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# Isolation and Characterization of Replication Forks from Discrete Regions of the Polyoma Genome

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Synthesis of polyoma DNA in nuclei isolated from virus-infected 3T6 mouse fibroblasts leads to the selective labeling of replicative intermediates. Digestion of these replicative intermediates with the restriction endonuclease *Hpa*II resulted in three highly labeled heterogeneous species in addition to the expected full-length fragments. These three species migrated more slowly in agarose than did any of the full-length restriction fragments and were shown to represent families of replication forks by criteria of sensitivity to S1 nuclease, kinetics of labeling both in vitro and in vivo, electron microscopy, and migration behavior during agarose gel electrophoresis. Subsequent digestion with other restriction enzymes showed that the two largest of the three fork bands originated from *Hpa*II fragments 1 and 2. These fragments flank the putative terminus located 180° relative to the origin. The third fork-containing band was less labeled and was derived from fragment 3, which is juxtaposed to the replication origin on the side corresponding to late transcription. A two-dimensional gel system revealed the presence of a fourth fork band, derived from fragment 4, that was obscured by full-length fragments 1 and 2 in the single-dimension electrophoresis. Resolution of the fork families revealed multiple discrete species within the major bands, implying the existence of stops or hesitations during replication of a given region of the genome. This conclusion is consistent with the presence of multiple species upon electrophoresis of the fork bands under denaturing conditions.

Replication of polyoma virus and simian virus 40 DNA begins at a unique origin and proceeds bidirectionally to a termination region approximately 180° relative to the origin (9, 10). Although intact intermediates of replication have been analyzed by sedimentation, gel electrophoresis, and electron microscopy (2, 4, 18, 19, 22, 26), each of these techniques can provide only limited information about fork movement through individual regions of the genome. Fork movement has been followed by incorporation of radioactive precursors into replicating DNA with subsequent analysis of the relative amount of label incorporated into regions already replicated (8, 17) or by measurement of the lengths of daughter strands (25). Neither of these approaches analyzes the intact replication forks or replication intermediates.

A powerful and technically simple approach described here entails isolation and characterization of intact replication forks from discrete regions of the genome. This analysis involves digestion of replicative intermediates with restriction nucleases and electrophoretic fractioning of the fork-containing fragments into groups or families. After digestion with *Hpa*II restriction endonuclease, three major clusters of fork-containing fragments were resolved that migrated more slowly than any of the full-length linear *Hpa*II fragments on agarose gels. These forks were localized to regions of the genome by digestion with multiple restriction endonucleases and by electrophoresis on the two-dimensional agarose gel system of Sundin and Varshavsky (24), modified to fractionate intact forks in the first dimension and then to resolve the (labeled) single-strand daughters in the second dimension. This approach provides a means for assessing the degree of heterogeneity of replicative intermediates with forks located in relatively small regions of the genome, and it may elucidate aspects of the processes of initiation and termination in addition to the mechanisms of fork movement.

(This work was performed in partial fulfillment of the requirements for the Ph.D. degree of A.J.B.-W.)

## MATERIALS AND METHODS

**Cells and virus.** A single plaque isolate of a large plaque strain of polyoma was kindly provided by M. Martin and was propagated at low multiplicity ($10^{-3}$) on primary baby mouse kidney cells. The 3T6 mouse fibroblasts were seeded at $2.5 \times 10^6$ cells per

150-mm culture plate (Falcon Plastics, Oxnard, Calif.) in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% calf serum (GIBCO). Cells were infected on the second day after seeding (while still subconfluent and actively dividing) at a multiplicity of infection of approximately 15.

**Preparation of in vivo labeled polyoma DNA.** In vivo labeled polyoma DNA was prepared by pulse-labeling cells for various times at 27 to 28 h after infection with 1 mCi of [$^3$H]thymidine (Amersham Corp., Arlington Heights, Ill.; 40 to 60 Ci/mmol) in 2 to 3 ml of medium. The pulse was stopped by three washes with ice-cold phosphate-buffered saline, followed by extraction of low-molecular-weight DNA by the method of Hirt (14).

**Preparation of nuclei and synthesis of polyoma DNA in vitro.** Cells were pulse-labeled with [$^3$H]thymidine (Amersham, 40 to 60 Ci/mmol) at either 4 or 25 μCi/ml of medium for 30 min before harvest at 27 to 28 h after infection. Nuclei were isolated at 4°C as described elsewhere (5) by blending cells in a Vortex mixer (approximately $3 \times 10^7$ cells per 5 ml) in isotonic HEPES buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 220 mM sucrose; pH 7.8) with a nonionic detergent, Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.; 0.5% [wt/vol]). After centrifugation ($600 \times g$, 5 min), the nuclei were resuspended in 0.9 ml of isotonic HEPES buffer, and 0.1 ml of incubation mix was added to give final concentrations of 40 mM NaCl, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 5 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 2 mM ATP, 70 μM unlabeled deoxyribonucleotides (Sigma), and 15 U of pyruvate kinase (Calbiochem, La Jolla, Calif.). The labeled deoxynucleotide (Amersham, 400 Ci/mmol) was either [α-$^{32}$P]dCTP at a concentration of 10 μM or [α-$^{32}$P]dGTP at 2 μM. After incubation for 5 to 30 min at 25°C, the reaction was stopped by 10-fold dilution in isotonic HEPES buffer, and the nuclei were recovered by centrifugation ($600 \times g$, 5 min).

