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**Okazaki pieces grow opposite to the replication fork direction during simian virus 40 DNA replication**

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Gabriel Kaufmann\*<sup>+</sup>, Rachel Bar-Shavit\* and Melvin L. DePamphilis\*\*

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\*The Biochemistry Department, The Weizmann Institute of Science, Rehovot, Israel and \*\*Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115, USA

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**ABSTRACT**

Simian virus 40 replicating DNA was pulse labeled with  $\alpha$ -<sup>32</sup>P-dATP using an acellular DNA replication system. Nascent DNA chains of less than 200 nucleotides (Okazaki pieces) were then isolated from the denatured replicating DNA by electrosieving through a polyacrylamide gel column. The purified Okazaki pieces were hybridized to separated strands of Bgl<sub>1</sub>+Hpa<sub>1</sub> simian virus 40 DNA restriction fragments immobilized on nitrocellulose filters. Only strands with polarity of the DNA replication fork direction hybridized with Okazaki pieces. Hence, Okazaki pieces in simian virus 40 are synthesized against the DNA replication fork direction.

**INTRODUCTION**

Short, nascent DNA chains are recurrently synthesized and ligated during the replication of numerous chromosome types. The discovery of these intermediates, termed Okazaki pieces, suggested how one daughter strand may be synthesized against the overall direction of DNA synthesis(1). Nevertheless, several investigators have reported that Okazaki pieces originate from both arms of the DNA replication fork. Thus, conflicting models explain the origin of Okazaki pieces in papova viruses. According to one view, papova virus DNA synthesis is discontinuous in both fork direction (2,3). Others suggested that DNA synthesis in polyoma (cf. 4) and SV40 (5) might occur semidiscontinuously, where Okazaki pieces originate from the "necessarilly discontinuous" (synthesized against the over-all direction of DNA synthesis) but not from the "potentially continuous" arm (5) although an intermediate case of partial discontinuous DNA synthesis on the "potentially continuous" arm has been considered too (4,5,6).

The present communication describes the hybridization of in vitro synthesized SV40 nascent DNA chains to an excess of single stranded DNA fragments of unidirectional fork polarities. The results of this experiment are consistent with semidiscontinuous DNA synthesis in SV40, in which Okazaki pieces are synthesized only against the fork direction.

METHODS

Preparation of single stranded SV40 DNA fragments of unidirectional fork polarity

SV40 (F1) DNA was digested by a combination of Bgl<sub>1</sub> and Hpa<sub>1</sub> restriction endonucleases (New England Biolab) and the resulting fragments were separated into single strands. The reaction mixture (0.4 ml) contained 64 µg of SV40 (F1) DNA, 60 units of Bgl<sub>1</sub>, 50 units of Hpa<sub>1</sub>, 10 mM MgCl<sub>2</sub>, 10 mM Tris HCl, pH 7.5; 1mM dithiothreitol and 6 mM KCl. Incubation was at 25°C for 16 hours, after which the DNA was precipitated with ethanol and dissolved in 10 mM NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA. Aliquots were separated by 1.8% agarose slab (150x200x1.5mm) gel electrophoresis in 0.1 M Tris borate pH 8.3, 2.5mM NaEDTA either as such (0.05 µg per an 8 mm slot, Fig. 1 right) or after alkali denaturation (0.5 µg aliquots were made up to 8 µl 0.2 M NaOH, incubated for 10 min at 25°C, chilled and mixed with 3 µl of 0.2% bromophenol blue in 50% sucrose - Fig. 1 left). Electrophoresis was conducted at 3V/cm at 2° for 16-20 hours until the tracking dye reached 0.8-1.0 of the gel length. The gel was stained with 0.5 µg/ml of ethidium bromide in electrophoresis buffer. Strand pair assignments were determined by parallel electrophoresis of denatured individual fragments. In addition to denatured fragments migrating as distinct bands we observed a small quantity of smear, apparently due to "trailing" and to non specific single stranded breaks that had occurred during the digestion and subsequent separation steps.

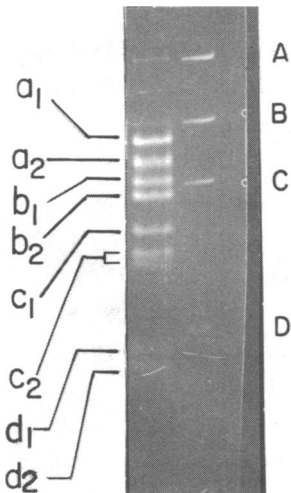


Fig. 1. Strand separation of Bgl<sub>1</sub>/Hpa<sub>1</sub> restriction fragments of SV40 DNA. Right column - native fragments, left - denatured fragments.

