Dynamics of Human DNA Topoisomerases I and II

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1. Introduction - DNA Topoisomerases

Already at the discovery of the double helix structure of DNA it was recognized that unwinding of intertwined DNA-strands would be necessary during semi-conservative replication of the molecule. With the discovery of ring-shaped double stranded DNA, the unwinding problem became a topological one: the two multiply linked circular parental strands must be unlinked after each round of replication. A unique class of enzymes known as DNA topoisomerases, which catalyse the breakage and rejoining of DNA strands by two successive transesterification reactions, are nature's tool for solving these topological problems of replication (reviewed in (Champoux, 2001; Nitiss, 1998; Wang, 1996; Wang, 2002)).

In this section, I will briefly introduce DNA topoisomerases in general, followed by a detailed description of human topoisomerases (topo) I and II, respectively. Subsequently, the current knowledge about the biological functions of these enzymes will be discussed in a joined section, since it is misguiding to separate these enzymes in this respect. Finally, present knowledge on the sub-cellular localization of the enzymes will be presented, with most attention paid to the topo II isoforms, because their localization has proven to be a particularly controversial subject.

1.1 DNA Topology and DNA Topoisomerases

Supercoiling of the genomic DNA is an essential feature of all living organisms and can be considered as one level of organization, which helps to pack the genome into a small volume so it fits the dimension of a cells nucleus. A prerequisite for supercoiling to occur is that the DNA must be constrained into closed structures. This is indeed the case for plasmids, bacterial chromosomes, mitochondria and chloroplasts, where the DNA exists as closed-circular DNA. Although eukaryotic chromosomes consist of linear DNA, the genome appears to be organized into constrained domains or loops that in a topological sense behave as closed-circular domains. These closed chromatin domains are mediated by the so-called scaffold/matrix attached regions (S/MARs) that are anchored to the nuclear matrix at a number of sites, thus generating chromatin loops of approximately 50-100 kb of DNA (reviewed in (Laemmli et al., 1992)).

The topological state of a constrained DNA molecule or domain is described by its linking number (Lk). The linking number is defined as the number of times that two DNA double helices are intertwined. It is the sum of twist (Tw) and writhe (Wr). Tw represents the total number of helical repeats and therefore determines the number of base pairs per turn in the basic Watson-Crick structure. Wr is a measurement of the contortion of the helix in space, describing the pathway of the DNA in space. In living cells, the characteristic organization of the DNA is a negative supercoil, in which the Wr is increased and Tw is lowered in

comparison to a DNA domain that is not supercoiled and has the same Lk (reviewed in (Bates and Maxwell, 1993)).

A common feature of all known eukaryotic topoisomerases is their ability to relax negatively supercoiled DNA. In addition, type II topoisomerases are able to catalyse knotting/unknotting- and catenation/decatenation reactions on intact duplex DNA. Knots are probably occurring rarely in nature, whereas catenates are found frequently. Indeed, all natural populations of DNA rings are to some extent interlocked as catenates (Bates and Maxwell, 1993). The process described best, in which catenation occurs, is the replication of genomic DNA, where the two sister chromatids are catenated after replication. It is the ability of type II topoisomerases to decatenate the intermediate interlocked DNA after replication that makes these enzymes essential in all living organisms. Fig. 1.1 summarizes the topological changes of the DNA that can be catalysed by these enzymes.

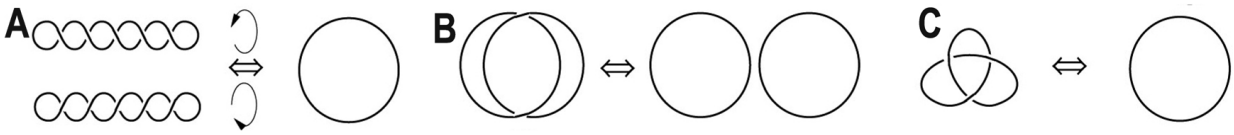

Figure 1.1: **Topological transformations performed by eukaryotic DNA topoisomerases on the example of circular plasmid DNA.** (A) Relaxation of supercoiled DNA (type I and type II topoisomerases). (B) Catenation/decatenation (type II topoisomerases). (C) Knotting/unknotting (type II topoisomerases). Figure is adapted from (Boege, 1996).

The first DNA topoisomerase was discovered in 1971 in *E.coli* by James Wang (Wang, 1971). Since then, extensive studies have focused on identifying new members of the family, and on elucidating their structures, enzymatic mechanisms, and cellular functions. DNA topoisomerases are classified according to their catalytic mechanism of action into four categories (see table 1.1): Type I topoisomerases relax DNA by introducing a single strand break, through which the other strand of DNA can pass, followed by religation of the broken strand. Thus, they change the linkage number of the DNA in steps of one. They are monomers and act in an ATP-independent manner. Type I topoisomerases fall into two subtypes, IA and IB. Type IA topoisomerases require Mg^{2+} for their activity and relax preferentially negative supercoils. This group comprises *E. coli* topo I and III, yeast topo III and mammalian topo IIIα and IIIβ. Type IB topoisomerases are also monomers and act also in an ATP independent manner. However, unlike type IA topoisomerases they are independent of Mg^{2+} and can relax positive as well as negative DNA supercoils. This group includes topo I of yeast and mammals.

Type IIA DNA topoisomerases catalyse double-strand breaks in DNA by transiently cleaving the two strands in one DNA duplex to form an enzyme-operated DNA gate, through which a second DNA helix can pass. Thus, the linkage number is changed in steps of two. The reaction of type IIA DNA topoisomerases is ATP dependent and requires the presence of

divalent cations, preferentially Mg^{2+}. The type IIA family includes *E. coli* DNA gyrase, yeast topo II and mammalian topos IIα and IIβ. Type IIB DNA topoisomerases include DNA topo VI from Archea and are ATP and Mg^{2+} dependent. This class of enzymes catalyses the relaxation of positively and negatively supercoiled DNA and possesses a potent DNA decatenase activity. The division of type II into type IIA and IIB is based on a lack in sequence and structure similarities. Common for the reactions of all types of DNA-topoisomerases is the formation of transient, covalent phosphor tyrosyl-linkages between DNA and enzyme, thereby preserving the energy from the broken phosphodiester bridge.

Type	Tyrosyl linkage	Enzymes	Catalytical activity	ATP	Mg^{2+}
IA	5'phosphate	Bacterial DNA topo I Bacterial DNA topo III DNA topo III Reverse gyrase	Weak relaxation of negative supercoils. Preference for single stranded DNA.	No	Yes
IB	3'phosphate	DNA topo I DNA topo V	Relaxation of posistive and negative supercoils. Decatenation of nicked Substrate.	No	No
IIA	5'phosphate	Bacterial DNA gyrase DNA topo II T4 DNA topo II	Relaxation of negative and positive supercoils. Catenation/decatenation And knotting/unknotting of double-stranded DNA.	Yes	Yes
IIB	5'phosphate	Archeal DNA topo VI	Generation of double strand breakage during meiosis.	Yes	Yes

Tabel 1.1: **Types of DNA Topoisomerases and Representative Examples.** ATP: requirement for ATP. Mg^{2+}: requirement for Mg^{2+}. (Bergerat et al., 1997; Bergerat et al., 1994; Confalonieri et al., 1993; Giaever et al., 1986; Goto and Wang, 1984; Goto and Wang, 1985; Hanai et al., 1996; Huang et al., 1988; Jenkins et al., 1992; Kato et al., 1990; Slesarev et al., 1993; Tsai-Pflugfelder et al., 1988; Tse-

This thesis is dealing with the two most prominent topoisomerase classes expressed in mammalian cells: topo I belonging to type IB, and topo IIα and IIβ belonging to type IIA. These enzymes will subsequently be introduced in more detail, whereas the other types (IA and IIB) will not be discussed further. Although this thesis is not about the catalytic mechanism of DNA-topoisomerases, I feel that it is necessary to introduce these complex enzymatic reactions since they are essential for an understanding of what these enzymes actually are doing in the cell. Therefore, the next chapters will summarise what is known about the domain organisation, crystal structure, and catalytic cycle of the two types of topoisomerases under investigation here.

1.2 Type IB DNA Topoisomerases

Eukaryotic topo IB are monomeric enzymes identified in a variety of eukaryotic species from yeast to human (Wang, 1996). In addition to the nuclear forms, variants of this enzyme have been identified in mitochondria (Brun et al., 1981; Castora and Lazarus, 1984), in chloroplasts (Fukata et al., 1991; Mukherjee et al., 1994) and in a number of poxviruses (Krogh et al., 1999; Shuman and Moss, 1987). The molecular weight of eukaryotic topo I typically ranges in size from 90 to 135 kDa. Contrary to type II enzymes, topo IB enzymes do not require high-energy cofactors in order to relax supercoiled DNA. Divalent cations stimulate these enzymes, but are not essential (Attardi et al., 1981; Riou et al., 1986; Rowe et al., 1981; Svejstrup et al., 1991). Whereas the domain and crystal structure of topo II is better described for the yeast enzyme, topo I from human is the type IB enzyme studied best. Accordingly, the following section will first introduce human topo I as a prototypic IB enzyme and then discuss its domain organization, protein structure and the catalytical cycle as an example for type IB topoisomerases in general.

1.2.1 Characteristics of Human DNA Topoisomerase I

Human topo I is encoded by a single copy gene located in chromosome region 20q12-13.2 (Juan et al., 1988). The cDNA encodes a protein with a size of 91 kDa, which is present in the nucleus at approximately 10^5 to 10^6 copies (Meyer et al., 1997). Topo I is a long-lived protein with a half-life time of 10 - 16 hours in mammalian cells (Desai et al., 1997), and it is expressed at a constant level throughout the cell cycle (Heck et al., 1988).

The catalytic activity of cellular topo I is partly regulated by post-translational modifications. It is known that topo I is poly(ADP-ribosylated) by poly(ADP-ribose)polymerase (PARP) resulting in a 3-5 fold decrease in its activity (Ferro et al., 1983; Ferro and Olivera, 1984; Jongstra-Bilen et al., 1983; Kasid et al., 1989). However, PARP has also a stimulatory effect on topo I. In the absence of NAD+ a direct binding of PARP to topo I coincides with an activation of the enzyme (Bauer et al., 2000; Bauer et al., 2001). The biological role of this interaction remains elusive. As PARP plays a role in DNA repair and requires nicks or free DNA ends in order to be activated (Jeggo, 1998), it has been suggested that it may be involved in repairing topo I trapped on DNA (Sastry and Ross, 1998). In keeping with this, cells deficient in PARP are hypersensitive to camptothecin - an agent stabilizing topo I covalently bound to DNA (see below)(Chatterjee et al., 1989).

A second post-translational modification of topo I is phosphorylation. The enzyme is found to be a phosphoprotein *in vivo* and a substrate for PKC and PK CK2 phosphorylation *in vitro* (Durban et al., 1985; Pommier et al., 1990). Phosphorylation stimulates the enzyme's relaxation activity and dephosphorylation almost abolishes enzymatic activity (Durban et al., 1983; Kaiserman et al., 1988). More recently, a cell cycle-specific and transcription-related phosphorylation of mammalian topo I was observed, which, however, did not to affect

catalytic activity (D'Arpa and Liu, 1995). The physiological relevance of this modification is unknown, but it seemed to be coupled to mitosis, which is supported by other investigations showing that exposure of cultured mammalian cells to mitogenic agents yield a similar effect on the phosphorylation state (Samuels et al., 1994). Although the mitosis-coupled phosphorylation of topo I has no effect on the catalytic activity of the enzyme, it is reasonable to suggest that this modification could regulate topo I function in other aspects, such as its interaction with DNA or other proteins.

Indeed it is becoming increasingly evident that topo I is regulated by its interaction with other proteins. Our group has recently shown that the RNA splicing factor PSF/p54nrb interacts with topo I thereby stimulating the relaxation activity of topo I by 16 fold (Straub et al., 2000). Moreover, the cleavage specificity of topo I was found to change markedly by its interaction with SV40 large T antigen during replication (Simmons et al., 1996; Simmons et al., 1998a; Simmons et al., 1998b). p53 has also been shown to interact with topo I, and cell extracts containing p53 have higher relaxation activity than cell extracts from p53-deficient cells (Gobert et al., 1996; Gobert et al., 1999; Mao et al., 2000b). A number of other proteins (histone H1, casein kinase II, non histone HMG, nucleolin, certain transcription factors and the WRN protein) have been shown to physically interact with topo I *in vitro* (Albor et al., 1998; Bharti et al., 1996; Haluska and Rubin, 1998; Kretzschmar et al., 1993; Lebel et al., 1999; Merino et al., 1993; Shaiu et al., 1999; Shykind et al., 1997; Wang and Roeder, 1998).

1.2.2 Domain Organization and Protein Structure of Topoisomerase I

Based on conservation of sequence, limited proteolysis and fragment reconstitution experiments, and crystallographic data, human topo I protein can be divided into four distinct domains (Redinbo et al., 1998; Stewart et al., 1996a; Stewart et al., 1997; Stewart et al., 1996b; Stewart et al., 1998): The enzyme comprises (i) a highly charged N-terminal domain, (ii) a highly conserved core domain, (iii) a linker region, and (iv) a highly conserved C-terminal domain, which contains the active site tyrosine (Fig. 1.2).

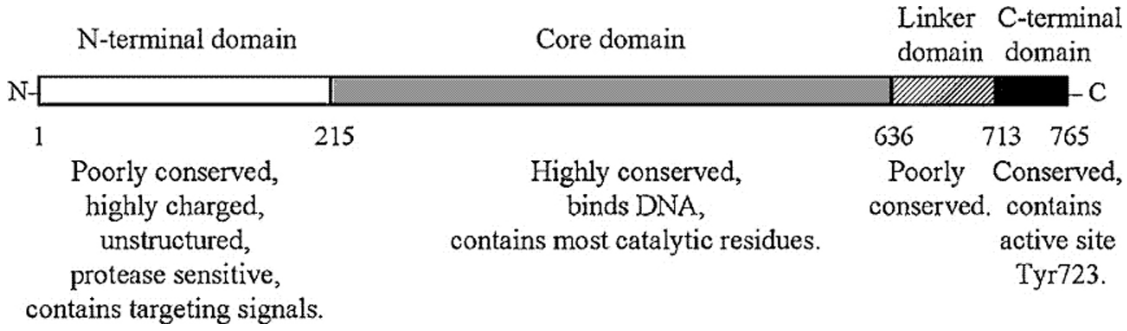

Figure 1.2: **Domain structure of human topoisomerase I.** Human topo I comprises an N-terminal domain (open box), a core domain (grey box), a linker domain (diagonally striped box), and a C-terminal domain (black box). The properties of the four domains are summarized in the lower portion of the figure. Figure reproduced from (Champoux, 2001).

The N-terminal domain (the first 214 amino acids) is dispensable for relaxation activity *in vitro* and it is poorly conserved through evolution (Stewart et al., 1997; Stewart et al., 1996b). Despite the fact it is not required for in vitro activity, it may have essential roles *in vivo*. Indeed, the N-terminal domain contains four NLS (Alsner et al., 1992; Mo et al., 2000a) and in *drosophila* this domain alone is sufficient to target topo I to transcriptionally active loci (Shaiu and Hsieh, 1998). Next to the N-terminal domain, the highly conserved core domain of 421 amino acids follows which contains all catalytic residues except the active site tyrosine (Cheng and Shuman, 1998; Jensen and Svejstrup, 1996; Megonigal et al., 1997; Redinbo et al., 1998). The core domain constitutes the principal DNA binding region. This domain alone exhibits DNA binding properties similar to that of the full-length enzyme. It interacts with high affinity and selectivity to supercoiled DNA (Madden et al., 1995). A protease-sensitive and poorly conserved linker domain comprising 77 amino acids connects the core to the C-terminal domain. The linker region is not required for relaxation activity *in vitro* (Stewart et al., 1997). The C-terminal domain (last 53 amino acids) contains the active site Tyrosine723 (Eng et al., 1989). Interestingly, catalytic topo I-activity can be reconstituted from fragments corresponding approximately to the core domain and the C-terminal domain (Stewart et al., 1997). In agreement with this, a topo I variant of Vaccinia virus consists of a reduced core and the C-terminal domain only (Redinbo et al., 1999a; Shuman and Moss, 1987). Thus, Vaccinia topo I is considered as the minimal functional unit of DNA topo I with respect to relaxation activity.