**Preparation of polyoma DNA.** Viral DNA was selectively extracted by the procedure of Hirt (14) and then was digested with RNase A (Sigma, type XII-A; 25 μg/ml) for 30 min at 37°C and with proteinase K (EM Laboratories, Elmsford, N.Y.; 25 μg/ml) for 60 min at 37°C. DNA was precipitated at −70°C with 2.25 to 3 volumes of ethanol. The precipitate was washed with cold 70% ethanol, desiccated, and dissolved in appropriate buffers for digestion with restriction endonucleases or S1 nuclease. Restriction endonucleases (*Hpa*II, *Pst*I, *Bam*HI, *Hha*I, and *Hin*dIII) were obtained from Bethesda Research Laboratories, Gaithersburg, Md., and were used under incubation conditions specified by the supplier. Digestion was terminated by the addition of 0.25 volume of 50% glycerol containing 0.05% bromphenol blue, 0.05% xylene cyanol, 1% sodium dodecyl sulfate, and 50 mM EDTA. S1 nuclease (Sigma) was incubated with DNA samples for 30 min at 37°C in 30 mM sodium acetate–75 mM NaCl–1 mM $ZnCl_2$ (pH 4.5).

**Isolation of replicating DNA by BND-cellulose chromatography.** After digestion with RNase A and proteinase K, samples were desalted by chromatography on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) and were adsorbed to benzoylated-naphthoylated DEAE-cellulose (BND-cellulose; Sigma) in 0.25 M NaCl–10 mM Tris–1 mM EDTA (pH 7.2). The completely double-stranded DNA fraction was eluted with 0.7 M NaCl–10 mM Tris–1 mM EDTA (pH 7.2), and then the partially single-stranded DNA was eluted with 2% caffeine in 1 M NaCl–10 mM Tris–1 mM EDTA (pH 7.2), a modification of the procedure of Levine et al. (16).

**Agarose gel electrophoresis of digested DNA.** Samples of DNA digested with various restriction endonucleases or S1 nuclease were analyzed on 4-mm-thick gels of either 1.2% agarose or 1.4% low-melting point agarose (Bethesda Research Labs) in 40 mM Tris–20 mM sodium acetate–1 mM EDTA (pH 7.8). Electrophoresis was performed at 5 V/cm for 8 to 12 h on horizontal submerged gels. Gels were dried onto filter paper on a gel drier (Bio-Rad), and autoradiographs were made with Kodak X-Omat R X-ray film (Eastman Kodak Co., Rochester, N.Y.). For samples labeled with [$^3$H]thymidine, the gels were impregnated for 3 h with En$^3$Hance (New England Nuclear Corp., Boston, Mass.), washed in water for an equivalent period, dried, and exposed to film at −70°C. Alternatively, gel tracks were divided into 1.2-mm slices and melted (100°C, 10 min) with 300 μl of water, and radioactivity was quantitated after the addition of 2.5 ml of scintillation fluor (HP Redisolv; Beckman Instruments, Fullerton, Calif.).

**Southern blot hybridization.** Replicating viral DNA was purified by chromatography on BND-cellulose. Samples of the DNA (≤0.25 μg) eluted with caffeine were digested with *Hpa*II restriction endonuclease and were electrophoresed on 1.2% agarose. DNA in the gel was transferred to a nitrocellulose membrane filter by the method of Southern (23) and was hybridized with polyoma DNA (~$10^6$ cpm) from the pBR322 plasmid containing the entire polyoma genome (15) labeled with $^{32}$P by nick translation (≥$2 \times 10^7$ cpm/μg) as described previously (5). The filter was exposed to film as described above.

**Second-dimension alkaline electrophoresis.** After first-dimension electrophoresis of a restriction digest on 1.2% agarose as described above, the wet gels were autoradiographed at 4°C overnight. Individual tracks were cut and then were soaked in alkaline buffer (30 mM NaOH, 2 mM EDTA, 1 mM EGTA) as described by Sundin and Varshavsky (24). The tracks were cast into second-dimension 1.5% agarose gels in alkaline buffer and were electrophoresed submerged at 2.5 V/cm for 20 h. The gels were then dried and autoradiographed as described above.

## RESULTS

**Electrophoretic separation of polyoma replication forks.** Digestion of mature polyoma DNA with restriction endonuclease *Hpa*II yields eight fragments (Fig. 1) ranging in size from 1,420 to 113 base pairs (11, 12). Digestion of an individual replicating molecule would yield six of the same full-length fragments (present as single copies from unreplicated regions and double copies from replicated regions of the molecule) plus two fragments each containing a replication fork. The relative population of individual fragments containing forks, of course, will depend

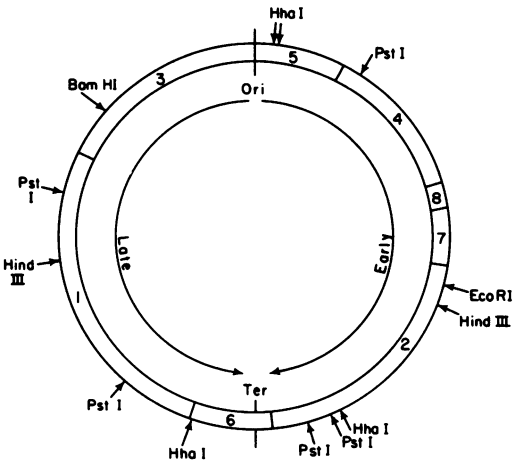


FIG. 1. *Hpa*II restriction map of polyoma DNA showing the cleavage sites for the restriction enzymes used in the experiments shown in Fig. 5 and 6. Location of the sites was determined from the sequence data of Friedmann et al. (12) and Deininger et al. (11). Arrows mark the regions corresponding to the early and late transcripts and indicate the direction of fork movement, assuming bidirectional replication from the origin.

