Strandedness of newly synthesized short pieces of polyoma DNA from isolated nuclei*

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ABSTRACT

     The discontinuous synthesis of the complementary strands of polyoma DNA in isolated nuclei has been studied by hybridization techniques. The relative amounts of the newly synthesized complementary strands were compared by separately annealing them to denatured Hpa_{II} restriction fragments. In every case an excess (1.4- to 2.4-fold) of short pieces of the strand growing in the 3' ⟶ 5' direction was found.

INTRODUCTION

     Since none of the known DNA polymerases extends the 5' end of DNA, a dilemma arises during replication when one of the daughter strands must grow in the 3' ⟶ 5' direction. Okazaki et al. (1) proposed that the extension of this strand occurs by a discontinuous mechanism whereby small pieces of DNA are synthesized in the 5' ⟶ 3' direction and then joined, resulting in overall growth in the 3 ⟶ 5' direction. Although not required by the above considerations, it is possible that both strands are synthesized in this fashion. The purpose of the present study was to compare the extent of discontinuous synthesis of the two strands of polyoma DNA at each growing fork during DNA synthesis in isolated nuclei.

     When nuclei from polyoma-infected 3T6 mouse cells are incubated in a system described by Winnacker et al. (2), labeled deoxynucleotides are incorporated into pre-existing replicative intermediates. Short RNA-primed pieces about 135 nucleotides long are synthesized and then joined to form longer chains (2, 3, 4). This process is similar to in vivo replication in that it proceeds bidirectionally from the same fixed origin (5 , 6, and Magnusson and Nilsson, manuscript in preparation).

A previous study (7) has shown that these short pieces self-
anneal to about 70% as judged by hydroxyapatite (HAP) chromato-
graphy.   In a different in vitro polyoma system, however, they
self-anneal to only 28% (8).   Both results are consistent with
discontinuous synthesis of both strands at each replicating fork,
and both suggest that unequal amounts of short pieces of the
two strands are found, with the strand bias being greater in the
latter system.   A method was therefore developed to measure
directly the proportion of short pieces of both strands at each
fork.   Since the polarities of strand growth are reversed for the
two forks in the replicative intermediate (Fig. 1), restriction
fragments were used to examine portions of the genome traversed
by only one fork.   The method involves separating the strands of
the short pieces and measuring the proportion of each strand
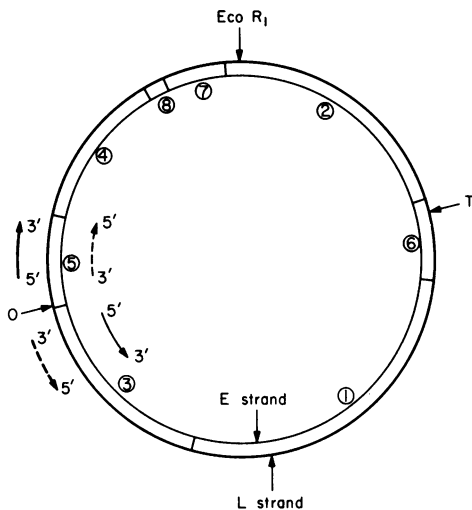which will anneal to an excess of a denatured restriction frag-
ment.

Fig. 1. Map of Polyoma DNA. The Hpa$_{II}$ restriction map of
polyoma DNA is shown, with the origin and terminus indicated by
"O" and "T" respectively (6). The polarities of the E (template
for early RNA synthesis) and L (template for late RNA synthesis)
strands are taken from ref. 12. Daughter strands growing in the
5' ⟶ 3' direction are indicated by solid arrows (⟶) and
those in the 3' ⟶ 5' direction by dashed arrows (--⟶).

MATERIALS AND METHODS
      Synthesis and isolation of short pieces: Nuclei from
polyoma-infected 3T6 cells were prepared as described previously
(9). Reaction mixtures were incubated for 5 min at 25°C under

