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Simian Virus 40 Deoxyribonucleic Acid Synthesis: Analysis by Gel Electrophoresis

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An agarose-gel electrophoresis technique has been developed to study simian virus 40 deoxyribonucleic acid (DNA) synthesis. Superhelical DNA I, relaxed DNA II, and replicative intermediate (RI) molecules were clearly resolved from one another for analytical purposes. Moreover, the RI molecules could be identified as early or late forms on the basis of their electrophoretic migration in relation to that of DNA II. The technique has been utilized to study the kinetics of simian virus 40 DNA synthesis in pulse and in pulse-chase experiments. The average time required to complete the replication of prelabeled RI molecules and to convert them into DNA I was approximately 10 min under the experimental conditions employed.

Simian virus 40 (SV40) deoxyribonucleic acid (DNA) molecules have been studied by a combination of techniques including dye buoyant-density centrifugation, velocity sedimentation, and electron microscopy (1, 3, 8, 9, 12). Virion particles have been shown to contain superhelical molecules (DNA I) which may be converted to relaxed molecules (DNA II) by a single-strand break (3). Cells infected by SV40 also contain a viral replicative intermediate (RI) which has both superhelical and relaxed regions (8, 12) and polymeric forms of viral DNA (7). However, no single technique has been utilized to clearly distinguish between the various forms of SV40 DNA and to determine the degree of replication of RI molecules. Thorne has shown that the superhelical and relaxed DNA of polyoma virus could be resolved by electrophoresis through an agar gel (14). The present communication describes the separation of the SV40 DNA I, DNA II, and RI molecules at different stages of replication with a high degree of resolution by a simple gel electrophoresis technique. In addition, the kinetics of the formation and processing of RI molecules were investigated.

MATERIALS AND METHODS

Cell culture. African green monkey kidney cell line AH (5) was cultivated in F12 medium containing 2 to 10% fetal calf serum (FCS).

Virus. SV40 strain WT (13), a cloned small-plaque strain, was used for all experiments. Virus stocks were prepared from cell line AH monolayers inoculated at a multiplicity of 0.01 plaque-forming units (PFU)/cell and plaque-assayed as previously described (13).

Infection of cells. AH monolayers grown to confluence in 8- or 32-o (45- or 150-cm^2 cell growing areas) prescription bottles were inoculated with 1.0 ml of stock virus at a multiplicity of 10 PFU/cell. After the virus was allowed to adsorb to the cells for 2 hr at room temperature, the inoculum was replaced with medium and the cells were incubated at 39 C.

Radioactive labeling of infected cells. After incubation of the infected cultures for 48 hr, the medium was aspirated, and 4 ml of prewarmed medium containing 50 to 100 μCi of ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass.; 15 to 20 Ci/mmole) per ml was added to each culture bottle which was then incubated underwater at the appropriate temperature. At the end of the pulse period, the culture bottles were rapidly filled with a prechilled, 4 C solution of 0.137 M NaCl, 0.005 M KCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, 0.001 M Na_2HPO_4, *p*H 7.4 (TBS). The cell monolayer was further washed once with 10 ml of cold TBS before extraction. In pulse-chase experiments, the medium containing isotope was replaced with prewarmed Hanks salt solution containing 2% FCS and an excess of unlabeled thymidine (100 μg/ml). Under these conditions, the addition of 10 μg of deoxycytidine/ml to the chase medium (11) did not alter the efficiency of the chase and was, therefore, not utilized. After the appropriate time period, the chase was rapidly terminated by the same method described above for termination of the pulse.

Extraction of DNA from infected cells. Viral DNA was selectively extracted from the infected cells as described by Hirt (6). Monolayers were lysed by 0.8 ml of a solution containing 0.01 M Tris, 0.01 M ethylenediaminetetraacetate (EDTA) and 0.6% sodium dodecyl sulfate, *p*H 7.4, for 20 min at room temperature. A 0.2-ml amount of 5 M NaCl was then added, and the bottle was gently inverted 10 times. The mixture was poured into a polyallomer centrifuge tube,

stored at 4 C overnight, and then centrifuged at 4 C for 20 min at 16,500 rev/min in a Spinco SW65 rotor. The supernatant fluid was dialyzed overnight in 0.01 M Tris and 0.01 M EDTA, *p*H 7.4. The dialyzed supernatant fluid is referred to as the Hirt supernatant fluid.

