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Both Parental Deoxyribonucleic Acid Strands at Each Replication Fork of Replicating Simian Virus 40 Chromosomes Are Cut by a Single-Strand-Specific Endonuclease†

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ABSTRACT: We have measured the relative accessibility to a single-strand-specific endonuclease of the single-stranded DNA on the leading and lagging sides of replication forks in replicating simian virus 40 (SV40) chromosomes. To do this we have digested replicating SV40 chromosomes with a single-strand-specific endonuclease (P1 nuclease) and then characterized the intermediate and final products of digestion by sucrose gradient sedimentation and agarose gel electrophoresis. P1 nuclease rapidly and specifically cleaves parental DNA strands at replication forks, yielding intermediate and final cleavage products which are consistent with an approximately equal rate of nuclease cleavage on both sides of the fork. Thus, single-stranded DNA is approximately as accessible to P1 nuclease on the leading side of the fork as on the lagging side; the simplest interpretation of this observation is that the stretch of single-stranded DNA on the leading side is as long as that on the lagging side.

When the two parental DNA strands unwind from each other at a replication fork, double-stranded DNA is converted to single-stranded DNA. On the lagging side of the replication fork (the side on which the direction of nascent strand synthesis is opposite the direction of fork movement), the length of single-stranded stretch depends on the frequency of initiation of Okazaki pieces (see Figure 1). Available evidence suggests that new Okazaki pieces are started at irregular intervals averaging 135 nucleotides, with a maximum interval of 290 nucleotides [reviewed by DePamphilis & Wassarman (1980)]. Thus a single-stranded stretch as large as 290 nucleotides may be found in front of or behind each Okazaki piece (Figure 1). On the leading side of the fork (the side on which nascent strand synthesis takes place in the same direction as fork movement), the length of single-stranded stretch is determined by the distance from the 3′ end of the nascent strand to the point at which parental strands separate (Figure 1). Nothing is known about this aspect of replication fork structure.

Herman et al. (1979) have previously shown that both deproteinized replicating simian virus 40 (SV40)[1] DNA and the DNA in replicating SV40 chromosomes are hydrolyzed by S1 nuclease (a single-strand-specific nuclease from *Aspergillus oryzae*) at least 10 000-fold more rapidly than are the corresponding form I, nonreplicating DNAs (deproteinized or in chromosomes), but the replicating DNA in chromosomes is cut about 200-fold more slowly than the deproteinized replicating DNA. Thus single-stranded stretches can be detected in replicating DNA, and when the replicating DNA is associated with chromosomal proteins, these stretches are partially protected from nuclease action. Herman et al. (1979) did not distinguish between hydrolysis of the leading side of the fork and hydrolysis of the lagging side.

In the studies described in this paper, we have analyzed the intermediate and final products of cleavage of SV40 chromosomes by P1 nuclease, a single-strand-specific nuclease from *Penicillium citrinum* (Fujimoto et al., 1974). This nuclease has a higher pH optimum than S1 nuclease. By using P1 nuclease, we were able to carry out nuclease digestions at our standard pH for SV40 chromosome preparations (pH 6.8).

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[1] Abbreviations: SV40, simian virus 40; RI DNA, replicating intermediate DNA; form I DNA, covalently closed circular, supercoiled DNA; form II DNA, circular DNA containing one or more nicks or small gaps; form III DNA, linear duplex DNA of full genome length; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane.

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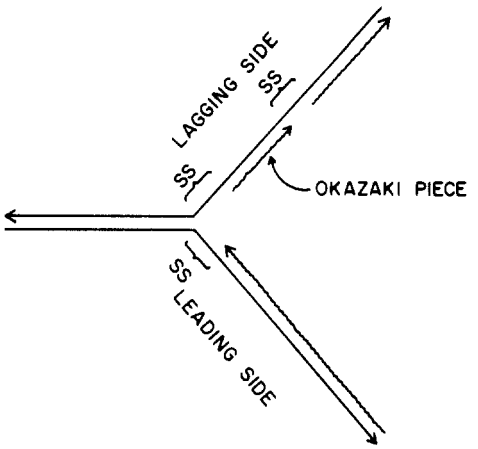

FIGURE 1: Single-stranded regions at a DNA replication fork. In this highly simplified diagram, solid lines represent parental DNA strands, and wavy lines represent daughter (nascent) strands. The arrowheads denote the 3′ ends of the DNA strands. Regions where the parental strand may be single stranded are denoted by "SS".