Recently the crystal structure of several forms of human topo I has been determined in the non-covalently and the covalently DNA-bound state (Redinbo et al., 2000; Redinbo et al., 1999b; Redinbo et al., 1998; Stewart et al., 1998). The entire N-terminal region is missing from the structure, because the X-ray density was not interpretable due to a very high flexibility. The structure of topo I in complex with DNA is shown in Fig. 1.3, including the subdivision of the core domain into core sub domain I, II and III. Human topo I is a bi-lobed protein that wraps completely around the DNA substrate which it buries in a DNA-binding pore. The central pore of the protein is 20 Å in diameter and provides an extensive, highly

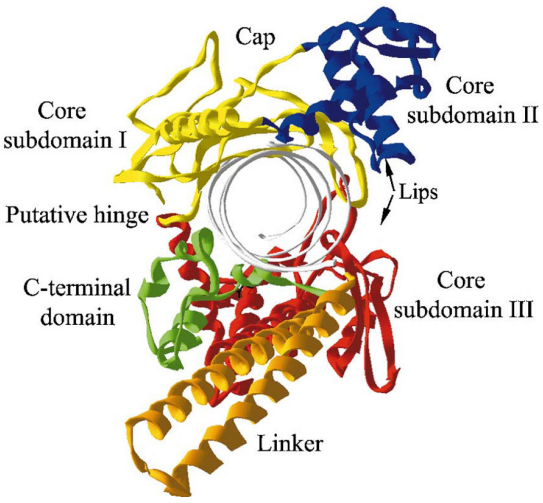

Figure 1.3: **Structure of human topoisomerase I in complex with DNA.** The bi-lobed protein tightly clamps around the DNA. Core subdomain I (residues 215-232 and 320-433), II (residues 233-319) and III (residues 434-633) are coloured yellow, blue and red, respectively. The linker (residues 641-712) and C-terminal domain (residues 713-765) are shown in orange and green, respectively. Core subdomains I and II forms the CAP lobe. Core subdomain II and C-terminal domain forms the base lobe. The lip regions where the protein opens during DNA binding and release, are marked by arrows, and the hinge region is labelled 'putative hinge'. Figure adapted from (Champoux, 2001)

positively charged region for binding of a DNA helix (Redinbo et al., 1998). The bi-lobed protein tightly clamps around the DNA with contacts between the protein and the DNA phosphate backbone extending over 14 base pairs. One of the lobes comprises core sub-domains I and II and forms what has been referred to as the "cap" of the protein (Redinbo et al., 1998). The other lobe forms a "base" that cradles the DNA and consists of core sub-domain II and the C-terminal domain. The two lobes are connected by a so-called hinge region believed to be involved in opening and closing of the protein clamp during DNA binding and release. Opposite to the hinge region are the cap lobe and the base lobe in close proximity, interacting via six amino acids and one salt bridge to connect the lobes, thereby forming the so-called "lips", which enclose the DNA helix. Opening and closing of the protein clamp during DNA binding or release must involve the breaking of this interaction between the lips. The dispensable linker domain protrudes from the base of the protein, and has been suggested to be involved in controlling the rotation of the DNA by interacting with DNA downstream to the cleavage site (Ireton et al., 2000; Redinbo et al., 1998; Stewart et al., 1999). Thus, topo I must be highly flexible to perform its catalytic function. Indeed non-isomorphous crystal structures have shown that there is a high structural elasticity in human topo I (Redinbo et al., 1999b). Especially the cap domain shows a large degree of conformational flexibility consistent with a separation of the lips and a rotation around the hinge region. Interestingly, the linker region showed the greatest degree of flexibility, consistent with the idea of controlled strand rotation as discussed in the next section.

1.2.3 Catalytic Cycle of Human Topoisomerase I

Extensive biochemical studies and the above described crystal structure have led to a model for the catalytic mechanism for topo I. According to this 'controlled rotation' model (Fig. 1.4) DNA relaxation catalysed by topo I can be divided into a number of consecutive steps. These are DNA binding (A to B), cleavage and covalent attachment of the enzyme to the scissile strand (B to C), strand rotation (D), religation (E to F) and DNA release from the enzyme (F to G).

Topoisomerization begins with the binding of topo I to the DNA duplex substrate (Fig. 1.4.A). The closed clamp structure of topo I in complex with DNA (Fig. 1.3) suggests that the enzyme must exist in an open conformation before DNA binding in order to allow entry of DNA into the central cavity of the enzyme (Fig. 1.4. A-B). This conformation is most likely achieved by a bending motion in the hinge region (Redinbo et al., 1999b; Redinbo et al., 1998). In support of this hypothesis, the hinge region has been observed to be sensitive to proteolysis in the absence of DNA and resistant to it upon binding of DNA (Stewart et al., 1996a). The binding event is directed in large part by the surface and charge complementarities of the enzyme cavity and the DNA, and culminates in the complete embrace of the DNA in a noncovalent complex (Fig. 1.4. B). The substrate specificity of eukaryotic topo I has been characterized at nucleotide sequence level and at the level of DNA tertiary structure. The preferred nucleotides in the scissile strand are 5'-(A/T)(G/C)(A/T)T-3'

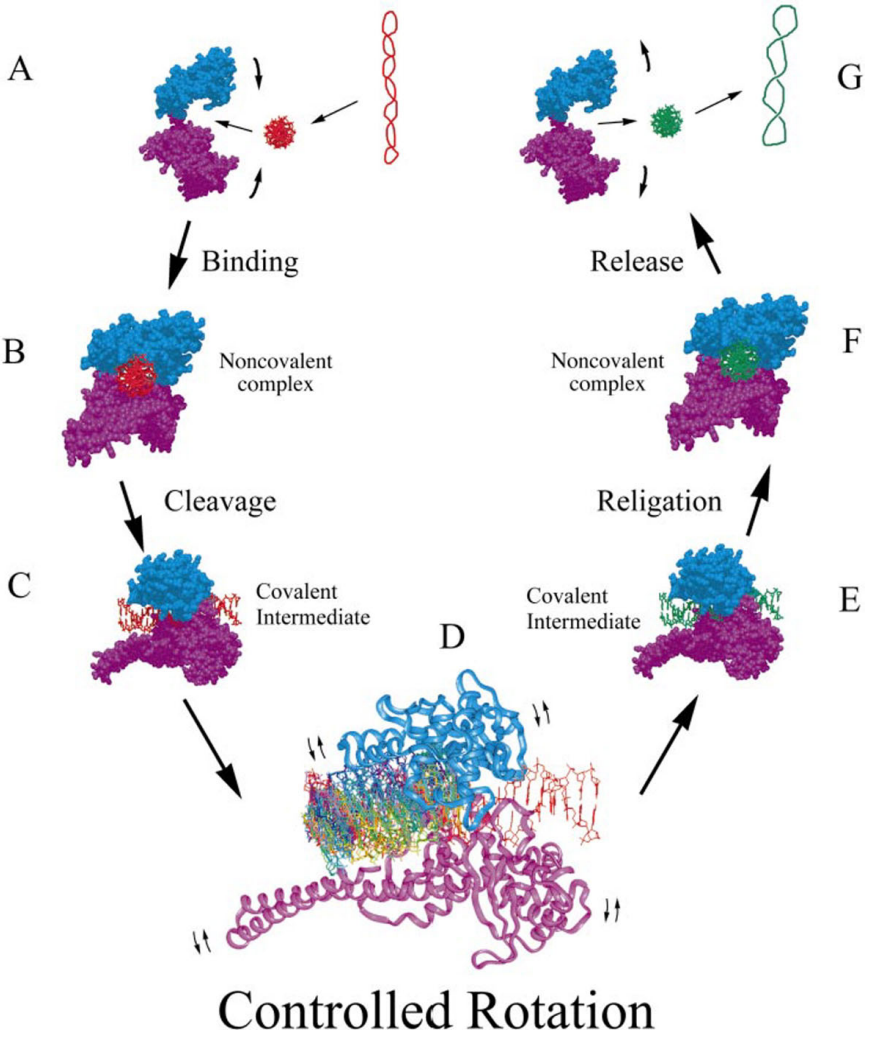

Figure 1.4: **The controled rotation mechanism of human topoisomerase I.** The cap and base lobes are coloured blue and magenta, respectively. The supercoiled substate DNA (red) becomes partially relaxed through steps (A) through (G) and is converted to the less supercoiled state depicted in green. (A-B) Binding of DNA. (B-C) Cleavage of scissile strand. (D) Strand rotation. (E-F) Religation of DNA. (F-G) Release of substrate DNA. Figure adapted from (Stewart et al., 1998)

with the enzyme covalently attached to the –1T residue (Been et al., 1984; Bonven et al., 1985; Tanizawa et al., 1993). A particular strong cleavage site for all eukaryotic topo I species was identified in the rDNA repeats of *Tetrahymena pyriformis* (Andersen et al., 1985). At the level of tertiary DNA-structure, a number of studies have shown that topo I prefers supercoiled over relaxed DNA as substrate (Camilloni et al., 1989; Muller, 1985), a property conferred by the core domain (Madden et al., 1995). Electron microscopy studies showed that topo I binds to supercoiled DNA at helix crossovers (Zechiedrich and Osheroff, 1990). Since the number of DNA crossovers increase with the degree of supercoiling, this result may explain the selectivity of topo I for supercoiled DNA.

As a result of topo I embracing a piece of DNA, the active site residues are brought into position for attack on the scissile phosphate, leading to cleavage of the scissile strand and to covalent attachment of the enzyme to the 3' end of the cleaved DNA (Fig 1.4.C). Topo I mediated cleavage proceeds by an in-line displacement reaction according to a classical S_N2 pathway: Topo I directs a nucleophilic attack of the active site tyrosine residue to the phosphorous of the phosphodiester linkage thereby mediating a transesterification reaction (Eng et al., 1989; Lynn et al., 1989; Lynn and Wang, 1989; Shuman et al., 1989). A number

of residues beside the active site tyrosine are required for the catalysis of transesterification ((Cheng and Shuman, 1998; Jensen and Svejstrup, 1996; Levin et al., 1993; Megonigal et al., 1997; Redinbo et al., 2000; Redinbo et al., 1998) and reviewed in (Champoux, 2001; Sherratt and Wigley, 1998)).

After the cleavage reaction, topo I may proceed right away to religation. Alternatively it performs a number of topoisomerization steps before resealing the broken strand (Fig. 1.4. D). Since ATP is not required, relaxation by human topo I is believed to be driven by topological tension in the DNA (Champoux, 1981). Previous studies have suggested that changes in linkage number (relaxation) mediated by topo I could precede either through strand passage or free rotation (reviewed in (Darby et al., 1985)). However, more recent biochemical and structural data suggest that topo I changes the linkage number of DNA by a mechanism of controlled relaxation (Stewart et al., 1998). In the controlled rotation model, the release of superhelical tension can occur through one or more cycles of controlled rotation (Fig 1.4.D). Stewart and colleagues suggested that the rotation of DNA is controlled by interaction of the enzyme with DNA downstream of the cleavage site. Indeed, filter binding assays showed that the N-terminal region of topo I binds to DNA, although with a significantly lower affinity than full-length topo I (Lisby et al., 2001). *In vitro*, religation experiments have shown that both the linker and the N-terminal domains are interacting with downstream DNA (Ireton et al., 2000; Lisby et al., 2001; Stewart et al., 1999). Taking all these findings together, it appears that the rotation of the DNA is controlled by interactions with DNA downstream to the cleavage site

Subsequent to strand rotation, the covalent intermediate (Fig. 1.4.E) is religated with concomitant release of the Tyr^{723} from the end of the DNA (Fig. 1.4.F). Finally, a DNA molecule with reduced superhelicity is released (Fig 1.4.G), allowing the enzyme to undergo another cycle of DNA binding and relaxation.

1.2.4 Camptothecin- a Drug Targeting Human Topoisomerase I

Topo I is the specific cellular target of a family of chemical compounds derived from the natural alkaloid camptothecin (CpT) that was originally identified in extracts of the Chinese tree *Camptotheca acuminata*, to which ancient Chinese drug lore has assigned anti-cancer activity for a long time. Biochemical studies showed that CpT inhibits topo I mediated relaxation by inhibiting specifically the religation half-reaction thereby inducing accumulation of covalent cleavage complexes (Svejstrup et al., 1991). The cytotoxic effect of CpT seems to be specifically linked to inhibition of topo I, since a point mutation of the enzyme can confer drug resistance in a mammalian cell line (Tamura et al., 1991). It has been proposed that topo I-mediated single stranded DNA nicks created by CpT are reversible unless they are processed into permanent double-strand breaks *in vivo* by at least two different mechanisms. The traditional mechanism involves collision of a DNA replication fork with the covalently trapped topo I thereby converting the transient nick into a double-strand break (Hsiang et al., 1989). It is now evident that CpT also has the capacity to induce DNA breaks in an S-phase

independent manner (Voigt et al., 1998): The authors suggest that these breaks could be generated by the transcription machinery or by several topoisomerases cleaving in close proximity of each other. In mammalian cells, the generation of significant amounts of double-stranded DNA breaks ultimately results in apoptosis or necrosis (Ryan et al., 1991; Solary et al., 1994). The high activity of topo I in rapidly dividing cells of most cancers results in a preferential killing of malignant cells (D'Arpa and Liu, 1989; Waldman et al., 1996).

Cellular resistance to CpT and other cytostatic drugs has turned out to be a significant complication in cancer treatment (Dingemans et al., 1998). Beside point mutations in topo I that confer resistance to CpT at the molecular level, a number of conditions have been identified that cause resistance to CpT on a higher level of the cell. In some cases, the resistance is found to correlate with reduced expression of the TOP1 gene. This was explained by reduced gene transcription, due to hyper-methylation at the gene locus (Fujimori et al., 1996). CpT resistance has also been observed in human cancer cells overexpressing the P-glycoprotein transporter (*MDR*), which presumably prevents CpT from accumulating intracellularly by actively exporting the drug out of the cell in an energy-dependent manner (Germann et al., 1993; Gottesman, 1993).

Treatment of cells with CpT results in a down regulation of topo I protein levels. This down regulation is believed to be mainly due to increased degradation bringing down half-life time to approximately 1-2 hours. CpT-induced degradation of topo I has been suggested to be mediated by multiple ubiquitination and subsequent degradation by the 26S proteosome pathway (Desai et al., 1997; Fu et al., 1999). However, the high molecular weight ladder of topo I immunoreactivity observed by Fu and colleagues did not correspond well with the 8.6 kDa adducts expected from ubiquitination which suggested that another pathway could be involved in topo I degradation. Accordingly, it was later reported that the topo I conjugated protein found in CpT treated cells is not ubiquitin but rather an ubiquitin related protein, SUMO-1/Smt3p (Mao et al., 2000a). CpT and similar drugs cause a relocation of topo I from the nucleoli to the nucleoplasm (Danks et al., 1996), and it has recently been suggested that sumoylation of topo I also plays a major role in this process (Mo et al., 2002).

Recently a specific repair enzyme has been discovered in *S. cerevisiae,* which has homologues in *C.elegans*, human, mouse and *D.melanogaster*. This enzyme, termed tyrosyl-DNA phosphodiesterase (Tdp1), may have important implications for the cellular effects of CpT (Yang et al., 1996). Tdp1 catalyses the hydrolysis of phosphotyrosyl linkage between tyrosine and 3'-ends of DNA thereby, releasing topo I from the DNA (Pouliot et al., 2001; Pouliot et al., 1999). Tdp1 is poorly active in releasing full-length topo I covalently attached to DNA, but is very efficient in releasing a small fragment of topo I left after proteolytic processing of the topo I•DNA complex (Debethune et al., 2002). This suggests that Tdp1 acts after topo I has been proteolyzed *in vivo*. The ability of Tdp1 to release topo I stalled on DNA suggests that Tdp1 may be involved in removing CpT induced topo I complexes, thus making the cells less sensitive to CpT. Indeed, survival of yeast after induction of covalent topo I DNA complexes is substantially reduced by inactivation of the *TDP1* gene (Pouliot et al., 2001). Moreover, unpublished results of our own group suggest that overexpression of Tdp1 renders human cells CpT-resistant. Thus, the development of a drug inhibiting Tdp1 is of

clinical interest, since it could increase cellular sensitivity to CpT, thereby acting as a sensitizer for CpT-analogues such as topotecan or irinotecan currently used in clinical cancer therapy.

1.3 Type IIA DNA Topoisomerases

So far, type IIA topoisomerases have been identified in a wide variety of eukaryotic organisms including *S.pombe* (Shiozaki and Yanagida, 1991; Uemura et al., 1986), *S. cerevisiae* (Giaever et al., 1986; Goto et al., 1984; Goto and Wang, 1984), Drosophila (Wyckoff et al., 1989) and mammals such as mouse (Adachi et al., 1992), rat (Park et al., 1993) and human (Jenkins et al., 1992; Tsai-Pflugfelder et al., 1988). In bacteria, several type II topoisomerases have been found, including DNA gyrase and topo IV (Gellert et al., 1979; Kato et al., 1990).

Since its discovery, eukaryotic topo II has attracted much attention because it became evident from early on that it is an essential enzyme. The discovery that human type II topoisomerases are the primary target of many anticancer agents already applied in the clinic has further increased the interest in the enzyme. Eventually, this has led to a detailed description of topo II on the level of DNA-sequence and protein structure. In addition to its enzymatic function, topo II is considered to play a structural role in the chromosomal scaffold and the interphase nuclear matrix. Throughout the introduction, I have put a certain emphasis on the presentation of data supporting this hypothesis (Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986; Strick et al., 2001), because recently, we have shown that such an architectural role for topo II is unlikely (Christensen et al., 2002b) and these findings are a major component of this thesis.

In this chapter the two human isoforms of topo II will be introduced, followed by a general characterization of the domain organization, protein structure and the catalytic cycle of type IIA topoisomerases, comprising of data from type II enzymes from *E.coli,* yeast, and human.

1.3.1 Characteristics of Human Topoisomerases IIα and IIβ

Whereas lower eukaryotes only encode one form of topo II, mammalian cells express two related but genetically distinct isoforms of topo II, designated IIα and IIβ (Chung et al., 1989; Jenkins et al., 1992; Tsai-Pflugfelder et al., 1988). The cDNAs for topo IIα and IIβ encode proteins with a molecular weight of 170 and 180 kDa, respectively. The human topo IIα gene has been mapped to chromosome 17q21-22 (Tan et al., 1992; Tsai-Pflugfelder et al., 1988) whereas the gene encoding the IIβ isoform is located on chromosome 3p24 (Jenkins et al., 1992; Tan et al., 1992). A small fraction of topo IIβ RNA has been reported to undergo differential splicing in various human cell lines generating a minor protein by-product named IIβ-2 (Davies et al., 1993), which has an insertion of additional 5 amino acids at its N-

terminus. No specific role could be assigned to the IIβ-2 splice variant so far, and data about its biochemical properties are lacking.

