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Increased Gene Amplification in Immortal Rodent Cells Deficient for the DNA-dependent Protein Kinase Catalytic Subunit[1]

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ABSTRACT

Gene amplification is one of the most frequent genome anomalies observed in tumor cells, whereas it has never been detected in cells of normal origin. A large body of evidence indicates that DNA double-strand breaks (DSBs) play a key role in initiating gene amplification. In mammals, DSBs are mainly repaired through the nonhomologous end-joining pathway (NHEJ) that requires a functional DNA-dependent protein kinase catalytic subunit (DNA-PKcs). In rodent cell lines, *N*-(phosphonacetyl)-L-aspartate (PALA) resistance is considered a measure of gene amplification because it is mainly attributable to amplification of the carbamyl-*P*-synthetase aspartate transcarbamylase dihydro-orotase (*CAD*) gene. In this paper we show that the radiosensitive hamster cell line V3, which is defective in DSB repair because of a mutation in the *DNA-PKcs* gene, displays also an increased frequency of gene amplification. In these cells, we found that the amplification of the *CAD* gene occurs with a frequency and a rate more than one order of magnitude higher than in control cell lines, although it relies on the same mechanisms. When the same analysis was performed in mouse embryo fibroblasts (MEFs) obtained from animals in which the *DNA-PKcs* gene was ablated by homologous recombination, a higher frequency of amplification compared with the controls was found only after cellular immortalization. In primary *DNA-PKcs*$^{-/-}$ MEFs, PALA treatment induced a block in the cell cycle, and no PALA-resistant clones were found. Our results indicate that the lack of DNA-PKcs increases the probability that gene amplification occurs in a genetic background already permissive, like that of immortalized cells, although it is not sufficient to make normal cells able to amplify.

INTRODUCTION

In mammalian cells, genome stability is maintained through different pathways mainly controlling either the cell cycle in response to DNA damage or the repair of the damage itself. Mutations in genes involved in these pathways cause the accumulation of genetic lesions that can lead to neoplastic transformation (reviewed in Ref. 1).

A frequent manifestation of the genomic instability of neoplastic cells is gene amplification, a process leading to the increase in the copy number of a portion of the genome (2–4). Amplification of proto-oncogenes has been found in several types of solid tumors, and many observations indicate that it plays an essential role in their development (reviewed in Ref. 5).

Gene amplification has never been detected in cells of normal origin, which suggests that either control mechanisms are active that prevent the occurrence of amplification or that cells carrying gene amplification are not allowed to survive (6, 7). On the other hand, the well-documented permissivity for amplification of tumor cells and of established cell lines has been related to their lack of a stringent control on genome integrity (reviewed in Ref. 4). In this regard, it was shown that the inactivation of *p53*, one of the most important genes involved in maintaining genome stability, is sufficient to render normal cells able to perform gene amplification (8, 9).

A large body of evidence indicates that DNA DSBs[3] can trigger gene amplification, probably through the activation of recombination-based mechanisms (10–17). Breakage-fusion-bridge cycles after a DSB are currently the favored mechanism for gene amplification (12, 18). In this view, it can be understood how the ability of a cell to efficiently counteract DNA breaks can play a major role in the control of gene amplification (4, 19, 20).

In mammalian cells, the main mechanism involved in the repair of DSBs is the NHEJ pathway (reviewed in Ref. 21). Among the factors operating in NHEJ, the best characterized are the DNA-PK complex (reviewed in Ref. 22), together with the XRCC4 and the ligase IV proteins (23, 24). The DNA-PK complex is constituted by a heterodimeric subunit with DNA end-binding activity, termed Ku, and a subunit carrying the catalytic activity (DNA-PKcs). The Ku proteins bind to a DSB and recruit DNA-PKcs to the break, whereas XRCC4 and ligase IV are involved in the final ligation steps. Cells defective in any of the NHEJ genes mentioned above share a common phenotype, *i.e.*, hypersensitivity to X-rays, impaired DSB repair, and V(D)J recombination (23, 25, 26).

