Structure of Chromatin at Deoxyribonucleic Acid Replication Forks: Prenucleosomal Deoxyribonucleic Acid Is Rapidly Excised from Replicating Simian Virus 40 Chromosomes by Micrococcal Nuclease†

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ABSTRACT: Replicating simian virus 40 (SV40) chromosomes were found to be similar to other eukaryotic chromosomes in that the rate and extent of micrococcal nuclease (MNase) digestion were greater with replicating than with nonreplicating mature SV40 chromatin. MNase digestion of replicating SV40 chromosomes, pulse labeled in either intact cells or nuclear extracts, resulted in the rapid release of nascent DNA as essentially bare fragments of duplex DNA (3–7 S) that had an average length of 120 base pairs and were degraded during the course of the reaction. In addition, nucleosomal monomers, equivalent in size to those from mature chromosomes, were released. On the other hand, MNase digestion of uniformly labeled mature SV40 chromosomes resulted in the release of only nucleosomal monomers and oligomers. The small nascent DNA fragments released from replicating chromosomes represented prenucleosomal DNA (PN-DNA) from the region of replication forks that encompasses the actual sites of DNA synthesis and includes Okazaki fragments. Predigestion of replicating SV40 chromosomes with both *Escherichia coli* exonuclease III (3′–5′) and bacteriophage T7 gene 6 exonuclease (5′–3′) resulted in complete degradation of PN-DNA. This result, together with the observation that isolated PN-DNA annealed equally well to both strands of SV40 restriction fragments, demonstrated that PN-DNA originates from both sides of replication forks. Over 90% of isolated Okazaki fragments annealed only to the retrograde DNA template. The characteristics of isolated PN-DNA were assessed by examining its sensitivity to MNase and single strand specific S_1 endonuclease, sedimentation behavior before and after deproteinization, buoyant density in CsCl after formaldehyde treatment, and size on agarose gels. In addition, it was observed that MNase digestion of purified SV40 DNA also resulted in the release of a transient intermediate similar in size to PN-DNA, indicating that a DNA–protein complex is not required to account for the appearance of PN-DNA. These and other data provide a model of replicating chromosomes in which DNA synthesis occurs on a region of replication forks that is free of nucleosomes and is designated as prenucleosomal DNA.

Replication of eukaryotic chromosomes requires the accurate duplication of both DNA sequence and chromatin organization [reviewed in DePamphilis & Wassarman (1980)]. For example, transcriptionally active genes differ in structure from inactive genes (Weintraub & Groudine, 1976; Weisbrod et al., 1980). Since this difference must be maintained at each cell division, chromosome replication must embody properties that allow accurate reproduction of those differences in chromatin structure that affect gene expression (Weintraub, 1979). To understand the mechanisms involved in this duplication process requires a detailed knowledge of chromatin structure at DNA replication forks.

Previous studies have demonstrated that specific endonucleases and exonucleases can be used to dissect each arm of DNA replication forks in simian virus 40 (SV40)[1] chromosomes (DePamphilis et al., 1978, 1980; DePamphilis & Wassarman, 1980). These studies have shown that (i) Okazaki fragments are initiated on nonnucleosomal DNA and then generally ligated to the 5′ ends of long nascent DNA chains prior to assembly into nucleosomes (Herman et al., 1979, 1981), (ii) the average distance from either 3′ or 5′ ends of long nascent DNA chains to the first nucleosome on either arm of replication forks is 125 nucleotides (Herman et al., 1981), and (iii) phasing of chromatin structure with respect to DNA sequence appears to be random on both arms of a replication fork, generating nonidentical sibling chromosomes (Tack et al., 1981). In addition, it has been generally observed that viral and cellular replicating chromatin is more sensitive to micrococcal nuclease than nonreplicating chromatin and that micrococcal nuclease digestion of replicating, but not nonreplicating, chromatin releases DNA fragments smaller than those associated with nucleosomal monomers (Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Knippers, 1979; Seale, 1978; Klempnauer et al., 1980; Yakura & Tanifuji, 1980). These measurements have begun to reveal the sequence of molecular events that culminate in the accurate duplication of chromosome structure.

In this paper, we have used replicating and mature SV40 chromosomes to demonstrate that the small DNA fragments rapidly released by micrococcal nuclease from replicating chromatin originate from prenucleosomal DNA (PN-DNA) at replication forks. This DNA comes from both the forward and retrograde sides of the fork and includes Okazaki fragments as well as portions of long nascent DNA chains. Prenucleosomal DNA is released as 3–7 S double-stranded DNA fragments (about 120 bp) that do not appear to be complexed with protein. These results, together with other data on replicating and mature SV40 and polyoma chromosomes

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[1] Abbreviations used: SV40, simian virus 40; SV40(I) DNA, covalently closed, superhelical mature form of viral DNA; SV40(II) DNA, duplex circular viral DNA containing at least one single-strand interruption; SV40(RI) DNA, normal replicating intermediates of SV40 DNA; EDTA, sodium ethylenediaminetetraacetate; Exo III, *E. coli* exonuclease III; Exo $T7_6$, *E. coli* bacteriophage T7 gene 6 exonuclease; MNase, micrococcal nuclease; bp, base pairs; PN-DNA, prenucleosomal DNA; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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(Cremisi, 1979; DePamphilis & Wassarman, 1980), have been used to construct a model for the structure of eukaryotic DNA replication forks that relates DNA replication, chromosome structure, and chromatin assembly.

Experimental Procedures

Growth of Cells and Virus. The small plaque strain of simian virus 40, Rh911, grown at a low multiplicity of infection, was used to infect an African green monkey kidney cell line (CV-1). Conditions for growth of cells and their infection with virus have been described previously (Wilson et al., 1976; Anderson et al., 1977).

Labeling and Preparation of Replicating and Mature SV40 Chromosomes. (*a*) *From Intact Cells* (*Isotonic Method*). Mature SV40 chromosomes containing SV40(I,II) [^{14}C]DNA were radiolabeled by adding [^{14}C]thymidine (0.4 μCi/mL, 50 mCi/mmol) to the medium of SV40-infected CV-1 cells at 24-h postinfection and then continuing the incubation until 36 h (Herman et al., 1979, 1981). Replicating SV40 chromosomes containing [^{3}H]DNA were prepared from virus-infected CV-1 cells at 36-h postinfection. Dishes of cells were first incubated with [^{3}H]thymidine (167 μCi/mL, 55 Ci/mmol) for 10 min at 0 °C to maximize the specific radioactivity of dTTP pools in the absence of DNA synthesis (Perlman & Huberman, 1977; Herman et al., 1979). The dishes were then shifted to 20 °C for 1 min at allow DNA replication to proceed. Replicating and mature SV40 chromosomes were then extracted from isolated nuclei in 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA, and 1% Triton X-100 as previously described (Herman et al., 1979; Shelton et al., 1980). The chromosomes were purified and fractionated into replicating and mature chromosomes by sedimentation through a linear 5–30% sucrose gradient in the same buffer minus Triton (Herman et al., 1979; Shelton et al., 1980). Gradients were fractionated from the top to avoid contamination with virions in the pellet. In some cases, chromosomes were sedimented through sucrose gradients prepared in hypotonic buffer (see below) plus 200 mM NaCl, but no difference was observed in the experimental results. Fractions containing mature (55 S) or replicating (65 S) SV40 chromosomes were pooled separately and then concentrated by pressure dialysis under N_2 gas against 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in preparation for digestion by various nucleases. This procedure routinely yielded about 5 μg of SV40 DNA per 6 $\times$ 10^6 cells. DNA concentrations were measured by a modification (Shelton et al., 1978a) of a standard fluorometric assay (Kissane & Robbins, 1958).

