A detailed analysis of duplications appearing during early, high multiplicity infections with polyoma virus

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Serial undiluted passage of polyoma virus derived from transfection of mouse fibroblasts with well defined wild-type genomes results in the appearance of a very heterogeneous population of defective virus particles. Many of these variants show duplications which not only contain the cis-acting region minimally required for efficient replication, but also other regions. A detailed analysis of the duplication patterns appearing in high multiplicity infections is presented. We performed heteroduplex analyses of duplicated fragments using mung bean nuclease and demonstrated that the pattern of duplication junctions is conserved qualitatively and quantitatively. However, the distribution of fragment sizes varied in a number of independently derived virus stocks. Amplification of viral nucleotide sequences is an early event in virus replication, occurring at least as early as 3 days post-transfection. The pattern of duplication did not change significantly in successive early passages at high multiplicity. Although duplication was accompanied by deletion of various parts of the viral genome, a sequence bordering the duplications at the late side of the origin of replication was retained as a single copy in all defective viruses. The relevance of these findings to the mechanism that creates the duplications and the biological activity of defective virus is discussed.

Introduction

Defective virus particles have been described for a number of animal viruses, such as negative-strand RNA viruses (for review see Lazzarini et al., 1981) and the DNA viruses polyoma virus (Py) and simian virus 40 (SV40) (Thorne & Warden, 1968; Blackstein et al., 1969; Brockman et al., 1973; Fried, 1974). They are characterized by four major properties. (i) They are enriched during serial passage at high multiplicity. (ii) They interfere with the replication of wild-type virus at higher passage numbers when defective particles accumulate; this may facilitate the establishment of persistent virus infections (Huang & Baltimore, 1970). (iii) Most defective virus particles contain a smaller genome than wild-type virus because of deletions that render them unable to multiply in a host cell; thus they need the presence of a homologous, complete virus as a helper. (iv) In many cases more complex rearrangements of viral nucleotide sequences can be identified (e.g. amplification of a part of the viral genome and acquisition of host sequences) (Lavi & Winocour, 1972; Rozenblatt et al., 1973; Martin et al., 1973; Folk & Wang, 1974; Griffin & Fried, 1975).

There are a number of different constraints on the structure of defective virus. The rearranged genome must contain all cis-acting elements that are required for replication, which may be the same as or better than that of wild-type virus. There are size limits for packaging the viral chromatin into a capsid, and cis-acting sequences may also be required for the association of viral nucleic acid with proteins to form viral chromatin, and to initiate encapsidation. Finally, different constraints put on the virus by the cellular environment and the machinery that rearranges the viral genome may contribute to the genotype of a defective virus. All these factors act repeatedly during serial passage of the virus to create the evolutionary variants observed.

In all isolates of Py and SV40 defective genomes having duplications, the amplified unit spans a segment around the origin of replication and contains multiple cis-acting sequence elements that function early and late in transcription, and in virus replication. Certain rearrangements within this region alter the host range of Py (Vasseur et al., 1982; Melin et al., 1985; Maione et al., 1985; De Simone & Amati, 1987). By dissecting the control region and by mutational analysis, the minimum length of genome sequence required for efficient replication of Py and SV40 has been defined. Interestingly, the duplications found in defective viruses are much larger than these segments, extending into the early region. This may be due to the mechanism that creates the duplications or selection for amplification of still unknown cis-acting elements within the early region.

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One way to characterize this mechanism and gain more insight into the complex interactions between the virus and the host cell is to define the endpoints of rearrangements in the defective virus; this has already been done for some cloned variants of SV40 (Lee et al., 1975; Wakamiya et al., 1979; Woodworth-Gutai, 1981a). We were interested in making a comprehensive survey of all duplications selected during high multiplicity infections by Py. Hence we examined the population of viral DNA extracted from cells after infection with independently derived virus stocks, rather than studying single variants.

