Structure of Chromatin at Deoxyribonucleic Acid Replication Forks: Okazaki Fragments Released from Replicating SV40 Chromosomes by Single-Strand Specific Endonucleases Are Not in Nucleosomes†

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ABSTRACT: The structure of replicating chromosomes at the sites of deoxyribonucleic acid (DNA) synthesis was characterized by digestion of replicating simian virus 40 (SV40) chromosomes with either S_1 or *Neurospora crassa* single-strand specific endonucleases. The products of digestion were compared with those from the digestion of nonreplicating SV40 chromosomes and purified replicating and nonreplicating viral DNA. While purified, covalently closed, superhelical DNA [SV40(I) DNA] was cleaved by these nucleases, SV40(I) DNA contained in mature SV40 chromosomes was completely resistant. On the other hand, all replicating SV40 DNA [SV40(RI) DNA], both in the form of purified DNA and replicating chromosomes, was cleaved by these single-strand specific nucleases. Although the DNA contained in replicating chromosomes was cleaved at a rate ~200 times slower than purified SV40(RI) DNA, the initial cleavage of either form of SV40(RI) DNA (25 S) generated a comparatively homogeneous population of 18S DNA. Continued digestion released a maximum of 50–60% of the Okazaki fragments as 4–6S duplex DNA. The 40–50% of the Okazaki fragments which remained with 18S DNA could result from the presence of single-stranded regions of DNA too small to be recognized by S_1 nuclease under the conditions used. At least 90% of the Okazaki fragments released by S_1 nuclease from formaldehyde-fixed chromosomes were not contained in nucleosomes, but were found as bare DNA. They were digested by micrococcal nuclease at the same rate and to the same extent as purified DNA. They sedimented at 4–6 S before and after treatment with Sarkosyl, proteinase K, and 1 M NaCl, and they had the same buoyant density in CsCl as purified DNA. Therefore, the synthesis of Okazaki fragments and their assembly into nucleosomes need *not* occur concomitantly. Rather, Okazaki fragments can be synthesized and ligated to longer nascent DNA chains prior to assembly into nucleosomes.

DNA nucleases have proven extremely valuable in demonstrating that mammalian chromosomes are organized into discrete particles, called nucleosomes, each containing ~200 base pairs of DNA coiled around an octamer of histones (Kornberg, 1977). While several of the nucleases employed digest single- as well as double-stranded DNA, the use of endonucleases which are solely single-strand specific has not been thoroughly explored. Such enzymes should be particularly appropriate for analysis of the organization of replicating chromosomes since single-stranded regions are expected to exist specifically at DNA replication forks (Figure 1) (Gefter, 1975; Edenberg & Huberman, 1975; Alberts & Sternglanz, 1977).

We have used S_1 and *Neurospora crassa* endonucleases to search for regions of single-stranded DNA in isolated replicating and mature SV40[1] chromosomes. SV40 chromosomes replicate in the nuclei of mammalian cells and are composed of nucleosomes whose structure and histone composition are strikingly similar to those of their host (Bellard et al., 1976; Cremisi et al., 1976; Varshavsky et al., 1977; Christiansen & Griffith, 1977; Ponder & Crawford, 1977; Müller et al., 1978; Shelton et al., 1978). Since it has been shown that replication forks in purified polyoma DNA are accessible to single-strand specific endonucleases (Bourgaux & Bourgaux-Ramoisy, 1971), we anticipated that they might also be accessible in replicating SV40 chromosomes. Among the products of the digestion, we expected to find a duplex form of Okazaki fragments, perhaps associated with chromosomal proteins or even assembled into nucleosomes. Furthermore, unique regions of single-stranded DNA might exist at the origins of DNA replication or transcription as a consequence of the interaction of chromosomal proteins with specific DNA sequences. If such regions were accessible to single-strand specific endonucleases, they would be detected by analysis of mature SV40 chromosomes.

Experimental Procedures

Growth of Virus and Cells. The small plaque SV40 strain Rh911, prepared by infection of MA-134 cells at multiplicities of less than 0.01, was propagated on a CV-1 monkey cell line obtained from P. Tegtmeyer. Cells were cultured in modified Eagle's medium (MEM medium) supplemented with 5% fetal calf serum as previously described by Anderson et al. (1977). Upon infection, the media was supplemented with 10% calf serum and antibiotics.

Radioactive Labeling of SV40 DNA. Uniformly labeled SV40 DNA was prepared by incubating SV40-infected cells with either 25 μCi of [^{3}H]thymidine (55.5 Ci/mmol) per 100-mm diameter dish of cells or 4 μCi of [^{14}C]thymidine (50 mCi/mmol) from 24 to 36 h after infection. The specific activity of the DNA obtained by this procedure was routinely

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[1] Abbreviations used: SV40, simian virus 40; SV40(I) DNA, covalently closed superhelical viral DNA; SV40(II) DNA, duplex circular viral DNA containing at least one single-strand interruption; SV40(III) DNA, full-length linear duplex viral DNA; SV40(RI) DNA, replicative intermediate of viral DNA; EDTA, sodium ethylenediaminetetraacetate.

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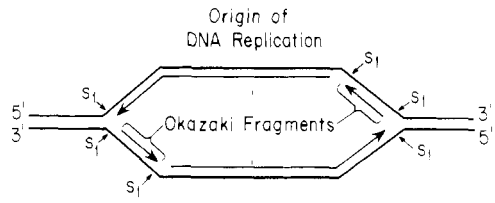

FIGURE 1: Potential S_1 nuclease cleavage sites at the replication forks of SV40 DNA. DNA replication occurs bidirectionally from a unique origin (Danna & Nathans, 1972; Fareed et al., 1972). Nascent DNA chains are elongated continuously in the direction of fork movement (forward arm) and discontinuously in the form of Okazaki fragments in the direction opposite that of fork movement (retrograde arm) (Hunter et al., 1977; Perlman & Huberman, 1977; Flory, 1977; Kaufmann et al., 1978). Predicted regions of single-stranded parental DNA which could serve as S_1 nuclease cleavage sites are indicated by $S_1 \rightarrow$.

50 000 cpm/μg of ^{3}H-labeled DNA and 20 000 cpm/μg of ^{14}C-labeled DNA.

Pulse-labeled replicating SV40 DNA was prepared by equilibrating the deoxyribonucleotide pools in virus-infected cells with [^{3}H]thymidine at 0 °C followed by DNA synthesis at 20 °C (Perlman & Huberman, 1977). SV40-infected cells were washed with 20 mM Tris-HCl (pH 7.4), 5 mM KCl, 0.5 mM $MgCl_2$, 137 mM NaCl, and 1.0 mM $CaCl_2$, and floated on ice–water. Thirty seconds later, 100 μCi of [^{3}H]thymidine in 0.6 mL of 20 mM Tris-HCl (pH 7.4), 5 mM KCl, 1 mM Na_2HPO_4, and 137 mM NaCl was added to each dish. After a 10-min incubation at 0 °C, each plate was then floated on water at 20 °C for 1.0 min. DNA synthesis was stopped abruptly by returning the plates to ice–water and adding 10 mL of ice-cold 15 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, and 25 mM NaCl. The specific activity of replicating SV40 chromosomes labeled in this way was consistently two- to threefold higher than that obtained if the cells were not preincubated with [^{3}H]thymidine on ice for 10 min prior to incubation to 20 °C.

