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Differential Synthesis and Replication of DNA in the *Neurospora crassa* Slime Mutant versus Normal Cells: Role of Carcinogens

M. Beljanski[a], *S.K. Dutta*[b]

[a] Laboratoire de Pharmacodynamie, Université Paris-Sud, Centre d'Etudes Pharmaceutiques et Biologiques, National de la Recherche Scientifique, Chatenay-Malabry, France;
[b] Laboratory of Molecular Genetics, Department of Botany and Cancer Research Center, Howard University, Washington, D.C., USA

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Abstract. Small quantities of carcinogens, *dl*-ethionine, thiotepa, actinomycin D, and 1-(2-chloroethyl-3-cyclohexyl)-1-nitrosourea (CCNU) stimulated in vitro deoxyribonucleic acid (DNA) synthesis of the slime mutant of *Neurospora crassa*, while there was practically no effect on the DNA from the normal wild type 74A strain. All of these compounds caused increased strand separation in the mutant DNA of *N. crassa*, but no separation of normal DNA strands. The growth (in vivo tests) of the *N. crassa* slime mutant, but not its wild type, was markedly increased when nontoxic concentrations of one of the carcinogens (*dl*-ethionine) tested were present in the growth medium. These observations suggest that, unlike the wild type *N. crassa*, the slime mutant allows an excessive and unscheduled replication, indicating destabilized nature of its DNA.

Introduction

It is universally accepted that gene activation and/or inactivation are related to deoxyribonucleic acid (DNA) transcription, synthesis, and/or replication, which can be regulated biochemically and environmentally [1, 7]. Carcinogens and other chemicals prevalent in our environment may play a role in DNA replication and thereby cell division. It is known that some chemicals can destabilize the double-stranded structure through their binding to DNA [3, 5]. In cancerous and normal animal [2, 3, 15] and plant cells [13] it has been shown that carcinogens can greatly influence the 'geometry of the DNA-compound complexes', leading to the opening or closing of DNA chains in certain areas [7, 15]. Low concentrations of carcinogens can induce in vitro local cancer DNA strand separations, accelerate DNA in vitro synthesis, and enhance cancer cell multiplication in vivo [3, 13]. DNAs from healthy cells respond to these carcinogens with low efficiency.

In this paper, we report that the in vitro and in vivo DNA synthesis of the slime mutant cells (which have lost their ability to differentiate markedly) of *Neurospora crassa* respond differently to carcinogenic compounds in comparison to DNA from the wild type.

Materials and Methods

Chemicals

Sources of chemicals used are as follows: deoxyribonucleoside-5'-triphosphates (Miles Laboratories, USA); ^{3}H-labelled thymidine triphosphates, specific activity 17.5 Ci/mol (Amersham, UK); Pancreatic RNase, RNase T1, DNase (Worthington Co., USA); *dl*-ethionine (Biochemicals Co., USA); actinomycin D (Merk, USA); 1-(2-chloroethyl-3-cyclohexyl)-1-nitrosourea (CCNU) and thiotepa (Laboratory Bellon, France).

Cultures

The wild type 74A strain of *N. crassa* (FGSC No. 987) and the slime mutant (FGSC No. 1118) were obtained from the Fungal Genetics Stock Center, Humboldt, Calif., USA. The wild type *N. crassa* 74A shows fluffy aerial growth, orange color on agar

medium, and mycelia grow like mat of compact cells. The colonies of slime mutant [8] of *N. crassa* (fz; sg; cs-1) grow like yeast cells instead of having a mycelia mat-like growth.

