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ISOLATION OF A MITOCHONDRIAL DNA TOPOISOMERASE FROM HUMAN LEUKEMIA CELLS

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SUMMARY. Mitochondria from human acute lymphoblastic leukemia cells contain an ATP-independent DNA topoisomerase which can relax negative and positive supercoils. This enzyme has been purified 200-fold by carboxymethyl-cellulose or double stranded DNA-cellulose chromatography. In contrast to the molecular weights reported for mitochondrial topoisomerases in other systems, the native leukemia enzyme has a molecular weight of 132,000 daltons as determined by gel permeation chromatography in buffer containing 0.4 M KCl. It also exhibits a sedimentation coefficient of 7.1 S when centrifuged through a 10-30% glycerol gradient in this high salt buffer. The enzyme is presumably a type I topoisomerase analogous to those found in rat liver and Xenopus laevis mitochondria.

DNA topoisomerases alter the topology of DNA molecules by breaking and rejoining the backbone bonds by a mechanism involving either a single strand (type I topoisomerases) or double strand (type II topoisomerases) cleavage. These enzymes catalyze relaxation, supercoiling, knotting-unknotting and catenation-decatenation of circular DNA. Topoisomerases appear to be ubiquitous, having been isolated from a variety of prokaryotic and eukaryotic sources (for recent reviews see ref. 1-3), including mitochondria (4-6). The involvement of DNA topoisomerases in mammalian mtDNA replication has been assessed indirectly by inhibitor studies (7,8). On the whole, however, our knowledge of the enzymology of mtDNA replication is not well developed.

Mammalian mtDNA exists predominantly as a monomeric, supercoiled circular molecule of approximately 16,000 bp. However, complex forms of dimer and oligomer catenanes (interlocked rings) and circular dimers (head-to-tail dimers) have been observed. In particular, the latter species occur in high

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Abbreviations: HALL, human acute lymphoblastic leukemia; PEG, polyethyleneglycol.

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proportions in neoplastic cells (9-11). Since circular dimers are topological isomers of catenated dimers, they may be somehow generated by the action of DNA topoisomerases. We have already reported the isolation of a type II topoisomerase from rat liver mitochondria that can relax, knot, catenate and decatenate circular DNA (6).

We have now begun an investigation into the occurrence of topoisomerases in mitochondria from human leukemia cells in order to understand the role played by these enzymes in the metabolism and biogenesis of mtDNA, particularly the complex catenane and circular dimer forms. In this early study we have used human acute lymphoblastic leukemia (HALL) cells obtained from patients by leukophoresis. We have detected DNA topoisomerase activity in mitochondrial extracts from these cells and have partially purified this enzyme by column chromatography. Interestingly the HALL topoisomerase appears to be much larger than analogous enzymes previously isolated from rat liver (4) and X. laevis (5).

MATERIALS AND METHODS

Peripheral blood was obtained from patients by leukophoresis and supplied by Dr. David Poplack of the Pediatrics Oncology Division of the Clinical Center at the National Institutes of Health, USA. Leukocytes were subsequently collected by ficoll centrifugation (12). These cells (5×10^8) were 95-99% blasts as judged by phase microscopy. The cells were washed twice in phosphate buffered saline, suspended in 40 mM Tris·HCl, 0.1 mM EDTA, 0.34 M sucrose, homogenized and the nuclear and mitochondrial fractions were separated by differential centrifugation. Mitochondria were collected (10,000 x g, 10 min) and washed three times. Mitochondrial protein was determined by biuret analysis (13), and the mitochondrial outer membrane was removed with digitonin (1 mg digitonin per 8 mg mitoprotein) (14). The resulting mitoplasts were pelleted washed twice, then lysed in lysing buffer (0.15 M potassium phosphate, pH 7.0/ 1 mM phenylmethyl sulfonyl fluoride/10 mM dithiothreitol/20% glycerol) containing 1 M NaCl and 1% NP40, a nonionic detergent. This lysate was treated with 6% polyethyleneglycol (PEG) and centrifuged at 10,000 x g for 10 min.

