-molecular-weight contaminant.

In vitro [methyl-^{3}H] replicative form (RF). To use the *Haemophilus* restriction endonucleases to cleave φX DNA into specific fragments, the DNA must be in the double-stranded form. Therefore, methyl-labeled viral DNA was used as template for in vitro synthesis of the complementary strand using *E. coli* DNA polymerase I, in the presence of nucleoside triphosphates. The complementary strand was labeled with [α-^{32}P]dXTP. Under the conditions described above, onefold synthesis was usually complete in about 7 h at 15 C, when the incorporation of the ^{32}P-radioactivity reached a plateau level.

This in vitro RF DNA was separated from unreacted [α-^{32}P]dXTP by exclusion chromatography on a porous glass-bead column. The RF DNA was eluted as a single narrow peak, well separated from the unreacted [α-^{32}P]dXTP which trailed behind (Fig. 2). The elution

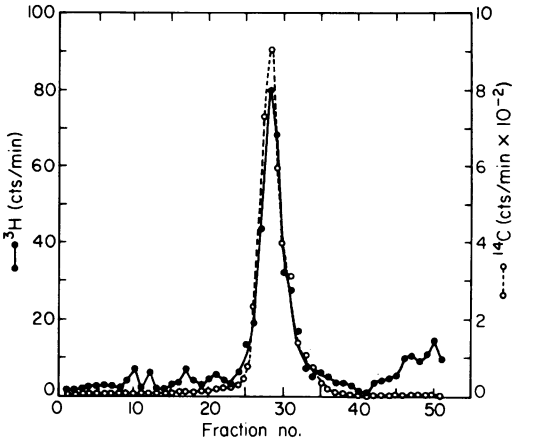

FIG. 1. *Zone sedimentation of methyl-labeled DNA through an alkaline CsCl gradient. φX viral DNA was labeled in vivo with [methyl-^{3}H]methionine and isolated. Labeled DNA (16 μliters) dissolved in Tris-EDTA was adjusted to 0.1 M KOH and 0.01 M EDTA, and then was layered onto a preformed, linear gradient of CsCl (ρ = 1.20 to 1.35 g/ml) in 4.8 ml of 0.1 M KOH plus 0.01 M EDTA. Centrifugation was carried out at 55,000 rpm at 4 C for 120 min in an SW65 rotor. Fractions of 0.1 ml were collected from the bottom of the tubes onto 2.3-cm GF/C filters. The filters were dried and counted in toluene-liquifluor scintillation fluid. Sedimentation is from right to left. The ^{14}C-single-strand phage DNA marker was run in a separate tube and is included in the same sedimentation profile for comparison. Symbols: ●, ^{3}H; ○, ^{14}C.*

