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Analysis of recombination in mammalian cells using SV40 genome segments having homologous overlapping termini

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ABSTRACT

Segments of SV40 DNA having homologous overlapping termini recombine to produce viable genomes in monkey cells. Frequencies of recombination on either side of a deletion marker are non-random; replication and palindromes do not appear to be essential. Since recombination involves host enzymes, a suitable system has been devised for analysing host cell recombination functions.

INTRODUCTION

Analysis of recombination events at a molecular level in mammalian cells, whose genomes are extremely large has so far proved to be difficult [1,2]. The different patterns of recombination observed in prokaryotes (including bacterial transformation and transduction) and eukaryotes and the mechanistic models proposed therefore have been countenanced as variations on one basic template-switching process [1]. This view, that generalised recombination involves breakage and reunion of DNA via the formation of heteroduplex joints followed by lateral migration of the bridge and cleavage to release helices with various arrays of flanking markers has been re-emphasised by Radding [2]. Observations in different genetic systems are then accommodated as minor variations. However, most observations have come from fungal genetics, or at a molecular level, from experiments utilising bacteria or their viruses and plasmids [2]. We have approached the analysis of recombination in mammalian cells by utilising the well characterised miniature chromosome of the simian papovavirus, SV40 [3].

The SV40 genome utilises host cell replication functions and is unlikely to encode information for recombination functions, since the genome specifies information for only five protein products, three of which are capsid proteins. Furthermore, the SV40 genome behaves as a 'mini-chromosome' and has been used for model studies of mammalian chromosome structure: the genome has been well characterised both genetically and physically [3]. If recombination could be shown to occur in the SV40 genome at a reasonable efficiency and with little

background contamination, the genome could be manipulated as a miniature chromosome for probing host cell recombination functions.

We describe 'generalised recombination' between two segments of the SV40 genome that have been generated specifically by restriction endonuclease cleavage. These segments have termini which overlap in homologous regions of the genome, such that the overlapping regions represent portions of homologous chromosomes (homologues) in the classical genetic sense, and that two cross-over events could generate a viable viral genome. One of the segments has been cleaved from a viable deletion mutant, dl 2006 [4], in such a way that one of the recombination events necessary to produce a viable genome would occur on one or other side of the deletion, thereby producing either wild type or dl 2006 - marked progeny. Plaques from the recombination events were picked and the genome structure of the isolates analysed by restriction endonuclease cleavage and gel electrophoresis. Of thirty plaques analysed in one experiment, sixteen demonstrated unique recombination products, and the others were a mixture of wild type and defective deleted genomes. There was also a preference (5:1) for production of wild type genomes over dl 2006 genomes.

MATERIALS AND METHODS

Preparation of transfecting DNA segments

Wild type SV40 strain 776 DNA was cleaved with PstI [5] at a concentration of 33 µg/ml in 90mM Tris HCl/ 10mM $MgSO_4$ buffer (pH 7.4) at 37°C for 60 min. SV40 deletion mutant dl 2006 [4] DNA was cleaved with HpaII [6] and EcoRI [7] in 10mM Tris HCl/ 10mM $MgCl_2$/ 6mM KCl/ 1mM dithiothreitol buffer (pH 7.4) at 37°C for 60 min. Each cleavage mixture was separated on 1.5% agarose gels in a horizontal slab gel apparatus at 14mA (70V) for 16h [8,9]. The gel was stained with ethidium bromide (1 µg/ml) for 60 min. and viewed while irradiated with UV light (Ultraviolet Products BlakRay UVL56). The required bands were excised and the agarose dissolved in 10 volumes of 100mM Tris HCl (pH 8.0) saturated with KI [10], then loaded onto hydroxyapatite (Biorad HTP), washed with 100 volumes of 0.05M potassium phosphate buffer (pH 6.7), then eluted with 0.4M potassium phosphate buffer (pH 6.7). The DNA was dialysed versus 10mM Tris HCl/ 1mM EDTA (pH 7.6). Yields were greater than 50%.

