Homologous recombination of SV40 DNA in COS7 cells occurs with high frequency in a gene dose independent fashion

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ABSTRACT

Homologous recombination between microinjected SV40 DNA fragments and endogenous SV40 DNA in COS7 cells was analysed by immunofluorescence staining and DNA blotting. Time course experiments revealed that recombination between the transferred (trans) DNA and the chromosomal DNA occurred about 8 hours after microinjection with high efficiency in a gene dose independent fashion. Deletions of up to 1018 basepairs (bp) within the early or the late SV40 region were efficiently repaired after the transfer of linear but not of circular DNA molecules. A 22 bp homology between the trans DNA and the endogenous DNA was sufficient to initiate recombination but 14 nonhomologous bp at one open end of the SV40 DNA fragments hindered gap repair.

INTRODUCTION

During the last 2-3 decades different methods have been developed for transferring genes into mammalian tissue culture cells. This includes various DNA transfection techniques, vesicle mediated gene transfer and microinjection (1-3). These techniques allow the direct study of many biological and biochemical questions with the DNA in its native environment, the living cell, which cannot be done in a cell free system (4). One goal of DNA transfer experiments namely gene repair, an aspect of central medical interest, has still not been achieved.

In order to cure a mutated cellular gene, the trans DNA has to repair the damage through homologous recombination. This prevents simultaneous expression of the endogenous and the trans gene and ensures correct regulation of transcription. In eukaryotic cells however, homologous recombination between circular trans DNA and the cellular DNA occurs with a low frequency of only one in 10^6 transfected cells (5). In contrast, nonhomologous recombination can be obtained with a high frequency. After intranuclear DNA injection, 25-30% of the recipient cells can be converted into transformed cells which permanently express the trans gene (6). This high transformation rate is independent of the DNA configuration, and integration into the host genome occurs in a non specific fashion (7-8).

A breakthrough towards more efficient homologous recombination in eukaryotic cells was obtained by Orr-Weaver's experiments with yeast cells (9,10). They demonstrated that the recombination efficiency of the trans DNA with a double strand break in the region of homology was about 3000 fold higher than that obtained with closed circular DNA molecules (for review see 11). Similar results were also reported for mammalian tissue culture cells (5, 12-21). The highest recombination rate

reported so far was obtained by Schaffner's group. Using linear enhancerless SV40 DNA as trans molecules and SV40 transformed COS cells as recipients 25-30 % of the transfected cells supported homologous recombination as indicated by regeneration of infectious SV40 virus (17). We initiated similar experiments independently by microinjecting enhancerless SV40 DNA fragments or viral DNA molecules with large deletions within the early or late coding region into COS7 cells. We observed homologous recombination between the trans DNA and the endogenous COS7-SV40 DNA in 1-3% of the injected cells. Recombination occurred early after injection of linear DNA molecules in a gene dose independent fashion. Our results further showed that the high rate of recombination was not restricted to the SV40 enhancer region. Also, large deletions within the early or late SV40 coding region were efficiently repaired after microinjection.

MATERIALS AND METHODS

Cells and microinjection.

The monkey cells (TC7, CV1 and COS7) were grown in Dulbecco's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (GIBCO Laboratories). The COS7 cells (CV1 cell transformed by an ori defective SV 40 DNA, (22)) were kindly provided by Y. Gluzman. Details of the microinjection technique are described elsewhere (4).

Substrates for recombination

The DNA fragments used for microinjection were prepared from the early SV40 884 deletion mutant (23). The 884 KpnI(nuc.nr.298)-SphI(nuc.nr.132) DNA A fragment was inserted into the corresponding sites of the pSP68 DNA (Pharmacia) and propagated as pSPSV5 (fig.1) in *E.coli* (HB 101). After isolation by standard procedures, the plasmid DNA was purified by CsCl gradient centrifugation. This plasmid DNA served as source for the tested recombination substrates: as pSPSV5 DNAI, as pSPSV5 linearised with SphI or KpnI, as the KpnI/SphI-884-fragment, the pSPSV5-HaeIII-A-fragment and the HaeIII/SphI-A fragment (fig. 3 left side). For the recombination experiments, the fragments were liberated from the plasmid DNA by cleavage with the appropriate restriction enzymes and separated by agarose gel electrophoresis. The recovery of the DNA from agarose gels was achieved by the method of Wende et al. (24). pBR 322 was used as a vector for cloning of the 884-NdeI-A(nuc.nr.3809-nuc.nr.4827)-DNA fragment (pSVN1, fig.1, fig.3 right side) (25)

DNA reextraction and blot analysis.