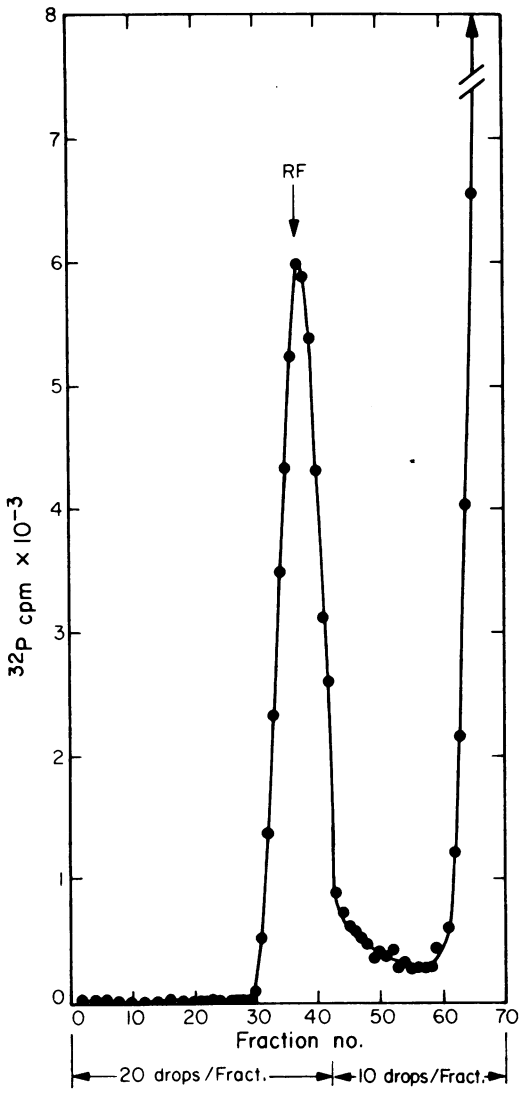

FIG. 2. *Exclusion chromatography of in vitro RF DNA on a porous glass-bead column. A column (1 × 54 cm) of porous glass beads (Sigma G2000-50, pore size 2000 nm) was washed with 50 ml of 0.2 N NH_4OH, 250 ml of 0.1 M sodium acetate, and 1 mM EDTA, pH 6.5. The in vitro synthesis reaction mixture (300 μliters) was adjusted to 0.2% sodium dodecyl sulfate and 15% sucrose and applied to the column. Elution was carried out at 4 C at a rate of 36 ml/h with acetate buffer described above. Fractions of 20 drops (~0.5 ml) were collected, and ^{32}P-radioactivity was monitored as Cerenkov radiation in a liquid scintillation counter.*

procedure was usually complete within an hour. (The glass-bead column can be regenerated for further use by 2 h of washing with the acetate buffer.)

The peak fractions containing the RF DNA were pooled into two SW41 polyallomer tubes. Two volumes of absolute alcohol were added, the solution was well mixed, and then it was stored at −20 C for at least 8 h.

The DNA was collected by centrifugation at 35,000 rpm for 1 h and resuspended in 50 μliters of 0.005 M Tris (pH 7.4) for the *Haemophilus* restriction enzyme digestions.

Digestion of in vitro methyl-labeled RF with H. influenzae restriction endonuclease. The *H. influenzae* restriction enzyme (*Hin*) produces specific cleavage in double-stranded DNA at a sequence of six nucleotides, G, T, Py ↓ Pu A, C (9). The fragments produced by the action of this enzyme on the replicative form of φX174 have been characterized (5, 11b). The R fragments range in size from 1,000 to 80 base pairs. The R6 band contains three distinct tracts (R6a, b, and c) very similar in size, and R7 contains two distinct tracts, R7a and R7b, which can be partially separated into two peaks on 5% polyacrylamide gels.

The in vitro RF containing a [*methyl*-^{3}H]-labeled viral strand and an [α-^{32}P]dTTP-labeled complementary strand was digested with the restriction endonuclease from *H. influenzae*. The results of a polyacrylamide gel electrophoresis of the resultant fragments are shown in Fig. 3. It is evident that the R6 band contained the largest amount of the ^{3}H-viral label. However, there was also a significant amount of the ^{3}H-label distributed in all the other fragments.

This background incorporation into all fragments was expected since, as reported earlier (13), the [*methyl*-^{3}H]methionine does not exclusively label the methylated base. Under the in vivo labeling conditions described above, only 25 to 35% of the ^{3}H-viral DNA label was found in the 5-methylcytosine spot by base composition analysis. The remainder of the ^{3}H-label was found mainly in thymine and guanine. The data of Fig. 3 suggest that this nonspecific label was randomly distributed among the fragments.

The small peak trapped in the 20% gel cushion may consist of R10 and small nucleotides, possibly derived from slight contamination of the enzyme with an exonuclease activity. The amount of ^{3}H-label found in this peak was less than 2% of the total.

It is interesting to note that the [α-^{32}P]dTTP label in the complementary strand was not uniformly distributed, as was evident in the R5 and R7 peaks which contained 50% more ^{32}P-counts than expected. This result suggests that some specific regions of the φX174 viral DNA were biased in their adenine composition.

