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7. Molecular Radiobiology: Plasmid pMTa4 as a Tool for Studying Effects of *g*-Radiation *in vitro* and *in vivo*

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Abstract Radiation induced damage to DNA molecule is known to be critical. Base or nucleotide damage, strand breaks and clusters of damages are some of the important lesions produced by radiation in a DNA molecule. However, molecular intricacies of such events are not clearly understood obscuring molecular mechanisms of radiation-induced damages and their repair. A well-defined plasmid DNA construct, pMTa4, along with selected restriction endonucleases have been utilized in this line of study to elucidate some events that follow irradiation *in vitro* and *in vivo*. The results reveal that GC-rich nucleotide sequences of pMTa4 were preferably affected by ^{60}Co γ-irradiation *in vitro* generating premutagenic lesions in a non-random way. The results also show that even under repair non-permissive conditions repair activities went on *in vivo*, albeit with very low fidelity causing misrepair. Under repair permissive conditions, in contrast, high fidelity repair was observed. These observations are likely to have significant bearing on our present understanding of inherent radiosensitivity and genome instability. Use of a simple plasmid DNA molecule can potentially provide clear insight into molecular consequences of irradiation on DNA and should be exploited further.

Introduction

Plasmid DNA, besides being the foundation of biotechnology, can be a potentially very useful tool for studying radiation-induced DNA damages. Its simple form, small size and known nucleotide (NT) sequence provides a convenient possibility of qualitatively and quantitatively determining the DNA lesions. In conjuncture with endonucleases, especially restriction endonucleases (RE), plasmid DNA can become an extremely powerful tool in analyzing the manifestation of DNA damage following irradiation. In recent past, this approach has been employed in some such studies of radiation-induced DNA damages. Limited studies using plasmids have provided new insights in studies of repair, mutation induction, alterations of DNA structures by alkylation, drug-DNA-interaction and in measurement of DNA strand breaks [19, 1, 22, 9]. RE is highly NT specific in its action and cleaves DNA only if is not modified at its restriction site. Therefore,

by analyzing the fragments of a plasmid produced by a RE, one can not only deduce the order of the segments within the original DNA molecule, but also possibly study the alterations or modifications of the NT sequence that might have been induced by radiation. Further, generation of single strand breaks (SSB) and double stranded breaks (DSB) in a DNA molecule can also be very conveniently monitored and analyzed.

Base or NT damage is one of several distinct lesions produced by radiation in a DNA molecule. It involves chemical alterations of the bases that can result from severance of a side group from the base or damage to the ring structure itself. A large number of base products have been identified and reported in literature [33]. Ionizing radiation has also been shown to induce clustered DNA damage containing oxidized purines and pyrimidines and abasic sites [29]. In recent years, studies on identification of such damage have been carried out to define specific molecular damages in an attempt to understand the non-random biological response to radiation. In these investigations repair enzymes like formamidopyrimidine-DNA glycosylase or endonuclease III have been used. These enzymes were found to be useful in characterization of DNA damage induced by reactive oxygen species and photosensitizers in cellular and cell-free systems [7] besides by γ-radiation [16]. The damages usually get converted to SSB, which can be then measured by several methods like nick translation [5], gel electrophoresis [21], or comet assay [26]. The use of RE offer several additional advantages due to its very high NT specificity and could be a potential tool for elucidation of molecular mechanism of radiation induced DNA damage [12].

Using a well-defined plasmid construct, designated as pMTa4, and selected RE, this piece of work was designed in an attempt to study influence of NT sequence on DNA damage induced by γ-radiation and its repair in a cellular (*in vivo*) as well as an acellular (*in vitro*) condition.

Materials and Methods

E. coli culture and preparation of plasmid DNA:

XL 1 strain of *E. coli* bacterium harboring a 6173 bp plasmid construct, designated as pMTa4 (kindly provided by Prof. C.H. Schroeder, DKFZ, Heidelberg), was grown at 37 °C in LB medium containing ampicillin (50 μg ml^{-1}). After overnight culture, the plasmids were isolated by High Pure Plasmid Isolation kit (Boehringer Mannheim, Germany). The yield and purity of the isolate were determined by measuring the absorption ratio A_{260}: A_{280}.

