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Physical Map of Polyoma Viral DNA Fragments Produced by Cleavage with a Restriction Enzyme from *Haemophilus aegyptius*, Endonuclease R·HaeIII

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Digestion of polyoma viral DNA with a restriction enzyme from *Haemophilus aegyptius* generates at least 22 unique fragments. The fragments have been characterized with respect to size and physical order on the polyoma genome, and the 5′ to 3′ orientation of the (+) and (−) strands has been determined. A method for specific radiolabeling of adjacent fragments was employed to establish the fragment order. This technique may be useful for ordering the fragments produced by digestion of complex DNAs.

Specific fragmentation of viral DNA has revealed considerable information concerning the structure of the DNA with respect to the origins and termini of DNA replication (3), the regions coding for early and late RNA (8), and the physical order of the genes (5). In addition, fragments may be characterized genetically and used as hybridization probes for the activity of specific genes.

We have specifically cleaved polyoma viral DNA with the restriction enzyme of *Haemophilus aegyptius*, endonuclease R·HaeIII (10), and determined the physical order on the genome of approximately 20 of the 22 fragments identified. Griffin et al. (6) have recently published a physical map of polyoma DNA produced by the restriction enzymes endonuclease R·HpaII and endonuclease R·HindIII.

MATERIALS AND METHODS

Virus. The Dulbecco large plaque strain of polyoma virus was cloned one time on a monolayer of secondary mouse embryo cells. Stocks were grown from this cloned isolate by infection of primary mouse kidney cells at a multiplicity of infection of 0.01 to 0.1 per cell. Virus was grown at 37 C.

Isolation of viral DNA. Viral DNA was prepared from baby mouse kidney cells infected at an input multiplicity of 5 to 20 PFU per cell and incubated in Dulbecco's modified Eagle medium containing 3% horse serum at 37 C for 48 h. Labeling with radioisotopes was performed between 24 to 48 h after infection. Viral DNA was extracted from infected cells by the method of Hirt (7), and then extracted one time with phenol-chloroform (1:1) and precipitated with 2.5 volumes of ethanol. The DNA was then banded to equilibrium in a cesium chloride gradient containing ethidium bromide and dialyzed against 10 mM Tris-hydrochloride (pH 7.0). ^{32}P-labeled viral DNA was prepared in cells incubated in the presence of 100 μCi of [^{32}P]orthophosphate per ml in the usual medium.

Isolation of single-stranded circular polyoma viral DNA. Covalently closed circular DNA (0.1 to 1.0 mg/ml; component I) was subjected to limited endonucleolytic cleavage with 0.025 μg of DNase I per ml (Worthington Biochemicals) in 0.5 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), and 10 mM $MgCl_2$ at 37 C for 15 min. This treatment converted approximately 50% of the DNA to the open circular form (component II). Component II was purified by banding in a cesium chloride gradient containing ethidium bromide and then dialyzed and concentrated. Single-stranded circular DNA was prepared from component II by sedimentation through an alkaline sucrose gradient. The leading band corresponding to the circular strands was collected, dialyzed to neutral pH, concentrated, and stored at 4 C. Single-stranded circular DNA was always incubated for 2 to 3 min in 0.2 M NaOH and then neutralized with an equal volume of 1 M Tris-hydrochloride (pH 7.0) before use to denature any DNA which may have self-annealed.

Endonuclease R·HaeIII digestions. Endonuclease R·HaeIII was a gift from John Newbold, University of North Carolina at Chapel Hill. Viral DNA, at a concentration of 1 mg/ml or less, was digested for 12 h at 37 C with a 10% dilution of the enzyme stock in 0.04 M Tris-hydrochloride (pH 7.0), 10 mM $MgCl_2$, 1 mM dithiothreitol.

