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Mealybug as a Model for Studying Responses to High Doses of Ionizing Radiation

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1. Introduction

Biological effects of exposure to different doses of ionizing radiation (IR) range from death within two days in case of high doses (>30 Gy) to various types of diseases such as cancers (due to gene mutations) in case of long-term exposure to sub-lethal doses (8-30 Gy). Further, IR-induced mutations when transmitted to the progeny can cause various types of cancer. At molecular level, IR results in double-strand breaks in DNA leading to loss of genetic material and cell death. To address the damaging consequences of exposure to radiation, recent research in radiation biology is focused on biological responses to IR in organisms showing resistance to high doses of ionizing radiation. For example, *Deinococcus radiodurans*, a bacterium that can survive radiation doses as high as 5,000 Gray can be potentially used for remediation of waste sites mixed with radioactive materials. However, investigation of radiation response in a higher eukaryotic organism that is resistant to high doses of ionizing radiation is desirable to offer treatment regimens to individuals exposed to IR in events of nuclear accidents. We propose here that mealybug (*Planococcus lilacinus*, Insecta; Coccoidea; Hemiptera) is an ideal model organism for studying responses to high doses of ionizing radiation. Among different organisms whose sensitivity to IR investigated so far, mealybug has been shown to tolerate radiation doses as high as 1100 Gy. Following exposure to such high doses, this insect has an extraordinary ability to survive and produce fertile offspring. Cytogenetic analysis of the embryos produced by irradiated parents showed that a number of tiny fragments of irradiated chromosomes are capable of metaphase alignment and anaphase segregation, suggesting that the centromere property is distributed throughout each chromosome. Taken together, mealybug has very efficient DNA repair machinery that ensures proper healing of double strand breaks induced by ionizing radiation. Because of the central role of telomeres in chromosome end-healing, the nature, distribution and maintenance of telomeric repeats in the mealybug were recently studied. Telomeric repeat has been shown to occur interstitially as well as at the ends of the mealybug chromosomes and telomerase which maintains these repeats is constitutively active. At radiation doses higher than 100 Gy, telomerase activity decreases, suggesting that telomerase-independent mechanisms may play a role in maintenance of telomeres. Based on these studies we

propose that in the mealybug, features such as diffuse centromere, constitutive telomerase activity and interstitial telomeres are components of machinery that confers resistance to high doses of ionizing radiation.

2. Biological effects of ionizing radiation

Ionizing radiation (IR) is a stream of high energy particles (such as electrons, protons or α -particles) or any of the short wavelength electromagnetic radiation (such as X-rays, γ -rays or ultraviolet rays) that is capable of removing an electron from an atom or a molecule in the substance through which it passes. The most common biological effect of IR is ejection of an electron from water molecule, resulting in the formation of highly reactive species:



In the reaction above, the dot before a radical indicates unpaired electron and * indicates an excited species. These free radicals are highly reactive and can alter other molecules in the cell (Reactor Concepts Manual, NRC). DNA is one such important target which can also be directly ionized by IR. Cells experiencing IR-induced DNA damage respond in different ways, depending on the extent of damage. In cases where the extent of DNA damage is minimal, the cells successfully repair the damaged DNA and as a result, there is no recognizable biological effect. At the other extreme is when the damage is severe, leading to a failure in the cells' ability to repair the DNA and as a result, the cells undergo programmed cell death or apoptosis so that a potential damage from the entire tissue is prevented. In the middle of these two extremes are detectable DNA mutations that occur when repair results in a non-lethal DNA mutation that can be transmitted to the daughter cells produced during successive cell divisions and these mutations may induce cancer subsequently. A fourth possible consequence of exposure to IR is when cells experience irreparable DNA damage leading to replication and transcriptional errors that predispose their daughter cells to premature aging and cancer (reviewed in Maynard et al., 2009).

2.1 Acute and chronic exposure to ionizing radiation

Similar to the effects at cellular level, the biological effect of IR at the organismal level is also dependent on the severity of the dose received and the duration of exposure. Acute radiation exposure is usually for a short period of time but involves high dose of exposure as in case of nuclear explosions (instantaneous flashes), exposures because of handling of highly radioactive resources, accidents in laboratories or manufacturing units and high medical doses that could be either intentional or accidental. It is relatively more difficult to determine the effects of chronic exposure to IR and the results have been conflicting. For instance, based on studies on an Indian population that is exposed naturally to thorium-containing monazite mineral in sand (1.5-3.0 roentgen per year), Kochupillai et al. (1976) reported higher prevalence of abortions, mental retardation, Down syndrome, chromosomal and chromatid abnormalities. However in a separate study, no significant difference was observed between the high-level exposed population and control population (Cheriyen et al., 1999). In addition, a recent report on mitochondrial DNA sequence analysis revealed that there is an increased rate of mitochondrial DNA mutations in the 'exposed population' than in the control population (Forster et al., 2002). Clearly more detailed studies are required to be done to conclusively associate genomic effects of chronic exposure to IR.

Exposure to high doses of IR has been associated with leukemia and cancers of thyroid, breast, bladder, colon, liver, lung, esophagus, ovarian, multiple myeloma, and stomach. There have also been suggestions that ionizing radiation exposure may cause cancers of prostate, nasal cavity/sinuses, pharynx and larynx, and pancreas (ICRP, 1991).

