Genet. Res., Camb. (1983), **41**, *pp.* 271–286
With 1 *plate and* 3 *text-figures*
Printed in Great Britain

Biochemical genetics of Neurospora nuclease I: Isolation and characterization of nuclease (*nuc*) mutants

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(*Received 30 November 1982 and in revised form 4 February 1983*)

SUMMARY

Isolation and characterization of five new nuclease (*nuc*) deficient mutants of *Neurospora* have been described. The new mutants are unable to utilize nucleic acids as the sole phosphorus source and possess growth characteristics similar to those *nuc* (*nuc-1* and *nuc-2*) mutants described previously. Two new mutants (*nuc-4* and *nuc-5*) were able to use RNA or predigested DNA (but not intact DNA) as phosphorus source and showed temperature sensitive growth at 37 °C. Based on the data from complementation and genetic analyses the five new *nuc* mutants (*nuc-3*, *nuc-4*, *nuc-5*, *nuc-6* and *nuc-7*) were found nonallelic to each other and to previously described *nuc* (*nuc-1* and *nuc-2*) mutants; the new *nuc* mutants mapped to the right of *arg-12* on linkage group II. On biochemical analyses, these *nuc* mutants were found to possess a lower level of extracellular nucleases and alkaline phosphatase as compared to the wild type strain. The ds DNase activity of the new mutants was only about 2–12% of that of the wild type strain; thus, the low level of these extracellular enzymes in the *nuc* mutants causes their inability to utilize nucleic acids as the sole phosphorus source. Wild type levels of these enzymes were restored in the complementing heterokaryons capable of full growth on the DNA medium. Data from intercrosses, mutagen sensitivity and spontaneous mutation-frequency studies (as discussed in a subsequent paper) indicated the involvement of the *nuc* genes in DNA repair and recombination.

1. INTRODUCTION

In the past, a combination of biochemical and genetic approaches have been used to elucidate the role of deoxyribonucleases in the different aspects of the DNA metabolism (such as repair, recombination, replication and restriction). Much of the current knowledge regarding the metabolism of DNA has been elucidated from studies of bacteria (Clark & Volkert, 1978; Witkin, 1976; Hanawalt *et al.* 1979), and to some extent from those of mammalian cells (Painter, 1976; Sierakowska & Shugar, 1977); information regarding the precise role of deoxyribonuclease in eukaryotic DNA metabolic processes has been limited (Schroeder, 1975; Cox &

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https://doi.org/10.1017/S0016672300021339 Published online by Cambridge University Press

Game, 1974; Stadler & Moyers, 1981; Delange & Mishra, 1981–82; Kafer & Fraser 1979; Kafer, 1981). Since *Neurospora crassa* is amenable to biochemical genetic methods of analysis, we have attempted the isolation and characterization of nuclease deficient mutants of this eukaryotic micro-organism. Earlier Ishikawa and his collaborators (1969) have described the isolation of two nuclease deficient mutants, (*nuc-1* and *nuc-2*) of *Neurospora* by the inability of these mutants to utilize DNA as the sole phosphorus source for growth. We have used a 'brute force' method for the isolation of nuclease deficient mutants of *Neurospora* since the method of filtration enrichment used for the isolation of the *nuc* mutants by Ishikawa *et al.* (1969) was limited in its scope. In this paper we describe the characterization of five such mutants. The new *nuc* mutants (designated as *nuc-3*, *nuc-4*, *nuc-5*, *nuc-6* and *nuc-7*) were found unable to utilize DNA (or RNA) as the sole phosphorus source and mapped to the right of *arg-12* (on linkage group II) of *Neurospora crassa.* All five *nuc* mutants were found deficient in extracellular nucleases (ds-DNase and ss-DNase specific for double and single stranded DNA as the substrates) and alkaline phosphatase. Preliminary evidences indicating the role of *nuc* genes in genetic recombination and DNA repair are discussed.

2. MATERIALS AND METHODS

Strains. The *Neurospora* strains used in the present study are listed in Table 1. All of them (except the *nuc* mutants) were obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, California. Each of the new *nuc* mutants was back crossed (3–4 times) to obtain a uniform genetic background. Later, they were crossed to strain FGSC 2248 or 2249 (*pan-2*) and to strain FGSC 2464 or 2465 (*sn cr*; *al-3 inl*) yield double mutants *nuc pan-2* and *nuc inl*; the *pan-2* and *inl* were used as forcing markers for heterokaryon formation during complementation analysis.

Media. Standard *Neurospora* media have been used throughout these studies (Davis & deSerres, 1970). Fries minimal medium (without inorganic phosphate) supplemented with 10–100 μg/ml of Salmon Sperm DNA as the sole phosphate source was used to screen mutagenized isolates for the *nuc* phenotype, as described by Ishikawa *et al.* (1969).

Mutagenesis. Mutagenesis was carried out by treating a conidial suspension with MNG (N-methyl-N'-nitro-N-nitroso-guanidine) as described previously (Malling & deSerres, 1970; Westrum & Vigfusson, 1973). The mutagenized conidia were plated onto Vogel's minimal medium N (containing Sorbose) and incubated at 25 °C for 3–4 days. All surviving colonies were individually examined for the *nuc* phenotype by plating onto medium with DNA as the sole source of phosphate. Any isolate which failed to grow on the DNA medium but exhibited wild type growth on medium containing inorganic phosphate (KH_2PO_4) was identified as putative *nuc* mutants. Fourteen *nuc* mutants were isolated using this method, of which five (listed in Table 1) have been analysed here in detail.

Genetic Mapping. Each of the *nuc* mutants was crossed to a multiply-marked linkage tester strain. When linkage to one of the seven markers was found, the *nuc* mutants were further crossed to other tester strains carrying additional biochemical markers for that particular linkage group.

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Test for allelism. The *nuc* mutants were checked for allelism and/or close linkage by a series of crosses among mutants. Such test was based on the concept that crosses involving non allelic mutants would yield recombinant wild type progeny capable of growth on selective medium. Another method used to examine the allelism (or non allelism) among *nuc* mutants was complementation analysis. A pairwise combination of *nuc* mutants (carrying either *pan* or *inl* mutation) was

Table 1. *Description of strains used*

	Stock no.	Allele*	Linkage group	Reference or comments
1.	2218	wild type (RL3-8A)		Barratt & Ogata, 1976
2.	986	wild type (74-OR23-1A)		Barratt & Ogata, 1976
3.	2464A 2465a	*sn*, *cr-1*; *al-3*, *inl*	IC, R; VR, R	Barratt & Ogata, 1976
4.	2248A 2249a	*pan-2*	VIR	Barratt & Ogata, 1976
5.	285	*inl*⁻ts	VR	Barratt & Ogata, 1976
6.	2053A	*al-2*; *trp-3*, *tyr-1*	IR, IIR	
	2054a	*pdx-1*; *inl*; *chol-2* *thi-2 ars* (101)	IIIR, IVR, VR VIL, VII	Metzenberg & Ahlgren, 1970
7.	2998a	*pyr-4 arg-12*	IIL, R	—
8.	I-1	*nuc-1*	IR	Ishikawa *et al.* 1969
9.	I-2	*nuc-2*	IIR	Ishikawa *et al.* 1969
†10.	701	*nuc-3*	IIR	—
†11.	51	*nuc-4*	IIR	—
†12.	276	*nuc-5*	IIR	† This Study
†13.	936	*nuc-6*	IIR	—
†14.	24	*nuc-7*	IIR	—

* Allele symbols are as designated by Barratt & Ogata 1976: Albino conidiation (*al-2*, *al-3*), aryl sulfatase (*ars*), Crisp morphology (*Cr-1*), snowflake morphology (*Sn*), nuclease (*nuc*). Growth requirements for arginine (*arg-12*), choline (*chol-2*), inositol (*inl*), pantothenic acid (*pan-2*), pyridoxine (*pdx-1*), pyrimidine (*pyr-4*), thiamine (*thi-2*), tryptophan (*trp-3*), tyrosine (*try-1*).