Electrophoresis. Digested DNA was fractionated by electrophoresis through tube gels (6 by 170 mm) of 5 or 8% polyacrylamide as described by Edgell et al. (5). Digests of 1 to 5 μg of polyoma DNA were visualized by staining the DNA with ethidium bromide (5 μg/ml) in distilled water and then viewing the florescent bands under a long wavelength ultraviolet lamp (11). For isolation of fragments, the portions of the gel containing DNA bands were cut out with a razor blade and crushed by passing them through an orifice prepared by cutting off a 23-gauge

needle. The DNA was eluted from the crushed gel slices with 0.05 M EDTA-NaOH (pH 7.0), and the DNA was precipitated with 2.5 volumes of ethanol. The precipitates containing EDTA were dissolved in deionized water and dialyzed againt 1 mM Tris-hydrochloride (pH 7.4). Isolated fragments were concentrated to a small volume and stored at −20 C. Slab gels for autoradiography were either 0.05 or 0.10 inches (ca. 0.13 or 0.25 cm) thick and 14 cm long (Savant Instruments, Inc., Hicksville, N. Y.).

DNA polymerase reactions. DNA polymerase I, purified to homogeneity, was a gift of Lawrence Loeb. DNA polymerase reactions were carried out in 0.1 M Tris-hydrochloride (pH 7.5), 7 mM $MgCl_2$, and 4 to 100 μM deoxynucleoside triphosphates (Pabst Laboratories). α-[^{32}P]dCTP and α-[^{32}P]TTP were purchased from New England Nuclear Corp.

Use of degraded calf thymus DNA primers. Complete conversion to component II of polyoma single-stranded circular DNA, primed by a single fragment at 15 C with 10 μg of DNA polymerase per ml and 1 μg of DNA per ml took 2 to 3 h. To increase the rate of conversion in cases where fully double-stranded component II was the desired product (see Table 2, Fig. 4), a complex mixture of oligonucleotides was added to the reaction. These oligonucleotides provided additional priming sites for repair type DNA synthesis (4). Oligonucleotides were generated by digestion of 5 mg of calf thymus DNA per ml with 70 μg of DNase I per ml in a reaction containing 10 mM Tris-hydrochloride (pH 7.4) and 10 mM $MgCl_2$ at 37 C for 2 h. DNase I was inactivated by heating at 121 C for 10 min. Degraded calf thymus DNA (1 μg) was found to provide approximately 14 priming events per molecule of viral DNA in a reaction with 0.1 μg of single-stranded circular polyoma DNA. Under these conditions, the conversion to double-stranded component II was complete within 20 min.

S-1 nuclease digestions. S-1 nuclease was prepared from Takadiastase according to Ando (1). ^{32}P-labeled DNA samples were digested in a buffer containing 0.025 M sodium acetate (pH 4.1), 5 mM $ZnSO_4$, 0.3 M NaCl, and 9×10^3 units (1) of S-1 nuclease per ml. After 30 min at 37 C portions were removed and applied to glass fiber filters. Filters were either counted directly in toluene scintillation fluid for total counts or washed with 0.5 M HCl and 95% ethanol and counted to determine the acid-insoluble radioactivity.

Polyoma cRNA. Polyoma complementary RNA (cRNA; 880 μg) was prepared by transcribing 50 μg of polyoma component I DNA with 25 μg of purified *Escherichia coli* RNA polymerase in a standard reaction mixture (2). Self-annealing of the cRNA resulted in a maximum level of 10% resistance to pancreatic RNase digestion in 0.3 M NaCl. A 40-fold excess of self-annealed polyoma cRNA was able to protect a maximum of 50% of single-stranded polyoma DNA from digestion with S-1 nuclease after hybridization.

RESULTS

HaeIII fragments of polyoma viral DNA. ^{32}P-labeled polyoma DNA was digested with endoR·HaeIII, and the digested DNA was fractionated by electrophoresis through 5 and 8% polyacrylamide gels. The gels were dried, and the bands were located by autoradiography (Fig. 1). At least 20 distinct bands were visible. Bands were excised from the gel, and the radioactivity in each band was determined. The square root of the distance of each band from the origin is plotted against the log radioactivity in each band in Fig. 2 (12). A linear proportionality is observed for all but the first band and one or more of the smaller bands. These deviations can be explained by the presence of two unresolved fragments in those bands with twice the expected radioactivity. In fact, the first band is seen to split into two bands on longer gels (data not shown). Some variability from stock to stock of virus is seen in the proportions of radioactivity in some of the smaller bands. This is probably due to the presence, in some stocks, of viral DNA with reiterated regions.