on the distribution of species at different stages of maturity and on the mode of replication. Since a restriction fragment containing a replication fork could be almost twice as large as its corresponding unreplicated full-length linear fragment, fork-containing fragments would be expected to migrate more slowly on agarose gels. As a test system, nuclei from polyoma-infected and mock-infected 3T6 cells were allowed to carry out DNA synthesis for 30 min with [$\alpha$-$^{32}$P]dGTP under conditions known to preferentially label replicating molecules (21, 27). Viral DNA isolated by selective extraction (14) was digested with the restriction endonuclease *Hpa*II and was electrophoresed on a 1.2% agarose gel. Polyoma *Hpa*II fragments 1–7 were labeled during the in vitro incubation (Fig. 2, lane 4; fragment 8 is not resolved on this gel system). In addition, a sizable amount of label appeared in three additional diffuse bands which migrated more slowly than fragment 1. These slowly migrating bands were not visible by ethidium bromide staining (Fig. 2, lane 2), suggesting that they were not the result of incomplete digestion by *Hpa*II. Furthermore, these bands did not coincide with the gel bands generated by the partial digestion of polyoma DNA at several different concentrations of enzyme (data not shown). The high specific activity of the slowly migrating bands (compare the relative intensities of $^{32}$P label with ethidium bromide stain) would be expected for replication forks since fewer than 5% of the polyoma DNA molecules are replicating at this time after infection (6). No corresponding $^{32}$P-labeled bands appeared in comparable digests of DNA from nuclei of mock-infected cells (Fig. 2, lane 3). Faint traces of *Hpa*II fragments originating from low-level contaminant mitochondrial DNA can be seen with ethidium bromide stain (Fig. 2, lane 1) (1), but these species are of very low specific activity when labeled in washed nuclei and do not contribute to the intensity of the highly labeled bands of viral origin from infected cells. These mitochondrial DNA fragments are visible in digests of DNA labeled in vivo and extracted from whole cells (Fig. 2, lane 7; see below).

The viral origin of the DNA fragments in the putative fork region of the gel was confirmed by Southern blot hybridization. For this purpose, replicating DNA from infected cells was purified by chromatography on BND-cellulose (16), digested with *Hpa*II, electrophoresed on 1.2% agarose, and transferred to a nitrocellulose filter by the method of Southern (23). The filter was hybridized with labeled DNA from plasmid pBR322 containing the entire polyoma genome (15). The labeled polyoma DNA hybridized (Fig. 2, lane 5) to each of the species labeled in vitro (compare Fig. 2, lane 4). The difference in the relative intensity of the bands in the two samples reflects the mode of labeling. In vitro, only short regions of daughter strands are labeled, but both daughter and parental strands of forks are labeled by hybridization. Thus, the relative intensity of isotopic labeling is affected by the respective amounts of individual fork species as well as by the degree of replication of the forks.

Pulse-labeling for 5 min in vivo with [$^3$H]thymidine (Fig. 2, lane 6) yielded a pattern of labeled forks similar to that produced from a 30-min incubation in isolated nuclei (Fig. 2, lane 4). After 30 min of labeling in vivo, the specific activity of the full-length fragments increased relative to that of the fork fragments (Fig. 2, lanes 7 and 8). The longer period of in vivo labeling changed the distribution of label within the major fork bands to a pattern similar to that produced by Southern blot hybridization (Fig. 2, lane 5), reflecting the labeling of parental as well as daughter strands of the forks during the longer pulse periods. At the same time, the amount of label in large-molecular-weight cellular DNA increased relative to the label in the fork bands.

**Verification of the presence of replication forks.** The slower migration rate, higher specific activity, and labeling patterns of these bands compared with those of the full-length restriction fragments were consistent with the predictions for polyoma replication forks. Additional verification of the identity of these species as replication forks was provided by several criteria,