Preparation of SV40 DNA. Purified SV40 chromosomes (50–100 μg of DNA/mL) in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA were digested with proteinase K (10 μg/mL) in the presence of 0.5% sodium dodecyl sulfate for 20 min at 37 °C. The digest was then extracted twice at ambient temperature with an equal volume of chloroform–isoamyl alcohol (24:1). Sodium chloride was added to the second aqueous phase to a final concentration of 0.3 M, and the DNA precipitated overnight at −20 °C with 2 volumes of ethanol. The DNA was resuspended in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA and stored at 4 °C. Alternatively, SV40 DNA was prepared directly from virus-infected cells by the method of Hirt (1967).

Preparation of SV40 Chromosomes. SV40 chromosomes containing either SV40(I) or SV40(RI) DNA were prepared 36 h postinfection when the rate of viral DNA synthesis was at a maximum by a modification of the procedure described by Green et al. (1971). SV40-infected cells were washed once with ice-cold 15 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, and 25 mM NaCl, scraped from the dishes with a rubber policeman, and lysed with three strokes of a tight-fitting Dounce homogenizer (Kontes Glass Co., B pestle). The homogenate was diluted with additional buffer (25 mL/10 plates of cells), and the nuclei were pelleted at 1500g for 5 min and resuspended in 0.25 mL of 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, and 200 mM NaCl per dish of cells. Triton X-100 was added to a final concentration of 1%. The nuclei were incubated at 30 °C for 20–30 min with frequent stirring and then pelleted by centrifugation at 2000g for 5 min at 4 °C. The supernatant containing the SV40 chromosomes was layered on a 5–30% neutral sucrose gradient in 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, and 200 mM NaCl and centrifuged for 4.5 h at 25 000 rpm, 4 °C, in a Beckman SW27 rotor. All gradients were collected from the top to avoid contamination with pelleted SV40 virions. Mature SV40 chromosomes (~55 S) containing either SV40(I) ^{3}H- or ^{14}C-labeled DNA were recovered and concentrated from the appropriate gradient fractions by vacuum dialysis against 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA. Replicating SV40 chromosomes (60–65 S) containing SV40(RI) ^{3}H-labeled DNA were recovered and concentrated in the same way. The above procedure routinely yielded ~5 μg of SV40 DNA per dish [(4–6) × 10^6 cells]. DNA concentrations were measured by a modification (Shelton et al., 1978) of the fluorometric method of Kissane & Robbins (1958).

Optimal Conditions for S_1 Nuclease Digestion of DNA. Optimal conditions for S_1 nuclease digestion of denatured DNA were found to be 30 mM sodium acetate, pH 4.6, 0.05 mM zinc acetate, 75 mM sodium chloride, 10 μg/mL single-stranded radiolabeled DNA, and 5 units/mL S_1 nuclease (Beard et al., 1973). All digestions were performed at 37 °C. One unit of S_1 nuclease converts 1 μg of heat-denatured DNA to an acid-soluble form in 1 min at 37 °C. S_1 nuclease was either purified from Taka–Diastase powder (Parke–Davis) by the method of Wiegand et al. (1975) or purchased (Sigma). All preparations of nuclease gave similar results.

Optimal Conditions for S_1 Nuclease Digestion of Chromatin. Optimal conditions for S_1 nuclease digestion of SV40 chromatin were found to be 30 mM sodium acetate (pH 5.3), 0.05 mM zinc acetate, 75 mM sodium chloride, 10–50 μg of DNA/mL of chromatin, and 2.5–10 000 units/mL S_1 nuclease. Reactions of 0.1 mL were routinely incubated at 37 °C for 30 min. The reactions were stopped by addition of EDTA and Tris-HCl (pH 7.5) to give final concentrations of 10 and 50 mM, respectively. When the DNA was analyzed by agarose gel electrophoresis, it was purified as previously described. When the DNA was analyzed by sedimentation in neutral sucrose gradients, the reactions were adjusted to 10 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.5% Sarkosyl, and 20 μg/mL proteinase K, incubated at 37 °C for 20 min, and then layered directly onto neutral sucrose gradients. This procedure eliminated the possible loss of small DNA fragments during chloroform–isoamyl alcohol extraction and ethanol precipitation.

Two parameters were considered in determining optimal conditions for the S_1 nuclease digestion of chromatin: solubility of the chromatin and activity of the nuclease. While isolated SV40 chromatin was completely soluble at pH 5.5, the fraction which remained in solution decreased markedly as the pH of the reaction mixture was lowered. All of the chromatin was precipitated at pH 4.6. At the same time, the initial rate of digestion of heat-denatured CV-1 cell DNA by S_1 nuclease decreased approximately 10-fold as the pH of the reaction mixture was increased from 4.6 to 5.3; at pH 7.0, nuclease activity was barely detectable. Under the conditions found to be optimal for digestion of chromatin (pH 5.3), S_1 nuclease exhibited ~10% of the activity observed at pH 4.6 and at least 80% of the chromatin was soluble.

The solubility of SV40 chromatin was also dependent upon the concentration of Zn^{2+} in the reaction mixture. At pH 5.3, the solubility of the chromatin increased steadily as the concentration of Zn^{2+} was lowered from 1.4 to 0.1 mM. At concentrations of Zn^{2+} lower than 0.1 mM, the chromatin exhibited its maximum solubility at pH 5.3. Decreasing the

concentration of Zn^{2+} to 0.05 mM did not significantly affect the activity of S_1 nuclease.

S_1 nuclease preparations were assayed for contaminating double-strand specific endonuclease activity by incubating the enzyme with SV40(III) ^{3}H-labeled DNA under the optimal conditions for the digestion of chromatin as described above. Some double- as well as single-stranded interruptions were detected by sedimentation analysis of the DNA in neutral or alkaline sucrose gradients, but only when concentrations of S_1 nuclease were in excess of those employed in the analysis of replicating DNA or chromosomes (e.g., 15 000 units). This amount of enzyme was at least 7-fold more than that required to convert SV40(II) DNA into SV40(III) DNA and 7000-fold more than that required to cleave SV40(RI) DNA.

Optimal Conditions for Neurospora crassa Nuclease Digestion of DNA or Chromatin. Conditions for *Neurospora crassa* nuclease digestion of either SV40 DNA or chromatin were 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM cobalt acetate, 10 μg of DNA/mL, and 0.5–32 units/mL nuclease (Linn & Lehman, 1965). Cobalt was necessary to overcome the inhibitory effect of low concentrations of EDTA (0.01–0.05 mM) contributed by the buffer in which the chromatin sample was stored. One unit is defined as that amount of enzyme which solubilizes 50 μg of denatured DNA in 30 min at 37 °C. Following the digestion, the reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The DNA was then extracted as described previously and analyzed by sedimentation in 5–20% neutral sucrose gradients.

Agarose Gel Electrophoresis of DNA. DNA extracted from S_1 nuclease digested SV40(I) DNA or mature chromatin was analyzed on 1.4% agarose slab gels. The gels were prepared in electrophoresis buffer which consisted of 40 mM Tris–acetate (pH 7.2), 20 mM sodium acetate, and 2 mM EDTA. Samples containing 0.1 μg of DNA in 20–30 μL of a 10-fold dilution of electrophoresis buffer were applied to each lane, and electrophoresis was carried out at 100 V/50 mA for 5 h at ambient temperature. This was approximately twice the time required for a bromphenol blue standard to migrate off the gel. Gels were stained with 0.001% ethidium bromide in H_2O for at least 30 min and photographed under ultraviolet light with Polaroid Type 51 film and a Kodak 23A filter. Microdensitometer tracings of photographic negatives of stained gels were obtained by using an Ortec microdensitometer, Model 4300. The areas under the peaks were integrated to quantitate the rate of conversion of SV40(I) DNA to SV40(II) and SV40(III) DNA.