**Methods**

**Virus and transfections.** The virus strains described in this study originate from wild-type (PyA3) or variant viruses which have been cloned into the BamHI site of pAT153. Nucleotide (nt) numbering is according to Tooze (1981), but corrected for strain A3. Py5116/5145 contains a duplication of the sequence between nts 5116 and 5145; PyPst5173 is probably identical to PyAT3 (Tseng & Acheson, 1986), both having a PstI site at nt 5173 owing to a duplication of the sequence between nts 5099 and 5173, and a deletion of the sequence between nts 5209 and 5262; PyXho5173 was constructed by replacing the PstI site at nt 5173 with an 8 bp Xho1 linker (see also Fig. 1a).

To prepare viral DNA containing a high proportion of defective genomes, virus was propagated as follows. Logarithmically growing Swiss 3T3 cells were transfected as described (Kovar & Wintersberger, 1985). With 1 µg total DNA (equivalent to 0.6 µg viral DNA) per 5 × 10^6 cells, a transfection efficiency of approximately 1% was achieved. Viral DNA was liberated from the plasmid by BamHI digestion followed by recircularization. The transfected cells were grown in Dulbecco's MEM (DMEM) containing 10% foetal calf serum for 16 h, and subsequently trypsinized and resed on a subconfluent monolayer of Swiss 3T3 cells. The medium was replaced by DMEM containing 5% horse serum and 1 µg/dexamethasone 20 h post-transfection and the cells were incubated at 37 °C in a CO_2_ incubator for 2 to 3 weeks until lysis was complete. The medium, containing virus at approximately 1 × 10^9 to 5 × 10^9 p.f.u/ml (as determined by plaque assay for PyA3), was collected, subjected to freezing and thawing, and used for reinfection of Swiss 3T3 cells. All successive virus infections were performed using culture supernatants after lysis was complete.

DNA was extracted from infected cells, essentially by the method of Hirt (1967), 24 h post-infection. Hirt supernatants were either extracted with phenol (Maniatis et al., 1982), followed by extensive dialysis against 10 mM-Tris–HCl, 1 mM-EDTA at 4 °C in the presence of RNase A, or centrifuged through a calcium chloride/ethidium bromide gradient.

**Heteroduplex analysis.** DNA fragments (10 ng), labelled at one end with ^32_P using polynucleotide kinase, were mixed with a five- to 10-fold excess of trimmed BglI fragments containing duplication junctions from defective virus DNA, as illustrated in Fig. 4(a). Denaturation was performed in a total volume of 10 µl containing 70% formamide, 10 mM-Tris–HCl pH 8.0, 1 mM-EDTA, 300 mM-NaCl at 75 °C for 5 min, as previously described (Oudet & Schatz, 1985) after which the reaction mix was rapidly chilled on ice. After renaturation for 1 h at 25 °C, 1 ml of mung bean nuclease buffer or S1 nuclease buffer was added. Aliquots (0.2 ml) of this mixture were digested with either mung bean nuclease (MBN) for 1 or 2 h at 37 °C, or with S1 nuclease for 30 min at 20 °C. MBN digestions were stopped by phenol extraction and S1 nuclease reactions by addition of 12 µl 2 M-Tris base and 40 µl 1 M-NaCl. DNA was ethanol-precipitated from the mixture at −20 °C in the presence of 6 µg tRNA as a carrier. Digestion products were resolved on 6% polyacrylamide sequencing gels containing urea and autoradiographed.

**Results**

**Defective Py genomes contain deletions and duplications**

To compare defective viruses, we first propagated virus stocks from 13 independent transfections of PyA3, Py5116/5145, PyPst5173 and PyXho5173. The structure of the transected viral genomes is presented schematically in Fig. 1(a); Fig. 1(b) shows the viral DNA extracted from cells infected with PyA3 at the third passage. The four virus Hirt extracts contained multiple supercoiled DNAs with a high percentage of genomes smaller than that of wild-type virus. Although originating from the same plasmid in the first passage, the restriction fragment pattern of defective DNAs varied significantly owing to independent evolution; this was true for all the virus strains tested. Preliminary analysis of defective genomes using restriction enzymes showed that most of those with a duplication of a segment around the origin of replication retained a single BglI site (nt 5021) flanking the control region at the late side of the origin of replication (data not shown). Sequences in the early region which contain the EcoRI site were preferentially deleted; this finding is in accordance with earlier reports (Fried, 1974; Folk & Wang, 1974; Griffin & Fried, 1975).

