The Cleavage of Polyoma Virus DNA by Restriction Enzymes KpnI and PstI

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SUMMARY

The action of two restriction endonucleases on polyoma virus DNA has been examined and the sites at which they cleave the DNA located. One of the enzymes, KpnI from Klebsiella pneumoniae OK8, cleaves polyoma DNA twice at about 11.6 and 59.2 % from the EcoRI site. The other enzyme, PstI from Providencia stuartii 164, cleaves polyoma DNA five times at about 14.8, 16.5, 32.6, 50.3 and 80.0 % from the EcoRI site. Some of the cleavages produced by these enzymes alone, or in conjunction with other endonucleases, may be of use in the isolation of regions of particular interest from the virus DNA.

INTRODUCTION

The anatomy of polyoma virus DNA is now well defined, largely as a result of restriction enzyme mapping. The positions of the cleavage sites of a substantial number of type II restriction enzymes have been located including those of HpaII, EcoRI and HindIII (Griffin, Fried & Cowie, 1974), HindII (Chen, Chang & Salzman, 1975; Folk, Fishel & Andersen, 1975; Griffin & Fried, 1975). (The nomenclature used here for the restriction endonucleases follows that suggested by Smith & Nathans, 1973, omitting the R for restriction.) Many regions of the virus DNA of particular interest can be isolated by making use of these enzymes in various combinations. Even so, due to the locations of the cleavage sites of these enzymes, there are several regions of the DNA which are difficult to isolate. For this reason enzymes from two further organisms were isolated and examined to determine whether they would prove useful in helping to dissect the virus DNA and understand its functions.

Klebsiella pneumoniae OK8 and Providencia stuartii 164 have both been shown to release restriction enzyme-like activity, as assayed on phage lambda DNA, when cells were subjected to osmotic shock (Smith, Blattner & Davies, 1976). We tested crude extracts of both organisms, prepared by osmotic shock, and found them to be active on polyoma DNA. Other non-specific activities were clearly present and these interfered with the use of the enzymes for obtaining specific DNA fragments. A simple purification procedure was devised which produced enzymes of sufficient purity to allow determination of their cleavage patterns on polyoma DNA and their subsequent use in isolation of specific DNA fragments. The location of cleavage sites was greatly facilitated by the restriction enzyme maps already mentioned and several of these enzymes were used here. The positions already determined for their cleavage sites were used as reference points for the location of the cleavage sites on polyoma virus DNA for KpnI and PstI, the major enzymes from Klebsiella pneumoniae OK8 and Providencia stuartii 164.
METHODS

Virus DNA. The polyoma virus strain used, large plaque A2 (Griffin et al. 1974) had recently been plaque purified and was free of detectable defective DNA molecules. Virus DNA was isolated by extraction of virus infected cells by the method of Hirt (1967). The supercoiled form I DNA was isolated by equilibrium centrifugation in CsCl gradients containing ethidium bromide (Radloff, Bauer & Vinograd, 1967). Virus DNA labelled with $^{32}$P by propagation in medium containing inorganic $^{32}$P-phosphate was purified by an additional sucrose velocity sedimentation step.

Nomenclature. The fragments produced by each enzyme are numbered in order of decreasing size, PstI-1, PstI-2 etc. Fragments produced by the sequential action of two enzymes are referred to as for example, KpnI:EcoRI fragments. The sizes of fragments are given as percentages of the size of the intact polyoma virus DNA molecules. Positions on the circular physical map are given as percentages from the single EcoRI site measured clockwise on the map as conventionally drawn (Griffin et al. 1974).

Enzyme purification and assay. Cultures of Klebsiella pneumoniae OK8 and Providencia stuartii 164 were obtained from Dr J. Davies. The enzymes from these strains were both purified by similar procedures, based on those developed for other restriction enzymes by Dr R. J. Roberts (e.g. Roberts et al. 1975).

