Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 53-59

A DUAL-CIRCULAR PLASMID STRUCTURE DEPENDENT ON DNA REPLICATION GENERATED IN MONKEY COS7 CELLS AND CELL EXTRACTS

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Received February 24, 1989

When monkey COS7 cells are transfected with plasmids pSVod or pSV2-neo, a DNA structure can be detected consisting of two circular forms linked by a duplex bridge. Generation of this structure is enhanced by camptothecin, an inhibitor of DNA topoisomerase I. Generation of dual-circles in vitro, using a DNA replication system with added T-antigen, requires template DNA with an SV40 origin. Heterogeneous dual-circles can be visualized involving two initially independent molecules of different size. Implications for in vitro studies of certain types of recombination are discussed.

Transfection of plasmid DNA into mammalian cells has been employed in several studies of replication initiated at the SV40 origin (1-3) or at potential genomic sequences (4-6). Relatively high rates of recombination have been observed among transfected plasmids replicating in monkey COS cells (7,8). Homologous recombination between coinjected plasmids peaks during S-phase in the cell cycle of rat fibroblasts (9). Linearized DNA fragments bearing an SV40 origin of replication have been reported to generate intermediates capable of recombining with other circular molecules through a process involving DNA synthesis (10,11). At this point there has been little analysis of plasmid replication intermediates that might be recombinogenic. We report here a novel dual-circular structure involving plasmids bearing an SV40 origin of replication. This structure can be generated in vitro to link two different, co-incubated molecules.

METHODS

Transfection of COS7 cells. Monkey COS7 cells were transfected with indicated plasmids, using a calcium phosphate

0006-291X/89 $1.50

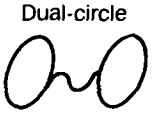
precipitation method with a chloroquine pulse, and low molecular-weight DNA extracted as previously described (4).

DNA templates and in vitro replication conditions. Plasmid templates containing an SV40 origin of replication were pSVod (3.3 kb [12]) and pSV2-neo (5.6 kb [13]). Plasmids pML-2 (3.0 kb; identical to the parent vector for pSVod [14]) and pBR322 were non-replicating controls. In some experiments purified SV40 DNA (5.2 kb) was used. All templates were >80% form I DNA. Reaction mixtures (50 µl) contained 300 ng of DNA in 30 mM HEPES, pH 7.5, 7.0 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 µM each dATP, dGTP and dTTP, 20 µM $\alpha[^{32}P]$dCTP (15-30 Ci/mmole), 200 µM each GTP, UTP and CTP, 4.0 mM ATP, 40 mM phosphocreatine, 10 ug creatine kinase, and 30 µL COS7 cell extract (15). Reactions were supplemented as indicated with T-antigen, purified from SV40-infected COS7 cells by immunoaffinity chromatography using monoclonal antibody PAb419 as described by Simanis and Lane (16). Reactions, at 37°C, were stopped by addition of EDTA to 25 mM and SDS to 0.5%, and labeled DNA was extracted as described (15). Extracted DNA was treated with restriction endonuclease DpnI or prepared for electron microscopy as previously detailed (4).

RESULTS AND DISCUSSION

Dual circular plasmid forms in transfected COS7 cells. We report here the presence in monkey COS7 cells of a novel dual-circular plasmid form, detected by electron microscopy within 24 hrs of transfection with either pSVod or pSV2-neo. This form consists of two circles of monomeric plasmid size connected by a duplex DNA bridge. Because the structure possesses two branch points, potential replication forks, we have compared its frequency to that of known plasmid replication forms (Table 1). The majority of replicating structures are theta forms at various

