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Biochemical genetics of Neurospora nuclease II: Mutagen sensitivity and other characteristics of the nuclease mutants

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SUMMARY

Five new *nuc* mutants of *Neurospora crassa* were characterized for their relative sensitivities to different mutagens (UV, MNG, MMS), to mitomycin-C and to histidine; latter has been shown to inhibit the growth of certain UV sensitive mutants. These mutants were also compared for their capabilities for spontaneous mutation as determined by resistance to p-fluoro-phenylalanine. Based on these characterization, the mutants seem to belong to two groups. The first group included *nuc-3* and *nuc-6* which showed sensitivity to all mutagen tested and possessed capability for a very high frequency of spontaneous mutation (i.e. mutator effect). The second group included *nuc-4*, *nuc-5* and *nuc-7*; these were as resistant to different mutagens as the wild type strain, but possessed an antimutator effect (i.e. the frequency of spontaneous mutation by these three mutants were at least 0·5–100 × less than the wild type strains). There was some variation in these properties of mutants belonging to the two groups. Among all the five *nuc* mutants, *nuc-3* was characterized by extreme sensitivity to all mutagens. None of the five *nuc* mutants were sensitive to histidine. The properties of *nuc* mutants are discussed in relation to their possible role in DNA repair and recombination.

1. INTRODUCTION

In prokaryotes, a combination of related approaches has elucidated the molecular mechanism of DNA repair, recombination and replication. These include biochemical genetic characterization of appropriate mutants including their complementation both *in vivo* or *in vitro*. An important aspect of these studies has been the discovery of common steps or participation by common enzymes (such as DNA polymerase, deoxyribonuclease and ligase) in these basic genetic processes (see Kornberg, 1980; Alberts *et al.* 1980; Hanawalt *et al.* 1979; Witkin, 1976; Howard-Flanders, 1981; Clark 1976; Radding, 1978; Stahl, 1980). Such an in-depth study of eukaryotic DNA repair or recombination has not yet been possible. However, a similar biochemical genetic approach has been made in order to understand the mechanisms of DNA repair in yeast (Cox & Game, 1974; Lawrence & Christensen, 1976; Haynes *et al.* 1978) and in *Neurospora* (Schroeder, 1979; Worthy & Epler,

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1973; Kafer, 1980; Delange & Mishra, 1981, 1982; Stadler & Moyer, 1981). In our laboratory we have employed two methods. First we have isolated a number of mutagen-sensitive mutants (Delange & Mishra, 1981, 1982) which are being characterized for possible biochemical defects in the enzymes (such as DNA polymerase, deoxyribonuclease and/or ligase) of DNA repair pathways. This method has been widely used by different investigators (Schroeder, 1979; Worthy & Epler, 1973; Fraser, 1979; Kafer, 1980; Delange & Mishra, 1981, 1982). Second, we have isolated a number of mutants which are deficient in deoxyribonucleases (Forsthoefel & Mishra see the first paper in this series); these mutants are being characterized for possible relation to a defective step in DNA repair as revealed by their sensitivity to different mutagen and their capabilities for spontaneous mutation. Earlier Ishikawa *et al.* (1969) described the isolation of two nuclease (*nuc-1* and *nuc-2*) deficient mutants of *Neurospora*; they have shown that *nuc-2* was sensitive to UV light thus suggesting its involvement in DNA repair. In this paper we present a detailed characterization of five *nuc* mutants with respect to their cross sensitivities to UV, MMS, MNG, mitomycin-C, and histidine. The frequency of spontaneous mutation leading to β-P-fluoro-phenylalanine resistance have been also compared. The cross sensitivity to mutagens and increased frequency of mutation suggest the involvement of the *nuc* mutation in the defective DNA repair. The mutator and anti mutator properties of the two groups of mutants are correlated to their adverse and favourable effects on the frequency of recombination. The data presented suggest that further biochemical analysis of these mutants should be useful in understanding the mechanism of DNA repair and recombination in eukaryotes.

