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Mammalian cell functions mediating recombination of genetic elements

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ABSTRACT

Recombination of segments of the SV40 genome by a variety of mechanisms is described. These include the faithful joining of linear segments that have flush termini as opposed to previously described cohesive or resected termini. Lack of involvement of viral proteins has been demonstrated for recombination of segments with homologous overlapping termini, but probably applies also to the other joining reactions.

Segments of the genome that have been cleaved in such a manner as to be unable to manufacture any known viral proteins are neutral elements of genetic information, incapable of selection by replication or biological function until recombined. These recombination functions presumably are available to the host cell and any element of genetic information that can be generated in that cell.

INTRODUCTION

We have approached the analysis of recombination of genetic elements in mammalian cells by defining various functions that mammalian cells specify in joining or recombining these elements. For this purpose we have utilised segments of the genome of the simian papovavirus, SV40, since successful recombination can be assayed in viable progeny whose genomes can be dissected [1], and the genome is very well characterised both genetically and physically [2]. The SV40 genome also behaves as a miniature mammalian chromosome [3,4]. We describe experiments showing that mammalian cells recombine segments of DNA in a variety of ways and that these events appear to be mediated by host cell enzymes.

MATERIALS AND METHODS

Preparation of transfecting segments

Wild type SV40 strain 776 DNA was cleaved with the appropriate combination of the following restriction endonucleases, HpaI, HpaII, PstI, EcoRI, BglI or TaqI [5] as described in the text. Each cleavage mixture was separated on 1.5% agarose gels in a horizontal slab gel apparatus. The gel was stained with ethidium bromide (1 μg/ml) and viewed while irradiated by long wavelength UV light.

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The required bands were extracted as described [1].

Transfection

Each appropriate segment, or mixture, was transfected into the CV-1P line of African green monkey kidney cells in 55cm^2 dishes as described [1,6].

Gel analysis of plaque isolates

Where required, one half of each plaque isolate was used to infect CV-IP cells in 25cm^2 flasks. 2ml of this stock were used to infect two 150cm^2 dishes and DNA was extracted by the method of Hirt [7]. The soluble DNA fraction after SDS-NaCl precipitation was treated with RNase and then phenol and ether extracted. Samples to be cleaved with restriction endonucleases were dialysed exhaustively against 10mM TrisHCl/ 1mM EDTA (pH 7.6). Samples were analysed by agarose gel or gradient polyacrylamide gel electrophoresis as previously described [1].

RESULTS

Monkey cells faithfully seal flush- and cohesive-ended SV40 genome segments

Mertz and Davis [8] demonstrated the cohesive nature of the termini generated by cleavage of the SV40 genome with the restriction endonuclease EcoRI when they transfected these unit length linear (fIII) SV40 genomes into African green monkey kidney cells. Plaques were generated at a frequency of 10% of those produced by supercoiled (fI) SV40 DNA. Resection of SV40 DNA by the lambda 5'-exonuclease by a length of 15-50 nucleotides after cleavage with different restriction endonucleases has been used to generate deletion mutants [9], and Cole et al. [10] have described the rejoining of SV40 DNA that was randomly cleaved with HaeIII (producing flush ends) to generate mutants having both small and extended deletions at the cleavage sites.

We describe data here which demonstrate that monkey cells will seal faithfully SV40 DNA that has been cleaved with a restriction endonuclease that generates flush ends. Full length linear SV40 DNA, generated after partial cleavage with the enzyme HpaI, which recognises four sites in the SV40 genome [4], was transfected into monkey cells after gel purification. Plaques were generated at one tenth the frequency of those generated by fI DNA (3x10^6 pfu/μg DNA). SV40 DNA prepared from six randomly chosen plaque isolates retained all four HpaI sites, thus demonstrating that the randomly generated, HpaI cleaved, fIII molecules were sealed faithfully at these sites at approximately the same frequency as molecules with cohesive termini [8] (gel data not shown). When SV40 DNA was cleaved with HpaII (which recognises one site at 0.73 in the SV40 genome in an area that can be deleted to produce viable viral genomes [9]) and subsequently treated with *E.coli* DNA polymerase I to generate flush ends by virtue of the 5'-3' pol activ-

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ity [11], molecules were again sealed, but with the expected loss of the HpaII site [12].

When the SV40 genome was cleaved with two restriction endonucleases that recognise unique but different sites in the genome and the products were transfected into African green monkey kidney cells, Wilson showed that these segments with cohesive termini resealed to produce plaques at a frequency of 0.4% of those produced by fI SV40 DNA [13]. In a similar approach, we have cleaved the SV40 genome into two segments that have flush ends as diagrammed in Figure 1.