To investigate whether a defect in the NHEJ pathway can affect the process of gene amplification, we analyzed gene amplification ability in rodent cells defective in the *DNA-PKcs* gene, and we showed that, after cellular immortalization, the lack of DNA-PKcs increases the probability that gene amplification occurs.

MATERIALS AND METHODS

Cells and Cell Culture. The radiosensitive Chinese hamster cell line V3 and the parental cell line AA8 were described elsewhere (27). The V3 147f cell line derives from a V3 clone in which the genetic defect was complemented by a human YAC containing the *DNA-PKcs* gene (28). Wild-type, heterozygous, and *DNA-PKcs*$^{-/-}$ MEFs were prepared from mice as described by Taccioli *et al.* (29). MEFs were analyzed at three stages in culture: at approximately passage 3 and, after immortalization, at approximately passage 30 (mid-passages) and passage 50 (late-passages). All of the cell lines were grown in DMEM (Hy-clone) supplemented with 10% FCS (Hy-clone) and maintained at 37°C in the presence of 3% CO_2. The V3 147f line was propagated in medium containing G418 (Life Technologies, Inc.) at a final concentration of 500 μg/ml.

PALA was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Experiments with PALA were carried out in the presence of 1 μM dipyridamol (Boehringer Ingelheim) a specific inhibitor of the uptake of uridine, which allows an increase in PALA toxicity (30). In our experimental conditions, 1 μM dipyridamol was not cytotoxic and reduced the uptake of uridine by $\sim$ 90%.

Measurement of Sensitivity to PALA. In the hamster cell lines, PALA sensitivity was evaluated by measuring the reduction in colony-forming ability.

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[3] The abbreviations used are: DSB, double-strand break; CAD, carbamyl-*P*-synthetase aspartate transcarbamylase dihydro-orotase; PALA, *N*-(phosphonacetyl)-L-aspartate; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; NHEJ, nonhomologous end joining; MEF, mouse embryo fibroblast; FISH, fluorescence *in situ* hybridization; DAPI, 4′-6diamidino-2-phenylindole.

Five hundred cells were plated in 10-cm dishes in medium containing different concentrations of PALA and 1 μM dipyridamol. After 1 week, the surviving colonies were fixed with methanol and stained with Coomassie Blue (1% Page Blue in 50% methanol and 7.5% acetic acid), and the LD_{50} of PALA was determined. Plating efficiencies in the untreated samples were ~40% in the AA8 line and ~35% in V3 and V3 147f.

PALA sensitivity of MEFs was determined by measuring the inhibition of growth in massive cell cultures. Cells (10^4 per 6-cm dish) were plated in medium containing different concentrations of PALA and 1 μM dipyridamol. After 5 days, the cells were fixed and stained as described above. For each dish, the number of cells contained in 20 independent microscope fields was counted, and the concentration of PALA inhibiting growth by one-half (RD_{50}) was then determined. For the late-passage MEFs, the number of cells was estimated by measuring the absorbance of the lysed samples (31). In all of the MEFs analyzed, the RD_{50} was ~8 μM PALA (data not shown).

Measurement of the Frequency of PALA-resistant Clones. Cells (10^5 per 10-cm dish) were seeded in selective medium containing 1 μM dipyridamol and PALA at concentrations ranging from 160 to 320 μM (4–8 $\times$ LD_{50}) for the hamster cells and from 12 to 40 μM (1.5–5 $\times$ RD_{50}) for MEFs. For the Chinese hamster cell lines, five dishes were seeded for each PALA concentration. For MEFs, the total number of cells analyzed in the different experiments is indicated in "Results." After 2 weeks, the surviving hamster clones and those obtained from late-passage MEFs were fixed and stained. The dishes with early- and mid-passage MEFs were examined *in vivo* under an inverted microscope, and after 3–4 weeks, clones were detected and isolated. The recovered cells were propagated in the presence of PALA to verify resistance.