For the DNA reextraction experiments the COS7 cells were grown on small glass slides (2x2 mm²). The microinjected cells were further cultivated under standard conditions until lysis (26,27). Before lysis, the slides with the cells were washed in PBS and transferred into an Eppendorf tube filled with 200 µl lysis buffer (0,6 % SDS, 10 mM Na₂EDTA, pH 7.5). After 15 minutes at room temperature, 50 µl of 5 M NaCl was added in order to precipitate the cellular DNA overnight at 4° C. Cellular DNA was spun down in an Eppendorf centrifuge. Low molecular weight DNA in the supernatant was precipitated by 3 volumes of ethanol. For further purification the dried DNA pellet

was dissolved in TE buffer (10mM Tris-Cl, pH8, 1mM EDTA), treated with proteinase K, extracted with phenol, and again precipitated with ethanol. After restriction endonuclease digestion, the DNA fragments were separated on an agarose gel, transferred to Amersham Hybond N membrane and

Fig.1 SV 40 genome with the early and late transcription and translation products (arrows). The small segment inside of the circle depicts the 884 deletion. The relevant restriction sites are shown and the SV40 nucleotide numbers are: BglI (5235); HaeII (832); HpaI (499,519, 2666,3733,4739); HpaII (346); KpnI (294); NdeI (3808,4826); PvuII (270,1716, 3506); SphI (128,200); TaqI (4739). The blow-up focuses on the region between BglI-site and HaeII-site with the early and late promoters, the two 72bp enhancer elements (nuc. nr. 107-179-250) and the origin of SV40 DNA replication (BglI site).

The solid box of the pSPSV 5 DNA represents the KpnI-SphI 884-DNA fragment inserted into the respective sites of the pSP68 DNA (Pharmacia). The early and late transcription orientations are indicated by the arrows.

The pSVN1 DNA contains the SV40 NdeI A-DNA fragment and the pBR322 DNA as vector.

hybridized with nick translated SV40 DNA (2×10^8 cpm / μ g DNA) (4).

DNA-sequencing

DNA-sequencing was done as described elsewhere (28).

RESULTS

It is possible to follow the biological activity and the fate of injected DNA molecules by biological and biochemical standard procedures using a small number of injected cells (27). In order to analyse homologous recombination between trans DNA and endogenous DNA we microinjected early SV40 884 DNA fragments into COS7 cells and monitored recombination by immunofluorescence staining and by DNA blotting. The DNA fragments were prepared from the early dl 54-59 SV40 884 mutant, because this DNA contains a 247 bp deletion (mp. 0,54-0,59) in the T-antigen intron part (23), which allowed the discrimination between the injected and the endogenous SV40 DNA. The first DNA fragment tested was the large SV40 884 KpnI-SphI DNA fragment. As shown in figure 1, cleavage of the SV40 DNA with the KpnI and SphI restriction endonucleases introduced a 166 bp gap into the 884-DNA which included the main part (122 bp) of the two 72 bp enhancer elements (29). As shown