*KpnI* cuts intact Py DNA at two sites, in the early and late regions; however, Fig. 1(c) shows that defective genomes lost one KpnI site. Most of the linearized viral DNA was larger than 4000 bp, a size range preferentially packaged into capsids (Ganem et al., 1976; Menck et al., 1990).

Digestion of Hirt extracts from high multiplicity infections with various restriction enzymes which cut at a single site close to the origin of replication created a number of accessory fragments 600 to 1400 bp in length due to duplication of these restriction sites (Fig. 2). The restriction fragment size pattern generated by cleavage with a single enzyme was retained in at least four successive passages for all virus strains tested. *BglI* digestion of low *M* DNA extracted as early as 3 days post-transfection with cloned PyA3 DNA revealed the individual patterns of duplication; this corresponds to approximately the second or third cycle of virus replication because, in our hands, infectious virus could be recovered from the culture supernatant as early as 24 h post-transfection. DNA extracted 10 h post-transfection showed significantly different patterns. Bands
Fig. 1. (a) Structure of the wild-type Py genome showing the coding regions for the three early transcribed tumour antigens and the three late transcribed viral coat proteins. Some of the restriction sites used for characterization of defective genomes are shown. The control regions of the variants used are shown at higher magnification. A and B, core regions of the Py enhancer; TA, the assumed TATA boxes; ori, origin of replication; E and L, initiation sites for early and late transcription.

(b) Hirt extracts from four independent third passage PyA3 infections (lanes 1 to 4). The position of wild-type supercoiled DNA is marked by I.

(c) Hirt extract from PyXho5173 (fourth passage), undigested (lane 1) and KpnI-digested (lane 2). A BamHI digest of plasmid-cloned PyXho5173 is shown as a size marker (lane 3).

Fig. 2. Characterization of duplications by digestion with restriction endonucleases which cut close to the origin of replication. (a) Comparison of XhoI digests of Hirt-extracted DNA after infection with three independently derived PyXho5173 stocks (lanes 1 to 3) in four successive passages (A to D). (b) Pattern of PyA3 DNA BglI fragments 10 h (lane 1) and 3 days (lane 2) post-transfection. Restriction digests were separated on a 1.5% agarose gel and hybridized to a radiolabelled BamHI–ApaI fragment. Positions of size markers are indicated.

Cluster around a fragment of approximately 750 bp generated by BglI digestion of BamHI-linearized, transfected DNA as a consequence of incomplete recircularization before transfection (Fig. 2b). Therefore, we do not think that the bands obtained 3 days post-transfection originate from rearrangements at the ends of linear viral DNA. Similar results were obtained from different stocks of PyA3, Py5116/5145 and PyPst5173. No differences in the distribution of size of duplicated fragments could be detected in stocks of a Py variant which has a strongly retarded infectious cycle owing to a mutation in the C-terminal part of the large T antigen (data not shown).

The design of the experiment presented in Fig. 3 allowed quantitative determination of the restriction sites contained in the whole population of duplicated sequences within a virus stock. First passage PyA3 DNA was digested with BglI and separated on a 1% agarose gel. Accessory digestion products ranging from 600 bp to 1400 bp in length were eluted from the ethidium bromide-stained gel, dephosphorylated and end-labelled with [γ-32P]ATP using polynucleotide kinase. Aliquots of these labelled BglI fragments were cleaved independently with different restriction enzymes in the presence of a 10-fold excess of well defined, unlabelled plasmid DNA as a control to ensure complete digestion. The
expected variation in the length of labelled products in the presence or absence of recognition sequences is outlined in Fig. 3(a); representative experimental data are shown in Fig. 3(b). Digestion of each of the end-labelled BglI fragments (contained in the smear in lanes 1 and 2) with PstI, SstI and AvaI, which cut wild-type DNA at nt 484, nt 569 and nt 653, respectively, should give rise to two cleavage products if the restriction site is present in the fragment: one of well defined length, 397 bp (PstI), 482 bp (SstI) and 566 bp (AvaI), and one of variable length which is dependent on the distance between the respective restriction site and the duplication junction (marked X/Y in Fig. 3a). The latter bands should be reduced to 117 bp when cleaved with PvuII, which cuts at the late region side and close to the BglI site. Comparison of the products of double digestions with PvuII and either PstI, SstI or AvaI with the products of single digestion with PvuII showed that there are many BglI fragments not cut by PstI, SstI or AvaI. From the relationship between the intensity of the well defined bands created by these enzymes and the intensity of bands generated by PvuII digestion only, we concluded that there was a high proportion of duplications that did not extend beyond nt 484, nt 569 or nt 653 on the early region side of the origin of replication.