standard conditions (2) modified by the addition of CTP, GTP, and UTP at a concentration of 200 μM and α-^{32}P-dGTP (Amersham Searle) at a concentration of 2 μM and a specific activity of 238,000 cpm/pmole. The reaction was stopped and the viral DNA was selectively extracted as described previously (2, 10). After digestion with proteinase K (Merck) and extraction with 1:1 phenol: chloroform, the DNA was alcohol precipitated and applied to a 1.5 x 90 cm Sepharose 4B column equilibrated with 0.25M NaCl, 10mM Tris (pH 7.5), 1mM EDTA. All of the label eluted in the void volume, indicating that the newly synthesized DNA was still base paired to the template. These fractions were pooled and applied to a benzoylated-napthoylated-DEAE cellulose (Serva) column. The column was washed with 1M NaCl, 10mM Tris (pH 7.5), 1mM EDTA, followed by 2% caffeine in this buffer. More than 99% of the label applied eluted in the caffeine fraction. Denatured sonicated calf thymus DNA (Worthington) was added at this and later points as carrier (the total amount added was 300 μg). Caffeine was removed by dialysis at room temperature versus 1M NaCl, 10mM Tris (pH 7.5), 1mM EDTA, and the DNA was alcohol precipitated. The overall recovery of radioactivity over this entire procedure was 70%. In particular, the recovery during the phenol-chloroform extraction step was 90%, so that the release of small DNA pieces from replicative intermediates observed with T7 DNA (11) did not occur.

The replicative intermediates were then sedimented through alkaline sucrose, and the peak of slowly sedimenting material was pooled (7). After neutralization and alcohol precipitation, short pieces were dissolved in 50mM Tris (pH 7.5), 1mM EDTA; denatured (100°C, 3 min); quenched on ice; and reapplied to a Sepharose 4B column under conditions identical to the previous chromatography. Since they were partially included, the short pieces could be separated from any longer chains surviving the previous step. The pooled fractions were subjected to alkaline hydrolysis in 0.5M NaOH for 16 hr at 37°C to remove any traces of RNA. This treatment did not significantly degrade sonicated polyoma I DNA as judged by alkaline sucrose sedimentation (data not shown). After neutralization, the preparation was alcohol precipitated and redissolved in 200 μl of 100mM Tris (pH 7.5),

2mM EDTA.

Separation of the strands of short pieces with cRNA: A modification of the method of Kamen et al. (12) was used. In some experiments the cRNA was a kind gift of Dr. Kamen; for others it was synthesized (12). Short pieces (8 ng in 170 μl of 3mM Tris (pH 7.5), 0.3mM EDTA) were denatured at $100^{\circ}C$ for 3 min. Three to five μg of cRNA (a 400- to 600-fold mass excess) was immediately added in 30 μl of buffer mixture which resulted in final concentrations of 0.12M NPB (sodium phosphate buffer, pH 6.8), 0.1% SDS (sodium dodecyl sulfate), and 2mM EDTA. After incubation at $71^{\circ}C$ for 4 hr, the samples were applied to 0.5 ml hydroxyapatite (HAP) columns (Bio-Rad) at $60^{\circ}C$ and eluted with 0.13M NPB and 0.4M NPB (both containing 0.1% SDS). About 60% of the ^{32}P counts were eluted with 0.13M NPB in the single stranded fraction ("E strands); the hybrids eluted with 0.4M NPB ("L" strands). Both fractions were treated 12 hr with 1M NaOH at $37^{\circ}C$ to hydrolyze the cRNA and then dialyzed at room temperature versus 1M NaCl, 10mM Tris (pH 7.5), 1mM EDTA to remove SDS and phosphate. They were concentrated by alcohol precipitation, dissolved in 75 μl of 1M NaCl, 0.12M NPB, 2mM EDTA, 0.1% SDS, and self-annealed at $70^{\circ}C$ for 50 hr (10 times the $t_{\frac{1}{2}}$ for renaturation if no strand separation had been achieved) to remove contaminating complementary strands from each of the two preparations. Aliquots were assayed on HAP to ascertain when the reaction had gone to completion. The samples were then chromatographed on HAP columns as before. Between 20 and 35% of the "E" and 6 to 9% of the "L" strands were discarded as duplex. The actual cross contaminations were, of course, less than half these values. The purified E and L strand short pieces were then dialyzed and alcohol precipitated as before.

Preparation of Hpa_{II} restriction fragments: ^{3}H-thymidine labeled polyoma I DNA was isolated from infected cells by the Hirt procedure (10). The covalently closed DNA was purified by banding in a propidium-diiodide-CsCl gradient followed by sedimentation through a neutral sucrose gradient. It was then cleaved with the Hpa_{II} restriction enzyme, a generous gift from Dr. Göran Magnusson. The resulting fragments were separated on a continuous elution gel system (13, and M. Vogt, personal

communication) as shown in Fig. 2.

