Mechanistic Characterization of the Mitochondrial Type I DNA Topoisomerase and a Study of Genes Containing Type I DNA Topoisomerase-Related Domains

Jaydee Dones Cabral
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MECHANISTIC CHARACTERIZATION OF THE
MITOCHONDRIAL TYPE I DNA TOPOISOMERASE AND
A STUDY OF GENES CONTAINING TYPE I DNA
TOPOISOMERASE-RELATED DOMAINS

by

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B.S. May 1994, College of William and Mary
M.A. August 1995, College of William and Mary

A Dissertation Submitted to the Faculty of
Old Dominion University and Eastern Virginia Medical School
in Partial Fulfillment of the
Requirement for the Degree of
DOCTOR OF PHILOSOPHY
BIOMEDICAL SCIENCES
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and
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August 2000

Approved by:

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ABSTRACT
MECHANISTIC CHARACTERIZATION OF THE MITOCHONDRIAL TYPE I DNA TOPOISOMERASE AND A STUDY OF GENES CONTAINING TYPE I DNA TOPOISOMERASE-RELATED DOMAINS

Jaydee Dones Cabral
Old Dominion University
and
Eastern Virginia Medical School
2000
Director: Dr. Frank J. Castora

The mitochondrial type I DNA topoisomerase (mt-topo I) serves an important function in the mitochondrion by relaxing mtDNA supercoils to allow for replication of the mitochondrial genome as well as for gene expression. The mt-topo I's role in essential processes, such as replication and transcription, makes it an ideal candidate as a target for antitumor or antifungal drugs. To gain further insight into mt-topo I mechanism, a cleavage assay and drug inhibition studies were performed. As well, a search for the mt-topo I gene or genes containing type I topoisomerase-related domains was conducted. To characterize the mt-topo I mechanism, the 5' or 3' binding of the mt-topo I to cleaved DNA was assessed by electrophoresis on an agarose gel after treatment with and without proteinase K (PK). The results of the cleavage assay revealed that the mt-topo I formed a covalent linkage to the 5' end of cleaved DNA. In addition, drug inhibition studies were performed to aid in distinguishing between the mt-topo I and the nuclear type I topoisomerase (nu-topo I). In agreement with earlier work with the calf thymus mt-topoI, the calf liver mt-topo I was found to be slightly more sensitive to inhibition by dimethyl sulfoxide (DMSO) than was the nuclear enzyme. In an attempt to identify new drugs that might selectively inhibit the mt-topo I, a series of seven compounds with
antifungal/antimicrobial activity were examined using standard DNA relaxation assays. One of these drugs, designated C1, was found to inhibit the nuclear topo I but not the mitochondrial enzyme and thus might serve as a useful tool to discriminate between these enzymes. To search for genes containing type I topoisomerase-related domains, reverse transcriptase polymerase chain reaction (RT-PCR) was performed to amplify conserved eukaryotic and prokaryotic topoisomerase I domains using primers that were homologous to these conserved regions. An amplified topo-related fragment, 575-1, which possessed a 57% similarity to a topo II signature, 56% similarity to a prokaryotic type I topo signature, and no similarity to the known nu-topo I, as indicated by prositescan searches, was used as a probe to screen a human cDNA library and in a northern hybridization. Fragment 575-1 detected a large 9 kb message which was three times the size of known type I topoisomerase messages. Due to the large message size, a further investigation to compare 575-1 to known eukaryote type I topoisomerase species was performed using the computational biology program, MEME. The program MEME finds conserved motifs in a group of DNA sequences. An RT-PCR positive control nu-topo I fragment, designated 575-6, was detected and included in the MEME run. 575-6, matched motif 1 out of 6 motifs identified by MEME. 575-1 contained none of six type I topoisomerase motifs identified. The program, Dnapars, revealed via an unrooted phylogeny tree that 575-1 was distantly related when compared to seven known eukaryotic type I topoisomerase species. In conclusion, the mt-topo I gene is fundamentally different from known eukaryotic type I topoisomerases. Degenerate primers developed from eukaryotic type I topoisomerase conserved domains were unable to amplify a region of the mt-topo I gene in RT-PCR reactions, although the known nu-topo I was detected.
To my grandmother,
Ligaya Morales, who taught me to value knowledge,
and
to my parents, Gil and Carmelita Cabral,
who showed me, by example, the merits of
hard work and perseverance.
ACKNOWLEDGMENTS

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Thanks are also given to Barbara Conyers and Jeffrey Stephens, for their friendship.

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And, thank you, Bret, for your encouragement, patience, and love.
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CHAPTER I
INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Topoisomerases are ubiquitous DNA-modifying enzymes found in prokaryotes, eukaryotes, viruses, and organelles, such as chloroplast and mitochondria (1-5). Topoisomerases catalyze a variety of DNA topology-altering reactions enabling them to effect many genetic processes. These processes include DNA replication (6,7), transcription (8,9), recombination (10,11), and transposition (12). They function by introducing or removing DNA superhelical turns, tying or untied DNA knots, and lastly, catenating or decatenating circular DNA by forming enzyme-bridged strand breaks for the passage of another strand (13).

There are two classes of topoisomerase (topo), type I and type II. Type I topoisomerases (top1) cleave a single strand of DNA and pass the intact strand though the break before resealing. Type II topoisomerases (top2) are ATP-dependent, and cleave both DNA strands, passing the intact strand from the same or different molecule through the break before resealing. In both cases, the same four steps in mechanism prevail. First, the topoisomerase binds DNA. Next, the DNA strand is broken. Subsequently, the strand is passed through the break, and finally, the strand is resealed. To date, all type I and type II topoisomerases studied have been found to be covalently linked to the cleaved DNA via a phosphotyrosine linkage (14-17).

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Subgroups of the type I topoisomerase family exist (Table 1). The two subgroups are monomeric, nonhomologous, and differ in the type of DNA adducts they form. The type IA topoisomerase subfamily exhibits a mechanism, homologous to bacterial type I topoisomerases, wherein the enzymes covalently link to the 5' end of cleaved DNA. Members of the type IA family include *E. Coli* topo I and topo III, eukaryotic topo III, and prokaryotic reverse gyrase (18). The type IB topoisomerase subfamily links to the 3' ends of cleaved DNA. Members of the type IB family include eukaryotic topo I, prokaryotic topo V, and the poxvirus type I topoisomerases.

**TABLE 1**

Subgroups of Type I DNA Topoisomerases

<table>
<thead>
<tr>
<th>IA</th>
<th>IB</th>
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<tr>
<td>5' covalent linkage</td>
<td>3' covalent linkage</td>
</tr>
<tr>
<td>Relax negative supercoiled DNA</td>
<td>Relax negative and positive supercoiled DNA</td>
</tr>
<tr>
<td>Not inhibited by camptothecin</td>
<td>Inhibited by camptothecin</td>
</tr>
<tr>
<td>Requires divalent cation</td>
<td>Does not require divalent cation, but is stimulated in its presence</td>
</tr>
<tr>
<td>2 classes: Prokaryotic topo I (reverse gyrase), topo III (prokaryotes and eukaryotes)</td>
<td>Eukaryotic topo I (nuclear topo I), prokaryotic topo V, poxvirus type I, mitochondrial topo I (5' or 3' linkage?)</td>
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The type II topoisomerase family, on the other hand, shows a mechanism where during cleavage, a pair of tyrosines attack opposite strands of the DNA duplex and become covalently attached to the DNA through the 5'-phosphotyrosine linkage.
Members of the type II topo family include the bacterial gyrase, yeast topoisomerase, and mammalian topoisomerases. *E. coli* DNA gyrase is a $\text{B}_2\text{A}_2$ heterotetramer whereas yeast topo II is a $(\text{BA})_2$ homodimer. The N-terminal region of the B region contains the ATP-binding and hydrolysis activities, while the A region contains the active-site tyrosine. Mammalian type II topoisomerases have two isoenzymes, $\Pi\alpha$ and $\Pi\beta$, that possess indistinguishable enzymatic properties. A subgroup of the type II topoisomerase family also exists which is comprised of the bacterial topoisomerase IV (19).

In addition to subgroups within the type I topoisomerase family, there exist distinct biochemical differences between the prokaryote and eukaryote varieties overall. The prokaryote topoisomerase I has an absolute requirement for Mg$^{2+}$, relaxes negative but not positive supercoils, is not inhibited by the anti-tumor plant alkaloid camptothecin, cleaves single stranded DNA, forms a phosphotyrosine linkage to 5' end of DNA, and does not cleave relaxed DNA. The eukaryotic nuclear topoisomerase I (nu-topo I), on the other hand, is stimulated by but does not require Mg$^{2+}$, relaxes both negative and positive supercoils, is inhibited by camptothecin, cleaves double and single stranded DNA, cleaves relaxed DNA, and forms a phosphotyrosine linkage to 3' end of DNA (20,21).

Most mitochondrial type I topoisomerases (mt-topo) like their eukaryotic counterparts, do not require Mg$^{2+}$ but are stimulated in its presence, are able to relax positive and negative supercoils, and form phosphotyrosine linkages to DNA. The yeast mt-topo I, unlike its nuclear counterpart, exhibits an absolute requirement for a divalent cation such as Mg$^{2+}$ or Ca$^{2+}$. In addition, it is slightly more sensitive to monovalent salts, with optimal activity obtained in 50-100 mM KCl (22).
Other factors that distinguish the mt-topo I from the nuclear enzyme are differences in their MW, pH profile, thermal stability, chromatographic properties, sensitivity to dimethyl sulfoxide, ethidium bromide, and the trypanocidal agent berenil (23-25). In addition, the mt-topo I also yields a protease digestion pattern distinct from that of the nuclear enzyme (26). Although differences in these biochemical properties have been observed, they can not eliminate the possibility that the mt-topo I may be a degraded nu-topo I fragment. It is unknown whether the mt-topo I phosphotyrosine linkage occurs at the 5' or 3' end. Determining the site of linkage may provide additional evidence to help delineate between the nu-topo I and the mt-topo I.

Interest in the discovery of a mt-topo was motivated by the fact that the mitochondrion possesses its own circular DNA consisting of 16569 nucleotide base pairs that define 13 polypeptides that are components of the respiratory chain, 22 transfer RNAs, and 2 ribosomal RNAs. Each mitochondrion contains 2 to 10 copies of mtDNA. In order to get expression of the mtDNA, the DNA must be exposed to replication and transcription machinery. The need for a swivel in front of the replication fork to remove supercoils led investigators to believe in the presence of some type of topoisomerase. The role of topoisomerases in eukaryotic DNA replication has been proven in studies investigating SV40 DNA in vitro (6), and in experiments dealing with the replication of kinetoplast DNA as found in trypanosomes (27). Topoisomerases also play a very important role in transcription as found in studies with ribosomal RNA genes and protein coding genes as demonstrated in yeasts (28), Tetrahymena (29), and Drosophila (30).

In terms of the mitochondrial type I topoisomerase, activity was detected for the first time in rat liver mitochondria in 1985 (31). Topoisomerase relaxation assays (fig. 4)
provided evidence for the ability of the mt-topo I to relax supercoiled plasmid DNA, lending strength to the argument of its participation in replication and transcription (32). Type I mt-topo activity has also been discovered in yeast (33), calf thymus and liver (34), human platelets (35), and human leukemia cells (36). Without the discovery of the mt-topo I gene, the question still remains whether the mt-topo I truly exists. Critics believe that nu-topo I contamination of mitochondrial extracts account for the observed activity. Isolating the mt-topo I gene would definitively confirm its existence.

The search for the mt-topo I gene occurs within the nuclear genome. Mitochondrial evolution is believed to have begun with endosymbiosis between respiring bacteria-like endosymbionts and pre-eukaryotic host cells (37, 38). The majority of mitochondrial proteins of animals are encoded by nuclear genes and imported into the organelle where they function under conditions that differ from those in the cytosol. Unfortunately, the large variety of proteins within the cytosolic and mitochondrial compartments have obscured the identification of traits specific for either population of proteins (39). Evaluation of amino-terminal residues of a collection of mitochondrial precursors have identified a few features that distinguish the amino termini of proteins destined for the mitochondrion: a nearly total absence of acidic amino acid residues; preponderance of arginine, serine, and leucine residues; and a segment with a large predicted helical hydrophobic moment (40). Due to the lack of an obvious pattern found in amino acid sequences of known mitochondrial leader peptides, the use of a mitochondrial localization signal as a probe to search for the mt-topo I was not feasible. Another approach was implemented which took advantage of new technology.
Computational biology tools, however, did prove useful in looking for the mt-topo I gene and type I topo-related genes. The employment of computational biology tools was preferred over more traditional gene isolation methods for several reasons. The primary reason was the inability to obtain enough mt-topo I protein for use in amino acid sequencing. A second reason was to take advantage of the sequence information available on known type I topoisomerases for the development of primers and probes. As well, computational biology provided a quick means to characterize putative mt-topo I DNA fragments. Database search results could be obtained within minutes.

Computational biology research or bioinformatics is a recently developed discipline or tool of molecular biology, not appearing in the literature until around 1991 (41). Bioinformatics has been described as the convergence of two technologies: the explosive growth of biotechnology and the paralleled growth in information technology (42). A nucleotide or protein sequence of interest can be used to search sequence databases. These similarity searches are akin to computer generated southern blots, a technique that detects DNA containing specific base sequences. A great deal can be learned from an uncharacterized string of DNA or protein sequence if it possesses significant sequence similarity to a well studied protein or protein domain. Protein searches performed with translated DNA sequence can be used to scan enzyme domain databases. As well, multiple sequence alignments and phylogeny trees can be developed to investigate the evolutionary relationship between known genes and genes containing similar domains. The use of bioinformatics technology was critical in finding type I topo-related genes.
Obtaining the mt-topo I gene would open doors to investigating cancer-related or mitochondrial disorder related research. The mt-topo I's role in essential processes such as replication and transcription makes it an ideal candidate for antitumor drugs, in addition to possible implications in the defective expression of mtDNA. In terms of cancer research, investigators have looked at chemically inhibiting topoisomerase I in cancerous cells. Such antitumor drugs would interfere with essential cellular processes, eventually leading to cell death.

Currently, there are topoisomerase inhibitors being investigated for clinical use with different drugs displaying specificity for the type I or the type II topoisomerases. Camptothecin is a type I topoisomerase inhibitor. Clinical studies show that camptothecin (CPT) and some of its derivatives are active in both slowly and rapidly proliferating tumors. The lactone form of CPT functions by reversibly stabilizing the cleavable complex, formed after the covalent attachment of the topoisomerase to the DNA through a phosphotyrosine linkage, resulting in single strand breaks and inhibition of religation. These events arrest DNA synthesis that leads to the arrest of the cell cycle in the S-G2 phase, which ultimately results in cell death. Other type I inhibitors include topotecan and CPT-11 (irontecan). Topotecan and CPT-11 are both semisynthetic analogs of the natural plant alkaloid, CPT, designed to be more water-soluble (43). Topotecan displays antineoplastic activity against refractory non-small cell lung cancer and ovarian cancer. CPT-11 is particularly active against a diverse group of tumors, including colon cancer, non-small cell lung cancer, cervical cancer, and non-Hodgkin's lymphoma (44). CPT has also been found to inhibit the mitochondrial type I DNA topoisomerase from bovine liver (45).
Type II inhibitors can be divided into two categories, nonintercalating drugs and intercalating drugs. Examples of nonintercalating topoisomerase II-interacting drugs include the epipodophyllotoxins, etoposide and teniposide. Structure-activity studies of epipodophyllotoxins derivatives confirmed that antileukemic effects and topoisomerase II interactions paralleled one another. DNA alkaline elution studies show that both etoposide and teniposide lead to DNA strand breaks. Etoposide has been shown to be clinically effective against small cell lung cancer and germ cell tumors of testicular origin. Teniposide's clinical role was confined to acute lymphoblastic leukemia (46).

Examples of topoisomerase II intercalators include m-AMSA (an acridine) (4), anthracyclines, ellipticenes, and adriamycin. Alkaline elution studies of the interaction of DNA with these agents documented a consistent association of DNA strand breaks with topoisomerase II. Anti-tumor activity of modest proportions have been noted against endometrial adenocarcinoma and breast cancer (47).

In addition to anticancer drugs, the contribution of mtDNA defects to tumor formation may be related to the misfunctioning of mt-topo I (48). More specifically, findings in the case of chronic myelogenous leukemias, reveal the presence of complete and partial duplications of mtDNA (49). These unicircular dimers may be formed by an internal recombination event assisted by the mt-topo I.

As mentioned previously, topoisomerases are not only implicated in cancer-related diseases but in mitochondrial disorders as well. Mitochondrial disorders may be defined as primary, due to defects in nuclear DNA encoding for mitochondrial protein or in mitochondrial DNA (50), or they may be secondary, due to all other causes. Various subgroups of mitochondrial disorders can include defects related to fuel transport and
metabolism, associated with disturbances in oxidative phosphorylation and mutations in mtDNA, and age-related degenerative diseases. In terms of the mt-topo I’s role in mitochondrial related disorders, disorders associated with mutated mtDNA and/or misexpressed mtDNA are of particular interest. A number of diseases with mutations in the mtDNA have been linked to dysfunction in electron transport (51). The following syndromes have been vital in learning more about mitochondrial medicine, Kearne-Sayre syndrome (KSS), chronic progressive external opthalmoplegia (CPEO), Pearson’s syndrome, Leigh disease, myoclonic epilepsy and ragged red fibers syndrome (MERFF), and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). More specifically, partial duplications and deletions of mtDNA have been found in mitochondrial myopathies and Pearson’s Syndrome (52). These mutations may be explained by the production of defective mt-topo I (53). Defective mt-topo I can be implicated in a number of important cellular events which result in abnormal mitochondrial function. Some examples include expression of mtDNA regions that are normally not expressed, a lack of expression of regions that normally are expressed, defective mt-topo I’s that cleave mtDNA, but cannot ligate, or the ligation of incorrect strands resulting in an abnormal recombination event. Future studies involving analysis of mt-topo I interfering with the normal processing of mtDNA and/or its gene products should be further investigated.
STATEMENT OF PROBLEM

The overall goal of this study was to characterize the mechanism of type I mitochondrial DNA topoisomerase (mt-topo I) and to search for the mt-topo I gene or genes containing type I topoisomerase-related domains.

The specific aims of this proposal were:

1. To search for the mt-topo I gene or genes containing type I topoisomerase-related domains, RNA was isolated from human placental tissue using a CsCl preparation. Reverse transcriptase polymerase chain reaction (RT-PCR) was then performed using primers homologous to conserved domains in type I topoisomerases found in eukaryotes and prokaryotes. These primers were used in various combinations under stringent conditions to favor more specific binding. PCR products were then sequenced, and the resulting sequence compared to sequence databases to aid in their identification. Any unique or uncharacterized RT-PCR sequence that possessed some topoisomerase homology was used as a radiolabeled probe to screen a human cDNA library in order to obtain the full-length mt-topo I gene. The unique sequence was also used to perform a northern blot for determination of message size.