Irradiation source and experimental protocols:

E. coli culture (2 ml of overnight culture) on ice or an aqueous solution of isolated pMTa4 (41.25 μg ml^{-1}) at room temperature was γ-irradiated in a ^{60}Co Gamma chamber 900 (~0.2 Gy sec^{-1}) to accumulate doses of 10, 20 and 30 Gy (cellular or *in vivo* condition) or 30, 60, 120 and 240 Gy (acellular or *in vitro* condition), respectively. The irradiated samples were stored on ice until analysis. From the irradiated *E. coli*, plasmid DNA was isolated similarly either immediately after irradiation (repair non-permissive) or after a post-irradiation repair incubation

of 60 minutes at 37 °C (repair permissive). The plasmid preparations were analyzed further.

Analysis of plasmid DNA

Irradiated and non-irradiated pMTa4 samples (~1 μg) were run on a one percent agarose gel electrophoresis using TAE buffer for monitoring the SSB and DSB induced by irradiation. In addition, the pMTa4 preparations were fragmented with different RE such as, *ACC I Hae II, Ksp I, Nci I, Bgl II, Dra I, Pvu II, Bgl I* and *Hinf I,* under appropriate conditions (Boehringer Mannheim, Germany), and analyzed by gel electrophoresis. Ethidium bromide stained bands on the gels were seen through an UV transilluminator and scanned (GS-690 Imaging Densitometer).

Strand break analysis and determination of fragment sizes

The relative amounts of DNA in each of the plasmid topological form resulting from irradiation were determined by scanning. The sum of the pixel intensities of the DNA bands were calculated using Molecular Analysts^{TM}/PC Windows software, version 1.5. The approximate sizes of the DNA fragments generated by RE were determined using the same software. Based on the Rf values of the different fragments, their molecular weights (sizes; bp) were obtained against known λ DNA double digest marker through third order cubic regression.

Results

Effect of γ-radiation on pMTa4 *in vitro:*

Figure 1 depicts the gel photograph and plot revealing the changes in the three

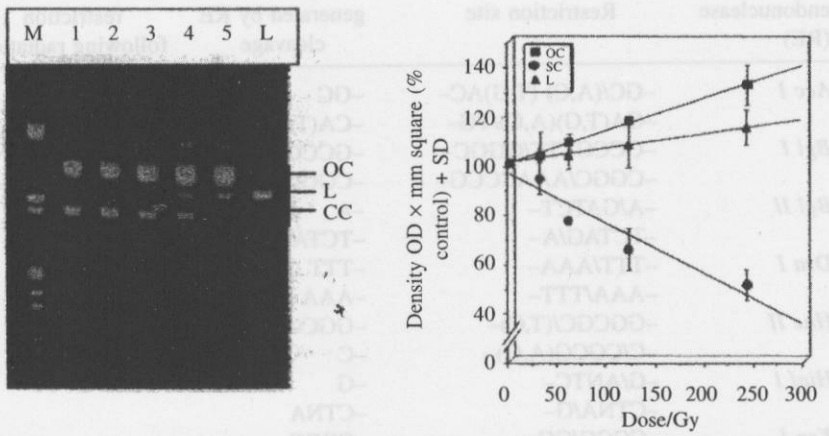

Figure 1. Agarose gel electropherogram of pMTa4 showing its supercoiled (CC), open circle (OC) and linear (L) topological forms (left). Lane 1 shows the control plasmid (unirradiated) while lanes 2 to 5 show *in vitro* γ-irradiated plasmid with 30, 60, 120 and 240 Gy, respectively. M represents λ DNA double digest marker while L represents *Nco I* linearized non-irradiated pMTa4. Densitometric analysis of 3 topological forms of pMTa4 is shown in the right panel; data points are from two independent experimental sets and represent the mean ± SD.

topological forms of pMTa4 in its non-irradiated (control) state and that follow irradiation. It indicates dose dependent reduction in the quantity of supercoiled (SC) form, while dose dependent increase in the quantities of open circular (OC) and linear (L) forms were observed.