Fragments 1-4 were separately concentrated by alcohol precipitation and then further purified by electrophoresis in 1.4% agarose gels (Bio-Rad) in 40mM Tris (pH 7.8), 5mM Na acetate, 1mM EDTA, 1 µg/ml ethidium bromide (0.6 x 16 cm gels, 7 hr, 50 volts). The DNA bands were detected with UV light and excised. The DNA was electrophoresed out of the gels, concentrated by alcohol precipitation, sonicated to an average size of 340 nucleotides, alcohol precipitated once more, and dissolved in 10mM Tris (pH 7.5), 1mM EDTA.

Hydroxyapatite (HAP) chromatography: Analytical incubations in small volumes (3-20 µl) sealed into siliconized glass capillaries were diluted into 1 ml of "S" buffer (0.13M NPB, 0.4% SDS) and stored at 2°C until analyzed (less than 24 hr). They were applied to 0.6 ml columns of HAP (Bio-Rad HTP), prewashed with "S" buffer and pre-equilibrated to 60°C. The single strand fraction was eluted with 3 x 1 ml of "S" buffer; the double strand fraction was eluted with 3 x 1 ml of "D" buffer (0.4M NPB,
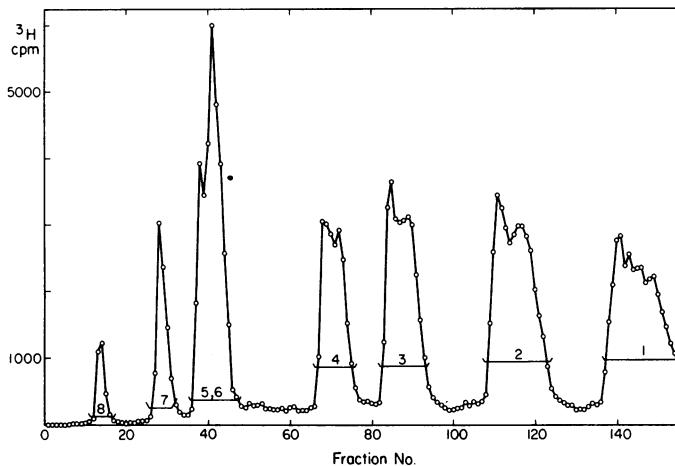

Fig. 2: Separation of Hpa_{II} Fragments by Continuous Gel Electrophoresis. 40 µg of ^{3}H-thymidine labeled polyoma I DNA was digested with Hpa_{II} enzyme, alcohol precipitated, dissolved in 50 µl of 10mM Tris (pH 7.5), 1 mM EDTA, and applied to a 1 x 12 cm gel of 2.2% acrylamide-0.11%-bis-acrylamide-0.7% agarose in 40 mM Tris, 20 mM sodium acetate, 1mM EDTA (pH 7.5). After 6 hr at 65 volts, the voltage was reduced to 30 volts and fractions were taken.

0.4% SDS). The latter was diluted with 1 ml of distilled water, 15 ml of Instagel (Packard) as added to each, and the samples were counted. The efficiencies of counting both fractions were the same, so that no systematic error was introduced. Recoveries were routinely 90-100% of input counts even with small amounts of DNA. The selectivity of this lot of HAP was checked; less than 1.4% of native DNA (^{32}P-polyoma I) eluted as single strands and less than 2.3% of denatured DNA (^{32}P-$\underline{E}$. $\underline{coli}$ DNA) eluted as duplex.

Preparative columns were run under similar conditions.

RESULTS

$\underline{Size\ analysis\ of\ short\ pieces}$: Short pieces were sedimented through alkaline sucrose together with Hpa$_{II}$ restriction fragments 7 (270 nucleotides) and 8 (95 nucleotides) as markers (Fig. 3). The profile indicates a heterogeneous distribution of sizes. The band center at half height corresponds to a mass
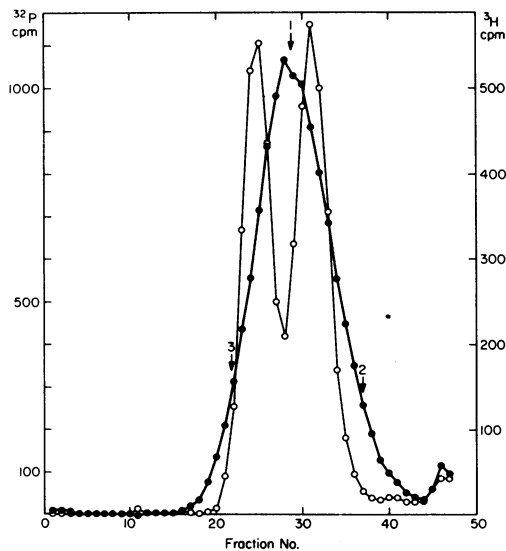

