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Deoxyribonuclease-deficient mutants of *Ustilago maydis* with altered recombination frequencies

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SUMMARY

Current molecular models of genetic recombination invoke steps mediated by deoxyribonucleases. It is likely therefore that some mutants deficient in these enzymes would have defective recombination mechnisms. Mutants of *Ustilago maydis* deficient in extracellular and intracellular DNase activity have been isolated. Three of the extracellular mutants are recessive and non-complementing, as are four of the intracellular mutants. The loci for extracellular and intracellular DNase deficiency have been named *nuc-1* and *nuc-2* respectively, and they are linked.

In crosses where each parent was deficient in both extracellular and intracellular DNase activity, meiotic allelic recombination (gene conversion) within the *nar* locus was abolished, although crossing-over in one tested interval was apparently normal in frequency. In DNase-deficient diploids, no mitotic allelic recombination in the *nar* locus was observed and the distribution of crossing over was abnormal. Nuclease deficiency did not appear to have any strong effect on radiation sensitivity.

1. INTRODUCTION

Breakage and reunion models of genetic recombination have been proposed by Whitehouse (1963), Holliday (1964) and others (see review by Davern, 1971). Holliday (1968) proposed a series of enzymic steps by which crossing-over and gene conversion might occur, but in fungi none of the enzymes and other proteins specifically required for these processes have been identified. At least two DNases were required by this model. Although many DNases have been purified from various organisms (more than eight in *Escherichia coli*), their physiological roles have rarely been discovered (Richardson, 1969). In procaryotes two DNases have been shown to be necessary for recombination. *red* mutants of λ phage which do not recombine vegetatively lack λ exonuclease (Radding, 1970), and an ATP-dependent exonuclease is absent from extracts of *recB* and *recC* mutants of *E. coli* (Buttin & Wright, 1968; Willetts & Clark, 1969). As well as being recombination-deficient, these *rec* mutants are also radiation-sensitive, which suggests that the missing DNase is shared by the recombination and repair systems. Most recombination-deficient mutants of procaryotes are radiation-sensitive, and many radiation-sensitive mutants have abnormal recombination (Howard-

Flanders, 1968). Since recombination-deficient mutants cannot be easily selected directly in eucaryotes, usually radiation-sensitive mutants have been tested for abnormal recombination. Among those showing effects are *uvs-1*, *uvs-2* (now designated *rec-1* and *rec-2*) and *uvs-3* of *U. maydis* (Holliday, 1967); *uvsB*, *uvsC*, *uvsD* and *uvsE* of *Aspergillus nidulans* (Fortuin, 1971, and references therein); *uvs-3* of *Neurospora crassa* (Schroeder, 1970) and several *rad* mutants of yeast (Hunnable & Cox, 1971). More recently Rodarte-Ramón & Mortimer (1972), using a disomic strain of yeast, were able to select *rec* mutants directly due to their deficiency in radiation-induced mitotic heteroallelic recombination. Yeast *rec-1* showed normal meiotic recombination, but meiosis was abortive in *rec-2* and *rec-3*. Strains carrying *rec-4* showed depressed meiotic conversion, though intergenic recombination was unaffected. These four mutants showed wild-type UV sensitivity and only *rec-2* was X-ray sensitive. In no case is the enzymic basis for the abnormal repair and recombination known.

The isolation of mutants deficient in enzymes of the type required for recombination might provide another indirect method of obtaining recombination deficient mutants. Nuclease-deficient mutants have been isolated from *N. crassa* (Ishikawa *et al.* 1969). The mutants at two unlinked loci *nuc-1* and *nuc-2* show reduced activity of an intracellular nuclease which digests both DNA and RNA. *Nuc-2* mutants are more sensitive to UV light than wild-type, but show no difference in X-ray sensitivity. No significant effect on crossing-over or allelic recombination was found in crosses homozygous for *nuc-1*, *nuc-2* or both genes. A mutant of *U. maydis* with reduced extracellular DNase activity was isolated by Holliday & Halliwell (1968); the phenotype is apparently the result of two mutations, and the effect of these on recombination is not known. The aim of this study was to isolate further DNase deficient mutants in *U. maydis* and to measure their effects on recombination and repair.

2. METHODS

The composition of media and the methods of genetical analysis were essentially those described by Holliday (1961*a*, *b*).

(i) *Strains*

All mutants were derived from a haploid wild-type of mating type a_2b_1. They were crossed to standard laboratory strains with the following markers: *ad 1–1*, *me 1–2*, *pan 1–1*, *nar 1–1* and *nar 1–6*. These indicate requirements for adenine, methionine, pantothenate and the inability to utilize nitrate as sole source of nitrogen. (For nomenclature of mutant strains see Unrau & Holliday, 1972.)

(ii) *Media*

Minimal medium was the same as previously described except that the nitrogen source was 3 g/l. potassium nitrate. Mutants were detected on Difco tryptic soy agar, to which filter-sterilized crude DNA was added after autoclaving, giving a

final concentration of 0·3 %. Mating medium was 1·7 g Difco cornmeal agar added to 100 ml liquid complete medium.

(iii) *Mating-type tests and the isolation of diploids*

This method is Holliday's (unpublished) modification of that devised by Puhalla (1968). Strains to be tested were streaked on mating medium and the four tester mating types streaked to within 2 mm. Plates were sealed with sticky tape and incubated for 2 days at 28 °C in an illuminated incubator. White heterocaryotic hyphae appeared along the interface between opposite mating types. Diploids were isolated by streaking together strains of complementary mating types, each having a different nutritional requirement. When white hyphae appeared, the plate was replica-plated to minimal medium on which heterocaryotic hyphae grew slowly whereas diploids formed vigorous colonies. Single colony isolates were made of each diploid, and diploidy was checked for solopathogenicity by inoculation into maize seedlings.

