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In Vivo Assay of p53 Function in Homologous Recombination between Simian Virus 40 Chromosomes

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To investigate a possible role of p53 in DNA exchange mechanisms, we have developed a model system which allows us to quantify homologous recombination rates in eukaryotic cells. We generated two types of simian virus 40 (SV40) whose genomes were mutated in such a way that upon double infection of monkey cells, virus particles can be released only after interchromosomal exchange of genetic material. This test system allowed us to determine recombination rates in the order of 10^{-4} to 10^{-6} for chromatin-associated SV40 genomes. To study the role of p53–T-antigen (T-Ag) complexes in this process, we designed viral test genomes with an additional mutation leading to a single amino acid exchange in T-Ag (D402H) and specifically blocking T-Ag–p53 interactions. Analysis of primary rhesus monkey cells endogenously expressing wild-type p53 showed a decreased recombination rate upon loss of efficient T-Ag–p53 complex formation. However, cells expressing mutant p53 (LLC-MK_2 cells), the introduction of mutant T-Ag did not affect the DNA exchange rates. Our data are interpreted to indicate an inhibitory role of wild-type p53 in recombination. In agreement with this hypothesis, p53–T-Ag complex formation alleviates the inhibitory effect of wild-type p53.

Germ line mutations in the p53 gene are associated with genetic instabilities which become manifested as aneuploidies, allelic losses, and gene amplifications (9, 15, 21, 41, 64, 67) and which contribute to tumor progression. Because of its ability to arrest cells in G_1 after DNA damage, p53 was ascribed a central role in promoting DNA repair before errors are being manifested upon DNA synthesis (33). However, p53 upregulation upon γ irradiation is delayed in cells from patients with ataxia telangiectasia, which are characterized by chromosomal instabilities, by misregulated V(D)J recombination, and by abnormally frequent spontaneous intrachromosomal recombination (29, 30, 32, 42, 45). This indicates that signals related to DNA recombination, such as DNA strand breaks, are required for the induction of the p53 response (47). Repair control exerted by wild-type p53 can partially be explained by transcriptional transactivation of certain genes containing p53 consensus-binding elements. Among the known target sequences, the gene coding for the Cdk inhibitor p21/WAF-1/CIP1/SDI1 (12, 20, 65) seems to be the primary candidate for executing the growth-inhibitory functions of p53. Furthermore, p21 binds proliferating cell nuclear antigen (PCNA), and thereby blocks PCNA-dependent simian virus 40 (SV40) DNA replication, but seems to allow PCNA-dependent repair DNA synthesis (37). Gadd45, which is induced by genotoxic stress via p53-regulated transcription, stimulates excision repair in vitro (55). Furthermore, p53 displays a physical interaction with ERCC3 (62), a helicase which participates in nucleotide excision repair and the initiation of transcription (11, 58).

Beyond the indirect involvement in repair processes through regulatory functions in the cell cycle, p53 was shown to be connected to recombination processes: expression of p53 mRNA in testes of mice is specific for the meiotic stage of pachytene during spermatogenesis, when chiasmatas are formed for the exchange of genetic material (52). In pre-B cells, wild-type p53 accumulation induces cell differentiation, which is manifested by immunoglobulin κ light-chain gene expression after successful V(D)J recombination (1). When immortalized fibroblasts from patients with Li-Fraumeni syndrome were tested for *CAD* gene amplification by *N*-(phosphonoacetyl)-L-aspartate (PALA) selection, a dramatic increase of spontaneous rates from $<10^{-7}$ to approximately 10^{-4} was discovered after loss of the wild-type p53 allele (41, 67). The same tendency was observed with fibroblasts from p53-knockout mice (41). Gene amplification is mediated by recombination events coupled to unequal sister chromatid exchanges and overreplications within the same cell cycle (53, 56). Strikingly, the C terminus of p53 displays several biochemical activities related to recombination (2, 6, 26, 36, 49): DNA reannealing, DNA strand transfer, and DNA damage recognition. Gene amplification can also be coupled to DNA replication, but conflicting data exist on direct effects of p53 on the latter process (7, 9, 14, 59). In former studies, inhibition of SV40 origin-dependent replication by full-length p53 (7, 14) and nuclear replication by C-terminally truncated p53 (9) were observed in vitro or upon overexpression, but no influence could be attributed to endogenous p53 in vivo (59).

SV40 lytic infection should provide a useful model system for studying homologous recombination in eukaryotic cells, as it can be used to physiologically introduce DNA substrates in their chromatin-bound form as well as to produce the recombination signal. The obvious advantage of applying SV40 virus is the ease in manipulating the recombination target, i.e., the small SV40 genome, in order to measure homologous recombination on DNA with defined indicator sites. Wild-type p53 protein was originally discovered in complex with the viral oncogene product SV40 large tumor antigen (T-Ag) (34, 39), which is known to increase the recombination frequencies of cellular and viral DNA by several orders of magnitude (25, 57). A recombination assay system based on SV40, therefore, offers the possibility to study a putative role of p53–T-Ag complexes with regard to this process. It is widely believed that T-Ag eliminates the growth-inhibitory functions of wild-type p53 via

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abrogation of its site-specific DNA binding and transcriptional activities through complex formation (3, 28). In analogy, T-Ag might inactivate putative functions of wild-type p53 in recombination. Loss of wild-type p53 has been described to facilitate genetic instabilities (8, 13, 21, 41, 64, 67), which might point to an inhibitory role with respect to the underlying mechanism. By use of SV40 derivatives which direct the expression of mutant T-Ag defective in complexing p53, our system allows us to investigate whether T-Ag neutralizes putative p53 functions inhibitory to recombination processes.

