

## A NEW METHOD TO DETECT POTENTIAL GENOTOXIC AGENTS USING MITOTIC CROSSING-OVER IN DIPLOID STRAINS OF *Aspergillus nidulans*

Lidia Terêsa de Abreu Pires<sup>1</sup> and Tânia Maria Araújo Domingues Zucchi<sup>2</sup>

### ABSTRACT

This paper presents a new method for detecting mitotic crossing-over in *Aspergillus nidulans*, based on the "homozygosity index" (HI) of recessive genes originally present in heterozygosis in diploid strains, which occurs after mitotic crossing-over between the marker in question and the centromere. Since homozygous diploids (-/-) for auxotrophic markers can not grow in MM, homozygotization can be demonstrated by distorted mitotic segregation of the alleles involved. Two similar diploid strains (UT 448//UT 184 and Z1//UT 184), which differ by a chromosomal duplicate segment transposed from chromosome II to I in the Z1 haploid strain, were used. This excess of genetic material confers to the Z1 mutant the *uvr* character and makes Z1//UT 184 more unstable and sensitive to genotoxic agents, as evidenced by its high spontaneous recombinational index.

After UV-treatment, the "HI" of both strains is significantly increased, especially for the Z1//UT 184 diploid strain.

Induced mitotic crossing-over detection through traditional methods requires that the event occurs in specific regions of the genome and involves only two markers. The novel aspect of the proposed method is that it abolishes such a restriction by rendering crossing-over a mere function of "HI" of any marker.

### INTRODUCTION

Nowadays, increasing awareness of the importance of several environmental factors upon life quality, motivates the need of evaluating the potential genotoxic (mutagenic, carcinogenic, recombinogenic, etc.) of several agents. The method here proposed is based on the detection of mitotic crossing-over in *Aspergillus nidulans* induced by physical or chemical agents. Mitotic recombination as a tool for screening carcinogenic and/or mutagenic agents is doubly attractive: first, it leads to homozygosis and consequently to the phenotypical expression of recessive genes originally present in heterozygous condition, an event related to carcinogenesis in higher eukaryotic cells (Bertoldi and Griselli, 1980; Roeder and Stewart, 1988; Wang *et al.*, 1988); second, mitotic recombination is related to a mechanism of DNA repair stimulated by lesions caused by genotoxic agents (Kunz and Haynes, 1981;

Orr-Weaver and Szostak, 1985; Bernstein *et al.*, 1987; Rudin and Haber, 1988). Indeed, large scale genomic alterations such as mitotic rearrangements seem to be more hazardous than point changes produced by conventional mutagenesis (Cairns, 1981; Marx, 1982; Wang *et al.*, 1988; Schiestl, 1989).

### MATERIAL AND METHODS

#### Strains

The strains were from Utrecht stocks (UT 448 and UT 184) and are described in Zucchi (1990b).

The other strain (Z1) was derived from UT 448 after MNNG treatment and is described in Zucchi (1990a), Castro-Prado and Zucchi (1991a), and in Marin and Zucchi (1991).

#### Diploid strains

The diploid strains UT 448//UT 184 and Z1//UT 184 were prepared according to Roper (1952). The mutant alleles were allocated to their linkage group by mitotic haploidization (Forbes, 1959), facilitated by treatment with UV irradiation for five seconds.

<sup>1</sup> Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brasil.

<sup>2</sup> Instituto de Ciências Biomédicas, Av. Prof. Lineu Prestes 1374, Cidade Universitária, 05508-900 São Paulo, SP, Brasil. Fone: (011) 818-7264, FAX: (55) (011) 813-0845. Send correspondence to T.M.A.D.Z.

## Culture media

Complete medium (CM) and minimal medium (MM) were as described by Van de Vate and Jansen (1978). Selective medium (SM) was MM supplemented according to the requirements of each strain. Solid medium contained 1.5% agar. Incubation was at 37°C.

## UV treatment

The UV source used was a 15 watt VIS germicidal lamp. The distance from the source to the material was 15.0 cm. The estimated applied dose was 0.32 m watts/cm<sup>2</sup>/sec.

## Procedures

Conidia from each diploid strain (UT 448//UT 184 and Z1//UT 184) were inoculated individually into the center of solid MM plates and irradiated for 0, 20, 40, 60 or 80 seconds with a UV lamp after 0, three, five or seven hours of pre-incubation at 37°C. After treatment, the plates were incubated for seven days at 37°C.