2. MATERIALS AND METHODS

The media and the methods for the routine manipulation of *Neurospora* strains were as described previously (Davis & DeSerres, 1970; Delange & Mishra, 1981, 1982). All *nuc* mutants used have been described previously (Forsthoefel & Mishra, see the first paper in this series), these included *nuc-3* (701), *nuc-4* (51), *nuc-5* (276), *nuc-6* (936), and *nuc-7* (24). The wild type strains used were the Oak Ridge type (FGSC 986) and the Rockefeller type (FGSC 2218). All strains bearing FGSC numbers were obtained from the Fungal Genetics Stock Centre, Humboldt State University, Arcata, California.

UV irradiation. Five day old conidia were irradiated in 0·5 ml sterile distilled water in a glass Petri plate at a density of 2×10^4/ml. This volume (0·5 ml) formed a thin film in Petri plate. The UV dose (measured by an UV light meter, UV products, San Gabriel, California) was constant at 3.44 Jm sec^{-1}. Irradiation and subsequent manipulations were carried out under dim light condition at 25 °C and the plates were incubated in dark at 25 °C until ready to score (usually 4–5 days).

MMS treatment. The conidia were given two different kinds of MMS treatment. In the first method conidia were treated with 0·5% of MMS for different time intervals and then plated on regular growth media (free of MMS), whereas, in the second method, conidia were plated on growth medium containing different concentrations of redistilled MMS (Eastman Kodak Co. Rochester N.Y.). The

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details of MMS treatment were as described previously (see Delange & Mishra, 1981). The plates containing MMS treated conidia following both methods of treatment were incubated at 25 °C and scored after 4–5 days of growth.

MNG treatment. Conidial suspension was plated on growth medium containing different concentration of MNG (0–1·5 μg/ml) and incubated in dark 25 °C for 4–5 days before scoring.

Mitomycin-C. Conidia of different strains were plated on growth medium containing different concentrations of mitomycin-C (Boehringer Mannheim); the only concentration used were 0, 2, 4, 8, and 16 μg/ml and plates were incubated in dark at 25 °C for 4–5 days to score the growing colonies as described by Chow & Fraser (1981).

Histidine sensitivity. This was determined by examining growth on medium containing histidine (500 μg/ml). The plates were scored for growth after 4–5 days.

Determination of spontaneous mutation frequencies. Mutation frequencies were determined by scoring forward mutations to resistance to P-fluorophenyl-alanine (PFA). For each strain tested, conidial suspension (5–7 days old) were spread (10^4–10^6 conidia/plate) on sorbose supplemented medium containing 10 μg/ml of PFA and incubated at 25 °C (Kinsey & Stadler, 1969). The number of variable conidia was determined separately by plating conidia on medium lacking PFA; about 95 % of conidia were found germinable. Plates were incubated at 25 °C and colonies were scored after 3–4 days (PFA free medium) or after 5–6 days (PFA containing medium). Usually a representative sample of colonies from the PFA plates were transferred to fresh PFA plates in order to distinguish between genuine resistant mutants and adaptively grown sensitive colonies, latter were detected only occasionally and were excluded from the computation to determine the mutation frequency.

Abbreviations. MMS, methyl methane sulphonate; MNG, *N*-methyl-*N′*-nitrosoguanidine; PFA, p-fluoro-phenyl-alanine and UV, ultraviolet.

3. RESULTS

Five nuclease (*nuc*) mutants and the wild type strain of *Neurospora crassa* were compared with respect to their sensitivities to several mutagens (UV, MNG, MMS and mitomycin-C) in order to elicit the relation of *nuc* mutation to possible defect in DNA repair pathways. The results of these experiments are described here.

UV-sensitivity. The wild type strains showed a typical survival curve with the characteristic shoulder as seen in Fig. 1. The mutant *nuc-7* was as UV-resistant as the wild type strain. There were certain marked differences among the remaining mutants (*nuc-3*, *nuc-4*, *nuc-5*, and *nuc-6*) in their UV-sensitivities; the *nuc-3* was extremely sensitive to UV light whereas the *nuc-4*, *nuc-5*, and *nuc-6* were much less sensitive and showed a shoulder characteristic of the wild type (see Fig. 1).