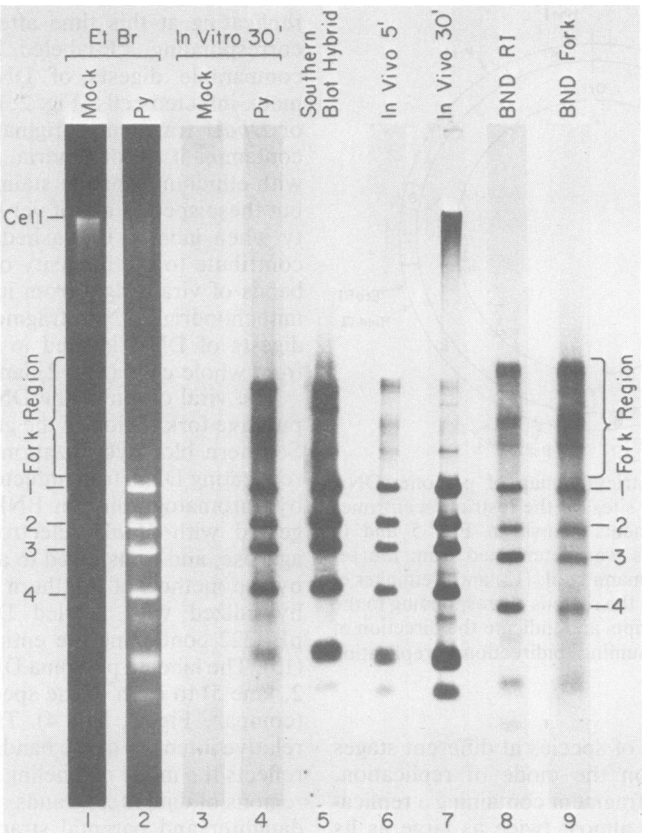


FIG. 2. Electrophoretic separation of polyoma replication forks. Viral DNA was labeled either in isolated nuclei with [α-$^{32}$P]dGTP for 30 min (lanes 2, 4, 8, and 9) or in vivo with [$^{3}$H]thymidine for 5 or 30 min (lanes 6 and 7, respectively). Nuclei from mock-infected plates were labeled in parallel with nuclei from infected cells. DNA, purified as described in the text, was digested with *Hpa*II and electrophoresed on 1.2% agarose before autoradiography or fluorography. Lanes 1 and 2 show the ethidium bromide (Et Br) fluorescent staining patterns of lanes 3 and 4, respectively, for mock-infected (Mock) and polyoma-infected (Py) cells. In lane 8, replicating DNA was purified by BND-cellulose chromatography (BND) before *Hpa*II digestion; in lane 9, chromatography was performed after *Hpa*II digestion. In lane 5, labeled polyoma DNA was hybridized to a Southern blot transfer of an *Hpa*II digest of BND-cellulose-purified replicating polyoma DNA, prepared as for lane 8. *Hpa*II fragment 1 was arbitrarily aligned in all lanes for comparison of the fork regions. The slight differences in the migration of the bands in the various lanes result from minor variations in voltage and length of electrophoresis of the different gels.

including elution behavior on BND-cellulose, sensitivity to a single-strand specific nuclease, the kinetics of labeling during active DNA synthesis, and visualization by electron microscopy.

BND-cellulose separates completely double-stranded DNA from DNA containing single-stranded regions (16). Thus, replicating molecules should be separated from mature molecules on the basis of the single-stranded regions adjacent to the forks. Purification of replicating DNA molecules on BND-cellulose before digestion with *Hpa*II enriched the level of label in forks relative to that in full-length linear fragments for molecules labeled in vitro (Fig. 2, lane 8) and in vivo (data not shown). As expected, much greater enrichment for forks was possible when the restriction digestion was performed before BND-cellulose chromatography, purifying the forks themselves rather than the entire replicating molecules (compare Fig. 2, lanes 4, 8, and 9). The small amount of label in full-length fragments for the forks purified with BND-cellulose may be due to the presence of small single-stranded regions, possibly owing to unligated Okazaki fragments, or it may represent the 10 to 20% cross-contamination of double-stranded and replicating DNA reported for these columns (16).

The single-stranded regions adjacent to replication forks also render them sensitive to the single-strand-specific nuclease S1 (3, 13). To test

for sensitivity to S1 in molecules in the fork region, samples of an *Hpa*II digest of DNA labeled with [α-$^{32}$P]dGTP for 30 min in isolated nuclei were subsequently digested with increasing amounts of nuclease (Fig. 3). The slowly migrating fork bands were preferentially digested at a concentration of S1 nuclease (1 U) that had little apparent effect on the linear *Hpa*II fragments. Products of the S1 digestion contributed to the background between the full-length *Hpa*II bands.

The labeling kinetics of the putative fork bands was examined by analyzing the viral molecules labeled in vitro for 5, 10, 20, and 30 min. During a short pulse in vivo with [$^3$H]thymidine or during the incubation of isolated nuclei with [α-$^{32}$P]dCTP, replicating molecules are labeled only in the daughter strands of the replicated portions (i.e., in the tines of each fork). A labeled restriction fragment migrates in its appropriate full-length position only after the fork passes completely through the fragment; thus, label should appear in a given fork fragment before it appears in the corresponding full-length linear fragment. The $^3$H label (introduced in vivo as [$^3$H]thymidine) was incorporated almost entirely into full-length restriction fragments and into large-molecular-weight cellular DNA (Fig. 4, fractions 10–20). In contrast, the bulk of the $^{32}$P label incorporated in vitro appeared in the three broad bands migrating more slowly than full-length fragment 1. This is particularly true for the short incubation periods. The high ratio of $^{32}$P to $^3$H for the slowly migrating species is consistent with the presence of replication forks within these three bands, and it verifies that the bands did not arise from incomplete digestion with *Hpa*II. In a similar experiment, polyoma DNA was labeled in vivo for 3 min and chased for 45 min in an excess of unlabeled thymidine. The fork bands were apparent in fluorographs of *Hpa*II digests of DNA labeled for either 3 or 45 min, although the fork bands represented a much smaller fraction of the label during the longer pulse. In contrast, after a 45-min chase the label appeared exclusively in the full-length restriction fragments (data not shown).

As a final confirmation that the three broad bands migrating more slowly than *Hpa*II fragment 1 contained replication forks, DNA from the fork region of a gel was extracted from agarose (7) and visualized directly by electron microscopy. Y-shaped molecules were observed whose dimensions were consistent with those of a fork migrating in this region of the gel (data not shown).