DNA Isolation

N. crassa mycelia cells were collected from liquid cultures by straining through 4-fold cheese cloth. *N. crassa* slime mutant cells were collected by centrifugation at 5,000 *g*. All of these cell masses were homogenized with 30 ml of buffer containing 100 m*M* Tris (pH 7.5), 10 m*M* ethylenediamine tetraacetate, and 1% Triton X-100 for 15 min, centrifuged for 12,000 *g* for 10 min, and the supernatant was saved. To the supernatant was added the same volume of water-saturated phenol (v/v); the mixture was shaken for 10 min and again centrifuged. The process was repeated 3 times more. The upper phase was treated with chloroform and the process repeated twice more. To the aqueous phase chilled 95% ethyl alcohol (v/v) with 0.1 *M* KCl was added and the mixture left over night at 4 °C. After centrifugation at 12,000 *g* for 10 min the pellet was dissolved in 2SSC (1SSC: 0.15 *M* NaCl + 0.015 *M* sodium citrate) solution and centrifuged at 1,000 *g* for 15 min. The supernatant containing DNA was dialyzed against 2SSC solution for overnight at 4 °C. Optical densities were taken at 260 and 280 nm to check ratios of 260/280 = 2 as an indication of purity. DNA in 2SSC solution was then treated with DNase-free RNase (25 µg/ml of DNA solution) for 40 min at 37 °C. RNase was then removed by 3 chloroform extractions, each of which was followed by centrifugation (5,000 *g* for 10 min) at 4 °C. DNA was precipitated with 2 vol of 95% alcohol, dissolved in 2SSC solution and dialyzed against this solution overnight at 4 °C. Purified DNA (absorbance at 260/280 = 2.0–2.1) was stored at –20 °C, without losing its polymerized form. DNAs were characterized by physical means [3] using Cs_2SO_4 buoyant density gradient and thermal denaturation curves. The hyperchromic shift in the presence of alkali was between 35 and 45%.

DNA Strand Separation (Hyperchromicity of DNAs)

UV absorbance at 260 nm of DNAs from *N. crassa* strains was measured at 24 °C before and after addition of a given compound, as described elsewhere [3, 13].

In vitro DNA Synthesis

The incubation conditions for in vitro synthesis have been described elsewhere [3]. The reaction mixture contains per 0.15 ml: Tris HCl buffer (pH 7.65), 25 µmol; $MgCl_2$, 2 µmol; 4 deoxyribonucleoside-5′-triphosphates, each 5 nmol (+ ^{3}H-labelled thymidine triphosphates; 50,000 cpm); 0.5–1 µg of purified DNA; 40–80 µg of partially purified DNA-dependent DNA polymerase I (EC 2.7.7.7) originated from *Escherichia coli* [3]. Incubation was done for 10 min at 36 °C. The amount of acid-precipitable ^{3}H-labelled DNA (trichloroacetic acid, 5% solution) was determined in the absence and presence of each tested compound. The acid precipitable product was filtered on a millipore GF/C glass filter, washed with 5% trichloroacetic acid solution and alcohol, and dried. The radioactivity was then measured with a Packard liquid scintillation counter.

Tests to determine the presence of ^{3}H-labelled thymidine in newly synthesized DNA were performed by routine verification of resistance to KOH and to RNase but sensitive (degradation) to DNase (RNase-free), and Cs_2SO_4 boyant density profiles as described by Dutta et al. [9, 10].

Results

Effect of Carcinogens on in vitro Synthesis of DNA from Wild Type and Slime Mutant of Neurospora crassa

Under identical conditions we attempted to evaluate the template responsiveness of DNAs from wild strain and slime mutant of *N. crassa* to carcinogens. The choice was based upon our previously described observations, showing that carcinogens strongly stimulated *Salmonella* mutant DNA in vitro synthesis (synthesis was poor or absent with DNA of the wild strain) [5]. Increased DNA synthesis in the presence of carcinogens correlates with DNA in vitro strand separation. The same was observed with DNA from cancerous and normal animal, human, and plant tissues [3, 13]. Figure 1 shows that *dl*-ethionine, thiotepa, and actinomycin D stimulated DNA in vitro synthesis of the slime mutant of *N. crassa,* while there was practically no effect on the DNA from the wild strain.

UV Absorbance Changes of Mutant DNA in the Presence of Carcinogenic Agents

Mutant DNA strand separation depends on the concentrations and nature of the drugs used (fig. 2). Actinomycin D induces an UV absorbance increase up to 24%, and thiotepa up to 12%. The additive effects (not shown in fig. 2) of CCNU, actinomycin D. and *dl*-ethionine resulted in a 30% increase in UV absorbance of mutant DNA. These carcinogens did not exhibit an effect on wild type strain DNA. It should be noted that in the presence of alkali (0.1 N NaOH), the UV absorbance (hyperchromicity) increase was 45% for both mutant and wild strain DNA.

