Polyoma DNA Synthesis in Isolated Nuclei: Evidence for Defective Replication Forks

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Nuclei, isolated from polyoma virus-infected mouse 3T6 cells, were incubated under conditions suitable for polyoma DNA synthesis. By using electron microscopy and standard regression statistics, it was shown that replication is mainly unidirectional in a large number of molecules, indicating the presence of inactive replication forks. The replication forks were inactivated randomly, and the defect seemed to be present from the beginning of the in vitro incubation.

Replication of polyoma DNA starts at a fixed origin on the circular molecule and proceeds from this site in both directions (5). Termination of DNA replication seems to occur at a site 180° from the origin (4, 5).

The elongation process involves the following steps. At certain positions on the polyoma genome, probably dependent on chromatin structure (13, 18), a 10-nucleotide-long RNA primer (initiator RNA) is synthesized (19). Using this primer, a DNA polymerase synthesizes a piece of DNA with a maximum length of about 200 nucleotides, the so-called Okazaki fragments (14). The RNA is subsequently removed, the resulting gaps between adjacent Okazaki fragments are filled in, and, finally, the fragments are ligated to each other. This kind of synthesis has been called "discontinuous synthesis" and seems to be predominantly asymmetric in the individual fork (8, 11, 16). Termination of replication of polyoma DNA is probably not determined by a site with a specific nucleotide sequence (1, 9, 14) but, rather, occurs when two forks meet.

The main objective of this study was to analyze how faithfully replication in a well-characterized nuclear system (22) follows the bidirectional pathway. For that purpose mouse 3T6 cells were infected with plaque-purified virus, and at 25 h postinfection [3H]thymidine was added to the cultures. One hour later nuclei were isolated according to the procedure described by Pigiet et al. (17). Viral DNA was immediately extracted (10) (in vivo) from a portion of the nuclei, and three other portions of nuclei were incubated together with [α-32P]GTP for 1.5, 5, and 30 min, respectively, before extraction of viral DNA. The samples were subsequently treated with pronase, phenolized, and precipitated with ethanol. The properties of the isolated 3H- and 32P-labeled DNA were identical to those described earlier (3, 20, 22), as judged by the analysis of total radioactivity and sedimentation through alkaline sucrose. The viral DNA was further purified by sedimentation through neutral sucrose gradients, and the peak fractions of viral DNA were pooled and dialyzed. Portions of this material were mounted for analysis by electron microscopy (6) (ethidium bromide was added to the spreading mixture to urtcoil F1 DNA and replicative intermediates [RI]). The analysis of the "in vivo sample" showed that of total viral DNA 3.6 ± 0.3% were of the θ form (normal RI) and 0.04 ± 0.03% were in the form of a circle with a tail (rolling circle-type molecule [7]). Throughout the in vitro incubation the amount of RI remained essentially unchanged. These data are similar to those presented earlier (1, 3, 15) and indicate that there is no substantial breakdown of replicating molecules during the in vitro incubation.

To enrich for replicating molecules, the samples were chromatographed on BND-cellulose (22), and recovery of total DNA was between 84 and 89%. To study the distribution of replicating intermediates at different degrees of replication, aliquots of this material were analyzed by electrophoresis on agarose gels (21). The results from this experiment are shown in Fig. 1. The DNA isolated from nuclei before incubation (in vivo) separated into three main peaks (Fig. 1A). The slowest peak was heterogeneous and contained mainly late RI. The intermediate peak corresponded to relaxed circular F2 DNA molecules, whereas the fastest peak had a mobility corresponding to that of F1 DNA. The presence of radioactivity in F1 DNA shows that F1 DNA was not completely removed by BND-cellulose chromatography. After 1.5 min of incubation, a

shift towards more replicated RI of both $^3$H- and $^{32}$P-labeled DNA could be observed as compared to material analyzed before incubation. This shift of label continued with increasing incubation time, and after 30 min of incubation the majority of label was found in a position corresponding to late RI. At this time an increased amount of in vitro labeled DNA was detected in material with a mobility corresponding to that of F2 DNA. Moreover, a small amount (about 2%) of the total radioactivity of $^{32}$P-labeled DNA was found in a peak corresponding to F1 DNA (agarose gel electrophoresis on aliquots before BND-chromatography; data not shown). Except for the $^3$H-labeled F1 DNA, there was a good correlation between the patterns of in vivo and in vitro labeled material in all cases.