† The mutants marked with asterisk originated during the present study and are subject of investigation discussed in this paper. (These mutants were isolated by Mishra.)

examined for its ability to grow on DNA medium. Mutants capable of growth on the selective medium as heterokaryons were considered as complementing mutants and were therefore inferred to be nonallelic.

Growth measurement. The wild type and the *nuc* mutants were compared for their rate of growth in a 'race tube' (Ryan, Beadle & Tatum, 1943) at different temperatures (25 and 37 °C) in order to detect any temperature sensitive variation in growth.

Growth on different phosphate sources. Shake cultures of the wild type and *nuc* mutant strains, were grown in media containing various concentrations of DNA, RNA, dCMP, AMP, or KH_2PO_4 to test their ability to utilize these compounds as a phosphorus source. The shake culture was started with conidia (1×10^6) and incubated at 25 °C for 3–4 days at 150 rev/min and the dry weights of the mycelial pads were then determined for comparison.

Assay of extracellular enzymes. The wild type and the *nuc* mutant strains were grown as shake culture (150 rev/min) in low (0·3675 mM) and high (7·35 mM)

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phosphate media (Ishikawa *et al.* 1969). After 3 days of growth at 30 °C, the culture filtrates were collected and used for the assay of nuclease and alkaline phosphatase activities. The dry weight of the mycelial pads was determined for comparison.

Nuclease activities were determined by an increase in OD_{260} due to the release of perchloric acid (PCA) soluble material, from substrates (dsDNA, ssDNA, or RNA). The reaction mixture for the assay of DNase activity contained 12·5 μmol of potassium phosphate pH 6·0, 5 μmol of $MgCl_2$, 1 μmol of $CaCl_2$, 100 μg gelatin, and appropriate aliquots of the culture filtrate in a total volume of 1·0 ml. Native or heat-denatured salmon sperm DNA (625 μg) was added as substrate. The assay mixture for RNase activity contained 12·5 μ mol of Tris-HCl, pH 9·0, 5 μmol of $MgCl_2$, 100 μg gelatin, yeast RNA (500 μg) and various aliquots of culture filtrate in a total volume of 1·0 ml. All reaction mixtures were incubated at 37 °C for 30 min and then the PCA soluble OD_{260} was measured by a Gilford Model 240 Spectrophotometer.

Repressible alkaline phosphatase activity was assayed as described by Nyc, Kadner & Crocken (1966). After incubation for 30 min at 37 °C, the reactions were terminated with half the volume of 1 M-KOH in 90% ethanol (Lehman *et al.* 1973) and the absorbance at 405 nm was taken according to the method of Bessey, Lowry & Brock (1946). All data presented are representative of three or more experiments.

Enzyme unit and specific activity. One unit of nuclease (ds-DNase, ss-DNase and RNase) activity was defined as the amount of enzyme which released one A_{260} unit of PCA-soluble material in 30 min under the assay conditions. A unit of alkaline phosphatase activity was defined as 1 μmol of p-nitrophenol released per min at 37 °C. Specific activity was defined as units of enzyme per mg of protein or as per mg of mycelial dry weight (Hasunuma, 1973).

Protein determination. Protein in the culture filtrates was assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Chemicals. All chemicals (except where specified) were purchased from Sigma Chemical Co. St Louis.

3. RESULTS

Isolation of nuclease (nuc) *deficient mutants.* Over 3000 colonies growing on minimal medium were isolated after mutagenesis with MNG. These were examined individually for their ability to grow on a medium with DNA as the only phosphorous source. Fourteen colonies unable to grow on DNA medium were isolated as presumptive nuclease (*nuc*) mutants. Five of these mutants (designated as *nuc-3*, *nuc-4*, *nuc-5*, *nuc-6* and *nuc-7*) showing Mendelian transmission of *nuc* phenotype in crosses with the wild type (*nuc+*) strain, were chosen for further analyses of allelism, linkage relationship, growth characteristics and elucidation of underlying biochemical defects. These data are presented below.

Complementation analysis. All pairwise combinations of different *nuc* mutants showed a full wild type growth and conidiation on the DNA medium, these data are presented in Plate 1. The complementing heterokaryons usually grew over 100 mm Petri plates in about 3 days and their growth-rate and conidiation-pattern were comparable to that of the wild type strain on a DNA medium or to that of a known complementing heterokaryon constructed between *nuc-1* and *nuc-2* (see

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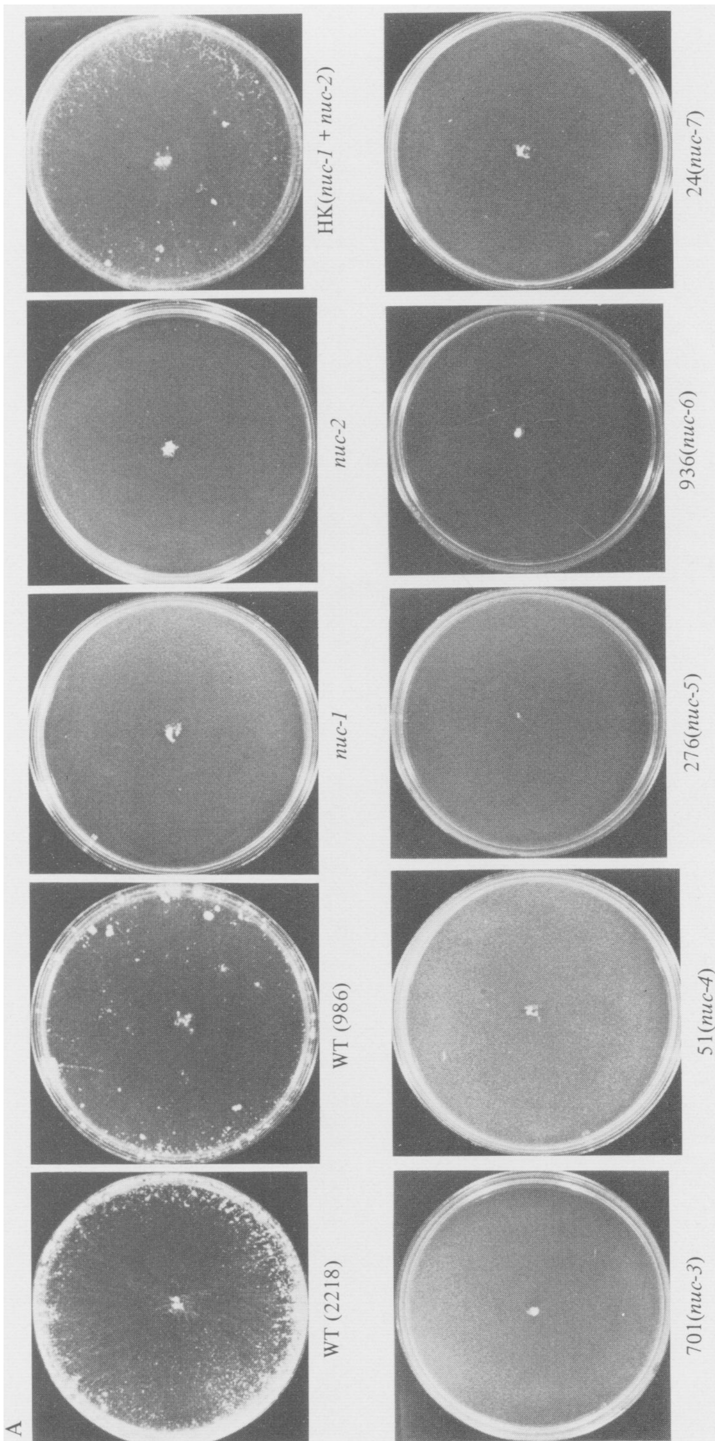

