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Quantification of DNA damage and repair in amino acid auxotrophs and UV-sensitive mutants of *Aspergillus nidulans* using an ELISA

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Abstract An ELISA used to investigate DNA repair in mammalian cells has been adapted to investigate mutagen-induced DNA damage and repair in protoplasts of *Aspergillus nidulans*. The assay shows a reduced rate of repair of DNA damage in methionine and arginine auxotrophs (*meth*G and *arg*B), which were shown previously to be hypersensitive to UV radiation and chemical mutagens. The assay also showed a considerably reduced ability to repair mutagen-induced damage in the *uv*-sensitive mutants *uvs*B and *uvs*H. The increased sensitivity of amino acids auxotrophs to mutagens is, therefore, correlated with a reduced capacity to repair mutagen-induced DNA damage.

Key words: DNA repair; Single-strand break assay; ELISA; *Aspergillus nidulans*

1. Introduction

The mechanisms of DNA damage and repair in eukaryotes can be readily investigated by the isolation and analysis of mutants to identify the genes involved. This can be done most easily with those lower eukaryotes such as the yeasts and filamentous fungi which have well-characterized genetic systems. For example, investigation of radiation-sensitive mutants of the yeast *Saccharomyces* has identified genes paralleling functions found previously in mammalian cells [1]). The lower eukaryotes are, therefore, excellent model systems for investigating fundamental processes such as DNA repair.

Radiation-sensitive (*uvs*) mutants have been isolated in the filamentous fungus *Aspergillus nidulans* [2–4]. These repair deficient *uvs* mutants are hypersensitive to uv radiation and to the alkylating agent methyl methanesulphonate (MMS), and have been mapped and investigated using a classical genetics approach [4]. Two of the *uvs* genes have been cloned and sequenced [5,6]. Mutagen-sensitive (*mus*) mutants have been similarly characterized [7].

Kafer [8] showed that auxotrophic strains of *A. nidulans* requiring amino acids for growth in minimal media are sensitive to the chemical mutagen MMS. We showed subsequently that they are also hypersensitive to uv radiation, nitroquinoline oxide, heat shock and oxidative stress [9]. The results show that germinating spores of amino acid auxotrophs are defective in a pathway which makes them sensitive to environmental stress and DNA damage, and we have suggested a defect in the RAS/cAMP pathway by analogy with *S. cerevisiae* where some mutants defective in this pathway are sensitive to stress [10]. The aim of this investigation was to quantify the DNA damage and repair capacity in *Aspergillus* amino acid auxotrophs using a sensitive ELISA previously developed to investigate DNA repair in mammalian cells [11,12].

2. Materials and methods

2.1. Strains, media and growth conditions

Routine growth and maintenance of *A. nidulans* was as described previously [13]. The strains used were R21 (*yA2 paba*A1), a methionine auxotroph (*yA2 paba*A1; *meth*G1) obtained by crossing FGSC A154 (*ad*E20 *bi*A1; *w*A2 *cnx*E16; *s*C12; *meth*G1; *nic*A2; *lac*A1; *cho*A1; *cha*A1) with R21, an arginine auxotroph G034 (*bi*A1; *arg*B2) and two UV-sensitive strains G423 (*an*A1 *yA2*; *acr*A1; *uvs*H311; *fw*A2) and G421 (*acr*A1; *uvs*B312 *pyro*A4; *ribo*B2). Strain G034, G423 and G421 were kindly provided by Professor A.J. Clutterbuck, Department of Genetics, University of Glasgow, Scotland, UK. Thick suspensions of conidia (10^{10} conidia/ml) were prepared freshly for each experiment as described previously [13] and stored in Tween-saline at 4°C. Viable spore titres were determined by plating suitable dilutions on to MEA medium (2% malt extract, 2% sucrose, 0.1% Bactopeptone, 1.5% agar), with nutritional supplements as appropriate.

2.2. Preparation of protoplasts

Protoplast preparation was based on the method of Ballance et al. [14] from conidia which were pregerminated for 6 h in fully supplemented YEPD medium (2% yeast extract, 0.1% peptone, 2% glucose). Following mutagenesis, the protoplasts were shaken at 37°C in a solution containing 0.6 M KCl, 0.05 M $CaCl_2$ as osmotic protector. Protoplast viability was determined by spreading dilutions on malt extract agar (MEA) medium containing 0.6 M KCl and 0.05 M $CaCl_2$ and incubating overnight at 37°C. The total number of protoplasts present was determined using a haemocytometer slide.