For column chromatography the PEG supernatant was dialyzed several hours against the appropriate column buffer. Carboxymethylcellulose and phosphocellulose were equilibrated in 0.1 M potassium phosphate, pH 7.0/solution A (solution A is 20% glycerol, 1 mM phenylmethylsulfonylfluoride, 10 mM dithiothreitol. Double stranded DNA cellulose was equilibrated in 0.1 M KCl/40 mM Tris·HCl, pH 7.0/solution A. Ecteola-cellulose and DEAE cellulose were equilibrated in 40 mM Tris·HCl, pH 7.0/1 mM EDTA/solution A. Hydroxyapatite was prepared in 50 mM potassium phosphate, pH 7.0/solution A. Sephadex G-100 was prepared in 8% glycerol/40 mM Tris·HCl, pH 7.5, containing 0.1 M or 0.4 M KCl.

Protein determinations of lysate or PEG-supernatant were by biuret analysis (13) using bovine serum albumin as standard, while protein determinations of column fractions were performed according to the method of Bradford (15).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (16). Markers were myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (43,000), and carbonic anhydrase (29,000).

Glycerol gradients (10-30%) were prepared in 40 mM Tris·HCl, pH 7.5 containing either 0.1 M or 0.4 M KCl. Centrifugation was for 19 hours at 40,000 rpm in an SW50.1 rotor at 4°C.

Novobiocin and Berenil were prepared as described previously (7).

Toposiomerase activity was assayed as described (6). One unit of activity was the amount of enzyme necessary to relax 50% of the supercoiled pBR322 DNA. Dilutions of enzyme samples were made with assay cocktail.

RESULTS

Detection and Isolation of Mitochondrial Topoisomerase Activity

Mitoplasts from 5 x 10^8 human acute lymphoblastic leukemia cells were prepared and lysed with a non-ionic detergent, yielding 2.4 mg mitochondrial protein. Aliquots of this mitochondrial lysate were assayed for topoisomerase activity in the absence of ATP. The results of an agarose gel electrophoresis of the reaction products of such assays are shown in Fig. 1. As can be seen, (lanes 2-5) the ability to relax negatively supercoiled pBR322 decreases as the lysate is diluted. This lysate contained approximately 10,000 units of relaxing activity (4,200 units/mg).

We have found no contamination of our mitochondrial preparations with nuclear topoisomerases adhering to the mitochondrial outer membrane, since removal of this membrane with digitonin results in no loss of topoisomerase activity. The use of the mitoplast preparation actually leads to an increase in specific activity since the mitochondrial proteins associated with the outer membrane and the intermembrane space are removed.

Treatment of the lysate with 6% PEG effected almost a three-fold increase in the specific activity (11,400 units/mg protein) as shown in Fig. 1 (lanes 6-9). The PEG removes mtDNA and some proteins but it also acts to stimulate the topoisomerase activity, perhaps by stabilizing the enzyme in aqueous solution.

The activity detected in these experiments was not increased by including 1 mM ATP. Occasionally, we have observed some stimulation in relaxing activity