Effect of γ-radiation on the pMTa4 restriction sequences for different RE

Table I lists different restriction endonucleases used in this study along with their restriction sequence and NT sequence at fragment-end in pMTa4 plasmid. Figure 2 shows the restriction fragmentation patterns of non-irradiated (control) pMTa4 generated by *Hae II* and *Nci I*, and that after exposure of the plasmid to varying doses of γ-radiation. The irradiated plasmid restricted with *Hae II* and *Nci I* showed additional slow migrating bands or large DNA pieces on the gel which were entirely absent in the non-irradiated control. The formation of the extra fragments (Fig. 2A lanes 2 through 5) remained unchanged even at the maximum dose of 240 Gy for *Hae II*. The *Nci I* restriction showed formation of four distinct additional bands (Fig. 2B). The band intensities increased with radiation dose exhibiting dose dependence (Table 2), as revealed by densitometric quantification. In contrast, the fragmentation pattern of pMTa4 generated by other RE did not show any difference between irradiated and non-irradiated DNA samples (results not shown).

Table 1. Characteristics of the different restriction endonucleases (RE): RE were selected depending on their restriction sites: blunt- and staggered-ends as well as their NT composition in the site.

Restriction endonuclease (RE)	Restriction site	Fragment-end generated by RE cleavage	Effect on the restriction following radiation
Acc I	–GC/(A,C) (T,G)AC–	–GC	–
	–CA(T,G)(A,C)/TG–	–CA(T,G)(A,C)	
Bgl I	–GCCGTTT/GGGC–	–GCCGTTT	–
	–CGGC/AAACCCG–	–CGGC	–
Bgl II	–A/GATCT–	–A	–
	–TCTAG/A–	–TCTAG	
Dra I	–TTT/AAA–	–TTT	–
	–AAA/TTT–	–AAA	
Hae II	–GGCGC/(T,C)–	–GGCGC	+
	–C/CGCG(A,G)–	–C	
Hinf I	–G/ANTC–	–G	–
	–CTNA/G–	–CTNA	
Ksp I	–CCGC/GG–	–CCGC	–
	–GG/CGCC–	–GG	
Nci I	–CC/(C,G)GG–	–CC	+
	–GG(G,C)/CC–	–GG(G,C)	
Pvu II	–CAG/CTG–	–CAG	–
	–GTC/GAC–	–GTC	

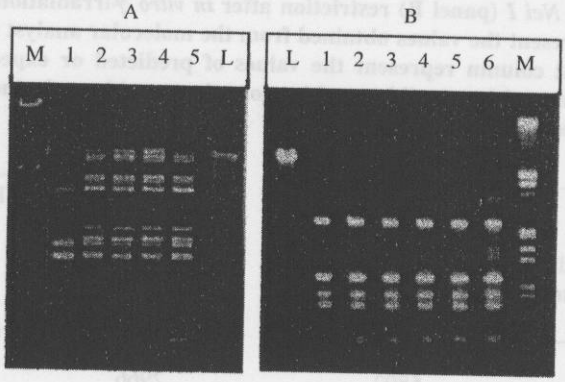

Figure 2. **Agarose gel electropherogram of pMTa4 restricted with *Hae II* (A) and *Nci I* (B). Restriction with *Hae II* was carried out after 0 (control), 30, 60, 120 and 240 Gy of *in vitro* γ-radiation (lanes 1 through 5), respectively. Restriction with *Nci I* was done following 0 (control), 15, 30, 60, 120 and 240 Gy of *in vitro* γ-radiation (lanes 1 through 6), respectively. M represents λ DNA double digest marker while L represents *Nco I* linearized non-irradiated pMTa4.**