Ten g of bacteria were disrupted by sonication in 20 ml of 0.01 M-tris buffer, pH 7.9, containing 0.01 M-$\beta$-mercaptoethanol. The supernatant fluid from centrifugation at 100000 g for 90 min was made 1 M with NaCl and passed over a Biogel A-0.5 m column (50 ¥ 2.4 cm). The column was developed with buffer containing 10% glycerol, 1 M-NaCl, 0.01 M-tris, pH 7.9, 0.01 M-$\beta$-mercaptoethanol and 1 mM-EDTA and 10 ml fractions collected. Enzyme activity was located by assaying 2 $\mu$l from each of the first 40 fractions on polyoma virus DNA (0.2 $\mu$g in 20 $\mu$l assay buffer, 0.01 M-tris, pH 7.5, 0.01 M-MgCl$_2$ for 30 min at 37 °C). The assay mixtures were analysed by electrophoresis on 1% agarose tube gels run in 0.04 M-tris, pH 7.8, 0.005 M-sodium acetate, 1 mM-EDTA containing 0.5 $\mu$g/ml ethidium bromide and DNA bands located by fluorescence of bound ethidium as described by Sharp, Sugden & Sambrook (1973). The active fractions (20 to 25) were dialysed into 0.01 M-potassium phosphate buffer, pH 7.4, 0.01 M-$\beta$-mercaptoethanol, 0.1 mM-EDTA containing 10% glycerol, applied to a P11 phosphocellulose column (12 ¥ 1.2 cm) and eluted with a gradient of 0 to 1 M-KCl in the same buffer.

KpnI eluted from phosphocellulose at 0.2 to 0.25 M-KCl. The flow-through from the column also contained a variable amount of activity but as this fraction was contaminated with much more non-specific endonucleolytic activity it was discarded. The enzyme eluting from phosphocellulose at 0.2 to 0.25 M-KCl was concentrated by dialysis against 50% glycerol, 0.25 M-NaCl, 0.05 M-tris, pH 7.5, mm-dithiothreitol and stored at $-20 \, ^\circ\mathrm{C}$.

The same procedure was used for PstI and the enzyme was found to be eluted from phosphocellulose at 0.2 to 0.3 M-KCl. There was little activity detectable in the flow-through of the column. The enzyme at this stage did not appear to contain substantial amounts of other activities. Digestion with a tenfold excess of enzyme did not alter the fragment pattern observed on agarose gels.

The same basic assay buffer (0.01 M-tris, pH 7.5, 0.01 M-MgCl$_2$, mm-dithiothreitol) was used for digestion with HpaII and HhaI. For the other enzymes, EcoRI, BamI, KpnI and PstI, 0.1 M-NaCl was added to the basic assay buffer and for HindIII the basic assay buffer plus 0.05 M-NaCl was used.

Separation and recovery of DNA fragments. Large DNA fragments were separated both
analytically and preparatively on 1\% agarose gels containing 0.5 µg/ml ethidium bromide (Sharp et al. 1973).

Fragments from \(^{32}\)P-labelled DNA were separated on acrylamide gels of various concentrations ranging from 4 to 10\% for the smallest fragments. Bands were located by radioautography of wet gels where fragments were to be recovered or of dried gels where increased sensitivity was required. The DNA fragments were eluted from excised pieces of gel by electrophoresis on to a small pad of benzoylated naphthoylated DEAE (BND) cellulose held between discs of glass fibre paper in plastic syringes. Electrophoresis at 200 to 300 V in 0.01 M-tris buffer, pH 7.5, 0.01 M-sodium acetate for 10 to 25 h was needed to get most of the DNA out of the gel slices. DNA was recovered from the BND cellulose by elution with 1 M-NaCl in 0.01 M-tris, 0.05 M-EDTA containing 1\% caffeine and precipitated with ethanol. This method routinely gave recoveries of 60 to 90\% of the radioactivity from acrylamide or agarose gel slices, the losses being greater for larger fragments. Fragments recovered by this procedure were fully susceptible to digestion with other restriction enzymes.

