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Structure of Chromatin at Deoxyribonucleic Acid Replication Forks: Location of the First Nucleosomes on Newly Synthesized Simian Virus 40 Deoxyribonucleic Acid†

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ABSTRACT: Exonucleases specific for either 3′ ends (*Escherichia coli* exonuclease III) or 5′ ends (bacteriophage T7 gene 6 exonuclease) of nascent DNA chains have been used to determine the number of nucleotides from the actual sites of DNA synthesis to the first nucleosome on each arm of replication forks in simian virus 40 (SV40) chromosomes labeled with [^{3}H]thymidine in whole cells. Whereas each enzyme excised all of the nascent [^{3}H]DNA from purified replicating SV40 DNA, only a fraction of the [^{3}H]DNA was excised from purified replicating SV40 *chromosomes*. The latter result was attributable to the inability of either exonuclease to digest nucleosomal DNA in native replicating SV40 chromosomes, as demonstrated by the following observations: (i) digestion with either exonuclease did not reduce the amount of newly synthesized nucleosomal DNA released by micrococcal nuclease during a subsequent digestion period; (ii) in briefly labeled molecules, as much as 40% of the [^{3}H]DNA was excised from long nascent DNA chains; (iii) the fraction of [^{3}H]DNA excised by exonuclease III was reduced in proportion to the actual length of the radiolabeled DNA; (iv) the effects of the two exonucleases were additive, consistent with each enzyme trimming only the 3′ or 5′ ends of nascent DNA chains without continued excision through to the opposite end. When the fraction of nascent [^{3}H]DNA excised from replicating SV40 DNA by exonuclease III was compared with the fraction of [^{32}P]DNA simultaneously excised from an SV40 DNA restriction fragment, the actual length of nascent [^{3}H]DNA was calculated. From this number, the fraction of [^{3}H]DNA excised from replicating SV40 *chromosomes* was converted into the number of nucleotides. Accordingly, the average distance from either 3′ or 5′ ends of long nascent DNA chains to the first nucleosome on either arm of replication forks was found to be 125 nucleotides. Furthermore, each exonuclease excised about 80% of the radiolabel in Okazaki fragments, suggesting that less than one-fifth of the Okazaki fragments were contained in nucleosomes. On the basis of these and other results, a model for eukaryotic replication forks is presented in which nucleosomes appear rapidly on both the forward and retrograde arms, about 125 and 300 nucleotides, respectively, from the actual site of DNA synthesis. In addition, it is proposed that Okazaki fragments are initiated on nonnucleosomal DNA and then assembled into nucleosomes, generally after ligation to the 5′ ends of long nascent DNA chains is completed.

Replication of eukaryotic chromosomes requires the accurate duplication of both DNA sequence and chromatin organization. Discrete particles, called "nucleosomes", each containing about 200 base pairs of DNA coiled around an octamer of histones H2A, H2B, H3, and H4, represent the primary level of chromatin organization (Kornberg, 1977; Lilley & Pardon, 1979; McGhee & Felsenfeld, 1980). The invariant portion of a nucleosome, the "core", consists of the histone octamer and 145 base pairs of DNA, while the remainder of the DNA is called the "linker" or "spacer" and consists of 10–100 base pairs of DNA depending upon the source of the chromatin (Thomas & Thompson, 1977; Prunell & Kornberg, 1978; Shelton et al., 1980; McGhee & Felsenfeld, 1980). When nucleosomes are not arranged contiguously, as in SV40[1] chromosomes, spacer DNA can include nonnucleo-

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received June 13, 1980.* This research was supported by grants from the National Cancer Institute (CA20841) and the National Science Foundation (PCM76-01963). M.L.D. was an Established Investigator of the American Heart Association, and T.M.H. was supported by a postdoctoral fellowship from the National Institutes of Health (GM06186).

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0006-2960/81/0420-0621$01.00/0

somal DNA that is randomly interspersed between nucleosomes (Shelton et al., 1978, 1980). Association of histone H1 with nucleosomes leads to their aggregation, a second level of chromatin organization in which higher order structures are generated (Thoma & Koller, 1977; Griffith & Christiansen, 1978; Müller et al., 1978; Lilley & Pardon, 1979; Thoma et al., 1979). Finally, a third level of chromatin organization is represented by extended loops containing 34–85 kilobases of DNA (Benyajati & Worcel, 1976; Pinion & Salts, 1977; Igo-Kemenes & Zachau, 1978) that are held together by nonhistone "scaffolding" proteins (Adolph et al., 1977; Paulson & Laemmli, 1977).

It is likely that chromosome structure can directly affect the replication and expression of genes in eukaryotic organisms (Weisbrod et al., 1980). Therefore, chromosome replication must allow for accurate reproduction of those differences in chromatin structure that affect gene expression (Weintraub et al., 1978). Since an understanding of the mechanisms involved requires a detailed knowledge of chromatin structure immediately before and after replication, we have undertaken to obtain such information through biochemical analyses of replication forks in simian virus 40 chromosomes. In this paper, we demonstrate that exonuclease III from *E. coli* (degrading one strand of duplex DNA in the 3′–5′ direction) and gene 6 exonuclease from bacteriophage T7 (degrading one strand of duplex DNA in the 5′–3′ direction) do not degrade nucleosomal DNA in native replicating SV40 chromosomes. These enzymes were used to estimate the average number of nucleotides from the 3′ and 5′ ends of long nascent DNA chains to the first nucleosome encountered on each arm of a replication fork and to determine the average fraction of Okazaki fragments associated with nucleosomes at replication forks. The results of these and other experiments were used to construct a model of the structure of eukaryotic DNA replication forks.

Experimental Procedures

Cells and Virus. In all experiments, a 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 and infection of virus and cells have been described previously (Wilson et al., 1976).

Preparation of SV40 Chromosomes. For preparation of mature SV40 chromosomes containing SV40(I) [^{14}C]DNA, SV40 infected CV-1 cells were incubated at 37 °C with [^{14}C]Thd (0.4 μCi/mL, 50 mCi/mmol) from 24 to 36 h postinfection (Herman et al., 1979). The resulting SV40(I) [^{14}C]DNA contained 15–20 × 10^3 cpm/μg. For preparation of the replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA, SV40 infected CV-1 cells were first equilibrated at 36 h postinfection with [^{3}H]Thd (167 μCi/mL, 55.5 Ci/mmol) for 10 min at 0 °C to maximize the specific radioactivity of dTTP pools in the absence of DNA synthesis and then shifted to 20 °C for 1–6 min to allow DNA replication to proceed (Perlman & Huberman, 1977; Herman et al., 1979). The resulting SV40 [^{3}H]DNA labeled for 1 min in this manner contained 15–20 × 10^2 cpm/μg. Viral chromosomes were then extracted in 10 mM Tris-HCl (pH 7.6), 200 mM NaCl, 5 mM EDTA, and 1% Triton X-100 as previously described (Herman et al., 1979; Shelton et al., 1980). Mature and replicating chromosomes were purified by sedimentation through a sucrose gradient in the same buffer minus detergent (Herman et al., 1979). Gradients were fractionated from the top to avoid contamination with virions in the pellet. Fractions containing mature SV40 chromosomes (55 S) or replicating SV40 chromosomes (65 S) were pooled and then concentrated by vacuum dialysis against 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA. These procedures routinely yielded 3–5 μg of SV40 DNA per 6 × 10^6 cells, as measured by a fluorometric assay (Shelton et al., 1978).