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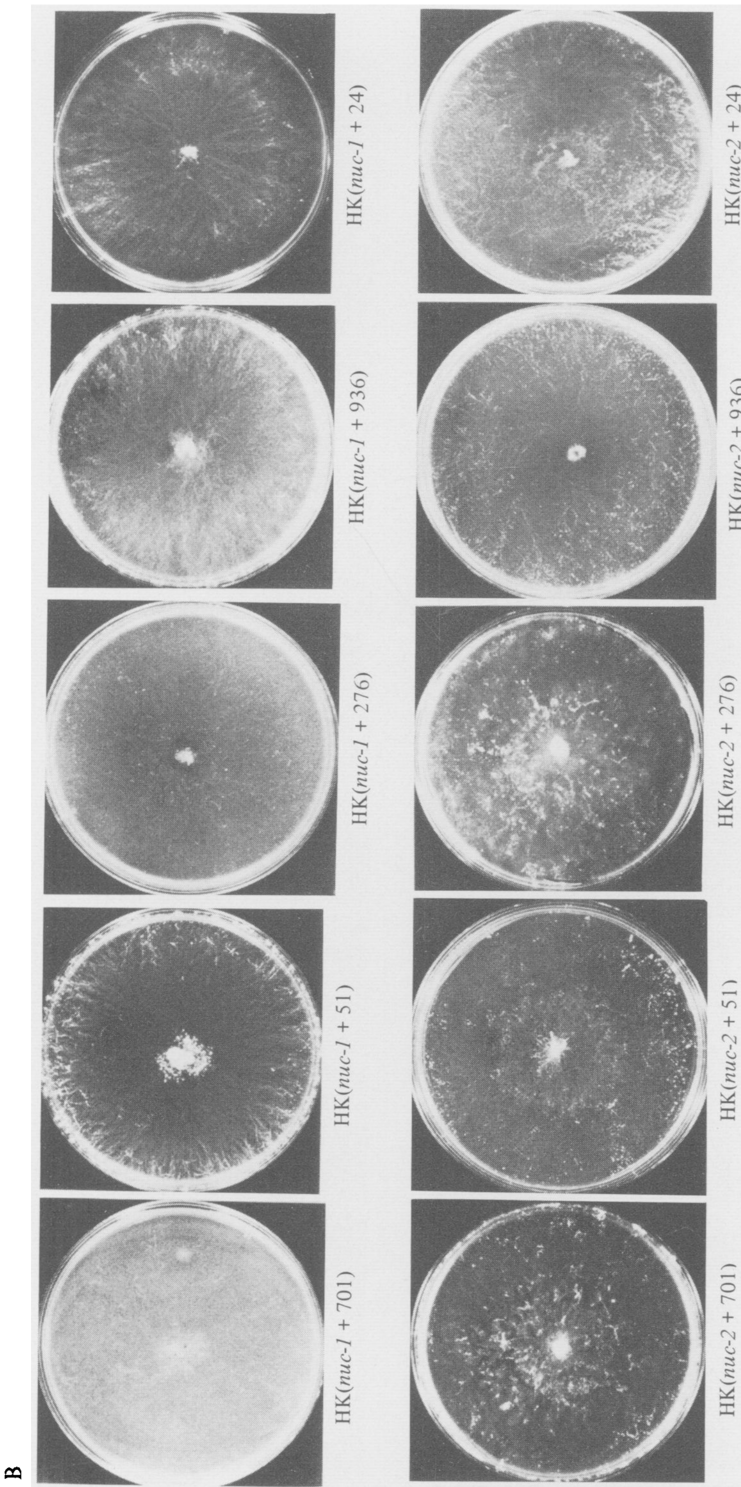