(iv) *Irradiation*

The UV source was a Hanovia low-pressure mercury lamp delivering 46 ergs per mm^2 per sec. Cells were irradiated at 10^6 per ml in water. γ irradiation was from a Gammabeam 650 ^{60}Co source delivering 40 krads per min. Cells were irradiated at 10^7 per ml in water with stirring.

(v) *Biochemical methods*

The extracellular DNase activity of culture supernatants was assayed on calf thymus DNA using an Ostwald viscometer (see Holliday & Halliwell, 1968). Cell-free extracts were prepared from stationary phase cells in 0·05 M Tris/HCl, pH 7·2. Washed cells were suspended at 2×10^9/ml. Half a volume of Ballotini (grade II) was added, and the suspension shaken in a Mickle disintegrator for 10 min at about 4 °C. The extract was centrifuged at 12000 rev/min at 2 °C for 20 min. The supernatant was used as a crude extract.

Intracellular DNase activity was assayed using ^{32}P-labelled *E. coli* DNA substrate. The reaction mixture contained:

^{32}P *E. coli* DNA (20–50 μg/ml)	10 μl
$1·5 \times 10^{-2}$ M-$MgCl_2$	10 μl
0·05 M Tris/HCl, pH 7·2	110 μl
Enzyme preparation	20 μl

After 30 min incubation at 32 °C the reaction was stopped by adding 100 μl ice-cold unlabelled carrier DNA (calf thymus 1·5 mg/ml) followed by 250 μl ice-cold 5 % TCA. After mixing, the tubes were held in ice for 10 min before centrifuging at 8000 rev/min at 2 °C for 10 min; 0·1 ml aliquots of the supernatant were counted in 10 ml scintillation fluid (Bray, 1960). Protein determinations were by the method of Lowry *et al.* (1951). DNA cellulose chromatography was carried out basically according to Alberts *et al.* (1968). Calf thymus DNA was used to prepare the DNA cellulose.

3. RESULTS

(i) *Isolation of mutants*

Two mutants deficient in extracellular DNase activity were isolated by the method used by Holliday & Halliwell (1968). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) mutagenized wild-type cells were plated on tryptic soy agar containing DNA (TSD) at approximately 80 cells per plate. After 3 days incubation the colonies were rescued by replica-plating to complete medium. The DNA in the medium was then precipitated by placing a filter paper in the lid of the dish and adding a few drops of concentrated hydrochloric acid. After 5–10 min haloes of digestion were clearly defined around wild-type colonies. Any colony having reduced activity was picked from the replica and retested. Many of these colonies were found to be wild-type. The apparent reduced activity was probably due to their being rather smaller colonies and consequently not producing much enzyme.

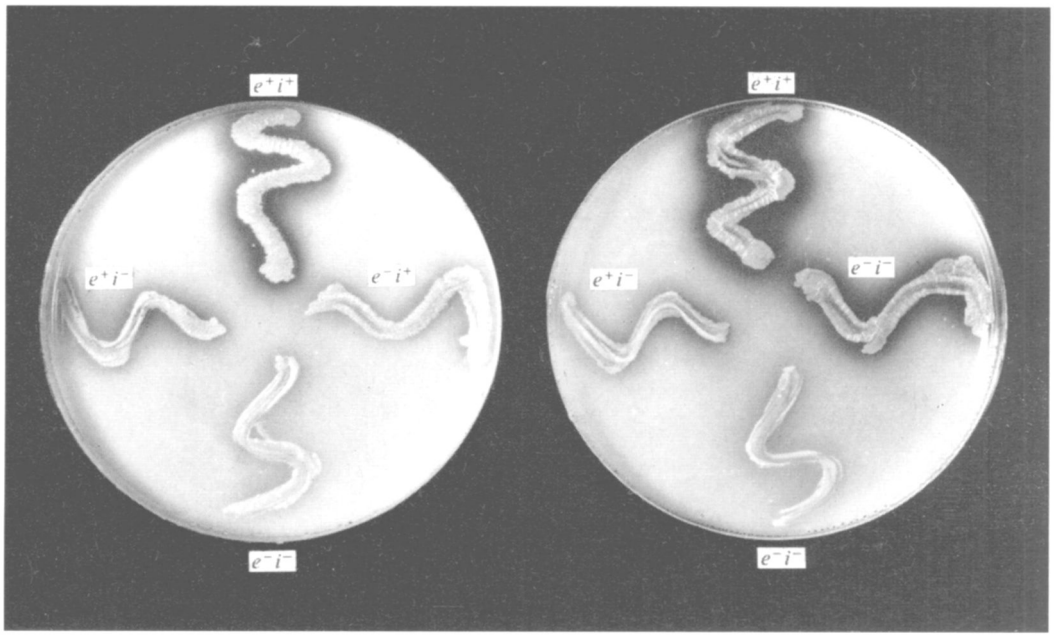
It was thought that DNases concerned with the recombination of DNA molecules would be intracellular. A method was therefore developed for scoring mutants deficient in intracellular DNase. After rescuing mutagenized colonies plated on TSD medium by replica-plating to complete medium the TSD plates were rinsed with toluene. After about 6 h incubation at 32 °C the DNA was precipitated in the usual way. Large haloes were seen around wild-type colonies. Five mutants deficient in intracellular DNase were isolated from the two extracellular mutants in this way. Two additional mutants deficient in intracellular and extracellular DNase activity were isolated after toluene treatment of mutagenized wild type cells. Table 1 shows details of the experiments in which mutants were isolated and Plate 1 the DNase activity of several strains on TSD medium.