Preparation of unidirectional strand - nitrocellulose filters

Agarose gel portions containing separated strands of Bgl<sub>1</sub>/Hpa<sub>1</sub> SV40 DNA restriction fragments (Fig.1) were destained by incubation in 0.9 M NaCl, 0.09 M sodium citrate (6xSSC) and "blotted" onto nitrocellulose filter strips (Schleicher and Schull BA-85) according to Southern (8), except that no prior alkali denaturation was required. The filters were air dried and baked for 2 hours at 80°C in vacuo.

Fork-polarity determination of the separate strands

Aliquots containing 1 µg <sup>32</sup>P-labeled SV40 DNA Bgl<sub>1</sub>/Hpa<sub>1</sub> fragments (about 20,000 cpm) were incubated in 10 µl formamide for 2 min at 50°C. The denatured samples were quickly mixed with 10 µl of 1.5 M NaCl, 0.2 M Tris HCl buffer, pH 7.5, 10 mM EDTA containing up to 10 µg of SV40 cRNA (7). The mixture was incubated at 37°C for 1 hour. Electrophoresis was carried out as described in the previous section. At the end of electrophoresis the gel was dried and autoradiographed. Those strands hybridizing with cRNA were not seen on the gel in mixtures containing high excess of SV40 cRNA. The direction of synthesis of the various DNA strands was deciphered by their ability to hybridize with cRNA (table 1)

Strand <sup>a</sup>	Hybridization <sup>b</sup> with cRNA	Fork <sup>c</sup> polarity	Fork polarity of complementary Okazaki pieces
a <sub>1</sub>	-	-	+
a <sub>2</sub>	+	+	-
b <sub>1</sub>	+	-	+
b <sub>2</sub>	-	+	-
c <sub>1</sub>	+	-	+
c <sub>2</sub>	-	+	-
d <sub>1</sub>	-	-	+
d <sub>2</sub>	+	+	-

Table 1 - Determination of fork polarity of unidirectional strands by their ability to hybridize with cRNA

a - Shown in Fig. 1. b - +hybridizing, -not hybridizing, c - +potentially continuous polarity, - necessarily discontinuous polarity.

Preparation of Okazaki pieces

SV40 Okazaki pieces were prepared from in vitro α-<sup>32</sup>P dATP (100 Ci/mmol) pulse-labeled SV40 (R1) DNA by a modification of a previous procedure (9). Following Sepharose 6B filtration the viral DNA was precipitated with ethanol and the pellet dissolved in 99% formamide containing 0.02% each

of Bromophenol blue and Xylene cyanol FF. The solution was heated 2 min at 50°C and applied on top of a column (4x0.6 cm) of 7.5% polyacrylamide gel in 7 M urea, 0.1 M Tris borate buffer pH 8.3, 2.5 mM EDTA made up in a pipette tip. To the tips bottom was attached a dialysis bag containing 0.5 ml of electrophoresis buffer. Electrophoresis was conducted for 4 hours at 100 V until the tracking dye XCFE has twice traversed the column. Under these conditions all DNA chains shorter than 200 nucleotides were well separated from longer chains (Fig. 2). Okazaki pieces were then precipitated with ethanol and carrier *E. coli* transfer RNA and served for subsequent hybridization experiments. The long chains were extracted from the finely ground top portion of gel-columns by shaking them with electrophoresis buffer. The extract was filtered through GF/C paper and the DNA precipitated with ethanol and transfer RNA and served for hybridization as indicated. The entire isolation procedure of Okazaki pieces from *in vitro* synthesized SV40 (R1) DNA lasted less than 24 hours. This reduced the background level of labeled short fragments resulting from radiochemical breaks of long chains.