(*b*) *From Nuclear Extracts* (*Hypotonic Method*). Mature SV40 chromosomes containing SV40(I,II) [^{3}H]DNA were radiolabeled by adding [^{3}H]thymidine (2.5 μCi/mL, 55 Ci/mmol) to the medium of infected CV-1 cells at 24-h postinfection and then continuing the infection until 36 h. Nuclear extracts containing both mature and replicating SV40 chromosomes were prepared from SV40-infected CV-1 cells at 36 h after infection by the hypotonic extraction procedure of Su & DePamphilis (1976, 1978), except that only half as much hypotonic solution was used for the extraction. The hypotonic extraction buffer contained 10 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol (DTT). Replicating SV40 chromosomes were radiolabeled for 1 min at 30 °C in the nuclear extract with [α-^{32}P]dCTP (100–200 Ci/mmol) in the presence of cytosol as previously described (Su & DePamphilis, 1978; Shelton et al., 1980), except that the concentration of dCTP was 5 μM and the concentration of the other deoxyribonucleotides was 20 μM. [α-^{32}P]dCTP was prepared by the method of Symons (1974) as modified by Rigby et al. (1977). Samples were placed on ice, and EDTA was added to 10 mM to stop replication. Mature and replicating SV40 chromosomes were then purified by sedimentation through sucrose gradients, pooled, and concentrated, as described above. This method routinely yielded 1–2 μg of SV40 DNA per 6 $\times$ 10^6 cells.

Nuclease Digestion of SV40 Chromosomes. (*a*) *Micrococcal Nuclease* (*MNase*). Isolated SV40 chromosomes (5–10 μg of DNA/mL) were incubated at 37 °C with MNase (Worthington, 0.1 unit/μg of DNA) in a 0.35-mL reaction volume containing 10 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Acid-soluble radioactivity was determined at various times during the digestion by adding an aliquot of the reaction mixture to 3 mL of 1 N HCl containing 0.5% sodium pyrophosphate. After 10 min on ice, the precipitate was collected on a Whatman GF/C filter, washed with cold 1 N HCl, and 0.5% sodium pyrophosphate, and then washed with ethanol. The filters were dried, and their radioactivity was measured in a standard toluene-based liquid scintillation cocktail. When nucleoprotein products were analyzed by velocity sedimentation, the digestion was stopped by adding EDTA to 10 mM, and then the sample was layered directly onto a neutral sucrose gradient. Sedimentation conditions are described below.

(*b*) *Escherichia coli Exonuclease III* (*Exo III*). SV40 chromosomes (5–10 μg/mL) were incubated at 20 °C with Exo III (New England Biolabs, 40 units/μg of DNA) in a 0.30-mL reaction volume containing 50 mM Tris-Hcl (pH 8.0), 0.26 mM $MgCl_2$, 0.06 mM EDTA, and 1 mM DTT (Herman et al., 1981). The digestions were stopped by adding EDTA to 5 mM, and then the reaction mixture was filtered through Sephadex G-50 (Pharmacia) in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in preparation for subsequent digestion with MNase; SV40 chromosomes were recovered in the void volume.

(*c*) *Bacteriophage T7 Gene 6 Exonuclease* (*Exo $T7_6$*). Exo $T7_6$ was purified to homogeneity by the procedure of M. Engler and C. C. Richardson (unpublished experiments). The final enzyme preparation contained 77 500 units/mL (93 000 units/mg of protein), with 1 unit defined as the amount of enzyme required to digest 1 nmol of DNA (*Hae*III restriction endonuclease fragments of SV40 DNA) to acid-soluble material in 15 min at 37 °C. SV40 chromosomes (5–10 μg/mL) were incubated at 20 °C with Exo $T7_6$ (16 units/μg of DNA) in a 0.30-mL reaction volume containing 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 0.15 mM $MnCl_2$, 0.05 mM EDTA, and 1 mM DTT (Herman et al., 1981). Digestions were stopped and analyzed as described above for Exo III. When SV40 chromosomes were digested concurrently with both exonucleases, the conditions for Exo $T7_6$ were used, since Exo III retains full activity under Exo $T7_6$ conditions.

(*d*) *Aspergillus S_1 Endonuclease.* SV40 or CV-1 DNA was digested with S_1 nuclease (Sigma, 0.5 unit/μg of DNA) under conditions previously described (Herman et al., 1979). Acid-insoluble radioactivity was determined as described above for MNase.

Velocity and Equilibrium Sedimentation Analysis. Nucleoprotein products of MNase digestions of SV40 chromosomes were routinely analyzed by velocity sedimentation in linear 5–20% neutral sucrose gradients containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 50 mM NaCl. Sedimentation was for 14 h at 35 000 rpm at 4 °C, in a Beckman SW41 rotor. Fractions were collected from the bottom of the tube, and their radioactivity was recorded as previously described (Herman et al., 1979).

Nucleoprotein products were sedimented to equilibrium in CsCl gradients containing 10 mM triethanolamine (pH 7.0) and 0.1 mM EDTA. Gradients were established by centrifugation at 40000 rpm for 48 h in a Beckman SW50.1 rotor (Herman et al., 1979; Shelton et al., 1980). For prevention of dissociation of chromosomal protein from DNA in CsCl gradients, nucleoprotein samples were fixed with 1% formaldehyde for 1 h at 0 °C. Before fixation, nucleoprotein samples were filtered through Sephadex G-50 equilibrated with 10 mM triethanolamine (pH 7.0) and 0.1 mM EDTA to remove Tris buffer which reacts with formaldehyde (Herman et al., 1979).

Gel Electrophoresis of Purified MNase DNA Digestion Products. DNA products from MNase digestions were extracted and purified as previously described (Shelton et al., 1978b, 1980). This method includes proteinase K digestion, extraction with chloroform, RNAse digestion, and ethanol precipitation. Electrophoresis was carried out in 2% agarose gels containing 40 mM Tris–acetate (pH 7.9), 20 mM sodium acetate, and 2 mM EDTA (Shelton et al., 1978a, 1980). Gels were sliced into 2-mm fractions, and each slice was solubilized by incubation overnight at 37 °C in 10 mL of Liquifluor (New England Nuclear) containing 3% NCS (Amersham) before determining the amount of radioactivity (Herman et al., 1981). Alternatively, gels were exposed to preflashed Kodak SB film at −70 °C against a Kodak intensifying screen (Laskey, 1980).

Blotting and Hybridization to Separated Strands of SV40 DNA Restriction Fragments. Highly purified SV40(I) DNA was digested by a combination of *Bgl*I and *Hpa*I restriction endonucleases (New England Biolabs) as previously described (Kaufmann et al., 1978). The resulting fragments were deproteinized, denatured in 0.2 M NaOH at 25 °C for 10 min, chilled to 0 °C, and then separated into their complementary single strands by electrophoresis through a 2% agarose gel in 90 mM Tris, 2.5 mM EDTA, and 90 mM boric acid (pH 8.3) (Kaufmann et al., 1978). The separated strands were then transferred to diazobenzyloxymethyl paper (DBM paper; Wahl et al., 1979) that was prepared as originally described (Alwine et al., 1977; Wahl et al., 1979) with nitrobenzyloxymethylpyridinium chloride (NBPC) purchased from BDH Chemicals (Gallard-Schlesinger). Following transfer of the DNA fragments, DBM paper was prehybridized as described (Wahl et al., 1979) for 20 h at 42 °C to reduce nonspecific adsorption of the [^{32}P]DNA samples. For hybridization, the [^{32}P]DNA samples, in 200 μL of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM NaCl, and 250 μg of sonicated salmon sperm DNA, were first denatured for 5 min at 100 °C. The samples were quickly cooled to 0 °C, added to 8 mL of prewarmed (42 °C) hybridization solution [50% formamide, 0.15 M NaCl, 15 mM trisodium citrate, 20 mM sodium phosphate (pH 6.5), and Denhardt's solution (0.02% bovine serum albumin, 0.02% poly(vinyl pyrrolidone), and 0.02% Ficoll)], sealed in a glass tube, and incubated at 42 °C for 16 h. Following hybridization, the strips of DBM paper were washed (Wahl et al., 1979), dried, and exposed to preflashed Kodak SB film at −70 °C against a Kodak intensifying screen (Laskey, 1980).