Table 2. **Densitometric quantification of the four extra fragments produced by *Nci I* after γ-irradiation (see Fig. 2B). The four fragments in order of decreasing size are denoted as F1, F2, F3 and F4. Data points are from a single experimental set.**

Dose (Gy)	Density OD x mm^{-1} (% control)			
	F1	F2	F3	F4
0 (control)	100	100	100	100
15	193.85	186.49	181.14	173.33
30	228.07	213.79	206.20	200.74
60	230.99	242.52	231.26	225.18
120	260.23	254.59	241.43	234.81
240	296.78	281.60	294.54	288.64

Analysis of *Hae II* and *Nci I* generated fragmentation pattern after γ-irradiation

Table 3 shows the sizes of *Hae II* and *Nci I* generated extra fragment that were calculated using MA software in comparison to the theoretical sizes that should be generated. Out of the several possible theoretical combinations of fragments generated by *Hae II*, the values falling nearest to the observed sizes calculated by software were taken as their actual fragment size (Table 3, A). An approach similar to that of the *Hae II* fragment analysis was employed in the identification of the extra fragments produced by for *Nci* (Table 3, B).

Effect of γ-radiation on the pMTa4 *in vivo*

The results of analysis of pMTa4 isolated from irradiated *E. coli* cells (without and with post-irradiation repair incubation) are shown in Figure 3. In absence of

Table 3. pMTa4 size analysis of the extra fragments generated by *Hae II* (panel A) and *Nci I* (panel B) restriction after *in vitro* γ-irradiation. Left columns represent the values obtained from the molecular analyst software, while right column represent the values of predicted or expected fragments generated by possible combinations that would result due to incomplete digestion.

A		B	
Approximate fragment Size (bp) calculated by MA software γ-radiation	Predicted actualy size in bp with their possible fragment comb. inations	Approximate fragment size (bp) calculated MA software γ-radiation	Predicted actual size in bp with their possible fragment combinations
6356	6173	3208	3272
6055	5802	2966	2901
5605	5589	1537	1514
4300	4234/4326	1387	1387
4126	3864/3856		
3721	3539		
2462	2588		
2261	2316/2303		

repair incubation, both OC (representing SSB) and L (representing DSB) forms of pMTa4 increased in a radiation dose dependent manner (Fig. 3 – minus). In addition, progressively slow migrating bands of DNA were observed between OC and L bands generating a 'ladder' (Fig. 3) The intensity of bands in the 'ladder' showed dose dependence. Both SSB and DSB were efficiently repaired upon repair incubation accompanied by almost total fading of the 'ladder' (Fig. 3–plus) The plasmid did not show any difference in restriction patterns by *Hae II* and *Nci I* before and after irradiation (results not shown).

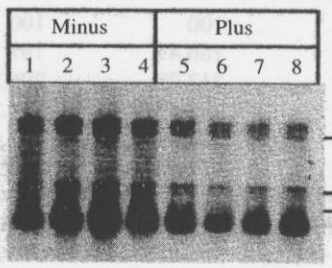

Figure 3. Agarose gel electropherogram of pMTa4 isolated from *E. coli* (*in vivo*) following irradiation accompanied with repair non-permissive (minus; lanes 1 through 4)) and permissive (plus; lanes 5 through 8) conditions. Lanes 1 and 5 show pMTa4 isolated from unirradiated *E. coli* cells while lanes 2 to 4 and 6 to 8 show the pMTa4 following 10, 20 and 30 Gy irradiation, respectively.

