CLONING OF A DEFECTIVE POLYOMA DNA MOLECULE WITH REARRANGEMENTS IN THE REGION CODING FOR MIDDLE AND LARGE T ANTIGENS

Carmen PARENT and Marcel BASTIN
Département de Microbiologie, Centre Hospitalier Universitaire, Sherbrooke, Québec, Canada J1H 5N4

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1. Introduction

Polyoma virus (Py) is a small tumor virus whose DNA can induce tumors in rodents and transform susceptible cells in culture [1]. Its transforming activity has been linked to the early region of the viral genome. This region, which is expressed early after infection and in transformed cells, codes for three proteins: the large (100 K), middle (55 K), and small (22 K) T antigens [2]. Although the transformed state of some rat cells has been reported to require the entire early region [3], several lines of evidence suggest that large T antigen is not essential for transformation. Many Py transformed cell lines fail to express a full-sized large T [4] or to produce full-sized viral early mRNAs [5]. Moreover, fragments of Py DNA that lack the sequences coding for the C-terminal segment of large T provide sufficient information to induce tumors in vivo [6] and to initiate and maintain cellular transformation in vitro [7–9]. Thus, these results suggest that the middle or small T antigens, or both, may be the most important proteins for the expression of the transformation phenotype.

To further define the sequences of Py DNA that are essential for transformation, we have cloned defective Py DNA molecules in pBR322, and we have tested the recombinant plasmids for their capacity to transform cells in culture. In this paper we show that a recombinant carrying the region coding for small T but lacking about 350 base pairs (bp) in the region coding for the carboxy terminus of middle T failed to transform cells.

2. Materials and methods

2.1. *Py DNA*

The ts-P155 strain of Py [10] was used. Details of the mapping of this strain will be described elsewhere. The physical map shown in fig.2 differs from that of the A2 strain [1] in three restriction enzyme sites: an additional *Hpa*II site at map position 40.0, an additional *Hin*fI site at map position 89.7 and a *Hin*fI site missing at map position 39.2. Defective molecules were isolated from mouse cells infected with high-multiplicity-passaged virus [11]. The DNA was extracted by the selective method of Hirt [12] from mouse 3T3 cells infected at a multiplicity of 10–100 plaque-forming units per cell, and the supercoiled DNA was purified by an ethidium bromide–cesium chloride equilibrium gradient [13].

2.2. *Molecular cloning*

Py DNA was digested with *Hin*dIII, and the fragments were ligated in the *Hin*dIII site of pBR322 with T4 DNA ligase. Portions of the ligation mixtures containing 0.1 μg of vector DNA were used to transform *E. coli* HB101 to ampicillin resistance, a selectable marker carried by the plasmid. Clones containing insertions of Py DNA were identified by screening for a tetracycline-sensitive phenotype as previously described [14].

3. Results and discussion

3.1. *Cloning of defective Py DNA molecules*

To screen for defective molecules, the recombinant plasmids were digested with *Hin*dIII, and shorter molecules were detected by agarose gel electrophoresis. Analysis of about 200 recombinant plasmids by endonuclease mapping showed that some of the recombinants comprised a full-length Py *Hin*dIII-A (3.0 kilobases) (kb) or Py *Hin*dIII-B (2.2 kb) fragment inserted into the *Hin*dIII site of pBR322. Two of the recombinants contained the complete genome presumably

because digestion of Py DNA with *Hin*dIII had not been carried out to completion. One of these recombinants, pH6, is analyzed in fig.1A and has its structure shown in fig.2A. Depending on the DNA preparation used in cloning, from 15 to 80% of the recombinants exhibited electrophoretic mobilities in agarose

