Volume 11 Number 12 1983 Nucleic Acids Research

Reversible decatenation of kinetoplast DNA by a DNA topoisomerase from trypanosomatids

Joseph Shlomai and Anat Zadok

The S.F. Kuvin Centre for the Study of Tropical and Infectious Diseases, The Hebrew University–Hadassah Medical School, Jerusalem 91010, Israel

Received 29 March 1983; Revised and Accepted 20 May 1983

ABSTRACT

DNA topoisomerase activity detected in cell extracts of the trypanosomatid Crithidia fasciculata interlocks kinetoplast DNA duplex minicircles into huge catenane forms resembling the natural kinetoplast DNA networks found in trypanosomes. Catenation of duplex DNA circles is reversible and equilibrium is affected by ionic strength, and by spermidine. The reaction requires magnesium, is ATP dependent and is inhibited by high concentrations of novobiocin. Extensive homology between duplex DNA rings was not required for catenane formation since DNA circles with unrelated sequences could be interlocked into mixed network forms. Covalently sealed catenated DNA circles are specifically used as substrates for decatenation. No such preference for covalently sealed duplex DNA rings was observed for catenane formation. Its catalytic properties and DNA substrate preference, suggest a potential role for this eukaryotic topoisomerase activity in the replication of kinetoplast DNA.

INTRODUCTION

Kinetoplast DNA (kDNA) is a unique DNA structure present in the single mitochondrion of flagellate protozoa of the order Trypanosomatidae. This unusual DNA structure consists of thousands of small duplex DNA rings heterogeneous in their nucleotide sequences, known as minicircles (0.8-2.5kb each, depending on the species), and a few larger DNA circles (20-40 kb) known as maxicircles. Minicircles and maxicircles are organized within the trypanosomatidial mitochondrion as a three dimensional network and it is generally believed that the circular DNA molecules are interlocked to form a catenane (reviewed in references 1-3). It was suggested by Englund and his colleagues (3-5) that during the S phase in the cell cycle, minicircles are released from the network and replicate as free minicircles through a Cairns type mechanism. The resulting progeny DNA minicircles which are nicked, reattach to the network periphery. The network increases in size until it doubles and then segregates into two daughter networks. Such a model for kDNA network replication assumes an

enzymatic system which releases minicircles from the network prior to their replication and subsequently reattaches the progeny minicircles to the DNA network (3). Enzymatic activities capable of interconverting monomeric circles and catenanes have been described in various systems including extracts of Xenopus laevis oocytes (6,7), T4-infected E. coli cells (8,9), Drosophila embryos (10), E. coli and M. luteus (11-15), HeLa cells (16) and Yeast (17) (reviewed by Gellert in reference (18). Furthermore, Marini et al. (19), have shown that T4 DNA topoisomerase as well as E. coli DNA gyrase catalyze the breakdown of kinetoplast DNA networks into individual covalently sealed minicircles, and revealed the catenane feature of the kDNA network.

Presuming the requirement for the release of replicating kDNA minicircles from the network and their subsequent post replication reattachment to it, we have searched for an enzymatic system in trypanosomatids which can carry out such "release and reattachment" functions. Considering the catenane nature of kDNA networks, and the specific requirements of kDNA minicircle replication, it was presumed that such an activity would be capable of: (i) catalyzing the interconversions of monomeric DNA circles and catenanes; (ii) functioning via a transient breakage of both strands of the mature duplex DNA rings in the network; (iii) carrying out catenation and decatenation of DNA molecules of heterogenous nucleotide sequences, and (iv) discriminating between newly replicated and unreplicated kDNA minicircles. We have used these criteria in the present study for the detection of a DNA topoisomerase activity in Crithidia cell extracts. The potential function of this activity in the process of kDNA replication is discussed.

MATERIALS AND METHODS

Nucleic Acids and Enzymes

Kinetoplast DNA was prepared from Crithida fasciculata according to Simpson and Simpson (20) and pBR322 as described by Davis et al. (21). pMB9 DNA and ϕX174 RF DNA were gifts of Dr. A. Cohen and Dr. A. Razin of this institute. Restriction endonucleases, E. coli DNA polymerase I, and T4 DNA ligase were obtained from New England BioLabs, and calf-thymus topoisomerase I from Bethesda Research Laboratories.

Cell Growth

Crithidia fasiculata cell cultures, were grown in Brain-Heart Infusion (Difco), containing 20 μg/ml Hemin (Sigma), 100 units/ml of penicillin, and

100 µg/ml of streptomycin sulfate, at 28°C with shaking. Cells were harvested at the density of 7.5-10x10^7 cells/ml.

