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THE NATURE OF X-RAY AND CHEMICALLY INDUCED MUTATIONS IN *DROSOPHILA* IN RELATION WITH DNA REPAIR

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Summary. - *This paper describes the spectrum of mutations induced by alkylating agents and ionizing radiation in* Drosophila. *Specifically, the genotoxic profile of the alkylating agents is set against their carcinogenic potency. Alkylating agents that react preferentially with N-atoms in the DNA are relatively poor mutagens, especially so in repair-competent (early) germ cells, and likewise weak carcinogens when compared to those that are more efficient in O-alkylation. Genetic techniques combined with molecular analysis of X-ray and neutron induced mutations show that ionizing radiation induces primarily break-type mutations in a repair proficient background. Both multi-locus deletions as well as small intragenic deletions of only a few base-pairs are observed. The small deletions occur between direct repeats of 2-3 nucleotides, one copy of which is retained in the mutant allele. Possibly, these deletions are the result of repair processes. The effect of changes in DNA-repair (excision repair deficient) is reflected by a "hypermutability" for alkylating agents specifically for N-alkylators, indicating that the normal efficient error-free repair of N-alkylation damage can explain the high exposure doses required for tumor induction in mammals. The frequency of X-ray induced whole-body* white *mutations, recovered in excision repair deficient* Drosophila, *is only slightly enhanced, when compared to the repair proficient situation. In contrast, mosaic mutations occur 3-4 times more frequent, indicating that part of the X-ray damage, normally removed by the excision repair process, is not a major impedement during replication. However, preliminary data concerning the molecular nature of the whole body mutations recovered in excision repair deficient background, indicate that the spectrum of mutations might be altered; relatively more small lesions are recovered compared to those induced in a repair proficient background.*

Riassunto (Caratterizzazione delle mutazioni indotte da raggi X e agenti chimici in *Drosophila* in relazione alla riparazione del DNA). - *Questo articolo descrive lo spettro di mutazioni indotto da agenti alchilanti e radiazioni ionizzanti in* Drosophila. *In particolare, il profilo genotossico degli agenti alchilanti è valutato in funzione della loro potenza cancerogena. Gli agenti alchilanti che reagiscono preferenzialmente con gli atomi di azoto nel DNA sono mutageni relativamente deboli, particolarmente in cellule germinali proficienti nella riparazione del DNA, ed anche deboli cancerogeni se paragonati a quelli che alchilano preferenzialmente gli atomi di ossigeno. Tecniche genetiche associate all'analisi molecolare delle mutazioni indotte da raggi X e neutroni mostrano che le radiazioni ionizzanti inducono principalmente mutazioni del tipo rotture in cellule proficienti nella riparazione del DNA. Si osservano sia delezioni multilocus che piccole delezioni intrageniche di poche paia di basi. Le piccole delezioni avvengono tra sequenze ripetute di 2-3 nucleotidi, delle quali una copia è mantenuta nell'allele mutante. Queste delezioni sono probabilmente il risultato di processi di riparazione del DNA. L'effetto di alterazioni nel processo di riparazione del DNA (difetto nella riparazione per escissione) si riflette sulla ipermutabilità indotta da agenti alchilanti che reagiscono preferenzialmente con gli atomi di azoto, indicando che una efficiente riparazione "error-free" del danno di alchilazione degli atomi di azoto può spiegare le alte dosi di esposizione richieste per l'induzione di tumori nei mammiferi. La frequenza di mutazioni di tutto il corpo* white *indotte dai raggi X, osservate in* Drosophila *difettive nella riparazione per escissione, è solo leggermente aumentata se confrontata con la situazione di riparazione efficiente. Le mutazioni mosaico si verificano invece 3-4 volte più frequentemente, indicando che parte del danno indotto dai raggi X, normalmente rimosso dai processi di riparazione per escissione, non è un grosso ostacolo durante la replicazione. Tuttavia, dati preliminari che riguardano la natura molecolare delle mutazioni di tutto il corpo osservate in ceppi difettivi nella riparazione, indicano che lo spettro di mutazioni potrebbe essere alterato; si osservano lesioni relativamente più piccole se confrontate con quelle indotte in ceppi proficienti nella riparazione.*