MNG sensitivity. The wild type showed a typical survival curve with a characteristic shoulder and then a sudden drop in the survival frequency at a concentration of 2 μg/ml (see Fig. 2). The LD_{50} for the wild type was 1·5 μg/ml. The *nuc* mutants showed 3 different levels of sensitivity to the mutagen. The mutant *nuc-3* showed extreme sensitivity with a LD_{50} of 0·25 μg/ml, whereas the

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mutants *nuc-6* and *nuc-7* were found to show intermediate level of sensitivity (i.e. more sensitive than wild type but less sensitive than *nuc-3*) with a LD_{50} of 0·5 μg/ml. The remaining two mutants *nuc-4* and *nuc-5* were as resistant as the wild type strain (with LD_{50} of 1·5 μg/ml). The MNG sensitivity of the mutant and wild type strain are depicted in Fig. 2.

MMS sensitivity. The MMS-sensitivity was determined in two different ways: First, the wild type and mutant conidia were treated with 0·5 % of MMS for different time intervals and were then plated on normal growth medium to examine

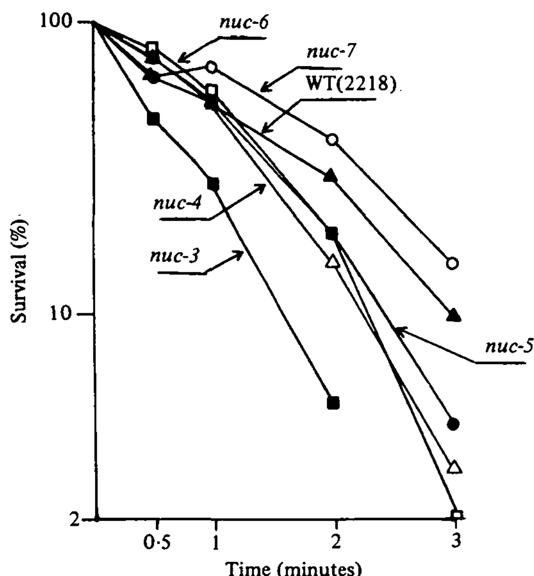

Fig. 1. Effect of ultraviolet light (UV) on the survival of the wild type and mutant strains.

their capabilities in repairing the damages inflicted on DNA, as estimated by their survival frequency. Second, the wild type and mutant conidia were plated on medium containing different concentration of MMS and then examined for their ability to repair the continuously inflicted damages by determining the frequency of the surviving colonies. These data are depicted in Figs 3 and 4. Under both conditions, the *nuc-3* was found to be the most sensitive to MMS; the remaining mutants were equally resistant to MMS as the wild type strain; however there were some variations in their level of resistance, the mutant *nuc-7* was the most resistant whereas *nuc-5* and *nuc-6* were the least resistant (see Table 2). Also the *nuc-5* was more sensitive when grown on medium containing MMS.

Sensitivity to mitomycin-C. Except for mutants *nuc-3* and *nuc-6*, all mutants were equally sensitive (or resistant) to mitomycin-C as the wild type strain. The mutants *nuc-3* and *nuc-6* were somewhat more sensitive to the drug when compared to the wild type; these two mutants (i.e. *nuc-3* and *nuc-6*) showed a survival frequency of 36 and 62 % respectively at a drug concentration of 16 μg/ml. The other three mutants (i.e. *nuc-4*, *nuc-5*, and *nuc-7*) and the wild type showed 100 % survival at this concentration of mitomycin-C (16 μg/ml). The other feature

