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Investigation of the Substrate Recognition Characteristics and Kinetics of Mammalian Mitochondrial DNA Topoisomerase I

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INVESTIGATION OF THE SUBSTRATE RECOGNITION CHARACTERISTICS AND KINETICS OF MAMMALIAN MITOCHONDRIAL DNA TOPOISOMERASE I

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ABSTRACT

Topoisomerases are DNA-modifying enzymes found in prokaryotes, eukaryotes, viruses and organelles such as chloroplast and mitochondria. Information about these enzymes in eukaryotic systems is mostly limited to nuclear enzymes, although our laboratory has been characterizing the biochemical and biophysical properties of the mammalian mitochondrial topoisomerases. We have determined the polarity of the attachment of mitochondrial topoisomerase I to its substrate DNA. To study the substrate preference and kinetic parameters of mitochondrial topoisomerase I, selected regions of mammalian mitochondrial DNA (mtDNA) were inserted into pGEM plasmid vectors following a series of modification and optimization experiments of currently available methods for PCR-cloning. These mtDNA containing recombinant plasmids were used in a kinetic analysis of the highly purified enzyme. Recombinant plasmids containing the bovine mtDNA heavy and light strand origins of replication (pZT-Hori and pZT-Lori, respectively), a major transcription termination region (pZT-Term) and a portion of

cytochrome b gene (pZT-Cytb) were prepared. Two other recombinant plasmids, containing non-mitochondrial DNA inserts (pZT-800 and pZT-400) served as control substrates. Southern hybridization using probes specific for either control or mtDNA-containing plasmids indicated a relative preference by the mitochondrial topoisomerase I to relax supercoils in pZT-Hori and pZT-Term. Quantitative determination of kinetic parameters derived from double-reciprocal Lineweaver-Burk plots showed that recombinant plasmids containing the heavy and light strand origins and the transcription termination region were preferentially relaxed by the mitochondrial enzyme with K_m values 2.3 to 3.3- fold lower than controls. The K_m values for pZT-Hori, pZT-Lori and pZT-Term were 21.0 ± 0.9 μM, 25.2 ± 1.0 μM and 17.0 ± 0.8 μM, respectively, while those for control plasmids were 57.5 ± 2.1 μM and 56.3 ± 2.3 μM. pZT-Cytb was not preferentially relaxed compared to the control plasmid (K_m = 53.4 ± 2.0 μM vs. 56.3 ± 2.3 μM, respectively) indicating that mitochondrial topoisomerase I preferentially interacts with certain mtDNA sequences but not others. Identical experiments with the purified nuclear enzyme did not differentiate between control or mtDNA containing plasmids.

DEDICATION

This work is dedicated to my parents,

Kadiriye and **Remzi Topcu**

for their encouragement and support.

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1.1 BACKGROUND AND SIGNIFICANCE

1.1.1 DNA topology and topoisomerases

1.1.1.1 Description of DNA topology

A circular double-stranded DNA molecule can exist in a number of isomeric forms that differ in the number of times one strand is linked with the other, a parameter termed linking number (Lk) (1-3). Linking number is related to two geometrical properties of the molecule; the twist (Tw), rotation of the strand around the helical axis, and the writhe (Wr), the measure of the path of the helix axis in space. These properties are related by Lk=Tw+Wr (3, 4). A DNA molecule that is completely relaxed has no writhe and makes a full helical turn every 10.5 bp under normal physiological conditions. The topological state of a DNA molecule can be changed only as a result of breaking and resealing the chain (5, 6). Reducing the Lk of a double-stranded ring induces twisting and coiling of the double helix itself. In this form, the ring is said to be supercoiled. The sense of supercoiling can, in principle, be either the same or opposite to that of the helical twist, conventionally designated as negative and positive supercoiling, respectively (7, 8).

Linking number is an inherent property of plasmids, bacterial chromosomes, mitochondrial and chloroplast DNA, and many viral genomes as their DNA molecules occur as closed-circular DNA (9, 10). However, the concept of topology is not limited to circular DNA molecules, it also applies to linear DNA if the ends are prevented from free rotation. Eukaryotic chromosomes and other DNAs such as some yeast plasmids, although consisting of linear DNA, appear to be anchored to a nuclear matrix (scaffolding) at a number of sites and the domains between such attachment sites behave in a topological sense as closed-circular loops (10).

A variety of methods has been used to study DNA supercoiling and relaxation including electrophoresis in agarose gels, ethidium fluorescence, sedimentation analysis and electron microscopy (11, 12).

1.1.1.2 Biological consequences of DNA supercoiling and topoisomerases

DNA supercoiling has many influences on how it functions (7, 13-15). The first and most quoted consequence of DNA supercoiling is that it allows its compaction into a very small volume (16). More important, perhaps, is that DNA supercoiling has a direct influence on many DNA-associated processes *in vivo*, which, for the most part, involve the interaction of specific proteins with DNA (10). The binding of proteins to DNA is often supercoil dependent. Negatively-supercoiled DNA is in a high energy conformation compared to unconstrained DNA (4). The excess energy may be relieved by protein binding. The excess free energy associated with supercoiling also influences the formation of Z-DNA, cruciforms, and H-DNA structures (10, 17). The helical structure of DNA and its closed-circular nature cause a number of complications in processes such as replication (18) and transcription (19) which require specific mechanisms for their resolution. Moreover, the activities of a number of DNA-specific proteins involve synapses (e.g., certain transcriptional activators and repressors, site-specific recombination proteins). Supercoiling provides a way of bridging two distant sites on DNA together (20-22).

Topoisomerases have probably evolved to solve the conformational changes in DNA (3, 5, 12, 23-26). DNA topoisomerases introduce or remove DNA superhelical tensions, tie or untie DNA knots and catenate and decatenate circular DNA molecules by forming enzyme-bridged strand breaks that act as transient gates for the passage of other DNA strands (6, 7, 23, 27, 28). The reactions catalyzed by DNA topoisomerases are given

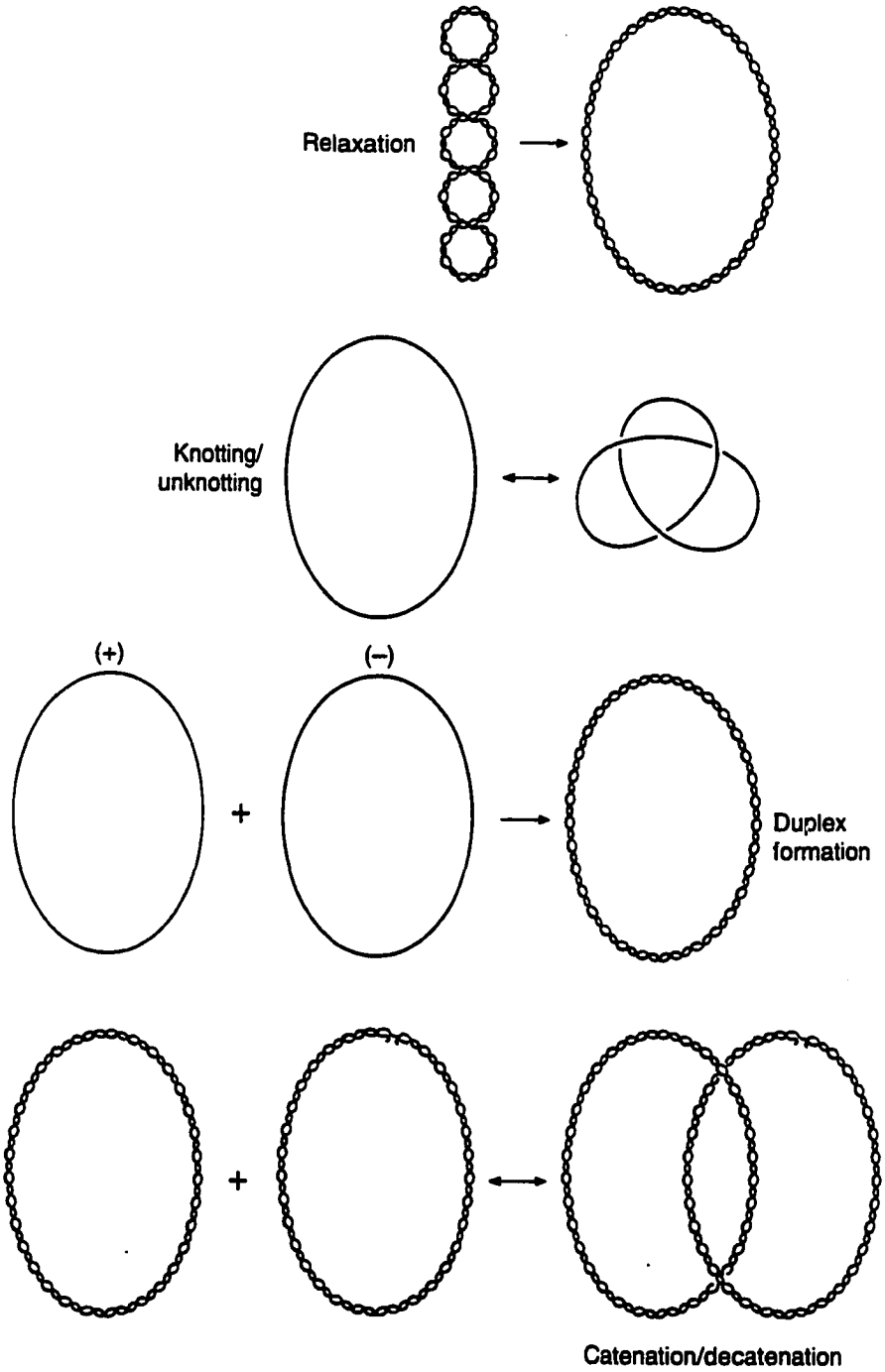
in Fig 1.

DNA topoisomerase activity was first observed by James Wang in *E. coli* in 1969 (29). In relatively short order other topoisomerases were isolated from a variety of prokaryotic and eukaryotic sources and virus-infected cells as well as from organelles such as chloroplasts and mitochondria (30-37). Their essential roles in DNA replication (18, 38), transcription (19, 39), recombination (40), and transposition (41, 42) have been amply demonstrated. The ascendancy of the DNA topoisomerases in nature can be viewed as a consequence of the selection of double-stranded DNA as genetic material (25). During the long history of evolution, DNA topoisomerases, through their manipulation of DNA topology, have been assigned additional roles in the optimization of the intracellular state of DNA. Plasmids and chromosomes, isolated from topoisomerase-mutants have been shown to contain altered levels of supercoiling (10, 25). On the other hand, the topoisomerase genes themselves are affected by supercoiling which represents a homeostatic mechanism of the enzyme (10). Over the past years, there has been a considerable interest in these enzymes as targets of certain classes of anti-cancer drugs (24, 43-46).

1.1.1.3 Classification of DNA topoisomerases

Some DNA transformations can be accomplished by cleavage of a single strand while others such as catenation of two closed circular duplexes require cleavage of both strands. This gives rise to the basis of classification of topoisomerases; type I topoisomerases act by making a transient break in one strand of DNA substrate while type II topoisomerases introduce transient double strand breaks (3,5, 47). A consequence

Fig 1. The reactions catalyzed by DNA topoisomerases (Reproduced from "Bates and Maxwell, 1993 (10).



of topoisomerase reactions via either single- or double- stranded breaks in DNA is that the former will alter the linking number in increments of one while the latter must proceed with linking number increments in multiplies of two (5, 8, 47).

Four topoisomerase have been identified from *E. coli*. Topoisomerase I (97 kD), the *top*A gene product (48) and topoisomerase III (74 kD), the *top*B gene product (49) are type I topoisomerases. DNA gyrase, the product of *gyr*A and *gyr*B genes (50) and topoisomerase IV (67 and 81 kD), the product of *par*C and *par*E genes (51) are type II topoisomerases. Both topoisomerase I and III are active as monomers. Their eukaryotic counterpart, nuclear topoisomerase I is also a monomer with a molecular mass of 100 kD (34, 52). DNA gyrase is a tetramer and is composed of four subunits (A_2B_2; Mr=107 kD). Eukaryotic topoisomerase II is a 170 kD multimeric protein. Recently, a distinct topoisomerase related to the eukaryotic enzyme has been identified from thermophilic bacteria, *Methanopyrus kandleri* (18, 53). The classification of DNA topoisomerases and general properties of individual enzymes are given in Table 1.

Type II topoisomerases, in general, are structurally and evolutionary related as evidenced from amino acid sequences in both prokaryotic and eukaryotic systems (8, 54). In contrast, bacterial and eukaryotic topoisomerases are distinct (5, 6).

1.1.1.4 Catalytic properties of type I DNA topoisomerases

All topoisomerases can relax negatively-supercoiled DNA; The ability to relax positively supercoiled DNA is less general (28, 54, 55). Besides the relaxing negative supercoils, the type I topoisomerases can also promote the following three kinds of topological interconversions of DNA *in vitro*; a) Intertwining and complete renaturation

Table 1
Properties of selected topoisomerases

Enzyme	Type	Source	Gene	Subunit MW(kDa)	Structure	DNA end	Protein-bound	Relaxation of Supercoils? (-)	Relaxation of Supercoils? Supercoiling	(+)
Procaryotic topoisomerase I (ω protein)	I	Bacteria (e.g., *E coli*)	*top*A	~97	Monomer	5'		No	Yes	No
Int	I	Phage λ	*int*	40	?	3'		Yes	Yes	No
Resolvase	I	Transposons λδ and Tn[3]	*tnp*R	21	?	5'		?	Yes	No
Nuclear topoisomerase I	I	Yeast, human	*MAK*1	90	Monomer	3'		Yes	Yes	No
		Rat liver HeLa cells, etc.	?	~100[a]						
Mitochondrial topoisomerse I	I	Bovine liver, calf thymus, HALL, platelets	?	78	?	3'[c]		Yes	Yes	No
Chloroplast topoisomerase I	I	Pea chloroplast		69	?	3'		Yes	Yes	No
Vaccina virus topoisomerase I	I	Vaccina virus	?	37	Monomer	3'		Yes	Yes	No
DNA gyrase (procaryotic Topoisomerase II)	II	Bacteria (e.g. *E.* coli)	*gyr*A *gyr*B	100	A_2B_2	5'		No	Yes	Yes
E. coli topoisomerase II'	II	*E. coli*	*gyrA* *gyr*B	90 50[e]	A_2B_2'	5'		Yes	Yes	No
T4 topoisomerase	II	Phage T4	Gene 39 Gene 52 Gene60	57 48 18	?	5'		Yes	Yes	No
Eukaryotic topoisomerase II	II	Yeast	*TOP*2	150	Dimer	5'		Yes	Yes	No
		Drosophila HeLa cells, etc.	?	~170[a]						

[a] Also active as proteolytic fragments.
[b] ATP may stimulate or inhibit DNA relaxation by some eukaryotic type I enzymes.
[c] Preliminary observations.
[d] Required for supercoiling reaction only.
[e] Proteolytic fragment (B') of gyrase B protein.
[f] ATP stimulates topoisomerase activity.

of two single-stranded, complementary DNA circles (52, 55, 56), b) Prokaryotic enzymes can catalyze the knotting of single-stranded circles (57), c) In the presence of a DNA aggregating agent, such as spermidine, type I enzymes can catenate duplex circular DNA molecules (58, 59). In a related reaction, the prokaryotic enzyme can knot a nicked or gapped duplex circle (55). Knotting by nuclear type I topoisomerase has not been reported.

If strand passage occurs between two different molecules of DNA (e.g., two plasmids), then the level of catenation of the two molecules is changed; that is they become more or less interlinked. When a topoisomerase acts upon two different segments of a single topological closed DNA molecule (e.g., a plasmid), passing one strand through the other, then the level of supercoiling is changed, because the number of times the strands cross each other has changed. Thus similar reactions can have very different results. Preference for a specific type of reaction (relaxing supercoils vs. decatenation) is presumably indicative of a substrate preference (one vs. two molecules) (10).

Prokaryotic topoisomerase I has a preference for binding single stranded DNA. (55). Positively-supercoiled DNA is not normally a substrate for relaxation by *E. coli* topo I, presumably due to its lack of single-stranded character. However, if a positively supercoiled DNA molecule, containing a single-stranded heterologus loop is constructed, it can be relaxed, indicating that the single stranded region allows the enzyme to bind. (60). The only enzyme so far shown to be able to introduce negative supercoils in DNA is the bacterial type II topoisomerase, DNA gyrase (32). The limitations on the activities of bacterial topoisomerase I is consistent with its *in vivo* role of preventing excessive supercoiling, introduced by DNA gyrase (23, 25). The reactions of type I topoisomerases are energy-independent (10, 23). Reverse gyrase from eubacteria is the only type I topoisomerase with a requirement for ATP (61). This enzyme is involved in the regulation

of supercoiling levels and stabilizing DNA against melting.

Prokaryotic type I topoisomerases require a divalent cation for their activity (23). These enzymes cause a gradual reduction in the number of superhelical turns in all molecules of a negatively-supercoiled DNA substrate population until a limit product is reached in which each DNA retains a significant number of superhelical turns (55). By comparison, the most closely analogues nuclear topoisomerase I catalyzes the complete relaxation of a fraction of the population of supercoiled DNA molecules soon after initiating the reaction (62). Nuclear topoisomerase I interacts primarily with duplex DNA and acts preferentially on curved DNA which implies that the enzyme senses Wr rather than Tw (52, 55). Since Wr is relatively independent of the sign of supercoiling, nuclear enzyme relaxes both positive and negative supercoils in DNA (34). In contrast, prokaryotic enzyme senses Tw due to a preference for single stranded DNA (12, 24, 60). The free energy associated with high negative supercoiling destabilizes the helix sufficiently to facilitate the formation of a single-stranded region when the *E. coli* topoisomerase I binds to the DNA (55).

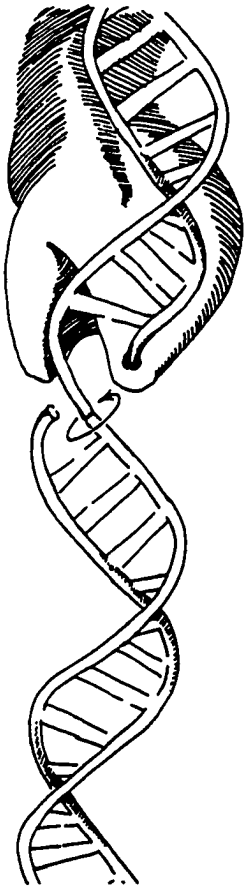
1.1.1.5 Proposed mechanisms of topoisomerase reactions

Topoisomerases represent a heterogeneous class of enzymes but nevertheless share the basic chemistry of DNA breakage and reunion. Considering the relaxation reactions in the light of what is known about linking number changes in DNA, it would seem logical that topoisomerases should work by a swivel mechanism. This would involve breaking of one (or both) strand of DNA, allowing the free end (or ends) to rotate about the helix axis and resealing the break (10, 63). However, consideration of the knotting/unknotting and catenation/decatenation reactions suggest that a swivel mechanism can not account for the full range of topoisomerase reactions. A different type of mechanism, called strand

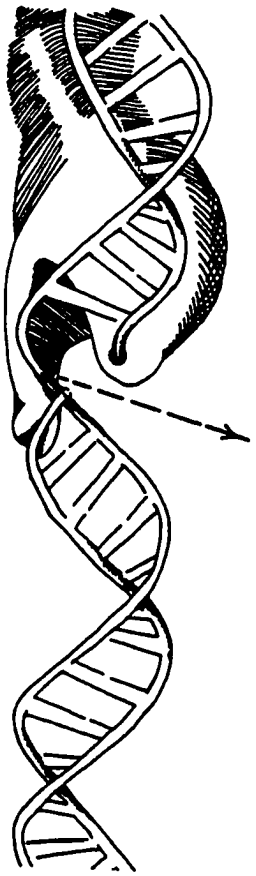
passage, can account for the ability of topoisomerases to catalyze all these reactions (55). In its simplest form, strand passage involves the cleavage of one or both strands of the DNA by the enzyme and the passing of a single- or double-stranded segment of DNA through the break which is then resealed. (6, 60, 64). These two mechanisms are diagrammed in Fig. 2.