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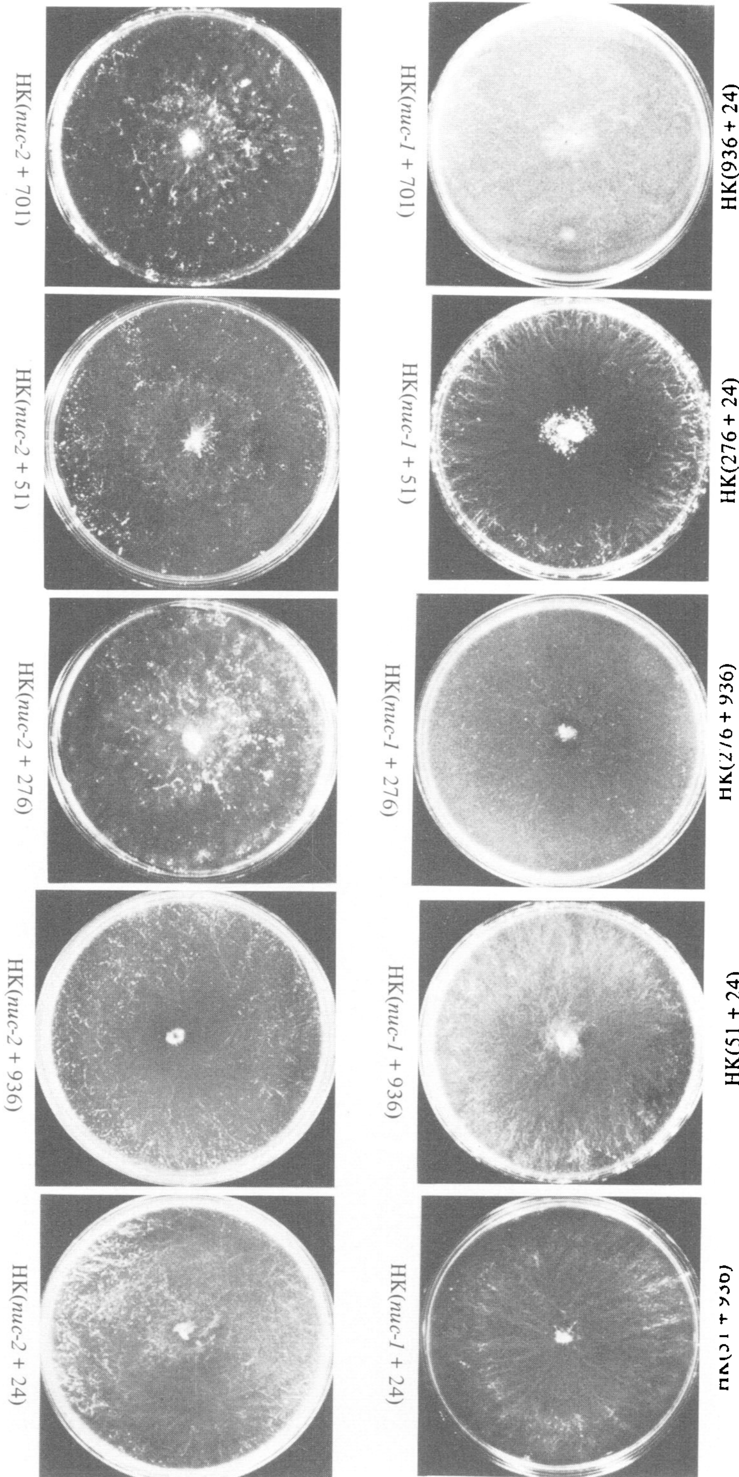

Plate 1

Growth pattern of the wild type, *nuc* mutants and their heterokaryons on DNA medium. (A) Growth of the wild type, *nuc-1* and *nuc-2* mutants and heterokaryon between *nuc-1* and *nuc-2* (top row) and the five new *nuc* mutants (bottom row). (B) Growth of heterokaryons constructed between each of the five new *nuc* mutants with *nuc-1* (top row) and with *nuc-2* (bottom row). (C) Growth of heterokaryons constructed among the new *nuc* mutants.

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Plate 1). Since the inability of *nuc* mutant to utilize nucleic acids as a phosphorus source is known to result from a deficiency in nucleases and alkaline phosphatases (Tohe & Oshikawa, 1971; Hasunuma, 1973), it is expected that a heterokaryon constructed between a pair of complementing *nuc* mutants should possess a wild type level of these enzymes. Three heterokaryons *nuc-1* + *nuc-2*, *nuc-1* + *nuc-4* and *nuc-3* + *nuc-4* were examined for alkaline phosphatase activity and they showed 60, 66 and 85% of the wild type level of the specific activity of this enzyme respectively (see Table 5) (when cultures were grown in a low phosphate medium as described in Materials and Methods). The level of complementation observed among *nuc* mutants was that one would expect for inter-genic (and *not* intra-allelic) complementation since almost the full wild type level of growth and the specificity of the enzyme (alkaline phosphatase) were restored in the complementing heterokaryons. These data clearly suggest that the new *nuc* mutants are nonallelic to each other as well as to the previously described *nuc-1* and *nuc-2* mutants. Thus, the new *nuc* mutants represent mutations in five distinct genes; this conclusion is further supported by their linkage analysis as discussed later in this paper.

The new *nuc* mutants were designated as *nuc-3*, *nuc-4*, *nuc-5*, *nuc-6* and *nuc-7* (see Table 1); this designation was based on the consideration that two *nuc* (*nuc-1* and *nuc-2*) mutants were described previously (Ishikawa *et al.* 1969).

Analysis of crosses involving nuc *mutants.* Since crosses among the non-allelic mutants are expected to yield a wild type recombinant progeny; the new *nuc* mutants (*nuc-3*, *nuc-4*, *nuc-5*, *nuc-6* and *nuc-7*) were crossed among themselves as well as with the previously described *nuc-1* and *nuc-2* mutants to determine their allelism. These data are presented in Table 2. All crosses except one (*nuc-3* × *nuc-6*) produced wild type recombinant progeny. The sum of these data (presented in Tables 2 and 3) confirmed the conclusions regarding the non-allelic nature of all seven *nuc* mutations. The frequency of the wild type recombinant obtained from the crosses among new *nuc* mutants (see Table 2) [*nuc-3* × *nuc-4* (1%), *nuc-3* × *nuc-5* (12·2%), *nuc-3* × *nuc-6* (0%), *nuc-3* × *nuc-7* (0·125%), *nuc-4* × *nuc-6* (2%), *nuc-4* × *nuc-5* (9·3%) and *nuc-6* × *nuc-7* (2·3%)] were much lower than 25% expected for non-linkage. These data therefore suggested that the new *nuc* mutants were nonallelic but linked to each other. Moreover, the results of certain crosses (*nuc-4* × *nuc-7*, *nuc-5* × *nuc-7* and *nuc-3* × *nuc-6*) showing a range of high (58–60%) to low (0%) number of recombinant progeny (see Table 2) imply the role of the *nuc* genes in controlling the process of genetic recombination.

The data presented in Table 2 indicate that none of the new *nuc* mutants (i.e. *nuc-3* to *nuc-7*) is linked to *nuc-1*. The recombination frequencies obtained from the crosses of the new *nuc* mutants to *nuc-2* show that, among the five *nuc* mutants, *nuc-3* had the closest linkage to *nuc-2* (with a recombination frequency of 18%). This observation is consistent with the data (presented later in this paper) in which *nuc-3* was found to show a close linkage to *arg-12* (see Fig. 1).

Absence of the pseudowild type among the progeny of nuc × nuc *crosses.* There are two obvious sources for the presence of the progeny with wild-type phenotype from the crosses involving two *nuc* parents; firstly, these may arise as a result of inter- or intragenic recombination, or secondly from complementation of mutants in pseudowild types arising by rare non-disjunction of chromosome (linkage group

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II) during meiosis. The distinction between a wild-type recombinant and pseudowild type can be made by conidial analysis. On such conidial analysis pseudowild type will yield both mutant and wild-type somatic segregants (Pittenger, 1964; Threlkeld, 1962), whereas a wild-type recombinant will yield only wild-type somatic segregants. Five or less wild-type progeny from such a cross (*nuc* × *nuc*) were examined by conidial analysis and none of them (over 100 examined) yielded

Table 2. *The occurrence of wild type recombinants among the progeny of intercrosses of the* nuc *mutants*