Because of difficulties in interpretation of consequences of chronic exposure to low doses of IR (below 0.01 Gy), researchers depended mainly on models of the process by which lower doses of radiation causes cancer. Data on effects of exposure to IR at low but higher than the normal background levels gave mixed results on carcinogenesis or transgenerational effects, but were consistent with estimates of risk based on atomic bomb survivors (Cardis et al., 2005). These results suggest that there is a small increase in chances of developing cancers among individuals exposed to low levels of IR for extended periods of time. A collection of reports on consequences of chronic and acute exposures to IR led to the linear dose-response model that suggests an increase in the risk to develop cancer with any increase in dose, however small. Agencies such as Nuclear Regulatory commission, Environmental Protection Agency, National Academy of Sciences Committee lent support to the linear no-threshold model. According to this model, IR from background levels natural and man-made resources) can cause cancer in ~ 1% of the exposed population (BEIR VII report).

2.2 DNA damage due to exposure to IR

IR-induced DNA damages include single strand and double strand breaks that are repaired by different DNA repair machineries. Single-strand breaks are repaired by the same enzymes that mediate base-excision repair. These include DNA glycosylase, AP-endonuclease, DNA polymerase β and DNA ligase. Here, damaged nucleotides are excised and repaired using undamaged sequence from the complementary strand. Double-strand breaks are particularly hazardous because they create 'sticky' chromosome ends that cause

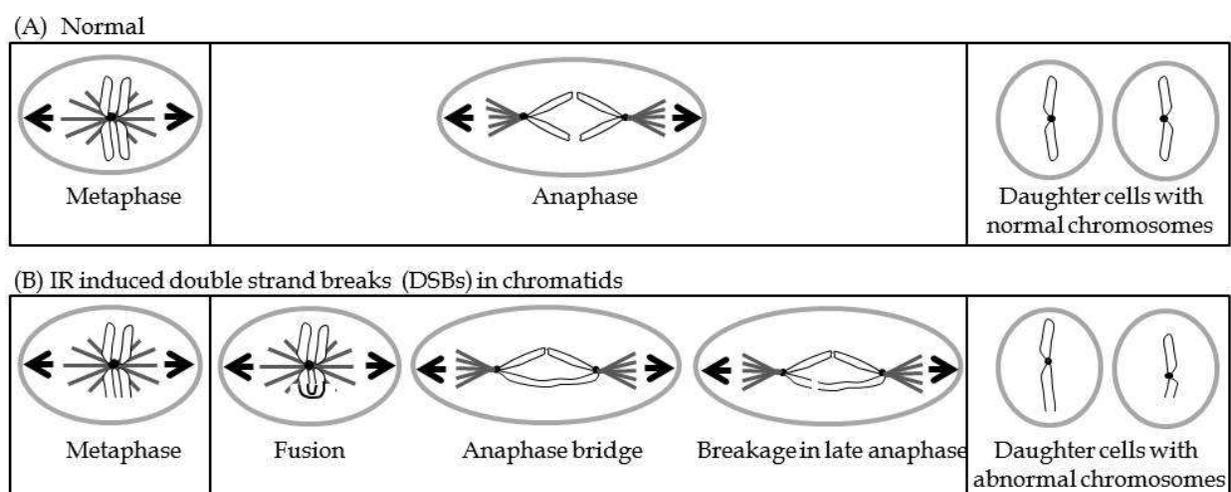


Fig. 1. IR-induced double strand breaks cause fusion-bridge-breakage-fusion cycles. IR induced double strand breaks in sister chromatids result in sticky ends that fuse and form anaphase bridges. Because of the force generated by spindle fibres, the sister chromatids in the bridge break unevenly leading to abnormal chromosomes at the end of cell division. One chromosome would have extra copies and the other would have lost a portion of the parent chromosome. Note that the daughter chromosomes also have sticky ends and participate in the fusion-bridge-breakage cycles again. These cycles result in complex genomic rearrangements.

fusion-bridge-breakage-fusion cycles (McClintock, 1938; reviewed in deLange, 2005; Fig.1) and lead to genomic rearrangements leading to cell death or transformation. DNA double-strand breaks can be repaired by non-homologous end joining (NHEJ; Moore & Haber, 1996), microhomology-mediated end joining (MMEJ; Liang et al., 1996) and homologous recombination (HR; Lundblad & Blackburn, 1993). In NHEJ, single strands of DNA ends showing microhomology are recognized and joined. If these overhangs are compatible, repair is usually accurate. A protein called Ku is essential for NHEJ. Other proteins such as DNA ligase IV, XRCC 4 participate in the process. MMEJ also depends on microhomology among the overhangs of the broken fragments of the chromosomes to be joined but these microhomologies are 'revealed' by chromosome end resection and therefore MMEJ usually results in deletion of the DNA sequences between microhomologies. In Homologous recombination, on the other hand uses the undamaged DNA sequence that is identical or highly related to the DNA sequence that has been damaged. Sister chromatids (after G2 phase) or a homologous chromosome (in G1 phase) serve as templates for recombination-mediated repair. Recombination-mediated repair uses the same machinery that is used in the process of meiotic recombination. Homologous recombination has been shown to be the process involved in the recovery from exposure to high doses of ionizing radiation in the radio-resistant bacterium *Deinococcus radiodurans* (see below).