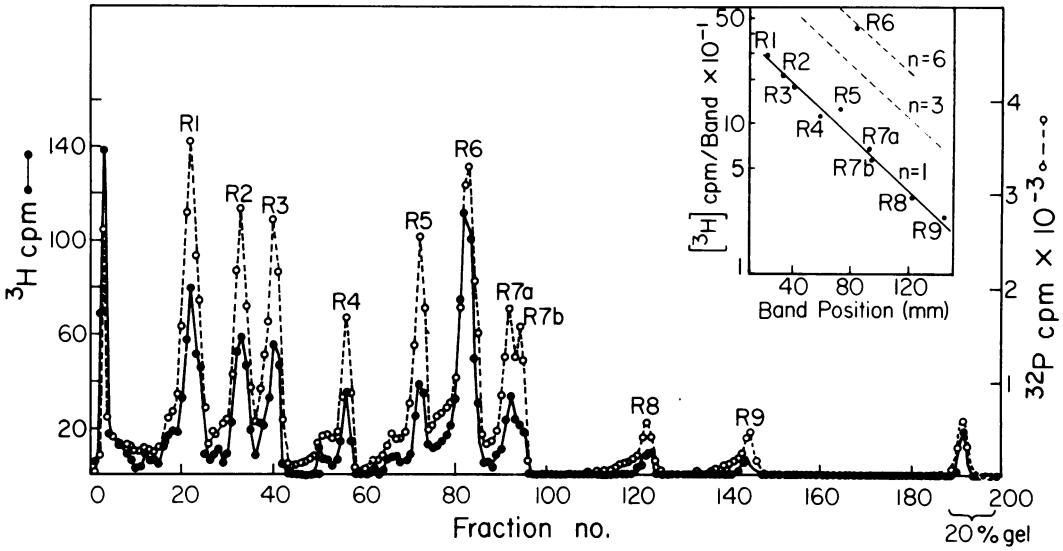

FIG. 3. *Polyacrylamide gel electrophoresis of DNA fragments produced by H. influenzae restriction endonuclease from methyl-labeled φX in vitro RF. The reaction mixture contained 40 μg of RF DNA, 7 mM each of Tris (pH 7.4), β-mercaptoethanol, and $MgCl_2$, 50 mM NaCl, and 30 μliters of H. influenzae restriction enzyme, in a total volume of 90 μliters. Incubation was at 37 C for 5 h. The reaction was stopped by adding EDTA to 10 mM. Sucrose and sodium dodecyl sulfate were added to final concentrations of 15% and 1%, respectively. The mixture was layered on a 5% polyacrylamide gel (1 × 19.5 cm; bottom 1 cm was 20% gel). Electrophoresis was carried out at a constant voltage of 60 V for 18 h. Symbols: ●, ^{3}H-methyl-labeled viral DNA; ○, ^{32}P counts/min incorporated into the complementary strand with the methyl-labeled viral DNA as template, in the presence of [α-^{32}P]dTTP and polymerase I. Insert: The integrated ^{3}H-counts in each band were plotted against mobilities (band position in mm). Band R6 has been shown to contain three tracts very similar in size and would be expected, if the label were randomly distributed, to contain three times (n = 3) the number of counts as those bands containing a single fragment (n = 1).*

The total ^{3}H-counts under each band were summed and plotted against their relative mobilities (Fig. 3 insert). A linear relationship was observed between the logarithm of the integrated counts and their electrophoretic mobility for every band except R6. Since band R6 has been shown to contain three distinct tracts very similar in size, it might be expected to contain three times the amount of counts as other bands. Instead, it contained about 30% of the total ^{3}H-viral label, twice as much as the expected value. This result leads us to conclude that the [*methyl*-^{3}H]-viral label was preferentially located in the R6 band.

Digestion of in vitro [methyl-^{3}H]RF with H. aegyptius restriction endonuclease. The restriction enzyme from *H. aegyptius* cleaved φX RF into 11 specific fragments (Z1 to Z10); Z6 consisted of two tracts of similar size (11b). This set of fragments was distinct from those produced from the *H. influenzae*. To resolve which of the three R6 fragments contained the methylated base, the in vitro RF containing a [*methyl*-^{3}H]-labeled viral strand and an [α-^{32}P]-dCTP-labeled complementary strand was digested with the *H. aegyptius* restriction enzyme. The distribution of the ^{3}H-viral label in the resultant fragments was analyzed by polyacrylamide gel electrophoresis. The Z2 fragment contained the largest amount of the ^{3}H-label in the viral strand (Fig. 4). When the ^{3}H-label under each band was summed and plotted against its relative mobility (Fig. 4 insert), Z2 was found to contain 36% of the total ^{3}H-counts recovered from the gel, 2.5 times more ^{3}H-label than otherwise expected. The ^{3}H-count in Z5 was also enhanced; this fragment contained about twice the expected label (9% of the total ^{3}H-radioactivity). Z6 has been shown to contain two tracts and, thus, has a normal content of ^{3}H-radioactivity with respect to other fragments. The peak trapped inside the 20% gel at the end of the gel may consist of Z10 and other nucleotides resulting from enzyme digestion; it contained less than 2% of the total ^{3}H-label.