Crosses	Number of progeny: Wild type	Total	*Frequency (%) of the wild type recombinants
(A) Involving new *nuc* mutants			
nuc-3 × *nuc-4*	1	100	1
nuc-3 × *nuc-5*	30	255	12·2
nuc-3 × *nuc-6*	0	420	0
nuc-3 × *nuc-7*	2	796	0·125
nuc-4 × *nuc-5*	7	74	9·4
nuc-4 × *nuc-6*	1	100	1
nuc-4 × *nuc-7*	34	113	30
nuc-5 × *nuc-6*	3	94	3·19
nuc-5 × *nuc-7*	56	200	28
nuc-6 × *nuc-7*	4	174	2·3
(B) Involving new *nuc* × *nuc-1* or *nuc-2*			
nuc-1 × *nuc-3*	22	66	30
nuc-1 × *nuc-4*	17	59	29
nuc-1 × *nuc-5*	17	57	30
nuc-1 × *nuc-6*	33	86	38
nuc-1 × *nuc-7*	30	87	35
nuc-2 × *nuc-3*	6	64	9
nuc-2 × *nuc-4*	9	50	18
nuc-2 × *nuc-5*	11	46	24
nuc-2 × *nuc-7*	26	100	26
nuc-2 × *nuc-7*	16	70	23

* The recombination frequency was estimated as twice the frequency of the wild type progeny (see Mishra, 1977).

any mutant isolate suggesting that all wild-type progeny examined were true wild-type recombinants (and never a pseudowild type).

Linkage relationship of the new nuc *mutants.* The *nuc* mutants showed no linkage to either of the markers *sn cr-l* (LGI); *al-3 inl* (LGV) or *pan-2* (LGVIR) used during the back crosses. Despite this fact, the new *nuc* mutants were individually crossed to a multiply-marked tester strain which facilitated their assignment to a particular linkage group; in such crosses each of the *nuc* mutants showed linkage to *trp-3*, located on the right arm of the linkage group II. Further mapping was carried out with the follow-up three-point crosses to a linkage group II tester strain no. 2998 (*pyr-4 arg-12 nuc*$^+$). Four of the new *nuc* mutants were located to the right of *arg-12* on linkage group II (see Table 4 and Fig. 1). The *nuc-7* showed a

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recombination of 45% with *arg-12*. However, the data from the cross to the multiply marked tester strain indicated that *nuc-7* is located approximately 31 map units to the left of *trp-3* on linkage group II (32 recombinants among 103 ascospores analysed). The data on linkage relationships among the *nuc* mutants and their linkage to *arg-12* on the LG II as presented in Fig. 1 show a general agreement regarding the map distance between *nuc* genes and their order on the linkage map. However, a number of intercrosses showed significant fluctuation in recombination frequency because of the role of the *nuc* genes on the process of genetic recombination (see discussion).

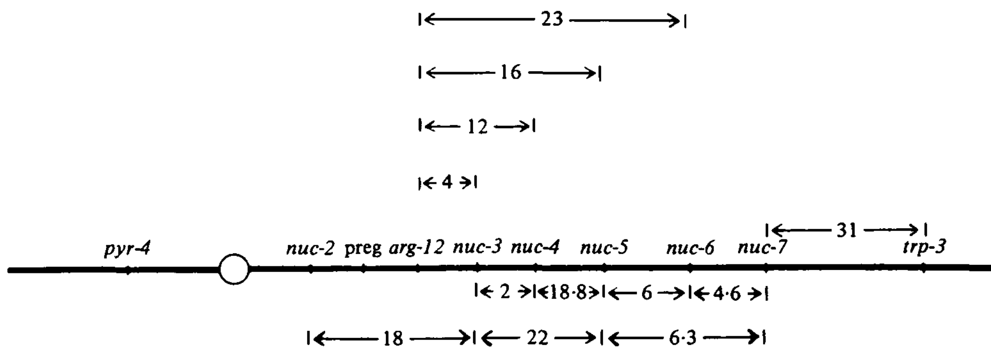

Fig. 1. Genetic map of *nuc* genes. Linkage group II. Recombination frequencies presented was determined from 3 point crosses (shown above the genetic map) or from intercrosses (shown below the genetic map).

Growth on different phosphate source. The two *nuc* mutants *nuc-1* and *nuc-2* were originally characterized by their inability to utilize RNA as a phosphate source (Ishikawa *et al.* 1969). Neither *nuc-1* nor *nuc-2* responded to DNA as a phosphate source but both produced wild type growth on media supplemented with a mononucleotide as a phosphate source. The new *nuc* mutants were, therefore, examined for their ability to utilize different phosphorus sources. The growth characteristics of the wild type and the five nuclease mutants in medium supplemented with various amounts of DNA, RNA, dCMP, and inorganic phosphate (KH_2PO_4) as a phosphorus source are presented in Fig. 2. From these data (see Fig. 2), it is obvious that the *nuc* mutants failed to exhibit wild type growth on a medium supplemented with DNA, even at a concentration 50-fold higher than that used for the initial screening of the *nuc* phenotype. However, on the RNA-supplemented medium (100 μg/ml), *nuc-4* and *nuc-5* exhibited growth which was approximately 38 and 53% of that of the wild type strain. The remaining *nuc* mutants showed negligible growth which ranged from 12·5% (*nuc-7*) to 5% (*nuc-3* and *nuc-6*) of the growth of the wild type strain on a RNA medium. All *nuc* mutants were able to utilize dCMP as the sole phosphate source (see Fig. 2); their growth ranged from 45 to 58% of that of the wild type strain. The ability of the *nuc* mutants to utilize dCMP became further obvious when these mutants were examined for growth on an agar plate containing nucleoside monophosphate as the sole phosphorus source. In such assay, the mutant showed no difference in their

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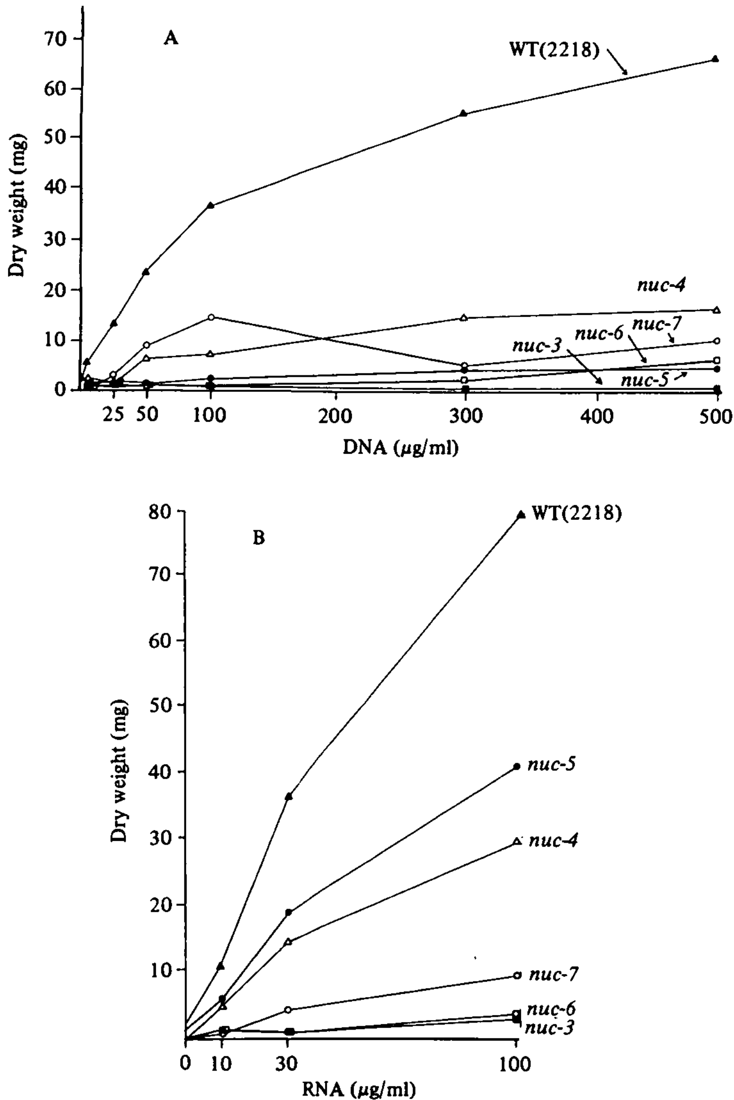