Velocity and Equilibrium Sedimentation Analysis. SV40 DNA digested by either S_1 or *Neurospora crassa* nuclease was routinely analyzed by velocity sedimentation in linear 5–20% neutral sucrose gradients containing 1 M NaCl, 10 mM Tris-HCl (pH. 7.5), and 2 mM EDTA. Gradients were centrifuged at 35 000 rpm in an SW41 rotor for 14 h at 4 °C to separate SV40(I) DNA (21 S) and SV40(II) DNA (16 S). Fractions were collected from the bottom of each tube. DNA was precipitated by the addition of 3 mL of cold 1 N HCl and collected on Whatman GF/A filters. Filters were dried, and the radioactivity present was measured in a toluene-based liquid scintillation fluor.

Nascent SV40 DNA was analyzed by velocity sedimentation in linear gradients containing 5–20% sucrose, 0.2–0.8 M NaOH (proportional to sucrose concentration), 0.8–0.2 M NaCl (to make Na^+ concentration up to 1 M), 2.5 mM EDTA, and 0.015% Sarkosyl. Gradients were centrifuged at 60 000 rpm in an SW60 rotor for 6 h at 4 °C to separate single-stranded circular (18 S) and single-stranded linear (16 S) SV40 DNA. Alkaline sucrose gradients were fractionated, and the DNA was measured as described for neutral sucrose gradients.

Replicating SV40 chromosomes were sedimented to equilibrium in CsCl gradients (mean ρ = 1.51 g/cm^3) containing 10 mM sodium phosphate (pH 7.0) and 0.02% Sarkosyl. Sarkosyl reduced the adsorption of ^{3}H-labeled chromatin to the walls of polyallomer tubes. Gradients were established by centrifugation at 40 000 rpm in an SW50.1 rotor for 40 h at 20 °C. Fractions were collected from the bottom of each tube directly into liquid scintillation vials. The refractive index of selected fractions was measured to determine the density. Aquasol (New England Nuclear) was then added to each sample, and the radioactivity was measured in a liquid scintillation counter.

Formaldehyde Fixation of Replicating SV40 Chromosomes. A modification of the formaldehyde fixation procedure described by Chalkley & Hunter (1975) was used to prevent the dissociation of chromosomal proteins from SV40 DNA. Replicating SV40 chromosomes were filtered through Sephadex G-50 equilibrated with 10 mM sodium phosphate (pH 7.0) to remove the Tris buffer which would otherwise react with the formaldehyde. SV40 chromosomes were incubated for 1 h on ice with 1% formaldehyde. When chromosomes were to be digested with nucleases subsequent to the fixation step, they were filtered through a Sephadex G-50 column equilibrated in 10 mM Tris (pH 7.5) and 0.1 mM EDTA to remove the unreacted formaldehyde.

Results

(*a*) *S_1 Nuclease Digestion of SV40(I) DNA and Mature SV40 Chromosomes.* S_1 nuclease, a single-strand specific nuclease from *Aspergillus oryzae*, was selected initially from a variety of similar nucleases because of its high specificity for single-stranded DNA and its ability to recognize small mismatched regions of single-stranded DNA within a duplex region (Shenk et al., 1975). However, while S_1 nuclease exhibits maximal activity at pH 4.6 (Ando, 1966; Vogt, 1972), the solubility of chromatin decreases dramatically below pH 5.5 (Phillips, 1971). Consequently, in order to use S_1 nuclease to analyze the structure of mature and replicating SV40 chromosomes and compare these results with those obtained with purified SV40 DNA, it was necessary to establish digestion conditions under which the chromatin remained soluble and the enzyme retained significant activity. As described under Experimental Procedures, the optimal conditions for digestion of chromatin were found to be 30 mM sodium acetate (pH 5.3), 75 mM sodium chloride, and 0.05 mM zinc acetate. Under these conditions, S_1 nuclease exhibited ~10% of the activity observed at pH 4.6 and at least 80% of the chromatin was soluble.

S_1 nuclease has been shown to cleave SV40(I) DNA into SV40(III) DNA; SV40(II) DNA appears as a transient intermediate in the digestion (Beard et al., 1973). Virtually all of the circular viral DNA was converted into linear DNA when the digestion was performed at pH 4.6. The reaction was strongly dependent upon the ionic strength of the digestion buffer; conversion of SV40(I) DNA to SV40(II) DNA was at least 5 times faster at 75 mM NaCl than at 250 mM NaCl (Beard et al., 1973). We have observed the same time course for the digestion of SV40(I) DNA under the optimal conditions for chromatin digestion. During a 1-h incubation with 150 units of S_1 nuclease in the standard 0.1-mL reaction mixture, SV40(I) DNA was rapidly converted to SV40(II) DNA which was then slowly converted to SV40(III) DNA (Figure 2A).

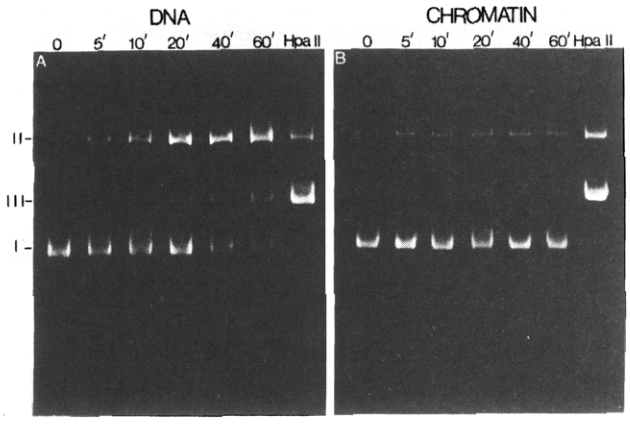

FIGURE 2: Time course of S_1 nuclease digestion of SV40(I) DNA and mature SV40 chromatin. SV40(I) DNA (A) or mature SV40 chromatin (B) was digested with S_1 nuclease under conditions optimal for chromatin digestion. Aliquots of the reaction were removed at the times indicated above each lane (minutes), and the DNA was purified and analyzed by agarose gel electrophoresis. The gels were then stained with ethidium bromide and photographed. All of the DNA applied to each lane entered the gel and migrated as either SV40(I), -(II), or -(III) DNA as indicated. A mixture of SV40(II) and -(III) DNA resulting from a partial *Hpa*II digestion of SV40 DNA is shown in the right-most lane of each gel.

