Location of the Origin and Terminus of Replication in Polyoma Virus DNA

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SUMMARY

The kinetic data obtained from examination of restriction enzyme fragments give a direct determination of the position of the origin of replication in polyoma virus DNA. The results obtained are consistent with the previous estimates based on electron microscopic measurement of replicating molecules cleaved by endonuclease EcoR1 (29 ± 2% from the EcoR1 site). The position of the terminus is diametrically opposite the origin, at approx. 79% clockwise from the EcoR1 site.

INTRODUCTION

In a previous paper it was shown that polyoma virus DNA replicates bidirectionally, starting from a specific region. The origin of replication was found to be 29% of the genome from the cleavage site of the restriction endonuclease EcoR1 (Crawford, Syrett & Wilde, 1973). Because of the circularity of the DNA, this positioning is in one sense ambiguous, in that there are two positions on the DNA 29% from the EcoR1 site, depending on whether measurement is made clockwise or anticlockwise, on any other map. To resolve this ambiguity the origin of replication was located by an independent method, in relation to the physical map based on the fragments produced by digestion with endonuclease HpaII (Griffin, Fried & Cowie, 1974), a restriction enzyme from Hemophilus parainfluenzae (Gromkova & Goodgal, 1972; Sharp, Sugden & Sambrook, 1973). This involves pulse labelling of the molecules during replication. In the molecules which complete replication during the pulse, regions of the DNA near the origin would be expected to be least radioactive and those near the terminus of replication most radioactive. Conversely, molecules which enter replication during the pulse will be more radioactive near the origin and less radioactive near the terminus. This technique, based on the kinetic analysis of haemoglobin synthesis (Dintzis, 1961), had been used to determine the origin and bidirectional mode of replication in SV40 (Danna & Nathans, 1972; Nathans & Danna, 1972). The results of this analysis, in conjunction with the previous measurements, define the positions of the origin and terminus of replication on polyoma virus DNA.

METHODS

Virus stocks. The small plaque virus, CSP, and the large plaque virus, DLP 1106, were those used in previous studies on replication (Crawford et al. 1973). Another large plaque strain, also derived from Dulbecco's large plaque virus, A2 (Griffin et al. 1974) was also used.
Preparation of radioactive virus DNA. Cultures of 3T6 mouse cells were grown at 37 °C in Dulbecco's enriched Eagle's medium (E4) with 5% foetal calf serum. When the cultures were nearly confluent they were infected with 10 p.f.u./cell of polyoma virus.

Forty hours after infection the cultures were pulse labelled with [3H]-thymidine (22 Ci/mmol), 200 μCi per 90 mm plate in 0.4 ml of medium without serum. After 10, 20 or 30 min at 34 °C the medium was removed, and the DNA extracted by the method of Hirt (1967). Superhelical DNA (form 1) was isolated by equilibrium sedimentation in CsCl containing ethidium bromide (Radloff, Bauer & Vinograd, 1967). Replicating molecules (RF), which band between the covalently circular and nicked circular plus linear DNA in the ethidium bromide/CsCl gradients, were collected separately. Further purification of form 1 and RF DNA was obtained by chromatography on benzoylated naphthoylated DEAE cellulose (Levine, Kang & Billheimer, 1970; Bourgaux, Bourgaux-Ramoisy & Seiler, 1971). Form 1 DNA was eluted with 1M-NaCl and RF DNA with 1M-NaCl containing 1% caffeine. As a check on the purity of the two types of DNA at the end of this procedure, samples were rechromatographed on BND cellulose. With form 1 DNA 98% of the radioactivity eluted again in the 1M-NaCl fraction and with RF DNA 90% eluted again in the caffeine fraction.

Uniformly labelled DNA was produced and purified by the same technique, except that the [3H]-thymidine was left on the cultures for 20 h. Uniformly [32P]-labelled DNA was produced in 3T6 mouse cells grown in E4 medium minus phosphate with 5% calf serum.

Nuclease digestion and separation of DNA fragments. Endonuclease EcoR₁ was isolated from Escherichia coli RY-13 by the method of Yoshimori (1971), endonuclease HpaII from Hemophilus parainfluenzae by the method of Sharp et al. (1973), and HaeIII endonuclease from Hemophilus aegyptius by the method of Middleton, Edgell & Hutchison (1972).

