CLONING AND PARTIAL CHARACTERIZATION OF THE 5'- FLANKING REGION OF THE HUMAN TOPOISOMERASE IIβ GENE

by

Nicholas R. Jones

A thesis submitted in conformity with the requirements for the Degree of Master of Science, Department of Pharmacology, University of Toronto.

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ABSTRACT

Mammalian DNA topoisomerase II (topo II) is an essential nuclear enzyme which changes the topology of DNA by passing an intact helix through a transient double-stranded break made in a second helix followed by religation of the DNA break. These interactions with DNA are important in almost every aspect of DNA metabolism, including replication, transcription, recombination and repair. Topo II is also the cellular target of several clinically relevant anti-cancer drugs. There are two isoforms of topo II, α and β, each differing in biochemical properties, cell-cycle expression, and sensitivity to antineoplastic agents. Various drug-resistant cancer cell lines demonstrate both qualitative and quantitative differences in both the α and β isoforms. To date, only the 5'-flanking region of the α isoform has been isolated and characterized. In order to investigate elements governing the expression of DNA topo IIβ, the 5'-flanking region of human topo IIβ has been cloned and partially analyzed. The transcription start site, 1183 bp of 5'-flanking sequence, and fifty-six potential putative regulatory elements have been identified. Whether these regulatory elements are functional or important, remains to be explored.

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TABLE OF CONTENTS

TABLE OF CONTENTS - FIGURES

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
°C	degrees Celsius
cDNA	complimentary deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
g	gravity units
kb	kilobase
kD	kiloDaltons
M	Molar
mg	milligram
mL	millilitre
mM	millimolar
mm	millimeter
mRNA	messenger ribonucleic acid
N	normal concentration
NAD	nicotinamide adenine dinucleotide
ng	nanogram
$O.D._{600}$	optical density at 600 nanometers

Pi	inorganic phosphate
pmol	picomole
RNA	ribonucleic acid
topo IIα	topoisomerase II, α isoform
topo IIβ	topoisomerase II, β isoform
μg	microgram
μL	microlitre
μM	micromolar

INTRODUCTION

I TOPOISOMERASE BIOLOGY

I.1 Topoisomerases govern DNA topology

The movement of RNA polymerase along DNA presents a topological problem for the cell. Either the RNA polymerase traverses a helical path around the DNA, or the DNA rotates about its long axis during replication (Gamper and Hearst, 1982). In the former case, the nascent RNA would become wrapped around the DNA along with any proteins that might be bound to it (ribosomes or ribonucleoproteins), and would eventually have to unravel (Sternglanz, 1989). Since the unwinding of such a large structure would probably be quite slow, it is considered more likely that the DNA rotates during transcription (Sternglanz, 1989). DNA rotation would lead to positive supercoiling ahead of the moving RNA polymerase and negative supercoiling behind it, creating topological constraints in the DNA that must be eliminated (Giaever and Wang, 1988; Liu and Wang, 1987; Wu *et al.*, 1988; Tsao *et al.*, 1989). Topoisomerases are essential for relaxing this type of torsional stress.

In 1969, *Escherichia coli* (*E. coli*) extracts capable of relaxing supercoiled DNA were first identified (Wang, 1969). Subsequent purification identified a single enzyme responsible for relaxing negatively supercoiled DNA without the requirement of a high-energy cofactor, such as ATP or NAD (Wang, 1971). This enzyme, originally designated as the ω protein (Wang, 1971), was characterized by the transient breakage and rejoining of one strand, allowing the passage of another strand through the break. In 1972, the eukaryote equivalent to the ω protein was identified (Champoux and Dulbecco, 1972), and in 1979, the ω protein

was renamed DNA topological isomerase I or topoisomerase I (topo I) for its ability to control DNA topology (Wang and Liu, 1979).

Two more relaxing enzymes were later identified, one in bacteria and the other in eukaryotes, designated DNA gyrase and topoisomerase II (topo II) respectively. Both enzymes preferentially relax positive supercoils (Wu *et al.*, 1988; D'Arpa and Liu, 1989), and both alter nucleic acid topology by passing an intact double helix of DNA through a transient double-stranded break made in a second DNA helix (Liu *et al.*, 1980; Gellert, 1981; Wang 1982). Unlike the bacterial enzymes, both eukaryotic type I and type II enzymes relax negative and positive supercoils at comparable rates (Goto and Wang, 1982; Benedetti *et al.*, 1983; Osheroff *et al.*, 1983; Schomburg and Grosse, 1986; D'Arpa and Liu, 1989).

I.2 Topoisomerase I

Sedimentation and gel filtration data suggest that both the bacterial and eukaryote type I enzymes, topo I and III, are monomeric in nature and do not require a divalent cation for activity, but are stimulated 10 to 20 fold in the presence of Mg^{2+} or Ca^{2+} (Goto *et al.*, 1984). The type I enzyme interconverts different topological forms of DNA by creating a transient single-stranded break in the nucleic acid backbone, passing the unbroken strand of the DNA through the nick, and resealing the original scission (Gellert, 1981; Wang, 1981, 1982, 1985; Vosberg, 1985; Osheroff, 1989a; Cozzarelli and Wang, 1990; Andersen *et al.*, 1994; Champoux, 1994; Gupta *et al.*, 1995; Sharma and Mondragon, 1995).

Although topo I is not necessary for the viability of eukaryotic cells (Uemura *et al.*, 1986), topo I does appear to play an important role in chromatin organization (Uemura and

Yanagida, 1984), mitosis (Maul *et al.*, 1986), DNA replication (Goto and Wang, 1985; Snapka, 1986; Brill *et al.*, 1987; Yang *et al.*, 1987), recombination (Halligan *et al.*, 1985; Bullock *et al.*, 1985; McCoubrey and Champoux, 1986), and transcription (Fleischmann *et al.*, 1984; Bonven *et al.*, 1985; Muller *et al.*, 1985; Gilmour *et al.*, 1986; Brill *et al.*, 1987).

I.3 Topoisomerase II

The type II enzymes, which encompass bacterial DNA gyrase, topoisomerase IV, and eukaryotic topo II, alter nucleic acid topology by passing an intact double helix of DNA through a transient double-stranded break made in a second DNA helix (Liu *et al.*, 1980; Gellert, 1981; Wang 1982, 1985; Vosberg, 1985; Osheroff, 1989a; Cozzarelli and Wang, 1990; Osheroff *et al.*, 1991; Andersen *et al.*, 1994; Watt and Hickson, 1994; Sharma and Mondragon, 1995).

Unlike the type I enzyme, topo II is essential to the survival of the eukaryotic cell (DiNardo *et al.*, 1984; Goto and Wang, 1984; Uemura and Yanagida, 1984; Holm *et al.*, 1985). Topo II is involved in many aspects of nucleic acid metabolism, including DNA replication (Sundin and Varshavsky, 1981; Nojuchi *et al.*, 1983; Jazwinski and Edelman, 1984; Weaver *et al.*, 1985; Nelson *et al.*, 1986; Snapka, 1986; Brill *et al.*, 1987; Yang *et al.*, 1987), transcription (Glikin and Blangy, 1986; Rowe *et al.*, 1986b; Brill *et al.*, 1987), mRNA processing (Schroder *et al.*, 1987), and chromosome segregation (Sundin and Varshavsky, 1981; DiNardo *et al.*, 1984; Uemura and Yanagida, 1984, Uemura *et al.*, 1986; Holm *et al.*, 1985; Snapka, 1986; Brill *et al.*, 1987; Uemura *et al.*, 1987b; Yang *et al.*, 1987). In addition, the type II enzyme plays an important role in chromosome structure (Earnshaw *et al.*, 1985;

Earnshaw and Heck, 1985; Cockerill and Garrard, 1986; Gasser *et al.*, 1986; Gasser and Laemmli, 1986; Newport, 1987; Newport and Spann, 1987; Uemura *et al.*, 1987b), structural tethering (Heck *et al.*, 1989), condensation (Uemura *et al.*, 1987b; Wood and Earnshaw, 1990; Adachi *et al.*, 1991; Hirano and Mitchison, 1993), and in the organization of the nuclear matrix (Berrios *et al.*, 1985).

Eukaryotic type II topo activity was first reported in 1980 (Baldi *et al.*, 1980; Hsieh and Brutlag, 1980; Liu *et al.*, 1980), and the isolation of the enzyme from mammalian cells soon followed (Miller *et al.*, 1981). Since the initial isolation, topo II has been purified and characterized from a wide variety of eukaryotic species, including protozoans (Riou *et al.*, 1986), fungi (Goto and Wang, 1982; Goto *et al.*, 1984), insects (Hsieh, 1983; Shelton *et al.*, 1983), amphibians (Benedetti *et al.*, 1983), and mammals (Halligan *et al.*, 1985; Riou *et al.*, 1985; Schomburg and Grosse, 1986)

The physical properties of all eukaryotic type II topoisomerases isolated are similar. The polypeptide molecular mass of the enzyme ranges from 160 kD to 180 kD, while hydrodynamic and sedimentation coefficient studies indicate that the enzyme exists in solution as a homodimer, and appears to have an elongated shape, rather than globular three-dimensional structure (Miller *et al.*, 1981; Sander and Hsieh, 1983; Shelton *et al.*, 1983; Goto and Wang, 1984; Halligan *et al.*, 1985; Schomburg and Grosse, 1986).

The type II topoisomerase has an absolute requirement for a divalent cation. Goto *et al.* (1984) identified Mg^{2+} as the most effective cation for topo II activity, whereas Mn^{2+}, Ca^{2+}, or Co^{2+} were found to support the reaction to a lesser extent. Goto *et al.* (1984) also discovered that ATP or dATP are required for topo II activity, whereas the nonhydrolyzable

ATP analogues [adenylyl imidodiphosphate and adenylyl (β,γ-methylene) diphosphonate] inhibit activity.

Although yeast (Goto and Wang, 1984) and *Drosophila* (Wyckoff *et al.*, 1989) apparently contain only a single type II topoisomerase, mammals possess two isoforms of the enzyme, α and β (Drake *et al.*, 1989; Watt and Hickson, 1994). Our understanding of these two isoforms, especially the β isoform, is still rudimentary. Why higher eukaryotes possess two distinct isoforms of the type II enzyme whereas other organisms survive with only one is not clear.

I.4 A probable evolutionary relationship between the domains of topoisomerase II and DNA gyrase

A comparison between the nucleotide sequence for the *Saccharomyces cerevisiae* (*S. cerevisiae*) gene TOP2, encoding DNA topo II, and the sequence for bacterial DNA gyrase revealed large regions of similar sequence (Lynn *et al.*, 1986). Although the two enzymes differ in both quaternary structure and activity, the homology between the two proteins indicates mechanistic as well as structural similarities, and a probable evolutionary relationship (Lynn *et al.*, 1986).

Based on this comparison with DNA gyrase A and B subunits, the eukaryotic topo II homodimer can be divided into three domains: an amino terminal domain (N-terminus) homologous to the *E. coli* gyrase B subunit (involved with ATP hydrolysis), a central domain homologous to the *E. coli* gyrase A subunit (involved with the DNA breakage and reunion

reactions), and a carboxyl terminus (C-terminus) with clusters of charged amino acids (Wang, 1985).

Topo II sequence homology between *S. cerevisiae*, *Saccharomyces pombe* (*S. pombe*), and *Drosophila melanogaster* is less than 50 %, and not evenly distributed throughout the protein. The highest sequence conservation between species is seen within the N-terminal domain, whereas the C-terminal domain shows the least degree of sequence conservation (Wyckoff *et al.*, 1989; Austin *et al.*, 1995). Thus, the C-terminal region present in topo II is extremely unlikely to contain residues required for formation of the catalytic active sites of the enzyme. This, however, does not imply that this domain is dispensable for function.

There are four potential roles of the C-terminal region: nuclear localization, dimerization, regulation of enzymatic activity, and aid in chromosome segregation. Shiozaki and Yanagida (1992), working with the *S. pombe* topo II enzyme, found that two nuclear localization signals exist: one in the N-terminal ATPase domain, and one in the C-terminus. Mirski and Cole (1995) also demonstrated that alterations in the COOH-proximal domain of topo IIα in human cells diminished the ability of the enzyme to localize in the nucleus. Since eukaryotic topo II is a homodimer, it must therefore contain defined areas vital for the dimerization of the individual subunits. In 1994, Caron *et al.* identified one of these areas located in the C-terminus of a *S. cerevisiae* enzyme. The C-terminal domain of the fission yeast topo II enzyme has also been identified as a target for phosphorylation, although additional sites of phosphorylation are found in the N-terminal region of this enzyme (Shiozaki and Yanagida, 1992). Finally, Watt *et al.* (1995) hypothesized that one function of

the C-terminal domain of topo II might be to direct specific interactions with other proteins required to effect chromosome segregation. Watt *et al.* (1995) identified a Slow Growth Suppressor (SGS) protein (thought to be a DNA helicase) that interacts specifically with a short region of the C-terminal domain. Strains lacking a functional SGS gene are unable to eliminate the supercoiling caused by the rotation of the DNA. These trains show a reduced fidelity of both mitotic and meiotic chromosomal segregation, leading to a diminished capacity to undergo productive cell division (Watt *et al.*, 1995).

I.5 The catalytic cycle of topoisomerase II

How the DNA segment being transported through the DNA gate enters and leaves the enzyme during a reaction cycle has been a question of long standing. Two types of models have been postulated. In the two-gate model, the DNA segment to be transported passes through the entire interfacial channel in between the two halves of the enzyme (Mizuuchi *et al.*, 1980; Wang *et al.*, 1980; Kirchhausen *et al.*, 1985; Reece and Maxwell, 1991). In such a model, the second DNA segment enters through a gate on one side of the enzyme and then exits through a second gate on the other side of the enzyme; the two gates must open at different stages to prevent the two halves of the enzyme from coming apart (Roca and Wang, 1994). In the one gate model, the DNA segment to be transported passes through the DNA gate, the gate then closes and the enzyme-bound DNA segment containing the DNA gate dissociates from the enzyme (Osheroff, 1989a). In either model, the DNA gate may form one gate or an integral part of one gate, and the second DNA strand segment must pass through the DNA gate only once during each reaction cycle.

Catalytic double-stranded DNA passage mechanism: the two-gate model

Although this reaction appears to be concerted, it can be broken into discrete steps as summarized (Roca and Wang, 1994) (Figure 1).

1) **DNA binding**. In the absence of bound ATP, the enzyme is in the form of an open clamp and can bind to or dissociate from a linear or circular DNA segment. The enzyme binding to the DNA creates the future DNA gate. This interaction requires no cofactors.

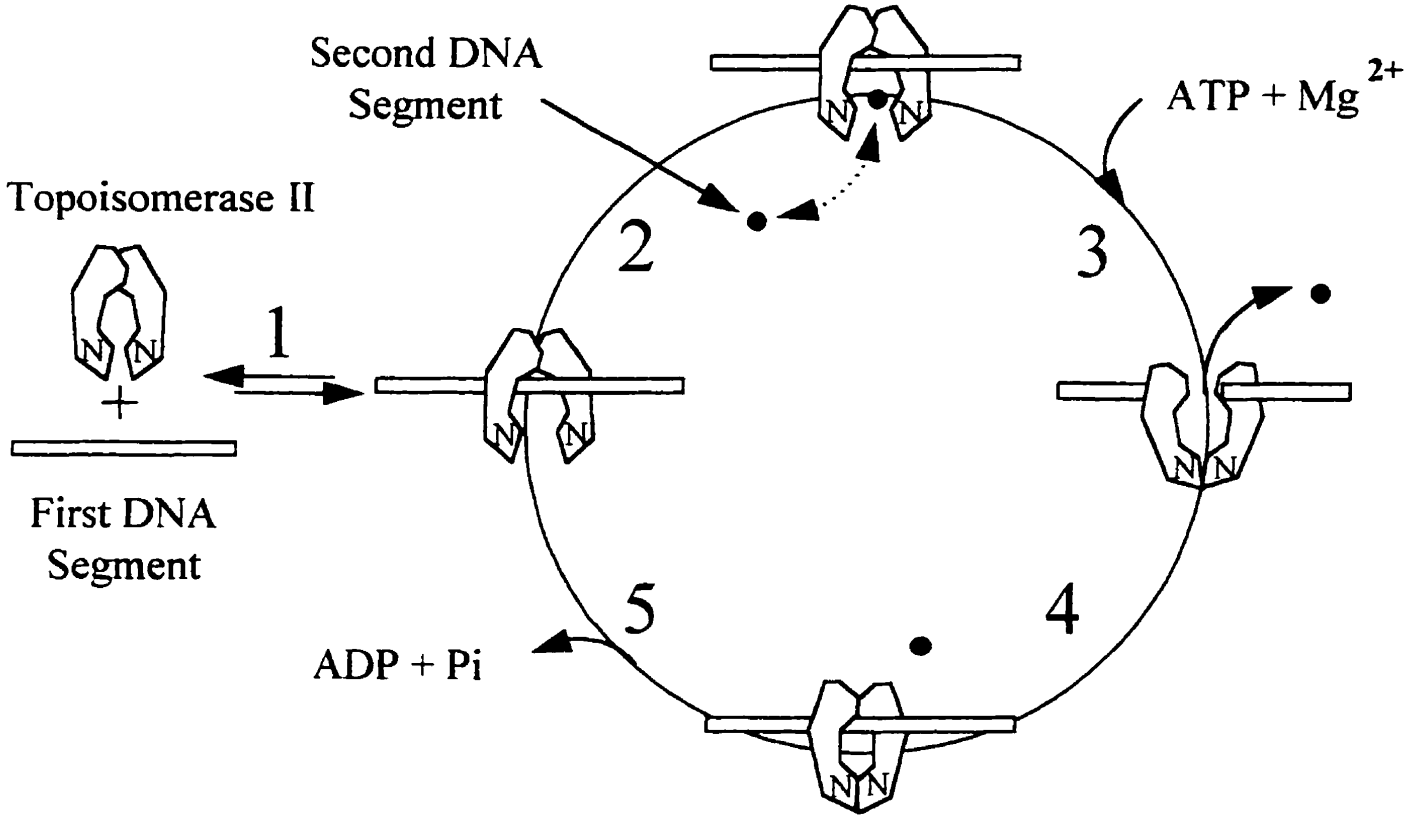
2) **Second DNA strand**. A second DNA segment can go in and out of the DNA-bound enzyme so long as it remains in the open form.

3) **DNA cleavage and double-strand DNA passage**. Upon the binding of ATP to the enzyme, the protein gate composed of the N-terminal domain (N-gate) closes. If the second DNA segment has entered the clamp before the N-gate is completely closed, a DNA gate on the opposite side of the N-gate opens. The opening of the DNA gate creates a transient double-stranded enzyme-bound break in the DNA backbone to allow the exit of the second DNA segment from the interior of the enzyme. ATP binding is likely to favor the entrance of the second DNA segment prior to the closure of the clamp. This reaction absolutely requires the presence of a divalent cation.

4) **Religation**. The DNA gate closes religating the cleaved DNA. The first DNA segment still remains associated with topo II in a noncovalent fashion.

5) **ATP hydrolysis and enzyme turnover**. Topo II hydrolyzes the bound ATP molecule to ADP and inorganic phosphate (hydrolysis of ATP probably occurs shortly before step 5), and topo II returns to the open-clamp form bound to the first DNA segment to complete one cycle of reaction.

Figure 1. The catalytic cycle of topoisomerase II: The two-gate model



Catalytic double-stranded DNA passage mechanism: the one-gate model

Although this reaction appears to be concerted, it can be broken into discrete steps as summarized (Osheroff 1989a) (Figure 2).

1) **DNA binding**. Topo II binds to its DNA substrate in a noncovalent fashion. This interaction requires no cofactors.

2) **DNA cleavage**. Topo II creates a transient double-stranded enzyme-bound break in the DNA backbone. This reaction absolutely requires the presence of a divalent cation.