Fig. 2 Agarose gel analysis of the pl-20, pl-6 and pl-2 DNA. The Hirt extracted DNA was digested with the HpaI-endonuclease and the cleavage products are shown in lane 1 for the pl-2 DNA, in lane 2 for the pl-20 DNA and in lane 3 for pl-6. Lane 4 contains the Hirt extract from COS7 cells after microinjection of the KpnI-SphI DNA. In this case recombination did not occur. DNA blot analysis confirmed that the DNA fragments shown are SV40 specific (data not shown). Lane 5-8 contain the DNA treated with the TaqI endonuclease: lane 5 contains wt SV40 DNA, lane 6 the pl-20 DNA, lane 7 the pl-6 DNA, lane 8 the pl-2 DNA. The position of DNAI, DNAII, DNAIII and the HpaI A,B,C-DNA fragments are indicated.

elsewhere, this deletion inactivates the SV40 enhancer thereby preventing T-antigen synthesis in monkey cells (TC7) and hence viral DNA replication and late SV 40 gene expression. The 884 KpnI-SphI DNA fragment was cloned in *E.coli* as pSPSV5 DNA (fig1). The purified plasmid DNA was restricted with the KpnI and SphI enzymes. The SV40 DNA fragment was separated by gel electrophoresis and microinjected into TC7 cells at a multiplicity of 1000-2000 DNA molecules per cell. Recipient cells were fixed and stained for T-and V-antigen at different time after injection (24, 48, 72 hrs) but viral gene expression was not obtained. In contrast V-antigen synthesis was demonstrable in COS7 cells. Twenty-four hours after microinjection of 2-4 KpnI-SphI DNA fragment molecules per cell, 26% of the recipient cells exhibited a strong intranuclear immunofluorescence when stained with SV40 anti V-serum. This showed that the T-antigen synthesizing COS7 cells supported replication of the injected KpnI-SphI DNA fragment and hence late viral gene expression. In order to prove whether homologous recombination occurred additionally under these conditions, we microinjected 100 COS7 cells each with 2-4 DNA fragment molecules in 25 independent experiments. Two weeks after microinjection the culture medium was collected and assayed on TC7 cells for infectious SV40 virus. As tested by T-and V-antigen synthesis 21 out of the 25 isolates contained SV40 virus, demonstrating that recombination occurred. To further characterize these isolates, TC7 cells were infected and the viral DNA was extracted by the Hirt method one week later. The DNA of each extract was then cleaved with the HpaI endonuclease and subjected to agarose gel electrophoresis. The criterion used for homologous recombination was the mobility of the HpaI B fragment, since the KpnI-SphI deletion and also the 54 - 59 deletion of the 884 DNA are within this DNA fragment. The results obtained indicated that three categories of recombinant molecules were

Fig. 3 shows the microinjected circular and linear DNA molecules with restriction sites of the used endonucleases. The pSPSV5-HaeII-fragment has 14 bp of bacterial origin adjacent to the SphI site of the 884 fragment part (according to the information about the vector pSPSV68 by the supplier Pharmacia) Homologous recombination after injection into COS7 cells is indicated by + and -

generated after microinjection. Out of the 21 positive isolates, 16 were of 884 DNA characteristics (e.g. pl-20) while the HpaI-B fragment of three isolates was shorter in size than that of the 884 (e.g. pl-6). DNA fragments of wild type (wt) SV40 specificity were demonstrable in 2 extracts (pl-2). Examples of each categories are given in fig. 2 (pl-20 lane 2, pl-6 lane 3 and the pl-2 DNA lane 1) .

DNA sequencing experiments showed that the pl-20 DNA regained exactly the deleted 166 bp with the two 72 bp enhancer elements, while the pl-6 DNA regained 94 bp with only one 72 bp element. The pl 2 DNA also regained the 166 bp of the KpnI-SphI deletion and additionally, the 247 bp of the early 54-59 deletion, specific for the 884 DNA (fig.1 and 2). Our experiments further confirm that the high frequency of homologous recombination requires linear trans DNA molecules with homology at both open ends. As shown in fig. 3, recombination of circular pSPSV5 DNA with endogenous SV40 DNA was not demonstrable after microinjection. Also the transfer of linear pSPSV5 DNA fragments with the entire vector DNA at the KpnI or the SphI site (fig.3) did not result in generation of infectious SV40 virus. We further reduced the portion of plasmid DNA at the SphI site of the SV40 DNA by treatment with different restriction enzymes and found that even 14 nonhomologous bp from the vector DNA (pSPSV5-HaeIII-A-fragment fig.1, fig.3) are still inhibitory. In an experiment which