Next we analyzed the pools of replicating molecules with respect to replication patterns at the different time points, and for that purpose portions of the samples were treated with EcoRI before analysis by electron microscopy. After cleavage the following molecules could be seen in the microscope: (i) linear molecules of polyoma length; (ii) linear molecules with a replication eye and double-Y molecules; (iii) Y-shaped molecules; and (iv) other linear molecules.

An analysis of the class (ii) molecules with respect to replication indicated that at zero time there was a rectangular or symmetric distribution of molecules from early to late RI ($\chi^2$ analysis: 0.50 > $P$ > 0.40). However, already after 1.5 min of incubation a significant ($\chi^2$ analyses: $P < 0.01$) decrease appeared in the amount of molecules having replicated less than 10% of the genome. At 30 min about 5% of the total amount of molecules were 30% or less replicated. These results are in good agreement with those previously published (3) and show that late RI accumulate during the incubation of infected nuclei.

Using an analysis similar to that of Fareed et al. (7), we next investigated whether the replication process also followed the bidirectional pathway under in vitro conditions. Only class (ii) molecules were included in this analysis, and molecules having replicated less than 5% were excluded because of difficulties in positioning the individual fork in such molecules. Only the in vivo sample and that incubated for 30 min in vitro were analyzed. The measurements for molecules with a replication eye and double-Y molecules are included in the same graph and were obtained by measuring the distance from the EcoRI site to the nearest replication fork in the direction of the origin (L1). This distance is defined as positive when replication has not yet reached the EcoRI site (eye-shaped molecules) and negative when the replication fork has passed the EcoRI site (double-Y molecules).

The values obtained are plotted against the total length of replicated DNA (L2). The theoretical regression line crosses the X and Y axes at points $(2\mu, 0)$ and $(0, \lambda)$, respectively, where $\lambda$ is the distance from the EcoRI site to the origin.

The results obtained from the in vivo and in vitro experiments are shown in Fig. 2A and B, respectively. The distance from the EcoRI site to the nearest replication fork ($\lambda$) and 95% confidence limits of this distance are given in Table 1.

Table 1 also gives the results of the statistical comparison of the observed regression coefficients with the expected value of 0.50, the mutual comparisons of the regression coefficients in A (in vivo) and B (in vitro), and, finally, a statistical comparison of the variation around the regression line (the unexplained variation) in A and B.

The distance from the EcoRI site to the origin was 28.9 ± 2% in the in vivo experiments, similar to the value of 29.2 ± 2% given by Crawford et al. (4, 5). Furthermore, the regression coeffi-
Fig. 2. Relative distances between the EcoRI site and nearest replication fork in cleaved replicating molecules. Replicating polyoma DNA was purified as described in Fig. 1, treated with EcoRI, and analyzed by electron microscopy. (A) Results of the analysis of in vivo polyoma DNA molecules; (B) corresponding results of material after 30 min of in vitro incubation. Graphs and statistical calculations were made on a CDC Cyber 173 computer. All length measurements were calculated as percentage of the length of the total genome. An XY graph was made with the length of replicated DNA (L2) along the abscissa and the distance from the EcoRI site to the nearest replication fork along the ordinate (L1). This distance (L1) is plotted as negative when the replication fork has passed the EcoRI cleavage site (double-Y molecules). The equations for the regression lines were calculated and compared mutually with the theoretical regression line, using standard regression statistics (see Table 1).

cients were very close to the theoretical value of 0.50, and the regression coefficient in the in vitro experiments was not significantly different from that found in the in vivo experiments.