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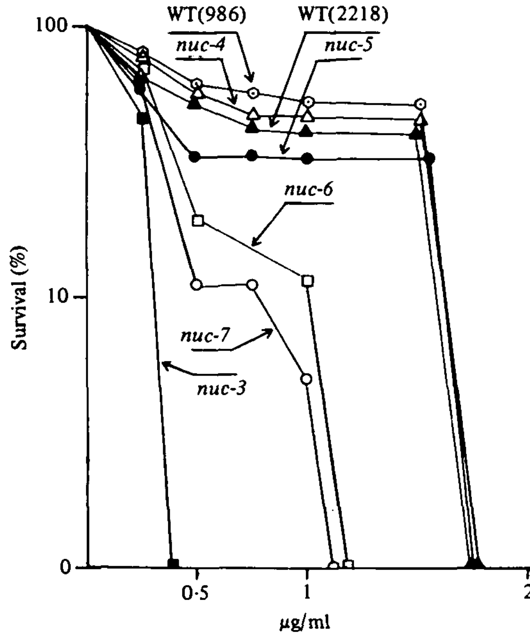

Fig. 2. Effect of MNG on the survival of the wild type and mutant strains.

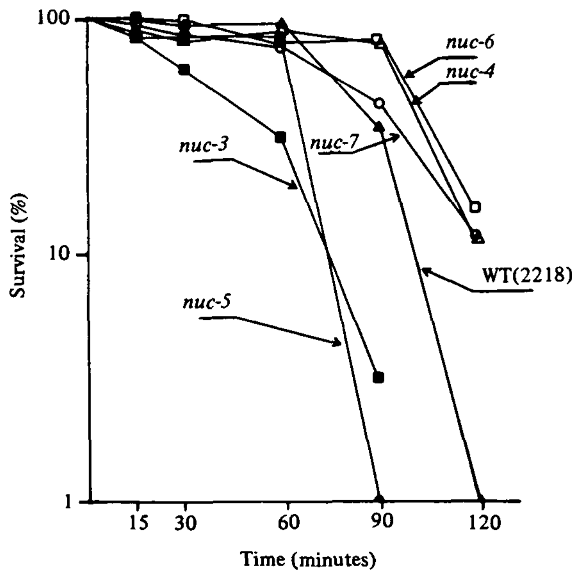

Fig. 3. Effect of MMS on the survival of the wild type and mutant strains. Conidia were first exposed to 0·05 % MMS for different time interval and then examined for frequency of survival.

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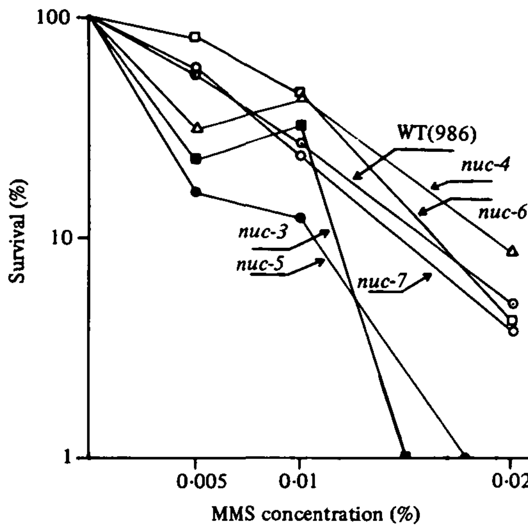

Fig. 4. Effect of MMS on the survival of the wild type and mutant strains. Conidia were directly plated on media containing different concentration of MMS (see Materials and Methods).

Table 1. *A comparison of the spontaneous mutation frequency at the PFA locus among different nuclease* (nuc) *mutants and wild type strain of* Neurospora crassa

Strain	Frequency of PFA resistant colonies		Mutation	
	10^5	10^6	Frequency (XWT)	Property
Wild type				
Oak ridge type	11	108	1X	
Rockefeller type	10	113	1X	
Mutants				
nuc-3	102	1123	10X	Mutator
nuc-6	80	690	7X	Mutator
nuc-4	0	1	0·01X	Anti Mutator
nuc-5	0	15	0·1X	Anti Mutator
nuc-7	5	70	0·5X	Anti Mutator

The conidial germination was about 95% in the different strains of *Neurospora* on medium without (PFA).

of the mitomycin-C was the lack of dose response up to 8 μg/ml of mitomycin-C. At this concentration of mitomycin-C (8 μg/ml) all strains showed 100% survival. Such a lack of the dose response has been reported earlier (Kafer, 1981).