(ii) *Assignment of mutants to genetic loci*

The two loci for extracellular and intracellular DNase deficiency have been named *nuc-1* and *nuc-2* respectively. Since diploids heterozygous for *nuc-1* and *nuc-2* had wild-type DNase activity, the mutations are recessive. On the basis of complementation tests three extracellular DNase-deficient mutants are allelic, the fourth was not tested. Four intracellular DNase mutants are alleles at another locus. The phenotypes and genotypes of the mutants isolated are shown in Table 2. Complementation between the extracellular DNase-deficient mutants and the mutant isolated by Holliday & Halliwell (1968) was not tested. Therefore it is not known if they are allelic.

(iii) *Biology of the DNase mutants*

None of the mutants showed significant differences from wild-type in cell shape or intracellular structure as examined by phase-contrast with a Zeiss Universal microscope. They grew more slowly than wild-type, with an average generation time in complete medium of about 135 min compared with 90 min. Growth curves of three mutants are shown in Fig. 1. Mutant strains inoculated into maize seedlings to perform crosses were less infectious than DNase-positive strains, which suggests that DNases may play an important role in infection.



(*a*) (*b*)

DNase activity of several strains on tryptic soy agar containing DNA after precipitation of the DNA hydrochloric acid. (*a*) without toluene, (*b*) after incubation with toluene. The reduced extracellular DNase activity of strain e^+i^- is due to slow growth.

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Table 1. *Isolation of deoxyribonuclease-deficient mutants of Ustilago maydis*

Experiment	Strain used	NG treatment (min)*	Survival (%)	Colonies scored	Mutant number
1, 2	a_2b_1	25	4, 9	2950	117 114
3, 4, 5	114	15–30	0·05–7·5	1500	4/131 4/96
6, 7	117	30	0·6, 1·0	2000	7/301 7/342 7/159
8	a_2b_1	30	0·04	400	162 175

* NG concentration = 20 μg/ml.

Table 2. *Phenotypes and genotypes of DNase-deficient mutants*

Mutant	DNase phenotype: Extracellular	DNase phenotype: Intracellular	Genotype
117	−	+	*nuc* 1–1
114	−	+	*nuc* 1–2
7/301	−	−	*nuc* 1–1 *nuc* 2–1
7/342	−	−	*nuc* 1–1 *nuc* 2–2
7/159	−	−	Complementation not tested
4/131	−	−	*nuc* 1–2 *nuc* 2–3
4/96	−	−	Not tested
175	−	−	*nuc* 1–3 *nuc* 2–4
162	−	−	Not tested

(iv) *Linkage of* nuc-1 *and* nuc-2

The linkage between *nuc-1* and *nuc-2* and of these mutants with auxotrophic markers was tested with crosses between mutants and DNase-positive auxotrophs. The scoring of extracellular DNase activity was usually unambiguous. Intracellular DNase activity was easily scored in colonies deficient in extracellular DNase, but it was difficult to identify intracellular DNase deficiency in the presence of extracellular DNase. With experience it proved possible to score these colonies on the basis of the size of the halo of DNA digestion.

The data obtained from the random products of meiosis of two crosses are shown in Table 3. In cross 1, linkage was demonstrated between *nuc-1* and *nuc-2* (recombination frequency 13·7 %). No linkage between these and any of the auxotrophic markers was found. The recombination between *ad 1–1* and *me 1–2* (19 %) fell within the normal range. Cross 2 involved different alleles for DNase deficiency. Linkage again was demonstrated between *nuc-1* and *nuc-2* (recombination frequency 26 %). The variation in the recombination frequency between *nuc-1* and *nuc-2* may have a similar basis to that between *ad-1* and *me-15*, which varies between 15 and 30 % (Holliday, 1967, references therein and unpublished observations). The relatively loose linkage between *nuc-1* and *nuc-2* may not have any functional significance.

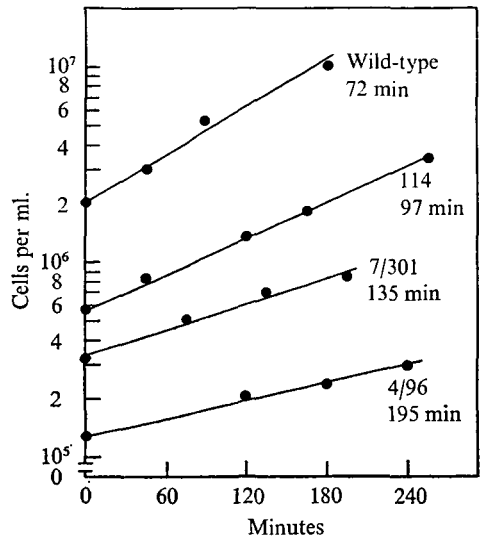

Fig. 1. Growth curves of wild type and DNase-deficient strains in complete medium. The generation time of each strain is shown in minutes.

