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Low frequency of spontaneous rearrangements during plasmid incorporation in CHO-KI mutant cells defective in DNA repair

Marcin Kruszewski,
Hanna Kruszewska,
Masahiko Mori,
Kiyomi Eguchi-Kasai,
Hiroko Inaba,
Iwona Zakierska,
Isamu Hayata

Abstract pLrec plasmid DNA was introduced into Chinese hamster cell lines defective in DNA repair (Pa13, Pb4, xrs6) and the parental CHO-K1 cell line. Clones with stable integrated plasmid were isolated and integrity of the incorporated DNA was checked by Southern blotting and PCR. Intact pLrec plasmid was found in 11% of the isolated CHO-K1 clones. In contrast, intact plasmid copies were found in 68.8%, 50%, 35.7% clones of Pa13, Pb4 and xrs6 cell lines, respectively. We conclude that certain DNA repair defects may facilitate intact plasmid integration. The higher frequency of integration of the intact vector into the genome of cell lines defective in DNA repair as compared to the parental cell line points to two possibilities, not mutually exclusive: (1) these cells possess a mechanism that facilitates the process of plasmid incorporation and hence, plasmid DNA is incorporated into the genome before extrachromosomal recombination takes place; (2) the vector is inserted into a less recombination prone site in the genome.

Key words CHO-K1 • Pa13 • Pb4 • plasmid incorporation • xrs6

M. Kruszewski✉
Department of Radiobiology and Health Protection,
Institute of Nuclear Chemistry and Technology,
16 Dorodna Str., 03-195 Warsaw, Poland,
Tel.: +48 22/ 811 07 36, Fax: +48 22/ 811 15 32,
e-mail: marcinkr@orange.ichtj.waw.pl,
and M.Skłodowska-Curie Memorial Cancer Center
and Institute of Oncology,
5 W. K. Roentgena Str., 02-781 Warsaw, Poland

H. Kruszewska
Drug Institute,
33/35 Chełmska Str., 00-725 Warsaw, Poland

M. Mori, K. Eguchi-Kasai, H. Inaba, I. Hayata
National Institute of Radiological Sciences,
9-1, 4-Chome, Anagawa, Inage-ku, Chiba 263, Japan

I. Zakierska
Polfa-Łódź S.A.,
43/55 Drewnowska Str., 91-002 Łódź, Poland

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Introduction

Transfection of DNA into mammalian cells is a widely used technique. However, information is scarce on the actual events involved in DNA integration and on factors that may influence the integration fidelity. Although certain techniques are now available for targeted DNA transfection [11, 14, 24], integration seems to be predominantly random [13, 17]. Several studies have demonstrated that foreign DNA incorporated into mammalian genome is highly unstable and many rearrangements may occur in the integrated DNA as well as in its vicinity [2, 9]. On the other hand, one study indicates that incorporation of viral DNA into mouse cell DNA can occur directly without additional rearrangement [5]. It has also been found that incorporation of plasmid DNA into the DNA of primary human fibroblasts takes place without major rearrangements; nevertheless, rearrangements of the cell DNA at the integration site were found in one clone [17].

Although, little if any sequence similarity is necessary for DNA integration, most integrations [13] occur primarily in regions of repetitive DNA [12, 17]. Repetitive DNA sequences have also been suggested to be a preferred site of retrotransposon insertion [22]. It is possible that unstable regions prone to retrotransposon insertion are hotspots for integration of the transfected DNA. Short deletions found in the cell DNA at the plasmid integration sites indicate that integration might occur by a mechanism similar to that used for repair of spontaneous or gamma ray-induced strand breaks [17].