Mixtures of [3H]-labelled and [32P]-labelled DNA (in the ratio of 3:1 to 10:1, by radioactivity) were suspended in tris buffer (0.01 M, pH 7.5) containing MgCl₂ (0.01 M) and 10 μg/ml gelatin. Each reaction mixture contained 1 to 10 μg DNA in 0.02 to 0.2 ml. A sample of endonuclease HpaII was desalted and 10 μl added per reaction mixture. After 2 h incubation at 37 °C one-tenth of each reaction mixture was analysed by electrophoresis on agarose gels (Sharp et al. 1973) to ensure that digestion was complete. Where necessary 10 μl more enzyme was added and the incubation repeated.

The DNA fragments were separated by electrophoresis in slab gels 0.4 x 20 x 40 cm using 4% acrylamide (Griffin et al. 1974), but omitting agarose. Fragments to be recut were eluted electrophoretically as described by Galibert, Sedat & Ziff (1974). After phenol extraction the DNA was precipitated with ethanol, resuspended in tris buffer (pH 7.6, 6 mM), containing 6 mM-MgCl₂ and 6 mM-β-mercaptoethanol for digestion with HaeIII. The recut fragments together with reference HpaII fragments were separated on 8% acrylamide gels.

The radioactive bands were located by radioautography, excised and dissolved in 30% H₂O₂ at 70 °C, and mixed with Aquasol (New England Nuclear). Samples were then counted in a scintillation spectrometer and after appropriate corrections for background and spillover, the relative amounts of [3H] and [32P] calculated for each sample.

Since [3H]-thymidine was used to pulse label the DNA the radioactivity in each fragment depends both on its size and base composition. To allow a correction to be made for the effect of variation in thymidine content, continuously labelled DNA was also analysed in parallel with the pulse labelled material.

Electron microscopy of replicating DNA. DNA preparations containing replicating molecules were prepared and digested with endonuclease EcoR₁ as previously described.
Replication in polyoma virus DNA

Fig. 1. The positions of the replication forks in replicating molecules cleaved by endonuclease EcoR1. (a) A preparation of A2 polyoma virus DNA containing early replicating molecules was digested with endonuclease EcoR1 and examined by electron microscopy. The contour lengths of the four elements of each molecule were measured and expressed as percent of the overall length \( \left( \frac{L_1 + L_2 + L_3 + L_4}{2} \right) \). In the diagram \( L_1 \) is plotted against percent replication \( \left( \frac{L_2 + L_3}{2} \right) \). (b) Late replicating molecules from the same preparation were also examined. In these replication had passed the EcoR1 cleavage site and the molecules had the appearance shown in the inset. Percent replication \( \left( \frac{L_5 + L_6 + L_7 + L_8}{2} \right) \) is plotted against the mean length of the long arms \( \left( \frac{L_5 + L_6}{2} \right) \).

(Crawford et al. 1973), except that the digestion buffer contained 0.2 M NaCl to inhibit nucleases other than EcoR1. All other details, of electron microscopy and length measurement were as previously described.

RESULTS

The strains of polyoma virus used in this and previous work differed in their fragment patterns after digestion with endonuclease HpaII, as described later. For this reason, it was necessary to check the position of the origin of DNA replication by electron microscopy on the A2 strain used by Griffin et al. (1974) for construction of their map of polyoma virus DNA. The techniques used were the same as described previously (Crawford et al. 1973). Replicating molecules were digested with endonuclease EcoR1 and the position of the replication loop on each molecule measured (Fig. 1a).

Several additional precautions were taken to ensure that the position determined was not biased by enzymic or electron microscopic artefacts. An internal standard of known length, bacteriophage PM2 DNA, was added to all preparations as a check on the actual magnification. Also the contour length of open ring polyoma DNA molecules not involved in replication was measured in the same preparation as the replicating molecules. Any attack on the replicating molecules by exonucleases and other endonucleases in the EcoR1 preparation would result in the RF becoming shorter than the open rings. The ionic
Polyoma DLP A2

Fig. 2. Relative specific activities of HpaII fragments of pulse labelled A2 polyoma virus DNA. Cultures of 3T6 cells were infected with A2 polyoma virus. Forty hours after infection [3H]-thymidine was added for 10, 20 or 30 min at 34 °C. Virus DNA was then isolated and separated into completed, form I DNA (labelled predominantly near the terminus) and replicating molecules (labelled predominantly near the origin). Each DNA was then mixed with uniformly [32P]-labelled A2 DNA and digested with endonuclease HpaII. The DNA fragments were isolated and their specific activities determined. The relative specific activity for each fragment is plotted against the map positions of the various fragments. The upper part of the diagram shows data from completed molecules and the lower part data from replicating molecules. • •, 10 min completed DNA; • •, 20 min completed DNA; ▲ — ▲, 30 min completed DNA; □ — □, 10 min replicating DNA.

conditions used in the EcoR1 digestion were designed to inhibit other enzymes and in fact there was no detectable difference between the lengths of open rings and the end to end length of the replicating molecules. This shows that there was no significant attack by enzymes other than EcoR1.

