Unidirectional Replication of a Minority of Polyoma Virus and SV40 DNAs

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SUMMARY

Polyoma DNA replication is initiated predominantly at a site which is 29% from the EcoRI cleavage site. Molecules replicating from this site, after digestion with EcoRI, appear as linear structures with a double stranded loop centred at the origin of replication. These forms constitute 90% of all replicating intermediates.

Approx. 10% of the replicating intermediates of polyoma and SV40 DNAs occur as Y-forms after treatment with EcoRI. These structures have probably resulted from unidirectional replication initiated at an additional origin of DNA replication which is located near the EcoRI cleavage site on the genomes of these viruses.

INTRODUCTION

Both polyoma and SV40 DNAs can be cleaved at specific sites with the restriction endonuclease EcoRI (Morrow & Berg, 1972; Folk & Wang, 1974; Robberson & Fried, 1974). EcoRI cleavage in one unique region of replicative intermediates of polyoma and SV40 provides a basis for the physical mapping of the origin and direction of DNA replication. The predominant mode of DNA replication that has been detected in these viruses corresponds to bidirectional synthesis initiated at sites which are 29 and 33% of the distance from the EcoRI cleavage sites on polyoma (Crawford, Syrett & Wilde, 1973) and SV40 (Fareed, Garon & Salzman, 1972) DNAs, respectively. The EcoRI cleavage sites in the two DNAs are probably not in functionally equivalent regions so that the similarity of the two values, 29% and 33%, may be fortuitous. In both cases, the two forks in the bidirectional replicating molecules grow at approximately equal rates with termination of DNA synthesis at a site which is diametrically opposite the initiation site. In addition to these major populations of Cairns forms, the pool of replicative intermediates contains minor species of replicating molecules which have the formal appearance of the letter Y, after cleavage with EcoRI. Y-forms were previously reported to constitute approx. 10% of the replicating intermediates of SV40 DNA (Fareed, et al. 1972). In this earlier study these structures were interpreted as replicative forms cleaved by EcoRI which had also been broken at one of the replication forks. An alternative explanation for this type of replication intermediate would be that it arises from unidirectional replication initiated at an origin near the EcoRI cleavage site.

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METHODS

Preparation of replicating DNA. Stocks of small plaque and large plaque polyoma virus were used to infect mouse embryo fibroblast secondary cells as previously described (Crawford et al. 1973). A stock of SV40 virus (SVL) was used to infect BSC-1 cells. These stocks had all been plaque-purified and grown at low multiplicity. In all cases, the infection was performed at a multiplicity of 10 to 20 p.f.u./cell. At about 30 h after infection, intracellular DNA was extracted by the Hirt procedure (Hirt, 1967) and banded in ethidium bromide CsCl gradients as described by Radloff, Bauer & Vinograd (1967). The gradients were then fractionated into lower, intermediate and upper band portions. After removal of ethidium bromide by extraction with isopropanol, the DNA collected from the intermediate region of the ethidium bromide CsCl gradients, enriched for replicative intermediates, was digested with EcoRI as previously described (Crawford et al. 1973). The endonuclease was prepared by a modification (Folk & Wang, 1974) of the method of Yoshimori (1971). Some properties of this preparation of EcoRI have already been described (Robberson & Fried, 1974). Non-replicating samples of SV40 and polyoma DNAs were prepared in the same manner and correspond to lower band samples isolated from cells 48 h after infection. Prior to EcoRI cleavage, these latter DNA samples contained less than 1% of the molecules as Cairns forms of the type previously described (Hirt, 1969).

Electron microscopy. Samples for electron microscopy were prepared by a modification of the Kleinschmidt basic protein film technique (Davis, Simon & Davidson, 1971). Grids were rotary shadowed with platinum: palladium and examined in a Siemens 101 electron microscope. Micrographs were taken at an instrumental magnification of 10000 and enlarged approx. 20 times. Length measurements on tracings of the molecules were performed with a map measure.

RESULTS

The predominant topological form apparent in the replication of polyoma and SV40 DNAs is a duplex circular molecule containing a single duplex circular loop (Fig. 1 a and 2). As replication proceeds, the size of this loop increases with each of the two replication forks moving at approx. equal rates. This loop is centred approx. one-third of the molecule from the region within which the EcoRI cleavage occurs, as measured on EcoRI treated SV40 and polyoma DNAs. These forms constitute approx. 90% of the replicative intermediates of SV40 and polyoma DNAs (Table 1). The remainder of the replicative forms after cleavage by EcoRI consist largely of single-fork molecules with a formal appearance that resembles a Y (Fig. 3 and Table 1). The Y-forms that occur in SV40 and polyoma DNAs possess two duplex arms with approximately equal lengths (Fig 4a) which appear to extend throughout the length of the virus genome (Fig. 4b) These structures may result from the EcoRI digestion of unidirectionally replicating molecules initiated near the cleavage sites (Figs. 1c, d). Y-forms are rarely seen in preparations of non-replicating DNA (see Methods). Those that are seen have presumably arisen through fortuitous sticking of linear fragments in the course of EcoRI digestion and specimen preparation for electron microscopy. These non-replicating structures occur as Y-forms with two arms of unequal lengths (Fig. 4c).