It was important to determine if carcinogens used at appropriate concentrations differentially stimulate the in vivo growth of *N. crassa* mutant and that of the wild strain. Results of such tests are summarized in figure 3. *Dl*-ethionine at a higher concentration (above 10 µg/ml) was clearly toxic (fig. 3b) to the growth of both normal and the slime mutant. At nontoxic concentrations (1 µg/ml or lower), growth of the wild type was constant, whereas that of slime mutant was increased several times in comparison to mutant growth in the absence of that carcinogen. The increased growth correlates with the effect of *dl*-ethionine on mutant DNA in vitro strand separation (fig. 2) and increased DNA synthesis (fig. 1).

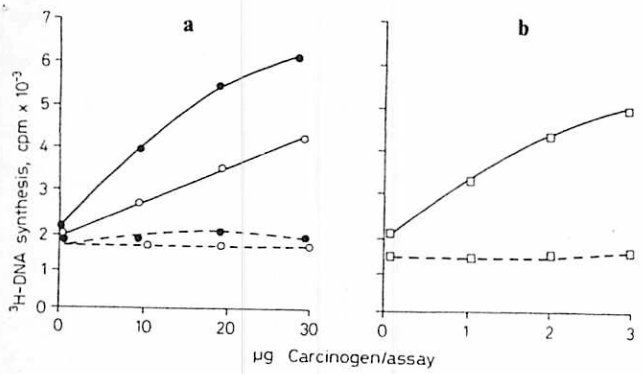


Fig. 1. Effects of different carcinogens on in vitro synthesis of DNA from wild type and slime mutant of *N. crassa*. **a** ●—● = *N. crassa* slime DNA + *dl*-ethionine; ●--● = *N. crassa* wild type DNA + *dl*-ethionine; ○—○ = *N. crassa* slime DNA + thiotepa; ○--○ = *N. crassa* wild type DNA + thiotepa. **b** □—□ = *N. crassa* slime DNA + actinomycin D; □--□ = *N. crassa* wild type DNA + actinomycin D.

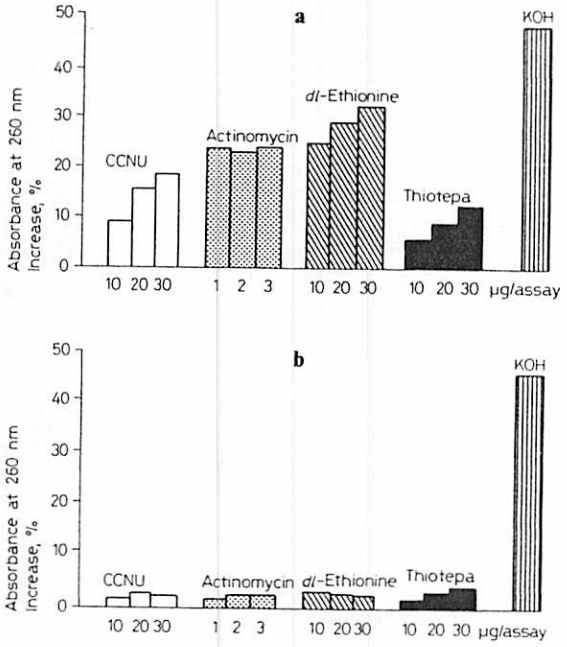
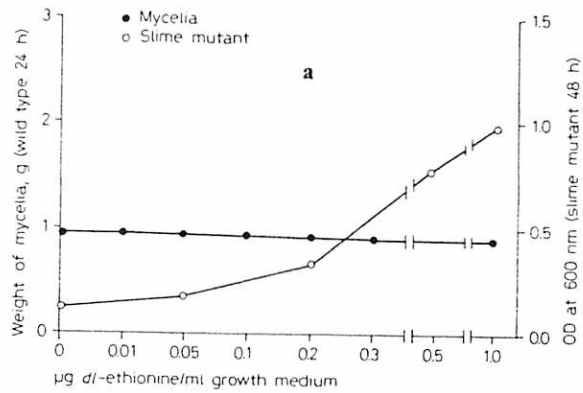


Fig. 2. Effects of carcinogens on the UV absorbance changes of DNAs: *N. crassa* slime mutant **(a)** and normal cells **(b)**.