2.3. Mutagen treatment of protoplasts

The protoplasts were irradiated (Camag-Universal-Lampe) at 0.02 $J\,s^{-1}\,m^{-2}$ in an open Petri dish in darkness, with gentle stirring to prevent sedimentation. Protoplasts were treated with MMS for 2 h at 30°C or with 1 μg/ml 4-nitro-quinoline-1-oxide (4-NQO) at 37°C. The 4-NQO was prepared by dissolving 10 mg of 4-NQO in 1 ml acetone and diluting with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01% Tween-20 [15].

2.4. Quantification of DNA damage and repair

Following mutagen treatment the amount of residual DNA damage was quantitated by means of a sandwich ELISA [11,12] previously used to investigate DNA repair in human blood cells after exposure to ionizing radiation. The amount of single-stranded DNA is measured with a monoclonal antibody (D1B) against single-stranded DNA. D1B stock was supplied by Dr. G.P. van der Schans (TNO Medical Cell Biology Laboratory, Rijswijk, The Netherlands). This was diluted to 10 μg/ml using phosphate buffered saline solution (PBS) and 100 μl was added to each well of a 96-well flat bottom, high binding Costar Microtiter plate. This was vibrated for 10 min and washed 3× with

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PBS. The plate was dried and 150 μl aftercoating (5% foetal calf serum in PBS) was added to each well and vibrated for 10 min. The plate was dried, packed in aluminium foil and stored at −20°C until required.

2.5 Controlled partial unwinding of DNA under alkaline conditions

Local single-stranded regions associated with strand breaks and with lesions converted into breaks in alkaline medium are converted by partial unwinding into longer single-stranded regions where the monoclonal antibody can bind. 0.8 ml alkaline solution (1.3 M NaCl adjusted to pH 12.3 with NaOH) was added to 30 μl. *A. nidulans* protoplasts (1×10^9 cells) and left at 20°C in red light for precisely 6 min. 145 μl neutralizing solution (0.25 M NaH_2PO_4) was then added and the sample immediately sonicated for 1 s at 20°C. The 100% single-stranded control for the ELISA was prepared by processing an aliquot of each sample further as follows; 20 μl of each sample was removed, 160 μl alkaline solution was added and then neutralized after 2 min by the addition of 20 μl neutralizing solution.

2.6 Sandwich ELISA for the detection of single-stranded DNA

DNB-coated 96-well plates were removed from the freezer and equilibrated to room temperature. To row A was added 120 μl of PBS and 70 μl PBS to rows B to H. 20 μl of each sample and the corresponding 100% single-stranded controls were added and serially diluted 1:1 through rows A to G inclusive. Row H was 'blank' i.e. contained no DNA. The plate was then vibrated for 10 min and washed 3 × in PBS.

The plate was dried and conjugated antibody-alkaline phosphatase (DNB-AP solution) added to each well, vibrated for 10 min, washed 3 × with PBS containing 0.05% Tween-20 (PT), then washed twice with 0.1 M diethanolamine, emptied and dried. 200 μl *p*-nitrophenylphosphate (PNP) containing 1 mg/ml PNP in 0.2 M Tris buffer, was added to each well and the plate was vibrated briefly before incubation at 25°C for 24 h. The absorbance from each well was then read in a Tikertek Absorbance ELISA plate reader at 405nm. The % single-stranded DNA present was calculated as the mean sample value (minus background), expressed as a percentage of the corresponding 100% unwound control.

3. Results

The purpose of this work was to determine the capacity of amino acid auxotrophs to repair mutagen-induced DNA damage. We chose to work initially with a methionine auxotroph (*methG*) lacking β-cystathionase activity [16] which had been used in a previous study on DNA repair [9]. The DNA repair mutant *uvs*H was chosen as a reference strain because it was the most sensitive of the *uvs* mutants tested to mutagens and heat shock [9].