Table 3. *The linkage of* nuc 1 *and* nuc 2

Cross 1: *nuc* 1–1 *nuc* 2–1 *pan* 1–1 $a_1b_2 \times$ *ad* 1–1 *me* 1–2 *nar* 1–6 a_2b_1

Random products of meiosis (226 progeny)

Allele frequencies

	ad	*me*	*nar*	*pan*	*e**	*i*†
+	117	120	127	137	97	100
−	109	106	99	89	129	126

Recombination frequencies

	ad	*me*	*nar*	*pan*	*e*
i	49·1	49·6	55·3	42·0	13·7
e	49·6	52·7	53·1	45·1	
pan	55·8	56·2	65·9		
nar	44·2	45·6			
me	19·0				

Cross 2: *nuc* 1–2 *nuc* 2–3 $a_2b_1 \times$ *nar* 1–1 *pan* 1–1 a_1b_2

Random products of meiosis (146 progeny)

Allele frequencies

	nar	*pan*	*e**	*i*†
+	57	90	62	89
−	89	56	84	57

Recombination frequencies

	i	*e*	*pan*
nar	46·5	50·0	39·0
pan	47·0	46·5	
e	26·0		

* Extracellular DNase-deficient.
† Intracellular DNase-deficient.

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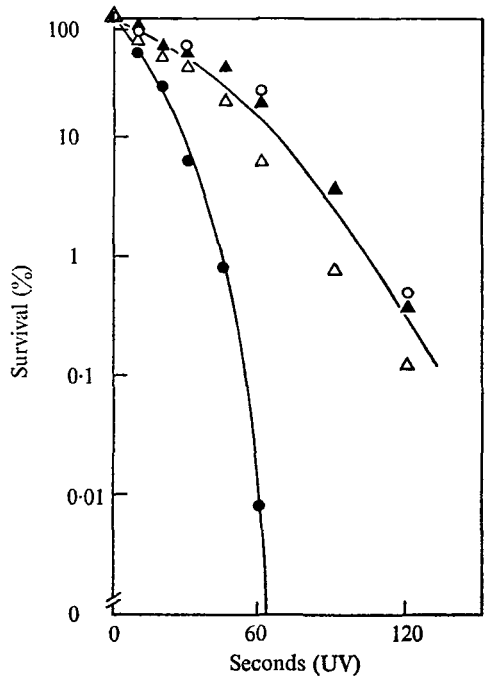

Fig. 2. UV survival of log phase wild-type and DNase-deficient haploid strains. ○——○, Wild type; ●——●, *nuc* 1–1 *nuc* 2–1 (7/301); △——△, e^-i^- (162); ▲——▲, e^-i^- (4/96).

Table 4. *UV sensitivity of random products of meiosis from cross 3:* nuc 1–1 nuc 2–1 $a_2b_1 \times$ nar 1–1 pan 1–1 a_1b_2

DNase activity	UV-resistant	UV-sensitive
e^+i^+	70	53
e^-i^-	60	50
e^+i^-	20	22
e^-i^+	24	16
	174	141

(v) *Effects of DNase deficiency on radiation sensitivity*

Since DNases are thought to be necessary for dark repair of UV and ionizing radiation damage, the sensitivity of the DNase-deficient strains to these treatments was tested. Fig. 2 shows the UV survival of several strains in log phase. No strains were more resistant than wild-type, and only one mutant, *nuc 1–1 nuc 2–1*, was found to be more sensitive. However, this sensitivity was found to be due to an independently segregating mutation as shown in Table 4. The γ-ray survival curves of the DNase-deficient diploids are compared with that of a DNase-positive control in Fig. 3. A proportion (30–80 %) of the population of DNase-deficient diploids was hypersensitive to the lowest dose of γ-rays (40 krads). The remaining cells were resistant to irradiation up to about 200 krads, above

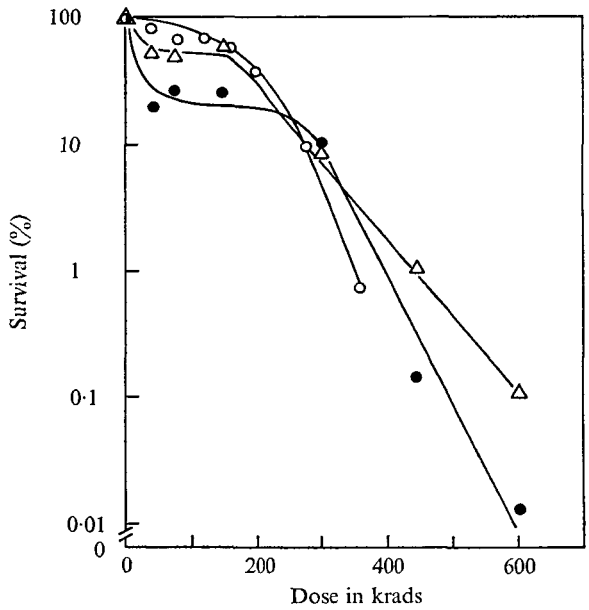

Fig. 3. γ-Ray survival of stationary-phase DNase-positive and DNase-deficient diploid strains. ○——○, DNase-positive (436); △——△, DNase-deficient (D 1); ●——●, DNase-deficient (D 2).

which survival was exponential and close to control levels. DNase-deficient haploids also showed the hypersensitivity and plateau region. All the cultures were irradiated in stationary phase, so it is unlikely that the hypersensitivity was due to a proportion of the cells undergoing DNA replication.