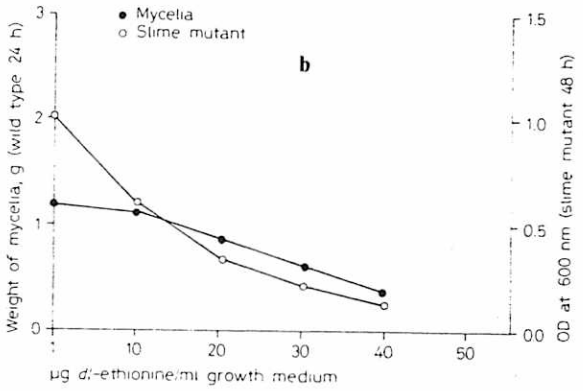


Fig. 3. Effect of *dl*-ethionine on in vivo growth of the *N. crassa* slime mutant and wild type cells. **a** Increased growth of slime cells versus no growth of wild type cells when small quantities of the carcinogen was given. **b** Toxic effect of the same *dl*-ethionine carcinogen when higher concentrations were used for cell growths. OD = Optical density.

Discussion

We have demonstrated the differential behavior of two types of DNAs from *N. crassa* strains (normal type and its slime mutant) using some known carcinogenic compounds. These carcinogens can distinguish DNAs from normal and cancer cells isolated from animal [3] and from plant cells [13]. *Salmonella typhimurium* mutant DNA reacts in vitro directly with carcinogens, while wild strain DNA apparently does not [5]. Here we have shown that carcinogens strongly stimulate the in vitro synthesis of DNA from slime mutant, but not that of the wild strain. DNA synthesis was dependent on the concentration of carcinogens used.

It is well established [6, 11] that increased in vitro or in vivo DNA synthesis requires DNA strand separation in order to allow DNA polymerase to replicate DNA strands. Data presented here demonstrate that the DNA chain opening, measured by the UV absorbance increase, is carcinogen concentration-dependent. In fact, *dl*-ethionine, CCNU, or actinomycin D, which all exhibit carcinogenic potential [3, 14, 15], induce strand separation in mutant DNA when

used at given concentrations, while they have practically no effect on DNA from the wild strain. An interaction between the above compounds and DNA from the mutant takes place, resulting in the break of hydrogen bonds that maintain the double strands of DNA. Our results confirm observation that *dl*-ethionine and other chemicals induce DNA strand separation, as previously shown for *S. typhimurium* [5]. Studies done by us [8] before with the slime mutant DNA of *N. crassa* did show more destabilization (i.e. mismatch of DNA duplexes) based on extensive studies of thermal elution profiles of DNA:DNA homo- and heteroduplexes of ^{32}P-DNA from normal *N. crassa* DNA and the slime mutant DNAs.

There is a good correlation between increased DNA in vitro synthesis and strand separation in mutant DNA and its increased in vivo growth in the presence of small doses of *dl*-ethionine. The differential behavior of mutant DNA is simply amplified in vitro and in vivo probably because of its destabilized physiochemical structure. Well-established animal cancer tissue DNAs, plant cancer DNAs, some bacterial mutants, and fungal DNAs all appear to have a destabilized secondary structure (an area of unpaired strands), making these DNAs susceptible to drugs or chemical compounds. Thus, 'destabilized DNAs' that have lost physiological control mechanisms may allow an excessive transcription of certain genes such as those coding for ribosomal or other types of RNAs. RNAs accumulated in excess for a given time might undergo cleavages by different nucleases, thus furnishing oligoribonucleotides that participate either as primers for DNA replication [4, 7] or as regulators of translation process [12]. Another study [3] has shown that some degradation products are excreted into the culture medium. Along these lines, it should be recalled that a small-size transforming (purine-rich) ribonucleic acid is excreted into the culture medium by *E. coli* showdomycin-resistant strain [4]. This ribonucleic acid possesses quite interesting biological properties, which have been recently reviewed and discussed [1, 4].

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S.K. Dutta, MD,
Department of Botany,
Howard University,
Washington, DC 20059 (USA)