Because of this variability the standard curve in Fig. 2, rather than the radioactivity of each fragment, has been used to calculate the relative sizes of the fragments according to their mobilities. The sizes determined in this way are independent of the variations in the radioactivity observed in the smaller fragments. A nominal size of the fragments can be calculated by assuming the sum of the sizes of all of the fragments equals the size of the polyoma genome, i.e., 5500 nucleotide pairs. The size of each fragment is then expressed as the percentage of the total genome or in nucleotide pairs. These calculated values are shown in Table 1. The absolute sizes calculated in this way agree well with those determined for other fragments. For instance, the polyoma HaeIII-C fragment, calculated to be 580 nucleotide pairs in length, almost comigrates with the simian virus 40 HaeIII-C fragment, reported to be 610 nucleotide pairs (8).

Physical order of the polyoma HaeIII fragments. The physical order of the fragments on the polyoma genome was determined by the following method. Isolated fragments were denatured and annealed to single-stranded circular polyoma DNA. The DNA concentrations and time of annealing were such that during the course of the annealing the fragments annealed to an extent of greater than 80%, whereas the single-stranded circles combined with each other to an extent of less than 20%. Thus, the major products of the annealing reaction were double-stranded reannealed fragments and single-stranded circles to which a fragment was

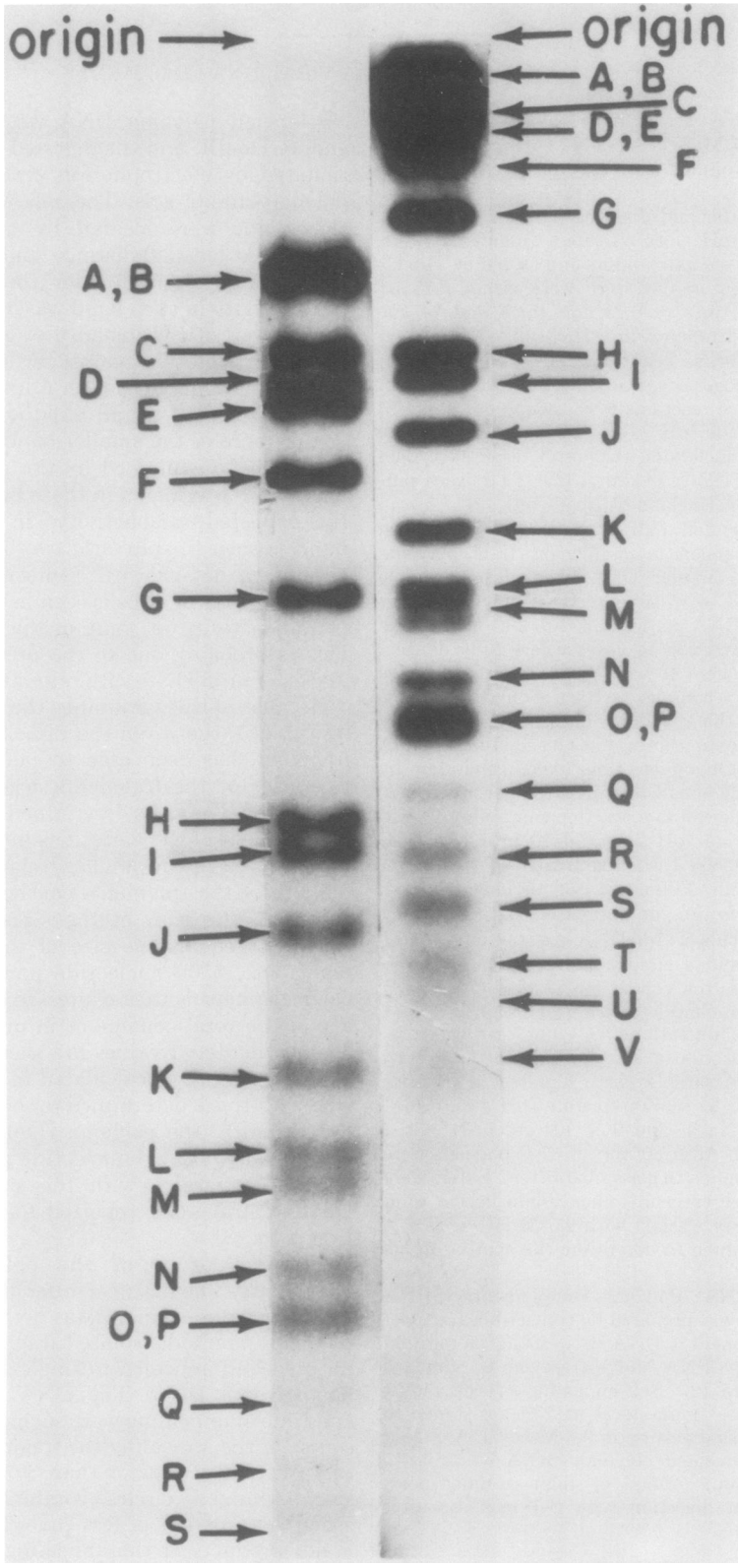