Fig. 4 Southern blot analysis of the reextracted SV40 DNA. COS7 cells (100 for each point) were microinjected with the KpnI-SphI DNA fragment (1000-2000 molecules/cell) and DNA was extracted by the Hirt method at different hours after gene transfer, digested with HpaI enzyme, separated on agarose gels, transferred to Hybond-N membranes (Amersham) and hybridized with nick translated SV40 DNA (specific activity 2×10^8 cpm/ μ g DNA). Lane 1 contains the DNA extracted directly after microinjection, lane 2: 10 hrs, lane 3: 24 hrs and lane 4: 36 hrs after injection. Lane 5 contains as marker the 884 DNA with HpaI enzyme cleavage product and lane 6 the 884 DNAl and DNAlI. Lane 7 contains the KpnI-SphI DNA fragment as marker, lane 8 the undigested fragment extracted directly after injection, lane 9: 10 hrs and lane 10: 24 hrs after injection. The mobility of the Hpa I B fragment reflects recircularisation of the injected KpnI-SphI fragment and homologous recombination.

was repeated three times, 1000 COS7 cells were microinjected with 1000-2000 HaeII fragment molecules each, but virus production was not demonstrable. However after transfection of this pSPSV5-HaeII-A-fragment into COS7 cells a low rate of recombination was observed (data not shown).

To learn when recombination occurred after microinjection of the SphI-KpnI 884 fragment, 100 COS7 cells were microinjected with 1000 - 2000 molecules per cell and the DNA was reextracted from the recipient cells at different times as described in material and methods (27). The extracted DNA was then fragmented with the HpaI endonuclease, separated by agarose gel electrophoresis and subjected to DNA blot analysis. As shown in fig. 4 lane 4, a HpaI fragment with the electrophoretic mobility of the 884 HpaI B fragment was first seen 36 hours after injection. A further event which occurred early after injection was recircularisation of the KpnI-SphI fragment. In contrast to oocytes (21), mammalian cells have the ability to ligate efficiently molecules with heterogenous

TABLE1: Percent of V-Antigen positive COS cells

		Hours after Injection				
		8	12	16	20	24
Injected DNA-molecules per cell	DNA-Species injected					
1000-2000	884 DNA I	26	54	70	80	80
1000-2000	884 DNA III (HaeII cut)	0	6	36	42	60
1000-2000	884 Hae II-SphI-Frag.	0	0	0	0	1-3
10-20	884 DNA I	6	14	26	62	70
10-20	884 DNA III (HaeII cut)	0	2	32	48	64
10-20	884 HaeII-SphI-Frag.	0	0	0	0	1-3
2-4	884 DNA I	0	10	22	36	52
2-4	884 DNA III (HaeII cut)	0	0	4	36	42
2-4	884 Hae II-SphI-Frag.	0	0	0	0	1-3

Data shown are mean values from three independent experiments, each with 100 injected cells. The number of injected cells were counted as 100%. At the time indicated, cells were fixed and stained with fluorescein conjugated anti V-serum.

Fig. 5 Southern blot analysis of the NdeI DNA. Lane 1 contains the NdeI-A-DNA fragment and lane 2 the PvuII digested NdeI fragment as marker. The lane 3 the wt SV40 DNAI and DNAIL, lane 4 the NdeI recombination product propagated in TC7 cells and lane 5 the PvuII digested DNA. Lane 6 contains the marker Pvu II wt SV40 DNA fragments. A,B,C indicates the position of the wt SV40 DNA PvuII fragments.

ends (27). As shown in fig. 4 lane 9, extracts obtained 10 hours after injection contained mainly DNAI and DNAIL molecules. Recircularisation of the SV40 DNA was also indicated by the change in the mobility of the HpaI B fragment on the agarose gel (fig. 4, lane 3). In spite of the fact that recombination was not demonstrable by Southern blot analysis at this early time, we have to assume that homologous recombination occurred before recircularisation of the injected DNA, since we showed that circular DNA molecules are poor substrates for homologous recombination (fig.3).