The ELISA had been developed for the quantitative detection of DNA damage in mammalian cells induced by ionizing radiation. The assay had not been used previously with a fungus so it was essential at the outset to determine the conditions to detect mutagen-induced DNA damage and repair in *Aspergillus*. Since the assay depends upon the binding of a monoclonal antibody to single-stranded DNA it was necessary to convert the mycelium, which has a rigid multilayered cell wall [17] into protoplasts lacking this barrier to protein penetration. It was also necessary to determine a suitable mutagen dose causing sufficient initial DNA damage to enable us to follow the time course of subsequent repair. *Aspergillus* protoplasts were treated with a range of doses of uv, MMS and 4-NQO, and the extent of DNA damage determined using the ELISA. The results (Fig. 1) show the expected dose-dependent increase in the percentage of single-stranded DNA (%ssDNA), from an initial value of approximately 12%, with all three strains irrespective of the mutagen used. For subsequent work, we chose mutagen doses giving rise (after controlled unwinding of the DNA in

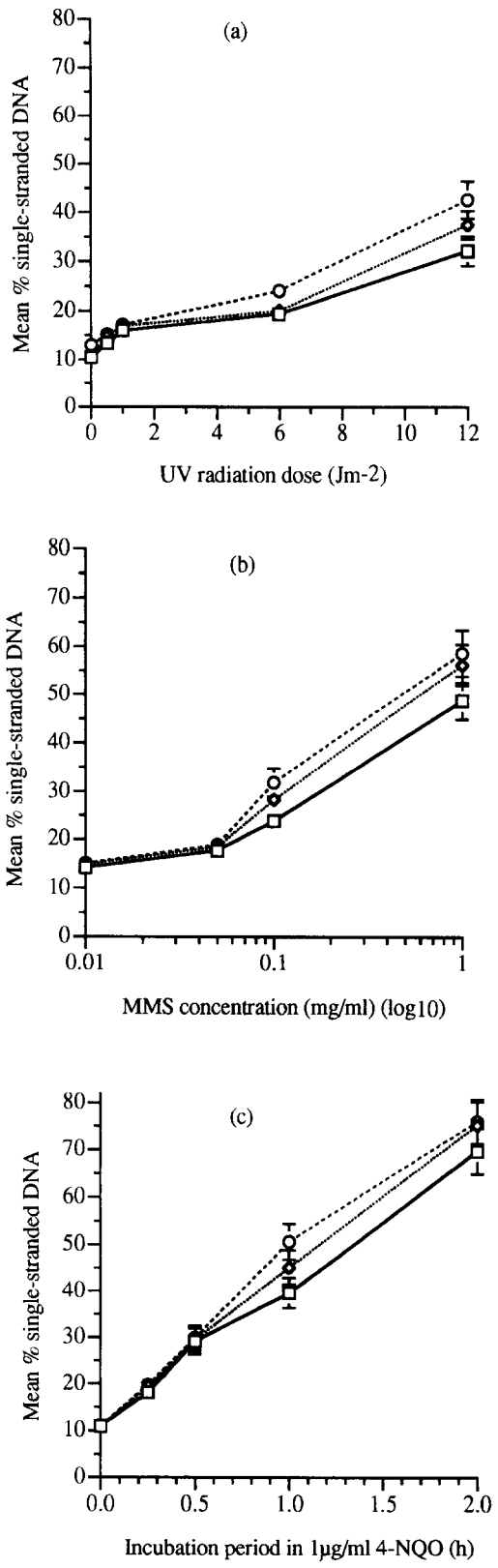

Fig. 1. The effect of different doses of (a) UV radiation, (b) MMS and (c) 4-NQO on the %ssDNA measured by the ELISA in protoplasts of R21 (–□–), *meth*G (–●–), and *uvs*H (–○–). The %ssDNA was calculated as the mean sample value (minus background) expressed as a percentage of the corresponding 100% unwound control. Each ELISA was carried out in duplicate and the values quoted are the mean values of two experiments.