Transfection

For the analysis of recombination each segment preparation was transfected into the CV-1P line of African green monkey kidney cells [11], either separately as a control for contamination by viable genomes, or as a mixture. For transfection the DNA preparations (100 µl) were mixed with 0.5ml DEAE-dextran ($2x10^6$

MW, Pharmacia) [12] and then 0.5ml MEM [11] containing 20mM HEPES (Calbiochem Ultrol) was added. Dilutions were executed in the DEAE-dextran solution. CV-1P cells that had been subcultured at a split ratio of 1:2 when confluent into 55cm^2 dishes approximately 16h previously [13] were washed with MEM and the DNA solution added. The dishes were rocked for 30 min. at room temperature, then the DNA solution removed, washed with MEM containing 500 µg/ml heparin [14] and finally with MEM. The transfected cells were maintained overnight in medium 199 containing 10mM HEPES and 2% fetal calf serum then overlaid with agar medium. Neutral red was added after 9d and plaques picked or counted for the next two days [11].

Gel analysis of individual plaque isolates

One half of each plaque isolate was used to infect CV-1P cells in 25cm^2 tissue culture flasks. 2ml of this stock were used to infect two 150cm^2 dishes and DNA extracted by the method of Hirt [15]. The soluble DNA fraction after SDS-NaCl precipitation [15] was treated with RNase (20µg/ml) for 4h, then phenol and ether extracted [8]. Samples to be cleaved by restriction endonucleases were dialysed exhaustively against 10mM Tris HCl/ 1mM EDTA (pH 7.6). 20µl (approximately 1µg) of each of the samples was analysed by agarose gel electrophoresis in 1.5% agarose [8]. The gels were stained as described above, observed while illuminated with short wavelength UV light (Ultraviolet Products C51), and photographed with Polaroid Type 55 positive-negative film through a red filter.

RESULTS

Experimental design

In 1972 Mertz and Davis demonstrated that linear (fIII) SV40 DNA which had been generated by cleavage with the restriction endonuclease EcoRI could reseal and form viable virus plaques at a frequency of 10% compared with supercoiled (fI) SV40 DNA [16]. Subsequent investigations demonstrated the closure of resected genomes to generate deletion mutants [17] and the resealing of flush-ended fIII SV40 genomes to generate viable virus at the same frequency as fIII genomes with cohesive termini [18]. These joining events appear to be manifested by host cell enzymatic machinery since cleavage of the SV40 genome in regions coding for genetic information essential for both viral replication and reproduction still yields viable progeny (Refence 19 and unpublished observations). As these host enzymes are DNA recognition and joining enzymes they presumably could be involved in repair and recombination. Similar functions may recognise and recombine DNA segments other than host cell DNA. To test this hypothesis we have used segments of the SV40 genome as probes and CV-1P African green

monkey kidney cells as the host [11].

One of the many possible choices for segments generated by restriction endonuclease cleavage of the SV40 genome is described in Figure 1. The two segments have homologous overlapping termini spanning 4% and 46% of the genome from 0 to 0.04 and from 0.27 to 0.73 respectively (Figure 1a). If a successful recombination event were to occur between the two segments one would predict two cross-over events, one between 0 and 0.04 and the other between 0.27 and 0.73 to be necessary to generate a recombinant genome (Figure 1b). However, the segment from dl 2006 contains a 5% deletion between 0.54 and 0.59 so that a cross-over between 0.27 and 0.54 would be expected to generate a wild type genome (c) and between 0.59 and 0.73 a genome marked with the dl 2006 deletion (d). The choice between these would be dependent upon any restrictions placed on recombination events in these two regions, or alternatively on any 'hot