The HpaI C segment was isolated after total HpaI cleavage of SV40 DNA. The large HpaI A+B+D segment was generated by partial HpaI cleavage. Both segments were purified by agarose gel electrophoresis. When these segments were transfected into CV-1P cells plaques were generated at a frequency of 0.08% of those produced by fI SV40 DNA (Table 1). This frequency is of the same order as the joining of two segments of the SV40 genome having cohesive termini [13] and recombination of two segments having homologous overlapping termini [1]. Each of the segments transfected separately generated plaques at a frequency of $<10^{-5}$ of those generated by fI SV40 DNA, thus demonstrating a low level of contaminating DNA. DNA isolated from six randomly chosen plaques [1] again showed the retention of all four HpaI sites (gel data not shown). We therefore conclude that monkey cells recombine linear SV40 genome segments whether they have cohesive termini, resected termini which presumably have weak interstrand homology, or, flush-ended termini. Monkey cells also recombine two separate segments of the SV40 genome that either have cohesive- or flush-ended termini. The frequency of joining

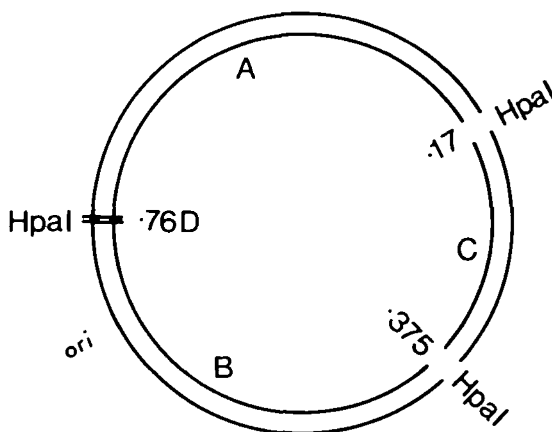

Figure 1. Cleavage of the SV40 genome into two segments, HpaI A+B+D and HpaI C, that have flush-ended termini.

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Table 1. Recombination of flush-ended SV40 genome segments.

DNA	Infectivity pfu/μg DNA	Plaque formation (%pfu/μg DNA)	
		Relative to the yield of recombinants in line 3.	Relative to fI ($3x10^6$pfu/μg DNA)
1.HpaI A+B+D	32±10(1-2/dish)	1.3%	0.001%
2.HpaI C	not detected	<0.1%	<0.0005%
3.Mixture of 1.+2.	$2.5±0.7$ x10^3	100%	0.08%

pfu = plaque forming units; fI = form I (supercoiled) wild type DNA
Segments 1. and 2. were prepared as described in Materials and Methods and are depicted in Figure 1. 40ng of each segment was used for transfection of CV-1P cells in $55cm^2$ dishes as described [1]. The data are the averages of three experiments, and are expressed as the mean ± standard deviation.

cohesive- or flush-ended segments is of the same order.

Recombination between genetic elements that express no biological function

The joining events described above are probably executed by host cell enzymes since cleavage of the SV40 genome in regions essential for viral replication and reproduction still yields viable progeny (e.g. Figure 1). Furthermore, it is unlikely that SV40 specifies information for its own recombination enzymes, since recombination is not known to be an important factor in its reproduction and the small size of the SV40 genome limits the capacity to encode non-essential genetic information [1,14].

Previous experiments describing recombination of linear segments of the SV40 genome having homologous overlapping termini utilised segments that were capable of replication, transcription and translation [1]. Figure 2 depicts another combination of segments where the choice of cleavage sites limits the capacity for transcription and translation. The TaqI/EcoRI segment is lacking the start site for transcription [14], and the PstI/PstI segment is lacking the terminal region of the early transcriptional unit, but contains the origin of replication. Although truncated transcripts could be made from the PstI/PstI segment, one would expect them to be unstable because of the lack of the terminal poly-adenylation region [15]. When these segments were transfected into CV-1P cells plaques were generated at a frequency of 0.1% of those generated by fI SV40 DNA (Table 2). Each segment transfected separately generated plaques at a frequency of <0.001% of those generated by fI SV40 DNA. Each segment was also transfected into CV-1P cells on cover-slips at a concentration thirty times greater than for plaque formation. These cells were fixed in acetone and fluorescently stained for the prescence of antigen reacting to mouse anti-T serum (i.e. serum raised against