The reactions of type II enzymes proceed via double-stranded break and involve covalent attachment at the 5'-phosphate (6, 65). Resealing the breaks by topoisomerase II is an ATP-dependent step (55). The strand passage mechanism of type II topoisomerases involves the translocation of DNA both through the double stranded break and through the protein complex. Crystallographic evidence suggests that, for DNA gyrase, this is facilitated by the existence of a "hole" between two of subunits (66). The DNA and protein linkages for both the eukaryotic and eukaryotic type-I enzymes have been found to be phosphodiester bonds between the ends of the broken strand and a tyrosine (Tyr) residue in the respective protein (67, 68). Substitution of either phenylalanine or serine for the active site Tyr resulted in an inactive enzyme (12, 68). The only exception is the resolvase protein of transposons, Tn3, which also exhibits a topoisomerase activity by carrying out site specific recombination between appropriately oriented sites in supercoiled DNA (6, 69). This protein was shown to bind its substrate DNA through a serine (Ser) residue (6). Chemically, breakage of the DNA by the enzyme is a transesterification reaction involving nucleophilic attack by the Tyr hydroxyl in the active site on a phosphodiester bond in the DNA (68) (Fig. 3A). Closure is similarly a transesterification reaction in which the attacking group is the hydroxyl group on the free end of the broken DNA strand. The protein-linked DNA breaks introduced by topoisomerases are fundamentally different from those induced by nucleases. Topoisomerase reactions are readily reversible, providing that the free energy of hydrolysis of the protein/DNA

Fig 2. Drawings showing two alternative mechanisms for type I topoisomerases. Model I depicts the free rotation model, and model II shows the enzyme-bridging model (Reproduced from "Champoux, J. J., 1990") (55). See text for detailed explanation of the models.



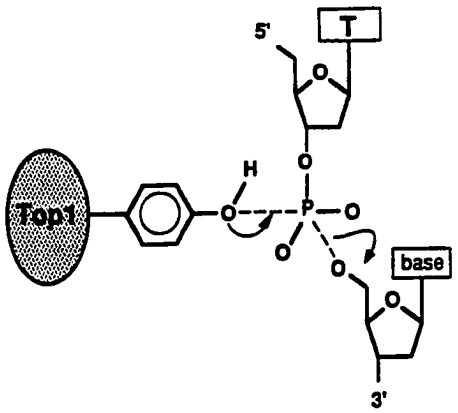
I



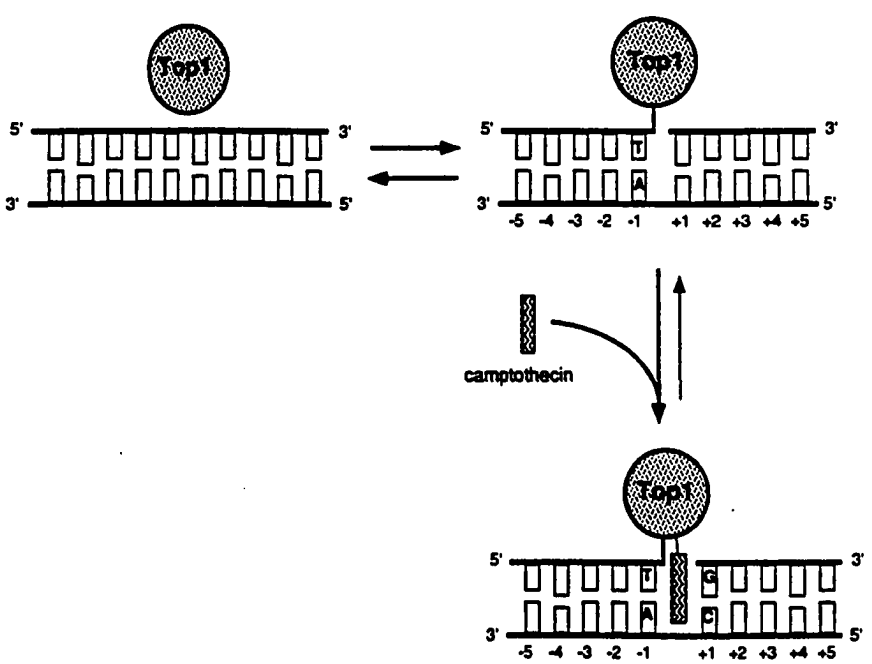
II

Fig. 3. Proposed model of transesterification reaction of nuclear type I topoisomerases and camptothecin-induced enzyme-DNA complexes. A). The tyrosine hydroxyl at the reactive site of topoisomerase I attacks the DNA phosphodiester bond and displaces the 5'-O of the nucleotide residue which will become the 5' terminus of the resulting break. The preferred base on the 5' side of the break (3' terminus) is a thymine (T). B) Under physiological conditions topoisomerase I-linked DNA breaks ("cleavable complexes") (upper right) are readily reversible to non-covalent enzyme-DNA complexes (upper left) before or after topoisomerization of the DNA strand breaks. Camptothecin forms a ternary complex (bottom) with enhanced stability which can not progress to topoisomerization of the DNA strands until the drug dissociates (Reproduced from "Pommier et al, 1994) (71).

A



B



phosphodiester bond is comparable to the free energy of hydrolysis of the DNA phosphodiester linkage (55). Demonstration of topoisomerase-linked DNA breaks requires the addition of a strong protein denaturant such as NaOH or an ionic detergent (70).

Prokaryotic topoisomerases make a transient covalent complex with the 5' end of the broken end of DNA. The eukaryotic nuclear type I topoisomerase, as well as the lambda integrase and the yeast 2-μm circle FLP recombinase (55, 72), all become linked to the DNA through a 3'-phosphoryl bond. Insights into DNA binding properties of topoisomerases have been greatly facilitated by procedures that prevent the reclosure reaction of the enzyme and allow a characterization of the nicked intermediate. Inducers of topoisomerase I-linked DNA breaks inhibit the enzyme by stabilizing a cleavable enzyme-DNA complex upon binding to the topoisomerase I or DNA component of the complex, or to the both, resulting a stabilized ternary complex impedes the resealing step (see Fig. 3B for illustration). Such cleavable complexes can be detected after protein denaturation with SDS (71). Among the most well-known inducers of topoisomerase I-linked DNA breaks is Camptothecin (CPT), a cytotoxic plant alkaloid, from *Camptotheca acuminata*, that is a potent inhibitor of both DNA, and RNA, synthesis in which leads to accumulation of single-stranded breaks in DNA *in vivo* (73). Different levels of partial inhibition in vitro have been observed for a variety of CPT derivatives (74).

Topoisomerase reactions have been shown to proceed via both distributive and processive models (13). Distributive action involves the dissociation of the enzyme from DNA after each catalytic cycle whereas processive action requires several catalytic cycles to occur before the enzyme dissociates (6, 13). The composition of the reaction environment influences the processivity. The enzyme, in general, is less processive with the increased salt concentration as high salt tends to dissociate it from its substrate DNA (34, 75). Several topoisomerases have been shown to be substrates for protein kinases

(76). Treatment with alkaline phosphatase dephosphorylates the enzyme and reduces its relaxation activity. Subsequent treatment with protein kinase restores the activity to its original level (76). The phosphorylated residues were found to be Ser. and threonine; Ser. was the predominant residue, phosphorylated in the topoisomerase reactions (77).

1.1.1.6 Physiological roles of DNA topoisomerases

Torsional stress occurs in double stranded DNA during both replication and transcription (18, 19, 38, 39). Negative superhelical tension facilitates the melting of DNA necessary for these processes. In *E. coli*, topoisomerase I is a specificity factor in the initiation of *oriC* plasmid replication, preventing the initiation at sites other than the origin of replication (78, 79). Topoisomerase III, the other type I enzyme of *E. coli*, has been shown to act catalytically on RNA (80). Whether this observation has a physiological relevance is not known. The report may suggest a role for the enzyme in "decatenation" of mRNA from DNA during transcription. *In vitro* evidence suggests that the cellular role of topoisomerase I is relaxing supercoils while topoisomerase III is decatenase (49). Intertwined progeny DNAs at the termination of replication are resolved by topoisomerases (10). Topoisomerase I and DNA gyrase are diametric, shown by compensation mutations in E. coli (25). Although, the principal function of bacterial topoisomerase I is to remove the excessive supercoils introduced by DNA gyrase, in highly-transcribing cells, DNA gyrase also acts as an enzyme removing supercoils rather than introducing them. Multiple topoisomerases with distinct functions may give the cell a more precise control over DNA topology by allowing tighter regulation of the principal enzymatic activities of these different proteins (25, 79). During transcription, as RNA polymerase transcribes a gene, the protein-RNA complex must follow the helical path of the DNA strands. The combination of the polymerase, the nascent RNA chain, and possibly even ribosomes translating the mRNA in a coordinated fashion in prokaryotes,

would form a complex so large that it would be unable to rotate around the DNA, and that instead , the DNA would rotate upon its axis (10). Such a rotation of the DNA around its axis, relative to the unpaired DNA region at the polymerase, causes an increase in twist ahead of the moving polymerase, and a reduction in twist behind which would manifest positive and negative supercoiling respectively (10, 80). The essential roles of topoisomerases in transcription elongation and gene expression have been demonstrated both by *in vivo* and *in vitro* studies (10, 13). Topoisomerases also have essential roles in genetic recombination (40). Because nuclear topoisomerase I can spontaneously break down single-stranded DNA and covalently transfer heterologus DNA strands, it has been implicated to be the key enzyme in illegitimate recombination (3, 81, 82). The movement of certain transposable elements in eukaryotes may be promoted via topoisomerase-mediated illegitimate recombination at sites flanking each transposable element (41).

On the other hand, there are some indications that *in vivo* a putative topoisomerase II plays a role in the assembly of torsionally strained transcriptionally active chromatin (83, 84). It is one of the proteins that make up chromosome scaffolds. These structures are believed to reflect the organization of chromatin into transcriptional units. Topoisomerase II was shown to bind rich MARs or SARs (for Matrix or Scaffold Association Regions) (85, 86).

1.1.2. Organization of mammalian mitochondrial DNA and involvement of topoisomerases in its biogenesis.

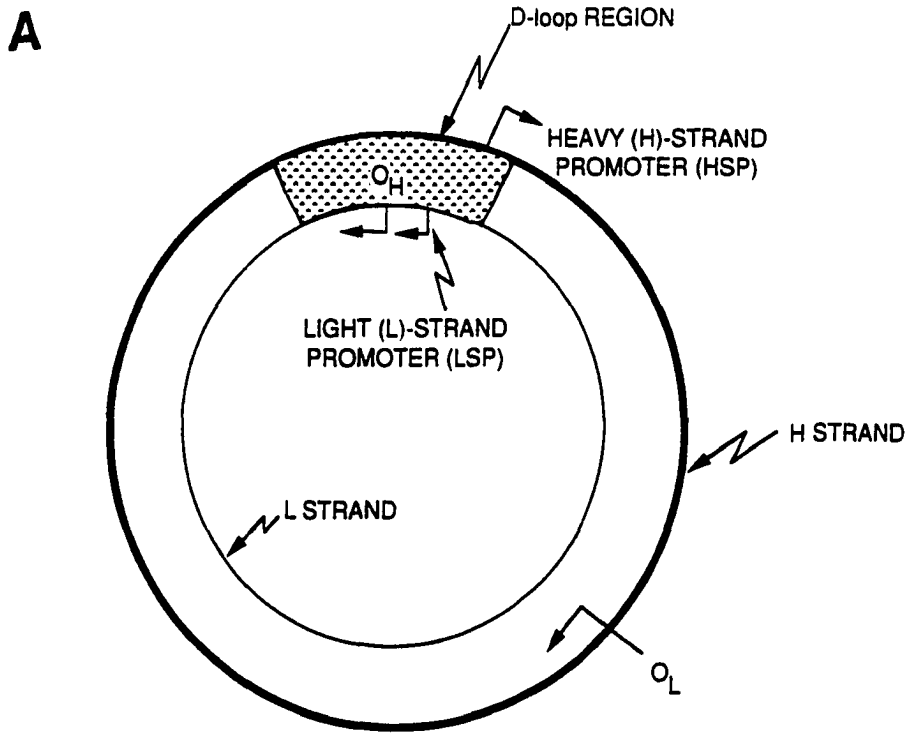
1.1.2.1 Basic organization of mammalian mitochondrial DNA

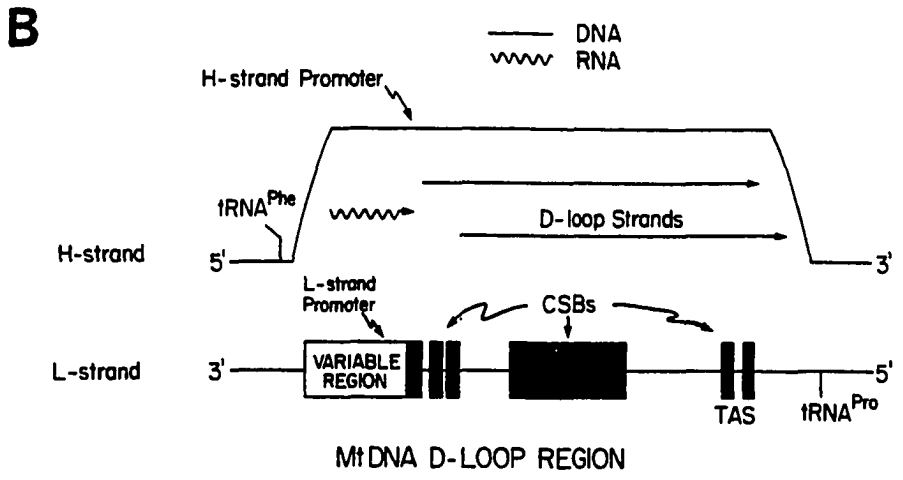
Mammalian mitochondria contain a covalently closed , circular double helix of naked DNA that is present at high cellular copy number (10^3-10^4 mtDNA per somatic cell) (87, 88). Mammalian mitochondrial DNA (mtDNA) consists of approximately 16500

base pair (bp). Given its small size, mtDNA is limited in its coding capacity. The biogenesis of mitochondria requires both mitochondrial and nuclear products (87). The complete nucleotide sequence of human (89), bovine (90), mouse (91), *Xenopus* (92), and *Drosophilia* (93) mitochondrial DNA have been determined. Mitochondria are believed to have descended from eubacteria via endosymbiosis and, in fact, numerous biochemical and genetic processes occurring in the mitochondria have decidedly prokaryotic characteristics (94-96).Replication of mtDNA is unrestricted with regard to cell cycle (97). Early studies on the mode of mtDNA replication revealed that each strand of the duplex could be distinguished on the basis of G+T base composition; a bias in composition results in different buoyant densities of each strand ("heavy; H" and "light; L") in alkaline cesium chloride ($CsCl_2$) gradients (87, 98, 99). A simplified picture of the organization of mammalian mtDNA is given in Fig 4A.

A peculiar replicative form has been observed in mitochondria of most species; DNA synthesis is initiated from RNA primers, transcribed from the L-strand promoter but terminating after partial replication of the H-strand. Partially replicated H-strand of mtDNA remains annealed to the L-strand, displacing the native H-strand segment which results in an unusual triplex DNA structure, known as displacement loop (D-loop) (98, 100, 101) (Fig 4B). The proportions of D-loop form to duplex form correlates directly with mtDNA copy number, RNA abundance and specific activity of mtDNA polymerase gamma (101). The D-loop region of vertebrate mtDNA has evolved as the control sites for both replication and transcription of the mitochondrial genome (88). Potential secondary structures were found near the D-loop strand origin in mammalian mitochondria (98, 102). Mammalian mtDNA has two separate and distinct origins of replication. The origin of heavy strand (H-or) synthesis is located within the D-loop region of genome and origin of light (L-or) strand synthesis is nested within a cluster of five t-RNA genes well away from the D-loop (103). The H-strand (leading strand) origin is the

Fig. 4. A simplified organization of mammalian mitochondrial genome and the displacement loop region. A). Diagram of control regions and coding potential of mammalian mtDNA. The D-loop region contains the origin of leading-strand (heavy [H]-strand) replication and the promoters for transcription of the H-strand template (HSP) and L-strand template (LST). The origin of lagging -strand (light [L]-strand) synthesis is at O_L. Genes encoded by the mtDNA H strand are: 12S rRNA; 16S RNA; tRNAs for the amino acids in the following order, phenylalanine, valine, leucine (UUR), isoleucine, methionine, tryptophane, aspartic acid, lysine, glycine, arginine, histidine, serine (AGY), leucine (CUN), threonine, cytochrome oxidase (CO) subunits I, II, and III; ATPase subunits 6 and 8; cytochrome *b* (cyt*b*); and subunits 1, 2, 3, 4L, 4 and 5 of complex I (NADH-ubiquinone oxido-reductase [ND]). Genes encoded by the mtDNA L strand are: tRNAs for the amino acids in the following order, glutamine, alanine, asparagine, cysteine, tyrosine, serine (UCN), glutamic acid, proline; and subunit 6 of Complex I. B) The D-loop region of mammalian mtDNA. An unusual triplex DNA structure is formed due to partial replication of H-strand segment, annealed to L-strand. The variable region is that sequence subject to varying degrees of deletion among mammalian mtDNAs. CBSs, conserved regions of sequence between mammalian mtDNAs; TAS, termination-associated sequences relative to D-loop strand synthesis (Reproduced from "Clayton, 1991 and Clayton, 1984) (cited in refs. #88 and 103)





Mt DNA D-LOOP REGION

dominant element for replication while the L-strand (lagging strand) origin plays an essential but secondary role. Activation of L-strand promoter mediates both priming of leading strand replication and initiation of light strand transcription (88). Accurate initiation of transcription from *in vivo* promoter sequences was shown to be enhanced by supercoiling of mtDNA (104).

1.1.2.2 Mammalian mitochondrial DNA and topoisomerases

The replication of the closed circular mitochondrial genome results in the production of torsional stress in the molecule as the replication fork traverses the DNA (98, 105). In the absence of a strain-relief mechanism, the unwinding of Watson-Crick turns during replication of the molecule, while initially energetically favorable because of negative supercoiling, would become increasingly less so owing to the gradual removal of negative supercoils (106). The rotation of the transcription ensemble relative to the DNA template generates positive supercoiling ahead of, and negative supercoiling behind the transcription ensemble (107). Furthermore, a decatenation activity is required to separate replication products of circular mtDNA (35). The first mitochondrial type I topoisomerase was isolated from rat liver in 1979 (36). Subsequently the enzyme has been isolated from *Xenopus laevis* oocytes (108), human acute lymphoblastic leukemia cells (HALL) (109, 110), calf thymus (111, 112), human platelets (113) *Neuspora crassa* (114), and *Saccharomyces cerevisiae* (115). The characterization studies of the enzyme from the above sources indicate considerable variability in molecular weight and other biochemical properties which may be due to species specific properties (105, 109, 112). Isolated mtDNA is negatively supercoiled. The presence of a mitochondrial gyrase-like activity has been shown in rat liver (35, 106, 116), HALL (109) and calf thymus (117).

Mitochondrial topoisomerase I (mt topo I) was shown to become covalently joined

to substrate DNA through a phosphoryl linkage (35, 112). Interestingly, the mitochondrial enzyme yields a protease digestion pattern distinct from that of the analogues nuclear enzyme (112). Like the nuclear enzyme, mt topo I relaxes both negative and positive supercoils and acts on both single and double stranded DNAs (106, 110). The supercoil-relaxation activity of mt topo I has been shown to be inhibited by CPT, however the effect was reported to be 10 to 20 times less efficient when compared to its nuclear counterpart (118). Mitochondrial topoisomerase I differs from the nuclear enzyme in its pH profile, thermal stability, sensitivity to ethidium bromide (EtdBr) and the trypanocidal agent, berenil (36, 109, 110). The nuclear and mitochondrial topoisomerases behave dramatically different upon chromotography with single stranded-DNA cellulose and several sephadex gels and appreciably differently with double stranded-DNA cellulose and phosphocellulose (35, 110). These two enzymes also differ from each other in terms of sensitivity to ATP inhibition (110). Since mitochondria are the generators of high energy carriers in cells, one can expect that the level and activity of mitochondrial topoisomerase I is controlled by the relative concentration of high energy carriers, ATP and ADP.