The [α-^{32}P]dCTP-labeled complementary strand, in this case, did not show any bias in its labeling pattern in any of the Z fragments.

There was a linear relationship between the logarithm of the integrated ^{32}P-counts in each peak and the electrophoretic mobility.

It can be concluded that the [*methyl*-^3H]-label in the viral DNA was primarily located in the Z2 fragment and, very likely, in one of the R6 fragments.

DISCUSSION

The data from these experiments are consistent with the restriction fragment map for ϕX174 (Fig. 5). That is, one of the R6 fragments (R6 contains the largest amount of [*methyl*-^3H]-label) overlaps with the Z2 fragment (which also contains the largest amount of ^3H-label).

Preliminary genetic data correlating the restriction enzyme fragments with the ϕX genetic map indicate that R6c and Z2 are located at gene *H* (Fig. 5), in agreement with the findings of Hutchison et al. (C. A. Hutchison, J. H. Middleton, and M. H. Edgell, Biophysical Society Abstr., 1972, No. 31a) that both Z2 and R6c fragments contain the mutant *ts4* locus of gene *H*.

From the in vivo methylation studies of ϕX reported earlier (14), it was concluded that replicating intermediates with single-strand tails are the substrates for methylation. In addition, it was found that the methyl-label sedimented as linear single-stranded DNA of almost double the length of the phage ϕX genome. This observation suggested that methylation occurred at a final stage of phage maturation, and that methylation in the phage ϕX system might serve as a signal for the termination of a round of replication.

Considering the continuous mode of phage ϕX DNA synthesis suggested by the rolling circle model (6) and the finding that single-strand DNA synthesis is initiated from a unique position, namely the restriction enzyme fragment R3 in gene *A*, and proceeds clockwise around the ϕX174 genetic map (genes *A* → *H*) (8), the location of the unique methylated base in gene *H* accords well with the earlier suggestion that methylation occurs at a final stage of phage maturation and may serve as a signal for termination.

The excess ^3H-label found in Z5 (4 to 5% of the total) is difficult to explain; it occurred in both preparations of methyl-labeled DNA with the different labeling conditions. Z5 overlapped in part with R6a and R7a in the restriction enzyme fragment map. One possible explanation is that the part of Z5 which overlapped with R6a was highly biased in its base composition, since considerable ^3H-label was also incorporated,