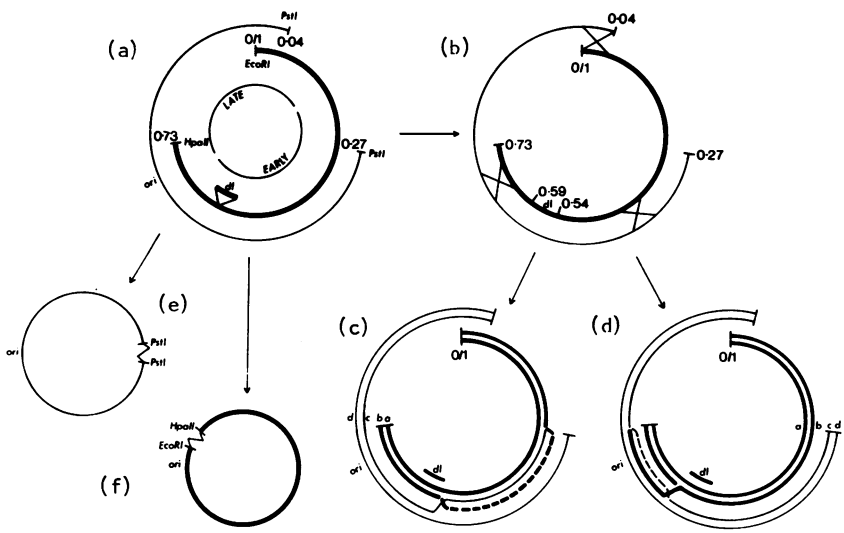

Figure 1. Diagrammatic representation of two segments of the SV40 genome having homologous overlapping terminal regions. (a) The segments are oriented with respect to the circular SV40 genome map [3,16], with the EcoRI site at 0/1 on the map. (b) demonstrates the possible cross-over events leading to either wild type or dl 2006 progeny as detailed in (c) and (d) (see text). (e) and (f) represent the two simplest products of the transfection, i.e. closure at the termini without recombination. (a), (b), (e) and (f) represent double stranded segments, but are drawn as single lines for clarity. The DNA sequence from 0.54 to 0.59 in the segment derived from dl 2006 (heavy lines) is actually deleted but is indicated to show the position and extent of the deletion in (b), (c) and (d).

spots' that might be present. The segment from 0 to 0.73 contains the entire early gene region [3] and information necessary for replication. The region from 0.59 to 0.73 contains the origin for replication, at 0.67 [20], and could be expected to favour strand displacement and exchange if the segments were able to replicate prior to recombination [2].

The model in Figure 1c is based on the review by Radding [2], including displacement of strand b by strand c thereby creating a D-loop in strand b, followed by uptake of strand c, perhaps D-loop cleavage, and assimilation of strand c as far as the deletion at 0.54. The model in Figure 1d is the alternative displacement of a wild type segment strand by a strand from the segment containing the dl 2006 deletion, with assimilation again as far as the deletion from the alternative side to 0.59. Similar events are predicted for a cross-over in the region 0 to 0.04. Figures 1e and f represent the two simplest possibilities for joining of the input segments. The linear PstI cleaved segment has cohesive termini, an origin for replication and an incomplete early gene region [3,21], but could be expected to replicate with helper virus when the termini are joined [22]. The segment derived from dl 2006 could replicate autonomously if the termini were covalently sealed, since it contains an origin for replication and the complete early gene region [3,21].

Recombination between segments of SV40 DNA

When the DNA segments described in Figure 1 were transfected into CV-1P cells (Tables 1 and 2) recombination was observed at a frequency of 0.1% of that observed with supercoiled wild type DNA. Background observed in this experiment was 2.9% for one segment and 0.6% for the other, relative to the yield of recombinants; in four other experiments it was less than 2%. These data demonstrate a low level of contamination by DNA able to produce viable virus in the absence of recombination. Thirty plaques were picked, propagated and the viral DNA analysed by agarose gel electrophoreseis (Figure 2). The genomes were analysed also by various restriction endonuclease cleavages and gradient polyacrylamide gel electrophoresis (Figure 3). Six of these isolates were identified as containing dl 2006 and ten as wild type in origin. The remaining fourteen consisted of a wild type genome and various defective deleted genomes (usually one per isolate). Since some of the defective genomes were of dl 2006 origin (i.e. they contained the dl 2006 deletion; Figure 3) in the presence of a wild type genome, they could not have arisen from the latter during propagation of the plaque isolates.

The lower frequency (0.1%) of plaque production from recombination per microgram of DNA compared to that produced by fl SV40 DNA presumably relates

Table 1. Efficiency of plaque formation: transfection of two overlapping segments of SV40 DNA into CV-1P cells.

DNA	Infectivity *pfu/μg DNA	Plaquing efficiency Relative to fI WT*	Relative to fIII WT*
dl 2006/ HpaII+EcoRI + WT/ PstI	$3x10^3$	0.1%	1%

* pfu = plaque forming units; fI WT = form I (supercoiled) wild type DNA; fIII WT = linear wild type DNA generated by unique restriction endonuclease cleavage.