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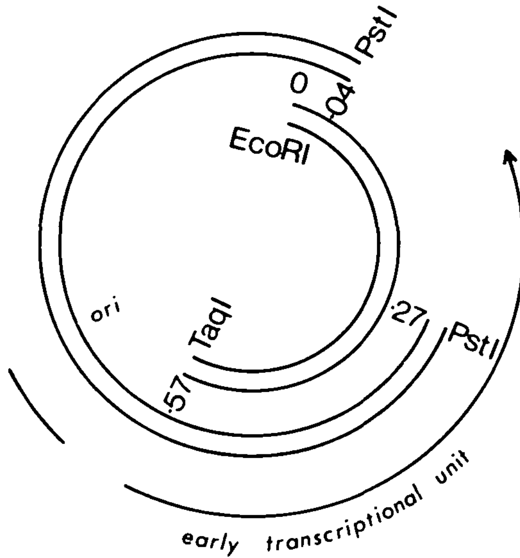

Figure 2. Cleavage of the SV40 genome into two segments, PstI/PstI (0.27-0.04) and EcoRI/TaqI (0-0.57), that have homologous overlapping termini.

cells transformed by SV40 and expressing early gene proteins). Neither of the segments generated any protein in the CV-1P cells which gave nuclear fluorescence expected of the SV40 T-antigen. However, Graessmann et al. [2] have demonstrated that when a PstI/HpaII (i.e. 0.27-0.73) segment was microinjected into the nucleus of African green monkey kidney cells, an antigen was detected which reacted with anti-T serum, but this antigen was incapable of complementing a tsA (early gene temperature-sensitive) mutant. Since the PstI/PstI and PstI/HpaII segments

Table 2. Recombination of SV40/EcoRI+TaqI and SV40/PstI DNA segments.

DNA	Infectivity pfu/µg DNA	Plaque formation (%pfu/µg DNA)	
		Relative to the yield of recombinants in line 3.	Relative to fI ($3x10^6$pfu/µg DNA)
1.EcoRI+TaqI	27±7(1-2/dish)	1%	0.001%
2.PstI/PstI	not detected	<0.07%	<0.0007%
3.Mixture of 1.+2.	$2.8±0.3 x10^3$	100%	0.1%

Segments are as described in Figure 2, and the data are the average of three experiments using 50ng of each segment for the transfection into CV-1P cells as described in Table 1.

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carry the same early gene information we conclude that the differences observed reflect the differences between transfection and microinjection, particularly in regard to the number of copies of DNA that can be delivered [2]. If a truncated A-gene protein were produced we were unable to detect its presence. Even if it were present in undetectable amounts it would be unable to support the replication of either segment. Since recombination occurred at the same frequency as observed for the segments previously described [1], this experiment substantiates the conclusions therein derived, that replication of the segments appears not to be involved in recombination of those segments.

To further analyse the possibility that segments of the SV40 genome are recombined by host cell enzymes, we constructed the segments depicted in Figure3. Recombination between these segments was observed at a frequency of 0.04% (Table 3), a figure not significantly different from the previous recombination experiments. Neither of these segments generated any nuclear early gene product detectable with fluorescent anti-T serum when transfected into CV-1P cells separately at a concentration thirty times higher than used for recombinant plaque formation. Mueller et al. [16] have shown that microinjection of the BamI/BglI segment (0.14-0.67) into African green monkey cells gives no detectable antigen which cross-reacts with anti-T serum, and they conclude that cleavage at the BglI site interferes with early SV40 transcription. The BglI/EcoRI segment encodes the same early gene information. The large TaqI/BamI segment carries only the leader

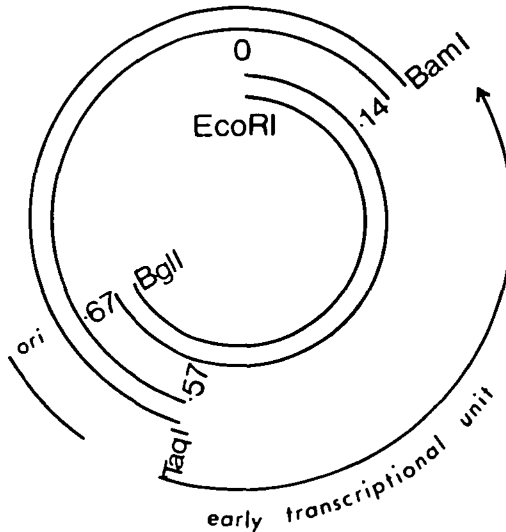

Figure 3. Cleavage of the SV40 genome into two segments, TaqI/BamI (0.57-0.14) and EcoRI/BglI (0-0.67) that have homologous overlapping termini.

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Table 3. Recombination of SV40/TaqI+BamI and SV40/BglI+EcoRI DNA segments.