MATERIALS AND METHODS

Cells and virus infection. Kidney cell lines from African green monkey (*Cercopithecus aethiops*), CV-1, TC7, and COS1 (15, 51), and rhesus monkey (*Macaca mulatta*) kidney cells, from primary isolates (PRK) and the established cell line LLC-MK_2 (23, 59), were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum at 37°C. For infection, cells were seeded at a density of 2×10^5 cells per 20-cm^2 plate 24 h prior to inoculation with 0.5 ml of SV40 suspension for 2 h at 39°C. Nonadhering virus particles were removed, and cultivation continued at 39°C for different time periods postinfection. SV40 derivatives of strain 776 were used in all experiments.

Cloning procedures and virus generation. Genomic SV40 DNA was isolated by the method of Hirt (22), *Kpn*I linearized, and cloned into the *Kpn*I site of Bluescript M13+ (Stratagene), using competent *Escherichia coli* XI1Blue (Stratagene). Additionally, SV40 DNA was *Kpn*I inserted into a modified version of pUC19 (New England Biolabs) whose polylinker had been deleted around the *Bam*HI site via *Sma*I- and *Pst*I restriction digestions, subsequent T4 DNA polymerase treatment, and religation by T4 DNA ligase. For oligonucleotide-directed mutagenesis, single-stranded DNA of the Bluescript construct pBS-SV40 was produced upon infection with the R408 helper phage (Stratagene) according to the manufacturer's protocol. Mutations were inserted with a mutagenesis kit from Amersham Buchler, using the oligonucleotides 5′-GCAGATGAACACT GACTACAAGGCTGTTTTGGAT-3′ for creating the mutation C2084T and 5′-GCAGTGGAAGGGACTTTCTAGATATTTTAAAATTACC-3′ for creating the double mutation C2354T C2356T in the late region of SV40 (mutated bases are underlined). Both mutations alter codons in the amino acid sequence of VP1, the former resulting in the amino acid exchange H196Y and plasmid pBS-SV40-tsVP1(196Y) and the latter resulting in P286S and pBS-SV40-tsVP1(286S). For analytical purposes, the mutation C2354T was coupled to the silent base exchange C2356T, which creates a unique *Xba*I site on the SV40 genome and therefore allowed us to check for the absence of reverting mutations in reamplified viral genomes by simple restriction digestions. Each mutated region was verified by DNA sequencing using a T7 polymerase kit from Pharmacia and recloned into pUC-SV40 by exchange of either the 905-bp *Acc*I-*Bam*HI fragment in the case of pBS-SV40-tsVP1(196Y) or the 275-bp *Apa*I-*Bam*HI fragment in the case of pBS-SV40-tsVP1(286S) to exclude extra mutations originating from replication errors during the mutagenesis procedure. Mutation C3614G in the early region of SV40 directs the amino acid exchange D402H in T-Ag. It was introduced into the early regions of pUC-SV40, pUC-SV40-tsVP1(196Y), and pUC-SV40-tsVP1(286S) by replacing the 2,701-bp *Sfi*I-*Bam*HI inserts in each by the corresponding insert from pBS/SV40-402DH, kindly provided by Daniel T. Simmons, University of Delaware (38). The resulting constructs were called pUC-T-Ag(402H)SV40, pUC-T-Ag(402H)SV40-tsVP1(196Y), and T-Ag(402H)SV40-tsVP1(286S). If not mentioned otherwise, all DNA-modifying enzymes were purchased from Boehringer Mannheim or New England Biolabs.

For virus production, SV40 genomes were separated from the bacterial part within the pUC constructs by *Kpn*I digestion and subjected to calcium phosphate transfection as described by Graham and van der Eb (16). To allow the assembly of infectious virus particles, cells transfected with *ts*VP1 derivatives had to be cultivated at 32°C; all other transfectants were kept at 37°C. Wild-type SV40, SV40-tsVP1(196Y), and SV40-tsVP1(286S) were raised in TC7 cells; T-Ag (402H)SV40, T-Ag(402H)SV40-tsVP1(196Y), and T-Ag(402H)SV40-tsVP1(286S) were raised in COS1 cells. Virus stocks were harvested from the tissue culture supernatants of transfected cells by freezing and thawing.

Determination of total virus yields. To determine infectious units in virus suspensions, we infected monkey cells grown on coverslips with serial dilutions of the virus stocks under recombination assay conditions (39°C) and continued culturing for 48 h at 39°C. The infected cells were then analyzed for SV40 T-Ag expression by immunofluorescence to calculate the multiplicity of infection (MOI) as described earlier (59). Dilutions leading to less than 10% T-Ag-positive cells were recruited for calculations of the final MOI in the virus stocks, with 100% of infected cells defined as an MOI of 1.

Plaque assays. Confluent monolayers of CV-1 monkey cells were prepared by spreading 3×10^6 cells on 64-cm^2 dishes and subsequent cultivation for 24 h at 37°C. Titers of plaque-forming virus particles (PFU) were determined by infecting the cell monolayers with appropriate dilutions of the tissue culture supernatants harvested during recombination assays (300 μl per plate). After a 2-h incubation period under restrictive conditions (39°C), cells and virions were directly overlaid with 9 ml of 1.2% SeaPlaque agarose (FMC Bioproducts) in Dulbecco modified Eagle medium plus 2% fetal calf serum. The same overlay mixture was used for feeding the cells 6 days postinfection. Plates were incubated for 12 days at 39°C for testing of wild-type SV40 release. For T-Ag(402H)SV40 release, 13- to 14-day incubations were preferred to ensure optimal plaque sizes for scoring. Plaques were visualized by staining with 0.2% crystal violet in 2% ethanol–11% formaldehyde–87% water and counted to calculate the PFU per microliter of virus-containing inoculation fluid.