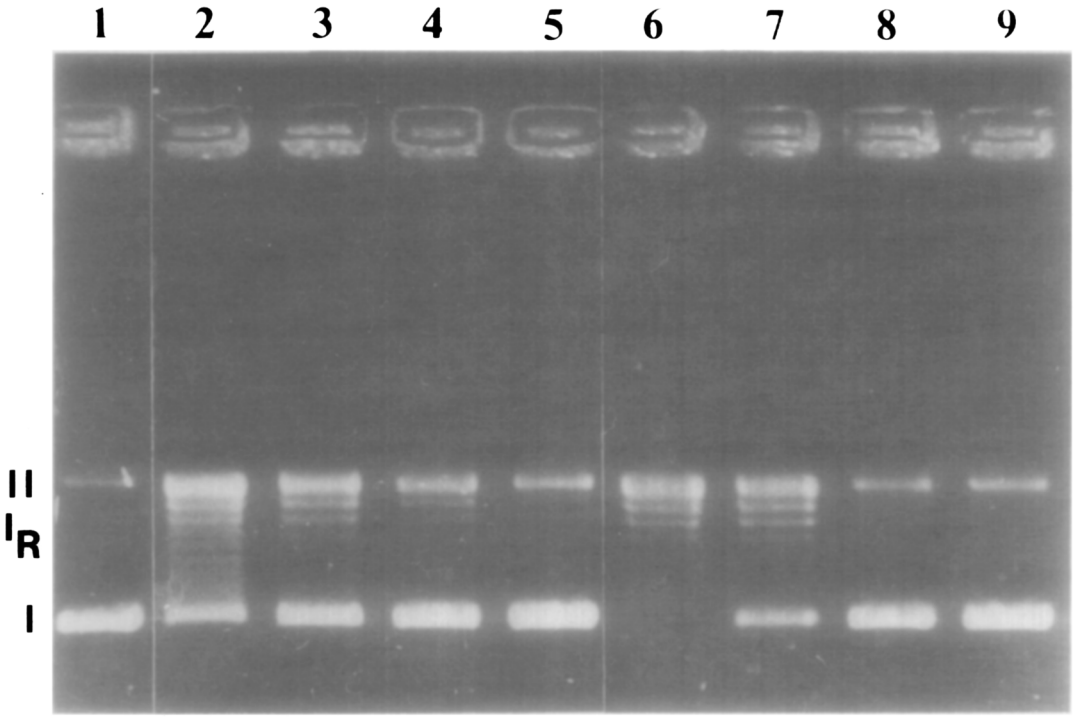

Fig. 1. Topoisomerase assays of mitochondrial lysate and PEG supernatant from human acute lymphoblastic leukemia cells. Lane 1, control pBR322, no enzyme added; lane 2→5; dilution series of 1 µl of undiluted, 1/2, 1/4, and 1/8 diluted samples from mitochondrial lysate: lane 6→9; dilution series of 1 µl of undiluted, 1/2, 1/4, 1/8 diluted sample from PEG supernatant. Form I, II and I_R are supercoiled, open circular and partially relaxed pBR322 DNA.

in the presence of ATP but this effect has been variable and not very reproducible. There are often faint bands of DNA with mobilities less than form II (open circular) pBR322 observed upon agarose gel electrophoresis. These species are presumbaly catenanes (17,18) but further characterization should unequivocally determine their identity.

The enzyme was further purified by cation-exchange chromatography on carboxymethyl-cellulose. The topoisomerase bound weakly to the column, eluting late in the 0.1 M potassium phosphate wash (Fig. 2A). This enzyme exhibited only ATP-independent relaxing activity. We attempted to stimulate any activity capable of generating catenanes by providing a condensing agent, histone H1, at 6.6 and 3.3 µg/ml. Instead of increasing catenanes, the added histone inhibited the topoisomerase relaxation reaction.