The results presented here allow us to conclude that single-stranded stretches are detectable on both the leading and lagging sides of the replication fork and that the rate of hydrolysis of the single-stranded DNA on the leading side is similar to that on the lagging side. Thus the possibility exists that the 3′ end of the nascent chain on the leading side of the replication fork may be separated from the fork by a single-stranded stretch of several hundred nucleotides!

Experimental Procedures

SV40 was grown on CV1 cells as previously described (Tsubota et al., 1978), and chromosomes were isolated from SV40-infected CV1 cells by the method previously described (Tsubota et al., 1978) with minor modifications. At 28–34 h after infection in some experiments, [^{14}C]thymidine was added to the cells, and growth was continued until 40 h after infection. At that time, the cells were labeled with [^{3}H]thymidine for 5 min at 37 °C (Tsubota et al., 1978). The labeling with [^{3}H]thymidine was terminated by washing the cells twice with ice-cold isotonic saline. The cells were then washed with buffer A (0.2 M glycerol, 2 mM Hepes, 0.5 mM $MgCl_2$, 0.5 mM PMSF, pH 6.8) and harvested in buffer A (10 mL/plate) with a rubber policeman. The cells were centrifuged and resuspended in 1 mL/plate of buffer A. An equal volume of 0.5% Triton X-100 was added, and the cells were gently homogenized with a Dounce homogenizer to release nuclei. The lysate was layered over an equal volume of buffer A and centrifuged at 3000*g* for 10 min. The pellet (mostly nuclei) was resuspended in buffer B (10 mM Hepes, 1 mM EDTA, 0.5 mM PMSF, pH 6.8), layered over an equal volume of buffer B supplemented with 5% glycerol, and then centrifuged at 3000*g* for 10 min. The pellet was again resuspended in buffer B (2 mL/10 plates) and incubated at 0 °C for 2–4 h. During this time the majority of SV40 chromosomes leached out of the nuclei. Finally, the nuclei were removed by centrifugation at 6000*g* for 10 min. The supernatant contained the SV40 chromosomes [as well as many impurities (Tsubota et al., 1978)]. If chromosomes were stored by freezing (at −20 or −70 °C), glycerol was added to 5%.

Purified P1 nuclease was a gift from Masao Fujimoto (Fujimoto et al., 1979) and was stored at 1 mg/mL in 10 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. Incubations with P1 nuclease were carried out in buffer B at 22–24 °C for 5–10 min or as indicated in the figure legends. We have found that the activity of P1 nuclease is unaffected by the 1 mM EDTA in buffer B.

Sedimentation of DNA through neutral 5–30% sucrose gradients in the SW41 rotor was carried out as previously described (Edenberg et al., 1977), as was sedimentation of denatured DNA through alkaline sucrose gradients in the SW41 rotor (Perlman & Huberman, 1977).

Agarose gel electrophoresis was carried out by using 20-cm-long 1.2% agarose gels. DNA was extracted from SV40 chromosomes by adding $NaDodSO_4$ (0.6%), sucrose (5%), and bromphenol blue (0.04%) and incubating at 37 °C for 30 min. Aliquots of 75 μL were placed in each well of the gel. The electrophoresis buffer (30 mM Tris, 36 mM sodium phosphate, 1 mM EDTA, pH 7.7) was recirculated between electrode chambers. Electrophoresis was carried out at 20 V for 18 h. After electrophoresis the gels were stained with ethidium bromide (1 μg/mL in electrophoresis buffer) and illuminated with a shortwave UV transilluminator. The fluorescence was photographed through a Kodak Wratten 23A filter on Polaroid type 55 positive/negative film. Fluorography of the agarose gels was performed according to Laskey et al. (1977).