Purification of SV40 virions and preparation of marker DNA. Stocks of virus were grown in the presence of 2 μCi of ^{14}C-thymidine per ml. The virus in the medium was concentrated by polyethylene glycol and isolated by isopycnic centrifugation in CsCl by the method of Friedmann and Haas (4). DNA was extracted from the purified virions by phenol containing 0.01 M Tris and 0.01 M EDTA (*p*H 7.4). The aqueous phase was dialyzed overnight at 4 C against TBS.

Deoxyribonuclease treatment. Bovine pancreatic deoxyribonuclease (electrophoretically pure) was obtained from Worthington Biochemical Corp. A 1-μg amount of DNA extracted from purified virions was exposed for 10 min at 37 C to 0.02 Kunitz units of deoxyribonuclease in 0.2 ml of TBS (*p*H 7.4) containing 0.001 M $MgCl_2$ and 0.001 M $CaCl_2$. The reaction was stopped by adding an equal volume of 4 C TBS containing 0.1 M EDTA.

Velocity sedimentation. Samples (0.1 ml) were layered onto 3 ml of CsCl (ρ = 1.50 to 1.52 g/cm^3) in 0.01 M Tris and 0.01 M EDTA, *p*H 7.4, covered with 1 ml of mineral oil, and centrifuged at 25 C for 3.5 hr at 35,000 rev/min in a Spinco SW65 rotor. Fractions (0.08 ml) were drop collected, precipitated with 5% trichloroacetic acid in the presence of 20 μg of carrier yeast ribonucleic acid, collected on nitrocellulose filters, and assayed for radioactivity in Triton-toluene (10) by liquid scintillation counting.

Dye buoyant-density centrifugation. Ethidium bromide (EB) and CsCl were added to the Hirt supernatant fluid to give a final concentration of 200 μg of EB/ml and a final density of 1.560 g/cm^3. Samples (4 ml) were centrifuged in a Spinco SW65 rotor at 40,000 rev/min for 65 hr at 4 C. Fractions (0.06 ml) were collected from the bottom of the tubes. A 10-μliter portion of each fraction was assayed for radioactivity on Whatman 3-mm filter discs by the method of Bollum (2). The fractions of interest were dialyzed overnight at 4 C against 0.01 M Tris and 0.01 M EDTA, *p*H 7.4.

Preparation of agarose gels. The appropriate concentrations (weight/volume) of agarose (Seakem) were autoclaved in electrophoresis buffer containing 0.036 M Tris, 0.03 M NaH_2PO_4, and 0.001 M EDTA (*p*H 7.5). The sterile agarose solution was divided into portions and stored at room temperature for as long as 1 year without change in electrophoresis characteristics. For use, the agarose was liquified in a boiling-water bath, cooled to 45 C, and distributed to 8-cm acrylic tubes (0.6-cm internal diameter). The gel tubes were cooled for 30 min at room temperature, and the top 1-cm portion of the gel was removed with a scalpel blade. The tubes were then covered at the bottom with wet dialysis tubing and immediately mounted in the electrophoresis apparatus (Model 70, Biox, West Lafayette, Ind.) to prevent drying of the gels.

Electrophoresis procedure. For electrophoresis, sodium dodecyl sulfate (SDS) was added to the electrophoresis buffer to a concentration of 0.2 g/100 ml. The gel tubes were subjected to pre-electrophoresis in the SDS buffer at room temperature for 30 min at a potential of 10 v/tube (power source: model 3-1014A, Buchler Instruments, Fort Lee, N.J.). Hirt supernatant fluid DNA samples (100 μliters) were mixed with 50 μliters of electrophoresis buffer containing 30% sucrose and 0.6% SDS and were warmed at 37 C for 5 min. Samples (100 μliters) were loaded into the top of the gel tubes and subjected to electrophoresis as described in each experiment. The maximum quantity of DNA added to a gel tube in the experiments to be described was 10 μg.