Extraction and immunoprecipitation of proteins. At the indicated times postinfection, around 2×10^5 cells per 20-cm^2 plate were harvested. Extractions and immunoprecipitations were performed as described earlier (59). Per lysate from one plate, 600 ng of affinity-purified antibodies was used for overnight incubations at 4°C. p53 was precipitated with the specific monoclonal antibody PAb421 (66); T-Ag was precipitated with PAb108 (18).

Western blot (immunoblot) analysis. Precipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and subsequent Western blotting (59). Proteins transferred to nitrocellulose were visualized by enhanced chemiluminescence (Amersham). Protein bands corresponding to T-Ag were detected by use of PAb108 (18) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad) as the secondary antibody. For the detection of protein bands corresponding to p53, biotinylated antibody PAb421 (66) and avidin-conjugated horseradish peroxidase (Bio-Rad) were applied. PAb421 was biotinylated by treatment of affinity-purified antibody preparations with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) according to the manufacturer's recommendations.

Quantitative evaluation of total amounts of SV40 DNA. Accumulation of viral DNA was quantitated by dot blot hybridization of Hirt DNA essentially as described previously (59). For hybridization, ^{32}P-labeled DNA fragments from positions 4739 to 294 on the SV40 genome were synthetized by PCR according to the protocol supplied with the *Taq* DNA polymerase (Gibco BRL). Primers used for the amplification reaction were 5′-AGGAGGTCGAGCGGCCGCTC TAGAAGCAGTAGCAATCAACCCACA-3′ and 5′-AGGAGGGGTACCGC GGCCGCTCTAGATTCTGAGGCGGAAAGAACCA-GC-3′.

Determination of recombination rates. To calculate the absolute recombination rates from plaque scorings (PFU per microliter of culture supernatant) after double infections, they had to be correlated to data from three parallel control infections with identical infectious units (Fig. 1): (i) infection with SV40-tsVP1 (196Y), (ii) infection with SV40-tsVP1(286S), (iii) double infection with SV40-tsVP1(196Y) and SV40-tsVP1(286S), and (iv) infection with wild-type SV40. Virus yields from single infections i and ii represented the background noise including spontaneous reversions of C2084T and C2354T from virus generation and amplification. Therefore, the mean of these values had to be subtracted from the virus yield obtained after double infection iii so as to give the mere recombination signal. Virus release after wild-type infection iv served as a reference which represented the total number of SV40 genomes within the cells compared with the few genomes having undergone successful recombination events within the locus considered. Consequently, the ratio between the recombination signal and the wild-type signal had to be determined in order to obtain the absolute rate of recombination events per locus and per virus. As a prerequisite for the reliability of rate calculations, a linear dependence between total virus input and output had to be guaranteed. For that purpose, we studied virus release by PRK and LLC-MK_2 cells in dose-response and time-response experiments. Cells were infected with dilutions of wild-type virus at defined MOIs ranging from 0.1 to 7.5, and virus yields were scored 48, 60, 84, 96, and 120 h postinfection (hpi). Finally, recombination rates calculated according to the formula explained above had to be corrected for the fraction of mutant SV40 genomes which had actually met mutant counterparts within the doubly infected cells. This was achieved by multiplying the recombination rates with the correction factor, which is approximated by the ratio between the infectious units corresponding to one of the two SV40-tsVP1 mutant viruses in double infections ($0.5 \times$ MOI) and the square of these infectious units.

RESULTS

Quantitative assay for homologous recombination. To study the phenomenon of homologous recombination between chromosomes in a quantitative manner, we have developed a cellular test system which exploits the physiological method of channeling DNA into cells by viral infection. In contrast to strategies which rely on the introduction of the recombination substrate by chemical transfection (4, 25), we consider infection to be superior for measurements of the exchange rates on foreign DNA, especially with respect to alterations in the p53 status. Not only is p53 accumulation observed after damage of the cellular DNA, but a sensitization of the cells for the p53 response can be noticed after transfection with merely single-

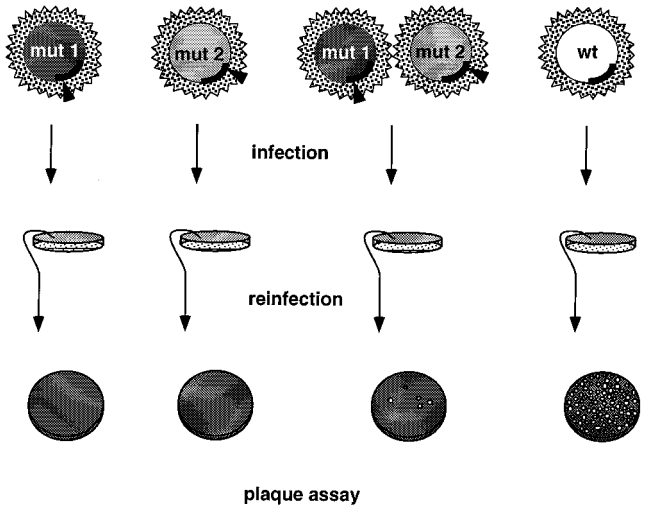

FIG. 1. Cellular test system for recombination based on SV40 infection and virus release. The recombination rate can be calculated from the virus yield in PFU by subtracting the mean value after single infections (mut 1 or mut 2) from the value obtained after double infection (mut 1 and mut 2) and dividing the difference by the virus release corresponding to the wild-type SV40 infection (wt).