1.1.3 Studies on sequence specificity of DNA topoisomerases

Numerous nuclear topoisomerase I (nc topo I) recognition sequences have been proposed by several authors (119-129). Most of these studies are based on the accumulation of nicked DNA-enzyme intermediates upon using protein denaturants and covalent linkage of the enzyme to its substrate DNA as the dissociation rate is much lower than the association rate (122, 126, 129-131). The majority of the cleavage fragments obtained in these studies were of discrete sizes and present on both single and double stranded DNA at the same nucleotide positions indicating that certain DNA sequences are essential for enzyme recognition (120, 122). Although the consensus sequences inferred from these analyses are degenerate and the enzyme is promiscuous to the proposed

sequences, statistical analysis of the sequences surrounding the cleavage sites has demonstrated that certain nucleotides have been found to be less favored at cleavage sites (123-125, 132). There is a high probability to exclude G from positions -4, -2, -1 and +1, T from position -3 and A from position -1 (6, 132). On the other hand, type I topoisomerases from both *E. coli* and *M. luteus* have been found to cleave several synthetic homopolymers and short oligomers, indicating the lack of an absolute cleavage specificity (58). However, the enzyme showed a preference for a C residue at the fourth position 5'- to the cleavage site on natural DNA substrates. When a nicked circular duplex substrate has been employed, the preferred sites of DNA cleavage were found in the intact strand at loci close to the nick, indicating that cleavage specificity could be shifted (134).

A relatively higher degree of sequence specificity was derived in the studies of nuclear topoisomerase I cleavage sites in the ribosomal genes of tetrahymena (121, 123, 124); The proposed recognition sequence was:

5'-A(G/A)ACTT*AGA(G/A)AAA(TA)(TA)(TA)-3'

(Order of alternative bases is from left to right, * shows the cleavage site). A bias in the location of cleavage sites was seen within the region flanking rRNA genes that can be associated with the involvement of the enzyme in the transcription of rRNA in nucleolus (121, 123-125, 132). Bonven *et al* also reported that the concentration of Ca^{2+} in the assay mixture affected the cleavage specificity of the enzyme (123). Been and Champoux have found that cleavage sites for the rat liver nuclear topoisomerase I can occur in the regions of single-stranded DNA with the potential for intramolecular base pairing (130). Paired hairpin loop structures formed by intrastrand base pairing, termed cruciforms, have been shown to influence several processes such as transcription regulation (135) and site specific recombination (136). The observation that several regulatory sites contain varying lengths of inverted repeat sequences highly encouraged these reports (127, 128, 137). These structures can experimentally be detected by gel electrophoresis and chemical

probing (138). Been and Champoux also reported an abolished cleavage specificity upon the deletions of sequences remote from the cleavage sites which disrupted potential hairpin structures (130). This indicates that the enzyme I requires at least a short region of duplex to execute the cleavage reaction (127, 130). Integrase protein of bacteriophage λ and resolvase protein of transposons Tn3 were also reported to show cleavage specificity in the regions of dyad symmetry (139, 140). The sequence at the cleavage site of resolvase was: 5'-TTAT*AA-3' (139).Sequence specificity of topoisomerase II was also extensively studied (60, 141-145). Some of these studies reported the elements of dyad symmetry and hairpin organization at or very near the cleavage site of type II enzymes. The enzyme was reported to show a preferential cleavage on alternating purine/pyrimidine repeats and GT, AC and AT repeats were better substrates for eukaryotic topoisomerase II than GC repeats (146, 147). Studies of cleavage by DNA gyrase have shown that the enzyme cleaves the DNA in both strands yielding a four base stagger between the cuts (60, 141). The proposed sequence was:

5'-RNNNRNRT*GRYC(T/G)YNYNGNY-3'

where N=any nucleotide, R=purine, Y=pyrimidine. This sequence shows elements of dyad symmetry about on axis midway between the cleavage sites in the two strands and is consistent with many of the sites found *in vivo* (6, 60). A consensus sequence for cleavage by the type II enzyme *Drosophilia melanogester* suggested following analysis of *in vitro* cleavage patterns of Drosophilia DNA were as:

5'-GTN(A/T)AY*ATTNATNNG-3' (144).

The occurrence of topoisomerase II cleavage sites was chiefly in intergenic regions which implicates the role of this enzyme in transcriptional control. Cleavage of cytosine-containing T4 DNA by the T4 topoisomerase was found to be relatively non-specific whereas native DNA containing glycosylated hydroxymethyl cytosine was cleaved at specific sites (143). The enzyme from T4 virus was also shown to cleave DNA at or near the hairpin structures (6, 143).

1.2 STATEMENT OF THE PROBLEM

Proteins that interact with DNA may show sequence selectivity ranging from stringent to fairly permissive, depending on the requirements imposed by their functions. In this regard DNA topoisomerases are no exception. Interaction of nuclear topoisomerases with DNA have been demonstrated by several groups of studies. On the other hand, since the first report of the presence of a DNA topoisomerase in mitochondria, the progress in the characterization of this enzyme has been slow. Sequence dependence and kinetic properties of mitochondrial topoisomerase I are two important aspects of its overall characterization. Our current isolation procedure for the enzyme employs ultracentrifugation to sediment complexes formed between the enzyme and mtDNA. In addition to providing the basis for purification step, this behavior suggests that there might be specific mtDNA sequences that the topoisomerase is recognizing and with which it is interacting. The issue of whether topoisomerases interact with specific DNA sequences is of significant interest as the specificity of such interaction may have implications for their in vivo actions, i.e., in DNA replication and transcription This question can be addressed by using a series of recombinant plasmids containing selected mtDNA regions as substrates for mt topo I. These recombinant plasmids will be used in substrate competition and supercoil relaxation assays to determine Km, Vmax, turnover numbers and specificity constants of the enzyme. These are the first kinetic studies performed with the highly purified mitochondrial topoisomerase and as such they provide important new information about the biochemical characteristics of this enzyme to distinguish it from its nuclear counterpart. This study contains the first report of the mammalian mitochondrial topoisomerase I interacting with specific mtDNA sequences. Together with the kinetic parameters of the enzyme, this study may have an impact on our understanding of the role of topoisomerases in the biogenesis of mtDNA.

1.3 OBJECTIVES OF THE STUDY

A) To determine the polarity of the attachment and kinetic parameters of mammalian mitochondrial topoisomerase I.

Prokaryotic and eukaryotic topoisomerases differ with respect to their transient covalent attachment to DNA during the catalytic cycle. Bacterial topoisomerase I becomes linked to the 5' end of DNA while eukaryotic enzymes have a 3' linkage. Although no known function is specifically attributed to the polarity of DNA linkage, this experiment may be an important parameter in the hypothesis of the prokaryotic origin of mitochondria and in discriminating between the nuclear and mitochondrial topoisomerases. This section also aims to compare the kinetic parameters of mitochondrial topoisomerase I to its nuclear analogues, using pUC19 as substrate DNA. The results of these studies will provide important data to distinguish the mitochondrial topoisomerase I from the nuclear type I topoisomerases.

B) To construct recombinant plasmid substrates having mitochondrial and non-mitochondrial DNA inserts.

Because of the documented role of DNA topoisomerases in the replication and transcription of DNA, we will chose to examine the interaction of mitochondrial topoisomerase I with recombinant plasmids containing heavy and light strand origins of replication and major transcription region of bovine mtDNA. We will also prepare recombinant plasmids containing general coding sequences as well as non-mitochondrial DNA fragments of the same sizes. The mtDNA inserts will be amplified by polymerase chain reaction (PCR). However, good yields of hybrid plasmids during the cloning of PCR-amplified, blunt-ended DNA inserts into plasmids have been proved to be very

difficult to obtain efficiently. In this regard, this section will not be limited to the construction of recombinant plasmids to provide experimental tools for our research but will also cover a number of experimental approaches to get the optimal conditions for ligation of blunt-ended DNA inserts into plasmid vectors and their efficient transformation into bacterial cells.

C) Southern hybridization and chemiluminescent detection of supercoil relaxation assays in paired reaction sets; Mitochondrial DNA Vs non-mitochondrial DNA.

This step aims to determine whether the mitochondrial topoisomerase I would preferentially interact with supercoiled DNA containing a particular region of mtDNA. We will perform standard relaxation assays using a mixture of size-paired experimental and control recombinant plasmids in single reaction sets. Southern hybridization using non-radioactivity tagged probes specific for either of the paired plasmid substrates will be used to visualize the relaxation of supercoiled DNA. The results will be compared to nuclear topoisomerase I-catalyzed reactions under the same conditions.

D) Estimation of Kinetic Parameters of Mitochondrial and Nuclear Type I Topoisomerases.

This section aims to determine the kinetic parameters, Km, Vmax , Kcat and specificity constants of both nuclear and mitochondrial type I topoisomerases using recombinant plasmid substrates. The plasmids will be studied in quantitative amounts in supercoil relaxing assays with assay products separated by electrophoresis, visualized by EtdBr staining and quantitated by densitometry. The quantitative values, obtained from this step will be compared to the kinetic parameters of the enzyme with pUC19 to highlight the contribution of the mtDNA sequences on the kinetics of the enzyme.

Chapter 2 MATERIALS AND METHODS

2.1 MATERIALS:

2.1.1 Equipment:

Cyclone DNA Synthesizer (Bioresearch, Inc), Thermocycler (Temp-Tronic, Inc), Ultrascan XL Microdensitometer (LKB), EC-103 Power supply (EC Apparatus Corporation), UV Stratalinker 1800 (Stratagene), UV Transilluminator (UPV, Inc), MP-4 Land Camera (Polaroid), Concentrator and refrigerated condensation trap (Savant), Slab dryer (Biorad) Spectrophotometer Ultraspec (LKB), Ultracentrifuge L7 (Beckman), X-ray films (Fuji).2.1.2. Chemicals; Calf intestinal alkaline phosphatase (CIAP), Klenow fragment of DNA Polymerase I, T4 DNA Polymerase, T4 Polynucleotide Kinase (PNK), pGEM-T vector and JM109 cells were from Promega (USA). Taq DNA Polymerase was from Perkin Elmer/Roche., DNA grade agarose was from Fisher Scientific (Pittsburgh, Pa), Phosphocellulose, hydroxyapatite, double-stranded DNA cellulose , Camptothecin (CPT), Proteinase K, Ampicillin, Isopropyl-1-thio-β-D-galactoside (IPTG), X-Gal were from Sigma Chemical Co (St Louis, Mo), [γ-^{32}P]ATP (3000 Ci/mmole) and [α-^{32}P]dATP (800 Ci/mmol) were purchased from New England Nuclear (Wilmington, De) and the chemiluminescence detection kit was from Boehringer Mannheim Co (Indianapolis, IN). All other reagents were of analytical grade.

2.2 METHODS

2.2.1. Cleavage assays

2.2.1.1 Preparation of end-labeled DNA

The procedure of end labeling of DNA has been described previously (142). Briefly, 100 ng of pUC19 was digested with EcoRI and dephosphorylated with CIAP (148). Linearized pUC19 was then either labeled at its 3'- ends with the large fragment of *E. coli* DNA polymerase and [α-^{32}P]dATP or labeled at its 5' ends with T4 Polynucleotide kinase and [γ-^{32}P]ATP (142). Following the extraction with phenol : chloroform : isoamylalcohol (25:24:1), unincorporated triphosphates were removed by two cycles of ethanol precipitation in the presence of 2.5 M ammonium acetate. The labeled DNAs were re-digested with BamH1 and the 20 bp fragment has been removed using Wizard columns (Promega).

2.2.1.2 Topoisomerase cleavage assays.

Reaction mixtures (40 µl each) containing 40 mM Tris-HCl (pH:7.0), 60 mM KCL, 10 mM $MgCl_2$, 0.5 mM Dithiothreitol (DTT), 10 ng uniquely end-labeled large fragment of EcoRI/BamH1-digested pUC19 DNA (2666 bp), and 25 units of bovine mitochondrial DNA topoisomerase I were incubated in the presence of 100 µM CPT at 37 ^{o}C for 15 minutes (142). The reactions were terminated by addition of 2 µl of 20 % SDS. Unless otherwise indicated, reaction mixtures were treated with 150 µg/ml proteinase K for another hr at 55 ^{o}C. DNA samples were denatured with 20 µl 0.45 N NaOH, 30 mM EDTA, 15 % sucrose and 0.25 mg/ml bromophenol blue and analyzed by electrophoresis in a 1.2% agarose gel.

2.2.2. Preparation of DNA inserts

Using the published bovine mtDNA sequence and a computer aided program "PRIMER DESIGN", the regions of interest (see Fig 4A for the locations of selected regions) were amplified using a multiplex PCR protocol (149) [25 cycles; one min at 94°C, two min at 65°C, and three min. at 72°C with a final extension of seven min. at 72°C using 100 ng template DNA, 20 pmole appropriate primers, 1.5 mM dNTP mixture and one unit of Taq DNA Polymerase in a buffer containing 83 mM $(NH_4)_2SO_4$, 335 mM Tris HCL (pH:8.0), 34 mM $MgCl_2$, 50 mM 2-mercapto ethanol, 34 mM EDTA and 850 μ g/ml BSA in a total volume of 50 μl]. Sequences of the primers used in the amplification of the bovine mitochondrial genome were as follows;

H-ori: Left: 5'-AACCAGAGAAGGAGAACAACTAACCTC-3';

Right: 5'-GCTCAAGATGCAGTTAAGTCCAGCTA-3'.

L-ori: Left: 5'-ACGACTCACGTATTCTACCACACTA-3';

Right: 5'-GAACAAGTCAGTTACCGAATCCTCC-3'.

Term: Left: 5'-TACGACCTCGATGTTGGATCAGGAC-3';

Right: 5'-AAGGAATGCTACGGCCAATAGGATG-3'.

Cytb: Left: 5'-AGCCCCATCAAACATTTCATCATGA-3';

Right: 5'-TGCTCCTCAGAATGATATTTGTCCT-3'.

Control inserts of known size were prepared from a 100 bp ladder molecular weight markers (Gibco BRL, USA). Ladder was resolved by 1.2% agarose gel electrophoresis. DNA was visualized by EtdBr staining and UV illumination. The appropriate size bands were excised and eluted as described (150).

2.2.3 Enzymatic manipulations of insert and vector DNAs

2.2.3.1 Dephosphorylation of 5'-ends of DNAs with CIAP

Calf intestinal alkaline phosphatase catalyzes the hydrolysis of 5'-phosphate groups from nucleic acids. (148). Dephosphorylation reactions have been carried out a mixture containing 50 mM Tris-HCL (pH; 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, 0.05 units of CIAP per pmole of 5' ends of DNA at 37 °C for 60 minutes in a volume of 50 µl (148, 151). The reaction was terminated by addition of % SDS and EDTA to final concentrations of 1% SDS and 1 mM EDTA.

2.2.3.2 Phosphorylation of 5'-ends of DNA with T4 PNK.

T4 Polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5'- termini of nucleotides bearing a 3'-phosphate group (152). The forty microliter of reaction mixture included 250 ng dephosphorylated DNA insert, 70 mM Tris-HCl (pH:7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM ATP, 20 units PNK. The reaction has been carried out at 37 °C for 30 min and stopped by the addition of 0.1 volume of 0.2 M EDTA.

2.2.3.3 Filling the 3'-protruding ends of DNA with dNTPs using Klenow fragment of *E. coli* DNA polymerase I.

To fill the 3'-protruding ends, PCR-amplified inserts were incubated in a reaction of 50 mM Tris-HCl (pH:7.2), 10 mM $MgSO_4$, 0.1 mM DTT, 1 mM dNTPs, 5 µg/ml acetylated BSA and one unit of Klenow fragment of *E. coli* DNA polymerase. The reaction was carried out at 37 °C for 30 min and terminated by heating to 70 °C for five min (153).

2.2.3.4 Blunting the 3'-overhangs with T4 DNA polymerase;

Like Klenow fragment of *E. coli* DNA Polymerase I, T4 DNA Polymerase possesses a 5'->3' polymerase and 3'->5' exonuclease activity (153). However the exonuclease activity of T4 DNA Polymerase is more than 200 times as effective as that of Klenow enzyme. Typical reaction mixture included 67 mM Tris-HCl (pH:8.8), 6.7 mM $MgCl_2$, 10 mM β-Mercaptoethanol, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM EDTA, 167 μg/ml BSA, 0.2 mM DNA and 2.5 units of T4 DNA polymerase. The reaction was carried out at room temperature for 20 min and terminated as above.

2.2.4 Plasmid construction

2.2.4.1. Construction of experimental recombinant plasmids

After amplification, PCR products were purified using Wizard columns (Promega) and were concentrated by ethanol precipitation. The products were then phosphorylated at their 5'-ends using T4 PNK and ATP (152). Because of non-template dependent addition of a single deoxyadenosine to the PCR products by Taq DNA polymerase, the inserts were ligated with 3'-T overhanging pGEM-T vector, linearized in its Lac-Z region with Eco RV. To obtain optimal ligation, a 1:3 molar ratio of vector to insert DNA was used. The appropriate amount of insert DNA has been calculated using the following equation;[(ng of vector)x(kb size of insert)/(kb size of vector)x[molar ratio of insert/vector]=ng of insert DNA. The ligation mixture contained 30 mM Tris-HCl (pH; 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 50 nag vector DNA, appropriate amount of insert DNA and one unit of T4 DNA ligase in ten μl volume (154). The ligation reactions were carried out overnight at 4 ^{o}C and heat inactivated. Competent JM109 cell were transformed with ten ng of ligated DNA and transformants were selected by overnight growth on Lauria-Bertani (LB) plates containing 50 μg/ml ampicillin, 0.5 mM IPTG and

40 μg/ml X-Gal. The plates were incubated at 37 °C and white colonies were analyzed by plasmid miniprep for the presence of recombinant DNA (153). Recombinant plasmids were recovered by the alkaline lysis method and further purified by ultracentrifugation in cesium chloride/EtdBr at 55,000 rpm at 20 °C (153).

2.2.4.2 Construction of control recombinant plasmids

For the control group, 3'-T overhangs of pGEM-T vector were removed with T4 DNA polymerase as described above. DNA were then hydrolyzed with CIAP using standard procedures (148). The 400 and 800 bp bands from 100 bp DNA molecular weight marker set were excised from EtdBr-stained agarose gel (150), purified, phosphorylated at their 5'-ends using T4 PNK and ATP (152) and ligated into dephosphorylated and T4 DNA polymerase-treated pGEM vector with T4 DNA ligase as described above (154). The ligation of control inserts into pGEM vector and the transformation of competent JM109 cell were carried out as above.