In this study plasmid DNA has been introduced into three Chinese hamster lines defective in DNA repair, and into

the parental CHO-K1 cell line. Mutant xrs6 cells are defective in DNA double strand break (DSB) repair. The defect is due to the lack of DNA-dependent protein kinase (DNA-PK) activity as a result of mutation in the XRCC5 gene. The gene codes the Ku80 subunit of DNA-PK [3]. Pa13 and Pb4 mutant cell lines have been isolated on the basis of sensitivity to reactive oxygen species (ROS)-generating agent, plumbagin [7]. These cell lines also are sensitive to another ROS generating agent, X-rays, and to DNA crosslinking agents. Although the nature of their sensitivity to DNA damaging agents is under investigation, it is speculated that Pb4 cells are exclusively defective in a pathway of DNA crosslink repair, whereas Pa13 may be defective in DNA repair pathway that is shared between DNA dsb and crosslink repair (for discussion see [7]).

The aim of this work has been to select the transfected clones of the above listed cell lines and to examine the integrity of the incorporated plasmid DNA with the use of PCR.

Materials and methods

Cell culture

All cell lines used in this study, Pb4 and Pa13, xrs6 and CHO-K1, were grown in HAM F12 medium supplemented with 7% FCS and antibiotics [7]. DNA construct, cell transfection and selection of single copy transfectants. The pLrec plasmid used in this study was developed by Dr M. S. Meyn [10]. The plasmid consists of two tandem copies of the bacterial *lacZ* gene separated by a mammalian selective marker – neo gene (Fig. 1), as well as pBR322 *ori* and *amp* sequences, HSV *tk* promoter, SV40 promoter/enhancer and poly(A) signal. The left-hand copy (3'*lacZ*) of the *lacZ* gene has an insertion of 8-bp Xho linker in the HpaI site, yielding a frame-shift mutation that makes *lacZ* gene dysfunctional. The right-hand copy (5'Δ*lacZ*) of the *lacZ* gene has a truncated 5'end part of the native *lacZ* gene [10].

SceI linearized plasmid was used for transfection. Cells were transfected by electroporation as described in [8] and plated on plastic Petri dishes. Two days after transfection the culture medium was changed to a medium supplemented with 500 μg/ml of G418 as the selective agent. The selective medium for Pb4 cells also contained 0.2% Bacto agar (Difco) because these cells did not attach firmly enough to the culture dishes. To prevent a spontaneous loss of the selective marker, the clones were maintained in the selective medium throughout the study. Two weeks after transfection individual clones were isolated (one per dish) and expanded.

The isolated clones were studied by Southern blotting in order to ensure that only one copy of the plasmid was incorporated. In brief, genomic DNA was digested with NaeI or HindIII and transferred to Hybond N(+) membrane (Amersham) according to the standard procedures [23] and manufacturer's recommendations. Blots were probed with a 3.8 kb HindIII-BamHI fragment of the pLrec plasmid that contained *lacZ* gene. NaeI and HindIII have only one recognition site in the pLrec plasmid sequence (Fig. 1). Clones whose genomic DNA, when digested with NaeI or HindIII and analysed by Southern blotting exhibited respectively only two or one fragment containing *lacZ* gene, were considered to be single copy transfectants. Additional digestions with BamHI, ScaI and XhoI were performed to ensure integrity of the plasmid.

Two pairs of PCR primers were designed to amplify regions specific for the left- and right-hand copy of the *lacZ* gene adjacent to the selective neo gene. Primer A: 5'-AAT CCG CCG TTT GTT CCC AC was specific for the left-hand copy of *lacZ* gene. Primer B: 5'-TGC GAA GTG GAC CTG GGA CC was specific for the right-hand copy of *lacZ* gene. Primer C: 5'-ACG ATC GCG CTG CAC CAT TC was specific to both copies *lacZ* genes. The primer pair A-C amplified the 1.01 kb fragment of the left-hand *lacZ* gene, whereas the primer pair B-C amplifies the 1.14 kb fragment of the right-hand *lacZ* gene (Fig. 1). Both PCR products could be resolved when electrophoresed through a 0.7% agarose gel (Fig. 2). Duplex PCR with all three primers was prepared in a programmable thermal cycler (Perkin Elmer PJ2000) for 30 cycles, each consisting of 30 s of denaturation at 94°C, annealing at 69°C and polymerisation at 72°C. Standard PCR conditions were as follows: 0.5 μg of genomic DNA was amplified enzymatically in a 10 μl reaction mixture which included 1 pg of each primer, 200 μM of each dNTP, 0.5 unit of DNA polymerase (AmpliTaq™ conjugated with TaqStart™ Antibody, Clontech, USA).