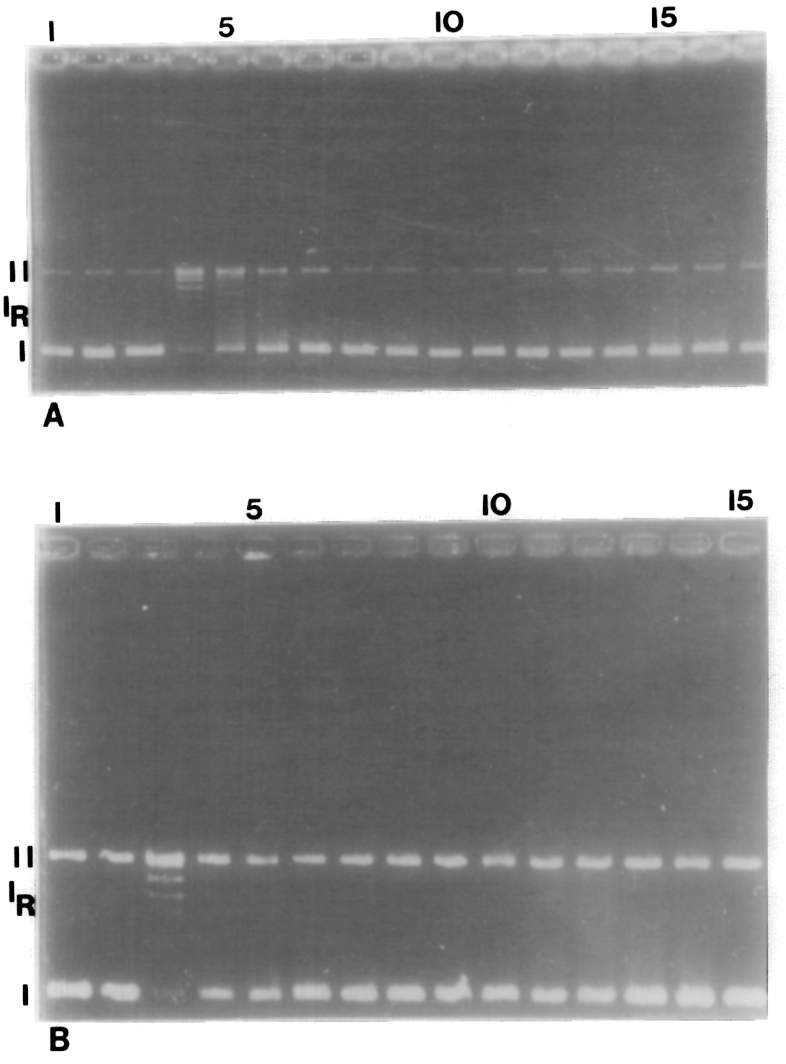

Fig. 2. Chromatographic separations of mitochondrial topoisomerase. A, carboxymethyl-cellulose chromatography of HALL mitochondrial PEG supernatant. Active fractions are in lane 4-6. Lane 1 is a control with no enzyme added. B, DNA cellulose chromatography of mitochondrial PEG supernatant. Active fractions are in lanes 3-5, eluting at 0.3 M KCl. Earlier 0.1 and 0.2 M salt step fractions are not included. Lane 1 is a control with no enzyme added. See Fig. 1 for DNA band assignments.

We have also obtained purification by affinity chromatography. Fig. 2B shows the results of a fractionation of PEG supernatant on double stranded DNA cellulose. The enzyme binds at low salt (0.1 M KCl) and, in a step gradient, elutes at 0.3 M KCl. This enzyme fraction was purified slightly more than 200-fold. Thus far, however, we have been unable to recover sufficient material from serial column chromatographic separations to fully characterize the enzyme.

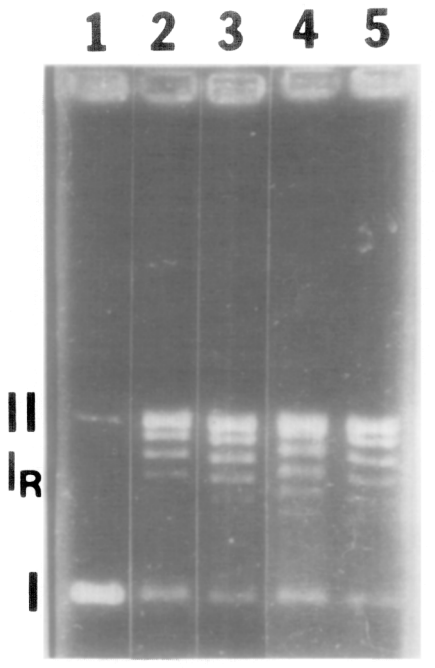

Fig. 3. Effect of inhibitors on mitochondrial topoisomerase relaxing activity. Lane 1, control pBR322, no enzyme added; lane 2-5, plus enzyme; lane 3, plus 20 μM Berenil; lane 4, plus 400 μg/ml novobiocin; lane 5, plus 400 μg/ml nalidixic acid.