3. Telomeres and sensitivity to IR

Unlike the genomes of bacteria which are circular with no 'exposed ends', eukaryote genome exists as linear chromosomal DNA molecules. This linearity also poses a problem in the completion of DNA replication, leaving a 20 nucleotide 'unfinished gap' in the lagging strand. Successive cell divisions lead to progressive shortening of the 3' ends of the chromosome ends. These ends of eukaryotic chromosomes are therefore 'exposed' and vulnerable to exonucleolytic attack yielding sticky ends which in turn mediate genomic rearrangements described above. In order to prevent loss of genetic material, and genomic rearrangements and, completion of replication at the chromosome ends, the ends of eukaryotic chromosomes are organized as telomeres (reviewed in Blackburn, 1991). Telomeres are specialized structures of DNA and proteins that have been characterized in a number of organisms. The nature of telomere-associated DNA sequences and the proteins associated with the telomeres are described in the next section. Telomere shortening was shown to result in increased sensitivity to ionizing radiation (Haber 1998; Ahmed & Hodgkin 2000). There is increasing evidence that enzymes involved in DSB repair also have a role at telomeres. Telomeres were shown to act as storehouses for proteins involved in DNA DSB repair (Ku70, Ku80, Sir2, Sir3, Mre11-RAD50-NBS1 complex) (Zhu et al. 2000). These proteins are relocated from telomeres to the damaged sites induced by IR (Martin et al. 1999; Mills et al. 1999). Genetic studies in *Caenorhabditis elegans*, yeast, and mice have shown that telomere shortening results in increased sensitivity to IR although DSB repair is unaffected (Haber 1998; Ahmed & Hodgkin 2000; Goytisolo et al. 2000). These results suggest a relationship between telomere length and sensitivity to IR.

3.1 Telomere structure

In several eukaryotes, telomeres are composed of simple tandem repeats at the end with complex subtelomeric repeats occurring next to these repeats (Blackburn 1991; Villasante et

al. 2008; Kojima et al. 2002). However, there are exceptions to this general rule. In *Bombyx mori* where the insect-specific pentameric TTAGG repeat was first discovered (Okazaki et al. 1993), the telomeric repeats are interrupted by non-long terminal repeat retrotransposons such as SART and TRAS (Fujiwara et al. 2005), HeT-A, TAHRE, and TART elements are found at the chromosome termini in *Drosophila* (Mason & Biessmann 1995; Biessmann et al. 1992; Levis et al. 1993) and complex long tandem repeats in the chromosome termini of *Chironomus* (Lopez et al. 1996). Studies in various insect species have suggested that TTAGG repeat is an ancestral motif in Arthropoda and that the TTAGG repeats might have originated from the TTAGGG repeats, also found at vertebrate telomeres (Sahara et al. 1999; Frydrychova et al. 2004; Vitkova et al. 2005). In some organisms, telomeres form structures called telomere loops or T-loops containing double-stranded DNA and a 3' single-stranded G-rich DNA overhang that is important for telomere maintenance (Fig. 2). The single-stranded DNA forms a circle and then disrupts a part of the double-stranded DNA in the telomere for base pairing with the C-rich strand, forming a displacement loop or D-loop (Griffith et al. 1999). The T-loop is held together by shelterin complex containing TRF1, TRF2, and POT1 that recognize TTAGG repeats and another three proteins TIN2, TPP1, and RAP1 that interconnect the first three proteins (reviewed in deLange 2005).

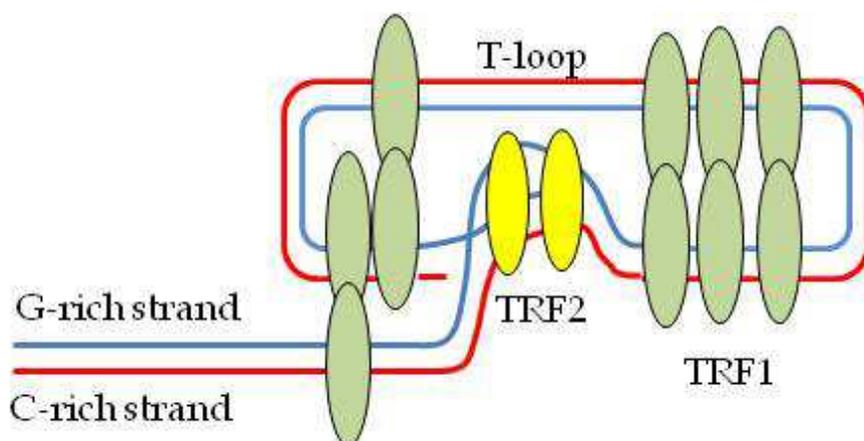


Fig. 2. Structure of telomeric DNA. The G-rich overhang (blue) forms a circle and then disrupts base pairing with the C-rich strand forming a displacement loop. The T-loop is held together by the shelterin complex containing proteins such as TRF1 and TRF2.

3.2 Telomere maintenance

In almost all organisms containing simple tandem repeats, telomeres are maintained by telomerase, an enzyme with reverse transcriptase property by which it mediates chromosome end-healing (reviewed in Blackburn 1991). This enzyme is induced soon after cells are exposed to ionizing radiation (reviewed in Crompton 1997). In humans, the telomerase contains two units each of reverse transcriptase (TERT), telomerase RNA (TR or TERC), and dyskerin (DKC1) (Cohen et al. 2007). TERT folds with TERC and forms a mitten-like structure that allows the enzyme to wrap around the chromosomes to extend the telomeric repeats. Using the telomerase RNA that is complementary to the telomeric repeat unit, the reverse transcriptase subunit synthesizes multiple telomeric repeats by continuous elongation and translocation steps to produce a long single stranded DNA containing telomeric repeats (Fig. 3).