or double-stranded DNA (50, 54). The overall principle of our recombination assay is outlined in Fig. 1. First, the cells of interest are simultaneously infected with mutant SV40 viruses containing two differently mutated genomes. Second, DNA exchange between the mutant viral chromosomes within the cells will result in the generation of wild-type viral genomes at a certain frequency and lead to the assembly and release of intact virus particles. Third, the number of virus particles containing wild-type SV40 genomes can be scored by performing plaque assays with the culture supernatants from the infected cultures. Several prerequisites have to be fulfilled to guarantee the success of this strategy. The nucleic acid sequences between the positions of the mutations must comprise at least 134 to 232 bp of uninterrupted homology, as mismatches have been shown to reduce the homologous recombination frequencies by 1 to several orders of magnitude (61). The indicator mutations must direct a phenotype which does not alter the replicative functions of the virus in order to permit recombination processes associated with or following DNA replication. Consequently, the production of intact virions from mutant genomes must be blocked at the stage of either virus assembly or release. On the other hand, conditions which allow the generation of virus particles carrying the same mutated SV40 genomes must exist. Finally, the defect displayed by one of the indicator viruses may not be compensated for in *trans* by functions of the other.

We chose to introduce indicator mutations into the late region of the SV40 genome for two reasons: first, they do not interfere with replication functions of the virus, and second, they allow further manipulations of the early region later on. Care was taken to verify the lack of mismatches in the intervening region by DNA sequencing after in vitro mutagenesis. The well-described mutations C2084T and C2354T were appropriate for our purpose, because they direct the amino acid exchanges H196Y and P286S in VP1, which confer thermosensitivity to this packaging protein at 39°C (48). The corresponding mutant viruses SV40-tsVP1(196Y) and SV40-tsVP1(286S) belong to the same complementation group, BC, and are blocked at 39°C at the same stage during viral assembly. The temperature-sensitive phenotype enabled us to produce infectious virus particles after calcium phosphate transfection of TC7 cells with the accordingly engineered SV40 genomes at the permissive temperature of 32°C. The virions collected from the culture supernatants could then be applied to recombination assays at the restrictive temperature of 39°C. The use of these viruses allowed the analysis of recombination rates as outlined in Fig. 1 in the different indicator cells (see below). For details of determining these rates experimentally, see Materials and Methods.

Recombination between SV40 chromosomes in primary monkey cells. SV40-specific homologous recombination was investigated by applying our test system to PRK cells, which we had demonstrated to support lytic infection of SV40 in earlier studies and which by definition express wild-type p53 (59). In time course experiments, we monitored virus release (measured as PFU per microliter of culture supernatant) in single and double infections with the respective SV40-tsVP1 mutant viruses and in wild-type SV40 infections. Reamplification of viruses with a period of 36 h provided a method for raising the sensitivity of the test system. The results of a representative experiment can be seen in Fig. 2A. None of the supernatants from control cultures after single infections contained plaque-forming virus particles beyond the limit of detection (between 10^{-7} and 10^{-6}). Consequently, plaque-forming virus detected in culture supernatants after double infections originated solely from successful recombination events in the VP1 locus. Virus particles signalling recombination could not be detected before 96 hpi and showed an increment of the signal at 120 hpi. Linear relationships between virus input and output were guaranteed for PRK cells under our assay conditions until 96 hpi (data not shown). This allowed us to calculate a specific recombination rate of 8×10^{-6} at 96 hpi in PRK cells (Table 1). To be absolutely sure that multicycle infection does not distort our recombination rates measured at 96 hpi, we applied high-titer virus stocks (MOI of ≥ 1) to identify the first generation of virions created by recombination. For the first generation released at 60 hpi, we obtained practically the same rate (10^{-5}) as for the second generation at 96 hpi. In comparison with the lower rates for spontaneous gene conversion in wild-type p53-expressing cells (ranging from 10^{-6} to $<10^{-9}$), these values indicate the stimulatory influence of SV40 with respect to this process (5, 57).

Recombination between SV40 genomes in the absence of wild-type p53–T-Ag complexes. To test our hypothesis that complex formation of T-Ag and p53 eliminates inhibitory functions of p53 in recombination, we introduced the mutation C3614G into the early region of wild-type SV40 and into both SV40-tsVP1 indicator genomes. The encoded amino acid exchange D402H in T-Ag (38) hampered complex formation between p53 and T-Ag. Mutating aspartate 402 in T-Ag was reported to interfere neither with the ability of SV40 to replicate in the established African green monkey kidney cell line BSC-1 nor with T-Ag-dependent stimulation of cellular DNA synthesis in quiescent CV-1P cells (10, 38). Therefore, T-Ag (402H) represents a suitable tool for dissecting putative cooperative effects between T-Ag and p53 without affecting unrelated viral or cellular functions. The results of recombination assays with PRK cells, using the newly generated indicator viruses T-Ag(402H)SV40, T-Ag(402H)SV40-tsVP1(196Y), and T-Ag(402H)SV40-tsVP1(286S), are shown in Fig. 2B. Experimental conditions, including infectious units (MOI of 0.5), were identical to those described above. Comparison of virus release upon T-Ag(402H)SV40 infection in Fig. 2B and upon wild-type virus infection in Fig. 2A confirms that D402H mutant viruses are fully able to propagate in the rhesus monkey