**RESULTS**

**Cleavage with KpnI**

Cleavage of polyoma virus DNA, in either superhelical or open circular form, with KpnI, gave two fragments of almost the same size, each corresponding to approximately half of the DNA. The mobilities of the fragments on agarose gels were between those of the two HindIII fragments which represent 55.6\% and 44.4\% of the DNA (Griffin et al. 1974). Thus both of the KpnI fragments are more than 44\% and less than 56\% of the DNA.
Fig. 2. Separation of restriction enzyme fragments of polyoma DNA on 1.8% agarose gel. The samples from left to right were digests of 32P-polyoma DNA with KpnI (52.4%, 47.6%); KpnI followed by EcoRI (47.6, 40.8 and 11.6%; HhaI (46.4%, 41.6% and 12%); HindIII (55.6% and 44.4%); KpnI and PstI (only the two largest fragments have been resolved). The 1.8% agarose gel was supported by 7% acrylamide gel and the interface is marked by arrows on either side. Fragments less than 1% of the DNA would not have been detected here.

(Fig. 1 and 2). However, it is difficult to give more exact sizes based on mobility alone. The positions of the two cleavages giving rise to these two fragments were determined in several ways. KpnI-1 was cleaved by EcoRI and therefore includes the EcoRI site used as a reference point on these maps. Separation of KpnI-1 and KpnI-2 followed by digestion with HpaII showed that HpaII-1 was entirely within KpnI-2. In the double digest of intact DNA with HpaII followed by KpnI, HpaII-2 and HpaII-3 were absent showing that the two KpnI cleavage sites were within the regions of the DNA corresponding to HpaII-2 and HpaII-3. The other HpaII fragments did not appear to be cleaved by KpnI and this was confirmed with each of the isolated HpaII fragments. To locate the KpnI cleavage within HpaII-3 more accurately, HpaII-3 was incubated with either KpnI, BamI or a mixture of both enzymes. This produced fragments with sizes as follows: KpnI, 11.5% and 5.3%; BamI, 12.3% and 4.5%; KpnI plus BamI, 11.5%, 4.5% and 0.8%. This positions one of the KpnI cleavage sites at 59.2% on the map, 0.8% from the BamI cleavage site at 58.4% (Griffin & Fried, 1975).
Cleavage of polyoma virus DNA

Cleavage of HpaII-2 with KpnI gave fragments of 13.0% and 8.3%; digestion with HhaI also gave two fragments, 15.4% and 5.9%. Further digestion of the mixture of these HpaII-2: HhaI fragments with KpnI, gave three fragments of 13.0%, 5.9% and a small fragment. The size of this fragment was estimated to be 2.4% by comparison with HpaII-7 and HpaII-8, and 2.2% by comparison with HaeIII-11 (2.2%, B. Griffin, personal communication). Since the HhaI cleavage site in HpaII-2 is at 14.0% and the KpnI site appears to be closer to the EcoRI, this places the KpnI site at 11.6% to 11.8% from the EcoRI site. Another estimate for the location of this site gave a similar result. The smallest fragment produced by digestion of intact DNA with KpnI followed by EcoRI ran just in front of HhaI-3. If HhaI-3 is taken to be 12.0% (Griffin & Fried, 1975), the KpnI:EcoRI fragment would then be about 11.8%, consistent with the estimate given above for the position of this site. The enzyme, purified as described in Methods, was sufficiently pure for location of its cleavage sites but contained at least one other activity. A 20-fold increase in the digestion time gave at least eight bands in addition to the two major fragments. The two KpnI sites in polyoma DNA were cleaved at different rates, the site at 59.2% being more susceptible than the site at 11.6%. This may indicate that either KpnI is a mixture of two enzymes eluting together from phosphocellulose or that the two cleavage sites have different sequences both including the basic recognition sequence for a single enzyme.