The two topo II isoforms in higher eukaryotes are believed to have arisen from a gene-duplication event relatively recent in evolutionary time (Coutts et al., 1993; Lang et al., 1998; Sng et al., 1999). Gene structure analysis has shown that the topo IIα (TOP2A) and IIβ (TOP2B) genes contain 35 and 36 exons, respectively (Lang et al., 1998; Sng et al., 1999). An alignment to the amino acid-sequence demonstrates that the intron/exon boundaries of human TOP2A and TOP2B are highly conserved between the two genes. Interestingly, the only cases where the introns are not conserved lie within regions that encode the more divergent NH_2- and COOH-terminal regions of the proteins. The high degree of conservation of the intron position in TOP2A and TOP2B supports the view that the two genes are derived from the duplication of a single genetic ancestor.

Human topo IIα and IIβ are close homologues with a sequence identity of 68% (Austin et al., 1993; Jenkins et al., 1992; Lang et al., 1998; Sng et al., 1999). Besides having a high sequence identity, both isoforms can complement the essential functions of yeast topo II in a temperature-sensitive *Saccharomyces cerevisiae* topo II-deletion mutant (Jensen et al., 1996b). In addition to human topo IIα and IIβ, several type II topoisomerases from other organisms are also able to complement a yeast topo II enzyme, including topo II from *Drosophila* (Wyckoff et al., 1989) and mouse (Adachi et al., 1994; Adachi et al., 1992). The fact that topo II from a wide variety of organisms is able to complement the yeast counterpart demonstrates an exceptional functional conservation of these enzymes. Indeed, the sequences of topo II from various organisms show a high degree of homology. However, although both, topo IIα and IIβ are able to complement the yeast topo II enzyme and can be considered as functional equivalents in this system, this might not be the case in their natural environment in higher eukaryotic cells, because isoform-specific functions could be missing or dispensable in the yeast system.

On the other hand, the fact that both human isoforms are able to complement a defective topo II in yeast proves that the two isoforms must possess the same basic enzymatic activities, although an early report showed that a topo II-specific inhibitor preferentially targeted the IIα isoform and that the two isoforms were biochemically distinguishable (Drake et al., 1989). Based on more recent *in vitro* studies using purified enzymes, it was, however, concluded that the enzymatic properties of the two isoforms are essentially indistinguishable (Austin et al., 1995; Wang, 1996). This view may however again be changing since a new topo II poison has been recently presented which only affected the IIβ isoform (Barthelmes et al., 2001). Based on the available data however, it is still reasonable to conclude that potential isoform specific functions of the two human isoforms must arise from other sources than their enzymatic properties. Different expression patterns, cellular localization and protein interaction partners have been suggested to mediate isoform specific functions.

In addition to differences in their gene sequence and structure, the expression levels of human topo IIα and IIβ differ as cells traverse through the cell cycle. These differences are mediated by different transcription rates and changes in mRNA and protein stability (reviewed in (Isaacs et al., 1998). Topo IIα undergoes significant cell-cycle dependent

alterations in both its amount and stability. The protein level starts to increase at the onset of replication, accumulates during S and G_2 phase, peaks in late G_2/M phase, which is then followed by a rapid decline after exit from mitosis (Goswami et al., 1996; Heck et al., 1988). Topo IIβ, on the other hand, has been reported to be present at a constant level throughout the cell cycle (Drake et al., 1989; Kimura et al., 1994b; Prosperi et al., 1996; Prosperi et al., 1992; Woessner et al., 1991). In addition, the expression of topo IIα in tissues of human, mouse, and rat is highest in proliferating compartments, such as thymus, spleen and bone marrow, whilst in non-proliferating tissue types the expression of topo IIα is barely detectable. The level of topo IIβ mRNA does not correlate with proliferation state but rather remains constant in all types of tissue (Boege et al., 1995; Capranico et al., 1992; Isaacs et al., 1996; Turley et al., 1997; Zandvliet et al., 1996). This suggests that the α-isoform is more involved in proliferation, whereas topo IIβ can be considered more as a housekeeping gene likely involved in processes like transcription or DNA-repair. The fact that expression levels of topo IIα are apparently higher in a variety of tumours in comparison with adjacent normal tissue (Hasegawa et al., 1993; Kim et al., 1991) further supports the view that topo IIα is linked to the proliferation state of the cell.

Even though the characteristic expression pattern of the two isoforms suggests isoforms-specific functions, direct evidence to whether topo IIα and IIβ fulfil different cellular roles is still lacking. This is partly due to the lack of mammalian cell mutants defective for either topo IIα or IIβ. This problem has been partly overcome by Wang and co-workers, who have constructed gene targeting vectors to knock out topo IIβ in mouse (Yang et al., 2000). They found that topo IIβ in mouse is essential for viability since homozygous TOP2β$^-$ embryos are stillborn. Interestingly, the embryos survive almost to term, indicating that most steps of embryogenesis can occur without the β-isoform. It was also possible to isolate mouse embryo fibroblasts from the TOP2β$^-$ embryos and propagate them in culture, indicating that topo IIβ is not required for the viability of all cells, but rather plays an essential function in some cell types. Since the enzyme constantly exists in cell types independent of proliferation, topo IIβ may play an essential function in differentiated cells, where topo IIα is not expressed. The high level of topo IIα in proliferating cells further suggests that knockouts of topo IIα would be lethal early in embryogenesis unless topo IIβ is able to substitute its functions. Further support of this view comes from the investigation of cells with an aberrant cytosolic expression of topo IIα, which causes aneuploidity and incomplete chromosome condensation (Grue et al., 1998). The reported differences between topo IIα and IIβ have led to the model of a division of labour. Whereas topo IIα is believed to be involved in cell division specific events, such as DNA synthesis, condensation and segregation, the topo IIβ isoform might maintain a housekeeping function possibly involved in DNA transcription (Capranico et al., 1992; Prosperi et al., 1992).

The investigation of isoform specific functions is further complicated by data suggesting that a heterodimeric form of mammalian topo II is formed in addition to the two homodimeric forms of the enzyme (Biersack et al., 1996; Gromova et al., 1998). Thus, cells may contain three distinct subclasses of topo II: α/α, β/β homodimers and α/β heterodimers. However, data about biochemical activity or cellular localization of topo II heterodimers are

lacking, and a more detailed investigation is required to assign a specific biological role to these otherwise possibly aberrant forms of the enzyme. Their existence, however, merits consideration whenever topo II *in vivo* functions and cell-cycle regulation are investigated.

1.3.2 Domain Organization and Protein Structure of Topoisomerase II

Limited protease-digestion of various topo II polypeptides suggests that the enzyme can be divided into three subdomains (Austin et al., 1995; Lee and Hsieh, 1994; Lindsley and Wang, 1991; Reece and Maxwell, 1989; Shiozaki and Yanagida, 1991). As shown in Fig. 1.5, *S.cerevisiae* topo II is cleaved by SV8 endoprotease at three sites, designated V8-A (residue 410), V8-B (residue 660) and V8-C (≈ residue 1164). V8-A and V8-C are the predominant cleavage sites whereas V8-B is only a minor site cleaved less efficient (Lindsley and Wang, 1991). Sites in a globular protein available to limited protease digestion must be accessible to the protease. The protease pattern therefore indicates that yeast topo II can be divided into three separate and spatial distinct domains that are termed: ATPase domain (amino acid 1-410), DNA binding/Cleavage domain (amino acid 410-1164) and C-terminal domain (amino acid 1164-1428).

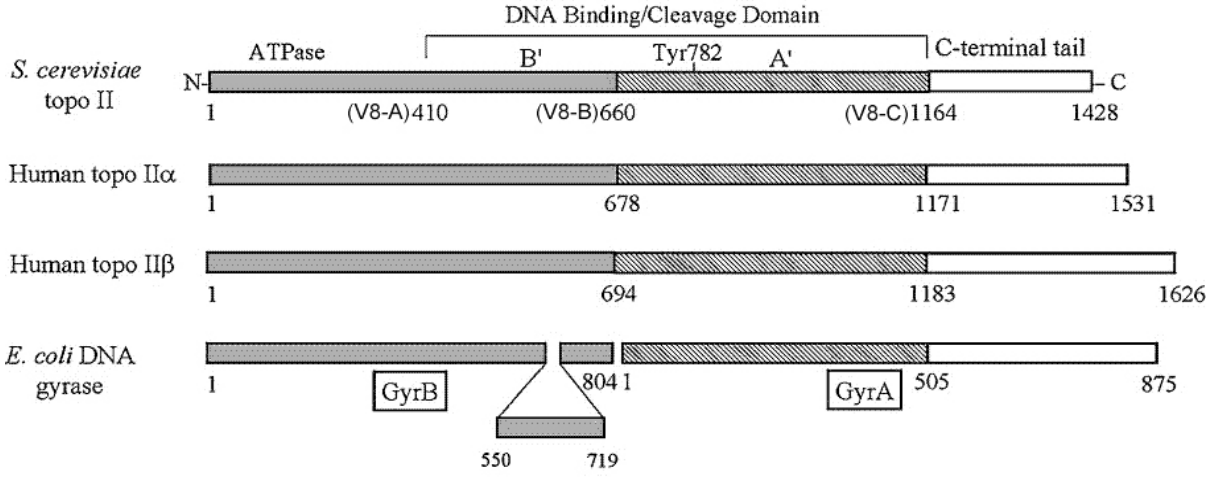

Figure 1.5: **Sequence comparison among type IIA topoisomerases.** The three-domain structure of the type IIA family is shown based on amino acid sequence homologies with the *E.Coli* DNA gyrase (Caron and Wang, 1994). Regions homologous to the Gyr B subunit (excluding the insertion 550-719) are shown with sequence coordinates and a grey shaded box. Regions homologous to the first 505 amino acids of GyrA are depicted by a box with diagonal striping. V8 protease sensitive sites in *S.Cerevisiae* topo II are shown below its respective panel.

The three-domain structure of eukaryotic topo II is further supported by comparison with bacterial type II topoisomerases. DNA gyrase, the *E.coli* counterpart of eukaryotic topo II, is structurally different from its eukaryotic counterparts. DNA gyrase is the product of two separate genes gyrB and gyrA and the enzyme is a heterotetramer of an A_2B_2 configuration, whereas eukaryotic topo II has a dimeric AB configuration. (Austin et al., 1993; Horowitz and

Wang, 1987). The three-domain structure is also in agreement with sequence comparison of various eukaryotic type II topoisomerases with DNA gyrase (see Fig. 1.5 for details): an N-terminal region homologous to the B subunit of DNA gyrase (GyrB), a central domain homologous to the GyrA subunit and a C-terminal region very divergent in nature with no equivalent in bacterial DNA gyrase (Austin et al., 1993; Caron et al., 1994; Jenkins et al., 1992; Lynn et al., 1986; Wyckoff et al., 1989).

The three domains described by proteolysis and sequence comparison can be assigned to different functions. The ATPase region is involved in ATP binding and hydrolysis. The central core-domain is responsible for DNA binding and cleavage, whereas the divergent C-terminal domain is dispensable for *in vitro* catalytic activity (Crenshaw and Hsieh, 1993; Jensen et al., 1996a). Of these three domains, only the divergent C-terminal domain has not yet been crystallized and resolved to high resolution. The structure of the central domain is now known for both DNA gyrase and yeast topo II, whereas the structure of the N-terminal domain has only been solved for DNA gyrase.

The high-resolution structural studies on topoisomerases over the past years have highly elevated our knowledge about the mechanism of topo II catalysis and drug interaction (reviewed in (Berger, 1998; Champoux, 2001)). The crystal structure of a 92 kDa yeast topo II fragment - spanning amino acids 410-1202 between V8 cleavage sites A and C - has been solved to 2.7 Å resolution (see Fig. 1.6) (Berger et al., 1996). The 92 kDa fragment contains the central DNA binding and DNA-breakage/reunion domain (see Fig. 1.5) but lacks the ATPase domain (residues 1-409) and the C-terminal domain. It can cleave DNA but cannot execute strand transfer, since it lacks the essential ATPase domain. The 92 kDa polypeptide fragment folds into two subfragments, denoted B' (residues 420-630) and A' (residues 682-1178) which are connected by a disordered, 50 amino acid linker. Interestingly, the V8-B protease cleavage site lies in this linker region. The two enzyme subunits form a pair $(A'B')_2$

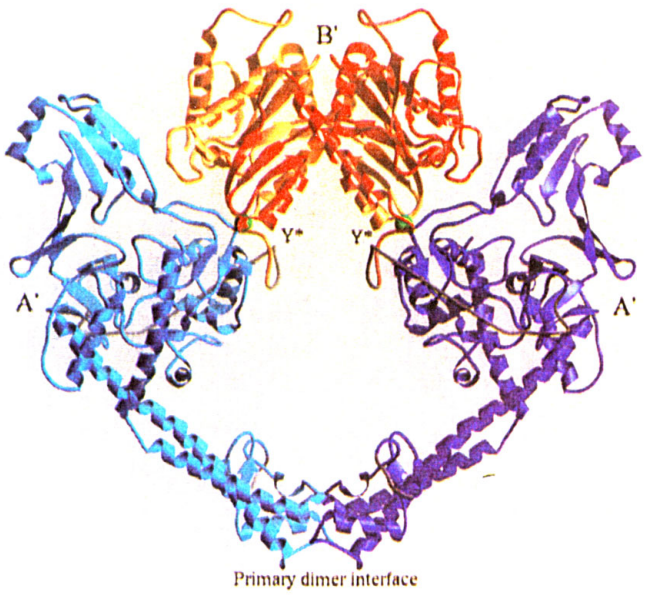

Figure 1.6: **Structure of the 92 kDa fragment of *S.Cerevisiae* topo II.** A ribbon representaion of the crystallized dimeric structure of the 92 kDa yeast topo II fragment showing an enzyme clamp with two sets of jaws and a large central hole. The two crescent-shaped monomers form a pair to make a heart shaped dimer with a large central hole. The B' fragments (residues 410-680) are coloured red and orange, whereas the A' fragments (residues 680-1202) are shown in purple and blue. The active site tyrosines (Y*) are situated 25 Å apart and are indicated by green spheres. The large hole is 55Å wide at its base, 25Å wide at the top and 60Å deep. Figure adapted from (Berger et al., 1996)

to make a heart shaped dimer of an overall dimension of 120 x 120 x 55 Å with a large central hole nearly 50 Å in diameter (see Fig. 1.6).

Two dimerization regions can be observed in the structure of yeast topo II: One between the two B' fragments and a second between the C-terminal end of the two A' fragments (Berger et al., 1996). The B'-B' contact takes place between two small but conserved hydrophobic patches on each sub fragment. The dimer contact in the B'-B' region buries an area of approximately 750 $Å^2$ surface in each fragment. A biochemical study has supported the existence of a B'-B' dimer contact (Olland and Wang, 1999). The structure further shows that part of the A' domain is involved in formation of the so-called primary dimer interface. The existence of the A'A' dimer interface is supported by a number of genetic and biochemical studies. Three cold-sensitive mutations of topo II have been mapped to the A'-A' dimerization region (Thomas et al., 1991) and appear to either increase the amount of buried hydrophobic surface or to create new salt bridges between monomers thus stabilizing the dimer. A biochemical approach has identified the primary dimerization region in human topo IIα (Bjergbaek et al., 1999) which was homologous to the A'-A' dimer interface identified in the yeast crystal.

A third dimer interface is formed at the ATPase domains. The crystal structure of *E.coli* gyrase together with biochemical data suggests that the ATPase domain forms a dimer in the presence of a non-hydrolysable ATP analogue (AMPPNP) (Ali et al., 1995; Wigley et al., 1991). Interestingly, in the presence of AMPPNP the protease pattern of yeast topo II is changed. The cleavage preference is shifted from site A to site B, which demonstrates that the binding of AMPPNP changes the structure of topo II (Lindsley and Wang, 1991). This phenomenon suggests a conformational change induced by the binding of ATP to the enzyme, which likely leads to the dimerization of the ATPase domain. This process seems to be important for proper strand passage (Bjergbaek et al., 2000)

When the structure of the 92 kDa yeast topo II fragment (Berger et al., 1996) is combined with the structure of the GyrB ATPase domain from *E.coli* DNA gyrase (Wigley et al., 1991), a three-dimensional picture of catalytic active topo II emerges: The $(A'B')_2$ yeast dimer forms a heart shaped structure representing the catalytic core of the enzyme, where the ATPase domain from DNA gyrase can be modelled to extend from the N-terminus of the catalytic core. The three-dimensional model is now generally accepted and further supported by electron microscopy (EM) studies producing the only images of intact type II topoisomerases obtained so far (Benedetti et al., 1997; Schultz et al., 1996): In the absence of ATP, both human and yeast topo II appear as multi-lobed structures in which two medium-sized objects extend from a large globular core. Upon binding of AMPPNP the smaller lobes will close together and dimerize which is consistent with biochemical and crystal data from the Gyrase ATPase domain (Ali et al., 1995; Wigley et al., 1991). These observations agree with the model that the ATPase domain is extending from the B' region, and that the ATPase domain will dimerize as a consequence of ATP binding.

