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Topoisomerase activity assays in Neurospora

DNA topoisomerases are enzymes capable of altering the topological conformation of DNA by inducing transient single (Topoisomerase I) and double strand (Topoisomerase II) breaks. These These activities appear to be involved in all aspects of DNA metabolism including replication, recombination, transcription and repair. All eukaryotes appear to have very similar topoisomerase activities (Wang, J.C. 1985 Ann. Rev. Biochem. 54:665-698). We have found that both topoisomerase activities are easily detected in Neurospora and appear to be concentrated in the nucleus.

Changes in topoisomerase II actitivy has been proposed to be a basic defect in the human genetic disease, Ataxia telangiectasia (A.T.). Activities both lower and higher than in normal cells have been observed (Davies, S.M. et al. 1989 Nut. Acid. Res. 17:1337-1352; Mohamed, R. et al. 1988 Biochem. Biophys. Res. Comm. 149:233-238) but these results are confounded by the effects of tissue type and transformation on topoisomerase II levels, as well as the large variability in activity in normal controls. Two mutagen-sensitive mutants of Neurospora, uvs-6 and mus-9 with the classical cellular properties of A.T.: X-ray and bleomycin sensitivity, chromosomal instability and failure of X-irradiation or bleomycin to cause normal inhibition of DNA synthesis, provide a model for understanding the cause of these changes without the problems of mammalian systems (Koga, S.J. and A.L. Schroeder 1987 Mutat. Res. 183:139-148). Therefore, topoisomerase activity was examined in these mutants. Wild type and uvs-2, a mutagen-sensitive mutant with normal chromosomal stability and DNA synthesis repression, served as controls.

METHODS: General--The wild type, 74-OR23-lA, and mutant strains have been described (Perkins, D.D. et al. 1982 Microbiol. Rev. 46:426-570). All mutant strains have been backcrossed several times to standard Oak Ridge wild-type strains. General methods and media for growing and harvesting conidia were those of Schroeder (Schroeder, A.L. 1970 Mol. Gen. Genet. 107:291-304). For the germinating conidia used for all extracts, minimal medium containing 10^7 conidia/ml was incubated for four hours at 30°C with 250 rpm shaking. These conditions give 80 to 95% germination in wild type. Extracts--For whole cell extracts, germinating conidia were washed and resuspended at a concentration of 5 x 10^9/ml in 0.5 ml extraction buffer containing 50 mM Tris-Cl pH 7.5, 25 mM KCl, 3 mM MgCl2, 0.25M sucrose, 1 mM PMSF, 2 mM DTT and 1 mM EDTA (Mohamed et al. 1987). They were transferred to a Mini-Bead-Beater (Biospec Products) vial. Glass beads (0.5 mm) were added to fill the vial and enough buffer to exclude all air. After cooling on ice, the conidia were broken by four 15 sec shakes with 45 sec on ice between each. The glass beads were removed using tubes with four perforations made with a dissecting needle. These were hung inside a second tube and centrifuged at 1000 x g for 5 min. The beads are retained in the upper tube. The supernatant was collected being careful not to include any of the pelleted unbroken conidia, adjusted to 0.4 M KCL, and centrifuged at 30,000 rpm in a 50 Ti Beckman rotor for 30 min. The supernatant was brought to 70% (NH4)2SO4 with a saturated solution of (NH4)2SO4 and left at 4°C for 30 min. The precipitated proteins were collected by centrifugation at 10,000 x g at 4°C for 15 min. and redissolved in 0.5 ml 40 mM Tris-Cl, pH 7.5, 2 mM DTT, 1 mM EDTA, 0.25 M sucrose (Dialysis Buffer). They were dialyzed against three changes of the same buffer. For nuclear extracts, 5 x 10^7 conidia in 0.5 ml were extracted using zirconium beads as described earlier (Schroeder, A.L. 1988 Use of Neurospora to study DNA repair, in: E.C. Friedberg and P.C. Hanawalt (Eds.), DNA Repair: A laboratory manual of research procedures, vol. 3, Marcel Dekker, Inc,. New York and Basel). The final nuclear pellets were resuspended in 0.5 ml of 0.4 M KCl to extract non-histone proteins and left on ice for 1 h. The extracts were centrifuged for 1 min. in a microfuge and the supernatants were dialyzed against dialysis buffer. Protein concentrations were determined by the method of Lowry et al. (Lowry O.H. et al. 1951 J. Biol. Chem. 193:265-275). Topoisomerase assays--The assays were based on those described by Liu and Davis (Liu, L.F. and J.L. Davis 1981 Nuc. Acid. Res. 9:3979-3989). Each 20 ul reaction contained 0-2 ug of extracted protein, 50 mM Tris-Cl, pH 7.7, 100 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA and 20 ug/ml DNA substrate. In topoisomerase I assays, covalently closed pUC19 DNA was used as substrate. In topoisomerase II assays, ATP was added to 1 mM and knotted P4 DNA was the substrate. Incubation was for 1 h at 37°C and the reaction was stopped by the addition of 4 ul of 2.5% SDS; 25 mM EDTA, 30% sucrose, 0.25% bromphenol blue. The reaction products were analyzed by electrophoresis on 0.75% agarose gels followed by staining with ethidium bromide.