Alternatively, replicating SV40 chromosomes were prepared by extraction of nuclei in a hypotonic buffer containing 10 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol (Su & DePamphilis, 1978) and then purified by sedimentation through a sucrose gradient in either the same buffer (Su & DePamphilis, 1978) or supplemented with 0.2 M or 0.4 M NaCl.

Nuclease Digestion of SV40 DNA and Chromosomes. (*i*) *E. coli Exonuclease III* (*Exo III*). Replicating SV40 DNA or chromosomes (50 μg/mL) were incubated at 20 °C with *E. coli* exonuclease III (New England Biolabs, 37 units/μg of DNA) in a 0.15-mL reaction volume containing 50 mM Tris-HCl (pH 8.0), 0.25 mM $MgCl_2$, 0.05 mM EDTA, and 1 mM dithiothreitol. These conditions suppressed the activity of endogenous nuclease that copurified with SV40 chromosomes. For example, in a standard Exo III incubation (37 °C, 66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$, and 0.1 mM dithiothreitol) as much as 10% of the [^{3}H]DNA in chromosomes labeled for 1 min could be digested by endogenous nuclease activities. Exo III digestions were terminated by placing the reaction on ice and adding an equal volume of 10 mM Tris-HCl (pH 8.0) containing 20 mM EDTA and 10 μg calf thymus DNA. Acid-insoluble radioactivity was determined by addition of 3 mL of 1 N HCl and 0.5% sodium pyrophosphate, and then, after 10 min on ice, collecting the precipitate on a Whatman GF/C filter, washing the filter three times with 5 mL of 1 N HCl and 0.5% sodium pyrophosphate and then twice with ethanol, drying the filters, and then analyzing the radioactivity in a standard xylene-based liquid-scintillation cocktail.

When Exo III was used to determine the number of nucleotides polymerized during a given labeling period with [^{3}H]Thd, purified SV40(RI) [^{3}H]DNA (3–5 μg, depending on its specific radioactivity) was incubated for 10 min at 0 °C in a 0.5-mL reaction containing Exo III (4.5 units/μg of DNA), 67 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 77 mM NaCl, 1 mM dithiothreitol, 30 μg of calf thymus DNA, and 0.05–1 μg of *Hin*dIII SV40 [^{32}P]DNA restriction fragment and then transferred to 37 °C to initiate DNA digestion by Exo III.

When nuclease-resistant DNA was subsequently analyzed by electrophoresis in agarose gels, reactions were terminated by addition of EDTA to a final concentration of 10 mM. Sarcosyl and proteinase K were then added to final concentrations of 0.5% and 20 μg/mL, respectively, and then the mixture incubated at 37 °C for 20 min. The reaction was extracted twice with an equal volume of chloroform–isoamyl alcohol (24:1), and the DNA was precipitated at −20 °C in 0.3 M NaCl and 67% ethanol. DNA was recovered by centrifugation at 12000*g* for 30 min and resuspended in 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA.

When Exo III resistant DNA was subsequently digested with micrococcal nuclease, reactions were terminated by addition of EDTA to a final concentration of 10 mM and then

[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; Exo III, *E. coli* exonuclease III; Exo $T7_6$, *E. coli* bacteriophage T7 gene 6 exonuclease.

the reaction mixture was filtered through Sephadex G-50 (Pharmacia) in 10 mM Tris-HCl (pH 7.4) and 0.1 M EDTA. Chromosomes were recovered in the void volume and then digested with micrococcal nuclease as described below.

(*ii*) *Bacteriophage T7 Gene 6 Exonuclease (Exo $T7_6$).* Replicating SV40 DNA or chromosomes (50 μg DNA/mL) were incubated at 20 °C with bacteriophage T7 gene 6 exonuclease (gift from Drs. M. Engler and C. Richardson; 0.35 milliunit/μg of DNA, with 1 unit defined as 1 nmol of 5′-terminal phosphate released in 30 min at 37 °C) in an 0.15-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 dithiothreitol. These conditions suppressed endogenous nuclease activity that copurified with SV40 chromosomes and could digest as much as 10% of the nascent DNA under standard Exo $T7_6$ conditions (37 °C, 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol). Digestions were terminated and analyzed as described for Exo III.

(*iii*) *Micrococcal Nuclease.* SV40 chromosomes (50 μg of DNA/mL) were incubated at 37 °C with micrococcal nuclease (Worthington, 0.2 unit/μg of DNA) in 0.15-mL reaction containing 10 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Acid-insoluble radioactivity was determined as described for Exo III. When nucleoprotein products were analyzed by sedimentation, the digestion was terminated in 10 mM EDTA at 2 °C and layered directly onto a neutral sucrose gradient.

Gel Electrophoresis of Denatured DNA. Purified SV40(RI) DNA was denatured in glyoxal and analyzed by electrophoresis in 2% agarose slab gels as previously described (Anderson & DePamphilis, 1979). Gels were sliced into 2 mm fractions, and each slice was solubilized by incubation overnight at 50 °C in 10 mL of Liquifluor (New England Nuclear) containing 3% NCS (Amersham).

Sedimentation of SV40 Chromosomes and Nucleoprotein Complexes. Substrates and products of micrococcal nuclease digestions were sedimented through neutral sucrose gradients containing 5%–20% sucrose, 10 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, and 50 mM NaCl for 14 h at 36000 rpm (4 °C) in a Beckman SW41 rotor.

Preparation of Intact SV40 DNA and DNA Restriction Fragments. Purified SV40(I) and SV40(II) [^{32}P]DNA and purified SV40(RI) [^{3}H]DNA were prepared as previously described (Anderson et al., 1977). *Hin*dIII restriction endonuclease (New England Biolabs) was used to prepare SV40 [^{32}P]DNA (200000 cpm/μg) fragments of known length. DNA fragments were separated by electrophoresis in 5% polyacrylamide slab gels (Tapper & DePamphilis, 1978), visualized under long-wavelength ultraviolet illumination against a background of Polygram Cel 300 PEI/UV 254 (Brinkman), and then recovered from gel slices by electroelution (Shelton et al., 1980). DNA fragments were then precipitated in 70% ethanol, resuspended in 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA, and checked for single-strand interruptions by denaturation in glyoxal followed by electrophoresis in 5% polyacrylamide gels (Anderson & DePamphilis, 1979).