Each treatment yielded six to eight visible sectors, with differentiated morphology and same colour of the original diploids (green), which were purified in MM and individually transferred to four points of 10 CM plates (40 diploid colonies). The material was incubated for five hours and then UV irradiated for five seconds in order to haploidize the recombinant diploids. The plates were then incubated again for 3-5 days at 37°C until colored (color of paternal haploids) haploid sectors were visible.

Conidia from the edge of each haploid sector were individually transferred to 25 defined positions (5 x 5 pattern) on CM plates, and so each treatment was separately grouped. After two days of incubation, the colonies were replicated on appropriate selective medium for determination of the haploid segregant genotype. The frequency of mitotic crossing-over was then determined.

## Measurement of mitotic crossing-over

Mitotic crossing-over promotes homozygotization of genes present in heterozygous condition. Thus, if the treatment induces mitotic crossing-over in the heterozygous diploids, +/+ , +/- and -/- diploids must be formed (Figure 1) independently for each marker. Since the treatment is applied in MM, only heterozygous (+/- or -/+) or homozygous (+/+) diploids can develop. Only the +/+ , +/- and -/+ diploids were really haploidized on CM. Consequently, the ratio of haploid segregants must be 4+ : 2-, as opposed to 4+ : 4-, when no mitotic crossing-over occurs.

The "homozygosity index" (HI) determined for a given marker, after one recombinational event, is 2.0 (HI

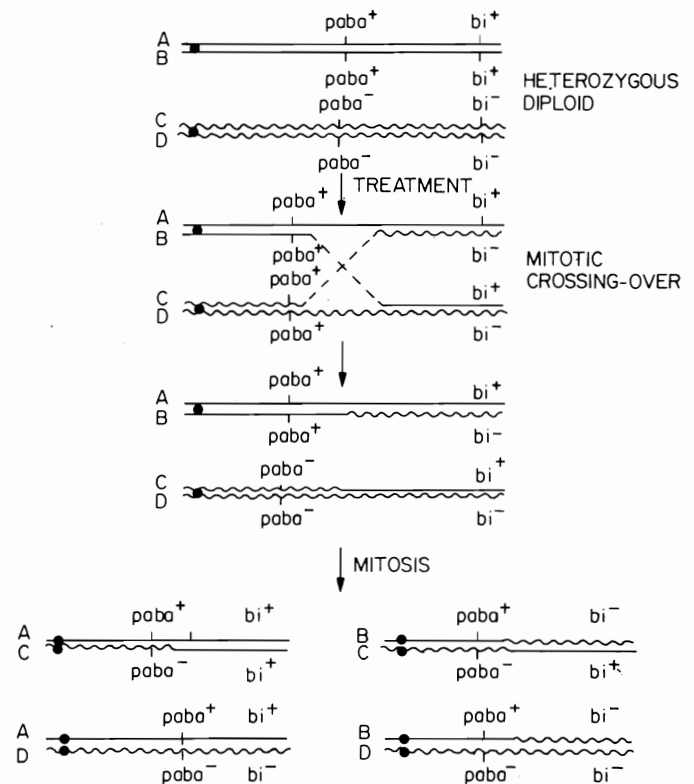


Figure 1 - Scheme of homozygotization of a heterozygous marker promoted by mitotic crossing-over between the marker and the centromere.

= % + / % -). Therefore, an "HI" higher than 2.0 will indicate the occurrence of more than one mitotic crossing-over for the given marker. Conversely, "HI" lower than 2.0 will indicate either the absence of crossing-over for the marker, or that it was not selected on MM, e.g., markers for conidia color. Therefore, HI demonstrates the distortion in the 1+ : 1- mitotic segregation rate caused by homozygotization of genes with consequent elimination of the recessive ones (-/-).

HI was determined for each marker at several doses for both diploids. A total of 12,000 haploid segregants were analyzed.

## RESULTS

### Analysis of the UT 448//UT 184 (uvs<sup>+</sup>//uvs<sup>+</sup>) diploid under UV-treatment

Most of the treatments induced a homozygosity index (HI) higher than that observed in the control (Table I). Measurement of induced mitotic crossing-over through the homozygosity of mutant alleles (for nutritional requirements) seems to be an efficient method for detecting a genotoxic effect.

The increase seems to be cell cycle-dependent, marker-dependent and dose-independent since higher "HI" was not invariably reached with higher doses. The same UV-dose promoted totally different responses when applied in the conidial phase, or during DNA and protein synthesis, or even after completion of DNA replication. In addition, each marker presented distinctive behaviour under different treatments. Higher "HI" values were found in the conidial phase (pre-incubation  $t = 0$  h) and after completion of DNA replication (pre-incubation  $t = 7$  h) (Table I).

