HAL
archives-ouvertes.fr

Mutant p53 proteins stimulate spontaneous and radiation-induced intrachromosomal homologous recombination independently of the alteration of the transactivation activity and of the G1 checkpoint

Yannick Saintigny, Danielle Rouillard, Brigitte Chaput, Thierry Soussi, Bernard Lopez

▶ To cite this version:

Yannick Saintigny, Danielle Rouillard, Brigitte Chaput, Thierry Soussi, Bernard Lopez. Mutant p53 proteins stimulate spontaneous and radiation-induced intrachromosomal homologous recombination independently of the alteration of the transactivation activity and of the G1 checkpoint. Oncogene, Nature Publishing Group, 1999, 18, pp.3553-3663. cea-01938157

HAL Id: cea-01938157
https://hal-cea.archives-ouvertes.fr/cea-01938157

Submitted on 28 Nov 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Oncogene (1999) 18, 3553–3563
© 1999 Stockton Press All rights reserved 0950–9232/99 $12.00
http://www.stockton-press.co.uk/onc

Mutant p53 proteins stimulate spontaneous and radiation-induced intrachromosomal homologous recombination independently of the alteration of the transactivation activity and of the G1 checkpoint

Yannick Saintigny[1], Danielle Rouillard[2], Brigitte Chaput[1], Thierry Soussi[2] and Bernard S Lopez*[,1]

[1]*Unité Mixte de Recherches CEA-CNRS 217, CEA, DSV, DRR, 60-68 av. du Général Leclerc, 92 265, Fontenay aux Roses, Cédex, France;* [2]*Institut Curie, 26 rue d'Ulm, 75 231, Paris Cédex 05, France*

We report here a systematic analysis of the effects of different p53 mutations on both spontaneous and radiation-stimulated homologous recombination in mouse L cells. In order to monitor different recombination pathways, we used both direct and inverted repeat recombination substrates. In each line bearing one of these substrates, we expressed p53 proteins mutated at positions: 175, 248 or 273. p53 mutations leading to an increased spontaneous recombination rate also stimulate radiation-induced recombination. The effect on recombination may be partially related to the conformation of the p53 protein. Moreover, p53 mutations act on recombination between direct repeats as well as between inverted repeats indicating that strand invasion mechanisms are stimulated. Although all of the p53 mutations affect the p53 transactivation activity measured on the WAF1 and MDM2 gene promoters, no correlation between the transactivation activity and the extent of homologous recombination can be drawn. Finally, some p53 mutations do not affect the G1 arrest after radiation but stimulate radiation-induced recombination. These results show that the role of p53 on transactivation and G1 cell cycle checkpoint is separable from its involvement in homologous recombination. A direct participation of p53 in the recombination mechanism itself is discussed.

Keywords: p53; radiation; cell cycle; homologous recombination

Introduction

DNA repair and recombination, associated with cell cycle control, participate in a network of pathways managing genome integrity. Homologous recombination is an essential mechanism involved in fundamental processes such as DNA repair (Friedberg *et al*., 1995), molecular evolution (Liebhaber *et al*., 1981; Arnheim, 1983), gene diversification (Baltimore, 1981; Reynaud *et al*., 1987; Becker and Knight, 1990) and chromosome segregation during meiosis (Roeder, 1990; Kleckner, 1996). Contrasting with its role in genome maintenance, homologous recombination between homologous sequences dispersed through the genome, may lead to profound rearrangements such as: inversions, deletions, duplications (Bollag *et al*., 1989). Additionally, gene conversion leads to loss of heterozygosity when acting between two heteroalleles (Cavenee *et al*., 1983; Xia *et al*., 1994) or to gene inactivation when acting between a gene and a related pseudogene (Amor *et al*., 1988).

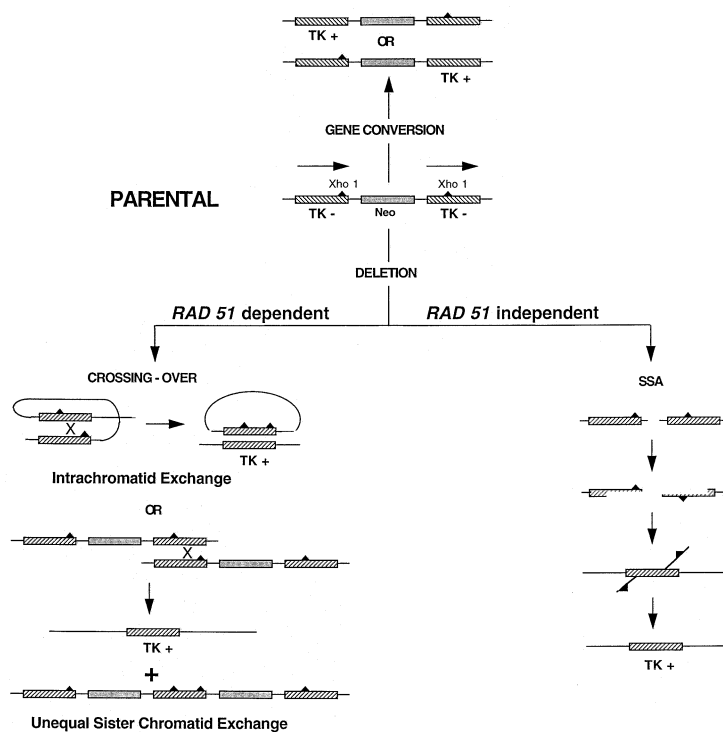
Cell cycle control has been shown to be essential to maintain genome integrity. For example, alteration of the G1 checkpoint would allow replication to take place on DNA templates bearing spontaneous or induced lesions. These lesions can block the progression of replication forks leading to the formation of single- and double-stranded breaks which are highly recombinogenic structures (Hartwell, 1992). The gene *p53*, which is the most frequently mutated gene in tumors, plays a pivotal role in the control of the G1 checkpoint (for reviews see Donehover and Bradley, 1993; Smith and Fornace, 1995; Hainaut, 1995; Ko and Prives, 1996; Tyler and Weinberg, 1996). Analysis of *p53* mutant cells show that (i) they have lost their capacity to inhibit cell growth after exposure to DNA damaging agents, (ii) they exhibit a high level of spontaneous chromosomal abnormalities (Bouffler *et al*., 1995), a higher likelihood of gene amplification (Livingstone *et al*., 1992; Yin *et al*., 1992) and higher levels of spontaneous recombination between SV40 genomes (Wiesmuller *et al*., 1996) or between intrachromosomal repeat sequences (Bertrand *et al*., 1997; Mekeel *et al*., 1997). Besides this indirect potential role on homologous recombination via cell cycle control, a direct participation of p53 protein in the recombination mechanism itself could be possible. Indeed, a physical interaction has been reported between p53 protein and Rad51 protein, a putative recombination protein in mammalian cells (Sturzbecher *et al*., 1996; Buchhop *et al*., 1997; Marmorstein *et al*., 1998). In addition, a role on the correction of heteroduplexes created during the recombination process, has been proposed for p53 protein (Dudenhoffer *et al*., 1998). Ionizing radiation produces DNA double strand breaks which are highly recombinogenic structures and strongly stimulates recombination in bacteria and yeast. In mammalian cells the situation appears to be more complex. For instance, according to different reports, opposite conclusions can be drawn, concerning the stimulation of recombination by ionizing radiation (Benjamin and Little, 1992; Park, 1995; Wang *et al*., 1988). It can be suggested that the stimulation of homologous recombination by γ-rays may depend on the type of substrates or on the genetic background of the cells. Since p53 protein plays an essential role in the cellular response to ionizing

*Correspondence: BS Lopez
Received 10 March 1999; revised 15 April 1999; accepted 29 April 1999

radiation, the question of the effect of the *p53* status on γ-rays induced homologous recombination is of particular interest.