Results

Digestion of Nascent DNA of Replicating SV40 Chromosomes by Exo III or Exo $T7_6$ Is Limited. On the basis of our current concepts of DNA replication and the structure of replicating DNA intermediates (DePamphilis & Wassarman, 1980), purified DNA replication forks should act as a substrate for either 3′- or 5′-exonucleases (Figure 1). However, in

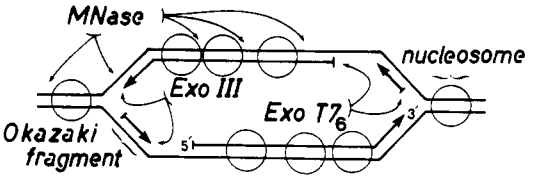


FIGURE 1: Potential nuclease cleavage sites at the DNA replication forks of replicating SV40 chromosomes. *E. coli* exonuclease III (Exo III) degrades duplex DNA only from 3′-OH or 3′-PO_4 termini and prefers recessed 3′ ends over matched ends of duplex DNA or single phosphodiester bond interruptions (Rogers & Weiss, 1980). Bacteriophage T7 gene 6 exonuclease (Exo $T7_6$) degrades duplex DNA only from 5′-OH or 5′-PO_4 termini (including 5′-terminal RNA primers) and prefers recessed 5′ ends (Okazaki et al., 1979; Richardson et al., 1979). Micrococcal nuclease (MNase) is an endonuclease that degrades duplex or single-stranded DNA or RNA and prefers single strands at AT-rich regions (Anfinsen et al., 1971). In this diagram, 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). Predicted regions of DNA at SV40 replication forks which could serve as Exo III, Exo $T7_6$, and/or MNase cleavage sites are indicated by arrows.

replicating chromosomes, histones, as well as other proteins, may protect all or part of newly synthesized DNA from exonucleolytic digestion (Lilley & Pardon, 1979; Cremisi, 1979). Therefore, exonucleases could serve as sensitive probes of the structure of chromatin at replication forks.

Exo III initiates digestion at any accessible 3′ ends on duplex DNA, regardless of the presence or absence of a phosphoryl group and digests one strand exonucleolytically, including any RNA sequences (Figure 1); the enzyme will not degrade single-stranded DNA (Rogers & Weiss, 1980). Exo $T7_6$ has similar properties except that it initiates digestion at 5′ ends of DNA or RNA (Figure 1) (Richardson et al., 1979; Okazaki et al., 1979). For determination of whether or not these exonucleases would specifically digest nascent DNA at replication forks, various forms of SV40 DNA of defined structure were exposed to each enyzme.

Mature SV40 chromosomes containing uniformly labeled SV40(I) [^{14}C]DNA and replicating SV40 chromosomes containing pulse-labeled SV40(RI)[^{3}H]DNA were purified from virus infected CV-1 cells. SV40(I) [^{14}C]DNA and SV40(RI) [^{3}H]DNA were extracted and purified from a portion of each chromatin sample. Samples containing both mature (^{14}C) and replicating (^{3}H) SV40 chromosomes, as well as samples containing both SV40(I) [^{14}C]DNA and SV40(RI) [^{3}H]DNA, were then incubated with either Exo III or Exo $T7_6$. As expected, neither enzyme digested covalently closed, mature SV40 DNA, but both removed at least 95% of the nascent [^{3}H]DNA in replicating DNA intermediates (Figure 2). Similarly, mature SV40 chromosomes were not digested by either exonuclease; however, a limit digest of replicating chromatin was obtained in which 50% of the nascent [^{3}H]-DNA was excised by Exo III and 35% by Exo $T7_6$ (Figure 2). Increasing the concentration of either nuclease by as much as 5-fold resulted in an increase in the rate but not in the extent of digestion of replicating chromatin. Furthermore, the extent of digestion of nascent [^{3}H]DNA by Exo III was only increased from 50% to 58% by sedimenting purified SV40 chromosomes through 0.4 M NaCl to remove nonhistone proteins. Nonhistone proteins as well as histones can protect against exonuclease digestion since only 20% of the [^{3}H]DNA was excised from SV40 chromosomes purified by a low salt (5 mM KCl and 0.5 mM $MgCl_2$) extraction procedure (Su & DePamphilis, 1978). These results indicate that chromatin

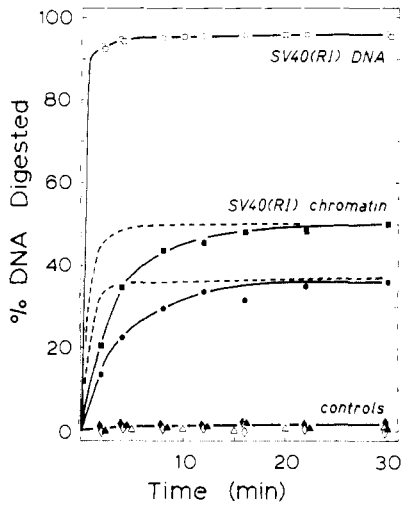


FIGURE 2: Determination of the extent of digestion of replicating SV40 DNA and replicating SV40 chromosomes by Exo III and Exo $T7_6$. Replicating SV40 chromosomes (●, ■) containing SV40(I) [^{3}H]DNA that had been labeled for 1 min in whole cells and SV40(RI) [^{3}H]DNA (○, □) that was purified from the replicating chromosomes were incubated with either Exo III (□, ■) or Exo $T7_6$ (○, ●). The broken lines represent digestions with 5-fold higher concentrations of either Exo III or Exo $T7_6$. Control incubations contained replicating chromosomes in the absence of Exo III (▲) or Exo $T7_6$ (▼) and SV40(I) [^{14}C]DNA in the presence of Exo III (△) or Exo $T7_6$ (◇) or Exo $T7_6$ (◇). Acid-insoluble readioactivity was determined as a function of time of incubation.

structure at replication forks prevents extensive digestion by either exonuclease.

Nascent Nucleosomal DNA of Replicating SV40 Chromosomes Is Not Digested by Exo III or Exo $T7_6$. The failure of either Exo III or Exo $T7_6$ to completely excise nascent DNA in replicating SV40 chromosomes (Figure 2) suggests that these exonucleases cannot digest through nucleosomal DNA (Figure 1). For assessment of this possibility, aliquots of a mixture of mature SV40 chromosomes containing SV40(I) [^{14}C]DNA and replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA were incubated with micrococcal nuclease alone to measure the fraction of radioactivity associated with nucleosomes (Figure 3A). In addition, separate aliquots were first digested to completion with either Exo III (Figure 3B) or Exo $T7_6$ (Figure 3C) and then incubated with micrococcal nuclease to determine whether or not nascent nucleosomal DNA had been excised by either of the exonucleases. In each case, the nucleoprotein digestion products contained nucleosomal monomers that sedimented at 11 S in sucrose gradients, as well as detectable amounts of nucleosomal dimers and trimers (Figure 3). Most importantly, comparison of the ^{3}H/^{14}C ratios of nucleosomal DNA in each experiment revealed no detectable change in the fraction of nascent nucleosomal DNA, despite the excision of 45% (Figure 3B) or 32% (Figure 3C) of the [^{3}H]DNA by predigestion with one of the exonucleases. Since the [^{3}H]Thd labeling period in these experiments was only 1 min, the total [^{3}H]DNA region is confined to replication forks (i.e., about 260 nucleotides; see below). Consequently, significant loss of nascent DNA from the first nucleosomes encountered by the exonucleases at replication forks would have been detected. These results indicate that Exo III and Exo $T7_6$ are not able to digest nucleosomal DNA from native replicating chromosomes; the nascent [^{3}H]DNA digested by these exonucleases is nonnucleosomal DNA at replication forks (Figure 1). These results were not an artifact attributable to the temperature shift from 0 to 20 °C used to increase the specific radioactivity of nascent DNA since similar results have been obtained with cells