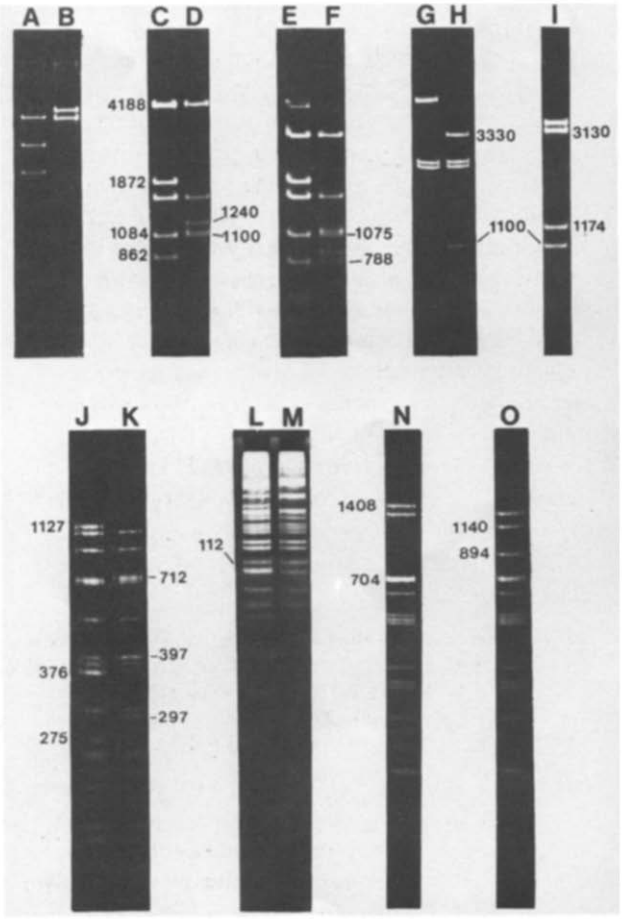

Fig.1. Restriction enzyme analysis of pHdf55 by electrophoresis in 1% agarose (A–I) and polyacrylamide (J–O) gels. Samples were electrophoresed for 16–18 h at 35 V in buffer containing 40 mM Tris–HCl (pH 7.9), 10 mM sodium acetate and 1 mM EDTA. The DNA was stained by immersing the gel in ethidium bromide (1 μg ml^{-1}) and visualized with an ultraviolet transilluminator. (A) pH6 digested by *Hin*dIII generating pBR322 and the two *Hin*dIII fragments of Py DNA. (B) pHdf55 digested by *Hin*dIII. (C) pH6 digested by *Pst*I. (D) pHdf55 digested by *Pst*I. (E) pH6 digested by *Pst*I plus *Hin*cII. (F) pHdf55 digested by *Pst*I plus *Hin*cII. (G) pH6 digested by *Bgl*I. (H) pHdf55 digested by *Bgl*I. (I) pHdf55 digested by *Pvu*II. (J and L) pH6 digested by *Hpa*II. (K and M) pHdf55 digested by *Hpa*II. (N) pH6 digested by *Hin*fI. (O) pHdf55 digested by *Hin*fI. The polyacrylamide concentration was 4% except for L and M, which was 8%. The size of a few fragments in bp is indicated in the margin.

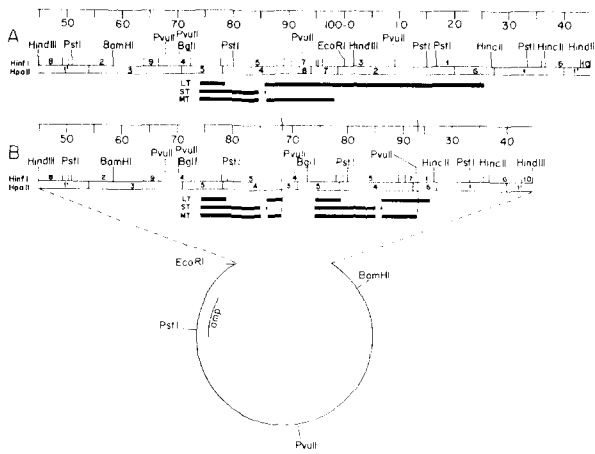

Fig.2. A. Physical map of Py DNA (ts-P155). The DNA is represented by a double line with the *Hpa*II sites indicated by small vertical lines inside the double line. The *Hin*fI sites are shown by small vertical lines outside the double lines. The putative coding regions for the 3 T antigens are indicated. In pH6 the Py genome is inserted into the *Hin*dIII site of pBR322 as shown in B for pHdf55. B. Structure of recombinant pHdf55. The pBR322 (circle) is interrupted in the *Hin*dIII site. The map units correspond to the units of the complete non-defective Py genome (shown in A) divided into 100 units starting at the *Eco*RI site.

gels higher than those expected for complete *Hin*dIII-A or *Hin*dIII-B fragments of Py DNA, or the entire Py genome. These plasmids appeared to carry deletions and rearrangements in their Py DNA moiety ranging in size from 0.15 to 2.0 kb. The deletions mapped in the early as well as in the late regions but never included the *Hpa*II-3/5 junction at 71 units (fig.2) identified as the origin of replication [15].