Analysis of gels. After electrophoresis, the gels were either analyzed immediately or stored at 4 C, for as long as 1 week, with similar results. Cooling was found to facilitate slicing of the gels. The gels were sliced into 2.5-mm discs which were placed into scintillation vials containing 10 ml of a Triton-toluene mixture (666 ml of toluene, 333 ml of Triton X-100, 0.25 g of dimethyl-1,4-bis-2-(5-phenyloxazolyl)benzene, and 8.25 g of 2,5-diphenyloxazole, Packard Instrument Co., Downers Grove, Ill.). The counting efficiency for ^{3}H-labeled DNA samples in the gel slices was less than 1% after 1 hr in the Triton-toluene mixture, but increased to stable levels of 15 to 20% after 2 days in the scintillation fluid at room temperature. During this two-day period, the opaque gel slices gradually became almost completely clear. More than 99% of ^{3}H-labeled SV40 DNA remained within the gel slices after 1 month in the scintillation fluid as determined by transferring the gel slice to a scintillation vial containing no Triton-toluene mixture and recounting the sample. The same procedure indicated that the scintillating events were actually occurring within the gel slice itself rather than in the total volume of the Triton-toluene mixture. The counting efficiency of ^{3}H-labeled DNA I samples in gels which had been dissolved in 0.2 ml of 30% H_2O_2 for 12 hr at 70 C before mixture with 10 ml of Triton-toluene was approximately 20%. For convenience, samples were routinely assayed in intact gel slices. Radioactivity of the samples was assayed in a liquid scintillation spectrometer (series 720, Nuclear-Chicago Corp., Des Plaines, Ill.). Quench corrections were made by the channels ratio method. Reconstruction experiments indicated that samples counted in intact or dissolved gels fit similar quench correlation curves.

RESULTS

Electrophoresis of DNA extracted from virions. SV40 DNA labeled with ^{14}C-thymidine was extracted from purified virions as described in Materials and Methods. DNA samples, with and without deoxyribonuclease treatment, were subjected to electrophoresis through 2% agarose gels as described in Fig. 1. Untreated virion-derived DNA migrated as a large, sharp, leading peak (DNA I) and as a smaller, trailing peak (DNA II). No heterogeneity of either peak was observed even when the gels were sliced at 1-mm intervals. No other DNA was observed on gels twice the length illustrated here. Controlled deoxyribonuclease treatment converted DNA I into DNA

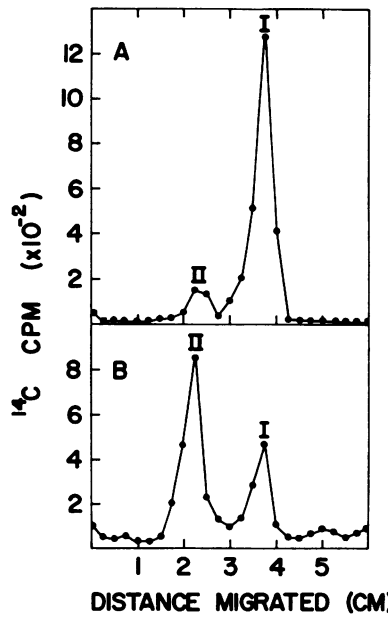

FIG. 1. *Gel electrophoresis of SV40 DNA I and II. DNA was extracted from purified SV40 virions and treated with deoxyribonuclease as described in Materials and Methods. DNA samples suspended in loading buffer containing 10% sucrose were subjected to electrophoresis for 2.5 hr through 2% agarose at 10 v/gel. Migration is from left (−) to right (+). The gels were sliced and analyzed as described in Materials and Methods. A, Untreated SV40 DNA; B, SV40 DNA treated with deoxyribonuclease.*