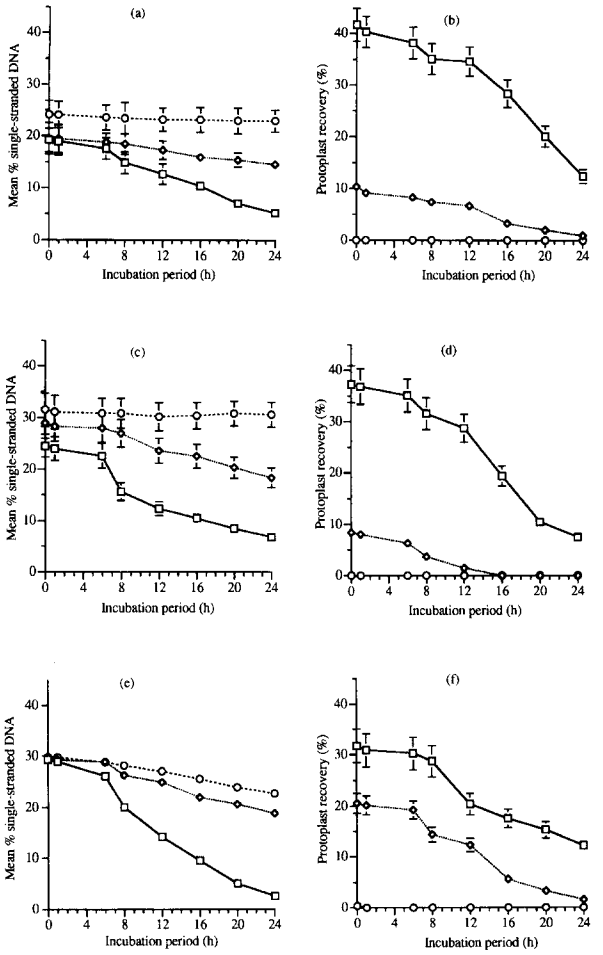

Fig. 2. The repair of mutagen-induced DNA damage in protoplasts of R21 (–□–), *meth*G (–◇–), and *uvs*H (–○–), measured over a 24-h period at 37°C in 0.5 M KCl, 0.05 M $CaCl_2$ solution, using an ELISA. The mutagen doses used were (a) UV, 6 J m^{-2}; (c) MMS, 0.1 mg/ml for 2 h; and (e) 4-NQO, 1 μg/ml for 30 min. The %ssDNA was calculated as the mean sample value (minus background) expressed as a percentage of the corresponding 100% unwound control. Each ELISA was carried out in duplicate and the values shown are the mean values of two experiments. The ability of the protoplasts to regenerate colonies was also determined (b,d,f) by plating out aliquots on MEA medium.

alkaline solution) to initially similar enhanced levels of %ssDNA with each of the *Aspergillus* strains.

In order to compare the rates of DNA damage repair in the three strains, protoplasts were exposed to the same mutagen dose and the extent of repair followed over a 24-h period in protoplast isolation solution (0.5 M KCl, 0.05 M $CaCl_2$). Samples were also removed to determine the proportion of protoplasts able to recover and regenerate colonies (Fig. 2). The mean %ssDNA measured in the wild type (R21) protoplasts decreased continuously over the 24 h period with all three mutagens, showing that these protoplasts were capable of repairing the damage induced by the mutagen treatment. In contrast, there was little decrease in %ssDNA in *uvs*H protoplasts after treatment with uv or MMS but a small decrease with 4-NQO. The rate of repair in protoplasts of the *meth*G strain was intermediate between the wild type and the DNA repair mutant. At the mutagen doses used few of the *uvs*H protoplasts formed colonies after the mutagen treatment (Fig. 2b,d,f). This is consistent with results obtained with germinating conidia [9] where the *uvs*H strain proved to be the most sensitive of the *uvs* mutants tested. For example, only 1% germinating conidia survived uv irradiation (data not shown) compared with 76% wild type, 27–72% *uvs*A, *uvs*B, *uvs*C, *uvs*D and *uvs*E, and 7% *uvs*F, at the same dose. The recovery of the methionine auxotroph protoplasts was intermediate between the wild type and the *uvs*H mutant, consistent with the effect of mutagens on the viability of germinating conidia [8,9]. In the absence of mutagen treatment, the protoplast viabilities of the three strains were comparable and decreased at similar rates over the incubation period (data not shown).