The apparent position of the origin of replication on A2 strain polyoma DNA was 29.7 ± 2% from the EcoR1 cleavage site calculated as the mean value of L1 + 1/3 (L2 + L3). Each point on Fig. 1a represents one of the molecules measured. The line indicates the trend expected if replication were bidirectional and equally rapid in the two directions. The two intercepts are 29.7% for L1 where percent replication equals zero, and 59.4% (twice 29.7%) for percent replication where L1 equals zero, i.e. one replication fork is at the EcoR1 site. As discussed previously (Crawford et al. 1973) the points fell close to this line, strongly supporting a bidirectional mode of replication with closely similar rates of elongation at the two forks, at least in the early stages of replication. Previous values
Replication in polyoma virus DNA obtained by this method for the position of the origin were 29.3% (DLP 1106 DNA and 28.7% (CSP DNA) (Crawford et al. 1973). None of these values differed significantly from each other, although the DNA fragment patterns of the three strains showed significant differences, as described later, and illustrated in Fig. 3.

Examination of late replicating molecules, cut with endonuclease EcoR1 in an analogous manner gave a position for the terminus of replication, calculated as the mean value of \( \frac{1}{3}(L_4 + L_5 + L_6) \). This was 79 ± 5% from the EcoR1 site (Fig. 1b). The trend is based on the same assumptions as before and the intercept at 100% replication corresponds to \( \frac{1}{3}(L_5 + L_6) \) equals 79%. The scatter of the values for the apparent position of the terminus in individual molecules was much larger than for the origin. This may be due to the fact that the two replication forks become increasingly out of step as replication proceeds. This does not necessarily mean that termination occurs wherever the forks happen to meet, rather than at a particular site or sequence on the DNA. The most rapidly moving fork may stop at a particular site and wait for the other fork to arrive. Replication could then be completed and the daughter molecules separated from each other.

Analysis of pulse labelled DNA

Endonuclease HpaII cuts A2 polyoma DNA into eight fragments separable by gel electrophoresis (Griffin et al. 1974). The kinetics of labelling of these fragments can be used to locate the origin of replication as has already been done for SV40 DNA using Hemophilus influenzae restriction endonucleases (HinIII) (Danna & Nathans, 1972). DNA preparations labelled for short periods with \(^{3}H\)-thymidine were separated into form I and RF mixed with \(^{32}P\) uniformly labelled DNA, digested with HpaII and the fragments separated by gel electrophoresis. The relative specific activities of the HpaII fragments of completed, form I, DNA molecules are shown in the upper part of Fig. 2, plotted at the map positions of the fragment centres. The least radioactive fragments from completed molecules were 3 and 5. This was sufficient to place the origin unambiguously on this side of the ring but, since the region around the origin was the least radioactive part of the molecule, these data are more useful for locating the terminus than the origin. Replicating molecules, i.e. those molecules which were still replicating at the end of the pulse, had the converse labelling pattern and gave much better data for the origin. The distribution of radioactivity in the HpaII fragments of replicating molecules is shown in the lower part of Fig. 2. The most radioactive fragment was HpaII fragment 5 and the relative specific activities of the fragments on either side showed clearly that this is the most probable location for the origin of DNA replication. This fragment extended from 21.7 to 29.4% from the EcoR1 site. The location of the terminus of replication, as judged from the labelling pattern of the completed molecules, seems to be close to the junction of fragments 2 and 6 at 80% from the EcoR1 site. Neither these data nor the electron microscopic measurements give an exact position for termination, if indeed it occurs at a precise location, rather than within a more extended region of the DNA.