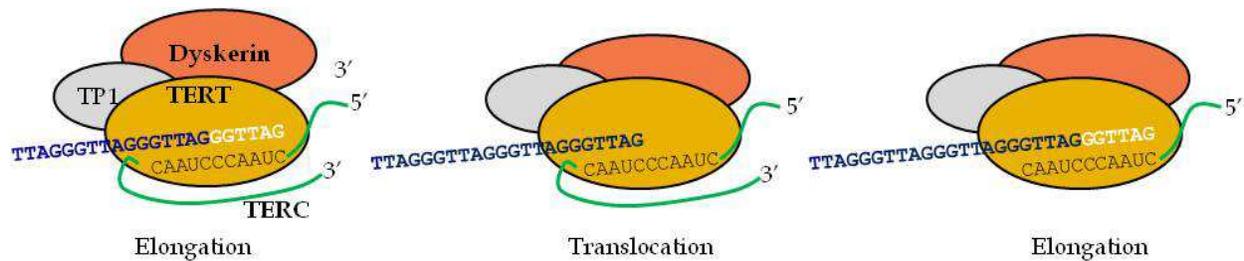


Fig. 3. Mechanism of elongation of telomeric repeats by telomerase. The RNA component of the telomerase pairs with the DNA to be extended and serves as a template for the elongation step. After elongation, there is a translocation step that brings the TERC to pair with the newly synthesized repeat unit to enable elongation again. Alternating elongation and translocation steps allows addition of multiple telomeric repeats.

In addition to telomerase-dependent mechanisms of chromosome end-healing, eukaryotic cells possess end-healing mechanisms that are telomerase-independent and are collectively called as alternative lengthening of telomeres (ALT). ALT, which was first demonstrated using telomerase-null yeast mutants, has been shown to be dependent on RAD52 that encodes a homologous recombination protein (Lundblad & Blackburn 1993). Such recombination-mediated chromosome end-healing mechanisms have also been demonstrated in human cells (Dunham et al. 2000). Additional studies have shown that in some cells lacking telomerase, there was a rapid increase as well as a decrease of telomeric repeats, suggesting that ALT may involve recombination at the telomeres (Murnane et al. 1994; Bryan et al. 1995). Unequal telomeric sister chromatid exchange and homologous recombination involving a damaged chromosome and an intact homologous chromosome have been proposed as the two mechanisms for recombination-mediated ALT (reviewed in Cesare & Reddel 2010). Unequal sister chromatid exchange results in two daughter chromosomes: one with longer telomeric repeats and another with shorter repeats. Preferential segregation of the chromosome with the longer telomeric repeats during cell division is expected, extending the cellular lifespan of one of the two daughter cells. Whereas there is no sufficient evidence for this model yet, down-regulation of homologous recombination promoting proteins such as the MRN complex (Jiang et al. 2005) and the SMC5-6 complex (Potts & Yu 2007) has been shown to result in telomere shortening, lending support to the homologous recombination model of ALT. NHEJ of two broken chromosomes, which does not involve recombination, is another telomerase independent mechanism that can restore chromosome integrity. In support of the evolutionary conservation of NHEJ as a means to maintain telomere integrity, a number of proteins involved in double-strand break DNA repair have also been shown to be involved in telomere maintenance (reviewed in Slijepcevic & Al-Wahiby 2005).

Several lines of evidence suggest that telomerase is absent in *Drosophila* species (reviewed in Pardue & DeBaryshe 1999). Telomerase activity is also not detectable in *B. mori* (Sasaki & Fujiwara 2000), a Lepidopteran insect, in which pentameric TTAGG repeats occur at the chromosome ends (Okazaki et al. 1993). Recent studies on cloning and expression analysis of the telomerase reverse transcriptase subunit from *B. mori* suggest that the reverse transcriptase gene is poorly transcribed and may be the reason for undetectable levels of its enzymatic activity (Osanai et al. 2006). It is however suggested that telomerase activity, even weak, is necessary for maintenance of telomeres in the domestic silk worm. In *Drosophila*, telomere length appears to be maintained by retrotransposition involving HeT-A, TAHRE, and TART elements (Biessmann et al. 1992). A role for proteins involved in doublestrand

break (DSB) repair in telomere maintenance has been suggested from findings in which inactivation of proteins such as Ku, ATM results in telomere shortening (Boulton & Jackson, 1998; Hande et al. 2001). From the description above, it is evident that insects have evolved different mechanisms of maintenance of telomeres and telomerase-dependent mechanisms are not indispensable for maintenance of genomic integrity.

4. Model organisms for studying response to ionizing radiation

As noted above, exposure to a radiation dose of > 10 Gy (1000 rads) results in 95-100% death in humans within two days to two weeks of exposure depending on the radiation dose. Similar levels of exposure (> 12Gy) is also lethal in mice. From the limited information available in other mammals, we can consider that a radiation dose of 20-30 Gy can result in certain death in most mammals. In order to obtain insights into biological responses to IR and mechanisms that may confer resistance to exposure to radiation a variety of in vitro models using cell lines and different organisms showing resistance to high doses of IR have been sought. One example is *Deinococcus radiodurans*, an extremophilic bacterium that can survive exposures of IR up to 5000 Gy with no loss of viability (Murray, 1992). Analysis of *D. radiodurans* response to IR showed that there are 4-10 copies of the genome per cell in this bacterium.