Preparation of Crithidia Cell Extracts

Cells were harvested by centrifugation and washed twice using 50mM Tris Cl pH 7.4, containing 10 sucrose. Cell suspensions (5x10^9 cells/ml) were made 5mM in EDTA, 20mM in spermidine (Sigma), and 5 (of saturation at 0°C) with ammonium sulfate. Cells were disrupted by French Press (1250 psi, at 0-4°C), and the crude extract was centrifuged at 25000xg for two hrs at 4°C. Supernatant collected is Fraction I. This fraction was further fractionated using ammonium sulfate precipitation. Crithidia ATP-dependent topoisomerase activity was recovered in the fraction precipitated at 40-60 ammonium sulfate (Fraction II).

DEAE-Cellulose Chromatography

70 ml of Fraction II (50.2 mg/ml protein) was applied to a 200 ml DEAE-cellulose (DE-52, Whatman) column equilibrated with buffer A (50mM Tris Cl pH 7.5, 50 (v/v) glycerol, 1mM EDTA and 5mM DTT). The column was washed with two column volumes of equilibration buffer and then eluted at a concentration of 60mM KCl in this buffer (Fraction III).

Phosphocellulose Chromatography

25 ml of Fraction III (3.3 mg/ml protein) was applied to a 7.5 ml phosphocellulose (P-11, Whatman) column equilibrated with buffer A containing 60 mM KCl. The column was washed stepwise with increasing concentrations of KCl in buffer A. Activity eluted at 450-600 mM KCl is Fraction IV.

Bio-Rex-70 Chromatography

12 ml of Fraction IV (0.1 mg/ml protein) was applied to a 0.5 ml Bio-Rex-70 (Bio-Rad) column equilibrated with buffer A, containing 200 mM KCl. The column was washed stepwise with increasing concentrations of KCl in buffer A. Activity eluted at 650-750 mM KCl is Fraction V.

Assay for Catenation and Decatenation Activities

Crithidia topoisomerase activity was assayed following the catenation of monomeric duplex rings into a network, or the decatenation of kDNA networks into the monomeric minicircles. One enzyme unit is defined as the amount of enzyme required to convert 1 pmol of pBR322 DNA into a network form in 60 min at 30°C under the standard assay conditions. Assays were carried out routinely in 25 µl reaction mixtures containing: 20mM Tris·Cl pH 8.3, 15% (v/v) glycerol, 10mM $MgCl_2$, 0.5mM EDTA, 1mM DTT, 30 µg/ml BSA and 2mM ATP. In addition, catenation assays contained 20mM KCl, 5mM spermidine and 0.7 µg pBR322 DNA, and decatenation assays contained 140mM KCl and 0.7 µg

Crithida fasciculata kDNA. Reactions started by the addition of 0.37 units of enzyme, were carried out at 30°C and stopped by the addition of 6µl of stopping solution containing: 5% SDS, 50% (v/v) glycerol, and 0.05% bromophenol blue. Reaction products were analyzed in 0.8-1% agarose gels at constant voltage (1V/cm) at room temperature in 40mM Tris acetate pH 7.8, 5mM sodium acetate, and 1mM EDTA.

ATPase Assay

The assay measures the production of labeled ADP from (^{3}H) ATP. Standard assays were carried out as above, except that (2,8-^{3}H) ATP (50 Ci/mmole, Amersham) was used. Aliquots of 2µl were applied to polyetheleneimine-cellulose strips (0.6x6 cm, Brinkman) together with unlabeled ATP, ADP and AMP markers. The strips were developed with 1M formic acid, 0.5M LiCl at room temperature, dried and examined with UV light to locate and cut out the ATP, ADP and AMP spots. Radioactivity was determined in scintillation fluid without elution.

RESULTS

An enzymatic activity from Crithida fasciculata catalyzes the reversible formation of DNA networks.

During our studies on DNA replication in fractionated Crithidia cell extracts, we observed an efficient process of aggregation of the double stranded DNA rings used as DNA templates. The aggregates formed failed to enter the 1% agarose gels (Fig. 1) and resembled the natural trypanosomatidial kDNA networks in the electron microscope (Fig. 2). It was further observed that the same enzyme fraction could decatenate the catenane structure of natural kDNA networks into their covalently sealed minicircle subunits (Figs. 1,2). These observations strongly suggested that we were assaying for a DNA topoisomerase activity. The enzymatic activity was almost undetectable in crude cell extracts, however, in cell supernatants made virtually free of DNA, cell membranes and large insoluble particles (Fraction I), formation of huge DNA complexes could be detected. Ammonium sulfate fractionation (at 40-60% of saturation at 0°C, Fraction II), and the subsequent steps of chromatography on DEAE-cellulose (Fraction III), phosphocellulose (Fraction IV), and Bio-Rex-70 (Fraction V), purified the enzyme about 6,000 fold over Fraction I and allowed a linear and sensitive assay for the crithidial topoisomerase to be carried out. Further purification of the Crithidia ATP dependent DNA topoisomerase to homogeneity is now in progress.