HgiAI cuts wild-type DNA at the SstI sites at nt 569 and nt 725. Thus, a fragment of a constant length of 482 bp (nt 87 to nt 569) should be created by HgiAI if the SstI site is contained in the duplicated sequence. The speed of migration of the variable length fragment containing the duplication junction is dependent on the existence of the HgiAI site at nt 725. Bands of equal $M_r$ in the SstI digestion and the HgiAI digestion, respectively, do not contain the HgiAI site at nt 725 whereas bands absent from the SstI digestion products but present in those of HgiAI digestion are created by cleavage at nt 725.
*Hind*I cleaves wild-type DNA at nt 385 and nt 5073. If both of these sites were present in the duplicated sequence, we would expect two bands of 306 bp and 298 bp, which cannot be resolved in the gel. Clearly, there was a high percentage of bands of higher *M*ₜ (Fig. 3b). *Hha*I cuts within the origin of replication (Fig. 3e), thus shortening the 298 bp fragment to 11 bp. Cleavage of *Hind*I-digested *Bgl*I fragments with *Hha*I shows that all the *Hind*I-resistant bands lack the cleavage site at nt 5073. On the other hand, all *Bgl*I fragments are completely resistant to digestion with *Sau*3A and *Bgl*I (Fig. 3b), which both cut wild-type DNA at nt 5021.

To summarize the data presented in Fig. 2 and 3, we may conclude that most of the duplications span a region from a site upstream of nt 5021 to different sites within the early region; only a few duplications include sequences beyond the *Hgi*AI site at nt 725. There may be additional rearrangements close to the origin of replication as revealed in some experiments (data not shown).

Fine mapping of rearrangement sites

From the previous data the question arises whether Py DNA contains preferred rearrangement sites and to what extent they contribute to the observed structure of duplications. To answer this question a technique was developed which allowed the determination of duplication sites in a heterogeneous population of duplications at the nucleotide level. Briefly, *Bgl*I fragments containing duplication junctions were eluted from the gel and trimmed with *Hpa*II, *Ava*I or *Pvu*II (Fig. 4a). These smaller fragments were denatured and then renatured in the presence of a 3²P-labelled fragment, homologous to the duplicated sequence at the labelled end but extending across the duplication junction either at the late region side or the early region side of the origin of replication.

We expected a heteroduplex to be formed which was double-stranded from the labelled end of the probe to any site of rearrangement, at which point single-stranded regions should form owing to lack of homology. The single-strand-specific MBN was employed to remove the unpaired ends from the heteroduplex. The resulting fragments were resolved on a sequencing gel and the ends mapped either by comparison with a size marker or a Maxam–Gilbert sequence of the probe run on the gel in parallel. Representative results obtained from some third passage virus are shown in Fig. 4(b to e).

*Bgl*I fragments trimmed with *Ava*I and hybridized to fragment M (nts 4965 to 5291*) (the asterisk marks the site of radioactive label) were digested with increasing amounts of MBN, resulting in a highly reproducible pattern of bands; the pattern was the same after a 2 h incubation with MBN. Using S1 nuclease, the pattern was essentially the same but appeared to be less reproducible (data not shown). Treatment of fragment M with MBN or S1 nuclease in the absence of trimmed *Bgl*I fragments did not result in a comparable pattern.