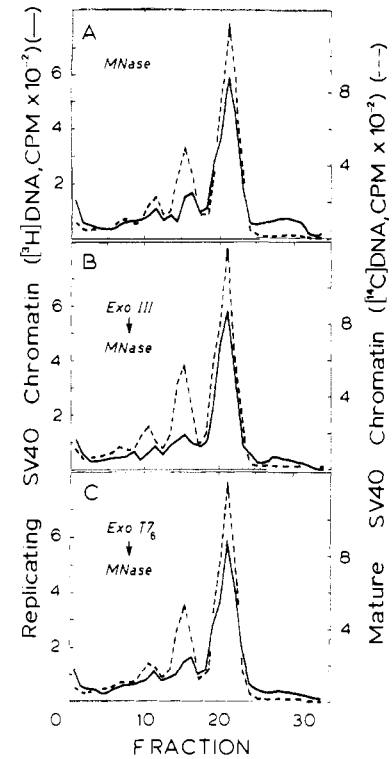


FIGURE 3: Sedimentation analysis of the effect of Exo III and Exo $T7_6$ on nucleosomal DNA of replicating SV40 chromosomes. A mixture of mature SV40 chromosomes containing uniformly labeled SV40(I) [^{14}C]DNA (---) and replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA (—) labeled for 1 min was digested with either micrococcal nuclease (MNase) alone (A) or first with Exo III and then with MNase (B) or first with Exo $T7_6$ and then with MNase (C), and then the digestion products were analyzed by sedimentation through a neutral sucrose gradient. Sedimentation if from right to left. Fractions were collected from the bottom of tubes, and acid-insoluble radioactivity was measured in each fraction. In each case (A–C), MNase digestion was stopped when 22 ± 1% of the mature label was acid soluble. Exo III and Exo $T7_6$ digestions were taken to completion (see Figure 2).

maintained at 37 °C (M. Cusick, M. DePamphilis, and P. Wassarman, unpublished results).

Exo III and Exo $T7_6$ Excise Nascent DNA Only from Replication Forks of SV40 Chromosomes. Exo III and Exo $T7_6$ appear to excise DNA from replicating chromosomes by initiating digestion at 3′ and 5′ ends of nascent DNA chains present at replication forks and proceeding until the first nucleosome is encountered. This was assessed in two ways. First, the size distribution of nascent [^{3}H]DNA chains on SV40(RI) [^{3}H]DNA purified from replicating SV40 chromosomes, labeled for 1 min, were analyzed by agarose–gel electrophoresis following denaturation of replicating DNA in the presence of glyoxal (Figure 4). Before exonuclease treatment, about 25% of the [^{3}H]DNA was present as Okazaki fragments having an average length of 135 nucleotides, and about 75% was found as long nascent DNA chains greater that 500 nucleotides in length (Figure 4, broken lines). Incubation of replicating chromosomes with either Exo III (Figure 4A) or Exo $T7_6$ (Figure 4B) resulted in the loss of about 80% of the ^{3}H label in Okazaki fragments which accounted for 20% of the total [^{3}H]DNA digested. In eight different experiments, including six in which the SV40(RI) [^{3}H]DNA was analyzed directly by sedimentation through alkaline sucrose gradients, Exo III removed 78 ± 5% of the radiolabeled Okazaki fragments; this result was not affected by first sedimenting the replicating chromosomes through 0.4 M NaCl. The remainder of the exonuclease-sensitive DNA came only from the ends of long

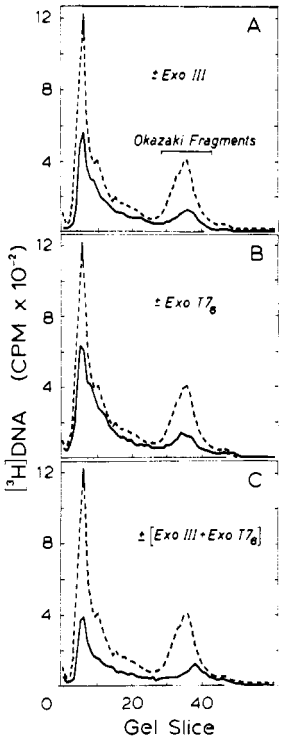


FIGURE 4: Electrophoretic analysis of the effect of Exo III and Exo $T7_6$ on nascent DNA of long chains and Okazaki fragments of replicating SV40 chromosomes. Replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA were incubated with either Exo III alone (A), Exo $T7_6$ alone (B), or Exo III and Exo $T7_6$ together (C), and then the purified SV40(RI) [^{3}H]DNA was denatured in glyoxal and analyzed by gel electrophoresis both before (---) and after (—) incubation with exonuclease. Gels were then sliced, and the radioactivity was measured in each slice. Electrophoresis is from left to right. Exonuclease digestion was carried to completion (see Figure 2).

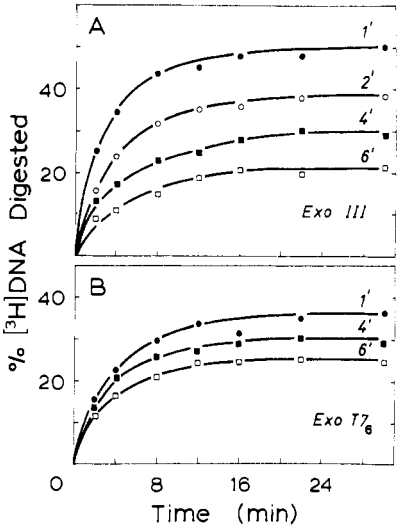


FIGURE 5: Determination of the fraction of exonuclease-sensitive nascent DNA in replicating SV40 chromosomes as a function of the extent of [^{3}H]thymidine incorporation. Replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA that was radiolabeled for 1 min (●), 2 min (○), 4 min (■), or 6 min (□) were incubated with either Exo III (A) or Exo $T7_6$ (B), and the amount of acid-insoluble radioactivity was measured as a function of time of incubation. Exonuclease conditions were the same as in Figure 2.

nascent DNA; Exo III excised about 40% (Figure 4A) and Exo $T7_6$ (Figure 4B) excised about 20% of the label from all nascent DNA chains greater than 500 nucleotides long without significantly affecting their size distribution. Digestion of replicating SV40 chromosomes with both Exo III and Exo $T7_6$ (Figure 4C) revealed that 15%–20% of the label in Okazaki fragments was resistant to attack from either the 3′ or 5′ ends but that the effects of Exo III and Exo $T7_6$ on long nascent DNA were cumulative, consistent with the model shown in Figure 1. Therefore, exonuclease digestion is limited to replication forks where at least 80% of the Okazaki fragments are not protected by nucleosomes.

A second type of experiment demonstrated that the fraction of labeled DNA susceptible to exonuclease digestion was proportional to that fraction present at replication forks. As the time period for DNA replication in the presence of [^{3}H]Thd was increased from 1 min to 2, 4, and 6 min, the fraction of [^{3}H]DNA digested by Exo III from purified replicating SV40 chromosomes decreased from 50% to 36%, 30%, and 22%, respectively (Figure 5A). Similarly, the fraction of [^{3}H]DNA digested by Exo $T7_6$ decreased from 36% to 25% (Figure 5B). These results can be explained on the basis of discontinuous DNA synthesis on the retrograde sides of replication forks where the direction of DNA synthesis is opposite to the direction of fork movement (Figure 9) and continuous DNA synthesis on forward sides; such a situation has been clearly demonstrated for SV40 DNA replication (Perlman & Huberman, 1977; Kaufmann et al., 1978). DNA synthesis on retrograde arms of replication forks necessarily lags behind synthesis on forward arms. Consequently, more labeled DNA will be accessible to Exo III, which digests long nascent DNA on forward arms and Okazaki fragments, than to Exo $T7_6$, which degrades the same fraction of Okazaki fragments but finds little label at 5′ ends of long DNA on retrograde arms. At longer labeling periods, the fraction of label in Okazaki fragments becomes insignificant, and the fraction of label released by either enzyme is proportional only to the distance from the ends of long nascent DNA to the first nucleosomes (Figure 1). Therefore, since both Exo III and Exo $T7_6$ digested about the same fraction of nascent DNA on replicating chromosomes that had been labeled for 6 min (less than 2% of the label was in Okazaki fragments), the first nucleosome on forward arms must be about the same distance from the 3′ ends of long nascent DNA as the first nuclesome on retrograde arms is from the 5′ ends of long nascent DNA (Figure 9).