We also analysed recombination by immunofluorescence staining after microinjection of the HaeII-SphI DNA fragment. Restriction of the pSPSV5 DNA with the HaeII and SphI enzymes introduced a 706 bp deletion into the 884 DNA which included not only the enhancer region but also a significant portion of the late SV40 coding part (fig.1). In order to obtain late SV40 gene expression in the COS7 cells, the deleted late viral sequence has to be regained by homologous recombination. To test at what time after microinjection V-antigen synthesis is first demonstrable, 2-4 HaeII-SphI fragment molecules were microinjected into the nuclei of COS7 cells. At different hours after injection the recipient cells were fixed and stained with fluoresceine conjugated anti-V-serum. As summarized in table 1, V-antigen formation was first demonstrable 24 hours after DNA transfer. However, after injection of 2-4 884 DNAILI molecules, which were linearised by HaeII enzyme, V-antigen formation was detected 16 hours later. In this case, late SV40 gene expression did not require recombination

but ligation at the *Hae*III site, since the *Hae*III site is within the V-antigen coding region (fig.1). Therefore the delay in late SV 40 gene expression observed after injection of the *Hae*III/*Sph*I-fragment (table 1), allowed us to conclude that homologous recombination between the injected *Hae*III-*Sph*I-884-fragment and the endogenous SV40 DNA of the COS7 cells occurred about 8 hours after microinjection.

The efficiency of late SV 40 gene expression cannot be increased by a higher copy number. Thus, after injection of 1000-2000 *Hae*III-*Sph*I fragment molecules per COS7 cell, only 1-3% of the recipient cells stained positively for V-antigen 24 hours after injection. A gene dose correlation was also not demonstrable 36 and 48 hours after transfer. In contrast, a clear gene dose correlation for late SV40 gene expression was observed after microinjection of 884 DNAI or 884 DNAIII (*Hae*III cut) molecules (table 1). Therefore, these results show that homologous recombination occurred in a gene dose independent fashion.

Finally, we investigated whether the high recombination efficiency of 1-3 % is specific for deletions which include the SV 40 enhancer element. For this purpose a large 1018 bp deletion was introduced into the early SV40 coding part by *Nde*I endonuclease treatment (fig.1). Following cloning of the SV40 DNA fragment into the *Nde*I site of pBR322 DNA (pSVN1-DNA) the purified *Nde*I-884 DNA fragment was microinjected into TC7 cells at a multiplicity of 1000-2000 molecules per cell. As expected, expression of the injected SV40 DNA fragment was not demonstrable.

To prove whether the 1018 bp deletion, which can be repaired by homologous recombination we microinjected 100 COS7 cells in three parallel experiments with 2-4 *Nde*I fragment molecules and collected the culture medium 7 days later. This medium was then added to TC7 cells, low molecular weight DNA was extracted by the Hirt method and subjected to Southern blot analysis as described above. We found that two out of the three isolates contained wt SV40 DNA. An example of a positive isolate is shown in fig.5. These results are in accordance to data reported elsewhere (25).

Discussion

Gene mutation is the cause for many different human diseases, such as sickle cell anemia, which cannot be cured by conventional medical methods. This is the reason why gene repair may become a method of great therapeutical interest. This kind of approach would involve the isolation of specific target cells, gene transfer and selection of cell clones which express the wild type gene. The donor person would be the recipient for these cured cells (31). The gene transfer method of choice is direct intranuclear microinjection, because this technique has the highest transformation efficiency among all gene transfer procedures (4). It allows the transfer of a defined number of test molecules without carrier or viral DNA (1, 32) and last but not least, requires only a small number of recipient cells. However, before practical application of gene therapy, numerous problems have to be solved, such as the isolation of suitable target cells and enhancement of homologous recombination.