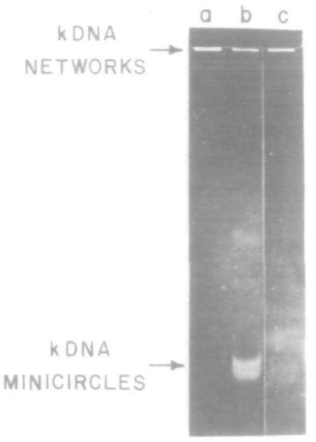

Figure 1. Catenation-decatenation activity displayed by a fraction isolated from Crithidia cell extract. 0.7 μg of kDNA (a) was incubated under standard decatenation (b) or catenation (c) assay conditions in the presence of 0.4 units of enzyme (Fraction III). Electrophoresis was in 1% agarose gel containing 1 μg/ml ethidium bromide.

Crithidia topoisomerase catalyzes the formation of heterogenous DNA networks.

Since kinetoplast DNA networks consist of kDNA minicircles of several different nucleotide sequences, one could speculate that the Crithidia catenating-decatenating activity like similar activities reported in other systems, does not express a very extensive sequence specificity. As it is demonstrated in Figure 3, apparently no extensive sequence specificity is involved in the reversible formation of DNA networks catalyzed by the crithidial enzyme, since double stranded circular DNA molecules from a variety of sources could be utilized as substrates. This included DNA from the bacterial plasmids pMB9 (data not shown), pBR322 (Figs. 3,6), bacterial virus ϕX RF DNA (Fig. 3) and crithidial DNA minicircles (Figs. 1,2). Furthermore, no homology between the duplex DNA rings involved was required. ϕX RF DNA circles could be linked to pBR322 plasmid DNA to form a heterogenous mixed DNA network (Fig. 3). An indication for the heterogenous nature of such DNA networks came from their analyses using restriction endonucleases. We have digested the DNA networks using EcoRI which cleaves at a single site pBR322 (but not ϕX RF DNA) and XhoI which has one cleavage site in ϕX DNA but none in pBR322. PstI digestion of both pBR322 and ϕX DNA at a single site was used as a marker for linearized DNA

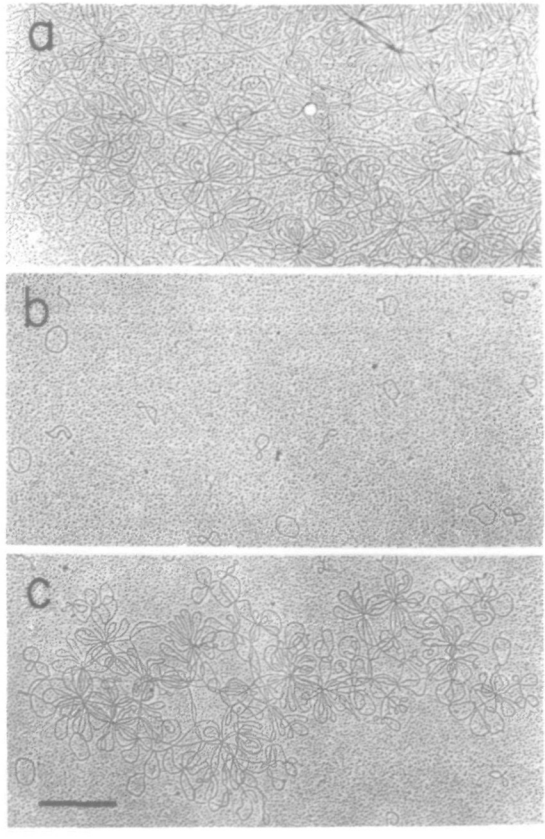

Figure 2. Electron microscopy of the products of reversible catenation of Crithidia kDNA networks by Crithidia DNA topoisomerase. kDNA networks isolated from Crithidia fasciculata ((a), represents an area in an isolated kDNA network) were treated with Crithidia DNA topoisomerase under the standard decatenation assay conditions as described in the Materials and Methods. Reactions were stopped by the addition of EDTA to 10mM, and the DNA products were phenol extracted (b). Minicircles resulting from the decatenation reaction were incubated with enzyme as above under standard catenation assay conditions to produce the product in (c). Phenol extracted DNA products of the above reactions were prepared for electron microscopy by the formamide technique (following Davis, Simon and Davidson, 1971 (22).