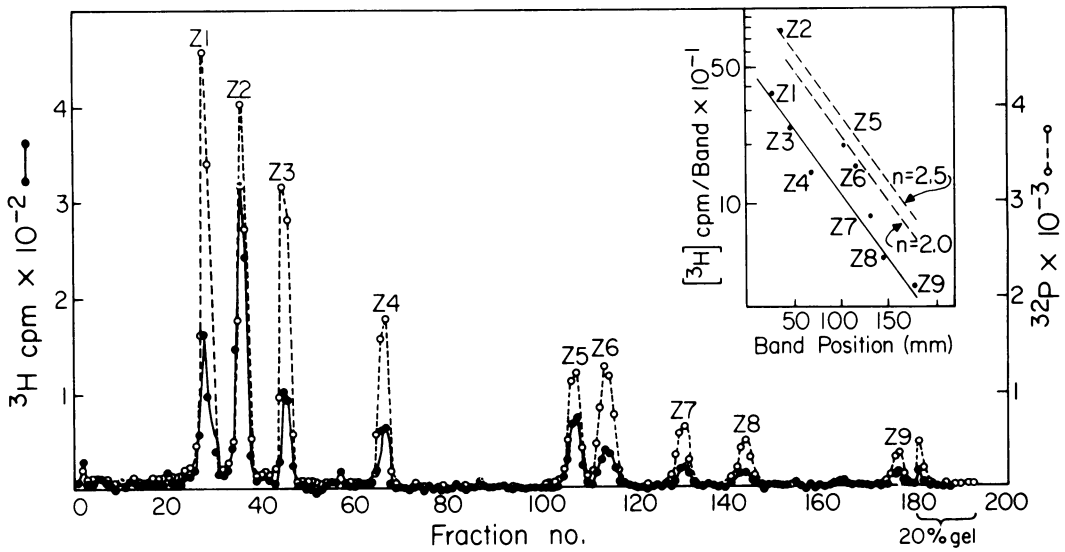

FIG. 4. *Polyacrylamide gel electrophoresis of H. aegyptius restriction endonuclease fragments produced from methyl-labeled ϕX in vitro RF. The reaction mixture contained 30 μg of RF DNA, 7 mM each of Tris (pH = 7.4), β-mercaptoethanol, and $MgCl_2$, 20 mM NaCl, and 15 μliters of H. aegyptius restriction enzyme, in a total volume of 70 μliters. Incubation was at 37 C for 4 h. The resultant fragments were separated on a 4% polyacrylamide gel (1 × 19.5 cm; bottom 1 cm was 20% gel). Electrophoresis was at a constant voltage of 60 V for 15 h. Symbols: ●, ^3H-methyl-labeled viral DNA; ○, [α-^{32}P]dCTP label in complementary strand. Insert: The integrated ^3H-counts found in each band were plotted against mobilities. Band Z6 has been shown to contain two tracts very similar in size and, therefore, contains two times (n = 2) the number of counts as those bands containing a single fragment.*

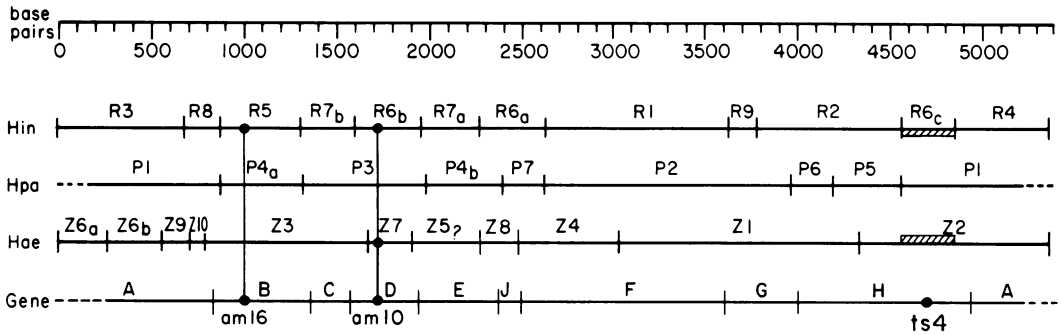

FIG. 5. *Restriction enzyme fragment map of φX174. The molecular size estimates and physical order of the φX DNA fragments produced by cleavage with the H. influenzae (Hin), H. parainfluenzae (Hpa) and H. aegyptius (Hae) has been described (11b). The order and size of the φX genes shown in the map are derived from the results of Benbow et al. (2). Using a modification of the genetic assays for φX fragments previously described (5, 18), R5 was found to contain the am16 locus in gene B, and R6b and Z7 to contain am10 in gene D (A. S. Lee, Ph.D. thesis, California Institute of Technology, Pasadena, 1974). The correlation of other fragments with the φX genetic map can be inferred, but it can only be considered as tentative. The location of the ^{3}H-methyl label in R6c and Z2 is indicated by the hatched area.*

preferentially into thymine and guanine. Alternatively, it cannot be ruled out that a minor population of the φX DNA has a different (additional?) methylation site than that in gene *H*. Unfortunately, the preparation of highly radioactive methyl-labeled DNA, with exclusive labeling in the methylated base, is so difficult that the obvious experiments to analyze this problem further are impractical at this time. Because of the limited radioactivity, it is impossible to recover enough ^{3}H-label from the restriction enzyme fragments for further analysis.