Fragment patterns of other strains of polyoma virus DNA

The patterns obtained by digestion of virus DNA with HpaII are shown in Fig. 3. The two large plaque strains were initially thought to be identical but in the course of these experiments on replication it became clear that they were not. The fragment 5 of the two strains differed in size by about 0.3%, or 20 base pairs. This was not due to DLP 1106 DNA having an additional HpaII site within the region corresponding to fragment 5 of A2, since no additional fragment of about the size expected was detected in digests of this DNA.
Fig. 3. Gel electrophoresis of DNA fragments produced by endonuclease HpaII digestion. DNA from polyoma virus strains DLP 1106, A2 and CSP were digested with HpaII and the fragments separated by electrophoresis on 4% acrylamide gel. Differences between the strains are arrowed. From top to bottom these are: the absence of fragment 1 in CSP, larger fragment 3 in CSP, additional fragments 1a and 1b in CSP, and smaller fragment 5 in DLP 1106. Fragment 6 is also slightly smaller in CSP but the difference in mobility is too small to be seen here.
Replication in polyoma virus DNA

Fig. 4. Relative specific activities of HpaII fragments of pulse labelled CSP polyoma virus DNA. Pulse labelled virus DNA was prepared and analysed as described for Fig. 2 but using small plaque CSP polyoma virus for production of both [3H]-labelled and [32P]-labelled DNAs. • •, 10 min completed DNA; ▲ ▲, 20 min completed DNA; ■ ■, 30 min completed DNA; ○ ○, 10 min replicating DNA; △ △, 20 min replicating DNA; □ □, 30 min replicating DNA.

DNA from the small plaque strain CSP showed a rather different pattern of HpaII fragments. Fragments corresponding in size to A2 fragments 2, 4, 5, 7 and 8 were present. Fragment 3 appeared to be larger and fragment 6 slightly smaller than the corresponding fragments of A2 virus. To ensure that the mobility differences of these fragments were genuine, double label experiments with mixtures of [32P]- and [3H]-labelled DNAs of the various strains were done. In each case the mixed DNAs were digested with HpaII and the fragments separated on gels. The area corresponding to the [32P]-fragment was located and cut out as three slices, the centre of the band and its leading and trailing edges. The ratios of [3H] to [32P] for the mixture of [3H]-A2 fragment 3 and [32P]-CSP fragment 3 were 0.25, 0.4 and 0.9 for the three slices in order of increasing mobility. The mixture of [3H]-CSP fragment 6 and [32P]-A2 fragment 6 gave ratios of 1.8, 2.6 and 3.8 across the band, consistent with CSP fragment 6 being slightly smaller than A2 fragment 6. The most striking difference between CSP and the large plaque DNA patterns was the absence of a fragment corresponding to A2 fragment 1 and appearance of two additional fragments. The mobilities of these fragments corresponded to 15% and 12% of polyoma virus DNA. Since A2 fragment 1 is 27.3% (Griffin et al. 1974), equal to the sum of these two fragments, the simplest explanation for these differences would be that CSP DNA has nine HpaII sites as compared
Fig. 5. The positions of the origin and terminus of replication on polyoma virus DNA. (a) This map shows the positions of the \( \text{Hpa}_{II} \) cleavage sites in DLP A2 polyoma virus DNA and the numbers of the fragments (Griffin et al. 1974). Outside the circle the positions of the origin and terminus, based on electron microscopic measurements, are shown with bars equal in length to \( \pm \) one standard deviation. Inside the circle the bars represent the positions and lengths of the \( \text{Hpa}_{II} \) fragments which appear to include the sites of initiation and termination of DNA replication.

(b) This shows the \( \text{Hpa}_{II} \) cleavage sites on CSP polyoma virus DNA. The position of the origin, based on electron microscopic measurements is shown outside the circle with a bar equal in length to \( \pm \) one standard deviation (Crawford et al. 1973).

with eight sites in A2 DNA, and that the additional site is in the region of the DNA corresponding to A2 fragment 1.