II. The untreated ^{14}C-labeled DNA was used as marker DNA in the remaining experiments.

Electrophoresis of SV40 RI. To examine the electrophoretic behavior of SV40 RI, AH cells were labeled for 10 min with ^{3}H-thymidine (100 μCi/ml) 48 hr after inoculation with SV40. The viral DNA was selectively extracted and examined by a variety of techniques (Fig. 2). Velocity sedimentation analysis (Fig. 2A) showed that most of the newly synthesized viral DNA sedimented as a broad peak ahead of marker DNA I and II, as previously described by others (8, 9, 12). Dye buoyant-density centrifugation (Fig. 2B) showed a banding of viral DNA at heterogeneous densities between the densities corresponding to marker DNA I and II, as also previously demonstrated by others (8, 12). Gel electrophoresis (Fig. 2C) of the same sample clearly resolved the DNA I and II peaks. Most of the newly labeled viral DNA migrated more slowly than DNA II. The trailing edge of this broad peak declined sharply, whereas the leading edge gradually extended through the DNA II peak to the base of the DNA I peak. Although the DNA I, II, and RI peaks overlapped

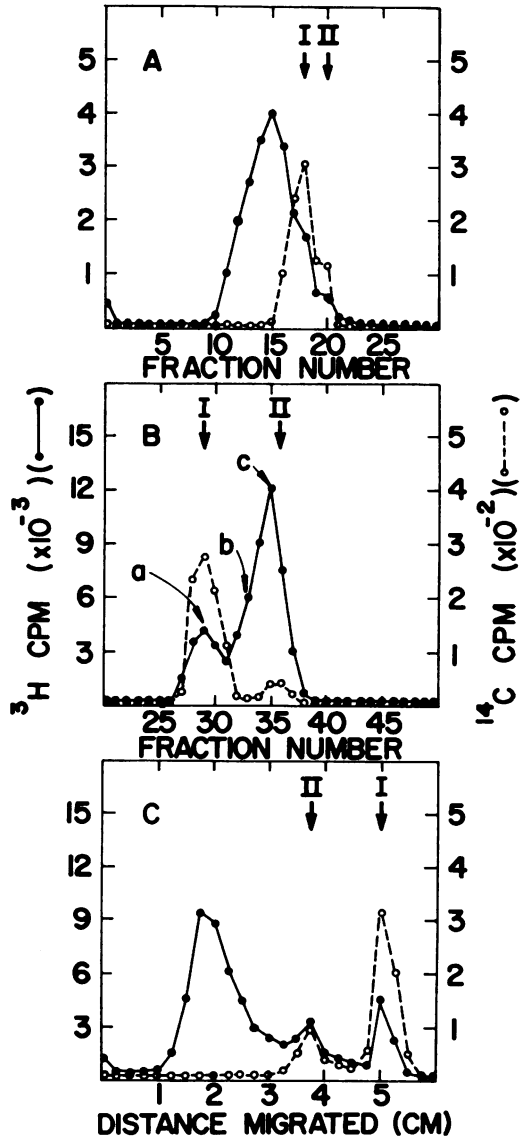

FIG. 2. *A comparison of replicating SV40 DNA as analyzed by velocity sedimentation, dye buoyant-density centrifugation, and gel electrophoresis. Cell line AH monolayers in 32-ounce (ca. 150 cm^2) bottles were inoculated with 10 PFU/cell, and 48 hours after infection at 39 C they were exposed to 100 μCi of ^{3}H-thymidine per ml for 10 min. The Hirt supernatant fluid was analyzed by velocity sedimentation in neutral CsCl (A), dye buoyant-density centrifugation (B), and electrophoresis through 1.5% agarose for 2 hr at 10 v/gel (C). See Materials and Methods for details of the techniques. Centrifugation fractions were collected from the bottom of the tubes and numbered in the order of collection.*

to some extent, an accurate estimate of the relative quantity of isotope incorporated into each of the three forms of viral DNA could easily be made on the basis of this single procedure. In parallel experiments, uninfected AH cultures produced no detectable DNA which migrated into the gels.