Fig. 3: $\underline{Alkaline\ Sucrose\ Sedimentation\ of\ Short\ Pieces}$. An aliquot of the pooled material after alkaline sucrose sedimentation was mixed with ^{3}H-labeled Hpa$_{II}$ restriction fragments 7 (270 nucleotides) and 8 (95 nucleotides) and layered onto an alkaline sucrose gradient. The gradient was centrifuged for 15 hr at 55,000 rpm in the SW56 rotor at 4°C, and then fractionated drop-wise and counted in Instagel. —●—●— , ^{32}P counts in short pieces; —o—o— , ^{3}H counts in restriction fragments. Sedimentation is from right to left. The center of the ^{32}P peak (arrow 1) corresponds to a mass average molecular weight of 136 nucleotides (14). The positions corresponding to 25 and 390 nucleotides are indicated by arrows 2 and 3.

average size of 136 nucleotides (14), assuming that the chains
are uniformly labeled. The long labeling time used, 5 min,
should ensure that this assumption is valid. In order to define
the limits of the size distribution better, the broadening
caused by diffusion and mixing during dripping was removed by
subjecting the data to a "de-smearing" Fourier analysis technique
(15 and D. Agard and M. Krieger, personal communication), using
the data for the monodisperse restriction fragments as standards.
This analysis shows that 95% of the label sedimented between
positions corresponding to 25 and 390 nucleotides. A more com-
plete analysis of the size of the short pieces using gel electro-
phoresis has been performed (Närkhammar, Magnusson, and Reichard,
manuscript in preparation).

Extent of self-annealing of short pieces: The kinetics of
self-annealing were examined before and after both the second
Sepharose 4B chromatography (Fig. 4a) and the alkaline hydrolysis
(Fig. 4b). This analysis shows that the Sepharose 4B step
removes unlabeled higher molecular weight polyoma DNA or RNA
which accelerates the annealing reaction about 2.2-fold, while
the alkaline incubation removes a small amount of low molecular
weight polyoma RNA which has a further 1.4-fold effect on the
reaction rate. The short pieces were not degraded by the alka-
line incubation, since identical treatment of ^{32}P-labeled
sonicated polyoma I DNA did not affect its sedimentation profile
in alkaline sucrose (not shown). It should also be noted that
95% of the label hybridized to polyoma DNA after the final step
of the purification, but that the maximum extent of self-
annealing observed was 75%. This result alone indicates that
there are differences in the amount of short pieces synthesized
from the two strands.

Specific activity of short pieces: The value used for the
specific activity, 59,000 cpm/pmole DNA nucleotides, was calcu-
lated from the specific activity of the dGTP used in the incu-
bation. This value was checked by determining specific activi-
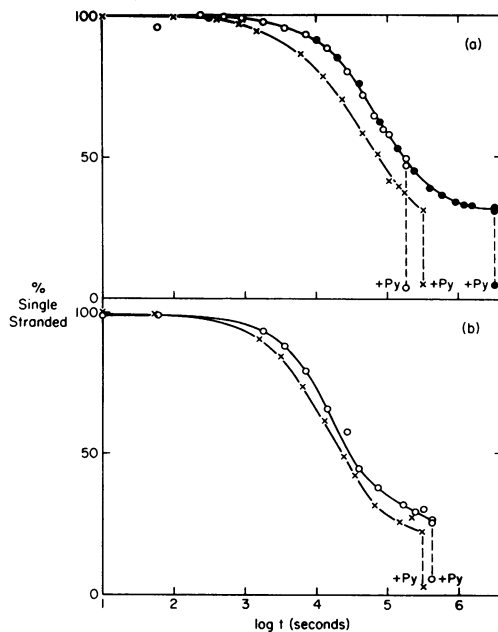ties from the kinetics of self-annealing (Fig. 4b) (16, 17).