In order to precisely characterize the relationships between homologous recombination, G1 checkpoint, and *p53* status, we performed here a systematic analysis of the effect of the expression of a set of different mutant p53 proteins on spontaneous recombination and also on γ-ray-induced recombination. Mutant p53 proteins have been shown to affect the activity of the wild type p53 protein via an inter-species co-dominant negative process (Milner and Medcalf, 1991). In the present work we took advantage of this strategy to study the effect of different p53 mutations on spontaneous or radiation-induced recombination between direct or inverted repeat sequences. We focused here on the three most frequently mutated positions of p53 protein found in human tumors: positions 175, 248 and 273. We expressed the p53 mutant proteins in cell lines with two different kinds of recombination substrates, in order to measure different pathways for intrachromosomal homologous recombination. We used here two parental lines both deriving from mouse L-cells; one line contains the recombination markers in direct repeat orientation (line pJS3–10) and the other line contains the recombination markers in inverted repeat orientation (line pJS4–7–1) (Liskay *et al*., 1984). Recombination between direct repeats results in two types of products: (i) gene conversion keeping intact the structure of the locus and arising via a strand invasion (SI) mechanism and (ii) deletion events (Figure 1). The latter events can result from different processes such as crossing over arising via SI, single strand annealing (SSA) and replication slippage (for review see Klein, 1995). In contrast, homologous recombination between inverted repeat sequences can only arise via SI. In yeast, the SI mechanism is dependent on the *RAD52* episthasis group including *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57* genes, whereas SSA does not require *RAD51*, *RAD54*, *RAD55*, *RAD57* genes. Beside Rad52, SSA is dependent on the nucleotide excision repair proteins

A / Direct Repeat Sequences



B / Inverted Repeat Sequences

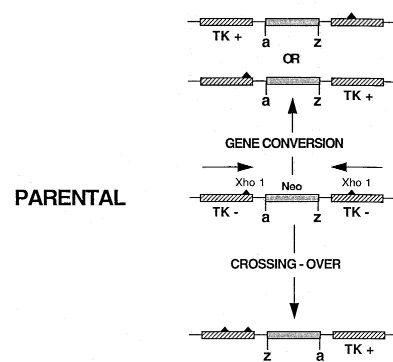


Figure 1 Organization of the recombination substrates to measure intrachromosomal recombination between tandem repeat sequences. The parental lines are mouse L*tk*$^{-}$ (sensitive to HAT medium) containing a unique copy of a chromosomal duplication of two Herpes Simplex type I (HSVI) *TK* genes (dashed boxes). Each *TK* sequence is inactivated by a linker insertion (▲). (**a**) Direct repeat orientation, (**b**) Inverted repeat orientation (the letters a and z show the orientation of the intervening sequence). The non-reciprocal transfer of genetic information (gene conversion) can restore one functional *TK* gene conferring the resistance to HAT medium. This mechanism can act on direct repeat (**a**) as well as on inverted repeat (**b**) sequences, is a conservative mechanism and requires a strand invasion process; it is thus *RAD51*-dependent in yeast (Klein, 1995; Lambert *et al*., 1999). (**a**) Alternatively, recombination can lead to deletion events between direct repeat sequences via several different mechanisms: (1) Crossing over between mispaired sister chromatids or intrachromatid crossing over, result from a strand invasion process (*RAD51*-dependent); (2) Single Strand Annealing (SSA). After the production of a double strand break, an exonucleolytic degradation creates two complementary single-stranded tails that can be annealed. This process does not require strand invasion and is consequently *RAD51*-independent. Resolution of the protruding non-paired single-stranded tails requires Rad1, Rad10, Msh2 and Msh3 proteins in yeast (Ivanov *et al*., 1996; Sugawara *et al*., 1997). Other mechanisms (not drawn here) such as replication slippage can also lead to deletion between direct repeats. These processes are also *RAD51*-independent (for review see Klein, 1995). (**b**) Crossing over between inverted repeats would lead to the inversion of the intervening sequence. Replication slippage cannot be measured with inverted repeats. SSA cannot work with inverted repeats because the exonucleolytic degradation would produce identical but non-complementary single-stranded DNA that are unable to anneal (Lin *et al*., 1987). The inverted repeat substrate mostly monitors recombination arising by strand invasion (*RAD51*-dependent) mechanism

Rad1 and Rad10, and on the mismatch repair proteins Msh2 and Msh3 (Ivanov *et al*., 1996; Sugawara *et al*., 1997). Homologues to all of these genes have been described in mammalian cells.

In the present work, we show that p53 mutations stimulate homologous recombination with both kinds of recombination substrates, thus that strand invasion mechanisms are stimulated. In addition, we show that the effect of p53 mutations on homologous recombination is independent of their effect on the G1 checkpoint, showing that these functions of p53 can be separable.

Results

Expression of different p53 mutations in the recipient lines

We used two mouse L-cell lines (pJS3–10 or pJS4–7–1) containing a unique intrachromosomal copy of a HSV-*TK* tandem repeat sequence. In the pJS3–10 line, the repeats are in direct orientation whereas in the pJS4–7–1 the repeats are in inverted orientation (Liskay *et al*., 1984). Thus recombination in pJS3–10 can arise by SI, SSA, replication slippage or other mechanisms (Figure 1). In pJS4–7–1, recombination arises only by SI (for review see Klein, 1995).

Mouse L-cells are wild type for *p53*. Overexpression of a mutant p53 protein has been shown to override the endogenous wild type p53 protein in a dominant negative manner by forming complexes with the wild type protein and functionally inactivating it. We previously used this strategy to show that the expression of the His175 mutant p53 protein in pJS3–10 line, resulted in a *p53* mutant phenotype with regard to the G1 block after irradiation and led to an increase of spontaneous recombination between direct repeat sequences (Bertrand *et al*., 1997). In the present work we expressed different p53 mutations in pJS3–10 as well as in pJS4–7–1 cell lines. The expression of the exogenous mutant as well as of the endogenous wild type p53 proteins was checked by Western blot using the monoclonal antibodies DO7 or HR231 (Figure 2). For each clone and whatever the mutation, the exogenous mutant p53 protein appears to be less expressed than the endogenous wild type p53 protein (Figure 2b). However, this interpretation postulates that the HR231 antibody presents an affinity for the wild type mouse p53 protein similar to that for the human mutant. In addition, the exogenous mutant p53 protein does not significantly affect the expression of the endogenous p53 protein. Finally, radiation leads to stabilization of both the