To confirm that MBN was acting as expected, we mixed 4 ng of labelled fragment M with a fivefold excess of a partially overlapping fragment (nts 5021 to 87). MBN digestion of this heteroduplex resulted in a 270 bp labelled fragment, as would be expected from cleavage of fragment M at nt 5021. The site of cleavage was determined by comparison with a Maxam–Gilbert sequence of fragment M. No comparable bands were obtained when a surplus of a non-overlapping fragment (nts 837 to 1100) was mixed with labelled fragment M; the same result was obtained using sheared calf thymus DNA, which was included as a negative control in all subsequent experiments (Fig. 4b).

The data presented show three clusters of rearrangement points within the eluted *Bgl*I fragments, around nt 5030, nt 5080 and nt 5100, respectively. Minor rearrangement points closer to the origin of replication were not detected by the method used, however their presence cannot be excluded.

We then compared the genomes of four PyA3 stocks derived independently (third passage) by studying their heteroduplex formation with either labelled fragment M or fragment B (nts 4965 to 5173*) (Fig. 4c). To our surprise, the patterns obtained from the genomes of the four independently derived virus stocks were essentially the same. The use of fragment B to form heteroduplexes with *Bgl*I fragments that had been trimmed with *Ava*I and *Hpa*II allowed the rearrangement points to be defined more precisely when compared with a sequence ladder of fragment B (not shown in the figure). A similar analysis was performed to define rearrangement points on the early region side of the origin of replication. For this purpose *Bgl*I fragments were trimmed with *Pvu*II and *Hpa*II, thus creating fragments which contained the duplication joint and extended to nt 399 and nt 5128 respectively. Fragment S (nts 436 to 778) was used as a hybridization probe and the results are outlined in Fig. 4(e). Again, the pattern obtained for the four virus stocks was essentially the same, except for slight variations in the relative intensities of certain bands, reflecting the varying representation of rearrangement points in the four virus stocks. Heteroduplex analysis revealed many accessory bands that were not seen in the negative controls; some of these were nearly as long as the undegraded fragment S, thus indicating that rearrangement points could occur downstream of nt 778. When fragment Q (nts 657 to 960) was used as a hybridization probe, we found that fragments no longer than approximately 97 bp, corresponding to cleavage at nt 753, could be detected (data not shown). This is in accordance with the data presented in Fig. 3. The results obtained from Fig. 4 are summarized in Fig. 5.
Fig. 4. Mapping of rearrangement points by heteroduplex analysis. (a) A representative BglI fragment containing a duplication junction (X/Y). Restriction sites used for trimming the BglI fragments are indicated: B, BglI; H, HpaII; A, AvaI; P, PvuII. Heteroduplexes formed between end-labelled wild-type fragments containing sequences outside those duplicated in defective Py genomes (M, B, S and Q), and trimmed BglI fragments containing a rearrangement site, are shown. The asterisk marks the site of radioactive label; vertical bars indicate the region of duplex formation. The protruding single-stranded ends of heteroduplexes were...
removed using either MBN or S1 nuclease, releasing double-stranded fragments that extend to the rearrangement point at either the late region (Y) or early region (X) side of the origin of replication. Fine mapping of the rearrangement points at the late region (b to d) and early region (e) side of the origin of replication of PyA3. (b) Digestion patterns obtained using 0, 5, 10, 20 and 50 units S1 nuclease (lanes 1 to 5), or 0, 7, 35, 70 and 140 units MBN (lanes 6 to 10). Heteroduplexes were formed between AatI-trimmed BglI fragments of third passage PyA3 and fragment M (lanes 1 to 10). As a control, renaturation was performed in the absence of PyA3 fragments (lanes 11 and 12) and the resulting heteroduplexes were digested with 5 units S1 (lane 11) or 35 units MBN (lane 12). (c) Demonstration of the specificity of MBN digestion. Fragment M (nts 4965 to 5291) was denatured and renatured in the absence of any other fragments (lanes 1 and 2), or in the presence of the partially overlapping (nts 5021 to 87) fragment K1 (lanes 3 to 5), the non-overlapping fragment (nts 837 to 1100) K2 (lanes 6 to 8), or AatI-trimmed BglI fragments from two independent PyA3 stocks (lanes 9 to 11 and 12 to 14). Heteroduplexes were digested with 45 units MBN for 1 or 2 h (lanes 2, 3, 4, 6, 7, 9, 10, 12 and 13) or 0 h (lanes 1, 5, 8, 11 and 14) at 37°C. A Maxam–Gilbert sequence gel of fragment M was run in parallel. (d) MBN digestion patterns of heteroduplexes formed between fragment M and AatI-trimmed BglI fragments (left), or between fragment B (nts 4965 to 5173) and AatI/HpaII-trimmed BglI fragments (right) of four independent third passage PyA3 stocks (lanes 1 to 4 respectively). As a negative control, sheared calf thymus DNA (lanes CT) was used instead of PyA3 fragments. Heteroduplexes were digested with 45 units MBN for 1 or 2 h (+), or were undigested (−). (e) Analysis of rearrangement sites in the early region side of the origin of replication in PyA3 stocks (lanes 1 to 4) using fragment S for heteroduplex formation.