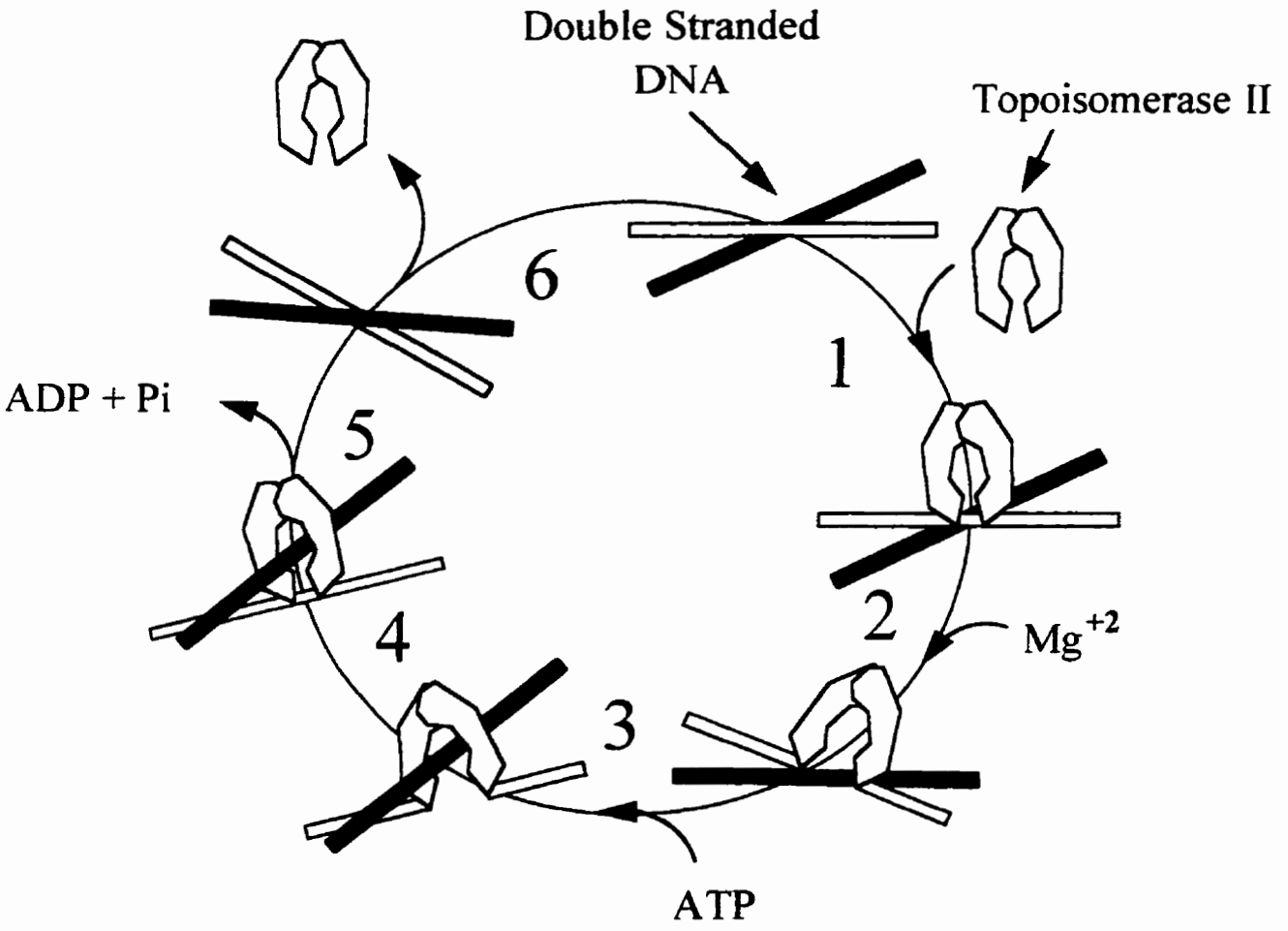
3) **Double-strand DNA passage**. Upon the binding of ATP to the enzyme, topo II passes an intact double-stranded DNA helix through the transient break made in the nucleic acid backbone.

4) **Religation**. Topo II religates the cleaved DNA and remains associated with its DNA product in a noncovalent fashion.

5) **ATP hydrolysis**. Topo II hydrolyzes the bound ATP molecule to ADP and inorganic phosphate.

6) **Enzyme turnover**. Following ATP hydrolysis, the topo II-DNA complex turns over and the enzyme regains its ability to initiate a new round of catalysis.

Figure 2. The catalytic cycle of topoisomerase II: The one-gate model



The two models described above, for the type II DNA topoisomerases, have been in parallel existence since 1980, owing to a lack of experimental tests. Recently, Roca and Wang (1994) showed clearly that when AMPPNP, the nonhydrolyzable βγ-imido analogue of ATP, was added to a yeast DNA topo II molecule bound to a supercoiled DNA ring singly linked to a nicked or relaxed DNA ring, the enzyme could transport the nicked or relaxed DNA ring through the supercoiled DNA with a high probability. Therefore, ATP-hydrolysis was not required for the release of the second DNA segment from the enzyme following its passage through the DNA gate. Because the N-gate of the enzyme is believed to be irreversibly locked by the binding of AMPPNP (Roca and Wang, 1992; Lindsley and Wang, 1993), the release of the unlinked nicked or relaxed DNA ring from the enzyme-supercoiled DNA complex upon binding of AMPPNP suggests that there is a second protein-protein gate in addition to the N-gate.

I.6 Two isoforms of topoisomerase II have been identified

Initial reports on the purification and characterization of topo II from human cells identified topo II as a 170 kD protein, now known as topo IIα. However, subsequent purification of topo II activity from both mouse and human cells revealed the presence of a larger 180 kD form, now known as topo IIβ (Miller *et al.*, 1981; Drake *et al.*, 1987, 1989). This newer isoform was initially purified in a 4'-(9-acridinylamino)-methanesulfon-*m*-aniside (*m*-AMSA) resistant P388 murine leukemia cell line providing some evidence that it might be of significance in cellular resistance to topo II agents (Miller *et al.*, 1981; Chung *et al.*, 1989).

The topo IIα and β genes appear to have arisen via a relatively recent gene duplication event which included several flanking markers including the retinoic acid receptor α and β genes (Coutts *et al.*, 1993). At the primary sequence level, the two isoforms in humans share 68% overall homology (Austin *et al.*, 1993), while other sequence comparisons indicate that the C-terminal region of eukaryotic topo II proteins show only 35% amino acid identity (Jenkins *et al.*, 1993; Watt and Hickson, 1994).

The two isoforms appear to be differentially regulated, and the ratio of their levels changes in response to cellular proliferation rate (Drake *et al.*, 1989), oncogenic transformation (Woessner *et al.*, 1990), and selection for resistance to topo inhibitors (Drake *et al.*, 1987) or alkylating agents (Tan *et al.*, 1988). The two isoforms of topo II also require different KCl concentrations for their optimal activity, show different heat denaturation levels and display different levels of association with chromatin or the nuclear matrix (Drake *et al.*, 1987, 1989). Finally, it has been shown that the 180 kD form is more processive suggesting that the enzyme dissociates more slowly from DNA than the 170 kD form (Osheroff, 1986).

I.7 Cell cycle expression of topoisomerase IIα and β

The two isoforms are differentially regulated, topo IIα is quantitatively associated with active cell growth and transformation, while topo IIβ is not (Woessner *et al.*, 1990). When mouse fibroblast NIH-3T3 cells are synchronized with serum starvation and then stimulated to enter the cell cycle by addition of fresh medium, topo IIα levels rise in late

S-phase, peak in G_2-M-phase, and decrease as cells complete mitosis (Woessner *et al.*, 1991). Heck *et al.* (1988) also demonstrated that the transition from mitosis into the subsequent G_1-phase is accompanied by a dramatic decrease in the stability of topo II (now thought to be the α isoform). The β isoform was present at relatively constant levels throughout the cell cycle (Woessner *et al.*, 1991).

I.8 Localization of topoisomerase IIα and β

In humans, the predominant localization of the topo IIα isoform is to the nucleoplasm and to a lesser extent to the nucleolus (Zini *et al.*, 1994). Topo IIβ, on the other hand, appears almost exclusively localized on the dense fibrillar component of the nucleolus (Zini *et al.*, 1992, 1994). In Chinese hamster fibroblasts, the prevalent localization of both the α and β isoforms is in the nucleoplasm and the nucleolus (Petrov *et al.*, 1993).

Studies on relative mRNA expression in a variety of murine tissues show high levels of topo IIα in proliferating tissues such as bone marrow and spleen, whereas topo IIβ is more widely expressed in non-proliferating tissues including uterus, ovary, adrenal glands, eye, bladder, and heart (Capranico *et al.*, 1992). A similar pattern of expression was confirmed by Holden *et al.* (1992), with the levels of topo IIα highest in human cancers with a high percentage of cycling cells. The highest levels of topo IIβ were found in the placenta, where there is a low percentage of cycling cells (Holden *et al.*, 1992).

The chromosomal location of genes encoding these isoforms also differs. The gene encoding the β isoform is located on the short arm of chromosome 3 at position p24 (Jenkins

et al., 1993), whereas the gene encoding the α isoform is located on the long arm of chromosome 17 at positions q21-22 (Juan *et al.*, 1988; Tsai-Pflugfelder *et al.*, 1988).

II THE ROLE OF TOPOISOMERASE II IN CHEMOTHERAPY

II.1 Topoisomerase II is a target for several clinically relevant antineoplastic agents

Topo II is the target of a number of antineoplastic drugs including DNA intercalating agents such as anthracyclines (e.g. doxorubicin and daunomycin), acridines [e.g. amsacrine (*m*-AMSA), *o*-AMSA (4'-(9-acridinylamino)-methanesulfon-*o*-aniside)], ellipticines (e.g. ellipticine and 2-methyl-9-hydroxyl-ellipticinium acetate), anthracenediones (e.g. mitoxantrone and bisantrene), actinomycin D, and non-intercalating epipodophyllotoxins (e.g. etoposide [VP-16], and teniposide [VM-26]). The evidence that supports the role of topo II in the action of these two classes of agents is comprehensive and consistent *in vitro* and *in vivo* (Ross *et al.*, 1978, 1979; Zwelling, 1981; Wozniak and Ross, 1983; Glisson *et al.*, 1984; Nelson *et al.*, 1984; Ross *et al.*, 1984; Rowe *et al.*, 1986a; Liu, 1989; Wasserman and Wang, 1994).

II.2 Different classes of antineoplastic agents stimulate topoisomerase II-mediated DNA cleavage at different sites

Pommier *et al.* (1983) found that intercalators from different chemical classes stimulated topo II-mediated DNA cleavage at different sites, whereas intercalators from the

same chemical class stimulated cleavage at similar sites. In the absence of drugs, topo II produces a cleavage pattern of its own, referred to as background cleavage. Topo II preferentially binds to DNA that contains A-T rich regions of DNA, matrix association regions (MARs) (Adachi *et al.*, 1991; Sperry *et al.*, 1989), or G-C rich regions flanked by arrays of oligo(dA) and oligo(dT) tracts characteristic of MAR sequences (Kas and Laemmli, 1992). These natural sites of action of topo II are primarily responsible for the background cleavage pattern. Many of the drug-stimulated cleavage sites overlap with these background cleavage sites (Pommier *et al.*, 1983), in particular, cleavage sites associated with the high-salt insoluble nuclear matrix (Gromova *et al.*, 1995). It seems possible that each drug selectively stimulates a subset of the background cleavage sites. This selectivity may reflect both specific drug-DNA interactions and enzyme-DNA interactions (Liu, 1989).

II.3 Topoisomerase IIα and β have different sensitivities to antineoplastic agents

Human topo II is an important anticancer drug target, but the role of the individual α and β isoforms is not known. Austin *et al.* (1995) discovered that topo IIβ is able to promote DNA cleavage with both *m*-AMSA and *o*-AMSA whereas topo IIα is only able to promote DNA cleavage with *m*-AMSA. In contrast, topo II α is 3-fold more sensitive to VM-26 and 8- to 10-fold more sensitive to merbarone than topo IIβ (Hochhauser and Harris, 1993). Therefore, despite their overall structural similarities by protease studies, human α and β isoforms can be distinguished by cleavage complex-forming compounds.

Whether specific targeting of human topo II isoforms is of benefit in cancer chemotherapy remains to be determined. A better understanding of the normal role of both

isoforms of topo II and their regulation may help to explain these differential sensitivities to chemotherapeutic agents.

II.4 Cellular content of topoisomerase II influences topoisomerase II sensitivity to antineoplastic agents

To better understand the regulation of topo II activity during the cell cycle and its relationship to drug sensitivity, Chow *et al.* (1988), examined DNA induced cleavage and topo II content in mouse fibroblast BALB / C 3T3 cells. Results indicated a marked increase in the drug-induced cleavage activity during the G_2 / M phase correlated well with increased topo II content. Maximal cytotoxicity of antineoplastic agents, however, occurred during S phase (Chow *et al.*, 1988). Other researchers also found a correlation between drug-induced DNA breakage and enzyme content in human, mouse, hamster, proliferating cells, and quiescent cells (Sullivan *et al.*, 1986; Chow and Ross, 1987; Estey *et al.*, 1987; Markovits *et al.*, 1987; Sullivan *et al.*, 1987; Zwelling *et al.*, 1987)

Kaufmann *et al.* (1994) discovered that estimates of topo II content in human acute myelogenous leukemia cell (AML) samples did not correlate with sensitivity to topo II-directed agents *in vitro*. But this lack of correlation appeared to result from marked cell-to-cell heterogeneity of topo II expression within leukemic marrow, a heterogeneity that obscured differences in topo II levels in clonogenic leukemia cells (Kaufmann *et al.*, 1994).

II.5 Topoisomerase II poisons stabilize the cleavable complex

Despite the important role of topo II in the treatment of human cancers, virtually nothing was known about the molecular mechanism by which antineoplastic drugs altered enzyme function until 1990. It was possible to demonstrate that antineoplastic agents stimulated enzyme-mediated DNA breakage by shifting the cleavage / religation equilibrium toward cleavage, but it was impossible to state with certainty whether these drugs acted by enhancing the forward cleavage reaction or by inhibiting DNA religation reaction (Osheroff, 1989a). Fortunately, recent advances have now made such delineation possible. Drugs appear to stabilize topo II-DNA cleavage complexes by two distinct mechanisms (Corbett and Osheroff, 1993; Wasserman and Wang, 1994). The first mechanism stabilizes the covalent enzyme-DNA reaction intermediates by inhibiting religation or enhancing cleavage, while the second mechanism inhibits the enzyme without trapping the covalent intermediates.

Epipodophyllotoxins (e.g. etoposide and teniposide), acridines (amsacrine), and anthracyclines (e.g. doxorubicin and daunomycin), act primarily by impairing the ability of topo II to religate cleaved nucleic acids (Ross, 1985; Osheroff, 1989a; Robinson and Osheroff, 1990, 1991; Sorensen *et al.*, 1992;).

In marked contrast, quinolones, nitroimidazoles, pyrimidobenzimidazoles, ellipticines (e.g. ellipticine and 2-methyl-9-hydroxyl-ellipticinium acetate), and genistein show little inhibition of enzyme-mediated DNA religation and appear to work by enhancing the forward rate of DNA cleavage (Robinson *et al.*, 1991; Corbett and Osheroff, 1993; Froelich-Ammon *et al.*, 1995).

Finally, Ishida *et al.* (1991) discovered that bisdioxopiperazines, ICRF-I59 and ICRF-193, have different effects on mammalian cells when compared to covalent complex-stabilizing agents such as etoposide. ICRF-193 appears to be an inhibitor of a conformational change of the enzyme during its catalysis of DNA transport. This conformational change is mechanistically distinct from drugs that stabilized enzyme-DNA covalent intermediates, often referred to as topoisomerase "poisons" (Roca *et al.* 1994).

II.6 Cellular consequences of the drug-topoisomerase II interaction

The potency of drugs, within a class, to induce cleavable complexes correlates well with their cytotoxic effects (Davies *et al.*, 1988; Holm *et al.*, 1989; D'Arpa *et al.*, 1990; Shin and Snapka, 1990). Two mechanisms seem possible to explain these results. First, the stabilization of the enzyme in the cleavable complex may render the enzyme catalytically inactive during a time when it is required. For example, topo II appears to be absolutely required for separating intertwined daughter molecules prior to mitosis in yeast (D'Arpa and Liu, 1989). Second, the cleavable complexes and their cellular response cause the majority of cell killing (D'Arpa and Liu, 1989). Most lines of evidence support the idea that drug cytotoxicity is related to the specific ability of the drug to stabilize the cleavable complex, rather than the inhibition of the catalytic activity of topo II (Zwelling, 1981; Nelson *et al.*, 1984; Tewey *et al.*, 1984; Rowe *et al.*, 1985; Bodley *et al.*, 1989), and that the cellular response to the stabilized cleavable complex ultimately leads to cytotoxicity (Long *et al.*, 1985; Li, 1987; Schneider *et al.*, 1989).

The potential lethality of drug-induced cleavage complexes occurs when replication machinery or helicases attempt to traverse the covalently bound "topoisomerase roadblock" in the DNA (Hsiang *et al.*, 1989; Howard *et al.*, 1994). When present at high concentrations, a portion of these transient enzyme-associated DNA breaks are converted to permanent untethered breaks during replication and transcription (Zhang *et al.*, 1990; D'Arpa *et al.*, 1990; Chen and Liu, 1994; Howard *et al.*, 1994; Liu, 1994). Once these untethered breaks are produced in the DNA, they become targets for recombination and repair pathways. This in turn stimulates sister chromatid exchange, the generation of large insertions and deletions, and the production of chromosomal aberrations and translocations (Corbett and Osheroff, 1993; Anderson and Berger, 1994; Chen and Liu, 1994; Ferguson and Baguley, 1994). When these permanent DNA breaks are present at sufficient concentration, they trigger a series of events that ultimately culminates in cell cycle arrest and / or apoptosis, leading to cell death (Duvall and Wyllie, 1985; Wyllie, 1987; Arends and Wyllie, 1991; Kaufmann, 1991; Pommier, 1993; Beck *et al.*, 1994; Chen and Liu, 1994; Liu, 1994; Pommier *et al.*, 1994; Gupta *et al.*, 1995).

III THE ROLE OF TOPOISOMERASE II IN DRUG RESISTANCE

III.1 Drug resistance is multifactorial

The simultaneous resistance to several classes of natural product drugs *in vivo* after exposure to a chemically dissimilar agent is termed multidrug resistance (MDR). Cells displaying this phenotype usually have a decreased internal steady state drug level, decreased

retention of drug (Beck, 1983), and specific changes in plasma membrane glycoproteins (Juliano and Ling, 1976). Several mechanisms may mediate MDR, primarily, altered expression of the MDR gene, the MRP gene, glutathione transferases, and topoisomerases.

MDR was originally closely associated with the over expression of P-glycoprotein (PGP), a 170 kD protein with six hydrophobic domains and a tandemly duplicated ATP binding domain (Endicott and Ling, 1989). PGP acts as an energy-dependent efflux plasma membrane pump, functioning to lower intracellular drug concentrations in resistant tumor cells (Kaye, 1990). Kaye (1990) and Beck (1983) discovered that the overexpression of PGP is responsible for cellular resistance to natural products such as anthracyclines, epipodophyllotoxins and vinca-alkaloids.

In 1992, Cole *et al.* identified a new member of the ATP-binding cassette super family of transporter genes, designated multidrug resistance-associated protein (MRP), that is highly overexpressed in a small cell lung cancer cell line, H69AR (Cole *et al.*, 1992; Evans *et al.*, 1994). This H69AR cell line displays resistance to VP-16 and doxorubicin that can not be explained by the level of expression of either topoisomerase or PGP, thereby invoking another resistance mechanism (Evans *et al.*, 1994). Thus the overexpression of the novel transporter gene MRP was strongly implicated.