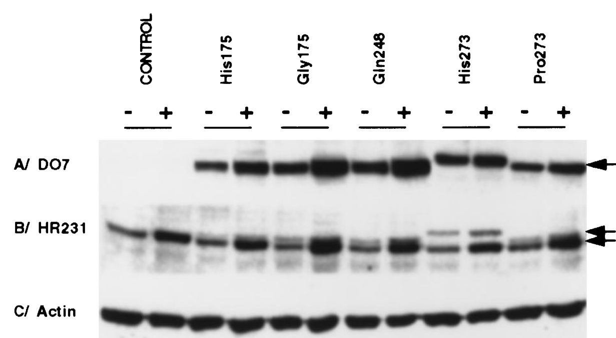


Figure 2 Expression of the exogenous p53 proteins analysed by Western blot. Extracts of non-irradiated cells (−) and irradiated cells (+) were prepared 4 h after exposure to 6 Grays. (**a**) with the DO7 anti-p53 antibody, that recognized only the human exogenous mutant p53 protein. (**b**) with the HR231 anti-p53 that recognizes both the exogenous (upper band) and the endogenous (lower band) p53 protein (indicated by the arrows). (**c**) Normalization using anti-actin antibody. The different mutant p53 proteins expressed are indicated on the top of each lane

Table 1 Cell line used

Structure of recombination substrates					
	Direct repeats			*Inverted repeats*	
Cell lines name	*Expression of an exogenous mutant p53 protein*[a]	*p53 mutation*[b]	*Cell lines name*	*Expression of an exogenous mutant p53 protein*[a]	*p53 mutation*[b]
pJS 3–10	–	None	pJS 4–7–1	–	None
CDR 1	–	None			
H175 DR 102	+	175 (Arg→His)	H175 ILR 8	+	175 (Arg→His)
H175 DR 211	+	175 (Arg→His)			
G175 DR 2	+	175 (Arg→Gly)	G175 ILR 3	+	175 (Arg→Gly)
			G175 ILR 5	+	175 (Arg→Gly)
Q248 DR 1	+	248 (Arg→Gln)	Q248 ILR 5	+	248 (Arg→Gln)
Q248 DR 3	+	248 (Arg→Gln)	Q248 ILR 6	+	248 (Arg→Gln)
Q248 DR 4	+	248 (Arg→Gln)	Q248 ILR 10	+	248 (Arg→Gln)
H273 DR 11	+	273 (Arg→His)	H273 ILR 4	+	273 (Arg→His)
H273 DR 17	+	273 (Arg→His)	H273 ILR 16	+	273 (Arg→His)
H273 DR 19	+	273 (Arg→His)			
P273 DR 8	+	273 (Arg→Pro)	P273 ILR 1	+	273 (Arg→Pro)
P273 DR 9	+	273 (Arg→Pro)	P273 ILR 6	+	273 (Arg→Pro)
			P273 ILR 8	+	273 (Arg→Pro)
			P273 ILR 11	+	273 (Arg→Pro)

[a]The parental lines are pJS 3–10 and pJS 4–7–1 (mouse L cells), they possess an endogenous wild type p53 protein. No exogenous p53 was expressed in these lines. CDR 1 is a pJS 3–10 line transfected with the empty expression vector. [b]All the lines express an endogenous wild type p53 protein. pJS 3–10, pJS 4–7–1 and CDR 1 do not express an exogenous p53 protein. The numbers indicate the position of the mutation on the exogenous p53 protein and the amino acid substitution is indicated

exogenous mutant and the endogenous wild type p53 proteins (Figure 2). Several clones were used for each mutation and with each orientation of the recombination markers. The cell lines used in the present study are listed in Table 1.

The consequences of the expression of the mutant p53 proteins was evaluated according to two criteria: the p53 transactivation activity and the G1 arrest after radiation. Transactivation was measured on the promoter of two genes controlled by p53: WAF1 and MDM2 (Figure 3). All the lines expressing the mutant p53 proteins exhibit a decrease in the transactivation capacities of both WAF1 and MDM2 gene promoters. Lines with Gln248 and Pro273 show the strongest decrease (Figure 3). This result shows that all the mutant proteins used here are able to titrate the endogenous wild type p53 protein, at least for the transactivation activity.

The effect on the G1 arrest was estimated by the percentage of cells in S phase after exposure to γ-rays. The control line pJS3–10 shows an 85% drop of the S phase-cells percentage, 24 h after irradiation at 6 Grays (Figure 4). In the line expressing the His175 mutant p53 protein, the fall is only of 20%. Thus, the expression of His175 impairs an efficient G1 arrest as already described (Bertrand *et al.*, 1997). However, none of the other p53 mutations significantly affect the G1 block after radiation (Figure 4).

Rates of spontaneous homologous recombination

Spontaneous rate of homologous recombination was determined by fluctuation analysis. The rate of recombination/cell/generation was calculated according to two classical methods: Luria and Delbruck (1943); Capizzi and Jameson (1973); Lea and Coulson (1948). Interestingly, p53 mutations act similarly on both tandem repeat systems. Mutations stimulating recombination between direct repeats also stimulate

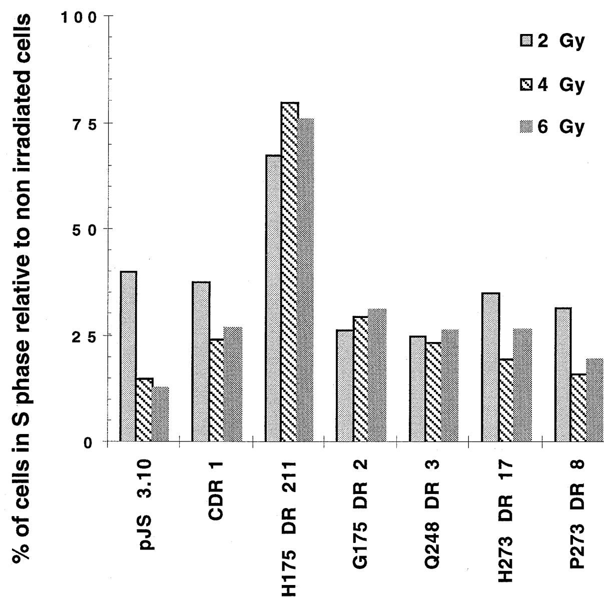
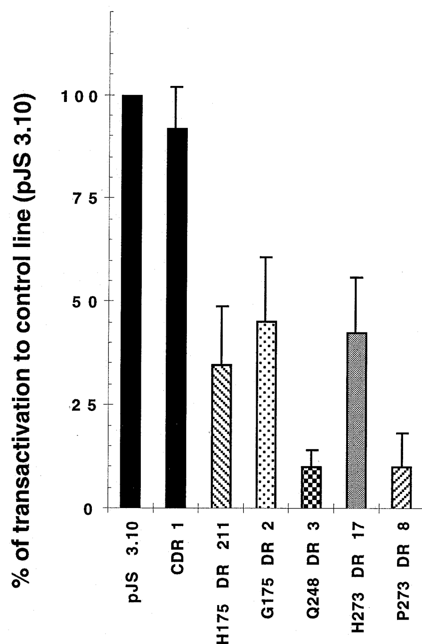