Introduction

One aim of studying induced mutagenesis is the assessment of the potential risks (heritable/somatic) humans incur from exposure to physical (X-rays/neutrons) and chemical agents. Many complementary approaches can be followed, one of which is the fundamental research of the mechanism of mutation formation. Several factors are important for the type of genetic alteration finally observed: the types of initial DNA adducts formed, the relative distribution and persistance in the DNA, the dose and the DNA-repair systems operating. Many specific questions with regard to the sequence of events leading from the initial DNA adduct or lesion to the mutations induced can be approached using the present DNA-technology which makes it possible to determine the exact nature of the mutations.

For various reasons *Drosophila* constitutes a very attractive system to study mutagenesis. Apart from being one of the genetically best characterized multicellular eukaryotes, many hundreds of thousands of offspring can be generated in a relative short period of time and practically the complete spectrum of somatic and heritable genetic alterations can be detected. Treatment of males can be followed by sampling different developmental stages of treated germ cells. In particular mature sperm, in which no repair is taking place, offers possibilities to study the factor time on the persistance of DNA-adducts formed, or the impact of the repair system provided by the oocyte, where after fertilization the damage in the sperm chromosomes is processed.

Several factors have to be considered in the analysis of germ cell mutations. First of all germinal selection may take place, especially after treatment of pre-meiotic stages. Secondly, the principle nature of the target gene(s) may influence the spectrum of mutations. Thirdly, the chromosomal environment (specific neighbouring genes) may have an effect on the spectrum by, preferentially, selecting (or not) particular mutations. Therefore, it will be difficult to draw general conclusions based on data obtained with one target gene only.

In *Drosophila* so far several target genes have been used to study systematically mutational spectra; *Adh* (alcohol dehydrogenase) and *ry* (rosy), both autosomal genes, and the X-linked genes *RpII215* (large subunit of polymerase), *w* (white) and *v* (vermilion). In this paper the results obtained using these last two genes as targets will be discussed.

The *w* gene most probably codes for a polypeptide involved in the deposition of the red and brown pigments, that determine the fly's eye color [1]. The complete gene, including regulatory elements, is about 8 kb long and produces a 2.6 kb mRNA. The sequence of the gene and several kb of flanking DNA is known [2]. Mutations of this gene are readily scored and the neighbouring genes are known, making a detailed genetic analysis possible. The *white* region of the X-chromosome is known to tolerate relatively large multi-locus deletions. The *v* gene is selected for its small size, facilitating the sequencing of small alterations. This gene codes for the enzyme tryptophan oxygenase, that catalyses the first step in the synthesis of the brown pigment in the eyes. The size of this gene is probably less than 3 kb and the mRNA encoded is 1.3 kb ([3, 4]; Searles, personal communication). Mutations at this locus change the red eye color to vermilion. The readiness of scoring mutations of this locus can easily be improved by manipulating the genetic background; including the mutant *bw* (brown) into the system changes the eye phenotype of vermilion mutants into white. The potential disadvantage of this gene is a reduced frequency of mutations due to its small size and the fact that many multi-locus deletions will not be tolerated due to the proximity of 1) a haplo-insufficient lethality gene and 2) a haplo-insufficient female fertility gene.

The mutational spectrum of alkylating agents (AA)

Alkylation of DNA is a frequent event in the induction of mutations and chromosomal aberrations and tumors. In this report some general findings on the effect of AA's (using conventional genetic tests) are described in addition to the determination of the molecular mutational spectrum, that are relevant in understanding the action principles of AA's. An attempt is made to correlate genotoxic effects of AA's in *Drosophila* in relation to their carcinogenic potency in rodents.