20ng of wild type SV40 DNA cleaved with PstI and 29ng of dl 2006 DNA cleaved with HpaII and EcoRI were transfected into CV-1P cells as described in Materials and Methods. Thirty plaques were picked at random on the tenth and eleventh days and stored after neutral red was added on the ninth day. The experiment was repeated five times at different DNA concentrations and with different DNA preparations. Plaque formation was linear with respect to DNA concentration in the range tested (0.5-50ng). Efficiency of recombination varied from 0.03 to 0.1% with different DNA prearations and transfections, but was constant for each experiment. Since the DNA from each of thirty plaque isolates was analysed in one experiment we have tabulated the data for that experiment here and not the averages.

to the probability of two molecules colliding as well as the necessary and subsequent cross-overs. However, 47% of the plaques analysed had two or more replicating genomes. It is unlikely that they were generated by a second recombination event at such a high frequency in the transfected cell, but

Table 2. Measurement of contaminating DNA able to yield viable progeny without recombination.

DNA	Plaque formation (% pfu/μg DNA) Relative to the yield of recombinants in Table 1	Relative to fI WT ($3x10^6$ pfu/μg)
dl 2006/ HpaII+EcoRI	0.6%	0.001%
WT/ PstI	2.9%	0.005%

Transfections were carried out as in Table 1 but with each segment separately. Levels of contamination without recombination (background) were less than 3% relative to the data for recombination involving simultaneous transfection with both segments (Table 1).

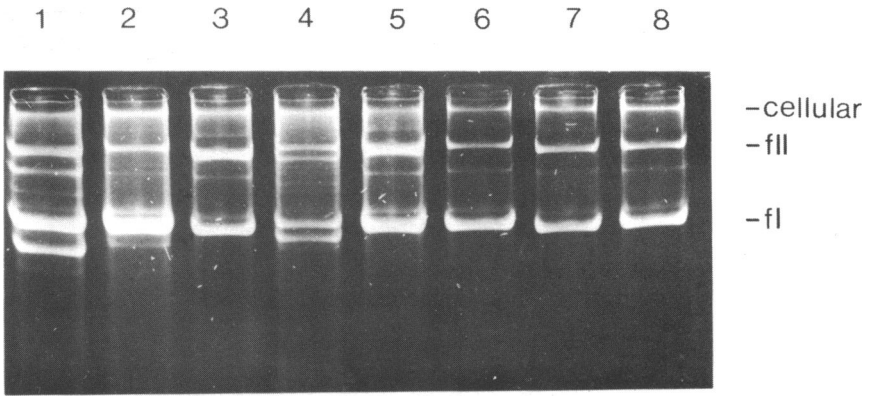

Figure 2. Agarose gel electrophoresis of recombinant viral DNA. Each of the thirty plaque isolates from the transfection described in Tables 1 and 2 was propagated in CV-1P cells. The gel shown here is a representative sample of DNA from eight of the thirty plaque isolates. Slots 1 and 4 have wild type plus a defective deleted genome; slots 3, 7 and 8 have dl 2006 DNA and slots 2, 5 and 6 have wild type DNA. These conclusions were reconfirmed by mixing wild type DNA with the samples followed by agarose gel electrophoresis or by gradient polyacrylamide gel electrophoresis (Figure 3).

probably arose from legitimate or illegitimate closure of independent transfected segments (Figure 1). The variety of defective deleted genomes scored also implies that simple closure of the PstI sites in the segment derived from wild type virus was not the major source. No isolates were detected that consisted of two defective complementing genomes; perhaps this is expected since closure of either of the transfected segments (Figure 1) would yield a deleted late gene region in both molecules, and hence incomplete information for virion production.

Lack of bias in the selection of recombinant plaques

Since the production of wild type recombinants relative to dl 2006 observed was five to one, it was necessary to exclude bias in the growth and selection of plaques in order to accept these data as meaningful.