Fig. 4: Self-Annealing of Short Pieces. (a) Comparison of
Self-Annealing Before and After Sepharose 4B Chromatography.
Aliquots of the pools after the preparative alkaline sucrose
gradient and second Sepharose 4B chromatography were denatured
at 100°C for 3 min, self-annealed at 58°C in 0.6M NPB, 5mM EDTA,
and then analyzed on HAP columns as described in Materials and
Methods. —x—x— , before Sepharose 4B; —o—o— , after
Sepharose 4B; —●—●— , after Sepharose 4B, but annealed at
approximately five times higher apparent concentration (^{32}P cpm
per ml). Since the actual DNA concentrations are not known, the
curves have been shifted laterally so that they correspond to
the same apparent concentration, using the apparent concentration
of the lower amount of short pieces after Sepharose 4B as the
standard. This method allows contaminating unlabeled polyoma
nucleic acids to be detected by the acceleration in annealing
which they cause. At the far right, the "+Py" points correspond
to aliquots incubated for 50 hr with 0.15 µg of denatured
sonicated polyoma I DNA under the same conditions. Each point
represents between 600 and 1400 cpm. (This material was a gift
from Peter Reichard.)   (b) Comparison of the Rate of Self-
Annealing Before and After Alkaline Incubation. Aliquots of the
pooled short pieces after Sepharose 4B chromatography (Fig. 3)
and of this pool after incubation in 0.5M NaOH for 16 hr at 37°C
were denatured, annealed (in 0.67M NPB), and analyzed on HAP
columns as above. —x—x— , before alkali treatment; —o—o— ,
after alkali treatment. Using the latter as a standard, the
curves have been shifted as above. These curves are not directly
comparable to those in (a) because the specific activities are
different. The "+Py" points correspond to aliquots incubated for
118 hr in the presence of 11 µg of denatured sonicated polyoma I
DNA. Each point represents between 1500 and 2500 cpm.

The formula,

$$\text{specific activity} = \frac{C^* \, t_{\frac{1}{2}}^*}{C_o \, t_{\frac{1}{2}}} \sqrt{\frac{\ell}{\ell^*}}$$

was used, where C^* is the ^{32}P counts per unit volume of the annealing mixture, $t_{\frac{1}{2}}^*$ is the measured time for half self-annealing of the short pieces, ℓ^* is the average length of the short pieces (135 nucleotides), and ℓ is 340 nucleotides. A value for $C_o t_{\frac{1}{2}}$ of $3.4 \pm 0.6 \times 10^{-3}\underline{M}$ sec in $0.12\underline{M}$ NPB was determined as an average of four measurements on ^{32}P-polyoma I DNA sonicated to 340 nucleotides. Using $t_{\frac{1}{2}}^*$ determined for the midpoint between 100% and 25% (the lowest value observed) single strands remaining, a value of 79,900 cpm/pmole DNA nucleotides was obtained, in reasonably good agreement with the value determined from the dGTP specific activity.

Analysis of the separated strands of short pieces: The E and L strand preparations separated by annealing with cRNA (Materials and Methods) were examined by the annealing methods shown in Table 1 in order to measure their ability to anneal to polyoma DNA and to check the extent of cross-contamination between them. Since the amount annealing to sonicated polyoma I DNA declined from the value of 95% obtained before strand separation, these values have been used to correct the results in Table 2. The two preparations did not self-anneal, indicating that they were not extensively cross-contaminated. The annealing time used was about three times the $t_{\frac{1}{2}}$ expected if each preparation consisted of equal amounts of the two strands. Much longer incubation times were not practicable. and the higher DNA concentrations necessary to reduce the time required for reannealing of a small contaminant could not be attained due to the small amount of material available. The annealings to cRNA and to the E strand of fragment 1 confirm that the E strand preparation was not seriously contaminated, since only 5-9% of it annealed in each case. These results together with the self-annealings at higher concentrations during the preparations indicate that the E and L strand preparations were at least 95% free from cross-contamination.

Table 1: Analysis of Separated Strands of Short Pieces

Short Piece Annealing To	Proportion of Total cpm Annealed	
	E	L
(1) sonicated polyoma I	77%	85%
(2) self, 24 hr	1-4%	0-8%
48 hr	4-6%	0-9%
(3) cRNA	5-9%	83-86%
(4) fragment 1, E strand, 25 hr	3%	26%
50 hr	5%	29%

Conditions:

(1) 0.01-0.02ng short pieces + 5 µg sonicated polyoma I in 5 µl 0.01$\underline{M}$ NPB, annealed 68°C, 3 hr.