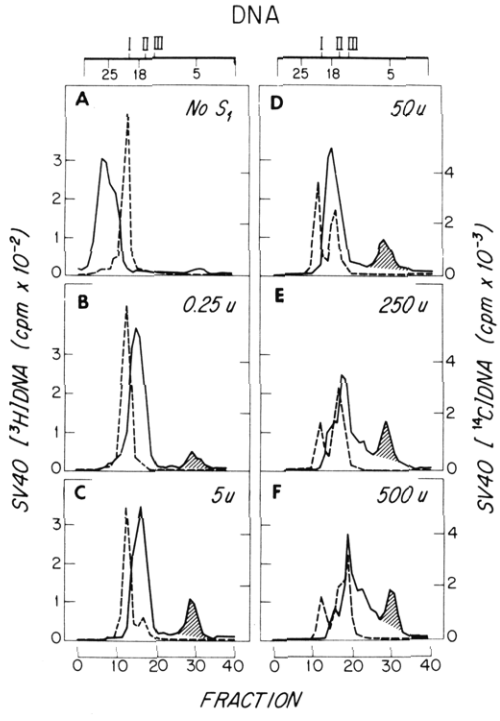

FIGURE 3: Sedimentation analysis of DNA isolated from S_1 nuclease digestion of SV40(I) and SV40(RI) DNA. A mixture of SV40(I) ^{14}C-labeled DNA (dashed line) and SV40(RI) ^{3}H-labeled DNA (solid line) was digested with increasing amounts of S_1 nuclease as indicated in each panel under conditions optimal for the digestion of chromatin. The DNA was extracted and analyzed by velocity sedimentation in neutral sucrose gradients. The positions of SV40(I) DNA (21 S), SV40(II) DNA (16 S), and SV40(III) DNA (14 S) as well as SV40(RI) DNA (~25 S), S_1 intermediate DNA (~18 S), and 4–6S DNA are indicated on the scale at the top of the figure. These sedimentation values were estimated by assuming a linear relationship between the top of each gradient and the position of the 16S SV40(II) DNA standard. The shaded areas represent minimum estimates of the amount of 4–6S DNA.

The same sequence of changes in the structure of the DNA was also observed by incubating SV40(I) ^{14}C-labeled DNA with increasing concentrations of S_1 nuclease for 30 min. The DNA was then analyzed by velocity sedimentation through

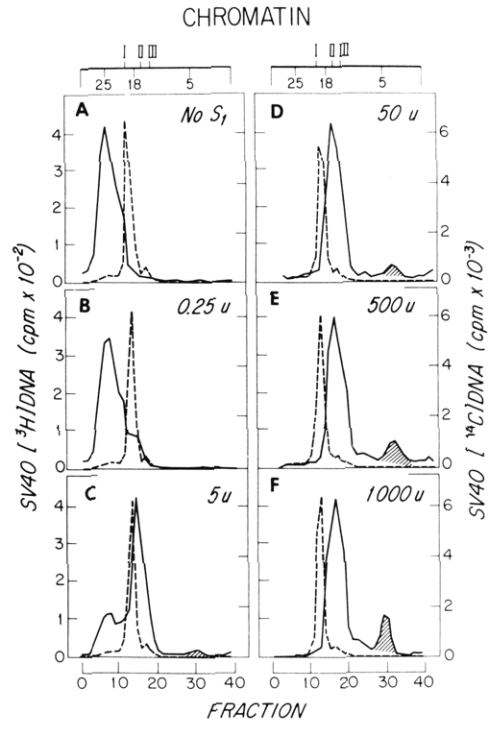

FIGURE 4: Sedimentation analysis of DNA isolated from S_1 nuclease digestion of mature and replicating SV40 chromosomes. A mixture of mature SV40 chromosomes containing SV40(I) ^{14}C-labeled DNA (dashed line) and replicating SV40 chromosomes containing SV40(RI) ^{3}H-labeled DNA was digested with increasing amounts of S_1 nuclease as indicated in each panel under conditions optimal for the digestion of chromatin. The DNA from each digestion was analyzed by sedimentation in neutral sucrose gradients. The positions of SV40(I) DNA (21 S), SV40(II) DNA (16 S), and SV40(III) DNA (14 S) as well as SV40(RI) DNA (~25 S), S_1 intermediate DNA (~18 S), and 4–6S DNA are indicated on the scale at the top of the figure. The shaded areas represent minimum estimates of the amount of 4–6S DNA.

neutral sucrose gradients (Figure 3) and quantitated by integrating the areas under the peaks of radioactivity (Figure 5A).

In contrast to the results obtained with SV40(I) DNA, mature SV40 chromosomes were completely resistant to the action of S_1 nuclease. Under experimental conditions that resulted in the cleavage of 90% of the purified SV40(I) DNA, both SV40(I) and SV40(II) DNA present as chromatin remained intact (Figures 2B, 4, and 5B). Identical results were obtained with mature SV40 chromosomes prepared by the method of Su & DePamphilis (1978) which have been shown to contain T antigen (Mann & Hunter, 1979).

(*b*) *S_1 Nuclease Digestion of SV40(RI) DNA and Replicating SV40 Chromatin.* S_1 nuclease cleaved SV40(RI) DNA at least 10 000 times faster than SV40(I) DNA. In these experiments SV40(I) ^{14}C-labeled DNA and SV40(RI) ^{3}H-labeled DNA were mixed and digested with increasing concentrations of S_1 nuclease under conditions optimized for the digestion of chromatin; the effects of S_1 nuclease on DNA and chromatin could then be directly compared. DNA was extracted from each sample and then analyzed by velocity sedimentation in neutral sucrose gradients (Figures 3 and 5C). While the lowest concentration of S_1 nuclease (0.25 unit) had no effect on the SV40(I) ^{14}C-labeled DNA, the SV40(RI) ^{3}H-labeled DNA, which originally sedimented as a broad peak between 22 and 28 S, was completely converted into a symmetrical peak sedimenting at ~18 S (parts A and B of Figure 3). In addition, smaller pieces of duplex DNA sedimenting between 4 and 6 S began to appear. Increasing the

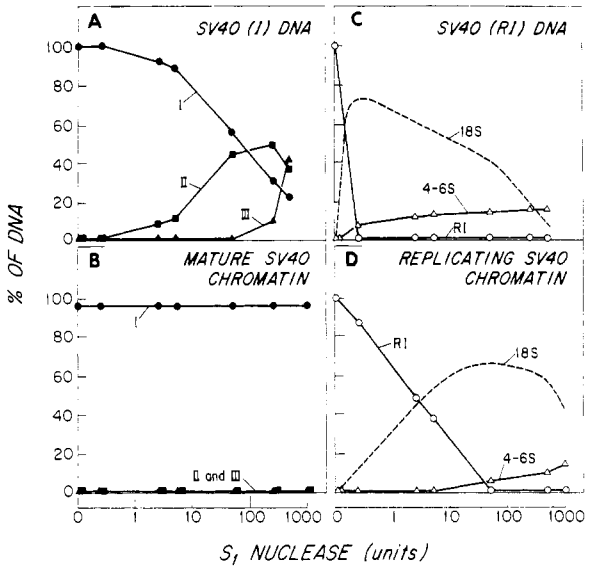

FIGURE 5: Quantitative analysis of data presented in Figures 3 and 4. The neutral sucrose gradient profiles shown in Figures 3 and 4 were analyzed to estimate the amount of radioactivity present in various forms of DNA. Panel A shows the fraction of ^{14}C-labeled DNA present as SV40(I), -(II), and -(III) DNA following digestion of purified SV40(I) ^{14}C-labeled DNA with increasing amounts of S_1 nuclease (Figure 3). Panel B shows the same analysis as panel A following digestion of mature SV40 ^{14}C-labeled chromosomes (Figure 4). Panel C shows the fraction of ^{3}H-labeled DNA present as SV40(RI)DNA, 18S DNA, and 4–6 S DNA following digestion of purified SV40(RI) ^{3}H-labeled DNA with increasing amounts of S_1 nuclease (Figure 3). Panel D shows the same analysis as panel C following digestion of replicating SV40 ^{3}H-labeled chromosomes (Figure 4). The areas under the appropriate peaks were integrated with a Hewlett-Packard digitizer. SV40(I) and -(II) DNA was generally well resolved except in parts E and F of Figure 3, where SV40(II) DNA was found as a shoulder on the peak of SV40(III) DNA. SV40(RI) DNA included ^{3}H-labeled DNA from fractions 1–11. The 18S DNA included ^{3}H-labeled DNA from fractions 12–16, the region between 21S and 16S DNA. The shaded areas designate the 4–6S DNA.