The relative distribution of methyl- or ethylgroups in DNA can be manipulated by using AA's with different nucleophilic properties [5]. The methylation of DNA by MMS (methyl methanesulfonate) will be predominantly (99%) on N-atoms (N-7 of guanine, N-3 of adenine) whereas few (1%) of the methylations will occur at the O^6 position of guanine [6]. The ratio O^6/N-7 methylation of guanine with MMS is as low as 0.004. In sharp contrast with MMS is the ethylation distribution by ENU (N-ethyl-N-nitrosourea). Preferentially the O^6 position of guanine is ethylated (and also the O^2 and O^4 of thymidine); the ratio of O^6/N-7 ethylation with ENU is in the order of 0.6-0.7 [7]. EMS (ethyl methanesulfonate) takes a position intermediate to MMS and ENU: 80% initial ring nitrogen alkylation and 20% O-alkylation giving an O^6/N-7 ethyl guanine ratio of 0.03. Alkylation by MMS will result in misrepair and depurination, leading to both "point mutations" and chromosomal aberrations [5, 8-10]. On the other hand, as a result of efficient alkylation at the O^6 position (ENU), guanine will mispair during replication with thymine and hence produce many GC to AT transitions [11, 12], leading only to "point mutations". The carcinogenic potency in rodents (determined by the dose producing

tumors in 50% of the test animals, TD_{50}) of AA's varies over a 10.000-fold range with MMS and ENU at the extreme ends of the range [13, 14]. The most efficient O-alkylators (such as ENU) are the strongest carcinogens. A positive correlation therefore is found between O^6-alkylguanine and overall carcinogenicity in rodents.

Data concerning the type of genetic changes induced by MMS and ENU were obtained using *Drosophila* genetic tests specific for chromosome aberrations (translocations) and mutations (sex-linked recessive lethals, SLRL). The results can be summarised as in Table 1, to illustrate that at comparable mutation frequencies (SLRL), MMS produced 65 translocations per 100 "point mutations", EMS 7 and ENU less than one. There is a strong correlation between products of N-alkylation in the DNA and the induction of chromosome aberrations such as translocations and chromosome loss, but also deletions [15, 16]. Point mutations by MMS-type of mutagens may arise from apurinic and apyrimidinic (AP-) sites, or misrepair. On the other hand, ENU-type mutagens may cause preferentially point mutations by mispairing of products of O-alkylation in the DNA [10]. The analysis of the molecular nature of MMS and ENU induced mutations may provide evidence for the proposed hypotheses.

Because of the small size, *vermilion* is very suitable for the determination of mutational spectra of agents inducing primarily base changes. A rapid cloning and sequencing method has been developed based on the recombinational screening method as described by Seed [17]. Eventually the *vermilion* mutants are recovered as a plasmid clone from which single stranded DNA can be obtained for dideoxy-sequencing [18]. Unpublished data from the sequencing of *vermilion* mutations induced by MMS and ENU are shown in Table 2. Among 11 mutations induced by MMS, one transition (GC to AT), 8 transversions and 2 deletions are found. ENU however, induced 20 transitions (mostly GC to AT) and only 7 transversions in 24 analysed mutants (in 3 mutants, two base-pair changes were observed). Thus the picture seen at the molecular level coresponds to the predictions made on the basis of "traditional" genetic analysis.

Table 1. - *The changes in the relative proportion of translocation to mutations as function of the change in the relative distribution of methyl- or ethyl adducts in DNA*

	MMS	EMS	ENU
Mutations	100	100	100
Translocations	65	7	1
N-alkylation (%)	99	80	20
O-alkylation (%)	1	20	80

Table 2. - *Molecular characterization of* vermilion *mutations induced in* Drosophila *by MMS or ENU*

	analysed	transition	transversion	deletions
MMS	11	1	8	2
ENU	27	20	7	0

Table 3. - *Genetic and molecular characterization of X-ray and neutron induced* white *mutants*