Clearly, it is important to extend this analysis to other amino acid auxotrophs and *uvs* mutants. We have examined DNA repair in two other strains, an arginine requiring mutant *arg*B, which lacks ornithine carbamyl transferase [18] and another mutant with an uncharacterized defect in DNA repair, *uvs*B. Both mutants had been used in a previous study [9]. As can be

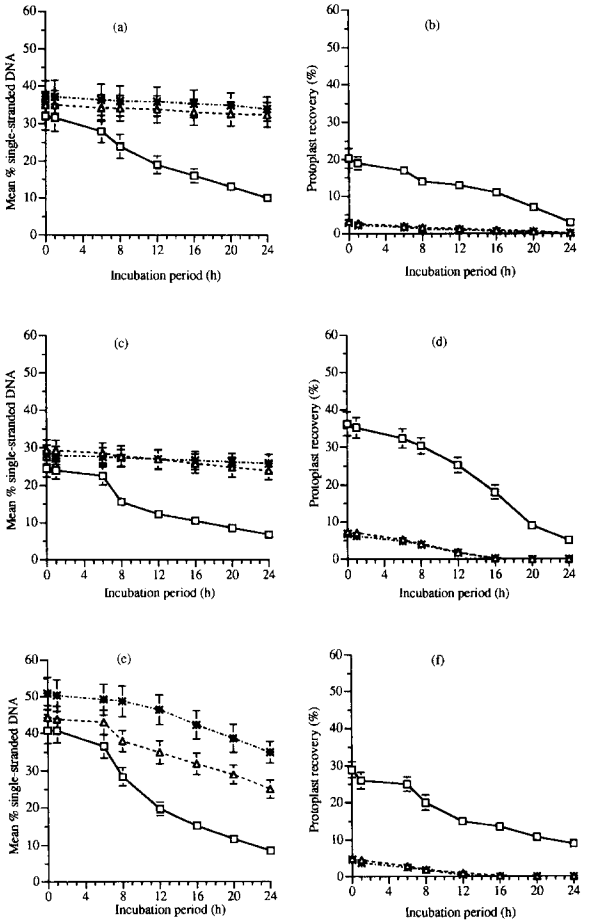

Fig. 3. The repair of mutagen-induced DNA damage in protoplasts of R21 (–□–), *arg*B (–△–), and *uvs*B (–×–) measured over a 24-h period at 37°C in 0.5 M KCl, 0.05 M $CaCl_2$ solution, using an ELISA. The mutagen doses used were (a) UV, 6 J m^{-2}; (c) MMS, 0.1 mg/ml for 2 h; and (e) 4-NQO, 1 μg/ml for 30 min. The %ssDNA was calculated as the mean sample value (minus background) expressed as a percentage of the corresponding unwound control. Each ELISA was carried out in duplicate and the values shown are the mean values of two experiments. The ability of the protoplasts to regenerate was determined by plating out aliquots of the suspension on MEA medium (b,d,f).

seen in Fig. 3, the pattern of DNA repair measured by the ELISA is similar to that found with *meth*G and *uvs*H. It should also be noted that the nutritional requirements of these strains are very different from the other mutants discussed above; the arginine auxotroph *arg*B requires biotin, the *uvs*B mutant requires pyridoxine and riboflavin. It is, therefore, very unlikely that DNA repair is affected by the vitamin requirements of the strains used.

4. Discussion

The ELISA assay [11,12] was developed for the detection of single-strand breaks and lesions that are converted to single-strand breaks in an alkaline solution, in human blood cells which had been exposured to ionizing radiation. The ELISA does not measure every type of DNA damage but only damage leading directly or indirectly to strand breaks or which is alkali-labile. The procedure is based on the enhancement of mutagen-induced single-strandedness which occurs in areas of DNA flanking strand breaks, by a time and dose-dependent controlled unwinding of DNA under alkaline conditions. Single- and double-strand breaks in DNA or mutational lesions resulting in these breaks in alkaline medium form the initiation points for the unwinding process. The degree of unwinding is a measure of the extent of initial DNA damage and this is determined by the use of an anti-single-stranded-DNA monoclonal antibody. Since the monoclonal antibody requires access to the DNA and since *A. nidulans* has a thick multiple-layered cell wall [17] unlike blood cells, it was necessary to convert the mycelium into protoplasts. Also because of the different effects of the mutagens on the viabilities of the strains it was necessary to incubate the protoplasts in protoplast isolation buffer rather than growth medium following mutagen treatment. The results of this study must, therefore, be interpreted with caution since protoplasts are very different from the actively growing cells found in mycelium. However, we are interested only in comparing the capacities of the enzymes present in the protoplasts to repair DNA damage and to that extent the approach is justified.