The involvement of glutathione and glutathione transferases (GSTs) in the multidrug resistance has been difficult to clarify. Glutathione transferases are enzymes involved in detoxification and are widely distributed (Pickett and Lu, 1989). Increases in the level of GST isozymes, particularly the Pi family, have been well correlated with the onset of drug resistance in several different cell lines (Cowan, 1989; Morrow and Cowan, 1990).

However, other studies have shown little, or no, correlation between GST levels and drug resistance (Bellamy *et al.*, 1989; Moscow *et al.*, 1989; Nakagawa *et al.*, 1990; Schisselbauer *et al.*, 1990).

III.2 Topoisomerase II is implicated in multidrug resistance

Topo II was first implicated in multidrug resistance because the only lesion identified in many human leukemic multidrug resistant cell lines was an altered topo II enzyme (Danks *et al.*, 1988). Danks *et al.* (1988) termed this phenotype altered topoisomerase II multidrug resistance, or atypical MDR (at-MDR). Hochhauser and Harris (1993) found that even though the phenomenon of at-MDR is associated with resistance to all types of topo II drugs, the degree of resistance varies between different classes.

III.3 Cellular resistance to antineoplastic agents is partially attributed to quantitative and / or qualitative alterations of the topoisomerase II enzyme

Atypical multidrug resistance appears to be caused by either a reduction in the level of expression of topo II or a qualitative alteration in the enzyme that confers drug resistance. Deffie *et al.* (1989b) provided evidence that in two Adriamycin resistant murine leukemic cell lines, P388/ADR/3 and P388/ADR/7 (Goldenberg *et al.*, 1986), a mutation in one of the alleles for topo II may be a major factor in the reduced levels of the native topo II mRNA and gene product. Other factors such as differences in transcription and stability of the native topo II transcript, may be involved in regulating the level of topo II in ADR-resistant P388 leukemia cells (Deffie *et al.*, 1989a). Other lines of evidence also suggest that topo II

resistance is attributed to a reduced level of enzyme (Glisson *et al.*, 1986; Per *et al.*, 1989; Potmesil *et al.*, 1988; Deffie *et al.*, 1989a; Zwelling *et al.*, 1989).

In 1989, Sullivan *et al.* described the first instance in which cellular resistance to topo II active drugs could be ascribed to a qualitative alteration in the enzyme. Other reports have identified a number of mutations in the gene encoding topo II that confer resistance to antineoplastic drugs, and a study of these mutations has now defined two regions in topo II that appear to be important for drug-enzyme interactions. The first region is located in the *gyrB* domain near the consensus ATP binding sequence (Danks *et al.*, 1989; Bugg *et al.*, 1991; Hinds *et al.*, 1991; Lee *et al.*, 1992; Chan *et al.*, 1993; Wasserman and Wang, 1994; Hsiung *et al.*, 1996), whereas the second region is located in the *gyr A* homology domain and spans approximately 200 amino acids flanking the active site tyrosine residue (Danks *et al.*, 1993; Jannatipour *et al.*, 1993; Patel and Fisher, 1993; Liu *et al.*, 1994; Hsiung *et al.*, 1996).

These findings make it difficult to draw conclusions concerning the potential microenvironment of drug binding with these regions or even to conclude whether drugs share a common site on the enzyme. Findings by Elsea *et al.* (1995) suggest that, in yeast, the interaction domains for most DNA cleavage-enhancing agents overlap one another, but the specific points of contact on the enzyme probably differ between drug classes.

III.4 The role of topoisomerase IIα and β as mediators of cytocidal activity

The identification of two topo II isozymes has raised questions about the nature of the functions previously attributed to the α isoform. Differences in expression of the two isoforms may be relevant to drug resistance clinically. Most studies report decreased levels

of topo IIα in drug resistant cell lines, suggesting that the α isoform mediates cytocidal activity (Bugg *et al.*, 1991; Hinds *et al.*, 1991; Lee *et al.*, 1992; Chan *et al.*, 1993; Danks *et al.*, 1993; Patel and Fisher, 1993; Adachi *et al.*, 1994; Feldhoff *et al.*, 1994; Kaufmann *et al.*, 1994; Liu *et al.*, 1994; Wasserman and Wang, 1994; Hsiung *et al.*, 1996). Several studies now indicate that a down regulation of the β isoform may be equally, if not, more significant at mediating cytocidal activity, in some cell lines, than a down regulation of the α isoform (Hill, 1993; Evans *et al.*, 1994; Hosking *et al.*, 1994; Harker *et al.*, 1995; Davies *et al.*, 1996).

In contrast, Brown *et al.* (1995) discovered that expression of topo IIα varies inversely with the level of expression of topo IIβ in six human acute lymphoblastic leukemia (ALL) cell lines, and that resistance to Adriamycin in the six ALL cell lines correlates directly with the level of expression of topo IIα and inversely with the level of expression of topo IIβ.

IV REGULATION OF GENE EXPRESSION

IV.1 Gene regulation in eukaryotes

The behavior of cells depends on the time at which genetic information is expressed, the precise portion of information that is expressed, and the extent to which this expression occurs. This ability of the cell to selectively control or regulate the various processes under its command is termed gene regulation. One of the most interesting aspects of gene control in multicellular organisms is that the patterns of expression of each individual cell serve the

need of the whole organism and not the survival of the individual cell (Darnell *et al.*, 1990). This control of a gene's expression can be categorized into four areas: transcriptional regulation, posttranscriptional regulation, translational regulation, and posttranslational regulation.

Regulation of transcriptional initiation is the most prevalent form of gene control in eukaryotes. Such control results in the increased or decreased synthesis of primary RNA transcripts, leading to a change in the level of specific mRNAs and their translation products (Darnell *et al.*, 1990). In eukaryotes, transcriptional initiation is regulated by proximal and distal regulatory sites, termed promoter and enhancer elements respectively, and by a TATA box, which is involved in the positioning of polymerase II (Darnell *et al.*, 1990). In genes containing a TATA box, protein factors help the RNA polymerase II to recognize the TATA box and assist in the initiation of transcription. The initiation of transcription is as follows (Darnell *et al.*, 1990). The TATA binding factor $TF_{II}D$ binds directly to the TATA box region; once this occurs, the transcription binding factor $TF_{II}B$ binds to RNA polymerase II and directs the polymerase to the $TF_{II}D$ bound to the DNA. Once the polymerase-$TF_{II}B$ complex binds to the DNA-$TF_{II}D$ complex, the resultant complex acts like ATPase and the energy released by ATP hydrolysis alters the conformation of certain proteins allowing transcription to begin. All that is required is the presence of the transcription binding factor $TF_{II}E$ to prevent premature termination of the polymerase II and ribonucleoside triphosphates to create the RNA.

Many genes do not contain a TATA box but contain an equivalent sequence necessary to start transcription, yet the rate of transcription of these genes tends to be low

compared with genes containing a TATA box (Strickberger, 1985). In genes lacking a TATA box, $TF_{II}D$ still plays a pivotal role as the primary factor directing the binding of the polymerase to the DNA (Darnell *et al.*, 1990).

Even when the TATA box, the TATA factor, and other transcription factors are present, initiation of transcription of eukaryotic genes by RNA polymerase II is not a frequent event. This process is improved by activators or enhancer sequences which may be located as far as one thousand bases or more from the genes they regulate (Strickberger, 1985). Gene-activating sites located over three-hundred bp from the start-site of transcription are called enhancer sites, whereas gene-activating sites located closer to the start-site of transcription are called promoter sites, yet there is little difference between the type of proteins that can bind to the enhancer sites versus the type of proteins that can bind to the promoter sites (Strickberger, 1985). The issue of distance may not even be an issue if one considers the possibility of DNA looping, and its potential to bring a distantly bound protein into proximity with the transcription start-site (Darnell *et al.*, 1982).

In eukaryotic cells most mRNA transcripts contain noncoding sequences, introns, that interrupt coding sequences called exons. The processing of these mRNA transcripts requires the removal of all introns, the addition of a methylated guanosine cap to the 5'-end, and the addition of a poly (A) tail to the 3'-end (Darnell *et al.*, 1990). The cap and tail provide protection from the cytoplasmic environment allowing the mRNA to remain stable for translation. These posttranscriptional modifications are important aspects of gene regulation since they enable control over the number, kinds, and stability of mRNA molecules that enter the cytoplasm for translation (Darnell *et al.*, 1990).

The appearance of mature mRNA does not necessarily mean that it will be translated into a protein. At the translational level various controls are possible including those that affect initiation, elongation, and termination factors; the ability of ribosomes to attach to the mRNA molecules; the half life of mRNA; and the kinds and amounts of tRNA molecules carrying the necessary anticodons to allow for translation (Darnell *et al.*, 1990).

Ribosomal translation of mRNA is not necessarily the last step that controls the appearance of functional protein. Both function and longevity may be affected by proteolytic enzymes that cleave polypeptide chains at specific places, as well as various kinases, methylases, and other enzymes that modify specific amino acids on specific polypeptides to affect activity (Darnell *et al.*, 1982).

IV.2 Transcriptional regulation of the topoisomerase IIα promoter

In 1992, Hochhauser *et al.* cloned and characterized the 5'-flanking region of the human topo IIα gene. Maximal promoter activity was observed 617 bp upstream of the start-site of transcription. Within this promoter sequence, a number of DNA elements that closely match the consensus sequences for transcription factor binding sites were identified, including two GC boxes (potential Sp1 factor binding sites), five inverted CCAAT boxes (ICBs), and a potential activating transcription factor (ATF) recognition sequence (Hochhauser *et al.*, 1992).

To assess the relevance of these putative *cis*-acting elements, Isaacs *et al.* (1996) designed a series of stably integrated promoter deletion constructs lacking one or more of the promoter's elements. The data suggest that all of the essential elements for transcriptional

repression are located in the region between (-144) and (-101) which contains only one transcription factor binding site, ICB2. Isaacs *et al.* (1996) destroyed the ICB2 site by site-directed mutagenesis and demonstrated that the mutation completely eliminated the down-regulation of expression. In contrast, Isaacs *et al.* (1996) discovered that ICB1 is neither necessary nor sufficient for growth regulation, yet ICB2 and ICB1 are distinguishable only by differences in spacing from other promoter elements and differences within specific sequences flanking each ICB. Furthermore, Isaacs *et al.* (1996) identified the NF-Y transcription factor as the protein that binds to ICB2, and subsequently inhibits ICB2's down-regulation. This NF-Y factor has been implicated in controlling the activity of numerous other promoters, including those required for the transcription of the major histocompatibility complex class II gene (Dorn *et al*, 1987) and the thymidine kinase gene (Chang and Liu, 1994).

The human topo IIα gene is transcriptionally regulated by the p53 protein. The p53 protein is one of the most important regulators of cell cycle progression in mammals, with an apparent dual role in the induction of cell cycle arrest following cytotoxic insults and in the regulation of the apoptotic cell death pathway. Ines *et al.* (1996) demonstrated that wild-type p53 is able to substantially decrease the activity of the full length topo IIα gene promoter. Using a series of deletion constructs, Ines *et al.* (1996) showed that this p53-specific regulation is independent of all characterized transcription factor binding sites and is directed at the basal transcription machinery. The precise down-stream consequences of this regulation have not yet been identified.

Topo IIα has also been shown to be transcriptionally regulated by retinoic acid (Tsao *et al.*, 1994), the phorbol ester phorbol-12-myristate-1-acetate (Zwelling *et al.*, 1990; Fraser *et al.*, 1995), and by methylation (Tan *et al.*, 1987). Since the topo IIβ promoter has not been isolated, it is not known whether any of the transcription factors associated with regulating the α isoform are involved in regulating the β isoforms.

Ng *et al.* (1995) analyzed the role of ICBs in the regulation of basal expression of the Chinese hamster topo IIα gene. The 400 bp promoter has a moderately high GC content, no canonical TATA box sequences, five potential inverted CCAAT boxes, a potential GC box, and transcriptional start-sites that are scattered in several discrete positions (Ng *et al.*, 1995). These are the characteristics of promoters of genes that have housekeeping and growth-related functions. The data from Ng *et al.* (1995) indicate that a 290 bp region of the 5'-flanking region contains the maximal promoter activity. Within this region, three ICBs were identified (equivalent to ICB1, 2, and 3 of the human gene) and were shown to contribute to basal promoter activity, although ICB1 and 3 were relatively more important for this effect than ICB2 (Ng *et al.*, 1995).

IV.3 mRNA stability of the topoisomerase IIα gene

Topo IIα expression is coupled to the cell cycle position with topo IIα levels rising in late S-phase, peaking in G_2-M-phase, and decreasing as cells complete mitosis (Woessner *et al.*, 1991). In 1996, Prabhat *et al.* demonstrated that this association between cell cycle position and topo IIα expression levels is mainly due to changes in topo IIα mRNA stability. Prabhat *et al.* (1996) found when cells are in G_2-M-phase and mRNA levels are maximal,

a half-life greater than four hours is observed. However, during G_1-phase, when cellular topo IIα levels are lowest, the half-life of topo IIα mRNA is approximately thirty minutes (Prabhat *et al.*, 1996). A similar decrease in mRNA stability was also induced by two external factors, heat shock and ionizing radiation, both of which are known to delay cell cycle progression (Prabhat *et al.*, 1996).

IV.4 Posttranslational modification of DNA topoisomerase II

Factors involved in posttranslational regulation of topo II activity include poly (ADP) ribosylation (Darby *et al.*, 1985), and phosphorylation by casein kinase II (Ackerman *et al.*, 1985; Cardenas and Gasser, 1993), protein kinase C, calmodulin-dependent protein kinase II (Sahyoun *et al.*, 1986; Kroll and Rowe, 1991), and protein tyrosine kinases (Ching *et al.*, 1984). Darby *et al.* (1985) provided evidence that the unknotting, relaxing and catenating activities of calf thymus type II topoisomerase are inhibited by poly (ADP) ribosylation. This finding suggests that poly (ADP) ribosylation could be a means by which eukaryotic cells achieve concerted regulation of topo II activity.

Heck *et al.* (1989) demonstrated that DNA topo II is phosphorylated *in vivo* in vertebrate tissue culture cells, and the level of phosphorylation increases gradually during the G_1 and S phases, and reaches a maximum in the G_2 phase. This elevated level of phosphorylation appears to be maintained until the cells enter mitosis. Casein kinase II appears to be the major kinase responsible for topo II phosphorylation *in vivo* (Ackerman *et al.*, 1985; Cardenas and Gasser, 1993). Ackerman *et al.* (1985) demonstrated that interactions between topo II and its nucleic acid substrates could be modulated by

posttranslational modification of the enzyme by casein kinase II. Evidence suggests that the effects of casein kinase II mediated phosphorylation results from the modification of a small number of specific amino acid residues on topo II (Ackerman *et al.*, 1985).

Sahyoun *et al.* (1986) discovered that DNA topo II, from *Drosophila*, is effectively phosphorylated by protein kinase C and calmodulin-dependent protein kinase II, but not cyclic AMP-dependent protein kinase. Phosphorylation of topo II by protein kinase C results in appreciable activation of topo II, suggesting that topo II may represent a possible target for the regulation of nuclear events by protein kinase C (Sahyoun *et al.*, 1986). Whether topo II is a direct target for protein kinase C remains to be determined. Ching *et al.* (1984) demonstrated that protein tyrosine kinases modulate the activity of topoisomerases by phosphorylating the tyrosine residue involved in DNA binding.

V RATIONALE AND OBJECTIVES

Both isoforms of the topo II gene are clearly important in several fundamental aspects of nucleic acid metabolism including DNA replication, mitosis, and transcription. In addition, each isoform plays a significant, but different, role in determining the response of cells to the cytotoxic effects of several clinically important classes of antineoplastic agents.

Since the human topo IIα 5'-flanking region has already been isolated and partially characterized, the objective of this thesis is to isolate the human 5'-flanking region of the

topo IIβ gene. The isolation of the 5'-flanking region will allow investigators to identify genetic elements involved in normal regulation of this important gene, and help to identify possible mechanisms that drug-resistant cells use to alter topo IIβ expression levels.

MATERIALS AND METHODS

I mRNA ISOLATION (Invitrogen, San Diego, CA)

To release the mRNA and digest unwanted proteins and ribonucleases, 5 x 10^6 HeLa cells were pelleted, then lysed in a detergent based buffer [200 mM NaCl, 200 mM Tris-Cl pH 7.5, 1.5 mM $MgCl_2$, 2% sodium dodecyl sulfate (SDS)] containing an RNase inhibitor and proteases at 45 °C for 20 minutes. The cellular matrix was then added directly to an Oligo (dT) Cellulose tablet, and nutated for 20 minutes to maximize absorption of RNA containing ATPs. The Oligo (dT) Cellulose was pelleted at x 4,000 g, resuspended in a high salt Binding Buffer (500 mM NaCl, 10 mM Tris-Cl pH 7.5), and transferred to a spun column. The DNA, proteins, and cell debris were removed with a high salt Binding Buffer, and centrifuged at 5,000 x g. Non-polyadenylated RNAs were washed off with a Low Salt Wash Buffer (250 mM NaCl, 10 mM Tris-Cl pH 7.5), and centrifuged as before. The $PolyA^+$ RNA was eluted with a salt free Elution Buffer (10 mM Tris-Cl pH 7.5), centrifuged at 5,000 x g, and precipitated with 20 μg of glycogen, 250 mM Sodium Acetate, and 100% ethanol (EtoH). The $PolyA^+$ RNA was stored at -70 °C and, when needed, centrifuged at 16,000 x g and resuspended in Elution Buffer.

II RAPID AMPLIFICATION OF cDNA ENDS (RACE) (Clontech, Palo Alto, CA)

II.1 Adaptor-ligated cDNA library synthesis from HeLa and Placental PolyA$^+$ RNA

To create single-stranded PolyA$^+$ RNA to allow a cDNA primer to anneal, 1 μg of each PolyA$^+$ RNA sample (placental PolyA$^+$ RNA supplied with kit as a positive control) was combined with 10 μM of a 52 bp Marathon cDNA synthesis primer (5'-TTCTAGAATTCAGCGGCCGC$(T)_{30}N_{-1}$-N-3', where N_{-1} = G, A, or C; and N = G, A, C, or T), denatured, and placed on ice. First-strand cDNA synthesis, from PolyA$^+$ RNA, was accomplished by combining first-strand buffer (50 mM Tris pH 8.3, 6 mM $MgCl_2$, 75 mM KCl), dNTP mix (dATP, dCTP, dGTP, dTTP, each at 1 mM), 100 units of Moloney murine leukemia virus reverse transcriptase, and incubating at 42 °C for 1 hour. The first-strand cDNA reaction was placed on ice to terminate the reaction. Second-strand cDNA synthesis was accomplished by combining second-strand buffer [100 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.15 mM β-NAD, 20 mM Tris pH 7.5, 4 μg bovine serum albumin (BSA)], dNTP mix (dATP, dCTP, dGTP, dTTP, each at 0.2 mM), second-strand enzyme cocktail (24 units *E. coli* DNA polymerase I, 4.8 units *E. coli* DNA ligase, 1 unit *E. coli* RNase H), and incubating at 16 °C for 1.5 hours. To allow the adaptor to ligate to the double-stranded cDNA (ds cDNA), the ds cDNA was blunt-ended with 10 units of T4 DNA polymerase, at 16 °C for 45 minutes. The blunt-ending reaction was terminated by the addition of 9.3 mM ethylenediaminetetraacetic acid (EDTA) and 8 μg of Glycogen. Each reaction was sequentially extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1),

1 volume of chloroform:isoamyl alcohol (24:1), then precipitated with 0.5 volumes of 4 M ammonium acetate, and 2.5 volumes of 95% EtoH. The ds cDNA was pelleted at 16,000 x g, washed with 80% EtoH, dissolved in dd-H_2O, and stored at -20 °C until needed.