Discussion

The experiments described were performed to define in more detail the duplications appearing during high multiplicity Py infections of mouse fibroblasts. It was necessary to start the analysis with genetically defined viral genomes. Thus, variants containing duplications were raised and propagated by high multiplicity infection of cells with supernatants from cells initially transfected with plasmid-cloned Py DNA; plaque-purified virus was not suitable as a starting point because the m.o.i. increases during plaque formation when virus particles spread from cell to cell, resulting in the presence of low amounts of defective virus (data not shown). We found that rearrangements can be detected as early as 3 days post-transfection with cloned DNA, corresponding to approximately two cycles of infection. Thus, simple duplications like those described in this paper and compensating deletions seem to be early events in the evolution of defective virus. The rearrangements observed 3 days after transfection of Py DNA differ significantly from those after transfection of processed DNA (Fig. 2b) and are similar to those described by others for virus infections at high multiplicities (Folk & Wang, 1974; Fried, 1974; Griffin & Fried, 1975). However, transfected circular Py DNA is subjected to recombination at high frequency (Kovar & Wintersberger, 1985), an event which might differ for virally introduced DNA.

The duplicated segment contains the origin of replication, but extends into the late and early regions, the majority of duplications spanning a region of between 600 and 1400 bp. The duplicated sequences in virus stocks derived independently vary significantly in size, clustering around 900 bp. However, we could show that the rearrangement points used for the generation of the
duplications are conserved qualitatively and quantitatively. This implies that there are certain sites at both sides of the origin of replication that are used to the same extent but in different combinations to create the duplication junctions observed. The pattern of duplications obtained did not change significantly within four successive virus passages.

The defective genomes described in this report contain only two copies of the repeat unit flanked by a single BclI site. The BclI site is of structural importance because it marks the end of a nucleosome-free gap within the viral chromatin which coincides with the non-translated control region for transcription and replication; this part is extremely sensitive to nucleases (Saragosti et al., 1980; Herbomel et al., 1981). Moreover, an altered DNA conformation in the control region has been reported for SV40 using different approaches (Azorin & Rich, 1985; Deb et al., 1986; Iacono-Connors & Kowalski, 1986; Ostrander et al., 1988). Brian & Folk (1986) have defined sequence elements within the Py enhancer that are responsible for DNase I hypersensitivity; these elements are included in the duplicated region of the defective genomes. The rearrangement points on the late region side of the origin of replication defined by our experiments using MBN are close to this sequence and duplication of this region may enlarge the nucleosome-free gap. One possible mechanism for the creation of duplications in defective virus is recombination and increased accessibility to nucleases may enhance recombination. Once the nucleasen-sensitive region has been enlarged by duplication, further rearrangements may be facilitated, allowing, in turn, further amplification (Griffin & Fried, 1975; Lee et al., 1975) and integration of host sequences until most of the viral DNA is replaced by cellular sequences as is seen at higher passage numbers (Lavi & Winocour, 1972; Rozenblatt et al., 1973; Martin et al., 1973; Folk & Wang, 1974). Such viral genomes might be a good substrate for recombinating with wild-type virus, producing self-replicating variants (Ruley & Fried, 1983); some of these alter the host range of Py. The duplication junctions of a number of these variants are similar to those described in this paper for defective viruses (Fig. 5). We therefore propose that replication-competent variants displaying small rearrangements within the Py enhancer region are generated by recombination between wild-type helper virus and defective virus within the duplicated region to create small duplications. Variants with an altered host range may be selected from a heterogeneous population contained in a virus stock by infection of foreign host cells.