Following exposure to IR, the bacterium is able to repair the DNA in 12-24 hours by reconnecting some chromosome fragments by a process called single-stranded annealing followed by homologous recombination (In: Clark et al. 2009). As a result, the entire genome is restored. Indeed homologous recombination and micro-homology mediated DSB repair is also observed in eukaryotes. It is however desirable that a eukaryotic multicellular organism that shows increased resistance to ionizing radiation is identified to study mechanisms conferring radiation resistance or a better response to ionizing radiation. Table 1 shows the lethal doses of IR in different eukaryotes. It is evident from the table that insects show a much higher level of resistance to IR than mammals. Among the three insect species shown, *Braconidae* show highest resistance. However, the nature of response has not been investigated either genetically or cytogenetically. Although the lethal dose has not been determined, *Planococcus citri* seems to be as resistant as *Braconidae*. It may also be noted that the radiation doses investigated in case of this insect are much higher than *Drosophila*.

Species	Lethal Dose (in Gy)	Taxonomic group
<i>Homo sapiens</i>	10	Mammals
<i>Mus musculus</i>	12	Mammals
<i>Daenio rerio</i>	40	Fish
<i>Drosophila melanogaster</i>	640	Insects
<i>Planococcus citri</i>	1200*	Insects
<i>Braconidae</i>	1800	Insects
<i>Caenorhabditis elegans</i>	800*	Worms
<i>Escherichia coli</i>	60	Bacteria
<i>Deinococcus radiodurans</i>	15000	Bacteria
<i>Thermococcus gammatolerans</i>	30000	Archaeobacteria

Table 1. Minimum lethal dose of IR in different organisms. * denotes that at the indicated dosage, the organism survives and reproduces. Information on lethal dose (expected to be higher) is not known.

4.1 Mealybug as a model for studying responses to IR

The mealybugs (*Planococcus* species) are lecanoid coccids (Coccoidea; Homoptera) whose high degree of resistance to ionizing radiation has been investigated at both the cytological and genetical levels (Chandra 1963a, b). After exposure to radiation doses as high as 1200 Gy (120,000 rads), male insects not only survive but mate and reproduce to produce viable progeny. Cytological examination of the progeny obtained from such paternal irradiation revealed very small chromosome fragments that appear to retain their individual chromosome identity such as replication, metaphase alignment, and anaphase segregation. No apparently significant loss of genetic material was observed. These results suggest that the mealybug genetic system possesses very efficient DNA repair machinery that ensures protection of the many tiny chromosome fragments produced by irradiation and prevents from fusing to one another via rapid healing of broken chromosome ends. Because of these extraordinary features we propose that mealybug is an attractive model system to study mechanisms underlying resistance to high doses of IR.

4.2 Telomeric repeats, their distribution and telomerase activity in unirradiated and irradiated mealybugs

Because telomeric repeat lengths are critical for double-strand DNA repair and shortening of telomeric repeats is associated with increased sensitivity to IR, in a recent study, we examined the nature and distribution of telomeric repeats in the mealybug *Planococcus lilacinus* (Mohan et al. 2011).

In order to isolate telomeric sequences from the mealybug, a polymerase chain reaction was conducted on genomic DNA using insect telomeric repeat (TTAGG)-specific primers (Fig.4A.). The PCR products obtained were cloned and sequenced to analyze the telomeric