To amplify unknown 5'- or 3'-ends of ds cDNA, an adaptor of known sequence must be ligated to the ends of the ds cDNA. To ligate the adaptor to the ds cDNA, 0.5 μg of each HeLa and placental reaction were combined with 10 μM of Marathon cDNA Adaptor

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5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
                                                3'-CCCGTCCA-5'
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(containing restriction sites *Not* I, *Srf* I, and *Xma* I), ligation buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 25% (w / v) Polyethylene glycol), 1 unit of T4 DNA ligase, incubated at 16 °C overnight, and heat inactivated at 70 °C. This step essentially creates adaptor-ligated ds cDNA libraries, for HeLa and Placenta, ready for 5'- or 3'-amplification. The ds DNA libraries were then diluted with Tricine-EDTA buffer (10 mM Tricine-KOH pH 8.5, 0.1 mM EDTA) to a suitable concentration for RACE, 0.2 μg / mL, and stored at -20 °C until needed.

II.2 Rapid amplification of 5'-cDNA ends (5'-RACE)

To identify the transcriptional start-site and untranslated region of topo IIβ, the 5'-end of topo IIβ was amplified from both HeLa and Placental ds cDNA libraries, by Polymerase Chain Reactions (PCR). Each PCR reaction contained 2 μg of appropriate cDNA template (HeLa or Placental), PCR buffer (67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 1.7 μg BSA, 16.6 mM $(NH_4)_2SO_4$), 10% dimethylsulfoxide, dNTP mix (dATP, dCTP,

dGTP, dTTP, each at 0.4 mM), and 50 pmol of each primer. Four amplification reactions were performed on each ds cDNA library and consisted of combinations of different primers; a sense oriented 27 bp Adaptor Primer 1 (AP1); a nested 23 bp sense oriented oligonucleotide, Adaptor Primer 2 (AP2); a 25 bp antisense oligonucleotide, Gene Specific Primer 1 (GSP1); and a 38 bp antisense oligonucleotide, Gene Specific Primer 2 (GSP2), with additional sequence added on the 5'-end to provide the restriction sites *Xho* I, *Sal* I, and *Cla* I to simplify cloning (Gibco BRL, Grand Island, NY) (Figure 3). The primer combinations for each HeLa and Placental cDNA template were AP1-GSP1, AP1-GSP2, AP2-GSP1, and AP2-GSP2. The PCR cycling conditions were as follows: the samples were placed inside a DNA Thermal Cycler (Perkin-Elmer-Cetus, Emeryville, CA, USA), heated at 95 °C for 5 minutes, then cooled to 72 °C and 5 units of *Taq*Plus (Stratagene, La Jolla, CA) were added. The samples were cycled 35x at 95 °C for 40 seconds, 55 °C for 1 minute, and at 72 °C for 3 minutes. When the PCR cycling was complete, 10% of each reaction was analyzed on a 1% agarose / ethidium bromide (EtBr) gel to determine the efficiency of each amplification and the size of the topo IIβ untranslated region.

III AMPLIFICATION OF KNOWN cDNA OR GENOMIC DNA (Ausubel, 1993)

The conditions for amplifying known cDNA or genomic DNA were identical to those of section **II.3**, (5'-RACE), except that different templates and primers were used. To create a probe (Probe 1) from the 5'-region of topo IIβ corresponding to the topo IIβ cDNA positions (-4 to +234), a 17 bp oligonucleotide (HT2B-A1), and a 17 bp antisense

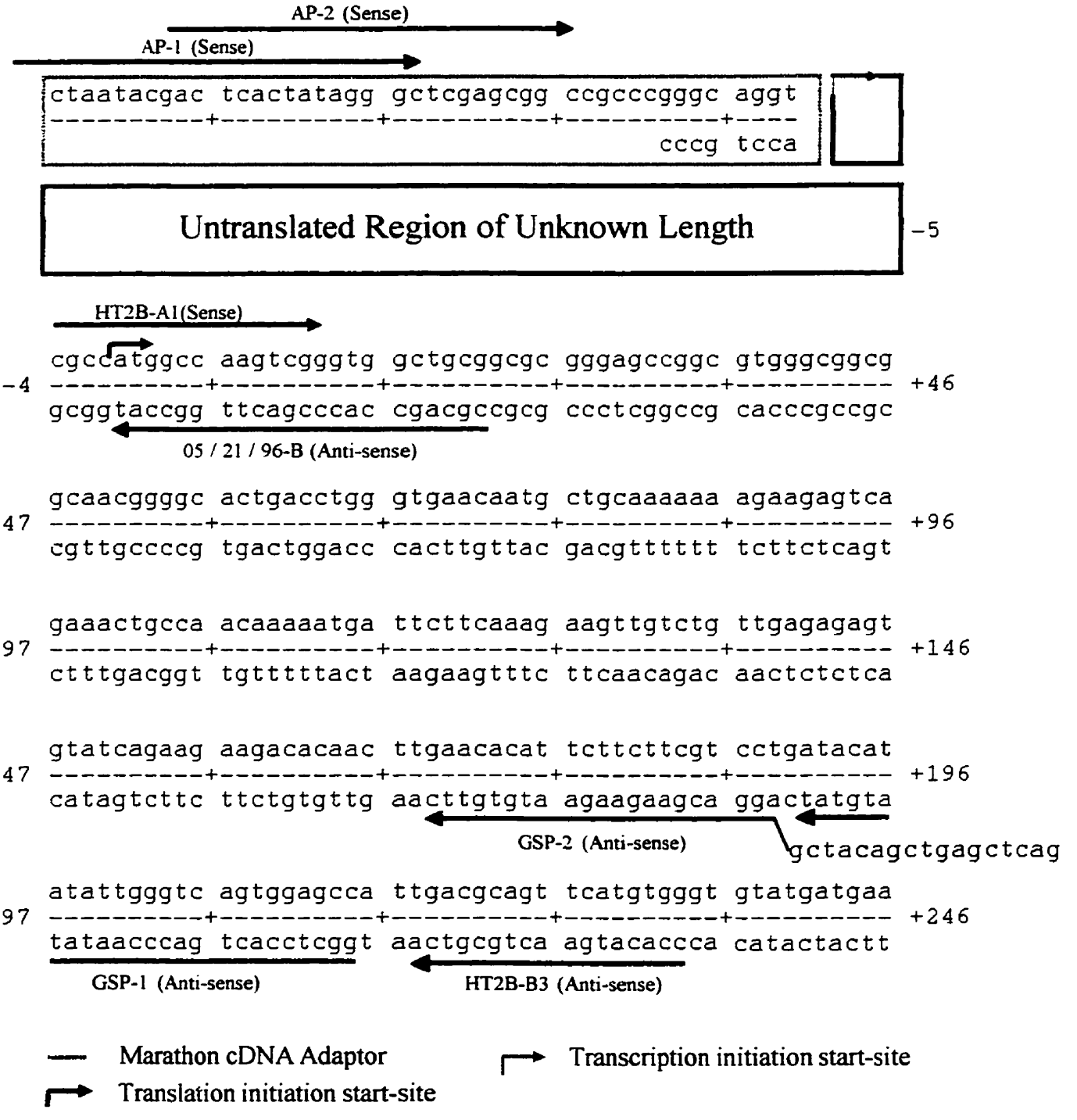
oligonucleotide (HT2B-B3) were constructed [General Synthesis and Diagnostics (GSD), Toronto, Ont.] (Figure 3). To create a probe (Probe 5) from the 5'-region of topo IIβ corresponding to the genomic topo IIβ positions (-219 to +22), a 22 bp sense oligonucleotide (05 / 21 / 96-A) (5'-CCTCGAGTTTGAGGGCAGCCGG-3') corresponding to the genomic topo IIβ positions(-209 to -188), and a 22 bp antisense oligonucleotide (05 / 21 / 96-B) (Figure 3) were constructed (Gibco BRL). When the PCR cycling was complete, 10% of each reaction was analyzed on a 1% agarose / EtBr gel to determine the fidelity and yield of each amplification by comparing the size of the PCR product with that expected.

IV RADIOLABELLING DNA PROBES (Sambrook *et al.*, 1989)

IV.1 Radiolabelling DNA by random-priming

DNA fragments larger than 100 bp were radiolabelled using the random-prime labelling technique. To create single-stranded DNA necessary for radiolabelling, DNA fragments were denatured and placed on ice. Radiolabelling the DNA was accomplished by combining 3 μg of random hexanucleotides, dNTP mix (dCTP, dTTP, and dGTP at 2 mM each), 10 μCi of 3000 Ci / mmol [α-^{32}P]dATP (ICN Biomedicals, Irvine, CA, USA), 5 units of Klenow fragment, incubating at 37 °C for 1 hour, and heat inactivating at 65 °C. The completed reaction was placed in a 1 c.c. spun column (Sambrook *et al.*, 1989) to remove non-incorporated nucleotides, centrifuged at 1,400 x g, boiled, and placed on ice until needed.

Figure 3. Location of oligonucleotides used in Polymerase Chain reactions.



Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

IV.2 Radiolabelling DNA by 5'-endlabelling an oligonucleotide probe

DNA fragments smaller than 100 bp were radiolabelled by the 5'-endlabelling technique. 5'-endlabelling of an oligonucleotide probe was accomplished by combining 30 pmol of oligonucleotide (DNA to be labelled), T4 Polynucleotide kinase buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 1 mM EDTA pH 8.0), 12.5 μCi of 5000 Ci / mmol [γ-^{32}P]ATP (ICN Biomedicals), 20 units of T4 Polynucleotide kinase, and incubating at 37 °C for 45 minutes. The 5'-endlabelling reaction was terminated with 19 mM EDTA and extracted with 1 volume of phenol:chloroform:isoamyl (25:24:1), precipitated with 2 volumes of 100% EtoH at -20 °C containing 300 mM NaAc, incubated at -70 °C for 2 hours, and centrifuged at 16,000 x g. The precipitated oligonucleotide was resuspended in TE8 (10 mM Tris-HCl pH 8.0, 1 mM EDTA), placed in a 1 c.c. spun column (Sambrook *et al.*, 1989), centrifuged at 1,400 x g, and stored at 4 °C until needed.

V GENOMIC DNA LIBRARY SCREENING (Sambrook *et. al.*, 1989)

V.1 Library titration, plating, and plaque lifts

To isolate pure populations of phage, serial dilutions were made from a human placental genomic library (cloning vector: Embl3 Sp6 / T7, cloning site: *Bam*H I, stock titer $\geq 10^8$ pfu / mL, *E. coli* host strains LE392 or K802) (Clontech) in SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 0.1% gelatin, 50 mM Tris-HCl pH 7.5) to achieve a suitable concentration of plaque forming units (pfus) for screening. The host strain LE392 was grown in Luria-Bertani

Broth (LB) (1% Tryptone, 0.5% Yeast Extract, 171 mM NaCl), 100 mM $MgSO_4$, and 2% maltose, at 37 °C to an $O.D._{600}$ = 2.0. Each serial dilution was added to the host strain, LE392, and incubated at 37 °C for 15 minutes, allowing the phage to infect the cells. Top agarose (1% Tryptone, 0.7% agarose, 138 mM NaCl) was added to each suspension and the mixture was poured onto LB agar plates (1% Tryptone, 0.5% Yeast Extract, 171 mM NaCl, 1.5% agar), and incubated at 37 °C overnight. The number of pfus per plate was determined, the concentration of the phage stock was calculated, and the stock library was diluted in SM buffer so that each plate would contain approximately 50,000 pfus. The Poisson distribution equation was used to identify how many pfus must be screened to obtain a 99% probability of finding a sequence of interest in a particular library. Enough pfus were plated out to obtain a 99% probability of finding the sequence of interest, and the library plating was carried out as before.

To transfer the phage DNA to membranes, to allow hybridization, plaques were lifted from LB agar plates using Nylon filters (Hybond-N, Amersham, Arlington Heights, IL, USA). Duplicate filters were used for each plate and each duplicate set was marked for orientation purposes. The filters were placed in denaturing solution (1.5 M NaCl, 0.5N NaOH) to create single-stranded DNA suitable for hybridization analysis, then neutralizing solution (1.5 M NaCl, 500 mM Tris pH 7.2, 1 mM EDTA) to reduce the pH to a level that the DNA can bind to the filter. Finally, the filters are placed in 2x SSC (300 mM NaCl, 30 mM Sodium Citrate pH 7.0) to remove any agar debris, and the phage DNA was permanently fixed to the filters by using an automated ultraviolet irradiation cross-linker (Stratagene)

V.2 Hybridization of Genomic library with radiolabelled DNA

Before the nylon filters, containing immobilized recombinant phage DNA, could be probed, the filters were first treated with a pre-hybridization solution (5x SSPE, 5x Denhardt's solution, 0.5% SDS, 2.25 μg denatured salmon sperm DNA) to provide an optimal environment for the radiolabelled probe to specifically bind to its target sequence, and to prevent non-specific hybridization. The filters were then incubated at 65 °C (for randomly-primed probes) or 55 °C (for 5'-end labelled probes) for 1 hour, and radiolabelled DNA probe was added to give a final hybridizing solution containing at least 2×10^6 cpm of probe per mL.

V.3 Filter washing, autoradiography, and isolation of positive plaques

To remove non-specifically bound probe, all filters were first washed with 2x SSC / 0.1% SDS, then the stringency of the wash was increased. Filters probed with randomly-primed DNA were washed 2x with 0.1x SSC / 0.1% SDS at 65 °C for 10 minutes, while filters probed with 5'-end labelled DNA were washed 2x with 2x SSC / 0.1% SDS, at 55 °C for 10 minutes. All filters were exposed to autoradiography film (Eastman Kodak, Rochchester, New York) overnight, and plaques that bound to the DNA probe were identified on the X-ray film autoradiographs. Once the autoradiographs were oriented with the filters, the areas of the plate corresponding to positive signals were removed and suspended in SM buffer. Since the area removed may contain unwanted plaques in very

close proximity to the plaque of interest, secondary and tertiary screens need to be performed to isolate the plaque of interest.

Dilutions of the phage isolated in the primary screen ranging from 10^{-1} to 10^{-6} were made in SM buffer, and plated out as before. The number of pfus per plate was determined, and an aliquot of the isolated phage was diluted in SM buffer so each plate would contain 1000 pfus. Each area that produced a positive in the primary screen was then plated out as before, at the appropriate dilution, filter lifted, hybridized, washed and exposed as before. By the end of three rounds of screening, purified pfus containing nucleotide sequences of interest were isolated, placed in SM buffer, and stored at 4 °C until needed.

VI **BACTERIOPHAGE LAMBDA DNA PREPARATIONS** (Grossberger, 1987)

To analyze the clones identified in the library screenings, lambda phage DNA was prepared as follows. A culture of LB containing 4% final volume LE392, 4% final volume lambda phage, 10 mM $MgCl_2$, and 10 mM $CaCl_2$, was incubated at 37 °C for 18 hours to create a high titer stock of the lambda bacteriophage clone. The high titer stock was centrifuged at 2,000 x g to pellet cellular debris, centrifuged at 218,000 x g to pellet the phage DNA, and resuspended in SM. To lyse the phage heads, the phage DNA was incubated with 200 µg of proteinase K at 37 °C for 2 hours. Phage DNA was extracted with 3 volumes of phenol, 1 volume of chloroform, then precipitated with 2 volumes of 100% EtoH at -20 °C containing 1.5 M NH_4OAc, incubated at -70 °C for 15 minutes, centrifuged at 16,000 x g, washed with 70% EtoH, and resuspended in TE8.

VII SOUTHERN BLOTTING (Ausubel *et al.*, 1993)

DNA from lambda bacteriophage clones were digested with appropriate restriction enzyme(s), run in an agarose gel with appropriate DNA size markers, stained with EtBr, and photographed upon UV illumination. If the DNA fragments of interest were larger than 5 kb in length, a depurination step (0.25 N HCl) was performed to cleave to DNA into smaller fragments suitable for transfer. The gel was placed in denaturing solution (1.5 M NaCl, 0.5N NaOH) to create single-stranded DNA suitable for hybridization analysis, then in neutralizing solution (1.5 M NaCl, 500 mM Tris pH 7.2, 1 mM EDTA) to reduce the pH to a level that the DNA can bind to the filter. Gels were transferred using 3MM Whatman filter paper (VWR Scientific, Toronto, Ontario) (Ausubel *et al.*, 1993) for 16 hours. Transferred DNA was fixed to the filters by using an ultraviolet irradiation cross-linker (Stratagene).

VIII SUBCLONING

VIII.1 DNA purification (Geneclean II Kit, Bio 101, La Jolla, CA, USA)

To allow DNA of interest to bind to Glassmilk, DNA fragments excised from a TAE agarose gel were combined with 3 volumes of 6 M NaI, and incubated at 65 °C to dissolve the agarose. 10 µL of glass suspension (Glassmilk, Bio 101) was added, the solution incubated on ice for 5 minutes, centrifuged at 16,000 x g, and the supernatant removed. The pellet was washed 3x with -20 °C New wash solution (Bio 101), and centrifuged at

16,000 x g to remove protein and RNA debris, then the Glassmilk pellet was resuspended in a sodium free buffer (TE8) and incubated at 65 °C for 10 minutes to elute the DNA from the Glassmilk. The glassmilk was pelleted at 16,000 x g, and the eluted DNA was stored at 4 °C until needed.

VIII.2 Cohesive and blunt end ligation (Ausubel *et al.*, 1993)

Cohesive and blunt end ligations were accomplished by combining a purified DNA fragment and purified vector, containing a gene coding for resistance to ampicillin, at a ratio of 5:1. DNA fragments were ligated together at 16 °C for 16 hours in T4 DNA ligase buffer (5 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM Dithiothreitol, 1 mM ATP, 0.375 μg BSA), and 1 unit of T4 DNA ligase.

VIII.3 TA overhang ligation (Invitrogen)

TA overhang ligations were accomplished by combining Ligase buffer (6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 1 μg BSA, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine), 50 ng of pCR II vector (containing a single 3'-T nucleotide overhang, and containing a gene coding for resistance to ampicillin), 6 ng of purified DNA fragment of interest (containing a single 5'-A nucleotide overhang), 1 unit T4 DNA ligase, and incubating at 14 °C for 18 hours. The T overhang of the vector and the A overhang of the insert create compatible sticky ends allowing the insert and vector to ligate together efficiently.

VIII.4 Transformation (Ausubel *et al.*, 1993)

To amplify the DNA of interest, ligation reactions were combined with competent DH5α cells (Gibco-BRL), the mixture was set on ice for 30 minutes, heat shocked at 37 °C for 45 seconds, then set on ice for 2 minutes. The DH5α cells were grown in antibiotic free, SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) at 37 °C for 1 hour, then plated onto LB agar ampicillin plates (1% Tryptone, 0.5% Yeast Extract, 171 mM NaCl, 1.5% agar, 1x ampicillin), and incubated at 37 °C overnight. Selected colonies were placed in LB containing 1x ampicillin, and incubated at 37 °C overnight, to propagate recombinant DNA plasmids.

IX PLASMID MINIPREPARATIONS (Ausubel *et al.*, 1993)

DH5α cells containing recombinant plasmids were centrifuged at 16,000 x g, and the pellet resuspended in Solution A (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA). The bacteria were lysed and any proteins, chromosomal DNA, and plasmid DNA were denatured upon addition of Solution B (0.2 N NaOH, 1% SDS). The mixture was neutralized with Solution C (2.4 M KAc, 11.5% glacial acetic acid), causing the plasmid DNA to reanneal and the chromosomal DNA and protein to precipitate. The precipitate was pelleted at 16,000 x g, and the supernatant extracted with 1 volume phenol:chloroform (1:1). RNA was removed from the supernatant by adding 40 μg of DNase-free RNase, incubating at 37 °C for 15 minutes, and re-extracting as before. The aqueous phase, containing purified plasmid

DNA, was precipitated with 100% EtoH, centrifuged at 16,000 x g, washed with 70% EtoH, and resuspended in TE8.