2.2.5 Enzyme purification and activity assays

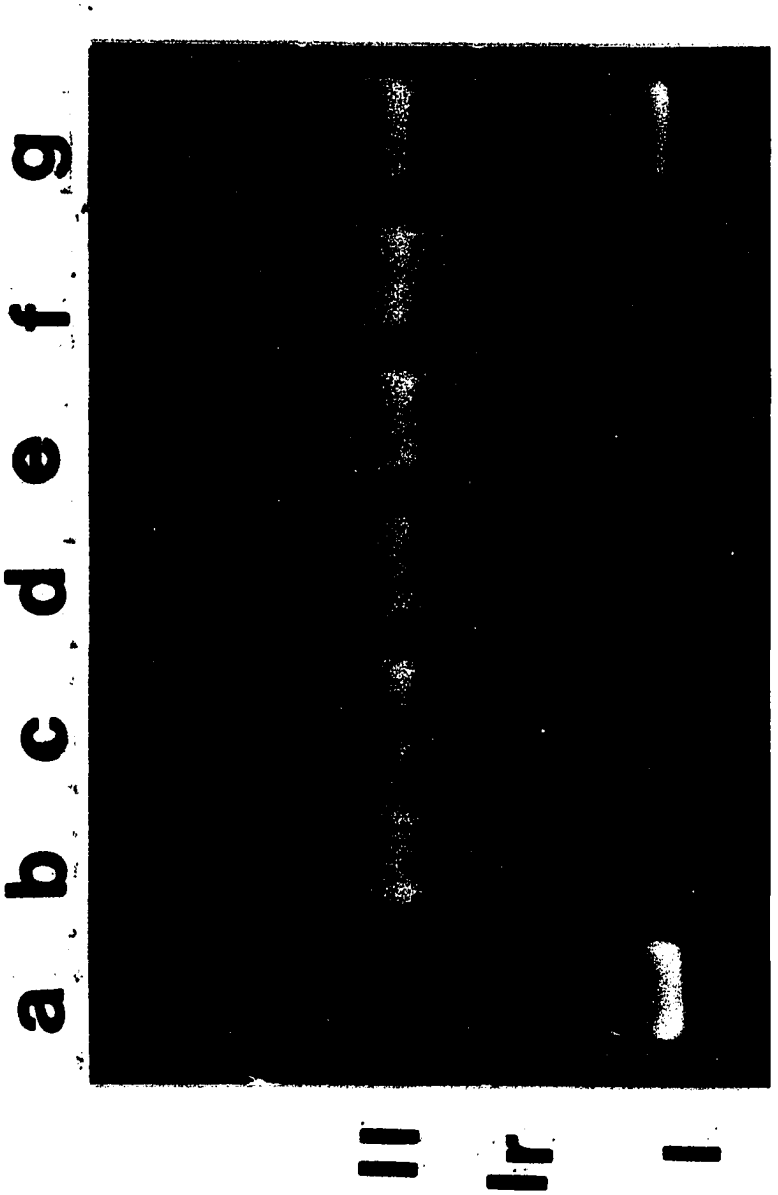
Mitochondrial DNA topoisomerase I was isolated from calf liver as described by Lin 1993 (118). Briefly, varying amounts of fresh calf liver (2.0 to 2.5 kg) was homogenized and applied to a two-step of sucrose gradient, composed of 75 ml of 25.0% and 90 ml of 42.5 % sucrose in TE (10 mM Tris-HCl, pH: 7.0/0.1 mM EDTA) for every 80 ml of crude mitochondrial suspension. The banded layers of mitochondria were collected at the interface of the two sucrose layers following centrifugation at 26,000 g for 75 minutes. The pellets were homogenized and the outer membrane of mitochondria were stripped by digitonine to obtain mitoplast. Mitochondrial DNA topoisomerase were isolated from mitoplast by lysis using nonidet and centrifugation with increased salt

concentration. The supernatants, containing topoisomerase I activities were applied to phosphocellulose, hydroxyapatite and double-stranded DNA-cellulose columns. Active fractions with more than four units per microliter were combined and stored in 50 mM potassium phosphate, pH 7.5/0.3 M KCl/50% glycerol at -20 ^{o}C. Nuclear topoisomerase I was isolated essentially according to Schmitt *et al* (157). The standard topoisomerase I relaxation assay and gel electrophoresis were described by Lazarus *et al.* (111). A typical relaxation assay using pUC19 plasmid and mitochondrial type I topoisomerase is given in Fig 5. The assay mixture contained 40 mM Tris-HCl (pH 7.0), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM Dithiothreitol (DTT), 0.5 mM EDTA and 30 μg/ml bovine serum albumin. One unit of enzyme activity removes 50% of the supercoils from 300 ng substrate in 30 minutes at 37 ^{o}C.

2.2.6 Chemiluminescence experiments

Experimental and control inserts were labeled by random primed incorporation of digoxigenin-labeled deoxyuridine triphosphate (DIG-dUTP). Each reaction set containing 0.3 μM of each of the pZT-Term/pZT-400 or pZT-Cytb/pZT-400 or pZT-Hori/pZT-800 or pZT-Lori/pZT-800 substrate mixtures was incubated with eight units of either mt-topo I or nuclear topoisomerase I in 200 μl final volume at 37 ^{o}C. Twenty microliter aliquots were removed at 0, 2, 4, 6, 8, 10, 15, 30 and 45 minutes and reactions were stopped by addition of 3 μl of 5% sodium dodecylsulfate/ 25% (w/v) sucrose/ 0.025% (w/v) bromophenol blue and applied to a 0.8% agarose gel. DNA was transferred to a nylon membrane by the capillary Southern blotting procedure (153) and UV-linked by using Stratalinker at 120,000 μJ/cm^2 for 30 sec. The membrane was prehybridized for one hr

Fig 5. Agarose gel electrophoresis of mitochondrial topoisomerase I relaxation assay visualized by EtdBr staining. Three hundreds microgram of plasmid pUC19 was incubated in each lane with 0 (lane a), 4 (lane b), 3 (lane c), 2 (lane d), 1 (lane e), 0.5 (lane f) and 0.25 (lane g) units of mitochondrial DNA topoisomerase I in the reaction mixture as described in "Materials and Methods" One unit of enzyme activity removes 50% of supercoils from 300 ng substrate in 30 min. at 37 ^{o}C. In each lane the slower migrating band is Form II or nicked circular DNA while the fast migrating band is Form I or highly supercoiled circular DNA. The ladder of bands between Form I and II DNA is the Form Ir, or partially relaxed circular DNA generated by removal of supercoils from highly supercoiled Form I DNA by the enzyme.

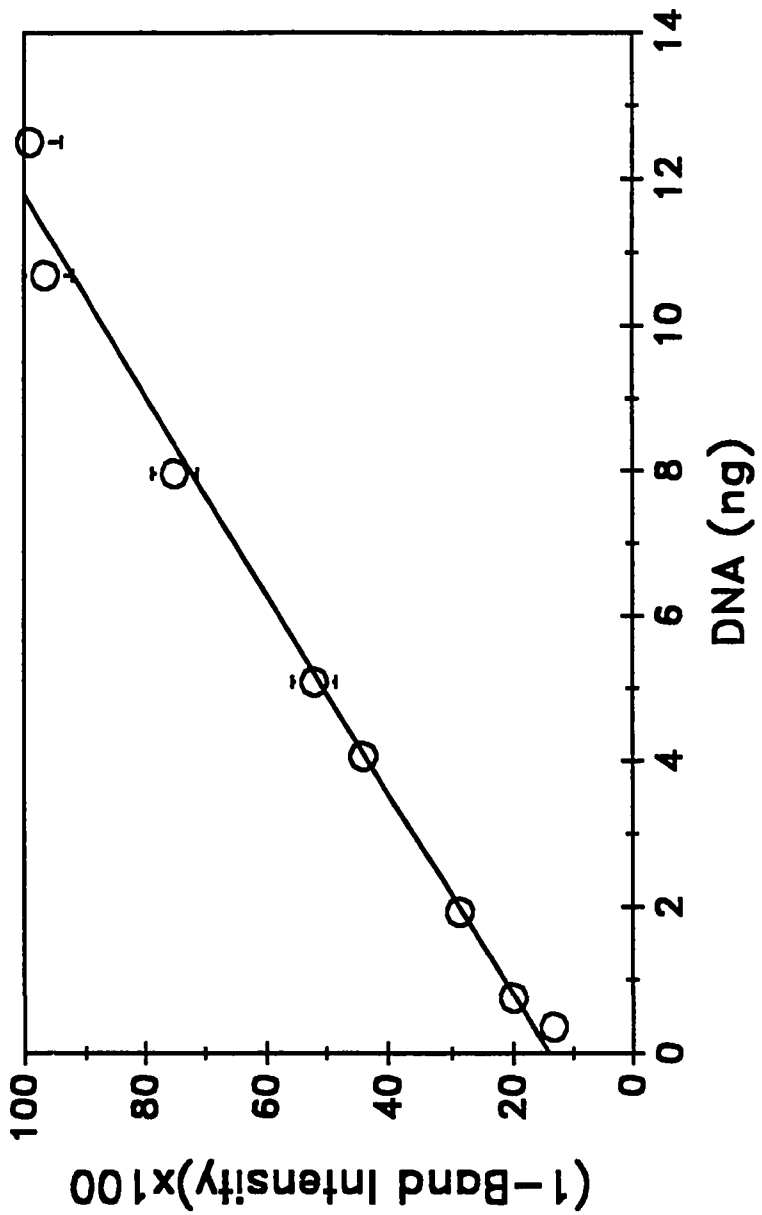


and relaxed DNA bands were visualized after hybridization of the membrane with DIG-labeled inserts under stringent conditions. Autoradiography was performed at -70 ^{o}C using an intensifying screen. Relative densities of the reaction products were quantified using an LKB Ultrascan XL Microdensitometer to scan the films. There was a linear relationship between the densitometric intensities and DNA concentration over the range of substrate concentrations (0.1 to 2.0 μM) used in chemiluminescence assays (see Fig 6).

2.2.7 Kinetic studies

The kinetic analysis was performed essentially as described by Castora *et al*, (158) Substrate concentrations ranged from 5.1 to 76.9 μM, based upon the size of the pUC19 or appropriate insert and the 3000 bp of the parent pGEM vector. Triplicate experiments were performed in standard assay mixture with 30 units of either mitochondrial or nuclear enzyme and varying amounts of DNA in 40 mM Tris-HCL (pH: 7.0), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM Dithiothreitol (DTT), 0.5 mM EDTA and 30 μg/ml bovine serum albumin (BSA) in a total of 200 μl final volume. The reactions were carried out at 37 ^{o}C. Twenty microliter aliquots were removed at 0, 2, 4, 6, 8, 10, 12, 20, and 45 minutes, terminated with 5% SDS/25% (w/v) sucrose/0.025% bromophenolblue and analyzed on 0.8 % agarose gels. DNA bands from EtdBr-stained agarose gels were photographed under UV illumination and relative DNA concentrations were determined by densitometry of photographic negatives. In DNA relaxation time-course experiments, the relative amount of supercoiled substrate, converted to relaxed form, was determined as; $1-(SC_0-SC_t)/SC_0$ where SC_0 was the area under the peak of supercoiled DNA at time zero and SC_t was the area of this peak at time t. Data from three independent experiments were averaged to produce values which represent relative amount of relaxation at each reaction interval. Time vs. product concentrations were plotted for each substrate concentration for both enzymes. Kinetics were examined by measuring initial velocities at different substrate

Fig. 6. The relationship between the band intensity and amount of DNA in densitometric quantification of X-ray films analyzed during chemiluminescence studies. The data points were obtained from the three separate measurements. The correlation coefficient is; 0.9250.



concentrations using both nuclear and mitochondrial topoisomerase I. Kinetic parameters (K_m, V_{max}) were derived from non-linear least-squares fitting of the experimental data to the Lineweaver-Burk plots using Sigmaplot 5.0. The turnover number (k_{cat}) was derived from the relationship of $V_{max}=k_{cat}x[E]$ where [E] was the total topoisomerase concentration of the individual reaction sets. The total enzyme concentration was estimated by the micro Bradford dye binding assay (155).

2.2.8 Presentation of data

Results are expressed as means ± standard error (rather than standard deviation) among the triplicate measurements for every substrate group. The differences in results between individual plasmid sets were analyzed by Student's t-test and enzymes were compared to each other by one way analysis of variance (ANOVA) test. The null hypothesis was rejected when $p < 0.05$; non significant (ns).

Chapter 3 RESULTS

3.1. Determination of polarity of DNA linking and kinetic parameters of mitochondrial topoisomerase I with pUC19

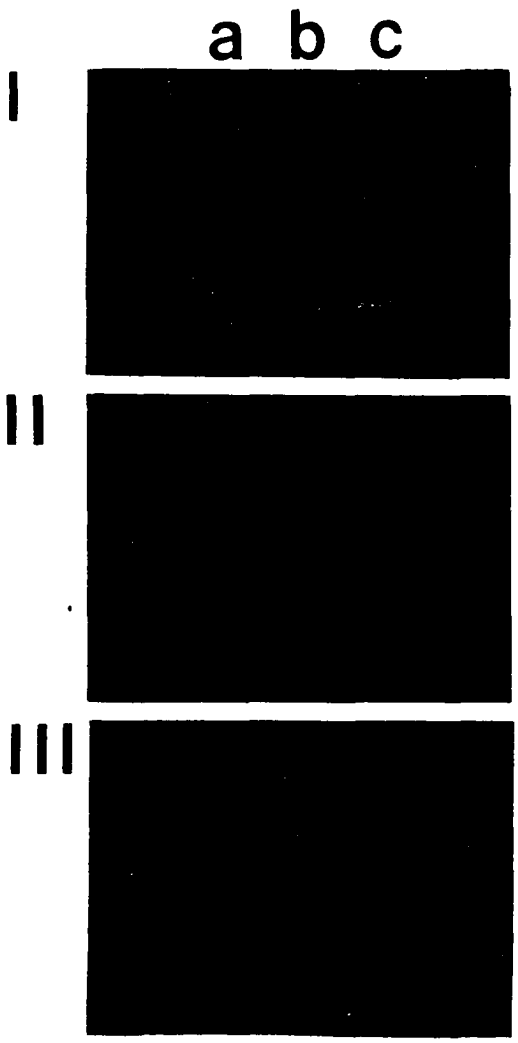
Previous studies on the biochemical and biophysical characterization of bovine mitochondrial topoisomerase I in our laboratory revealed that mitochondrial enzyme had both eukaryotic and prokaryotic properties (118). A preliminary result in DNA-binding studies of this enzyme by Dr. Lin has shown that, like prokaryotic topoisomerase I, the mitochondrial enzyme was linked to the 5' terminus of its substrate DNA (118). Dr. Lin's study employed the analysis of migration rate of the CPT-induced, topoisomerase-generated cleavage products of 5' end-labeled pUC19 in agarose gel following DNA cleavage with exonuclease III degradation and Klenow extension assays. To address the same question, we followed the procedure described previously by Liu *et al* (142) which was designed to determine the polarity of the DNA binding of nuclear topoisomerase I from calf thymus. We have prepared two sets of uniquely-end labeled DNAs having ^{32}P at either 5'- or 3'- ends (see "Materials and Methods" for the preparation of end-labeled DNAs). In the assay, mitochondrial topoisomerase I-mediated DNA cleavage was induced by CPT and the enzyme-DNA complex was trapped following the addition of SDS as denaturing agent. Hence, the enzyme remains covalently linked to the phosphorylated end of the nick because the resealing activity of mitochondrial topoisomerase I is blocked by the drug. If the 3' end-labeled pUC19 was used as a substrate for cleavage, and assuming that the mitochondrial topoisomerase I is linked to the 3' end of the broken DNA strand, one would expect the enzyme to be linked to the unlabeled DNA strand. In contrast, if 5' end-labeled DNA was used, one would expect the enzyme to be linked to the labeled DNA strand. When the DNA samples following CPT and mitochondrial topoisomerase I treatment were denatured by SDS, protein linked DNA strands migrate slower than

deproteinized DNA strands in agarose gel. As seen in Fig 7, when 5' end-labeled pUC19 was treated with CPT and 250 units of mitochondrial topoisomerase I, small DNA fragments were produced which migrated slower than their proteinase K-treated counterparts (Fig. 7 lanes E and F). No mobility shift was detected in 3' end labeled DNA (Fig. 7 lanes B and C). The drug did not produce a DNA cleavage in the absence of the enzyme (data not shown). This migration pattern indicates that the mitochondrial DNA topoisomerase I is linked to the 3' end of the broken DNA strand in CPT-induced cleavage assay.

We also initiated a kinetic analysis of the two enzymes, mitochondrial and nuclear type I topoisomerases, using pUC19, the plasmid which served as the substrate in the DNA binding experiments described above. We have performed standard supercoil relaxing assays with assay products separated by gel electrophoresis, visualized by EtdBr staining and quantitated by densitometry. Substrate concentrations between 5.8 to 76.9 μ M were investigated. Since the quantitative data were generated from the densitometric estimation of relative amounts of supercoiled plasmid to relaxed form using EtdBr-stained DNA bands, we, first, wanted to assess if there was any differential binding of EtdBr by closed circular and nicked DNA. This concept has been brought to our attention by an early study of Bauer and Vinograd (9) which indicates a difference in the dye binding of these two DNA population at certain EtdBr concentrations. We electrophoresed the samples of plasmid DNA on agarose, stained with 1 μg/ml EtdBr and photographed the gel. We then left the gel on the UV light box under illumination with short wave UV light for 30 and 60 minutes before rephotographing (Fig. 8) The supercoiled DNA band becomes completely nicked by 30 and 60 min UV irradiation and any change in band intensity would reflect the change in binding of open circular DNA relative to closed circular DNA. The result of this experiment was that we found no statistically significant difference in the binding of EtdBr by open or closed circular DNA at the concentrations of

Fig. 7. Mitochondrial DNA topoisomerase I was covalently linked to the 3' end of the broken DNA strand. Both 3' end labeled (lanes A-C) and 5' end labeled DNA (lanes D-F) were used in mitochondrial topoisomerase I cleavage assays. DNA samples were analyzed in 1.2% agarose. Lanes B, C, E and F contained 100 μM CPT and 250 units of mitochondrial topoisomerase I and lanes C and F contained plus 150 μg/ml proteins K. No enzyme was present in lanes A and D.

Fig. 8. Comparison of relative amounts of EtdBr binding by closed-circular and nicked forms of DNA. One hundred (lane A), 200 (lane B) and 300 (lane C) ng of pUC19 plasmid having both superhelical forms were electrophoresed on a 0.8 % Agarose gel, stained with 1μg/ml EtdBr and photographed (Panel I). The gel was left on the UV light box under illumination with short wave for 30 (Panel II) and 60 (Panel III) minutes before re-photographing. Dye binding were analyzed by densitometric quantification and one way ANOVA test was found to be p:0.1567.



dye that we were using to stain our gels (p;0.1567).

In order to obtain accurate initial velocities for the relaxation reactions, we generated plots of product formation versus time at 5.8, 11.5, 19.2, 30.8, 57.7 and 76.9 μ M supercoiled pUC19 concentrations for both mitochondrial and nuclear topoisomerase I. We have examined the kinetics by transforming the non-linear least-squares fitting of the initial velocities into double reciprocals of Lineweaver-Burk plots. The results of this experiment are given in Fig. 9. As seen in Fig. 9, the mitochondrial and nuclear type I topoisomerases gave different slopes which indicates different affinities of the two enzymes for pUC19. The Km values, derived from the slopes of Lineweaver-Burk plots were found to be 46.5±3.2 μM and 29.7±2.2 μM for mitochondrial and nuclear enzymes, respectively (see Table 2). On the other hand, Vmax values derived from the same plots were 4.10±0.28 for mitochondrial topoisomerase I and 5.62±0.37 for nuclear topoisomerase I (Table 2). Therefore the substrate affinity and maximal velocity of nuclear topoisomerase I were 1.6 and 1.4 times higher, respectively, than for the mitochondrial enzyme using pUC19 as substrate.

3.2. Preparation of recombinant plasmids containing selected regions of bovine mtDNA.

Evidence supporting the involvement of mitochondrial DNA topoisomerases in the replication and transcription of the mammalian mitochondrial genome has been reported previously (35, 112). We therefore chose to examine the interaction of the mt-topo I with supercoiled plasmids containing the mitochondrial heavy and light strand origins of replication as well as a region of mtDNA containing the major transcription termination signal to determine if the presence of mtDNA sequences would alter the kinetic parameters of mitochondrial topoisomerase I. We also selected a portion of the

Fig. 9. Lineweaver-Burk plots of mitochondrial and nuclear type I topoisomerases with pUC19 as substrate. The slopes of the linear part of the product formation versus time plots at 5.8, 11.5, 19.2, 30.8, 57.7 and 76.9 μM substrate concentrations were transformed into double reciprocal plots of 1/[S] versus 1/v (see "Materials and Methods" for detailed description of experiment).