In the present study we showed that homologous recombination can be obtained at a high efficiency rate. After intranuclear injection of SV40 DNA fragments, 1-3% of the COS7 cells supported

Fig. 6 Scheme for homologous recombination events observed after transfer of the KpnI-SphI-884-fragment in COS7 cells.

At the top a section of the COS7 SV40 DNA with the 72 bp repeats (boxed, I, II). The position of the SphI site (nuc.nr. 128) and the KpnI site (nuc.nr. 294) are indicated. The triangle symbolizes the 6 bp deletion at the origin of replication in the endogenous SV40 DNA. Beneath the KpnI-SphI-884-fragment with the remaining 22 bp of the enhancer element II (nuc.nr. 107-128). Generation of the pl 20 DNA with two enhancer elements (a) and of the pl 6 DNA with one 72 bp enhancer element (b) are illustrated.

homologous recombination and generated infectious SV40 virus. This approach is suitable for quantitative analyses because homologous recombination is the rate limiting step. As shown elsewhere (12), SV40 DNA replication is not essential for recombination, but avoids the time consuming cell line selection for further studies (14). Our experiments showed that homologous recombination can be seen shortly after DNA transfer by immunofluorescence staining and by DNA blot analysis. Using late SV40 gene expression (V-antigen synthesis) as an indirect marker for recombination, we calculated that gene repair occurred about 8 hours after injection of the HaeIII-SphI DNA fragment. DNA blot analysis and DNA sequencing experiments confirmed that the injected trans DNA fragments regained the deleted sequences from the endogenous COS7 SV40 DNA. Analysis of the 21 SV40 positive isolates revealed that three different categories of recombinant molecules were obtained after injection of the KpnI-SphI fragment. The majority of the recombinants namely 16 were of 884 DNA type and regained exactly the deleted 166 bp with two enhancer elements. Three isolates regained 94 bp with only one 72 bp element. Generation of 884 DNA with only one enhancer element reveals that 22 homologous bp are sufficient to initiate recombination as illustrated in fig. 6. The two wild type isolates (e.g. pl 2) could be either repaired trans DNA or repaired endogenous COS7 SV40 DNA. In both cases, regeneration of the wild type virus required two distinct recombination events, because the COS7 SV40 DNA contains two separate deletions, one

at the origin of replication and another deletion within the late coding part (22, Y.Gluzman, pers. comm.). Our experiments confirm further, that the high frequency of homologous recombination requires linear trans DNA molecules with homology at both ends (13,17,21,33) and that a few nonhomologous bp (33) at one end are inhibitory (fig 3).

We observed that homologous recombination occurred in a gene dose independent fashion. As summarized in table 1, the number of V-antigen positive cells was similar after injection of 2-4 or 1000-2000 HaeII-SphI DNA fragment molecules per cell. The copy number of the endogenous SV40 DNA in COS cells also does not seem to influence the recombination efficiency significantly (17). Similar results were observed for nonhomologous recombination. We found that the transformation efficiency of the SV40 and the HSV-TK DNA was unrelated to the copy number of the injected DNA molecules (6, 7, 30). These results support the assumption that a cellular factor limits recombination. The nature of this factor(s) is still a matter of speculation and will not be further discussed here.

It is obvious from our results as well as from experiments reported elsewhere (17), that in COS cells, homologous recombination occurs with a frequency which is at least one order of magnitude higher than in other cell systems (12-16,18-21). One feature of the COS cells is the high level of permanent T-antigen synthesis. It was shown that T-antigen synthesis is not essential for recombination (12), however it can be that one of the various T-antigen functions (34) facilitates homologous recombination. If so, coinjection of the trans DNA with early SV40 RNA would allow transient T-antigen synthesis in T-antigen negative cells. We have shown that in vitro synthesized early SV40 RNA (cRNA) can be efficiently processed into mature mRNA and translated into wt T-antigen after microinjection into tissue culture cells (35-37). The short half life of the cRNA would avoid the T-antigen induced oncogenic cell transformation.

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