X DNA SEQUENCING (Sanger *et al.*, 1977)

X.1 DNA sequencing reactions (Pharmacia Biotech, Uppsala, Sweden)

5 µg of minipreparation DNA was denatured in 400 mM NaOH / 0.4 mM EDTA for 10 minutes. The DNA was precipitated by adding 447 mM NaAc pH 5.2 and -20° C 100% EtoH, incubating at -70 °C for 15 minutes, spinning at 16,000 x g, and washing with 70% EtoH. The DNA pellet was resuspended in dd-H_2O and annealed to sequencing primers in the presence of 20% final DMSO to inhibit hydrogen bond formation, 300 ng of appropriate primer, and Annealing Buffer (286 mM Tris-HCl pH 7.6, 28.6 mM $MgCl_2$, 45.7 mM DTT). This mixture was incubated at 65 °C for 5 minutes, to inhibit self-annealing, then 37 °C for 10 minutes and room temperature for 15 minutes, to allow annealing of the primer to the template. DNA sequence reactions were initiated by combining dATP Labelling mix (0.206 µM of each dCTP, dGTP, dTTP, 50 mM NaCl), 3.2 units of T7 polymerase, 1.6 µL of enzyme dilution buffer (1.6 mM Tris-HCl pH 7.5, 0.4 mM DTT, 0.16 µg BSA, 5% glycerol),1 µCi of 1500 Ci / mmol [α ^{35}S]dATP (ICN Biomedicals), and incubating at room temperature for 5 minutes. To generate the four sequencing ladders of a dideoxy-sequencing reaction, aliquots from each labelling reaction were added to four possible dideoxynucleoside triphosphate solutions (Pharmacia) that terminate chain elongation, and incubated at 37 °C for 5 minutes. The sequencing reaction was stopped by adding Stop

Solution (0.3% each Bromophenol Blue and Xylene Cyanol FF, 4.7 mM EDTA pH 7.5, 97.5% deionized formamide).

X.2 **Polyacrylamide gel electrophoresis and autoradiography** (Ausubel *et al.*, 1993)

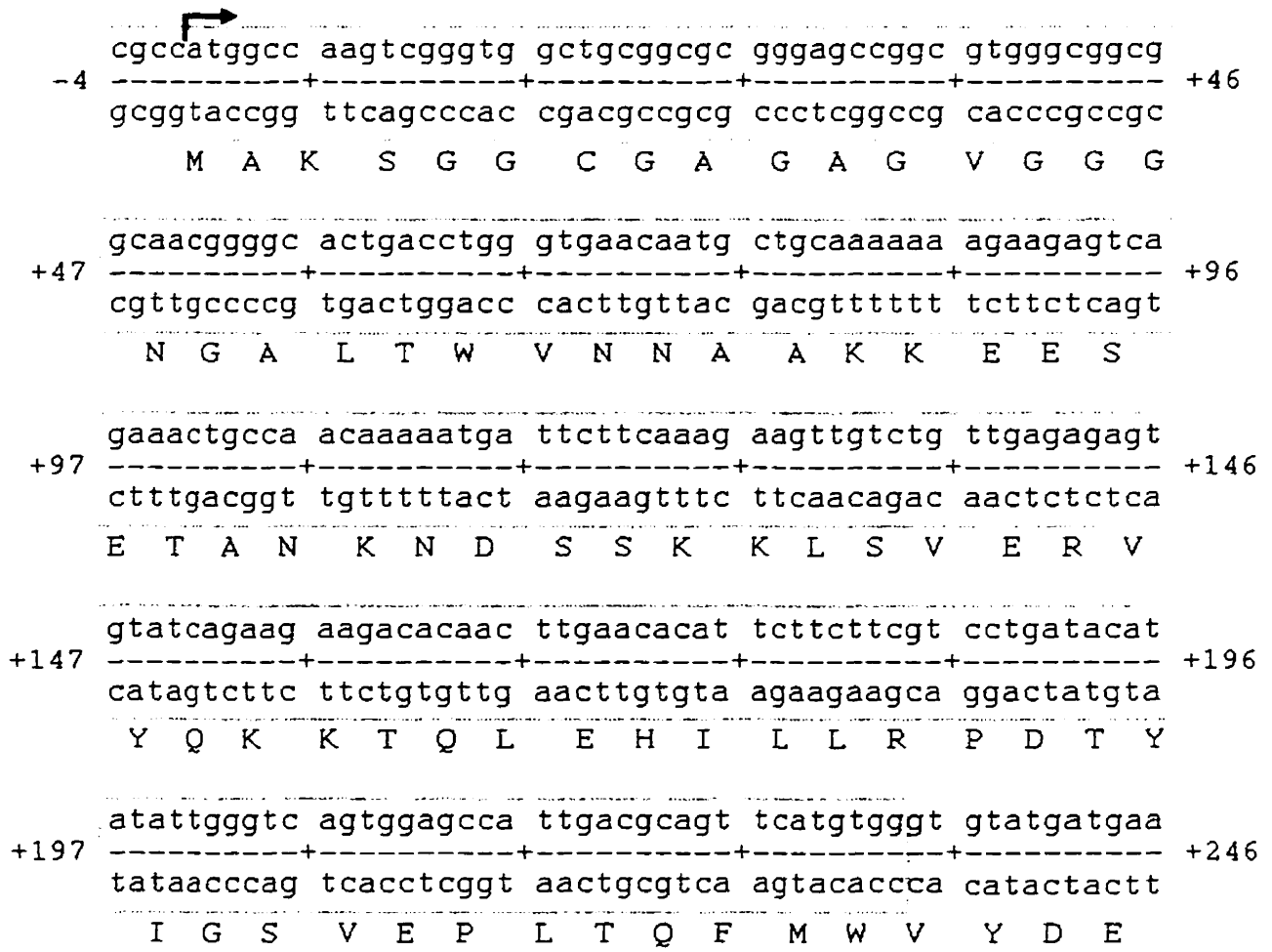
Sequencing reactions were heated at 95 °C for 5 minutes, to denature the single-stranded DNA, and 30% of each reaction was electrophoresed on an 0.75 mm, 8% polyacrylamide sequencing gel [8% acrylamide, 17.5 mM N, N'-bisacrylamide, 6.8 M urea, 1x TBE (890 mM Tris, 890 mM Boric acid, 20 mM EDTA pH 8.0)], polymerized with 0.054% ammonium persulfate and 3.3 mM of TEMED. Electrophoretic separation was allowed to occur for a desired period of time, usually 3 hours for a short sequence run and 6 hours for a long sequence run. The gel was dried on a vacuum gel dryer (Savant, Bluffton, Indiana) at 80 °C for 2 hours, and exposed to X-ray film (Kodak X-OMAT AR) overnight.

RESULTS

I SCREENING A GENOMIC LIBRARY USING PROBES COMPLEMENTARY TO THE 5'-END OF THE KNOWN TOPOISOMERASE IIβ cDNA SEQUENCE

To isolate clones containing the 5'-flanking sequence of human topo IIβ, a 238 bp fragment (Probe 1), spanning human topo IIβ cDNA (Jenkins *et al.*, 1993) positions (-4 to +234), was amplified by PCR and sequenced using the dideoxynucleotide chain termination method for fidelity confirmation (Figure 4). Probe 1 was radiolabelled by the random-priming technique and used to screen 1 x 10^6 pfus from a human placental genomic library. Three potential clones were identified (Clone 1, 2, and 3) from the primary library screening, purified by secondary and tertiary screenings, and amplified by liquid lysate phage preparations for further analysis. DNA from each clone was digested with the restriction enzymes *Bam*H I, *Sac* I, *Eco*R I, *Sal* I, *Xho* I, *Hind* III, and *Nco* I, then analyzed on a 1% agarose / EtBr gel. A Southern blot of the gel was performed and the membrane, containing the transferred DNA, was hybridized with Probe 1 to identify the fragments from each clone that contained the nucleotide sequence of interest. The fragments hybridizing to Probe 1 in Clone 1 were a 4.75 kb *Sac* I fragment [Clone 1, fragment 1 (1-1)], and a 2.0 kb *Hind* III fragment (1-2). The fragment hybridizing to Probe 1 in Clone 2 was a 1 kb *Eco*R I fragment [Clone 2, fragment 1 (2-1)], and the fragments hybridizing to Probe 1 in Clone 3 were a 1.5 kb *Eco*R I fragment [Clone 3, fragment 1 (3-1)], and a 700 bp *Eco*R I fragment (3-2). All of these fragments were subcloned into the phagemid BlueScript II KS^+ using the

Figure 4. 5'-portion of human topoisomerase IIβ cDNA sequence



238 bp double-stranded fragment (Probe 1) spanning topo IIβ cDNA positions (-4 to +234)

Translation start-site

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

cohesive-end ligation method, and amplified using the minipreparation method. Dideoxy-sequencing reactions were performed on fragments 1-1, 1-2, 2-1, 3-1, and 3-2, using primers specific to the 5'-end of topo IIβ cDNA (Figure 5). From these sequencing reactions, only fragment 1-1 and 1-2 produced sequence results. Fragments 1-1 and 1-2 were taken for further study, and digested with the restriction enzymes *Eco*R I, *Hind* III, *Sac* I, *Xba* I, *Sal* I, *Pst* I, *Bgl* II, *Eco*R V, *Pvu* II, *Xho* I, and *Nco* I, analyzed on a 1% agarose / EtBr gel, and a restriction map was established (Figure 6). Fragment 1-2 was identified as a sub-fragment of fragment 1-1, therefore only fragment 1-1 was taken for further analysis. Fragment 1-1 was sequenced using the dideoxynucleotide chain termination method, and found to contain topo IIβ exon sequence corresponding to the topo IIβ cDNA sequence (+70 to +225), and flanked by intronic sequence (Figure 7).

To investigate whether any of the three clones contained topo IIβ exon sequence corresponding to the topo IIβ cDNA sequence (-4 to +69), two antisense oligonucleotide probes were synthesized upstream of the two identified introns (Figure 8). The two oligonucleotide probes, a 30 bp antisense oligonucleotide (Probe 2) corresponding to the topo IIβ cDNA positions (-4 to +26) and a 24 bp antisense oligonucleotide (Probe 3) corresponding to the topo IIβ cDNA positions (+46 to +69) were radiolabelled using the 5'-endlabelling technique and hybridized to filters containing positive and negative controls, to determine each probe's specificity. These test filters were washed at different stringencies to identify the conditions maximizing each probe's signal while minimizing background signal (Figure 9). Once optimal conditions for both probes were established, Southern blots of Clones 1, 2, and 3. Clones 1, 2, and 3 did not hybridize to Probe 2. Probe 3 was Probe 2

Figure 5. Sequencing primers specific to the 5'-portion of human topoisomerase IIβ cDNA sequence

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

Figure 6. Restriction map of fragments 1-1 and 1-2.

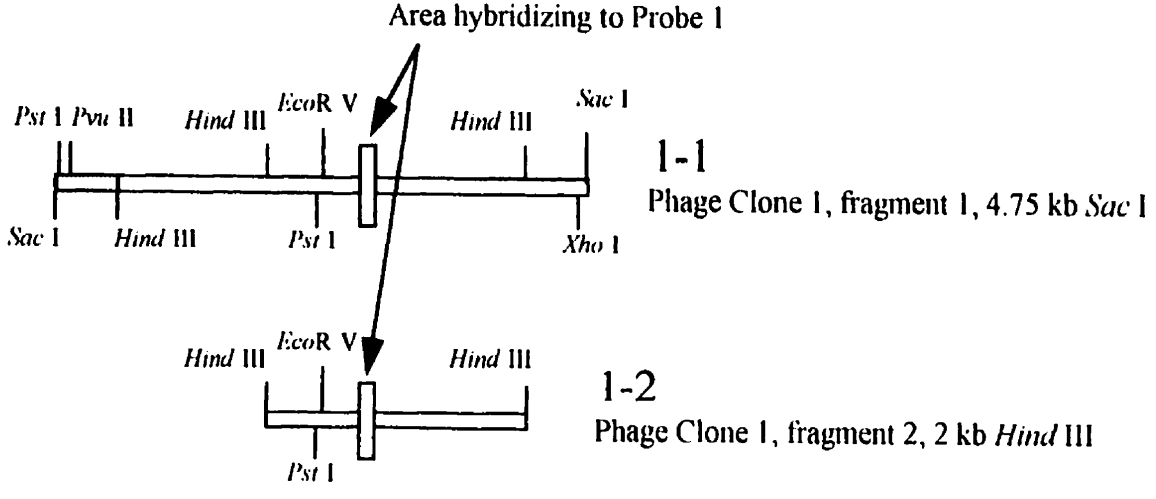
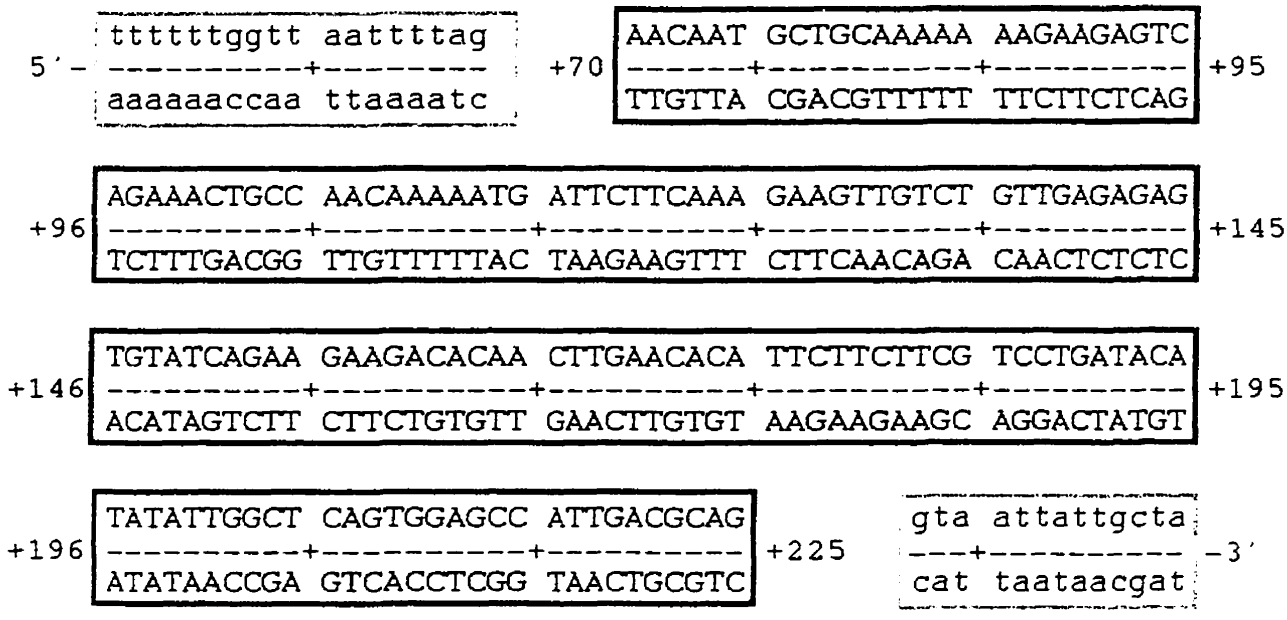


Figure 7. Exon sequence data from fragment 1-1



Exon sequence,corresponding to topo IIβ cDNA positions (+70 to +225), and flanked by intron sequence

Intron sequence

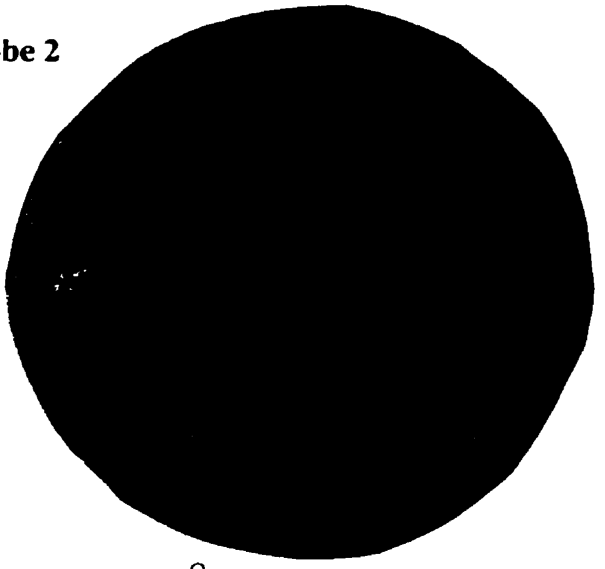
Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

Figure 8. 5'-portion of human topoisomerase IIβ cDNA sequence containing known intron / exon boundaries

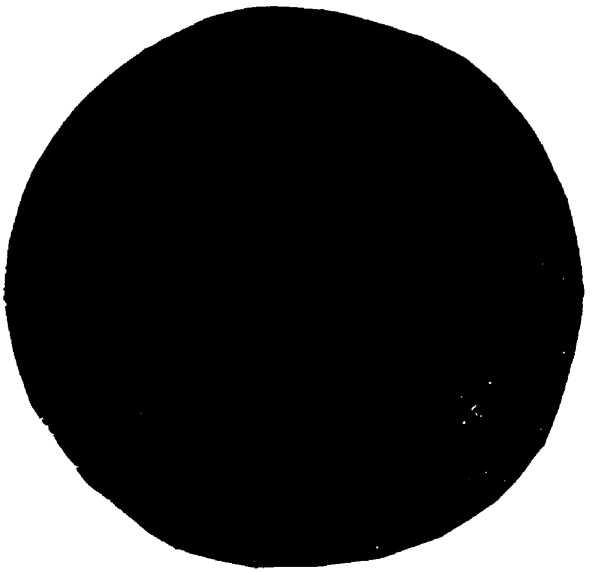
Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

FIGURE 9. Test filters containing positive and negative controls for Probe 2 and Probe 3

Probe 2

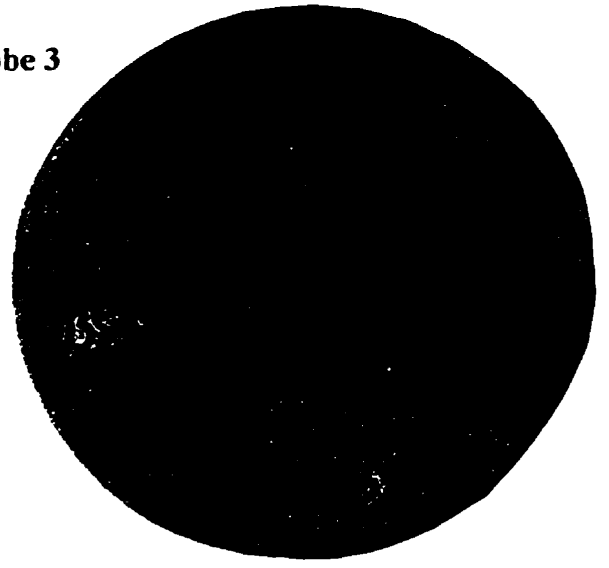


65 ^{O}C hybrization and wash temperatures

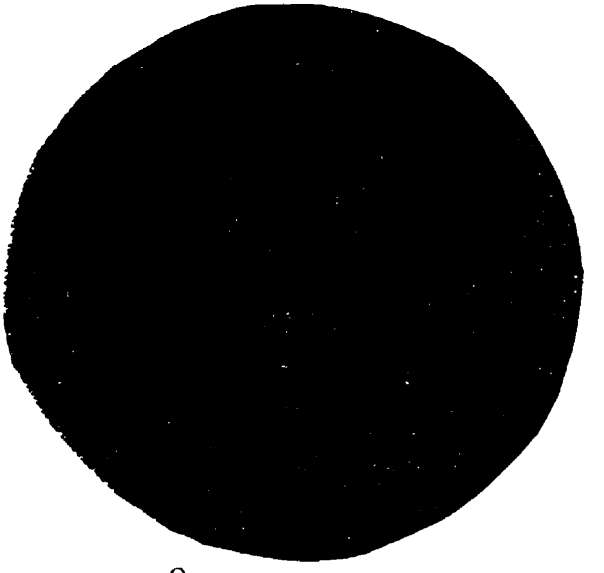


55 ^{O}C hybrization and wash temperatures

Probe 3



65 ^{O}C hybrization and wash temperatures



55 ^{O}C hybrization and wash temperatures

5'	5'-portion of topoisomerase IIβ (positive control)
3'	3'-portion of topoisomerase IIβ (negative contol)
BS	Bluescript II KS^{+} (negative control)

was radiolabelled using the 5'-endlabelling technique and hybridized to the radiolabelled using the 5'-endlabelling technique and hybridized to the Southern blots of Clones 1, 2, and 3. Clones 1, 2, and 3 also did not hybridize to Probe 3. These results indicated that Clones 1, 2, and 3 did not contain any upstream exon sequence corresponding to topo IIβ cDNA positions (-4 to +69).