2. To characterize the relationship between known topoisomerase type I genes and genes containing topoisomerase type I-related domains multilevel consensus sequence analysis (MEME), and phylogeny tree (dnapars) programs were performed using a database of seven known eukaryotic type I topoisomerases and RT-PCR product sequences generated from type I topoisomerase conserved domain primer sets.
(3) The determination of 5' or 3' binding of the mt-topo to cleaved DNA was assessed by electrophoresis on an agarose gel after treatment with and without proteinase K (PK).
CHAPTER II
MATERIALS AND METHODS

EQUIPMENT

E-C power supply by E-C Apparatus Corporation was used for agarose gel electrophoresis. MBP 3000D Electrophoresis power supply and DNA sequencing system by IBI were used for DNA sequencing. Stratagene UV stratalinker 1800 was used for DNA transfer. UV transilluminator was from UVP, Inc. The UV spectrophotometer used was a Beckmen model DU-50. The camera system was an MP-4 Land camera from Polaroid. A Savant speed-vac concentrator connected to a Savant refrigerated condensation trap was used. A Biorad model 83 slab dryer was used for gel drying. The thermal cycler used for PCR was a Barnstead Thermolyne Temp Tronic. A Labconco purifier Class II Safety cabinet was used for culture work. All microfuges were from Beckmen. All balances were from Mettler.

MATERIALS

Plasmid pUC19 DNA was isolated from HB101 cells harboring the plasmid by lysing the cells with alkaline-SDS followed by cesium chloride centrifugation in the presence of ethidium bromide (32). Mitochondrial DNA was recovered from purified bovine liver mitoplasts. Taq DNA polymerase, T4 polynucleotide kinase, and Proteinase K were purchased from Promega. Random primer labeling system was from Gibco BRL. Various restriction enzymes, pGEM-T Vector System, helper phage R408, and fmol DNA sequencing system were purchased from Promega. DNA primers were synthesized.
by Integrated DNA Technologies, Inc. Camptothecin, cellulose phosphate, hydroxyapatite, double stranded DNA cellulose, and Sigmacote were from Sigma. All radioactive isotopes were from NEN. DNA grade agarose, acrylamide, and bis-acrylamide were from Fisher. All other chemicals were of analytical grade or higher.

For PCR purification Prep-a-gene purification kits from Biorad were used. The Genius Chemiluminescent Detection kit was from Boehringer Mannheim. Polaroid type 55 film and Fuji RX autoradiography film were from VWR.

METHODS

Human Placental Tissue RNA Isolation

A CsCl preparation was used as follows. Flash frozen human placental tissue was homogenized using a polytron in a guanidinium isothiocyanate and β-mercaptoethanol solution. The sample was spun and the supernatant decanted into new sterile, polypropylene tubes. The pellet was discarded. A chloroform /isoamyl alcohol (24:1) solution was added to the supernatant, mixed, and then spun. The aqueous layer was transferred to a new tube. The solution was pulled back and forth several times with a syringe in order to shear the DNA. The sample was then layered on top of a CsCl gradient solution (0.96 g/ml), and centrifuged at 39,000 rpm for 21 hours. The supernatant was removed and discarded. A clear, white pellet of RNA remained. Sodium acetate was added to resuspend the RNA. RNA samples were quantitated and stored in a sodium acetate/ethanol solution at -20°C. Samples were analyzed spectrophotometrically (260:280 ratio) and visualized via formaldehyde gels. RNase contamination of tubes, buffers, etc. was kept at a minimum to prevent degradation. All
aqueous solutions were made with 0.1% diethylpyrocarbonate (DEPC) treated water. All glassware was oven baked overnight at 200°C. Pipet tips were autoclaved prior to use. Gel boxes were soaked in 3% hydrogen peroxide solution for 3 hours. Powder-free gloves were worn at all times when working with RNA in a specified RNA work zone.

**Probe and Primer Design**

a. **mt-topo I gene isolation** - DNA was amplified from human RNA using primers homologous to type I topoisomerase conserved regions from various eukaryotic or prokaryotic species. There exist four highly conserved domains, A, B, C, and D in eukaryotic topo genes (fig. 1) (54). Primers were chosen from areas just outside these conserved regions using DNA sequences that represent codons with minimum degeneracy. Topoisomerase I gene sequences from *Drosophila*, cow, rat, frog, and human were compared to determine the most frequently used base pairs in degenerate codons. Degenerate primer sets were designed to represent each possible permutation in a selected conserved region. Primers were used in different combinations under stringent conditions to favor more specific binding (Table 2 and 4). Prokaryotic primers were also used. Prokaryotic primers were considered due to the fact that the mt-topo I resembled the prokaryotic type I topoisomerase in its mechanism of action, exhibiting 5’ phosphotyrosine linkage to cleaved DNA verses nu-topo I’s 3’ phosphotyrosine linkage. In addition, the suggestion of prokaryote homology was not unheard of due to the endosymbiont theory, which suggests a prokaryotic origin of the mitochondrion (55). Lastly, an exact human nu-topo I primer set was used to serve as a RT-PCR positive control.
FIG. 1. Four conserved domains in topo I eukaryotic species.

TABLE 2

RT-PCR primers

<table>
<thead>
<tr>
<th>Eukaryotic Degenerate topo I primers</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region A_L</td>
<td>5’ AAR TGG AAA YGG TGS SA 3’</td>
</tr>
<tr>
<td></td>
<td>Region C_L</td>
<td>5’ ATG GAR MAC AAG SAG SS 3’</td>
</tr>
<tr>
<td></td>
<td>Region C_R</td>
<td>5’ SSC TSC TTG TKY TGC AT 3’</td>
</tr>
<tr>
<td></td>
<td>Region D_R</td>
<td>5’ YCM YAY TTY TTR CAM CM 3’</td>
</tr>
<tr>
<td>S= C,G; K= G,T; M= A,C; R= A,G; Y= T,C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exact Human nu-topo I primers</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region A_L</td>
<td>5’ AAG TGG AAA TGG TGG GA 3’</td>
</tr>
<tr>
<td></td>
<td>Region C_R</td>
<td>5’ GGC TGC TTG TTC TCC AT 3’</td>
</tr>
<tr>
<td></td>
<td>Region D_R</td>
<td>5’ CCC CAC TTC TTG CAC CA 3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prokaryotic topo I primers:</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Region A_L</td>
<td>5’ GAG TCG CCC GCA AAA GC 3’</td>
</tr>
<tr>
<td></td>
<td>Region C_R</td>
<td>5’ TTC CAT CTG CGC GGT GAA 3’</td>
</tr>
</tbody>
</table>

b. mt-topo II gene isolation- Topoisomerase type II probes were designed in a similar manner as the type I topo primers in that they were derived from conserved regions. The probes were selected from conserved sequences from *Saccharomyces cerevisiae*,

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Schizosaccharomyces pombe, Drosophila melanogaster, and Homo sapien. Topo II probes, denoted A-E (Table 3), were used to screen a human cDNA library.

**TABLE 3**

Topo II probes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5' TTTIGC ACC RTTİ CI ACC IGT 3'</td>
</tr>
<tr>
<td>B</td>
<td>5' ATCTTG ATC IGT CAT RAT CAT 3'</td>
</tr>
<tr>
<td>C</td>
<td>5' GAI GTA CCT ARA CCT TTR TAR TAT TT 3'</td>
</tr>
<tr>
<td>D</td>
<td>5' CAT TTICIT TGA CCT GGT TT 3'</td>
</tr>
<tr>
<td>E</td>
<td>5' ACC IGT ACC RAT ACC TTC IGC ACC RTT 3'</td>
</tr>
</tbody>
</table>

Exact topo II primers were generated using the known human topo II sequence. The software, Primer Design, was used to generate three primer sets at the 5', middle, and 3' portion of the human topo II gene. The exact topo II primers were used in RT-PCR as well as in plaque PCR. The expected size nu-topo II fragments generated from each primer set are as follows: 517 bp (5' primer set), 314 bp (mid primer set), and 787 bp (3' primer set).

**TABLE 4**

Topo II primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'_L</td>
<td>5' TGC ACT AAT GGT CAG AAG AG 3'</td>
</tr>
<tr>
<td>5'_R</td>
<td>5' GTA ACC GAC ACC ATA ACA TC 3'</td>
</tr>
<tr>
<td>mid_L</td>
<td>5' GGG TAC TAT TGA AGA ACT GG 3'</td>
</tr>
<tr>
<td>mid_R</td>
<td>5' GAG TGT ACG TTG AGA TAC CA 3'</td>
</tr>
<tr>
<td>3'_L</td>
<td>5' TCA GAA TCA GAT AGG AGC AG 3'</td>
</tr>
<tr>
<td>3'_R</td>
<td>5' GGA CTT CGA GAA TTG AAG GA 3'</td>
</tr>
</tbody>
</table>
**RT-PCR**

a. **RT (Reverse Transcriptase)**- Human liver mRNA (100 ng, Origene Technologies, Inc.) and right primers (Drev, Crev) were heated at 70°C, then chilled on ice. The following components were added: AMV RT 10X reaction buffer, 10 mM dNTP mix, RNasin ribonuclease inhibitor (40 u/μl), 100 mM oligo(dT)$_{15}$, AMV-RT (5 u/μl), and DEPC-treated (0.1%) water. In order to track the reaction, one-fifth of the reaction mixture was placed into another tube containing [$\alpha$-32P]dCTP (3,000 μCi/mmol). The radioactive and nonradioactive samples were then incubated at 37°C for 2 hours, placed on ice, with the subsequent addition of EDTA. EDTA was added to chelate the RT enzyme and inhibit its function. In addition to the original sample, two negative control samples were analogously performed, one containing all components except the RNA; the other, containing all components except the AMV-RT. A southern blot was also performed to confirm that DNA synthesis had occurred. Unlabeled RT sample was run on a 1% agarose gel, the DNA transferred to a nylon membrane, and the membrane hybridized with the radiolabeled primers $A_L$ and $C_L$. Primers were 5' end labeled using [$\gamma$-32P]ATP (3,000 μCi/mmol) and T4 polynucleotide kinase (5 units). Hybridization of these probes to the DNA showed that the DNA strands synthesized in the RT are long enough to produce the expected PCR products from the nuclear topoisomerase gene, and, presumably, from a putative homologous mt-topo I gene.

b. **PCR**- The above reaction mixtures were ethanol precipitated overnight at -20°C. The samples were then spun at 4°C for 30 minutes. The supernatant was removed and the pellets resuspended in 20 μl of water. A standard PCR reaction mixture (10X RT-PCR
buffer, 20-400µM forward and reverse primers, 2 units Taq polymerase) was used optimising for Mg\(^{2+}\). PCR was performed at the following temperatures cycling 40 times:

\[
\begin{align*}
30\text{s} & \quad 95^\circ \text{ denaturation} \\
30\text{s} & \quad 55-57^\circ \text{ annealing} \\
2\text{ min} & \quad 68^\circ \text{ synthesis}
\end{align*}
\]

Annealing temperatures and [Mg\(^{2+}\)] varied depending on the primer set used (Table 5). Moles of DNA synthesized from the RT were calculated using the specific activity of the radiolabel, the conversion factor 1 Ci is equivalent to 2.2 \times 10^{12} \text{ cpm} (counts per minute), and the cpm obtained from spotting a one microliter portion of the labeled RT products on Whatman DE-81 disks. The disks were washed 6 times with a sodium phosphate buffer, followed by 2 washes with water, and 2 washes with ethanol. The dried disks were then placed in a vial containing 3 mL of scintillant and counted. In addition, the labeled portion of the RT mixture was run on a 1% agarose gel for 5 hours at 60 volts in 1X TBE (0.089 M Tris base, 0.089 M boric acid, 20 µM EDTA, pH 8.0) buffer, dried under vacuum at 60°C, and exposed overnight to film at -70°C. PCR products were analyzed on 1% agarose gels stained with ethidium bromide (10 µg/µl).

**TABLE 5**

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Annealing T (°C)</th>
<th><a href="mM">Mg(^{2+})</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Deg. A-C</td>
<td>57</td>
<td>2</td>
</tr>
<tr>
<td>Deg. C-D</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>Pro. A-C</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>S(_{LR})</td>
<td>57</td>
<td>2</td>
</tr>
<tr>
<td>mid(_{LR})</td>
<td>57</td>
<td>2</td>
</tr>
<tr>
<td>3(_{LR})</td>
<td>57</td>
<td>2</td>
</tr>
</tbody>
</table>
**Plaque PCR**

Plaque PCR was used to determine if positives generated from λgtll cDNA library screens contained conserved eukaryotic or prokaryotic topo regions. Potentially positive plaques were cored out using a pasteur pipet and then placed in SM buffer (10 M NaCl, 8 mM MgSO₄, 50 mM Tris, 0.01% gelatin). Negative plaques were also cored out to serve as negative controls to eliminate PCR products generated from the λgtll vector itself. Ten µL of the SM mixture was placed in a 0.5 µL microfuge tube, boiled for 5 minutes, and then placed on ice. One ml of the denatured SM mixture was used as the DNA template source for a standard PCR reaction.

The primers shown in Table 6 were used to screen potential positives from the λgtll cDNA screen using 575-1 as the probe. 575-1 was a RT-PCR product generated from the eukaryotic degenerate topo I primer set Cₕ and Dₜ. 575-1LR represent primers designed directly from obtained sequence information for the 575-1 RT-PCR product.

<table>
<thead>
<tr>
<th>Table 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plaque PCR primers</strong></td>
</tr>
<tr>
<td>Name</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>575-1L</td>
</tr>
<tr>
<td>575-1R</td>
</tr>
</tbody>
</table>

**pGEM Vector Cloning**

PCR products were purified using Biorad Prep-a-Gene. Purity was assessed by gel electrophoresis and ethidium bromide (0.1 ng/ml) staining. For the ligation reaction the following components were mixed, then incubated at room temperature for 1 hour or overnight at 4°C: 2X Rapid Ligation Buffer, pGEM-T vector, PCR product, T4 DNA

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ligase, and water. For the transformation, two LB/ampicillin (AMP, 100 μg/ml)/IPTG (0.5 mM)/X-Gal (800 μg/ml) plates per ligation reaction were equilibrated to room temperature prior to plating. An aliquot of the ligation reaction was added to thawed JM109 High Efficiency Competent Cells, mixed, and placed on ice for 20 minutes. The cells were then heat-shocked for 50 seconds at 42°C and placed on ice for 2 minutes. SOC medium (for 100 ml solution: 2.0g bacto-tryptone, 0.5g bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20mM filter-sterilized Mg$^{2+}$, 20 mM filter-sterilized glucose) was added to the transformation mix and incubated for 1.5 hours at 37°C with shaking. The transformation culture was plated using LB-agar onto duplicate plates. Plates were incubated overnight at 37°C.

a. rapid plasmid preparation- Isolation of plasmid DNA was performed using the Alkaline Lysis method. Three milliliters of LB media-AMP (for 1 liter solution: add 10 g agar bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl, adjust pH to 7.0 with NaOH, autoclave, cool to 50°C, and add AMP to final concentration of 100 μg/ml) was inoculated with a single bacterial colony and shaken overnight at 37°C. The overnight culture was then collected by spinning down the bacteria for 3 minutes in a 4°C microfuge at maximum speed. The supernatant was decanted, the pellet resuspended in 4°C Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8.0), and incubated at room temperature for 5 minutes. Freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added, mixed by inversion, and incubated on ice for 5 minutes. Ice-cold potassium acetate (Solution III, 0.3 M) was added, the sample vortexed, and incubated on ice for 5 minutes. Samples were centrifuged and the supernatant transferred to a new tube. Solution IV (phenol/chloroform/isoamyl alcohol/1% hydroxyquinoline) was added, the
samples vortexed and spun in a microfuge for 2 minutes at maximum speed. The aqueous supernatant was transferred to a new tube and ethanol precipitated overnight at -20°C.

b. ssDNA isolation- For induction of ssDNA production, recombinants were infected with helper phage R408. The plasmid enters the f1 replication mode, and the resulting ssDNA is exported as an encapsulated virus-like particle. The ssDNA was purified from the supernatant using a simple precipitation and extraction procedure (Promega Protocols and Application Guide, catalog #P1610). An overnight culture of the recombinants was prepared by picking colonies off M9 plates, inoculating in 1 ml of LB-AMP media, and incubated at 37°C with vigorous shaking. The next morning, 25 ml of LB-AMP media was inoculated with 0.5 ml of the overnight culture, followed by a 30 minute incubation at 37°C. The culture was then infected with helper phage at a multiplicity of infection of 10 (10 helper phage particles per cell) and allowed to shake for an additional 6 hours at 37°C. The supernatant was harvested by pelleting the cells at 12,000 x g for 15 minutes. The supernatant was poured into a fresh tube and centrifuged again for 15 minutes. The phage were precipitated by adding phage precipitation solution (3.75 M Ammonium acetate pH 7.5, 20% PEG 6000) and incubating overnight at 4°C. The sample was centrifuged, the pellet thoroughly drained, and resuspended in 400 µl TE buffer (10 mM Tris·Cl, pH8.0, 1 M EDTA, pH 8.0). To lyse the phage, 400 µl of a 24:1 chloroform:isoamyl alcohol solution was added, vortexed for 1 minute, and centrifuged at 12,000 x g for 5 minutes. The upper, aqueous phase was transferred to a fresh tube, 400 µl of 25:24:1 phenol/chloroform/isoamyl alcohol solution was added, the mixture was vortexed for 1 minute, and centrifuged at 12,000 x g for 5 minutes. This phenol
extraction was repeated twice. The upper, aqueous phase was transferred to a fresh tube. The ssDNA was precipitated by overnight ethanol precipitation (add 0.5 volume of 7.5 M ammonium acetate, 2 volumes of 100% ethanol, mix, and store at -20°C overnight). To recover the ssDNA, the sample was centrifuged at 12,000 x g for 5 minutes, the supernatant removed, the pellet was rinsed with 70% ethanol, and the pellet was allowed to air dry. The ssDNA was resuspended in 20 μl nuclease-free dH2O. The amount of DNA can be estimated by agarose gel electrophoresis and ethidium bromide staining. Two major bands were usually seen: helper phage and ssDNA.

**Cycle Sequencing**

Promega’s fmol DNA Cycle Sequencing System was designed for thermal cycle sequencing, which incorporates Sanger’s dideoxy sequencing method and utilization of PCR (67). Purified PCR products or subclones were sequenced using nanogram quantities of template. Sequencing reactions included a Taq DNA polymerase (5 units) to synthesize a DNA strand complimentary to the DNA template, a short synthetic nucleotide primer which was homologous to a portion of the template and was 5’ end labeled using [γ-32P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase (5 units), and 2 μl of each dideoxynucleoside triphosphates (ddNTPs) that randomly terminated chain elongation when incorporated at the end of a growing chain. The sequencing reaction was then placed in a thermocycler using a thermal cycle sequencing profile. The chosen profile was designed for primers <24 bases or with a GC-content <50%. The sequencing profile was as follows:

- 95° 2 minutes, then:
- 95° 30 seconds
- 42° 30 seconds
- 70° 1 minute
35 cycles total, then 4°C

After the thermocycling program was complete, 3μl of fmol sequencing stop solution was added to 6μl of reaction mix. The sequencing products were then loaded in three intervals on an 8% polyacrylamide gel for 2 hours at 65 watts each in 1X TBE running buffer. The fragments, having been separated in parallel lanes in the gel, gave a pattern of radioactive DNA bands from which the DNA sequence was read. The polyacrylamide gel was exposed overnight to film at -70°C. The sequence was compared to the Genbank database in order to determine its originality and approximate position relative to the full length nu-topo I gene.

a. 5'end-label primers- The following were combined in a microcentrifuge tube: 10 pmol primer (Table 2, 4, 6), 10 pmol [γ-32P] labeled ATP, 1μl 10X T4 Polynucleotide Kinase buffer, T4 Polynucleotide Kinase (5 units), and sterile water. The mixture was incubated at 37°C for 30 minutes and the kinase inactivated at 90°C for 2 minutes. The end-labeled primers were stored at -20°C.

b. single-stranded DNA templates- ssDNA templates were synthesized as previously described. ssDNA templates were used in the hopes of generating more legible sequence.

c. double-stranded DNA templates- Double-stranded DNA was generated as previously described in the rapid plasmid preparation.