Measurement of the Rate of Occurrence of PALA-resistant Clones. The rate of appearance of PALA-resistant clones, *i.e.*, the rate of generation of new mutants for cell for generation, was determined in the hamster cell lines using the fluctuation test (32). For each cell line, 15 samples with 100 cells each were seeded in multiwell plates in nonselective medium. The cultures were grown until a cell density of ~5–7 $\times$ 10^4 cells was reached. The cells were then trypsinized, dispersed, and replated in 10-cm dishes with selective medium containing 1 μM dipyridamol and PALA at concentrations ranging from 160 to 480 μM PALA (4–12 $\times$ LD_{50}). After 2–3 weeks, the dishes were fixed and stained. Colonies containing more than 50 cells were counted. The mutation rate was calculated from the median number of colonies among the cultures (r_0) and from the proportion of cultures without colonies (p_0) according to Lea and Coulson (33). When the median was lower than 1, the rate was calculated only from p_0.

Chromosome Preparations and FISH. In hamster cells, chromosome spreads and FISH were performed as described in Bertoni *et al.* (13). In mouse cells, slides where pretreated with pepsin [(pH 2) 10 mg/ml for 10 min at 37°C] instead of with proteinase K, and washes were performed at 37°C because hybridization was carried out with the hamster probe for the *CAD* gene. Hamster PALA-resistant clones were propagated in selective medium for 4–5 passages before preparing chromosome spreads; the mouse clone was propagated for ~8 passages.

RESULTS

Elevated Frequency and Rate of *CAD* Gene Amplification in a DNA-PKcs-deficient Hamster Cell Line. In rodent cell lines, resistance to PALA, is mainly achieved through the amplification of the *CAD* gene (34, 35). Therefore, the frequency and the rate of appearance of PALA-resistant clones are considered a measure of gene amplification ability.

To unravel the role that DNA-PKcs might play in gene amplification, we analyzed the frequency and the rate of *CAD* gene amplification in a hamster cell line, V3, defective in *DNA-PKcs*. We used as controls the parental cell line AA8, and a V3 cell line complemented by the human *DNA-PKcs* gene, named V3 147f (28).

Preliminary experiments were performed to test PALA sensitivity in the three cell lines. The very similar response in all of the lines, with a PALA LD_{50} of ~40 μM (data not shown), allowed us to compare the frequency and the rate of resistant clones at the same doses of PALA.

The frequency of resistant clones was measured at 160, 240, and 320 μM PALA (which correspond to 4, 6, and 8 $\times$ the LD_{50}, respectively); the results of two independent experiments are displayed in Table 1. As expected, cells rapidly died on PALA treatment, and resistant clones arose in all of the cell lines. The frequency of resistant clones decreased with increasing PALA doses, and, at each dose of PALA, it was higher in V3 cells compared with AA8 cells. The ratio between the two values ranged between 12 and 53 in the first experiment and between 18 and 64 in the second experiment. In V3 147f, in which the V3 genetic defect has been complemented, the frequencies of PALA-resistant clones were in the same range as those found in the parental line.

To test whether this higher frequency of cells carrying gene amplification was attributable to a higher rate of generation of new mutants during cellular propagation, we performed a fluctuation test (see "Materials and Methods"). The results of two independent experiments are shown in Table 2. In the first experiment, the doses of PALA used were the same as in the frequency assays. In AA8 and in V3 147f, the mutation rate was similar and decreased with increasing PALA doses. In V3, the mutation rate did not decrease with increasing doses of the selective agent, and it was from 18 to 152 times higher than in parental cells. These results were confirmed in the second experiment in which a PALA dose up to 12 $\times$ the LD_{50} was tested. At this highest dose of PALA, the mutation rate in V3 was ~40 times higher than in AA8 and in V3 147f. In this second experiment at the lower doses (240 μM and 320 μM PALA), mutation rates in V3 were not calculated because in several dishes the clones were too numerous to be counted precisely.