Results

Micrococcal Nuclease Degrades Replicating Chromatin More Rapidly Than Mature Chromatin. Micrococcal nuclease (MNase) has been shown to degrade chromatin from a wide variety of sources by rapidly digesting the "linker" and internucleosomal DNA regions in an essentially random manner (Figure 1), leaving the "core" DNA regions fragmented, but relatively intact [reviewed by McGhee & Felsenfeld (1980)]. Therefore, the rate of chromatin digestion

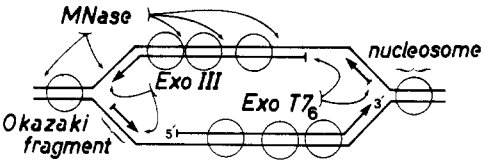

FIGURE 1: Potential nuclease sites at DNA replication forks of replicating SV40 chromosomes. DNA replication occurs bidirectionally from a unique origin (Fareed & Davoli, 1977; Kelly & Nathans, 1977). Nascent DNA chains are elongated continuously in the direction of fork movement (forward arm) and discontinuously in the form of Okazaki fragments in the direction opposite to that of fork movement (retrograde arm) (Perlman & Huberman, 1977; Kaufmann et al., 1978; this report). The sites at which *E. coli* exonuclease III (Exo III), bacteriophage T7 gene 6 exonuclease (Exo T7$_6$), and micrococcal nuclease (MNase) degrade replicating chromosomal DNA are indicated by arrows. Exo III degrades duplex DNA in the 3′–5′ direction from 3′-OH or 3′-PO$_4$ termini, preferring recessed 3′ ends over matched ends of duplex DNA or single phosphodiester bond interruptions (Rogers & Weiss, 1980). Exo T7$_6$ degrades duplex DNA in the 5′–3′ direction only from 5′-OH or 5′-PO$_4$ termini (including 5′-terminal RNA primers), preferring recessed 5′ ends (Kerr & Sadowski, 1972; Shinozaki & Okazaki, 1978; M. Engler and C. C. Richardson, unpublished experiments). MNase is an endonuclease that degrades duplex or single-stranded DNA or RNA, preferring single strands at AT-rich regions (Anfinsen et al., 1971).

serves as a measurement of DNA accessibility and the extent of digestion a measurement of DNA protection by association with nucleosomal "cores". When these measurements were made with a mixture of replicating and mature forms of SV40 chromosomes, it was apparent that DNA in chromatin at active replication forks was more accessible to MNase than DNA in mature, nonreplicating chromatin.

SV40 chromosomes were prepared by two methods: (i) from intact cells by an isotonic method and (ii) from nuclear extracts by a hypotonic method. Replicating SV40 ^{3}H-labeled chromosomes and mature SV40 ^{14}C-labeled chromosomes, both containing DNA that was radiolabeled in intact cells, were extracted and purified under isotonic salt conditions that included EDTA and Triton X-100. In addition, cells containing mature SV40 ^{3}H-labeled chromosomes were used to prepare nuclear extracts in low salt concentrations without EDTA or detergent. Replicating SV40 ^{32}P-labeled chromosomes were then obtained by incubating a nuclear extract with [α-^{32}P]dCTP under conditions that permit faithful continuation of DNA replication and chromatin assembly (Su & DePamphilis, 1976, 1978; Shelton et al., 1980). Chromosomes prepared by both methods were purified by sedimentation in netural sucrose gradients containing 200 mM NaCl, and then samples of replicating and mature chromosomes were mixed and digested with MNase (Figure 2). Nascent DNA in replicating SV40 chromosomes was digested several times faster and to a greater extent (about 25% more) than DNA in mature SV40 chromosomes, regardless of whether they were isolated directly from intact cells or were first incubated in a nuclear extract. Their relative responses to MNase were also the same when purified in sucrose gradients containing 50 mM NaCl. Therefore, the nascent DNA in newly replicated viral chromosomes is more accessible to MNase than is viral DNA in mature chromosomes.

Prenucleosomal DNA Is Rapidly Released from Replicating Chromatin and Then Degraded by Micrococcal Nuclease. Two striking differences between replicating and mature SV40 chromosomes were revealed by sedimentation analysis of MNase nucleoprotein digestion products. First, newly replicated chromatin is more rapidly degraded to nucleosomal monomers; however, the monomers containing nascent DNA cosedimented with those from mature chromosomes. Second, a fraction of nascent DNA that sedimented

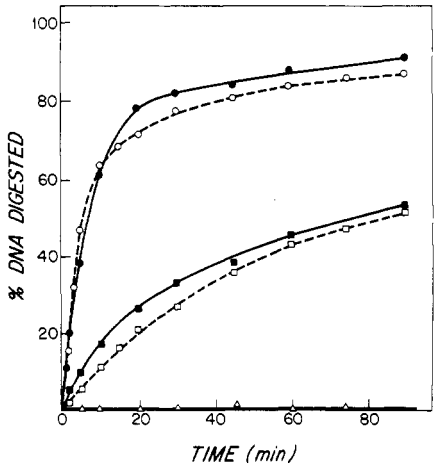

FIGURE 2: Time course of MNase digestion of replicating and mature SV40 chromosomes. In one experiment, replicating SV40 chromosomes were labeled with [^{3}H]thymidine (●) and mature SV40 chromosomes with [^{14}C]thymidine (■) in intact cells. These chromosomes were prepared by the isotonic method, mixed together, and then digested with MNase. In a separate experiment, replicating SV40 chromosomes were labeled with [α-^{32}P]dCTP in a nuclear extract supplemented with cytosol (○). The nuclear extract was prepared from cells containing mature SV40 chromosomes labeled with [^{3}H]thymidine (□). These chromosomes were prepared by the hypotonic method, mixed together, and then digested with MNase. In each case, control digestions were carried out in the absence of MNase (△).

more slowly than nucleosomal monomers was released from replicating, but not mature, chromosomes (Figure 3). While as much as 45% of the nascent DNA appeared in this fraction, there was never more than 3% of the radiolabeled DNA released from mature chromosomes in this region of the gradient (Figure 3). These results were essentially the same whether SV40 chromosomes were radiolabeled in intact cells or nuclear extracts, or when the sucrose gradients contained 0.2, 0.4, or 0.6 M NaCl. Experiments in which DNA standards were included in the same gradients (Figure 8A) revealed that the monomers sedimented at 11 S, typical of eukaryotic nucleosomes [reviewed by McGhee & Felsenfeld (1980)]. Increasing the extent of digestion generated more monomers at the expense of oligomers, and the rapidly released, slowly sedimenting fraction containing nascent DNA was degraded (Figure 3). The data to be presented here will identify the latter, transient, nascent DNA fraction as nucleosome-free DNA, present on both sides of replication forks between the actual sites of DNA synthesis and the first nucleosome encountered. This material has been designated as prenucleosomal DNA (PN-DNA; Figure 11).

In addition to sedimentation analysis of the nucleoprotein digestion products, DNA was purified from MNase digests of mature and replicating SV40 chromosomes and subjected to gel electrophoresis. Nucleosomal monomers and dimers, released from replicating chromosomes by MNase, contained DNA equivalent in size to DNA products from mature chromosomes present in the same digest (Figure 4). This relationship was observed throughout the digestion time course, suggesting that the spacing of nucleosomes in newly replicated SV40 chromatin is very similar to that in nonreplicating chromatin; the latter contains an average of 188 bp of DNA per nucleosome (Shelton et al., 1980). The DNA fragments observed after gel electrophoresis of purified DNA (Figure 4) correspond to nucleosomal trimer, dimer, and monomer, and to PN-DNA. This was demonstrated directly by purifying DNA from the appropriate sucrose gradient fractions (Figure 3) and analyzing the DNA by gel electrophoresis together with