Bioinformatics Programs

A variety of bioinformatics programs were used. They included basic similarity searches, multilevel consensus analysis, and phylogenies estimations. Multilevel consensus analysis, multiple alignments, and phylogenies estimations were performed.
using a worldwide web based system for DNA and protein sequence analysis called Bionavigator (56).

a. similarity searches- Similarity searches were performed in order to compare new sequence against all other known sequences in the hopes of deducing its function and/or identity. A variety of search programs available via the internet were used to scan DNA and protein databases. Examples of programs used were BLASTn (Basic Local Alignment Tool), BLASTp, BLASTx, tBLASTx, PROSITESCAN, and LALIGN. BLASTn, BLASTx, and tBLASTx were designed for nucleotide sequence queries. BLASTp was designed for protein sequence queries (57-61). PROSITESCAN was used to aid in the identification of uncharacterized protein sequence by searching for similarity to core patterns of known protein families (90). LALIGN was used to find the best local alignments between two sequences (62).

b. MEME (Multilevel Consensus Analysis)- Multilevel consensus sequence analysis (MEME) (63) uses the method of Bailey and Elkan (64) to find conserved motifs in a group of unaligned sequences. By default, the program assumes each motif occurs only once in each sequence in the search set. The resulting motifs are output as profiles. A profile is a logs-odd matrix used to judge how well an unknown sequence segment matches the motif. A score is assigned to quantify the relationship of each sequence relative to the motif found. Minimum width of motifs to search for was 8 nucleotides and the maximum width of motifs was set at 57 nucleotides. The maximum width was set at 57 due to the fact that the eukaryotic topo I active site is 19 aa or 57 nucleotides long.

c. dnapars- Dnapars estimates phylogenies by the parsimony method using nucleic acid sequence (65, 66). The method of Fitch (67) is used to count the number of changes of
base needed on a given tree. The assumptions of this method are that each site evolves independently, different lineages evolve independently, the probability of a base substitution is small over the lengths of time involved in a branch of a phylogeny, the expected amounts of change in different branches of the phylogeny do not vary by so much that the two changes in a high-rate branch are more probable than one change in a low-rate branch, and the expected amounts of change do not vary enough among sites that two changes in one site are more probable than one change in another. Change from an occupied site to a deletion is one change. Reversion from a deletion to an occupied site is allowed and is also counted as one change. This in effect assumes that a deletion N bases long is N separate events (68). Phylogeny trees were viewed using the software TreeView (69).

d. ClustalW(accurate)- The program ClustalW (accurate) is used for multiple alignment of nucleotide or protein sequences. Pairwise similarity scores are calculated from slow, accurate, global alignments. These initial alignments are re-scored to give percent identity scores. Percent identity scores can then be converted to distances for phylogeny trees which are used to guide the final alignment, i.e. sequence alignment results of ClustalW are used as input in the dnapars program (70).

Northern Hybridization

Two different protocols were used in northern blotting experiments:

a. biotinylated probe- Total human liver RNA purchased from Origene Technologies, Inc. was run on a 1.2% formaldehyde/agarose gel at 50 volts for 2 hours. The RNA was transferred to a Magnacharge nylon transfer membrane (MSI, Inc.) via capillary action. The RNA was UV linked to the membrane by using a UV Stratalinker set at 120,000
μJ/cm² for 30 seconds. The membrane was prehybridized at 42 °C for 1 hour, followed by an overnight hybridization at the same temperature in a mixture of Ambion’s Ultrahyb™ Ultrasensitive hybridization buffer and 50 ng/ml biotinylated probe RNA or DNA. The membrane was washed as follows before detected as described below: one 5 minute wash at room temperature (R.T.) in wash 1 [2X SSC (0.3 M NaCl, 0.03 M Na₃citrate·2H₂O, adjust pH to 7.0 with HCl)/0.1% SDS], one 5 minute wash at R.T. in wash 2 (0.2X SSC/0.1% SDS), one 5 minute wash at 42 °C in wash 1, one 5 minute wash at 42 °C in wash 2, and a final wash in 2X SSC.

b. radiolabeled DNA probe- Human liver mRNA purchased from Clontech was run on a 1.5% formaldehyde/agarose gel at 100 volts until the sample entered the gel, then at 22 volts overnight. The electrophoresis was stopped when the dye front reached the end of the gel. The RNA was transferred to a neutral nylon transfer membrane (MSI, Inc.) via capillary action. The RNA was UV linked to the membrane by using a Stratalinker at 120,000 μJ/cm² for 30 seconds. The membrane was prehybridized at 42 °C for 1.5 hours followed by an overnight hybridization at the same temperature in Ambion’s Ultrahyb™ Ultrasensitive hybridization buffer and 83 ng total random primer labeled DNA. The probe was radiolabeled using Promega’s Prime-a-gene labeling system and [α-³²P] dCTP (3,000 Ci/mmol). The membrane was washed as follows before being wrapped in saran and exposed overnight to film at -70°C with an intensifying screen: one wash for 30 seconds at room temperature (R.T.) in wash 1 (1X SSC/0.1% SDS), one 5 minute wash at R.T. in wash 1 with gentle shaking, and two 10 minute washes in wash 2 (0.25X SSC/0.1% SDS) at 42 °C.
Transcription Synthesis, Labeling, and Detection

a. transcription synthesis—RNA probes were synthesized using Ambion’s MAXIScript™ SP6/T7 *in vitro* transcription kit. The following transcription reaction was assembled at room temperature: add nuclease-free water to a 20 μl final reaction volume, 1 μg DNA template, 2 μl 10X Transcription buffer, 1 μl each of 10 mM ATP, CTP, GTP, UTP, and 2 μl RNA polymerase. The mixture was incubated for 1 hour at 37°C. To remove template DNA, 1 μl DNase I was added, then the mixture was incubated at 37°C for 15 minutes. To stop the reaction, 1 μl of 0.5M EDTA was added. Free nucleotides were removed by precipitation with ammonium acetate (add one-tenth volume 5.0 M) and ethanol (2 volumes 100%). The solution was chilled at -20°C for 30 minutes or longer, spun for 15 minutes at maximum speed in a 4°C microcentrifuge. The supernatant was discarded and the pellet was washed once with 70% ethanol. The pellet was resuspended in 50 μl of DEPC-water. The RNA probe was stored in 5 μl aliquots at -70°C.

b. labeling—Probes were biotinylated using Ambion’s BrightStar™ Psoralen-Biotin nonisotopic labeling kit. To label 0.5 μg of RNA in a 10 μl reaction, a clean, non-coated microtiter plate was placed on an ice bath. The following steps were performed in reduced light. One μl of Psoralen-Biotin reagent was added to 10 μl of RNA sample, mixed, and transferred to a well of the microtiter plate. A 365 nm UV light source was placed directly on the plate over the sample and irradiated for 45 minutes. The sample was diluted to 100 μl by adding 89 μl of 1X TE. The mixture was transferred to a clean microcentrifuge tube. Two hundred μl of n-butanol was added. The sample was then vortexed for 1 minute and centrifuged for 1 minute at 7,000 x g. The n-butanol layer was removed with a pipet. The RNA probe can be stored at -20°C for short term storage or at
-80°C for long term storage. DNA probes were 5' end labeled using T4 PNK and [γ-32P]ATP.

c. detection- After the northern hybridization was performed, Ambion's BrightStar™ Biodetect™ kit was used to detect the biotinylated probe. The positively-charged nylon membrane was washed twice for 5 minutes each in 1X wash buffer (use 1 ml/cm²), was washed twice for 5 minutes each in blocking buffer (use 0.5 ml/cm²), was washed once for 30 minutes in blocking buffer (use 1 ml/cm²), and was washed once for 10 minutes in conjugate solution (10 ml blocking buffer plus 1 µl streptavidin-alkaline phosphatase conjugate/100 cm²). The membrane was then washed once for 10 minutes in blocking buffer (use 0.5 ml/cm²), washed three times for 5 minutes each in 1X wash buffer (use 1 ml/cm²), washed two times for 2 minutes each in 1X assay buffer (use 0.5 ml/cm²), and then washed once for 5 minutes in CDP-Star™ (use 5 ml/cm²). The excess chemiluminescent reagent was shaken off, the membrane wrapped in plastic wrap, and exposed to film at room temperature for 2 hours.

cDNA Library Screen

A unique sequence was used as a radiolabeled probe to screen a λgt11 cDNA library from human fetal liver tissue (ATCC). The cDNA library from human fetal liver tissue was selected because young, developing cells are mitochondria-rich; therefore, possessing a high amount of mt-topo I. The first step in the nucleic acid hybridization screening was to grow large numbers of the recombinant phage in our library on agar plates. In plating the bacteriophage the titer of the cDNA library was determined by serial dilution. The recombinant phage and plating bacteria, LE392 E. Coli cells, were then mixed in a culture tube and incubated at 37°C. Top agar was added to the culture
tube and transferred to LB plates. The plates were incubated at 37°C until the plaques covered the plate but were not confluent. They were then incubated at 4°C for 1 hour before applying filters. To transfer to nitrocellulose filters, the filters were laid down, and left on the plates for a few minutes. The filter was removed from the plate and allowed to dry face up on the bench top. Duplicate lifts were obtained to eliminate false positives due to nonspecific binding. To denature the DNA, the filters were immersed using blunt-end forceps in the following solutions in the order shown using shallow trays: 1 minute wash in 0.5 M NaOH/1.5 M NaCl, 5 minute wash in 0.4 M Tris-HCl, pH 7.6/2X SSC, and a final rinse in 2X SSC for 2 minutes. The filters were then dried overnight in an oven at 42°C. Filters were stored at room temperature until needed for hybridization. The filters were then incubated overnight with the unique sequence radiolabeled probe at a hybridization temperature of 65°C. After incubation, the filters were rinsed with low stringency wash buffer followed by a washing with high stringency wash buffer.

The number of plaques that were screened in order to get a 99% probability of finding the mt-topo I gene was obtained using the following equation, \( R^e^{-Rh!} \). To simplify the equation, we can say that \( e^R = 0.01 \) because there is a 1% chance that the mt-topo gene will not be found. Solving the equation for \( R \), gives \( R = 4.6 \). Since a typical mammalian cell contains 160,000 mRNAs, by multiplying \( R \) with the number of messages, I needed to screen 736,000 plaques in order to get a 99% probability of finding the mt-topo I gene. Filters were exposed to film with an intensifier for a few hours at -70°C when using fresh radiolabel. As the radioactivity of the \( ^{32}\)P-label decreased over time, I increased the length of time the film was exposed to the filter.
a. probes- Degenerate primers that were developed for RT-PCR and topo II probes, as previously described in tables 2 and 3, were used to screen the cDNA library. RT-PCR products of interest, synthesized from topo I degenerate primers, were also used as probes.

b. phage plate stocks- Phage stocks of putative positives from the tertiary cDNA screen were made in the following manner: Phage (10^5 pfu) were mixed with 0.1 mL of exponentially growing plating bacteria. The mixture was incubated for 20 minutes at 37°C to allow phage to adhere to the *E. Coli* cells (LE392). Three mL of top agarose (per liter: 10 g tryptone, 8 g NaCl, 6 g agarose, autoclave) at 55°C was added, mixed, and poured onto a labeled 85-mm plate containing 30 mL of hardened, dry LB/0.2% maltose. The plates were inverted and incubated for 8-12 hours, until lysis was confluent. The plate was inverted, 5mL SM buffer was added, and the plates stored at 4°C for several hours with intermittent, gentle shaking. With a sterile pasteur pipet, the SM buffer was harvested. Fresh SM buffer (1 ml) was added and the plate was stored for 15 minutes in a tilted position at 4°C to allow all the fluid to drain to one area. Again, the SM buffer was removed and combined with the first harvest. The plate was discarded. One mL of chloroform was added to the pooled SM, vortexed, and centrifuged 4000 x g for 10 minutes at 4°C. The supernatant was recovered and chloroform added to 0.3% final concentration. The titer of the bacteriophage was determined by serial dilution. The titer was determined to be 10^10 pfu/mL. Phage were stored at 4°C.

*Fluorescence In Situ Hybridization*

The putative mt-topo I clone was amplified to generate large quantities of DNA. This was done by RT-PCR amplifying the putative mt-topo I gene or genes containing
topoisomerase domains by using degenerate primers developed from conserved topoisomerase domains. The amplified RT-PCR products were then random primer labeled (Gibco) with digoxigenin (DIG: 5β,20[22]-cardenolide-3β,12β,14-triol; 3β,12β,14-trihydroxy-5β, 20[22]-cardenolide). The DIG labeled DNA and Cot-1 DNA (100 μg/ml) were ethanol precipitated and resuspended in hybridization mix (50% formamide, 2X SSC, 10% dextran sulfate, pH 7.0). To enable specific hybridization to the chromosomal target site, the probe was denatured together with an excess of unlabelled Cot-1 DNA. Cot-1 DNA was used to suppress unspecific hybridization to human repetitive sequences. Leukocyte cells were denatured at 72° C in 70% formamide and 2X SSC. Probes were denatured at 72° C for 5 minutes. Hybridization was carried out at 37° C for approximately 20 hours before washing. Cytochemical detection was performed using FITC-avidin and biotinylated antiavidin amplification (Vector Laboratory, Burlingame, CA). Probe sequences that bound with the greatest homology should withstand more stringent washing than those that bound less closely matched sequences (91). Finally, slides were counterstained with DAPI (4',6-diamidine-2'-phenylindole dihydrochloride), mounted in antifade medium (VectaShield), and viewed under a Zeiss Axioscope microscope. Computer based image collection and enhancement were used to compensate for reduced signal intensity. The appearance of hybridization signals on slides of specific chromosomes should locate the topoisomerase domain containing genes.

3' Rapid Amplification of cDNA Ends

3’ Rapid amplification of cDNA ends (RACE) system (Life Technologies™) was used to amplify the 3’ end mRNA of 575-1. This system is useful for rapid amplification
of cDNA ends (RACE) and anchored PCR between a defined point within mRNA and the 3' poly(A) end (72, 73). First strand cDNA synthesis and amplification of the target cDNA were performed as directed by the 3' RACE instruction manual. To generate first strand cDNA, 50 ng of human liver poly(A)+ RNA, DEPC water, and 500 nM adapter primer were combined in a microcentrifuge tube. The contents were heated to 70°C for 10 minutes and chilled on ice for 1 minute. Following a brief spin in a microcentrifuge set at maximum speed, 10 X PCR buffer, 2.5 mM MgCl₂, 10 mM dNTP, and 10 mM DTT were added. The mixture was equilibrated to 42°C for 5 minutes. Two hundred units of reverse transcriptase was then added and the mixture incubated at 42°C for 50 minutes. The reaction was terminated by incubating at 70°C for 15 minute. The reaction was chilled on ice, collected by brief centrifugation, RNase H was added, and incubated at 37°C for 20 minutes. The mixture was stored at -20°C. To amplify the target cDNA, the following were combined: 10X PCR buffer, 1.5 mM MgCl₂, 200 μM each dNTP, 200 μM gene specific primer [575-1L (Table 5), 575-1L nested primer 1, 5' GCT AAG AAA CTG GGA TAG GA 3', or 575-1L nested primer 2, 5'CTT CGA TAT TTC TAA AGT TGT CTT T 3'], 200 μM abridged universal amplification primer, 2 μl of cDNA synthesis reaction, and 5 units Taq DNA polymerase. A layer of sterile mineral oil was then added and the reaction collected by brief centrifugation. The reaction was then placed in a thermocycler set to the following parameters:

- 94°C for 3 minutes, then cycled 35 times at
  - 95°C for 30 seconds
  - 48°C for 30 seconds (575-1L, 575-1L nested primer 1) or
  - 62°C for 30 seconds (575-1L nested primer 2)
- 70°C for 2 minutes
followed by an extended synthesis incubation of 72°C for 3 minutes.

After the amplification was complete, the sample was analyzed using agarose gel electrophoresis and ethidium bromide staining.

*Type I DNA Topoisomerase Isolation from Calf Liver*

Mitochondrial DNA topoisomerase I was purified from calf liver by the procedures listed in appendix I and II (74).

*5' and 3' End Labeling of dsDNA*

To phosphorylate the 5' ends of dsDNA, dsDNA and 10X T4 polynucleotide kinase (T4 PNK) buffer were incubated at 70°C for 1 minute, then quickly cooled on ice. Two μl of [γ-32P]ATP (3,000 μCi/mmol) and 5 units of T4 PNK were added to the mixture, followed by a 30 minute incubation at 37°C. The reaction was stopped by the addition of 2 μl of 0.5 M EDTA. The sample was phenol/chloroform extracted to remove protein and ethanol precipitated overnight at -20°C. To radiolabel 3' ends of dsDNA, dsDNA, 10X T4 DNA polymerase buffer, 2 mM dNTPs (N= A, T, or G), T4 DNA polymerase, and [α-32P]CTP were mixed, then incubated at 37°C for 5 minutes. One microliter of unlabeled 2 mM dCTP was added to the mixture. The mixture was heated at 70°C for 5 minute. Unincorporated dNTPs were separated by two rounds of ethanol precipitation.

*Standard Topoisomerase Relaxation Assay*

Standard DNA topoisomerase activity was measured by the extent of relaxation of supercoiled pUC19 DNA (75). The following components were combined and incubated at 37°C for 30 minutes: 10X assay cocktail, pUC19 DNA, topoisomerase, and dH2O. Topo stop solution was then added, and samples were run at 40 volts for 12 hours on a
medium sized 0.8% agarose gel. DNA was visualized via ethidium bromide staining. One unit of relaxing is equivalent to the amount of activity to relax 50% of plasmid DNA within 30 minutes at 37°C.

![FIG. 2. Standard mt-topo I relaxation assay. Lane 1, control supercoiled plasmid DNA (I); lane 2, mt-topo I at 2 units; lane 3, 1 unit; lane 4, 0.25 units of activity. The DNA bands labeled II, I₀, Iᵣ, and I are nicked, linearized, enzymatically relaxed, and supercoiled plasmid DNA, respectively.]

**Cleavage Assay**

To determine whether the mt-topo I phosphotyrosine linkage occurs at the 5’ or 3’ end of cleaved DNA, a cleavage assay was performed. Topoisomerase was added to the following mixture and incubated at 37°C for 15 minutes: 5’ or 3’ uniquely end labeled DNA, 10X assay cocktail, and 25 μM camptothecin unless otherwise noted. The reaction was stopped with the addition of SDS (final concentration 0.5%) followed by digestion with proteinase K (PK, final concentration 0.5 mg/ml). The sample treated with PK was incubated an additional 30 minutes at 55°C. Alkaline topo stop solution was added to all samples. Samples were loaded onto a 2% agarose gel run at 70 volts for 2.5 hours. The
gel was dried under vacuum and analyzed by autoradiography. Cleavage assay design is depicted in fig. 3.