concentration of S_1 nuclease 20-fold resulted in a significant increase in the amount of 4–6S DNA, as well as a slight decrease in the sedimentation value of the remaining DNA, from 18 to 16 S (Figure 3C). Higher concentrations of nuclease did not result in a significant increase in the amount of 4–6S DNA, although the size of the remaining DNA continued to decrease (parts E and F of Figure 3). At the highest concentration of S_1 nuclease used (500 units), virtually all of the DNA which had originally sedimented at 18 S was converted to a population of duplex DNA molecules of heterogeneous size, 16 S and smaller.

SV40(RI) DNA present as replicating chromosomes was digested in a similar manner, but at a rate that was at least 200 times slower than that observed with purified DNA. The slower rate of digestion revealed more clearly the order in which DNA products appeared during the digestion; SV40(RI) DNA was initially converted into 18S DNA (Figures 4A–C and 5D), from which the 4–6S DNA was subsequently released (Figures 4D–F and 5D). The highest concentration of nuclease used (1000 units) resulted in the release of the same amount of 4–6S DNA from replicating chromosomes as from the purified SV40(RI) DNA (Figure 4F). In the same reactions, SV40(I) ^{14}C-labeled DNA in mature chromosomes was not affected by these concentrations of nuclease (Figure 4).

(c) Neurospora crassa Nuclease Digestion of SV40(RI) DNA and Replicating SV40 Chromosomes. Experiments similar to those described for S_1 nuclease were also performed with *Neurospora crassa* nuclease, a single-strand specific endonuclease with a pH optimum of 8.0 and a requirement for Mg^{2+} instead of Zn^{2+}. The DNA products generated by *Neurospora crassa* nuclease, the order in which they appeared, and the relative rates of digestion were analagous to those observed with S_1 nuclease. The maximum amount of 4–6S DNA released from replicating SV40 chromosomes was the same as that released from purified SV40(RI) DNA. Therefore, the observed features of the digestion of replicating SV40(RI) DNA and chromosomes are not unique to one particular enzyme or set of digestion conditions. We continued to use S_1 nuclease in these studies because it contained less contaminating double-stranded nuclease activity and was reported to be more efficient in recognizing small single-stranded regions in duplex DNA than *Neurospora crassa* nuclease (Beard et al., 1973; Shenk et al., 1975).

(d) The 4–6S DNA Released from Replicating SV40 DNA or Chromosomes by S_1 Nuclease Contains Okazaki Fragments. SV40 DNA synthesis proceeds discontinuously through the synthesis of Okazaki fragments on the retrograde arm of the replication fork (Figure 1). These short nascent DNA chains of 50–300 nucleotides are rapidly joined to the 5′ ends of growing daughter strands (Edenberg & Huberman, 1975; DePamphilis & Berg, 1975; Anderson et al., 1977; DePamphilis et al., 1979). Under the labeling conditions used here, 26% of the radioactivity in isolated SV40(RI) ^{3}H-labeled DNA or replicating SV40 ^{3}H-labeled chromosomes was in Okazaki fragments which were rapidly converted into longer DNA chains. These were identified as 4S single-stranded DNA by velocity sedimentation of purified SV40(RI) DNA in alkaline sucrose gradients (Figure 6A). This 4S DNA was never found in purified SV40(I) or SV40(II) DNA. Therefore, the 4–6S duplex DNA (equivalent to 100–300 base pairs of linear duplex DNA; Studier, 1965) released by S_1 nuclease digestion of replicating but *not* mature SV40 DNA or chromosomes was assumed to contain Okazaki fragments. However, of the total Okazaki fragments present on SV40(RI) ^{3}H-labeled DNA or replicating SV40 ^{3}H-labeled chromosomes, no more than 58% was released as 4–6S duplex DNA regardless of the amount of S_1 nuclease used (parts C and D of Figure 5). The remaining Okazaki fragments were shown to be present on the higher molecular weight DNA. In the example shown in Figure 6, SV40(RI) ^{3}H-labeled DNA (Figure 6B) and replicating SV40 ^{3}H-labeled chromosomes (Figure 6D) were digested with 50 and 500 units of S_1 nuclease, respectively, and then the DNA was analyzed by velocity sedimentation in neutral sucrose gradients. In the case of replicating DNA, 93% of the radioactivity present as Okazaki fragments in undigested DNA (Figure 6A) could be accounted for as the sum of the 4–6S duplex DNA (58%; Figure 6B) and the 4S single-stranded DNA which was released by sedimentation of the high molecular weight (18 S) DNA fraction in an alkaline sucrose gradient (35%; Figure 6C). Similarly, 93% of the Okazaki fragments in replicating chromosomes (Figure 6A) were accounted for as the sum of 4–6S duplex DNA (39%; Figure 6D) and Okazaki fragments that had not been released by S_1 nuclease (54%; Figure 6E). The difference between the amount of Okazaki fragments released from DNA and chromatin simply reflects the amounts of S_1 nuclease used in the experiment (parts C and D of Figure 5).

To evaluate whether preincubation of SV40-infected CV-1 cells on ice caused an uncoupling of DNA synthesis and nucleosome assembly, we radiolabeled cells at 20 °C without a preincubation step and then treated replicating SV40 chromosomes with either S_1 nuclease or micrococcal nuclease.