To further correlate the behavior of viral DNA in dye buoyant-density centrifugation and gel electrophoresis, relevant fractions from the density gradient were dialyzed and subjected to electrophoresis (Fig. 3). Fraction a (Fig. 2B), corresponding to superhelical DNA in the EB-CsCl gradient, comigrated through an agarose gel with marker DNA I for the most part. However, the fraction also contained ^{3}H-DNA which migrated somewhat more slowly than DNA I. This DNA probably represented RI molecules early in the replication process which were not completely resolved from DNA I in the gradient fraction examined. Fraction b (Fig. 2B) of the gradient, corresponding to RI molecules intermediate in the replication process (8, 12), migrated to an intermediate position in the area of the agarose gel representing RI molecules. Fraction c (Fig. 2B) of the gradient, corresponding to RI molecules late in the process of replication and to an undetermined portion of DNA II (8, 12), migrated as a small, sharp peak of DNA II and a large well-defined peak of RI molecules clearly behind DNA II on the gel. Thus, the gel technique separated RI molecules in various stages of replication. In addition, DNA II could easily be identified as a small, but well-defined, peak superimposed on a flat portion of the broad, RI peak.

When infected cells were first pulsed for 10 min with ^{3}H-thymidine and then were subsequently chased for 1 hr with an excess of unlabeled thymidine, most of the newly synthesized DNA was present in form I DNA as determined by all three methods. A small remaining peak corresponding to RI DNA could be identified by gel electrophoresis analysis (Fig. 4). Studies by others have shown that ^{3}H-labeled DNA I may reenter the replicating pool as the parental strands of replicating molecules (8, 12). Thus, a chase would not be expected to be complete.

Replicative intermediate DNA was also examined by electrophoresis through agarose gels of varying concentrations. At an agarose concentration of 0.5%, all forms of viral DNA migrated as a single peak. At a 1.0% concentration, forms I and II were poorly resolved from each other but could be distinguished from RI. At a 2% concentration, RI did not completely enter the gel under the conditions of electrophoresis, but DNA I and II were well separated.

Kinetics of viral RI synthesis. The high degree of resolution of DNA I, II, and RI by a single technique provided a means to measure accurately the rate of synthesis of RI. AH monolayers were infected at an input multiplicity of 10 PFU/cell and labeled with ^{3}H-thymidine for varying periods of time 48 hr after infection. The viral DNA in the Hirt supernatant fluid was examined by gel electrophoresis (Fig. 5). With an increasing duration of the pulse period up to 40 min, there

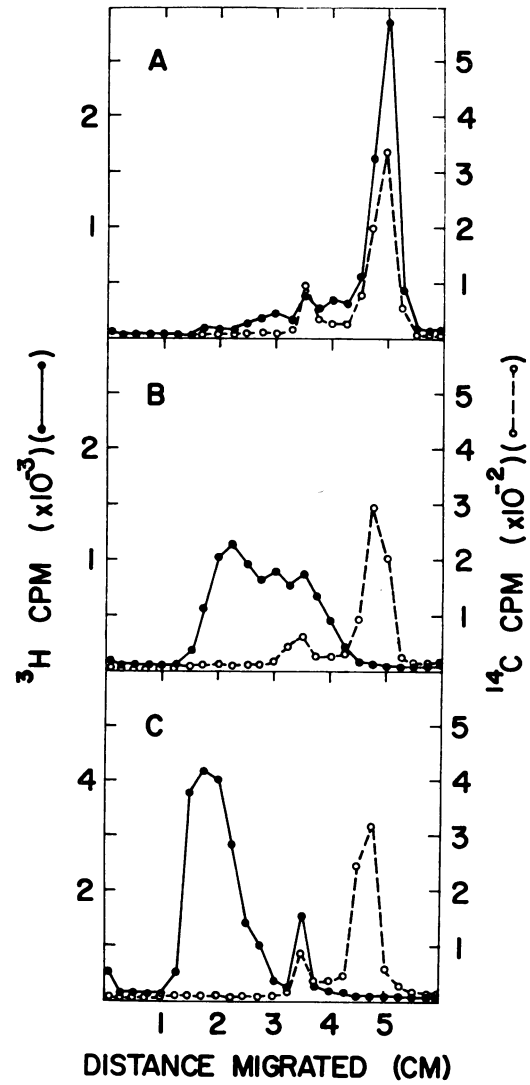

FIG. 3. *Electrophoresis of fractions a, b, and c from the dye buoyant-density centrifugation as indicated in Fig. 2B. Each fraction was dialyzed against 0.01 M Tris and 0.01 M EDTA, pH 7.4. A portion of the dialyzed fractions was subjected to electrophoresis through 1.5% agarose at 10 v/gel for 2 hr. A, Fraction a from Fig. 2B; B, fraction b from Fig. 2B; and C, fraction c from Fig. 2B.*