Results

Predicted Intermediate and Final Structures during the Hydrolysis of Replicating SV40 DNA by a Single-Strand-Specific Nuclease. In order to distinguish replicating SV40 DNA from nonreplicating DNA in our experiments, we pulse-labeled SV40-infected cells with [^{3}H]thymidine for 5 min immediately before lysing the cells and isolating SV40 chromosomes. During a 5-min period, [^{3}H]thymidine is incorporated predominantly into the long nascent strands of replicating DNA molecules (Tsubota et al., 1978).

If one assumes that hydrolysis of replicating DNA by a single-strand-specific nuclease can occur only at single-stranded stretches of the kind diagrammed in Figure 1, then hydrolysis of SV40 DNA must occur according to one of the pathways diagrammed in Figure 2. In this diagram, only structures containing labeled nascent strands are shown; structures containing only unlabeled parental strands would not be detected in our analyses. Because the experiments to be described do not distinguish between nuclease cuts ahead of or behind Okazaki pieces and because Okazaki pieces would not be preferentially labeled during a pulse as long as 5 min, Okazaki pieces are not shown in Figure 2.

Replicating SV40 DNA ("RI" in the figure) contains two replication forks. Thus there are four possible sites where nuclease cuts could occur, one on each side of the two replication forks (note that the single-stranded regions behind and ahead of Okazaki pieces on the lagging side of each fork are considered to be one site in this discussion). Those four possible sites are labeled "P_1" in the figure. Regardless of where the first cut is located, the product must be a gapped circle with double-stranded tail (X in Figure 2). The circle must have unit SV40 genome length, but the tail may vary from zero to full genome length depending on the extent of replication.

The strand polarities in the structure X shown in Figure 2 are drawn as they would be if the first nuclease cut were on the lagging side of a fork. If the second nuclease cut were on the lagging side of the other fork (site "b" in structure X of Figure 2), then the product would be structure B. Structure B represents a family of gapped linear molecules whose length would vary from SV40 unit genome length to twice SV40 genome length, depending on the extent of replication. Note that the family B would also be generated if the first and second cuts were both on the leading side of replication forks.

There are two other possible sites for the second nuclease cut. Cleavage at site "a" in X would generate the family of

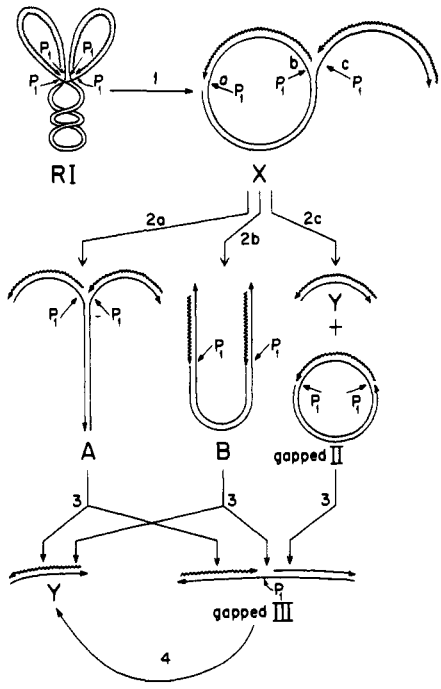

FIGURE 2: Diagram showing likely pathways for the degradation of replicating SV40 DNA by P1 nuclease. The first cut is pictured as taking place on the lagging (discontinuous) side of a replication fork. However, the same intermediates would be produced if the first cut were on the leading (continuous) side. Arrows labeled "P_1" indicate sites at which cutting by P1 nuclease is possible in each structure. Only products of P1 nuclease digestion which contain ^{3}H-labeled, nascent DNA (shown with wiggly lines) are shown. Note that all pathways lead to structure "Y" as the final structure. See text for further details and legend to Figure 1 for further explanations of symbols.