DNA-DNA hybridization

Nitrocellulose filters of "unidirectional" DNA strands were preincubated

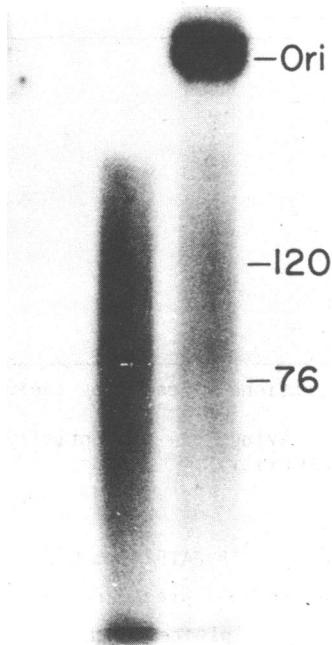
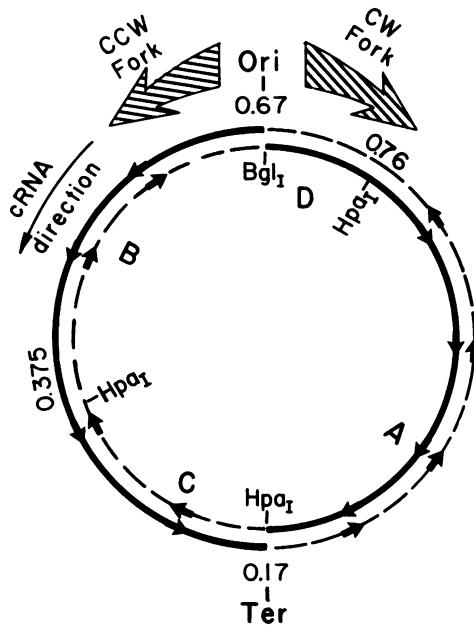


Fig. 2: Purity of electrosieved Okazaki pieces - Aliquots of denatured <sup>32</sup>P-SV40(R1) DNA (right) and of "electrosieved" Okazaki pieces (left) were separated by 7.5% polyacrylamide - urea gel electrophoresis and autoradiographed. Chain length markers were yeast 5S RNA and tRNA<sup>phe</sup>

in 50% formamide containing 0.75 M NaCl, 0.1 M Tris HCl pH 7.5, 5mM EDTA, 0.5% sodium lauryl sulfate and 0.02% each of ficoll, polyvinylpyrrolidone and bovine serum albumin (10) for at least 30 minutes at 37°C. The filters were drained and the hybridization medium containing Okazaki pieces was added. The medium was prepared as follows: The pellet containing <sup>32</sup>P Okazaki pieces was dissolved in formamide containing 1% SDS and heated for 2 minutes at 50°C. Afterwards, it was quickly mixed with an equal volume of 1.5 M NaCl, 0.2 M Tris HCl pH 7.5, 10 mM EDTA and 0.4% each of ficoll, polyvinylpyrrolidone and bovine serum albumin. Usually the final concentration of Okazaki pieces was 5x10<sup>5</sup> cpm/ml and 0.4 - 0.6 ml of solution were applied to filter strips measuring 1x8 cm. The filters and solution were shaken in sealed plastic envelopes for 24 hours at 37°C. At the end of incubation the solution was removed by centrifuging the pierced envelope at low speed. The filter was removed, washed with 50 ml each of hybridization buffer followed by 6xSSC. The filter was drained and autoradiographed. By a similar procedure the filters were hybridized with in vitro labeled long DNA chains.



Scheme 1 -SV40 genome map with "unidirectional strand" polarities. --- "necessarily discontinuous"polarity. \_\_\_ "potentially continuous"polarity, arrows point in 5' to 3' direction. The cleavage sites are expressed in distance from the Eco RI site.

RESULTS AND DISCUSSION

Our aim was to determine the fork polarity of SV40 Okazaki pieces. This was achieved by measuring the extent to which Okazaki pieces were able to hybridize with single stranded SV40 DNA fragments of different replication fork polarities. Such fragments, termed unidirectional strands, were prepared by a two step procedure. First, SV40 (F1) DNA was digested by a mixture of Bgl<sub>1</sub> (11) and Hpa<sub>1</sub> (12) endonucleases. The fragments thus produced (Fig. 1) are essentially synthesized by either the clockwise (A and D) or counter clockwise (B and C) DNA replication fork (scheme 1). Therefore each of these fragments consists of a pair of unidirectional strands synthesized either with or against the replication fork movement. Next, the fragments were denatured by alkali and resulting unidirectional strands separated by agarose gel electrophoresis (Fig. 1). The fork polarity of each strand was then determined by its ability to hybridize with cRNA. It was found that the fast migrating strand in each pair is synthesized with the direction of the replication fork (Table 1). The unidirectional strands were then blotted onto nitrocellulose sheets (8) and served in this form for DNA-DNA hybridizations with pulse labeled nascent DNA. The strand assignments (Table 1) were recently confirmed by hybridizing either E or L strands of SV40 DNA to these filters (Laub and Aloni, unpublished).