These results suggest that DNA-PKcs deficiency confers a higher amplification ability to immortalized hamster cells.

To study the mechanisms underlying gene amplification, PALA-resistant clones were isolated from the three cell lines, and *CAD* gene amplification was analyzed in single metaphases by FISH with a probe for the *CAD* gene (Fig. 1). Analysis of four PALA-resistant clones from the AA8 cells and of three clones from V3 and V3 147f showed that the *CAD* gene was amplified and that amplification was intrachromosomal. The amplified DNA was organized in ladder-like structures, prevalently with two (Fig. 1, *C* and *E*) or three hybridization bands (Fig. 1*D*) or in condensed arrays (Fig. 1, *F–H*). These results suggest that gene amplification occurs through the same mechanisms both in the presence and in the absence of DNA-PKcs.

Table 1 *Frequency of PALA-resistant clones in AA8, V3, and V3 147f cell lines*

PALA (μM)	Experiment No.	Frequency of PALA-resistant clones $\times 10^{-4}$ (ratio)[a]		
		AA8	V3	V3 147f
160	1	1.26	31.70 (25.2)	1.66 (1.3)
	2	3.58	66.46 (18.5)	13.66 (3.8)
240	1	0.24	12.84 (53.3)	0.78 (3.2)
	2	2.58	44.40 (17.2)	1.48 (0.6)
320	1	0.22	2.64 (12.0)	0.34 (1.5)
	2	0.46	29.62 (64.4)	0.76 (1.6)

[a] Ratio between the mutation frequency either in V3 or in V3 147f, and that in AA8 at each PALA dose.

Table 2 *Rate of PALA-resistant colonies in AA8, V3, and V3 147f cell lines*[a]

Cell line (μM PALA)	Exp. no.	No. of colonies per culture: Range	Mean	Median (r_0)	Fraction of cultures without colonies (p_0)	Mutation rate $\times 10^{-4}$[a] (ratio)[c]: from r_0	from p_0
AA8 (160)	1	2–124	40.0	28	0.0	1.10	
AA8 (240)	1	1–42	10.3	7	0.0	0.39	
	2	20–233	82.1	44	0.0	2.16	
AA8 (320)	1	0–7	1.9	2	0.3	0.17	0.21
	2	23–129	51.1	37	0.0	1.89	
AA8 (480)	2	0–167	23.9	1	0.3		0.25
V3 (160)	1	177–1320	662.6	528	0.0	19.9 (18.1)	
V3 (240)	1	196–996	738.1	736	0.0	26.5 (67.9)	
V3 (320)	1	336–1400	763.6	716	0.0	25.9 (152.3)	
V3 (480)	2	191–590	394.3	356	0.0	11.0 (44.0)	
V3 147f (240)	1	4–37	12.1	7	0.0	0.40 (1.0)	
	2	6–247	53.7	31	0.0	1.43 (0.7)	
V3 147f (320)	1	0–130	12.5	2	0.3	0.17 (1.0)	0.18 (0.8)
	2	1–87	14.6	5	0.0	0.38 (0.2)	
V3 147f (480)	2	0–13	2.0	1	0.4		0.28 (1.1)

[a] No. of cells seeded per culture: experiment 1 (Exp. 1), AA8: 7.6×10^4; V3: 4.6×10^4; V3 147f: 7.5×10^4; experiment 2 (Exp. 2) AA8: 5.5×10^4; V3: 6.0×10^4; V3 147f: 6.3×10^4.

[b] The mutation rate was calculated from the median number of colonies per culture (r_0) and from the fraction of culture without colonies (p_0) according to Lea and Coulson (33). When the median was below 1, the mutation rate was calculated only from p_0.

[c] Ratio between the mutation rate either in V3 or in V3 147f and that in AA8 at each PALA dose. The ratio was obtained from mutation rates calculated with the same method.