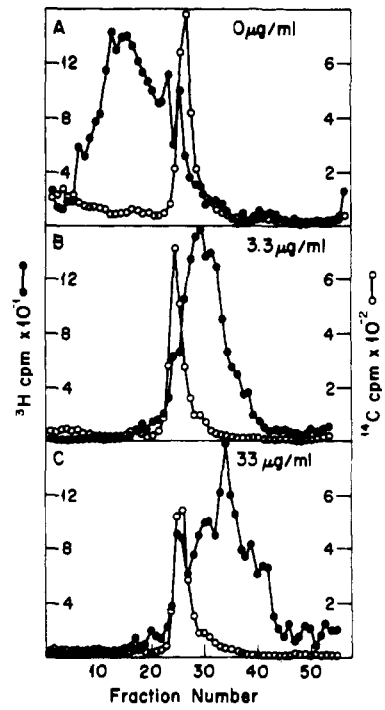

FIGURE 3: P1 nuclease selectively cuts the DNA in replicating SV40 chromosomes. SV40-infected cells were labeled with [^{14}C]thymidine overnight (to label nonreplicating DNA) and with [^{3}H]thymidine for 5 min (to label replicating DNA), and then SV40 chromosomes were isolated as described under Experimental Procedures. Samples (0.4 mL) of crude SV40 chromosome extract (50 μg of DNA/mL) were incubated with the indicated amounts of P1 nuclease at 25 °C for 10 min. The reactions were stopped by addition of EDTA to 10 mM and cooling to 0 °C. Half of each sample was treated with 0.6% $NaDodSO_4$ for 30 min at 37 °C in order to separate proteins from DNA, then layered on a neutral sucrose gradient, and centrifuged as described. Fractions were collected from the bottom, and acid-precipitable radioactivity was determined on samples of equal volume from each fraction. Sedimentation in this and the next figure is from right to left.

structures A, while cleavage at site "c" would yield a gapped form II circle of unit genome length plus a family of linear molecules, Y, which would vary in length from zero to one SV40 genome, depending on the extent of replication. A third nuclease cut in structures A or B, or in gapped form II molecules, would yield gapped form III molecules plus structures Y. After a fourth cut in the gapped form III molecules, another set of Y structures would be generated. Thus, if nuclease cuts can occur on both sides of each replication fork, Y structures would be the final products of nuclease digestion, while if cuts can occur *only* on the lagging or leading side, B structures would be the final products. If cuts occur much more rapidly (but not exclusively) on the lagging or leading side, then B structures would accumulate as intermediates, but Y structures would be the final products of digestion.

P1 Nuclease Cuts Only the Parental DNA Strands in Replicating SV40 Chromosomes. If P1 nuclease cuts only single-stranded DNA, then P1 nuclease should cut the DNA in replicating chromosomes but not in mature chromosomes, and within replicating chromosomes, P1 nuclease should cut only the parental strands. The data in Figure 3 show that when a mixture of replicating (labeled with [^{3}H]thymidine) and nonreplicating (labeled with [^{14}C]thymidine) SV40 chromosomes is treated with P1 nuclease, the replicating DNA is cut but the nonreplicating DNA is not. Note that Figure 3 shows the sedimentation of deproteinized *DNA*. The effects of P1 nuclease on the sedimentation of SV40 *chromosomes* will be presented in another paper (M. A. Waqar et al., unpublished results). The peak sedimentation rate in neutral sucrose gradients of intact replicating SV40 DNA is about 28 S; in these experiments the peak sedimentation rate is reduced by P1 nuclease to 17 S (Figure 3B) or 14 S (Figure 3C), depending on the amount of P1 nuclease used.

To test whether P1 nuclease cuts the parental or daughter DNA strands in replicating chromosomes, we treated a preparation of chromosomes whose daughter strands were labeled with [^{3}H]thymidine with P1 nuclease, then deproteinized the DNA, and sedimented half the DNA through a neutral sucrose gradient (as in Figure 3) and the other half of the DNA through an alkaline sucrose gradient. The data in Figure 4A,B show that when the DNA is analyzed by neutral sucrose gradient sedimentation (so that the sedimentation rate of the daughter strands is primarily influenced by the intactness of the parental strands), P1 nuclease causes a pronounced decrease in sedimentation rate (to about 16 S) as in Figure 3. However, if the DNA is denatured and sedimented through alkaline sucrose gradients (so that the sedimentation of the daughter strands can be measured independently of the parental strands) no change in sedimentation rate can be detected (Figure 4C,D). Thus the nuclease cuts responsible for the changes in sedimentation rate detected in neutral sucrose gradients (Figures 3 and 4A,B) must be cuts in parental, not daughter, strands. We cannot rule out frequent cuts near the ends of daughter strands nor infrequent cuts—much less than one cut per chromosome—near the middles of daughter strands. However, neither of these could be responsible for the dramatic decrease in sedimentation rate seen in Figures 3 and 4.