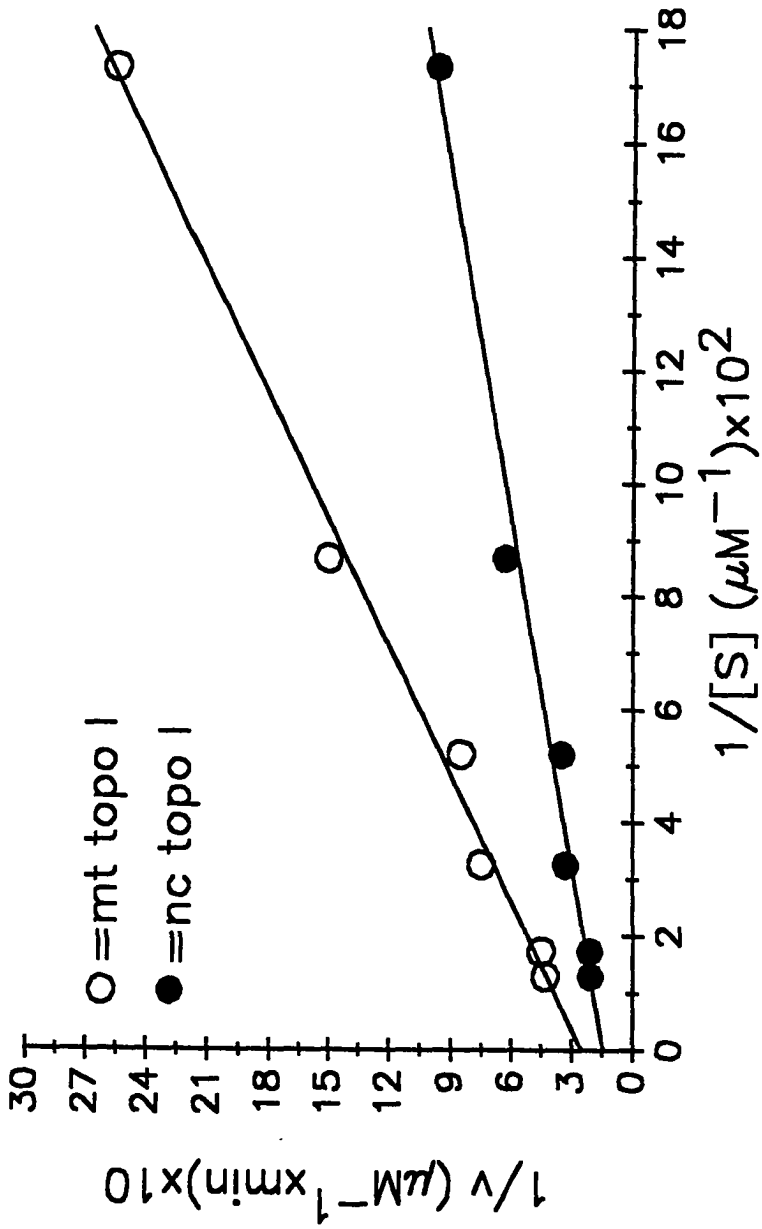


Table 2

Kinetic parameters of mitochondrial and nuclear type I topoisomerases with pUC19 substrate.

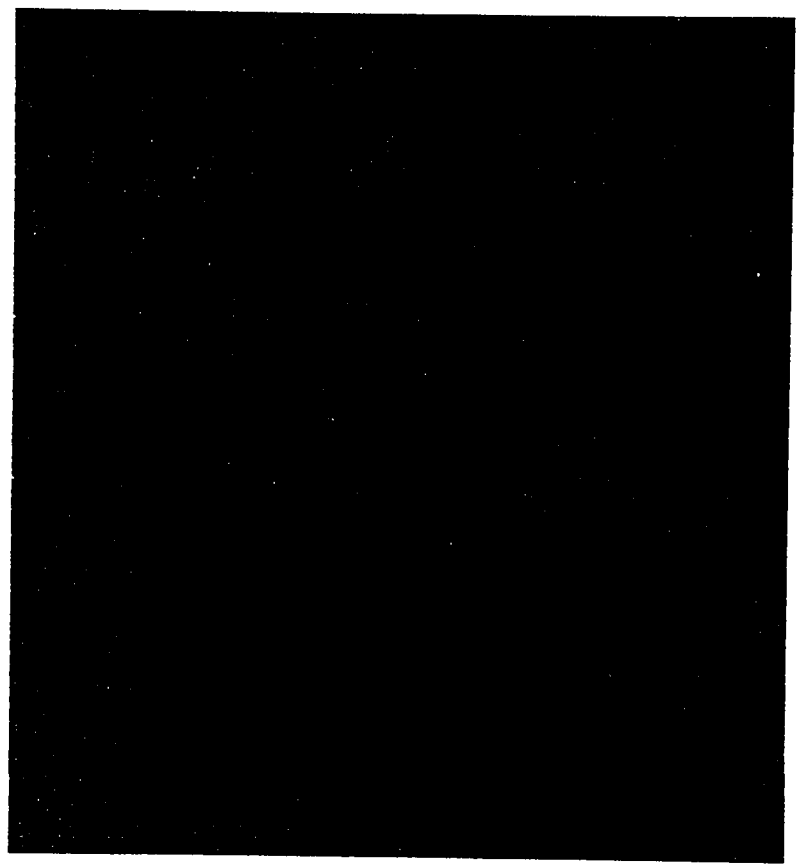
	Mitochondrial topoisomerase I	Nuclear topoisomerase I
Km (μM)	46.5 ± 3.2	29.7 ± 2.2
Vmax ($\mu M.min^{-1}$)	4.10 ± 0.28	5.62 ± 0.37

cytochrome b gene as being representative of a general mtDNA sequence.

Fig. 10A shows a schematic diagram of the circular mtDNA and the regions selected for amplification and insertion into the pGEM vector. The heavy strand origin fragment (H-ori) and light strand origin fragment (L-ori) are and 790 bp in length, respectively, while the transcriptional termination sequence (Term) and cytochrome b gene (Cyt b) coding region are 387 and 65 bp, respectively. These regions were amplified by PCR and inserted into pGEM-T plasmid as described in "Materials and Methods"

An agarose gel of the purified recombinant plasmids is shown in Fig. 10B. As a control for the two largest recombinants, pZT-Hori and pZT-Lori, we inserted an 800 bp sequence, isolated from a DNA molecular weight marker set, into pGEM. This control plasmid, pZT-800, is seen in lane D. Likewise, a 400 bp molecular weight marker fragment was inserted into pGEM vector to yield the plasmid pZT-400 (lane F) which served as control for two smaller recombinants, pZT-Term and pZT-Cytb. The proportions of supercoiled DNA in the purified plasmid DNA preparations was found to range from 86.5% to 94.0%. Since the rate of relaxation of supercoils is dependent upon the initial number of supercoils in the substrate (80), kinetic analysis was performed only on experimental and control groups (e.g.,, pZT-Hori and pZT-800) that possessed equivalent superhelical densities.Because of the well known low ligation and transformation efficiencies of cloning PCR-amplified DNA molecules into plasmid vectors, we have extended our effort to optimize ligation and transformation processes. The main difficulty arose because of the template-independent nucleotide addition by Taq DNA polymerase of a dATP to 3'-ends of double stranded DNA during PCR (159). The following strategies have been tested during the cloning experiments; a) Treatment of the ends of PCR products with Klenow fragment of *E. coli* DNA polymerase I and T4 DNA polymerase; b) Phosphorylation of PCR-amplified DNA inserts with T4 PNK and

Fig. 10. Schematic representation of PCR-amplified bovine mitochondrial DNA and agarose gel separation of the purified recombinant plasmids used in this study. a) Circular bovine mtDNA genome with the locations of the amplified regions. The nucleotide positions spanned by the PCR primers are indicated and the lengths of the amplified regions are: 797 bp and 790 bp for the heavy (H-ori) and light (L-ori) strand origins of replication, respectively; 387 bp for the transcription termination region (Term); and 365 bp for the cytochrome b coding region (Cyt b). b) Recombinant plasmid substrates (200 ng each) visualized on EtdBr-stained 0.8% agarose gel. Lane A, pGEM-T plasmid with no insert; B, pZT-Hori; C, pZT-Lori; D, pZT-800; E, pZT-Term; F, pZT-400; and G, pZT-Cytb.



dephosphorylation of vector DNA with CIAP prior to ligation; c) Optimum insert to vector ratio in ligase reaction during ligation; d) Optimum temperature, time, and enzyme concentration in T4 DNA ligase reaction; e)Effect of DNA concentration on recombinant plasmid recovery following the transformation of JM109 cells. The experimental procedures are detailed in "Materials and Methods". The results of individual steps were monitored in terms of the ratio of recombinants obtained per μg DNA for measuring the transformation efficiency.

Table 3 summarizes recombination efficiencies obtained in four different experimental groups treated with either Klenow or T4 DNA polymerase among the phosphorylated or unphosphorylated PCR inserts. As seen in the Table 3, neither Klenow nor T4 DNA polymerase treatments made the inserts compatible for ligation. The results show that phosphorylation of PCR-amplified DNA ,alone, was enough to give the highest yield although some recombinant colonies were also seen in PNK-untreated group (83% vs. 16 %). The ligation conditions for these four groups were essentially the same (see "Materials and Methods"). Molar ratio of insert to vector DNAs has been tested in three groups with optimized DNA ligase reaction (Table 4). The insert:vector ratios of 1:3 or 1:1 gave a significantly low transformation efficiencies ($1.2x10^3$ and $1.0x10^3$ per μg of recombinant DNA) while an insert to vector ratio of 3:1 resulted in the highest number of transformants ($3.5x10^4$ per μg DNA). Further increase in the relative insert concentration did not improve the ligation efficiency (data not shown). The optimal temperature, time and enzyme concentration during the ligation of experimental and control inserts are summarized in Table 5. Two out of these three parameters were hold constant in the analysis of individual experiments. The optimum conditions were found to be 4 °C, 12 hrs and 20 units enzyme during ligation as reflected with the higher number of transformants per μg DNA ($1.3x10^4$, $2.4x10^4$ and $3.5x10^4$ transformants per μg DNA, respectively) (Table 5). Table 6 shows the effect of the optimum DNA concentration during the

Table 3

Compatibility of PCR products for ligation following the treatments using Klenow fragment of DNA polymerase I, T4 DNA polymerase and T4 polynucleotide kinase. (ND = not detected).

Group	Klenow fragment of DNA Polymerase I	T4 DNA Polymerase	T4 Polynucleotide kinase	# of clones analysed	Positive Clones	Recombination efficiency (%)
1	-	-	-	6	1	16
2	+	-	+	9	ND	0
3	-	+	+	6	ND	0
4	-	-	+	12	10	83

Table 4

Effects of insert to vector ratios in the T4 DNA ligase reactions.

Group	Molar ratio of insert to vector	Number of transformants per μg of recombinant DNA
1	1:3	1.2×10^3
2	1:1	1.0×10^3
3	3:1	3.5×10^4

Table 5

Parameters affecting the ligation of PCR products (NA = not applicable).

Group	Definition	Parameter Studied	Number of Transformants per μg of recombinant DNA		
			A	B	C
1	Constant ligase concetration and time (20 μ/ml and 4 hrs)	Temperature 4°C (=A), 15°C (=B)	1.3×10^4	2.1×10^3	NA
2	Constant temperature and time 4° and 4 hrs)	Ligase concentration 10 (=A), 20 (=B), 50 (=C)	1.3×10^3	2.4×10^4	1.6×10^4
3	Constant temperature and ligase concentration (4° and 20 μ/ml)	Time (hr)	$1/4 \times 10^2$	3.5×10^4	NA

Table 6

Effect of DNA concentration in the trasformation efficiency of JM109 cells.

DNA concentration (μg/ml)	Number of transformants (per μg of total DNA)
2	1.4×10^3
5	1.6×10^3
10	3.5×10^4
50	1.8×10^3

transformation. As seen in Table 6, 10 μg DNA/ml gave appreciably higher number of transformants ($3.5x10^4$) than 2, 5 and 50 μg/ml concentrations ($1.4x10^3$, $1.6x10^3$ and $1.8x10^3$, respectively). The overall results, obtained from these experiments have been the basis of the experimental strategies during the construction of recombinant plasmids.

3.3 The mitochondrial topoisomerase I preferentially relaxes DNA containing the transcription termination sequence and the heavy strand origin of replication.

In order to determine whether the mt-topo I would preferentially interact with supercoiled DNA containing a particular region of mtDNA, standard relaxation assays were performed using a mixture of selected recombinant plasmids, e.g.,, pZT-Term and its appropriate control plasmid, pZT-400. If the mt-topo I preferred the plasmid with the mtDNA insert, then supercoils would be removed more rapidly from pZT-Term than pZT-400. With both plasmids in the reaction mix, the reaction products were distinguished using Southern hybridization. To specifically visualize the relaxation of pZT-Term a probe of digoxigenin-labeled, PCR-amplified Term (DIG-Term) (Fig. 10A, np 2774-3160) was used while the relaxation of control plasmid, pZT-400 was detected by hybridization to digoxigenin-labeled 400 bp molecular weight marker probe (DIG-400) complementary to the 400 bp insert in pZT-400. These results are shown in Fig. 11. Neither the DIG-Term nor the DIG-400 probes hybridized to pGEM vector (data not shown). As seen in the upper left panel of Fig. 11A, the supercoils in pZT-Term are relaxed more rapidly than those in pZT-400 (upper right panel). Densitometric quantification of reaction products revealed that the extent of relaxation of pZT-Term DNA was almost four times greater in the first eight minutes than that of pZT-400. On the other hand, there was little difference in the rate of removal of supercoils from pZT-400 and pZT-Cytb (Fig. 11A, lower left and right panels) indicating that the mitochondrial enzyme does not have a preference for the

Fig. 11A. Chemiluminescent detection by Southern hybridization of substrate specific products in 400 bp insert-containing group by mitochondrial topoisomerase I. Each reaction set contained 0.3 μM of each substrate. Upper left panel is the topoisomerase assay with a mixture of pZT-Term and pZT-400. Products were visualized by hybridization to a digoxigenin-labeled probe specific for Term. Upper right panel is the same assay products detected with a digoxigenin-labeled probe specific for the 400 bp molecular weight marker insert. The lower panels are similar assays using a mixture of pZT-Cytb and pZT-400 with visualization on the lower left using a probe specific for Cytb and, on the right, the 400 bp marker insert specific probe. Relative densities of relaxed DNA were quantified with LKB Ultrascan XL (see Figs 12A, 12B and 12C)

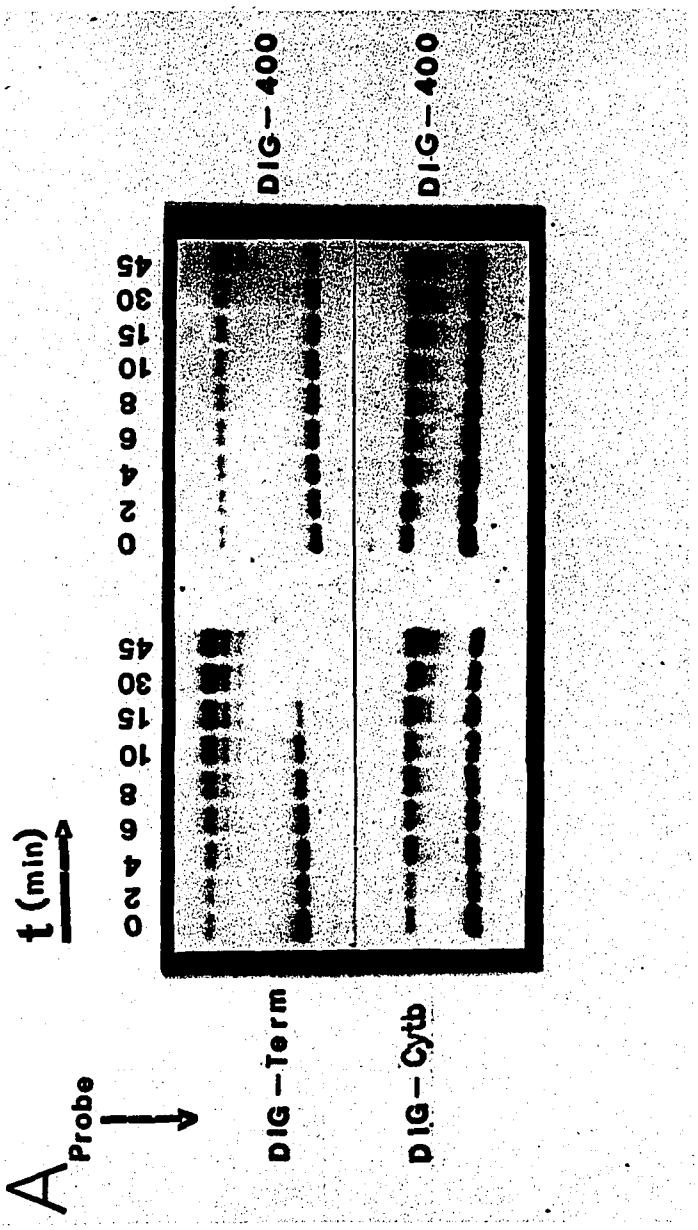


Fig. 11B. Chemiluminescent detection by Southern hybridization of substrate specific products in 800 bp-insert containing group by mitochondrial topoisomerase I assays. Time course of topoisomerase assay products detected as in 11A except using digoxigenin-labeled probes specific for pZT-Hori (upper left), pZT-Lori (lower left) and pZT-800 (upper and lower right). See Fig 11A legend for details of the assays.