Table 1. Electron microscopic analysis of pSV2-neo replication forms and dual-circles in transfected COS7 cells. Transfection and DNA isolation at 24 hrs are described in Methods. Numbers represent the contents of 2 grids, approximately 12,000 circular pSV2-neo molecules. Numbers in parentheses are per cents relative to theta forms

	pSV2—neo FORMS: Number of molecules		
	Theta 	Sigma 	Dual-circle 
No treatment	160 (100%)	33 (21%)	17 (11%)
Plus camptothecin	145 (100%)	55 (38%)	50 (37%)

stages of completion. Sigma form structures are also seen. The origin of sigma forms is not known, but at least a portion of them may represent broken theta forms since the majority of DNA "tails" protruding from circles are of less than one plasmid length. With transfected pSV2-neo, sigma forms are seen at a frequency of 21% relative to theta forms. The connected dual-circles are seen with a frequency of 11% relative to theta forms. This form is not seen among plasmids as isolated from E. coli. We sought to determine whether dual circle formation involves DNA replication.

It is conceivable that the generation of dual-circles proceeds through an intermediate with an available free end such as a sigma form or broken theta form. The antitumor agent camptothecin specifically inhibits DNA topoisomerase I, which is preferentially associated with replicating SV40 DNA in infected CV-1 cells (17). The agent reportedly breaks replication forks in growing Cairns structures on SV40 DNA (18,19). Immediately after transfection with pSV2-neo, we treated COS 7 cells with 2 μM

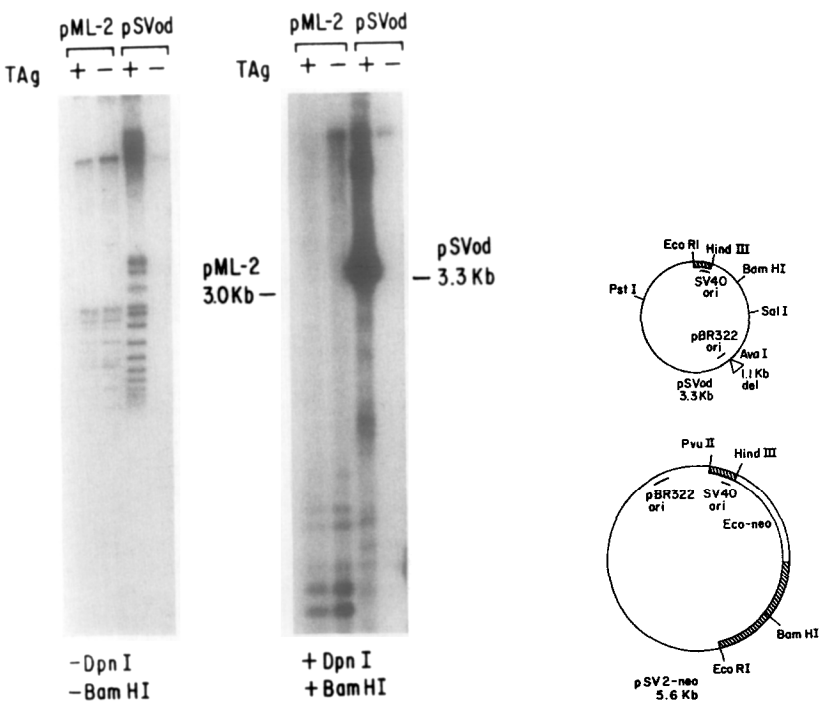


Figure 1. Replication of plasmid pSVod in vitro. The DNA was labeled for 2 hrs in the COS7 replication system described in Methods, ±T-antigen at 0.5 μg/reaction, subjected to 1.4% agarose gel electrophoresis and autoradiographed 24 hrs. Maps of plasmids pSVod and pSV2-neo are shown in approximate scale at right. Shaded bars = SV40 sequences; open bars = the E. coli neomycin resistance gene; lines = pBR322 sequences.

camptothecin, a concentration which inhibits thymidine incorporation 10-25% while having little effect on cell mortality at 24 hrs. At 24 hrs camptothecin enhanced the frequency of sigma forms from 21% to 38% relative to theta forms. Camptothecin enhanced the level of pSV2-neo dual-circles nearly 4-fold, from 11% to 37% relative to theta forms. Approximately 20% of sigma forms seen had tails of 1 full plasmid length. It has been reported that after fork breakage by camptothecin, DNA synthesis can continue unidirectionally on circular SV40 molecules to generate tails of one full viral length (18). Results summarized in Table 1 indicate an effect of camptothecin on replicating plasmid molecules and suggest a link between replication and formation of the dual circular structures.