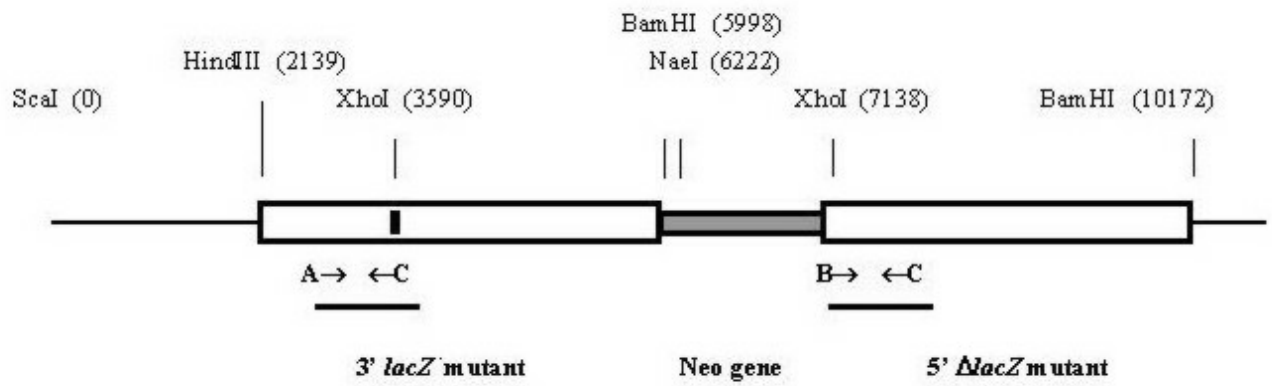

Fig. 1. Schematic diagram of pLrec plasmid (11444 bp) linearized with ScaI. Position of HindIII, XhoI, BamHI and NaeI sites are indicated. Vertical black bar corresponds to the position of Xho I linker that makes 3'*lacZ* gene copy not functional. Horizontal bars correspond to the position of AC and BC PCR products.

Table 1. Southern blot analysis of isolated clones.

Cell line	Single copy not rearranged	Single copy rearranged	Multicopy	Total analysed
CHO-K1	24	4	4	32
Pa13	15	1	1	17
Pb4	16	2	2	20
xrs6	14	0	1	15

Statistical analysis

The term "rearrangement" is used hereafter to denote that incorporated plasmid underwent after transfection some processing that changed its original structure. Although the actual nature of these processes has not been studied, at least two distinct mechanisms have to be taken into consideration. These are true rearrangements, i.e. changes in DNA sequence and distal degradation of plasmid ends.

Differences in the distribution of the intact and rearranged plasmids among the studied cell lines were evaluated by Yates corrected Chi-square, V-square and Fischer exact tests using commercially available software (Statistica v.5.1, StatSoft Inc., Tulsa, USA). Since 2×2 table frequencies were low in most cases, differences were considered as significantly important if all three tests gave a positive answer.

Results

Plasmid rearrangements resulting from incorporation into the mammalian cell genome were studied in the parental CHO-K1 and in the mutant cell lines with various defects in DNA repair (see Introduction). Linearized pLrec plasmid was transfected into the studied cells and the transfected clones were isolated. Integrity of the incorporated plasmid was checked using appropriate PCR primer pairs specific for plasmid DNA at the left and right side of the selective marker (as indicated in Fig. 1).

Table 1 shows the results of Southern blot analysis of the isolated clones. Extra bands that could be expected in case of multiple insertions were found in four CHO-K1 clones, one Pa13 clone, two Pb4 clones and one xrs6 clone. These clones were considered to be multicopy transfectants. Since the presence of several copies of pLrec plasmid could distort the results of PRC analysis (false positive results) these clones were excluded from further analysis. Some clones, however, exhibited extra bands, which were distinct in size or number from the bands that could be expected from multiple pLrec plasmids incorporations. These clones were considered to be rearranged.