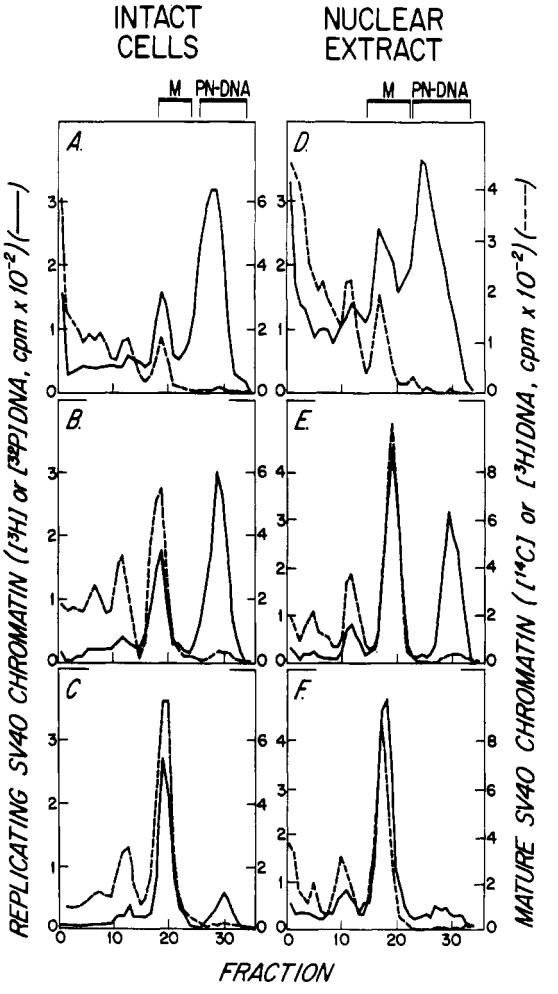

FIGURE 3: Sedimentation analysis of the nucleoprotein products from an MNase digestion of SV40 chromosomes. (A–C) Intact cells: Replicating SV40 chromosomes (—), labeled with [^{3}H]thymidine in intact cells, were mixed with mature SV40 chromosomes (---), uniformly labeled with [^{14}C]thymidine, and digested with MNase. (D–F) Nuclear Extract: Replicating SV40 chromosomes (—), labeled with [α-^{32}P]dCTP in a nuclear extract supplemented with cytosol, were mixed with mature SV40 chromosomes (---), uniformly labeled with [^{3}H]thymidine, and digested with MNase. Digestion products in both cases were then analyzed directly by sedimentation through a neutral sucrose gradient. Acid-insoluble radioactivity was measured in each fraction. Sedimentation is from right to left. The positions of nucleosomal monomers (M) and of prenucleosomal DNA (PN-DNA) are indicated. The percentage of mature DNA digested was (A) 3, (B) 10, (C) 20, (D) 8, (E) 30, and (F) 45. The percentage of replicating DNA digested was (A) 19, (B) 41, (C) 64, (D) 21, (E) 41, and (F) 75.

SV40 restriction fragments (Figure 5). As seen in Figure 5, the purified DNA fragments from MNase digests of replicating chromosomes exhibited a broad size distribution, with maxima at 670, 400, 200, and 120 bp for nucleosomal trimer, dimer, and monomer and PN-DNA, respectively.

Prenucleosomal DNA Originates from Both Sides of Replication Forks. PN-DNA was shown to originate from both sides of replication forks by its ability to anneal equally well with either strand of an SV40 DNA restriction fragment. SV40(I) DNA was digested with *Hpa*I and *Bgl*I restriction endonucleases to create four unique DNA fragments that represented DNA templates from both clockwise (A and D) and counterclockwise (B and C) replication forks in bidirectionally replicating DNA molecules (Figure 6). The complementary strands in each fragment were separated by denaturation in alkali, followed by agarose gel electrophoresis; the polarity of the faster migrating strand of each fragment

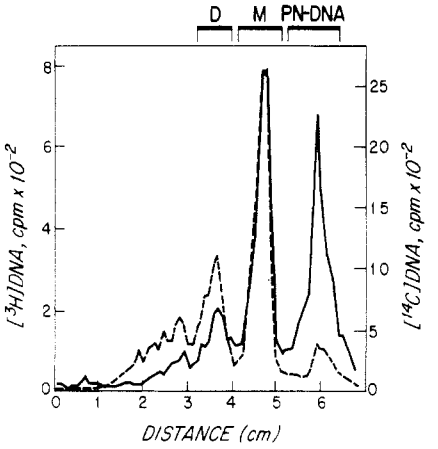

FIGURE 4: Electrophoretic analysis of the DNA products from an MNase digestion of SV40 chromosomes. Replicating SV40 chromosomes (—) labeled with [^{3}H]thymidine in intact cells were mixed with mature SV40 chromosomes (---) uniformly labeled with [^{14}C]thymidine and digested with MNase. Following digestion, the DNA fragments were purified and analyzed by agarose gel electrophoresis. Electrophoresis was carried out for 16 h at 6 V/cm at 4 °C. After electrophoresis, the gel was sliced, and the radioactivity was measured in each slice. The direction of electrophoresis is from left to right. The positions of nucleosomal dimer DNA (D), nucleosomal monomer DNA (M), and prenucleosomal DNA (PN-DNA) are indicated. When the digestion was terminated, 10% of the [^{14}C]DNA and 26% of the [^{3}H]DNA had been rendered acid soluble.

was previously shown to represent the retrograde DNA template (Kaufmann et al., 1978). The separated DNA strands were then transferred to diazobenzyloxymethyl (DBM) paper (Alwine et al., 1977; Wahl et al., 1979). Replicating SV40 chromosomes were radiolabeled with [α-^{32}P]dCTP in a nuclear extract and digested with MNase, and the digestion products were fractionated by sedimentation in a sucrose gradient. DNA from the PN-DNA fraction, nucleosomal monomers, and a sample of the total unfractionated MNase digest was purified and then hybridized to the separated strands of SV40 DNA restriction fragments immobilized on DBM paper (Figure 6). The DNA from all three samples annealed equally well to both the forward and retrograde DNA templates of fragments A, C, and D but showed a slight preference for the retrograde strand of fragment B. The reason for this preference is not known, but it may reflect a difference in secondary structure that allowed the two strands to separate during electrophoresis. Although the retrograde strand of fragment C sometimes migrated as two bands, both bands hybridized with equivalent amounts of [^{32}P]DNA. Variation in the amount of [α-^{32}P]DNA used in the experiments did not change the results, demonstrating that hybridization was done under saturating conditions.

In contrast to DNA released by MNase digestion of replicating SV40 chromosomes, at least 85% of Okazaki fragments from purified SV40(RI) DNA, radiolabeled at their 5′ ends with [α-^{32}P]ATP and polynucleotide kinase and purified by gel electrophoresis, annealed to the retrograde template strand of each DNA fragment (Figure 6D) as previously reported (Kaufmann et al., 1978; DePamphilis et al., 1978; Perlman & Huberman, 1977). Similar results were obtained when Okazaki fragments were purified by chromatography on Sepharose 6B (95% hybridized to the retrograde template) or sedimentation through an alkaline sucrose gradient (88% hybridized to the retrograde template). In each case, an effort was made to avoid contamination by long DNA chains that arise from continuous DNA synthesis on forward arms of replication forks, and by radiolytic breakdown products (Kaufmann et al., 1978).

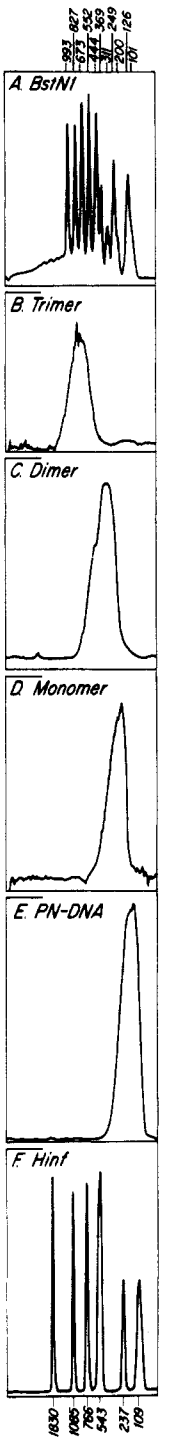

FIGURE 5: Sizes of nucleosomal DNA and PN-DNA isolated from a sucrose gradient. Replicating SV40 ^{32}P-labeled chromosomes were prepared and digested with MNase as described in Figures 2 and 3. The DNA from each peak in the sucrose gradient was purified and then analyzed by electrophoresis in a 2% agarose slab gel for 18 h at 6 V/cm, 4 °C. The results were visualized by autoradiography and densitometry tracings of each gel lane are shown. (A) *Bst*N1 SV40 DNA fragments labeled at their 5′ ends with ^{32}P (Chaconas & Van de Sande, 1980), (B) nucleosomal trimer DNA, (C) nucleosomal dimer DNA, (D) nucleosomal monomer DNA, (E) PN-DNA, and (F) *Hinf*I SV40 DNA fragments labeled at their 5′ ends with ^{32}P (Chaconas & van de Sande, 1980).

Therefore, since PN-DNA, monomer DNA, and total SV40 DNA patterns of hybridization were indistinguishable from one another and symmetrical with respect to the forward and retrograde templates, under conditions that detected asymmetrical hybridization of Okazaki fragments, PN-DNA originates from *both* arms of replication forks.