Formation of dual circular plasmid structures in vitro. To examine any link between replication and the formation of dual circles, we employed an *in vitro* replication system capable of selective initiation at the SV40 origin (15). This system replicates both plasmids pSVod and pSV2-neo. Fig 1 shows that pSVod replicates to generate full-length DpnI-resistant DNA. DpnI

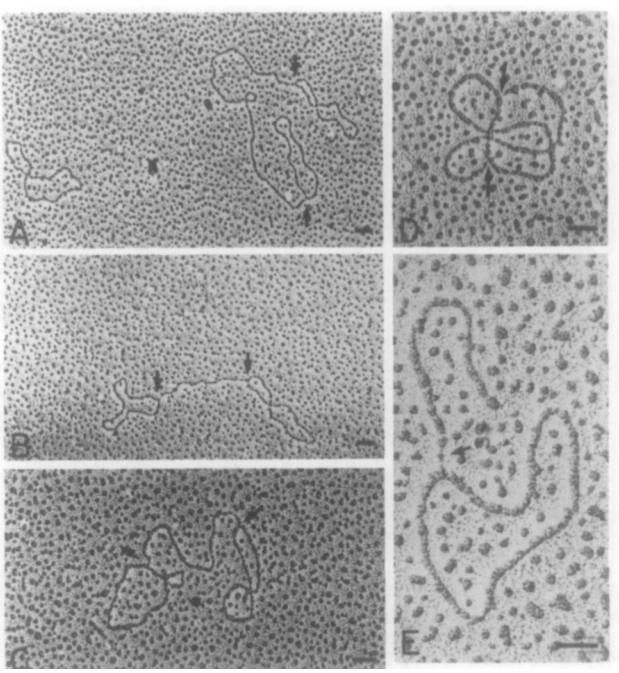


Figure 2. Representative dual-circular structures and replication forms generated by plasmid pSVod. The replication reaction was carried out with T-antigen as described for Fig 1. A-C: dual-circles; D: late Cairns (theta) structure; E: sigma structure. Arrows point to fork junctions. Bars = 100 nm.

resistance is a standard criterion for replication of bacterial plasmids in mammalian systems (4,6,12). Labeling of pSVod is entirely dependent on the addition of purified T-antigen. Without restriction cleavage the system labels primarily form I pSVod DNA, seen in this gel, run in the absence of ethidium, as a series of bands representing different degrees of supercoiling. After treatment with BamHI and DpnI, this label is seen primarily in the full-length plasmid band of 3.3 kb. Labeled material that migrates more slowly is thought to represent partially-replicated molecules (15). There is slight labeling of pML-2, the control parent vector for pSV0d, but upon DpnI treatment, no label is seen at the position of full-length pML-2 while most label appears at the position of digestion products. Occasionally this slight labeling, most likely due to repair, was also seen with pSVod in the absence of T-antigen. (A labeled, slowly migrating DpnI-resistant band is derived from a minor amount of E.coli chromosomal DNA isolated with pML-2.) Plasmid pML-2 does not contain an SV40 origin of replication. Previous workers have reported that with SV40 DNA this system generates theta structures, characteristic of bidirectional replication, as well as a significant percentage of sigma forms visible by electron microscopy (15). We detect similar intermediates with either plasmid pSVod or pSV2-neo as well as a significant percentage of dual circles. Representative dual circles, as well as theta and sigma forms of pSVod are shown in Fig 2. Table 2 documents the

Table 2. Plasmid molecular forms after 2 hrs incubation in the *in vitro* replication system. Plasmids were subjected to replication conditions in the presence of added T-antigen (0.5 µg/50 µl reaction) for 2 hrs, purified and spread for electron microscopy as described in Methods. 1000 molecules of each plasmid species, containing clearly-identified circles, were analyzed