Clones of single copy transfectants were analysed by PCR to see whether both copies of *lacZ* gene were present in the insert. In spite of the positive Southern blot signal, several clones revealed lack of PCR-detectable copies of *lacZ* gene. This discrepancy might be due to the different size of probed fragments. While S/B analysis covered entire 3.8 kb HindIII-BamHI fragment of plasmid DNA, the PRC reaction was warranted by the presence of short DNA sequences homologous to the PCR primers. Hence, small rearrangement or terminal deletions that impaired PCR reaction had no influence on the S/B analysis.

No correlation has been found between the amount of DNA used for transfection and the number and position of the *lacZ* gene copies in the clones isolated from transfected CHO cells (Table 2). However, it seems that in the case of Pa13 and Pb4 cell lines higher DNA concentrations gave higher frequencies of plasmid rearrangements, probably due to recombination between the plasmids before or after integration. The number of rearranged clones revealed by PCR was higher than the number of rearranged clones revealed by Southern blot analysis. This discrepancy might be due to small rearrangements and/or a partial distal degradation of the transfected DNA that, as mentioned above, did not affect Southern blot analysis but affected PCR.

Figure 3 shows the overall frequency of rearrangements resulting from incorporation of plasmid DNA into the genomes of the examined CHO cell lines. After transfection with pLrec plasmid we isolated 28 CHO-K1, 16 Pa13, 18 Pb4 and 14 xrs6 clones. Intact pLrec plasmid was only found in 3 single copy clones (11%) of CHO-K1 cells. In contrast, the intact plasmid was found in 11 clones of Pa13 (68.8%), 9 clones of Pb4 (50%) and 5 clones of xrs6 cells (35.7%) (Fig. 3). Statistical analysis revealed that the frequency of rearrangements of the pLrec plasmid in the parental CHO-K1 cell line significantly differed from those in all the other cell lines ($p<0.05$). No significant differences in this respect were found between Pa13, Pb4 and xrs6 cell lines (Table 2).

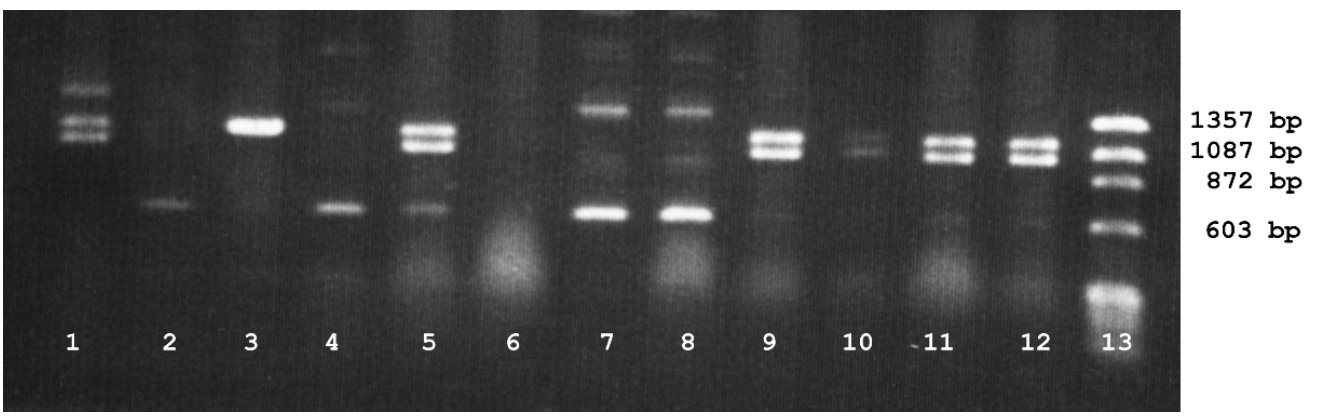

Fig. 2. PCR analysis of pLrec transfected clones. PCR amplification was performed as described in Materials and methods. Intact plasmid gave two bands (1.01 kb and 1.14 kb) lines 1, 5, 9, 10, 11, 12. Rearranged plasmid gave one band (line 3) or none (lines 2, 4, 6, 7, 8). Size markers (ΦX174 DNA digested with HaeII) were run in the line 13.