(2) 0.01-0.02ng short pieces in 5 µl 0.1$\underline{M}$ NPB, annealed at 68°C for 24 and 48 hr (The latter is equivalent to 3 times the $t_{\frac{1}{2}}$ expected if the strands were not separated at all.).

(3) 0.01-0.02ng short pieces + 0.6 µg cRNA in 5 µl 0.1$\underline{M}$ NPB, annealed at 68°C for 3 hr.

(4) 0.04-0.08ng short pieces + 0.13ng fragment 1, E strand (a kind gift from Monica Närkhammar) in 12 µl 0.33$\underline{M}$ NPB, annealed at 70°C for 25 and 50 hr.

HAP analyses were performed as described in Materials and Methods.

Annealing of separated strands of short pieces to denatured restriction fragments: The proportions of the E and L strand preparations arising from different portions of the genome were determined by separately mixing each with an excess of each of sonicated Hpa$_{II}$ fragments 1-4, denaturing, and annealing. The fraction of short piece label annealed as a function of time was determined by analysis of aliquots by HAP chromatography (Fig. 5). It is clear from Fig. 5 that more E than L strand short pieces annealed to fragments 2 and 4, and that the reverse was true for fragments 1 and 3. During replication, the E strand of fragments 2 and 4 and the strand of fragments 1 and 3 grow in the 3' $\longrightarrow$ 5' direction and are therefore expected to be synthesized discontinuously (Fig. 1).

The kinetics of the annealing were measured in order to use plateau values to quantitate these biases. The curves do not plateau as expected, however, because a slow annealing reaction
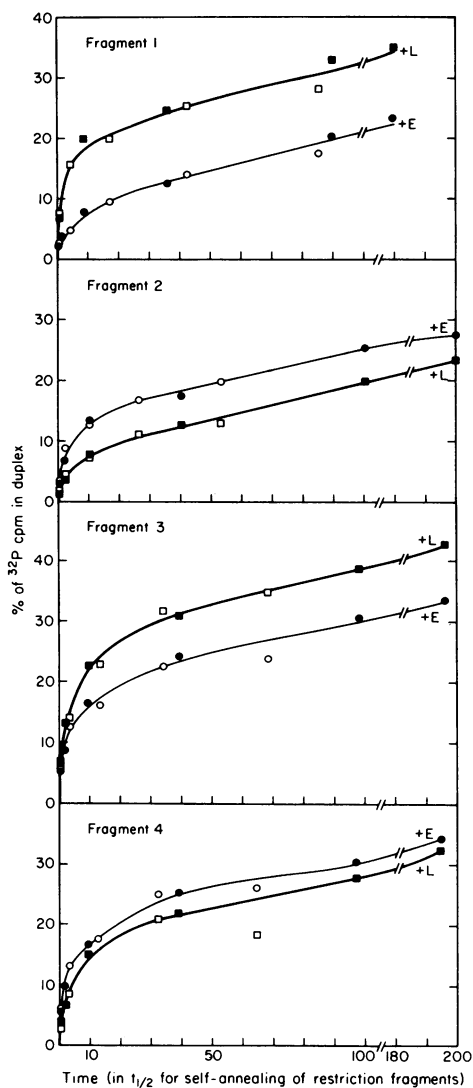
Fig. 5: <u>Annealing of the Separated Strands of Short Pieces to Excess Denatured Restriction Fragments.</u> E or L strand short pieces were mixed with an excess of one of the restriction frag-ments, denatured (100°C, 3 min), and annealed at 68°C in 0.3$\underline{M}$ NPB. At various times samples were analyzed by HAP chromatography (Materials and Methods) and the fraction of ^{32}P-label annealed was determined. Each point corresponds to 1400-1600 cpm (fragments 1 and 2) or 900-100 cpm (fragments 3 and 4). The concentrations of short pieces were the same in every annealing. Two different ratios of restriction fragments to short pieces were used: in each case, the amount of restriction fragment strand used was between 14 and 22 (open points) or 46 and 64 (solid points) times the proportion of these short pieces which would be expected to come from the restriction fragment region if they were synthesized uniformly over the genome. The time scale is expressed in $t_{\frac{1}{2}}$ units for the renaturation of the restriction fragments, since this method allows the comparison of results between both the different restriction fragments and the different ratios of each used. This simplification assumes that the rate of annealing of short pieces to restriction fragment strands is roughly the same as the rate of reannealing of the restriction fragments. The restriction fragments were sonicated to an average size of 340 nucleotides, which was as close as possible to the 135 nucleotide average size of the short pieces. Since the rate of renaturation is proportional to the square root of the length (17), the rate of annealing of short pieces to restriction fragment strands should be roughly 60% of the rate of the annealing of restriction fragment strands to each other. The amounts of the short pieces are so much less than those of the restriction fragments that they should not affect the $t_{\frac{1}{2}}$'s for reannealing of the latter.—o-o— and —●-●—, E strand pieces annealed with low and high amounts of restriction fragments; —□-□— and —■-■— , L strand pieces annealed with low and high amounts of restriction fragments.