Notice that we have selectively labeled the replicating DNA by pulse labeling with [^{3}H]thymidine for 5 min at 37 °C. That pulse-labeling time is so long compared to the half-life of

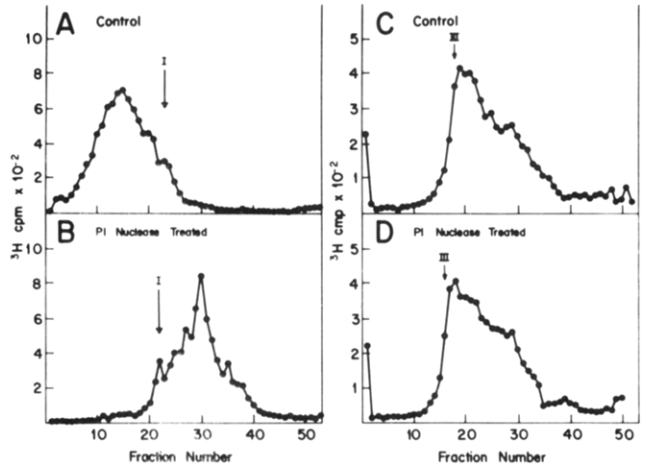

FIGURE 4: P1 nuclease does not cut the long nascent DNA strands in replicating SV40 chromosomes. SV40 chromosomes (2.0 mL) were isolated from cells which had been labeled for 5 min at 37 °C with [^{3}H]thymidine. The chromosomes were then incubated for 10 min at 23 °C with or without 13 μg/mL P1 nuclease. The chromosomes were deproteinized by incubation with 0.6% $NaDodSO_4$ at 37 °C for 15 min, and then aliquots of 0.5 mL were sedimented through neutral sucrose gradients (A, B) or alkaline sucrose gradients (C, D). The arrows marked "I" in A and B mark the positions to which marker ^{14}C-labeled SV40 form I DNA sedimented in the same tubes; the arrows marked "III" in C and D denote the positions to which genome-length linear single-stranded SV40 DNA (generated by denaturation of *Eco*RI-cleaved ^{14}C-labeled SV40 DNA) sedimented in the same tubes.

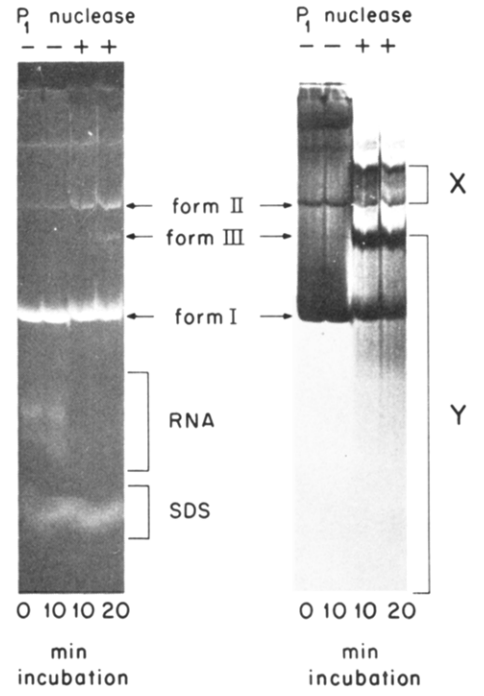

FIGURE 5: Effects of P1 nuclease on the agarose gel electrophoresis of DNA from SV40 chromosomes. SV40 chromosomes were extracted from cells labeled with [^{3}H]thymidine for 5 min, and to the chromosome extract at 0 °C were added sodium acetate buffer (pH 6.0) and $MgCl_2$ to final concentrations of 7.4 and 1.5 mM, respectively. To one-fourth of the extract was immediately added EDTA to 10 mM. The second one-fourth of the extract was incubated at 37 °C for 10 min before addition of EDTA and chilling to 0 °C. The third and fourth quarters of the extract were incubated at 37 °C for 10 or 20 min in the presence of 22 μg/mL P1 nuclease before addition of EDTA and chilling. Vertical agarose gel electrophoresis was performed as described under Experimental Procedures. The left lanes show a photograph of the ethidium bromide fluorescence generated by DNA in nonreplicating chromosomes and by RNA and $NaDodSO_4$. The right lanes show the fluorogram of the same gel. Only ^{3}H-labeled DNA is visible.