Lack of DNA-PKcs Function Is Not Sufficient to Allow Gene Amplification to Occur in Early-Passage MEFs. In normal MEFs, gene amplification has never been detected (8). To test whether a defect in the *DNA-PKcs* gene was sufficient to render normal cells prone to amplify, we analyzed PALA resistance in two independent MEF strains obtained from mice in which the *DNA-PKcs* gene had been ablated by homologous recombination (*DNA-PKcs*$^{-/-}$; Ref. 29), in one strain from a heterozygous mouse (*DNA-PKcs*$^{+/-}$) and in one strain from a wild-type homozygous mouse (*DNA-PKcs*$^{+/+}$). For each strain at passage 3, 10^6 cells were treated with 12, 24, and 40 μM PALA (1.5, 3, and 5 $\times$ RD_{50}, respectively; see "Materials and Methods"). In all of the strains and at all of the PALA concentrations, no clones were found, and flattened and enlarged cells remained attached to the dish. This result was confirmed in a second experiment in which 3×10^6 heterozygous and 3×10^6 mutant MEFs, obtained from embryos generated by an independent heterozygous cross, were treated with 24 μM PALA.

These results indicate that the lack of the DNA-PKcs function is not sufficient to allow gene amplification to occur in early-passage MEFs.

Increased Frequency of Gene Amplification in DNA-PKcs$^{-/-}$ MEFs Immortalized *in Vitro*. It is well known that after a limited number of passages in culture (~10) MEFs enter a phase of crisis. During this phase, most of the cells, die and those that survive give rise to established cell lines formed by immortal cells capable of dividing indefinitely (36). To test whether the lack of DNA-PKcs had an effect on gene amplification in an immortalized cellular background, we analyzed PALA resistance in a second group of MEFs that had overcome crisis. At this stage, as expected for immortalized populations, only <10% of the cells had 40 chromosomes, the majority of the cells being hypotetraploid, regardless of the *DNA-PKcs* genotype (data not shown).

Each genotype (2×10^6 cells) at passage 30 was challenged with doses of PALA corresponding either to 3 or to 5 $\times$ the RD_{50} (24 and 40 μM PALA, respectively), and after 3 weeks, the response to PALA was analyzed. As in early-passage MEFs, in all of the lines, flattened and enlarged cells were found attached to the dish. However, resistant clones arose in the *DNA-PKcs*-deficient MEFs, both at 24 μM and at 40 μM (Table 3A), whereas no clones were present in the wild-type cells at either of the PALA doses tested. At 24 μM PALA, clones were also detected in the heterozygous strain, although with a lower frequency compared with the *DNA-PKcs*$^{-/-}$ cells (Table 3A).

To confirm PALA resistance in the clones grown in the presence of PALA, a total of 22 clones were isolated from the *DNA-PKcs*$^{-/-}$ cells (19 grown in 24 μM PALA and 3 in 40 μM PALA), and 4 from the *DNA-PKcs*$^{-/+}$ cells. Seventeen *DNA-PKcs*$^{-/-}$ clones grew in PALA, confirming their resistance; 5 did not grow and neither did any of the four *DNA-PKcs*$^{-/+}$ clones, which indicated that they were not truly PALA resistant.

CAD gene copy number was analyzed by Southern blotting in eight PALA-resistant clones (seven resistant to 24 μM PALA, and one to 40 μM PALA); and it was found that, in two clones, the number of copies of the genes was increased. The average increase in the number of copies of *CAD* determined in three independent experiments was 2.5 in one clone and 2.2 in the other one (data not shown). In one of these clones, *CAD* gene amplification was also demonstrated by FISH on mitotic chromosomes (Fig. 1, *H–J*). These results indicate that, in mid-passage DNA-PKcs$^{-/-}$ MEF *CAD* gene amplification can occur, although PALA resistance can also be caused by other mechanisms.