**Cleavage Assay**

![Diagram](image)

* = $[^{32}P]$ dCTP

T4 DNA pol

FIG. 3. Topoisomerase cleavage assay schematic. Doubled stranded fragments of DNA that contain known mt-topo I cleavage sites are uniquely end-labeled. The fragments are then incubated with mt-topo I in the presence of CPT (which traps the mt-topo I to the cleaved DNA). A portion of this reaction mixture is then treated with PK (degrades the protein). Samples with and without PK treatment are run on an agarose gel. DNA is analyzed via autoradiography. By comparing migration patterns of samples with and without PK treatment, the site of mt-topo I binding can be determined. Expected migration results are depicted in bottom row of boxes. Lines within boxes represent radiolabeled, cleaved DNA fragments with or without the mt-topo I attached.
K^+ SDS Precipitation

As schematic of the procedure is depicted in fig. 4. Topoisomerase was added to a mixture containing 5' or 3' end labeled DNA, 10X assay cocktail, and 25 μM camptothecin. The samples were incubated at 37°C for 10 minutes, then divided in half. To one half, the DNA was denatured into ssDNA by adding a stop solution containing 2% SDS, 0.2 M NaOH, 2 mM EDTA, and 0.5 mg/ml salmon sperm DNA. In the other half, the DNA remained double stranded by adding a stop solution that contained the same components as above minus the NaOH. The samples were incubated further, then precipitated by the addition of 0.25 M KCl. The samples were spun, the supernatant removed, and the pellet washed with a solution containing 10 mM Tris, pH 8.0, 100 mM KCl, 1 mM EDTA, and 100 μg/μl salmon sperm DNA solution. After incubation at 65°C for 10 minutes, the samples were spun in a microcentrifuge set at maximum speed, the supernatant removed, and the pellet resuspended in water. Samples were transferred to vials containing scintillation cocktail to be analyzed.
OR

5' end labeled DNA  OR  3' end labeled DNA

\[ + \]
\[ \text{topo} \]
\[ + \text{CPT} \]

5' linked or 3' linked

Spotted onto disks, then washed

Expected results:
Low counts  High counts  High counts  Low counts

FIG. 4. Radioactive filter binding/K^+SDS^- precipitation schematic. CPT = camptothecin.

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Drug studies

Drug inhibition studies were performed in the hopes of further distinguishing between the mt- and nu-topo I. Quantitative analysis was performed via densitometry using Image-1/AT version 4.14 software.

a. Berenil- Berenil is an antitypanosomal agent that binds to nucleic acid duplexes. Berenil (1,3-bis (4'-amidinophenyl)triazene) was added in increments of 10, 40, or 100 μM to a standard topoisomerase relaxation assay.

b. DMSO- Dimethyl sulfoxide was added in increments of 1%, 5%, or 20% to a standard topoisomerase relaxation assay. Previous studies have shown that 5% DMSO inhibits mt-topo I 60% with no inhibition of nu-topo I (75).

c. Antifungal/antimicrobial agents- Seven antifungal drugs (A1, A6, B1, C1, C5, D1, F2) were analyzed at varying concentrations (2X LC50, 1X LC50, 0.5X LC50, 0.1X LC50, Table 7) in a standard topoisomerase relaxation assay.

| TABLE 7 |
| Antifungal/antimicrobial drug LC50s |

<table>
<thead>
<tr>
<th>Drug</th>
<th>LC50 (μg/Ml)</th>
<th>Microorganism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>8</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td>B1</td>
<td>8</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td>C1</td>
<td>64</td>
<td>Aspergillus fumigatus</td>
</tr>
<tr>
<td>D1</td>
<td>16</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td>A6</td>
<td>4</td>
<td>Micrococcus luteus</td>
</tr>
<tr>
<td>C5</td>
<td>64</td>
<td>Aspergillus fumigatus</td>
</tr>
<tr>
<td>F2</td>
<td>2</td>
<td>Bacillus subtilis, Myobacterium smeg</td>
</tr>
</tbody>
</table>
FIG. 5. Structures of seven antimicrobial/antifungal drugs.
CHAPTER III
RESULTS

The study began from a mechanistic standpoint to determine the mt-topo I's site of phosphotyrosine linkage at the 5' or 3' end of cleaved DNA. Determining the linkage site would provide additional evidence to differentiate the nu-topo I from the mt-topo I or, in the event of possessing the same linkage site, further characterize the mt-topo I. To address the 5' or 3' linkage of the mt-topo I, various groups have performed different experiments to elucidate this particular aspect of topo I mechanism (44-46). I performed the more widely accepted or conventional method, the cleavage assay. By observing gel shift patterns of samples treated with and without proteinase K, the cleavage assay revealed the mt-topo I linkage site. Another mechanistic study involved the inhibitory effect of various drugs on the mt-topo I and nu-topo I. Drug inhibition assays were performed in hopes of further delineating the nu-topo I from its mitochondrial counterpart, and to compare the sensitivity of topoisomerases isolated from calf liver with those isolated from rat liver or calf thymus. Previous studies have reported the mt-topo I from rat liver to be sensitive to dimethyl sulfoxide (DMSO) and to the trypanocidal drug berenil (75). Drug studies were performed using DMSO and berenil to discriminate between the nu-topo I and mt-topo I isolated from calf liver. As well, seven antimicrobial/antifungal drugs were assayed to find additional drugs that could discriminate between the nu-topo I and mt-topo I.

The molecular component of the study, involved the investigation of genes containing topoisomerase domains with the hope of finding the mt-topo I gene. By using primers developed from conserved topoisomerase domains in RT-PCR reactions,
followed by sequencing of the RT-PCR products, and their subsequent characterization via similarity searches, an uncharacterized stretch of sequence containing some identity to topoisomerases was found. The sequence, designated 575-1, was used as a probe to screen a human cDNA library, as a probe in a northern blot, and as a gene specific primer in 3' rapid amplification of cDNA ends (RACE) reactions.

In addition, to further characterize 575-1 and the other RT-PCR products generated from topo conserved domains, an evolutionary molecular analysis against seven known eukaryote type I topoisomerase genes was performed. Multilevel consensus sequence analysis (MEME), and phylogeny tree (dnapars) bioinformatics programs were performed to show what relationships, if any, exist between known eukaryotic type I topoisomerases and RT-PCR products generated from a eukaryotic topo I primer set, C-D. The C-D primer set was of particular interest, due to the fact that they flank the type I topoisomerase catalytic domain.

MECHANISTIC ANALYSIS

Purification of the Mitochondrial Type I DNA Topoisomerase

The mitochondrial DNA topoisomerase I has been shown to be a 78 kDa protein (p78) (75). As described in the methods section, starting with 1 kg of calf liver, mt-topo I activity was tracked after each column purification by means of a standard topoisomerase relaxation assay. Protein concentrations were determined by Bradford analysis. Fig. 6 shows column fractions displaying topo activity. Fractions showing topo activity were combined and loaded onto the next column. Total topoisomerase activity detected for each column fraction is shown in Table 8. Evidence of topo activity was seen as a ladder...
of bands which represented varying levels of plasmid relaxation. In the phosphocellulose column a faint ladder was seen in fractions 60 through 93. Ideally, topoisomerase activity should be captured over a much smaller range, but due to the fact that the phosphocellulose bed volume was too large in comparison to the amount of protein loaded, the protein came off the column at a much slower rate. These fractions were combined and loaded onto a hydroxyapatite column. In the hydroxyapatite column, fractions 41 through 57 were collected and loaded onto the dsDNA cellulose column. The topoisomerase activity appeared to peak at fraction 45 and then decrease with each subsequent fraction. In the dsDNA cellulose column, fractions 7 and 8 were combined and applied to a centricon-10 concentrator. The purified mt-topo I had a short half-life with topoisomerase activity decreasing over a period of a few weeks. Previously isolated mt-topo I fractions were concentrated by dialysis and used in the following mechanistic experiments (74).

**TABLE 8**

Topoisomerase Purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein Weight (mg)</th>
<th>[Protein] (mg/ml)</th>
<th>~Total Topo Activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Mitochondria</td>
<td>490</td>
<td>18914</td>
<td>38.6</td>
<td>ND</td>
</tr>
<tr>
<td>Pure Mitochondria</td>
<td>240</td>
<td>468</td>
<td>1.95</td>
<td>ND</td>
</tr>
<tr>
<td>S2</td>
<td>40</td>
<td>ND</td>
<td>ND</td>
<td>169,200</td>
</tr>
<tr>
<td>PC</td>
<td>225</td>
<td>ND</td>
<td>ND</td>
<td>52,500</td>
</tr>
<tr>
<td>HA</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>48,000</td>
</tr>
<tr>
<td>dsDNA cellulose</td>
<td>4.1</td>
<td>ND</td>
<td>ND</td>
<td>3,075</td>
</tr>
</tbody>
</table>

*Note.* S2 (supernatant 2), PC (phosphocellulose column), HA (hydroxyapatite column), ND (not determined, protein concentration below level of detection by Bradford analysis)
Cleavage Assay

A distinguishing feature of type IB eukaryotic topoisomerases is that they become linked to the 3’ terminus of a cleaved DNA strand (16), while the type IA bacterial topoisomerases become linked to the 5’ terminus of the broken strand (76). One method of determining the linkage site is by observing whether the topoisomerase becomes radiolabeled when using uniquely end-labeled substrates. This is often assessed by the following three experiments.

FIG. 6. Active topo I fractions from calf liver. pUC19 alone (control).
The results of two different experiments suggested that the mitochondrial topoisomerase is linked to the 5' terminus via a phosphotyrosine linkage. The first experiment involved the use of uniquely end-labeled substrates, which were assessed by measuring radioactivity after filter binding (77) or potassium sodium dodecyl sulfate precipitation (SDS) (figure 4, schematic diagram) (78). Radioactive filter binding involved spotting a reaction mixture of end-labeled DNA incubated with and without topoisomerase. The filters bound high molecular weight (MW) molecules such as DNA linked to topoisomerase. Subsequent washings of the filters removed lower MW molecules. If the DNA were 3' labeled, for example, and the topoisomerase was 5' linked, a high amount of radioactivity would be detected on the filter. Analogously, if the DNA were 5'-end labeled, and the topo was 5' linked, low amounts of radioactivity would be detected. Low levels of radioactivity would result because the topoisomerase would be bound to the unlabeled portion of the DNA cleavage product, and therefore, be undetected by the counter. The potassium-SDS precipitation method, on the other hand, worked along similar lines, except that instead of using filters, SDS bound the topo while it was still linked to the radiolabeled DNA. This SDS/topo/DNA complex was then precipitated and counted. End-labeled DNA with no topoisomerase attached to it would be detected with little or no counts because the radiolabeled DNA cannot bind to the SDS alone.

My own radioactive filter binding experiments did not provide reliable results. The inconsistency in data was believed to be due to the observed high background levels. I was also unable to obtain expected results for a positive control. Nu-topo I was used to serve as a 3' end linkage positive control. Using a 3' end labeled substrate, low counts
were expected when nu-topo I was used in this assay. For both ssDNA and dsDNA substrates (sample 6-9, table 9) higher then expected counts, relative to the background reading (sample 5, table 9), were seen. Background levels were determined by spotting a mixture consisting of all assay components minus the topoisomerase onto DE-81 disks. High background counts, as shown in sample 5 of table 9, indicated that the radiolabeled substrate was somehow interacting with K⁺SDS⁻ without the presence of the topoisomerase to link it. BSA contained in the 10X assay cocktail and/or the presence of too high a concentration of K⁺SDS⁻ was thought to contribute to the high background readings. The procedure was therefore repeated using in assay cocktail that did not contain BSA and decreased amounts of K⁺SDS⁻. The hope was that by reducing the background more consistent readings would be obtained.

**TABLE 9**

K⁺SDS⁻ Precipitation Assay Using 3' End-labeled H-ori as the Substrate

Total reaction volume was 50 μl.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mt-topo (μl)</th>
<th>Nu-topo (μl)</th>
<th>Counts (cpm) ssDNA</th>
<th>Counts (cpm) dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>---</td>
<td>6332</td>
<td>3318</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>---</td>
<td>8995</td>
<td>7912</td>
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<tr>
<td>3</td>
<td>5</td>
<td>---</td>
<td>7130</td>
<td>5320</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>---</td>
<td>8402</td>
<td>7197</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5199</td>
<td>4034</td>
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<tr>
<td>6</td>
<td>---</td>
<td>1</td>
<td>5734</td>
<td>4703</td>
</tr>
<tr>
<td>7</td>
<td>---</td>
<td>2.5</td>
<td>7120</td>
<td>5170</td>
</tr>
<tr>
<td>8</td>
<td>---</td>
<td>5</td>
<td>9118</td>
<td>5184</td>
</tr>
<tr>
<td>9</td>
<td>---</td>
<td>10</td>
<td>6885</td>
<td>5840</td>
</tr>
</tbody>
</table>

The next K⁺SDS⁻ precipitation assay attempt, as previously mentioned, focused on reducing the background counts. Three samples were prepared. The first tested
whether BSA contributed to high background counts by eliminating it from the assay cocktail. The second tested whether the [SDS] in the stop solution was high by reducing it from 2% to 1.5%. The last sample tested for both variables by containing no BSA and 1.5% SDS. As seen in table 10, the overall background was reduced as compared to the first background reading (sample 5, table 9). The ssDNA sample 2 had a 11% lower background then sample 1, and a 3.6% lower background then sample 3. The 11% decrease in background may be due to the fact that there was simply more SDS available to interact with the radiolabeled substrate. The dsDNA sample 2 was 88% lower then sample 1, and 41% lower then sample 3. The 88% difference observed in sample 2 may be due the fact that less than 1.5% SDS was added to this particular sample due to pipetting error. After spinning the samples down, I observed a smaller pellet in sample 2 as compared to the other samples. However, in both ssDNA and dsDNA samples, where no BSA was present and SDS amounts were reduced, there was an overall decrease in background. The assay was therefore repeated using these modifications.

**TABLE 10**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Counts (cpm) ssDNA</th>
<th>Counts (cpm) dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>753</td>
<td>537</td>
</tr>
<tr>
<td>2</td>
<td>668</td>
<td>66</td>
</tr>
<tr>
<td>3</td>
<td>693</td>
<td>112</td>
</tr>
</tbody>
</table>

The third K⁺SDS⁺ precipitation assay attempt using no BSA and 1.5% SDS was performed in duplicate. Once again, inconsistent data was observed. Background levels continued to be high with an average value of 4562 cpm between the two sets of assays.
Trends observed in one set; for example, increase in counts with increase in amount of topoisomerase added, were not seen in the corresponding duplicate set. Therefore, the K+SDS precipitation assay was abandoned.

As an alternative approach, the exonuclease III method performed by Jessica Lin proved to be more reliable (92). Exonuclease III (Exo III) is a 3'→5' exonuclease that produces 5' mononucleotides, and is specific for double-stranded DNA that has a free 3' hydroxyl terminus at blunt or 5' overhanging ends or at a nick (75). If the 3' end of the DNA break is free of enzyme attachment, the Exo III will degrade the DNA strand. This decrease in size of the topo-Exo III products would increase the migration rate upon gel electrophoresis compared to topo cleavage products not treated with Exo III. If the 3' end of the DNA break is blocked by enzyme, no change in the migration pattern of topo cleavage products would occur upon Exo III treatment. The results of the cleavage assay after treatment with Exo III showed that the migration rates of the nu-topo I products seem to be unaffected by treatment with Exo III. This is expected since it has been established that the nu-topo I becomes linked to the 3' terminus of cleaved DNA (16). In the case of the mt-topo I, the Exo III treatment increased the migration rate, reflecting a decrease in size of mt-topo cleavage products. The data from this experiment suggested that mt-topo I is linked at the 5' end of the cleavage site, leaving a free 3' hydroxyl group on the other side of the break which would serve as a substrate for Exo III.

Two additional assays used to support the 5' end linkage results are variations of the topoisomerase/Exo III assay. The results from these studies indicated a blocked 5' terminus and a free 3' hydroxyl group being generated by the mitochondrial enzyme (74). The suggestion from these three independent, less conventional methods was that the mt-
topo I was covalently linked through a phosphotyrosine to the 5' end of the cleaved DNA.

To determine the mt-topo I using the more widely accepted method, I performed cleavage assays using 3' end or 5' end labeled H-ori (mitochondrial heavy strand replication origin, 797 bp) or TERM (mitochondrial terminator region, 387 bp). H-ori and TERM DNA fragments were used as substrates because they contained known preferential mt-topo I cleavage sites (74). These fragments were PCR amplified from recombinants that contained H-ori or TERM inserts (fig. 7).

FIG. 7. PCR amplified H-ori and TERM DNA fragments. Lane 1, A and B, 100 bp MW ladder; lane 2, A, H-ori; lane 2, B, TERM.

H-ori and TERM were then 3’ or 5’ end labeled using two different techniques. To produce uniquely 3’ end labeled substrates, T4 DNA polymerase and [α-32P] dCTP were used to replace a unique C at the 3’ end of the H-ori or TERM DNA fragment (fig. 8). To produce uniquely 5’ end labeled substrates, T4 polynucleotide kinase and [γ-32P] ATP were used. This reaction was then followed by a Hinf I digestion, which specifically cleaves one of the two radiolabeled 5’ ends generated from the T4 PNK
reaction (fig. 9). H-ori and TERM possess unique Hinf I restriction enzyme sites near one of their 5' ends.

FIG. 8. Autoradiograph of 3' end labeled TERM. Underlined cytosine is removed and replaced with radioactive cytosine shown in bold. Arrow indicates migration of radiolabeled Term.

FIG. 9. Autoradiograph of 5' end labeled H-ori. Lane 1, undigested 5' end labeled H-ori; lane 2, Hinf I digested 5' end labeled H-ori.
Before performing the cleavage assay, a serial dilution of the mt-topo I was tested in order to determine the optimal units of activity needed to perform the cleavage assay. Both 5' and 3' end labeled substrates were tested. Fig. 10 shows the results of a mt-topo I dilution using 5' end labeled H-ori. At 100 units of mt-topo I activity the DNA is digested away, at 50 units a few cleavage products are evident, and at 25 units products are evident but with slightly less intensity. For 5' end labeled H-ori, 50 units of mt-topo I activity was determined to be the optimal amount of activity for use in the cleavage assay.

![Lane 1 2 3](FIG 10. Mt-topo I serial dilution using 5' end labeled H-ori. Lane 1, 100 units mt-topo I/25 μM CPT; lane 2, 50 units mt-topo I/25 μM CPT; lane 3, 25 units mt-topo I/25 μM CPT. Arrows indicate cleavage products.)

Fig. 11 shows the results of a mt-topo I dilution using 3' end labeled term. At 100 units of mt-topo I activity some cleavage products are visible, at 50 units cleavage products were completely digested, and at 25 units more concentrated cleavage products
are evident. For 3' end labeled term, 25 units of mt-topo I activity was determined to be the optimal amount of activity for use in the cleavage assay. It is unknown as to why products were digested away at 50 and not 100 units of topo activity. The inability to cleave was attributed to low stock concentrations of active mt-topo I.

![Diagram](image1)

**FIG. 11.** Mt-topo I serial dilution using 3' end labeled TERM. Lane 1, 100 units mt-topo I/25 μM CPT; lane 2, 50 units mt-topo I/25 μM CPT; lane 3, 25 units mt-topo I/25 μM CPT. Arrows indicate cleavage products.

Cleavage assay attempts using 5' end labeled DNA were unsuccessful. No cleavage products were evident using 50, 75, or 100 units of mt-topo I activity. The inability to cleave was attributed to low mt-topo I stock concentrations.