The results of a linear least squares analysis of measurements for the segments of SV40 and polyoma Y-forms in Fig. 4b is presented in Table 2. If Y-forms have arisen through unidirectional replication events initiated at origins within the region that is subsequently cleaved by our preparation of EcoRI, then we would expect length measurements on the
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Fig. 1. Diagrammatic drawings of replicative forms before (on the left) and after (on the right) cleavage with EcoRI. The region within which each molecule is cleaved by the EcoRI preparation is indicated by the two vertical lines in (a). (a) Bidirectional replication initiated approx. one-third of the molecule from the EcoRI site. (b) Bidirectional replication initiated near the EcoRI site. (c) Unidirectional replication initiated near the EcoRI site that proceeds to the right. (d) Unidirectional replication initiated near the EcoRI site that proceeds to the left. (e) Bidirectional replication initiated as in (a) plus unidirectional replication initiated as in (c). (f) Bidirectional replication initiated as in (a) plus unidirectional replication initiated as in (d). (g) Bidirectional replication initiated at both origins O and O'.
Fig 2. Examples of bidirectional replicating molecules of polyoma before (on the left) and after (on the right) cleavage by EcoRI. Magnification: \(1 \mu m = 3.1\) cm.

Table 1. Frequencies of polyoma and SV40 replicative forms after treatment with EcoRI

<table>
<thead>
<tr>
<th>% of replicating intermediates as</th>
<th>Polyoma (large plaque)</th>
<th>Polyoma (small plaque)</th>
<th>SV40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bidirectional forms*</td>
<td>86 (10)</td>
<td>87 (12)</td>
<td>89 (8)</td>
</tr>
<tr>
<td>Unidirectional forms†</td>
<td>13</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Double initiation forms‡</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Molecules classified</td>
<td>252</td>
<td>143</td>
<td>203</td>
</tr>
</tbody>
</table>

* The predominant structure classified here contains a loop located approx. one-third of the genome from the EcoRI site (Fig. 2). The numbers of terminal loop molecules (Fig. 5) are given in parentheses. These have been included in the totals for bidirectional replication together with a small number of double Y-molecules (Fig. 6).
† Presumed unidirectional replicative forms initiated near the EcoRI site.
‡ Replicative forms which appear to have been initiated at the predominant origin for bidirectional synthesis and at a second origin positioned near the EcoRI site (Fig. 7 to 9).

presumed replicated segments of these single-fork molecules to conform to a linear relationship that intercepts within approx. 5% of the measured position of the EcoRI cleavage site. In replicating samples of polyoma and SV40 DNA, the lengths of the replicated arms of the Y-forms are fitted to linear relationships with slopes and intercepts that are close to those expected for unidirectional replication initiated near the EcoRI site. In contrast to this rather close agreement found for replicating Y-forms, measurements on the non-replicating Y-forms do not fit a unique linear relationship. This is evident in the large error in the slopes determined for lines that do not intercept the expected position for EcoRI cleavage (at \(a = 1.0\)) (Fig. 4c and Table 1).

In addition to Y-forms we have noted the occurrence of linear replicative forms which terminate in a duplex circular loop or bubble (Fig. 5). These have been included in our scorings as part of the population of bidirectional replicating molecules initiated at the predominant origin, although they may equally well have come from unidirectional replication (Table 1). The contour length of the loop in these terminal loop molecules was seldom equal to one genome. About half of these forms had total lengths close to one genome, as though they were non-replicating DNA in which one end of the unit length molecule had become looped back. The other half were probably replicating molecules since, in these molecules, half the length of the loop plus the length of the tail equalled one genome. These could have come from unidirectional or bidirectional replicating forms in which one fork was near the EcoRI site.

Bidirectional replicative forms initiated near the EcoRI site will, after cleavage by EcoRI, give rise to double Y-molecules in which all the replicated arms are the same length (Fig. 1b and 6). Only two such replicative forms were detected in the pool of replicative molecules.
in polyoma and SV40 examined in this study. These forms are to be contrasted with the double Y-molecules derived by cleavage of bidirectional replicating molecules initiated at the predominant origin of replication described for polyoma and SV40 DNAs in which the bidirectional replication event has proceeded past the EcoRI cleavage site (Fareed et al. 1972; Crawford et al. 1973) (Fig. 6).