(vi) *Effects of DNase deficiency on recombination*

Mitotic recombination was measured according to the method of Holliday (1961*b*) in the following DNase-deficient diploids:

$$\text{D 1}\ \frac{nar\ 1\text{–}1}{nar\ 1\text{–}6}\ \frac{pan\ 1\text{–}1}{+}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{nuc\ 1\text{–}1\ nuc\ 2\text{–}2}{nuc\ 1\text{–}1\ nuc\ 2\text{–}1},$$

$$\text{D 2}\ \frac{nar\ 1\text{–}1}{nar\ 1\text{–}6}\ \frac{pan\ 1\text{–}1}{+}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{nuc\ 1\text{–}3\ nuc\ 2\text{–}4}{nuc\ 1\text{–}1\ nuc\ 2\text{–}1},$$

$$\text{D 3}\ \frac{nar\ 1\text{–}1}{nar\ 1\text{–}6}\ \frac{pan\ 1\text{–}1}{+}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{nuc\ 1\text{–}1\ nuc\ 2\text{–}1}{nuc\ 1\text{–}1\ nuc\ 2\text{–}1}.$$

Another diploid was heterozygous for *nuc 1–1* and *nuc 2–1* and was DNase positive:

$$\text{D 4}\ \frac{nar\ 1\text{–}1}{nar\ 1\text{–}6}\ \frac{pan\ 1\text{–}1}{+}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{nuc\ 1\text{–}1\ nuc\ 2\text{–}1}{+\quad +}.$$

This was used as a control together with another heteroallelic diploid (strain 436) which carried similar auxotrophic markers and contained no *nuc* mutants. *Nar 1–1* and *nar 1–6* are non-complementing alleles in the structural gene for

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Table 5. *Spontaneous mitotic allelic recombination (gene conversion) in DNase-deficient diploids and a DNase-positive diploid*

Strain	Total cells plated	No. of populations	Recombinants per 10^7 cells		
			Range	Mean	Median
436 (DNase+)	$1{\cdot}2 \times 10^9$	20	65–810	215	202·5
D 1	$8{\cdot}3 \times 10^8$	20	0	0	0
D 2	$2{\cdot}9 \times 10^9$	20	0–1	0·28	< 1·0

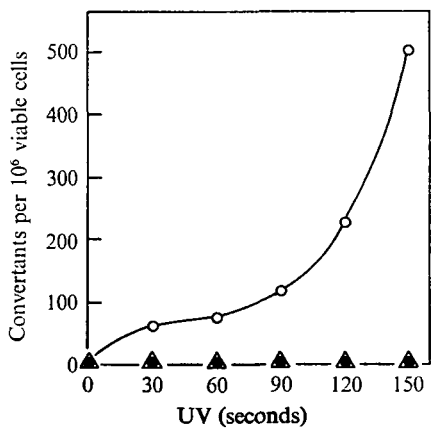

Fig. 4. UV-induced mitotic gene conversion in stationary-phase DNase-positive and DNase-deficient heteroallelic diploids. ○——○, DNase-positive diploid (436); △——△, DNase-deficient diploid (D 1); ●——●, DNase-deficient diploid (D 2).

nitrate reductase (Holliday, 1966), therefore the diploids require ammonia for growth. *Me 1–2* is situated between *ad 1–1* and the centromere.

The frequency of spontaneous allelic recombination (gene conversion) in the *nar* locus was measured by a fluctuation test. Twenty cultures, inoculated with a small number of cells from one starter culture, were grown to stationary phase (about 14 generations). The cells were washed and plated on minimal medium to select protrophic recombinants. The recombination frequency was calculated by the method of the median (Lea & Coulson, 1949). The results are shown in Table 5. Spontaneous allelic recombination in the DNase deficient diploids was extremely low. UV-induced allelic recombination in log phase cells is shown in Fig. 4. Survival after 60 sec averaged 40 % and after 150 sec 6 %. D 3 was similar to D 1 and D 2, whereas D 4 was similar to the control. Very few recombinants were obtained from DNase-deficient strains. The recessive character of the DNase-deficient mutants was confirmed by the wild-type recombination in D 4 heterozygous for *nuc-1* and *nuc-2*.

One explanation of the lack of recombination in some strains is that they were no longer heteroallelic at the *nar* locus. To check that this was not so, diploids were innoculated into maize seedlings and the resulting tetrads isolated.

Table 6. *Genotype of a meiotic tetrad from D 1*

	ad 1–1	*me* 1–2	*pan* 1–1	*nuc* 1–1	*nuc* 2–1	*nar* 1–1/6
1	+	−	−	−	−	1
2	−	+	+	−	−	1
3	−	−	−	−	−	6
4	+	+	+	−	−	(6)

Table 7. *Spontaneous and UV-induced mitotic segregation in DNase-deficient diploids*

						Phenotypes and simplest derivation of segregants					
				Segregants		Single exchanges			Multiple exchanges		
Diploid	UV (sec)	Survival* (%)	Colonies scored	no.	%	*ad*	*ad me*	*pan*	*me*	*me pan*	*ad me pan*
D 1	0	100	2150	1	0·05	0	0	0	1	0	0
	150	0·1	1760	23	1·31	10	1	3	6	1	2
D 2	0	100	1850	9	0·49	0	3	4	0	1	1
	150	0·04	1360	45	3·31	14	4	1	10	8	8
D 3	0	100	1007	0	0·1	0	0	0	0	0	0
	150	7·2	2500	8	0·36	1	0	7	0	0	0

* Differences in UV survival were due to cells being irradiated at various stages of late log phase.