In the wild-type and in the mutant MEF line, as well as in two additional *DNA-PKcs*$^{-/-}$ lines, the analysis was repeated at approximately passage 50. At this passage, in all of the lines regardless of the genotype, the response to PALA was similar to what was described for the established cell lines, *i.e.*, the majority of the cells rapidly died when challenged with PALA, and several resistant clones were observed. The frequency of PALA-resistant clones was higher in the DNA-PKcs-deficient cells compared with wild-type MEFs (Table 3B).

DISCUSSION

In this study, we show that the radiosensitive hamster cell line V3, which is defective in DSB repair because of a mutation in the *DNA-PKcs* gene (27, 37), displays also an increased gene amplification ability compared with parental cells and with the V3 147f cell line, in which the genetic defect had been complemented by transfection of the human gene. This result indicates that, in the absence of a functional DNA-PKcs protein, gene amplification is more frequent than in DNA-PKcs-proficient cells.

FISH analysis of the *CAD* gene in PALA-resistant clones that were isolated from the V3, V3 147f, and AA8 cell lines showed a similar organization of the amplified DNA, suggesting that gene amplification occurs through the same mechanisms regardless of the presence of a functional DNA-PKcs. In particular, the structure of the ampli-

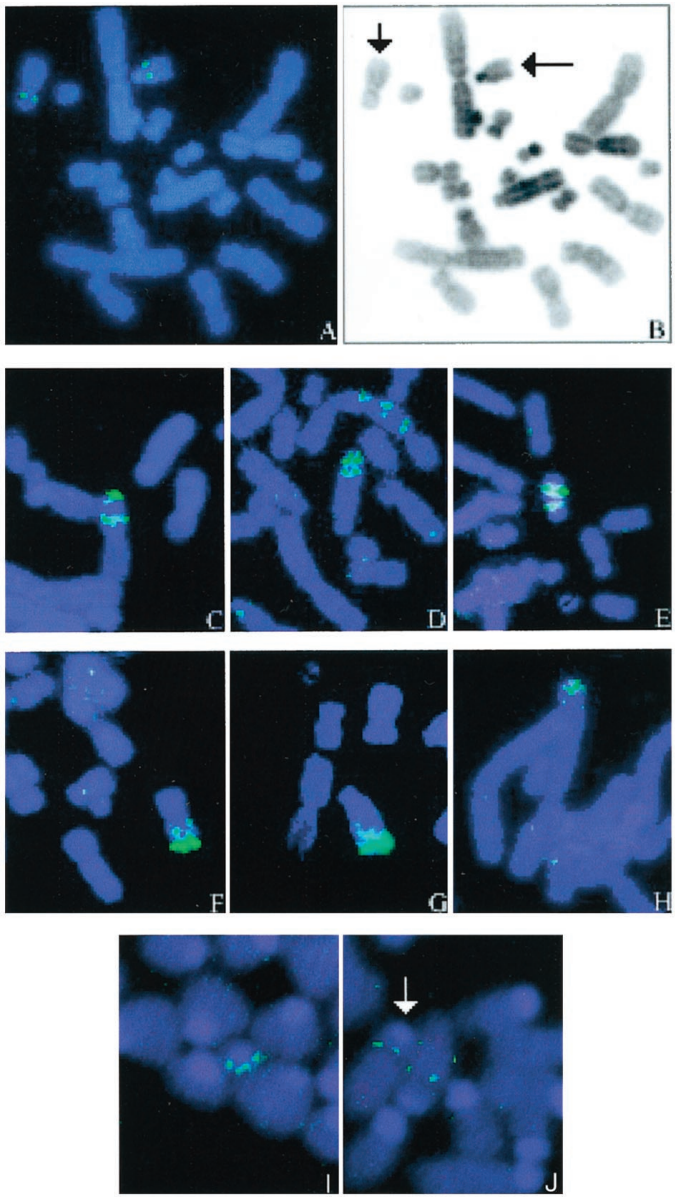

Fig. 1. *A–H*, localization of the *CAD* gene on the chromosomes of the V3 cell line and of hamster PALA-resistant clones. *A*, mitosis of the V3 cell line: the *yellow* hybridization signals attributable to the single copy gene are on chromosome 7 (*long arrow*; Ref. 46) and on chromosome Z8 (*short arrow*; Ref. 47); *B*, the same mitosis is shown after DAPI staining; *C–E*, examples of ladder-like amplified structures in PALA-resistant clones isolated from AA8 (*C*), V3 (*D*), and V3 147f (*E*); *F–H*, examples of condensed amplified structure in PALA-resistant clones from AA8 (*F*), V3 (*G*), and V3 147f (*H*). *I* and *J*, amplification of the *CAD* gene in one MEF PALA-resistant clone: examples of two marker chromosomes bearing an amplified *CAD* gene. *J*, the chromosome with the single copy gene is also shown (*arrow*).

fication-bearing chromosomes is compatible with an origin of the amplified DNA through bridge-breakage-fusion cycles (4).

The hamster cellular system, which is permissive for gene amplification, allows us to evaluate the role of DNA-PKcs in influencing the level of gene amplification; however, it does not allow us to determine whether a defect in the *DNA-PKcs* gene renders normal cells able to amplify. To address this problem, we analyzed PALA resistance in early-passage MEFs derived from mice in which the *DNA-PKcs* gene was ablated by homologous recombination (29). No PALA-resistant clones were found of the 9×10^6 *DNA-PKcs*$^{-/-}$ cells analyzed in the different experiments, which indicated that the deficiency of DNA-PKcs is not sufficient to allow gene amplification to occur with a detectable frequency in primary cells.