RESULTS AND DISCUSSION: Both toposiomerase I and II activities were easily detected with standard assays in both whole cell and nuclear extracts of Neurospora (Fig. 1). Nuclear extracts contained at least as much activity as whole cell extracts suggesting that these enzymes are concentrated in the nucleus in Neurospora as in other eukaryotes. Topoisomerase I activity was detected by the appearance of bands moving more slowly than the covalently closed circular pUC molecules (Fig. 1a). The activity was the same in wild type and mutant strains, with activity becoming detectable at 0.5 ug protein/assay in nuclear extracts. The presence of ATP, which should activate topoisomerase II made little difference in the amount of activity detected. Topoisomerase II activity, which was assayed by the decrease in the fast moving smear of knotted P4 DNA and an increase in the slow moving circular form, was detectable at 1.0 ug protein/assay in wild type (Fig. lb). Activity in all three mutant strains was somewhat greater than in wild type, being present at 0.5 ug protein/assay in uvs-2 and uvs-6 and at 0.25 ug protein/assay in mus-9 (Fig. 1b, c). It appears that large changes in topoisomerase II activity are not necessary for the increase in X-ray and bleomycin sensitivity, chromosomal instability, and failure to repress DNA synthesis after X-irradiation seen in uvs-6 and mus-9, nor do small increases in activity, as seen in uvs-2 necessarily lead to these properties.

Fig. 1. DNA topoisomerase activities in nuclear extracts of Neurospora. a. DNA topoisomerase I activity in wild type measured as the unwinding of pUC19 covalently closed supercoiled DNA. Amounts of protein/assay: lane 1, 0 ug; lane 2, 0.01 ug; lane 3, 0.05 ug; lane 4, 0.1 ug; lane 5, 0.5 ug; lane 6, 1.0 ug; lane 7, 2.0 ug; lane 8, 2.0 ug and 1 mM ATP. b. DNA topoisomerase II activity in wild type and uvs-2 measured as the unknotting of knotted P4 DNA in the presence of 1 mM ATP. Amounts of protein/assay: lane 1, 0 ug; lane 2, 0.25 ug; lane 3, 0.5 ug; lane 4, 1.0 ug; lane 5 2 ug. c. DNA topoisomerase II activity in uvs-6 and mus-9 measured as in b.

This work was supported by NSF Grant DMB-8502176 to ALS - - - ^1 Program in Genetics and Cell Biology, Washington State University, Pullman, WA 99164 and ^2 Department of Biochemistry, University of Queensland, St. Lucia, Queensland, 4067, Australia.