Discussion

The four NT of DNA molecule (G, A, T and C) are constant targets of damage by chemical processes such as methylation, depurination, deamination and

oxidation. Induction of DNA damage by radiation is mostly caused by oxidation via ·OH mediated indirect effect [33]. The 5-C and 6-C of the pyrimidines and the 4-C and 8-C of purines are the most probable sites known for the attack resulting into a plethora of modified bases [8]. Such damages induce predominantly SSB. Fig. 1 shows the changes in the pMTa4 after exposure to γ-radiation representing SSB and DSB. The increase in the OC form at higher doses of radiation is an indicator of induction of SSB. ·OH has been reported to be much more effective in the production of SSB than ·H and e^-_{aq} that are formed when DNA is γ-irradiated in an aqueous solution [16]. Since in this study pMTa4 was irradiated in an aqueous solution (*in vitro*), it will be reasonable to assume that induction of SSB was primarily due to the water derived ·OH and to a lesser extent ·H [16], and some direct effect. This is in good agreement with data on dose dependent response to radiation [20, 32]. On the other hand, in cellular condition (*in vivo*), radiation induced significant increase in quantities of both OC and L forms suggesting that both SSB and DSB were being induced in pMTa4 when *E. coli* cells were γ-irradiated (Fig. 3-minus). When the irradiated *E. coli* cells were incubated at 37 °C for 60 minutes permitting repair prior to pMTa4 isolation, there was significant drop in both OC and L forms suggesting that most damages could be repaired in this situation (Fig. 3–plus). It has to be noted that aqueous pMTa4 (*in vitro*) had no such repair possibility (Fig. 1).

The restriction capability of RE was observed to vary after *in vitro* plasmid DNA was exposed to γ-rays (Fig. 2). While efficiency of the plasmid restriction by *Acc I, Ksp I, Bgl II, Dra I, Pvu II, Bgl I* and *Hinf I,* used in this study, remained unchanged (not shown), it was significantly reduced for *Hae II* and *Nci I* (Fig. 2A and B) after γ-irradiation. Such sub-optimal restriction can result when either the restriction site is shielded by DNA binding chemical compound [9] or due to possible radiation-induced base modification [24]. The former possibility can be ruled out for the experimental set-up used in this investigation since it lacks any other component which may interact with the plasmid DNA in an aqueous solution. The partial restriction by *Hae II* appears to be dose independent as the intensities of fragments generated after the lowest dose of 30 Gy did not change even after the dose of 240 Gy (Fig. 2A, lanes 2 through 5). Result from *Nci I* restriction (Fig. 2B; lanes 2 through 6) also followed a similar pattern. The increase in the intensity of the additional fragments (Table 2) may, on the other hand, indicate a dose dependent manifestation of effect. The non-random generation of fragments due to sub-optimal restriction suggest that all restriction site for *Hae II* and *Nci I* in pMTa4 were not equally susceptible to the effect by radiation. For such sub-optimal restriction to occur, it is likely that pMTa4 underwent certain specific alteration or modifcation in selected *Hae II* and *Nci I* restriction sites such that only these sites became resistant to RE. This assumption logically requires substantiation by looking for the specific NT composition of the restriction sites for *Hae II* and *Nci I* in order to explore the possibilities of identifying changes that may be responsible for such partial fragmentation. A closer look into *Hae II* and *Nci I* restriction site revealed its GC-richness (Table I). Unlike the others, except *Ksp I,* it was notable that *Hae II* and *Nci I* produced 100 percent GC staggered-ended DNA pieces (Table I). The other RE that generated

either staggered- or bluntended pieces did not show the GC-motif feature. The NT composition in the flanking region around the restriction sequence of *Hae II* and *Nci I* too indicated high GC content [13].

Although the type of modified base was not determined in this study, a wide range of radiation-induced bases damages are known [30]. Of the several types, 8-oxoguanine (OG) has been identified as the major product [15]. Fuciarelli *et al.* [10] found that when DNA was γ-irradiated in oxic condition, high amount of OG was formed. Under conditions where the $^{\cdot}OH$ are the only damaging species, formamidopyrimidine glycosylase sensitive sites were formed in high amount [16] indicating a high formation of guanine product. Deamination or demethylation of C nucleotide is also known to occur following irradiation. Since the NT sequences effected are GC-rich, the resistance to restriction after irradiation may be due to modification of G and/or C nucleotides. Regardless of the type and nature of modification, the observations indicate that GC-rich motif was more frequently modified or damaged upon interaction with radiation.