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LITERATURE CITED

1. Arber, W., and S. Linn. 1969. DNA modification and restriction. Annu. Rev. Biochem. **38**:467-500.
2. Benbow, R. M., A. J. Zuccarelli, G. C. Davis, and R. L. Sinsheimer. 1974. Genetic recombination in bacteriophage φX174. J. Virol. **13**:898-907.
3. Billen, D. 1968. Methylation of the bacterial chromosome: an event at the "replication point?" J. Mol. Biol. **31**:477-486.
4. Dumas, L. B., G. Darby, and R. L. Sinsheimer. 1971. The replication of bacteriophage φX174 DNA *in vitro* temperature effects on repair synthesis and displacement synthesis. Biochim. Biophys. Acta **228**:407-422.
5. Edgell, M. H., C. A. Hutchison, and M. Sclair. 1972. Specific endonuclease R fragments of bacteriophage φX174 deoxyribonucleic acid. J. Virol. **9**:574-582.
6. Gilbert, W., and D. Dressler. 1968. DNA replication: the rolling circle model. Cold Spring Harbor Symp. Quant. Biol. **33**:473-484.
7. Johnson, P. H., A. S. Lee, and R. L. Sinsheimer. 1973. Production of specific fragments of φX174 replicative form DNA by a restriction enzyme from *Haemophilus parainfluenzae*, endonuclease HP. J. Virol. **11**:596-599.
8. Johnson, P. H., and R. L. Sinsheimer. 1974. Structure of an intermediate in the replication of bacteriophage φX174 deoxyribonucleic acid: the initiation site for DNA replication. J. Mol. Biol. **83**:47-61.
9. Kelly, T. J., and H. O. Smith. 1970. A restriction enzyme from *Haemophilus influenzae*. II. Base sequence of the recognition site. J. Mol. Biol. **51**:393-409.
10. Lark, C. 1968. Effect of the methionine analogs, ethionine and norleucine on DNA synthesis in *E. coli* 15 T⁻. J. Mol. Biol. **31**:401-414.
11. Lark, C. 1968. Studies on the *in vivo* methylation of DNA in *Escherichia coli* 15 T⁻. J. Mol. Biol. **31**:389-399.
11a. Lee, A. S., and R. L. Sinsheimer. 1974. A continuous electro-elution method for the recovery of DNA restriction enzyme fragments. Analyt. Biochem. **60**:640-644.
11b. Lee, A. S., and R. L. Sinsheimer. 1974. A cleavage map of bacteriophage φX174 genome. Proc. Nat. Acad. Sci. U.S.A. **71**:2882-2886.
12. Middleton, J. H., M. H. Edgell, and C. A. Hutchison. 1972. Specific fragments of φX174 deoxyribonucleic acid produced by a restriction enzyme from *Haemophilus aegyptius*, endonuclease Z. J. Virol. **10**:42-50.
13. Razin, A., J. Sedat, and R. L. Sindheimer. 1970. Structure of the DNA of bacteriophage φX174. VII. Methylation. J. Mol. Biol. **53**:251-259.
14. Razin, A., J. Sedat, and R. L. Sinsheimer. 1973. *In vivo* methylation of replicating bacteriophage φX174 DNA. J. Mol. Biol. **78**:417-425.
15. Scarano, E., M. Iaccarino, P. Grippo, and E. Parisi. 1967. The heterogeneity of thymine methyl group origin in DNA pyrimidine isostichs of developing sea urchin embryos. Proc. Nat. Acad. Sci. U.S.A. **57**:1394-1400.
16. Sinsheimer, R. L., B. Starman, C. Nagler, and S. Guthrie. 1962. The process of infection with bacteriophage φX174. I. Evidence for a "replicative form." J. Mol. Biol. **4**:142-160.
17. Smith, H. O., and K. W. Wilcox. 1970. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. J. Mol. Biol. **51**:379-391.
18. Weisbeek, P. J., and J. H. Van De Pol. 1970. Biological activity of φX174 replicative form DNA fragments. Biochim. Biophys. Acta **224**:328-338.