FIG. 1. *Autoradiograms of 5% and 8% polyacrylamide slab gels of ^{32}P-labeled polyoma DNA digested with endonuclease R·HaeIII. Approximately 20,000 counts/min of digested viral DNA were applied to each gel and subjected to electrophoresis at 50 V for 9 h. The gels were dried on a sheet of filter paper (Whatman No. 3) and placed face down on a sheet of Kronex 4 medical X-ray film (Dupont de Nemours and Co., Inc., Wilmington, Del.). The dried gel and film were pressed between two glass plates and wrapped in aluminum foil. The film was developed 48 h later.*

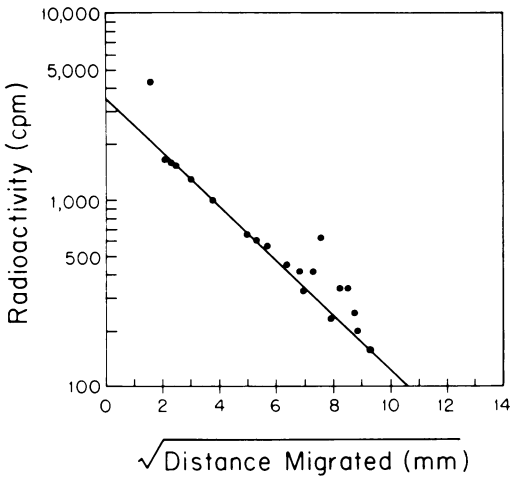

FIG. 2. *Plot of the log radioactivity in each band from the gels in Fig. 1 versus the square root of the distance migrated from the origin in the 8% gel. Eighty-nine percent of the radioactivity applied to the 5% gel was recovered in the bands, and 93% was recovered from the 8% gel.*

TABLE 1. *Sizes of the HaeIII fragments of polyoma viral DNA.*

Fragment	% of Genome	Nucleotide pairs
HaeIII-A	12.8	704
HaeIII-B	12.8	704
HaeIII-C	10.6	583
HaeIII-D	9.93	546
HaeIII-E	9.43	518
HaeIII-F	7.91	435
HaeIII-G	6.08	334
HaeIII-H	4.02	221
HaeIII-I	3.65	201
HaeIII-J	3.23	178
HaeIII-K	2.55	140
HaeIII-L	2.20	121
HaeIII-M	2.13	117
HaeIII-N	1.90	105
HaeIII-O	1.70	94
HaeIII-P	1.70	94
HaeIII-Q	1.53	84
HaeIII-R	1.37	75
HaeIII-S	1.24	68
HaeIII-T	1.15	63
HaeIII-U	1.10	61
HaeIII-V	0.99	54

annealed. The 3′ OH group of the fragment was utilized as a primer for repair type DNA synthesis by DNA polymerase I, directed by the single-stranded circular template. [^{32}P]TTP and cold dATP, dCTP, and dGTP were used as substrates for the polymerase to label the nucleotide sequences adjacent to the priming fragments. After a short extension of the priming fragment, the remaining template was completed with unlabeled deoxynucleoside triphosphates with the addition of degraded calf thymus DNA primer (see above) and a 2,000-fold excess of cold TTP.