Fig. 2.

growth rate as compared to that of the wild type. All five mutants showed a wild type growth in medium containing inorganic phosphate (see Fig. 2D). The data in Figure 2D further indicate that *nuc-4* and *nuc-5* can grow at the wild type rate at much lower levels of inorganic phosphate than the other three mutants (*nuc-3*, *nuc-6* and *nuc-7*). Also, at the inorganic phosphate concentration of 0·25–0·5 mM, the growth rate of mutants approximate that of the wild type. These data suggested that of the two phosphorus transport systems of *Neurospora* (Lehman *et al.* 1973; Lowendorf, Bazinet & Slayman, 1975; Lowendorf & Slayman, 1975), the high phosphorus affinity system was still operative in these *nuc* mutants.

Other growth characteristics. Three *nuc* mutants, *nuc-3*, *nuc-6* and *nuc-7*, showed wild type growth rate (11 cm/day) both at 25 and 37 °C. The growth of *nuc-4* and

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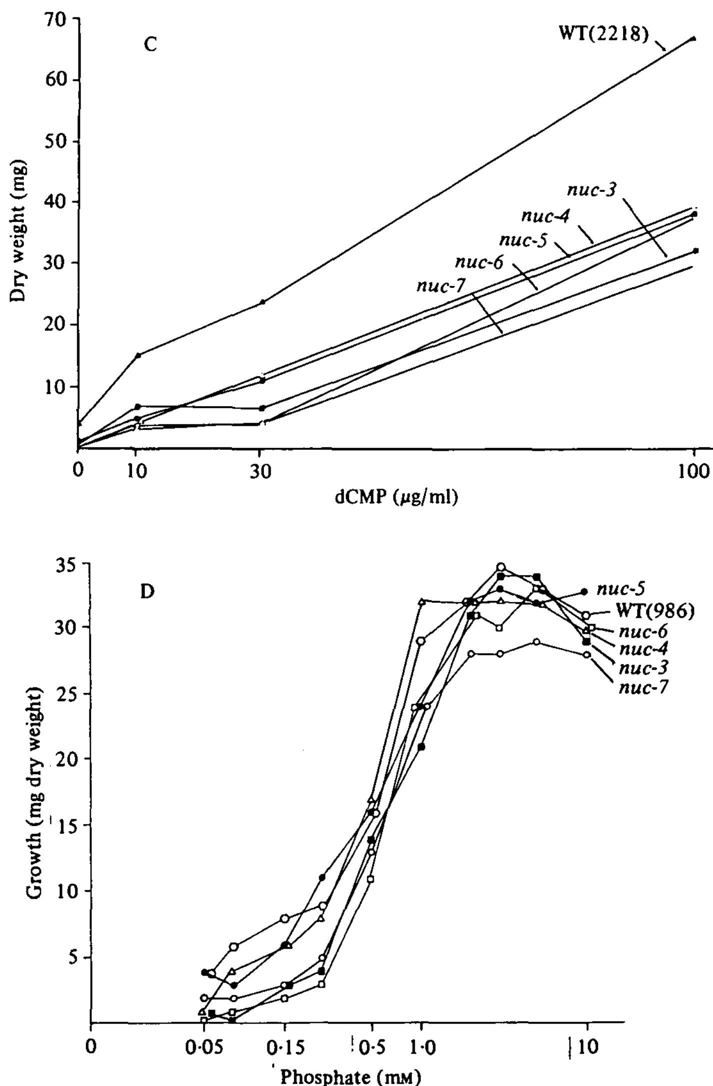

Fig. 2. Growth of the wild type and nuclease mutants on different phosphorous source (A) DNA, (B) RNA, (C) dCMP, and (D) inorganic phosphate (KH_2PO_4).

nuc-5 was drastically reduced at 37 °C; these two mutants showed a growth of 4 cm/day (see Fig. 3). Even though the temperature sensitive growth was found to segregate with *nuc* phenotype this may be controlled by closely linked gene(s).

Other characteristics. The intercrosses among the various *nuc* mutants yielded perithecia with black spores indicating no sign of infertility or ascospore abortions. These data suggest that the meiotic behaviour was not adversely affected by *nuc* mutation and thus eliminated the possibility of the production of pseudowilds among the progeny of such crosses (as discussed earlier in this paper).

Enzymatic defect (level of nucleases and alkaline phosphatases). Earlier it has been shown that *nuc-1* and *nuc-2* mutants of *Neurospora* are deficient in the enzymes

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involved in the utilization of phosphates (Toh-e and Ishikawa, 1971; Hasunuma & Ishikawa, 1972; Hasunuma, 1973; Hasunuma, Toh-e & Ishikawa, 1976). Therefore the level of some of these enzymes (DNase, RNase, and alkaline phosphatase) were examined in the new *nuc* mutants and wild type strain grown under conditions of low (0·3675 mM) and high (7·35 mM) phosphate concentrations.

Table 3. *Mapping of nuclease* (nuc) *mutants of* Neurospora crassa

		Number of progeny			
			Recombinant type		
			Single crossover		Double crossover
nuc mutant involved in the cross	Zygote genotype and recombination (%)	Parental type	Region I *pyr-arg*	Region II *arg-nuc*	Regions I and II *pyr-nuc*
nuc-3	*pyr-4* — *arg-12* — + / + 40 % + 4 % *nuc-3*	52	38	4	0
nuc-4	*pyr-4* — *arg-12* — + / + 29 % + 12 % *nuc-4*	62	26	9	3
nuc-5	*pyr-4* — *arg-12* — + / + 35 % + 16 % *nuc-5*	50	29	11	4
nuc-6	*pyr-4* — *arg-12* — + / + 33 % + 25 % *nuc-6*	48	27	19	6
nuc-7	*pyr-4* — *arg-12* — + / + 22·4 % + 45·9 % *nuc-7*	39	14	37	8

Individual ascospore isolates were examined for the marker phenotype as described in Materials and Methods, *arg*, and *pyr* indicated the alleles controlling arginine and pyrimidine requirements.