Histidine sensitivity. An apparent relation between histidine sensitivity and defect in DNA repair and/or recombination has been suggested by Newmeyer and her collaborators (Newmeyer, Schroeder & Galeazzi, 1978). We have therefore examined this possibility by comparing the growth rate of the *nuc* mutants and the wild type strain on medium with and without histidine. In such study both

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Table 2. *A summary of the properties of Neurospora* nuc *mutants as compared to the wild type strain*

Strai n	LG	ds-DNase* relative activity (%)	Sensitivity to						Spontaneous mutation		
			UV conidia	MNG mycelia	MMS Conidia	MMS Mycelia	Histidine mycelia	Temp (37 °C)	Relative frequency	Nature of effect	Effect on recombination*
Wild type	—	100	R	R	R	R	R	R	IX	—	—
Mutant											
nuc-3	II	9·6	SS	SS	SS	SS	R	R	10X	Mutator effect	Prohibit recombination
nuc-6	II	10·1	R–S	R	R	S	R	R	7X		
nuc-4	II	2·8	R–S	R	R	R	R	S	0.01X	Anti mutator effect	Promotes recombination
nuc-5	II	12·38	R–S	R	R	S	R	S	0·1X		
nuc-7	II	2.88	R	S	R	R	R	R	0·5X	—	—

* Based on preliminary data presented in the preceding paper (Forsthoefel & Mishra, 1983). R, resistant; RS, moderately sensitive; S, sensitive; SS, extremely sensitive.

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the mutant and wild type strain were found to grow equally well on medium with or without histidine.

Frequency of spontaneous mutation. Kinsey & Stadler (1969) have earlier shown that forward mutation in several genes can cause resistance to p-fluorophenylalanine (PFA). We have used this system to compare the mutational capabilities of the wild type and the *nuc* mutant strains by determining the frequencies of PFA resistant colonies as presented in Table 1. Compared to wild type, the *nuc* mutants appear to belong to two groups; the group I which included *nuc-3* and *nuc-6* have much increased spontaneous mutation frequencies (10 × the wild type). In contrast, the group II which consists of *nuc-4*, *nuc-5* and *nuc-7* showed much reduced (0·01–0·5 × the wild type) frequency. The mutation frequency of the wild type strain is identical to the values reported earlier from this laboratory (Delange & Mishra, 1982). In all cases, the PFA-resistant colonies were retransferred to fresh PFA plates in order to determine whether these were indeed PFA-resistant mutants and not just adaptively growing sensitive isolates; more than 95% of the transferred colonies were able to grow on the fresh PFA plates confirming the mutant nature of the PFA resistant colonies.

4. DISCUSSION

On the basis of mutagen sensitivities the present mutants show remarkable variation in their properties. The *nuc-3* was characterized by extreme sensitivities to all three mutagen (UV, MMS and MNG). Both *nuc-4*, *nuc-5*, *nuc-6* were characterized by sensitivities to UV, MNG and mitomycin-C but resistance to MMS. The mutant *nuc-7* was found least sensitive to UV, MMS and mitomycin-C. It is of interest to mention that *nuc-5* showed MMS-sensitivities when grown on a MMS-containing medium but not when the MMS-treated conidia were plated on normal growth medium. These data suggest that *nuc*-5 is unable to repair continuing damage to DNA by MMS.

A number of genes are known to control mutagen sensitivities in *Neurospora crassa* (Schroeder, 1975; Kafer, 1981; Fraser & Kafer, 1979; Delange & Mishra 1981, 1982). The present *nuc* mutants do not appear to be allelic to either of the previously described mutants because of their particular location on the linkage group II (right to the *arg-12*). Among the five *nuc* mutants described here, only *nuc-3* compares with the *mus-14* in its extreme sensitivity to a number of mutagen and in its high mutator effects (see Delange & Mishra, 1981, 1982). The effect of *mus-14* on recombination is not yet determined, however, the homozygous crosses are sterile (Delange & Mishra, 1982).