Okazaki pieces and longer nascent DNA chains were isolated from  $\alpha$ -<sup>32</sup>P-dATP, in vitro synthesized SV40 (R1) DNA (9). In the in vitro system employed, SV40 replicative DNA is ultimately converted into mature, covalently closed superhelical DNA. It is expected therefore that intermediate steps in the replication process, such as Okazaki piece synthesis, are faithfully performed as well (13). In the present experiment the method of Okazaki piece isolation (9) was modified however, to minimize the duration of isolation and improve the degree of resolution. Thus, Okazaki pieces were recovered from denatured SV40 (R1) DNA by electrosieving through a small column of polyacrylamide-urea gel (Fig. 2).

The hybridization of Okazaki pieces to unidirectional DNA strands immobilized on nitrocellulose filters was found to be highly asymmetrical. Thus, by inspection of the autoradiogram ( Fig. 3) and of the distribution of radioactivity along the filter (Fig. 4), it appeared that Okazaki pieces hybridized predominantly to the fast bands in each of the pairs A, B and C. A similar pattern, although of a less-pronounced asymmetry was seen with pair D. Since the polarity of the fast strands was of the fork direction, it follows that the bulk of Okazaki pieces that hybridized were synthesized



Fig. 3. Hybridization of Okazaki pieces with unidirectional strands immobilized on a nitrocellulose filter. Letters mark strand position

against the fork direction. However, it was not possible to calculate accurately the ratio between the Okazaki chain populations hybridizing to either strand in each of the 4 pairs because of band overlap and non specific background hybridization. The latter was largely due to the DNA smear in the agarose gel (Methods). Nevertheless, by considering these factors we have estimated these ratios to be 1:20 and 1:4 in pairs A and D respectively.

Of the input Okazaki pieces, about 70% hybridized with unidirectional DNA strands immobilized on filter (Fig. 4). The inability of the unbound fraction to hybridize could be attributed to several factors. Thus, it might have happened due to the presence of a small, albeit significant, Okazaki piece population of the potentially continuous fork polarity, preferentially annealed to its complementary counterpart in solution, rather than be hybridized to the complementary DNA on the filter. This possibility does not seem likely, however, since the DNA on the filter was present in vast excess over that in solution (about 500 ng vs. 1.5 ng). Furthermore, in experiments to be discussed below, short chains of the potentially continuous polarity, obtained by radiolysis of long chains, hybridized efficiently with the immobilized DNA on the filter although they were mixed with an excess of

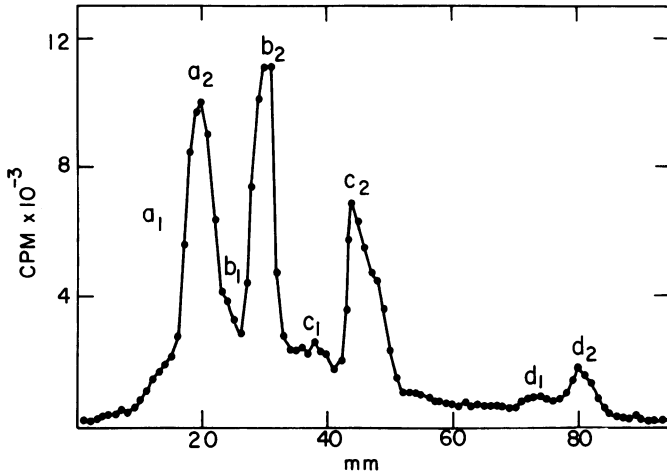


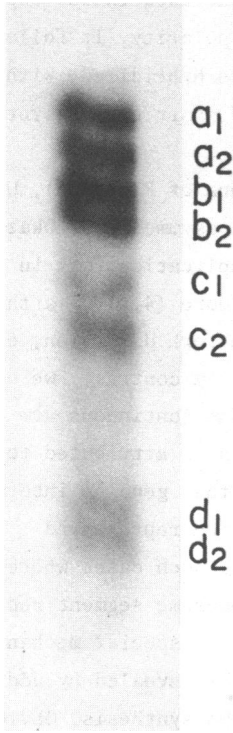
Fig. 4. Hybridization of Okazaki pieces to unidirectional DNA strands immobilized on a nitrocellulose filter. The filter of Fig. 3 was cut into 1 mm slices which were counted. The input radioactivity was 300,000 cpm, of which 216, 550 cpm, were bound.

pieces of the opposite polarity. Unequal amounts of slow and fast DNA on the filter could also contribute to the apparent asymmetry. However, this possibility was excluded by the above consideration and by the symmetrical hybridization of the long nascent DNA chains to a unidirectional DNA - nitrocellulose filter (Fig. 5). It was concluded therefore that the highly asymmetrical mode in which Okazaki pieces hybridized to the unidirectional DNA- nitrocellulose filters reflected the original preponderance of Okazaki pieces derived from the necessarily discontinuous arm of the replication fork.