Three mutagens were used in this work, MMS, 4-NQO and uv. These mutagens induce a wide variety of mutational lesions in DNA including strand breaks and other alkali-labile sites which may be processed under alkaline conditions to render them into stretches of single-stranded DNA which are detected in the ELISA. UV radiation produces a variety of photoproducts many of which (including pyrimidine dimers and 6–4 photoproducts) can be classified as alkali-labile lesions [19] and although they are chemically stable under normal physiological conditions their decomposition under alkaline conditions leads to the cleavage of the DNA backbone through the formation of abasic sites [20]. Other UV-induced DNA lesions known to act as alkali-labile sites include thymine glycols [21] and 8-hydroxyguanine [22]. Exposure of cells to alkylating agents such as MMS also results in the formation of DNA modifications that can be detected as single-stranded DNA after denaturation of the DNA in alkaline buffer and this was demonstrated using the alkaline filter elution technique [23]. These DNA strand breaks can result from direct DNA breakage in vivo or from the generation of alkali-labile sites [24]. The transient apurinic/apyrimidinic sites due to enzymatic or chemical hydrolysis of alkylated bases are alkali-labile and are converted to strand breaks in a pH-dependent reaction [25]. It has also been shown that treatment of Chinese Hampster Ovary cells with MMS results in far more alkali-labile sites than direct DNA strand breaks [26]. DNA damage induced by the other mutagen used in this study 4-NQO can also be detected as single-stranded DNA after alkaline treatment. 4-NQO is known to produce lesions such as AP sites [27] and 8-hydroxydeoxyguanosine [28] both of which are known to be alkali-labile sites. 4-NQO is also a strong inducer of oxidative stress conditions [29] which can give rise to strand breakage [30]. It is apparent, therefore, that DNA damage caused by the three mutagens used in this study may be processed under alkaline conditions into single-stranded DNA. Such single-stranded DNA may be detected with the ELISA by a monoclonal antibody against single-stranded DNA.

The repair of DNA damage in protoplasts appears to extend over a prolonged period compared with the measured time (2 h at 25°C) between successive mitoses in normal growing mycelial cells [31]. However, as pointed out earlier, protoplasts are very different from the actively growing cells found in mycelia. The protoplasts used in this work must also be considered a very heterogeneous population. A high proportion of newly isolated *A. nidulans* protoplasts fail to regenerate (perhaps 20 to 80%, depending on the the preparation) and those that do, regenerate asynchronously [32]. Furthermore in this work the protoplasts were incubated in a salts solution devoid of nutrients. It has also been shown that mycelial cells of *uvs* and *bim*D mutants exhibit a mitotic catastrophe when treated with DNA damaging agents [31,33]. This probably occurs when cell cycle progression resumes in the presence of DNA damage, after a temporary delay and results in extensive chromatin abnormalities and fragmentation. Given the presence of fragmented chromatin, the high proportion of non-regenerating protoplasts and the absence of nutrients, it is perhaps not surprising that DNA repair can be measured over an extended period in these protoplasts.

We have demonstrated here that DNA repair can be measured using a sensitive ELISA previously applied to quantitating DNA repair in mammalian cells [11,12] and we have used this assay to show that the ability of two amino acid auxotrophs to repair mutagen-induced DNA damage is considerably reduced in comparison with a repair-proficient strain. Under the same circumstances, repair in the UV-sensitive strains *uvs*H and *uvs*B is similarly reduced. The *Aspergillus uvs*H gene has recently been sequenced [6] and shown to encode a product homologous to the yeast RAD18 and *Neurospora* UVS-2 genes. The yeast RAD18 gene binds single-stranded DNA and probably directs the product of the DNA repair gene RAD6 to damage-containing DNA regions [34]. The lack of DNA repair in the *uvs*H mutant suggests that the gene product may play an essential role in several pathways of DNA repair.

All the amino acid auxotrophs of *A. nidulans* investigated are hypersensitive to UV radiation, chemical mutagens, heat shock and hydrogen peroxide [9]. We have argued elsewhere [9] that the amino acid auxotrophs are probably defective in the RAS/cAMP pathway and that this pathway might also control the ability to repair DNA damage. We have shown here that in the case of two *Aspergillus* amino acid auxotrophs (*meth*G and *arg*B), sensitivity to radiation and chemical mutagens is correlated with a real reduction in the capacity of cells to repair DNA damage.

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