Drug sensitivity of mitochondrial topoisomerase

The trypanocidal drug, Berenil, is a potent inhibitor of mtDNA replication (19) and has been shown to reduce topoisomerase activity by 50% in rat liver (4) and Xenopus laevis (5) mitochondria, at concentrations of 4 μM and 19 μM, respectively. The leukemia cell enzyme, on the other hand, is unaffected by Berenil at 20 μM (Fig. 3).

A second topoisomerase, presumably a type II enzyme, has been isolated from rat liver mitochondria (6). Since type II topoisomerases are often sensitive to bacterial DNA gyrase inhibitors, we tested the effects of novobiocin and nalidixic acid on the leukemia cell mitochondrial topoisomerase. There was essentially no inhibition of relaxing activity by 400 μg/ml novobiocin, and, at most, a 10-15% reduction in the presence of 400 μg/ml nalidixic acid.

Molecular Weight of Native and Denatured Enzyme

The leukemia cell mitochondrial topoisomerase has a native molecular weight of 132,000 daltons as determined by gel permeation chromatography on

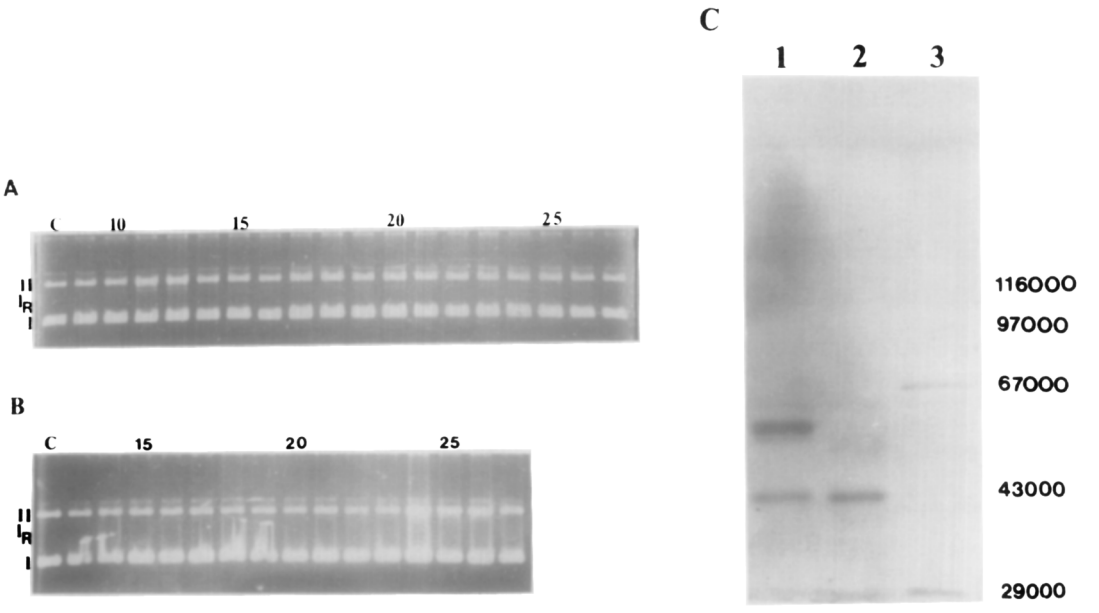

Fig. 4. Determination of molecular weight of the mitochondrial topoisomerase from leukemia cells. A, Topoisomerase profile after Sephadex G-100 chromatography in 0.4 M KCl containing buffer; relaxing activity is present in fractions 11 and 12. Lane C is a control, no enzyme added. B, Topoisomerase profile after centrifugation through 10-30% glycerol gradeints in buffer containing 0.4 M KCl. Relaxing activity is present in fractions 24 and 25. C, Sodium dodecyl sulfate-polyacrylamide gel electrohphoresis pattern of selected fractions from the glycerol gradients. Lane 1, fraction 24 from 0.4 M KCl gradient; lane 2, fraction 24 from 0.1 M KCl gradient; lane 3, molecular weight markers.