Exo III and Exo $T7_6$ Digest Different Domains of Replication Forks of SV40 Chromosomes. Given the specificities of Exo III and Exo $T7_6$ on bare DNA, their inability to digest through nucleosomes on replicating chromosomes, and the finding that each enzyme digests 78% of the Okazaki fragments, one would anticipate that nascent DNA released by each enzyme from native replicating chromosomes represents two separate, but overlapping, domains (Figure 1). Therefore, the total amount of DNA excised by both enzymes together should be greater than that released by either enzyme alone. This expectation was confirmed with replicating SV40 chromosomes labeled for either 1 min (Figure 6A,B) or 6 min (Figure 6C,D). Furthermore, the fraction of radioactivity at either the 3′ ends (region a, Table IA) or 5′ ends (region c, Table IA) of long nascent DNA chains could be calculated from these data (Table IB) by the following two independent methods (Table IC): (i) by subtracting the fraction of Okazaki fragments accessible to the exonuclease or (ii) by first removing the fraction of accessible Okazaki fragments with one exonuclease and then digesting the remaining accessible DNA with the other exonuclease. Exo III should excise all nonnucleosomal DNA from 3′ ends of long nascent DNA chains plus 78% of Okazaki fragments, while Exo $T7_6$ should excise 78% of Okazaki fragments plus any nonnucleosomal DNA at 5′ ends of long nascent DNA chains (Figure 1). After a 1-min

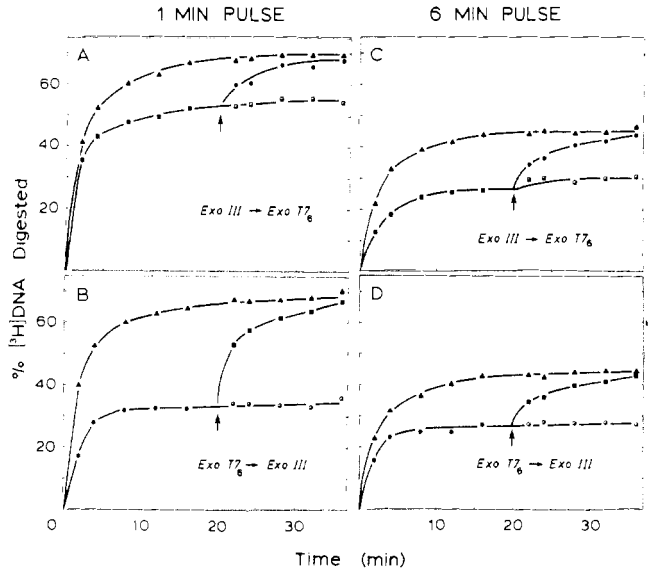


FIGURE 6: Determination of the sites of digestion by Exo III and Exo $T7_6$ in replicating SV40 chromosomes. Replicating SV40 chromosomes containing SV40(RI) [^{3}H]DNA that was labeled for either 1 min (A and B) or 6 min (C and D) were digested sequentially with either Exo III first followed by Exo $T7_6$ (A and C) or Exo $T7_6$ first followed by Exo III (B and D). Arrows mark the time (20 min) when either the second enzyme or an additional aliquot of the first enzyme was added. The effects of Exo III alone (■), Exo $T7_6$ alone (●), Exo III plus Exo $T7_6$ together (▲), or a second aliquot of the same exonuclease (◪, ◒) are shown. Acid-insoluble radioactivity was determined as a function of time of incubation. Enzyme conditions were the same as in Figure 2.

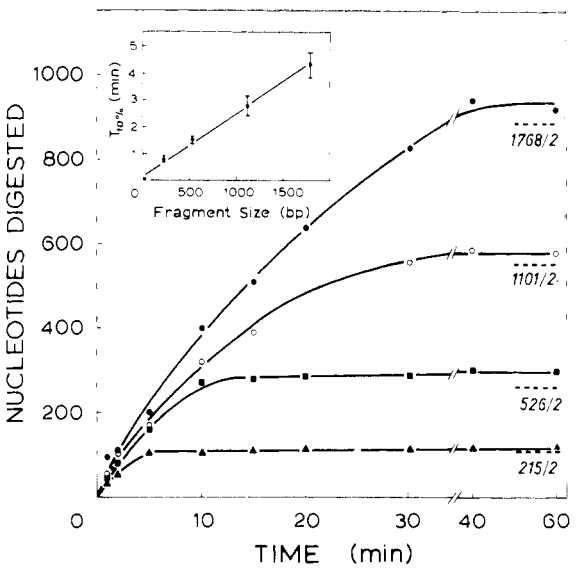


FIGURE 7: Evaluation of the relationship between the time required for Exo III digestion of DNA and DNA chain length. Individual, uniformly labeled *Hin*dIII [^{32}P]DNA restriction fragments were incubated with Exo III (7 units/μg of DNA). The fraction of acid-insoluble radioactivity was determined as a function of time of incubation and then converted into nucleotides digested based on the number of nucleotides present per sequenced fragment (van Heuverswyn & Fiers, 1979). The broken lines indicate the theoretical limit of digestion, assuming 50% of each fragment (i.e., 1768 (●), 1101 (○), 526 (■), and 215 (▲) base pairs of DNA divided by 2) was digested. Inset: The time required for 10% digestion by Exo III ($T_{10\%}$) as a function of DNA fragment size in base pairs.

labeling period, about 24% of the label was in Okazaki fragments (b), 17% was at the 5′ ends of long DNA chains (c), 34% was at the 3′ ends of long DNA chains (a), and the remaining 25% was protected by nucleosomes (Table I). However, with a 6-min labeling period, 3% or less of the radioactivity was in Okazaki fragments (b), about 20% was at the 5′ ends (c), and 20% was at the 3′ ends (a) of long DNA chains with the remaining 75% protected by nucleosomes. The difference between the amount of radioactivity released by exonuclease digestion of native replicating chromosomes and the amount released by the same enzyme from chromosomes preincubated with the other exonuclease could be accounted for by the fraction of label in Okazaki fragments (compare Figure 6A with 6B, and 6C with 6D; calculations shown in Table IC).

Number of Nucleotides from 3′ and 5′ Ends of Nascent DNA to the First Nucleosome on Each Arm of the Replication Fork of SV40 Chromosomes. The fraction of radioactivity released from nascent DNA by either Exo III or Exo $T7_6$ should be proportional to the average distance in nucleotides traveled by the exonuclease. The fraction of [^{3}H]DNA released from the 3′ ends (distance a, Table IA) or 5′ ends (distance c, Table IA) of long nascent DNA chains was converted into nucleotides by first measuring the total number of nucleotides incorporated into nascent DNA during a given labeling period and then multiplying that number (Table IB) by the percent counts per minute located either at the 3′ ends or 5′ ends (Table IC).