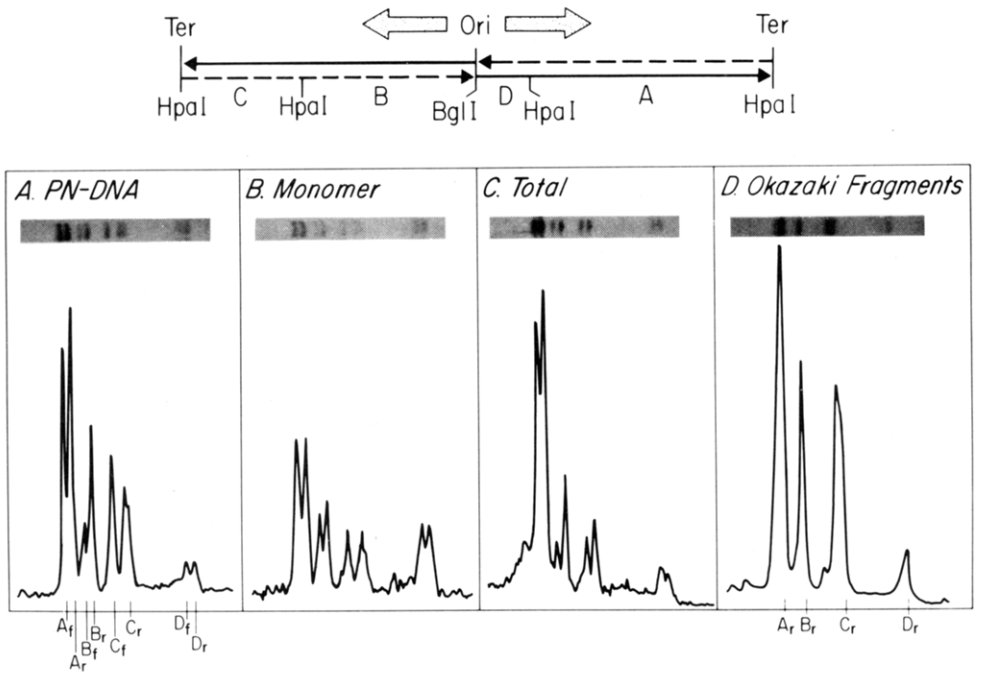

FIGURE 6: Hybridization of PN-DNA to separated strands of SV40 DNA restriction fragments. SV40(I) DNA was cut into four unique segments with *Hpa*I and *Bgl*I restriction endonucleases. The locations of the four DNA fragments are indicated on a genomic map of SV40 opened at the terminus of replication (Ter). DNA replication is bidirectional (open arrows) from the origin of replication (Ori), and one strand is synthesized continuously (solid line) on the forward arm of replication forks, while the other strand is synthesized discontinuously (broken line) on retrograde arms (Figure 1). The DNA strand complementary to nascent DNA on forward (f) or retrograde (r) arms is indicated for each fragment in panels A and D. The two strands of each restriction fragment were separated by gel electrophoresis, transferred to DBM paper, and hybridized with various purified [^{32}P]DNA probes. Three of the probes were isolated from SV40 ^{32}P-labeled chromosomes digested with MNase as described in Figures 2 and 3: (A) PN-DNA, (B) nucleosomal monomer, and (C) total MNase-resistant DNA. Okazaki fragments (D) were obtained from purified SV40(RI) DNA and labeled at the 5′ ends with ^{32}P (R. Hay and M. L. DePamphilis, unpublished results). Results were visualized by autoradiography, and densitometer tracings were recorded.

Prenucleosomal DNA Originates between the Fork and the First Nucleosomes. Previous studies have demonstrated that the 3′–5′ *E. coli* exonuclease III (Exo III) and the 5′–3′ exonuclease of phage T7 gene 6 (Exo $T7_6$) initiate digestion on the ends of nascent DNA strands at replication forks (Figure 1) and continue until the first nucleosome is encountered; no radiolabel was excised from newly synthesized nucleosomal DNA or uniformly labeled DNA in mature chromosomes (Herman et al., 1981). Therefore, as a determination of whether or not nascent DNA present prior to nucleosome assembly is released by MNase, a mixture of replicating and mature SV40 chromosomes was digested first with either Exo III or Exo $T7_6$, or with both, and then with MNase (Figure 7). The fraction of nascent DNA released from replicating chromosomes by MNase in nucleosomal monomers and oligomers was not decreased as a result of prior exonuclease treatment (compare the $^3H/^{14}C$ ratio in Figure 7A with that in Figure 7B). Instead, predigestion of replicating SV40 chromosomes with either Exo III alone (Figure 7B,D) or Exo $T7_6$ alone (Figure 7E) removed much of the PN-DNA, whereas Exo III and Exo $T7_6$ together (Figure 7F) removed all of the PN-DNA. Results were essentially the same for replicating chromosomes radiolabeled in intact cells (Figure 7A,B) or in nuclear extracts (Figure 7C–F). These data demonstrate that PN-DNA originates from nascent DNA present on both sides of replication forks between the fork and the first nucleosome encountered on either arm.

Prenucleosomal DNA Is Not Released as a Nucleoprotein Complex. In contrast to nucleosomal monomers, the PN-DNA fraction released from replicating SV40 chromosomes by MNase contained a broad size distribution of double-stranded DNA that behaved as bare DNA. Sample of nucleosomal monomers and PN-DNA were isolated from sucrose gradients similar to those shown in Figure 3, and one-half of each sample was deproteinized with proteinase K in the presence of sarkosyl. This treatment reduced sedimentation of nucleosomes from 11 S to 6 S (Figure 8A) but had no effect on sedimentation of PN-DNA which remained at about 5 S (Figure 8B). The sedimentation behavior of deproteinized DNA from nucleosomes and PN-DNA was consistent with the sizes of purified DNA samples determined by gel electrophoresis [200 bp for monomers and 120 bp for PN-DNA (Figure 5)]. Samples of nucleosomal monomers and PN-DNA were also treated with formaldehyde to prevent dissociation of DNA and protein and then sedimented to equilibrium in CsCl. Monomers had a buoyant density of 1.47 g/cm^3 (Figure 8C), typical of SV40 chromatin (Su & DePamphilis, 1978; Herman et al., 1979; Shelton et al., 1980), whereas PN-DNA had a density of 1.70 g/cm^3 (Figure 8D), typical of purified SV40 DNA. The small fraction of monomer DNA present in the nondeproteinized control sample (Figure 8A), with a buoyant density of bare DNA (Figure 8C), resulted from partial dissociation of low concentrations of nucleosomal monomers during their isolation (Cotton & Hamkalo, 1981).

MNase digestion of PN-DNA mixed with either purified SV40 DNA or mature SV40 chromosomes also showed that PN-DNA was indistinguishable from bare DNA (Figure 9A). Since MNase digests both double- and single-stranded DNA, PN-DNA was also exposed to single strand specific S_1 endonuclease in the presence of either native or heat-denatured CV-1 cell DNA (Figure 9B). At least 90% of the PN-DNA was resistant to digestion, whereas heat-denatured PN-DNA was as sensitive to S_1 endonuclease as denatured CV-1 DNA. Extraction of DNA from the PN-DNA fraction did not affect the results obtained with either MNase or S_1 endonuclease.

Micrococcal Nuclease Digestion of Bare DNA Proceeds through an Intermediate Similar to Prenucleosomal DNA. PN-DNA was released as bare DNA, suggesting that it rep-