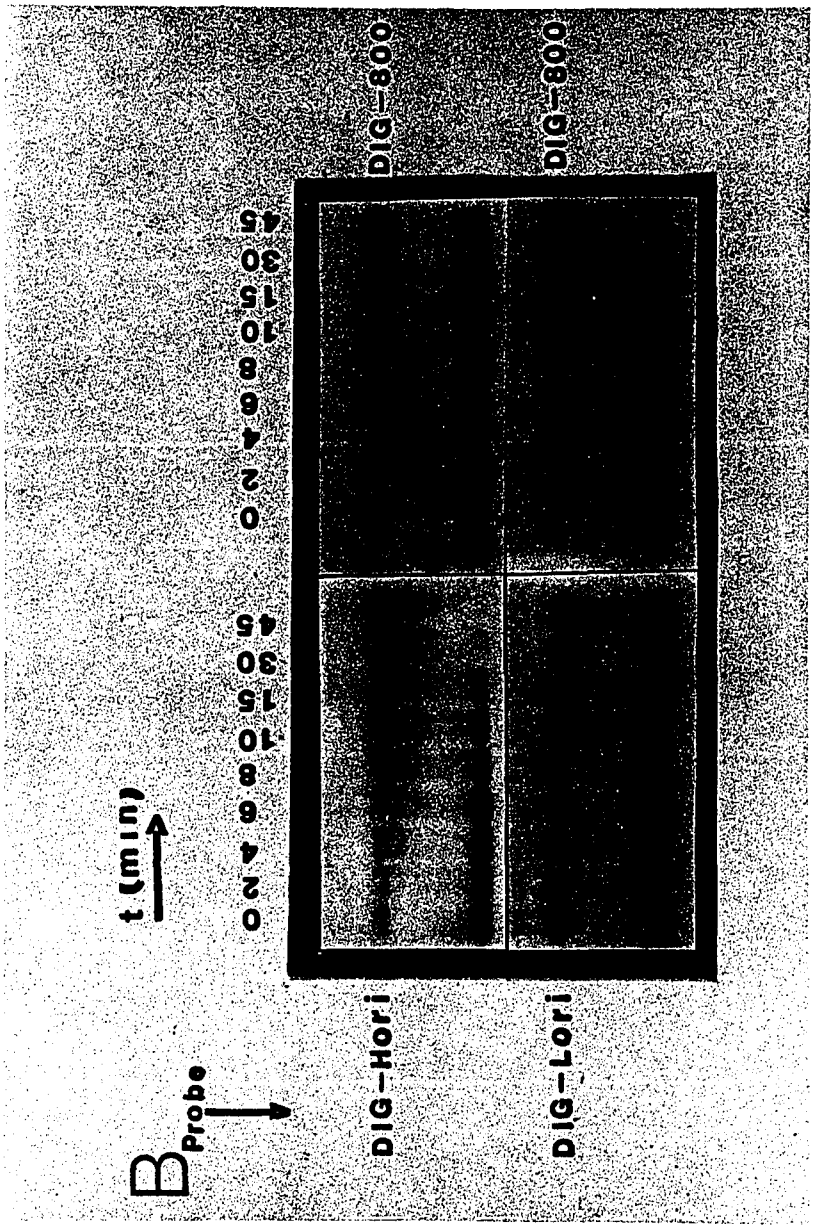
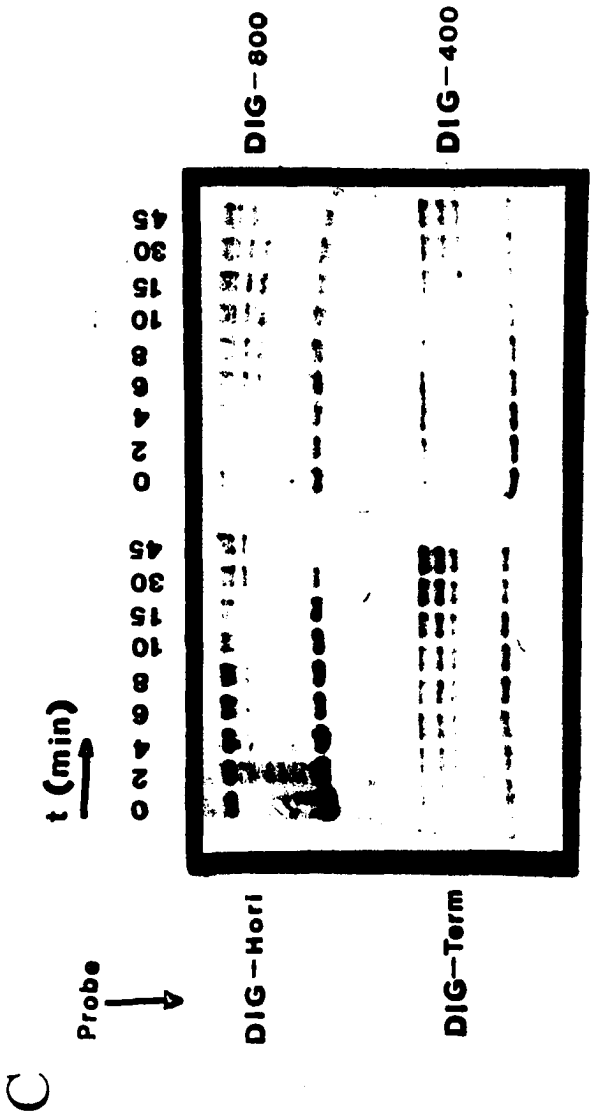


Fig. 11C. Chemiluminescent detection by Southern hybridization of substrate specific products in selected substrate groups by nuclear topoisomerase I. Time course of topoisomerase reaction products generated by the nuclear topoisomerase I detected by probes specific for pZT-Hori (upper left), pZT-Term (lower left), pZT-800 (upper right), and pZT-400 (lower right). See Fig 11A legend for details of the assays.



C

cytochrome b region compared to pZT-400. The amount of substrate loaded on the gel and detected by the hybridization to the membrane was slightly greater in the DIG-400 samples in the lower panel. This resulted in somewhat greater levels of slowly migrating nicked circular DNA being visualized. After densitometric quantitation and normalization for the amount of DNA on the membrane, there was no significant difference in the removal of supercoils from the 400 bp control-containing plasmid or the cyt b-containing plasmid. The same experiment was performed with the control plasmid pZT-800 and the two origin-containing plasmids, pZT-Hori and pZT-Lori. As Fig. 11B (upper left and right panels) shows, the mt-topo I removes supercoils preferentially from the plasmid containing the heavy strand origin of replication (compare time points 15, 30, and 45 min in both upper panels). Under these conditions there appears to be little, if any, preferential relaxation of pZT-Lori compared to pZT-800 (Fig. 11B, lower panels).

Figures 12A and 12B give the histograms of the densitometric estimation of the relative percent values of relaxed DNA, detected with DIG-labeled individual probes for paired substrate groups, presented in Figs 11A and 11B, respectively. In the 400 bp insert group, a significant difference in the relative amounts of relaxed DNA was obtained for pZT-Term (88.6% vs. 23.0% of pZT-Term and pZT-400, respectively; p=0.0039, and 36.0% vs. 37% of pZT-Cytb and pZT-400, respectively; p=0.2526) (Fig 12A) The amount of relaxed pZT-Hori in pZT-Hori/pZT-800 set reaches up to 89.5 % after 30 min while only 37.4 % pZT-800 has been relaxed at the same time interval in mt topo I-catalyzed reactions. (p=0.0070). The relaxed DNAs were comparable in pZT-Lori/pZT-800 pair for the same enzyme (39% vs. 32.8%; p=0.4770 after 30 min.) (Fig 12B). The above results suggest that the mitochondrial enzyme may prefer to interact with and relax supercoils in the regions of mtDNA containing the heavy strand origin of replication and the major transcription termination region. If this DNA-topoisomerase interaction was truly important for the mitochondrial enzyme, then we would not expect a nuclear

Fig. 12A. Graphical representation of the time course of percent relaxation of mitochondrial topoisomerase I in 400 bp insert pairs, given in Fig. 11A., as quantified with LKB Ultrascan XL. The paired t-test result at 95% confidence interval for pZT-Term/pZT-400 and pZT-Cytb/pZT-400 pairs were p;0.0039 and 0.2526, respectively.

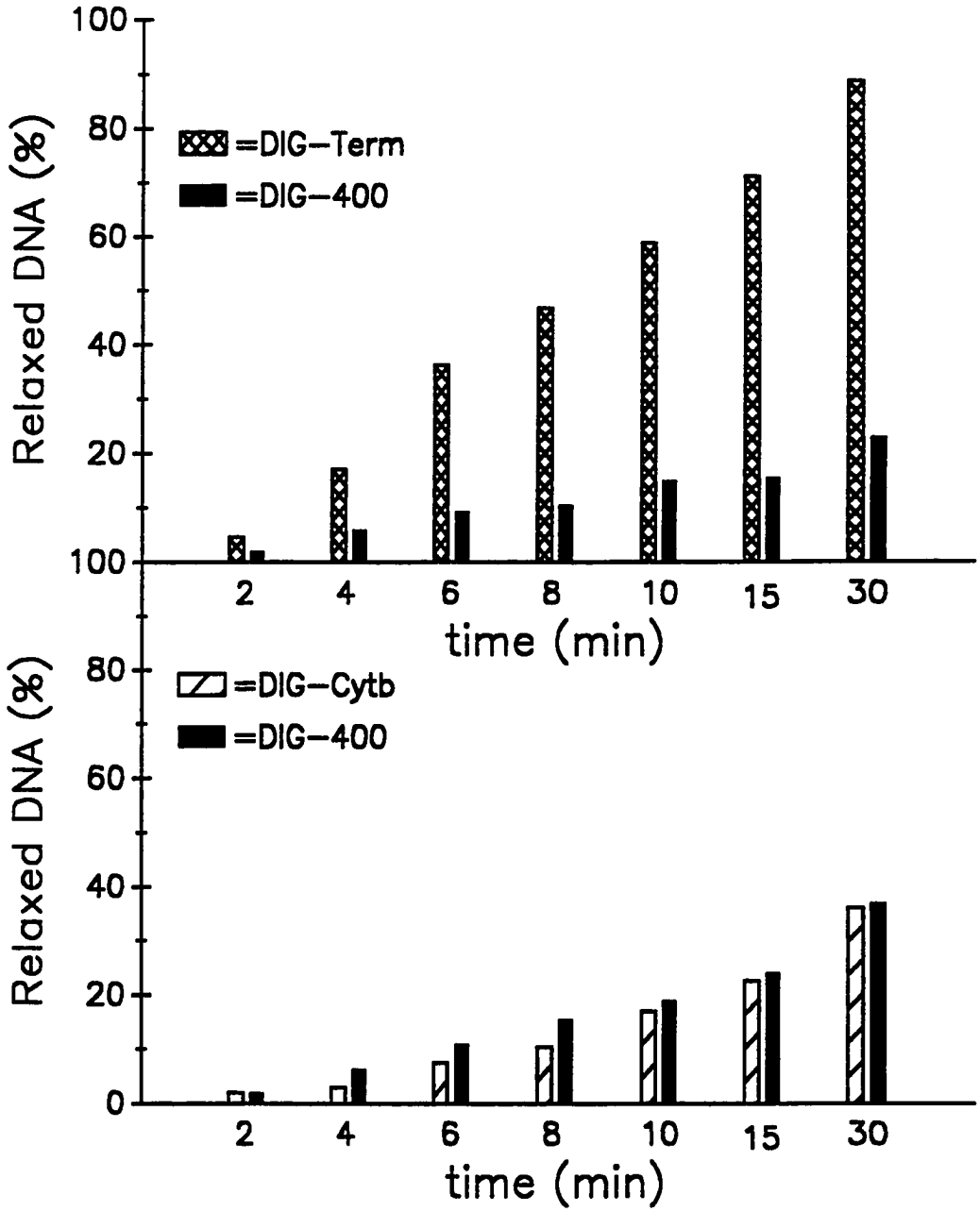


Fig. 12B. Graphical representation of the time course of percent relaxation of mitochondrial topoisomerase I in 800 bp insert pairs, given in Fig. 11B., as quantified with LKB Ultrascan XL. The paired t-test result at 95% confidence interval for pZT-Hori/pZT-800 and pZT-Lori/pZT-800 pairs were p;0.0070 and 0.1555, respectively.

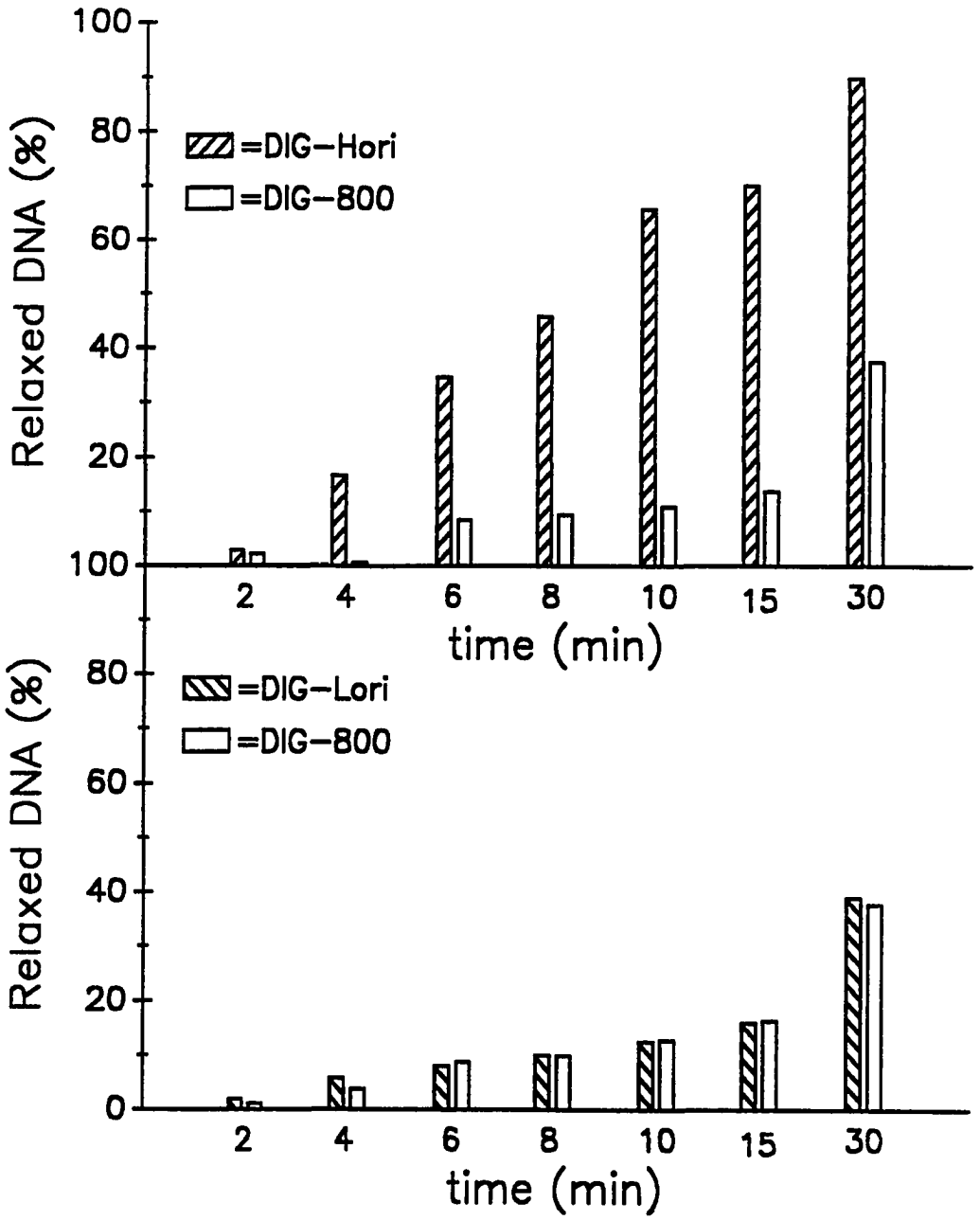
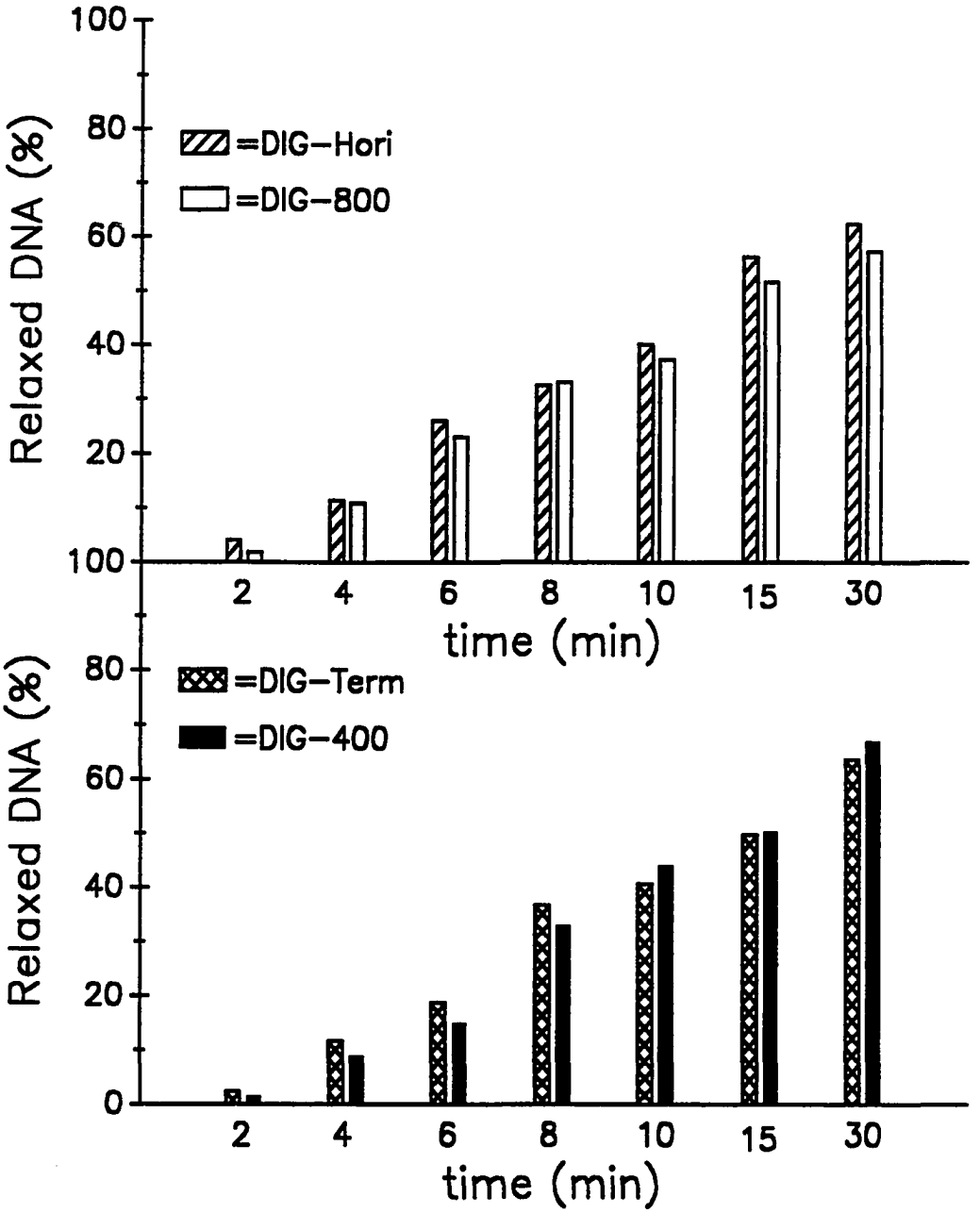


Fig. 12C. Graphical representation of the time course of percent relaxation of nuclear topoisomerase I in selected insert pairs, given in Fig. 11C., as quantified with LKB Ultrascan XL. The paired t-test result at 95% confidence interval for pZT-Hori/pZT-800 and pZT-Term/pZT-400 pairs were p;0.4620 and 0.4767, respectively.

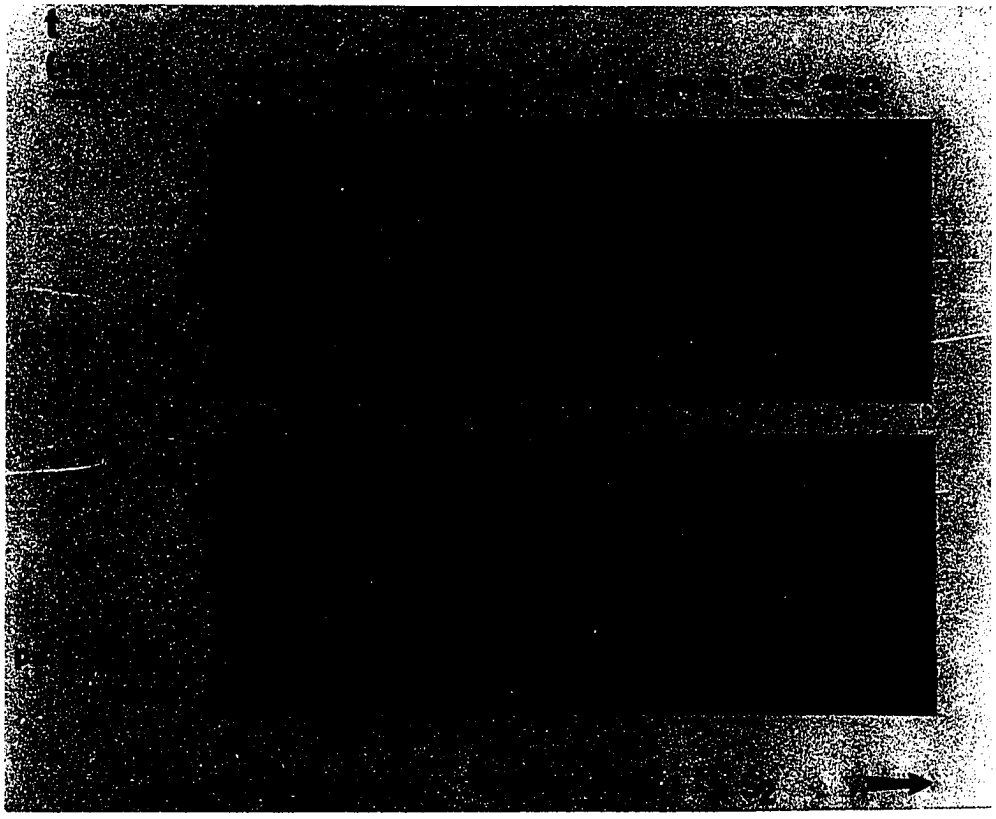


topoisomerase to manifest such a preference. Fig. 11C shows the results of performing the above experiment with the nuclear type I topoisomerase and the two substrates, pZT-Hori (Fig. 11C upper panel) and pZT-Term (Fig. 11C lower panel), seen to interact preferentially with the mt-topo I. Equal amounts of DNA were loaded in each lane on these gels, but the gels were blotted separately and there was unequal transfer and hybridization of DNA when comparing one membrane to another. However, we have normalized these differences by determining the percent supercoiled DNA relative to total DNA in each lane using densitometric analysis. Quantitation by densitometric analysis of the upper and lower panels reveals that the relaxation of supercoils in pZT-Term and pZT-Hori occurs at a rate equivalent to or very similar to that of the control pZT-800 or pZT-400 (Fig 12C). Neither the 800 bp nor the 400 bp plasmid groups showed more than minor differences in relaxation of DNA per unit time 62.0% vs. 57.0% of pZT-Hori and pZT-800, respectively; p=0.4620, and 63.7% vs. 67.0% of pZT-Term and pZT-400, respectively; (p=0.4767) (Fig 12C) Thus, the nuclear topoisomerase I shows no preference for interaction with these same mtDNA regions that seem to be preferred by the mitochondrial topoisomerase I.