SV40 was also found to be cleaved by KpnI but at a single site. This was close to the HpaII site (73.5% on the conventional map; Sharp et al. 1973) as also found by Dr R. J. Roberts (personal communication). Digestion with KpnI, HpaII, and HpaII followed by KpnI, gave similar patterns on agarose gels, converting form I DNA to more or less full length linear molecules. However, comparison of double digests of SV40 DNA with EcoRI in conjunction with HpaII or KpnI, showed that the sites for HpaII and KpnI were not coincident, KpnI cleaving 1.1% from the HpaII site at about 72.4% (data not shown).

Cleavage with PstI

Digestion of polyoma virus DNA with PstI gave five fragments (Fig. 3). The sizes of the fragments were determined relative to other restriction enzyme fragments of known sizes and were approx. as follows: PstI-1, 35%; PstI-2, 29%; PstI-3, 18%; PstI-4, 16%; PstI-5, 1.7%. The positions of the five cleavage sites were determined as follows. PstI-1 was cleaved by both EcoRI and KpnI restriction enzymes, and contained HpaII fragments 7 and 8. PstI-2 was cleaved by both BamHI and KpnI and contained HpaII fragments 3 and 5. This positions the two largest PstI fragments roughly on the map, PstI-1 either side of 0% and PstI-2 around 60% on the map. Isolated HpaII fragments were cleaved by PstI as follows: HpaII-1 twice to give three fragments 17.7%, 6.0% and 3.6%; HpaII-2 twice to give three fragments, 16.2%, 3.4% and 1.7%, the smallest fragment corresponding to the whole of PstI-5; HpaII-4 once to give two fragments, 11.6% and 1.6%. The other HpaII fragments HpaII-3, 5, 6, 7 and 8 were not cut. This accounts for all five PstI cleavages. PstI-3 was cut by HindIII but not by HpaII or HhaI and was entirely contained within HpaII-1, being the 17.7% fragment which appeared in the digest of HpaII-1 with PstI. PstI-4 contained HpaII-6 and was cleaved by HhaI to fragments of about 10% and 6%. These results together establish the order of the PstI fragments as 1, 5, 4, 3, 2 and locates the cleavage sites at 14.8%, 16.5%, 32.6%, 50.3% and 80.0%, as shown in Fig. 1.

SV40 DNA was also cleaved by PstI but at only two sites, in agreement with previous observations (R. J. Roberts, personal communication).
Fig. 3. Separation of restriction enzyme fragments of polyoma DNA on acrylamide gel. The upper part of the gel was 4% here and the lower part (below arrows) 10%. Samples from left to right were digests of 32P-polyoma DNA with HpaII; PstI; PstI followed by EcoRI; PstI followed by BamI; HpaII followed by PstI, and HpaII alone. The film of the upper part of the gel was exposed for 1 day and that of the lower part 4 days to show up the smaller fragments.

DISCUSSION

The two restriction enzymes studied here do not appear to share specificity with any of the other enzymes previously characterized in so far as they have been tested on polyoma virus DNA. In neither case has the specific sequence recognized been determined but the
number of cleavage sites found, two and five on polyoma DNA and one and two on SV40 DNA, make it more likely that the sequence contains six nucleotide pairs rather than a smaller number.

The fragments of polyoma DNA produced by KpnI may be useful, each comprising approximately half the DNA. One fragment, KpnI-1, includes the origin of replication at 71 ± 2% (Crawford, Syrett & Wilde, 1973; Crawford et al. 1974; Griffin et al. 1974) and most of the region of the genome expressed early after infection (Kamen et al. 1974).

PstI may also produce a useful fragmentation of the virus DNA, especially in conjunction with other enzymes. The double digestion with PstI and BamI produces a fragment PstI: BamI-2 which is well separated from other fragments and comprises the region from 58.4% to 80%. This includes the origin of DNA replication and the region around it, corresponding to the 5' ends of the large early and late messenger RNAs (Kamen et al. 1974). Although it is still too large for current sequencing techniques the fragment has several potential uses in examining the interaction of proteins with this important region of the DNA.

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REFERENCES


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