If origins for DNA replication do in fact exist at two positions on the virus genomes, one might also expect to find examples of replication forms in which initiation had occurred at both origins of the same molecule. The topological form of these replicative intermediates expected after cleavage with the EcoRI preparation is shown in Fig. 1 e, f. Examples of replicative forms with evidence of this type of double initiation were detected among the replicative intermediates of both SV40 and polyoma, and comprised 1% of all replicative forms (Fig. 7, 8 and 9). Unidirectional replication apparently proceeds to either the left or right of the minor origin on these molecules. Replicative forms containing evidence of bidirectional synthesis initiated at both origins of the same molecule (Fig. 1 g) were not detected in this study.

Virus preparations, passed at high multiplicity, accumulate defective DNA molecules in which sequence rearrangements have occurred (Tai et al. 1972; Brockman & Nathans, 1974; Folk & Wang, 1974; Robberson & Fried, 1974). If these rearrangements brought an origin of replication near to the EcoRI site this could lead to the production of Y-forms, from the
Fig. 4. Length measurements of replicating and non-replicating Y-forms. (a) Drawing of Y-form with arms designated A, B, and C. (b) Selected Y-forms in which the sum of measured lengths for either A plus B or A plus C was within 11% of the average length measured for bidirectional replicative forms cleaved by EcoRI (Fig. 2) that were present in the sample. Measurements were made on Y-forms detected among the replicative intermediates of large plaque polyoma virus (●), small plaque polyoma virus (○) and SV40 (□). The dashed lines indicate the expected lengths for arms B and C for unidirectional replication initiated at the EcoRI site. (c) Selected Y-forms in non-replicating samples (see Methods) of large plaque polyoma virus (●), small plaque polyoma virus (○) and SV40 (□). The sum of measured lengths for A and B or A and C on each Y-form was within 11% of the average length of the polyoma or SV40 EcoRI linear DNA molecules present in the sample. Molecules with lengths which deviated by more than 11% from that of EcoRI linear molecules have not been included here. These comprised of 8 molecules of large plaque polyoma, 4 molecules of small plaque polyoma and 4 molecules of SV40.
Table 2. Linear least squares analysis of length measurements on segments of single fork molecules*

<table>
<thead>
<tr>
<th>Sample</th>
<th>-B/A</th>
<th>-C/A</th>
<th>B_0 †</th>
<th>C_0 †</th>
<th>-B_0A/B§</th>
<th>-C_0A/C§</th>
<th>No. of molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicating polyoma (large plaque)</td>
<td>0.94 ± 0.12</td>
<td>0.87 ± 0.16</td>
<td>0.95 ± 0.08</td>
<td>0.91 ± 0.10</td>
<td>1.01</td>
<td>1.05</td>
<td>14</td>
</tr>
<tr>
<td>Replicating polyoma (small plaque)</td>
<td>0.86 ± 0.22</td>
<td>0.93 ± 0.17</td>
<td>0.94 ± 0.09</td>
<td>0.95 ± 0.07</td>
<td>1.09</td>
<td>1.02</td>
<td>13</td>
</tr>
<tr>
<td>Replicating SV40</td>
<td>0.96 ± 0.16</td>
<td>0.92 ± 0.15</td>
<td>1.0 ± 0.10</td>
<td>0.98 ± 0.09</td>
<td>1.04</td>
<td>1.06</td>
<td>18</td>
</tr>
<tr>
<td>Non-replicating SV40 and polyoma</td>
<td>0.54 ± 0.42</td>
<td>0.52 ± 0.39</td>
<td>0.91 ± 0.22</td>
<td>0.91 ± 0.20</td>
<td>1.68</td>
<td>1.75</td>
<td>15</td>
</tr>
</tbody>
</table>

* Calculated from data presented in Fig. 4b, c.
† The error in the slope indicates the interval that contains the true mean at a level of confidence of 90%.
‡ Measured intercepts and standard deviations on vertical coordinates of Fig. 4b, c.
§ Calculated intercepts on horizontal coordinates of Fig. 4b, c.

Fig. 5. Replicating forms of EcoRI-treated small plaque polyoma virus DNA which terminate in a duplex circular loop. Magnification: 1μm = 3.4 cm.