After characterization of the auxotrophic markers, diploids were synthesized using tetrad members and DNase-positive *nar-1–1* and *nar-1–6* strains. UV-induced allelic recombination was then tested to identify the *nar* allele carried by each tetrad member. Diploids carrying unlike *nar* alleles would produce recombinants, those carrying like alleles would not. The full genotype of one tetrad is shown in Table 6. The *nar 1–6* allele in 4 is inferred. Since it carried no auxotrophic markers, a diploid carrying one known *nar* allele could not be obtained. It can be seen that D 1 must have been heteroallelic at the *nar* locus.

The frequency of segregation of auxotrophs was taken as a measure of mitotic crossing over (Holliday, 1961*b*; Holliday, 1967). The results are shown in Table 7. The spontaneous recombination frequency for D 1 fell within the normal range (0·05–0·1 %). However, the phenotype of the single sergregant isolated apparently indicated a double cross-over event (since *me 1–2* is proximal to *ad 1–1*). UV irradiation stimulated crossing-over as expected, but an unusually large proportion of segregants due to multiple events were found. The frequency of *me 1–2* segregants is usually about 10 % (Holliday, 1961*b*; Holliday, 1967), and segregants due to multiple events involving more than one linkage group are normally rare.

D 2 appeared to show a high frequency of spontaneous crossing-over which was stimulated by UV light. There was again a high proportion of segregants attributable to multiple cross-overs. No spontaneous segregants were isolated from D 3, although examination of a larger number of colonies would be necessary

Table 8. *DNase activity of auxotrophic mitotic segregants from DNase-deficient diploids*

Diploid	UV (sec)	Segregant phenotypes: Single cross-overs DNase⁻	Single cross-overs DNase⁺*	Multiple cross-overs DNase⁻	Multiple cross-overs DNase⁺*
D1	0	0	0	0	1
	150	13	1	4	5
D2	0	7	0	1	1
	150	15	4	15	11
D3	0	0	0	0	0
	150	8	0	0	0
		43	5	20	18

* Intracellular DNase-positive. Some segregants of D2 were also extracellular DNase-positive. Since the other diploids were homoallelic for *nuc* 1, there was no possibility of recombination or complementation.

Table 9. *Meiotic allelic recombination in DNase-deficient diploids*

Diploid	Random products tested	Recombinants	Rate
D1	$5{\cdot}04 \times 10^7$	0	$< 2 \times 10^{-8}$
D2	$4{\cdot}95 \times 10^7$	0	$< 2 \times 10^{-8}$

to demonstrate a deficiency. All the UV-induced segregants were attributable to single cross-overs.

The DNase activity of mitotic segregants was also scored, although they were expected to be DNase-deficient, since mitotic allelic recombination had been shown to be absent. The phenotypes are shown in Table 8. About half of the multiple segregants were intracellular DNase-positive, but only 10 % of the single cross-over segregants. This result is very surprising, since allelic recombination at the *nar* locus did not occur. Possible explanations will be considered in the Discussion section.

Meiotic recombination was measured by innoculating DNase-deficient diploids into maize seedlings and analysing the random products of meiosis. Allelic recombination was measured by plating random spores on minimal medium supplemented with adenine, methionine and pantothenate. On this medium only *nar*⁺ recombinants would grow. The recombination frequency in DNase-positive diploids is $1{\cdot}2 \times 10^{-3}$ (Holliday, 1967). Table 9 shows that meiotic allelic recombination within the *nar* locus was completely deficient in D 1 and D 2.

The frequency of crossing-over between *ad-1* and *me-15* in meiosis varies from 15 to 30 % in DNase-positive strains (Holliday, 1961*a*; Holliday, 1967). Table 10 shows that crossing-over between these markers during meiosis of D 1 and D 2 fell within this range. However, since the range is so wide, abnormal recombination might not be detected.

Table 10. *Meiotic crossing over in DNase-deficient diploids*

Diploid	Parentals		Recombinants		Recombination frequency (%)
	adme	++	*ad*+	+*me*	
D 1	127	127	24	65	25·9
D 2	104	164	38	32	24·05

Table 11. *DNase activity of meiotic progeny of D 2*

	DNase phenotype			
	e^+i^+	e^-i^-	e^+i^-	e^-i^-
Number	33	17	15	226
Frequency (%)	11·3	5·8	5·2	78·7

Table 12. *Mitotic allelic recombination in D 5*

U.v. dose (sec)	Survival (%)	*nar*+ recombinants	Recombinants per 10^6 viable cells
0	100	206	6·1
30	84	483	169
150	0·059	79	39500

The random products of meiosis of D 1 and D 2 were tested for DNase activity. Of 243 products of D 1, 6·4% were intracellular DNase-positive. None were extracellular DNase-positive as expected since D 1 was homoallelic for *nuc-1*. DNase-positive progeny were also recovered from D 2 as shown in Table 11. These frequencies are exceedingly high if they represent recombination between supposed alleles. This will be discussed later.

Some further evidence was obtained which indicated that the DNase deficiency and the recombination deficiency were due to the same mutation. A diploid, D 5, heteroallelic at the *nar* locus, was obtained from a DNase-deficient strain (*nuc 1–1 nuc 2–1*) and a DNase-positive 'recombinant' which was one of the meiotic progeny of D 2. D 5 was DNase-positive as expected. Table 12 shows that D 5 had wild-type spontaneous and UV-induced allelic recombination in the *nar* locus. Thus a strain which had regained DNase activity had also regained recombination ability.