Analysis of digests of pulse labelled CSP DNA gave the results shown in Fig. 4. The arrangement of the fragments is based on the assumption given above, that CSP fragment 1a plus fragment 1b correspond to A2 fragment 1. The order of the two fragments is also clear from consideration of their relative specific activities. Reversing the order, or the placing of the fragments elsewhere would not be consistent with their relative specific activities in either completed or replicating molecules. Confirmation of this positioning was obtained by the use of another restriction endonuclease and from pyrimidine tract fingerprints. Endonuclease \( \text{Hin}_{III} \) cuts polyoma virus DNA at two sites, one of which is 8.5% from one end of \( \text{Hpa}_{II} \) fragment 1 of A2 DNA (Griffin et al. 1974). Digestion of CSP DNA with \( \text{Hpa}_{II} \) followed by \( \text{Hin}_{III} \) gave a pattern in which fragment 1a was absent and two fragments about 9 and 5% in size appeared. This showed that CSP fragment 1a has a \( \text{Hin}_{III} \) site 9% from one end, similar to A2 fragment 1, and that CSP fragment 1b lacked a \( \text{Hin}_{III} \) site. Fragment 1a may have a second \( \text{Hin}_{III} \) site in the end nearest to fragment 1b. This could account for the difference between the size of fragment 1a (15%) and the sum of the two \( \text{Hin}_{III} \) fragments (9% plus 5% = 14%). A fragment about 1% may therefore have been cut from one end of the smaller \( \text{Hin}_{III} \) fragment. Some comparisons of CSP and A2 DNA fragments have also been made by means of pyrimidine tract fingerprints (Ling, 1972), and we are indebted to Dr E. Ziff for these analyses. The fingerprint of CSP fragment 1a showed several of the characteristic spots of A2 fragment 1, whereas CSP fragment 1b gave spots not found in A2 fragment 1. In fact several of the major fingerprint differences between CSP and A2 DNA appeared to be in the fragment 1b region. The fingerprints of CSP fragment 3 and A2 fragment 3 were very similar with only a few obvious differences, consistent with their being the corresponding regions of the two DNAs. The overall distri-
bution of radioactivity for pulse labelled CSP DNA was very similar to that already shown for A2 DNA. The origin of replication was again in fragment 5 and the terminus close to the junction of fragments 2 and 6. These positions are shown in Fig. 5 for DLP A2 and CSP DNAs.

DISCUSSION

When the initial work on the location of the origin of DNA replication was carried out (Crawford et al. 1973), there was little information on the anatomy of polyoma virus DNA beyond denaturation mapping (Follett & Crawford, 1968). It would have been difficult to relate the position determined for the origin from measurements of replicating molecules cleaved by EcoR1 endonuclease to such a map. The elucidation of the map of fragments produced by HpaII digestion of the DNA (Griffin et al. 1974) gave a much better opportunity for relating the position of the origin to other reference points in addition to the EcoR1 cleavage site. With the knowledge of the distance of the origin from the EcoR1 site allocation of the origin to one side of the ring and the terminus to the other in fact requires only a small amount of additional information. The fact that HpaII fragments 3 and 5 have low specific activities (and fragments 2 and 6 high specific activities) in pulse labelled superhelical DNA is sufficient for an unambiguous allocation. The other data from both replicating and completed molecules provides substantial confirmation of this. It also gives an independent confirmation of the correctness of the positioning of the HpaII fragments since any error, such as the inversion of a pair of fragments, might be expected to show up as a deviation from the gradient of specific activity from origin to terminus on each side of the ring.

In small plaque polyoma virus DNA the data from pulse labelling allows the elucidation of differences in the HpaII pattern of the DNA, as well as providing information on the position of the origin of replication. This appears to be in fragment 5, as for large plaque polyoma virus DNA, consistent with the electron microscopic determinations of the positions of the origins in the two DNAs. The region between the EcoR1 cleavage site and the origin seems to be the same in the two DNAs, the differences between the strains being mainly in other parts of the molecule, HpaII fragments 1 and 3. The size of fragment 5 is such that sequencing of the DNA, to provide information about the nucleotide sequence at which DNA replication starts would be difficult. Further dissection of this region as a preliminary to sequencing and to provide a more precise location for the origin would be very useful. Preliminary data from redigestion of the HpaII fragments with endonuclease HaeIII suggests that the origin is in a HaeIII fragment about 300 nucleotide pairs long. This fragment comes from the end of HpaII fragment 5 next to the junction of HpaII fragments 3 and 5.

Since DNA replication is bidirectional, proceeding in both directions at similar rates, the point at which DNA replication terminates would be expected to be opposite the origin. This is the case in SV40 (Danna & Nathans, 1972; Fareed, Garon & Salzman, 1972) although it is not clear whether there is a specific sequence for termination or whether the two replication forks just happen to meet at about this point. Digestion of HpaII fragments from completed DNA molecules with HaeIII has also been used to give more information on the position of the terminus. The specific activities of the HaeIII fragments from HpaII fragment 2 and 6 are consistent with termination occurring in the region corresponding to the end of HpaII fragment 2 next to the 2/6 junction.
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REFERENCES


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