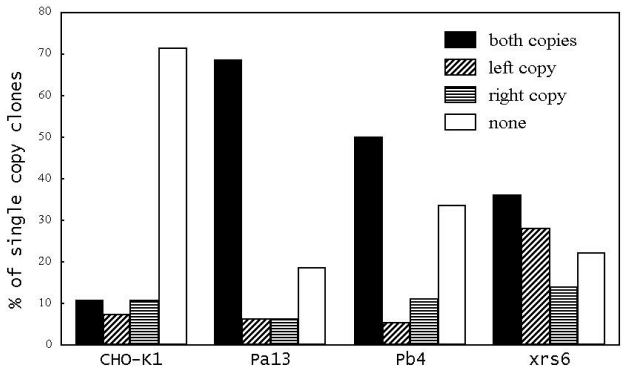

Fig. 3. Number and position of copies of *lacZ* gene in CHO-K1 cell lines transfected with pLrec plasmid. All clones were isolated from separate Petri dishes and contained only one copy of pLrec plasmid. The presence of both copies of *lacZ* gene in isolated clones was tested by PCR, as shown in Fig. 2 (cf. Materials and methods).

Discussion

The events leading to integration of nonhomologous plasmid DNA into a mammalian host chromosomal DNA are not well understood. Usually, a supercoiled plasmid used for transfection has to be linearized by a nuclease within the cell. To prevent random linearization, a previously linearized plasmid can be used. The linearized plasmid molecule is susceptible to further attack of nucleases, which may degrade most of the plasmid molecules before they have the opportunity to integrate into the genome. Another cellular enzymatic activity that is involved in foreign DNA integration into host cell genome is that of nuclear ligase. A number of DNA ligases are involved in maintaining integrity of the mammalian genome. It is likely that these enzymatic activities would act on all DNA molecules with "free ends", including linear plasmid molecules.

The general agreement is reached nowadays that primary integration of foreign DNA into the mammalian cell genome proceeds via recombination between the foreign DNA and homologous or nonhomologous sequences in the host genomic DNA [13, 17]. The cell lines that are described in this paper have been also used to study homologous recombination (Kruszewski *et al*., in preparation). No correlation has been found when the frequency of spontaneous homologous recombination was compared with the frequency of rearrangements of the integrated plasmids. The frequency of spontaneous homologous recombination differed among the studied cell lines. The highest frequency was observed in xrs6 cells, whereas in CHO-K1 cell line was 100 times lower. Interestingly, in Pb4 cell line the frequency of spontaneous homologous recombination was similar to that in CHO-K1 cells (Kruszewski *et al*., in preparation). In contrast, the rearrangement frequency was much higher in Pb4 cells than in CHO-K1 cells. This supports the previous findings that nonhomologous (illegitimate) recombination is the major mechanism of foreign DNA integration into the mammalian genome [16]. Even when the integrating DNA is homologous to the native target site the integrated plasmid sequences are, in the majority of cases, randomly distributed. Richard *et al*. [21] report that only 2% of over 400 distinct integration events of vector containing a repetitive LINE-1 element occurs through a homologous recombination mechanism. These data indicate that factors other than the cell's capability to carry out

Table 2. Number and position of *lacZ* gene copies in the single copy transfectants isolated clones as a function of amount of DNA used for transfection.