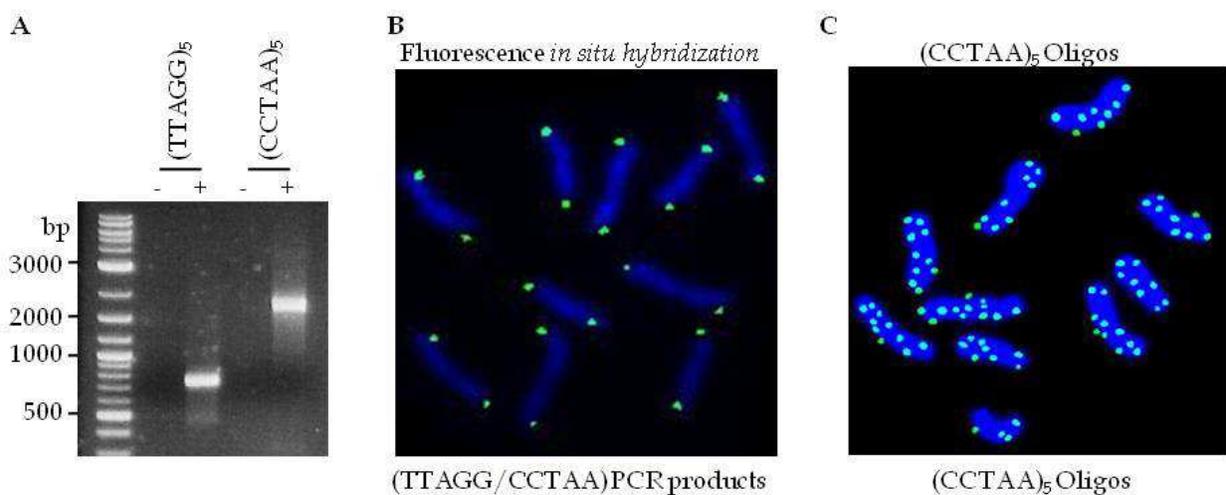


Fig. 4. Amplification and detection of TTAGG telomeric repeats in the mealybug *Planococcus lilacinus*. (A) Amplification of TTAGG sequences using (TTAGG)₅ and (CCTAA)₅ oligos. (B) Hybridization of metaphase chromosomes to a cloned TTAGG repeat sequence. The probe was labeled with digoxigenin (DIG)-UTP and hybridized to the chromosomes. Hybridization signals were detected using DIG-signal amplification kit. (C) Primed in situ hybridization of mealybug chromosomes with (CCTAA)₅ oligos. The oligos were hybridized to the chromosomes and an extension reaction was carried out with Taq DNA polymerase and DIG-labeling nucleotide mix. Signals were detected as in (B).

repeat-associated sequences. Two categories of sequences were identified: (1) Tandem repeats of TTAGG ranging from 100-200 repeats and (2) sequences containing 5 to 15 TTAGG repeats at the ends with a highly similar low-copy unrelated repeats of 400-450 bp. The unrelated low copy number repeats are interrupted by a variable number of TTAGG repeats. This sequence analysis suggested that whereas TTAGG repeats primarily occur at the ends of mealybug chromosomes, some of the repeats occur interstitially and are associated with certain low-copy repeats. Consistent with these two possibilities, FISH (fluorescent in situ hybridization) with a cloned TTAGG sequence (containing 96 TTAGG repeats) mainly hybridizes to the ends of mealybug chromosomes (Fig.4B) whereas primed in situ hybridization (PRINS) with TTAGG-specific oligos shows labeling at interstitial locations of the chromosomes (Fig.4C). Taken together, these results reveal a unique distribution of the telomeric repeats in the mealybug chromosome - they are distributed both terminally and interstitially. Because of this distribution, the interstitial telomeric repeats in this insect belong to the type I category in which short direct telomeric repeats interrupt precisely repetitive elements. Estimation by slot blot hybridization of different amounts of genomic DNA with (TTAGG)₅ probes suggest that the TTAGG repeats constitute ~0.03% of the mealybug genome (220 Mb; Mohan et al, 2002) and there are about 1275 TTAGG repeating units per each chromosome end. From these data, it may be noted that there is no significant difference in the length of telomeric repeats in the mealybug and other eukaryotic species and that resistance to high doses of IR is not due to unusual telomeric repeat lengths in this insect. To study the distribution of telomeric sequences in mealybug cells after exposure to IR, a series of experiments were performed in which gravid females (carrying embryos) were exposed to different amounts of IR, ranging from 30 Gy to 300 Gy. Whereas the number of chromosomes/fragments detectable under the microscope is relatively less at 30Gy (average number of fragments is about 14), at 300 Gy, the number of chromosome fragments is high and not estimable because of their tiny size. However at a radiation dose of 70 Gy, we were able to obtain an average chromosome/fragment number of 20 per metaphase, providing an adequate number of DSBs (about 10) per cell. This number of newly generated chromosome ends therefore provides a reasonable platform to study the distribution of TTAGG telomeric repeats in the context of IR and their terminal versus interstitial occurrence.

Analysis of chromosome fragments recovered after two days of exposure to a radiation dose of 70Gy by FISH showed that many of the chromosome ends induced by IR are labeled with the (TTAGG)₆ probe (Fig. 5A). Whereas in unirradiated chromosomes there are 20 labeled ends, in irradiated chromosomes with an average of 40 chromosome ends, 29 ends were labeled with the probe. It may be noted that at least some of the unlabeled chromosome ends may have extended TTAGG repeats but these repeats may not be long enough to be detectable by the FISH probe. Taken together, these results suggest that there is an efficient generation of TTAGG repeats at the recovered ends following IR.

To gain more insights into the origin of the telomeric signals in irradiated chromosomes, PRINS was performed on them. As shown in Fig. 5B, more than 95% of the ends of the irradiated chromosomes were labeled by PRINS. When the results in Fig. 5A and 5B are taken together, it is recognizable that there is an overlap in the signals between FISH and PRINS at half of the ends of irradiated chromosomes. It is also recognizable that there is a decrease in the number of interstitial signals in the irradiated chromosomes. Taken together, these results suggest that there is an extension of TTAGG repeats near the interstitial telomeric repeats that occur close to the DSBs induced by IR. It may be noted that there are

no additional telomeric or centromeric probes available at present to further clarify on the involvement of interstitial telomeric repeats in the generation of FISH-detectable signals at the radiation-induced chromosome ends.