(vii) *Biochemistry of DNase-deficient mutants*

The extracellular DNase-deficiency of *nuc-1* mutants was confirmed by viscometric assay of culture supernatants by the method used by Holliday & Halliwell (1968). The results are shown in Fig. 5. It was not possible to release intracellular DNase activity into aqueous solutions using toluene. Instead, crude extracts were made with a mickle disintegrator. Crude extracts contained considerable DNase activity, particularly on heat-denatured DNA, as is shown in Fig. 6. Significant qualitative or quantitative differences between the mutants and wild-type were

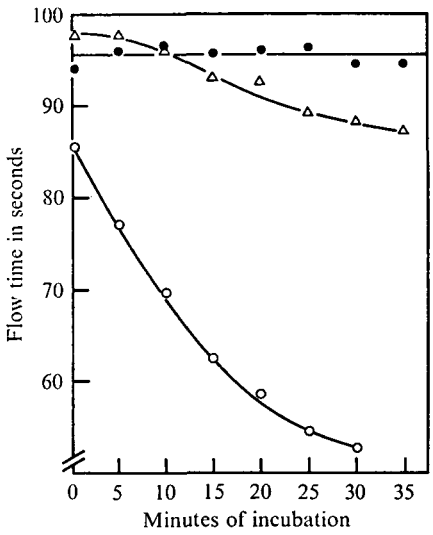

Fig. 5

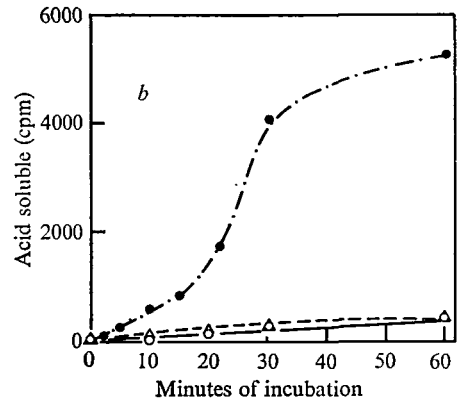

Fig. 6

Fig. 5. DNase activity of culture supernatants of wild-type and DNase-deficient mutants measured by viscometry. ○——○, Wild-type; ●——●, *nuc* 1–1; △——△, *nuc* 1–2.

Fig. 6. DNase activity of a wild-type crude extract on ^{32}P-labelled *E. coli* DNA. ○——○, Native DNA; ●—·—●, heat-denatured DNA; △---△, UV-irradiated DNA.

not found. This is in contrast to the plate DNase assay by which the mutants were identified. Two explanations are possible. The toluene may have affected the cells in such a way as to release only one active enzyme into the medium. Alternatively, only one of several intracellular DNases may have been active in the environment of the tryptic soy medium.

The complex nature of the curve for denatured DNA provides evidence for more than one active enzyme in the extract. The linear increase in the acid-soluble material released during the first 15 min may be de to an exonuclease, possibly mixed with an endonuclease specific for single-stranded DNA. The rapid increase in the rate of release of acid-soluble material during the next 15 min could be due to such an endonuclease beginning to release acid-soluble products. Alternatively, there may be an endonuclease present which is specific for very large molecules of DNA whose products are not acid-soluble. Such an enzyme would create additional sites for exonuclease activity and it may be the products of this which are observed. The activity on native and UV-irradiated DNA is probably due to another distinct enzyme.

Further evidence for the presence of multiple enzyme activities in the crude extract was found. The DNase activity on several types of DNA substrate (native, heat-denatured, UV-irradiated) over a range of pH varied apparently at random. DNase activity was slightly enhanced by $MgCl_2$, but the optimum concentration was different for each substrate. Similarly the inhibiting concentration of EDTA was different for the three substrates (see Badman, 1971).

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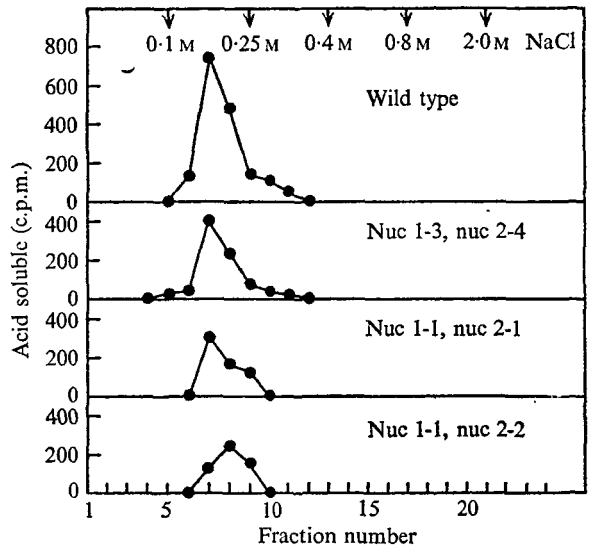

Fig. 7. DNase activity on denatured DNA recovered after DNA cellulose chromatography of crude extracts of wild-type and DNase-deficient strains.

It was clear that fractionation of the crude extract was necessary to identify the enzyme deficient in *nuc-2* strains. DNA cellulose chromatography (Alberts *et al.* 1968) seemed to provide a direct method of fractionating the unknown number and type of DNases in *U. maydis*. It was hoped that comparison of the elution patterns of wild-type and mutant extracts would demonstrate the DNase deficiency of mutant strains. Many variations of the method of Alberts *et al.* (1968) were used in attempting to characterize wild-type extracts (see Badman, 1971). Fig. 7 shows the DNase activity on denatured DNA recovered from chromatography of wild-type and three mutants in one of the experiments. No DNase deficiency was apparent in the mutants. It was eventually discovered that a proportion of the crude extract protein was being discarded when the endogenous DNA was removed. It therefore seems likely that the DNase which was deficient in the mutants was being lost from wild-type extracts at this stage.