3.4 Kinetic analysis of mitochondrial and nuclear type I topoisomerases

Although the above experiments indicate a preferred relaxation of supercoils in pZT-Term and pZT-Hori, the amount of substrate used (0.3 μM) was too low to calculate reliable kinetic parameters. Preliminary experiments using pUC19 revealed that the K_m for the mt-topo I was significantly higher than 0.3 μM, so we performed a kinetic analysis using a standard supercoil relaxing assays. Fig. 13 shows a typical result for the analysis of relaxation of pZT-Term and its control pZT-400 using mt-topo I. Aliquots removed at

Fig. 13. A representative supercoil relaxation reaction catalyzed by mitochondrial topoisomerase I using pZT-Term and pZT-400 in 5.1 μM [S1] and 10.2 μM [S2] concentrations. Triplicate experiments were performed in standard assay mixture with 30 units of enzyme and varying amounts of DNA in 200 μl total volume at 37 ^{o}C. Twenty microliter aliquots were removed at 0, 2, 4, 6, 8, 10, 12, 20, and 45 minutes, terminated with 5% SDS/ 25%(w/v) sucrose/ 0.025% bromophenol blue, and analyzed by electrophoresis on a 0.8% agarose gel. The gel was stained with EtdBr and photographed under UV light. Relative densities of relaxed DNA were quantified with LKB Ultrascan XL.



every time point show enhanced relaxation of pZT-Term relative to pZT-400 at either substrate concentrations S1 (5.1 μM) or S2 (10.2μM). Similar gel analyses were obtained for the other plasmids, pZT-Hori, pZT-Lori, pZT-Cytb and their controls pZT-800 and pZT-400. Likewise, similar analysis was performed using the nuclear type I topoisomerase. In order to obtain accurate initial velocities for the relaxation reactions, we generated plots of product formation versus time at 5.1, 10.2, 18.0, 30.0 and 45.6 μM recombinant substrate concentrations for both mitochondrial and nuclear topoisomerase I. A representative plot of data obtained at 5.1 μM substrate concentration for each of the recombinant plasmids with mt-topo I is shown in Fig. 14. After a linear increase in the removal of supercoils from the substrate at early time points, the reaction plateaus as the relaxed DNA molecules accumulate and begin to compete for binding of the topoisomerase. For example, this level of product inhibition results in a plateau at approximately 3.5 μM product when a starting substrate concentration of 5.1 μM was used. As can be seen, both plasmids containing the mitochondrial origins of replication were relaxed more rapidly than the 800 bp insert-containing control. Likewise, pZT-Term is seen to be relaxed more readily than either the 400 bp insert-containing control or the cytochrome b-containing plasmid, pZT-Cytb. Determination of the initial slopes of these curves allowed calculation of the initial velocities of the reactions and subsequent analysis by double-reciprocal plots. A Lineweaver-Burk plot of the mitochondrial enzyme relaxing supercoils in these plasmids is shown in Fig. 15. The group of substrates containing the 400 bp inserts are shown in Fig. 15A while Fig. 15B shows the group containing the 800 bp inserts. Examination of Fig. 15A shows that the V_{max} values were similar so that the differences in the slopes mainly reflect differences in the K_m values (Slope=K_m/V_{max}). Thus, relaxation of supercoils in pZT-Term would appear to be much preferred relative to control pZT-400. This was confirmed by statistical analysis of the slopes by one way

Fig. 14. A representative plot of product formation as a function of time at 5.1 μM substrate concentration. (npZT-Hori; lpZT-Lori; OpZT-800;p pZT-Term; pZT-Cytb; pZT-400). Correlation coefficients of the average values of triplicate measurements are: pZT-Term, 0.9260; pZT-Cytb, 0.9860; pZT-400, 0.9580; pZT-Hori, 0.9294; pZT-Lori, 0.9170; pZT-800, 0.9060.

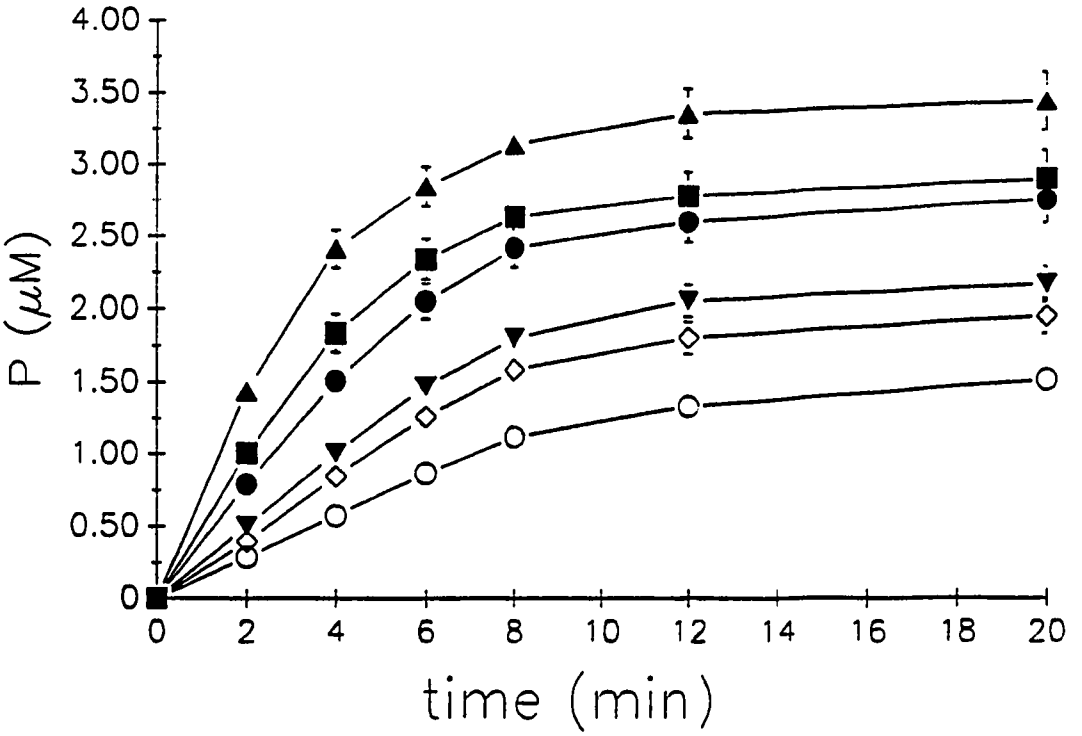
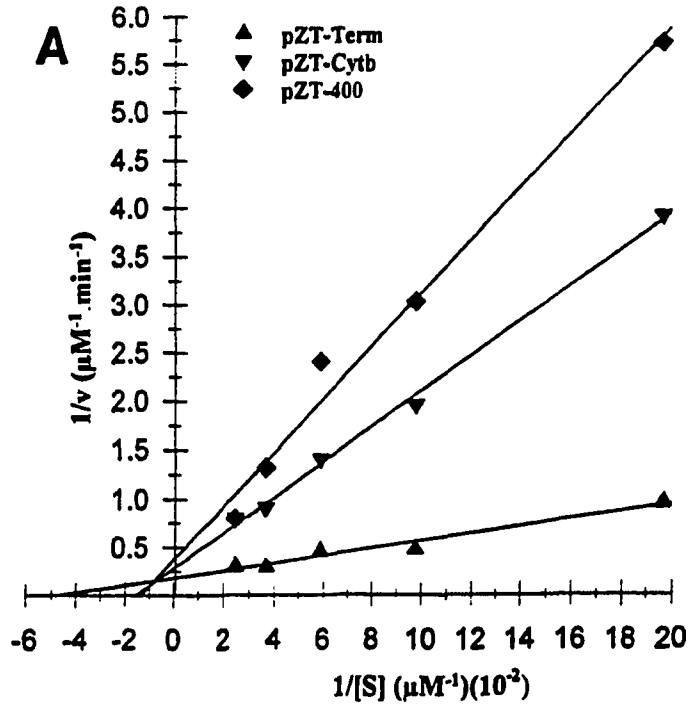
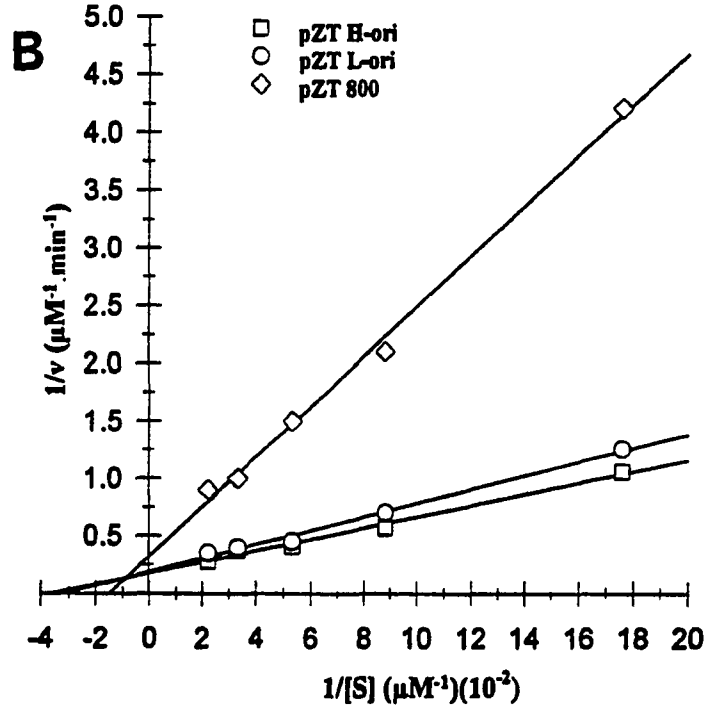


Fig. 15. Lineweaver-Burk plots for mitochondrial topoisomerase I using the 400 and 800 bp-containing recombinant plasmid groups. A, the 400 bp-containing plasmids pZT-400, pZT-Cytb, and pZT-Term. B, the 800 bp-containing plasmids pZT-800, pZT-Hori, and pZT-Lori. The slopes of the linear part of the product concentration versus time plots (See Fig. 14) for each substrate concentration were transformed into double reciprocal plots of 1/[S] versus 1/v. The slopes of substrate groups were compared to each other using the one way ANOVA test (see explanations in section 3.3 for the results of statistical analyses).



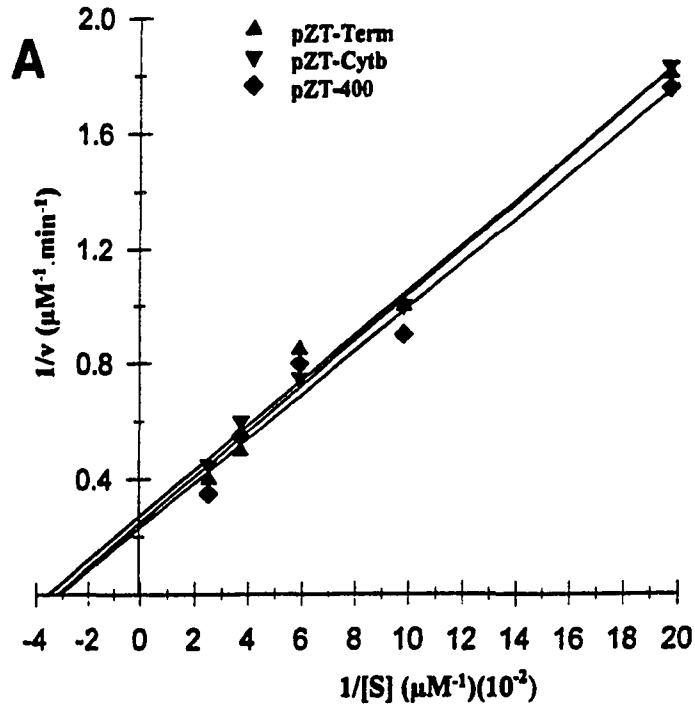


ANOVA, which revealed a significant difference between pZT-Term and pZT-400 ($p=0.03$) while the difference of the slopes of pZT-Cytb and pZT-400 was not significant ($p=0.66$) at a 0.05 confidence interval. Similar examination of the 800 bp group, shown in Fig. 15B, indicates that both pZT-Hori and pZT-Lori have significantly different slopes compared to pZT-800 ($p=0.04$). Although the above results indicated a preference of the mt-topo I for substrates containing specific mtDNA regions, to test whether this interaction was specific for the mitochondrial enzyme, the above kinetic experiments were repeated using the nuclear type I enzyme. Fig. 16A and 16B show that the nuclear enzyme exhibited no preference for relaxation of any of the substrates containing these specific mtDNA sequences. Statistical analysis of the slopes of the plots for pZT-Term and pZT-Cytb (Fig. 16A) and pZT-Hori and pZT-Lori (Fig. 16B) showed no significant difference from their controls, pZT-400 and pZT-800 ($p=0.35$ and $p=0.16$ for the 400 bp and 800 bp insert groups, respectively). Table 7 summarizes the kinetic parameters derived from the Lineweaver-Burk analyses. pZT-Term has a K_m of 17.0 ± 0.8 μM. This is 3.3-fold lower than the K_m for the control plasmid pZT-Term (56.3 ± 2.3 μM). Likewise, pZT-Hori and pZT-Lori have K_m values of 21.0 ± 0.9 μM and 25.2 ± 1.0 μM, respectively, which are 2.7 and 2.3 times lower than the K_m for their control, pZT-800 (57.5 ± 2.1 μM). By comparison, the plasmid containing the Cytb gene region (pZT-Cytb) has a K_m value of 53.4 ± 2.0 μM which differs from the control (pZT-400; 20.0 ± 0.7 μM) by only 5%.

The kinetic analysis of the interaction of the nuclear topoisomerase I with the same substrates indicates that this enzyme has no preference for any plasmid containing mtDNA sequences. K_m values ranged from 31.8 ± 1.8 μM to 33.6 ± 1.7 μM for mtDNA-containing substrates while those for the controls were 33.7 ± 1.8 μM to 34.7 ± 1.7 μM.

Table 7 also lists values of V_{max} attained for each substrate. For the 800 bp insert

Fig. 16. Lineweaver-Burk Plots for the nuclear topoisomerase I using the 400 and 800 bp-containing recombinant plasmids. A, the 400 bp-containing plasmids, pZT-400, pZT-Cytb, See Fig. 16 legend for the derivation and pZT-Term. B, the 800 bp-containing plasmids, pZT-800, pZT-Hori, and pZT-Lori.



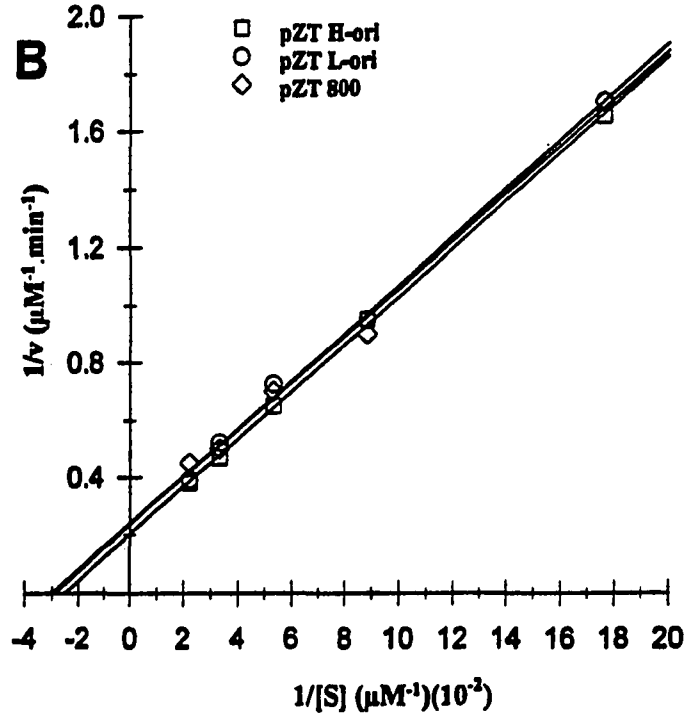


Table 7

Kinetic parameters of bovine mitochondrial and nuclear type I topoisomerases with recombinant plasmid substrates.

	Mitochondrial topoisomerase I		Nuclear topoisomerase I	
Substrate	[a]K_m (μM)	[b]V_{max} ($\mu M^{-1}min^{-1}$)	[a]K_m (μM)	[b]V_{max} ($\mu M^{-1}min^{-1}$)
pZT-Hori	21.0 ± 0.9	5.20 ± 0.23	32.6 ± 1.8	3.80 ± 0.18
pZT-Lori	25.2 ± 1.0	5.20 ± 0.23	31.8 ± 1.8	4.15 ± 0.19
pZT-800	57.5 ± 2.1	3.00 ± 0.15	33.7 ± 1.8	3.90 ± 0.18
pZT-Term	17.0 ± 0.8	4.85 ± 0.23	33.6 ± 1.7	4.10 ± 0.17
pZT-Cytb	53.4 ± 2.0	3.40 ± 0.16	31.8 ± 1.8	3.76 ± 0.18
pZT-400	56.3 ± 2.3	2.95 ± 0.15	34.7 ± 1.7	4.00 ± 0.19

[a] The differences between the two enzyme groups means were found to be significant in one way ANOVA test (two-tailed); [a] p=0.0063 [b] p=0.030. The variations of experimental plasmids, pZT-Hori, pZT-Lori, pZT-Term and pZT-Cytb relative to control plasmids, pZT-800 and pZT-400 for a given parameter were normalized for obtaining the relative percent differences, therefore, the p values in the ANOVA test represent the significance for mitochondrial and nuclear type I topoisomerases for the same plasmid groups.

groups, both pZT-Hori and pZT-Lori had a V_{max} value of 5.20 ± 0.23 μM.min^{-1} which is 73% higher than the V_{max} attained with the control plasmid pZT-800 (3.00 ± 0.15 μ M.min^{-1}). Likewise, V_{max} for pZT-Term (4.85 ± 0.23 μM.min^{-1}) was 64% greater than the V_{max} seen with pZT-400 (2.95 ± 0.15 μM.min^{-1}). In contrast, pZT-Cytb showed a V_{max} which was only 15% greater than the V_{max} of control plasmid, pZT-400.