The number of nucleotides incorporated (N) into replicating SV40 DNA was determined after a 1-, 2-, 4-, or 6-min incubation of SV40 infected CV-1 cells in the presence of [^{3}H]Thd. Exo III digestion of replicating SV40 [^{3}H]DNA was carried out in the presence of an SV40 [^{32}P]DNA restriction fragment containing 3′-OH recessed ends (usually the *Hin*d-III-D fragment) as an internal standard and an excess of *Bgl*I cut linear SV40 DNA to ensure that small variations in the amounts of labeled DNA would not affect their rates of digestion. For example, *Hin*dIII-D [^{3}H]DNA and [^{32}P]DNA, one at 10 × the concentration of the other, were digested at the same rate and to the same extent. Similarly, variations in SV40(RI) DNA concentration did not affect the results. Under these conditions, a maximum of 50% of the [^{32}P]DNA restriction fragments was digested (Figure 7) since Exo III only degrades duplex DNA (Weiss, 1976; Clements et al., 1978), and the time required to digest 10% of a given DNA fragment ($T_{10\%}$) was found to be proportional to its length (Figure 7, insert). Therefore, with the assumption that the same number of nucleotides was released from all 3′ ends during the initial period of digestion (10%–30% of the labeled DNA), percent [^{3}H]DNA digested was converted into nucleotides by comparison with the internal standard [[^{3}H]nucleotides = [^{32}P]nucleotides (% ^{3}H cpm/^{32}P cpm)]. From these data, the total number of nucleotides synthesized (N) was then calculated. As expected, the number of nucleotides incorporated during SV40 DNA replication in whole cells was proportional to the time of exposure to [^{3}H]Thd (Figure 8, inset). However, the rate of incorporation of radioactivity was faster during the first minute after shifting cells from 0 °C to 20 °C than during the subsequent period (Figure 8, insert). This presumably resulted from a decrease in the specific radioactivity of internal [^{3}H]dTTP pools as a new steady-state distribution was established between synthesis and utilization of dTTP. With the lower enzyme concentrations used in these studies (compare Figure 8 with Figure 2), the initial rates and extents of Exo III digestion decreased with replicating [^{3}H]-DNA that had been radiolabeled for increasing periods of time (Figure 8). This phenomenon was a consequence of bidirectional DNA replication from a unique origin via semidiscontinuous DNA synthesis (Figure 1). The fraction of ^{3}H label in Okazaki fragments is significantly greater in brief labeling periods relative to longer labeling periods (Table IB); therefore,

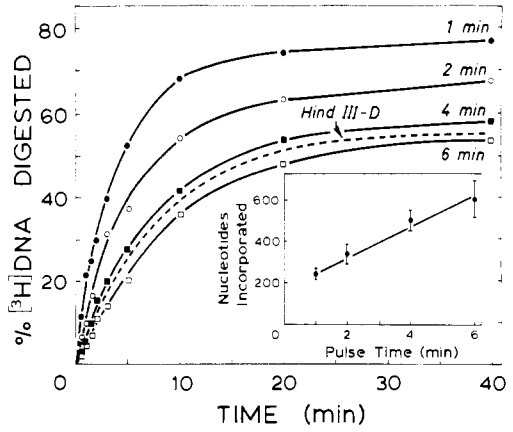


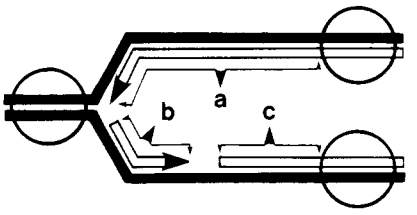
FIGURE 8: Determination of the number of nucleotides synthesized on SV40(RI) DNA as a function of time. Purified SV40(RI) [^{3}H]DNA, isolated from cells incubated for 1 (●), 2 (○), 4 (■), or 6 (□) min, was mixed with SV40 *Hin*d-D [^{32}P]DNA fragment (---) and digested with Exo III (7 units/μg of DNA). Acid-insoluble radioactivity was determined as a function of time of digestion. The time course for digestion of *Hin*d-D fragments (526 bp) was the same in each SV40(RI) DNA sample. Insert: The number of nucleotides incorporated into SV40(RI) [^{3}H]DNA was calculated as a function of time of labeling period in the following manner: [^{3}H]nucleotides digested = [^{32}P]nucleotides digested × (% [^{3}H]DNA digested/% [^{32}P]DNA digested); therefore total [^{3}H]nucleotides incorporated = ([^{3}H]nucleotides digested × 100/% [^{3}H]DNA digested).

a greater fraction of the radiolabel is immediately accessible to Exo III. As the labeling period increases, ^{3}H-labeled Okazaki fragments join to the 5′ ends of long nascent DNA chains. Therefore, the fraction of label in Okazaki fragments becomes insignificant, and the total radioactivity is divided equally between 3′ and 5′ ends of long nascent DNA, separated by comparatively long stretches (up to 5200) of unlabeled nucleotides. This results in a decrease in the initial rate of Exo III digestion and an apparent plateau of 50% digestion (e.g., 6-min sample, Figure 8); the remaining [^{3}H]DNA can be digested by the addition of more Exo III.

The average number of nucleotides from 3′ or 5′ ends of long nascent DNA chains to the first nucleosome on the forward and retrograde sides of the replication fork, respectively, was calculated for each of the four samples of replicating SV40 chromosomes; only date from 1- and 6-min samples are illustrated in Table I. As described in the previous section, two independent calculations were made (Table IC): One calculation (i) was based on subtracting the fraction of label in Okazaki fragments whereas the other (ii) relied on prior excision of all accessible Okazaki fragments by the alternative exonuclease. Results from both methods of calculation were in good agreement and were therefore combined to give *125 nucleotides* as the average distance to the first nucleosome, either from the 3′-OH ends of long nascent DNA chains on the forward side of replication forks (a) or from the 5′-PO_4 ends of long nascent DNA chains on the retrograde side (c). Although results from replicating chromosomes labeled for 4 min were essentially the same, distances calculated from 2- and 1-min samples were shorter in proportion to the brevity of the labeling period. The explanation for this apparent discrepency is 2-fold. First, the data is Figure 7 reveal an initial burst of radioactivity incorporated into nascent DNA, which means that DNA synthesized during the first minute will not be uniformly labeled. The concentration of radioactivity will be greater where replication forks were located at the beginning of the labeling period and lower at the 3′-OH ends on forward sides where the last nucleotides were incorporated. Consequently, the fraction of label associated with

Table I: Quantitative Analysis of the Digestion of Replicating SV40 Chromosomes by Exonuclease III and Exonuclease $T7_6$