Most rearrangement joints revealed by MBN digestion of heteroduplexes are found in AT-rich sequences. These sequences, as well as sequences of limited homology, have been proposed to play a role in viral recombination in SV40 (McCutchan et al., 1979; Woodworth-Gutai, 1981b; Sheflin et al., 1983). A-T-rich sequences in supercoiled DNA have been shown to be a good substrate for cleavage by MBN (Iacono-Connors & Kowalski, 1986; Sheflin & Kowalski, 1984, 1985). The results presented in this paper do not reflect this specificity because short DNA fragments were used instead of torsionally stressed DNA.

It is commonly suggested that the accumulation of defective virus during serial undiluted passage is due to the amplification of sequences necessary in cis for viral replication. In the case of Py these sequences are the origin of replication, at least one of the three binding sites for large T antigen on the early region side, and sequences contained within the transcription control region on the late region side of the origin of replication (Cowie & Kamen, 1984; de Villiers et al., 1984; Dailey & Basilico, 1985). All these sequences, which have been defined by deletion analysis, are restricted to a region of less than 500 bp. Replicative advantage can be conferred to the virus by duplicating part or all of this region, as can be shown in transient replication assays. Amplified units in the evolutionarily developed defective variants described in this paper extend far beyond this minimal region on both sides of the origin of replication and include the transcription initiation sites of late and early mRNAs; one border of the duplicated segments lies somewhere in the early region, upstream of nt 753. Thus, most duplications contain part or all of the coding region for Py small t antigen. No replication-enhancing cis-acting sequences are known to exist in this part of the Py genome. The other border of the duplicated segments is located within a rather short sequence between nt 5021 and approximately nt 5100. It has been proposed that the process of duplication leading to defective SV40 genomes is driven directly by encapsidation of viral chromatin (Chang & Wilson, 1986). Studies with infectious SV40-based mammalian shuttle vectors revealed that cis-acting encapsidation signals are probably located close to or overlapping the SV40 regulatory region (Menck et al., 1990). Nothing is known about the presence of similar sequences in Py.

The BclI site at nt 5021 is not contained in the duplicated region. Although deletions of various sequences outside the duplicated segment can be observed in defective Py DNA, the single BclI site is retained in most or all of the genomes. No role in viral replication has been assigned to this part of the genome. The only known function is that of a splice donor site that links the leader sequence between nts 5020 and 5076 to the late mRNA for VP1 (nt 4122) or VP3 (nt 4707) (Legon et al., 1979). This leader sequence has been shown to serve an essential spacer
function necessary for the viability of and late gene expression by wild-type DNA (Adami & Carmichael, 1986). We think that late transcription may take place from the defective Py genomes because all sequences necessary are present within the promoter–enhancer elements duplicated. Recently, a segment within the coding region for SV40 VP1 was identified which confers replicative advantage to defective genomes. Furthermore, this region was shown to be important in interfering with the growth of wild-type virus (O’Neill et al., 1987). We do not know whether a similar sequence is retained by defective Py.

Our data and the considerations alluded to above suggest that unduplicated virus infections result in a very heterogeneous population of viral genomes present in one cell. The biological activity of defective viruses still remains to be defined. At least they may serve as a pool for genetic variability. As has been stressed by Huang & Baltimore (1970), these defective virus particles do not appear only during propagation in vitro but also may play an important role in the process and evolution of virus-associated disease.

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