Figure 4 G1 arrest after radiation was estimated by the diminution of the frequency of cells in S phase after treatment with γ-rays. In a non-irradiated and non-synchronous population, cells in S phase represent 30–50% of the total cell population. In the figure, the frequency of cells in S phase after irradiation is normalized to that of the non-irradiated population. DNA content was estimated by flow cytometry 24 h after irradiation at the doses indicated. The name of the lines is indicated on the figure



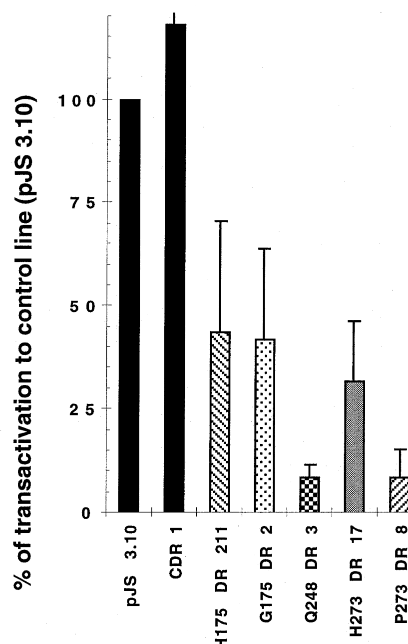


Figure 3 Effect of the expression of the different mutant p53 proteins on the p53-dependent transactivation. Plasmid bearing the luciferase gene under the control of either the WAF1 (**a**) gene promoter or the MDM2 (**b**) gene promoter. The activity of these two promoters was measured by the enzymatic detection of the luciferase. The activity of the parental line (pJS3–10) is 100% and the activity of the p53 mutant lines are normalized to this value

recombination between inverted repeats. Reciprocally, mutations inefficient to significantly stimulate recombination between direct repeats are also inefficient with inverted repeats (compare Tables 2 and 3). The mutation His175 shows a 3–5-fold increase of recombination between direct repeats, as we have previously described (Bertrand *et al*., 1997). We show here that recombination between inverted repeats is also stimulated to a similar extent. In contrast Gly175, a mutation in the same amino acid but with a different substitution, does not significantly stimulate spontaneous recombination either between direct repeat or between inverted repeat sequences. Similarly, mutations at the residue 273 lead to differing effects, depending on the amino acid substitution. Pro273 stimulates recombination 3–9 times between direct repeats as well as between inverted repeats, while His273 is without significant effect on the recombination rate in both recombination systems. In two independent clones expressing the His273 mutation, recombination between inverted repeats seems to be slightly increased (2–3 times) when using the Lea and Coulson test, however it was not significantly increased when calculation was made according to the Capizzi and Jameson method. Finally, Gln248 also stimulates recombination (direct and inverted repeats) from 3–7 times, i.e. to similar extent than His175.

When comparing the different results, it appears that the spontaneous rate of recombination (Tables 2 and 3) cannot be directly correlated either with the level of expression of the exogenous mutant p53 proteins (Figure 2), or with the transactivation activity (Figure 3) or with the G1 block after radiation (Figure 4).

Homologous recombination after ionizing radiation

It has previously been shown that ionizing radiation did not stimulate recombination between direct repeats in a similar cellular system (Wang *et al*., 1988). We

Table 2 Effect of the p53 mutant proteins on spontaneous recombination between direct repeat sequences

Cell lines name	*p53 protein mutation*	*Number of independent cultures*	*Recombination rate* ($\times 10^{-6}$/cell/generation) *Capizzi and Jameson*[a]	*Lea and Coulson*
pJS 3–10	None	18	1.5±0.8	1.9
CDR 1		18	2.6±0.9	2.7
H175 DR 102	175 (Arg→His)	19	6.5±0.9	6.9
H175 DR 211		18	9.7±0.7	11.7
G175 DR 2	175 (Arg→Gly)	12	2.1±0.7	2.9
Q248 DR 1	248 (Arg→Gln)	12	14.4±0.6	16.4
Q248 DR 3		12	9.2±0.6	7
Q248 DR 4		6	7.8±0.7	8.1
H273 DR 11	273 (Arg→His)	10	1.9±0.6	1.9
H273 DR 17		11	2.7±0.7	2.6
H273 DR 19		10	2±0.6	1.8
P273 DR 8	273 (Arg→Pro)	11	12.2±0.5	15
P273 DR 9		12	8.3±0.6	9.7

[a]Corresponds to the test of Luria and Delbrück

Table 3 Effect of the p53 mutant proteins on spontaneous recombination between inverted repeat sequences

Cell lines name	*p53 protein mutation*	*Number of independent cultures*	*Recombination rate* ($\times 10^{-6}$/cell/generation) *Capizzi and Jameson*[a]	*Lea and Coulson*
pJS 4–7–1	wild type	18	2.3±0.9	1.9
H175 ILR 8	175 (Arg→His)	8	5.4±0.5	5.8
G175 ILR 5	175 (Arg→Gly)	12	2.5±0.7	3
Q248 ILR 5	248 (Arg→Gln)	12	16±0.6	16.7
Q248 ILR 6		12	12.7±0.6	7.6
Q248 ILR 10		6	6.3±0.6	7
H273 ILR 4	273 (Arg→His)	11	3.9±0.6	4.6
H273 ILR 16		12	2.8±0.7	3.9
P273 ILR 1	273 (Arg→Pro)	11	15.9±0.5	19.1
P273 ILR 6		11	11.1±0.5	11.9
P273 ILR 8		6	8.6±0.5	8.4
P273 ILR 11		6	9.4±0.5	7.3

[a]Corresponds to the test of Luria and Delbrück

investigated here whether the status of *p53* may affect this process with direct as well as with inverted repeats. We confirmed that ionizing radiation does not stimulate recombination between direct repeats in the parental line pJS3–10 (Figure 5). We show here in addition, that expression of the mutant His175 p53 protein does not modify the radiation sensitivity of the cells (Figure 5a) but results in a stimulation of homologous recombination following exposure to γ-rays, in a dose-dependent manner (Figure 5b). The radiation stimulation ranges from 3–6 times at a dose of 6 Grays corresponding to a survival frequency comprised between 4 and 8% (Figure 5). Gln248 and Pro273, the other mutations resulting in an increase of spontaneous recombination, also lead to a stimulation of recombination after irradiation but to a lower extent for Gln248 (Figure 6). The two mutations that do not stimulate spontaneous recombination (Gly175 and His273) exhibit different behavior. Four independent clones carrying the His273 mutation fail to significantly stimulate recombination after radiation. In contrast, in the Gly175 clones, radiation induces recombination from 2–3 times, i.e. in similar range than the Gln248 mutation (Figure 6). Interestingly the different p53 mutations exhibit the same behavior with inverted repeat substrates and with direct repeat substrates. His175 and Pro273 are the two more efficient recombination stimulators, Gly175 and Gln248 stimulate recombination to a lesser extent and His273 is unable to increase recombination after radiation (Figure 6b).