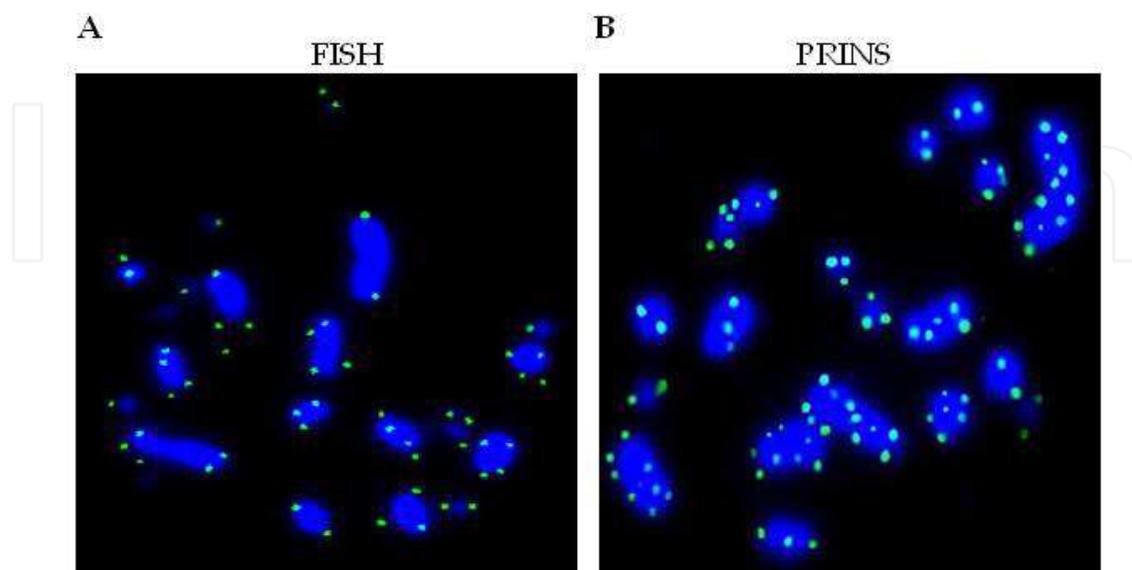


Fig. 5. Distribution of TTAGG telomeric repeat sequences in irradiated mealybug chromosomes. The chromosomes/chromosome fragments were subjected to FISH (A) and PRINS (B) as described in Fig.4.

4.3 Telomerase activity in unirradiated and irradiated mealybugs

For identification of components of machinery that maintains telomeric repeats in the mealybug, cell extracts were prepared from unirradiated and irradiated insects and telomerase assays were performed using TTAGG oligonucleotides as templates (Fig.6A and 6B). RNase-treated cell extracts were used as negative controls. As an internal size control, an end-labeled 30-bp oligonucleotide was used. The results shown in Fig. 6A suggested that in both unirradiated and irradiated insects, cell extracts produced ladder-like extensions of the TTAGG oligonucleotides which is suggestive of telomerase activity both prior to and after irradiation. However, we observed approximately six times more telomerase activity in irradiated insects than in the unirradiated insects. As one would expect for telomerase activity which is RNA-dependent, RNase-treated cell extracts did not produce detectable extensions. We also compared the levels of telomerase activity in insects exposed to different doses of ionizing radiation (30-300 Gy; Fig. 6B). Whereas there is an increase in the telomerase activity with an increase in the radiation dose up to 60 Gy, at higher levels there was a reduction in telomerase activity, and at 300 Gy, the telomerase activity approaches to the levels that are lower than that the unirradiated insects. Taken together, our results suggested that unlike in other organisms where telomerase activity ceases in adulthood, the mealybug telomerase is constitutively active. Such constitutive activity of telomerase was also reported in cockroaches, which are also radiation-resistant (Sasaki and Fujiwara 2000). It may be also noted that lower telomerase activity at doses >60 Gy highlights the possibility of occurrence of chromosome end-healing mechanisms that are telomerase-independent such as MMEJ-, NHEJ- and homologous recombination-mediated repairs (discussed in the section below).

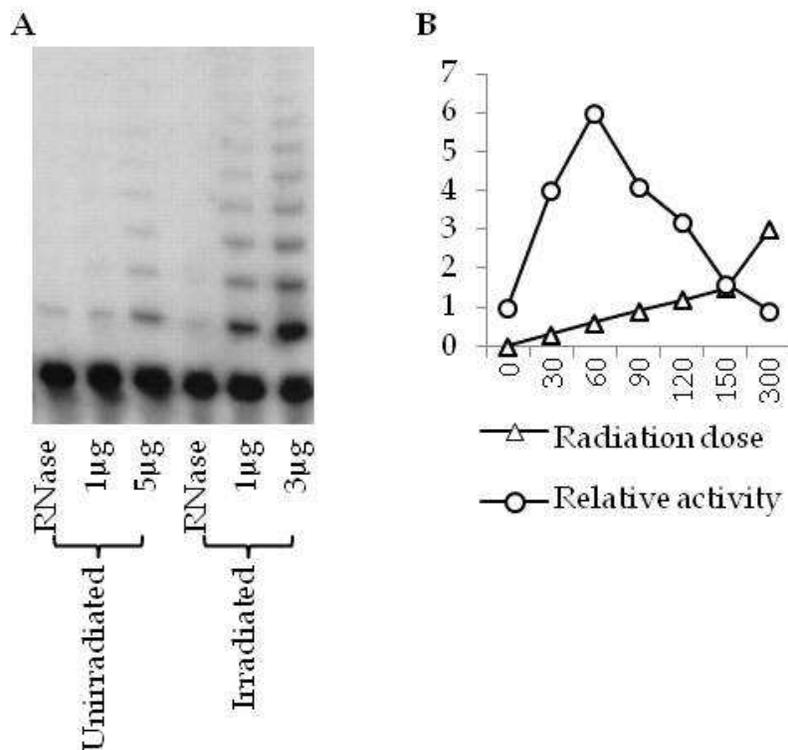


Fig. 6. Telomerase activity in mealybugs exposed to different doses of IR. (A). Telomerase activity in insects irradiated with a dose of 70 Gy is six-times the activity in unirradiated insects. (B) Progressive increase of telomerase activity was observed till 60 Gy. Exposure to higher doses was associated with a decrease in the level of activity.