Cleavage assay attempts using 3' end labeled H-ori showed the mt-topo cleavage products aggregating together, unable to enter the agarose gel in the absence of PK (fig. 12, lane 5). Addition of PK resulted in migration of numerous cleavage products into the gel (lane 6). When the topoisomerase is removed from the 5' end by digestion with proteinase K, cleavage products were free to migrate. This shows that the topoisomerase
is linked to the 5' end of the cleaved DNA. This agrees with the data from the exonuclease III assay result. The presence of the enzyme on the 5' end of DNA is catenating or holding broken strands together causing them to form one large structure which could not pass through the agarose gel.

The overall difficulty in obtaining cleavage products for 5' end labeled substrates was attributed to the low stock concentration of the mt-topo I. As cleavage assay experiments were performed, the enzyme, possibly due to repeated exposure to freezing and thawing, lost more than half of its activity over a few months. Therefore, to maintain the levels of mt-topo I activity needed, I increased the volume of enzyme added to the cleavage assay reaction mixture. Originally, the mt-topo I was assayed at 10 units/µl. After a few months, the activity was assayed at 4 units/µl. Instead of adding 5 µl to a 30 µl total volume to obtain 50 units of activity, I had to more than double the volume. This large increase in enzyme volume may have altered the cleavage assay cocktail final concentrations; therefore, contributing to the inability of the mt-topo I to properly cleave. Two attempts were made to isolate fresh mt-topo I from calf liver to use in the cleavage assay. As previously described, the isolated mt-topo I had a short half-life. Topoisomerase used in these assays were from previous calf liver preparations (74).
FIG. 12. 3' end labeled cleavage assay. Lane 1, 3' end $^{32}$P labeled H-ori alone; lane 2, 3' H-ori and CPT; lane 3, 3' H-ori and PK; lane 4, 3' H-ori and SDS; lane 5, 3' H-ori/CPT/SDS and mt-topo I; lane 6, 3' H-ori/CPT/SDS/mt-topo I and PK. Arrows indicate released cleavage products.

Drug Inhibition Assay

Drug inhibition assays were performed in hopes of further delineating the nu-topo I from its mitochondrial counterpart, and to compare the sensitivity of topoisomerases isolated from calf liver with those isolated from rat liver or calf thymus. The enzymes were serially diluted and topo activity determined by a standard topoisomerase relaxation assay. The same serially diluted set of enzymes were used in subsequent mixtures.
containing the drug of interest at varying concentrations. By comparing topoisomerase activity with and without the drug, differences in activity could be observed.

Five percent DMSO had previously been shown to inhibit mt-topo I activity isolated from calf thymus by 60% and result in almost total loss of activity with addition of 20% DMSO. Calf thymus nu-topo I was found to be uninhibited by 5% DMSO, but at a higher concentration of 20% DMSO, ~50% inhibition was observed (75). In my DMSO inhibition studies, I added DMSO to final concentrations of 1%, 5%, and 20% in standard topoisomerase relaxation assays using a serial dilution of mt-topo I or nu-topo I isolated from calf liver.

In assaying the nu-topo I, there appeared to be an average value of 3.7% inhibition using 1% DMSO (see Graph 1, fig. 13, and Table 11, lane 5-7 compared with lanes 2-4, percentages are based on densitometry results from photographs of agarose gels). Using 5% DMSO, there appeared to be increases in nu-topo I activity in lane 8 (1.9 units topo activity, 16% increase), and in lane 9 (2.0 units of topo activity, 5% increase). A slight decrease in activity (5.9% inhibition) was observed in lane 10. This apparent stimulation of activity by 5% DMSO has been observed in a previous study (75). As depicted in lanes 11-13 (fig. 13), nu-topo I activity was inhibited by an average value of 28% with addition of 20% DMSO.

The nu-topo I did not completely correlate with previous studies. The calf liver nu-topo I was inhibited 28% as compared to 50% inhibition in the presence of 20% DMSO, and showing a slight inhibition verses no inhibition in the presence of 1% DMSO. But, as in the previous study, the nu-topo I displayed some stimulation of activity in the presence of 5% DMSO.
TABLE 11

DMSO Percent Inhibition
Densitometry results for figures 16 and 17, respectively.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Nu-topo I Activity (units)</th>
<th>% Inhibition (average for each DMSO set)</th>
<th>%DMSO added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>3</td>
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</tr>
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<td>4</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>0</td>
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<td>1</td>
</tr>
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<td>7</td>
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</tr>
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<td>1.9</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
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<td>11</td>
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<td>19</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>1.7</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>0.75</td>
<td>53 (28)</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lane</th>
<th>Mt-topo I Activity (units)</th>
<th>% Inhibition (average for each DMSO set)</th>
<th>%DMSO added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>0</td>
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<tr>
<td>5</td>
<td>1.9</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1.2</td>
<td>20 (21)</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1.7</td>
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<td>5</td>
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<td>1.2</td>
<td>37</td>
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<td>10</td>
<td>0.98</td>
<td>35 (29)</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>0.83</td>
<td>58</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>0.24</td>
<td>87</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>0.34</td>
<td>77 (74)</td>
<td>20</td>
</tr>
</tbody>
</table>

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FIG. 13. DMSO inhibition assay of nu-topo I. Lane 1, pUC19 alone; lane 2, ~2 units nu-topo I; lane 3, ~1 unit nu-topo I; lane 4, ~0.5 units nu-topo I; lane 5, ~2 units nu-topo I 1/1% DMSO; lane 6, ~1 unit nu-topo I 1/1% DMSO; lane 7, ~0.5 units nu-topo I 1/1% DMSO; lane 8, ~2 units nu-topo I 1/5% DMSO; lane 9, ~1 unit nu-topo I 1/5% DMSO; lane 10, ~0.5 unit nu-topo I 1/5% DMSO; lane 11, ~2 units nu-topo I 1/20% DMSO; lane 12, ~1 unit nu-topo I 1/20% DMSO; lane 13, ~0.5 units nu-topo I 1/20% DMSO.

FIG. 14. DMSO inhibition assay with mt-topo I. Lane 1, pUC19 alone; lane 2, ~2 units mt-topo I; lane 3, ~1.75 unit mt-topo I; lane 4, 1 unit mt-topo I; lane 5, ~2 units mt-topo I 1/1% DMSO; lane 6, ~1.75 unit mt-topo I 1/1% DMSO; lane 7, ~1 unit mt-topo I 1/1% DMSO; lane 8, ~2 units mt-topo I 1/5% DMSO; lane 9, ~1.75 unit mt-topo I 1/5% DMSO; lane 10, ~1 unit mt-topo I 1/5% DMSO; lane 11, ~2 units mt-topo I 1/20% DMSO; lane 12, ~1.75 unit mt-topo I 1/20% DMSO; lane 13, ~1 unit mt-topo I 1/20% DMSO.

The calf liver mt-topo I, on the other hand, was inhibited by an average value of 21% in the presence of 1% DMSO (Graph 1, fig. 14 and Table 11, lanes 5-7) and an average value of 29% inhibition in the presence of 5% DMSO (lanes 8-10). 29% inhibition did not correlate with previous reports of 60% inhibition. Mt-topo I activity, as
in previous studies, was highly inhibited by 20% DMSO possessing an average value of 74% inhibition (lane 11-13).

Less inhibition was observed than expected for the mt-topo I in the presence of 5% DMSO, 29% verses 60% inhibition. In the presence of 20% DMSO, mt-topo I did correlate with previous results, displaying high levels of inhibition.

The overall conclusion was that the mt-topo I was more sensitive to inhibition by DMSO than its nuclear counterpart. DMSO may be used to distinguish calf liver mt-topo I from calf liver nu-topo I. In addition, each enzyme followed the overall trends of the previous study. The slight differences seen in my experiments may be attributed to minor structural differences between enzymes isolated from calf liver verses rat liver.

**GRAPH 1.** DMSO inhibition of nu-topo I and mt-topo I
(nu-topo I is represented by the left bar, mt-topo I by right bar)

In the case of the trypanocidal drug, berenil, previous reports have found that, like DMSO, berenil can differentiate between the mt-topo I and nu-topo I. Calf thymus nu-topo I was found to be unaffected by 5-50 μM berenil. While calf thymus mt-topo I was inhibited 37% in the presence of 5 μM berenil and inhibited 63% by 20 μM berenil (75). In order to observe various levels of berenil inhibition with mt-topo I and nu-topo I,
samples purified from calf liver, 10, 40, and 100 μM final concentrations of berenil were added to standard topoisomerase relaxation assays. Fig. 15 shows the berenil assay results for mt-topo I (lanes 2-9) and nu-topo I (lanes 10-17). By comparing the same levels of mt-topo activity without berenil (lane 2,3) verses with 10 μM berenil (lane 4,5), an average value of 5% inhibition of relaxation activity was observed. Inhibition of mt-topo activity increased to 23% when 40 μM berenil was added (lane 6,7). Twenty-eight percent inhibition was observed with the addition of 100 μM berenil (lane 8, 9). As compared to previous studies with calf thymus mt-topo I, much less inhibition was observed for calf liver mt-topo I. The nu-topo I, exhibited some inhibition in the presence of berenil. Average inhibition values of 11% in the presence of 10 μM berenil, 16.5% in the presence of 40μM berenil, and 11% in the presence of 100 μM berenil were detected (lanes 10-17). Calf liver nu-topo I, in contrast to calf thymus nu-topo I, was slightly inhibited by berenil. Calf liver nu-topo I was inhibited less than calf liver mt-topo I in the 40-100 μM berenil range.

FIG. 15. Berenil assay. Lane 1, pUC19 alone; lane 2, ~2 units mt-topo; lane 3, ~2 units mt-topo; lane 4, ~2 units mt-topo/10μM berenil; lane 5, ~2 units mt-topo/10μM berenil; lane 6, ~2 units mt-topo/40μM berenil; lane 7, ~2 units mt-topo/40μM berenil; lane 8, ~2 units mt-topo/100μM berenil; lane 9, ~2 units mt-topo/100μM berenil; lane 10, 1 unit nu-topo; lane 11, 0.5 unit nu-topo; lane 12, 1 unit nu-topo/10μM berenil; lane 13, 0.5 unit nu-topo/10μM berenil; lane 14, 1 unit nu-topo/40μM berenil; lane 15, 0.5 unit nu-topo/40μM berenil; lane 16, 1 unit nu-topo/100μM berenil; lane 17, 0.5 unit nu-topo/100μM berenil.
TABLE 12

Berenil Percent Inhibition
Densitometry results for figure 18. Mt-topo activity shown in lanes 2-9, nu-topo I activity shown in lanes 10-17. Average value written in parentheses.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Topo I Activity (units)</th>
<th>% Inhibition</th>
<th>Berenil Added (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>10</td>
<td>10</td>
</tr>
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<td>5</td>
<td>1.9</td>
<td>0 (5)</td>
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<td>10</td>
<td>1.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0.8</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1.4</td>
<td>22</td>
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<td>0 (11)</td>
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<td>14</td>
<td>1.2</td>
<td>33</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>0.95</td>
<td>0 (16.5)</td>
<td>40</td>
</tr>
<tr>
<td>16</td>
<td>1.4</td>
<td>22</td>
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<tr>
<td>17</td>
<td>1.8</td>
<td>0 (11)</td>
<td>100</td>
</tr>
</tbody>
</table>

In the antimicrobial/antifungal inhibition studies, six (A1, A6, B1, C5, D1, F2) of the seven drugs did not inhibit the mt- or the nu-topos. The mt-topo I was not inhibited by the drug C1, but the nu-topo I was inhibited in a dose-dependent fashion. At a concentration of 2X LC50 (46 μM), C1 inhibited nu-topo I activity by %60. Fig. 16 and Graph 2 shows a decrease in topoisomerase activity with increased addition of C1. The nu-topo I was found to be more sensitive to inhibition by C1 than its mitochondrial counterpart.
FIG. 16. Nu-topo I C1 inhibition assay. Lane 1, nu-topo I alone; lane 2, nu-topo I and 2X LC50 C1; lane 3, 1X LC50; lane 4, 0.5X LC50; lane 5, 0.1X LC50.

GRAPH 2. Effects of C1 on the mt-topo I and nu-topo I (bars from left to right correspond with labels in box starting from the top)
MOLECULAR ANALYSIS

*Human Placental Tissue Isolation*

To prepare a probe to screen a cDNA library for the mt-topo I gene, RT-PCR was performed on total RNA isolated from human placenta. From a 600 mg sample of human placental tissue, 93 µg of total RNA were recovered. A 260:280 ratio of 1.88 indicated minimal protein contamination of the RNA sample. Formaldehyde gels revealed intact 28S and 18S rRNA bands with minimal degradation (fig. 17).

![FIG. 17. Total RNA isolation from human placental tissue. Lane 1, total human placental RNA samples (2 µg); lane2, RNA MW Markers (3 µg).](image)

*RT-PCR*

DNA was successfully synthesized from RNA using primers homologous to conserved regions in eukaryote and prokaryote type I topoisomerases (fig. 18 and 19). A through D represent the four conserved eukaryotic type I topoisomerase domains. Products produced using region D were of particular interest due to the fact that D

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contains the topoisomerase catalytic domain. Figure 18 shows the expected size fragments that are generated from the known human nu-topo I. A 1590 bp nu-topo I PCR product would be generated using the primer set $A_L$ and $D_{rev}$. A 1090 bp nu-topo I PCR product would be generated using the primer set $A_L$ and $C_{rev}$. A 531 bp nu-topo I PCR product would be generated using the primer set $C_L$ and $D_{rev}$. Figure 20 shows the expected size fragment for the known bacterial topo I, using $A_{proL}$ and $C_{proR}$. Degenerate conserved type I topoisomerase primers from five different eukaryotic species were used with the idea that messages that contained type I topoisomerase conserved domains would be amplified. Several RT-PCR products were purified, sequenced, and subjected to a Genbank search for identification. The hope was to find a stretch of sequence that was uncharacterized yet possessed some prokaryotic and/or eukaryotic topoisomerase identity.

FIG. 18. Primers and expected RT-PCR DNA products of eukaryotic primers. Sizes seen on right represent expected size fragments for the known human nu-topo I.
A B C D

consensus prokaryotic sequence

A C

517 bp

FIG. 19. Primers and expected RT-PCR DNA products of prokaryotic primers. Sizes seen on right represent expected size fragments for the known E. Coli topA.

As a positive control for RT-PCR, an expected 300 bp region of α-actin was amplified. The primers were provided by Dr. William Kearns of the Center for Pediatric Research at Eastern Virginia Medical School. Fig. 20 shows the results of PCR amplification of actin. The expected 300 bp product is seen in lane 2.

Lane 1 2

500 bp ← actin product

FIG. 20. RT-PCR product positive control. Lane 1, 100 bp MW marker; lane 2, 300 bp actin product.
One RT-PCR product of particular interest, 575-1, was generated using the degenerate C<sub>l</sub>-D<sub>R</sub> primer set (fig. 18, 21). The two products seen in fig. 21, in addition to other RT-PCR products generated from different conserved topo primer sets, were purified (fig. 22). Purified RT-PCR products were cloned into pGEM vectors, sequenced, and then characterized via similarity searches. Out of 6 colonies selected and grown from the purified degenerate C-D 575 ligation mixture, the sequence of the clone from the first colony, called 575-1, when subjected to computer generated similarity searches was found to possess both topo I and topo II signature similarity. Sequence information obtained of the clone from the sixth colony, designated 575-6, was found to be the known human nu-topo I. Finding the known nu-topo I served as a more specific positive control by confirming that the use of the degenerate type I topoisomerase conserved primer sets in RT-PCR reactions can generate a topoisomerase gene. The RT-PCR product sequenced and their corresponding similarity search results are displayed in Tables 8-16.
FIG. 22. Purified RT-PCR products. Lane 1, 100 bp MW ladder; lane 2, Deg A\textsubscript{L}C\textsubscript{R} 425 bp; lane 3, Pro A\textsubscript{L}C\textsubscript{R} 450 bp; lane 4, Pro A\textsubscript{L}C\textsubscript{R} 400 bp; lane 5, Deg C\textsubscript{L}D\textsubscript{R} 700 bp; lane 6, Deg C\textsubscript{L}D\textsubscript{R} 575 bp.

Since it was unknown how related the mt-topo I gene was to the known nuclear topo, each RT-PCR was initially conducted under less stringent conditions, i.e. low annealing temperature and varying Mg\textsuperscript{2+} concentrations. After obtaining several products from one primer set, the stringency was gradually increased in order to decrease the production of products generated from nonspecific binding. Fig. 23 shows an optimization of RT-PCR product synthesis by modifying the MgCl\textsubscript{2} concentration and annealing temperature. Each RT-PCR product in lane 4 was subsequently cut from an agarose gel, purified, and sequenced. Not all fragments were sequenced due to difficulties inherent in using degenerate primers. These difficulties are discussed in greater detail in the sequencing results section.
FIG. 23. Optimized RT-PCR product synthesis. Lane 1, 100 bp MW Marker; lane 2, Pro A_l-C_R 3 mM MgCl_2; lane 3, Pro A_l-C_R 4 mM MgCl_2; lane 4, Pro A_l-C_R 4 mM MgCl_2 + increase in annealing T by 1°C.

Plaque PCR

This procedure was developed in order to provide a fast and easy way to screen potential cDNA positives. Plaques, that were cored out with pasteur pipet tips and resuspended in SM buffer, served as template sources in standard PCR reactions.

Since 575-1 was uncharacterized, yet possessed some topoisomerase similarity it was used as a probe to screen a human cDNA library. Plaque PCR was used to determine if tertiary cDNA screen positives did, in fact, contain a portion of the 575-1 probe. A 575-1 internal primer set was developed using PCR Primer Design software to amplify an expected 274 bp region (results, fig. 24; diagram, fig. 29). Clone 6A was the only sample to produce the expected fragment.
Plaque PCR was also performed to quickly screen potential topo II cDNA positives. A mixture of five topo II probes developed from conserved type II topoisomerase regions were used to screen a human cDNA library. To eliminate extraneous DNA bands that were due to amplification of the bacterial vector, a “negative” plaque was amplified along with positives (fig. 25, lanes 6-8). For example, by comparing bands seen in lane 6 (negative control) against PCR products generated using the same primer sets, as in lanes 2 and 5, irrelevant products can be eliminated.

As shown in fig. 25, several PCR products were generated using 3 different topo II primer sets. Clone 10-2 produced products for all three primer sets, making it a potential mt-topo II candidate. An expected size nu-topo II fragment was seen for the 5’ primer set (~517 bp) in addition to many other products for clones 10-2 and 19-1A (lane 2 and 5). Clones 10-2 and 19-1A lost their viability after a few weeks, ie. would not grow, before sequence information of the inserts could be obtained. Attempts were made to sequence plaque PCR products. A 400 bp plaque PCR product generated using topo II 5’LR primer set was sequenced (Table 14).
FIG. 25. Plaque PCR products using topo II primers. Lane 1, 100 bp MW Markers; lane 2, plaque 10-2 topo II 5'LR primers; lane 3, plaque 10-2 topo II mid LR primers; lane 4, plaque 10-2 topo II 3' LR primers; lane 5, plaque 19-1A 5' LR primers; lane 6, negative control topo II 5' LR primers; lane 7, negative control topo II mid LR primers; lane 8, negative control topo II 3' LR primers.