To isolate clones containing topo IIβ exon sequence, corresponding to the topo IIβ cDNA sequence (-4 to +69) and the 5'-flanking sequence of human topo IIβ, Probe 2 was radiolabelled using the 5'-endlabelling technique and used to screen 5 x 10^6 pfus from a human placental genomic library. No potential clones were identified. Probe 3 was then radiolabelled using the 5'-endlabelling technique and used to screen 3 x 10^6 pfus from a human placental genomic library. No potential clones were identified.

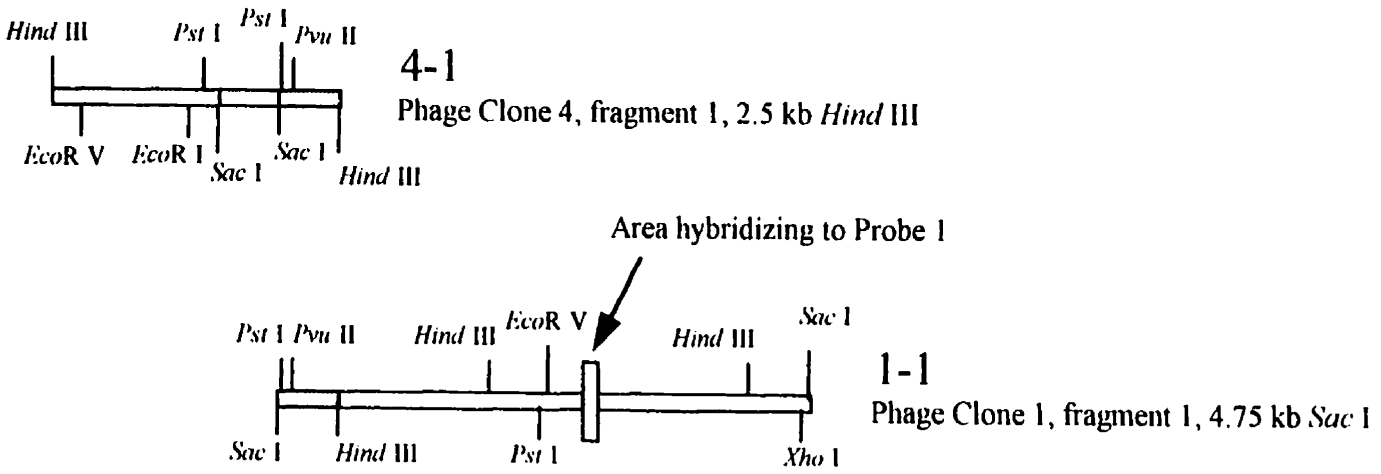
II WALKING UPSTREAM OF THE KNOWN EXON IN THE TOPOISOMERASE IIβ GENE

Since the first strategy, using three different cDNA probes, was unsuccessful in isolating any clones containing topo IIβ exon sequence upstream of the topo IIβ cDNA position +70, or the 5'-flanking sequence of human topo IIβ, a new strategy was implemented, which involved walking upstream along the gene to identify the translation and transcription start-sites. Using this strategy, a 700 bp *Sac* I / *Hind* III fragment was isolated from the 5'-end of fragment 1-1 and purified for use as a probe (in green, Figure 6). The

700 bp *Sac* I / *Hind* III fragment was radiolabelled by the random-priming technique and used to screen 1 x 10^6 pfus from a human placental genomic library. Three potential clones were isolated (Clones 4, 5, and 6) from the primary library screening, purified by secondary and tertiary screenings, and amplified by liquid lysate phage preparations for further analysis. Each clone was digested with the restriction enzymes *Bam*H I, *Sac* I, *Eco*R I, *Sal* I, *Xho* I, *Hind* III, and *Nco* I, then analyzed on a 1% agarose / EtBr gel. Preliminary restriction digests identified phage Clone 5 and phage Clone 6 as sister clones, therefore only Clone 4 and Clone 5 were further examined. A Southern blot of the gel was performed and the membrane, containing the transferred DNA, was hybridized with the 700 bp *Sac* I / *Hind* III fragment to identify the fragments from each clone that contained the area of interest. The fragment that hybridized to the 700 bp *Sac* I / *Hind* III fragment in Clone 4 and Clone 5, was a 2.5 kb *Hind* III fragment [Clone 4 and Clone 5, fragment 1 (4-1)]. Fragment 4-1 was subcloned into the *Hind* III site of BlueScript II KS^+. Fragment 4-1 was digested with the restriction enzymes *Eco*R I, *Hind* III, *Sac* I, *Xba* I, *Sal* I, *Pst* I, *Bgl* II, *Eco*R V, *Pvu* II , *Xho* I, *Bam*H I, *Nde* I, *Sca* I, *Bst*E II, *Kpn* I, *Nhe* I and *Nco* I, analyzed on a 1% agarose / EtBr gel, and a restriction map was established (Figure 10).

To continue the walk along the gene, a 1.4 kb *Hind* III / *Sac* I fragment was isolated and purified from the 5'-end of fragment 4-1 (in orange, Figure 10). To identify the fragments from each clone that contained the area of interest, the 1.4 kb *Hind* III / *Sac* I fragment was radiolabelled by the random-priming technique and hybridized to the Southern blots of Clones 4, 5, and 6. The fragment that hybridized to the 1.4 kb *Hind* III / *Sac* I fragment in Clone 4 was a 4.0 kb *Sac* I fragment [Clone 4, fragment 2 (4-2)]. The fragment

Figure 10. Restriction map of fragment 4-1, and comparision of overlap with fragment 1-1(▭).



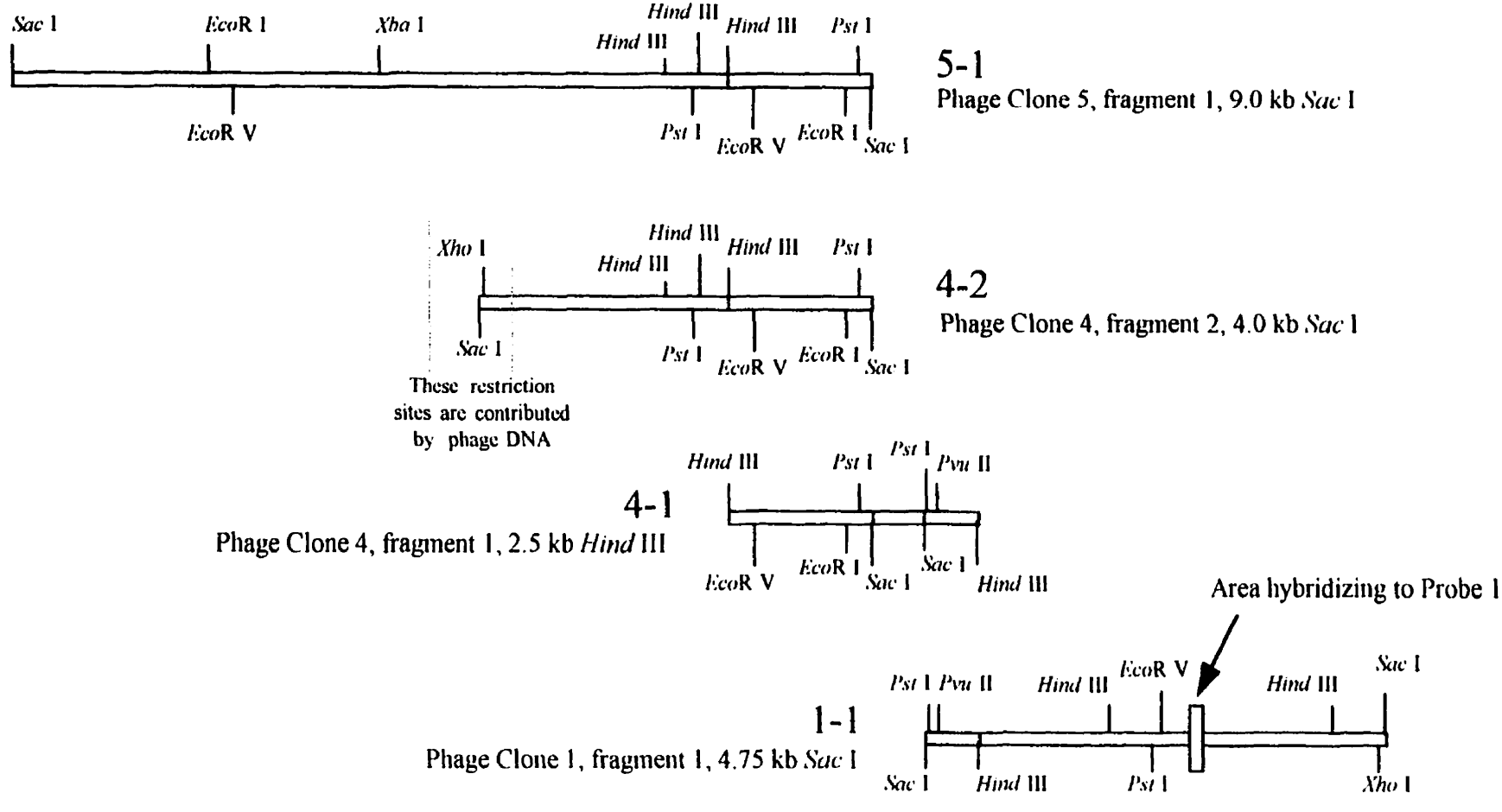
that hybridized to the 1.4 kb *Hind* III / *Sac* I fragment in Clone 5 was a 9.0 kb *Sac* I fragment [Clone 5, fragment 1 (5-1)]. Both fragments were subcloned into BlueScript II KS^+ using the cohesive-end ligation method, and amplified using the minipreparation method. Both fragments, 4-2 and 5-1, were digested with the restriction enzymes *Eco*R I, *Hind* III, *Sac* I, *Xba* I, *Sal* I, *Pst* I, *Bgl* II, *Eco*R V, *Pvu* II, *Xho* I, *Bam*H I, *Sma* I, and *Nco* I, analyzed on a 1% agarose / EtBr gel, and a restriction map was established (Figure 11).

All three fragments, 4-1, 4-2, and 5-1, derived from the short chromosomal walk did not contain an *Nco* I restriction site, an important indicator since the translation start-site of topo IIβ was expected to contain an *Nco* I restriction site based on cDNA sequence information. All three fragments were sequenced using the dideoxynucleotide chain termination method using primers specific to the 5'-end of topo IIβ cDNA, HT2B-A1, HT2B-A2, and HT2B-B1 (Figure 5). Finally, both Probe 2 and Probe 3 were radiolabelled using the 5'-endlabelling technique and hybridized to Southern blots of Clones 4, 5, and 6. Neither probe hybridized to any fragments, suggesting that no topo IIβ exon sequence corresponding to the topo IIβ cDNA sequence (-4 to +69) was present in these Clones.

III USING 5'-RACE TO IDENTIFY THE START-SITE OF TRANSCRIPTION AND TO GENERATE NEW PROBES TO SCREEN A GENOMIC LIBRARY

To identify the transcription start-site of topo IIβ, HeLa and human placental $PolyA^+$ RNA were isolated, converted to ds cDNA, and the 5'-end of each topo IIβ cDNA was amplified. PCR products were subcloned into the pCR II vector using the TA ligation

Figure 11. Restriction map of fragments 4-2 and 5-1, and a comparision of their overlap with fragment 4-1(▭).

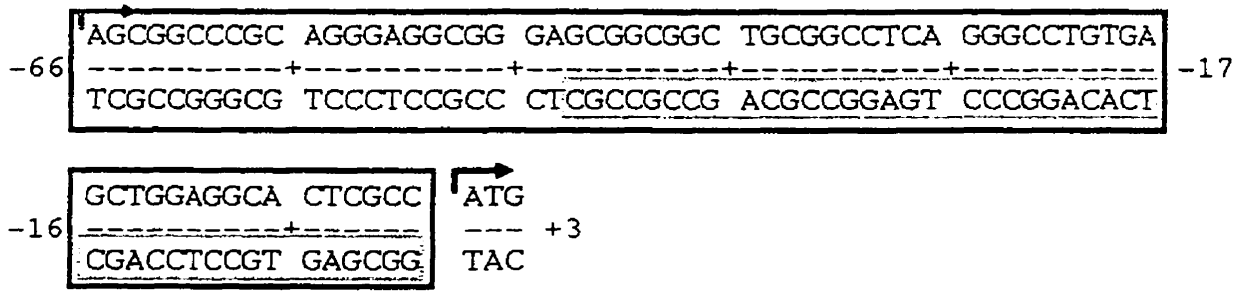


method and sequenced using the dideoxynucleotide chain termination method. Both the HeLa and human placental cDNA untranslated regions, PCR 1 and PCR 2 respectively, spanned topo IIβ cDNA positions (-1 to -66) and contained identical sequence (Figure 12).

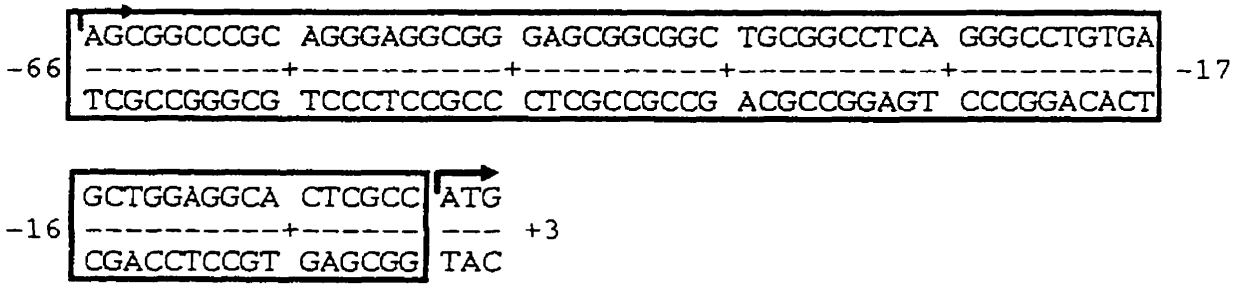
To isolate clones containing topo IIβ exon sequence, corresponding to the topo IIβ cDNA sequence (-66 to +69), and the 5'-flanking sequence of human topo IIβ, a 44 bp antisense oligonucleotide (Probe 4) corresponding to the topo IIβ cDNA positions (-1 to -44) was synthesized (Figure 12). Probe 4 was radiolabelled using the 5'-endlabelling technique and hybridized to a filter containing positive and negative controls, to determine the probe's specificity. The test filter was washed at different stringencies to identify the conditions maximizing the probe's signal while minimizing background signal. Before screening a genomic library, Probe 4 was radiolabelled using the 5'-endlabelling technique and hybridized to the Southern blots of Clones 4, 5, and 6. The probe did not hybridize to any fragments, suggesting that no topo IIβ exon sequence corresponding to the topo IIβ cDNA sequence (-44 to -1) was present in these Clones. Probe 4 was radiolabelled using the 5'-endlabelling technique and used to screen 1 x 10^6 pfus from a human placental genomic library. One potential clone was isolated (Clone 7) from the primary library screening, purified by secondary and tertiary screenings, and amplified by liquid lysate phage preparations for further analysis. Clone 7 was digested with the restriction enzymes *Bam*H I, *Sac* I, *Eco*R I, *Sal* I, *Xho* I, *Hind* III, and *Nco* I, then analyzed on a 1% agarose / EtBr gel. A Southern blot of the gel was performed and the membrane, containing the transferred DNA, was hybridized with Probe 4 to identify the fragments that contained the area of interest. The fragments that hybridized to Probe 4 in Clone 7 were 2.5 kb *Hind* III fragment [Clone 7,

Figure 12. Transcription start-sites, untranslated regions, and translation start-sites of HeLa and human placental cDNA

5'-end of HeLa topo IIβ cDNA (PCR 1)



5'-end of human placental topo IIβ cDNA (PCR 2)



Transcription start-site

Translation start-site

Untranslated region
(5'-region of topo IIβ Exon one)

44 bp anti-sense oligonucleotide
(Probe 4)

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

fragment I (7-1)], and a 750 bp *Eco*R I fragment (7-2) as illustrated in Figure 13. These fragments were subcloned into *Hind* III and *Eco*R I sites of BlueScript II KS^+ respectively. Minipreparations containing fragments 7-1 and 7-2 were digested with the restriction enzymes *Eco*R I, *Hind* III, *Sac* I, *Xba* I, *Sal* I, *Pst* I, *Bgl* II, *Eco*R V, *Pvu* II, *Xho* I, *Bam*H I, *Nde* I, *Sca* I, *Bst*E II, *Kpn* I, *Nhe* I and *Nco* I, analyzed on a 1% agarose / EtBr gel (Figure 13). Results of restriction mapping indicated that fragment 7-2 was contained within fragment 7-1, therefore only fragment 7-1 was further examined. Fragment 7-1 was sequenced using the dideoxynucleotide chain termination method, and found to contain no topo IIβ exon sequence, instead, a repeat sequence was identified containing sequence identical to portions of Probe 4, corresponding to topo IIβ cDNA positions (-4 to -15) and (-7 to -15) (Figure 14).