The final product of this reaction was component II polyoma DNA, in which short regions adjacent to the priming fragment were labeled with [^{32}P]TTP. Labeled component II DNA from each reaction was separated from unannealed fragments (which also incorporate radioactivity) by sedimentation through a neutral sucrose gradient (Fig. 3). To determine which fragments were adjacent to the priming fragment, the purified component II DNA was digested with endoR·HaeIII, and the resulting fragments were subjected to electrophoresis through a slab gel of 5% polyacrylamide. The gel was dried and exposed to X-ray film to determine the distribution of the radioactivity among the fragments.

The autoradiogram from this experiment is shown in Fig. 4. It is apparent that with each fragment used as a primer of DNA synthesis no radioactivity appears in the priming fragment, indicating that the 3′ exonuclease activity of DNA polymerase does not act extensively under these conditions of limited synthesis. Theoretically, since both (+) and (−) strands of circular

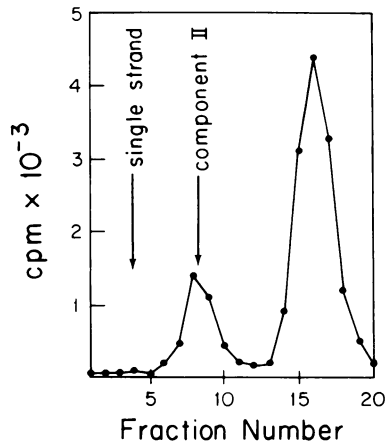

FIG. 3. *Separation of ^{32}P-labeled component II DNA from excess ^{32}P-labeled fragments. The product of a DNA polymerase reaction, as described in the legend to Fig. 4, was sedimented through a 3.8-ml, 5 to 20% sucrose gradient containing 0.15 M NaCl, 10 mM Tris-hydrochloride (pH 7.4), and 1 mM EDTA in a Beckman SW56 rotor at 55,000 rpm for 100 min at 20 C. The arrows denote the sedimentation positions of single-stranded circular DNA and double-stranded component II DNA markers. Sedimentation is shown from right to left. The entire ^{32}P-labeled component II peak was pooled and used for subsequent endoR·HaeIII digestion.*