A. Model



parameter	pulse-labeling time: 1 min	6 min	
B. Data			
total nucleotides incorporated (N)	258 ± 32	619 ± 76	
% cpm in Okazaki fragments (b)	24 ± 6	3 ± 0.5	
% cpm digested by			
Exo III alone (E_3)	51 ± 1	24 ± 1	
Exo $T7_6$ alone (E_6)	35 ± 2	25 ± 1	
Exo III after Exo $T7_6$ ($E_{6\to3}$)	36	18	
Exo $T7_6$ after Exo III ($E_{3\to6}$)	17	18	
C. Calculations			
forward side of fork			
% cpm in (a)			
(1) $a = E_3 - 0.78b$	32	22	
(2) $a = E_{6\to3}$	36	18	
nucleotides in (a)			
(1) $a = N(E_3 - 0.78b)$	83 ± 22	135 ± 20	
(2) $a = N(E_{6\to3})$	93	111	123 ± 20
retrograde side of fork			
% cpm in (c)			
(1) $c = E_6 - 0.78b$	16	23	
(2) $c = E_{3\to6}$	17	18	
nucleotides in (c)			
(1) $c = N(E_6 - 0.78b)$	42 ± 19	140 ± 20	
(2) $c = N(E_{3\to6})$	44	111	126 ± 20

nucleosomes will be greater than the fraction accessible to Exo III, and therefore the length of DNA excised by Exo III will be underestimated. Second, DNA synthesis is discontinuous predominatly, if not exclusively, on retrograde arms of SV40 replication forks. Therefore, retrograde DNA synthesis "lags" behind forward DNA synthesis as replication forks advance and new Okazaki fragments are initiated. Consequently, the number of labeled nucleotides on retrograde sides will not be equivalent to forward sides until the fraction of label in Okazaki fragments is insignificant. This phenomenon is clearly seen in Figure 6 where Exo $T7_6$ excised less [^{3}H]DNA than Exo III from replicating chromosomes labeled for 1 min whereas both enzymes excised the same fraction of [^{3}H]DNA from chromosomes labeled for 6 min. In theory, if a single nucleotide were incorporated at each 3′ end (Figure 1), Exo III could remove 100% of the label while Exo $T7_6$ could remove only 50% of the label. Therefore, calculations based on replicating chromosomes labeled for 6 min (3% or less of the label is in Okazaki fragments) should give the best estimate of the location of nucleosomes at replication forks because no assumptions are necessary concerning the distribution of labeled nucleotides at replication forks.

Discussion

During each S phase of the cell cycle replication of eukaryotic chromosomes takes place, resulting in duplication of both DNA sequence and chromosome structure. Since quiescent and actively transcribed regions of chromosomes have different structures (Weintraub et al., 1978; McGhee & Felsenfeld, 1980), the process of replication must include an accurate duplication of each structure such that concurrent and/or subsequent gene expression is unaltered. This suggests that

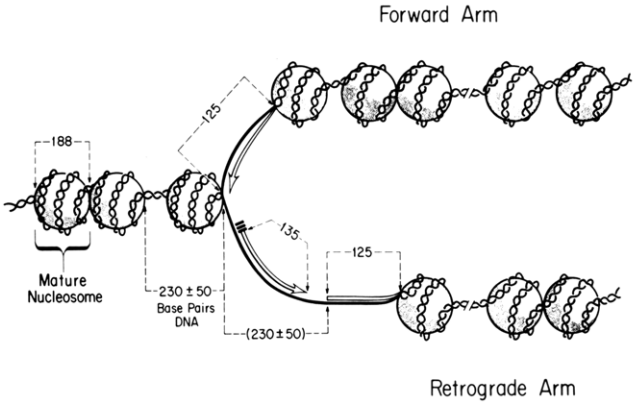


FIGURE 9: A diagrammatic representation of the DNA replication fork of SV40 chromosomes. The numbers given are from results presented here and from Shelton et al. (1978, 1980). An RNA-primed Okazaki fragment (⇒) is present on the retrograde arm of the replication fork. Note: Figure 3 in DePamphilis & Wassarman (1980) is similar to this figure but, due to an editorial error, shows slightly smaller values for the distances to the first nucleosomes.

assembly, spacing, and phasing of nucleosomes on newly synthesized DNA are important parameters in any consideration of chromosome replication as it relates to gene expression (e.g., Weintraub et al., 1978).

One fundamental question concerning chromatin replication deals with the proximity of nascent nucleosomes to the replication fork: how close to the the actual sites of DNA synthesis are nucleosomes assembled on forward and retrograde arms of replication forks? Analysis of the bouyant densities of replicating and mature SV40 (Goldstein et al., 1973; Herman et al., 1979), polyoma (Seebeck & Weil, 1974; Cremisi et al., 1976), sea urchin (Levy et al., 1975), and mammalian (Fakan et al., 1972; Seale & Simpson, 1975; Hancock, 1978) chromatin either failed to detect a difference in the protein to DNA ratio (Goldstein et al., 1973; Seebeck & Weil, 1974; Cremisi et al, 1976) or else found that some newly replicated chromatin contained *more* protein than nature chromatin (Fakan et al., 1972; Seale & Simpson, 1975; Levy et al., 1975; Herman et al., 1979). These results suggest that long stretches of bare DNA are not present at replication forks. Electron microscopy of replicating *Drosophila* (McKnight & Miller, 1977; McKnight et al., 1978) and SV40 (Seidman et al., 1978; Cremisi et al., 1978) chromosomes has revealed that prereplicative and newly replicated chromatin have a "beaded" appearance that is indistinguishable, with respect to bead diameter and periodicity, from that of nonreplicating chromatin. Extended regions of bare DNA do not accumulate at replication forks under normal conditions, suggesting that assembly of newly syntheszed DNA into nucleosomes or nucleosome-like particles is a relatively rapid process. However, stretches of DNA as long as 500 nucleotides would have gone undetected by electron microscopic examination, and longer regions of DNA may have been lost by redistribution of nucleosomes during sample preparation.

The results presented here extend the previous observations by demonstrating that the first nucleosomes encountered on either forward or retrograde arms of replicating SV40 chromosomes are an average of 125 nucleotides from the 3′ and 5′ ends of long nascent DNA chains (Figure 9). Their exact locations, however, may be broadly distrubuted about this mean value (Table I). This conclusion is drawn from three findings: (i) Exo III and Exo $T7_6$ do not excise nucleosomal DNA on native replicating chromosomes, (ii) at least 80% of the radiolabel in Okazaki fragments is accessible to either exonuclease, and (iii) the number of nucleotides excised by these exonucleases can be estimated by including DNA restriction fragments of known size and appropriate termini in the digest. These conclusions are not affected by the presence of an approximately 300-base-pair region adjacent to the origin of replication that apears to be free of nucleosomes in about 20% of mature SV40 chromosomes (Saragosti et al., 1980; Jakobovits et al., 1980). Our data represent the average of all replication forks which are distributed throughout the genome, and the pool of replicating chromosomes is biased toward molecules late in replication (Tapper & DePamphilis, 1978, 1980; Seidman & Salzman, 1979; D. Tapper, S. Anderson, and M. DePamphilis, unpublished results).