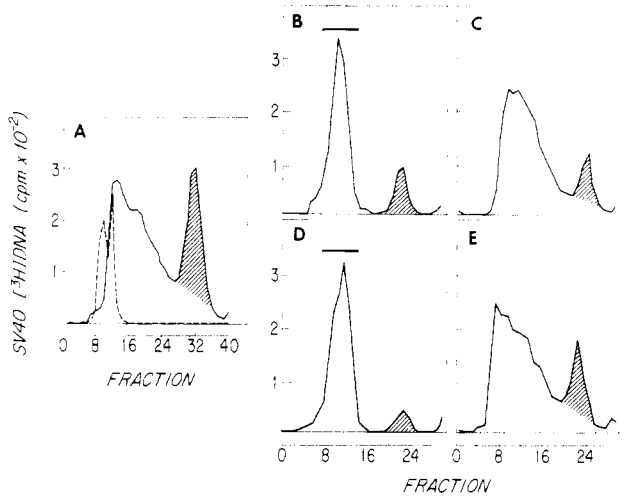

FIGURE 6: Quantitative analysis of the distribution of Okazaki fragments before and after S_1 nuclease digestion. ^{3}H-Labeled DNA was extracted from replicating SV40 ^{3}H-labeled chromosomes and sedimented in an alkaline sucrose gradient (A) to determine the fraction of radioactivity present as Okazaki fragments (shaded area, 26%). The same preparation of SV40(RI) (B) or the replicating chromosomes from which it was purified (D) were then digested with 50 and 500 units of S_1 nuclease, respectively, and the DNA was analyzed by sedimentation in neutral sucrose gradients. The amount of 4–6S ^{3}H-labeled DNA released from SV40(RI) DNA was 15% (shaded area, B), and the amount released from replicating SV40 ^{3}H-labeled chromosomes was 10% (shaded area, D). The fractions containing the high molecular weight DNA in panel B (bar) were then pooled, and the DNA was sedimented in an alkaline sucrose gradient (C). The high molecular weight DNA from the gradient in panel D (bar) was similarly analyzed (E). The fraction of radioactivity sedimenting as Okazaki fragments (shaded areas) was 9% in panel C and 14% in panel E. The sum of the amount of Okazaki-sized DNA fragments found on the corresponding neutral and alkaline sucrose gradients was 24% of the total radioactivity in the case of both purified SV40(I) DNA and replicating SV40 chromosomes. This value is in good agreement with the percent of the total ^{3}H-labeled DNA present in Okazaki fragments (26%, A).

S_1 nuclease digestion resulted in the release of the same fraction of Okazaki fragments as was released from chromatin prepared from cells which had been preincubated on ice. Furthermore, micrococcal nuclease digestion revealed that the same fraction of nascent DNA was assembled into nucleosomes whether or not the cells were preincubated on ice (T. Herman, M. DePamphilis, and P. Wassarman, unpublished experiments).

The failure to release all of the Okazaki fragments from purified SV40(RI) DNA (Figure 5) suggested that resistance to S_1 digestion resulted from the structure of the replicating DNA itself. Since at least 20% of the 3′-hydroxyl termini of Okazaki fragments present on SV40(RI) DNA are separated from the 5′-phosphoryl termini of the growing daughter strands by a single interruption of the phosphodiester backbone (S. Anderson, G. Kaufmann, and M. L. DePamphilis, unpublished experiments; DePamphilis et al., 1979), we investigated the ability of S_1 nuclease to recognize this type of "nick" in duplex DNA and to cleave the DNA strand opposite such sites. SV40(I) DNA was digested with DNase I in the presence of ethidium bromide (Greenfield et al., 1975) to generate SV40(II) DNA containing only phosphodiester bond interruptions. The resulting SV40(II) DNA, in which 50% of the molecules could be converted into covalently closed circular DNA by the action of *Escherichia coli* DNA ligase, was treated with S_1 nuclease. Under optimal conditions for digestion of DNA, 500 units of S_1 nuclease converted all of the SV40(II) DNA into SV40(III) DNA. However, under conditions optimal for the digestion of chromatin, the same concentration of nuclease cleaved only 50% of the DNA. We assume, therefore, that under these conditions (Figures 3–7 and 9) S_1 nuclease would not release those Okazaki fragments separated from the daughter DNA strands by a single nick or, perhaps, even by a small gap. The same amount of 4–6S DNA was released from replicating chromosomes by S_1 nuclease at pH 4.8 (conditions under which viral chromosomes were completely insoluble) or by *Neurospora crassa* nuclease at pH 7.5 as was released by S_1 nuclease under optimal conditions for chromatin digestion (pH 5.3). S_1 nuclease digestion of purified SV40(RI) DNA at pH 4.8 resulted in substantial fragmentation of nascent DNA chains which obscured the actual fraction of Okazaki fragments released.

(*e*) *Characterization of Okazaki Fragments Released by S_1 Nuclease Digestion of Replicating SV40 Chromosomes.* The duplex form of Okazaki fragments released from replicating SV40 chromosomes by S_1 nuclease was analyzed by velocity and equilibrium sedimentation, as well as by micrococcal nuclease digestion, to determine whether they were released as bare DNA or as a DNA–protein complex. All of these analyses indicate that the Okazaki fragments are released as bare DNA.

Replicating SV40 ^{3}H-labeled chromosomes were treated with formaldehyde to prevent dissociation of proteins from DNA and then digested with S_1 nuclease. The products were then analyzed directly by sedimentation in a neutral sucrose gradient. Under the conditions of centrifugation, all of the digested ^{3}H-labeled chromosomes (60 S) were pelleted. In three separate experiments, 500 units of S_1 nuclease released 9–12% of the ^{3}H label as 4–6S DNA and 0.5–1% as 10–11S material (Figure 7A). This is equivalent to the release of 35–46% of the Okazaki fragments as bare DNA and 2–4% as a DNA–protein complex. The material sedimenting at 10–11S represents a DNA–protein complex since it was not present when DNA from the same digestion mixture was purified before sedimentation. For comparison, nucleosomes released from replicating chromosomes by micrococcal nuclease were sedimented in a parallel gradient (Figure 7B). Similar results were obtained in experiments using un-cross-linked chromosomes and either S_1 nuclease or micrococcal nuclease.

DNA–protein complexes, such as nucleosomes, are digested by micrococcal nuclease more slowly and to a lesser extent than bare DNA. Therefore, we compared the kinetics of micrococcal nuclease digestion of bare SV40 ^{32}P-labeled DNA, mature SV40 ^{3}H-labeled chromatin, and the duplex form of Okazaki fragments released from replicating SV40 ^{3}H-labeled chromosomes by S_1 nuclease. The results presented in Figure 8 show that while the kinetics of digestion of SV40 chromatin and bare SV40 DNA are clearly different, the rates and extents of digestion of the duplex Okazaki fragments and bare DNA are the same.

Finally, the duplex Okazaki fragments released by S_1 nuclease were analyzed by equilibrium sedimentation in CsCl gradients to compare their density with the densities of both bare SV40 DNA and replicating SV40 chromosomes (Figure 9). Since most chromosomal proteins dissociate from DNA in CsCl gradients, it was necessary to fix replicating SV40 ^{3}H-labeled chromosomes with formaldehyde prior to centrifugation. For example, un-cross-linked replicating SV40 ^{3}H-labeled chromosomes sedimented at a density of 1.68 g/cm^3, the density characteristic of bare SV40 DNA. On the other hand, cross-linked replicating SV40 ^{3}H-labeled chromosomes sedimented at two distinct densities, 1.38 and 1.43 g/cm^3 (Figure 9A); the latter value corresponds exactly with

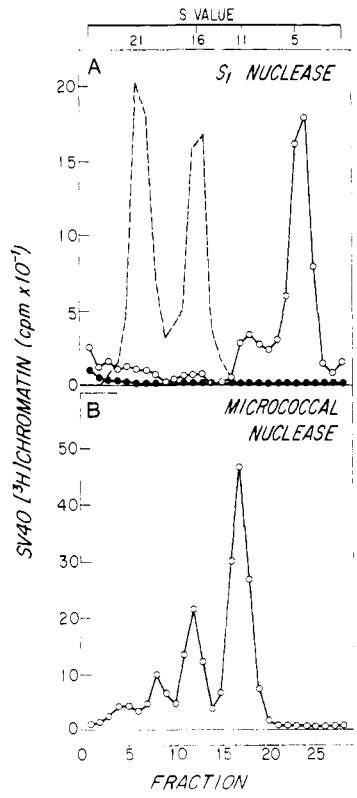

FIGURE 7: Sedimentation analysis of replicating SV40 chromosomes digested by S_1 nuclease. (A) Replicating SV40 ^{3}H-labeled chromosomes were fixed with formaldehyde, digested with 500 units of S_1 nuclease (open circles), and analyzed immediately by velocity sedimentation in neutral sucrose gradients containing 50 mM NaCl. An identical aliquot of fixed replicating SV40 ^{3}H-labeled chromosomes was incubated in the digestion buffer minus S_1 nuclease (closed circles) and then analyzed. SV40(I) and -(II) ^{32}P-labeled DNAs were mixed with the terminated digests immediately prior to centrifugation to serve as internal sedimentation standards (dashed line). The approximate positions of 11S and 5S DNA (indicated by the scale at the top of the figure) were estimated by assuming a linear relationship between the top of the gradient and the position of the 16S SV40(II) DNA standard. (B) Formaldehyde-fixed replicating SV40 ^{3}H-labeled chromosomes were digested with micrococcal nuclease (Shelton et al., 1978) and analyzed immediately by velocity sedimentation as in (A). The sedimentation values indicated by the scale above (A) also apply to (B).