1.3.3 Catalytic Cycle of Eukaryotic Topoisomerase II

The catalytic cycle of topo II has been extensively studied in biochemical assays and, with the aid of the crystal structure, a two-gate model was proposed (Berger et al., 1996; Cabral et al., 1997; Fass et al., 1999; Roca et al., 1996; Roca and Wang, 1994). This model (Fig. 1.7) proposes a concerted and complex process, involving several events including DNA-binding (step 1), DNA cleavage (step 2), ATP binding (step 3), DNA strand passage (step 3-4), DNA religation (step 4), ATP hydrolysis and enzyme turnover (step 5).

The catalytic cycle is initiated by binding of the DNA segment to be cleaved, the so-called G-segment (the DNA segment to be cleaved and opened to form a gate) (step 1 in Fig. 1.7). The G-segment is bound to the central DNA breakage/reunion domain. The N-terminal parts of the two A' domains create a semicircular groove of 20-25Å which funnels down to a tunnel leading to the active site of DNA cleavage. Electrostatic calculations show that the semicircular groove formed by the two A' regions has a strong positive electrostatic potential, which might attract the G-segment. Binding of the G-segment to the A'A' groove is also found in footprinting, crosslinking and DNase protection assays (Hung et al., 1996; Lee et al., 1989; Li and Wang, 1997; Thomsen et al., 1990). EM studies of the GyrA-DNA complex came to a similar conclusion (Kirchhausen et al., 1985). In addition, the active site tyrosines of topo II reside in this A'A' grove (Berger et al., 1996). In summary, these data imply that this grove is responsible for the recognition of the DNA substrate.

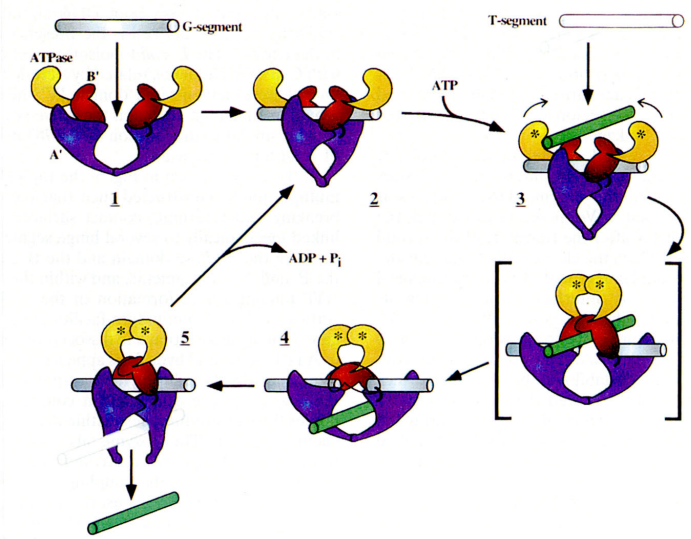

Figure 1.7: **The Current Model for the Catalytic Reaction of Eukaryotic Topoisomerase II.** The ATPase domain, B' and A' subfragments are colored yellow, red and blue, respectively. The G-segment DNA (containing the DNA gate) is grey, and the transported T-segment is an open cylinder or a green cylinder. (1) Binding of the G-segment. (2) Cleavage of the G-segment. (3) Binding of ATP (represented by asterisks) and capture of the T-segment and opening of the G-segment. (4) Storage of the T-segment in the central cavity. (5) Religation of the G-segment and release of the T- and G-segment, ATP is hydrolysed thereby regenerating the starting state. For clarity, the DNA transport step is shown to proceed through a hypothetical intermediate (brackets). Presentation reproduced from (Berger et al., 1996).

The recognition of DNA is mainly determined by the topological structure of the DNA, since topo II does not have any preferred cleavage sequence (Osheroff et al., 1991; Sander et al., 1987). The lack of choice for a specific DNA sequence may have an important physiological role, since it permits the enzyme to act all over the genome. The recognition of topological forms of the DNA is exemplified by the preferential binding of the enzyme to supercoiled over relaxed DNA (Osheroff, 1986; Osheroff et al., 1983) and the enzyme's interaction with curved DNA and DNA crossovers (Bechert et al., 1994; Zechiedrich and Osheroff, 1990). The preferential recognition of certain topological states of DNA is important for the function of topo II, allowing the enzyme to discriminate between the substrates and products of its catalytic reaction.

Following recognition and binding of a G-segment, the enzyme undergoes a range of conformational changes. These changes include a concerted movement of the A' and B' domains which brings the two A' domains into close proximity and the active site tyrosines into an attacking position for DNA-cleavage (step 2 in Fig. 1.7). This structural arrangement of topo II at the beginning of the catalytic cycle is termed T2C. Cleavage of the G-segment occurs in the T2C conformation (step 2 in Fig. 1.7) (Fass et al., 1999). The cleavage is mediated by a coordinated nucleophilic attack of the DNA backbone by the active site tyrosines in the two subunits of the dimeric enzyme (Zechiedrich et al., 1989). The attack by the two tyrosines generates a double-stranded break 4 base pairs apart in the G-segment, and the enzyme is covalently linked to the 5'-end of the cleaved substrate through its active site tyrosines (Rowe et al., 1986). The covalent linkage between the DNA and the enzyme prevents the release of a DNA double-stand break. The cleavage reaction requires the presence of a divalent cation, preferentially Mg^{2+} (Osheroff, 1987; Osheroff and Zechiedrich, 1987), but occurs in the absence of ATP (Lindsley and Wang, 1993; Tingey and Maxwell, 1996).

The protein-operated gate allows the transport of a second DNA strand necessary for the separation of catenated DNA. The transport of a second DNA strand, named T-segment (the DNA segment transported through the gate in the G-segment), through the gate is a complex process and requires the presence of ATP and a dynamic opening and closing of the three dimerization regions. Following cleavage of the G-segment and binding of ATP at the ATPase domain, the enzyme binds a T-segment and the complex process is initiated which transports the T-segment from the "top" of the enzyme through the dimeric interface until the T-segment finally exits a the "bottom" of the enzyme (step 3-5 in Fig. 1.7) (Berger et al., 1996; Fass et al., 1999).

From analysis of crystal structures of the breakage-reunion domain a model for the transport of the T-segment has been proposed (Berger et al., 1996; Cabral et al., 1997; Fass et al., 1999). Cleavage of the G-segment and binding of ATP is believed to induce a changed conformation for both the A' and the B' regions and dimerization of the ATPase domains. The changed conformation includes a rotation of the B' sub-fragments and a separation of the A' sub-fragments allowing for the opening of the DNA gate and the transport of the T-segment through the DNA gate into the central cavity. The conformation of topo II after strand passage is called T2O (Berger et al., 1996; Fass et al., 1999). This model is in

agreement with a range of biochemical data. Binding of ATP induces dimerization of the ATPase domain (step 3 in Fig. 1.7) (Ali et al., 1995; Roca and Wang, 1994; Wigley et al., 1991) changing the enzyme from an open clamp to a closed clamp. Interestingly, binding of AMPPNP changes the sites in the structure accessible to proteases supporting the described model that binding of ATP induces a changed conformation of topo II. The previously described preference for cleavage at sites A and C is changed to cleavage at site B and C (V8-B in Fig. 1.5) (Lindsley and Wang, 1991). Furthermore, the binding of AMPPNP also induces the formation of a wide hole in the large core. The central hole present in the structure is interesting because it is of an appropriate electrostatic nature to attract a DNA duplex. Therefore, transport of the T-segment into the central cavity is believed to be partly due to electrostatic attraction and partly steric repulsion (Berger et al., 1996). After strand transfer, the T-segment is stored in the central cavity of the enzyme (step 4 in Fig. 1.7).

Structural data suggest that, after the T-segment has entered the central cavity, the A' domains move towards each other to allow religation of the cleaved G-segment. The accompanying constriction of the central hole destabilizes the A'-A' interface sufficiently to open and expel the stored duplex (step 5 in Fig. 1.7) (Berger et al., 1996; Fass et al., 1999; Maxwell, 1996; Roca and Wang, 1992). Indeed, biochemical studies show that the A'-A' primary dimer interface opens to allow the escape of the T-segment (Roca et al., 1996; Roca and Wang, 1994).

The final step of one catalytic cycle is the hydrolysis of ATP bound to the ATPase domains and reformation of the primary dimer interface between the two A' sub-fragments (Berger et al., 1996). The hydrolysis of ATP facilitates enzyme turnover and reopening of the ATPase domain and allows the initiation of a new catalytic cycle (Osheroff, 1986; Osheroff et al., 1983). The above-described two-gate model emphasizes that the T-segment enters from one end of the enzyme, passes through the central axis between the two monomers, and exits at the other end.

1.3.4 Drugs Targeting Topoisomerase II

Topo II is the major cellular target of several anti-tumour drugs. The knowledge of the catalytic cycle has greatly increased our understanding of how topo II targeting drugs exert their anticancer effect. According to the mechanistic differences in their mode of action, the drugs known to target topo II can be divided into two classes: poisons and catalytic inhibitors (Froelich Ammon and Osheroff, 1995). The topo II poisons function by stabilizing the transient enzyme-DNA complex formed upon DNA cleavage, thus increasing the number of topo II molecules bound covalently to DNA (Robinson et al., 1991; Robinson and Osheroff, 1991; Sorensen et al., 1992). It is thought that cellular processes such as replication and transcription convert the drug-stabilized covalent complex into double stranded DNA breaks, which ultimately lead to cell death (Chen and Liu, 1994; Pommier et al., 1994). Catalytic inhibitors, on the other hand, inactivate the enzyme by inhibiting the catalytic activity of the enzyme without inducing double stranded breaks. These substances probably kill the cells, as

they induce traverse through mitosis without a functional topo II enzyme (Holm et al., 1989a; Wang, 1994).

When topo II poisons are used in clinical anticancer treatment, resistant cancer cells emerge typically during later stages of chemotherapy. Similarly, resistant cells emerge in cell culture when they are exposed to the drug for an extended period of time. Several mechanisms are responsible for the development of drug resistance, including mutations in the enzyme, altered drug transport and attenuated expression of topo II. Development of drug resistance to poisons is commonly associated with a down-regulation of mRNA and protein expression of one or both of the mammalian topo isozymes (reviewed in (Beck et al., 1993; Froelich Ammon and Osheroff, 1995; Pommier, 1993)). The observation that elevated expression of topo II leads to a hypersensitivity of cell lines to these drugs is consistent with a correlation between drug sensitivity and the level of topo II (Davies et al., 1988; Fry et al., 1991). Direct evidence for a role of down-regulation of topo IIα mRNA level in the generation of drug resistance was provided by expression of anti-sense RNA fragments, which reduces the level of cellular topo IIα protein, thus leading to higher resistance to etoposide in mammalian cells (Gudkov et al., 1993).

Drug resistance can as well be due to mutations in the topo IIα gene. A number of point mutations affecting either the catalytic properties of topo II or the enzyme DNA drug-interaction have been reported (Bugg et al., 1991; Danks et al., 1993; Lee et al., 1992; Patel and Fisher, 1993). In addition to point mutations, deletions in TOP2A associated with drug resistance have also been identified in human cells (Grue et al., 1998; Matsumoto et al., 1997; Mirski and Cole, 1995; Wessel et al., 1997; Yu et al., 1997). Interestingly, of the deletion mutants reported, many are not affected in their catalytic cycle. Instead, the resistance is caused by a changed cellular localization. Several deletions in the divergent C-terminal domain lead to improper nuclear localization and will be further discussed in the next section.

1.3.5 Function of the C-terminal Domain in Eukaryotic Topoisomerase II

Several studies indicate that the N-terminal and the core domain of topo II are sufficient for its *in vitro* catalytic activity. Thus, other functions must be ascribed to the divergent C-terminal region (Crenshaw and Hsieh, 1993; Jensen et al., 1996a; Shiozaki and Yanagida, 1991). Of interest in this respect, the overall amino acid sequence identity of 68% between the two human isoforms is not evenly distributed: While the N-terminal three quarters are 78% identical, the similarity in the C-terminal quarter is only 34% (Austin et al., 1993; Lang et al., 1998; Sng et al., 1999). This suggests that the C-terminal domain might mediate isoform-specific functions.

In higher eukaryotes several nuclear localization sequences (NLS) have been identified in the C-terminal domain of topo IIα and IIβ. The last 168 amino acids in murine topo IIα are essential for proper nuclear localization and a similar region is found in the IIβ isoform (Adachi et al., 1994). In human topo IIα, a sequence spanning amino acids 1454 to 1497 has been shown to be sufficient for nuclear localization, and in the human IIβ isoform,

the NLS sequences were mapped to amino acids 1522-1573 (Mirski et al., 1999; Mirski et al., 1997).

A second role for the divergent C-terminal domain is its interaction with other cellular proteins. Topo II has functions in virtually all aspects of DNA metabolism. In addition, topo II has a rather broad substrate specificity. Thus, its activity *in vivo* must be guided or controlled, probably by the aid of co-factors and/or other proteins. Indeed, several proteins interacting with the C-terminal region have been identified. Although many roles of such interactions are postulated, functional assays proving such roles are mostly lacking so far. Nevertheless, the following listing of putative interacting partners of topo II was sorted according to their putative function.

Only two studies have revealed a relation between topo II and transcription. Far western assays suggested that topo II interacts with transcription factors such as CREB, ATF-2 and c-Jun. *In vitro*, topo II-decatenation activity furthermore appeared to be slightly stimulated by CREB and c-Jun (Kroll et al., 1993). It has also been shown that topo II co-purifies with the chromatin accessibility complex (CHRAC) (Varga-Weisz et al., 1997), although the same group recently stated that topo II should not be considered as a stable subunit of CHRAC (Eberharter et al., 2001).

Several other interactions likely play a role during mitosis. The *Drosophila* barren protein, which is essential for sister chromatid segregation in mitosis, interacts with topo II throughout mitosis, as shown by yeast two hybrid and immunoprecipitation. It stimulates topo II relaxation activity *in vitro* (Bhat et al., 1996). Since mutations in the barren gene cause a failure of proper chromosome segregation at anaphase, it has been proposed that the barren - topo II association facilitates the decatenation of chromatids at anaphase, and thus is required for proper chromosomal segregation. A similar role has been suggested for the retinoblastoma protein (Rb). Rb is shown by immunoprecipitaion to interact with topo IIα and to inhibit topo IIα decatenation activity. Rb is a phosphoprotein and it is the non-phosphorylated form that seems to interact with human topo IIα and inhibits its activity (Bhat et al., 1999). Since Rb and topo II are both phosphorylated during G2/M phase, a model could be envisaged, in which phosphorylation regulates the activity of topo IIα in a cell-cycle specific manner. Phosphorylation of Rb in G2/M phase would disrupt Rb-topo II interaction thereby activating topo II during mitosis (Bhat et al., 1999). Thus, both barren and Rb might stimulate topo II decatenation activity during chromosome condensation and segregation. An interaction between topo II and pat1 (protein associated with topo II) has also been implicated in regulating topo II during mitosis. Screening of a yeast library using the C-terminal domain of yeast topo II revealed that pat1 interacts with this domain. Pat1 has functions in chromosome segregation and interacts with the highly conserved leucine-rich region in yeast topo II (Wang et al., 1996). It is possible that similar protein interaction partners in higher eukaryotes mediate an isoform specific function, since only topo IIα but not topo IIβ contains a leucine zipper motif (Austin et al., 1993). Yeast two-hybrid experiments and immunoprecipitation also demonstrated an interaction between topo II and sgs1, an interaction suggested to be important for faithful segregation of newly replicated chromosomes (Watt et al., 1995). It is not known if pat 1 and sgs1 affects the catalytic activity of topo II. Interestingly, topo IIα has

been shown to be part of the purified UB2 complex in mouse, which consists of several proteins including scaffold protein 2 (sc2). In the UB2 complex topo IIα preferentially binds a specific sequence, whereas free topo IIα has a low sequence specificity. The higher sequence stringency of UB2 over that of free topo IIα could be an important factor in limiting the number of DNA sequences bound by topo IIα (in the form of UB2) *in vivo* (Ma et al., 1993). The authors furthermore suggest that UB2 may be involved in mitotic chromosome condensation.

Topo IIα may also interact with CAD nuclease (Durrieu et al., 2000) connecting topo IIα to chromatin condensation during apoptosis. Indeed, it has recently been suggested that topo II is involved in caspase-independent excision of DNA loop domains during apoptosis (Solovyan et al., 2002). The authors suggest that this cleavage is performed by topo II located at the nuclear matrix.