appeared at times longer than $10t_{\frac{1}{2}}$, when more than 99% of the
restriction fragment DNA should have reannealed. Plots of
(fraction single stranded)$^{-1}$ versus time^{-1}, which normally are
linear, are biphasic in every case (not shown). The slow
reaction probably represents a low (< 5%) contamination of the
restriction fragment by DNA from other portions of the genome,
despite the purification of the restriction fragments by two
gel electrophoreses. The fraction of each short piece strand
complementary to each restriction fragment was therefore
measured using the value at $10t_{\frac{1}{2}}$ and applying a correction for
the small amount of annealing caused by the slow reaction
during this period. A further correction for the portion of the
short pieces which did not hybridize to sonicated polyoma I
(Table 1) was then applied, giving the results shown in Table 2.

Table 2: Annealing of Separated Strands of Short Pieces to
Sonicated Hpa$_{II}$ Restriction Fragments 1, 2, 3 and 4

Fragment	Fragment Size (% Genome)	Proportion of Short Piece Strand Hybridized		Strand Bias (E/L)
		E	L	
1	27.3%	8.4%	20.3%	0.41
2	21.4%	15.4%	7.1%	2.17
3	16.8%	15.2%	24.7%	0.62
4	13.2%	21.8%	15.9%	1.37
Total	78.7%	60.8%	68.0%	0.89

The data were taken from Fig. 5 by the method described in
Results and corrected for both the background slow hybrid-
ization and the proportion of short pieces of each strand
which did not anneal to sonicated polyoma I DNA (Table 1).
The fragment sizes are those given in ref. 6.

The strand biases for fragments 1 and 2 correspond to 2.4- and
2.2-fold excesses (respectively) of pieces of the strand growing
in the 3' ⟶ 5' direction. The biases for fragments 3 and 4
correspond to lesser excesses of 1.6- and 1.4-fold of this strand.
It also appears that more total synthesis occurs in fragments 3
and 4 than in 1 and 2 under the conditions used here. The overall

bias of 0.89 indicates that the biases on one side of the genome
are nearly counterbalanced by the opposite biases on the other.

Since the origin of replication is in fragment 5 near the
5-3 junction (6), young growing strands (resulting from the
joining of several very short pieces) from fragment 3 might be
included in the preparation and in the proportion of short pieces
annealing to fragment 3. Fragment 3 is 870 nucleotides long,
however, and most of the short pieces are less than 390 nucleo-
tides (Fig. 3). Therefore, most of the region of fragment 3
should be free from young growing strands. Unless there is a
large excess of very young growing strands, the data for fragment
3 should reflect mainly the distribution of label in short pieces.
Fragment 4, on the other side of the origin (Fig. 1), presents
fewer difficulties since young growing strands would be at least
300 nucleotides long before they include any portion of fragment
4.

In summary, the results indicate that short pieces of both
strands are found in all regions of the genome examined, but that
there are more from the strand which grows in the 3' $\longrightarrow$ 5'
direction at each fork.

DISCUSSION

Previous experiments (7) have shown that purified short
pieces of polyoma DNA synthesized in isolated nuclei self-anneal
to about 70-80% as judged by HAP chromatography. This result is
not due to contamination with unlabeled viral nucleic acids
since the present work shows that Sepharose 4B chromatography
(Fig. 4a) and alkali treatment (Fig. 4b) each remove small amounts
of contaminating nucleic acids without affecting the extent of
self-annealing. The extensive self-annealing suggests that the
short pieces arise by discontinuous synthesis of both strands of
the DNA but that the amount of short pieces of the two strands
may not be equal. About 20% of the short pieces do not self-
anneal, although they do anneal to polyoma DNA. If this material
were simply an excess of one strand, an overall strand bias of
1.5:1 would result. Since polyoma DNA replication is bidir-
ectional (5), however, the strand bias should be reversed on
opposite sides of the genome from the origin if the same
mechanisms are used for replication at each fork. The experi-

ments described here were designed to test this interpretation.