To detect deletions affecting sequences encoding the C-terminal portion of middle T antigen, the Py DNA inserts were screened for *Eco*RI resistance, and analyzed further by endonuclease mapping. pHdf55 is one such recombinant lacking the *Eco*RI site at 100 units (not shown) as well as one of the 2 *Hin*dIII sites (fig.1B). The DNA was cleaved by *Pst*I and by *Pst*I plus *Hin*cII. The presence of 2 *Pst*I fragments of 4188 and 1084 bp (fig.1D) indicated that df55 was inserted in pBR322 by its *Hin*dIII site at map position 44.6 and that it had the same orientation as pH6 (fig.2). This was also confirmed by the *Pst*I plus *Hin*cII digest showing two fragments of 1075 and 788 bp in both pH6 (fig.1E) and df55 (fig.1F). *Pst*I fragments of 1872 bp and 862 bp were missing in df55 and replaced by two other fragments of about 1240 and 1100 bp (fig. 1D). This indicated that the deletion occurred within

the region comprised between the 2 *Pst*I sites at map positions 79.7 and 33.1. Furthermore, there was a rearrangement of sequences within the same region containing an additional *Pst*I site. The *Pst*I plus *Hin*cII digestion (fig.2F) located this *Pst*I site at about 848 bp from the *Hin*cII site at map position 26.5. Digestion by *Pvu*II showed that df55 contained a fragment of 1174 bp (fig.1I) i.e., the sequence between 70.0 and 92.1 units.

To map the deletion in more detail, pH6 and df55 DNAs were digested with *Hpa*II and *Hin*fI and the products were analyzed by electrophoresis in 4% polyacrylamide gels. According to the restriction patterns of fig.1. (J and K), df55 lacked *Hpa*II fragments 2 (1127 bp), 6 (376 bp) and 7 (275 bp). When smaller fragments were visualized by performing the electrophoresis in 8% polyacrylamide gel (fig.1, L and M), it appeared that fragment *Hpa*II-8 was also missing in df55. Three additional fragments of 712 bp, 397 bp and 297 bp were observed in the df55 digest (fig.1K). The 397 bp fragment was detected by an increased density of the band corresponding to *Hpa*II-5. Fragments *Hin*fI-1 (1408 bp) and *Hin*fI-3 (704 bp) were missing in df55 (fig.1O) and replaced by two fragments of 1140 bp and 894 bp.

Our data indicate that the Py DNA sequence in df55 is interrupted from about 88 to 24 units (fig.2), before the *Hpa*II-4/8 junction and the *Hin*dII site in *Hpa*II-6. Such a deletion leaves the sequence encoding small T intact but removes about 1800 bp from the large T antigen gene and about 530 bp from the middle T antigen gene. There is a tandemly repeated copy of the 5′ portion of the early gene region which includes fragments *Hpa*II-5 and 4 as well as the *Pvu*II site at 92.1 units but not the entirety of fragments *Hpa*II-8 and *Hin*fI-7. The additional *Pst*I fragment of 1100 bp in df55 (fig.1D) is generated by a duplication of the *Pst*I site at 79.7 units. We have also found, as expected, that the duplication of the *Pvu*II and *Bgl*I sites at 70.0 and 72.2 units generates *Pvu*II and *Bgl*I fragments of the same size (fig.1 ,H and I).

3.2. *Transformation with cloned DNA*

An interesting feature of the structure of df55 is the loss of about 300 bp from the C-terminal portion of the middle T antigen gene. To study the importance of this region in transformation, we evaluated the ability of df55 DNA to transform cells in culture. Fischer rat 3T3 cells were transfected with purified recombinant plasmids by the calcium chloride–DMSO method [16]. Transformants were scored as dense foci after 10 days of incubation at 39°C. Plasmids containing an intact 5′ portion of the early region (the region mapping between 70 and 100 units) from either the A2 wild-type or the ts-P155 strains transformed cells with an efficiency of 50–60 foci per dish for a DNA concentration ranging between 0.2 and 1.0 μg. pHdf55, the recombinant lacking the DNA sequences encoding the C-terminal portion of Py middle T antigen failed to transform in eight different experiments, even though cultures were kept for periods as long as 3–4 weeks at 39°C. The inability of pHdf55 to transform is most likely due to the absence of a functional middle T antigen. Although our results do not exclude the possibility that small T antigen may play some role in transformation, they indicate that this antigen alone cannot be responsible for inducing the expression of the transformed phenotype.

Acknowledgements

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