molecules of both species. If DNA networks were composed of both species of duplex DNA rings linked together (rather than a mixture of two types of networks, each composed of only one DNA species), the results presented in Figure 3 would be expected. A single cut introduced in each ϕX RF DNA circle within the network (by XhoI) releases not only linearized ϕX RF DNA

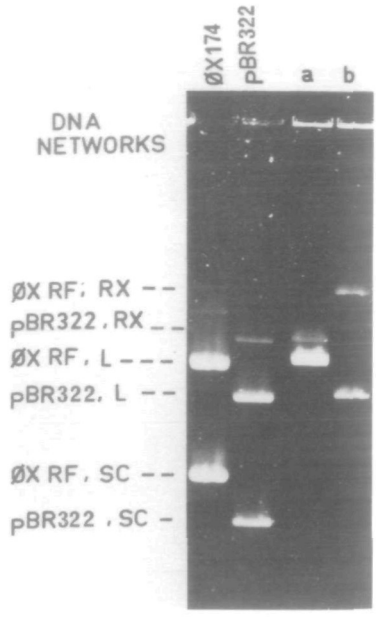

Figure 3. Restriction endonuclease analysis of heterogenous DNA networks produced by Crithidia fasciculata DNA topoisomerase. DNA networks composed of 0.5 µg pBR322 and 0.7 µg øX RF (øX174 replicative form DNA) were prepared as described in the Materials and Methods. DNA was phenol extracted from reaction mixtures and was submitted to digestion with 3 units of XhoI (a) or 3 units of EcoRI (b) for 60 min at 37°C. Reactions were stopped and submitted to gel electrophoresis as described in Materials and Methods, along with pBR322 and øX DNA markers as follows: RX, relaxed form; SC, supercoiled form; L, linear form.

molecules, but also free pBR322 circles (Fig. 3). Similarly, exposing the same DNA networks to EcoRI digestion yields linearized pBR322 DNA, as well as circular øX RF DNA molecules (Fig. 3). Subsequent exposure of such EcoRI-treated networks to XhoI, digests the remaining DNA networks and yields linearized DNA molecules of both species (data not shown).

DNA networks generated are interlocked catenanes.

The reduction in the electrophoretic mobilities of the duplex DNA circles in agarose gels (Fig. 1) and the formation of huge networks as observed by electron microscopy (Fig. 2), could not be explained by the association of proteins with DNA and the formation of nucleoprotein aggregates. Neither the electrophoretic mobility nor the network forms observed, were affected by treatment with SDS, pronase or phenol extraction prior to their analysis by electron microscopy (Fig. 2) or gel electrophoresis (Figs. 1, 3).

Having exluded the possibility of network formation through protein-DNA associations, at least two types of linkages between the duplex DNA circles in the network could be considered (15,10). The first assumes covalent linkage by one or two DNA strand exchanges, or by the formation of linear tandem array of joined DNA molecules. The second assumes topological, rather than covalent, linkage of the duplex DNA circles to form an interlocked DNA catenane.

Restriction enzyme analysis of the homogenous and heterogenous DNA networks is in support of the later model. Digestion of homogenous networks using a restriction endonuclease, which introduces a single cut in each of the DNA rings in the network, yields one unit length linear DNA molecules. Such linear DNA molecules were also produced upon digestion of heterogenous DNA networks (Fig. 3), as was described in the preceding section. Linear DNA oligomers which were expected if the two species of DNA molecules were covalently joined in a linear tandem array, were not observed. Instead, a quantitative release of free duplex DNA circles from the heterogenous networks was observed. No joined structures which would support a model of covalent linking through a single strand exchange were observed in these experiments, and the possibility of such linkage through double strand exchanges could be eliminated here because of the lack of homology of the molecules involved (discussed by Hsieh and Brutlag in reference 10). Based on these results we suggest that DNA circles within the networks formed in vitro are linked topologically, rather than covalently to yield an interlocked catenane.

Direction of the reversible catenation-decatenation reaction is affected by ionic strength and by spermidine.

The reaction catalyzed by Crithidia topoisomerase activity is affected by the ionic strength in the same fashion as most other known topoisomerases (7,10,14-17). Varying potassium chloride concentrations at the optimal range of 10-40mM have little or no effect on the rate of catenane formation. However, a decrease of 50% in the rates of catenation was observed in the presence of 75mM of the salt. A narrow range of optimal potassium chloride concentrations for decatenation was observed at around 140mM. A decrease of 50% in the rates of decatenation was measured at both 55 and 160mM of the salt (Fig. 4).