**Restriction nuclease localization of replication forks.** The bands migrating more slowly than *Hpa*II fragment 1 ranged from 1,420 base pairs (the size of fragment 1) to approximately 2,800 base pairs relative to standards of double-stranded linear DNA. Assuming that a fragment containing a replication fork could contain almost twice as much DNA as its parent fragment, the region above fragment 1 could contain forks from fragments 1, 2, and 3. A fork almost through fragment 4 (702 base pairs) would not be as large as full-length fragment 1. To analyze which of the fork bands were derived from regions of the genome corresponding to fragments 1, 2, and 3, samples of an *Hpa*II digest were digested with a second restriction endonuclease that cleaves within these fragments. Four additional restriction endonucleases were chosen for this purpose: *Hin*dIII, *Pst*I, *Hha*I, and *Bam*HI (Fig. 1). *Hin*dIII cleaves polyoma twice, once in *Hpa*II fragment 1 and once in fragment

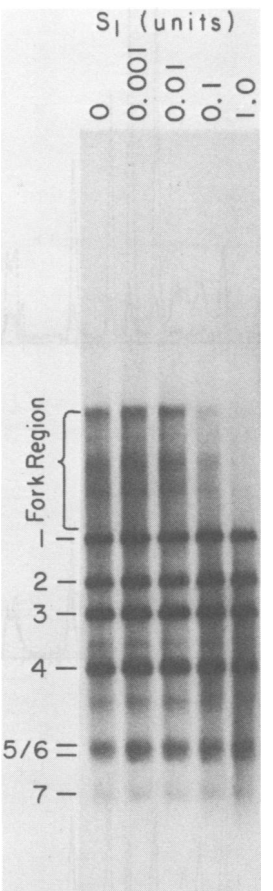


FIG. 3. S1 nuclease sensitivity of *Hpa*II-digested polyoma DNA. Viral DNA was labeled in isolated nuclei for 30 min with [α-$^{32}$P]dGTP. Samples of an *Hpa*II digest of purified DNA were incubated in the presence of increasing amounts of S1 nuclease and were analyzed by electrophoresis on 1.2% agarose and by subsequent autoradiography. Positions of *Hpa*II fragments 1–7 are indicated.

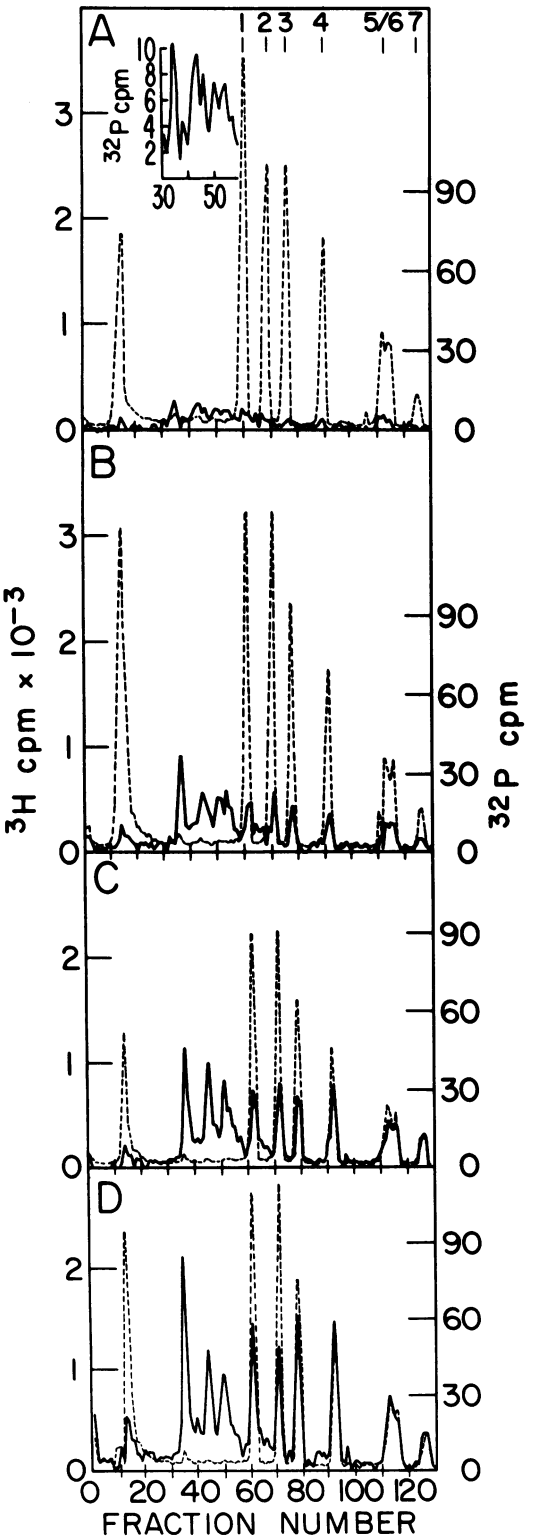


FIG. 4. *Hpa*II restriction pattern of polyoma DNA labeled in vitro. Viral DNA was labeled in vivo with [$^3$H]thymidine (30 min) and further labeled in isolated nuclei with [$\alpha$-$^{32}$P]dCTP for (A) 5, (B) 10, (C) 20, or (D) 30 min. DNA was purified, digested with *Hpa*II, and electrophoresed on 1.4% low-melting point agarose. Radioactivity was quantitated in 1.2-mm slices of the gel. Positions of *Hpa*II fragments 1–7 are indicated. Symbols: - - - -, $^3$H-labeled DNA; ——, $^{32}$P-labeled DNA.