Discussion

The strategy used here gives us the opportunity to compare the effect of different mutations of p53 in genetic backgrounds as similar as possible. Rather than comparing cell lines from various sources, bearing different p53 mutations, we expressed the p53 mutations in a common recipient cell line. Furthermore all the different lines derive from the same parental line containing a single copy of the recombination substrates. Thus, in each condition recombination rates are calculated for one copy of substrate, located at the same locus.

We measured recombination between direct repeats and between inverted repeats in all the conditions analysed here. The importance of distinction between direct *vs* inverted repeats becomes apparent when intrachromosomal recombination mechanisms are discussed (Klein, 1995). Indeed, direct repeats recombination refers to a mix of different mechanisms: SI, SSA, replication slippage and others (see Figure 1 and Klein, 1995). Inverted repeats recombination allows to focus on SI mechanisms. These concepts mainly derive from work in yeast, however these mechanisms have been also described in mammalian cells. The molecular pathways and the genes involved in these mechanisms have been identified in yeast and often present homologues in mammalian cells. For instance, SSA is dependent on the nucleotide excision repair Rad1 and Rad10 proteins, as well as the mismatch repair proteins

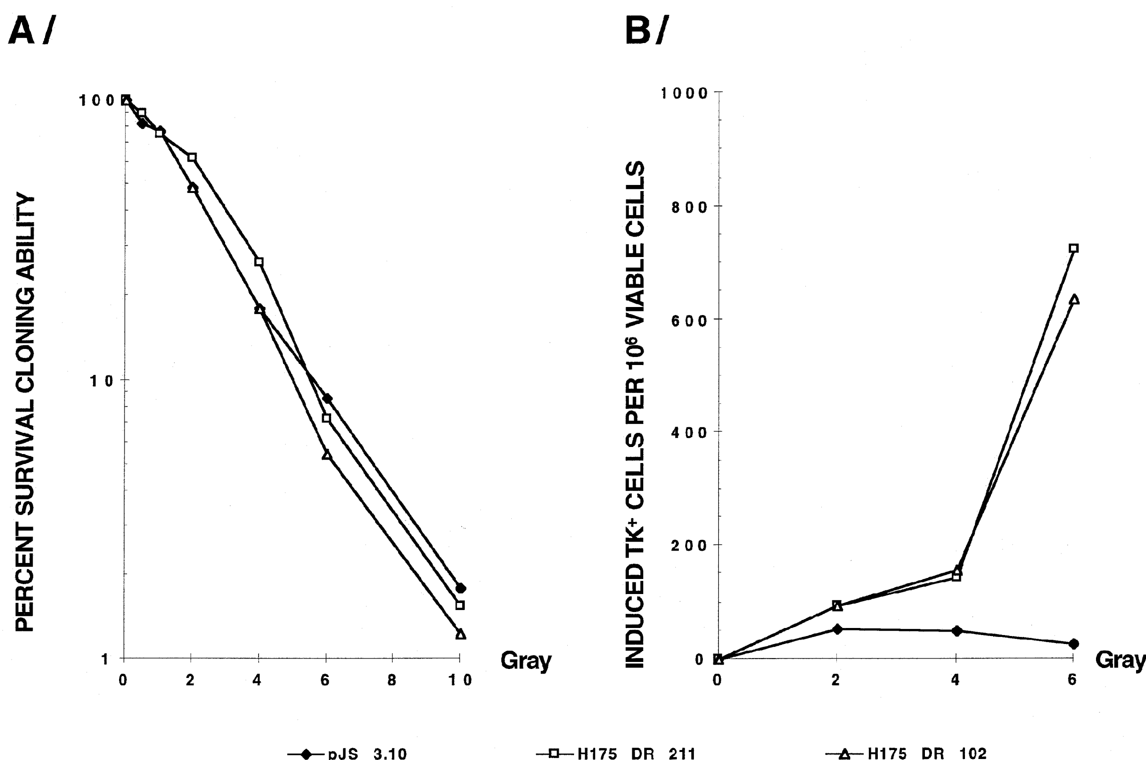


Figure 5 Cell killing and induction of recombination as a function of the dose of ionizing radiation. The lines used are indicated. pJS3–10 corresponds to the control line (no mutant p53 protein is expressed), H175 DR 211 and H175 DR 102 are two different lines both expressing His175 p53 mutant protein. (**a**) Per cent of survival measured by the plating efficiency following irradiation at the indicated doses. (**b**) Induction of recombination by γ-rays. The values correspond to the number of TK^+ clones in 10^6 surviving cells after radiation, following subtraction of the number of TK^+ clones in 10^6 non-irradiated cells

Msh2 and Msh3. *RAD10* corresponds to *ERCC1*, *RAD1* to *XP-F*, *MSH2* and *MSH3* correspond to *hMSH2* and *hMSH3* respectively in human cells. Remarkably, alteration of these genes leads to tumor predisposition (for review see Friedberg *et al*., 1995). SI in yeast, is dependent on the *RAD52* epistasis group including *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, all of these yeast genes presenting putative homologues in mammalian cells. In addition, the *RAD51* pathway may also be connected to the cancer prevention and may have developed specific characteristics in mammalian cells. Indeed, hRad51 protein has been shown to interact with the tumor suppressor proteins Brca1, Brca2 and p53 (Sturzbecher *et al*., 1996; Scully *et al*., 1997; Mizuta *et al*., 1997; Marmorstein *et al*., 1998). This conservation of genes and of molecular mechanisms, justifies a strategy using in parallel direct and inverted repeat substrates to identify candidate pathways involved in the genetic control of homologous recombination in mammalian