Catenane formation catalyzed by Fractions I, II, and III of the enzyme preparation was not enhanced in the presence of spermidine, at the range of 0.5-15mM. However, upon further purification, it was found that the

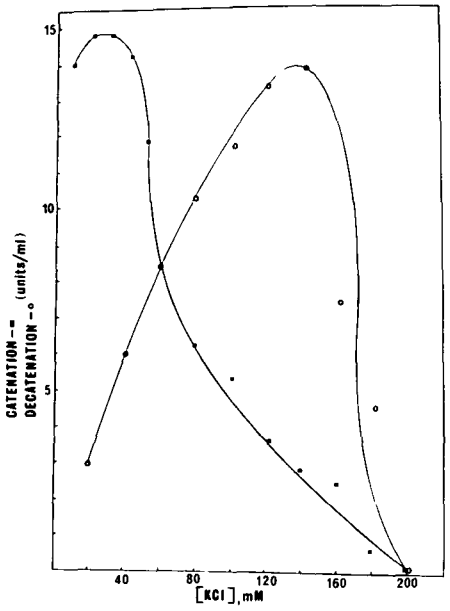

Figure 4. Effect of the ionic strength on the direction of the catenation-decatenation reaction catalyzed by Crithidia DNA topoisomerase. Catenation of 0.7 µg pBR322 DNA and decatenation of 0.7 µg kDNA networks were carried out as described in the Materials and Methods, except that KCl concentration was varied as indicated. Rates of the reactions were measured using microdensitometry as described in the legend to table I.

catenation activity becomes absolutely dependent upon the presence of the polyamine (at optimal levels of 5-10 mM). A DNA binding protein which is eluted from the phosphocellulose column at lower salt concentration (at 350 mM KCl) can substitute for the polyamine (data not shown). It is suggested that the spermidine dependence observed in more purified enzyme preparations is the result of the removal of this DNA binding protein, and thus the requirement for its substitution by an alternative aggregating factor (10,23,17).

Requirements of the reaction and its inhibition.

Magnesium ions are required for the reactions catalyzed by Crithidia DNA topoisomerase with an optimum at around 10mM. pH optimum in Tris and Hepes buffers is at around 8.3. The reactions are inhibited (>95%) by 50mM potassium phosphate and by 10mM N-ethyl maleimide (10 min, 30°C).

Reversible decatenation reaction is ATP-dependent and is inhibited by novobiocin.

Reversible decatenation by Crithidia DNA topoisomerase activity is ATP

Table 1. Nucleotide specificity of Crithidia fasciculata topoisomerase

Nucleotide	Relative rates of decatenation (%)
ATP	(100)
CTP	< 5
GTP	< 5
UTP	< 5
dATP	48
AMP-PCP	< 5

Decatenation reaction was carried out as described in the Materials and Methods, except that ATP was substituted in the reaction mixtures with 2mM of each of CTP, GTP, UTP, dATP (Sigma) and β,γ-methelene diphosphonate (Boehringer). Decatenation was measured by microdensitometry of negative photographs of the DNA bands fluorescence after ethidium bromide staining. Rates of decatenation in the presence of 2mM ATP was used as 100%.

dependent. None of the other common ribonucleoside triphosphates assayed (up to 2mM) could support the reaction. Deoxy-ATP could substitute for ATP, but was much less efficient (Table I.) Quantitative hydrolysis of ATP was observed, with the conversion of 50% of the ATP substrate into ADP within 30 min in a standard reaction mixture (Materials and Methods). Although it is not yet clear if ATP hydrolysis has any role in the reversible decatenation reaction, the lack of activity with the nonhydrolyzable β,γ-methelene analog of ATP (Table I) suggests that hydrolysis of ATP is required.

As has been reported for most other ATP dependent topoisomerases (24-26,7,10), our results show that the crithidial DNA topoisomerase activity is inhibited by the antibiotic drug novoviocin (Fig. 5). A concentration of 70 μg/ml of the drug reduces the rates of decatenation (Fig. 5) or catenation (data not shown) by 50% in the presence of 0.5mM ATP. Under these conditions the rate of the reaction decreases by only 15% when the ATP concentration is raised to 2mM. These observations are similar to those reported for several other eukaryotic ATP-dependent DNA topoisomerases, and suggest competition between the inhibitory drug and ATP for the same site in the enzyme molecule.

Crithidia topoisomerase specifically releases covalently sealed minicircles from kDNA networks.