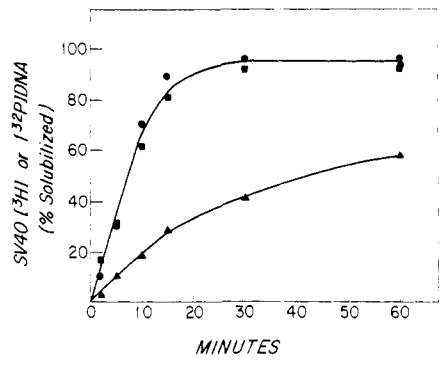

FIGURE 8: Micrococcal nuclease digestion of duplex Okazaki fragments released from replicating SV40 chromosomes. ^{3}H-Labeled duplex Okazaki fragments (closed squares) resulting from the S_1 nuclease digestion of replicating SV40 ^{3}H-labeled chromosomes were isolated from a low ionic strength (50 mM NaCl) neutral sucrose gradient and mixed with purified SV40 ^{14}C-labeled DNA (closed circles). The mixture was digested with micrococcal nuclease (Shelton et al., 1978), and the rate at which the DNA was made acid soluble was measured. SV40 ^{14}C-labeled chromosomes were digested with micrococcal nuclease under identical conditions in a separate tube (closed triangles).

the density of cross-linked mature SV40 chromosomes (the presence of a second component may be attributable to

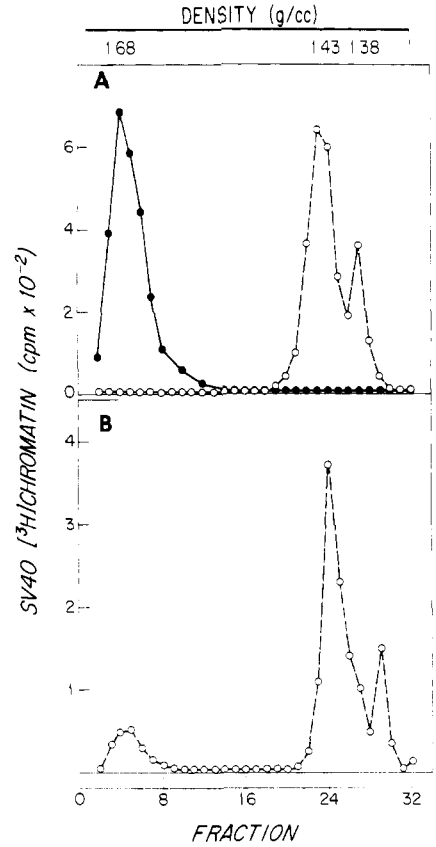

FIGURE 9: Equilibrium sedimentation analysis of duplex Okazaki fragments released by S_1 nuclease digestion of replicating SV40 chromosomes. (A) Replicating SV40 ^{3}H-labeled chromosomes were sedimented to equilibrium in CsCl gradients before (closed circles) or after (open circles) formaldehyde fixation. The refractive index of selected fractions was measured to establish the density scale indicated above the figure. (B) Formaldehyde-fixed replicating SV40 ^{3}H-labeled chromosomes were digested with 500 units of S_1 nuclease and sedimented to equilibrium in a CsCl gradient as in (A). The density scale above (A) also applies to (B).

variation in the amount of histone H1 associated with replicating chromosomes). When cross-linked replicating SV40 ^{3}H-labeled chromosomes were digested with S_1 nuclease, 90% of the radioactivity sedimented at the density of replicating chromosomes and 10% sedimented at the density of bare DNA. The amount of bare DNA accounted for at least 40% of the total radioactivity present in Okazaki fragments.

Discussion

Digestion of nonreplicating (mature) and replicating SV40 chromosomes with either S_1 or *Neurospora crassa* single-strand specific endonuclease has revealed regions of single-stranded DNA in replicating, but not in mature, chromosomes. SV40(I) DNA in the form of mature chromatin is resistant to cleavage by S_1 nuclease because the free energy required to melt A-T rich regions in purified SV40(I) DNA is provided by the topological strain from superhelical turns (Beard et al., 1973) and this strain is relieved when DNA is coiled around histones. Similarly, treatment of mature SV40 chromosomes with nicking–closing enzyme has no effect on the number of superhelical turns found per molecule when the SV40(I) DNA is subsequently purified (Young & Champoux, 1978). If any uniquely located single-stranded regions exist in mature SV40 chromosomes, they were not accessible to S_1 nuclease. Since mature SV40 chromosomes are completely resistant to S_1 nuclease, cleavage of replicating chromosomes resulted from the presence of DNA replication forks. Furthermore, the actual sites of nuclease digestion must be at the replication

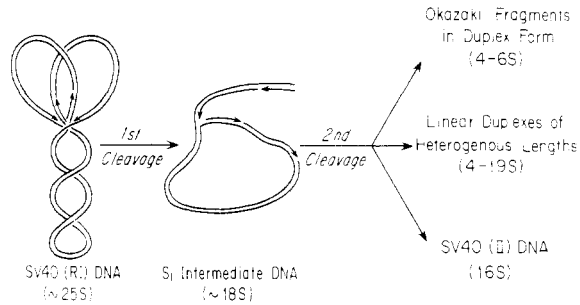

FIGURE 10: Possible products of cleavage of SV40(RI) DNA by S_1 nuclease. S_1 nuclease rapidly converts SV40(RI) DNA (22–28 S) to a slower sedimentating intermediate form (18 S) as a result of cleavage of the parental DNA strand at one of several possible sites at the replication fork (Figure 1). The initial cleavage by S_1 allows the superhelical unreplicated portion of the molecule to relax, accounting for the dramatic decrease in the sedimentation rate of the S_1 intermediate DNA. One possible structure of the 18S intermediate DNA, a circle with a tail, is indicated. Subsequent cleavages of the 18S intermediate DNA by S_1 nuclease occur at a somewhat slower rate and result in a variety of products. Note that to release the duplex form of an Okazaki fragment, S_1 nuclease must cleave the parental DNA both in front of and behind the fragment.

forks themselves since these endonucleases do not cleave nonreplicating chromosomal DNA (mature chromosomes) and since the bulk of newly replicated DNA is both free of superhelical constraints and rapidly assembled into nucleosomes (T. Herman, M. DePamphilis, and P. Wasserman, unpublished experiments).