*pGEM Vector Cloning*

RT-PCR products of interest were inserted into pGEM vectors to facilitate cycle sequencing. Inserts were flanked by SP6 and T7 polymerase promoters, and therefore, could be amplified and directly sequenced using primers representing either promoter sequence. The pGEM vector's multiple cloning region was within the coding region of β-galactosidase. Insertional inactivation of β-galactosidase allowed recombinants to be directly identified by blue/white screening. Successful insert ligation was indicated by a white bacterial colony. A blue colony indicated unincorporation of the insert. Rapid plasmid preparations were performed with samples run on agarose gels to confirm successful ligation of insert. ssDNA isolations were also performed in an effort to provide a purer template source for cycle sequencing.
**Rapid Plasmid Preparation**

Fig. 26 shows a 1% agarose gel of double stranded DNA isolated from individual clones recovered after plating PCR insert/pGEM vector cloning reactions. As expected, slower migrating bands were seen for recombinants that contain inserts (lanes 1, 3) and a faster migrating band for the vector alone (lane 2).

![Lane 1  2  3]

**FIG. 26.** Rapid plasmid preparation. Lane 1, Deg. C-D 290 bp insert white colony; lane 2, no insert blue colony; lane 3, Deg. A-C 900 bp insert white colony.

**ssDNA Isolation**

Fig. 27 shows a 1% agarose gel of single stranded DNA isolated from pGEM vectors with 575-1 and 575-6 inserts.

![Lane 1  2]

**FIG. 27.** ssDNA isolation. Lane 1, degenerate conserved topo primer set C-D 575-1; lane 2, degenerate conserved topo primer set C-D 575-6. Faint upper band is the helper phage DNA.
Cycle Sequencing

Initially, RT-PCR products were commercially sequenced. The sequencing lab successfully sequenced only 2 out of the 8 samples sent. The low success rate was attributed to the unpredictable nature of working with degenerate oligomers. In lab sequencing was more productive because it allowed for the optimization of parameters needed in order to obtain legible sequence. Higher concentrations of degenerate primers were needed in order to obtain the appropriate concentration for the one primer in the degenerate set that was specific to the RT-PCR product of interest. Sequencing of products produced from degenerate primers was further complicated by the fact that contamination of a comigrating product was common. Insertion of the RT-PCR product into a vector eliminated the problem of comigrating products due to the fact that only one product can be inserted into the vector. Cycle sequencing using these cloned recombinants as templates provided more legible sequence.

Fig. 28 shows a cycle sequencing diagram for a commercially sequenced RT-PCR product of ~1100 bp. Having ligated purified RT-PCR products into a pGEM vector, the inserts were sequenced with SP6/T7 primers. SP6 and T7 promoters flank the pGEM insertion site. Since these primers annealed to regions just outside of the insert, they eliminated sequencing problems due to degenerate primer use. Several RT-PCR products were sequenced. Tables 13-15 show the primer sets used, product sizes, and sequences obtained.
FIG. 28. Cycle sequencing diagram of 1100 bp Deg A_L-C_R RT-PCR product. Four different color waves are used to represent G, A, T, or C.

FIG. 29. Diagram of 575-1 primers. Primers generated from known sequence information obtained for 575-1. The primers 575-1_L and 575-1_R were used in plaque PCR reactions to screen potential cDNA positive clones. 575-1 internal forward primer was developed as an internal sequencing primer. Nested primer 2 and nested primer 1 were used in 3' RACE reactions.
<table>
<thead>
<tr>
<th>Primer (seq. primer)</th>
<th>Size (bp)</th>
<th>DNA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>700</td>
<td>TAATACGACTCACTATAGGGGGAATTGGGCCCCAGTCCGA TGCTCCCGCCCGCCTGAGCTGGGAGATATGACGACAGGA GCTCCTGAAAGAATGGGGAACATTCGAGACTC CTAACCTCTGCCCCTTCCTTCGAGGGAGATCTATAGAAGA</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>575</td>
<td>ATGGGAACAAAGGACCGGGAGATCGTTGGATGATGGGATCCGCACACCCACTGCTG CATTCTTCTTCTTAATATTAGTGATGGGAACAAGTCTTCTTCGAC AAGTAATCTCTGTCAGGCTAACTCAGAGGAAATTATCACATATGCCTTCA</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>575-1</td>
<td>TTA TGG AAC ACA GCA GCA TAA ATA CAG AGA AAG AAT AGA GTG CAG ACT GTA GCT TTC TTT GTG CTGT CAG TAT TAT TTA ATT TAT ATT ACA TTA CAT TAC A</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>600</td>
<td>TAT CTT GTT GAT AGC ATC TTC AGC TAT AGC TTC GAC GAG ATT CAT ACA TCT TAA CCA AGA TAC AAG TCACTTCAGGCTACAGCAGCCAAGATGTCATGACATGCTGAGGAGGAGTCTCTTTAGAAACA</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>290-2</td>
<td>TA TGG AGC ACA AGG AGG CTG TGC AAA CTG TTG TGA CAT AGT GGC TGC TGC CAG AGC GCT ATC GAC TAA ACT GCT GGT AGA GTT GTG ACT TCA TAG TCA TAG TCA A</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>290-3</td>
<td>TATT GGA GCA CAA GGT AGG GGG AGG AGG AGG GTA TTG GTA GGC AGT CCC ATG TTA CTA TCA TCA TAT TGT GAC TCG AGC TGT CAC TTA GTT GCA TCA</td>
</tr>
<tr>
<td>Deg A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>900</td>
<td>T GGC TCC TTG GTG CTG CAT GAC TCA TGC TCT GAG GCA ACA AGG AAA CTG TGC CTC GCT TCG ACT TCC AGA TGT AGC CCTG AGC GAG ACA TCCA GCT CTT GAC TAT A</td>
</tr>
<tr>
<td>Exact A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>1100</td>
<td>GCTANCCCTGAANCGATCAAGTGGGGAATTCTCATGAAACATA AAGGTCCAGATTNTGNCCCAAAATTGNAACCTGCTCAGNAG AATGTCAGTCTTATTANTATGAGTTAAAGTCANAGCTGAN CAAAATCTCAGGGANNTATGAGCTCAGTCTTATGNAAAATAGGC NCCNACCAGGAATATANTATNANGAATANTANNTAGAAAAC ACTTCTTATAACGACTGGCTCAANGGAAAANACCAGAAGTCA ATAAAAACANTNTACNATNCTAAAAANANTTNGATNTGNNN CATNTATNCNCTNTNAAAANNCCCCAGNNGNNAACTCG AGGCCCCATNAXNNAGCACAANTNCGCTATAGTNNNGGANT</td>
</tr>
</tbody>
</table>

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### TABLE 14

Prokaryote RT-PCR Sequences

<table>
<thead>
<tr>
<th>Primer (seq. primer)</th>
<th>Size (bp)</th>
<th>DNA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro A\textsubscript{L}-C\textsubscript{R} (Creverse)</td>
<td>425</td>
<td>TCMTTRTCMCCCCGTTCGAGAGCTACATCTACGTGAAGAGGTGTCATNNNAAAGYTCTGTCGGCATAMCATTTCTGCTA TKCKCGTCAMAMAGAGGATGTGTTAGGTCATGCACCACATYYAACAAGG GSATAAGASAGACAGA</td>
</tr>
<tr>
<td>Pro A\textsubscript{L}-C\textsubscript{R} (Creverse)</td>
<td>450</td>
<td>GAGGTTGCTCACTCAGATGAGCTCTCTACGAGCTCCAAATTATGAGGAGGATAGTGCAATG ATCCAGGCTCGGCCAGATGAGCTCTCTACGAGCTCCAAATTATGAGGAGGATAGTGCAATG</td>
</tr>
<tr>
<td>Pro A\textsubscript{L}-C\textsubscript{R} (Cforward)</td>
<td>575</td>
<td>TGCATTTCTTCTCACTCAGATGAGCTCTCTACGAGCTCCAAATTATGAGGAGGATAGTGCAATG</td>
</tr>
</tbody>
</table>

### TABLE 15

Topo II Plaque PCR Sequence

<table>
<thead>
<tr>
<th>Primer (seq. primer)</th>
<th>Size (bp)</th>
<th>DNA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textsuperscript{L} (5\textsuperscript{*}L)</td>
<td>400</td>
<td>CGCCCGGNTAAGTCTCTCACAGCGGTACGTTGGAGCGTTG NCTNTACAAGTGCAGACAGATGAGCTACAGTGTTCNCCATTT ATGTCGNCNAGCTGATNCTNGANNTCTTCTAAATCTTC NACGGGACAGCGTCTCCCGTNGANCGNNGGTNNCGATA NACNNNTNCTNNAGCANCNGGANNCNGNANCNACCGN GNGGTTAGGTAGGTAGCTCGNGNCCNGTNNCTNGTAGATTC AGITNNGGCTTCACAA</td>
</tr>
</tbody>
</table>

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**TABLE 16**

Similarity Search Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Size (bp)</th>
<th>Search Program</th>
<th>Similarity Search Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>700</td>
<td>Tblastx</td>
<td>95% Homo sapien cDNA clone h03001 5' end similar to glutathione S transferase T19407</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>600</td>
<td>Tblastx</td>
<td>56% Homo sapien STS genomic sequence, sequence G51046</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>575-1</td>
<td>Blasnt</td>
<td>no significant topo similarity</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>575-1</td>
<td>Tblastx</td>
<td>99% Homo sapien cDNA clone N54001</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>575-6</td>
<td>prositescan</td>
<td>57% similarity topo II signature</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>575-6</td>
<td>prositescan</td>
<td>56% similarity Pro topo I signature</td>
</tr>
<tr>
<td>Deg A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>900</td>
<td>blastn</td>
<td>95% similarity to human mu-topo I U07804</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>290-2</td>
<td>tblastx</td>
<td>95% human rectifier potassium channel 4 HS434P1</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>290-3</td>
<td>blastn</td>
<td>95% human DEAD-box protein p72 U59321</td>
</tr>
<tr>
<td>Deg C&lt;sub&gt;L&lt;/sub&gt;-D&lt;sub&gt;R&lt;/sub&gt; (reverse)</td>
<td>290-3</td>
<td>prositescan</td>
<td>no significant topo similarity</td>
</tr>
<tr>
<td>Exact A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>1100</td>
<td>blastn</td>
<td>94% similarity to human DNA topoisomerase I</td>
</tr>
<tr>
<td>Pro A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>575</td>
<td>tblastx/blastn</td>
<td>no significant similarity</td>
</tr>
<tr>
<td>Pro A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>425</td>
<td>tblastx/blastn</td>
<td>no significant similarity</td>
</tr>
<tr>
<td>Pro A&lt;sub&gt;L&lt;/sub&gt;-C&lt;sub&gt;R&lt;/sub&gt; (forward)</td>
<td>450</td>
<td>tblastx</td>
<td>96% similarity to α-actinin</td>
</tr>
<tr>
<td>topo II 5'&lt;sub&gt;L&lt;/sub&gt; (5')</td>
<td>400</td>
<td>blastn</td>
<td>no significant similarity</td>
</tr>
</tbody>
</table>

*Based on e value = expectation value, the number of distinct alignments with scores equivalent or better than the one of interest, that are expected to occur in a database search purely by chance, i.e. the lower the e value, the more significant the score is. E values less than 1e<sup>-6</sup> were considered significant.
Similarity Searches

Each RT-PCR product sequence was subjected to database searches and sequence alignments. The tblastx search program was used more frequently than other search programs because it was specifically designed to find distantly related genes. Similarity significance was dependent upon the expectation value (e value) of a match. The expectation value is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. The e value is a convenient way to create a significance threshold for reporting similarity results (79). Similarity search results can be seen in Table 16. A brief description of BLAST programs used can be seen in Table 19.

The search tool PROSITESCAN was used to scan protein sequence for occurrence of core patterns of known protein families (90). The Prosite database consists of protein families and domains that include biologically significant regions such as catalytic sites, prosthetic groups, metal binding sites, and molecule binding sites. As shown in Table 16, a PROSITESCAN usually accompanied a BLAST search if inadequate information was attained at the BLAST level. Table 17 shows topoisomerase consensus patterns. Table 18 shows which frame, out of a six frame translation of nucleotide sequence, possessed topoisomerase pattern similarity.
### TABLE 17

Prosite Topoisomerase Consensus Patterns

<table>
<thead>
<tr>
<th>Protein Family/Domain</th>
<th>Prosite Consensus Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eukaryotic DNA</td>
<td>[DEN]-x(6)-[GS]-[IT]-S-K-x(2)-Y-[LIVM]-x(3)-[LIVM].</td>
</tr>
<tr>
<td>Topoisomerase I Active Site</td>
<td>[DEQS]</td>
</tr>
<tr>
<td>Prokaryotic DNA</td>
<td>[EQ]-x-L-Y-[DEQT]-x(3,12)-[LI]-[ST]-Y-x-R-</td>
</tr>
<tr>
<td>Topoisomerase I Active Site</td>
<td>[ST]-[DEQS]</td>
</tr>
<tr>
<td>DNA topoisomerase II</td>
<td>[LIVMA]-x-E-G-[DN]-S-A-x-[STAG]</td>
</tr>
</tbody>
</table>

*Note: Amino acids contained in square brackets indicate any one amino acid at that position. X represents any amino acid. Numbers in parentheses indicate the number of X’s possible at that position.*

### TABLE 18

Prosite Search Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Translation Frame</th>
<th>% Similarity</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>575-1</td>
<td>5'3' Frame 2</td>
<td>57%</td>
<td>DNA topoisomerase II signature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LNsnGNSyHx</td>
</tr>
<tr>
<td></td>
<td>5'3' Frame 3</td>
<td>56%</td>
<td>Prokaryotic DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>xGLypAHHCPLFFLTISvExTS</td>
</tr>
<tr>
<td>575-6</td>
<td>5'3' Frame 2</td>
<td>51%</td>
<td>DNA topoisomerase II signature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sQEGxtAKv</td>
</tr>
<tr>
<td></td>
<td>5'3' Frame 3</td>
<td>60%</td>
<td>Prokaryotic DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>kELtaPDENIPAKILSYN</td>
</tr>
<tr>
<td></td>
<td>3'5' Frame 1</td>
<td>58%</td>
<td>Eukaryotic DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LFRIPVLSSKKiLGLLV</td>
</tr>
<tr>
<td></td>
<td>3'5' Frame 3</td>
<td>50%</td>
<td>Prokaryotic DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hCmYEYLGCLalLRSx</td>
</tr>
</tbody>
</table>

*Note: Amino acids shown in capital letters represent exact matches to the specified protein domain. E values less than 1e-7 were considered significant.*

The program LALIGN was used to eliminate the possibility that the putative mt-topo I sequence obtained, 575-1, was not one of the two human type I topoisomerase pseudogenes. 575-1 had a 57% identity score in a 199 nucleotide overlap with human...
topoisomerase pseudogene 1 and a 55% identity score in a 194 nucleotide overlap with human topoisomerase pseudogene 2. 575-6, which is 96% homologous to nu-topo I, had a 96% identity in 1805 nucleotide overlap with pseudogene 1, and a 95% identity in 1229 nucleotide overlap with pseudogene 2. 575-1 was not one of the two human type I topoisomerase pseudogenes.

LALIGN was also used to determine the percent identity of 575-1 to a prokaryotic type I topoisomerase, \textit{E. coli} topA gene, and a eukaryotic type I topoisomerase, human nu-topo. There was a 56% identity match in a 157 nucleotide overlap to the \textit{E. coli} topA gene and a 57% identity match in a 200 nucleotide overlap to the human nu-topo I. Due to the greater than 50% similarity of 575-1 to both a prokaryotic and eukaryotic type I topoisomerase, but otherwise being uncharacterized, 575-1 was used as a probe to screen a human liver cDNA library. A northern blot was performed to determine the size of the 575-1 message. A type I topoisomerase conserved motif analysis and the phylogenetic relationship of 575-1 to known type I topoisomerase species were also conducted.

\textbf{TABLE 19}

\textbf{BLAST Programs Used}

\begin{tabular}{lll}
\hline
Program & Query Sequence/Database & Comments \\
\hline
BLASTN & nucleotide/nucleotide & Not intended for finding distantly-related coding sequences, parameters optimized for speed \\
TBLASTX & Nucleotide/protein & Very useful for preliminary data, translates DNA query \\
\hline
\end{tabular}

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MEME

To further characterize the putative mt-topo I fragment, 575-1, and the other RT-PCR products generated from the C-D primer set, the bioinformatics program MEME was utilized. MEME finds conserved motifs in a group of unaligned sequences. Sequence information of RT-PCR products generated from the degenerate primer set, C-D, which flanks the type I topo catalytic domain, were analyzed along with seven known eukaryotic type I topo species. This allowed a comparison of RT-PCR sequence fragments against full-length topo sequences to see if any conserved motifs existed between them. It was interesting to see if the RT-PCR products contained any of the type I topo motifs found. More specifically, the sequences MEME analyzed for conserved motifs were HUMTOPI (human type I topo), XELTOPOIS (frog type I topo), DROTOPISO (drosophila type I topo), YSCTOPI (yeast type I topo), CETOPOI (C. elegans type I topo), MUSTOPI (mouse type I topo), CGDNTPIIB (chinese hamster type I topo), and six RT-PCR products, CD575-1, CD575-6, CD290-2, CD290-3, CD700, and CD600.

MEME found six motifs. To determine what these motifs were, each motif was subject to a blastn search. A prositescan was also performed using the motifs translated sequence. In all six motifs, type I topoisomerases were detected in blastn searches. In a few cases, other seemingly unrelated proteins were detected. Motif sequence information and RT-PCR products included in the motif can be seen in Table 20. Table 21 shows motif blastn and prositescan search results.
# TABLE 20

MEME Motif Results

<table>
<thead>
<tr>
<th>Motif #</th>
<th>DNA Sequence</th>
<th>RT-PCR Sequences Included in Each Motif&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5' TTC CGT ACA TAC AAT GCC TCC ATC ACA CTA CAG CAG CAG CT 3'</td>
<td>575-6 (58.93)</td>
</tr>
<tr>
<td>2</td>
<td>5'GCCCTGGGAACCTCCAAACTC AATTATCTGGACCTAGGATCA CAGTGGCATGGGTG 3'</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>5'GTTCGGCAGAGAGCTGTAGCT CTGTACTTGAATGGCTTGC TCTGAGACAGG</td>
<td>290-2 (24.11) [82.55]</td>
</tr>
<tr>
<td>4</td>
<td>5'TACAACCGTGCCCAATCGAGCT TTGCAATCCTTTTGTAAACCATCA 3'</td>
<td>none</td>
</tr>
<tr>
<td>5</td>
<td>5'TGGAAAGAAGTCCGACATGAT AACAAGGTTACTTGGCTGTTC TGGACAGAGA 3'</td>
<td>none</td>
</tr>
<tr>
<td>6</td>
<td>5'GCAGATACCTGCGGTGGTTTGC TCACCTCGAGTGAGACAT3'</td>
<td>none</td>
</tr>
</tbody>
</table>

<sup>a</sup> All seven known topo species were found in each motif. RT-PCR scores are listed in parentheses. For reference, HUMTOPO1 score is listed in brackets.
**TABLE 21**

MEME Motif Similarity Search Results

<table>
<thead>
<tr>
<th>Motif #</th>
<th>Search Program</th>
<th>Similarity Search Results*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>blastn</td>
<td>97% Homo sapiens type I DNA topoiso merase gene, exon 17; 95% Human clone 798A17 prositescan N-glycosylation site, Casein kinase II phosphorylation site</td>
</tr>
<tr>
<td>2</td>
<td>blastn</td>
<td>99% Homo sapiens type I DNA topoiso merase gene prositescan PKC phosphorylation site</td>
</tr>
<tr>
<td>3</td>
<td>blastn</td>
<td>100% C. griseus topo I, 94% Homo sapiens type I DNA topo prositescan Tyrosine Kinase Phosphorylation site</td>
</tr>
<tr>
<td>4</td>
<td>blastn</td>
<td>97% Human topoiso merase pseudogene 1 prositescan N-glycosylation site</td>
</tr>
<tr>
<td>5</td>
<td>blastn</td>
<td>98% Mouse topo I mRNA complete cds, 96% Human topo I mRNA, exon 13 prositescan Casein kinase II phosphorylation site, PKC phosphorylation site</td>
</tr>
<tr>
<td>6</td>
<td>blastn</td>
<td>91% Human topo I, exon 15 prositescan Casein kinase II phosphorylation site, PKC phosphorylation site</td>
</tr>
</tbody>
</table>

*For blastn searches, E values less then 1e-4 were considered significant.