It has been reported that newly replicated kDNA minicircles, either free or reattached to the network, are nicked, while unreplicated ones are cova-

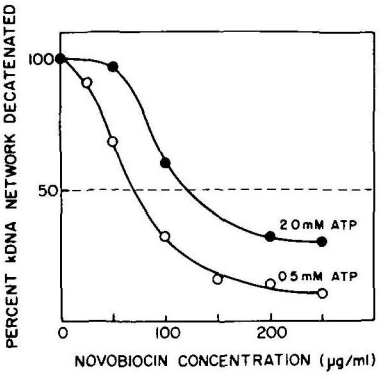

Figure 5. Inhibition of Crithidia fasciculata ATP dependent topoisomerase activity by novobiocin. Decatenation of 0.7 μg samples of kDNA network was carried out in the presence of 0.5mM or 2mM ATP and increasing concentrations of novobiocin (Sigma) as described in Materials and Methods. Decatenation was detected and quantitated using microdensitometry as described in the legend to Table I. Values represent rates of decatenation relative to the rate measured in the absence of the drug.

lently sealed (27-30,4,5). It was suggested that nicking could provide the signal for discrimination between replicated and unreplicated minicircles to insure replication of each minicircle only once in every generation

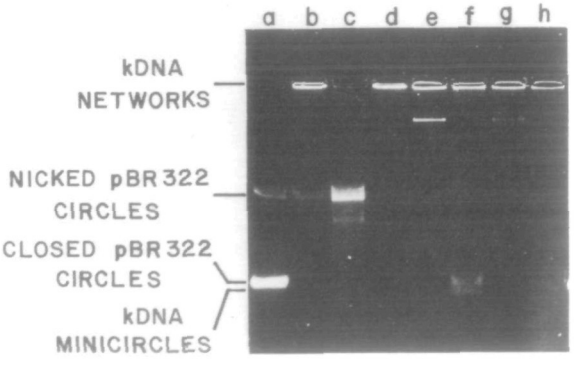

Figure 6. Reversible catenation of nicked DNA substrate by Crithidia topoisomerase. In a-d, samples of 0.7 μg of pBR322 DNA either untreated (a) or nicked (using Crithidia nicking activity) (c), were incubated with Crithidia topoisomerase under catenation assay conditions (b and d, respectively). In e-h, 0.7 μg samples of kDNA networks either untreated (e) or nicked (g) were incubated with Crithidia topoisomerase (as above) under decatenation assay conditions (f and h, respectively). Products of the reactions were electrophoresed on a 1% agarose gel containing 1 μg/ml ethidium bromide as described in Materials and Methods.

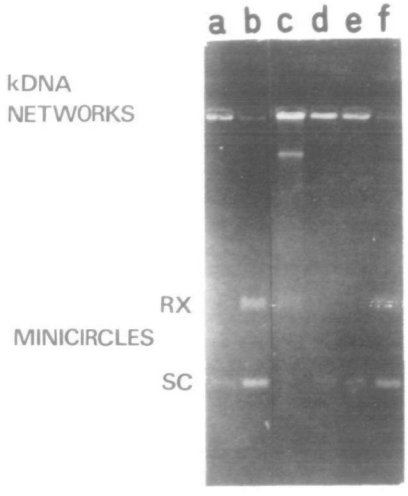

Figure 7. Decatenation of covalently sealed relaxed kDNA networks by Crithidia topoisomerase. 0.7 µg samples of kDNA networks which were untreated (a), nicked (as in Fig. 5) (c) or relaxed using 2 units of calf thymus type I DNA topoisomerase (e), were incubated with Crithidia DNA topoisomerase (0.4 units, Fraction V), under decatenation assay conditions (b, d, and f respectively). Products of the reactions were electrophoresed on a 1% agarose gel containing 1 µg/ml ethidium bromide as described in the Materials and Methods.

(3). Therefore, we have studied the effect of the existence of nicks in the DNA substrate on the reversible catenation activity catalyzed by Crithida fasciculata topoisomerase. Nicking of free DNA circles or DNA networks was carried out using a nicking activity, which was partially purified from Crithida fasciculata cell extracts. The scissions introduced in the DNA molecucules using this nuclease activity were characterized as nicks which could be sealed by the action of T4 DNA ligase. (Zadok and Shlomai, unpublished data). Figure 6 shows the results of an experiment in which pBR322 DNA circles were nicked prior to their use as substrates for catenation. It was found that nicked DNA circles were utilized as substrates for catenation. However, when nicked kDNA networks were compared with covalently sealed networks as substrates in a decatenation reaction, it was found (Fig. 6) that while covalently sealed kDNA networks were efficiently decatenated, no significant decatenation could be measured after prolonged incubations when nicked kDNA networks were used. kDNA networks which were nicked-closed by calf thymus DNA topoisomerase I, were found to be as suitable substrates for decatenation by Crithidia topoiso-

merase as are the untreated networks (Figs. 6,7). It seems therefore, that the inefficiency of interrupted DNA rings as substrates for decatenation does not result from their relaxation per se, and it is probably due to the existence of interruptions in the DNA ring.