Hunter, Francke, and Bachelor (manuscript submitted) have used a kinetic method for measuring the strand bias in short pieces from different regions of the genome. Their method compares the rates at which the two separated strands of restriction fragments anneal to an excess of unlabeled short pieces. The direct measurement described here is essentially the reverse of theirs. The separated strands of short pieces are annealed to an excess of denatured Hpa_{II} restriction fragments. Only those short pieces arising from each restriction fragment region should anneal, so that the proportion annealing at saturation should be the proportion arising from the restriction fragment region. Comparison of the results for the two strands of the short pieces gives a direct measurement of the strand bias in each region.

The results show that short pieces from <u>both</u> strands are found in all regions of the genome examined, but that the amounts from the two strands are unequal. There are more E strand short pieces in the fragment 2 and 4 regions and more L strand in the fragment 1 and 3 regions. The results obtained by different methods by Hunter, Francke, and Bachelor (manuscript submitted) and Närkhammar, Magnusson and Reichard (manuscript in preparation) for fragments 1 and 2 are in agreement with those presented here, although larger strand biases were observed in the former case. During replication, the L strand of fragments 1 and 3 and the E strand of fragments 2 and 4 grow in the 3' ⟶ 5' direction and are therefore expected to be synthesized discontinuously (Fig. 1). These are precisely the regions in which a greater amount of short piece label is observed, so these experiments indicate that there is a 1.4- to 2.4-fold excess of short pieces from the strand growing in the 3' ⟶ 5' direction.

These biases in the amount of short pieces could reflect either a difference in the size of the pieces isolated from the two strands or a difference in their number. If the short pieces synthesized on the strand growing in the 5' ⟶ 3' direction were longer than those from the other strand, a large portion of the completed ones might be removed during the isolation, leaving only smaller, nascent, short pieces from this strand and

resulting in a bias in the amount of label. Alternatively, the short pieces from the two strands could be the same size but the joining of completed ones to longer chains could occur more rapidly for the 5' ⟶ 3' strand, removing them from the pool. In the former case, a difference in the size of the short pieces from the two strands could be expected; in the latter no difference would be observed. The results described here do not allow a choice between these two possibilities.

REFERENCES

1   Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A. and Iwatzuki, N. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 129-143
2   Winnacker, E. L. Magnusson, G., and Reichard, P. (1972) J. Mol. Biol. 72, 523-537
3   Magnusson, G., Pigiet, V., Winnacker, E. L., and Reichard, P. (1973) Proc. Nat. Acad. Sci. 70, 412-415
4   Reichard, P., Eliasson, R., and Söderman, G. (1974) Proc. Nat. Acad. Sci. 71, 4901-4905
5   Crawford, L. V., Syrett, C., and Wilde, A. (1973) J. Gen. Virol. 21, 515-521
6   Griffin, B. E., Fried, M., and Cowie, A. (1974) Proc. Nat. Acad. Sci. 71, 2077-2081
7   Pigiet, V., Winnacker, E. L., Eliasson, R., and Reichard, P. (1973) Nature New Biol. 245, 203-205
8   Francke, B. and Vogt, M. (1975) Cell 5, 205-211
9   Pigiet, V., Eliasson, R., and Reichard, P. (1974) J. Mol. Biol. 84, 197-216
10 Hirt, B. (1967) J. Mol. Biol. 26, 365-369
11 Paetkau, V., Langman, L., and Miller, R. C. (Jr.) (1975) J. Mol. Biol. 98, 719-737
12 Kamen, R., Sedat, J., and Ziff, E. (1976) J. Virol. 17, 212-218
13 Lee, A. S. and Sinsheimer, R. L. (1974) Analyt. Biochem. 60, 640-644

14 Studier, F. W. (1965) J. Mol. Biol. 11, 373-390
15 Brault, J. W. and White, O. R. (1971) Astron. and Astrophys. 13, 169-189
16 Britten, R. J. and Kohne, D. E. (1966) Carnegie Inst. Wash. Yearbk. 65, 78
17 Wetmur, J. G. and Davidson, N. (1968) J. Mol. Biol. 31, 349-370