The Replication of Polyoma DNA

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SUMMARY

Measurement of replicating molecules of polyoma virus DNA after digestion with endonuclease R₁ shows that DNA replication is bidirectional, starting predominantly at a specific site. In both large plaque and small plaque polyoma virus DNA this site is 29 ± 2 % of the total length of the DNA from the cleavage site of the endonuclease R₁.

INTRODUCTION

It has been established that polyoma virus DNA and Simian virus 40 (SV 40) DNA replicate by the mechanism first proposed by Cairns (1963) and Hirt (1969). Further studies (Bourgaux & Bourgaux-Ramoisy, 1971, 1972) have elucidated some of the properties of the intermediates in replication. The replication of SV 40 DNA has been shown to start at a specific site, proceed bidirectionally, and terminate at a specific site (Danna & Nathans, 1972; Fareed, Garon & Salzman, 1972; Nathans & Danna, 1972). It is of interest to know whether the replication of polyoma virus DNA follows the same pattern.

METHODS

Virus stocks. One stock of small plaque (Crawford, 1962) and one stock of large plaque (Dulbecco, Hartwell & Vogt, 1965), polyoma virus were used for this study. Both stocks had been recently plaque purified and grown at low multiplicity of infection. The quality of the virus was checked by determining that the DNA produced in one cycle of growth at high multiplicity was more than 90% sensitive to the restriction endonuclease R₁.

Isolation of replicating DNA. Mouse embryo fibroblast secondary cells were infected at an input multiplicity of 10 to 20 p.f.u./cell and grown for 24 or 30 h at 37 °C in Dulbecco’s enriched Eagle’s medium (E 4), with 10% calf serum. Medium was removed from the cultures and the DNA extracted with sodium dodecyl sulphate by the method of Hirt (1967). The small DNA in the Hirt supernatant fraction was phenol extracted, precipitated with ethanol and centrifuged in ethidium bromide/CsCl gradients (200 µg/ml ethidium bromide; density 1.58 g/ml) in a 50 Ti angle rotor (Beckman L 2 65 B centrifuge), at 40,000 rev/min for 48 h. After centrifuging the tubes were examined in u.v. light to locate the lower (covalently closed circular DNA) and upper (linear and relaxed circular DNA) bands. These two bands, and the region between them, were collected separately.

The ethidium was removed from the samples by repeated extraction with isopropanol, saturated with 50 % (w/w) CsCl, and the samples dialysed against tris buffer (0·01 M, pH 7·5) containing mm-EDTA. In some cases, the DNA was concentrated by precipitation with two vol. of ethanol and the precipitate resuspended in the same buffer.

R₁ endonuclease cleavage. The R₁ endonuclease was isolated by Dr W. R. Folk, following
the procedure of Yoshimori (1971) from *Escherichia coli* RY-13, an endonuclease 1− strain carrying the resistance transfer factor R1. DNA was digested in tris-buffered (0.05 M, pH 7.5) saline (0.1 M-NaCl) containing MgCl₂ (0.01 M). Digestions were carried out at 37 °C usually for 15 min, with the amount of enzyme adjusted to cleave 50 to 90 % of the polyoma DNA molecules, as determined by preliminary experiments. Since the R₁ endonuclease may not be completely specific nor completely free from other less specific endonucleases it was considered desirable to avoid treating the DNA with an excess of enzyme, since this might cause cleavage of the replicating molecules at sites other than the R₁ site. Most DNA preparations contain defective molecules which are resistant to digestion by the R₁ endonuclease. Cleavage of such molecules by endonucleases other than R₁ would be expected to give rise to molecules in which the apparent origin of replication was not in its normal position.