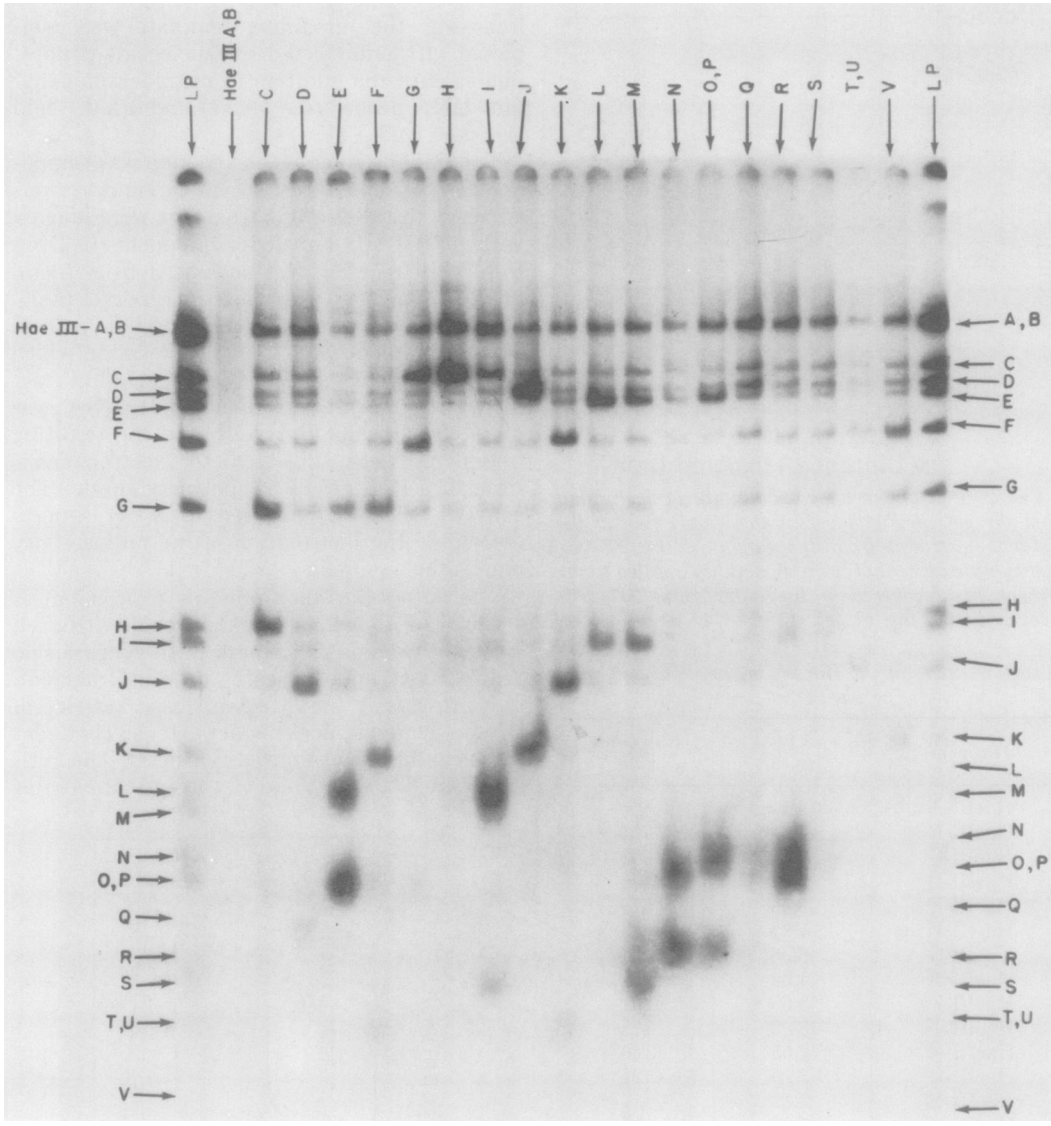

FIG. 4. *Autoradiogram of a polyacrylamide gel separation of endoR·HaeIII digestion products of polyoma viral DNA synthesized by priming DNA synthesis in vitro with the specific HaeIII fragments. Single-stranded circular polyoma DNA (0.1 μg) was annealed to 0.1 to 0.3 μg each of 20 denatured fragments in 0.04 ml of 0.3 M Tris-hydrochloride (pH 7.5) at 65 C for 1 min. [^{32}P]TTP (5 pmol; specific activity, 121 Ci/mmol) was added along with 0.5 nmol each of dATP, dCTP, and dGTP, 0.7 μmol of $MgCl_2$, and 0.5 μg of DNA polymerase I in a final volume of 0.1 ml. The reactions were incubated at 15 C for 20 min, at which time 10 nmol each of dATP, dCTP, dGTP, and TTP were added along with 1 μg of degraded calf thymus DNA primer. After 60 min, the reactions were stopped by the addition of 0.02 M EDTA-NaOH (pH 7.0) and 0.1% sodium dodecyl sulfate. ^{32}P-labeled component II was separated from ^{32}P-labeled fragments by sedimentation through neutral sucrose gradients as shown in Fig. 3. The component II peaks were precipitated with 2.5 volumes of ethanol. The precipitates were collected by centrifugation and dissolved in 0.05 ml of digestion buffer containing one-tenth volume of endo R·HaeIII. After 12 h of incubation at 37 C, portions of the digested DNA were layered on a 5% polyacrylamide slab gel, subjected to electrophoresis for 3 h at 100 V, dried, and exposed to X-ray film for 12 days. The specific HaeIII fragment used as primer for DNA synthesis in each reaction is indicated along the top of the autoradiogram. The migration positions of each HaeIII fragment, located by running a digest of in vivo labeled DNA (LP), are indicated on either side of the autoradiogram. Each sample contained approximately 1,000 counts/min of ^{32}P-labeled DNA, prepared in vitro as described. The samples labeled LP contained approximately 1,000 counts/min of endoR·HaeIII digested ^{32}P-labeled DNA, prepared in vivo.*