For the nuclear enzyme, where no significant difference was seen in K_m values, no significant variation in V_{max} was observed for either control or mtDNA-containing substrates (between 3.76 ± 0.18 μM.min^{-1} and 4.10 ± 0.17 μM.min^{-1} in the 400 bp insert set and 3.80 ± 0.18 μM.min^{-1} and 4.15 ± 0.19 μM.min^{-1} in the 800 bp insert set).

From the K_m and V_{max} values we can also calculate several other meaningful kinetic parameters. As seen in Table 8, the turnover numbers of the mitochondrial topoisomerase acting on the preferred substrates pZT-Hori and pZT-Lori are 26.26 ± 1.16 s^{-1}. These values are about 1.73 times larger than the value for the non-preferred substrate pZT-800 (15.15 + 0.76 s^{-1}). Similarly, the turnover number for the recombinant plasmid pZT-Term (24.49 ± 1.16 s^{-1}) is 1.6 times larger than k_{cat} for the 400 bp insert-containing control pZT-400 (14.90s^{-1}). Because the physiologic concentration of an enzyme's substrate is typically close to the K_m, it is unlikely that the topoisomerase will be operating in an environment where the substrate is near V_{max} and hence k_{cat} differences may be over interpreted. In the situation where [S] < K_m, a better measure of the ability of an enzyme to distinguish the substrates would be the specificity constant, k_{cat}/K_m. As seen in Table 8, pZT-Hori and pZT-Lori have specificity constants 4.8 and 4.0 times higher, respectively, than that of pZT-800. The mitochondrial enzyme has the greatest preference for pZT-Term, where the specificity constant is 5.5 times greater than that for its control pZT-400.

Table 8

Turnover numbers and specificity constants of the mitochondrial and nuclear type I topoisomerases with recombinant plasmid substrates.

	Mitochondrial topoisomerase I		Nuclear topoisomerase I	
	[a]k_{cat}	[b]k_{cat}/K_m	[a]k_{cat}	k_{cat}/K_m
Substrate	(s^{-1})	($M^{-1}s^{-1}$) (10^6)	(s^{-1})	($M^{-1}s^{-1}$)(10^6)
pZT-Hori	26.26±1.16	1.25	10.64 ± 0.50	0.33
pZT-Lori	26.26 ± 1.16	1.04	11.85 ± 0.50	0.36
pZT-800	15.15 ± 0.76	0.26	10.92 ± 0.50	0.32
pZT-Term	24.49 ± 1.16	1.44	11.48 ± 0.50	0.34
pZT-Cytb	17.17 ± 0.81	0.32	10.48 ± 0.50	0.33
pZT-400	14.90 ± 0.76	0.26	11.20 ± 0.50	0.32

The differences between the two enzyme group means were found to be significant in one way ANOVA test (two-tailed); [a]: p=0.0470 [b] p= 0.0257. The variations of experimental plasmids, pZT-Hori, pZT-Lori, pZT-Term and pZT-Cytb relative to control plasmids, pZT-800 and pZT-400 for a given parameter were normalized for obtaining the relative percent differences, therefore, the p values in the ANOVA test represent the significance for mitochondrial and nuclear type I topoisomerases for the same plasmid groups.

CHAPTER FOUR DISCUSSION

Our laboratory has been studying DNA topoisomerases isolated from mammalian mitochondria for a number of years (35, 109-112, 117, 160) and recent improvements in purification and recovery have allowed us to perform extensive enzyme characterization. In the present study we have found that the mitochondrial topoisomerase I demonstrates a significant preference for removing supercoils from substrates that contain specific mtDNA sequences. The bovine mitochondrial enzyme exhibited the strongest preference for substrates containing the bovine mitochondrial heavy strand and light strand origins of replication and the major transcription termination sequence located within the tRNALeu gene located immediately downstream from the 16S ribosomal RNA gene. Specific preferred interactions of these mitochondrial DNA sequences with a mitochondrial topoisomerase might not be unexpected since prior evidence (35, 105, 106) has indicated the involvement of the mitochondrial topoisomerase in the processes of replication and transcription of the mitochondrial genome.

4.1 Kinetic parameters and the polarity of DNA linking of mitochondrial topoisomerase I. DNA with pUC19 as substrate.

Use of anti-tumor drugs that target DNA topoisomerases has greatly facilitated the characterization studies on the mechanism of topoisomerase-catalyzed reactions. We have employed CPT in our study to detect the cleavable complexes formed between mitochondrial topoisomerase I and pUC19 after protein denaturation with SDS (142). Our result indicates that the mitochondrial enzyme binds to 3' end of pUC19 (Fig 7). Although our laboratory has several lines of biochemical and biophysical data indicating that mitochondrial topoiomerase I has similarities to both the prokaryotic and eukaryotic type I topoisomerases, the DNA linkage property of this enzyme has been found to be similar to

its nuclear counterpart. This is in agreement with a study by Mukherjee *et al* (37) who reported the binding of pea chloroplast topoisomerase I to the 3' end of its DNA substrate. No obvious pattern emerges that suggests a possible functional significance to the different polarities of attachment. It seems likely therefore that the different polarities represent equivalent, independent solutions to the same mechanistic problem. The initial kinetic experiments were part of the characterization studies of the mitochondrial enzyme. The data, obtained from this set of experiments served as the basis of our further kinetic studies that involved using mtDNA-containing recombinant plasmids to determine if the Km and Vmax parameters of mitochondrial topoisomerase I were sensitive to the presence of certain mtDNA sequences in its substrate DNA.

4.2. Preparation of recombinant plasmids containing mtDNA

The experiments during the cloning of mitochondrial and non-mitochondrial DNA inserts have been extended to search for an optimum set of conditions for ligation of PCR-amplified DNA into plasmid vectors and transformation of bacterial cells with recombinant plasmids. The investigation of alternative approaches, outlined in Tables 2-5, arose because of the inefficiency of the standard ligation process due to 3'-A overhangs of the PCR products which were introduced by the terminal transferase activity of Taq DNA polymerase (159). pGEM-T vectors were found to be a convenient system for cloning of PCR products as single 3'-T overhangs on the vector take advantage of the 3' protruding A on the PCR product. One strategy for high-efficiency cloning of blunt end DNA molecules involves dephosphorylation with CIAP. Using this strategy in the ligation of control inserts was necessary to prevent vector self-ligation, a parameter frequently addressed as a cause of lowering ligation and transformation efficiencies (148, 151). The ligation of gel-excised DNA inserts required removal of 3'-T overhangs from vector DNA, thereby increasing the potential of vectors for self-ligation. Dephosphorylation with CIAP

in this group resulted a considerably higher success in ligation and transformation (data not shown). However, we also found CIAP reaction as a factor increasing the efficiency of ligation of PCR-amplified inserts into pGEM-T vectors although vector DNAs were not expected to undergo a self-ligation due to the presence of 3'-T overhangs. This observation could be attributed to a possible removal of overhanging dTMPs by contaminating exonucleases and/or the presence of vectors without 3'-T overhangs in the pGEM-T population.Treatment of the PCR-amplified DNAs with Klenow fragment of DNA polymerase I did not make the DNA fragments compatible for ligation (Table 3). Likewise, treatment of PCR products with T4 DNA polymerase did not result in the formation of recombinant colonies (Table 3). The 3'-exonuclease activity or proof reading activity of the T4 DNA polymerase acts non-processively and indiscriminately on all 3'-OH termini whether the ends are blunt or have either 3'- or 5'- overhangs. The failure to obtain successful ligation could be attributed to the insufficient saturation of 3'-ends of DNA occurs due to low affinity of T4 DNA polymerase for 3'-ends (153). Optimal conditions for T4 DNA ligase concentration, reaction temperature and time were 100 u/ml, 4^0C and 12 hr, respectively (Table 4). The optimal molar ratio of insert to vector DNA was 3:1 (Table 5). On the other hand, maximal efficiency was seen with DNA concentrations of 10 μg/ml (Table 6). This was observed by the yield of recombinant bacterial colonies after ligation. Therefore, the insert to vector ratio and the total DNA concentration seem to be essential parameters for efficient ligation and recombinant plasmid recovery. However, we have limited the number of parameters due to time consideration as the recombinant plasmids would serve as experimental tools in the characterization of mt topo I. A more definitive tabulation of the procedures, followed, and the characteristics of individual parameters need further detailed examination in a study, specifically addressing the "efficiency" of ligation PCR products and their efficient transformation into bacterial cells.

4.3. Substrate competition assays with paired recombinant plasmids

The first set of experiments using recombinant plasmids with Southern hybridizations and chemiluminescence detection clearly revealed pZT-Term and pZT-Hori as preferential substrates for mt-topo I (see Fig. 11A and 11B). Studies were carried out in paired-substrate mixtures with appropriate plasmid controls (pZT-400 and pZT-800) verifying the specificity of the preference of the mitochondrial enzyme for the substrates containing the heavy strand origin of replication and the transcription termination region. Relaxation rates of the same control and mtDNA-containing substrates with the nuclear topoisomerase I were not significantly different, further supporting the specificity of the mitochondrial enzyme for the mtDNA-containing plasmids, pZT-Term and pZT-Hori (Fig. 11C). Quantification of relative relaxed DNA and statistical evaluation of individual groups confirmed this conclusion (see Fig 12A, B, and C). As seen in Fig 12A, the relaxation rate of pZT-Term was 3.9 times more than that of pZT-400 as detected by their appropriate probes at 30 minute aliquots (Fig 12A). Likewise, a 2.4 times more relaxed pZT-Hori was detected when compared to pZT-800 (Fig 12B). Although pZT-Lori was slightly more preferential for mitochondrial enzyme, relative to pZT-800 (15 % at 30 minute time interval), this difference was not equally significant since the nature of the Southern blotting and chemiluminescence detection also carries some degree of inequality in the amount of DNA transfer and/or detection of bands during hybridization as much as 10%. On the other hand, the difference in the relaxation of these substrate pairs by nuclear topoisomerase I were found to be between 4.6 and 4.9 % for 800 and 400 bp groups, respectively (12C).

4.4. Kinetic analysis of mitochondrial topoisomerase I using recombinant substrates.

The second set of experiments with recombinant plasmids employed quantification of relaxed DNA by microdensitometer to determine the kinetic constants. In this set, pZT-Lori was found to be a third preferential substrate as manifested by its low K_m value (25.1 ± 1.0 μM), comparable to those for pZT-Term and pZT-Hori (21.0 ± 0.9 μM and 17.0 ± 0.8 μM, respectively). The appearance of pZT-Lori as a preferential substrate relative to the control plasmid in the second set of experiments, but not in the chemiluminescence experiments, was due most probably to the low substrate concentration (0.3 μM) used in the latter set of experiments. The determination of reliable kinetic parameters generally requires the use of substrate concentrations at or near the K_m and, in the chemiluminescence experiments, a substrate concentration (0.3 μM) was not sufficient to allow detection of any preferential mitochondrial topoisomerase-DNA interaction. However, with substrate concentrations ranging from 5.1 to 45.6 μM in the kinetic experiments, pZT-Lori was seen to be a preferred substrate in the same category as pZT-Hori and pZT-Term. The specificity of the interaction of the topoisomerase with these mtDNA regions was indicated by the enhanced relaxation of supercoils in pZT-Hori, pZT-Lori, and pZT-Term relative to the 800 and 400 bp containing control plasmids. In addition, the absence of similar stimulated relaxation of supercoils in pZT-Cytb indicates that the mitochondrial enzyme does interact preferentially with certain mtDNA regions and not with others. This is also evident when the Km and Vmax parameters of mitochondrial topoisomerase I obtained with pZT-Term, pZT-Hori and pZT-Lori (Table 2), are compared to the ones with pUC19 (Table 7). Although these two sets of plasmids were different from each other in size, utilization of mtDNA inserts in substrate DNAs resulted an increase in substrate affinity by almost 2.7 times. Although, the H-ori, L-ori, and Term regions are A-T rich regions of the mtDNA, we do not believe that this preferential interaction with the mt-topo I is merely due to a fortuitous A-T rich base

composition in certain plasmids. Table 9 shows that the average A-T content for pZT-Hori, pZT-Lori, and pZT-Term is 60.3% which is only 5.5% higher than the A-T rich Cytb region (54.8%). This small difference in A-T content is unlikely to be responsible for the two to three-fold differences we see in K_m or the four to five-fold changes observed in the specificity constants. In fact, as seen in Table 8, the mitochondrial preferred region Term, which has an A-T composition closest to that of Cytb, has the lowest K_m, 17.0 +0.8 μM.

Although there was a sizable variation in K_m values (2.3 to 3.3-fold), the variation in V_{max} was somewhat smaller (64 to 73%). It should be noted that, unlike K_m, V_{max} is not a kinetic constant. This value depends on the catalytic rate constant for product formation (K_p) and the total concentration of the enzyme in the assay mixtures. The compromise in our study was to utilize a relatively low concentration of enzyme in order to monitor reaction products at early time points when the product concentration was less than one tenth of the initial substrate concentration. This approach minimized possible product inhibition and provided accurate initial velocity calculations for individual reaction sets.

Turnover numbers, given in Table 8, represent the theoretical V_{max} in the presence of one molar enzyme or, more practically, the number of reaction processes (turnovers) that each active site on the enzyme catalyzes per unit time. Interpretation of turnover number is further complicated by the fact that the number of active sites and subunit structure of mitochondrial topoisomerase I has yet to be clarified. Moreover, the kcat determinations are based upon the enzyme operating at V_{max} when sufficiently high substrate concentrations are present. In the more general case where $[S] < K_m$, a better indicator of catalytic efficiency would be the specificity constant.The specificity constant,

Table 9

Base composition of PCR-amplified mtDNA regions inserted into pGEM

Region	Nucleotides	A-T(%)	G-C(%)	K_m (μM)
H-ori	15,691-149	60.8	39.2	21.0 ± 0.9
L-ori	5,147-5,936	60.3	39.7	25.2 ± 1.0
Term	2,774-3,160	59.7	40.3	17.0 ± 0.8
Cytb	14,579-14,943	54.8	45.2	53.4 ± 2.0

K_m/k_{cat}, represents how fast the reaction of a given substrate would occur when the substrate is bound to the enzyme (k_{cat}) and how much of the substrate is required to reach half of the V_{max} (K_m). This ratio essentially establishes that the rate of the reaction will vary directly with how often the enzyme and substrate come together in solution. As expected from their lower K_m values, pZT-Term, pZT-Hori and pZT-Lori were found to have higher specificity constant relative to their controls (see Table 8).

The interaction of topoisomerases with specific DNA sequences has mainly been addressed by examination of the cleavage reaction catalyzed by these enzymes (122, 125, 132). These studies have, for the most part, been performed with the nuclear topoisomerase I. The consensus sequences inferred from these analyses are highly degenerate and the enzyme is reported to be highly promiscuous to the proposed sequences. Although there are clearly discrete cleavage sites along a substrate DNA molecule, a strong bias for a specific sequence has been shown in only one case, that of the region flanking the Tetrahymena ribosomal RNA genes. This sequence interaction may be associated with the involvement of the topoisomerase in the transcription of rRNA genes in the nucleolus (125, 132). The regions of single stranded or double stranded DNA that have the potential for intramolecular base pairing to form cruciforms have also been proposed as favorite recognition sites for both the nuclear type I and type II topoisomerases (142, 144, 161). DNA molecules used in these cleavage studies have varied from short oligomers and synthetic polymers of 40-mer to natural DNA molecules of several kilobases.In one other study by Busk *et al*, (124), the interaction of Tetrahymena nuclear topoisomerase I with the rRNA-topoisomerase preferred cleavage site was investigated by inserting the proposed sequence motif into pBR322. These authors reported a preferential relaxation of the recombinant plasmid substrate by the nuclear topoisomerase. However, this preference was estimated by comparison to native pBR322 control plasmid which differed from the experimental substrate in size. No kinetic

parameters were determined or reported in the Busk *et al.* study (124). Comparisons in our study have been based on the control substrates, pZT-400 and pZT-800, which are recombinant plasmids having the same size inserts as the experimental substrates, pZT-Hori, pZT-Lori, pZT-Term, pZT-Cytb thus eliminating size-dependent differences in the observed rates of relaxation. The studies by Dr. Lin (118), using drug-induced DNA cleavage patterns of mitochondrial topoisomerase I have reported a preference for T residue at the -1 position at cleavage sites and a purine rich region 5'-adjacent to the T. Of the cleavage sequences, identified by Dr. Lin, three sequences in two regions of the lower strand of Term fragment have been reported as preferential interaction sites. These sequences have an unusual cleavage of G at -1 position. All three sequences in Dr. Lin's results have sequence elements which could potentially lead to the formation of cruciform structures. Therefore, appearance of pZT-Term plasmid as the most preferred one is in close confirmation with Dr. Lin's results. Examination of results obtained in studies with other DNA binding proteins suggests that the differences in kinetic parameters obtained with the mt-topo I in the present work are physiologically significant. For example, the affinity of RNA polymerase II to three different TATA box promoters in adenovirus was reported to vary three to four-fold as monitored by the relative strengths of productive transcripts (162). In addition, several point mutations 5' to the Xenopus tRNA selenocysteine TATA box were found to alter RNA polymerase III transcription rates within a range of 50 to 70% (163). Therefore, the 2.3 to 3.3-fold lower K_m, as well as the 60-75% higher V_{max} values, should be considered as physiologically significant characteristics of the ability of the mitochondrial topoisomerase I to interact with important mtDNA sequences.

Protein DNA interactions play essential roles in many cellular activities, including restriction and modification of DNA, chromatin assembly, replication, transcription and recombination. Various studies have indicated that different DNA binding proteins exhibit

different sequence requirements for their DNA substrates. At one end of the spectrum are proteins such as the bacterial type II restriction enzymes that recognize unique DNA sequences. Other proteins, however, bind sites with certain degrees of sequence degeneracy. This class of proteins includes phage 434 repressor (164), *E. coli* catabolite activator protein (CAP) (165) and mammalian CCAAT-binding proteins (166) etc. At the other end of the spectrum are enzymes such as DNase I (167) which bind to DNA helices with no apparent sequence specificity. Our overall results indicate that the presence of certain DNA sequences significantly increases the affinity of mitochondrial topoisomerase I for a DNA substrate. Our attempts for footprinting the minimum DNA-binding sites of enzyme did not give an interpretable result to explore the sequence (data not shown). On the other hand, the enzyme does exhibit a certain level of catalytic activity on supercoiled plasmid substrates that lack mitochondrial DNA sequences. This result can be interpreted as a limited specificity for nucleotide sequence in DNA, rather than a digital recognition or an absolute sequence dependence. Another way of concluding the relative preference of certain defined substrates by mt topo I would be that the presence of certain DNA sequences appreciably enhances the catalytic activity of the enzyme but the secondary structure of DNA is another parameter to be taken into consideration for the dictation of the enzyme's affinity.

Is the limited sequence specificity displayed by the mitochondrial enzyme of any biological significance? Given the low degree of specificity and the corresponding wide range of substrate utilization, it would appear that the sequence preference of the mt topo I does not bring a digit limit on the access of the enzyme to DNA. Indeed, the enzymes may have been designed by evolution to act on supercoiled DNA. If this speculation is true, than the observed preference of mt topo on the plasmids containing certain mtDNA sequences is probably based on the best steric fit between the active site regions of the enzyme and the DNA strand to be broken. Taken together, our laboratory currently has

sequence data as short as individual preferential nucleotide positions at the cleavage site of mitochondrial topoisomerase I and 400 and 800 bp sequences revealing considerably high affinity for the enzyme. The further step of our study might be footprinting analysis to estimate the minimum binding region out of the mtDNA sequences, covered in this study. Indeed, we have initiated preliminary footprinting experiments in our laboratory. The kinetic parameters, estimated in our study will be a parameter to distinguish the mitochondrial topoisomerase I from its nuclear counterpart. Considering the fact that the mitochondrial enzyme preparations are frequently questioned for a possible nuclear contamination, this will provide a precise analytical criteria to distinguish the origin of the enzyme. Kinetic parameters will also be helpful in further studies addressing the factors in determining DNA binding properties of the enzyme as such studies need the half-life of association/dissociation to be taken into account.

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