#### Analysis of the Z1//UT 184 (*uvs//uvs*<sup>+</sup>) diploid under UV-treatment

Except for the higher mitotic crossing-over frequency found for all markers (for nutritional requirements) under all treatments, the behaviour of this diploid was similar to that of the normal one (Table II). In this strain the same recombinational levels were induced by lower doses as in the wild-type strain, indicating that pre-recombinogenic lesions remain longer in the mutant diploid.

In this mutant strain, spontaneous mitotic recombination rate was very high, because it bears inherent mitotic instability. Again there was no linear dose-response relationship; and cell cycle and marker dependence appeared as in the normal diploid UT 448//UT 184.

## DISCUSSION

Two similar diploid strains, bearing several markers distributed all over the eight linkage groups, were used. One was composed of two normal haploid strains (UT 448//UT 184) and the other was formed by a normal strain and a strain bearing a duplicate segment transposed from chromosome II to I (Z1//UT 184) (Castro-Prado and Zucchi, 1991a,b; Marin and Zucchi, 1991).

The presence of the duplicate segment confers to Z1 an inherent mitotic instability; also Z1 bears a *uvs* character (Marin and Zucchi, 1991), due to deficiencies in photoreactivation and excision repair (Bonilha *et al.*, submitted). So, lesions inflicted on Z1//UT 184 are preferentially removed by recombinational repair, which raises the homozygosity rates. Other short-term tests use repair-deficient microorganisms (Thomas and MacPhee, 1985; Leifer *et al.*, 1991; Siede and Friedberg, 1990) or mammalian repair-deficient cells (Darroudi and Natarajan, 1989; Menichini *et al.*, 1991) for screening chemical and physical mutagens. Such mutant properties seem to increase the sensitivity of the method since it can lead to more defined results. The special sensitivity of the diploid Z1//UT 184 is shown in Figures 2 and 3, which compare the maximum "HI" values under UV-induction with control values for both strains. It can be seen that "HI" values for the normal diploid obtained by induction are similar to those "HI" values of the mutant diploid obtained without induction.

Table I - Increased "Homozygosity Index" of several markers of UT 448//UT 184 exposed to several UV doses during different phases of cell growth in comparison to control values.

Hours of pre-incubation	Group linkage	I	I	IV	VI	VII	VI	V	II			
	Markers	<i>paba</i>	<i>bi</i>	<i>pyro</i>	<i>s</i>	<i>nic</i>	<i>lac</i>	<i>fac</i>	<i>w</i>	Number analyzed		
	Treatments										Diploids	Haploids
0	0 sec. UV (control)	2.5	2.7	3.0	2.1	1.2	1.5	2.5	0.8	7	262	
	40 sec. UV	5.3	15.2	5.3	15.1	0.8	5.9	11.2	1.0	4	111	
	80 sec. UV	13.3	11.4	15.8	21.7	13.4	20.2	29.4	2.1	6	241	
5	20 sec. UV	2.8	3.1	2.2	7.6	6.4	2.9	9.9	1.8	7	221	
	40 sec. UV	7.5	7.6	4.9	9.0	14.0	4.8	11.4	0.9	6	194	
	80 sec. UV	6.6	5.0	9.5	9.3	6.8	10.5	8.1	1.4	3	40	
7	20 sec. UV	19.2	5.0	18.3	11.3	14.2	15.3	9.3	0.8	3	123	
	40 sec. UV	20.2	18.5	18.8	11.6	3.6	4.3	12.1	1.2	4	120	
	80 sec. UV	1.4	1.8	1.5	5.3	1.2	1.7	1.8	0.7	6	96	
										Total	39	1,408

Table II - Increased "Homozygosity Index" of several markers of Z1//UT184 exposed to several UV doses during different phases of cell growth in comparison to control values.