New information concerning the human topo IIβ promoter region was presented at the Annual Meeting of the American Association for Cancer Research, April 1996 (Ng *et al.*, 1996). This information included a portion of the topo IIβ promoter region and the entire topo IIβ exon one sequence, together corresponding to cDNA positions (-224 to +69). The sequence information from Ng *et al.* (1996) was used to design a 5'-primer to amplify a 241 bp double-stranded fragment (Probe 5), corresponding to cDNA positions (-219 to +22), from a human placental genomic DNA library. Probe 5 was sequenced using the dideoxynucleotide chain termination method for fidelity confirmation, then radiolabelled by the random-priming technique and used to screen 1 x 10^6 pfus from a human placental genomic library. Six potential clones were identified (Clones 8, 9, 10, 11, 12, and 13) from the primary library screening, purified by secondary and tertiary screenings, and amplified by liquid lysate phage preparations for further analysis. Each clone was digested with the

Figure 13. Restriction map of fragments 7-1 and 7-2

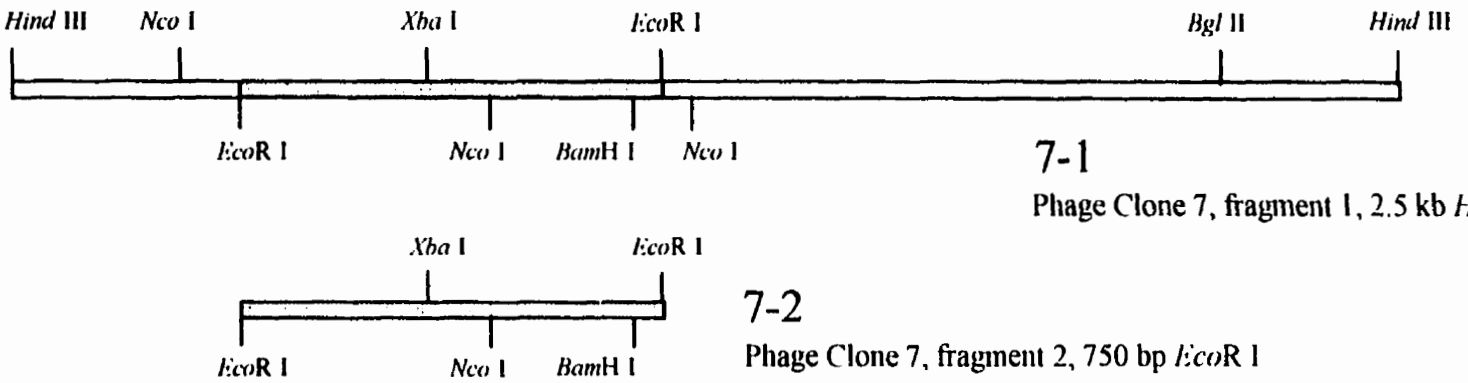


Figure 14. Sequence comparison between Probe 4 and fragment 7-1

Probe 4 (Anti-sense)

```
-44 ----------+----------+----------+----------+---- -1
3'- GCGGCGGCTG CGGCCTCAGG GCCTGTGAGC TGGAGGCACT CGCC -5'
```

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

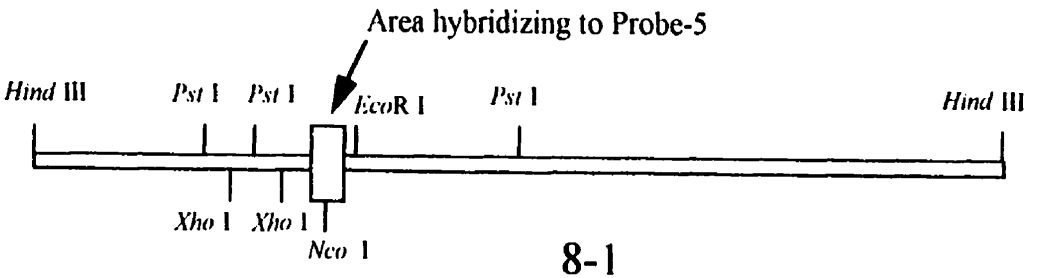
Sequence from fragment 7-1

```
.......... ctggaggcat tcacagtttg gaagcactca cactctggag
----------+----------+----------+----------+----------
.......... gacctccgta agtgtcaaac cttcgtgagt gtgagacctc

gcattcacac tctggaggca ttcacactct ggaggcattc actctctgga
----------+----------+----------+----------+----------
cgtaagtgtg agacctccgt aagtgtgaga cctccgtaag tgagagacct

ggcattcact ctggaggcat tcacactctg gaggcactca cactctggag
----------+----------+----------+----------+----------
ccgtaagtga gacctccgta agtgtgagac ctccgtgagt gtgagacctc

gcactcacac tctggacact cacattctgg aggcattcac ..........
----------+----------+----------+----------+----------
cgtgagtgtg agacctgtga gtgtaagacc tccgtaagtg ..........
```

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

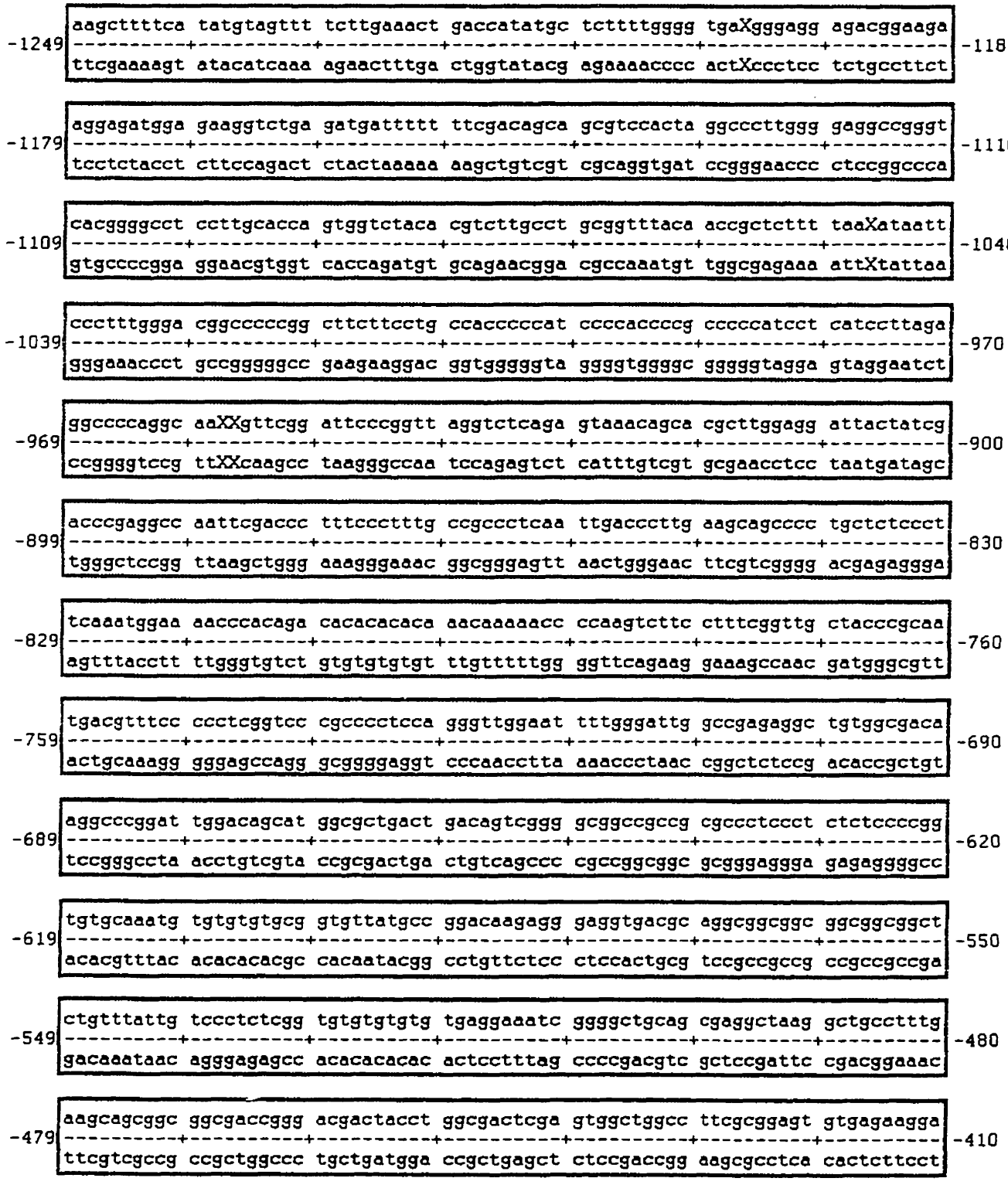
restriction enzymes *Bam*H I, *Sac* I, *Eco*R I, *Sal* I, *Xho* I, *Hind* III, and *Nco* I, then analyzed on a 1% agarose / EtBr gel. From these preliminary restriction digests, phage Clones 8, 9,10, and 11 were identified as sister clones, and phage Clone 12 and Clone 13 were identified as sister clones, therefore, only Clone 8 and Clone 12 were further examined. A Southern blot of the gel was performed and the membrane, containing the transferred DNA, was hybridized with Probe 5 to identify the fragments from each clone that contained the nucleotide sequence of interest. The fragment hybridizing to Probe 5 in Clone 8 and Clone 12 was a 4.35 kb *Hind* III fragment [Clone 8 and Clone 12, fragment 1 (8-1)]. Fragment 8-1 was subcloned into the phagemid BlueScript II KS^+ using the cohesive-end ligation method, and amplified using the minipreparation method. Fragment 8-1 was digested with the restriction enzymes *Eco*R I, *Hind* III, *Sac* I, *Xba* I, *Sal* I, *Pst* I, *Bgl* II, *Pvu* II, *Xho* I, *Bam*H I, *Nde* I, *Sca* I, *Bst*E II, *Kpn* I, *Nhe* I, *Cla* I and *Nco* I, analyzed on a 1% agarose / EtBr gel, and a restriction map was established (Figure 15). Fragment 8-1 was sequenced using the dideoxynucleotide chain termination method, and found to contain the entire sequence of topo IIβ exon one (-66 to +69) and approximately 1183 bp of 5'-flanking sequence (Figure 16). This sequence was analyzed using Transcription Element Search Software (TESS) from the Baylor College of Medicine, Houston, Texas, and the TRANSFAC version 3.0 database. Fifty-six potential mammalian transcription factor binding sites were identified (Figure 17).

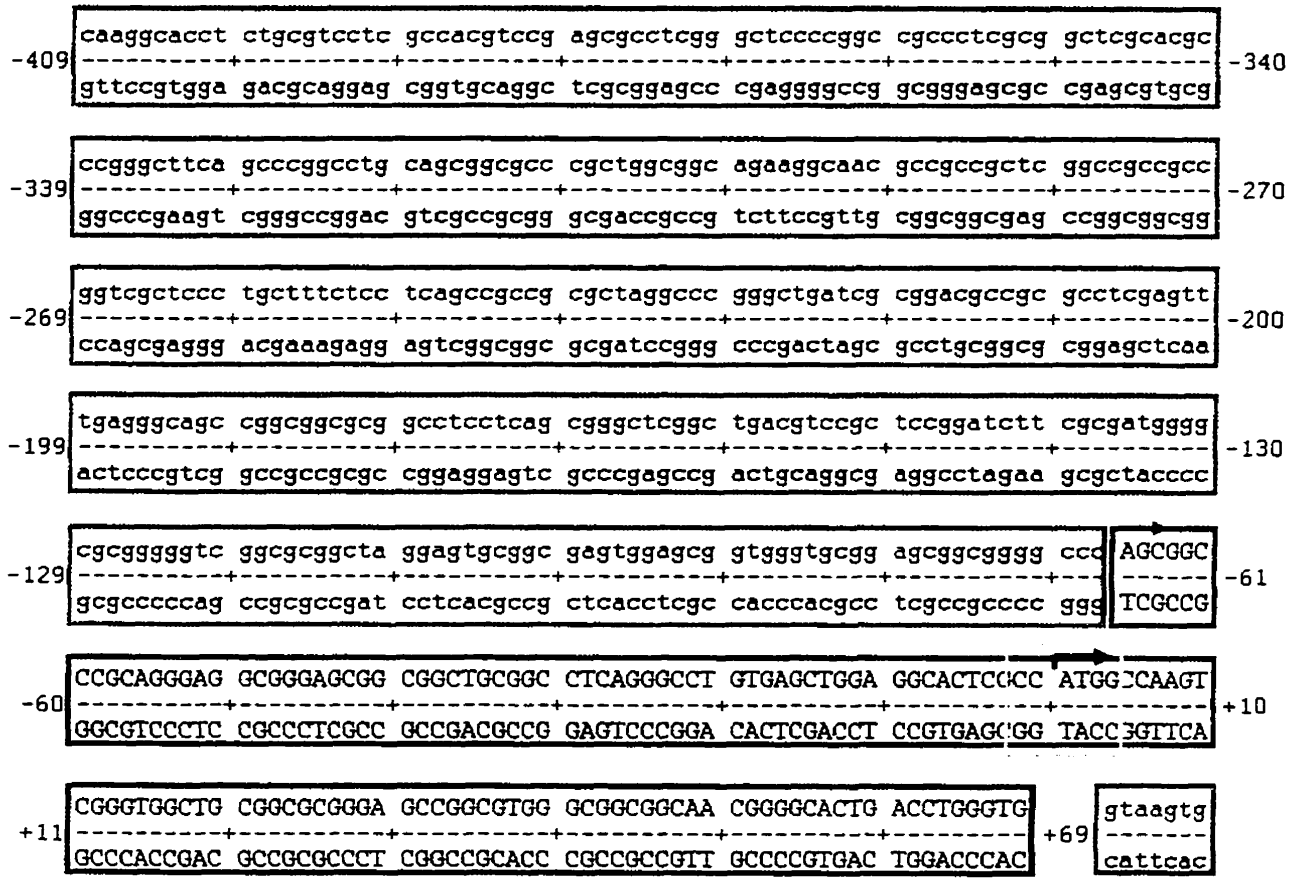
Figure 15. Restriction map of fragment 8-1



500 bp

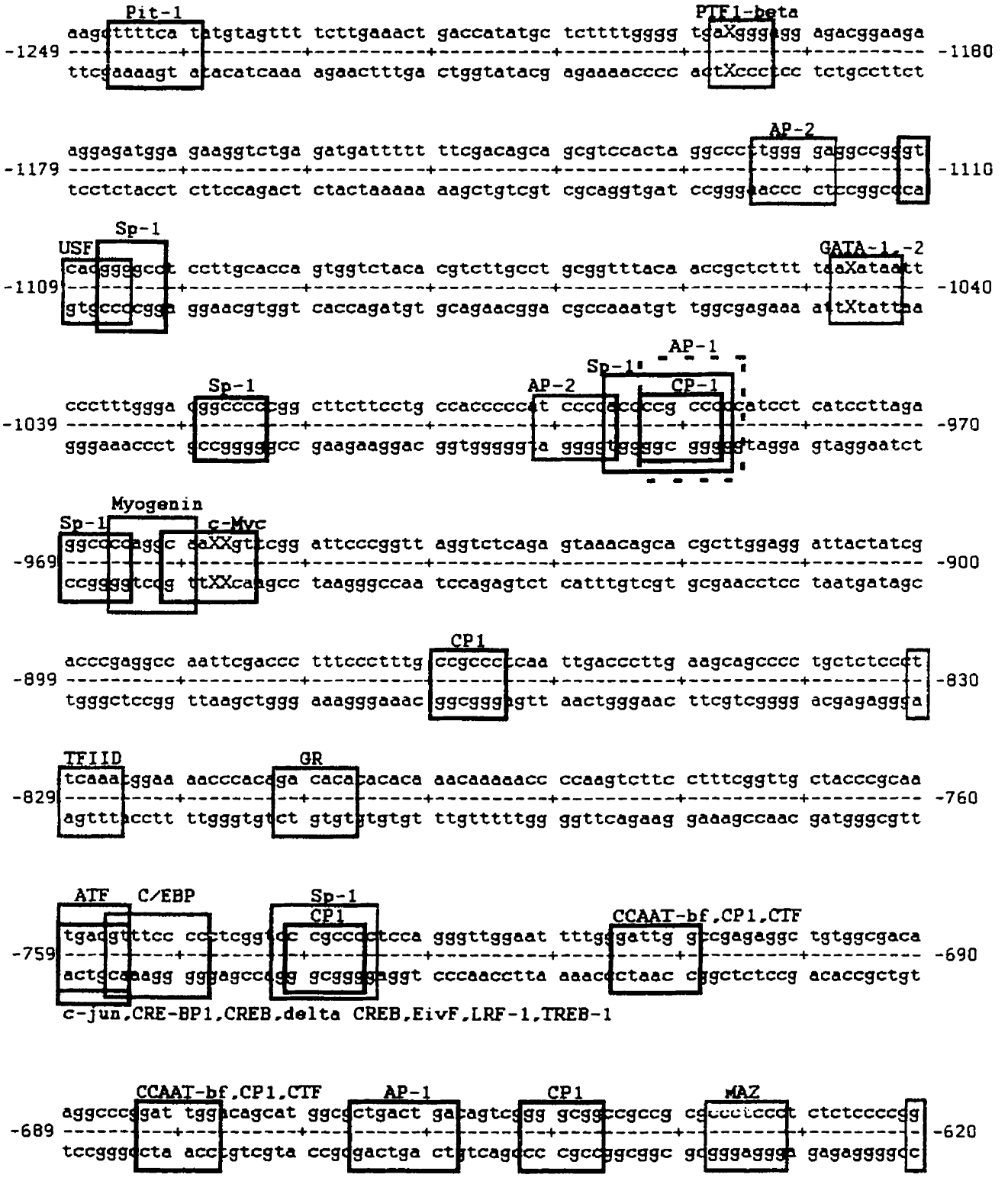
Figure 16. Sequence data of fragment 8-1

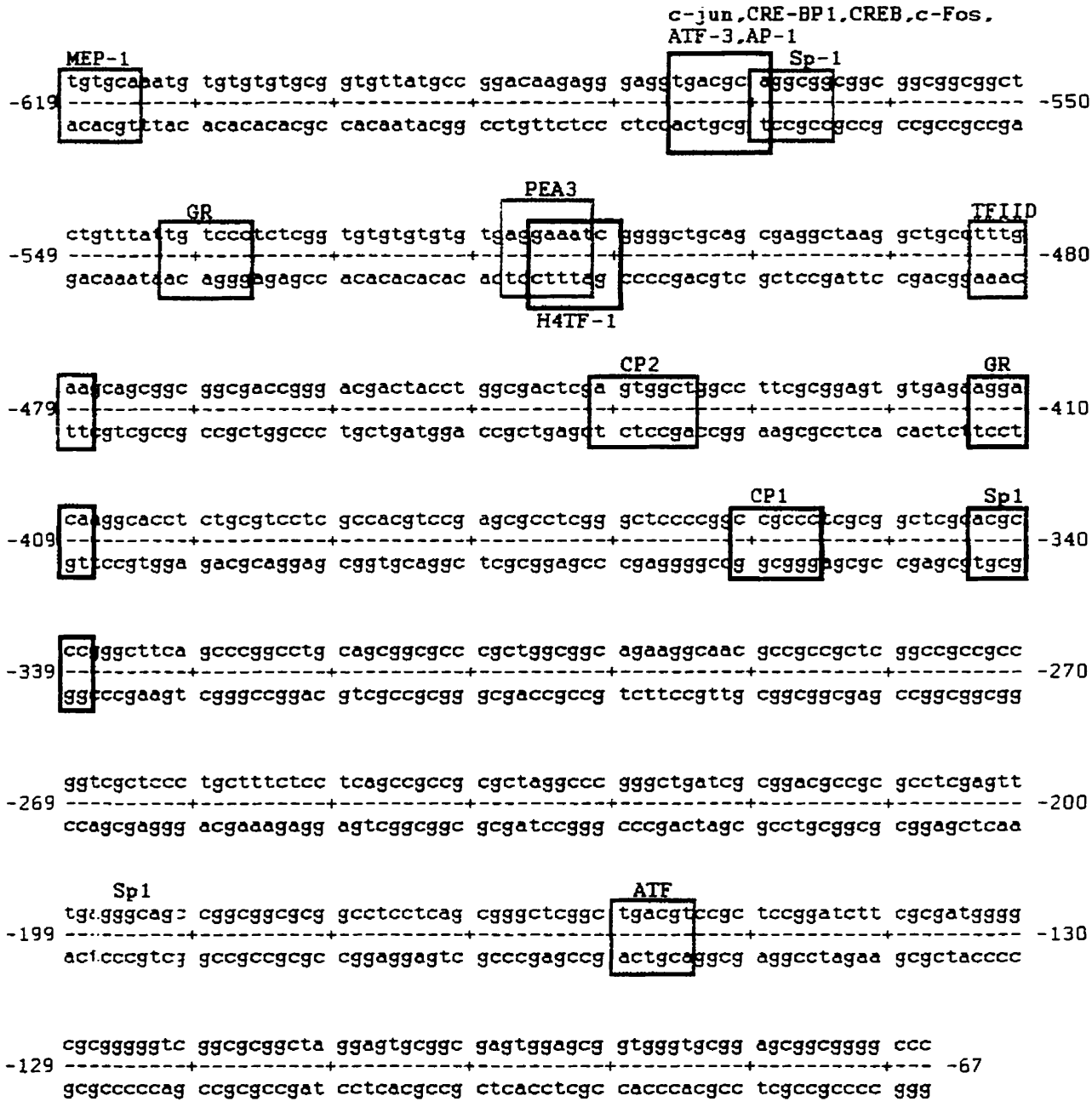




Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

Figure 17. Potential mammalian transcription factor binding sites identified on the 5'-flanking region of topoisomerase IIβ from fragment 8-1





— Human transcription factor binding site (tfbs)

— Chicken tfbs

— Mouse tfbs

Pig tfbs

— Rat tfbs

X Unknown nucleotide

Note: Nucleotide numbering system was derived from Jenkins *et al.* (1993) with +1 representing the start-site of translation for topo IIβ

DISCUSSION

A conventional method for isolating that portion of genomic DNA that transcriptionally regulates gene expression is to screen a genomic library using probes complementary to the 5'-end of the known cDNA sequence. Before a probe was selected, properties of the transcription start-site and untranslated region of human topo IIα were taken into consideration. The transcription start-site of human topo IIα was identified 90 bp upstream of the translation start-site, and the 90 bp untranslated region of human topo IIα was not interrupted by introns. Therefore, it was predicted that a probe spanning the translation start-site of topo IIβ could be used to isolate clones that included the topo IIβ initiation start-site and 5'-flanking sequence. Accordingly, a 238 bp fragment (Probe 1) (Figure 4) spanning topo IIβ cDNA positions (-4 to +234) was amplified from known human topo IIβ cDNA sequence, spanning (-4 to +4866) (Jenkins *et al.*, 1993), and pre-existing primers, HT2B-A1 and HT2B-B3 (Figure 5). Initial genomic library screens yielded a 4.75 kb *Sac* I fragment (1-1) that hybridized to Probe 1. Restriction mapping revealed that fragment 1-1 did not contain an *Nco* I restriction site (Figure 6), an important indicator since the start-site of translation of topo IIβ was expected to contain an *Nco* I restriction site based on cDNA sequence information. When dideoxy-sequencing reactions were performed on fragment 1-1, using primers specific to the 5'-end of topo IIβ cDNA (Figure 5), only two primers, HT2B-B2 and HT2B-B3, produced sequencing results. These sequencing results could not be identified as topo IIβ, which suggested that either the two primers were non specific, or that fragment 1-1 might contain intron / exon boundaries in the 5'-region of topo IIβ corresponding to the location of the two sequencing primers.