5. Possible mechanisms of genomic stability in mealybugs exposed to high doses of ionizing radiation

From the results described here and the collection of prior data on the behavior of mealybug chromosomes after exposure to high doses of ionizing radiation, we suggest that there is highly efficient protection of radiation-induced chromosome ends that prevent the onset of fusion-bridge-breakage-fusion cycles and this machinery is excellently complemented by the centromere property in the mealybug chromosomes. As mentioned in the section 4.1, radiation-induced chromosome fragments which are very tiny and small, are capable of metaphase alignment and anaphase segregation. These results suggest that the centromere property is distributed throughout the chromosomes, or in other words the centromere is 'diffuse'. An obvious advantage of possessing diffuse centromere property is that there would not be a significant loss of genetic material among the collection of the chromosome fragments in the irradiated cells. These cells would be by and large viable and unless radiation-induced double strand breaks disrupt haplo-insufficient genes that are crucial for development or survival. Therefore, the combination of rapid chromosome end healing and diffuse centromere offer a great advantage to the mealybug in successful survival and reproduction upon exposure to high doses of IR.

What are the advantages in possessing constitutively active telomerase? As noted above, telomerase-mediated extension of telomeric repeats is one of the different mechanisms that operate in presumably all eukaryotes with the exception of Diptera. The existence of

telomerase even before irradiation in mealybugs provides an opportunity to extend telomeric repeats whenever required for maintenance an optimal length of telomeric repeats (which is higher than the critical length) at newly broken ends of irradiated chromosomes. Such maintenance of optimal length creates a high probability to restore the original chromosome in the presence of a radiation-induced chromosome double-strand break. It may be noted that in cells containing critically short telomeres, the number of unprotected chromosome ends is higher and as a result restoration of the original chromosome is more unlikely and results in genomic instability. An additional advantage in efficient maintenance of optimal number of telomeric repeats is the availability of sufficient number of binding sites for the DSB repair proteins per cell. In this context, interstitial telomeric repeats when they occur at DSBs have the potential to serve as templates for formation of new repeats as part of protection of newly formed chromosome ends. An alternative mechanism is 'exposure' of the interstitial repeats akin to the MMEJ-mediated search for homology/micro homology by resection.

As discussed in section 4.3, telomerase activity decreases at doses higher than 60 Gy and at 300 Gy, the activity in irradiated insects is lesser than unirradiated insects. It is therefore clear the telomerase-dependent mechanism of end-healing may not be efficient at higher doses that mealybugs have been shown to survive. Therefore at these doses, chromosome end healing mechanisms that are telomerase-independent should be functional. These mechanisms are collectively called as alternative mechanisms of telomere maintenance (ALT). As mentioned previously, non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ) and homologous recombination-mediated DNA repair are the three mechanisms which enable telomere maintenance. NHEJ and MMEJ, as mentioned before require microhomology between the damaged ends to be joined whereas homologous recombination requires a sequence homology from a homologous chromosome or a sister chromatid. In this context, it is conceivable that repeat sequences associated with the interstitial TTAGG repeats may play a role in one or more of the ALT pathways.

6. Conclusion

In summary, constitutively active telomerase, diffuse centromere and the unusual distribution of telomeric repeats together constitute components of efficient DNA repair machinery in the mealybug. In particular, interstitial telomeric repeats appear to be attractive candidate sequences that may help in the acquisition of additional telomeric repeats at DSBs either through telomerase-dependent or independent mechanisms. Direct cloning and analysis of healed chromosome ends induced by radiation and their comparison with 'intact' unirradiated homologs should shed more light on the role of interstitial telomeric sequences.

Future research on the isolation and analysis of telomerase encoding genes should provide important insights on regulation of telomerase and mechanisms underlying telomerase-dependent maintenance of telomeric repeats. A comparison of the DNA regulatory elements and analysis of the primary amino acid sequence should reveal any differences from the telomerase enzymes described in other insects. As in case of other eukaryotes, alternative lengthening of telomeres also appears to play an important role in the response to exposure to IR. In this context, isolation and analysis of isolation of genes involved in ALT pathways, their expression patterns and localization in cells following irradiation should provide more

insights in the chromosome end-healing and DNA repair mechanisms in the mealybug. For a better understanding of the nature of biological response to IR, experiments such as mRNA expression profiling, protein profiling and protein modification profiling in insects before and after exposure to high doses of IR should provide more insights into the mechanisms conferring radiation resistance to the mealybug.

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8. References

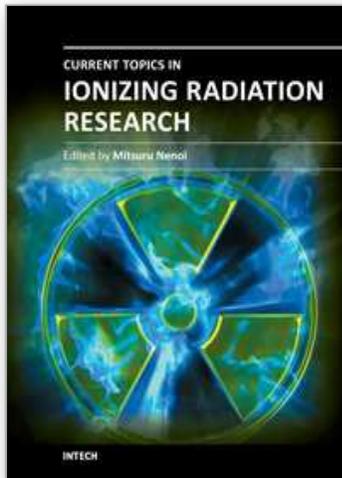
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