	X-ray	neutron
mutations recovered	31/94.398	35/102.756
multilocus deletions	15	26
large intragenic lesions (del.'s + tr.'s + inv.'s)	5/11 analysed	6/6 analysed
small intragenic deletions	5/11 analysed	0/6 analysed
base change	1/11 analysed	0/6 analysed

The mutational spectrum of X-rays and neutrons

The mutational spectrum induced by ionizing radiation was determined using *white* as the target gene. The *zeste-white* region of the X-chromosome is genetically well known and many reference genes (lethal and visible mutations) neighbouring the *white* gene are available that make a detailed genetic analysis for multi-locus deletions possible. In a series of identical experiments 31 X-ray- and 35 neutron-induced *white* mutants were recovered from irradiated mature germ cells in males [19]. As shown in Table 3, genetic experiments revealed multi-locus deletions in 50% of the X-ray induced and 75% of the neutron induced mutants. Most of the remaining intra-genic mutations were analysed by blot-hybridization permitting the detection of rearrangements larger than 50-100 bp. Deletions and other rearrangements like translocations or inversions could be identified in a further 20% of the mutants induced by X-rays and also in 20% of the neutron induced mutations [20]. These results are comparable with those obtained using autosomal *Drosophila* genes *Adh* and *ry* [21-24]. Also in mammalian cells about 70% of the mutations induced by ionizing radiation at the *hprt* locus show complete or partial loss of the coding sequences of this gene [25]. Six X-ray induced intragenic *white* mutations that did not show a changed restriction enzyme profile, were further subjected to nuclease S_1 protection analysis to identify small (larger than 5 bp) deletions [18, 26]. Five of these are indeed caused by small deletions (ranging in size from 6 to 29 bp) in the 3' half of the coding sequence of the gene. Two, independent, events resulted in exactly the same 29 bp deletion. The remaining three are clustered in a 100 bp sized region. The clustering indicates non-random distribution of damage or preferential repair.

Most remarkable is the fact that all five deletions occurred between two direct repeats of 2-3 base-pairs, one of which is retained in the mutant allele. Similar deletions between small direct repeats have been reported for both spontaneous and induced mutations at the *Adh* gene of *Drosophila* [27, 28], the *aprt* locus in hamster cells cultured *in vitro* [29-31] and the *lacI-Z* fusion gene in *E. coli* [32, 33]. These deletions may have arisen by the formation of "slipped mispairs" or recombinational events supposedly during the removal of damage from the DNA. This may implicate that possibly the initial DNA-lesion was not a break but base-damage or damage to the sugar/phosphate backbone.

The results of the combined genetic and molecular analysis of X-ray and neutron-induced *white* mutations show that the majority of lesions induced by ionizing radiation may result from DNA breaks (direct or indirect). However, it cannot be excluded that at least these six small X-ray induced deletions have resulted from the repair of base/sugar damage. It should be noted that for the recovery of larger deletions the chromosomal environment is important. The presence of essential genes in the vicinity of for example the *aprt* gene in hamster may result in an underestimation of deletions [34]. The recovery of a high percentage of multi-locus deletions, as in the case of *white* in *Drosophila*, does not imply that these are the major lesions induced by ionizing radiation, because the number of small lesions recovered in a single locus as for example *white* should be multiplied by the total number of genes present in the region uncovered by all detected multi-locus deletions (in our analysis of X-ray induced *white* mutants, the number of genes between the most distal break of one of the multi-locus deletions and the most proximal break of another deletion is in the order of 20). Assuming that the six intra-genic small lesions are due to base/sugar damage, than the relative ratio of break-type damage (multi-locus deletions and large intragenic deletions and translocations and inversions) and the total of small lesions in the uncovered region can be estimated to be in the order of 1:1. Deficiencies in DNA-break repair or excision repair may result in a shift in the mutational spectrum in both directions.