4. DISCUSSION

It has been shown that DNase-deficient mutants of *U. maydis* can easily be obtained by a two-step process. Colonies of double mutants showed no extracellular or intracellular DNase activity when examined on tryptic soy agar containing crude DNA. Three extracellular DNase-deficient mutants were shown to be allelic, as were four intracellular DNase-deficient mutants at a different locus. The loci for intracellular and extracellular DNase deficiency were found to be linked. The mutants have reduced growth rates, but no effect on morphology was seen. No strong effect on repair was found. UV survival was the same as wild-type, but altered survival after γ-ray treatment may indicate a minor repair

deficiency. On the other hand the recombination processes were seriously abnormal.

Allelic recombination in the *nar* locus was abolished during both mitosis and meiosis. It is assumed that the abnormal recombination is caused by the intracellular DNase deficiency due to the *nuc-2* mutation rather than *nuc-1*, but this is not known. Crossing-over was in the normal range in meiosis, but in mitosis an abnormally high frequency of segregants attributable to multiple crossing over was found. This suggests that the distribution of exchanges may have been affected. Further genetic analysis of crossing-over and allelic recombination in different loci should resolve this.

A surprisingly high proportion of mitotic segregants and random meiotic products were DNase-positive. These were unexpected, as allelic recombination in the *nar* locus did not occur. There are several possible origins for these DNase-positive strains. The *nuc* mutants may not be closely linked alleles, and therefore the DNase-positive strains could be explained by crossing-over. Loosely linked non-complementing mutants are known in *Coprinus lagopus* (Lewis, 1961; Morgan, 1966). Alternatively the DNase activity may be due to modifiers cr suppressors. This is not altogether unlikely, as the laboratory strains of *U. maydis* are believed to be very heterozygous. The hypothesis could be tested by screening for DNase-deficient meiotic progeny of a cross between a DNase-positive 'recombinant' and wild-type. Another explanation is that the distribution of recombination is affected by *nuc-1* and *nuc-2*. Although allelic recombination within the *nar* locus is abolished, it may be enhanced in the *nuc-1* and *nuc-2* loci. An altered distribution of exchanges could also explain the more frequent regaining of DNase activity in mitotic segregants where multiple events have occurred rather than in those due to a single cross-over. An altered distribution of exchanges might also affect the expression of recessive suppressors in mitotic segregants. It has been suggested that the distribution of exchanges may be determined by the action of DNases at specific sites along the chromosome called recombinators (Holliday, 1968). Catcheside and his associates (see Catcheside & Austin, 1971) have studied in considerable detail the genetic control of recombination in different regions of the *Neurospora* genome, and their overall results certainly suggest that there may be several kinds of sites which influence recombination in their vicinity. Therefore, if the *nuc-1* and *nuc-2* mutations reduce the number of nucleases acting on such sites or alter their specificity, recombination might fail to be initiated near the *nar* locus, whilst occurring with high frequency at the *nuc* loci.

Alternatively, the effects of *nuc-1* and *nuc*-2 on recombination can be partly explained on the basis of current models of recombination. The complete absence of allelic recombination might be due to mis-matched base pairs remaining uncorrected because of a deficiency in an endonuclease specifically causing single strand breaks close to mis-matched pairs. Allelic recombination might also be reduced if the region of hybrid DNA formed during crossing-over was very short, or alternatively if the strands involved in hybrid DNA formation tended to reassociate rapidly with their original partners. This might be caused by the

deficiency of a DNase acting at half chromatid chiasmata. Another possibility is that the enzyme deficiency could allow a very long region of hybrid DNA to be formed. In this case co-conversion might be extensive (Fogel & Mortimer, 1969; Fincham & Holliday, 1970), and therefore allelic recombination might not be observed.

It has been assumed that the *nuc* mutations cause a DNase deficiency. It is possible that in fact they cause the synthesis of increased amounts of an inhibitor, or an inhibitor with altered specificity. (A protein inhibitor of DNases is known in *B. subtilis*; Strauss & Marone, 1967.) An inhibitor could abolish allelic recombination by binding to the excision enzyme and could alter the distribution of exchanges by altered binding to DNases or recombinators.

It has been shown that *nuc-1* mutants lack an extracellular endonuclease similar to the strain isolated by Holliday & Halliwell (1968). It has not yet been possible to characterize the DNases of wild-type *U. maydis* or to identify the deficiency of *nuc-2* mutants. The *nuc-2* mutants had considerable intracellular DNase activity, whereas none was evident on DNA-containing agar. It was hoped that by varying the conditions of extraction or assay it would be possible to show a difference between the mutant strains and wild-type and thus to identify the DNase deficiency. In these experiments attempts to demonstrate an enzyme deficiency in mutant strains were not successful. However, more recently Holloman (personal communication) has been able to identify two intracellular *Ustilago* DNases and show that one of them is deficient in *nuc-2* strains. Characterization of this enzyme is essential if one is to distinguish between the alternative explanations of the genetic data which have been considered.

Despite the relative lack of information on the biochemical defect of the *nuc-2* mutants, it has been shown, for the first time in eucaryotes, that the deficiency of a DNase causes recombination deficiency. Also, the particular stage of the recombination process which is blocked is unique to recombination and is not shared by the repair processes.

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