Complete sequence information from fragment 1-1 revealed a 156 bp exon fragment corresponding to topo IIβ cDNA position (+70 to +225) flanked by two introns (Figure 7). Whether more introns existed further upstream of these two introns was not known at this time. Therefore, two antisense oligonucleotide probes, Probe 2 and Probe 3, were synthesized 1 bp and 44 bp, respectively, upstream of the most 5' known intron to identify if any isolated clones contained topo IIβ exon sequence corresponding to topo IIβ cDNA positions (-4 to +69). Two distinct probes were synthesized to minimize the possibility of another intron existing within topo IIβ cDNA positions (-4 to +69) and destroying one probe's capability to hybridize to the target sequence.

The length and location of Probe 2 (Figure 8), corresponding to topo IIβ cDNA positions (-4 to +26), was chosen to create an appropriate G + C nucleotide composition. The G + C nucleotide composition determines the melting temperature of the probe, and thereby influences the hybridization and wash temperatures. The hybridization and wash temperature must be high enough to minimize any non-specific hybridization. The length and location of Probe 3 (Figure 8), corresponding to topo IIβ cDNA positions (+46 to +69), was chosen for two reasons. First, the length and location were chosen to create an appropriate G + C nucleotide composition. Second, exon fragment sizes are thought to be at least 30 bp in length. Therefore, it was predicted that the topo IIβ exon sequence, corresponding to cDNA positions (+29 to +69), would not be interrupted by another intron when one intron already exists between topo IIβ cDNA positions (+69 and +70).
When both Probe 2 and Probe 3 specifically hybridized to the test filters but failed to hybridize to Clones 1, 2 or 3, it was concluded that the 5'-sequence, corresponding to

topo IIβ cDNA positions (-4 to +69), did not exist in the three clones. When Probe 2 and Probe 3 were unable to isolate any potential clones after 2 million pfus were screened, positive and negative test filters were included in the hybridization process to test radiolabelling efficiency. An additional 6 million pfus were screened, and again Probe 2 and Probe 3 failed to identify any potential clones, even though the results obtained using the positive and negative controls were appropriate.

The reason Probe 2 and Probe 3 failed to select potential clones is not clear, but at the time, several possible explanations existed. First, it was thought that both probes might be interrupted by introns located within the 5'-region of topo IIβ corresponding to cDNA positions (-4 to +69), but this seemed highly unlikely. Second, the concentration of target sequence might have been too low for efficient hybridization to occur, but oligonucleotides have been used previously to isolate clones from libraries. Finally, the most likely explanation was that other, unknown, factors were responsible. It was concluded that the difficulties encountered with Probe 2 and Probe 3 would probably be encountered with any oligonucleotide synthesized within the known topo IIβ exon region corresponding to cDNA positions (-4 to +69) and therefore this strategy was abandoned.

A new strategy was implemented, focusing on walking upstream along the gene, through any introns 5' of the exon region corresponding to topo IIβ cDNA positions (+70 to +225), and eventually identifying the translation and transcription start-sites of topo IIβ. The chromosomal walk entailed using a segment of DNA derived from the 5'-end of some fragment of the gene and using it as a probe to identify overlapping clones that contain adjacent 5'-sequence. Using the strategy of walking along the chromosome provided

genomic fragments spanning 12.2 kb upstream of the known exon sequence, corresponding to topo IIβ cDNA positions (+70 to +225). None of the three fragments derived from the chromosomal walk contained an *Nco* I restriction site, indicating that the topo IIβ translation start-site was not present. None of the primers specific to the 5'-end of topo IIβ cDNA (Figure 5) produced sequencing results, also Probe 2 and Probe 3 failed to hybridize to any of the clones. The results suggested that none of the genomic clones, upstream of the known exon sequence, contained any exon sequence corresponding to topo IIβ cDNA positions (-4 to +69). Therefore, the 12.2 kb upstream region, derived from the chromosomal walk, was thought to represent a substantial intron in the gene. Continuing the walk could potentially entail walking through a very large intron, which would take a long time to isolate the translation and transcription start-sites. Without knowledge of the size of this intron, or whether other introns exist further upstream in the untranslated region or translated region of topo IIβ, the decision was made to abandon the chromosomal walk.

The next strategy employed a commonly used method for isolating the 5'-flanking region of a gene, screening a genomic library using probes complementary to the 5'-end of the known cDNA sequence. This time the cDNA sequence would be derived from the transcription start-site and untranslated region of topo IIβ. To isolate the transcription start-site and provide more 5'-cDNA sequence for probe construction, amplification of 5'-HeLa and human placental topo IIβ cDNA was performed. The decision to amplify the cDNA ends of both HeLa and human placenta was made because both sources were readily available and there was uncertainty regarding efficiency of the 5'-RACE reaction using HeLa

or placental mRNA. Both the HeLa and human placental mRNA isolations, and 5'-end cDNA amplifications were performed independent of one another.

From the newly identified transcription start-site and untranslated region of topo IIβ cDNA, a 44 bp antisense oligonucleotide (Probe 4) (Figure 12), corresponding to the topo IIβ cDNA positions (-1 to -44), was synthesized. The length and location of Probe 4 within the untranslated region, was chosen to create an appropriate G + C nucleotide composition. Screening a primary library yielded a 2.5 kb *Hind* III fragment (7-1) that hybridized to Probe 4. Restriction mapping (Figure 13) revealed that fragment 7-1 contained two *Nco* I restriction sites, an important indicator that the topo IIβ translation start-site may be present. Fragment 7-1 was sequenced using the dideoxynucleotide chain termination method, revealing that fragment 7-1 did not contain topo IIβ exon sequence, but did contain a repeat sequence identical to portions of Probe 4. The repeat sequences, corresponding to topo IIβ cDNA positions (-4 to -15) and (-7 to -15) (Figure 14), accounted for 27% and 20.5% of Probe 4 respectively, providing enough homology to create a false positive signal. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program and the National Center of Biotechnology Information GENBANK database, but no sequence similarity matches were identified. A new probe would have to be constructed corresponding to sequence within the untranslated region, but without the topo IIβ sequence corresponding to the repeat sequences found in fragment 7-1.

Before a new probe was synthesized, new information concerning the human topo IIβ promoter was presented by Dr. Alex Ng *et al.* (1996) of Harvard Medical school at the Annual Meeting of the American Association for Cancer Research in Washington, April

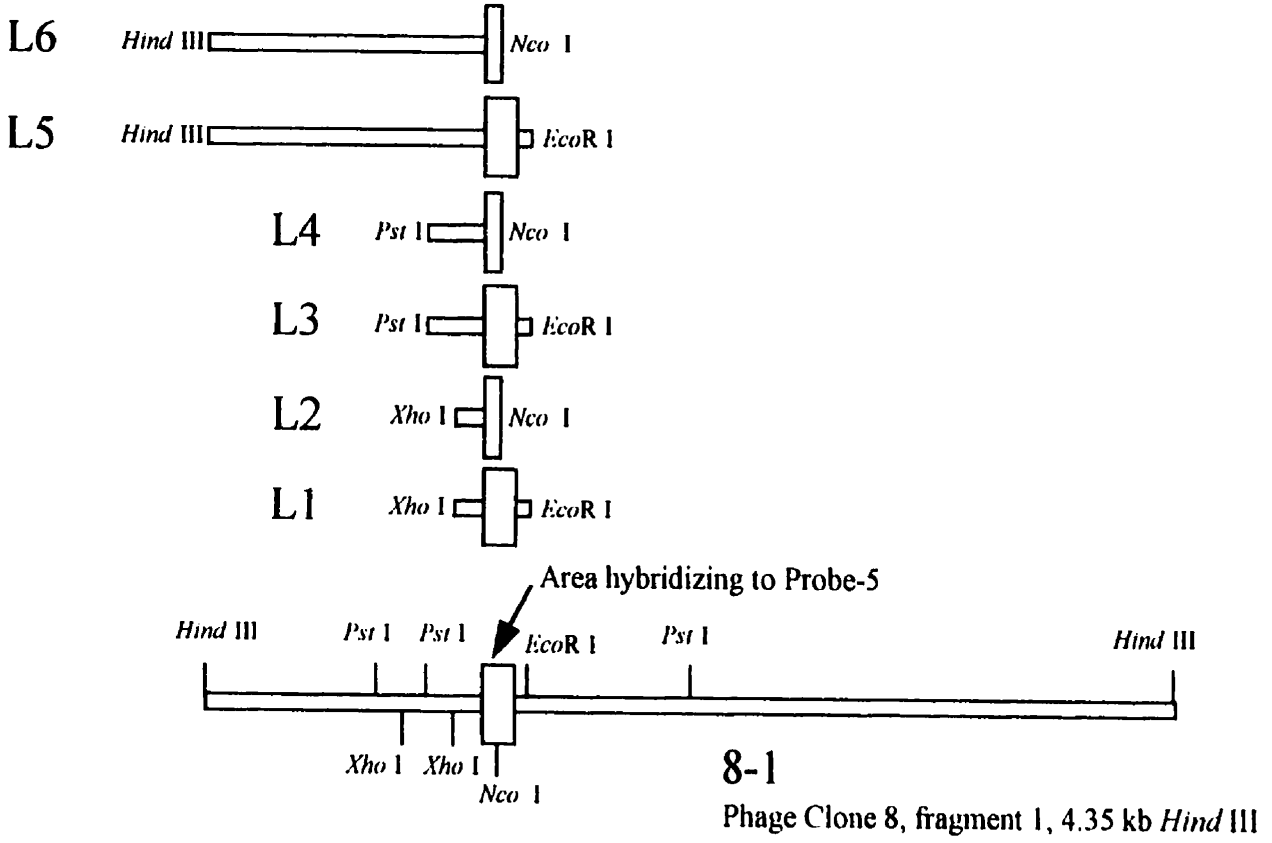
1996. The information included topo IIβ 5'-flanking sequence corresponding to cDNA positions (-224 to -89), two transcription start-sites corresponding to topo IIβ cDNA position -88 and -190, and the entire sequence of the first exon of topo IIβ corresponding to cDNA positions (-88 to +69). The human placenta transcription start-sites identified by Ng *et al.* (1996) are 22 bp and 124 bp further upstream than the single transcription start-site identified here for both HeLa and human placenta topo IIβ. The transcription start-sites identified in human genomic DNA by Ng *et al.* (1996) were established by 5'-RACE and confirmed by S1-nuclease mapping. To clarify this discrepancy, an S1-nuclease protection assay for both HeLa and human placental mRNA is required. It is difficult to comment on this discrepancy, however it is tempting to speculate that more than two transcription start-sites may exist for the β isoform of human topoisomerase II.

Regardless of the discrepancy between transcription start-sites, the new topo IIβ sequence information from Ng *et al.* (1996) was used to design a probe (Probe 5) spanning the untranslated region, the transcription start-site, and part of the 5'-flanking sequence. Probe 5, a 231 bp double-stranded fragment corresponding to cDNA positions (-219 to +22), was amplified from a human placental genomic DNA library. Screening of a primary library yielded a 4.35 kb *Hind* III fragment (8-1) that hybridized to Probe 5. Restriction mapping revealed that fragment 8-1 contained an *Nco* I restriction site, indicating that the fragment may contain the start-site of translation of topo IIβ (Figure 15). Complete sequence information from fragment 8-1 revealed topo IIβ exon one (-66 to +69) and approximately 1183 bp of 5'-flanking sequence (Figure 16).

To establish functional activity of the putative promoter of topo IIβ, preliminary luciferase activity assays were conducted on several constructs derived from fragment 8-1 (Figure 18). The CMV promoter was cloned into the luciferase vector, pGL2-basic, as a positive control, and the pGL2-basic vector alone was used as a negative control. All the constructs were sequenced using the dideoxynucleotide chain termination method to confirm insert orientation, transfected into an Acute Lymphoblastic Leukemia (ALL) cell line, and tested for luciferase activity. Unfortunately, none of the constructs, including the positive controls, demonstrated luciferase activity. Ng *et al.* (1996) provided preliminary evidence of promoter activity in a 137 bp fragment of the 5'-flanking region of human topo IIβ using luciferase assays. Given that Ng *et al.* (1996) demonstrated promoter activity on similar portions of the 5'-flanking region of topo IIβ, and the fact that our positive controls were unable to elicit a response, it was concluded that the transfection conditions for the luciferase assays were simply not optimized. Future experiments should manipulate cell type, cell number, transfection voltage, transfection current, and incubation times to identify the optimal transfection conditions.

The sequence obtained from fragment 8-1 was analyzed using Transcription Element Search Software (TESS) from the Baylor College of Medicine, Houston, Texas, and the TRANSFAC version 3.0 database. Fifty-six potential mammalian transcription factor binding sites were identified (Figure 17).

Figure 18. Portions of fragment 8-1 used in preliminary luciferase activity assays



FUTURE RESEARCH DIRECTIONS

Clearly further studies would be required to establish which of the potential putative binding sites found in the 5'-flanking region of topo IIβ are of biological relevance. Studies should also be performed to help elucidate the role that the 5'-flanking region of topo IIβ plays in drug resistant cell lines.

Firstly, a series of deletion constructs lacking one or more elements in the 5'-flanking region would be constructed. Elements affecting transcriptional activity might then be destroyed by site-directed mutagenesis in an attempt to correlate alteration of transcriptional activity with destruction of specific transcription factor binding sites. Secondly, DNase footprint analysis would be used to identify proteins associated with the 5'-flanking region of topo IIβ. Thirdly, the normal topo IIβ 5'-flanking region would be transfected into drug resistant cell lines to provide specific insight into the role that transcriptional regulation plays in drug resistance. Finally, the 5'-flanking region of topo IIβ in resistant cell lines would be cloned and compared to the normal functioning topo IIβ 5'-flanking region in an effort to identify possible mutations.

Previously, p53 proteins (Ines *et al.* 1996), phorbol esters (Zwelling *et al.*, 1990), retinoic acid (Tsao *et al.*, 1994), and methylation (Tan *et al.*, 1989) have been shown to transcriptionally regulate topo IIα. Whether these previous observations are found solely in the α isoform or in both isoforms is not known at this time, but an investigation of the topo IIβ 5'-flanking region is now possible to assess the involvement of these agents in transcriptional regulation.

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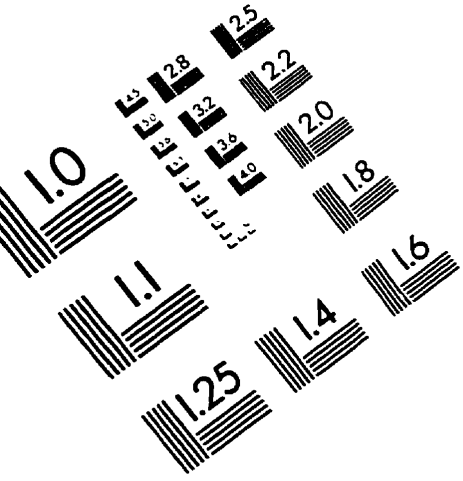
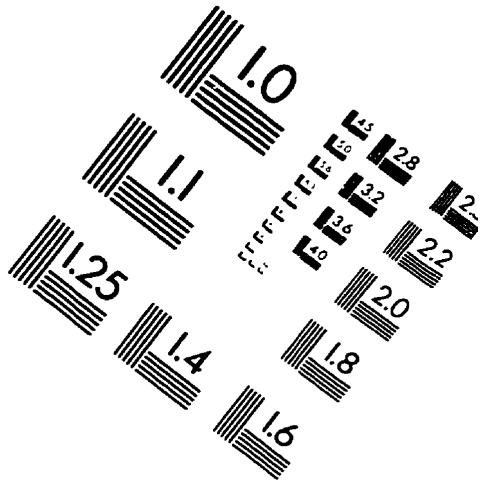
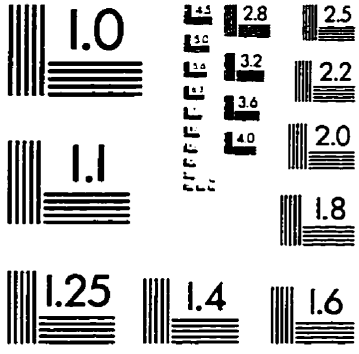
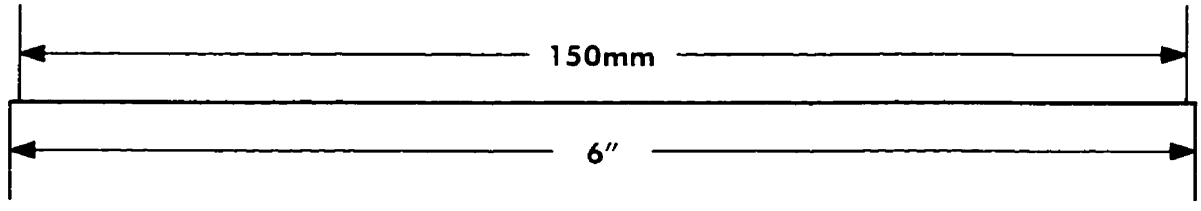
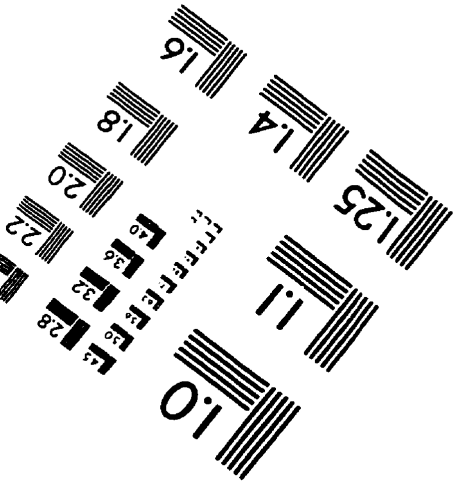
IMAGE EVALUATION TEST TARGET (QA-3)

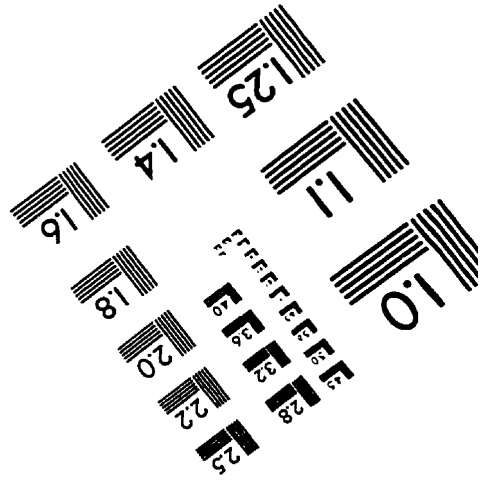












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989