2. After sequential digestion with *Hpa*II followed by *Hin*dIII, *Hpa*II bands 1 and 2 disappeared, and two new bands appeared migrating between bands 3 and 4 to a position corresponding to the expected products (Fig. 5, lane 4). The two slowest major fork bands were also sensitive to *Hin*dIII, as indicated by their increased mobility to a position just above *Hpa*II fragment 1. If these forks within *Hpa*II fragments 1 and 2 had already passed through the *Hin*dIII site, the products of *Hin*dIII digestion of *Hpa*II band 1 or 2 would be small linear pieces derived from the replicated arm of the fork fragment plus the corresponding truncated fork. The linear pieces generated from the fork fragments would comigrate with the corresponding pieces from the full-length *Hpa*II fragments, but the new shortened forks would still migrate more slowly than band 1, because of both their molecular weight and their Y shape. In fact, most of the forks resulting from the double restriction digestion appeared as broad bands above the position of *Hpa*II band 1, corresponding to a linear length of approximately 1,620 to 1,940 base pairs (compared with the full length of 1,420 for band 1; see below).

Similar results were obtained after sequential digestion of the DNA with *Hpa*II and with *Pst*I, which cleaves twice each in *Hpa*II fragments 1 and 2 and once in fragment 4. The fork bands also shifted to positions of lower apparent molecular weight after digestion with *Pst*I (Fig. 5, lane 3). However, because of the multiple cleavages by this enzyme, some possibly quite close to the fork branch point, the migration of the resulting forks could not be predicted. The third enzyme used, *Hha*I, cuts once each in fragments 2 and 6 and twice in 5 but does not cut fragment 1. The slowest migrating fork band remained unaltered after the double digestion, and therefore it is derived from *Hpa*II fragment 1, as predicted from its sensitivity to *Hin*dIII and *Pst*I. However, most of the labeled DNA corresponding to the other two major fork bands now migrated more rapidly, and so it must be derived from fragment 2 (Fig. 5, lane 2). The DNA remaining in the region of the two fastest *Hpa*II fork bands after digestion with *Hha*I could represent, in part, fragment 1 forks with relatively small replicated regions. At least a portion of the label in this region of the gel is derived from

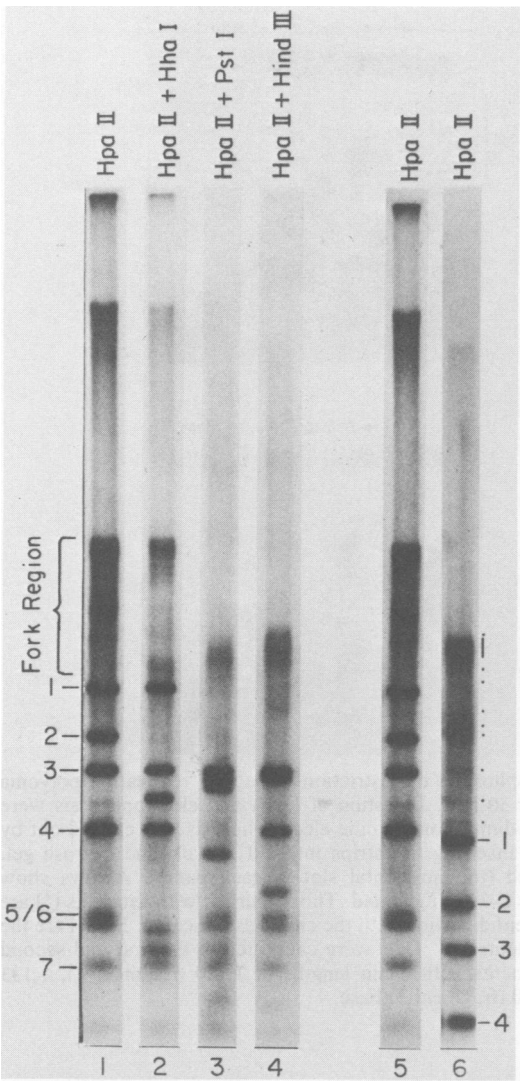


FIG. 5. Restriction analysis of polyoma DNA labeled in vitro. Viral DNA was labeled in isolated nuclei for 30 min with [α-$^{32}$P]dGTP. Purified DNA was digested with *Hpa*II (lanes 1, 5, and 6) and then was digested with *Hha*I (lane 2), *Pst*I (lane 3, or *Hin*dIII (lane 4). Positions of the *Hpa*II bands and fork region are indicated. Electrophoresis was performed at 5 V/cm for 10 h (lanes 1–5) or 14 h (lane 6).

*Hpa*II fragment 3 based on its sensitivity to *Bam*HI, which cleaves polyoma only in fragment 3. These assignments of the major fork bands to *Hpa*II fragments 1, 2, and 3 were confirmed by Southern blot hybridization with the individual cloned *Hpa*II fragments (data not shown) and by resolution of the fork bands into families from each fragment after two-dimensional gel electrophoresis.