**Electron microscopy.** Samples were prepared for electron microscopy by the basic protein film technique of Kleinschmidt, using the aqueous method of Davis, Simon & Davidson (1970). The DNA sample in 0.5 M-ammonium acetate with 100 µg/ml cytochrome C was layered on to a hypophase of 0.25 M-ammonium acetate. The film was picked up on parlodion-coated copper grids, stained with 10⁻⁸ M-uranyl acetate in 90 % ethanol, dried with ethanol and rotary shadowed with platinum: palladium (80:20). Micrographs were taken with a Siemens 101 at an instrumental magnification of ×10000, and the DNA molecules measured at a final magnification of ×90000.

**RESULTS**

The techniques used here were based on those developed for SV 40 by Fareed *et al.* (1972). Replicative intermediates were isolated by centrifuging to equilibrium in CsCl gradients containing ethidium bromide and then cleaved at a specific point with the restriction endonuclease R₁ (Yoshimori, 1971). This enzyme cuts DNA at a specific sequence, which has been determined (Hedgpeth, Goodman & Boyer, 1972) and, as this occurs only once in SV 40 DNA (Morrow & Berg, 1972), converts circular DNA into linear DNA of the same length. The same is true for polyoma virus. Polyoma DNA is cleaved by endonuclease R₁ to full length linear molecules and these give rise to linear molecules, not circles, when denatured and reannealed, showing that they were all cut at the same point (D. Robberson, personal communication). The results reported here confirm this, since the distance of the origin of replication from the cleavage site would be constant (see below) only if both the following conditions were satisfied. Firstly, that the endonuclease R₁ always cleaves the replicating molecules at the same site and, secondly, that the origin of replication is at a specific site on the DNA. The R₁ site forms a convenient reference point on the DNA and the positions of the replication forks in the replicative intermediates can then be determined relative to this site.

In CsCl gradients containing ethidium bromide, replicating circular duplex DNA has a buoyant density between that of non-replicating covalently closed circular DNA (1.59 g/ml), and that of open circular DNA (1.55 g/ml). DNA, extracted from cells infected with small plaque polyoma virus, was centrifuged to equilibrium in a CsCl-ethidium bromide gradient. The interband DNA, when examined by electron microscopy, contained many replicating molecules, although it was contaminated with supercoiled DNA from the lower band and linear plus open circular DNA from the upper band.

Before R₁ endonuclease digestion, many of the replicating molecules were too tightly supercoiled for the entire contour length of the DNA to be discerned. This was previously
observed in the replication of SV 40 by Sebring et al. (1971). After R₁ cleavage, the molecules assume the linear configuration shown in Fig. 1 with a replication loop, a long arm and a short arm. Since R₁ endonuclease cleaves polyoma virus DNA at a specific site, these forms must have been derived from Cairns type circles, all of which have been opened at the same site. Cleavage at this site then generates the two free ends of each molecule.

Measurements were made on molecules of the type shown in Fig. 1, with the results summarized in Fig. 2. As the replicated region becomes larger, both arms (L₁ and L₄) become shorter, i.e. the replication forks approach the R₁ site. After replication passes through the R₁ site, the appearance of the molecule changes to that of a double Y (Fig. 3).

The distance of the origin of DNA replication from the R₁ cleavage site was estimated as $\left[\frac{1}{2}(L_2 + L_3)\right] + L_1$ for the set of molecules measured for Fig. 2. For small plaque polyoma
virus this origin occurs at a position corresponding to $29 \pm 2\%$ from the R₁ site, taking the length of the molecule $(L₁ + \frac{1}{2}(L₂ + L₃) + L₄)$ as 100%. The actual value obtained was 28.67% and since the standard deviation was $\pm 2\%$ this has been rounded off to 29%. This is the origin of the line shown on Fig. 2, extrapolating to $L₁$ equals 29% at zero replication and to $L₁$ equals zero at 58% replication. All the points in Fig. 2 fall close to the line as expected for R₁ endonuclease cleavage at a unique site, when the replication is bidirectional from a unique origin with both replication forks moving at similar rates. The point at which replication terminates is not localized precisely but it is likely to be the opposite side of the circle from the origin, i.e. 79% of the molecule from the R₁ site. The small number of double Y molecules observed are consistent with this interpretation. Some molecules, similar in general appearance to those shown in Fig. 1, have not been included in Fig. 2 or subsequent calculations. When the two sides of the replication loop were of unequal length (by more than 2% of the overall length of the molecule) (about 20% of the
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Fig. 3. Replicating molecules of small plaque polyoma virus DNA. These molecules are from the same preparation as shown in Fig. 1 but differ from them in that replication has extended beyond the R₁ cleavage site.