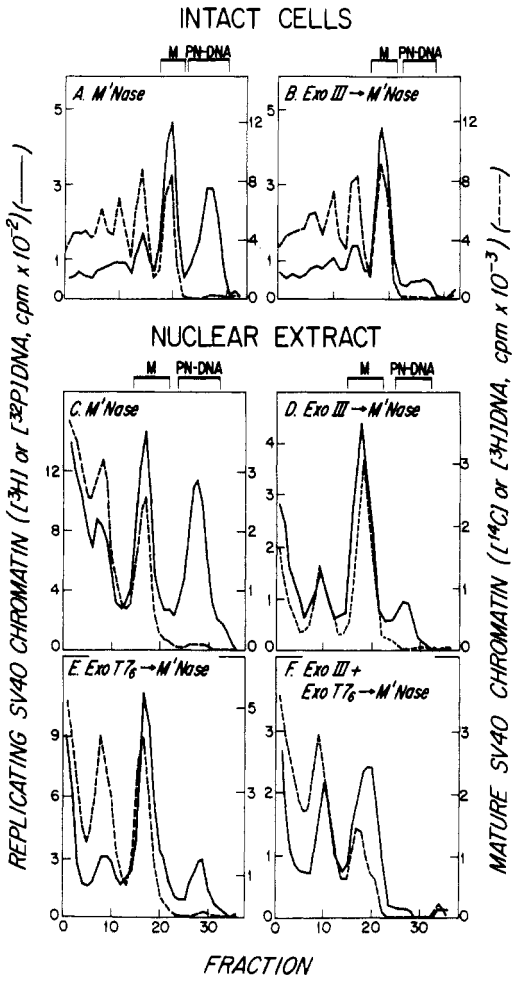

FIGURE 7: Effects of predigestion of SV40 chromosomes with Exo III and Exo $T7_6$ upon the digestion products released subsequently by MNase. Intact cells: Replicating SV40 ^{3}H-labeled chromosomes (—) and uniformly labeled mature SV40 ^{14}C-labeled chromosomes (- - -) labeled in intact cells (Figure 2) were digested together with either MNase (A) or Exo III followed by MNase (B). Nuclear extract: Replicating SV40 ^{32}P-labeled chromosomes (—), labeled in a nuclear extract plus cytosol, and uniformly labeled mature SV40 ^{3}H-labeled chromosomes (- - -), labeled in intact cells (Figure 2), were digested together with either MNase (C), Exo III followed by MNase (D), Exo $T7_6$ followed by MNase (E), or both Exo III and Exo $T7_6$ followed by MNase (F). Exonuclease digestions were taken to completion in each case as previously described (Herman et al., 1981). Sedimentation is from right to left, and conditions are as in Figure 3. The positions of nucleosomal monomers (M) and prenucleosomal DNA (PN-DNA) are indicated.

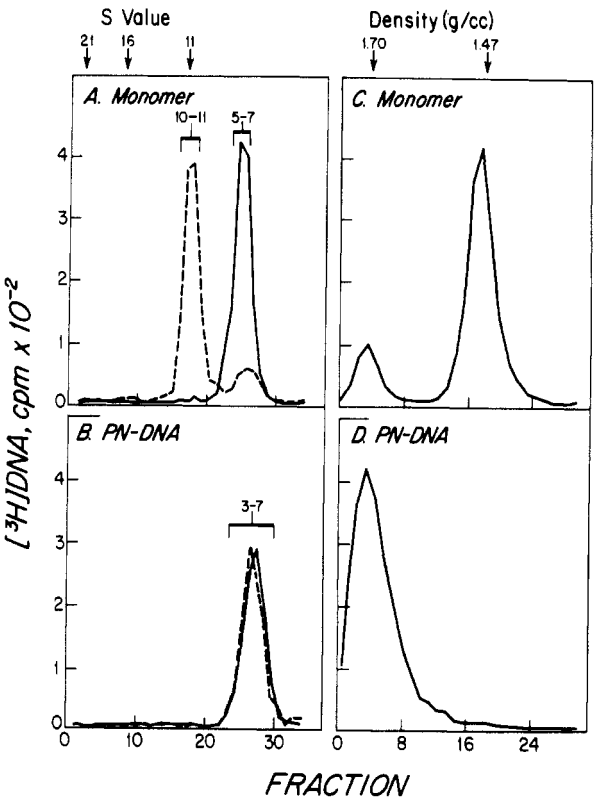

FIGURE 8: Sedimentation analysis of nucleosomal monomers and PN-DNA. Replicating SV40 ^{3}H-labeled chromosomes, labeled in intact cells, were digested with MNase, and the nucleosomal monomers and PN-DNA were purified by sedimentation through a neutral sucrose gradient. Velocity sedimentation analysis: Isolated nucleosomal monomers (A) were resedimented before (- - -) and after (—) treatment with proteinase K (20 μg/mL) in 0.2% sarcosyl at 37 °C for 1 h. Similarly, PN-DNA (B) was resedimented before (- - -) and after (—) the proteinase K–sarcosyl treatment. Sedimentation conditions are as in Figure 3. The positions of ^{32}P-labeled sedimentation markers analyzed in the same gradient are indicated above the figure: SV40(I) DNA (21 S), SV40(II) DNA (16 S), and the SV40 *Hin*fA restriction fragment (11 S). Equilibrium sedimentation analysis: Isolated nucleosomal monomers (C) or PN-DNA (D) were cross-linked with formaldehyde and then sedimented to equilibrium in CsCl gradients. The refractive index of selected fractions was measured to establish the density scale indicated above the figure.

resents a region at replication forks that is essentially free of tightly bound protein under our conditions of chromatin isolation. One might expect MNase to digest such regions into a continuous size distribution of DNA products, ranging from large oligomers to mononucleotides, without the appearance of a transient DNA intermediate of defined size. In fact, MNase digestion of a mixture of purified SV40 DNA and either replicating SV40 chromosomes (Figure 10A) or calf thymus DNA (Figure 10B) was rapidly converted into a broad peak of DNA that cosedimented with PN-DNA. As the digestion proceeded, the DNA was completely converted into acid-soluble material not visible on the gradients. Therefore, the characteristics of PN-DNA are consistent with the concept that replicating chromosomes, isolated as described under Experimental Procedures, contain nonnucleosomal, essentially bare DNA at their replication forks (Figure 11).

Discussion

Analyses of replicating eukaryotic chromosomes by electron microscopy and MNase digestion have identified nucleosomes directly behind DNA replication forks on both of the two sibling chromosomes, as well as directly in front of forks [reviewed in DePamphilis & Wassarman (1980)]. The average distance from the 3′ and 5′ ends of long nascent DNA strands to the first nucleosomes on either arm of a replication fork has been determined from exonuclease digestion of replicating SV40 chromosomes to be 125 ± 20 nucleotides (Herman et al., 1981). However, despite the presence of nucleosomes, comparison of the rates, extents, and products of MNase digestion revealed that newly replicated chromatin was more sensitive to MNase than nonreplicating chromatin in four ways: (i) a 2–6-fold increase in the initial rate of DNA digestion, (ii) a 25–50% increase in the extent of DNA digestion, (iii) a faster release of nucleosomal monomers, and (iv) a concurrent appearance of nascent DNA in a form significantly smaller than that which is associated with nucleosomes (DePamphilis & Wassarman, 1980; Klempnauer et al., 1980; Yakura & Tanifuji, 1980). Previous investigators have suggested that the small nascent DNA fragments ("subnucleosomal" fragments) rapidly released by MNase from replicating chromosomes originate either from nucleoprotein intermediates in nucleosome assembly (Hildebrand & Walters, 1976; Schlaeger & Knippers, 1979; Klempnauer et al., 1980)

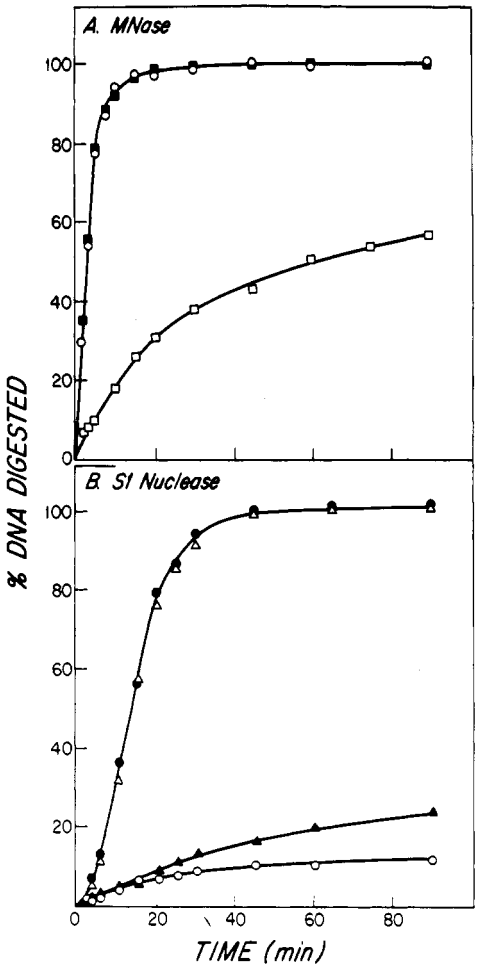

FIGURE 9: Time course of MNase and S_1 nuclease digestion of PN-DNA. Replicating SV40 ^{32}P-labeled chromosomes, labeled in a nuclear extract, were digested with MNase, and [^{32}P]PN-DNA was purified by sedimentation through a neutral sucrose gradient. (A) MNase digestion of [^{32}P]PN-DNA (○) mixed together with either *Hae*IIII DNA restriction fragments from SV40 [^{3}H]DNA (■) or mature SV40 ^{3}H-labeled chromosomes (□). (B) S_1 nuclease digestion of native [^{32}P]PN-DNA (○) or denatured [^{32}P]PN-DNA (●) mixed together with either native (▲) or denatured (△) CV-1 [^{3}H]DNA. DNA was denatured by heating at 100 °C for 5 min.