DNA and fragment are involved in the annealing, elongation of the 3′ OH* of the priming fragment with radioactive substrates should label the regions adjacent to and on either side of the priming fragment. As a result, labeling patterns should be reciprocal among neighboring fragments. This pattern is consistently observed in the autoradiogram, i.e., fragments HaeIII-F and HaeIII-C, as primers, stimulate incorporation into each other. Although not seen in Fig. 4, in other experiments priming by a mixture of fragments HaeIII-A and -B resulted in the appearance of radioactivity in fragments HaeIII-A, -B, -H, and -O.

From the pattern of labeling it appears that elongation with the radioactive precursor proceeded for 60 to 70 nucleotides. For example, elongation of fragment HaeIII-M proceeded into HaeIII-S but not into HaeIII-Q. Therefore, elongation was less than 70 nucleotides (length of HaeIII-S). However, elongation HaeIII-K proceeded through HaeIII-T (or -U) and into HaeIII-F. In this case, elongation proceeded for at least about 60 nucleotides (length of HaeIII-T or -U).

The autoradiogram was interpreted to construct the physical map shown in Fig. 5. Although priming by HaeIII-A and -B resulted in the labeling of HaeIII-O and -P, the reciprocal is not true. Direct linkage between HaeIII-O or -P is therefore not certain, and this is indicated by a dashed line in Fig. 5. Priming by a combination of HaeIII-T and -U results in the labeling of HaeIII-F and -K. Since these fragments apparently map at only one position, it is possible that *both* HaeIII-T and -U are located between HaeIII-F and -K and this is indicated by the parenthetical inclusion of HaeIII-U in Fig. 5. HaeIII-V has not been located. Although it is labeled by priming with fragment HaeIII-G and is therefore presumably adjacent, it was not labeled by the fragments adjacent to HaeIII-G, i.e., HaeIII-C or -F.

Orientation of the (+) and (−) strands. *E. coli* RNA polymerase transcribes polyoma DNA asymmetrically (Kamen et al., Cold Spring Harbor Symp. Quant. Biol., in press). By convention, the RNA transcript is designated the (+) strand and the DNA strand complementary to it is referred to as the (−) strand. The 5′ to 3′ orientation of the (+) and (−) strands of polyoma DNA was determined by the following rationale. When DNA synthesis is primed with specific fragments, the fragments contiguous to and on either side of the priming fragment are labeled. On one side,

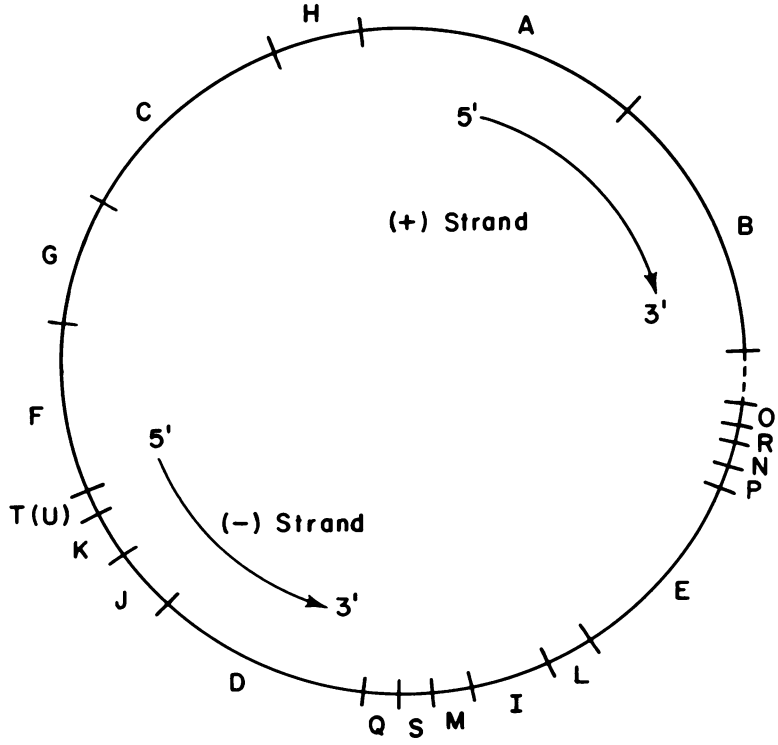

FIG. 5. *Map of the HaeIII fragments of polyoma viral DNA and the strand orientation.*