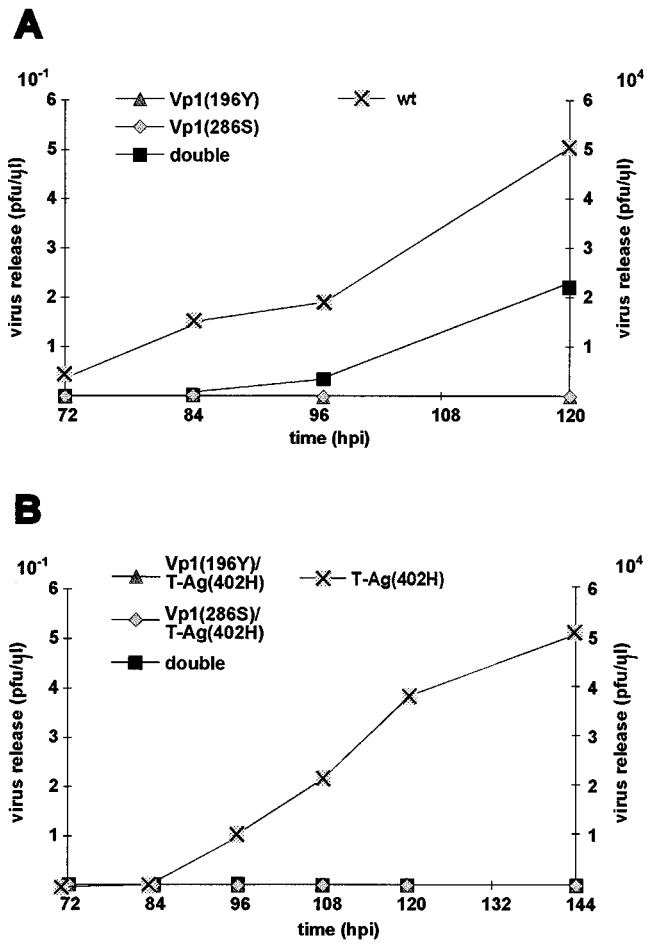

FIG. 2. Comparison of virus release by PRK cells upon infections with wild-type T-Ag or T-Ag(402H)SV40 derivatives according to the test system. Release of plaque-forming virus particles by PRK cells at 39°C between 72 and 120 h hpi was measured by performing plaque assays with the corresponding culture supernatants at the same temperature. Each point represents the mean value from two independently treated culture dishes. (A) Infections with wild-type T-Ag indicator viruses. Virus yields on the left *y* axis relate to infections with SV40-tsVP1(196Y) [Vp1(196Y)], SV40-tsVP1(286S) [Vp1(286S)], and both (double). Values on the right *y* axis refer to virus infections with wild-type SV40 (wt). (B) Infections with T-Ag(402H) indicator viruses. Virus yields on the left *y* axis relate to infections with the indicator viruses T-Ag(402H)SV40-tsVP1(196Y) [Vp1(196Y)/T-Ag(402H)], T-Ag(402H)SV40-tsVP1(286S) [Vp1(286S)/T-Ag(402H)], and both (double). Values on the right *y* axis refer to virus infections with T-Ag(402H)SV40 [T-Ag(402H)].

cell system (38). However, we were unable to detect any virus release from PRK cells until 144 h after double infection with T-Ag(402H)-expressing VP1 mutant viruses. From this, we estimated a recombination rate clearly below 10^{-6} (Table 1). In comparison with the PRK-specific recombination rate calculated for VP1 mutant viruses expressing wild-type T-Ag (8×10^{-6} to 10×10^{-6}), it becomes evident that the amino acid exchange 402H in T-Ag causes a reduction of the DNA exchange rate by at least 1 order of magnitude. This result strongly supports the idea of an inhibitory role of wild-type p53 in recombination processes and indicates that efficient complexing by T-Ag neutralizes the respective function of p53.

Recombination with respect to the T-Ag status in cells carrying mutant p53. The effect of neutralizing wild-type p53 functions in recombination was analyzed further by making use of a cellular system which can be distinguished from PRK cells and other monkey cell lines with respect to the endogenous p53 status (59). The p53 protein from LLC-MK_2 cells shows all of the biochemical characteristics of mutant p53 (prolonged half-life, PAb240 antigenicity, loss of T-Ag complex formation) and is discernible at the primary sequence level by a deletion of three amino acids (positions 237 to 239) which are located within the T-Ag binding region (27, 43). Mutant p53 in these cells was expected not to inhibit recombination, similar to

TABLE 1. Recombination rates upon viral infection

Time (hpi)[a]	Recombination rate in infected cells[b]	
	PRK	LLC-MK_2
84		
Wild-type T-Ag	$\leq 10^{-6}$	4×10^{-4}
T-Ag(402H)	$<10^{-6}$	4×10^{-4}
96		
Wild-type T-Ag	8×10^{-6}	10^{-4}
T-Ag(402H)	$<10^{-6}$	2×10^{-4}

[a] Hours postinfection until the quantitation of interchromosomal recombination by virus release. SV40 used for infections carried indicator mutations in the VP1 gene as described in the text. The mutation T-Ag(402H) additionally introduced into the early region was examined.

[b] Rhesus kidney cells tested for recombination between SV40 chromosomes differ with respect to their endogenous p53 status. Primary cells (PRK) carry wild-type p53; LLC-MK_2 cells carry mutant p53.