Okazaki pieces [see Perlman & Huberman (1977)] that Okazaki pieces cannot be detected in these experiments. Our experiments investigating the effects of P1 nuclease on Okazaki pieces will be described elsewhere (M. A. Waqar et al., unpublished results). Herman et al. (1979) have shown that S1 nuclease releases a significant fraction of Okazaki pieces from replicating SV40 chromosomes in double-stranded form, as would be predicted from Figure 1.

Analysis of the products of P1 nuclease treatment of replicating SV40 chromosomes by sucrose gradient sedimentation allowed us to conclude that P1 nuclease specifically cuts parental DNA strands, but the resolution of sucrose gradient sedimentation is not sufficient to distinguish between the products predicted in Figure 2. To achieve higher resolution we turned to agarose gel electrophoresis.

P1 Nuclease Cuts Both Parental DNA Strands at Each Replication Fork. When SV40 chromosomes were extracted from cells labeled for 5 min with [^{3}H]thymidine and half the chromosomes were treated with P1 nuclease, the DNA extracted from those chromosomes migrated in 1.2% agarose gels as shown in Figure 5. The left lanes of Figure 5 show the distribution of ethidium bromide fluorescence in these gels. Because nonreplicating DNA was present in about 100-fold excess over replicating DNA, the fluorescence shows the distribution of nonreplicating DNA. This DNA is mostly form I DNA, with smaller amounts of form II DNA and a third unidentified species which migrates more slowly than form II DNA. Incubation for 10 min with 22 μg/mL P1 nuclease led to slight conversion of form I DNA to form II DNA. After an additional 10 min of incubation, additional form II DNA and a small amount of form III DNA were formed. Thus, under these conditions, unlike the conditions used in Figure 3, form I DNA in nonreplicating chromosomes *was* slowly nicked to forms II and III by P1 nuclease. Note that the low level of ethidium bromide fluorescence due to contaminating RNA in these crude chromosome preparations was eliminated by P1 nuclease.

When the distribution of *replicating* DNA in the agarose gel was made visible by fluorography (right lanes of Figure 5), a much more pronounced effect of P1 nuclease could be seen. Replicating DNA from control chromosomes (not treated with P1 nuclease) was distributed in a smear from the position of form I DNA to the top of the gel. As shown by others (Tapper & DePamphilis, 1978; Sundin & Varshavsky, 1980), this smear represents chromosomes which have completed increasing extents of replication, from the form I position (chromosomes initiating replication) to the heavy band near the top of the gel (chromosomes which have completed 90–97% of their replication); a kinetic block after 90–97% replication causes accumulation of replication intermediates at this position (Tapper & DePamphilis, 1978, 1980; Seidman & Salzman, 1979; Sundin & Varshavsky, 1980). After P1 nuclease digestion, nearly all label is removed from the top region of the gel, and most label is now found in (a) a smear of fragments running from the bottom of the gel to a dark band which migrates slightly faster than form III DNA (region Y), (b) a smear of fragments running from the form II position upward and terminating in a dark band (region X), and (c) a dark band in the form I position which is due to incorporation of [^{3}H]thymidine into form I DNA during the 5-min labeling period.