Sephadex G-100 in Tris buffer containing 0.4 M KCl. Fig. 4A shows the profile of enzyme activity eluting from the G-100 column soon after the void volume. Interestingly, identical samples chromatographed in lower salt buffer (0.1 M KCl) were devoid of any relaxing activity.

We also sedimented the enzyme through 10-30% glycerol gradients containing Tris buffer and 0.4 M KCl. In this centrifugation experiment, the mitochondrial topoisomerase was found to sediment at 7.1 S, (Fig. 4B) in good agreement with the gel chromatography results. As in the previous G-100 experiment, sedimentation through glycerol gradients containing 0.1 M KCl resulted in a complete loss of activity.

The results of denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 4C. They indicate the presence of a number of bands, many of them faint, using this partially purified enzyme sample. However, there is a major band at 60,000 daltons in the active fractions from

the glycerol gradient run in 0.4 M KCl. This band is totally absent from the corresponding fractions in the 0.1 M KCl glycerol gradient. Although preliminary, these results suggest that the mitochondrial topoisomerase may exist as a dimer of 60,000 dalton polypeptides.

Relaxation of positive and negative supercoils

We tested the ability of the leukemia cell enzyme to relax positive supercoils in pBR322. The substrate was prepared by addition of ethidium bromide to a solution of relaxed, closed circular pBR322. We found that the mitochondrial topoisomerase was able to remove all the positive supercoils as effectively as it relaxed negatively supercoiled pBR322 (data not shown).

DISCUSSION

An ATP-independent DNA topoisomerase capable of relaxing positive and negative supercoils was detected in and isolated from mitochondria from human acute leukemia cells. This enzyme was partially purified (about 200-fold) by carboxymethyl-cellulose or double stranded DNA cellulose chromatography. A more extensive purification scheme has been developed from other pilot experiments and further characterization of this mitochondrial enzyme is in progress (Castora and Lazarus, in preparation).

This topoisomerase was insensitive to up to 400 μg/ml novobiocin and nalidixic acid and 20 μM Berenil, properties distinct from known eukaryotic type II or mammalian mitochondrial type I topoisomerases, respectively.

Preliminary results using gel filtration or glycerol gradient centrifugation indicate that the native enzyme isolated from leukemia cells (132,000 daltons) is larger than analogous enzymes isolated from Xenopus laevis (5) or rat liver (4) mitochondria (65-70,000 daltons). In addition, the results of denaturing gel electrophoresis indicate that the native leukemia cell enzyme is probably a dimer of 60,000 dalton subunits.

Recently it has become evident that protease inhibition and rapidity of purification are important elements in affecting the observed molecular weight of DNA topoisomerases (20,21). Although we have worked quickly and included protease inhibitors in our buffers, it is interesting that even with frozen

leukemia cells we have been able to observe the 132,000 form of the enzyme. An important question to address in the light of these findings is whether these various molecular weight forms are simply proteolytic degradation products which still maintain topoisomerase activity or whether they are specific, processed forms which have particular functions or locations (e.g., nuclear vs mitochondrial compartmentalization). We have isolated several topoisomerases from nuclei of human leukemia cells and have begun investigations into this question (Castora, Kunes and Lazarus, in preparation).

The need for a swivel-like mechanism during replication of the mitochondrial genome has been evident for some time. In the rat liver system, inhibitor studies have provided evidence for the involvement of mitochondrial topoisomerases in this process (4,7). Indeed, both the isolated type I (4) and type II topoisomerases (unpublished observation) from rat mitochondria, as well as the Xenopus topoisomerase (5), can relax positively supertwisted DNA. The ability of the leukemia enzyme to relax positive supercoils suggests its possible involvement in the "swivel-ase" function during human mtDNA replication as well. Further studies using these purified mitochondrial topoisomerases and isolated mtDNA will hopefully shed light on this aspect of mtDNA biogenesis.

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