Hours of pre-incubation	Group linkage	I	I	IV	VI	VII	VI	V	II		
	Markers	<i>paba</i>	<i>bi</i>	<i>pyro</i>	<i>s</i>	<i>nic</i>	<i>lac</i>	<i>fac</i>	<i>w</i>	Number analyzed	
	Treatments										Diploids
0	0 sec. UV (control)	21.8	21.7	21.7	17.0	11.7	18.2	18.2	1.2	9	184
	20 sec. UV	58.0	58.0	58.0	16.2	16.0	16.1	58.0	2.5	2	121
	40 sec. UV	41.7	41.7	34.4	29.2	30.0	25.6	24.0	1.0	7	306
	60 sec. UV	22.3	37.8	15.2	9.7	20.1	20.5	22.0	2.2	6	236
	80 sec. UV	35.7	37.6	37.6	32.2	27.0	29.7	26.0	2.5	6	236
3	20 sec. UV	36.1	26.1	36.5	20.3	23.7	37.6	46.1	1.8	5	238
	40 sec. UV	27.8	30.6	39.6	23.2	24.5	7.8	2.5	1.0	7	251
	60 sec. UV	41.2	41.2	23.2	31.1	7.7	5.0	5.7	1.3	5	245
	80 sec. UV	45.9	26.6	29.2	33.8	26.4	17.4	8.4	1.6	9	413
5	20 sec. UV	22.1	21.6	10.7	27.9	10.9	19.1	19.1	0.9	3	89
	40 sec. UV	6.7	8.2	11.5	18.7	11.3	6.4	19.2	1.2	7	279
	60 sec. UV	7.4	65.6	4.6	8.7	5.1	8.7	15.9	1.1	1	68
	80 sec. UV	10.0	18.4	12.6	13.9	9.5	8.2	13.4	1.2	6	184
7	20 sec. UV	13.8	27.9	6.3	18.1	6.9	6.6	11.2	1.0	5	168
	40 sec. UV	28.2	35.2	12.5	17.6	12.5	9.2	35.2	0.8	2	111
	60 sec. UV	10.7	15.5	15.5	9.6	12.7	8.6	10.8	0.5	4	67
	80 sec. UV	9.2	22.9	23.3	13.8	24.1	7.4	18.7	0.8	7	210
Total									92	3,406	

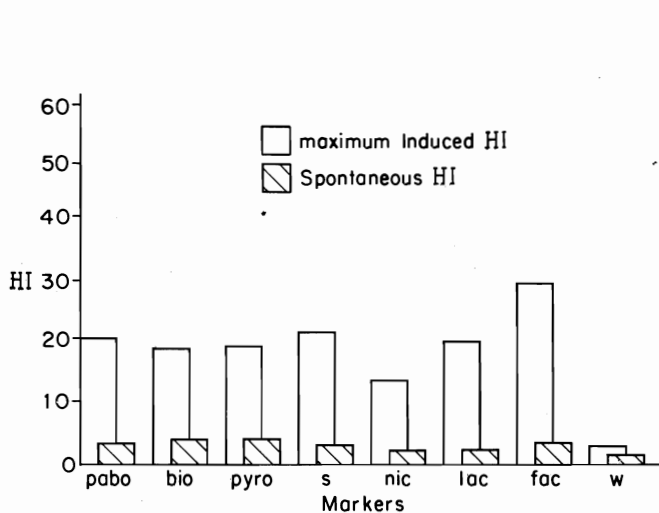


Figure 2 - Induced and spontaneous "Homozygotization Index" in the UT 448//UT 184 diploid strain.

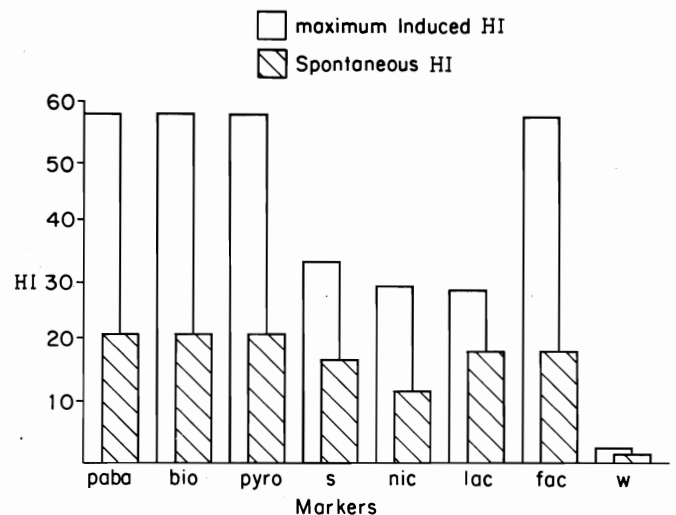


Figure 3 - Induced and spontaneous "Homozygotization Index" in the Z1//UT 184 diploid strain.