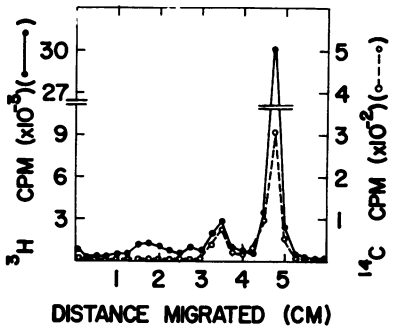

FIG. 4. *Gel electrophoresis of SV40 DNA after a ^{3}H-thymidine pulse and a 1-hr chase. Cell line AH monolayers in 32-ounce (ca 150 cm^2) bottles were inoculated with 10 PFU/cell, and 48 hr after infection at 39 C they were exposed to 100 μCi of ^{3}H-thymidine per ml for 10 min at 39 C and subsequently were chased with an excess of unlabeled thymidine for 60 min. The Hirt supernatant fluid was subjected to electrophoresis through 1.5% agarose for 2 hr at 10 v/gel.*

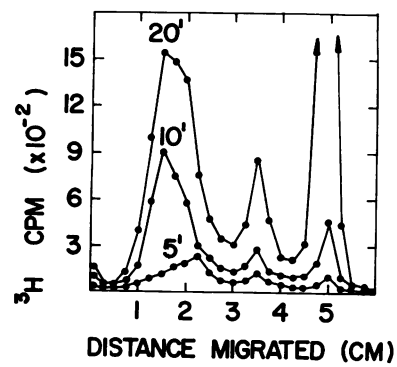

FIG. 5. *Composite diagram of the gel electrophoresis patterns of replicating SV40 DNA extracted from infected cells after exposure to ^{3}H-thymidine for increasing periods of time. Cell line AH monolayers in 8-ounce (ca 45 cm^2) bottles were inoculated with 10 PFU/cell and incubated at 39 C for 48 hr. The cultures were labeled with medium containing 50 μCi of ^{3}H-thymidine per ml for periods of from 5 to 20 min. Hirt supernatant fractions were subjected to electrophoresis through 1.5% agarose for 2 hr at 10 v/gel. The numbers in the figure indicate the length of the labeling period in minutes.*

was a concomitant increase in the quantity of RI. However, the shape of the RI peak, as determined by gel electrophoresis, was different depending on the duration of the pulse period (Fig. 5). After a 5-min pulse, most of the ^{3}H-labeled DNA was present in the region of the gel which was previously shown to correspond to the location of RI molecules intermediate in the replication process. In contrast, after a pulse of 10 min or longer, RI molecules late in the process of replication contained most of the radiolabel. The shift of ^{3}H-DNA to the late region of the RI peak probably coincided with the average time period required to label a newly replicated strand of maximal length. The continued increase in the quantity of ^{3}H-DNA in the RI region of the gel observed in pulses longer than 10 min could be explained, in part, as a reutilization of recently labeled ^{3}H-DNA I molecules as the parental strands of newly initiated RI molecules and possibly as a result of a depletion of endogenous thymidine pools.

Processing of RI molecules. Pulse-chase experiments were utilized to study the rate at which RI molecules were completed and converted into DNA I. Infected AH cells were pulse-labeled with ^{3}H-thymidine for 5 min. The incorporated label was then chased with an excess of unlabeled thymidine for varying periods of time (Fig. 6). RI disappeared rapidly during the first 10 min of the chase with a reciprocal increase in DNA I. Thereafter, the conversion of RI into DNA I appeared to occur at a much slower rate. When the length of the initial pulse period was varied, the efficiency of the chase, but not the kinetics of the chase, depended on the length of the preceding pulse (Fig. 7). The rapid phase of the chase has been interpreted to parallel the completion of replication of the new progeny strands of RI molecules, whereas the slow phase of the chase reflected, in part, the continued reutilization of prelabeled DNA I as the parental strands of newly initiated