CV-1P cells were infected with mixtures of equal plaque forming quantities of wild type and dl 2006 virus stocks at multiplicities of infection of $2x10^{-5}$, 10^{-4} and $2x10^{-4}$ in order to emulate the transfection conditions. Thirty plaques were picked at random ten or eleven days later and stored. Each plaque isolate was propagated, then viral DNA was isolated and analysed by agarose gel electrophoresis. Of the thirty isolates, fifteen were shown to be dl 2006, twelve were wild type, two were wild type plus a defective deleted genome and

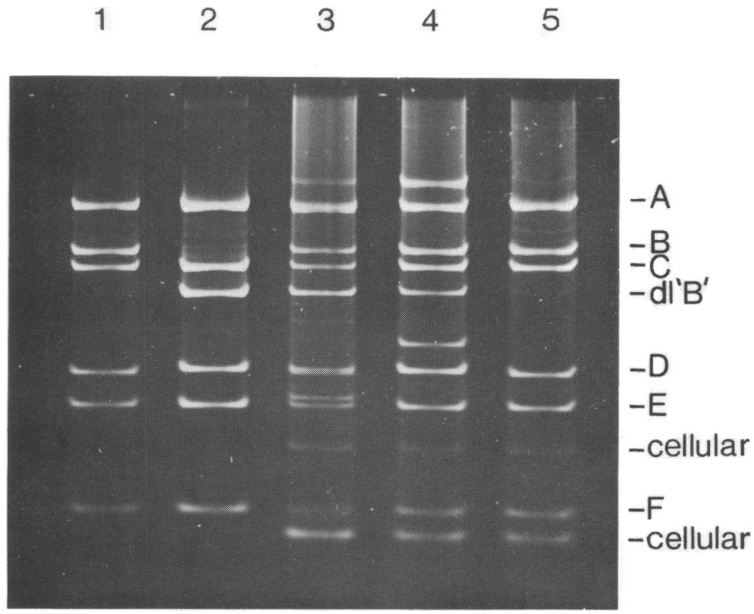

Figure 3. (a) Gradient polyacrylamide gel electrophoresis of DNA from recombinant plaque isolates. DNA from mixed plaque isolates (Figure 2) and samples detected as wild type or dl 2006 were cleaved with HindIII [23] and electrophoresed on gradient polyacrylamide gels. Vertical gels (3.5-10% [24] gradient, 3% stack; 30:1 acrylamide: bisacrylamide in Tris-borate-EDTA [24]) were poured in the apparatus described by Studier [25]. Slot 1 contains wild type marker DNA and slot 2 dl 2006 marker DNA. The 1169 base pair HindIII B fragment spans the deletion in dl 2006 (from 0.426 to 0.655 in wild type DNA) [3,21], and is the only HindIII fragment affected by the deletion. Slots 3 and 4 contain DNA from two representative plaques showing wild type plus a defective deleted genome by agarose gel electrophoresis (Figure 2). Both contain the wild type HindIII B fragment and its deleted counterpart from dl 2006. The remaining bands are common to both wild type and dl 2006 virus in some cases, but some are different also, indicating differences in the deleted genomes, as was predicted from agarose gel electrophoresis. Slot 5 contains DNA from a plaque shown to be wild type on agarose gels. The two extra bands compared to wild type DNA are derived from host cell DNA (Figure 3b); the smaller correlates with the 172 base pair reiterated sequence found in HindIII cleavages of BSC-1 African green monkey cell DNA [26]. Each of the DNA samples prepared in the manner described for Figure 2 had approximately equal quantities of host cell DNA and f1 viral DNA soluble in the Hirt supernatant [15].

one was a mixture of wild type and dl 2006 in origin. As 50% of the plaque isolates were dl 2006 we conclude that dl 2006 propagates at a rate equal to wild type virus and that there was no bias in the selection or growth of wild type over dl 2006 plaques. The difference observed between the yields of