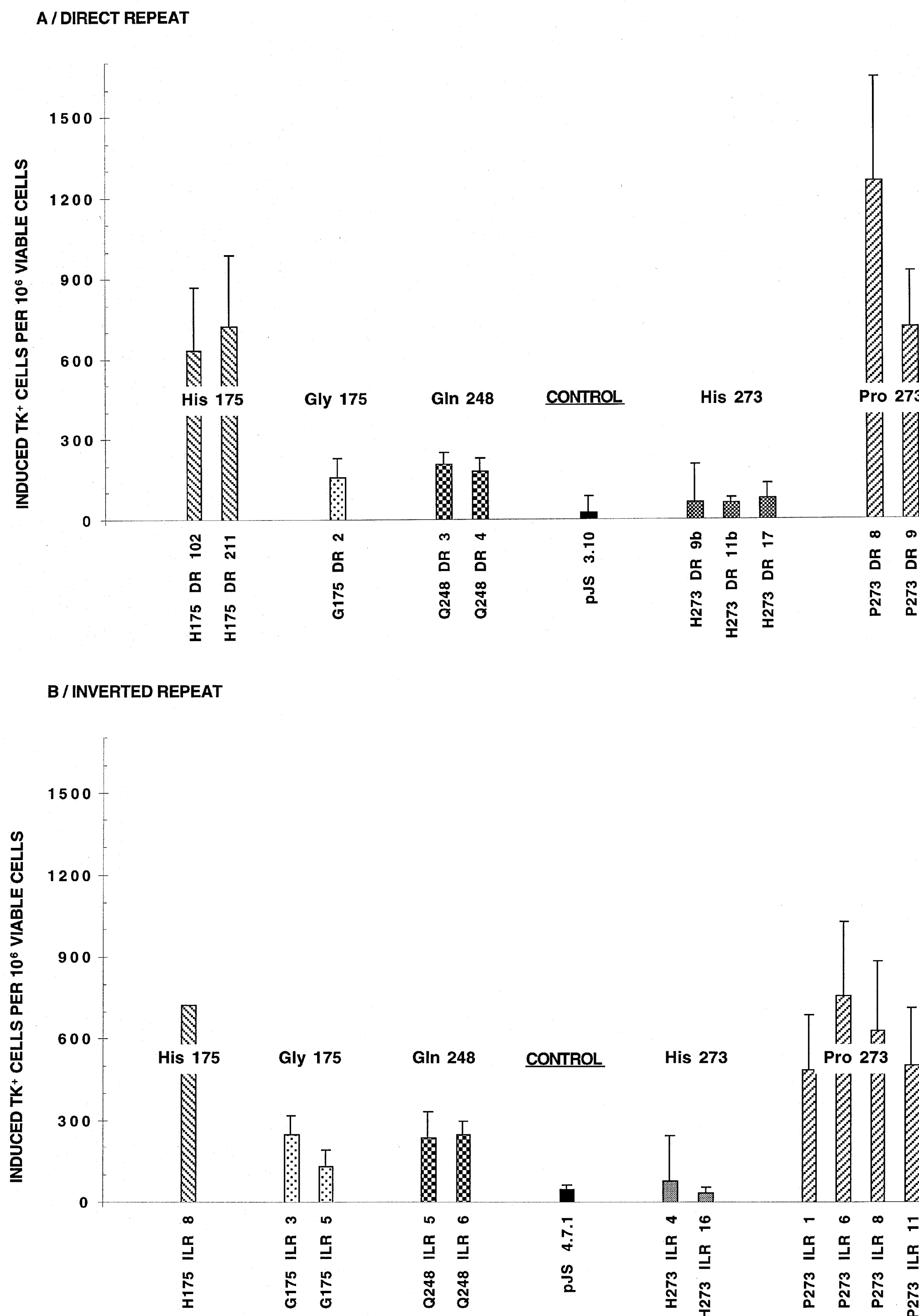


Figure 6 Induction of recombination by ionizing radiation in lines expressing the different p53 mutations. The dose was 6 Grays for each line. No differences in the survival have been observed between the different lines. The name of the lines and the expressed mutant p53 proteins are indicated on the figure. (**a**) Recombination between direct repeats. (**b**) Recombination between inverted repeats

Table 4 Recapitulation of the characteristics and effects of the different p53 mutations

Exogenous p53 mutation	*Transactivation activity*[a]	*G1 arrest after γ-rays*[b]	*Homologous recombination Spontaneous*	*Homologous recombination Radiation induced*	*Mutant p53 conformation*[c]
None[d]	+	+	–	–	
Gly 175	–/+	+	–	+	?[c]
His 175	–/+	–	+ +	+ + +	mutant
Gln 248	–	+	+ +	+	wild type
Pro 273	–	+	+ +	+ + +	mutant
His 273	–/+	+	–	–	wild type

[a]see Figure 3; [b]see Figure 4; [c]from Ory *et al.* (1994); Gly 175 has been shown to be unstable; [d]Corresponds to the control cell line (see Table 1)

cells. These strategies have been used here to study the effect of the status of *p53* protein on spontaneous and radiation-induced homologous recombination.

In our experiments, recombination between direct repeats or between inverted repeats varies similarly: stimulation of recombination acts on both types of substrates. Importantly, the fact that most of the mutant p53 proteins stimulate spontaneous and/or radiation-induced recombination between inverted repeats permits the conclusion that SI is stimulated. This conclusion is backed up by the observation that the ratio gene conversion *vs* deletion (80% *vs* 20%) remains unchanged in cells expressing the His175 p53 protein although the total rate of recombination is increased. This shows that gene conversion (i.e. SI mechanism) events are increased in these cells (Bertrand *et al*., 1997).

The p53 mutations analysed here exhibit differing capacities to stimulate homologous recombination (see Table 4). In the present experiments, we cannot know whether mutant p53 proteins act directly on the homologous recombination process itself or whether they inhibit the endogenous wild type p53 activity. The latter hypothesis would agree with reports showing that the wild type p53 protein inhibits homologous recombination (Wiesmuller *et al*., 1996) and that its inactivation leads to stimulation of homologous recombination (Mekeel *et al*., 1997). In the present report, p53 mutations leading to a stimulation of spontaneous recombination also lead to an increase of radiation-induced recombination. Indeed, mutations showing the more pronounced effect on spontaneous recombination (His175, Pro273) also show the strongest stimulation of radiation-induced recombination. However, the extent of radiation-induced recombination is not directly correlated to the extent of spontaneous recombination stimulation: in contrast to Gln248, Gly175 does not stimulate spontaneous recombination, but is as efficient as Gln248 in stimulation of radiation-induced recombination. In addition, differing mutations at the same position in p53 may differently affect homologous recombination rate. Mutations at the residues Arg175 and Arg273 show different effects on homologous recombination, according to the substituting amino acid: His175 stimulates both spontaneous and radiation-induced recombination whereas Gly175 has no effect on spontaneous recombination. This result is in agreement with a previous report showing that (i) the mutant His175 adopts a mutant conformation and is affected in all its biological activities; (ii) the mutant Gly175 is an unstable protein in SAOS-2 cells (Ory *et al*., 1994). However, our results have shown in addition, that radiation can stabilize the Gly175 p53 protein (see Figure 2). This radiation-dependent stabilization of the Gly175 protein may explain its mutant phenotype for homologous recombination after treatment with γ-rays. At the position 273, the replacement of the Arg amino acid by a His is without effect on homologous recombination but the substitution by a Pro produces the more pronounced effects on recombination. This result could be associated to the fact that the His273 protein keeps a wild type conformation whereas the Pro273 protein adopts a mutant conformation as described (Ory *et al*., 1994). The conformation of the mutant p53 proteins constitutes an explanation of our results, but only partially. Indeed, the two mutant p53 proteins most efficient in stimulating spontaneous and radiation-induced recombination are His175 and Pro273, both of which exhibit a mutant conformation. In contrast, Gln248 exhibits a wild type conformation (Ory *et al*., 1994) and is able to stimulate radiation-induced recombination to a much lesser extent, for radiation-induced recombination.