DISCUSSION

The Crithida fasciculata topoisomerase activity described here, resembles the eukaryotic type II DNA topoisomerases and T4 DNA topoisomerase in several of its properties. Its activity is strictly dependent upon ATP, and it is inhibited, (unlike the yeast type II DNA topoisomerase) by novobiocin. It catalyzes the catenation and decatenation of covalently sealed duplex DNA rings and the relaxation of negatively supercoiled DNA. However, as was likewise reported for the other eukaryotic DNA topoisomerases and that of T4, it failed to catalyze the supercoiling of relaxed DNA circles under the assay conditions employed. As was also reported for the catenation reaction catalyzed by other eukaryotic topoisomerases (10,17), catenane formation by the Crithidia enzyme is dependent upon the presence of a DNA aggregating protein in the reaction mixture. This double-stranded DNA binding protein, whose purification from Crithidia cell extracts is now in progress, could be substituted for by the polyamine spermidine as an alternative aggregating factor.

The DNA topoisomerase described here is located within a cell which contains a most complex topological structure - the kinetoplast DNA network. However, it is not yet known if this enzyme is associated with the cell mitochondrion. Replication of the kinetoplast DNA minicircles as free individual DNA rings (31-33, 3-5) implies the requirement for an enzymatic activity which catalyzes their release from the network prior to replication and their subsequent reattachment once replication is terminated. It is suggested that the DNA topoisomerase described here could potentially fit the role for the "release and reattachment" enzyme in the course of kDNA replication.

(A) In accord with the catenane nature of kDNA networks, the enzyme catalyzes the interconversion of monomeric DNA circles and catenanes (Figs. 1,2)

(B) Its lack of nucleotide sequence specificty (Fig. 3) likewise shared by other known DNA topoisomerases (18), could ensure the random decatenation and catenation of minicircles of various nucleotide sequences, thereby conserving the heterogeneity of kDNA networks.

(C) The crithidial topoisomerase activity described here, could utilize covalently sealed DNA circles as substrates for both catenation and decatenation (Figs. 3,6,7). It is implied therefore, although not directly shown, that it can operate via a mechanism in which transient breakage of both strands of the DNA helix is involved. Breakage of both DNA strands would be required for the release of mature kDNA minicircles from the kDNA network prior to their replication.

(D) Our results suggest that the activity described here has the specificity to strictly discriminate between newly replicated DNA minicircles as substrates for catenation, and nonreplicated ones as substrates for decatention. Figure 6 shows that this topoisomerase activity efficiently catenates nicked duplex DNA rings and decatenates covalently sealed minicircles (interlocked into kDNA networks). However, it fails to release nicked minicircles from the catenane. It has been reported that E. coli and M. luteus DNA gyrases as well as T4 DNA topoisomerase unlink nicked catenated or knotted DNA ineffeciently in comparison to the unknotting and decatenation of the covalently sealed forms (9,13). Preference for covalently sealed DNA rings might bear a special significance in the system described here, by confering the capability of the crithidial topoisomerase to distinguish between replicated and unreplicated kDNA minicircles. Since newly replicated DNA minicirles, either free or reattached to the network, were found to contain nicks (27-30), it has been previously suggested (3) that nicking might provide the signal for discriminating between replicated and unreplicated minicircles. The mechanism by which this nicking is carried out is not yet clear. It has been previously suggested that the nicks either remained in the replicated minicircles due to inefficient ligation, or were introduced after replication was terminated, by a special nicking enzyme (3). Our results are in accord with the notion that nicking of minicircles might play an important role in the regulation of kDNA replication, by insuring that each minicircle is decatenated and thereby replicated only once in every generation.

The observation that free covalently sealed DNA circles could also be utilized in vitro as substrates for catenation is not necessarily inconsistent with the "release and reattachment" model postulated by Englund (3-5). The specificity of the enzymatic system, as observed in the in vitro reaction, may be based upon the abundance of nicked and covalently sealed minicircles under physiological conditions. As has been reported, free replicated minicircles are nicked. It is also reasonable to assume that the replication of minicircles is either coupled to their

decatenation or is taking place shortly after their release. Therefore, free covalently sealed minicircles are not expected to be largely available for catenation. In support of this argument is the observation (5) that free minicircle populations consist mostly of nicked duplex DNA rings.