In the cellular (*in vivo*) situation, there was a dose dependent increase in OC as well as L forms of pMTa4 after irradiation and there was apparent repair of most strand breaks under post-irradiation repair permissive condition (Fig. 3). Under repair non-permissive condition, it also showed a 'ladder' due to progressively slow migrating DNA bands between OC and L bands, which faded significantly upon repair incubation. Further, the plasmid DNA did not show any resistance to *Hae II* and *Nci I* restriction (not shown) in sharp contrast to the *in vitro* results (Fig. 2). These results suggest some very interesting insight into induction of radiation damage to pMTa4 DNA. At first, partial repair activity must have been on even under repair non-permissive condition (on ice), which could repair, at least, the base modifications efficiently since no resistance to RE restriction was observed *in vivo*. Secondly and as expected, under repair permissive condition, both SSB and DSB were repaired which was not the case in absence of repair incubation (Fig. 1). Thirdly, there was formation of a 'ladder' in repair non-permissive condition, which faded significantly upon repair incubation.

The cause of 'ladder' formation needs further explanation. It is known that plasmid DNA in its native conformation is supercoiled (CC form) and migrates fast on agarose gel. This band becomes significantly slow migrating upon relaxation of the conformation (OC form) caused by nicks or SSB, which reduces its extent of supercoiling. The L form, caused by DSB, migrates in between the two bands (see Fig. 1). The 'ladders' represents a collection of plasmid DNA molecules with intermediate states of supercoiling resulting in progressively slow migrating bands on agarose gel. Since there are indications of partial repair activity under repair non-permissive condition, it can be assumed that some of the radiation-induced nicks or SSB were also being repaired. However, the fidelity of this repair seemed to be poor, falling short of complete repair. This may cause wrong joining of DNA strands (or misrepair) creating different degrees of supercoiling in different pMTa4 molecules. This could lead to collection of pMTa4 molecules with different degrees of supercoiling and produce the 'ladder'. Apparently some critical components of repair machinery were absent in repair non-permissive condition. Upon repair incubation, the critical components also became available,

perhaps due to induction, leading to high fidelity of repair exhibited by fading of the 'ladder'.

While confirmation of this hypothesis may involve some additional work, there are indirect supports available in literature. Large numbers of studies have reported high mutagenecity at GC rather than AT [23, 3]. Mutation studies using MSH2 deficient mice have indicated a greater percent of mutation in GC than AT [25, 27]. Chemically modified G or C causes mispairing, where an error-prone polymerase frequently introduces a wrong base opposite these NT. OG preferentially pairs with A and causes G to T transversion [14]. 5-methyl C in a CpG motif can undergo deamination to produce C to T transition [26]. C could also spontaneously deaminate to produce uracil [17] or be oxidized to 5-hydroxycytidine [31] leading to a C to T transition. With such evidences of GC-vulnerability to radiation, it is likely that radiomodified GC nucleotides would form important premutagenic lesions. This indication also points that clusters of GC in the DNA molecule may very likely form hotspots for radiation-induced damages. While further detailed investigation would be required, it opens up a likely possibility that inherent radiosensitivity and genome instability may be at least partly determined by the GC-richness of NT sequence in the DNA. Similarly, GC-rich sequences, referred to as 'CpG islands', have been reported to occur frequently in many human genes [6, 11, 28]. The results from this piece of work suggests that most genes (rich in CpG islands), regardless of its state (transcribing or non-transcribing), might be effected by radiation with equal probability. This perhaps partially explains the high radiosensitivity of humans and current observation of induction of almost uniform damage in active or inactive genes [2, 18].

While more work, especially with mutants of repair system in *E. coli,* is currently on, it is clear that plasmid DNA has all potentials to become highly useful in studies in the domain of molecular radiobiology. The simple systems provides deep and clear insight into the molecular consequences of irradiation on DNA and should be exploited to elucidate molecular intricacies of radiation induced damages.

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