Finally, the C-terminus of topo II plays a major role in the regulation of topo II by phosphorylation. Topo II is phosphorylated on multiple serine and threonine residues, the majority of which are located in the C-terminal domain (reviewed in (Isaacs et al., 1998)). It has been proposed that phosphorylation may regulate the activity of the enzyme and its interaction with other cellular proteins. However, the exact role of phosphorylation in regulating topo II activity is still under debate. Phosphorylation has been reported to stimulate the *in vitro* activity of topo II 2-3 fold (Ackerman et al., 1985; Corbett et al., 1992; Corbett et al., 1993; DeVore et al., 1992). In agreement with this, soluble mitotic extracts from HeLa cells, which are known to be hyper-phosphorylated, contain a higher topo II activity than S-phase extracts (Estey et al., 1987). However, several observations have seriously challenged any significant role of phosphorylation in regulating topo II catalytic activity. For example, the enzymatic activity of fission yeast topo II is not affected by phosphorylation or dephosphorylation *in vitro* (Shiozaki and Yanagida, 1992). In addition, topo II interacts with the β subunit of protein kinase casein kinase 2 (PK CK2) in yeast (Bojanowski et al., 1993; Leroy et al., 1999), but Kimura and co-workers reported that PK CK2 has no effect on the activity of mouse topo IIα. More importantly, the authors concluded that their previously reported results (Saijo et al., 1990), showing a stimulation of topo IIα activity by PK CK2, could not be attributed to the kinase, but to the composition of the incubation buffer (Kimura et al., 1996b). Instead, a new mechanism by which PK CK2 could regulate human topo IIα function has been suggested. PK CK2 phosphorylated the enzyme and increased its *in vitro* activity, but the cause of the activation was not phosphorylation, but rather stochiometric stabilization against thermal inactivation of human topo IIα during the incubation (Redwood et al., 1998). Of possible significance in this regard is the observation that the β subunit of PK CK2 confers thermal stability upon the PK CK2 α subunit, in which the catalytic activity of the dimer resides (Meggio et al., 1992). Since the topo II - PK CK2 interaction is also mediated by the β subunit (Bojanowski et al., 1993; Leroy et al., 1999), it can be speculated that the β subunit of PK CK2 is involved in stabilizing topo IIα thereby increasing its *in vitro* activity. Taking all these findings together, it seems appropriate to conclude that the major role of topo II phosphorylation does not appear to be the regulation of enzyme activity.

In vitro studies of topo II have implicated that phosphorylation of the enzyme plays a role in chromosome condensation. Yeast topo II is able to form multimers *in vitro* and this multimerization is dependent on phosphorylation (Cardenas and Gasser, 1993; Vassetzky et al., 1994). Topo II preferentially binds and aggregates S/MAR containing DNA. The interaction is highly cooperative and, with increasing concentrations of topo II the enzyme titrates first S/MAR containing DNA and then non S/MAR DNA (Adachi et al., 1989). Similar to multimerization, the specific topo II S/MAR interaction is abolished when the enzyme is dephosphorylated (Dang et al., 1994; Vassetzky et al., 1994). These *in vitro* studies lead to a model in which topo II plays a structural role, fastening DNA loops to the scaffold via specific interactions with S/MAR sequences. Multimerization of topo II binding S/MAR elements could tether the chromatin fibre together thus facilitating an ordered chromosome condensation.

The fact that topo II multimerization and its interaction with S/MAR elements require phosphorylation of the enzyme strongly indicates an important biological function of topo II phosphorylation in chromosome organization. Indeed, phosphorylation of topo II is cell cycle specific, reaching a maximal level during G_2-/M-phase (Cardenas et al., 1992; Heck et al., 1989; Kimura et al., 1996a; Kimura et al., 1994a; Saijo et al., 1992; Wells et al., 1995; Wells and Hickson, 1995). Thus, one function of phosphorylation of topo II in G_2-phase could be to stimulate topo II mediated chromosome condensation.

In summary, it is clear that the N-terminal and central domains of topo II are required for basic enzymatic activity, whereas the C-terminal domain is most probably mediating the specific biological roles of the enzyme. It is involved in transport to the nucleus, interaction to other proteins and phosphorylation which all are important for the biological functions of topo II.

1.4 Biological Functions of Eukaryotic Topoisomerases I and II

Already from their catalytic properties it is evident that topo I and II must be involved in most if not all processes of DNA metabolism. Many of the biological roles of topoisomerases in the cell are possibly shared among the different types. However, the ability to decatenate interlinked DNA is a unique feature of topo II. Thus, the enzyme must have unique biological functions. Topoisomerases are required in interphase for processes like transcription, replication, recombination, and in mitosis for chromosome condensation and segregation. In addition, it has been suggested that topo II plays an important role in the architectural organization of the nucleus, because it is part of the nuclear matrix and the chromosomal scaffold. Emphasis in this section is put on the role of topo I and II in transcription and replication, and another major focus will be on their role during mitotic cell division. The role of topo I and II in meiosis, recombination, DNA damage/repair and apoptosis is less well understood and will not be discussed further.

1.4.1 Transcription and Replication

According to the twin domain model of Liu and Wang, transcription of a right handed double DNA helix generates positive supercoils ahead of the moving RNA-polymerase complex and negative supercoils behind it (Liu and Wang, 1987). Similarly, positive supercoils are generated during semi-conservative replication in front of the replication complex as the parental strands come apart behind it (Cook, 1999; Wang, 1991). A swivel activity, which removes supercoils, is therefore required during these processes (reviewed in (Andersen et al., 1996; Nitiss, 1998; Wang, 2002)). Recent evidence suggests that supercoils can diffuse across the replication fork, resulting in intertwined replicated strands called precatenanes (reviewed in (Postow et al., 1999; Postow et al., 2001)). This implies that not only relaxation (swivelation) but also decatenation activity is required during replication.

To date, topo I is believed to be the most likely candidate for the swivelase activity during transcription. Plasmids carrying actively transcribed genes were found to be extremely negatively supercoiled when isolated from yeast lacking topo I but not topo II (Brill and Sternglanz, 1988). Consistent with this, injection of the drug VM 26, which inhibits topo II activity, into human HeLa cells or Xenopus oocytes did not decrease RNA polymerase transcription rate, indicating that topo II is not essential for efficient transcription (Dunaway, 1990; Schaak et al., 1990). On the other hand, the topo I-specific inhibitor camptothecin decreased the rate of transcription significantly, further supporting the view that mainly topo I is responsible for the swivelase activity during transcription (Schaak et al., 1990). Localization studies showing topo I to colocalize with transcribed regions (Fleischmann et al., 1984; Gilmour et al., 1986; Shaiu and Hsieh, 1998) are in agreement with the view that topo I is responsible for resolving topological problems generated during transcription.

Until quite recently it was believed that topo I is also the enzyme responsible for the removal of supercoils generated during replication. *S.cerevisiae* mutants lacking both topo I and topo II, can initiate replication but elongation requires a topo activity. Inactivation of topo I leads to a temporary delay in DNA chain elongation, whereas inactivation of topo II had little effect on the rate of chain elongation (Kim and Wang, 1989). Studies on the replication of Simian Virus 40- DNA confirmed these results and moreover implicated that topo II is only required during replication of the final 5% of the genome (Sundin and Varshavsky, 1981; Yang et al., 1987). It is now generally held that topo I removes supercoils, thus driving elongation, whereas topo II is required to remove catenane crossings persisting after replication. In addition, the discovery that precatenanes are formed behind the replication complex (Sogo et al., 1999) suggests that these structures are also resolved by topo II. In keeping with this, it was recently demonstrated that topo II acts behind the forks at all stages of elongation (Lucas et al., 2001).

Topo II was previously recognized as the major non-histone protein component of the chromosome scaffold (Earnshaw et al., 1985; Gasser et al., 1986) and the interphase nuclear matrix (Berrios et al., 1985), insoluble nuclear structures believed to play an architectural role. In keeping with this, topo II was detected by immunocytochemistry along the longitudinal axes of gently expanded chromosomes (Earnshaw and Heck, 1985; Gasser et al., 1986) and

shown to bind preferentially to AT-rich DNA elements (S/MARs) believed to specify the bases of chromatin loops (Adachi et al., 1989). Ever since, topo II is considered to play a structural role in chromosome organization in addition to its enzymatic functions (Adachi et al., 1991). This view is supported by findings that topo II inhibitors induce cleavage at S/MARs *in vivo* (Gromova et al., 1995), and that S/MAR sequences are preferential cleavage sites for topo II both *in vitro* and *in vivo* (Cockerill and Garrard, 1986; Kas and Laemmli, 1992; Miassod et al., 1997). An important biological function of topo II appears thus to be that it helps organizing the interphase chromatin into constrained topological domains, which contain genes independently regulated (reviewed in (Laemmli et al., 1992)).

In this context, it is worth mentioning that the presence of flanking S/MARs consistently stimulated the expression of stably integrated transgenes, as compared to the same transgenes integrated without flanking S/MAR sequences (Blasquez et al., 1989; Klehr et al., 1991; Poljak et al., 1994; Stief et al., 1989). S/MAR elements are believed to mediate their stimulatory effect by altering the chromatin structure in such a way that displacement of histone H1 is facilitated (Poljak et al., 1994) and/or that spreading of the closed chromatin structure form adjacent gene domains to the transcribed gene and *vice versa* is blocked (Laemmli et al., 1992).

In vitro studies have suggested that, besides being involved in binding of S/MAR elements to the nuclear matrix, topo II could also play a more direct role in S/MAR-stimulated expression. As elaborated above, transcription generates supercoils that need to be removed by the swivelase activity of topo I. S/MARs and topo II could also be involved in relieving these negative supercoils. S/MAR sequences alone have been proposed to remove negative supercoils generated during transcription based on their ability to base-unpair under superhelical strain (Bode et al., 1992). This could release negative supercoils and could be considered as a storage place or sink for thermodynamic energy in the form of negative supercoiling. Interestingly, a number of eukaryotic genes are optimally transcribed when supercoiled (Hirose and Suzuki, 1988). For example, a certain degree of negative supercoiling enhances the transcription of the fibroin gene (Mizutani et al., 1991a; Mizutani et al., 1991b). The thermodynamic energy stored at nuclear matrix bound S/MARs could then at other times be converted into negative supercoiling in order to enhance transcription within the domain (Bode et al., 1992). Alternatively, the negative supercoiling stored in the S/MARs could be released by topo II that binds S/MARs at the nuclear matrix.

1.4.2 Chromosome Structure and Condensation

In human interphase cells, chromosomal DNA molecules (having a total length of ≈1.4 m) are packaged into nuclei that are only ≈10 μm in diameter. At mitosis, the chromosomes become further condensed to an overall 10,000-fold compaction. Compaction of the DNA is accomplished by a hierarchy of DNA and chromatin packing devices (reviewed in (Earnshaw, 1991; Koshland and Strunnikov, 1996)). At the lowest level, the DNA fibre is compacted six- to seven-fold by winding around the histone core of the nucleosome, generating fibres of ≈10

nm in diameter (Kornberg, 1974). At the second level, association of histone H1 causes the fibre to shorten and thicken to a diameter of ≈30 nm effecting an overall compaction of ≈40-fold (Finch and Klug, 1976; Horowitz et al., 1994; Thoma et al., 1979). The remaining 250-fold compaction is achieved by more complex architectural measures. The most widely accepted model, the so-called loop model, proposes that the 30 nm-fibre is gathered into loops each containing ≈50-100 kb of DNA. In this model, the chromatin fibre is presumably organized into domains that are constrained by a residual chromosomal scaffold (Adolph et al., 1977; Laemmli et al., 1978; Paulson and Laemmli, 1977). The loop domains are further organized into rosettes, which are compacted into coils that finally form the two chromatids (Boy de la Tour and Laemmli, 1988; Filipski et al., 1990).

Topo II is the most abundant non-histone protein of isolated metaphase scaffolds (Earnshaw et al., 1985; Earnshaw and Heck, 1985; Gasser et al., 1986). Roughly three copies of topo II per DNA loop are present in an average human metaphase chromosome (Gasser et al., 1986). The architectural role of topo II in chromosome structure is also indicated by its proposed ability to form multimers and to bind S/MAR fragments in a cooperative manner (Adachi et al., 1989; Vassetzky et al., 1994). Some localization studies of topo II in metaphase chromosomes have shown the enzyme at the longitudinal axes of the chromosome arms, which is consistent with the scaffold model. However, other studies have reported a uniform distribution of topo II through the entire diameter of the chromosomes, which argues against the scaffold model. Thus it remains uncertain, whether topo II resides at the base of the chromosome loops or is dispersed over the entire chromatin. Most importantly, the observation that topo II is uniformly distributed over the chromosomes does not exclude that topo II is also present in the chromosomal scaffold.

Lohka and Masui have developed an elegant *in vitro* assay for studying chromosome condensation, and have thus studied the role of topo II in chromosome condensation extensively. They found that incubation of chromatin with extracts from frog eggs induces condensation (Lohka and Masui, 1983). By using extracts from *Xenopus* eggs immuno-depleted of topo II, Adachi and co-workers examined the role of topo II in chromosome condensation. In chicken erythrocyte nuclei, which contain very low levels of topo II, the depleted extracts were incompetent in forming condensed chromosomes, whereas addition of purified topo II restored their ability to support chromosome condensation (Adachi et al., 1991). Analogous results have been obtained with extracts from mammalian cells (Wood and Earnshaw, 1990). In summary, these experiments precipitated the conclusion that topo II is essential for proper chromosome condensation. Hirano and Mitchison further examined the role of topo II in condensation, and confirmed this notion. Surprisingly, however, they showed that all detectable topo II could easily be extracted from the chromosomes under mild salt conditions without dramatically changing their shape. After salt extraction, topo II was no more detectable in the chromosomes by topo II specific antibody staining, whereas the chromosome core as delineated by the MPM-2 antibody remained intact. From these examinations the authors concluded that topo II is essential for mitotic chromosome assembly, but does not play a scaffolding role in the structural maintenance of chromosomes after their assembly (Hirano and Mitchison, 1993). Later it was shown that a phosphorylated form of

topo II is the major target of the MPM-2 antibody in mitotic chromosome scaffolds (Taagepera et al., 1993). Thus the question arises whether in the study of Hirano and Mitchison the MPM-2 antibody was after all detecting topo II and, thus, residual enzyme at the chromosomal axis escaped investigation. If the epitope(s) detected by MPM-2 in the extracted chromosomes indeed were topo II, this would indicate that a small, salt resistant fraction remained at the chromosomal scaffold and that this fraction is essential for maintaining the chromosomal structure. Such an interpretation is consistent with the loop model and the proposed function of topo II in organizing the chromosomal loops.

Additional evidence for an involvement of topo II in chromosome condensation comes from genetic data obtained in yeast. Chromosomes of fission yeast are known to undergo condensation to some degree. In a strain carrying double mutations in the topo II and β-tubulin genes, however, chromosomes appeared to be less condensed (Uemura et al., 1987). And finally, when topo II was inhibited by specific drugs in mammalian cells, proper chromosome condensation was inhibited leading to the same conclusion that topo II is essential for chromosome condensation (Anderson and Roberge, 1996; Buchenau et al., 1993; Downes et al., 1991; Ishida et al., 1994; Newport and Spann, 1987; Roberge et al., 1990).

The role of topo I in chromosome condensation is less clear. Direct evidence linking topo I to condensation is lacking altogether. However, genetic studies in yeast have implicated an involvement of topo I in condensation. Although, topo I is not essential in yeast (Goto and Wang, 1985; Thrash et al., 1985), it has been shown that yeast with *topo 1 trf4* double mutants are defective in mitotic chromosome condensation (Castano et al., 1996a; Castano et al., 1996b). These strains fail to establish and maintain chromosome condensation in the rDNA at mitosis (Castano et al., 1996a). This may indicate a special role of topo I in the condensation of rDNA, an interpretation consistent with immunofluorescent findings suggesting that topo I is concentrated in NOR's of metaphase chromosomes (Suja et al., 1997). Finally, the involvement of both topo I and topo II in chromatin condensation during mitosis is indicated by their physical interaction with the condensins and the SMC-complex (Kimura and Hirano, 1997; Kimura et al., 1999).

1.4.3 Chromosome Segregation

At anaphase, the two sister chromatids are separated and equally distributed between the two daughter cells. Separation is a complex process and requires the enzymatic function of topo II to ensure the disentanglement of the two sister chromatids. ΔTOP2 mutants of *S.cerevisiae* and *S.pombe* fail to segregate the replicated sisterchromatids and a substantial level of chromosome nondisjunction and breakage can be observed, consequently leading to cell death (Holm et al., 1985; Holm et al., 1989b; Uemura et al., 1987). In addition, the use of nocodazole, an inhibitor of tubulin polymerization that prevents the formation of the mitotic spindle, rescued cell viability of ΔTOP2 mutants of *S.cerevisiae*. Thus, yeast cells lacking the ability to segregate the chromosomes can survive without a functional topo II which implicates that topo II is only essential for the segregation of the chromosomes (Holm et al.,

1985). In yeast cells lacking an active topo II, small chromosomes (with arms less than 380 kb) can segregate most likely simply by unravelling at their ends, whereas larger chromosomes are inclined to fragmentation (Spell and Holm, 1994). The crucial role of topo II in segregation has also been observed in *Drosophila* and mammalian cells, where inhibition of topo II activity prevents the separation of sister chromatids during anaphase (Buchenau et al., 1993; Clarke et al., 1993; Downes et al., 1994; Downes et al., 1991; Ishimi et al., 1995).