These results presented in Table 4, show that in all mutants, the enzyme levels are greatly reduced as compared to those of the wild type. The ds-DNase activities of the mutants grown at the low phosphate concentration (0·3675 mM) ranged from about 3 % (for *nuc-5* and *nuc-7*) to 15 % (for *nuc-6*) of that found in the wild type strain grown under the same conditions (see Tables 4 and 5).

At low phosphate concentration, the mutants showed more ss-DNase activity than ds-DNase although the range of activity was still reduced, (extending from nearly 7 % for *nuc-7*) to 34 % (for *nuc-5*)) as presented in Table 5. The RNase activity of the *nuc* mutants was found to vary greatly (such as 37 % for *nuc-7* to 53 % for *nuc-5*) from that found in the wild type strain (Table 5).

When grown at a high phosphate level (7·35 mM-PO_4), the wild type ds-DNase,

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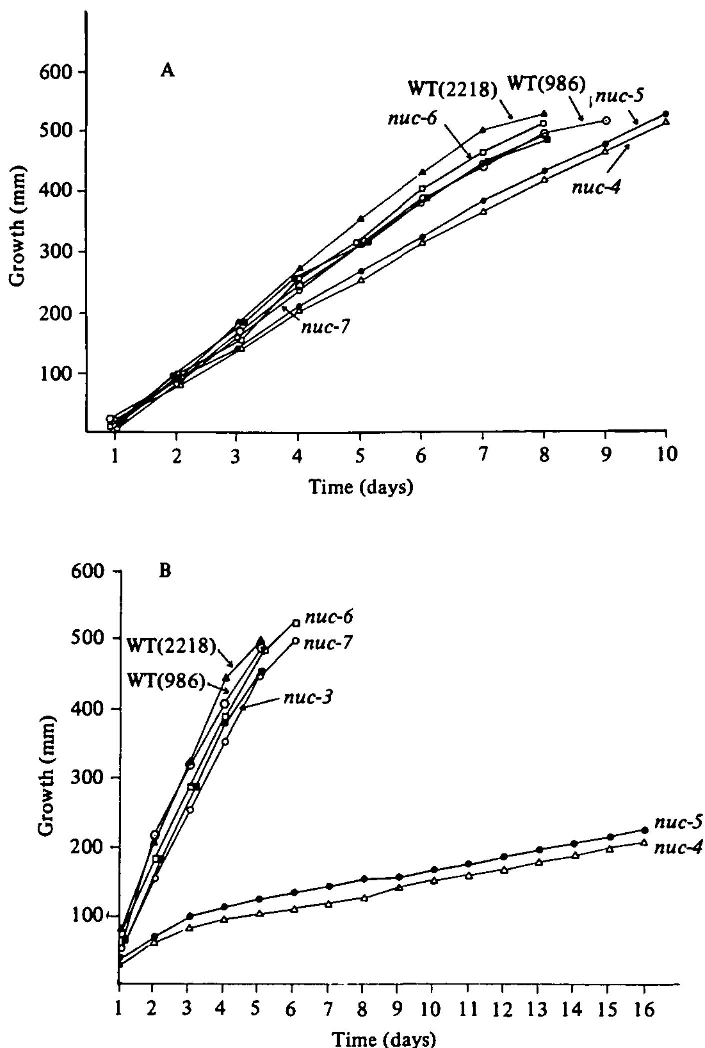

Fig. 3. Growth of the wild type and nuclease mutants at different temperature (A) 25 °C and (B) 35 °C. Symbols same as in Fig. 3.

ss-DNase and RNase activities were significantly reduced as compared with the activities in the low-phosphate medium (0·3675 mM-PO_4) as shown in Table 4. Reduced levels of extracellular nuclease activity were exhibited by each of the *nuc* mutants at the high phosphate concentration, which indicated the ability of the mutants to repress these enzymes under conditions of phosphorus sufficiency (see Table 4).

All of the *nuc* mutants showed appreciable decrease in alkaline phosphatase activity as compared to the wild type when grown on low phosphate concentrations (Tables 4 and 5). This inability to derepress alkaline phosphatase synthesis under conditions of phosphorus limitation is a property shared by the mutants *nuc-1* and *nuc-2*. The slightly elevated levels of alkaline phosphatase noted in the *nuc* mutants

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Table 4. *A comparison of the repressible extracellular ds-DNase, ss-DNase, RNase and alkaline phosphatase activities in culture filtrates of wild type and nuclease mutant strains of* Neurospora *grown at low and high inorganic phosphate concentrations*

	Specific activity of enzymes (unit/mg dry weight)*							
	ds-DNase		ss-DNase		RNase		Alkaline phosphatase	
Strain	Low Pi	High Pi	Low Pi	High Pi	Low Pi	High Pi	Low Pi	High Pi
Wild Type								
RL3-8A	42·00	5·00	41.33	17·71	40·93	20·39	0·98	0·088
Mutants								
nuc-3	4·06	0·28	8·15	2·79	20·29	3·78	0·20	0·053
nuc-4	4·25	0·51	8·68	2·44	17·93	2·05	0·24	0·039
nuc-5	1·20	2·22	13·97	3·24	21·87	9·59	0·14	0·046
nuc-6	5·20	0·31	6·78	2·14	20·40	5·31	0·15	0·034
nuc-7	1·21	0·92	2·84	0·60	15·00	6·73	0·20	0·054

Low Pi and high Pi denote low-phosphate (0·367 mM-PO_4) and high-phosphate (7·35 mM-PO_4) media respectively. The experimental details are presented in the text.

Table 5. *A comparison of the repressible extracellular ds-DNase, ss-DNase, and RNase and alkaline phosphatase activities in culture filtrates of the nuclease mutants and the heterokaryons*

	Specific activity of the different enzymes			
Strain	ds-DNase	ss-DNase	RNase	Alkaline phosphatase
Wild type				
RL3-8A	100	100	100	100
Mutants				
nuc-1	5	10	32	18·5
nuc-2	10	15	57	9·6
nuc-3	9·67	19·72	49·57	20·4
nuc-4	10·12	21·00	43·81	24·48
nuc-5	2·86	33·80	53·43	14·28
nuc-6	12·38	16·40	49·84	15·3
nuc-7	2·88	6·87	36·65	20·4
Heterokaryons				
nuc-1 + *nuc-2*	ND	ND	ND	58·0
nuc-1 + *nuc-4*	ND	ND	ND	66·0
nuc-3 + *nuc-4*	ND	ND	ND	86·0

* All strains grown in low phosphate (0·3675 mM-PO_4) medium. ND, not determined.

on high-phosphate media during this study was reported by Lehman *et al.* (1973) in a similar study of *nuc-1* and *nuc-2*.

The above conclusion that *nuc* mutants are defective in nucleases and therefore unable to utilize DNA as the sole phosphorus source is further supported by the fact that they were able to utilize predigested DNA. In such experiments DNA was first digested by a commercially available deoxyribonuclease (i.e. pancreatic

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DNase I) and then added to the growth medium as the sole phosphorus source. Results of such experiments showed that at least two new mutants (*nuc-4* and *nuc-5*) were able to utilize predigested DNA as the sole phosphorus source; the level of growth in such medium was the same as that of the wild type strain in a medium containing inorganic phosphorus.