No clear-cut response curve was demonstrated, suggesting lack of a direct relationship between number of lesions and number of recombination events, as reported for other systems measuring recombination (Kunz *et al.*, 1985; Ferguson, 1990). According to Kunz *et al.* (1985) the absence of linearity is inherent to recombination mechanisms, being related to induction of recombinational enzymes by the lesions promoted by the UV treatment; such enzymes would derepress the mitotic recombination (Holliday, 1968, 1971; Fabre and Roman, 1977; Ganesan *et al.*, 1982; Walker *et al.*, 1985). The absence of clear-cut dose-response poses no restriction, since any dose is enough to enhance "HI".

The present method permits the detection of homozygotization of any heterozygous gene of an *A. nidulans* diploid strain, which provides a versatile detection of recombination events, in comparison with determination of the recombination frequencies between two linked genes by the traditional methods.

### ACKNOWLEDGMENTS

Financial support by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Proc. 90/0227-1) and by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Proc. 50007590-5) is acknowledged. Special thanks to Dr. Ronaldo Zucchi for useful discussion and revision of the text. We are indebted to Mrs. Sônia Mathias da Silva for technical assistance and to Mr. Cassiano Pereira Nunes for illustrations.

Publication supported by FAPESP.

### RESUMO

Apresentamos um teste para detecção de crossing-over mitótico em *Aspergillus nidulans*, baseado na taxa de homozigotização de genes recessivos, presentes originalmente em heterozigose em linhagens diplóides; a homozigotização ocorre após crossing-over mitótico entre um marcador em estudo e o centrômetro. Desde que diplóides homozigotos (-/-) para qualquer dos marcadores envolvendo auxotrofia não podem se desenvolver em MM, a homozigotização pode ser demonstrada pela distorção na taxa de segregação mitótica dos alelos em questão. Duas linhagens diplóides similares (UT 448//UT 184 e Z1//UT 184), que diferem por um segmento cromossômico duplicado e transposto do cromossomo II para o I na linhagem haplóide Z1, foram usadas. Esse excesso de material genético confere ao mutante Z1 um carácter *uvr* e torna Z1//UT 184 mais instável e sensível a agentes genotóxicos, como foi evidenciado por seu alto índice recombinacional, mesmo em condições normais.

Após tratamento com luz UV, a "taxa de homozigotização" (HI) de ambas as linhagens é significativamente aumentada, durante as várias fases do ciclo celular estudadas, especialmente no diplóide Z1//UT 184.

Detecção de indução de crossing-over mitótico através dos métodos tradicionais requer que o evento ocorra na região específica do

genoma, entre os dois marcadores em estudo. O método proposto abole tal restrição, desde que "HI" pode ser calculado para qualquer marcador em heterozigose, em qualquer linhagem diplóide de *A. nidulans*.

### REFERENCES

- Bernstein, H., Hopf, F.A. and Michod, R.E. (1987). The molecular basis of the evolution of sex. *Ad. Genet.* 24: 323-369.
- Bertoldi, M. and Griselli, M. (1980). Different test systems in *Aspergillus nidulans* for the evaluation of mitotic gene conversion, crossing-over and non-disjunction. *Mutation Res.* 74: 303-324.
- Cairns, J. (1981). The origin of human cancers. *Nature* 289: 353-357.
- Castro-Prado, M.A.A. and Zucchi, T.M.A.D. (1991a). Stabilization of a duplicated segment *Dp* (II-I) in an *uvr* mutant of *A. nidulans* through genetic mechanisms. *Brazil. J. Genetics* 14: 239-248.
- Castro-Prado, M.A.A. and Zucchi, T.M.A.D. (1991b). Meiotic segregation of a recessive gene (*wA2*) in a *Dp* (II-I) of *A. nidulans*. *Brazil. J. Genetics* 14: 249-260.
- Darroudi, F. and Natarajan, A.T. (1989). Cytogenetical characterization of chinese hamster ovary X-ray-sensitive mutant cells *xrs 5* and *xrs 6*. III. Induction of cell killing, chromosomal aberrations and sister-chromatid exchanges by bleomycin, mono- and bifunctional alkylating agents. *Mutation Res.* 212: 123-135.
- Fabre, F. and Roman, H. (1977). Genetic evidence for inducibility of recombination competence in yeast. *Proc. Natl. Acad. Sci. (USA)* 74: 1667-1671.
- Ferguson, L.R. (1990). Mutagenic and recombinogenic consequences of DNA-repair inhibition during treatment with 1,3-bis (2-chloroethyl)-1-nitrosourea in *S. cerevisiae*. *Mutation Res.* 241: 369-377.
- Forbes, E. (1959). Use of mitotic segregation for assigning genes to linkage group in *A. nidulans*. *Heredity* 13: 67-80.
- Ganesan, A.K., Cooper, P.K., Hanawalt, P.C. and Smith, C.A. (1982). Biochemical mechanisms and genetic control of DNA repair. In: *Progress in Mutation Research* (Natarajan, A.T., Obe, G. and Altman, H., eds.). V. 4, Elsevier, Amsterdam, pp. 313-323.
- Holliday, R. (1968). Genetic recombination in fungi. In: *Replication and Recombination of Genetic Material* (Peacock, W.J. and Brock, R.D., eds.). Austral. Acad. of Sciences, Canberra, pp. 157-174.
- Holliday, R. (1971). Biochemical measure of the time and frequency of radiation induced allelic recombination in *Ustilago*. *Nature, Neur. Biol.* 232: 233-236.
- Kunz, B.A. and Haynes, R.H. (1981). Phenomenology and genetic control of mitotic recombination in yeast. *Ann. Rev. Genet.* 15: 57-80.
- Kunz, B.A., Eckardt, F. and Haynes, R.H. (1985). Analysis of nonlinearities in frequency curves for UV-induced mitotic recombination in wild-types and excision-repair-deficient strains of yeast. *Mutation Res.* 151: 235-242.
- Leifer, Z., Kada, T., Mandel, M., Zeiger, E., Stafford, R. and Rosenkranz, H.S. (1991). An evaluation of tests using repair-deficient bacteria for predicting genotoxicity and carcinogenicity. *Mutation Res.* 87: 211-297.