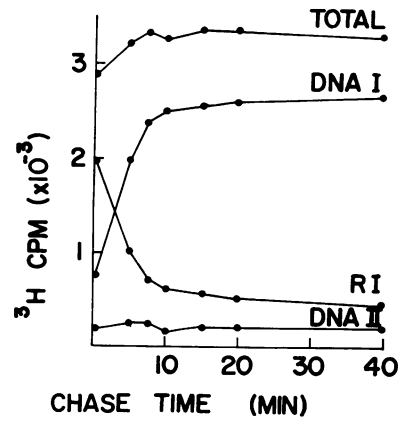

FIG. 6. *Kinetics of processing of ^{3}H-thymidine-labeled SV40 DNA during chase periods of from 0 to 40 minutes. Cell line AH cultures in 8-ounce (ca 45 cm^2) bottles were infected and pulse labeled for 5 min as described in Fig. 5. At the end of the pulse, the medium containing ^{3}H-thymidine was replaced with medium containing an excess of unlabeled thymidine. DNA was extracted from the cultures and analyzed by gel electrophoresis as described in Fig. 5. The total counts/min of the SV40 DNA components were plotted above.*

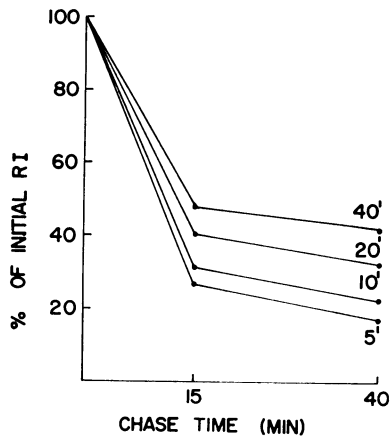

FIG. 7. *Effect of the length of the pulse period on the efficiency and kinetics of the chase. Cell line AH monolayers in 8-ounce (ca 45 cm²) bottles were inoculated with 10 PFU/cell, and 48 hr after infection at 39 C they were pulse labeled with ³H-thymidine for periods of from 5 to 40 min. At the end of the pulse, the medium containing 50 μCi of ³H-thymidine per ml was replaced with medium containing an excess of unlabeled thymidine. DNA was extracted from the cultures after 15- and 40-min chase periods and analyzed by gel electrophoresis as described in Fig. 5. The numbers in the figure indicate the duration of the initial pulse period in minutes.*

RI molecules. Although the factors responsible for the slow phase of the chase have not been fully investigated, pulse-chase studies provide a useful means to measure the rate of synthesis of replicative intermediates and their conversion into DNA I under standard conditions.

DISCUSSION

A new and simple technique utilizing electrophoresis through SDS-agarose gels has been developed to obtain a high degree of resolution of the various forms of SV40 DNA. Accurate estimates of the relative quantities of radiolabeled DNA I, II, and RI could easily be made, and RI molecules at different stages in the replication process could be identified. In the presence of SDS, the migration of DNA molecules through gels would be expected to depend primarily on the size and shape of the molecules. Previous studies by others utilizing density gradient and electron microscopy techniques have shown that the RI molecules, early in the process of replication, remain predominantly superhelical in configuration (8, 12). These molecules would be expected to migrate through gels more slowly than DNA I because of their slightly larger size and less superhelical configuration. RI molecules, late in the process of replication, have been shown by others to be up to twice the size of DNA I and II and to be predominantly or completely relaxed in configuration (8, 12). These molecules would be expected to migrate more slowly than DNA II. Some RI molecules, intermediate in the process of replication, would migrate like DNA II. The behavior of fractions derived from dye buoyant-density centrifugation on subsequent gel electrophoresis strongly supported these interpretations.

In contrast, dye buoyant-density centrifugation cannot be used to quantitate DNA II in the presence of late RI molecules in a relaxed configuration. Velocity sedimentation fails to separate early and late RI molecules (12), because the concurrent increase in molecular weight and the relaxation of replicating molecules affect the sedimentation of the molecules in opposing directions. Thus, gel electrophoresis provides favorable resolution for monitoring SV40 DNA synthesis by a single and simple technique.

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