In many cases, the status of p53 affects spontaneous and radiation-induced recombination in parallel. This may indicate that p53 acts on spontaneous and radiation-induced recombination via common general mechanisms. Several mechanisms may account for the effect of p53: (i) an indirect effect via the transactivation activity and the control of the G1 checkpoint or (ii) a direct effect on the recombination mechanism itself. A negative effect of *RAD51* on the transactivation activity of p53 has recently been reported (Marmorstein *et al*., 1998). This observation may suggest that the effect of p53 on recombination could act via its transactivation activity. The transactivation experiments first confirmed the p53 wild type status of the control cell lines, i.e. pJS3–10, pJS4–7–1 and CDR1. These experiments also show that all the mutant p53 proteins used here are able to partially inhibit the endogenous p53 activity. In addition, His175 is as efficient as Gly175 and His273 in inhibiting transactivation but is, in contrast to them, able to stimulate recombination. Taken together, these results show that no correlation can be drawn between the transactivation activity and the extent of spontaneous as well as radiation-induced recombination. In addition, our results do not fit with the hypothesis involving the absence of G1 block for the stimulation of recombination. Indeed, some p53 mutant proteins do not alter the G1 block after

radiation (for instance: Gln248, Pro273), but are able to stimulate spontaneous and radiation-induced recombination. This observation reveals that the control of the G1/S transition and the control of the recombination process are separable activities of the p53 protein. A heterogeneous response to different p53 mutations has already been described for other p53 end-points. For instance, the activation of apoptosis and of cell cycle arrest have been shown to be separable activities indicating that p53 protein can control the choice between different alternatives: cell cycle arrest or apoptosis (Ryan and Vousden, 1998).

Since p53 alteration stimulates homologous recombination independently of the transactivation activity and of the G1 arrest, our results may argue in favor of a direct action of p53 protein on the recombination mechanism itself. Several other lines of evidence support this conclusion. Firstly, a recognition of the Holliday junctions by the p53 tetramer may suggest an involvement of p53 protein in the resolution of the recombination intermediates and would thus abort the progression of the branch migration (Lee *et al*., 1997). Secondly, it has been shown that the wild type p53 protein inhibits recombination between SV40 genome by correcting the heteroduplexes created during the recombination process (Dudenhoffer *et al*., 1998). Expression of the mutant p53 proteins might thus affect p53 heteroduplex correction, leading to an increase in the number of recombinant clones. Thirdly, the interaction of the wild type p53 protein with Rad51 would inhibit homologous recombination (Sturzbecher *et al*., 1996). Mutant p53 proteins seem to be unable to interact with Rad51 (Sturzbecher *et al*., 1996; Buchhop *et al*., 1997). The residue 273 or p53 protein is located in the region of interaction with Rad51 (Buchhop *et al*., 1997) and possibly the mutation His273 conferring a p53 wild type conformation (Ory *et al*., 1994) would not alter the interaction with Rad51 while the Pro273, leading to a mutant conformation (Ory *et al*., 1994), would destabilize the interaction with rad51.

Finally, if we assume that HR231 antibody has a similar affinity for the exogenous and for the endogenous p53 proteins, this would imply that mutant p53 protein acts on recombination, even when its expression level was much lower than that of the wild type p53 protein. This observation may suggest that p53 heterozygous cells would exhibit a general stimulation of genetic recombination in the entire genome. However, additional experiments would be required to assess this hypothesis. Nevertheless, this consideration is of particular importance with respect to the involvement of homologous recombination in genome rearrangements (inversion, deletion, translocation) and the implication of gene conversion in the propagation of genetic alterations (even point mutations). The consequence of this would be that mutation of only one allele of p53 would be sufficient to increase the probability of genome modification, even in absence of cell cycle control alteration.

In conclusion (see Table 4), cells expressing mutant p53 proteins generally show a higher likelihood of spontaneous and of radiation-induced recombination. More specifically, among the processes of homologous recombination, the strand invasion mechanism is stimulated and the type of p53 mutation may modify this phenotype. More importantly, our results show that the increase of recombination is not correlated with the transactivation activity and can be independent of an absence of G1 arrest after radiation, suggesting a direct action of p53 protein on the recombination process itself.

Materials and methods

DNA manipulations

All DNA manipulations were performed as described (Sambrook *et al*., 1989).

Cells and plasmids

Mouse L cells (pJS3–10, pJS4–7–1 and their derivatives) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. TK^+ clones were selected in HAT medium (100 μM hypoxanthine, 2 μM aminopterin, 15 μM thymidine) as described (Liskay *et al*., 1984). The different p53 cDNA were driven by the CMV promoter and constructed as described (Ory *et al*., 1994). These plasmids were cotransfected with pHYG (Clontech) containing the hygromycin resistance gene. Clones resistant to hygromycin (200 μg/ml) were selected and screened for the expression of the human mutant p53 protein, by Western blot.

Measure of the cell cycle after γ-irradiation

For each point, 10^6 cells were plated in DMEM and incubated for 24 h at 37°C. Cells were then washed in PBS buffer and irradiated (in PBS) at the indicated doses using a ^{60}Co irradiator (2.5 Grays/min). PBS was then replaced by DMEM and the cells were incubated at 37°C. Twenty-four hours after irradiation, cells were trypsinized, collected by centrifugation (5 min at 2000 *g*), re-suspended in 500 μl PBS and fixed by adding 1.5 ml of cold ethanol. The DNA content was estimated by propidium iodide fluorescence and DNA Flow Cytometry (FACSstar, Becton).

Measure of the transactivation activity on the WAF1 and MDM2 gene promoters

Cells were transfected with the WWp-Luc plasmid containing the luciferase reporter gene under the control of the WAF1 gene promoter (El-Deiry *et al*., 1993) or with the pGLaBasic plasmid containing the luciferase gene under the control of the MDM2 gene DNA binding sequence (provided by M Oren). As a transfection control, we also transfected the cells with a plasmid containing the EGFP gene (Clontech) under the control of the PGK gene promoter. No differences in the transfection efficiency was recorded between each line. The transactivation of the WAF1 and MDM2 promoters was measured using the luciferase activity. Seventy-two hours after the transfection, cell extracts were prepared and the luciferase activity was measured with the Luciferase Assay System kit (Promega) and using the Microlumat LB 96P (Berthold EG & G instrument) luminometer.

Western blot analysis

All extract preparation steps were performed at 4°C. After washing with PBS, cells were suspended in lysis buffer A (25 mM Tris, pH 7.5, 5 mM EDTA, 600 mM NaCl, 1 mM DTT, 0.1% NP40, 5 μg/ml Leupeptin, 2 μM Pepstatin, 1 mM PMSF, 10% glycerol) and incubated for 30 min. Extracts

were centrifuged 30 min at 15 000 *g*, supernatant was retrieved and protein concentration was determined using the Biorad Protein Assay (Biorad). Fifty μg/well of the bodied samples were loaded on a 10% polyacrylamide gel electrophoresis (PAGE) in presence of SDS. After migration, the proteins were electrotransferred on a nitrocellulose membrane and probed with specific antibodies. Standard procedures were used for the electrophoresis, transfer and Western blotting (Laemmli, 1970). HR231 antibodies detect human exogenous as well as mouse endogenous p53 proteins. DO7 antibodies are specific for human p53 protein and thus detect the mutant exogenous human p53 protein. Antibodies were revealed using the ECL detection kit (Amersham).

Measure of the recombination

The cell lines used: we used the cell lines and the strategy developed by Liskay and coworkers to measure homologous recombination (Liskay *et al*., 1984). The recipient lines are Ltk^-, sensitive to the HAT selective medium. These lines contain a unique copy of a tandem repeat of Herpes Simplex Virus type I (HSV1) *TK* gene, integrated into the cellular genome. Each HSV-*TK* sequence is inactivated by linker insertions; the cells are thus tk^- and thus sensitive to the HAT medium. Recombination between the two HSV-*TK* sequences can restore a functional *TK* gene. The recombinant cells become TK^+ and resistant to the HAT medium. The number of HAT resistant clones on the total number of plated cells give the frequency of recombination.