Interpretation of the products of P1 digestion of replicating chromosomes is aided by reference to the predictions in Figure 2. The structures formed by the first P1 cut, structures X, would be expected to migrate in an agarose gel as a smear

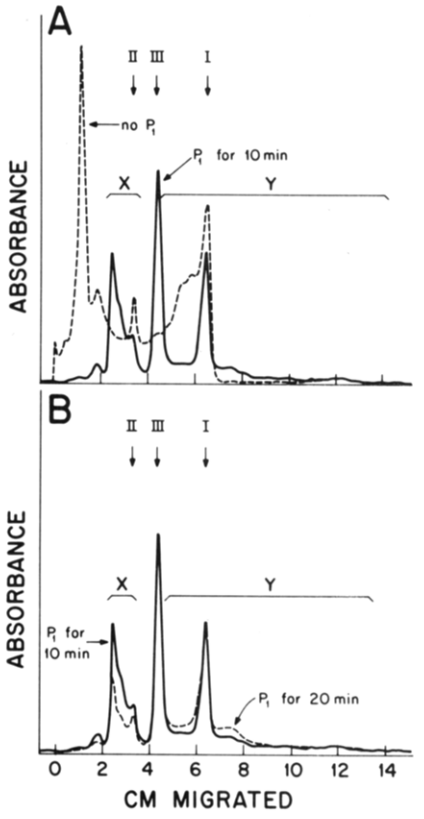

FIGURE 6: Densitometric scanning of the fluorogram from Figure 5. The scans were made by using a Corning Model 750 scanning densitometer. The solid line in panels A and B is the scan of the sample incubated *with* P1 nuclease for *10* min. The dashed line in panel A is the scan of the sample incubated *without* P1 nuclease for *10* min, while the dashed line in panel B is the scan of the sample incubated *with* P1 nuclease for *20* min.

running from the form II position upward, depending on the extent of replication. The region marked X in Figure 5 may well correspond to this predicted smear. The family of structures labeled Y in Figure 2 (formed by two, three, or four nuclease cuts) would be expected to migrate in an agarose gel as a smear running from the bottom of the gel up to the form III position, again depending on the extent of replication. The region marked Y in Figure 5 appears to correspond to this predicted smear, and the dark band which migrates slightly faster than form III appears to correspond to the Y structure which would be generated by complete P1 nuclease digestion of the replicating DNA molecules accumulated at 90–97% completion of replication.

Notice that the family of structures which would be generated by two successive cuts, one at each replication fork, with both cuts on the lagging side or both cuts on the leading side (family B), would be expected to migrate as a smear running from the form III position upward to the position of form III dimer, depending on the extent of replication. Detection of such a smear is rendered difficult by the presence of the smear due to the X family. However, if present, the B family smear must be present at low level. Likewise, the smear predicted for the A family of Figure 2 (which should migrate in an agarose gel over the same range as the B family) is not detectable. The intermediates predicted if the second cut were at site c in Figure 2 (Y structures and gapped form II molecules) may be present in Figure 5. Gapped form II molecules would migrate at the form II position.

The scans of a less heavily exposed fluorogram shown in Figure 6 indicate that the region X material decreases during prolonged nuclease digestion while the region Y material increases, consistent with the region X material being intermediate structures which can be further cut to generate Y structures as final products. The fluorogram of a similar experiment, in which P1 nuclease digestion was carried out for a longer time, is shown in Figure 7. Here, too, the region X material decreases with time of digestion while the region Y material increases (see Table I). Furthermore, the presence of labeled form III marker DNA in Figure 7 allows one to see that the heavy bands at the top of the Y smears in Figure 7 have migrated slightly faster than form III DNA, consistent with those heavy bands being due to linear fragments cut from replicating SV40 chromosomes which accumulated at 90–97% completion of replication.