The segregation of sister chromatids at anaphase instead of during S- or G_2-phase lead to a model proposing that a driving force such as pulling by microtubules is a necessary requisite for topo II to untangle the chromosomes. This model is supported by the study of Uemura *et al*. (1987) where a TOP2 temperature-sensitive and NDA3 cold-sensitive double mutant was used to separate the functions of topo II in condensation and segregation. By lowering the temperature to inactivate the NDA3 gene encoding β-tubulin the cells were arrested at metaphase and it was shown that in this situation chromosomes could be condensed to completion in the presence of an active topo II. However, when temperature was subsequently raised, thereby inactivating topo II and activating mitotic spindle formation, the chromosomes became fragmented (Uemura et al., 1987). This experiment demonstrates that fully condensed chromosomes remain catenated in the presence of active topo II if a pulling force such as the mitotic spindle is absent. However, the idea of segregation being dependent on microtubule forces has been challenged by the observation that sister chromatid separation can occur in the absence of microtubules (Straight et al., 1996). The lack of a requirement for microtubules indicates that another kind of directional force than the spindle is responsible for mediating topo II decatenation of the two sister chromatids. Based on the observation that many topo II interacting proteins are involved in chromosome condensation and segregation, it seems reasonable to speculate that topo II interaction partners could mediate such a function. Indeed, it has been suggested that the *Drosophila* barren protein may have such a function in segregation, by regulating the activity of topo II during anaphase (Bhat et al., 1996). It should also be mentioned that the process of chromosome condensation has also been proposed as a driving force for chromosome disentanglement (Haglund and Rothblum, 1987; Holm, 1994; Holm et al., 1989b).

In conclusion, the ability of topo II to decatenate the two sister chromatids makes the enzyme essential during segregation. However, the complete function and regulation of topo II in chromosome dynamics does most likely involve a complex interaction with other chromosomal proteins such as the SMC complex and cohesion proteins.

1.5 Subnuclear Distribution of Mammalian Topoisomerases

1.5.1 Topoisomerase I

The subnuclear localization of topo I is not entirely settled but much less controversially discussed than that of the two topo II isoforms (see below). Therefore, this

topic will subsequently be discussed in less detail. Indirect immunofluorescence studies have shown that during interphase topo I is located in the nucleoplasm and in the nucleoli (Buckwalter et al., 1996; Danks et al., 1996; Kaufmann et al., 1995; Meyer et al., 1997; Muller et al., 1985). Recently, this notion has been partially confirmed by transient expression of GFP topo I fusion proteins (Mo et al., 2000b). However, in the latter study, only 15% of cells displayed nucleolar localization. This may be due to a cell-cycle effect or to the relatively brief expression period that might not be sufficient for the expressed protein to get properly allocated in the cell. Interestingly, a number of studies have shown that the catalytically dispensable N-terminal region of topo I is important for correct cellular targeting of topo I. The N-terminal region targets topo I to transcriptional active loci in Drosophila (Shaiu and Hsieh, 1998), and to the nucleoli in human cells (Mao et al., 2002; Mo et al., 2000a; Mo et al., 2000b). The recruitment to nucleoli was independent from an interaction with nucleolin (Mao et al., 2002), which previously was believed to be responsible for proper nucleolar localization of topo I (Edwards et al., 2000). Instead, Mao *et. al*. (2002) suggested that ongoing rDNA transcription recruits topo I to the nucleoli.

Topo I is closely associated with mitotic chromosomes throughout mitosis (Meyer et al., 1997; Mo et al., 2000b) in a manner independent from the enzymes catalytic activity (Mo et al., 2000b). Furthermore, it has been observed that the enzyme concentrates in NORs of mitotic cells (Guldner et al., 1986; Suja et al., 1997), which fits its physical interaction with RNA-polymerase I (Rose et al., 1988).

1.5.2 Topoisomerase II

In an approach to identify possible isoform specific functions, the subcellular localization of DNA topo IIα and IIβ has been studied *in extenso*. However, the results obtained so far are rather equivocal and sometimes contradictory to each other. A clear image of the localization of topo IIα and IIβ at the subnuclear level is still lacking. Especially for the IIβ isoform different results have been reported. To fully understand the biological role of topo IIα and IIβ in the cell, it is essential to determine their precise nuclear distribution. In the following sections the current knowledge regarding this subject will be summarized and possible reasons for the apparent contradictions in previous results will be discussed.

The localization of topo IIα in interphase has been thoroughly investigated by indirect immunofluorescence and the results are in general consistent. During interphase, DNA topo IIα has a nuclear localization and is found in both the nucleoplasm and the nucleoli (Cowell et al., 1998; Meyer et al., 1997; Petrov et al., 1993; Rattner et al., 1996). The staining of the nucleoplasm has been reported to be either homogeneous (Chaly et al., 1996; Cowell et al., 1998; Sugimoto et al., 1998; Zini et al., 1992; Zini et al., 1994) or homogeneous with some speckle formation (Chaly and Brown, 1996; Meyer et al., 1997; Petrov et al., 1993). The presence of topo IIα in the nucleolus is supported by most of the data (Cowell et al., 1998; Grue et al., 1998; Meyer et al., 1997; Negri et al., 1992; Petrov et al., 1993; Rattner et al., 1996; Sugimoto et al., 1998; Zini et al., 1994), although some studies claim an exclusion from

the nucleolus (Cabral et al., 1997; Chaly and Brown, 1996; Chaly et al., 1996; Zini et al., 1992). Those studies demonstrating a nucleolar localization of topo IIα show different staining patterns at the subnucleolar level. Intensity of nucleolar staining ranges from less intense than the nucleoplasm (Negri et al., 1992; Sugimoto et al., 1998; Zini et al., 1994) to a slight increase in intensity as compared to the nucleoplasm (Meyer et al., 1997), and one study found nucleoli to be even more intensely stained than the nucleoplasm (Cowell et al., 1998).

The localization of topo IIβ in the interphase nucleus is even less clear. In one series of studies, antibodies against topo IIβ were reported to stain only the nucleolus (Negri et al., 1992; Zini et al., 1992; Zini et al., 1994; Zweyer et al., 1995), whereas other studies using a different set of antibodies have reported topo IIβ to be present in both the nucleoplasm and the nucleolus (Chaly and Brown, 1996; Chaly et al., 1996; Cowell et al., 1998; Petrov et al., 1993; Sugimoto et al., 1998; Turley et al., 1997). However, the extent of nucleolar staining in these reports is not the same, showing an up-concentration in the nucleolus (Cowell et al., 1998; Petrov et al., 1993), a similar level in the nucleolus and the nucleoplasm (Sugimoto et al., 1998) or a very low level in the nucleolus (Chaly and Brown, 1996; Chaly et al., 1996). Interestingly, a study has even reported an exclusion of topo IIβ from the nucleolus (Meyer et al., 1997), which further complicated our understanding of the cellular localization of the enzyme.

As discussed above, topo II has functions at various stages of chromosome formation and separation. Thus, numerous studies were undertaken to describe its precise chromosomal localization. One approach used living cells of *Drosophila* embryos micro-injected with rhodamine-labeled topo II to investigate the distribution of mitotic topo II (Swedlow et al., 1993). This study indicated that there are at least three pools of topo II in the chromosome: one that leaves the chromosome after prophase, one that leaves after metaphase, and a third that remains associated with the chromosomes throughout the mitotic cycle. Since *Drosophila* only expresses a single topo II isoform, it is possible that in higher eukaryotes the role of one of these pools is conducted by only one of the two isoforms.

All studies on the localization of mammalian topo II isoforms during mitosis have been performed on dead and fixed cells. Immunohistochemical staining methods revealed that during mitosis, topo IIα is associated with metaphase chromosomes (Chaly et al., 1996; Earnshaw et al., 1985; Earnshaw and Heck, 1985; Meyer et al., 1997; Rattner et al., 1996; Sugimoto et al., 1998; Sumner, 1996) although two reports have shown chromosomes not stained by topo IIα specific antibodies (Fischer et al., 1993; Petrov et al., 1993). However, questions still remain on the precise distribution of topo IIα on mitotic chromosomes. Immunostaining for topo IIα of hypotonically treated chromosomes revealed a more or less axial distribution through the center of chromosomal arms (Earnshaw and Heck, 1985; Gasser et al., 1986; Saitoh and Laemmli, 1994; Strick et al., 2001) and a concentration of the enzyme at centromers (Taagepera et al., 1993). In contrast, histone depleted HeLa and *Drosophila* chromosomes showed a more general distribution of topo IIα over the chromosome arms (Boy de la Tour and Laemmli, 1988; Buchenau et al., 1993; Swedlow et al., 1993). Two studies have reinvestigated the precise chromosomal localization of topo IIα with particular

interest to cell-cycle effects (Rattner et al., 1996; Sumner, 1996). In prophase, topo IIα was present at high concentrations throughout the chromosomes. At metaphase, topo IIα was lost from the chromosome arms, but still stained the axial cores and concentrated at the centromers. In anaphase, centromeric staining was lost and only the chromosome core was stained. The presence of multiple populations of topo IIα and a temporal shift in location of these populations during mitosis (Rattner et al., 1996; Sumner, 1996) parallels the behaviour of *Drosophila* topo II during mitosis (Swedlow et al., 1993). The apparent axial distribution of topo IIα has been suggested to reflect its location at the base of the chromosomal loop domains, where topo II attaches to S/MAR sequences. Recently, the transient expression of EGFP tagged topo IIα has been reported (Mo and Beck, 1999). The authors show that GFP-topo IIα is closely associated with mitotic chromosomes in living cells. Based on fixed cells stained with Hoechst dye they furthermore conclude that GFP-topo IIα is distributed in an axial pattern along chromosome arms.

The precise distribution of topo IIβ in mitotic cells is not satisfactory described. Several investigations have shown topo IIβ to be excluded from metaphase chromosomes (Chaly and Brown, 1996; Chaly et al., 1996; Meyer et al., 1997; Petrov et al., 1993; Turley et al., 1997; Zini et al., 1992). However, after careful examination of the data in two of these studies (Chaly and Brown, 1996; Chaly et al., 1996) staining of telophase chromosomes cannot be excluded. In all these studies, topo IIβ was detected in the cytosol as a homogeneous staining surrounding the chromosome (Chaly and Brown, 1996; Chaly et al., 1996; Meyer et al., 1997; Petrov et al., 1993; Turley et al., 1997; Zini et al., 1992). Contradictory to these studies, topo IIβ has recently been found to colocalize with condensed chromosomes, although to a lesser extent than the IIα isoform (Sugimoto et al., 1998). The presence of topo IIβ at the chromosomes is in agreement with a previous analysis showing that topo IIβ is part of the chromosomal scaffold (Renzi et al., 1997; Taagepera et al., 1993).

When discussing cellular distribution of topo II it seems somehow surprising that so many different results have been reported. It is now generally held that these localization results have been subjected to the influence of many marginal factors including differences in cell lines, different fixation and permeabilization methods and different mono- or poly-clonal antibodies employed. As discussed below, all these factors have influenced the results reported in the literature and biased the conclusions drawn from them.

The effects that cell fixation and permeabilization have on immunohistochemical localization of topo II is very well exemplified by an examination of Sumner (Sumner, 1996). Even with the method that gave the best and most consistent results, considerable variation within a single preparation of metaphase chromosomes was observed. The staining of the chromosomes by a topo IIα specific monoclonal antibody (OM-11-930) ranged from no staining at all to a general staining of the whole chromosome arms. Chromosomes with a centromeric up-concentration and axial distribution could be observed in the same sample. Phase contrast images revealed that unstained chromosomes were surrounded by a significant amount of cytoplasm, suggesting that the fixation/permeabilization protocol is the most likely cause for this variation. This notion is also illustrated in a recent study reporting an axial localization of topo IIα in spread chromosomes. The authors demonstrate that depletion of

divalent cations during spreading causes a 10-fold reduction of antibody staining for topo IIα (Strick et al., 2001).

In addition, the immunohistochemical localization of topo II is to a considerable extent influenced by the antibody used for staining. This is exemplified by comparing two studies using the same fixation/permeabilization protocol (4% PFA and 0.5% T-X-100), but different sets of antibodies. One study observed an exclusion of topo IIα from the chromosomes (Petrov et al., 1993), whereas the second report shows chromosome staining (Chaly et al., 1996). One possible explanation for such differences could be that some antibodies preferentially or exclusively recognize a posttranslationally modified form of the enzyme. Cell line-specific localization differences could as well be the cause of inconsistent results. In summary, it becomes clear that available immunohistochemical data on the localization of topo IIα and IIβ are to be interpreted with outmost care and can not be trusted with respect to the differentiation of nuclear ultrastructures and subnuclear compartments.

2. Aims of Study

As described above, the precise localization of topo I, IIα, and IIβ, in the cell nucleus and their association with various defined nuclear substructures is still an unsettled subject. Since this is most likely due to uncontrollable variations caused by fixation methods and antibody preparations inherent in classical immunohistochemical methods, the first goal of this study was to study cellular localization of these enzymes by an alternative approach. I wanted to investigate localization of the enzymes in living mammalian cells, thereby not only avoiding the use of permeabilization, fixation and antibody techniques, but also being able to tackle questions of dynamic reallocation of the enzymes during the cell-cycle. The discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has provided the tool for such a study (Chalfie et al., 1994). GFP can be fused to proteins presumably without influencing their function (reviewed in (Ludin and Matus, 1998; Stearns, 1995)) and such GFP-linked proteins can be directly traced in cells living under a fluorescent microscope. Therefore, I set out to investigate the cellular disposition of mammalian topoisomerases by expressing GFP-chimera of these enzymes in human cells.

A second subject of this thesis was the question, whether topo II plays a role in building the interphase nuclear matrix and the chromosomal scaffold (Adachi et al., 1991; Berrios et al., 1985), structures that have been considered to provide an immobile element of cellular architecture. This subject is highly controversial. If only a fraction of topo II was part of such immobile structures, it should be immobile in a living cell. Thus, I set out to address the question of an architectural role of topo II by determining the mobility of topo II-GFP at various locations in the living cell nucleus by confocal microscopy and photobleaching techniques.

The third objective of my thesis is one of lesser scientific controversy but a more pronounced impact on medical praxis. Topo I and II are cellular targets of some of the most potent and most widely used anti cancer agents. It is well established that most of these poisons stabilise covalent complexes between DNA and topoisomerases I or II, which are otherwise quite transient. While this mechanism of drug action has extensively been characterised *in vitro,* it is still unclear to what extent it actually applies to the situation encountered in a living cell. Where in the living cell nucleus are such covalent DNA•topoisomerase complexes formed; how immobile are they actually; and how long do they persist? My thesis aimed at addressing these question by treating cells expressing GFP-chimera of topoisomerases with such drugs and then studying their impact on localization and mobility of GFP-topoiosmerases in the living cell by confocal microscopy and photobleaching.

The aims of this study may be summarized as follows:

1. **Generation and characterization of cell lines stably expressing GFP chimeras of topoisomerases I, IIα and IIβ.**
2. **Description of the precise localization of topo I, IIα and IIβ in the interphase nucleolus and in mitotic chromosomes.**
3. **Study of the *in vivo* mobility of topo II with special emphasis on the question of whether the enzyme participates in the formation of an immobile nuclear matrix/chromosomal scaffold**
4. **Study of the effects of topo poisons on the behaviour of topoisomerases in a living cell.**

3. Materials and Methods

3.1 Plasmid Construction

For the expression of GFP chimera of human topoisomerases I, IIα, and IIβ, bicistronic expression vectors for mammalian cells were constructed from the plasmid pMC-2P (Mielke et al., 2000), in which the puromycin resistance gene (pyromycin-N-acetyl-transferase, *pac*) constitutes the second cistron, followed by the *simian virus 40* (SV40) polyadenylation signal. In front, it is linked by an IRES element to a multiple cloning site (MCS), where the cDNAs of topo-GFP chimera were inserted. GFP was attached to the N-terminus of topo I (Fig. 3.1.B) and to the C-terminus of topo IIα and IIβ (Fig. 3.1.A), because previous experiences (Jensen and Svejstrup, 1996; Mo et al., 1998; Mo et al., 2000b) suggested that at these positions GFP-fusions would less likely disrupt enzymatic functions. A *cytomegalovirus* promotor (CMV) fused to the *myeloproliferative sarcomvirus* (MPSV) LTR-enhancer repeat ensures a high transcription level of the bicistronic message in various mammalian cells and the transcriptional linkage ensures a fixed simultaneous expression of *pac* and the topoisomerase (Fig. 3.1.C).

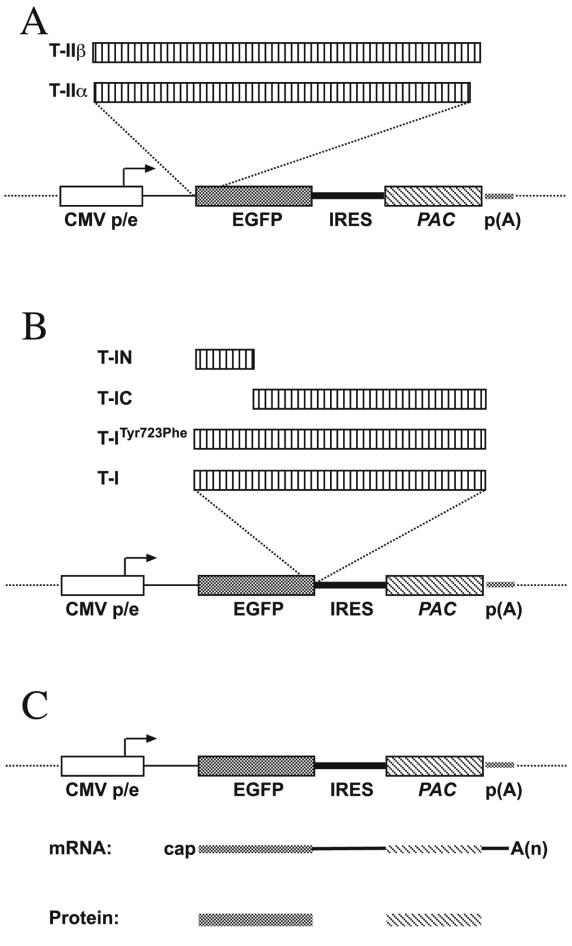

Figure 3.1: **The pMC vector system.** pMC harbours a CMV promoter element (open box), EGFP (grey box), an IRES element (black bar), *pac* (diagonally striped box), and a SV40 p(A) site (grey bar). (A) Topo IIα and IIβ (vertically striped boxes) were inserted in front of EGFP. (B) Topo I, topo I^{Tyr723Phe}, topo I-C, and topo I-N (vertically striped boxes) were fused to the C-terminus of EGFP. (C) Expression from the pMC vector system: After transfection pMC produces one bicistronic mRNA. Translation of the first cistron is mediated by the normal CAP-dependent translation initiation. The IRES element mediates translation initiation of the second cistron.