However, when we analyzed PALA resistance in MEFs that were adapted in culture by successive passages (24–32 passages), we found a higher frequency of PALA-resistant clones in the *DNA-PKcs*$^{-/-}$ cells compared with controls. In fact, in the mutant cell line, the frequency of PALA-resistant clones was close to 10^{-4}, whereas no clones were found in wild-type cells treated with PALA. In the heterozygous cell line, clones were found with a frequency lower than 10^{-5}; however, the frequency of truly resistant clones has to be lower because none of the clones that we isolated survived in subsequent cultures in PALA. In contrast, more than 70% of the clones isolated from the *DNA-PKcs*$^{-/-}$ MEFs were able to grow in PALA. Moreover, in two *DNA-PKcs*$^{-/-}$ clones, an increase in the *CAD* gene copy number was confirmed by Southern blot analysis and by FISH. It is worth pointing out that, because gene amplification was not detected in all of the resistant clones, other mechanisms besides the increase in *CAD* gene copy number can be responsible for PALA resistance in this cellular system (38).

The results obtained in these mid-passage MEFs suggest that cellular immortalization *per se* is not sufficient to promote an increase in the amplification frequency great enough to make gene amplification detectable by analyzing a few millions of cells. However, when immortalization is associated with a defect in the *DNA-PKcs* gene, the absence of the functional protein boosts gene amplification to levels that allow it to be detected in tissue culture.

Regardless of the genotype, immortalized MEF lines displayed a heteroploid karyotype with a similar distribution of chromosome number (data not shown); therefore, the amplification detected in the mutant cells cannot be related to the heteroploid karyotype.

It is worth noting that, after PALA treatment, the morphology of both the early- and the mid-passage MEFs changed. Independently of the *DNA-PKcs* genotype of the populations, flattened and enlarged cells remained attached to the dish suggesting that the selective agent induced a block in the cell cycle. This cellular response to PALA is typical of cells of normal origin; it has been related to the lack of gene amplification (7–9) and to the presence of a functional p53 (39). Although in the early-passage MEFs, the block in the cell cycle induced by PALA correlates, as expected, with the absence of gene amplification, in the mid-passage MEFs, amplification was detected at least in the mutant cells. This apparent contradiction with the data of the literature can be explained by assuming that, at this stage of immortalization, the cellular populations are a mixture of permissive and non-permissive cells for gene amplification. If this were the case,

Table 3 *Frequency of PALA-resistant clones in (A) mid- and (B) late-passage MEFs with different DNA-PKcs genotype*