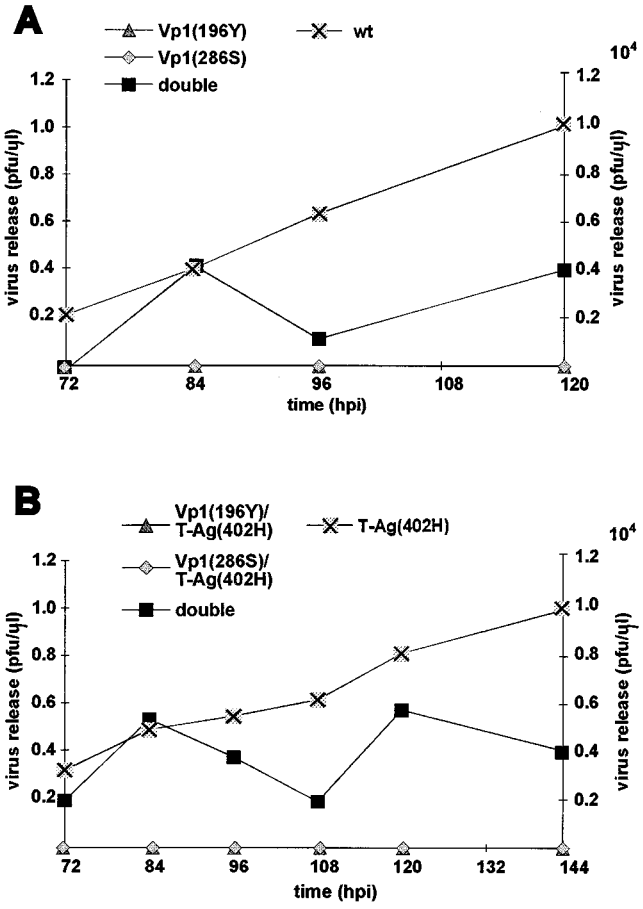

FIG. 3. Comparison of virus release by LLC-MK_2 cells upon infections with wild-type T-Ag or T-Ag(402H)SV40 derivatives according to the test system. (A) Infections with wild-type (wt) T-Ag indicator viruses. (B) Infections with T-Ag(402H) indicator viruses. Release of plaque-forming virus particles by LLC-MK_2 cells at 39°C was determined and plotted as described for Fig. 2.

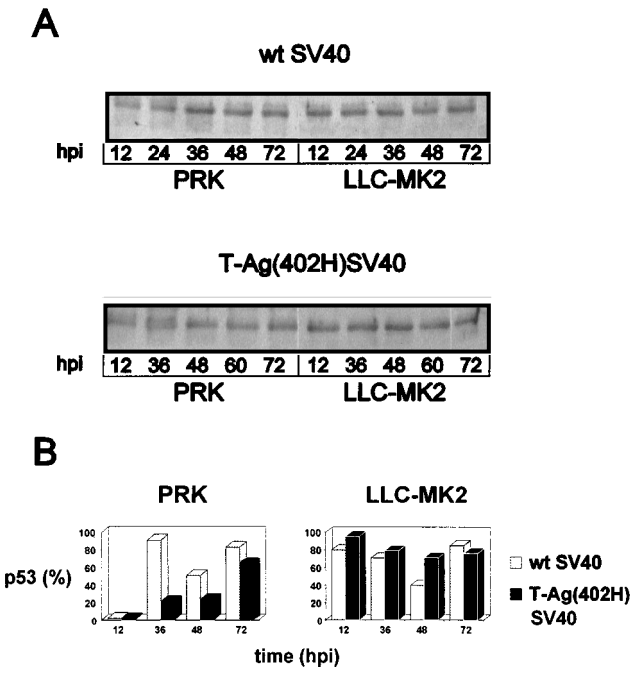

FIG. 4. Time course of p53 protein expression in PRK and LLC-MK_2 cells after infection with wild-type SV40 or T-Ag(402H)SV40. Steady-state levels of p53 were determined at different times after infection of 10^5 PRK and LLC-MK_2 cells with wild-type (wt) wt SV40 or T-Ag(402H)SV40. PAb421 (66) was used for immunoprecipitation from cellular extracts, and biotinylated PAb421 was used for subsequent chemiluminescence detection of precipitated p53 in Western blots (A). p53-specific signals were quantitatively evaluated by densitometry and correlated to the band intensities of equal amounts of purified baculoviral p53, and relative amounts were plotted diagrammatically (B).

wild-type p53 complexed to T-Ag. Recombination assays in LLC-MK_2 cells with indicator viruses coding for either wild-type T-Ag or T-Ag(402H) were performed under the same conditions as in PRK cells (MOI of 0.5). Double infections of LLC-MK_2 cells with both VP1 mutant virus types caused virus release in peaks which reappeared with a period of 36 h as a result of reinfection of cells on the same culture dish (Fig. 3). The peaks at 84 and 120 hpi represent the second and third generations of virions created by DNA exchange mechanisms in LLC-MK_2 cells. Genetic exchange between the genomes of SV40-tsVP1(196Y) and SV40-tsVP1(286S) occurred with a maximal rate of 4×10^{-4} at 84 hpi (Fig. 3A). Again, the recombination rate determined for the first generation at 48 hpi (3×10^{-4}) obtained from recombination assays with high-titer virus suspensions deviated very little. Assaying LLC-MK_2 cells with the corresponding viral T-Ag(402H) counterparts resulted in the same maximum at 84 hpi (Fig. 3B) and an identical recombination rate (summarized in Table 1). In comparison with rhesus monkey cells endogenously expressing wild-type p53, recombination of SV40 genomes in LLC-MK_2 cells was resistant to modulation of recombination activities by exchange of wild-type T-Ag for T-Ag(402H). This is consistent with the idea that inhibitory activities of wild-type p53 can be eliminated either by mutation or by complex formation with T-Ag.