As mentioned above, the asymmetry of Okazaki pieces hybridization was less pronounced with pair D. Nevertheless, this exception can be reconciled with the foregoing conclusion about the highly asymmetrical origin of Okazaki pieces. Namely, in this short (350 nucleotides) region near the origin of DNA replication one expects a considerable proportion of continuously synthesized chains to be in the size range of Okazaki pieces (4,5). The same argument applies, although to a much lesser extent, to pair B.

Numerous factors may artefactually decrease the apparent asymmetry of Okazaki pieces (cf. 4,5,14). We shall discuss two which were largely overcome and one still inherent in our experiment. First, sedimentation in alkaline sucrose gradient does not sufficiently resolve Okazaki pieces from longer nascent DNA chains (5). Such cross contamination did not occur, however, when Okazaki pieces were isolated by gel electrophoresis (Fig.2).





**Fig. 5.** Hybridization of long nascent DNA chains with unidirectional strands immobilized on a nitrocellulose filter. Letters mark strand position

Secondly, radiochemical breaks in long chains produce artefactual short DNA chains. Thus, a considerable proportion of DNA chains synthesized from  $\alpha$ - $^{32}\text{P}$ -dNTP of high specific radioactivity contains more than one labeled atom. Upon radioactive decay these chains will be converted into shorter labeled fragments. Indeed, with "aging" of  $\alpha$ - $^{32}\text{P}$ -dATP *in vitro* labeled SV40 (R1) DNA preparations, we have observed an increasing proportion of "Okazaki pieces" with a decreasing asymmetry of hybridization to unidirectional strands. For example, short chains derived from an "aged" preparation of replicating DNA, in which the ratio of short to long chains shifted from the original 52:48 to 63:37, hybridized to the  $a_1:a_2$  unidirectional strand pair at a 1:4 ratio (results not shown) compared to about 1:20 obtained with "fresh" Okazaki pieces (Figs 3 and 4). We estimated the level of short chains produced by radiochemical breaks under the standard conditions of Okazaki pieces isolation (METHODS) to be less than 2% of the total radioactivity in long chains, assuming the latter were extended on the average by 200 nucleotides during the radioactive labeling period.

Finally, it is not known whether  $\text{Bgl}_1$  and  $\text{Hpa}_1$  restriction endonucleases sites 0.67 and 0.17 (scheme 1) coincide with the origin and terminus of DNA

replication. Consequently, each unidirectional strand used in our experiment may have contained a short segment of the opposite polarity. It follows that strands  $a_1$ ,  $b_1$ ,  $c_1$  and  $d_1$  (Fig. 1, Table 1) may have hybridized, within the range of this uncertainty, with a small fraction of short chains synthesized against the fork direction.

Our data agree in part with previous conclusions of Flory (6), Hunter et al (4) and of Perlman and Huberman (5) about the asymmetry of Okazaki pieces distribution between the two arms of the replication fork in papova virus. Yet in their experiments about one fifth or more (4,5) or a third (6) of Okazaki pieces seemed to be synthesized with the fork direction, on the potentially continuous arm of the replication fork. By contrast, we observed a much lower proportion of Okazaki pieces of apparent continuous arm polarity which were dismissed as radiochemical decay products or attributed to the incomplete unidirectionality of our probes, rather than genuine intermediates of DNA replication. Conversely, if this small fraction represented discontinuous forward synthesis, we have to envisage such cases where less than one Okazaki piece is synthesized per chromosome segment replicated by one fork, or that some DNA molecules replicate by a special mechanism.

The asymmetry of the DNA replication fork may be revealed by additional aspects related to the semidiscontinuous mode of DNA synthesis. Obviously, in such a mechanism initiator RNA of Okazaki pieces (9,15) can arise only from the "necessarily discontinuous" arm. Consequently it may be asked whether the same DNA polymerase(s) extends both the all DNA chain on the continuous arm and the RNA-DNA hybrid chain on the discontinuous arm(16). Similarly, in the conservative model of chromatin replication, in which only one of the daughter chromosomes becomes associated with the parental histones (17,18), the continuous arm may be the more probable candidate for retaining the parental nucleosome structure because its double-strandedness is least perturbed.

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<sup>†</sup>To whom all correspondence should be addressed

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