Amount of DNA	Copies of *lacZ* present	Cell line			
		CHO-K1	Pa13	Pb4	xrs6
1 µg	both copies	1 (16.5%)	6 (100%)	3 (50%)	–
	left copy	0 (%)	0 (0%)	0 (0%)	–
	right copy	1 (16.5%)	0 (0%)	0 (0%)	–
	none	4 (67%)	0 (0%)	3 (50%)	–
3 µg	both copies	ND	5 (50%)	3 (75%)	ND
	left copy	ND	1 (10%)	0 (0%)	ND
	right copy	ND	1 (10%)	0 (0%)	ND
	none	ND	3 (30%)	1 (25%)	ND
5 µg	both copies	1 (7%)	ND	ND	5 (36%)
	left copy	1 (7%)	ND	ND	4 (29%)
	right copy	2 (14%)	ND	ND	2 (14%)
	none	10 (72%)	ND	ND	3 (21%)
25 µg	both copies	1 (12.5%)	ND	3 (37.5%)	ND
	left copy	1 (12.5%)	ND	1 (12.5%)	ND
	right copy	0 (0%)	ND	2 (25%)	ND
	none	6 (75%)	ND	2 (25%)	ND
Total	intact	3 (11%)	11 (69%)*	9 (50%)*	5 (36%)*
	not intact	25 (89%)	5 (31%)*	9 (50%)*	9 (64%)*

– no transfectants obtained, ND – not determined, * – significantly differ from CHO-K1 cell line (by Yates corrected Chi-square, V-square and Fischer exact tests, $p < 0.05$).

homologous recombination may also play an important role in foreign DNA integration into mammalian cell genome. Since it is suggested that integration might occur by a mechanism similar to that used for repair of DNA strand breaks [13], it is possible that some malformation of DNA structure, e.g. DNA DSBs, may promote the integration of foreign DNA into the genome. DNA DSBs are known to stimulate gene targeting in embryonic stem cells [26] or integration of a foreign DNA into mouse zygotes [25]. DNA DSBs promote also intrachromosomal homologous recombination [1]. It has been also shown that pretreatment with genotoxic agents that generate DNA DSBs, such as topoizomerase II inhibitors or bleomycin, enhance the rate of plasmid integration [4, 18]. Hence, the presence of the more persistent "free DNA ends" in cells defective in DNA damage repair may also facilitate a foreign DNA integration. On the other hand, such persistent free ends may faciliate DNA degradation by exonucleases. This factor seems to be essential in the case of xrs6 cells; these cells lack Ku80 protein that can bind the free ends of DNA and thus, probably protects them against degradation. Because of this defect, xrs6 cells exhibit excess degradation of extrachromosomal DNA [15]. Indeed, we needed 5 times higher DNA concentration during electroporation to get transfected xrs6 clones, as compared to the parental cell line. The excessive degradation of the extrachromosomal DNA in xrs6 cells also was reflected in the pattern of rearrangements. True rearrangements were found only in 4 (16%), 1 (20%), 2 (22%) and 0 (less than 11%) of the total (25, 5, 9, 9) incomplete ("not intact") plasmids isolated from CHO-K1, Pa13, Pb4 and xrs6 cells, respectively (Tables 1 and 2). These data point to the distal degradation of the plasmid ends as a major process that affects the transfection efficiency in mammalian cells.

The assumption that malformations of DNA structure may promote foreign DNA integration is supported by the fact that nonhomologous DNA introduced to the normal mammalian cells is preferentially integrated into the chromosomal fragile sites [20], repetitive DNA elements [12, 13] or unstable regions [16]. Integration of a foreign DNA into these sites usually is accompanied by a high frequency of rearrangements and chromosome instability [9, 16, 19]. On the other hand, DNA of these regions is highly unstable and a high frequency of DNA rearrangements was found in mouse satellite DNA [2], fragile sites [6] and in integration sites [16] in native, not trasfected cells. Hence, it is not clear whether genomic instability and the high frequency of rearrangements are associated with the integration event itself or with the specific structure of these regions. In this context, it is of interest that we found a lower frequency of rearrangements of the genome-integrated vector in cell lines defective in DNA repair as compared to the parental repair competent cell line. Even in the case of xrs6 cells that suffer from an enhanced degradation of DNA in cell milieu (see above), the majority of integrated plasmids (36%) were intact, indicating that in spite of the enhanced degradation of the transfecting DNA, some plasmids can integrate correctly. It is plausible to assume that the mechanism leading to incorporation of the foreign DNA into the genome of the repair-defective cells is different from that in repair-competent cells. Alternatively, DNA may be incorporated into different sites, which are not recombination prone. It is not yet possible to discern between these possibilities and further investigations are warranted.

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