The principal difference between the in vivo and in vitro experiments is the fact that the variation around the regression line is significantly larger in the in vitro experiment than in the in vivo experiment (see Table 1).

From the distribution of data around the regression line it is concluded that in a considerable fraction (probably >40%) of all RIs the two forks move at different rates. An alternative interpretation would be that replication forks are subject to inactivation, because in some cases there was an indication of completely inactive forks (RI with one fork still at the origin). The presence of molecules with one fork at the origin of replication, but having replicated more than 20%, also indicates that defective forks are present from the beginning of the incubation of isolated nuclei. However, additional inactivation of forks might occur throughout the incubation.

The regression coefficients were the same in the in vivo and in vitro samples and not significantly different from the theoretical value of 0.5. This means that there is no preferential inactivation of forks moving in a certain direction from the origin, and thus forks are impaired randomly. Moreover, the finding that replication in vitro is asymmetric with respect to bidirectional replication supports the idea of a nondiffusible "replication complex" at an individual fork. If, on the other hand, "replication enzymes" were freely diffusible, no increase in the variation around the regression line would be observed, because all forks should have the same chance of being engaged in DNA replication.

In this context we want to emphasize the importance of analyzing the total pool of replicating molecules, i.e., both linear molecules with a replication eye and double-Y molecules, and including both types in the same graph. Otherwise, because of asymmetric synthesis, an analysis of, for example, only double-Y molecules will give rise to a significant difference between regression coefficients.

In this paper we demonstrate that (i) each DNA segment from the origin to the terminus consists of forks moving at different rates and (ii) many forks pass the termination site. These results suggest that it might be difficult to interpret the results of annealing studies on the symmetry of Okazaki fragment synthesis (8, 11). First, impaired forks might produce "artificial" Okazaki fragments, and, second, the HpaII-generated fragments 1 and 2 contain forks moving in opposite directions. Moreover, the discrepancy with respect to symmetry of Okazaki fragment synthesis between the nuclear systems (8, 11) might to some extent be due to differences in extent of inactivation of forks.

After 30 min of incubation it was shown by both electrophoresis on agarose gels and electron microscopy that late RI accumulates, but only a limited amount of the radioactive DNA was converted to F1 or F2 DNA due to defective termination. Since it was demonstrated here that a significant fraction of the RI contains one
TABLE 1. Statistical evaluation* of the results of the analysis of in vivo and in vitro replicating polyoma DNA molecules

<table>
<thead>
<tr>
<th>DNA molecules</th>
<th>Statistical comparison of coefficients with theoretical value of 0.50</th>
<th>Statistical comparison of regression coefficients in vivo and in vitro</th>
<th>Distance (%) from EcoRI site to origin with 95% confidence limits</th>
<th>Statistical comparison of unexplained variation in vivo and in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>$Y = 28.935 - 0.49131 \times X$</td>
<td>$t = 0.8448$ 0.40 &lt; $P &lt; 0.50$</td>
<td>28.935 29.584 0.50 &lt; $P &lt; 0.50$</td>
<td>28.368 29.584 0.50 &lt; $P &lt; 0.50$</td>
</tr>
<tr>
<td>(n = 98)</td>
<td></td>
<td></td>
<td>$F = 10.0$ P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td>$Y = 28.805 - 0.51483 \times X$</td>
<td>$t = 0.5384$ 0.50 &lt; $P &lt; 0.60$</td>
<td>28.805 30.220 0.50 &lt; $P &lt; 0.60$</td>
<td>27.390 30.220 0.50 &lt; $P &lt; 0.60$</td>
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<tr>
<td>(n = 126)</td>
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* See legend to Fig. 2.

impaired fork, we suggest that two active forks are needed for proper termination.

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LITERATURE CITED