Fluctuation analyses for spontaneous recombination were performed as previously described (Liskay *et al*., 1984; Bertrand *et al*., 1997). For each line analysed, several independent cultures were plated and cultured to confluence. Cells were then trypsinized, counted and one portion was used for plating efficiency estimation. The remaining cells were plated under HAT selection and the resulting number of TK^+ clones allowed us to calculate the recombination frequency. The rate of recombination per cell per generation was calculated by using fluctuation tests of Luria and Delbruck (1943); Capizzi and Jameson (1973) or of Lea and Coulson (1948).

Recombination frequency after γ-rays: Cells were irradiated (in PBS) at the dose indicated, using a ^{60}CO irradiator (2.5 Grays/min). After irradiation the PBS was replaced by DMEM and the cells were incubated at 37°C for 24 h. The cells were then trypsinized and divided in two fractions. The first fraction was used to calculate the viability by measuring the plating efficiency. The second fraction was plated under HAT selection to measure the frequency of TK^+ clones. The recombination frequency was estimated by the ratio: number of TK^+ clones on the total number of surviving clones.

Acknowledgments

Thanks are due to Drs E May, M Liskay and C Ory for their generous gift of materials. We are grateful to Dr N Green for providing us with the computer application for the fluctuation analysis. We thank Drs P May, C Ory, C White and the members of our laboratory for helpful and stimulating discussions. Yannick Saintigny was supported by an EDF/INSTN fellowship. This work was supported by ARC (1366), ANRS and Electricité de France.

References

Amor M, Parker LK, Globerman H, New ML and White PC. (1988). *Proc. Natl. Acad. Sci. USA*, **85,** 1600–1604.
Arnheim N. (1983). In: *Evolution of genes and proteins*. Koehn RK and Nei M (eds), Sinauer Associates: Sunderland, MA, pp. 38–61.
Baltimore D. (1981). *Cell,* **26,** 295–296.
Becker RS and Knight KL. (1990). *Cell,* **63,** 987–997.
Benjamin MB and Little JB. (1992). *Mol. Cell. Biol.,* **12,** 2730–2738.
Bertrand P, Rouillard D, Boulet A, Levalois C, Soussi T and Lopez BS. (1997). *Oncogene,* **14,** 117–1122.
Bollag RJ, Waldman AS and Liskay RM. (1989). *Annu. Rev. Genet.,* **23,** 199–225.
Bouffler SD, Kemp CJ, Balmain A and Cox R. (1995). *Cancer Res.,* **55,** 3883–3889.
Buchhop S, Gibson MK, Wang XW, Wagner P, Sturzbecher H-W and Harris CC. (1997). *Nucleic. Acids Res.,* **25,** 3868–3874.
Capizzi RL and Jameson JW. (1973). *Mut. Res.,* **17,** 147–148.
Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, Strong LC and White RL. (1993). *Nature,* **305,** 779–784.
Donehover LA and Bradley A. (1993). *Biochimica. Biophysica. Acta.,* **1155,** 181–204.
Dudenhoffer C, Rohaly G, Will K, Deppert W and Wiesmuller L. (1998). *Mol. Cell. Biol.,* **18,** 5332–5342.
El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.
Friedberg EC, Walker GC and Siede W. (1995). *DNA repair and mutagenesis*. ASM Press, Washington, DC.
Hainaut P. (1995). *Curr. Opin. Oncol.,* **7,** 76–82.
Hartwell L. (1992). *Cell,* **71,** 543–546.
Ivanov EL, Sugawara N, Fishman-Lobell J and Haber JE. (1996). *Genetics,* **142,** 693–704.
Kleckner N. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 8167–8174.
Klein HL. (1995). *BioEssays,* **17,** 147–159.
Ko LJ and Prives C. (1996). *Genes Dev.,* **10,** 1054–1072.
Laemmli U. (1970). *Nature,* **227,** 680–685.
Lambert S, Saintigny Y, Delacote F, Amiot F, Chaput B, Lecomte M, Huck S, Bertrand P and Lopez BS. (1999). *Mutation Res.,* (in press).
Lea DE and Coulson CA. (1948). *J. Genet.,* **49,** 264–248.
Lee S, Cavallo L and Griffith J. (1997). *J. Biol. Chem.,* **14,** 7532–7539.
Liebhaber SA, Goosens M and Kan Y-K. (1981). *Nature,* **290,** 26–29.
Lin F, Sperle K and Sternberg N. (1987). *Mol. Cell Biol.,* **7,** 129–140.
Liskay RM, Stachelek JL and Letsou A. (1984). *Cold Spring Harbor Symp. Quant. Biol.,* **49,** 183–189.
Livingstone LR, White A, Sprouse J, Livanos E, Jacks T and Tlsty TD. (1992). *Cell,* **70,** 923–935.
Luria SE and Delbruck M. (1943). *Genetics,* **28,** 491–511.
Marmorstein LY, Ouchi T and Aaronson SA. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 13869–13874.
Mekeel KL, Tang W, Kachnic LA, Luo C-M, DeFrank JS and Powell SN. (1997). *Oncogene,* **14,** 1847–1857.
Milner J and Medcalf EA. (1991). *Cell,* **65,** 765–774.
Mizuta R, LaSalle JM, Cheng H-L, Shinohara A, Ogawa N, Copeland N, Jenkins NA, Lalande M and Alt FW. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 6927–6932.
Ory C, Legros Y, Auguin C and Soussi T. (1994). *EMBO J.,* **13,** 3496–3504.

Park MS. (1995). *J. Biol. Chem.,* **270,** 15467 – 15470.
Reynaud CA, Anquez V, Grimal H and Weill J-C. (1987). *Cell,* **48,** 379 – 388.
Roeder SG. (1990). *TIG,* **6,** 385 – 389.
Ryan KM and Vousden KH. (1998). *Mol. Cell. Biol.,* **18,** 3692 – 3698.
Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.
Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T and Livingston DM. (1997). *Cell,* **88,** 265 – 275.
Smith ML and Fornace AJ. (1995). *Curr. Opin. Oncol.,* **7,** 69 – 75.
Sturzbecher H-W, Donzelmann B, Henning W, Knippschild U and Buchhop S. (1996). *EMBO J.,* **15,** 1992 – 2002.
Sugawara N, Pâques F, Colaiàcovo M and Haber JE. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 9214 – 9219.
Tyler J and Weinberg RA. (1996). *Nature,* **381,** 643 – 644.
Wang Y, Maher VM, Liskay RM and McCormick JJ. (1988). *Mol. Cell. Biol.,* **8,** 196 – 202.
Wiesmuller L, Cammenga J and Deppert WW. (1996). *J. Virol.,* **70,** 737 – 744.
Xia F, Amunson SA, Nickoloff JA and Liber HL. (1994). *Mol. Cell. Biol.,* **14,** 5850 – 5857.
Yin Y, Tainsky MA, Biscoff FZ, Strong LC and Wahl GM. (1992). *Cell,* **70,** 937 – 948.