**Characterization of replication forks by two-dimensional gel electrophoresis.** The precise location of the fork within the DNA molecules corresponding to each band could not be accurately determined by the one-dimensional gel system since the mobility of the forks in agarose is influenced in a complex manner both by molecular weight and by the configuration of the branches within each fork (6; A. J. Buckler-White and V. Pigiet, manuscript in preparation). Since both parameters vary as functions of the percentage replicated, the total number of base pairs included in both the replicated and unreplicated portions of the fork cannot be obtained reliably by comparison of gel migration relative to that of linear DNA standards. A two-dimensional gel system involving denaturing conditions in the second dimension, however, does provide a way to determine the length of the labeled (i.e., replicated) portion of the forks at each position in the first-dimension gel. Tracks excised from a 1.2% agarose gel were soaked in alkali to denature the DNA and then were mated to a 1.5% alkaline agarose gel (24). After electrophoresis in the second dimension, single strands from the full-length *Hpa*II fragments formed a diagonal series of spots (Fig. 6A). The faint downward streaming from each band may result from nicking owing to isotope decay or from the presence of gaps between unligated Okazaki fragments. Although labeled strands from the fork-containing fragments migrated more slowly than the corresponding full-length fragments in the first dimension, they now appeared as a series of diagonal bands migrating more rapidly than their corresponding full-length *Hpa*II fragments. Each diagonal family of forks represented a continuum of molecules from a given restriction fragment varying in the position of the fork within the molecule.

We verified the genomic origin for each of the diagonal fork families by digestion with additional restriction nucleases. Digestion with *Hha*I, which cleaves once within *Hpa*II fragment 2, caused the second major fork family to shift to a position between the third fork family and *Hpa*II fragment 1 (see F2* in Fig. 6B). This shift revealed another faint spot migrating slightly faster than fork 1 in both dimensions (labeled F3′ in Fig. 6B) and also allowed the resolution of the fork 1 family into a series of approximately eight discrete spots. Five of these spots are clearly visible in Fig. 6B and D, and three additional spots became visible by comparison of autoradiographs of longer and shorter exposure. The spot migrating slowest in the first dimension had a single-strand length almost equal to full-length fragment 1, indicating that it originated from forks with very short unreplicated portions. Forks with replicated portions of decreasing size (i.e., with smaller arms) should migrate faster in the first dimension and release

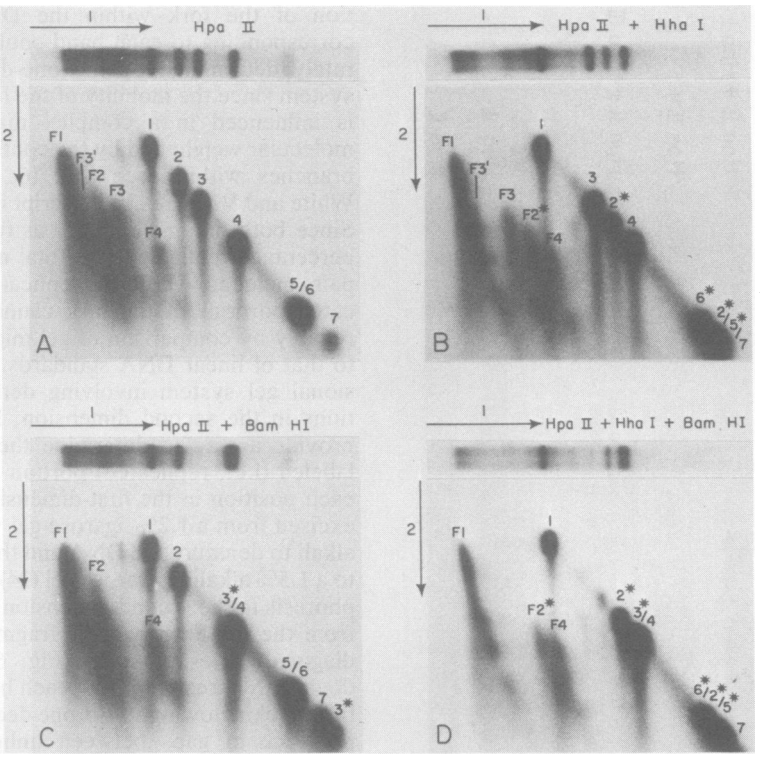


FIG. 6. Two-dimensional neutral → alkaline electrophoresis of restriction digests of replicating polyoma DNA. DNA was labeled in vitro with [α-$^{32}$P]dGTP for 30 min. Digestion of DNA and electrophoresis were performed as described in the legend to Fig. 5. Second-dimension alkaline electrophoresis was carried out by denaturing the DNA in the gel tracks with alkali and inserting the strips into a 1.5% alkaline agarose gel. Duplicate first-dimension tracks were autoradiographed (top horizontal slot in each panel). Arrows show direction of electrophoresis. *Hpa*II restriction fragments 1–7 are indicated. Those marked with asterisks (*) are the products from a given *Hpa*II fragment after subsequent digestion with the enzymes indicated. F1–F4 are the families of fork-containing molecules from *Hpa*II fragments 1–4. Gels were calibrated in the first and second dimensions with respect to the full-length *Hpa*II fragments with chain lengths of 1,420 (fragment 1), 1,133 (fragment 2), 955 (fragment 3), 702 (fragment 4), and 400 (fragment 5) base.

correspondingly shorter single-strand fragments. The resulting diagonal would thus approach the migration position of the full-length fragment. This is in fact observed for forks originating from fragment 1, indicating that some of the forks contained only very small replicated portions. Between these extremes lies a series of discrete spots from fragment 1 with an average spacing corresponding to approximately 150 (±27) bases as determined by comparison with the migration of the full-length restriction fragments. The other three diagonal fork bands also show the same discontinuities, although the proximity of these bands complicated attempts to determine accurately the number of distinct fork species within these regions of the genome.