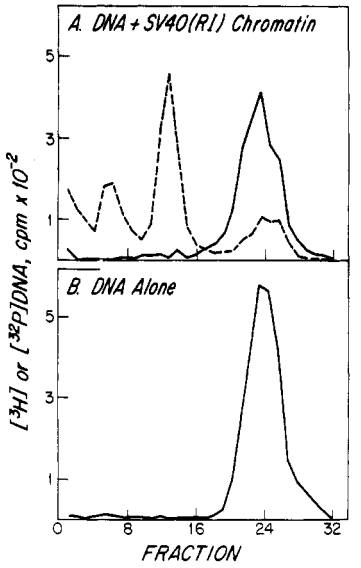

FIGURE 10: MNase digestion of purified SV40 DNA. Purified SV40(I) [^{32}P]DNA (—) was mixed with replicating SV40 ^{3}H-labeled chromosomes (---) labeled in intact cells and isolated by the isotonic method (A), or with an equivalent amount of unlabeled calf thymus DNA (B), and digested with MNase. The digestion products were then analyzed by sedimentation through a neutral sucrose gradient as in Figure 3. In (A), 33% of the nascent chromosomal DNA and 37% of the purified DNA were rendered acid soluble. In (B), 35% of the purified DNA was rendered acid soluble.

or from immature nucleosomes (Levy & Jakob, 1978; Seale, 1978). In this report, we have described the results of experiments demonstrating that these DNA fragments in SV40 actually originate from the nascent DNA found on both arms of replication forks before the first nucleosomes. Accordingly, this material, which includes Okazaki fragments, has been designated prenucleosomal DNA (PN-DNA; Figure 11).

PN-DNA is unambiguously recognized by its sensitivity to Exo III and Exo $T7_6$, two exonucleases whose opposing specificities allow complete excision of nascent DNA strands from replication forks (Figure 1). Since neither Exo III nor Exo $T7_6$ excises DNA from mature SV40 chromosomes or removes nascent DNA from nucleosomes at native replication forks (Herman et al., 1981), excision of nascent DNA from replicating chromosomes is limited to the region before the first nucleosome on each arm of the fork. When replicating SV40 chromosomes were digested with MNase, both nucleosomal monomers and PN-DNA were rapidly released (Figure 3). Predigestion of replicating chromosomes with either Exo III or Exo $T7_6$ decreased in part the amount of PN-DNA released by MNase; however, predigestion with both exonucleases resulted in a complete removal of MNase-sensitive PN-DNA (Figure 7). In these experiments, the amount of radiolabeled DNA in nascent nucleosomal monomers relative to monomers released from mature SV40 chromosomes present in the same reaction remained constant (Figure 7). These data demonstrate that PN-DNA originates from the sites of DNA synthesis at replication forks and, apparently, from both arms of the forks. The second conclusion was confirmed by hybridization of PN-DNA to separated strands of SV40 DNA restriction fragments. While isolated Okazaki fragments annealed predominantly, if not exclusively, to DNA fragments representing the retrograde template, PN-DNA (like total SV40 DNA) annealed equally well to DNA fragments from throughout the genome representing both forward and retrograde templates (Figure 6). Furthermore, PN-DNA represented at least twice as much nascent DNA as found in Okazaki fragments, and was observed even when Okazaki fragments represented an insignificant fraction of the labeled DNA in replicating chromosomes (data not shown). These data demonstrate than PN-DNA does not simply represent a preferential release of Okazaki fragments as 5 S duplex DNA.

PN-DNA was always recovered as a heterogeneously sized population of duplex DNA, resistant to single strand specific S_1 endonuclease, but as sensitive to MNase as purified DNA. Gel electrophoretic analysis of purified PN-DNA revealed a range of lengths from about 65 to 245 bp, with a maximum at 120 bp. PN-DNA sedimented at 5 S in neutral sucrose gradients whether or not it was pretreated with proteinase K and sarcosyl, and, following fixation in formaldehyde, PN-DNA had the same isopycnic density in CsCl as purified DNA. On the other hand, nascent nucleosomal monomers from replicating chromosomes were resistant to MNase, had a buoyant density similar to that of chromatin, and sedimented at 11 S prior to deproteinization, but at 5–6 S afterwards. Therefore, isolated PN-DNA appears to be free of protein.

DNA replication proteins that were presumably associated with the PN-DNA region of replication forks inside the cell (DePamphilis & Wassarman, 1980) may have been lost when chromosomes were sedimented in 200 mM NaCl (Su & DePamphilis, 1978) or were not cross-linked to DNA by form-

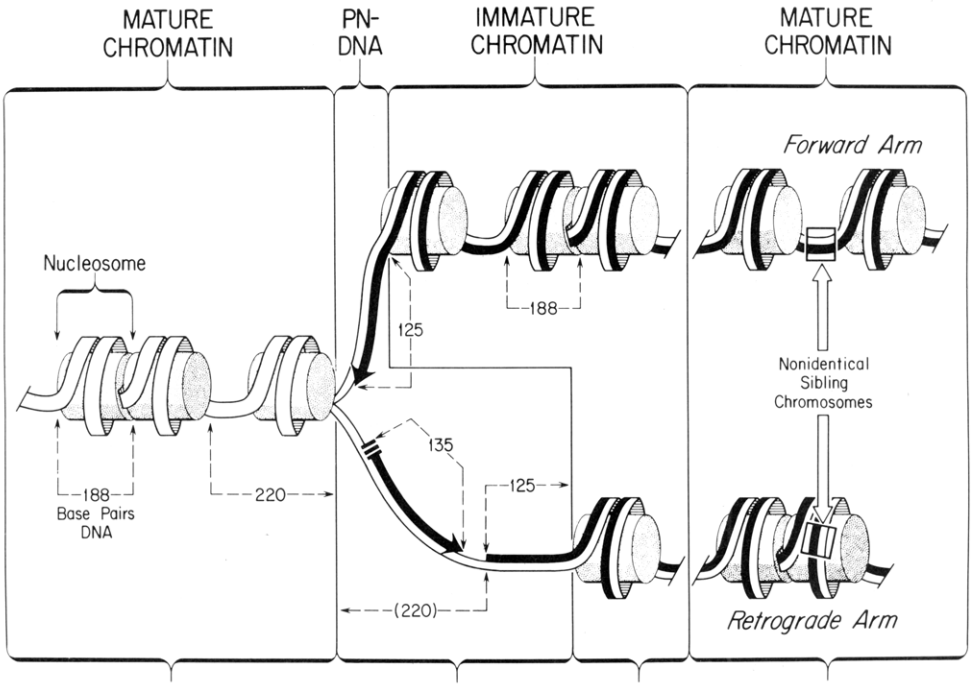

FIGURE 11: Model for replication forks in SV40 chromosomes. Numbers give the average distance in nucleotides. Nascent DNA is represented by a black ribbon, with an average of one RNA-primed Okazaki fragment per fork represented on the retrograde side. The dimensions and shape of a nucleosome (110 × 55 Å cylinder consisting of a 1.75 turn of duplex DNA, 20 Å in diameter, coiled around a histone octamer) were taken from Klug et al. (1980). The prenucleosomal DNA region (PN-DNA) contains that portion of *nonnucleosomal* nascent DNA which is rapidly released and then digested by MNase, excised by Exo III and Exo $T7_6$, and sensitive to S_1 or *Neurospora crassa* nucleases, characteristics unique to replicating chromatin. Immature chromatin contains that portion of *nucleosomal* nascent DNA that is hypersensitive to MNase. Mature chromatin in replicating chromosomes appears to be identical with chromatin in nonreplicating chromosomes. See Discussion for further details.

aldehyde. In any event, it is not necessary to postulate the presence of a DNA–protein complex in order to explain the transient accumulation of PN-DNA during MNase digestions, since MNase digestion of purified SV40 DNA initially released DNA fragments equivalent in size to PN-DNA (Figure 10). As PN-DNA originates from replication forks distributed throughout the genome (Figure 6), MNase digestion of bare DNA at replication forks should lead to the same result. There are two explanations for the size of PN-DNA: first, MNase initially cuts bare DNA at a large number of preferred sites (Ponder & Crawford, 1977; Fittler & Zachau, 1979), and second, random endonuclease cuts in the prenucleosomal DNA region (Figure 11) should generate DNA fragments with an average size of about 100 bp.