The coding sequence of GFP and for chimera of human topoisomerases and GFP were inserted in front of the IRES by linker PCR. Thus, topo GFP chimeras were encoded by the first cistron and the puromycin resistance gene (puromycin-N-actyl-transferase (*pac*)) by the second cistron. Sequences of PCR primers used for cloning are listed in section 3.1.1.

pMC-EGFPP and pMC-EGFPP-N: EGFP was PCR-generated from pEGFP (Clontech, Heidelberg, Germany) using primer sets K/J or F/B respectively and cloned (EcoR I/HindIII) into pMC-2P. The plasmid pMC-EGFPP (Fig 3.1.A) and pMC-EGFPP-N (Fig. 3.1.B) thus obtained contain MCS's for the insertion of various open reading frames in front of, or behind EGFP, respectively.

The sequence encoding the last 531 amino acids of human topo IIα was PCR generated from pHT300 (Jensen et al., 1996b), (primer set G/M). pHT300 carries the wild-type human topo IIα cDNA. The PCR generated fragment was then cloned into pMC-EGFPP (SpeI/ApaI), thereby generating pMC-TIIα-c531-EGFPP. From this pMC-TIIα-EGFPP was created by inserting the N-terminal part of topo IIα (MluI/SpeI). The same strategy was used to clone pMC-TIIβ-EGFPP using primer sets L/N and pTIIβKOZ (carries the wild-type human topo IIβ cDNA; Larsen MK, unpublished) as template.

For the yeast complementation assay plasmids pHT300 and pHT400 (Jensen et al., 1996b) carrying cDNAs of human topo IIα and IIβ under the control of the yeast triose phosphate isomerase (TPI) gene promoter were modified to express human topo II-GFP fusion proteins by replacing 3'-terminal restriction fragments of topo II reading frames by PCR-generated fragments containing the respective GFP chimera of the mammalian expression vectors. Briefly, the sequence encoding a C-terminal fragment of topo IIα and GFP was generated by PCR amplification using the primer set G/A and pMC-TIIα-EGFPP as template. The fragment was then inserted (SpeI/NotI) into pHT300, thereby generating pHT300-EGFP. The sequence encoding a C-terminal fragment of topo IIβ and GFP was generated by PCR amplification using the primer set E/A and pMC-TIIβ-EGFPP as template. The fragment was then inserted (BamHI/NotI) into pHT400, thereby generating pHT400-GFP.

Human topo I was PCR amplified from pHT-100 (Alsner et al., 1992) using the primer set H/C, and then inserted (MluI/ApaI) into pMC-EGFPP-N, thereby generating pMC-EGFP-TIP. pHT100 carries the wild-type human topo I cDNA. pMC-EGFP-TI^{Tyr723Phe}P, was cloned using the same strategy, with the exception that a plasmid encoding the active site tyrosin mutant of human topo I (Tyr^{723}Phe) (Madden et al., 1995) was used as a template for the PCR reaction. pMC-EGFP-TINP was PCR cloned, using pHT100 as template and the primer set H/D. This plasmid encodes the N-terminal domain (amino acids 1-210). The C-terminal domain of human topo I (amino acids 210-765) supplemented with an SV40 NLS was cloned into pMC-EGFP-N using pHT100 as template and the primer set I/C, thereby generating pMC-EGFP-TICP.

3.1.1 PCR

All PCR fragments intended for subsequent cloning were generated using the Expand™ high fidelity PCR system (Roche, Basel, Switzerland). The reactions were carried out on a Perkin Elmer Cetus DNA thermal cycler, programmed as follows:

1x	5 min 96°C
25x	30 s 94°C
	30 s 55°C
	3-5* min 72°C

*: Depending on the length of the substrate

Oligonucleotides used as PCR-primers
(in alphabetic order)

A) 3'G/BFP-Not I
5' GGG CGC GGC CGC TTA CTT GTA CAG CTC GTC 3'

B) 3'GFP-PL-EcoRI
5' GGA ATT CGG GCC CAC TAG TCC TCA GGA CGC GTC CAC CGC TTG TAC AGC TCG TCC ATG 3'

C) 3'TopI-ApaI
5' GCG TAG GGC CCC TAA AAC TCA TAG TCT TC 3'

D) 3'TopI-210-ApaI
5' GCG TAG GGC CCC TAC TTG ATG CCT TCA GGA TA 3'

E) 5'BamHI Topo2b
5' ACG AAT GCT AGA TGG CCT 3'

F) 5'GFP-HindIII
5' GGC GGG AAG CTT CCA TGG TGA GCA AGG GCG 3'

G) 5'Spe I-h:
5' GAG AGA GTT GGA CTA CAC 3'

H) 5'TopI-MluI
5' GGG ACG CGT ATG AGT GGG GAC CAC CTC 3'

I) 5'TopI-MluI-SV40
5' GGG CGG ACG CGT ATG TGT ACA CCT CT AAG AAG 3'

J) EGFP-3-EcoN:
5' CGA ATT CTT ACT TGT ACA GCT CGT CC 3'

K) EGFP-5-Pl:
5' CGC AAG CTT GCG GCC GCA CGC GTC CTA AGG ACT AGT AGG GCC CAC CGG TCG CCA TGG 3'

L) hTβBsu-f:
5' CTG GCT GGA ATG TCT GGA AAA G 3'

M) Top2α-Fus-Apa:
5' CGC GGG CCC AAC AGA TCA TCT TCA TCT G 3'
N) Top2β-Fus-Apa:
5'CGC GGG CCC TTA AAC ATT GCA AAA TCA AC 3'

Before restriction digestion all PCR products were treated with proteinase K (1mg/ml proteinase K in 1 x modified bradley solution (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 10 mM NaCl, 0.5 % SDS)) for 1 h at 37°C. Subsequently, 1 volume of phenol-chloroform-isoamyl (25:24:1) was added and the phases were separated by centrifugation (14000 x g, 10 min, 20° C). DNA was precipitated from the water phase by adding 2 volumes of 0.6 M LiCl-EtOH. PCR-products were finally dissolved in 10 µl TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

3.1.2 Gel Electrophoresis and Recovery of DNA from Agarose Gels

The DNA fragments were dissolved in loading buffer (3 % Ficoll, type 400, 8 mM Tris-HCL pH 8.5, 8 mM Glacial acetic acid, 0.4 mM EDTA, 0.05 % Bromphenol blue) and separated by electrophoresis through an agarose gel (0.8-1.2% agarose (depending on fragment size) in TAE buffer (40 mM Tris-HCl pH 8.5, 40 mM Glacial acetic acid, 2 mM EDTA)). After electrophoresis, DNA in the gels was stained with 1 µg/ml ethidium bromide (EtBr). The fragments were excised and recovered from the agarose by electroelution. DNA was precipitated from the eluates with EtOH and redissolved in 10 µl TE.

3.1.3 Ligation

To insert PCR or restriction fragments into vectors, T4 DNA ligase (New England Biolabs, Beverly, USA) was used. Ligation was carried out at RT for 30 min. Prior to transformation, the ligation sample was heated (10 min, 65° C) to inactivate the ligase.

3.1.4 Sequencing of Plasmids

Sequencing of the constructs was done using the Dye Terminator Cycle Sequencing Kit (Perkin Elmer) according to manufacturer's protocol.

3.2 *E.coli* Strains, Transformation, and Isolation of Plasmid DNA

3.2.1 E.coli Strain

DH5α (supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) (Hanahan, 1983).

3.2.2 Generation of Electrocompetent E.Coli cells

E.coli were grown in 1 L TB media (1.2 % Tryptone, 2.4 % Yeast extract, 0.4% Glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, pH 7.2) and harvested (4000 x g, 20 min, 4°C), when the OD_{600} of the culture reached a value of 0.5-0.8. The cells were gently resuspended , washed twice with ice-cold ddH_2O (1 L and 0.5 L, respectively), and finally again sedimented (4000 x g, 20 min, 4°C). Cells were washed with 20 ml ice-cold ddH_2O, containing 10% Glycerol and finally resuspended in a final volume of 2-3 ml the same medium. Such competent cells were frozen in dry ice, and stored at - 80 °C.

3.2.3 Transformation of E.coli by Electroporation

2μl ligation reaction mixture was mixed with 8μl ddH_2O and placed for 30 min on a 0.05 μm filter (Millipore, Bedford, USA) swimming on a reservoir of ddH_2O to remove salt. Subsequently, the ligation reaction was mixed with 40 μl competent DH5α cells, transferred to a pre-chilled electroporation cuvette (internal width = 2 mm), and subjected to a charge of 2.5 kV. Immediately hereafter 0.7 ml TB-media (RT) was added. After incubation for 1 h at 37°C, the cells were transferred to LB-agar plates containing 50μg/ml ampicillin.

3.2.4 Plasmid Preparation at a Small Scale (vulgo: Minipreps)

The cell pellet of a 2 ml over night culture was resuspended in 400 μl lysis solution (0.2N NaOH, 1% SDS), neutralized with 300 μl 7.5 M NH_4oAC, kept for 10 min on ice, and then sedimented again (14000 x g, 20 min, 4°C). The DNA was precipitated form the supernatant with 500 μl 2-Propanol, sedimented (14000 x g, 20 min, 4°C), resuspended in 80 μl TE, and the sequence was finally confirmed by restriction digestions and sequencing.

3.2.5 Plasmid Preparation at a Large Scale (vulgo: Maxipreps)

E.coli cells grown in 1 L TB media over night were harvested (4000 x g, 20 min, 4°C), resuspended in 18 ml GTE (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA), lysed by adding 2 ml 20mg/ml freshly prepared lysozyme (Sigma Chemie, Deisenhofen, Germany) in 40 ml lysis solution (0.2 N NaOH, 1%SDS), neutralized with 30 ml 7,5 M NH_4oAC, and incubated (20 min on ice). Subsequently, the lysis solution was cleared by centrifugation (20000 x g, 30 min, 4°C). Plasmid DNA was precipitated from the supernatant with 0.625 vol 2-propanol, sedimented (20000 x g, 30 min, 4°C), and dissolved in 4 ml TE, 100 μl 2M Tris.

4 ml of this solution was mixed with 4.75 g CsCl and shaken 1 h at 37°C. 50 µl EtBr (5mg/ml) was added, the solution was mixed, cleared (2500 x g, 5 min, 20°), and 4 ml of the supernatant was transfered into a Beckman quick-seal tube. The tube was filled with a CsCl-TE solution (100 g CsCL + 100 ml TE) and a CsCl-gradient was established (50000 rpm, 18 h, 25°C). The plasmid DNA was extracted from the gradient under optical control in UV light. Plasmids were transferred to a fresh Beckman quick-seal tube and the procedure was repeated. EtBr was removed from the plasmids by repeated extraction with NaCl saturated 2-propanol. Salt was removed by dialyzing 3 times against TE. Finally the DNA was precipitated with EtOH and resuspended in TE.

Alternatively, plasmid DNA was isolated using Qiagen maxi prep (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

3.3 Mammalian Cells

3.3.1 Mammalian Cells

We used the cell line HEK 293 established from human primary embryonal kidney cells by transformation with adenovirus type 5 (Graham et al., 1977). This cell line was obtained from DSMZ, Braunschweig, Germany and will subsequently be referred to as 293 cells.

3.3.2 Maintenance of Mammalian Cells

293 cells were maintained as subconfluent monolayer cultures in Duldecco's Modified Eagle's Medium (DMEM with Glutamax-I (GibcoBRL/Life Technologies, Karlsruhe, Germany)) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C under a humidified 5% CO_2 atmosphere. For passage, cells layers were washed once with PBS, detached by a short treatment with 0.675 mM EDTA in PBS and reseeded upon dilution (1:6).

3.3.3 Transfection and Selection of 293 Cells

24 h before transfection a monolayer culture covering 80% of the substratum (i.e. 80% confluent) was divided 1:3. Cells were transfected using Lipofectamine (GibcoBRL/Life Technologies, Karlsruhe, Germany) according to the manufacturer's instructions. Transient expression could be observed after 12-24 hours with a transfection efficiency varying between 20-90 % depending on the construct used (pMC-EGFPP had the highest efficiency). After 36

hours stable cell lines were selected and maintained thereafter in medium containing 0.35 µg/ml puromycin (Sigma Chemie, Deisenhofen, Germany).

3.4 Western Blotting

To obtain an efficient extraction of proteins we used an extraction/loading buffer containing 5% SDS and 4 M urea. Cells were harvested at 80 % confluency, sedimented (600 x g, 5 min, 4°C), resuspended in 100 µl ice-cold PBS, mixed with 100 µl 2 X lysis buffer (25 mM Tris-HCl pH 6.8, 10 % SDS, 8 M urea, 20 % glycerol, 0.04 % bromophenol blue, 10 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 mM PMSF, 20 µg/ml aprotinin, 10 µg/ml pepstatin A), and homogenised by exposure to ultrasound for 10 s. Subsequently, samples were boiled (95°C, 5 min), aliquots equivalent to 5 x 10^5 cells were then loaded into the slots of SDS-polyacrylamide gels while still hot, and electrophoresis was started immediately.

For topo II, were the proteins in the sample separated in 1 mm gradient gels containing 3-8% polyacrylamide and Tris-acetate running buffer or 4-20% polyacrylamide and Tris-glycine running buffer (both obtained from Life Technologies, Karlsruhe, Germany). A mix of prestained recombinant proteins (Life Technologies, Karlsruhe, Germany) was used as a size marker. Electrophoresis was at 120 V for approx 1 h, or until the bromo phenol blue of the samples reached the bottom of the gel. For experiments involving subsequent silver staining, a homogeneous gel containing 6% polyacrylamide and Tris-glycine running buffer was used. For topo I the same procedure was used as for Topo II, except that homogeneous gels (1 mm thick) were used containing 9% polyacrylamide and Tris-glycine running buffer.

Proteins were electrophoretically transferred from the gel to a PVDF membrane (Immobilon P; Millipore, Bedford, Maryland) by the semi-dry method. Briefly, four 3MM paper filters soaked in K-buffer (70 mM CAPS-NaOH pH 10.5, 10% MeOH) were stacked on the anode side of the gel, whereas the PVDF membrane soaked in MeOH, one 3 MM paper filter soaked in A2-buffer (25 mM Tris-HCl pH 10.4, 20% MeOH), and two paper filters soaked in A1-buffer (300 mM Tris-HCl pH 10.4, 20 % MeOH) were stacked on the catode side of the gel. Subsequently, the stack was placed between two graphite plates (NovaBlot, Amersham Pharmacia Biotech, Freiburg, Germany) and 0.8 mA/cm^2 were applied for 2 h. Finally the PVDF membrane was detached from the stack and left over night to dry.

3.4.1 Western Blot

The PVDF membrane was soaked in MeOH for 5 s, washed for 5 min with CMF-PBS (167 mM NaCl, 25.5 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5), containing 0.05 % Tween 20, and blocked for 1-2 h with CMF-PBS, containing 0.05% Tween 20 and 2% BSA. The membrane was then incubated for 1 h with the primary antibody diluted in CMF-PBS, 0.05% Tween 20, 2% BSA. This was followed by rinsing and 4 washes (1 x 15 min, 3 x 5 min) with CMF-PBS, containing 0.05% Tween 20. The membrane was then incubated for 45 min with

the secondary antibody conjugated to the horseradish peroxidase and diluted in CMF-PBS, containing also 0.1% Tween 20, 2% BSA. This was again followed by rinsing and 4 washes as above. Protein bands labelled by the secondary antibody were visualized by chemiluminescence using the ECL Plus system (Amersham Pharmacia Biotech, Freiburg, Germany).

Human topo IIα was detected with a rabbit peptide-antibody specific for the last 18 amino acid residues of the enzyme (Genosys, Cambridge, UK), which was diluted 1:10000. Human topo IIβ was detected with a rabbit peptide-antibody specific for the last 12 amino acid residues of the enzyme (Boege et al., 1995), which was diluted 1:2000. GFP and GFP-fusion proteins were detected with a mouse monoclonal antibody (clone JL8, Clontech, Heidelberg, Germany), which was diluted 1:5000. α-tubulin serving as a loading control was detected with a mouse monoclonal antibody (Sigma Chemie, Deisenhofen, Germany), diluted 1:1000. Human Topo I was detected with a rabbit peptide antibody specific for the last 14 amino acid residues of the enzyme (OA-11-754, Genosys, Cambridge, UK), which was diluted 1:10000.

Secondary antibodies were goat anti-rabbit (diluted 1:10000) or goat anti-mouse IgG (diluted 1:50000) coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Freiburg, Germany).