	Frequency of PALA resistant clones $\times 10^{-5}$ (PALA) μM		
DNA-PKcs genotype	24 μM[a]	40 μM[a]	24 μM[a]
A. Mid-passage MEF			
Wild-type (MEF #23)	<0.05	<0.05	<0.05
−/+ (MEF #22)	0.05	<0.05	0.83
−/− (MEF #26)	3.80	0.25	11.26
B. Late-passage MEF			
	24 μM[b]	40 μM[b]	40 μM[b]
Wild-type (MEF #23)	0.8	4.5	<0.2
−/− (MEF #20)	71.0	11.0	8.8
−/− (MEF #24)	22.0	31.6	10.2
−/− (MEF #26)	96.0	86.0	84.6

[a] The results reported in columns 2 and 3 were obtained treating the cells with either 24 or 40 μM PALA in the same experiment; the results reported in the last column were obtained in an independent experiment two $\times 10^6$ cells from each strain were analyzed in each assay.

[b] The results reported in each column were obtained in independent experiments.

the fraction of permissive cells would be too small to allow the detection of amplified mutants in the wild-type population, but sufficient to allow their detection in the *DNA-PKcs*-deficient population in which the probability of gene amplification is higher.

When we extended our studies on PALA resistance to MEFs at later passages in culture (between passage 52 and 62), we found that the cellular response to PALA was different from that detected at earlier passages and was very similar to that described for established cell lines (4, 8, 16). On PALA treatment, cells did not block in the cell cycle; most of the cells rapidly died and detached from the dish. In agreement with this observation, evidence was obtained that p53 is defective in these cells. In fact, large amounts of nuclear p53, indicative of the presence of a mutant protein, were detected by indirect immunofluorescence (40) in the *DNA-PKcs*-deficient MEF 26 (data not shown). In the late-passage MEFs, PALA-resistant clones arose with higher frequency than in mid-passage MEFs, and, in particular, their frequency was higher in the *DNA-PKcs*$^{-/-}$ lines than in wild-type cells. These results indicate that, after crisis, MEFs undergo a continuous evolution leading to the acquisition of several different characteristics, among which is a higher amplification ability, which is further increased in the absence of DNA-PKcs.

Taken together our results allow us to conclude that the deficiency in DNA-PKcs is not sufficient to make primary cells permissive for gene amplification, whereas in immortalized cell lines, it increases the probability of gene amplification. We are tempted to speculate that the permissivity for gene amplification depends on mutations in genes deputed to the control of genome integrity, like, *e.g.*, *p53*, whereas the probability that gene amplification occurs in a permissive background is related to the efficiency of the DSB repair mechanisms as well. Broken DNA ends that are not repaired correctly could undergo promiscuous recombination events triggering gene amplification.

It has recently been reported that proteins involved in the NHEJ, like DNA-PKcs, Ku70, Ku80, XRCC4, and DNA ligase IV, play an essential role in maintaining genome stability and in preventing tumor formation (41–44). Spontaneous chromosome aberrations have been observed in fibroblasts from mice in which the genes coding for these proteins were ablated by homologous recombination. This suggests that, during normal cellular growth, DNA breaks are generated that, if not repaired correctly, lead to chromosome anomalies. Moreover, mice carrying double mutations in a NHEJ gene and in *p53*, such as *Ku80*$^{-/-}$*p53*$^{-/-}$ and *XRCC4*$^{-/-}$ *p53*$^{-/-}$ mice, develop multiple lymphomas at an earlier age compared with *p53*$^{-/-}$mice (42, 43). These lymphomas show several chromosome aberrations and often harbor amplification of the *c-myc* gene. This body of evidence suggests that the NHEJ proteins be added to the list of "genome guardians" (45).

Our data, showing the involvement of *DNA-PKcs* in gene amplification, one of the most important manifestations of genome instability in tumor cells, confirm and underline the role of this protein in the maintenance of genome integrity.

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