Digestion with *Bam*HI in addition to *Hpa*II (Fig. 6C) resulted in the disappearance of the third fork family, but it also removed the faint band between fork families 1 and 2 (labeled F3′ in Fig. 6A). Its susceptibility to *Bam*HI identifies it as fragment 3 material (also verified by Southern blot hybridization with cloned *Hpa*II fragment 3), but it did not migrate within the fork 3 family. A fourth fork family, migrating between full-length fragments 1 and 2, was derived from fragment 4, based on a maximum size in the first dimension of approximately twice that of fragment 4 and a maximum single-strand length slightly less than that of fragment 4 in the second dimension (also verified by Southern blot hybridization with cloned *Hpa*II fragment 4).

The presence of multiple discrete fork bands from a given restriction fragment was verified by extracting the DNA from agarose in the fork region of 1.2% agarose gel followed by electrophoresis after denaturation with glyoxal (20). The multiple species within each fork band were also discerned by using longer electrophoresis times to effect greater resolution in the fork

region (Fig. 5, lane 6). The migration pattern of these bands was reproducible for different preparations of DNA labeled in vitro for 30 min. The same individual species were partially resolved and appeared as shoulders on the major peaks of fork species at all incubation times (Fig. 4), even at the shortest incubation time (5 min), when label was incorporated almost exclusively into the fork species. Furthermore, the major clusters of fork bands were apparent after digestion and electrophoresis of DNA labeled in vivo (Fig. 2) and were resolved into series of forks by two-dimensional electrophoresis (data not shown).

## DISCUSSION

The technology presented here provides a means for isolating intact replication forks from any region of the genome after digestion with appropriate restriction endonucleases. Control experiments negate the alternative possibilities that these labeled species are derived from cellular sequences or result from partial cleavage products (Fig. 2). That they are derived from replication forks is shown by their migration behavior in neutral and denaturing conditions, their rapid labeling in vivo and in vitro, their susceptibility to single-stranded nucleases, and their enrichment on BND-cellulose. Moreover, these forks are active in replication, as shown by the occurrence of the same gel patterns with materials labeled in vivo and in vitro, by detection with the Southern blot method, and by the labeling patterns observed in pulse-labeling and pulse-chase experiments.

Because a full-length fragment is labeled only after the fork has passed entirely through it, analyzing the relative amounts of labeled nucleotides incorporated into individual restriction fragments during replication (8, 17) can only provide a measure of regions already replicated rather than a direct way to analyze the movement of the actual replication fork. For example, labeled fragments near the origin may be derived from early, middle, or late replicative intermediates as well as from mature form I molecules terminated during the labeling period. Furthermore, full-length fragments from the termination region will be labeled only after termination has occurred. As illustrated here, under some labeling protocols most of the label may appear in fork species, not in full-length fragments (Fig. 4). Furthermore, labeling of the full-length fragments is not uniform. For example, fragments 3 and 4 are labeled to a much greater extent than are fragments 1 and 2 in vitro (Fig. 2, lane 8; Fig. 4), presumably because of an accumulation of late replicative intermediates (26). As a result, analysis of label incorporated only into the full-length restriction fragments may underestimate the late replicative intermediates and consequently may overestimate the proportion of the label incorporated into regions close to the replication origin. This caveat is particularly germane to analyses based on data solely from the full-length restriction fragments as a measure of de novo initiation (8, 17).

The discontinuous distribution of isotopes associated with the individual fork families (Fig. 6) when resolved by two-dimensional electrophoresis suggests forks do not exist as continua, but as collections of relatively discrete species. These multiple fork species are apparent on high-resolution electrophoresis in neutral gels (see particularly Fig. 5, lane 6). The species in these spots originate from the fork families with which they comigrate based on restriction endonuclease digestion patterns. For example, the spots associated with the F2 family disappear concomitantly with the full-length fragment 2 on treatment with *Hha*I (Fig. 6); conversely, the spots associated with the F1 family are unaffected by *Hha*I or by any other nuclease that does not cleave in fragment 1. The genomic origin of the spectrum of species for the F1 family was confirmed by Southern blot hybridization with a probe of *Hpa*II fragment 1 cloned into plasmid pKH-47 (data not shown).

The simplest interpretation of these data is that a replication fork proceeding through any given region of the genome may momentarily hesitate at a limited number of distinct sites. An analogous accumulation of replicating molecules at specific hesitation sites juxtaposed to the termination region has been reported for simian virus 40 (25). Studies here with polyoma confirm analogous hesitations in the same region but suggest that hesitation sites are present throughout the genome (see F3 and F4 in Fig. 6). Furthermore, all of the species labeled in vitro were also visualized after labeling in vivo. It is therefore unlikely that fork hesitation occurs solely during in vitro DNA synthesis or only because of rate-limiting events immediately preceding or during termination. Such a pattern of hesitation in fork movement could be due to either a sequence-specified event or some structural periodicity of the chromatin such as nucleosome spacing. Either of these models is compatible with the existence of forks in every region of the genome.

A simple extension of methods described can provide a means for isolating forks within the origin region and may provide a convenient assay for dissecting the detailed processes of initiation. Likewise, the choice of an appropriate restriction endonuclease should allow isolation of fragments containing one or more forks approaching termination. Thus, analysis of individual intact forks or of the distribution of individual members of fork families may provide a

valuable insight into some of the observed complexities of termination and daughter segregation (24, 26).

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