Besides the *nuc* mutants described here only other mutants which seem to be deficient in deoxyribonucleases are those described by Fraser (1979) and by Fraser & Kafer (1979); however it remains to be established whether or not the deficient nucleases are identical in these mutants. The other mutants in fungi which have been shown to possess reduced levels of nucleases are *nuc-1* and *nuc-2* in *Utilago* (Holloman & Holliday, 1973); *rad-1* in yeast (Bryant & Haynes, 1978). However in none of these cases a direct gene-enzyme relationship has yet been established.

The study of the frequency of spontaneous mutation show a clear cut grouping

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of these mutants: group I included *nuc-3* and *nuc-6* which showed high mutator effect; group II included the remaining 3 mutants (*nuc-4*, *nuc-5*, and *nuc-7*) which were characterized by their antimutator effect. The three mutants varied in their antimutator effect to a great extent. The *nuc-4* showed prominent antimutator effect (almost 100 × less than the wild type), the *nuc-5* showed a moderate antimutator effect (only 10 × less than the wild type) where as the *nuc-7* showed a weak antimutator effect (with a mutation frequency 0·5 times of that of the wild type). Such changes in the mutation frequency at the PFA locus among the *nuc* mutants are significant and compare with similar changes described in the MMS-sensitive mutants of *Neurospora* (Delange & Mishra, 1981, 1982; Kafer, 1981).

It is remarkable that among the *nuc* mutants, a mutator effect is correlated to a decreased recombination frequency where as an antimutator effect is related to an increased recombination frequency. The *nuc-3* and *nuc-6* mutants with high degree of mutator effect appeared to represent mutation in genetic elements controlling recombination (Stahl, 1980) and were characterized by extremely reduced frequency of recombination in heterozygous crosses involving *nuc-3* and *nuc-6* mutations (Forsthoefel & Mishra, see first paper in this series). The intercrosses between *nuc-3* and *nuc-6* yielded no recombinant progeny even though they are several map units apart (Forsthoefel & Mishra, see the previous paper). Where as the mutants *nuc-4*, *nuc-5*, and *nuc-7* which showed different degree of antimutator effect were found to promote the frequency of the wild type recombinant in crosses involving these mutations (Forsthoefel & Mishra, see the previous paper). It is proposed here that (unlike *nuc-3* and *nuc-6*), this group of mutant (i.e. *nuc-4*, *nuc-5*, and *nuc-7*) represent mutation in genetic elements which in their wild type form ordinarily binds with an inhibitor of recombinogenic signal and that they (*nuc-4*, *nuc-5*, and *nuc-7*) promote recombination by relief of this inhibition (of a recombinogenic signal). However these ideas regarding the nature of *nuc* mutation must be tested by tetrad analysis using easily detectable outside markers. The mitomycin-C sensitivity seems to be restricted to the group of mutants which are sensitive to a wide spectrum of mutagen (UV, MMS, and MNG). It is of interest to mention here that the sensitivity of the *nuc-3* and *nuc-6* mutants toward mitomycin-C is consistent with their possible roles in recombination. Mitomycin-C has been used to select mutants of Escherichia which are deficient in recombination (Clark, 1976). The other *Neurospora* mutants which are sensitive to mitomycin-C are *uvs-3*, *uvs-6*, *mei-3*, and *nuh-4* (Chow & Fraser, 1978) and the *mus* mutants described by Kafer (1981). The properties of the mutants discussed here suggest that the *nuc* mutations adversely affect a biochemical step common to DNA repair, recombination and mutation induction pathways. A defective deoxyribonuclease can cause the observed changes in the DNA repair, recombination and mutation frequency among the nuclease mutants. Additional biochemical characterization of the *nuc* genes and their products using methods of molecular cloning now available in fungi (see Mishra, 1982 for a review on this subject, also see Schablik *et al.* 1983) can provide a better insight into these mechanisms. The nature of these mutation in relation to mutagen sensitivities and possible involvement in DNA repair can also be probed by rescue methods recently

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described by Stadler & Moyer (1981). The properties of *nuc* mutants described in this paper clearly point out their usefulness in future studies to elucidate the mechanism of DNA repair and recombination in *Neurospora*, a lower eukaryote.

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