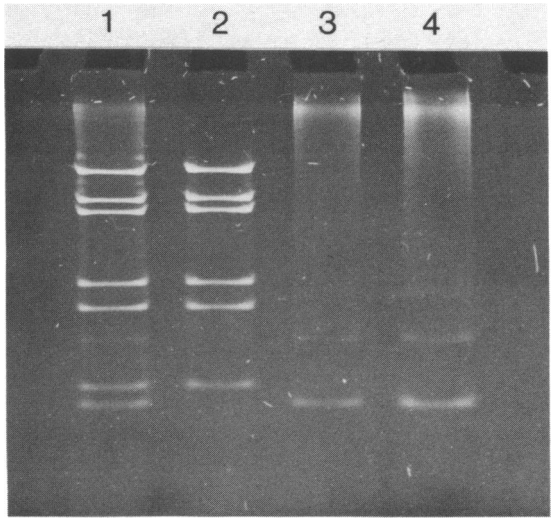

Figure 3. (b) HindIII cleavage of recombinant plaque and host cell DNA. Slot 1 contains DNA from the isolate shown in slot 5 of Figure 3a, and slot 2 contains DNA from wild type virus purified by the Hirt procedure, but continuing through to the CsCl-ethidium bromide step which removes linear and open-circular DNA [15]. Slots 3 and 4 contain 0.5 and 1 µg respectively of DNA isolated from uninfected CV-1P cells [8].

recombinant plaque types appears to reflect a difference in frequency of representative recombination events (Figure 1).

DISCUSSION

We have described the recombination of two specific segments of SV40 DNA that have homologous overlapping termini in African green monkey kidney cells at a frequency of 0.1% relative to fI DNA. Recombination between two temperature sensitive mutants of SV40 has been observed at a similar frequency (2×10^{-3}) [27]. When Wilson cleaved the SV40 genome with two different restriction endonucleases that recognise unique but different sites and transfected the resulting segments into African green monkey kidney cells, plaque formation occurred at a frequency of 0.37% compared with fI DNA [19]. Recombination between two segments having overlapping termini occurs at a similar frequency. Since recombination occurs at a low frequency between genetically marked viable SV40 viruses [27], it appears not to be essential for replication which is dependent upon host functions [3]. Furthermore, since the small size of the SV40 genome limits the capacity to encode non-essential genetic information [21], it

is unlikely that SV40 specifies information for its own recombination enzymes. Although we cannot exclude transcription and translation of a minor SV40 protein from the transfected segments that could be involved in recombination we propose that recombination is mediated by host functions in joining the deleted and non-viable segments to generate a viable genome.

It has been proposed that replication is the motive force for strand displacement during recombination [2]. Both the segments described in Figure 1 contain origins of replication and perhaps could replicate as linear molecules [28]. If replication were an important factor in the experiments described one would predict that the more frequent recombination product would be generated close to the origin of replication, since branch migration is probably hindered by the deletion at 0.54-0.59 (Figure 1) [2,29]. However, our data indicate that the more frequent (5:1) event arose distal to both origin and deletion. Data not presented here using two overlapping segments, one of which does not contain the origin of replication, show that replication of both segments is not essential for recombination. We feel a more reasonable assumption would be that recombination is initiated by strand invasion from the free segment termini [1,2]. Sobell [30] and Wagner and Radman [31] have postulated that inverted repetitions or palindromes constitute special sites which lead to the initiation of strand exchange. The 622 base pair sequence surrounding the origin of replication and lying between the early and late gene coding sequences is unusually rich in palindromes, long repeats, blocks of DNA with high AT-content and unusual alternations of AT- and GC-rich regions [21], but the more frequent recombination event did not occur in this region. If replication and palindromic sequences were essential to generalised recombination a significant bias towards the yield of dl 2006 recombinants should have been detected, but the reverse was observed.

Although the SV40 genome may not be totally representative of the host genome, the ability to analyse its structure at a molecular level, its dependence on host replication and recombination functions, and its mini-chromosome behaviour should allow insight into mechanisms of recombination of the host chromosomes. The appropriate choice of various SV40 DNA segments for recombination as described here should demonstrate the presence of specific sequences in the SV40 genome either enhancing or interfering with recombination. In principle, the deletion between 0.54 and 0.59 could be replaced by specific host cell inserts such that DNA sequences of interest, e.g. highly repetitive sequences or regions involved with DNA splicing, could be analysed for their recognition by host cell recombination functions. This could be achieved by

restriction endonuclease removal of the 0.54-0.59 region from both segments, followed by ligation to cloned host DNA segments of appropriate length to generate overlapping homologous host cell DNA termini. Since these sequences are in a region which is dispensable (dl 2006) the progeny of recombination should be viable but contain the host cell DNA sequence. We are presently testing the feasibility of the system as a more general recombination assay.

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