The interconversion of topological isomers of kDNA described here is ATP dependent (Table I), and is inhibited by novobiocin as was likewise found for most other known eukaryotic type II DNA topoisomerases (Fig. 5) (7,10,16). So far, there is no direct indication for the involvement of eukaryotic DNA topoisomerases in DNA replication. The inhibition of DNA replication in some mammalian cell systems (34,35) by novobiocin could be explained by the effect of the drug on the cellular polymerases (36) and does not necessarily indicate the involvement of topoisomerases in chromosome replication. We believe that the data presented here provide some indications linking the activity of a eukaryotic DNA topoisomerase to the process of replication of an extrachromosomal DNA unit in a eukaryotic cell.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dianne Casuto for her excellent assistance during various stages of this work and Dr. A. Friedman from the Department of Genetics, Hebrew University for his help and advice with the E.M. studies. This work was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, and by the Fund for Basic Research Administrated by the Israel Academy of Sciences and Humanities.

REFERENCES

1. Simpson, L. (1972) Int. Rev. Cytol. 32, 139-207.
2. Borst, P., and Hoeijmaker, J.H.J. (1979) Plasmid 2, 20-40.
3. Englund, P.T. (1980) in Biochemistry and Physiology of Protozoa, eds. Levandowsky, M., and Hunter, S.H. (Academic Press, New York, N.Y.) 2nd edition, vol. 4, pp. 334-383.
4. Englund, P.T. (1978) Cell 14, 157-168.
5. Englund, P.T. (1979) J. Biol. Chem. 254, 4895-4900.
6. Gandini Attardi, D., Martini, G., Mattocia, E., and Tocchini-Valentini, G.P. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 554-558.
7. Baldi, M.I., Benedetti, P., Mattocia, E., and Tocchini-Valentini, G.P. (1980) Cell 20, 461-467.
8. Liu, L.F., Liu, C.-C, and Alberts, B.M. (1979) Nature 281, 456-461.
9. Liu, L.F., Liu, C.-C, and Alberts, B.M. (1980) Cell 19, 697-707.
10. Hsieh, T. and Brutlag, D. (1980) Cell 21, 115-125.
11. Brown, P.O., and Cozzarelli, N.R. (1979) Science 206, 1081-1083.
12. Cozzarelli, N.R. (1980) Science 207, 953-960.
13. Mizuuchi, K., Fisher, L.M., O'Dea, M.H., and Gellert, M (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1847-1851.
14. Kreuzer, K.N., and Cozzarelli, N.R. (1980) Cell 20, 245-254.

15. Tse, Y., and Wang, J.C. (1980) Cell 22, 269-276.
16. Miller, K.G., Liu, L.F., and Englund, P.T. (1981) J. Biol. Chem. 256, 9334-9339.
17. Goto, T., and Wang, J.C. (1982) J. Biol. Chem. 257, 5866-5872.
18. Gellert, M. (21982) Ann. Rev. Biochem. 50, 879-910.
19. Marini, J.C., Miller, K.G., and Englund, P.T. (1980) J. Biol. Chem. 255, 4976-4979.
20. Simpson, A.M. and Simpson, L. (1974) J. Protozool. 21, 774-781.
21. Davis, R.W., Botstein, D., and Roth, J.R. (1980) Advances Bacterial Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 116-117.
22. Davis, R.W., Simon, M., and Davidson, N. (1971) Methods in Enzymology 21D, L. Grossman and K. Moldave, eds. (Academic Press, New York, N.Y.) pp. 413-428.
23. Krasnow, M., and Cozzarelli, N.R. (1982) J. Biol. Chem. 257, 2687-2694.
24. Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4474-4478.
25. Mizuuchi, K., O'Dea, M.H., and Gellert, M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5960-5963.
26. Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L., and Cozzarelli, N.R. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4838-4842.
27. Simpson, L., Simpson, A.M., and Wesley, R.D. (1974) Biochem. Biophys. Acta 349, 161-172.
28. Simpson, A.M., and Simpson, L. (1976) J. Protozool. 23, 583-587.
29. Benard, J., Riou, G., and saucier, J.-M. (1979) Nucleic Acids Res. 6, 1941-1952.
30. Englund, P.T., DiMaio, D.C., and Price, S.S. (1977) J. Biol. Chem. 252, 6208-6216.
31. Brock, C., Delain, E., and Riou, G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1642-1646.
32. Wesley, R.D., and Simpson, L. (1973) Biochem. Biophys. Acta 319, 237-253.
33. Manning, J.E., and Walstenholme, D.R. (1976) J. Cell. Biol. 70, 406-418.
34. Mattern, M.R., and Painter, R.B. (1979) Biochem. Biophys. Acta 563, 306-312.
35. D'Halluin, J.-C., Milleville, M., and Boulanger, P. (1980) Nucleic Acids Res. 8, 1625-1641.
36. Edenberg, H.J. (1980) Nature 286, 529-531.