The fraction of nascent DNA that is released as PN-DNA depends upon both the length of the radiolabeling period (i.e., the number of labeled nucleotides per fork) and the extent of digestion by MNase. The longer the time period used to radiolabel replicating SV40 (data not shown) or cellular (Schlaeger & Knippers, 1979; Klempnauer et al., 1980) chromatin, and the longer the time of digestion with MNase (Figure 3), the less PN-DNA observed. Cellular DNA replication, which proceeds at a faster rate than SV40 replication (Edenberg & Huberman, 1975; Tapper et al., 1979), requires shorter radiolabeling periods than SV40 in order to observe PN-DNA (Klempnauer et al., 1980). These parameters could account for differences in the amount of PN-DNA seen in experiments from different laboratories (Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Klempnauer et al., 1980).

The PN-DNA region has also been suggested as the location of salt-labile 11 S nascent nucleosomes. Sedimentation of MNase digestion products from replicating lymphocyte chromatin in 2 mM EDTA (pH 7.5) and 10 mM mercaptoethanol showed only nucleosomal monomers and oligomers, while addition of 0.5 M NaCl revealed a DNA fraction equivalent to PN-DNA (Schlaeger & Knippers, 1979). However, these data could also have resulted from adventitious association of PN-DNA with nucleosomes after release by MNase under these exceptionally low ionic strength conditions. Our results were essentially the same in 50 mM NaCl as in 200 mM NaCl gradients, and lower salt concentrations resulted in decreased resolution of nucleosomal monomers and oligomers.

On the basis of results of experiments reported here and elsewhere, native replication forks in replicating SV40 chromosomes can be divided into at least four regions (Figure 11): (i) prereplicative mature chromatin, (ii) prenucleosomal DNA that encompasses the actual sites of DNA synthesis and includes Okazaki fragments, (iii) immature chromatin that encompasses regions of newly assembled nucleosomes that are more sensitive to nonspecific endonucleases than are regions of mature chromatin, and (iv) postreplicative mature chromatin consisting of newly assembled nucleosomes that cannot be distinguished from nucleosomes in nonreplicating mature chromosomes. Nucleosomes of mature SV40 chromosomes, that are indistinguishable in histone composition and nuclease sensitivity from those of their monkey cell host, each contain an average of 188-bp DNA with a "core" consisting of 146 bp (Shelton et al., 1978a, 1980). Improved electron microscopic techniques have revealed 24 ± 0.2 nucleosomes per mature SV40 chromsome (Saragosti et al., 1980), with an average spacing of 220 bp from one nucleosome to the next [based upon 5243 bp per genome; van Heuverswyn & Fiers, 1979]. Since these nucleosomes are arranged in a nearly random fashion around the genome (Cremisi et al., 1976; Persico-DeLauro et al., 1977; Liggins et al., 1979; Shelton et al., 1980; Tack et al., 1981), the internucleosomal distance varies from 0 to 60 bp. These variable regions of internucleosomal DNA appear in intranuclear (Shelton et al.,

1978a; Hallick et al., 1978) as well as isolated SV40 chromosomes (Bellard et al., 1976; Shelton et al., 1980).

Comparison of replicating and mature SV40 chromosomes by analysis with MNase (Figures 3 and 4; Klempnauer et al., 1980), restriction endonucleases (Tack et al., 1981), and electron microscopy (Cremisi et al., 1977; Seidman et al., 1978) demonstrated that the size and arrangement of nucleosomes on newly replicated chromatin were essentially the same as those on mature chromatin. Furthermore, the nearly random phase relationship between DNA sequence and chromatin structure is established within 400 bp of replication forks and is present on both arms of the same replication fork (Tack et al., 1981), consistent with the presence of nucleosomes an average of 125 bp from the 3′ and 5′ ends of long nascent DNA strands (Herman et al., 1981). The above data demonstrate that newly replicated DNA is rapidly assembled into nucleosomes and that chromatin assembly on one side of the fork does not appear to be directed by assembly on the other side. On the basis of our characterization of PN-DNA, the sensitivity of Okazaki fragments to Exo III and Exo $T7_6$ (Herman et al., 1981), and the properties of Okazaki fragments released from replicating SV40 chromosomes by S_1 nuclease (Herman et al., 1979), Okazaki fragments appear to be synthesized on nonnucleosomal DNA followed by ligation to long DNA strands and assembly into nucleosomes. The size range and metabolism of Okazaki fragments (Anderson & DePamphilis, 1979) have been previously related to chromatin structure by suggesting that initiation of Okazaki fragments occurs stochastically within an "initiation zone" through de novo synthesis of an RNA primer complementary to the retrograde strand DNA template; the size of the initiation zones (ca. 220 bp) is determined by nucleosome spacing in prereplicative mature chromatin, assuming that nucleosome "disassembly" is rate limiting for replication (DePamphilis et al., 1978, 1980; Anderson & DePamphilis, 1979; DePamphilis & Wassarman, 1980).

A region of immature chromatin is included in the model of eukaryotic replication forks (Figure 11) to account for the observation that replicating SV40 chromosomes, predigested with Exo III and Exo $T7_6$ to remove PN-DNA (Figure 6), still exhibit enhanced sensitivity to MNase. Furthermore, nascent nucleosomal dimers and trimers excised from replicating SV40 chromosomes are also more susceptible to MNase digestion than oligomers from mature chromosomes (M. Cusick, M. DePamphilis, and P. Wassarman, unpublished results). The basis of chromatin maturation is not known but may involve changes in histone composition and/or modification [reviewed in DePamphilis & Wassarman (1980)]. The characteristics of PN-DNA and immature chromatin disappear with time as nascent chromatin completes its maturation process, between 5 and 40 kb from the ongoing replication forks (Hildebrand & Walters, 1976; Levy & Jakob, 1978; Worcel et al., 1978; Schlaeger & Klempnauer, 1978; Schlaeger & Knippers, 1979; Klempnauer et al., 1980; Yakura & Tanifuji, 1980; M. Cusick, M. DePamphilis, and P. Wassarman, unpublished results).

A major difficulty in studies of chromatin structure at native replication forks has been the limited amount of radiolabel that can be incorporated by using whole cells. One solution is to increase the specific radioactivity of precursor pools in the absence of DNA synthesis by preincubation of cells with radiolabeled DNA precursors at 0 °C (Perlman & Huberman, 1977; Herman et al., 1979, 1981). Another solution is to use subcellular systems that faithfully continue DNA replication and chromatin assembly (Shelton et al., 1978b, 1980; Schlaeger & Klempnauer, 1978; Seale, 1978). Data in this paper demonstrate that the *initial state* of SV40 chromatin synthesized in nuclear extracts supplemented with cytosol is identical with that of SV40 chromatin recently synthesized in intact cells. The rates and extents of digestion by MNase (Figure 2), the sedimentation behavior of nucleoprotein particles released by MNase (Figure 3), the sizes of DNA from nucleosomes (Figures 4 and 5), the presence of PN-DNA (Figure 3), and the effects of Exo III and Exo $T7_6$ on PN-DNA (Figure 6) were the same for chromatin synthesized in vitro and in vivo. Using similar criteria, we previously demonstrated that the *final state* of cellular chromatin synthesized in nuclei (Shelton et al., 1978b) and SV40 chromatin synthesized in nuclear extracts (Shelton et al., 1980; M. Cusick, M. DePamphilis, and P. Wassarman, unpublished results) supplemented with cytosol is essentially the same as that of mature chromatin synthesized in intact cells. Neither mature SV40 chromosomes nor cellular chromatin prelabeled in intact cells underwent any changes during the in vitro incubations (Shelton et al., 1980). Our results with replicating SV40 chromosomes radiolabeled in nuclear extracts are in complete agreement with our results with chromosomes labeled in intact cells.

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