Fig. 4. The relationship of the extent of replication and the position of the replication loop in endonuclease R₁ cleaved large plaque polyoma virus DNA. Replicating DNA molecules were isolated from cells infected with large plaque polyoma virus and treated with endonuclease R₁. The relative length of the short arm L₁ has been plotted against the extent of replication, as for Fig. 2.
molecules), or when the length of the molecule was less than that of the open circular or linear polyoma DNA measured on the same grids (about 10% of the molecules), the molecule was not included in the analysis. Most of this variation is probably due to uneven spreading of the molecules during preparation for electron microscopy. Some of the smaller molecules may be defective DNAs in which parts of the virus DNA are missing or have been rearranged.

Similar experiments with large plaque polyoma virus gave the results shown in Fig. 4. Replication appears to follow the same course as seen with small plaque virus and the origin of replication is located at 29±2% from the R1 site. This is, therefore, not significantly different from the value obtained for small plaque polyoma virus.

DISCUSSION

The DNA replication of the two strains of polyoma virus studied appears to be similar in several respects to that of SV40 DNA (Danna & Nathans, 1972; Fareed et al. 1972; Nathans & Danna, 1972).

The replicating polyoma virus DNA molecules, after R1 digestion, can be arranged in an orderly series, indicating a unique origin for the bidirectional replication, assuming that the R1 endonuclease cleaves at the corresponding site in each molecule or at a small number of sites very close to each other. The average overall length of all the replicating molecules observed was not significantly different from that of non-replicating open circular DNA. Thus no detectable length of DNA was removed by R1 treatment.

The symmetry of this array of replicative forms indicates that replication proceeds in both directions from a unique origin at comparable rates. Unidirectional replication would give rise to molecules in which either L1 or L4 would remain constant as replication proceeded, and this is clearly not the case here.

The positions determined for the origin of replication, at 29% of the genome from the R1 site, are similar for large plaque and small plaque polyoma virus. The origin for SV40 DNA replication occurs at 33% from the R1 site (Fareed et al. 1972). Since the virus DNAs are circular there is an ambiguity in positioning the origin of replication by these studies alone. The origin located at 29% of the genome from the R1 site may in fact be positioned at 71% (100-29) from the R1 site as measured in the opposite direction. To resolve this ambiguity it is necessary to locate some other marker on the DNA and experiments are now in progress to locate the attachment site for phage T4 gene 32 protein (Delius, Mantell & Alberts, 1972) on replicating molecules relative to the R1 site and the origin of replication. This should allow an unambiguous positioning of the replication origin in the two strains of polyoma virus DNA. Preliminary experiments indicate that the gene 32 protein attachment site in polyoma virus DNA extends from approximately 20 to 25% of the total length of the DNA from the R1 cleavage site (M. Yaniv, O. Croissant & F. Cuzin, personal communication; and our unpublished results with Dr J. Monjardino). This is true for both large plaque and small plaque virus. In R1 cleaved replicating molecules gene 32 bubbles have not been seen in the long arm, L4 of the molecules. This indicates that, measuring in the same direction from the R1 site, the gene 32 site is likely to be at 20 to 25% and the origin of replication at 29%.

The comparison of polyoma virus DNA with SV40 DNA is more difficult since it is not known whether the R1 site is located in a region with equivalent biological function in the two viruses. With the accumulation of additional positional markers on polyoma virus DNA it should be possible to make more meaningful comparisons for the functional anatomies of the two virus DNAs.
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REFERENCES


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