PLASMID MOLECULAR FORM	PERCENT OF MOLECULES		
	pSVod	pSV2-neo	pML-2
Circles, no replication evident	76.9	85.0	93.8
Theta forms	7.7	5.3	0
Sigma forms	9.0	5.3	0
Catenated dimers	0	1.1	3.1
Circular dimers	5.1	2.2	3.1
Dual-circles (2 equal circles)	1.3	1.1	0

extent of dual-circle formation with pSVod or pSV2-neo *in vitro* and shows pML-2, lacking an SV40 origin, forms no dual-circles.

Heterogeneous dual circles. When plasmids pSVod and pSV2-neo are coincubated in the *in vitro* replication system, heterogeneous dual-circles are formed that include both molecules (Fig 3). No dual-circles are formed with pML-2 and pBR322, neither of which replicate in the system. Heterogeneous dual-circles are seen only when both participating plasmids contain an SV40 origin of replication and only in the presence of added T-antigen. Under the conditions described in Methods, heterogeneous dual-circles were detected with co-incubated pSVod and pSV2-neo at a frequency of 0.15% relative to all circular pSVod molecules, as determined by scoring 12,000 molecules. With pSVod and SV40 DNA, heterogeneous dual-circles were seen at a frequency of 0.7%. Using pre-labeled SV40 DNA subjected to the replication system with pSVod, we have recently devised means of analyzing the levels of dual-circle formation by gel electrophoresis

Our results implicate a mechanism of interaction between two circular molecules in which at least one of the two is a replication intermediate. Observations with camptothecin suggest that breakage of normal bidirectional replication intermediates could play a role. Topoisomerase I cleavage sites have been implicated

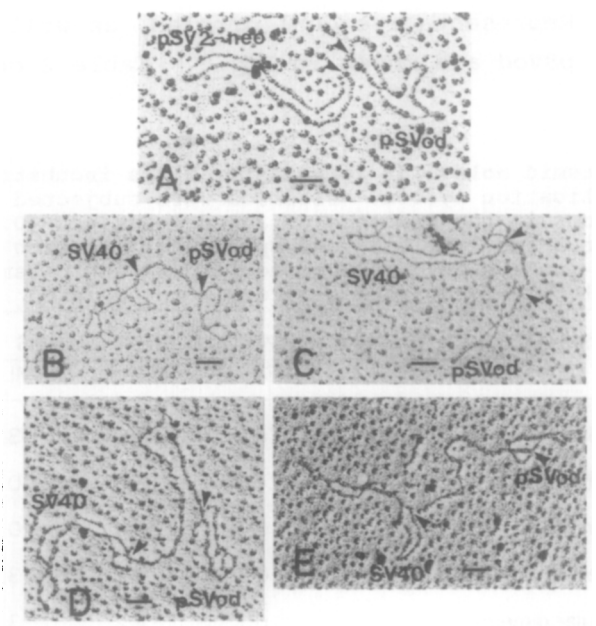


Figure 3. Heterogeneous dual-circles formed with pSVod and pSV2-neo or with pSVod and SV40 DNA. Plasmid and viral DNAs (300 ng each) were co-incubated as described for Fig 2. Arrows point to fork junctions. Bars = 100 nm.

as points for nonhomologous recombination involving plasmid (20) or SV40 (21) DNA. Free ends of SV40 DNA sigma structures have been hypothesized to play a role in recombination between transfected plasmid species (10,11). Further work may reveal whether dual-circles are intermediates in genetic recombination. If so, optimizing their *in vitro* formation could provide a model for studies of types of strand invasion involving DNA replication.

ACKNOWLEDGMENTS

Aided by American Cancer Society grant CD-318A. Initial samples of immunoaffinity-purified T-antigen were graciously supplied by Dr. Peter Tegtmeyer. Plasmid pSVod was a gift of Dr. Tom Maniatis. This is publication No. 18 from the Brookdale Center for Molecular Biology, Mount Sinai Medical Center.

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