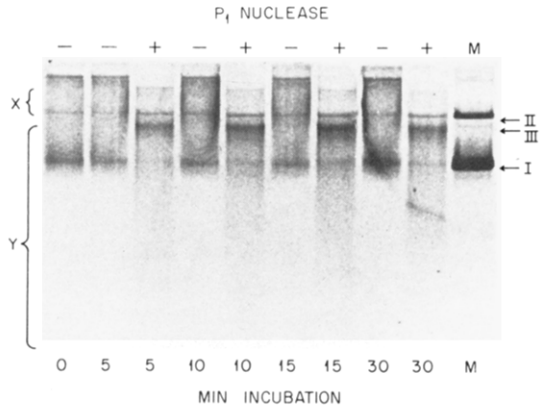

FIGURE 7: Analysis by agarose gel electrophoresis of the effects of incubating replicating SV40 chromosomes with P1 nuclease for varying time intervals. SV40 chromosomes were prepared from cells labeled for 5 min with [^{3}H]thymidine. The crude chromosome extract was incubated in the absence or presence (20 μg/mL) of P1 nuclease for the indicated length of time at 22 °C. At the indicated times, samples of 0.1 mL were taken, and EDTA and sodium dodecyl sulfate were added to final concentrations of 10 mM and 0.6%, respectively. The samples were mixed and then incubated at 37 °C for 30 min. Samples of 60 μL were mixed with sucrose (5%) and bromphenol blue (0.04%) and then electrophoresed in a horizontal gel apparatus. The lane labeled "M" contains a mixture of ^{3}H-labeled SV40 form I, form II, and form III DNAs.

Table I: "X" Structures (Intermediate Products) and "Y" Structures (Final Products) of P1 Nuclease Digestion of Replicating SV40 Chromosomes[a]

time of incubation with P1 nuclease (min)	total ^{3}H in gel lane (%)	
	X	Y
5	13	68
10	7	77
15	6	79
30	4	85

[a] The fluorogram in Figure 7 was scanned, and the proportions of total grain density above background in each lane represented by regions X and Y were determined. The grain density due to form II DNA was excluded from the X region measurement by starting the X region measurement immediately above the form II peak, and the grain density due to form I DNA was excluded from the Y region measurement by subtracting the form I peak (above base line) from the Y region measurement.

In summary, the data in Figures 5–7 and Table I are consistent with the intermediate and final structures predicted in Figure 2 if P1 nuclease can cleave both parental strands at both replication forks. Since B structures do not accumulate, P1 nuclease must attack both the leading and lagging sides of the replication fork at approximately equal rates.

Discussion

The results of the experiments described here suggest that there are single-stranded stretches of parental DNA, of ap-

proximately equal overall accessibility to P1 nuclease, on both the leading and lagging sides of the replication forks in replicating SV40 chromosomes. The "approximately equal overall accessibility" of these single-stranded stretches to P1 nuclease can be explained in several ways. The simplest explanation is that the single-stranded stretches on both sides of the fork have approximately the same conformation and approximately the same length. Another possibility is that the lengths of single-stranded DNA on the two sides of the fork differ significantly, but that the shorter stretch is in a conformation which renders it significantly more exposed to P1 nuclease than the longer stretch. Yet a third possibility is that the single-stranded region on one side of the fork *is* significantly more accessible to P1 nuclease so that the *first* nuclease cut almost always occurs on that side of the fork, and once the first nuclease cut has occurred, a conformational change takes place which makes the opposite sides of the forks very accessible to P1 nuclease so that the *second* cut almost always occurs on the opposite side of the same fork (2a in Figure 2) or on the opposite side of the other fork (2c in Figure 2). Although this third possibility is consistent with the data presented in this paper, it is not supported by our observation that, under certain conditions, the sedimentation rate of replicating SV40 chromosomes is not altered after one or more cuts have been made at the replication forks by P1 nuclease (M. A. Waqar et al., unpublished results). A significant conformational change would, if it occurred, be likely to affect the sedimentation rate.

Additional experiments will be needed to distinguish between these possibilities. Additional experiments will also be necessary to answer some of the other questions raised by the observations in this paper. Exactly how long are the single-stranded stretches on each side of the fork? What proteins are responsible for keeping these stretches single stranded? Are the single-stranded stretches on opposite sides of the fork in phase with each other so that rewinding could potentially occur (as in the case of the two fork-proximal stretches in Figure 1) or out of phase (as in the case of the fork-distal stretch on the lagging side of the fork in Figure 1)?

The presence of stretches of single-stranded DNA on the leading as well as on the lagging side of replication forks can provide a possible explanation for the observation of Okazaki pieces on the leading side of the fork (Narkhammar-Meuth et al., 1981a,b). The enzymes responsible for initiating Okazaki pieces may well recognize *any* sizable stretch of single-stranded DNA without regard to whether that stretch is on the leading side or the lagging side of the fork (DePamphilis & Wassarman, 1980).

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