TABLE 2. *Direction of the (−) strand of polyoma viral DNA*[a]

Priming by fragment:	Radioactivity (%) in (−) strand of fragment:						
	HaeIII-A	HaeIII-H	HaeIII-C	HaeIII-F	HaeIII-K	HaeIII-J	HaeIII-D
HaeIII-H	36		92				
HaeIII-K				8		81	72
HaeIII-J				11	6		88

[a] HaeIII-H, -K, and -J were annealed to 0.1 μg of single-stranded circular polyoma DNA in three separate reactions and elongated with 1 μg of DNA polymerase In in a 0.1-ml reaction containing 0.4 nmol each of TTP, dATP, and dGTP and 7 pmol of [^{32}P]dCTP (141 Ci/mmol) at 15 C. After 30 min, 10 nmol each of dATP, dCTP, dGTP, and TTP and 1 μg of degraded calf thymus DNA primer were added. The reaction was stopped after 60 min, and the component II DNA was separated and digested with endoR·HaeIII as described in the legends to Fig. 3 and 4. The HaeIII fragments were separated by electrophoresis through a 5% polyacrylamide gel, and the radioactivity was located by autoradiography. Radioactive fragments were eluted from the dried gel by soaking the excised bands in 0.1 M NaCl and 10 mM EDTA·NaOH (pH 7.0) at 4 C for 24 h. The eluted radioactive fragments were precipitated with 2.5 volumes of ethanol and 100 μg of yeast RNA and redissolved in 0.04 ml of 1 mM EDTA-NaOH (pH 7.0). The fragments were denatured by heating to 100 C for 10 min and annealed with 3.5 μg of unlabeled polyoma cRNA in a 0.05-ml volume of 0.6 M NaCl at 65 C for 30 min. The radioactive DNA fragments hybridized with cRNA were incubated with S-1 nuclease to determine the fraction of the total radioactivity which was protected from digestion by hybridization to cRNA. This fraction, expressed in the table, is the fraction of radioactivity in the (−) strand.

label is introduced by elongation of the 3′ OH of the (+) strand of the primer, whereas regions on the other side of the priming fragments are labeled by elongation of the (−) strand of the primer. Radioactivity in the (+) or (−) strands can be distinguished by its ability to hybridize to polyoma cRNA. By demonstrating the direction, with respect to the fragment order, in which the (−) strand is elongated one establishes the 5′ to 3′ direction of that strand.

Table 2 shows the ability of radioactivity introduced into fragments adjacent to three different priming fragments to hybridize to polyoma cRNA. Fragments which are located clockwise on the map (Fig. 5) from the priming fragment are labeled preferentially in the (+) strand, as shown by the ability of the radioactivity to hybridize with polyoma cRNA, whereas fragments which are located counterclockwise from the priming fragment are labeled preferentially in the (−) strand. This labeling pattern establishes the 5′ to 3′ direction of the (−) strand to be counterclockwise and the direction of the (+) strand to be clockwise.

DISCUSSION

The physical order of DNA fragments produced by digestion of homogeneous DNA with restriction enzymes is commonly determined by analyzing the cleavage products of fragments isolated from incomplete digests, or by generating large fragments with one restriction enzyme, to be cleaved by a second enzyme which attacks at different and more numerous sites. Either method places characteristic fragments arising by cleavage of larger fragments in adjacent positions on the intact genome. The ordering method used in this study may be of use in cases where the number of fragments is so large that incomplete digests are too complex to yield unambiguous results, or when other restriction enzymes are not available. Particularly, the cleavage of more complex genomes, even with the known restriction enzymes which break DNA at relatively rare sites, will produce a large number of fragments which may be difficult to arrange in order by the more conventional methods.

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