The effect of DNA-repair on the mutational spectrum

The effect of DNA-repair can be studied by changes in the mutational spectrum of a particular agent when DNA repair deficient mutants are used. There are at this moment in *Drosophila* over 30 different mutants known [35], all of which in some way alter the efficiency of repair. Among these, the excision repair deficient mutants *mei-9* and *mus201* are the best characterized, biochemically [36, 37] and genetically [9, 38, 39]. In *Drosophila*, repair of premutagenic lesions occurs in early male germ cells (paternal repair), and in the egg after fertilization (maternal repair). No repair is observed in mature spermatozoa [40]. The impact of DNA repair can be studied by mating treated males with repair deficient females and collecting then only those eggs that are fertilized by sperm that at the time of treatment already had matured, and isolating the mutations arising. Comparing the mutation induction in excision repair deficient *(exr⁻)* and excision repair proficient *(exr⁺)* genotypes, a drastic increase can be found using *exr⁻* females with MMS-type mutagens (N-alkylators). No, or only small increases are observed using ENU-type mutagens (O-alkylators) [9]. These results indicate that products of N-alkylation are efficiently repaired in *exr⁺* cells. The observed hypermutability in *exr⁻* mutants can even be increased when the time between treatment and repair is prolonged (Vogel, unpublished observations). The mutational spectrum of small AA's therefore may be expected to shift in the direction of more transversions when recovered using *exr⁻* females. The mutational spectrum determined for a particular mutagen might in part reflect efficient/inefficient repair of certain DNA-adducts of the biological system used.

Preliminary data have been obtained on the mutational spectrum of X-ray induced *white* mutants that have arisen in an *exr⁻* background. Irradiated males are mated to *mus201* females, mutations are scored in the *yellow*, *white*, *vermilion* and *forked* gene. Whole body mutations and mosaic mutations can be detected at all loci in a one generation test, with the exception of *vermilion*. Mosaic mutations arise some what later during development than whole body mutations. When a mosaic mutation includes also some germ cells, the mutation can be recovered in a subsequent generation, enabling the molecular analysis. The frequency of whole body mutations is only slightly affected by *mus201*. In contrast, the frequency of mosaic mutations is enhanced by a factor 3-4. This indicates that part of the X-ray induced damage, normally removed by the excision repair pathway, is not a major impedement in the first rounds of replication after fertilization. The molecular analysis of the whole body *white* mutations show a decrease in the number of multi-locus deletions and an increase in the number of mutations due to small (< 50 bp) lesions as compared to the X-ray induced *white* mutations recovered in an *exr⁺* background (detailed data will be published elsewhere).

Conclusion

The use of molecular techniques makes it possible to determine the nature of mutations at the level of the DNA. This approach complements existing genetic, biochemical and cytological analysis and greatly helps in understanding basic mechanisms in mutagenesis and carcinogenesis. Some examples of the molecular analysis of induced mutations in combination with a geneti-

cal approach, in *Drosophila*, clearly shows the possibilities of this organism to study processes of mutation formation in the *in vivo* situation.

The findings indicate that AA's that alkylate predominantly N-sites in the DNA, are relatively poor mutagens in *Drosophila* due to efficient repair systems present (absent in *exr*$^-$). N-alkylation of the DNA causes structural chromosome aberrations and "point mutations" arising from AP-sites or misrepair. Since these AA's are also weak carcinogens, it has recently been hypothesized that this is due to the presence of an efficient error-free repair in rodents [41]. O-alkylators, on the other hand, may lead to, among others, O^6-alkylguanine, that by mispairing will predominantly result in point mutations due to the transition of GC to AT.

Ionizing radiation induces predominantly break-type of mutations (chromosome aberrations, multi-locus deletions). However, the detected small intra-locus deletions (< 50 bp), characterized by the fact that they arise between two direct repeats of 2-3 basepairs, may in fact arise during a repair process where recombinational events are involved in the removal of damage.

Evidence is accumulating that excision repair in *Drosophila* is very efficient and can remove N-alkylation products from DNA, whereas it is not involved in the repair of O-alkylproducts of DNA. The effect of a deficiency of this repair process results in the recovery of X-ray induced mutations that are smaller in nature than those recovered when normal repair is operating.

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