Fig. 6. Double Y-molecules with all arms of similar lengths (on the left) and with pairs of arms of dissimilar lengths (on the right). The molecule on the left was observed in a sample of replicating large plaque polyoma virus and the molecule on the right was observed in a sample of replicating small plaque polyoma virus. Magnification: 1μm = 3.4 cm.

EcoRI digested replicating molecules. Defective DNA molecules were therefore considered as a possible source of Y-forms. Since most defective polyoma DNA are resistant to EcoRI (Folk & Wang, 1974; Fried, 1974), the fraction of a DNA preparation which is resistant to EcoRI is a good indication of its content of defective DNA. Only 1% of the non-replicating large plaque polyoma DNA preparation examined was EcoRI resistant (Table 3). Such EcoRI resistant molecules are therefore unlikely to have contributed significantly to the
Fig. 7. Molecules of replicating SV40 DNA treated with EcoRI containing a loop corresponding to bidirectional synthesis and a single-fork Y-structure corresponding to unidirectional synthesis initiated near the EcoRI site. Magnification: $1 \mu m = 3.9$ cm.

Fig. 8. The molecule at the top was observed in replicating large plaque polyoma DNA treated with EcoRI and contains a loop corresponding to bidirectional synthesis as well as a single-fork Y-structure corresponding to unidirectional synthesis initiated near the EcoRI site. The molecule at the bottom was observed in replicating small plaque polyoma virus and represents a Y-form, one arm of which contains a loop indicative of a second initiation event in the replication of this molecule. Magnification: $1 \mu m = 3.9$ cm.

Fig. 9. Molecules of replicating SV40 DNA treated with EcoRI in which two initiation events appear to have occurred. The molecule on the left contains a single fork Y-structure and a loop which is not at a position corresponding to initiation of bidirectional synthesis. The molecule on the right contains a loop corresponding to bidirectional synthesis and a small single-fork Y-structure. Magnification at left: $1 \mu m = 4.2$ cm. Magnification at right: $1 \mu m = 3.4$ cm.
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Table 3. Examination of large plaque polyoma virus DNA for defective genomes

<table>
<thead>
<tr>
<th>%duplex molecules as</th>
<th>Before first EcoRI digestion</th>
<th>After first EcoRI digestion</th>
<th>After second EcoRI digestion</th>
<th>After denaturation-renaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular forms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closed</td>
<td>85.7</td>
<td>0.8</td>
<td>0.7 (1.1)*</td>
<td>0.6</td>
</tr>
<tr>
<td>Open</td>
<td>12.8</td>
<td>0.7†</td>
<td>1.1 (0.1)</td>
<td>0.5‡</td>
</tr>
<tr>
<td>Linear forms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without inhomology region</td>
<td>1.5</td>
<td>98.5</td>
<td>98.2 (98.8)</td>
<td>98.7</td>
</tr>
<tr>
<td>With inhomology region</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2§</td>
</tr>
<tr>
<td>Molecules classified</td>
<td>943</td>
<td>1006</td>
<td>940 (820)</td>
<td>1959</td>
</tr>
</tbody>
</table>

*Numbers in parentheses and in the last column on the right refer to frequencies of molecules determined on samples prepared by the formamide modification of the Kleinschmidt technique (see Methods).
† The majority of these forms are hydrogen bonded circles of EcoRI cleaved DNA.
‡ These forms are either renatured defective DNAs that had been randomly nicked in the preparations or defective DNAs nicked in the course of the renaturation itself.
§ The region of inhomology in these renatured molecules occurs at one terminus and represents less than 10% of the genome length.

population of Y-forms described. Defective DNAs which are sensitive to cleavage by EcoRI and in which a sequence rearrangement had brought an origin of DNA replication close to the EcoRI site would be expected to display a corresponding region of inhomology at this site in heteroduplexes with non-defective virus DNA. A search for such heteroduplexes after denaturation-renaturation of the EcoRI-treated sample described above revealed the presence of only 0.2% of the renatured molecules with any evidence of a region which had not hybridized (Table 3).

DISCUSSION

Replication of polyoma and SV40 DNAs is normally bidirectional, starting from a site about one-third of the genome from the EcoRI cleavage site. This is based on examination of replicating molecules cleaved by EcoRI (Fareed et al. 1972; Crawford et al. 1973) and on pulse-labelling of the DNA, followed by digestion with restriction enzymes (Danna & Nathans, 1972; Crawford, Robbins & Nicklin, 1974) The results of pulse labelling experiments would not be significantly affected by a low frequency of replication initiated elsewhere in the DNA.