Relationships between homologous recombination, p53 and T-Ag expression, and DNA replication. Alternative explanations for the observed differences in recombination frequencies would be provided by differences in protein expression of p53 and T-Ag, or in viral DNA synthesis, during the course of infections. Therefore, these parameters were analyzed after infection with either wild-type or T-Ag(402H) SV40 in PRK and LLC-MK_2 cells.

p53 protein expression. Steady-state levels of p53 during the course of infection were determined by Western blot analysis, applying identical conditions for infection as during the recombination assays (Fig. 4). Mutant p53 has a prolonged half-life by definition, whereas wild-type p53 is metabolically stabilized by T-Ag interactions (34). Accordingly, p53 levels were approximately the same after infection of LLC-MK_2 cells with either virus type. PRK cells infected with wild-type SV40 accumulated approximately three times more p53 than PRK cells infected with T-Ag(402H)SV40. This finding demonstrates that wild-type p53 is significantly stabilized even without permanently binding to T-Ag in T-Ag(402H)SV40-infected cells.

T-Ag protein expression. When we compared wild-type T-Ag with T-Ag(402H) accumulation in Western blots, negligible deviations were observed throughout the 96-h assay period (Fig. 5). Between PRK and LLC-MK_2 cells, we saw minor differences in T-Ag expression, with on average 1.5-fold-higher T-Ag amounts produced in LLC-MK_2 cells. In summary, protein expression patterns for p53 and T-Ag displayed maximally threefold differences after virus infections with either wild-type or T-Ag(402H)SV40 in either wild-type or mutant p53 cells. Thus, the drastic changes observed with respect to DNA exchange activities do not simply reflect altered protein expression of p53 or T-Ag.

Viral DNA synthesis. Because there is ample evidence for a functional interrelationship between replication and recombination, information on viral DNA synthesis is important for the interpretation of the recombination data obtained. In agreement with a previous report (38), we found that T-Ag(402H)SV40 replicates as effectively as wild-type SV40 in wild-type p53-expressing PRK cells, as demonstrated by dot blot analysis of SV40 DNA accumulation (Fig. 6). In LLC-MK_2 cells, differences between the levels of DNA accumula-

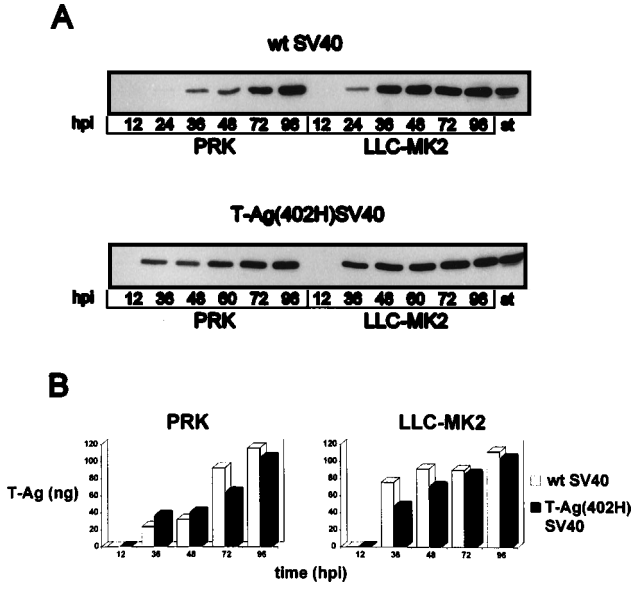

FIG. 5. Time course of T-Ag protein expression in PRK and LLC-MK_2 cells after infection with wild-type (wt) SV40 or T-Ag(402H)SV40. Steady-state levels of T-Ag were determined and evaluated as described for p53 in the legend to Fig. 4. PAb108 (18) was used both for immunoprecipitation and for chemiluminescence detection (A). T-Ag-specific signals were correlated to the band intensities of known amounts of purified baculoviral T-Ag (st), and the absolute amounts are presented in panel B.

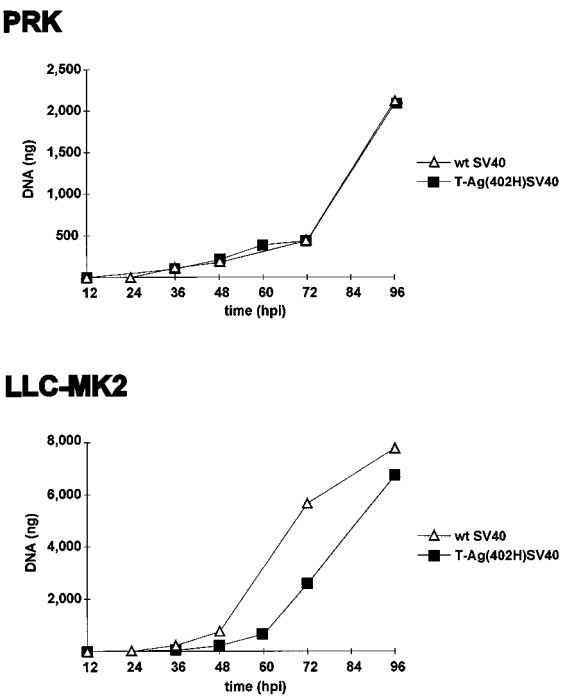

FIG. 6. Comparison of SV40 DNA accumulation in PRK and LLC-MK_2 cells after infection with wild-type SV40 or T-Ag(402H)SV40. PRK and LLC-MK_2 cells (2×10^5 of each) were infected with wild-type (wt) SV40 or T-Ag (402H)SV40, and viral DNA was isolated by the Hirt procedure (22) at various times postinfection. Total amounts of viral DNA were evaluated by dot blot hybridization with a ^{32}P-labeled SV40 DNA fragment (positions 4739 to 294 on the SV40 genome).