4. DISCUSSION

Data presented in this paper provide evidence for the presence of five additional genes controlling the level of nucleases (deoxyribonucleases and ribonuclease) and alkaline phosphatase in *Neurospora*. The use of a 'brute force' method for the mutant isolation can explain the high success in obtaining a large number of mutants during the present study. It is possible that the design of our experiment was instrumental in retaining the mutants which would have been otherwise lost during the process of the filtration enrichment, a method used by the previous workers to isolate *nuc* mutants (Ishikawa *et al.* 1969). The data from the complementation (see Plate 1) and genetic analyses (see Tables 2–3) are in agreement in establishing that the new *nuc* mutants are nonallelic and represent mutation in distinct genes and in providing their linkage to *arg-12* on the linkage group II. The location of the new *nuc* mutants to the right of *arg-12* on the linkage group II (see Fig. 2) eliminates any possibility of their allelism either to *nuc-2* and to *preg* (both situated to the left of *arg-12* on linkage group II) or to *nuc-1* (situated on linkage group I) (Perkins & Bjorkman, 1980). Therefore the sum of the genetic data confirms the conclusion that the new *nuc* mutants represent mutations in non-allelic genes. Thus there are altogether eight genes (seven *nuc* genes discussed here and the *preg* gene described by Lehman *et al.* 1973) controlling the *nuc* phenotype and/or phosphorus utilization in *Neurospora*. Of these, all except *nuc-1* were found to be located on LG II; the *nuc-1* has been shown to map on LG I (Ishikawa *et al.* 1969). It is plausible that their linkage on the same region of a chromosome may provide a means for their coordinate regulation during phosphate utilization.

The data presented in this paper (see Tables 4–5) clearly demonstrate that all five new *nuc* mutants are deficient in nucleases and are thus similar to *nuc* mutants as described previously (Ishikawa *et al.* 1969; Hasunuma & Ishikawa, 1972). The data presented confirms the conclusion that new *nuc* mutants are deficient in deoxyribonuclease and therefore unable to use DNA as the sole phosphorus source. This conclusion is further substantiated by the fact that they (*nuc-4*, *nuc-5*) were able to use predigested DNA as phosphorus source (Forsthoefel and Mishra unpublished results). It seems unlikely that the new *nuc* loci described here are the structural genes for enzymes with diverse properties (such as ds-DNase, ss-DNase, RNase, and alkaline phosphatase). Instead it seems more probable that they represent mutations in regulatory genes which control the level of enzymes involved in DNA degradation and/or phosphorus utilization by *Neurospora*. The fact that the number of genes controlling nucleases of *Neurospora* has swelled to eight would require a modification in the model of cascade regulation of phosphorus utilization as proposed earlier (Metzenberg & Nelson, 1977). However, this model

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must wait any serious consideration before the *nuc*$^{+}$ genes and their products are fully characterized which is now feasible by the technique of molecular cloning of genes in *Neurospora* (see review by Mishra, 1982).

The new *nuc* mutants appear to belong to two different groups with distinct characteristics: one group includes *nuc-3*, *nuc-6* and *nuc-7*, whereas another group includes *nuc-4* and *nuc-5*. The second group is characterized by the ability to utilize RNA and predigested DNA as phosphorus source (see Figure 2B) and by their temperature sensitive growth (see Figure 3A and 3B). The two groups of *nuc* mutants are also characterized by difference in their mutagen sensitivities and spontaneous mutation frequencies (see the accompanying paper).

The high frequency of wild type recombinants observed among the progeny of certain crosses involving *nuc* mutants may be explained on the following basis: (*a*) the production of pseudowilds; or, (*b*) a distortion in segregation ratio (of the wild type and mutant progeny) due to poor germinability of the double (*nuc*) mutant spores; or (*c*) a high frequency of gene conversion. The first explanation seems implausible in view of the facts that no evidence for pseudowilds among the recombinant wild type progeny was found even after extensive conidial analysis of the recombinant progeny [since *no* segregation of *nuc*$^{+}$ and *nuc*$^{-}$ phenotype was seen on the conidial isolates of the recombinant (wild type) progeny]. However, the role of *poor* spore germination and gene-conversion cannot be ruled out at this time; latter should be further examined by tetrad analysis using appropriate outside markers. Data presented in this paper (see Table 2) show that *nuc-3* and *nuc-6* produced an adverse effect on genetic recombination; whereas, the *nuc-7* (also *nuc-4* and *nuc-5* to some extent) had a stimulating effect. It is possible that *nuc-3* and *nuc-6* may cause adverse effect in a manner similar to previously described genetic elements (see Stahl, 1980) and that *nuc-7* may promote recombination by relief of its inhibition. Furthermore, such an adverse effect of *nuc* genes can account for any disparity seen in the linkage data between a set of two *nuc* genes obtained by two different methods (such as two point and three point crosses, see Tables 2–3); it is possible that in a two point cross involving both *nuc* parents, the frequency of recombination may differ adversely from that in a three-point cross involving only one *nuc* parent. Furthermore, a possible poor germinability of the recombinant double mutant class of progeny in a *nuc* × *nuc* cross can distort the segregation ratio of the mutant and wild type phenotypes. Such a segregation ratio can cause an apparent increase in the frequency of the wild type recombinants as seen among the progeny of *nuc* × *nuc* cross (see Table 2). However any further speculation on their nature as controlling elements for genetic recombination must await a detailed analysis of their effect on the recombination of a number of other genetic loci. Such study should be carried out by tetrad analysis of crosses homozygous for a particular *nuc* mutation. It is of interest to mention here that Catchside (1981) has recently reported the natural occurrences of *Neurospora* alleles which produce variation in genetic recombination by interfering with the process of synapsis.

It is of further interest to point out here that *nuc-3* and *nuc-6* are characterized by high mutagen-sensitivity and mutator effects; whereas, the remaining mutants *nuc-4*, *nuc-5* and *nuc-7* (which causes relief of inhibition of recombination) are

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characterized by a reduced mutagen-sensitivity, and anti-mutator effect (Mishra and Forsthoefel, see accompanying paper). The role of nuclease deficiency in the DNA repair among the new *nuc* mutants is suggested by their mutagen-sensitivity and high mutation frequency. Fraser & Kafer (1979) have recently described a similar correlation between nuclease deficiency and DNA repair in certain *nuh* mutants of *Neurospora*. It is our belief that an in-depth biochemical and genetic analysis of these mutants, aided with new methods for the analysis of DNA repair (Mishra, 1982; Stadler & Moyer, 1981) will provide a better understanding of the role of deoxyribonucleases in the metabolism of DNA (including DNA repair in eukaryotes).

This research was performed under a contract from the U.S. Department of Energy (Contract no. DE-AS-09-1071). One of us (NCM) wishes to thank A. A. Shums for his excellent technical assistance and Ms Marie Nasif for help in some of the experiments.

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