A unidirectional mode of replication initiated near the EcoRI site is now suggested to account for the appearance of a minority of the replicative intermediates of both SV40 and polyoma. After treatment with EcoRI, these molecules appear as Y-forms in which the two arms of the Y have approximately equal lengths. This type of Y-form is essentially absent from samples of non-replicating DNA. Furthermore, initiation of DNA replication appears to have occurred at both origins in a few of the replicating molecules examined. It should be pointed out that since this study is based entirely on the appearance of the DNA in the electron microscope there is no direct proof that these were replicating molecules. However, the most likely explanation of their appearance is that these molecules were in the process of replication when isolated. Under conditions similar to those used here, this preparation of EcoRI cleaves large plaque polyoma DNA to linear forms with expression of additional nuclease activities (Robberson & Fried, 1974). The additional nucleases appear to act preferentially to one side of the EcoRI site on polyoma DNA and within a distance of approx. 5% of the EcoRI site. Thus, this postulated second origin of DNA replication is
expected to occur within a distance of approx. 5% from the EcoRI site on polyoma DNA. The occurrence of Y-forms in replicating SV40 DNA treated with EcoRI implies a similar pattern of additional nuclease digestion as observed for polyoma DNA and perhaps something special about the EcoRI site itself.

Since unidirectional replication from a site near the EcoRI cleavage site has not previously been suggested for these DNAs, it is important to consider alternative explanations for these results. The explanation given by Fareed et al. (1972) for the Y-forms they observed was that they were molecules broken at one of the replication forks and cleaved by EcoRI. This would account for molecules which were less than full length but not for full length molecules. Most of the molecules discussed here were within 11% of the full length and the two arms of the Y were approx. equal (Fig. 4). These could only be produced from replicating molecules which were not cleaved by EcoRI but which were broken simultaneously on both sides of a fork. It seems unlikely that this would occur often unless the EcoRI preparation contains an enzyme with a preference for the fork itself. Breakage on only one side of the fork would seem much more likely and molecules in which this had occurred were seldom seen, either here or previously (Fareed et al. 1972).

Some of the Y-forms could have arisen through cleavage of replicating circular dimeric molecules of SV40 and polyoma DNAs if the EcoRI sites on the monomer genomes of these dimers are arranged appropriately. Approx. 1% of the intracellular forms of both SV40 (Rush, Eason & Vinograd, 1971) and polyoma (our examination of 500 molecules) occur as circular dimers. Total replicative forms constitute about 1% of the intracellular DNA. Thus, the frequency of circular dimers is considerably lower than the frequency of the Y-forms reported here and they are not likely to be the source of this particular replicating intermediate. Similarly, defective molecules either sensitive or resistant to EcoRI, are not frequent enough to account for the number of Y-forms seen. None of these alternative explanations seem satisfactory and we therefore conclude that the Y-forms arise from unidirectional replication initiated near the EcoRI site. This unusual mode of replication might, when abortive, lead to the formation of defective DNAs as suggested recently (Robberson & Fried, 1974).

Note added in proof.

An examination of replicating SV40 DNA molecules cleaved by endonuclease HpaII, carried out with the help of Paul Nicklin, has now provided further evidence for a unidirectional mode of replication. Endonuclease HpaII cleaves SV40 DNA once, 74% from the EcoRI site (Sharp, Sugden & Sambrook, 1973). This therefore avoids the complications caused by the use of a cleavage site close to the apparent minor origin of replication. Molecules in which replication was initiated as the predominant origin (67% from the EcoRI site) are mostly cleaved to double Y forms. Measurement of thirty such molecules showed that the origin was 7 ± 2% (74 minus 67) from the HpaII cleavage site, as expected.

Molecules in which replication had been initiated at a position away from the normal origin may be converted to forms with a single duplex circular loop by cleavage with HpaII (Figs. 1a and 2). Measurement of seventeen such molecules showed that in fourteen of them one end of the loop was located at 26 ± 4% from the end of the molecule. This is consistent with this fork being close to the EcoRI cleavage site (26% from the HpaII site). In eleven of the molecules replication appeared to be going away from the HpaII site i.e. clockwise on the map as normally drawn, and in three molecules towards the HpaII site. In the remaining three molecules the position of the internal loop was not consistent with
unidirectional replication as above or bidirectional replication from the normal origin. Thus the configuration of fourteen out of seventeen of these internal loop molecules was consistent with their coming from unidirectional replication starting at a position $26 \pm 4\%$ from the HpaII site, i.e. within $4\%$ of the EcoRI site.

We are indebted to Alan Robbins for his help with preparation of virus DNA. We would also like to express our gratitude to Dr J. Sinkovics for the use of his electron microscope laboratory, the gift of Mr and Mrs L. Lippman, in the completion of these studies.

REFERENCES


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