Motif 1 included RT-PCR products, 575-6 and 290-3. A blastn search of motif 1 against the NR database resulted in 97% similarity to Human topo I, exon 17. Motif 1 was also 95% similar to a human DNA clone 798A17 on chromosome 1q24 that contains the 3' part of the FMO1 (Flavin-containing Monooxygenases) gene and the FMO4 gene, three pseudogenes of High Mobility Group protein gene 1, DNA topoiso merase I, and a Signal Recognition Particle. 575-6 (96% similar to human nu-topo I), as expected, scored higher in relation to motif 1, then CD 290-3 (95% similar to human DEAD-box protein). Motif 3 included CD 290-2 (95% similar to human inwardly rectifying potassium channel), although 290-2 scored ~3.5 times lower than the human nu-topo I.

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sequence. Motifs 2, 4, 5, and 6 did not contain any of the RT-PCR generated products. CD575-1, CD600, and CD700 were not found in any of the 6 motifs. The type I topoisomerase catalytic domain was not detected by MEME. The weak homology of the six RT-PCR products as compared to the seven known type I topoisomerase species may have interfered with producing an accurate depiction of topo I motifs.

Phylogeny Tree (dnapars)

An unrooted phylogeny tree of seven known eukaryotic type I topoisomerase species C-D domains and degenerate C-D RT-PCR products was constructed. The distance matrix used to calculate divergence time between species is shown in Table 22. The lower the number, the more closely related the 2 sets of nucleotide sequence. The tree (fig. 30), which is a diagrammatic representation of the distance matrix data seen in Table 22, was constructed to show the overall evolutionary relationship of known type I topoisomerase species C-D domains as compared to type I topo RT-PCR products generated using the degenerate C-D primer set.

As expected, Chinese hamster and mouse topos was more closely related to each other than to the other fragments, possessing a low distance ratio of 0.021. The humtopol was most closely related to xeltopol (0.302). 575-6 was more closely related to cetopol (0.575), than to the human topo I (2.13). Although 575-6 was found to be 96% similar to the human nu-topo I in a BLAST search, cetopol was the next most closely related known eukaryotic topoisomerase after xetopol to humtopol (1.51). 575-1 was most closely related to 290-2 (rectifier potassium channel, 1.27). 290-3 (DEAD box protein/ATP dependent RNA helicase) was most closely related to ysctopol (1.89). 700 (glutathione-S-transferase) was most closely related to cetopol (1.82). Although the mt-
topo I gene was not detected, type I topoisomerase-related domains were found in rectifier potassium channel, DEAD box protein/ATP dependent RNA helicase, and glutathione-S-transferase.

**TABLE 22**

Phylogeny Tree Distance Matrix*

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<th>MUS</th>
<th>CGD</th>
<th>HUM</th>
<th>XE</th>
<th>CE</th>
<th>575-</th>
<th>600</th>
<th>290-3</th>
<th>290-2</th>
<th>575-1</th>
<th>700</th>
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*The distance is a maximum likelihood estimate of the divergence time (total branch length) between the two sequences.
FIG. 30. Eukaryotic type I topo unrooted phylogeny tree.
An unrooted phylogeny tree was also developed for RT-PCR products generated from the prokaryotic topo primer set, A-C, and three known prokaryotic topoisomerases (fig. 31). Since RT-PCR products 425 and 575 were uncharacterized, it was interesting to see how related or unrelated they were to known prokaryotic topos. There was a closer relationship between α-actinin (AC450) and the bacillus topo strains (BACTO, BACSMF) as compared to the other prokaryote topo, E. Coli (ECTOPA). AC 425 and AC 575 were both distantly related to the known pro topos. Prokaryote conserved topo domain primers, which were used to generate DNA from human message, failed to amplify a protein with topoisomerase characteristics or signature pattern.
**FIG. 31.** Prokaryotic topo unrooted phylogeny tree.

*Northern Hybridization*

A northern blot was performed in order to obtain the full-length 575-1 message. The putative mt-topo I purified PCR product, 575-1, was used as a probe to screen human liver mRNA (Clontech). The DNA probe was random primer labeled using [α-32P]dCTP. As shown in fig. 32, a 9 kb message hybridized with the 575-1 probe. Message size was
determined by plotting RNA MW marker distance migration verses log of each MW marker in base pairs (Graph 3). Measurements of distance migrated were taken relative to the bottom edge of the formaldehyde/agarose gel.

**FIG. 32.** Northern blot. Autoradiograph depicting 9 kb 575-1 mRNA.
GRAPH 3. RNA MW marker size vs. distance migrated. Standard curve used to determine 9 kb message size.
Transcript Synthesis/Psoralen-Biotin Labeling

Approximately 6.5 μg of RNA was successfully synthesized from 575-1 DNA with an absorbance 260:280 ratio of 1.8.

575-1 RNA and DNA probes were successfully biotinylated as shown in figure 39. The PCR product of interest, 575-1, was cloned into a pGEM vector which contained SP6 and T7 promoter regions. RNA could be transcribed from the SP6 promoter using SP6 RNA polymerase or from the T7 promoter using T7 RNA polymerase.

![FIG. 33. Dot blot of biotinylated 575-1 RNA and DNA probes.](image_url)

cDNA Library Screen

a. topo I containing genes- Approximately 800,000 plaques were screened. Three cDNA screens were performed with 3 different probes.

The first screen used a combination of eukaryotic and prokaryotic primer sets as probes (Table 2). The primary screen produced 22 putative positive signals. These putative positive clones were then subject to plaque PCR in order to determine if they contained conserved eukaryotic or prokaryotic regions. Three out of the 22 positives produced plaque PCR products. The secondary screen was performed using the 22 positives from the primary screen. Twelve positives were detected. Two clones

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produced plaque PCR products. Sample 13C produced a 350 bp Deg. C-D band. Sample 20-2 produced 11 products ranging in size from 1.3 kb to 300 bp. Cored positive plaques suspended in SM buffer/2% chloroform and stored at 4°C should be stable without loss of viability. The plaques, in fact, no longer grew after a few weeks, therefore, DNA in significant quantities could not be recovered and sequence information could not be obtained.

The second cDNA screening attempt used 575-1 as the probe. An example of cDNA library plating is shown in fig. 34. The primary or initial cDNA screen produced 11 putative positives. A sample autoradiograph from the primary cDNA screen is shown in fig. 35. Each positive plaque was cored, regrown on a fresh LB plate, and rescreened. This secondary screen produced eight putative positives (fig. 36). The tertiary cDNA screen produced six positives (fig. 37).

These six were then subject to plaque PCR reactions in order to determine if they contained 575-1. A 575-1 left and right primer set was used to amplify an expected 274 bp product of 575-1 (fig. 29, diagram). As shown in fig. 25, clone 6A was the only one to produce a band at the expected size. Clones 6A appeared to contain the amplified region of 575-1.
FIG. 34. cDNA library plating: serial dilution.

FIG. 35. Primary 575-1 cDNA screen positive. Positive clone circled. (dots at edge of membrane mark needle pokes for LB plate orientation)
b. **topo II containing genes** - The initial cDNA library screen using topo II probes (Table 3) produced 30 putative positives. These were recovered from the original plate, replated, and rescreened. Although many of these proved to be false positives, nine produced positives at the secondary screen level. Out of these nine, six were still viable to continue on with a tertiary screen. The tertiary screen failed to work, resulting in no detectable positive signals. Plaque PCR was then used to screen putative positives from the secondary screen. Topo II primers used are shown in Table 4. Topo II primers were used with the hope of amplifying expected nu-topo II regions so as to eliminate these
clones as prospective mt-topo II candidates. Fig. 25 shows plaque PCR results for two putative topo II cDNA screen positives, 10-2 and 19-1A.

**Fluorescence In Situ Hybridization**

Fluorescence In Situ Hybridization (FiSH) experiments were performed with a mixture of purified RT-PCR products ranging in size from ~800-200 bp using degenerate A-C and C-D primer sets. No distinct signals were discerned due to high background levels.

**3' Rapid Amplification of cDNA Ends**

This system was used for its ability to amplify rare messages for which little sequence information is available. Three gene specific primers (GSP) were used, 575-1L, 575-1L nested primer 1, and 575-1L nested primer 2 (diagram, fig. 29). 575-1L occurred at base pair position 61 out of the 410 bps sequenced of 575-1L. 575-1L nested primer 1 occurred at base pair position 321, ie. 260 bps downstream of 575-1L. 575-1L nested primer 2 occurred at base pair position 277, ie. 210 bps downstream of 575-1L.

![FIG. 38. 3’ RACE using 575-1L (gene specific primer). Lane 1, 100 bp MW Marker, highest band is 1.5 kb; lane 2, 3’ RACE product.](image)

The 575-1L primer produced an ~1.4 kb 3’ RACE product (fig. 38). The 575-1L nested primer 1 produced an ~1.3 kb 3’ RACE product (fig. 39). Sequencing attempts of this product failed due to possible contamination of comigrating products. Cloning
attempts of these products also proved unsuccessful as is described in more detail later in this section.

**FIG. 39.** 3' RACE using 575-1L nested 1 (gene specific primer). Lane 1, 100 bp MW Marker, highest band is 1.5 kb; lane 2, 3' RACE product.

After repeated failed attempts to reproduce products synthesized from 575-1L and 575-1L nested primer 1, the 575-1L nested primer 2 was developed. The 575-1L nested primer 2 had a $T_m$ which more closely matched the $T_m$ of the right universal amplification primer used in the 3' RACE kit. In addition to having a matching $T_m$ value, the second nested primer was longer in length, being a 25-mer verses a 20-mer. The 25-mer was hoped to provide more specificity when PCR amplifying from the 1.4 kb 575-1L generated 3' RACE product. The 575-1L nested primer 2 produced a ~1.2 kb PCR product (fig. 40). The ~1.2 kb product was purified and used as a template in cycle sequencing reactions with 5' end labeled nested primer 2 or nested primer 1. Two sequencing attempts were conducted at low annealing temperatures and varying salt concentrations for each primer. No legible sequence was produced.

3' RACE was performed using 575-1L nested primer 2 and human liver cDNA as the template. The hope was that by using a primer set with matching $T_m$'s the specificity of the PCR reaction would increase. Several PCR reactions were performed at various
annealing temperatures and Mg$^{2+}$ concentrations. Even at an annealing temperature as high as 63°C and using a range of Mg$^{2+}$ concentrations, a few distinct products and smearing were evident. Smears indicated the production of several products at varying sizes due to nonspecific priming. Since the PCR parameters could not be optimized for the production of a single product, the PCR reactions were run on an agarose gel and the expected size fragment excised and purified. Attempts were then made to sequence these purified products directly, and to further purify by ligation into a pGEM vector, followed by transformation into JM109 competent bacterial cells.

Failure to produce sequence information was thought to be due to dirty template, insufficient template, and/or poor annealing of the primer template. Annealing temperature adjustments were made below the temperature used to produce the PCR product. The template concentrations used were well beyond recommended amounts as suggested in the fmol DNA sequencing system technical manual. Dirty template was believed to be the most likely reason due to contamination of comigrating products. Cloning efforts were performed with the hope of alleviating this problem.

![Figure 40](image)

**FIG. 40.** 3' RACE using 575-1L nested 2 (gene specific primer). Lane 1, 100 bp MW Marker, highest band is 1.5 kb; lanes 2 and 3, purified ~1 kb 3' RACE product.
Cloning attempts of these products also proved unsuccessful. The ligation reaction may have been inhibited by sodium iodide carried over from the agarose gel slice purification. A different purification kit (Qiagen Qiaquick) was then used that did not contain sodium iodide. In addition, the insert to vector ratio was increased to 6:1 and the ligase concentration quadrupled. Although several white colonies were observed with a low percentage of blue, recombinants isolated from rapid plasmid preparations appeared to contain no insert. Recombinants were difficult to see due to RNA contamination. To confirm insert was not incorporated, PCR was performed on RPP samples and from colonies directly picked; ie. colony was picked with a sterile toothpick, resuspended in water, boiled for 5 minutes, and quick cooled on ice (fig. 41). PCR products from white and directly picked colonies near expected insert size were purified (fig. 42) and used in cycle sequencing reactions. In fig. 42 lane 2, three products were amplified from a blue colony RPP. These serve as a negative control representing DNA generated from the vector or from bacterial genome contamination. The \(\sim 850\) bp product, produced from a white colony RPP, seen in lane 3 was a potential insert, since it was a different size than the negative control. This product was isolated for sequencing. White colony RPP depicted in lanes 4, 5, and 7 produced no products. This could be due to the fact that no DNA was recovered in the original RPP. DNA bands of RPP samples were difficult to see due to RNA contamination. The \(\sim 1.1\) kb products seen in lanes 6, 8, and 9 were isolated and purified for sequencing.
FIG. 41. PCR products using 575-1 nested 2 on RPP samples and direct colony picks. Lane 1, 100 bp MW marker; lane 2, blue colony RPP; lane 3-7, white colony RPPs; lane 8, direct colony 1; lane 9, direct colony 2.

FIG. 42. Purified RPP and colony PCR products. Lane 1, 100 bp MW marker; lane 2, figure 47/lane 8; lane 3, figure 47/lane 9; lane 4, figure 47/lane 6; lane 5, figure 47/lane 3.

Purified PCR products were used in sequencing reactions at 41°C and 37°C annealing temperatures with 10 to 4 times the amount of recommended template. Despite changes in annealing temperature and template concentration, sequence information was not obtained.
CHAPTER IV
CONCLUSIONS

MECHANISTIC ANALYSIS

In terms of the mt-topo I proteins mechanistic characterization, a 5’ phosphotyrosine linkage to cleaved DNA was determined by cleavage assay and confirmed by the exonuclease III assay. In the 3’ end labeled cleavage assay, aggregated mt-topo cleavage products were unable to enter the agarose gel in the absence of proteinase K (PK) (fig. 12, lane 5). Addition of PK resulted in migration of numerous cleavage products into the gel (fig. 12, lane 6). When the topoisomerase was removed from the 5’ end by digestion with proteinase K, cleavage products were free to migrate. This showed that the topoisomerase was linked to the 5’ end of the cleaved DNA. The presence of the enzyme on the 5’ end of DNA was catenating or holding broken strands together, causing them to form one large structure which could not pass through the agarose gel.

The nu-topo I is known to covalently link to the 3’ phosphate of a phosphodiester bond when cleaving DNA (16, 80). The prokaryotic top I links to the 5’ phosphate of cleaved DNA (81, 82). The ability of mt-topo I’s to link to the 5’ end of cleaved DNA not only serves as a discerning mechanistic difference between the nu-topo I and the mt-topo I, but also suggests a closer evolutionary link of the mt-topo I to prokaryotic type I topoisomerases. Future attempts to isolate the mt-topo I gene should include a more extensive selection of prokaryotic topo primers for use in RT-PCR.
A problem encountered in performing the cleavage assay was the inability of the mt-topo I to cleave due to its low storage concentration. The enzyme was dialyzed in the hope of concentrating the topoisomerase. Dialysis, itself proved risky, because topoisomerase activity could be lost altogether.

As for the K+ SDS' precipitation experiment, high background counts were detected. The high background counts suggested that the radiolabeled DNA was able to interact with the K+ SDS' without the presence of the topoisomerase. Bovine serum albumin (BSA) contained in the assay cocktail and/or the use of too high a concentration of SDS may have contributed to the problem.

Drug inhibition studies were also performed to further distinguish the mt-topo I from its nu-topo I counterpart. DMSO, berenil, and seven antimicrobial/antifungal drugs were studied. DMSO and berenil have previously been found to discriminate between the nuclear and mitochondrial type I topoisomerases isolated from rat liver and calf thymus (75). My study looked at differences in DMSO and berenil sensitivity of type I topoisomerases isolated from calf liver.

In terms of the DMSO inhibition assays, less inhibition was observed than expected for the mt-topo I in the presence of 5% DMSO, 29% verses 60 % inhibition. In the presence of 20% DMSO, mt-topo I did correlate with previous results displaying high levels of inhibition.

The nu-topo I did not completely correlate with previous studies. The calf liver nu-topo I was inhibited 28% as compared to 50 % inhibition in the presence of 20% DMSO, and showing a slight inhibition verses no inhibition in the presence of 1%
DMSO. But, as in the previous study, the nu-topo I displayed some stimulation of activity in the presence of 5% DMSO.

Each enzyme followed the overall trends of the previous study. The nu-topo I did correlate with previous studies displaying some stimulation of activity in the presence of 5% DMSO and inhibition in the presence of 20% DMSO. Slightly less inhibition was observed than expected for the mt-topo I in the presence of 5% DMSO. In the presence of 20% DMSO, mt-topo I did correlate with previous results with activity almost completely reduced.

As for the berenil inhibition assays, the mt-topo I exhibited a decrease in activity in the presence of 10 μM berenil. Inhibition of mt-topo activity increased when 40 μM berenil was added. Inhibition was apparent at the addition of 100 μM berenil, but not much more than the 40 μM samples. The nu-topo I, as expected, showed little or no inhibition of activity in the presence of berenil.

The slight differences seen in my experiments may be attributed to minor structural differences between enzymes isolated from calf liver versus rat liver. As well, it was more difficult to quantitate inhibition when observed topoisomerase activities were so close to 2 units.

As for the antifungal/antimicrobial agents, only one drug, C1, inhibited the nu-topo I in a dose-dependent fashion. The mt-topo I was not inhibited by the drug C1. The drug, C1, could potentially be used to discriminate between the nu-topo I and mt-topo I. More studies need to be performed to extend the preliminary findings with C1.
GENES CONTAINING TOPO-RELATED DOMAINS

A key technique used in examining topo-related genes was RT-PCR. For each eukaryotic topo primer set used, at least one product, representing the known human nuptopo I, was expected to be made. Other DNA bands generated were potential mt-topo I candidates until proven otherwise by sequencing and computational similarity searching. Speculation as to the types of products generated from the prokaryotic primer sets were unknown, due to the fact that prokaryotic primers were being used to amplify from human message.

To reduce the problem of nonspecific binding of primers, which could result in the synthesis of several bands that did not represent amplified topoisomerase regions, annealing temperatures were increased and/or MgCl₂ concentrations varied. Increases in annealing temperature forced more specific binding of the primers. Weaker bonds formed from mismatches can not successfully anneal at higher temperatures. Since it was unknown exactly how related the mt-topo I was to currently characterized type I topoisomerases, the decision to use high verses low stringency conditions was difficult. Amplification of regions due to primer mismatch were undesirable, but too high a stringency could eliminate the amplification of the mt-topo I gene.