About 200–300 nucleotides of DNA were synthesized per replication fork when SV40 was pulse labeled with [^{3}H]thymidine for 1 min at 20 °C. Since virtually all of this label could be excised from bare SV40 DNA by either Exo III or Exo $T7_6$, the limited digestion observed with replicating chromosomes resulted from the presence of chromosomal proteins. Histones appear primarily responsible for preventing complete exonucleolytic excision of nascent DNA from replicating chromosomes since chromosomes prepared in either 200 or 400 mM NaCl to remove most nonhistone proteins (Elgin & Weintraub, 1975) were digested to about the same extent. Chromosomes were routinely prepared in 200 mM NaCl to avoid possible nucleosome sliding or translocation that can occur at higher salt concentrations (Thomas, 1978). In the experiments reported here, the number of radiolabeled nucleotides per replication fork was similar to the number of base pairs per nucleosome (about 200). Therefore, only the first nucleosome on each arm of the fork could contain a significant fraction of [^{3}H]TMP. Consequently, if either Exo III or Exo $T7_6$ excised DNA from the first nucleosome encountered at the fork, it would have been easily detected in our experiments (e.g., Figure 3). Although Exo III cannot digest nucleosomal DNA on native replicating SV40 chromosomes, it can degrade both core and linker DNA in nucleosomal monomers and dimers, hesitating during the course of digestion every ten nucleotides along the DNA (Prunell & Kornberg, 1978; Riley & Weintraub, 1979; T. Herman, M. DePamphilis, and P. Wassarman, unpublished results). Therefore, steric constraints imposed by replication forks may prevent exonucleases from penetrating nucleosomal DNA.

On the basis of results of experiments reported here and elsewhere (Shelton et al., 1978, 1980; Herman et al., 1979), a model for the replication forks of SV40 chromosomes has been constructed (Figure 9). This model presumably suits polyoma virus as well, since both SV40 and polyoma chromosomes have a histone composition and nucleosome structure indistinguishable from that of their host (Cremisi, 1979; Shelton et al., 1980) and both viruses rely upon the host cell to carry out virtually all steps in DNA replication and chromatin assembly (Fareed & Davoli, 1977; Kelly & Nathans, 1977; Cremisi, 1979). In this model, viral chromosomes consist of 22 ± 2 nucleosomes, each containing about 188 base pairs of DNA, and are separated by highly variable regions of nonnucleosomal DNA that account for about 20% of the genome, thus making the average internucleosomal spacing about 230 ± 50 base pairs (Shelton et al., 1978, 1980). Analysis of nucleosome locations with respect to DNA sequence provided no evidence that nucleosomes are located at unique sites anywhere in the SV40 genome, either on mature or on replicating chromosomes (Shelton et al., 1980; DePamphilis et al., 1980). In addition, analysis of replicating chromosomes revealed that the structure of the two sibling molecules in a single replicating SV40 chromosome were not

identical (DePamphilis et al., 1980). The date were consistent with a near-random distribution of nucleosomes on each sibling molecule. During replication, the first nucleosome encountered on either arm of SV40 replication forks is an average of 125 nucleotides from the 3′ and 5′ ends of long nascent DNA chains. SV40 Okazaki fragments have a broad length distribution of 40–290 nucleotides with an average length of 135 nucleotides (Anderson & DePamphilis, 1979). They are separated from longer chains by about 40 nucleotides (Anderson & DePamphilis, 1979) and are found predominantly, if not exclusively, on retrograde arms of replication forks (Perlman & Huberman, 1977; Kaufmann et al., 1978). Consequently, the first nucleosome on retrograde arms is found, on average, 300 nucleotides from the 5′ ends of Okazaki fragments (135 + 40 + 125). Since a small fraction of Okazaki fragments may be associated with nucleosomes [10% (Herman et al., 1979) to 20% (Figure 4)] and nucleosomes do not occupy unique locations on the genome, it must be assumed that the range of values for the location of nucleosomes about a replication fork is broad. Therefore, synthesis of Okazaki fragments and their assembly into nucleosomes need not occur concomitantly; Okazaki fragments can be first synthesized and then assembled into nucleosomes. This would explain why single-stranded interruptions were not detected in newly assembled nucleosomes from sea urchin embryos (Levy & Jakob, 1978). However, it has also been reported that all Okazaki fragments are in nucleosomes (Hildebrand & Walters, 1976; Schlaeger & Klempnauer, 1978). This apparent contradiction can be explained if the fraction (about 50%) of nascent DNA released as nucleosomes in one study (Hildebrand & Walters, 1976) was not the same fraction of nascent DNA found as Okazaki fragments (about 50%); Okazaki fragments may have been completely degraded during micrococcal nuclease digestion. In the other study, virtually all of the nascent DNA appeared as Okazaki fragments, but only a fraction (about 30%) was shown to be released as nucleosomes. Furthermore, all of these short nascent DNA chains may not be true Okazaki fragments (DePamphilis & Wassarman, 1980).

This view of replication forks (Figure 9) is consistent with a stochastic model for initiation of Okazaki fragments in which nascent DNA chains have a certain probability of being initiated at many sites within an approximately 290 nucleotide region of exposed retrograde DNA template (the "initiation zone"; DePamphilis et al., 1979, 1980; Anderson & DePamphilis, 1979). If the rate-limiting step in DNA replication is assumed to be the unwinding of nucleosomal DNA in front of replication forks, then as a fork moves from one nucleosome to the next, continuous DNA synthesis would maintain the forward arm as duplex DNA, while the resulting single-stranded DNA on the retrograde arm would provide an initiation zone of 230 ± 50 nucleotides, the average nucleosomal spacing in front of replication forks. This model predicts that Okazaki fragments will have an average length of about 115 nucleotides, a broad size distribution with a maximum length of about 280 nucleotides, and a heterogeneous RNA primer sequence; all three predictions are borne out by experimental results (DePamphilis & Wassarman, 1980). This model neither requires nor excludes Okazaki fragments on forward arms. Thus, the regularity of Okazaki fragment initiation and the heterogeneity in their lengths could be imposed on DNA replication by chromatin structure rather than by DNA sequence.

The presence of nucleosomes, or nucleosome-like particles, so close to the actual site of DNA synthesis demonstrates that newly synthesized DNA becomes rapidly associated with chromosomal proteins and does not accumulate as long stretches of "bare" DNA at replication forks. Experimental support for conservative segregation of intact histone octamers during chromosome replication has come from studies of chromosomes replicated in the presence of cycloheximide (Riley & Weintraub, 1979; Seidman et al., 1979). The results of these and other experiments (Leffak et al., 1977; Prior et al., 1980) strongly suggest that old (parental) histones segregate with forward arms of replication forks, without mixing of old and new histones within individual octamers, while newly synthesized histones associate exclusively with retrograde arms. In addition, it is possible that assocation of histones with nascent DNA occurs via a stepwise process, with H3 and H4 being deposited first, followed by H2A and H2B, and then by H1 (Worcel et al., 1978). The idea that newly assembled nucleosomes undergo a "maturation" process as the sites of DNA synthesis move further away is supported by a variety of biochemical data (DePamphilis & Wassarman, 1980). Regardless of the exact mode and sequence of events, it is clear from the results reported here that nascent DNA on both arms of the replication fork is rapidly complexed with histones such that it cannot be degraded by either Exo III or Exo $T7_6$. It is possible, of course, that $(H3)_2(H4)_2$ tetramers alone, which can organize DNA into particles resembling nucleosomal cores (McGhee & Felsenfeld, 1980), are responsible for the protection of nascent DNA from exonucleolytic digestion.

Acknowledgments

We are indebted to Eileen Herlihy for expert technical assistance and to Dr. Earl Shelton for constructive comments during the course of this work. We thank Dr. Michael Engler and Dr. Charles Richardson for generously contributing purified *E. coli* bacteriophage T7 gene 6 exonuclease and Jean Baschnagel for help in the preparation of the figures.

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