Our current conception of the structure of SV40(RI) DNA replication forks is presented schematically in Figure 1 in order to illustrate the products resulting from cleavage of SV40 chromosomes by single-strand specific endonucleases. DNA synthesis proceeds continuously on the "forward arm" of the replication fork (Perlman & Huberman, 1977; Kaufmann et al., 1978) and discontinuously in the form of Okazaki fragments on the "retrograde arm". There is approximately one Okazaki fragment on the retrograde arm of each replication fork (Francke & Hunter, 1974; S. Anderson and M. L. DePamphilis, unpublished experiments). Endonucleolytic cleavage of any one of the single-stranded DNA regions in either of the two replication forks of SV40(RI) DNA would immediately yield molecules resembling a "circle with a tail" (Figure 10). The tails should vary in length in the same manner as the extent of replication varies throughout the population of SV40(RI) DNA molecules. These molecules correspond to the 18S DNA that rapidly appeared when SV40(RI) DNA, either purified or in the form of chromatin, was digested by S_1 nuclease (Figures 3 and 4); such "circles with tails" were the predominant DNA form observed by electron microscopy (data not shown; Bourgaux & Bourgaux-Ramoisy, 1971). Subsequent cleavage of single-stranded DNA regions should yield circular SV40(II) DNA (16 S), linear duplex DNA of various lengths (4–19 S), and Okazaki fragments in the form of duplex DNA (4–6 S) (Figure 10). The appearance of the latter product was easily recognized; as much as 58% of the ^3H label in Okazaki fragments was released from either purified replicating SV40 DNA or replicating SV40 chromosomes by S_1 or *Neurospora crassa* endonuclease. While the DNA products of nuclease digestion were identical, replicating chromosomes were at least 200 times less sensitive than replicating DNA to the action of either endonuclease. This suggests that the single-stranded DNA regions in replicating SV40 chromosomes are partially protected, either by association with single-stranded DNA binding proteins or as the result of a compacted chromatin structure.

A maximum of 50–60% of the Okazaki fragments present in replicating SV40 chromosomes was released by either S_1 or *Neurospora crassa* endonuclease (Figure 5D). The inability to release all of the Okazaki fragments is not due to protection of a fraction of the single-stranded regions of the DNA by chromosomal proteins since identical results were obtained by using purified replicating SV40 DNA (Figure 5C). Consequently, the action of these endonucleases is apparently limited by the size of the single-stranded DNA targets at the SV40 replication forks. Such a situation is consistent with a previous report that the ability of S_1 nuclease to digest single-stranded DNA decreases rapidly when the target size is smaller than six nucleotides in length (Dodgson & Wells, 1977). Although S_1 nuclease will recognize single base pair mismatches in DNA heteroduplexes under conditions optimal for digestion of DNA (pH 4.6; Shenk et al., 1975), it apparently cannot recognize small single-stranded regions in duplex DNA under conditions optimal for digestion of chromatin (pH 5.3). We found that while duplex DNA containing a single phosphodiester interruption was cleaved by S_1 nuclease under conditions optimal for digestion of DNA, it was not cleaved under conditions for digestion of chromatin. Therefore, it would appear that ~50% of the Okazaki fragments in replicating SV40 chromosomes are separated from longer nascent DNA chains by a single-stranded region of DNA too short to be recognized by S_1 or *Neurospora crassa* endonuclease. In this connection, S. Anderson and M. L. DePamphilis (unpublished experiments) have found that at least 20% of the Okazaki fragments in SV40(RI) DNA can be joined to longer nascent DNA chains by *E. coli* DNA ligase alone, demonstrating that such fragments are separated from longer chains by the absence of a single phosphodiester bond ("nick"). Furthermore, their data suggest that an additional 30% of the Okazaki fragments are separated from longer nascent DNA chains by a "gap" which may be either too small to be attacked by S_1 or *Neurospora crassa* endonuclease or filled with an RNA primer.

At least 90% of the Okazaki fragments released from replicating SV40 chromosomes by S_1 nuclease behaved, under a variety of conditions, as bare DNA. Micrococcal nuclease digested the isolated duplex Okazaki fragments at the same rate and to the same extent as purified DNA, whereas replicating and mature chromosomes were digested much more slowly and resulted in the release of nucleosomes which sedimented at 11 S in neutral sucrose gradients. Furthermore, S_1 nuclease digestion of isolated replicating SV40 chromosomes, treated with formaldehyde to prevent dissociation of any protein that may have been associated with the DNA, resulted in the release of duplex DNA that sedimented at 4–6 S (100–300 base pairs) and had the same isopycnic density in CsCl as purified DNA (1.68 gm/cm^3). Removal of protein from replicating chromosomes before digestion with S_1 nuclease did not affect the sedimentation of the 4–6S DNA fragments in sucrose gradients, but did convert a small amount of 10–11S DNA–protein complex into 4–6S DNA. Since the SV40 chromosomes were prepared in 0.2 M NaCl, many nonhistone chromosomal proteins including DNA replication proteins are presumably absent (Su & DePamphilis, 1978).

On the basis of the experimental results reported here, we conclude that 50–60% of the total population of Okazaki fragments present in replicating SV40 DNA, either purified or in the form of chromosomes, can be released by single-strand specific endonucleases. At least 90% of the released Okazaki fragments are not contained in nucleosomes. The possibility that DNA synthesis and nucleosome assembly were

uncoupled as a result of the pulse-labeling procedure was ruled out by the demonstration that preincubation of the virus-infected cells with [^{3}H]thymidine on ice prior to transfer to 20 °C had no effect on either the sensitivity of the replicating chromosomes to S_1 nuclease or the assembly of the newly synthesized DNA into nucleosomes.

Hildebrand & Walters (1976) reported that ~50% of the briefly labeled DNA in intact CHO cells sedimented as Okazaki fragments and that ~50% of the nascent DNA was associated with nucleosomes released by micrococcal nuclease. They concluded from these observations that nucleosome assembly preceded the joining of Okazaki pieces to longer DNA chains. However, they did not consider the possibility that all of the Okazaki fragments had been digested to acid-soluble products by the time sedimentation analysis was performed. Consequently, newly synthesized DNA contained in nucleosomes could have originated not from Okazaki pieces but from continuously growing DNA strands. Schlaeger & Klempnauer (1978), using isolated lymphocyte nuclei labeled in vitro, also concluded that Okazaki fragments are assembled into nucleosomes prior to their ligation to longer DNA chains. However, in these experiments the investigators found that virtually all of the newly synthesized DNA was present as Okazaki pieces, consistent with solely discontinuous DNA synthesis during the in vitro pulse. Tseng & Goulian (1975), on the other hand, using lymphocyte lysates and intact lymphocytes, found only 70 and 25%, respectively, of the radiolabel present in Okazaki pieces during a short pulse. The latter results strongly suggest that DNA synthesis in lymphocytes does not take place in a purely discontinuous manner and may bring into question the significance of the results obtained with isolated lymphocyte nuclei.

The data presented here support a structural pathway for chromosome replication in which Okazaki fragments are first synthesized and then assembled into nucleosomes following ligation to longer nascent DNA chains; the synthesis of Okazaki fragments and their assembly into nucleosomes need *not* occur concomitantly. This is consistent with the observation that a purified mammalian DNA ligase is incapable of sealing nicks in duplex nucleosomal DNA (Zimmerman & Levin, 1975).

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