DNA	Infectivity pfu/μg DNA	Plaque formation (%pfu/μg DNA)	
		Relative to the yield of recombinants in line 3.	Relative to fI ($3x10^6$ pfu/μg DNA)
1.TaqI+BamI	not detected	<0.2%	<0.002%
2.BglI+EcoRI	not detected	<0.2%	<0.002%
3.Mixture of 1.+2.	1.2 ± 0.1 x10^3	100%	0.04%

Segments are as described in Figure 3. 25ng of each segment was transfected into CV-1P cells as described in Table 1. The experiment was repeated three times.

sequence of T-antigen and is lacking the terminal portion of t-antigen [4,14]. Deletion mutants such as dl 2006, which totally lack t-antigen show no impairment and hence no involvement of t-antigen in lytic propagation functions [1,17,18]. Although we cannot exclude the possibility that some unknown minor protein is made at an undetectable level from these segments, no known SV40 early gene protein could be made from these segments [10,18,19] and none is detected in transfected cells by fluorescent staining with serum raised against early functions expressed in transformed mouse cells. Since a threshold level of T-antigen that is detectable by fluorescent staining is required for late gene synthesis, no late gene proteins would be manufactured either [2]. We conclude that recombination of these linear SV40 genome segments is mediated by host cell functions.

DISCUSSION

Recombination of segments of the SV40 genome by a variety of mechanisms has been described. These include the joining of linear segments that have flush- as well as cohesive-termini [13], resected termini [9], or homologous duplex terminal regions simulating two homologous regions of the host chromosome. The lack of involvement of viral proteins has been demonstrated for recombination of segments with homologous overlapping termini, but, as discussed above, probably applies to the other joining reactions. Segments of the genome that have been cleaved in such a manner as to be unable to manufacture any known viral proteins are, in effect, neutral elements of genetic information, incapable of selection by replication or biological function until recombined. The recombination functions that we can define in the end product as progeny virus are available to the host cell and presumably to any element of genetic information that can be generated in that cell. Although replication, unusual DNA sequences and supercoiled structures

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have been implicated in generalised recombination, none of these factors appears necessary for the mechanism of recombination of SV40 genome segments that have homologous overlapping termini. However, these segments already possess free terminal DNA strands that can invade a region of homology in another (or the same) DNA duplex. Perhaps a free DNA strand, however generated, is sufficient to initiate a recombination event including integration of SV40 DNA during transformation. A rapid assay, where recombination can be detected in nonpermissive as well as permissive cells, has been described in Figure 3: here recombination leads to the production of T-antigen which can be monitored by immunofluorescent staining. We are extending this approach with the use of microinjection [2] to increase the efficiency of events in a variety of cell systems.

At present we can only speculate upon the specific enzymes involved in the abovementioned recombination events, but presume that they are host cell functions which are required for DNA repair and recombination. Such functions are for example: E.coli DNA and RNA ligase are known to be involved in the joining of flush-ended DNA in vitro [20], double-stranded DNA breaks caused by ionising radiation are efficiently repaired in mammalian cells [21] and Subramanian has described deletion mutants of SV40 where filling of cohesive termini followed by covalent closure has apparently occurred [22]. Gene amplification has been associated with the production of homogeneously staining regions of chromatin and double-minute chromosomes [23,24], the rearrangement of exons, it has been proposed, may occur independently by recombination [25], and although sister chromatid exchange has been associated with mutagenesis and carcinogenesis, Bradley et al propose that definitive interpretations of sister chromatid exchange data await a molecular mechanism for their formation [26]: the use of SV40 and other miniature chromosome probes should provide insight into such exchange mechanisms [1].

Our observations with SV40 described here may not be too far removed from the behaviour of its host cell since recombination of SV40 genome segments appears to be totally dependent upon host functions, replication is very much dependent except for initiation, unusual functional similarities are found between SV40 and its host cell DNA such as RNA splicing and sequences around the origin of replication [14,27] and the SV40 genome is a miniature chromosome [3].

Botchan et al. [28] have proposed the 'onion-skin' model for excision of SV40 DNA from the genomes of transformed cells: the maturation of the linear excised genome could readily be accommodated by the experiments described here for homologous overlapping termini, as can the maturation of linear concatamers of SV40 DNA [29,30]. Other variations of recombination almost certainly occur (e.g. the joining of V and C regions of immunoglobulins [31]), but recombination experiments

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described here and further suitable manipulations [1] of SV40 genome segments should lead to some understanding at a molecular level of DNA recombination mechanisms in mammalian cells.

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