- Marin, J.M. and Zucchi, T.M.A.D.** (1991). Genetic analysis of some factors affecting mitotic and meiotic behaviour of a mutant of *A. nidulans*. *Brazil. J. Genetics* 14: 9-20.
- Marx, J.L.** (1982). The case of a misplaced gene. *Science* 218: 983-985.
- Menichini, P., Vrieling, H. and Van Zeeland, A.A.** (1991). Strand-specific mutation spectra in repair-deficient hamster cells. *Mutation Res.* 251: 143-155.
- Orr-Weaver, T.L. and Szostak, J.W.** (1985). Fungal recombination. *Microbiol. Rev.* 49: 33-58.
- Roeder, J.A. and Stewart, S.E.** (1988). Mitotic recombination in yeast. *Trends in Genetics* 4: 263-267.
- Roper, J.A.** (1952). Production of heterozygous diploids in filamentous fungi. *Experientia* 8: 14-15.
- Rudin, N. and Haber, J.E.** (1988). Efficient repair of HO-induced chromosomal breaks in *S. cerevisiae* by recombination between flanking homologous sequences. *Mol. Cell. Biol.* 8: 3918-3928.
- Schiestl, R.H.** (1989). Non-mutagenic carcinogens induce intra-chromosomal recombination in yeast. *Nature* 337: 285-288.
- Siede, W. and Friedberg, E.C.** (1990). Influence of DNA repair deficiencies on the UV sensitivity of yeast cells in different cell cycle stages. *Mutation Res.* 245: 287-292.
- Thomas, S.M. and MacPhee, D.G.** (1985). Frameshift mutagenesis by 9-aminoacridine in ICR 191 in *E. coli*: effects of *uvrB*, *recA* and *lex A* mutations of plasmid pKM 101. *Mutation Res.* 151: 49-56.
- Van de Vate, C. and Jansen, G.I.O.** (1978). Meiotic recombination in a duplication strain of *A. nidulans*. *Genetic Res.* 31: 29-52.
- Walker, G.C., Marsh, L. and Dodson, L.A.** (1985). Genetic analysis of DNA repair: inference and extrapolation. *Ann. Rev. Genet.* 19: 103-126.
- Wang, Y., Maher, V.M., Liskay, R.M. and McCornick, J.J.** (1988). Carcinogens can induce homologous recombination between duplicated chromosomal sequences in mouse L cells. *Mol. Cell. Biol.* 8: 196-202.
- Zucchi, T.M.A.D.** (1990a). Isolation of a putative recombination mutant of *A. nidulans*. *Brazil. J. Genetics* 13: 409-424.
- Zucchi, T.M.A.D.** (1990b). Location of the suppressor of *meth A17* mutation in the 30 mutant of *A. nidulans*. *Brazil. J. Genetics* 13: 425-443.

(Received May 24, 1993)