tion of wild-type SV40 and T-Ag(402H) SV40 were observed, although recombination rates were the same. Wild-type SV40 DNA levels in cells expressing wild-type p53 (PRK) were on average threefold lower than in cells expressing mutant p53 (LLC-MK_2). [^{3}H]thymidine incorporation was measured in parallel for each virus and cell type combination. The resulting de novo DNA synthesis rates precisely reflected the pattern of DNA accumulation (data not shown).

DISCUSSION

To analyze the involvement of p53 in recombination events, we established a cellular test system for homologous recombination which minimizes distortions of the in vivo situation but still allows manipulations of key molecules and rapid quantitation. To that end, we avoided genetic selection procedures which are routinely used for quantitative studies on rearrangements of endogenous chromosomal DNA as well as chemical or physical methods for the introduction of exogenous substrate DNA. The physiological method of SV40 virus infection represented an appropriate tool for this purpose: virus particles are taken up by the natural process of endocytosis, and viral DNA enters and leaves the cell in a chromatin-bound form. Amplification of the signal is achieved by viral DNA replication and reinfection. The comparison of recombination frequencies between SV40 DNA derivatives after transfection (25, 60) with those obtained by our assay after viral infection revealed that DNA exchange on naked SV40 genomes is apparently 10^2- to 10^4-fold more frequent than on whole SV40 chromosomes. The dependence of recombination rates on the transfection method used caused Jasin and coworkers (25) to postulate that reciprocal exchange of DNA takes place prior to packaging of the introduced DNA into nucleosomes. It is conceivable that the presence of naked DNA on its own triggers repair mechanisms. In support of this view, transfection was shown to sensitize cells for p53 upregulation (50, 54); thus, subtle changes in p53-regulated recombination activities might not become obvious. In conclusion, chromatin components are important targets for recombination control and are accounted for by our assay system. With this SV40-based method, recombination frequencies in nonmanipulated primary cells were in the order of 10^{-5}. This is clearly below the recombination rates published for SV40 T-Ag or polyomavirus T-Ag by others (25, 35, 57). The differences can partially be explained by distinct experimental conditions as discussed above. In addition, they reflect the confinement to selectively detect double-crossover and gene conversion events in our system. Nevertheless, the recombination frequencies remain at least 10-fold higher than the spontaneous gene conversion rates (5, 57) and thus again confirm the recombinogenic character of SV40, though in a quantitatively different manner.

Our data allow the interpretation that the stimulatory effect of T-Ag on recombination (25, 57) can be explained by alleviating the inhibitory effect of p53 via complex formation. This conclusion is based on our finding that recombination rates are lowered by at least 1 order of magnitude when T-Ag(402H), impaired in its ability to bind p53, is expressed by SV40 instead of wild-type T-Ag in PRK cells (which express wild-type p53). No effect on the recombination capacities of LLC-MK_2 cells can be seen in response to T-Ag(402H), as wild-type p53 activities in these cells are already inactivated by mutation and cannot further be modulated by T-Ag binding. Similarly, stable p53–T-Ag complex formation is sufficient to convert REF52 cells to the permissive state for gene amplification, as was demonstrated by experiments using the temperature-sensitive mutant T-Ag(tsA58) (24). These findings define a new function for p53–T-Ag complexes in promoting genetic instability, whereas permanent binding does not seem to be required for stabilization of p53 or the elimination of its growth-inhibitory functions (44). Further support for our interpretation that wild-type p53 is a negative regulator of recombination comes from a recent report on the human lymphoblast cell line WTK1, which endogenously expresses mutant p53 and shows an approximately sevenfold-higher capacity for interplasmid recombination in transfection assays compared with wild-type p53 cells from the same donor (63, 64). p53 in these cells carries a homozygous mutation leading to the amino acid exchange M2371, whose counterpart in p53 from LLC-MK_2 cells is deleted (31, 43). Although these data nicely complement our recombination rate measurements, further experiments are needed to solve the question of whether the 30- to 50-fold-higher recombination rates in LLC-MK_2 cells than in PRK cells can be interpreted as representing stimulatory functions of mutant p53 in recombination.

It is interesting that the increase of interchromosomal recombination rates per locus and virus corresponds to the rise of gene amplification rates per locus and clone after loss of wild-type p53 function (24, 41, 67). At least with respect to SV40 DNA replication, we could not relate any influence on the process of DNA replication to loss of wild-type p53 function. This is not consistent with a role of p53 in overreplication as one possible initiator of gene amplification (53). The failure to arrest replication in the presence of PALA, possibly leading to chromosome breakage at replication bubbles, was suggested to account for stimulated gene amplification in cells lacking wild-type p53 (67). On the basis of our data, we favor the idea that the primary events of gene amplification which are affected by p53 begin with homologous recombination. This in-

terpretation is compatible with bridge breakage-fusion cycles underlying gene amplification in REF52 cells expressing SV40 T-Ag (24). These cycles are thought to be initiated by recombination between the highly conserved sequences in centromeres or near telomeres (56). Interestingly, the gene product mutated in patients with ataxia telangiectasia shows significant homology to a protein with functions in the maintenance of telomere integrity (17, 19, 46).

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