Another level of specificity or purification was attained through pGEM vector cloning. pGEM vector cloning was performed in order to eliminate contamination from comigrating RT-PCR products that interfered with obtaining legible sequence. A universal primer set that annealed to regions flanking the pGEM vector insertion site eliminated problems encountered when trying to cycle sequence using degenerate primers. Cycle sequencing with degenerate primer sets was difficult due to the multiple
primers present that could anneal to more than one area of the template at a time. After successfully cloning an RT-PCR product into the pGEM vector, the recombinant plasmid DNA was isolated and purified to serve as a template for cycle sequencing reactions.

Rapid plasmid preparations provided a pure, more concentrated stock of an RT-PCR product. Single stranded DNA (ssDNA) isolations of pGEM vectors were also produced in order to provide a cleaner template source for use in cycle sequencing. There was no notable difference in band clarity, as visualized on an autoradiograph, between using ssDNA verses double stranded DNA as the template source in cycle sequencing reactions.

Overall, thirteen products were generated and sequenced. Seven proved to be previously characterized genes. The remainder possessed various enzyme signatures, but otherwise, were not completely characterized. Putative mt-topo I RT-PCR products were used in various experiments in attempts to further characterize the fragments.

One experiment, Fluorescence In Situ Hybridization (FISH), was performed in order to determine the location of the putative mt-topo I gene and/or topo-related genes on human chromosomes. The chromosomal location of a putative mt-topo I gene would be an important determination to make since the location of the known nu-topo I gene and its pseudogenes is already known. A difference in chromosomal location could be used to further distinguish the mt-topo I from its nuclear counterpart. FISH attempts resulted in nonspecific binding of the probe producing a high background. A mixture of RT-PCR products were used as probes with their sizes ranging from ~200-800 bp. The use of a larger probe may have produced a stronger, more concise signal with minimal background.
One RT-PCR product in particular, 575-1, based on similarity search information was the most interesting. It possessed a 57% similarity to the topo II signature and a 56% similarity to the prokaryotic topo I signature in prositescan searches. This putative mt-topo I fragment was later used as a probe to screen a human liver cDNA library.

In cDNA library screening, plaque PCR proved to be an instrumental technique in confirming positive cDNA clones. It provided a quick and easy means to find topo-related genes. By using plaque PCR, potential positive cDNA clones were successfully screened for regions that contained an area of sequence related to the primer set being studied. Having obtained a positive clone, the next step was to sequence it. A drawback, however, in doing this type of PCR was that the viability of the resuspended plaque was finite. Therefore, the integrity of the recombinant DNA rapidly decreased over a matter of a few weeks. Unfortunately, as in the topo II and 575-1 cDNA screens, clones of interest lost their viability before sequence information could be obtained.

However, some legible sequence information had been obtained from direct sequencing of the RT-PCR products. The next step was to identify or characterize them. Use of bioinformatics technology was critical to finding topo-related genes. Sequence databases and search tools were not only used to characterize RT-PCR products generated, but were also used in the beginning stages of the project to design RT-PCR primers and cDNA screen probes. Through similarity searching, the putative mt-topo I sequence, 575-1, was revealed.

Similarity searching is the process of comparing a new sequence against all other known sequences and then attempting to infer the function of the new sequence by assessing the matches (83). Table 16 shows a variety of similarity search results ranging
from identities in the 90 percentile range to cases of no significant sequence similarity. Several explanations may account for cases in which search results indicate no significant similarity. This may be due to the use of a sequence query that is too short, inaccuracies in reading an autoradiograph, the query sequence not being characterized, or that the query sequence contained regions with low-complexity. Regions with low-complexity sequence; for example, the nucleotide sequence AAA TAA AAA ATA AAA AT, have an unusual composition that can create problems in sequence similarity searching due to their ability to produce artificial hits (84). Inaccuracies when reading cycle sequencing autoradiographs may also explain lower than anticipated percentile scores; for example, 96% verses 100% identity, for the expected nu-topo I fragments generated from degenerate primer sets.

In an effort to obtain the entire 575-1 gene, a cDNA library screen was performed using 575-1 as the probe. Selected cDNA clone positives were believed to represent portions of the mt-topo I gene. If the sequence was similar to the nu-topo I and/or a topoisomerase pseudogene, yet different, the sequence was a potential mt-topo I gene candidate. A dark spot on each of the duplicate lifts represented the hybridization of the probe and a particular cDNA. To ensure the dark spot was not a false positive, the individual plaque was isolated, replated at a more dilute concentration, and a second screen was performed. If the signal persisted, and was found in all or most of the replated plaques, this cDNA most likely consisted of a portion of the 575-1 fragment. The cDNA clone was isolated and sequenced using the fmol DNA Cycle Sequencing System. Several thousands of plaques were screened in order to increase the probability of finding the low frequency mt-topo gene. Some false positives were detected due to
nonspecific interactions. Where false positives were detected, filters were rewashed under more stringent conditions, ie. at higher temperatures and/or lower salt concentrations to minimize nonspecific binding.

The cDNA screen produced one positive clone. More positive clones were expected due to the 9 kb message size, as indicated by a northern blot, and due to the 2 kb insert capacity of the λgt11 vector. Attempts to sequence the cDNA screen clone failed due to the inability to properly purify the template. Phenol/chloroform extractions, a Biorad DNA purification kit, and a Promega lambda DNA purification kit failed to produce pure template. Difficulty in sequencing the clone, 6A, was compounded by the fact that the phage containing the recombinant would not grow. A possible explanation for the lack of growth may be the addition of too much chloroform when resuspending the cored plaque in SM buffer or that the protein expressed by this particular recombinant had some lethal effect. Because sequence information could not be obtained from the clone 6A, 3' RACE was performed. Since 410 bp of 575-1 had been successfully sequenced, the 3' RACE system was used in the hopes of gathering more sequence from the 3' end of the 575-1 message. The 3' end of the message was of particular interest due to the fact that the eukaryotic type I topoisomerase catalytic domain, D, was contained in this region.

3' RACE was chosen versus 5' RACE also due to the 575-1's 9 kb message size. The 5' RACE system was not designed to amplify such large messages. Since 575-1 was produced from a primer set located near the 3' end of conserved topoisomerase domains (Deg. C_L-D_R), 575-1 was more likely to be found closer to the 3' end of the message.
Being closer to the 3' end of the message would allow for a more reasonably sized amplification product.

The 575-1 fragment was closer to the 3' end, as suspected, because the 3' RACE experiment produced a ~1.4 kb product using 575-1L as the gene specific primer. This was confirmed when the primary PCR product was reamplified using the 575-1L nested primer 1 which produced a ~1.2 kb product. The ~1.2 kb product was around the expected size since the 575-1L nested primer anneals 260 bps downstream from 575-1L (figure 29, 575-1 primer diagram). The use of a nested primer confirmed that the PCR product generated was a specific amplification product representing a portion of the 575-1 message. Attempts were made to sequence both the ~1.4 kb and ~1.2 kb products. Cycle sequencing directly from purified samples of each resulted in comigrations at specific regions of the gel in all four lanes (G, A, T, C) or no bands at all. These results suggested contamination of a similarly sized fragment in the purified samples or dirty template. To circumvent these problems, attempts were made to ligate the 1.4 kb 3' RACE product into a pGEM vector. By successfully creating this clone, the contamination problem would have been eliminated because only one fragment can be inserted into each vector at a time. In addition, the clone would have provided an easy means to generate more template as needed. Repeated cloning attempts proved unsuccessful. Although positive control samples were successfully ligated, increases in ligase concentration, template concentrations, and incubation periods did not ligate the 3' RACE product samples.

Bioinformatics technology was used to further characterize the 575-1 sequence information available. The program, MEME, was used to search for motifs or consensus
sequence patterns from a pool of seven known eukaryotic type I topoisomerase species and six RT-PCR products. The goal was to determine if any of the RT-PCR products possessed shared motifs in common with the already characterized topo species. Only three, 575-6, 290-2, and 290-3 possessed some similarity to six motifs found by the program. 575-6, which represents a portion of the human nu-topo I, was expected to be included in a motif. 575-6 was found in motif 1 possessing a relatively high score. 290-3 was also grouped with motif 1, but had a low relative score. Motif 1 was found to be homologous to exon 17 of the human nu-topo I. The 290-3 result was interesting because similarity search results revealed it to be the human DEAD-box protein, p72. P72 is a novel human member of the DEAD box family of putative RNA-dependent ATPases and ATP-dependent RNA helicases (85). Although 290-2 had a low score in relative comparison to the other sequences, 290-2 had some similarity to motif 3. This was surprising since blast search results indicate that 290-2 to be the human inwardly rectifying potassium channel J4. 575-1 was not found in any of the six motifs. Although the mt-topo I gene was not detected, type I topo-related proteins were found: DEAD-box protein, human inwardly rectifying potassium channel J4, and glutathione-S-transferase.

In addition to a consensus sequence analysis, a comparison at the molecular evolutionary level was investigated. The purpose was to determine if a relationship, if any, existed between known type I topo species and the RT-PCR products. The program, dna parsers, was used to construct an unrooted phylogeny tree of the seven known topoisomerase species C-D domains and six RT-PCR products generated using the degenerate C-D primer set. A tree was also constructed for three known prokaryotic topoisomerases and three RT-PCR products generated from a prokaryotic topo primer set.
In the eukaryotic tree, 575-6, was the most related to the known topoisomerase, CETOPOI. 575-1, on the other hand, was the most related to the RT-PCR product 290-3 (DEAD box protein). In the prokaryotic tree, none of the RT-PCR products were significantly related to the known prokaryotic topo species. Prokaryote conserved topo domain primers, which were used to generate DNA from human message, failed to amplify DNA products encoding a protein with topoisomerase characteristics or signature pattern.

Another piece of evidence to further characterize 575-1 was obtained from a northern blot. The large 9 kb message size, in addition to the distant relationship to known eukaryotic type I topoisomerase species indicated that the 575-1 putative mt-topo I fragment was highly unlikely to be part of the mt-topo I gene. A literature search indicated no known topoisomerase possessing a message of that size. A typical type I topoisomerase message is around 3 kb. Prositescan results of 575-1 contained 105 other enzyme patterns in addition to the topo signature patterns when the stringency of the scan was set to 50% similarity. A literature search was performed looking for a correlation between a 9 kb message and any of the other enzyme patterns seen on the prositescan. Only 2 patterns, an N-glycosylation site and a tyrosine kinase phosphorylation site, had proteins associated with these signatures that possessed a 9kb message. They were a human pre-sialomucin complex (membrane-associated glycoprotein) and a human trkB receptor (membrane-associated tyrosine kinase receptor). 575-1 may represent a portion of these proteins.

575-1 is not the mt-topo I gene. The putative mt-topo I fragment, 575-1, possessed a message size three times the size of known type I topoisomerases, contained
none of six type I topoisomerase motifs, and was distantly related when compared to seven characterized eukaryotic type I topoisomerase species in the unrooted phylogeny tree.

In conclusion, the mt-topo I gene is fundamentally different from known eukaryotic type I topoisomerases. Degenerate primers developed from eukaryotic type I topoisomerase conserved domains were not able to amplify a region of the mt-topo I gene in RT-PCR reactions, although the known nu-topo I was detected. Future investigations should include a more thorough development of prokaryotic type I topoisomerase primers.

FUTURE DIRECTIONS

In order to find the mt-topo I, better primers and probes need to be developed. The motif information generated from the MEME program may be used to design varying primer sets for use in RT-PCR. In addition, further investigation into prokaryotic type I topoisomerases primer sets should be conducted. The mt-topo I, as indicated by its 5' covalent linkage to cleaved DNA, possesses prokaryotic characteristics as well as eukaryotic. Recent evidence indicates the obligate anaerobe, Rickettsia, to be the closest known eubacterial relative of the mitochondria (86). Further investigation of the rickettsia genome should be conducted for RT-PCR primer development.

In future RT-PCR reactions, if a DNA sequence of interest does arise, the putative mt-topo I gene may be verified by translating its cDNA and assaying for mt-topo I activity. The active protein would be expressed in bacterial cells. A bacterial system
would be preferred due to the fact that the bacterial topoisomerase and mt-topo I activity can be easily distinguished from one another in a standard topoisomerase relaxation assay. To distinguish endogenous bacterial topoisomerase activity from recombinant topoisomerase activity the relaxation assay would be performed in the presence and absence of Mg\(^{2+}\). A potential difficulty or limitation may be that some recombinants may express low levels of proteins making it difficult to detect enzyme activity. To further distinguish the mt-topo I from the bacterial topo I, a western blot would be performed using a topoisomerase specific antibody that recognizes the mitochondrial but not the bacterial topoisomerase. Topoisomerase-specific antibody has been found to recognize both the nu-topo I and the mt-topo I (35).

The pET E. coli expression system (Stratagene) can be used. The pET E. coli expression system is a widely used in vivo bacterial expression system, due to the strong selectivity of the bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level of activity of the polymerase, and the high level of efficiency of translation mediated by the T7 gene 10 translation initiation signals (87, 88). In the pET system, protein coding sequences of interest are cloned downstream of the T7 promoter and gene 10 ribosome binding site, and then transformed via electroporation into E. coli. The coding sequence for the putative mt-topo I will be processed for adding BamHI linkers to the ends, followed by insertion at the BamHI cloning site in the pET3b expression vector. The pET3b recombinant plasmid will be transformed into E. coli cells with protein expression achieved by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase was expressed from the lacUV5 promoter (89). Upon induction, the highly active polymerase will produce an abundant number of transcripts with the
target protein constituting the majority of the cellular protein after a few hours. *E. coli* cells will be harvested and stored at -70°C. The bacterial cell paste will be thawed and lysed by the presence of endogenous T7 lysozyme. The cell lysate will then be centrifuged to remove cell debris and tested for mt-topo I activity. Successful mt-topo synthesis will be determined in two ways: using a standard topo relaxation assay, and via immunodetection of the mt-topo I protein expressed in bacterial cells to topoisomerase-specific antibody.

To perform the western blot, bacterial cell lysates expressing the mt-topo I protein will be run on a polyacrylamide gel, the separated proteins will then be transferred to nitrocellulose, and incubated with topoisomerase-specific antibody. Recognition of the antibody to the mt-topo I protein will be detected on the blot by using an enhanced chemiluminescence system (ECL, Amersham) which incorporates Protein A-linked horseradish peroxidase. The western blot will be performed on bacterial cells not containing the mt-topo I protein expression vector. Since bacterial cells do not express the nu-topo I, one would expect to get no detection using the topo-specific antibody. This negative result can be compared to an expected positive result when using cells containing the mt-topo I expression vector. By probing with an antibody that recognizes both the nu-topo I and the mt-topo I in a bacterial system where nu-topo I does not exist, any observed topo I activity present in the absence of Mg²⁺ would provide further evidence that distinguishes the mitochondrial topoisomerase from its nu-topo counterpart.

To ensure expression of the full message, cDNA insert sizes will be compared to the full length message as obtained via northern blot. Expression of a partial message
may explain low levels or lack of mt-topo I activity in topoisomerase relaxation assays or lack of detection in western blots.
REFERENCES


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79. BLAST FAQs (www.ncbi.nlm.nih.gov/BLAST/blast_FAQS.html) National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland, 20894, USA.


APPENDIX A

MITOCHONDRIAL PREPARATION FROM CALF LIVER

1. Trim the fat and grind fresh calf liver.

2. Suspend the ground volume in 6 volumes (w/v) of 1X MIB (0.04 M Tris, 0.25M sucrose, 0.1mM EDTA) and blend in a blender with a 10 seconds on and off burst twice.

3. Centrifuge for 2000 g for 30 minutes.

4. Collect the supernatant and centrifuge at 8500 g for 35 minutes.

5. Collect the pellets and homogenize in 6 volumes (v/v) of 1X MIB.

6. Homogenize in a final volume of 600 mL per kg of the liver with 1X MIB.

7. Apply 80 mL of the crude mitochondrial suspension to a two-step sucrose gradient composed of 75 mL of 25% sucrose in TE and 90 mL of 42.5% sucrose in TE.

8. Centrifuge at 26,000 g for 75 minutes.

9. Collect the banded mitochondria at the interface of the two sucrose layers.

10. Dilute with 2 volumes of TE and centrifuge at 22,000 g for 20 minutes.

11. Collect the pellets and homogenize in 6 volumes of 1X MIB.

12. Determine the mitochondrial protein suspension by Biuret analysis.

13. Add 1 mg of digitonin per 8 mg of mitochondrial suspension and stir occasionally for 15 minutes on ice.

14. Dilute with 2 volumes of 1X MIB.

15. Centrifuge at 10,000 g for 15 minutes.

16. Collect the pellets and homogenize in 6 volumes of 1X MIB.

17. Repeat step 15.
18. Collect the pellets and homogenize in 100 mL per kg of liver with 50 mM KPi (potassium phosphate)/0.1 M KCL/15% glycerol for direct lysis.

19. Add Nonidet P40 to the mitochondrial suspension to a final concentration of 1%.

20. Collect the supernatant as S1.

21. Homogenize the pellet with PiK-1.0 (50 mM Kpi/1.0 M KCL/15% glycerol).

22. Stir for 30 minutes on ice.

23. Centrifuge at 250,000 g for 3 hours. Collect the supernatant and label S2.
APPENDIX B

MT-TOPO I ISOLATION: COLUMN CHROMATOGRAPHY

1. Phosphocellulose (PC) column (3 x 20 cm):
   a. pack 12.5 Ml of PC per gram of mitochondrial protein in PiK-0.3 (50 mM Kpi/0.3 M KCL/15% glycerol).
   b. dilute S2 with PiK-0 (50 Mm/15% glycerol) to 0.3 M KCL.
   c. load the diluted sample to the PC column and wash with 3 column volumes of PiK-0.3.
   d. elute the column with a gradient of PiK-0.3 to PiK-1.5 (50 mM Kpi/1.5 M KCL/15% glycerol) in a total of 10 column volumes.
   e. collect in each fraction one tenth of the column volume.
   f. combine the active fractions with more than 4 units (1 unit of topo activity = amount of activity to relax 50% of plasmid DNA within 30 minutes at 37°C) per µl (PC-1).

2. Hydroxyapatite (HA) column (2 x 20 cm):
   a. pack 4 Ml of HA per gram of mitochondrial protein in PiK-0.3 (50 Mm Kpi/0.3 M KCl/15% glycerol/pH 7.8).
   b. dilute PC-1 with PiK-0 to 0.3 M KCL
   c. load the diluted sample to the HA column and wash with 3 column fractions of PiK-0.3.
   d. wash with 3 column volumes of Pi-200 (200 mM/15% glycerol/pH 7.8).
   e. elute the column with a gradient of Pi-200 to Pi-1000 (1 M KPi/15% glycerol/pH 7.8) in a total of 10 column volumes.
f. collect in each fraction one tenth of the column volume.

g. combine the active fractions with more than 4 units of relaxing activity per μl (HA-1).

3. dsDNA-Cellulose (dsDNA-C) column (1 x 9 cm):

a. pack 1 ml of dsDNA-C per gram of mitochondrial protein in Tris-0.1 (50 mM Tris-HCl/0.1 M KCl/15% glycerol).

b. dialyze the HA-1 (combined active fractions from HA column) with at least 200 volumes of Tris-0.1 for 3 hours twice.

c. load the dialyzed sample and wash with 3 column volumes of Tris-0.1.

d. elute the column with a gradient of Tris-0.1 to Tris-0.6 (50 mM Tris-HCl/0.6 M KCl/15% glycerol) in a total of 10 column volumes.

e. collect in each fraction one tenth of the column volume.

f. combine the active fractions with more than 4 units of relaxing activity per μl (ds-DNA-1).
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