3.5 Banddepletion Assay

Band-depletion was performed as described in (Meyer et al., 1997) with minor modifications. Briefly, cells were harvested (600 x g, 37°C, 5 min), gently resuspended in medium (37°C) containing topo poisons and 1% DMSO as a solvent aid, and incubated at 37°C. Subsequently, cells were lysed by adding 1 vol of 2 X lysis solution, also containing topo poisons and 1% DMSO as a solvent aid. From this point on, samples were treated as described in section 3.4. For depletion of topo II cells were exposed to 200 μM VM 26 for 1 hour. For depletion of topo I cells were exposed to various concentrations of CpT for 20 min. Controls were treated similarly with medium just containing 1% DMSO.

3.6 Complementation Assay

Yeast complementation was carried out as described in (Jensen et al., 1996b). Briefly, *LEU2* expression plasmids for human topo II isoforms and the respective GFP chimera were transformed into the haploid Δ*top2* strain BJ201 (pHT173 (*S.pombe* TOP2/URA-ARS/CEN) MATα *ura3 trp1 leu2 his3 pep4::HIS3 prb1Dcan1 top2::TRP1 GAL*) which contains a *URA3* plasmid carrying the *S. pombe TOP2* gene to substitute for the essential topo II activity. Colonies from selection plates (without *Leu*) were then streaked onto media containing 1

mg/ml 5-fluoro acetic acid (5-FOA) to counterselect against the plasmid carrying the *S. pombe TOP2* gene.

3.7 Extraction and Immunoprecipitation of Active Topoisomerase II from Mammalian Cell Nuclei

Exponentially growing cells were harvested, sedimented (600 x g, 3 min), carefully resuspended ($5x10^9$ cells/3 ml) in lysis-buffer (15 mM Hepes pH 7.5, 1 mM EGTA, 60 mM KCl, 15 mM NaCl, 30 µg/ml Spermine, 7.5 µg/ml Spermidine, 5 µg/ml Leupeptin, 5 µg/ml Pepstatin A, 1 mM Benzamidine, 10 µg/ml Aprotinin, 1 mM DTT, 1 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride, 1 mM PMSF), and incubated for 5 min on ice to allow for cell swelling. Cells were then lysed by addition of Triton-X-100 to a final concentration of 0,5%, followed by a brief incubation (2 min in ice). Nuclei were then sedimented (1000 x g, 5 min, 4°C), washed once with lysis buffer, and finally resuspended ($5x10^9$ nuclei/200 μl) in extraction buffer (50mM Hepes pH 7.5, 0.5 mM EDTA, 100mM NaCl, 50µg/ml Leupeptin, 5 µg/ml, Pepstatin A, 1 mM Benzamidine, 10µg/ml Aprotinin, 1 mM DTT, 1 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride, 1 mM PMSF, 10 % Glycerol). Subsequently, NaCl was added until a final concentration of 500 mM followed by incubation (10 min on ice). The salt-insoluble fraction was sedimented (20000 x g, 30 min, 4°C) and the supernatant (i.e. the nuclear extract) was mixed with 1vol of water free glycerol, frozen in liquid N_2, and stored at –80°C until further use.

Magnetic particles (Dynabeads M-500 "Subcellular", Dynal, Oslo, Norway) were covalently coated with anti-antibodies according to the producer's manual. Briefly, 8μg of goat anti mouse- or goat anti rabbit-IgG (Dianova, Hamburg, Germany) were mixed with $1x10^7$ beads (resuspended in 0.1 M borate buffer pH 9.5) and incubated under slow top over bottom rotation for 48 h at 20°C. Subsequently, the beads were collected with a magnet, washed (2 x 15 min with PBS pH 7.4 with 0.1% BSA), and remaining free tosyl-groups were blocked (24 h at 20°C with 0.2 M Tris-HCl pH 8.5 and 0.1 %BSA). Beads were then washed and incubated with primary antibodies (5 μg IgG/$1x10^7$ beads, 60 min at 4°C, slow top over bottom rotation). After washing again, the beads were ready to use. They were stored at 4°C in PBS pH 7.4 with 0.1% BSA until the actual experiment. Primary antibodies were: rabbit antibodies against topo IIα (Genosys, Cambridge, UK), mouse monoclonal antibodies against topo IIβ (clone 3H10, (Meyer et al., 1997)), and mouse monoclonal antibodies against GFP (Roche, Indianapolis, USA).

For immuneprecipitation of topo II, $2x10^7$ beads were incubated for 2h at 4°C with nuclear extracts obtained from 10^9 cells in binding buffer (175 mM NaCl, 6 mM HEPES, pH 7.5, 2 mM EDTA, 14 % glycerol, 0.2 mM DTT, 0.5 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride, 10 µg/ml aprotinin, 5 % FBS). This amount of extract was sufficient to ligand all specific binding sites of the beads. Subsequently, the beads were collected with a magnet and washed with binding buffer (4 x 15min) and assay buffer (1 x 15 min) (120 mM KCl, 50 mM

Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1mM ATP, 0.5 mM DTT, 0.5 mM EDTA, 30µg/ml BSA). Topo II bound to the beads was subsequently assessed for topo II – specific DNA decatenation activity as described in section 3.8. It should be noted that Dynabeads M-500 Subcellular were found optimal for these experiments, whereas smaller variants of magnetic beads or sepharose gave less good separations.

3.8 Assessment of Topo II Activity

Immunoprecipitates were subjected to serial dilution and assessed for decatenation of *Crithidia fasciculata* kinetoplast DNA (kDNA, TopoGen Inc., Columbus, Ohio, USA). Beads were incubated for 2 h at 37°C with 1 µg kDNA in a final volume of 40 µl assay buffer (120 mM KCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1mM ATP, 0.5 mM DTT, 0.5 mM EDTA, 30µg/ml BSA). Negative controls included addition of 300 µM Na_3VO_4 (inhibiting topo II activity *via* poisoning the ATPase activity) and beads not incubated with nuclear extracts. The reactions were terminated by addition of SDS (0,5%) and subsequently digested with Proteinase K (60 min, 37°C). Finally samples were subjected to agarose gelelectrophoresis.

For a quantitative determination and comparison of the amounts of immunoprecipitated enzyme, $3x10^6$ beads were loaded with extract and eluted with 100 mM Na_3PO_4, 100 mM NaCitrate (pH 3) and 3 M urea. The eluted material was subjected to SDS-PAGE (6% homogeneous gels) and visualized by silver staining (Heukeshoven et al., 1986).

3.9 Microscopy

In all cases cells were grown on glass coated with poly-L-lysine (Sigma Chemie, Deisenhofen, Germany) until 40-60% of the substratum was covered.

3.9.1 Immunocytochemistry

Cells were grown on microscopic coverslips, washed in PBS, fixed in 2 % paraformaldehyde in PBS (15 min, 37°C), and permeabilized with 0.25 % Triton X-100 in the presence of 1% paraformaldehyde in PBS (10 min, RT). All subsequent steps were carried out at ambient temperature. After washing with PBS, cells were first blocked for 1 h in PBS, 2 % BSA, 5 % goat serum and then incubated for 1 h with topo IIα antibody KiS1 (mouse), topo IIβ antibody 3H10 (mouse), topo I antibody Scl70 (human) (Dunn laboratories, Asbach, Germany) or RNA polymerase I antibody S18 (human autoimmune serum, (Reimer et al., 1987)). All antibodies were diluted 1:500 in blocking solution (S18, was diluted 1:100). After washing, bound antibodies were counterstained by incubation for 1 h with Cy3™-conjugated goat anti-mouse or anti-human $F(ab')_2$ fragments (Dianova, Hamburg, Germany) diluted 1:1000. Coverslips were then washed three times for 5 min in PBS, the first washing cycle

including 80 ng/ml of 4,6-Diamidino-2-phenylindole (DAPI) to counter stain the genomic DNA. Slides were finally mounted in antifade solution (PBS containing 1.5% N-propyl-galate and 60% glycerol), and immediately inspected (see section 3.9.3).

3.9.2 Spreading of Native Chromosomes

10^7 exponentially growing cells were harvested, collected by centrifugation for 5 min at 160 x g, carefully resuspended in 6 ml hypotonic solution (0.075 M KCl), sedimented again, and resuspended in 400 µl hypotonic solution. Drops of about 50 µl were then spotted on a slightly tilted glass slide. Prior to this, slides were carefully cleaned in methanol and then soaked in double distilled water. After spreading, unattached cells were removed by letting 200 µl freshly prepared isotonic CSK buffer (100 mM KCl, 300 mM Sucrose, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride, 1 mM PMSF, 20 µg/ml Aprotinin, 10 µg/ml Pepstatin A) containing 80 ng/ml DAPI run down the slightly tilted slide. Spread chromosomes were then carefully mounted in the same buffer and imaged immediately (see section 3.9.3).

3.9.3 Fluorescence Microscopy

Epifluorescent images were acquired with appropriate filter sets (AHF Analysentechnik, Tübingen, Germany) at 630X magnification using an inverted microscope (Axiovert 100; Carl Zeiss, Göttingen, Germany) equipped with a cooled charge-coupled device camera (Sensys, Photometrics Ltd., München, Germany) and an additional 4X magnification lens.

For observation of living cells, they were grown and inspected in CO_2-independent medium (GibcoBRL/Life Technologies, Karlsruhe, Germany) using live-cell chambers (Bioptechs Inc., Butler, Pennsylvania) to keep the cells under microscope at 37 ˚C. It should be emphasized that strict temperature control was crucial for localization and mobility of topo II especially.

3.9.4 Confocal Laser Scanning Microscopy

Confocal imaging, FRAP and FLIP analyses of living cells were performed at 37 °C with a Zeiss LSM 510 inverted confocal laser scanning microscope equipped with a CO_2-controlled on-stage heating chamber using a heated 63x/1.4 NA oil-immersion objective and the FITC filter setting (488 nm / 515 nm) (Carl Zeiss, Göttingen, Germany).

3.9.5 Photobleaching

For fluorescence recovery after photobleaching (FRAP) experiments, a single optical section was acquired with 5.6 x zoom. Images were taken before and at 1.577 s time intervals after bleaching of a circular area at 20 mW nominal laser power with 3 iterations. The imaging scans were acquired with a laser power attenuated to 0.1 – 1 % of the bleach intensity. For quantitative FRAP analysis, fluorescence intensities of the bleached region, the entire cell nucleus and background were measured at each time point. Data were corrected for extracellular background intensity and for the overall loss in total intensity as a result of the bleach pulse itself and the imaging scans. Unless stated otherwise, FRAP recovery curves were generated by calculating the relative intensity of the bleached area I_{rel} as described (Phair and Misteli, 2000):

$$I_{rel} = (I_{\text{bleached spot}} \times I_{\text{entire cell nucleus at time 0}})/(I_{\text{bleached spot at time 0}} \times I_{\text{entire cell nucleus}})$$

The same laser settings were used for FLIP experiments where cells were repeatedly bleached and imaged at intervals of 15 s. FLIP depletion curves of selected areas outside the bleach spot were just corrected for the loss of fluorescence intensity caused by the imaging scans by comparison with the fluorescence intensity of a neighbouring cell nucleus.
FRAP and FLIP of mitotic cells was performed in the presence of 0.1 μM paclitaxel to reduce movements of the chromosomes.

3.9.6 Kinetic Modelling-Topo I

The computer software Prism (GraphPad Software Inc., San Diegeo, CA) was used for nonlinear regression analysis and plotting of FRAP-kinetics. Kinetic models assuming the coexistence of one, two, or three individual enzyme fractions with different mobility were tested. In all cases, best fits (according to R^2-value and F-test significance) were obtained when assuming the coexistence of two enzyme fractions with different mobility. Values of maximal recovery derived from nonlinear regression were used to calculate the proportion of the two enzyme fractions with different mobility, whereas a third, immobile enzyme fraction was calculated by adding up the two fractions to 100%.

4. Results

First I will describe in some detail our strategy of expressing bio-fluorescent chimera of topo I, IIα, and IIβ in a constitutive manner. I believe this to be important, because other groups trying to do the same experiment with different vector systems have encountered insurmountable problems. Subsequently, I will present data of the localization of topo IIα and IIβ in living cell nuclei at interphase, the relocation of the enzymes during cell division, the *in vivo*- mobility of the enzymes as determined by various photobleaching approaches, and the changes of these parameters, induced by specific topo II poisons. Finally I will present similar data obtained for GFP-chimera of topo I and various truncated versions of the enzyme that are suited to highlight the biological role of the non-conserved N-terminus of the enzyme. Most of these results have been published (Christensen et al., 2002a; Christensen et al., 2002b). Whenever published data are presented, a reference is included in the Figure legends.

4.1 Construction of Bicistronic Expression Constructs

The generation of stable cell lines demands the simultaneous expression of a selectable marker and the gene of interest, classically achieved with two independent sets of regulatory elements. Thus, the physical and functional independence of the selection marker from the gene of interest allows the cell to express the one but not the other.

In the case of topo II this problem has been encountered in a particularly severe manner. Attempts at stable heterologuous expression of topo II in mammalian cells have produced cells that either expressed topo II in a transient manner or at levels to low for observation (Asano et al., 1996; Eder et al., 1993; McPherson et al., 1997). The apparent counterselection against stably transfected cells was explained with a pronounced sensitivity of the cells to alterations in the total amount of active topo II in the cell nucleus. Current belief holds that cell-cycle-related regulation of topo II expression is essential for the enzyme to function properly in the context of the living cell nucleus. Override of cell-cycle-related modulations of topo II levels by an unregulated source of expression is therefore likely to harm the cells. Not unexpectedly, deregulation of topo IIα expression has indeed been found to induce apoptotic cell death, most probably by disrupting the temporal order of events mediated by the enzyme during cell division (McPherson and Goldenberg, 1998). Taking all this into account, it seemed an essential prerequisite of this study first to devise an expression system that would overcome these problems.

In order to do so, I used a vector system that provides a fixed linkage between the expression of the selection marker and the expression of the gene of interest. The discovery of the function of the 5'-untranslated regions of transcripts made by picornaviruses provided the tool for this purpose. This region triggers translational initiation of the uncapped viral transcript by directing initiating ribosome's to a defined start codon about 600-1200 nucleotides downstream and was consequently termed internal ribosome entry site (IRES).

The IRES element has been shown to function in artificial bicistronic mRNAs. When placed between two open reading frames, it mediates the translational initiation of the distal cistron (Molla et al., 1992; Pelletier and Sonenberg, 1988). Using this property of the IRES element, I constructed a vector system where expression of topoisomerases is physically linked to expression of the selection marker *via* the translation of a single bicistronic mRNA. This linkage ascertains that cells have to maintain heterologous coexpression of the selection marker and a topoisomerase. Thus, they are forced to compromise between expressing enough of the one and not too much of the other, when put under selection pressure.

4.2 Dynamics of Human DNA Topoisomerases IIα and IIβ in Living Cells

Human embryonal 293 kidney cells were transfected with pMC-EGFPP, pMC-TIIα-EGFPP and pMC-TIIβ-EGFPP, bearing in the first cistron GFP alone or hybrid genes of topo IIα or topo IIβ fused at their C-termini to GFP, and in the second cistron the selection marker *pac*. Stable cell lines expressing either GFP alone, topo IIα/GFP, or topo IIβ/GFP were then generated by selection of the transfected cells in the presence of 0.35 μg/ml puromycin. These three cell lines were maintained as mixed populations and not separated into individual clones. Significantly less puromycin-resistant cells emerged from transfection with topo II-GFP than from transfection with the GFP gene alone, attesting to a narrow tolerance margin for transgenic expression of topo II. This observation might reflect that the cells are unharmed by a high expression of GFP, whereas cells expressing a high level of topo II-GFP undergo cell-death, and only cells with a low expression survive. However, cell lines supporting constitutive expression of topo IIα-GFP or topo IIβ-GFP had growth rates and morphologies similar to untransfected cells or cells expressing GFP alone. This suggested a *quasi* physiological expression of the GFP-tagged topo II species suitable for fluorescence studies in living cells, provided that the fusion proteins were active, not over expressed, and colocalized with their endogenous counterparts. Control experiments addressing these issues are addressed in the following 3 sections.

4.2.1 Expression of Full-length Topoisomerase IIα and IIβ-GFP Chimeras

We performed immunoblotting of the cell lines expressing topo IIα-GFP (Fig. 4.1, lane 3) and topo IIβ-GFP (Fig. 4.1, lane 4). Untransfected cells (Fig. 4.1, lane 1) and GFP-expressing cells (Fig. 4.1, lane 2) served as controls. To assess the integrity of the fusion proteins and to compare their relative expression levels, the blots were probed with GFP-antibodies (Fig. 4.1, top panel). In transfected cells (lanes 3 and 4), chimeric topo IIα and IIβ were detected as single protein bands of the expected size (arrow), but were absent in untransfected cells (lane 1) and in cells expressing GFP alone (lane 2). Thus, we could exclude rearrangements of the chimeric genes, and ascertain that green fluorescence of the cells was due to full-length topo II-GFP chimera. Topo IIα-GFP and topo IIβ-GFP appeared to be expressed in similar amounts (compare lanes 3 and 4), allowing a comparison of data between these cell lines.

To compare expression levels of GFP-fused topo II isozymes with the endogenous enzymes, blots were probed with isoform-specific antibodies against topo IIα (Fig. 4.1, second panel) or topo IIβ (third panel). The GFP chimera could clearly be discriminated from the corresponding endogenous enzymes as additional bands of slower migration (arrows).

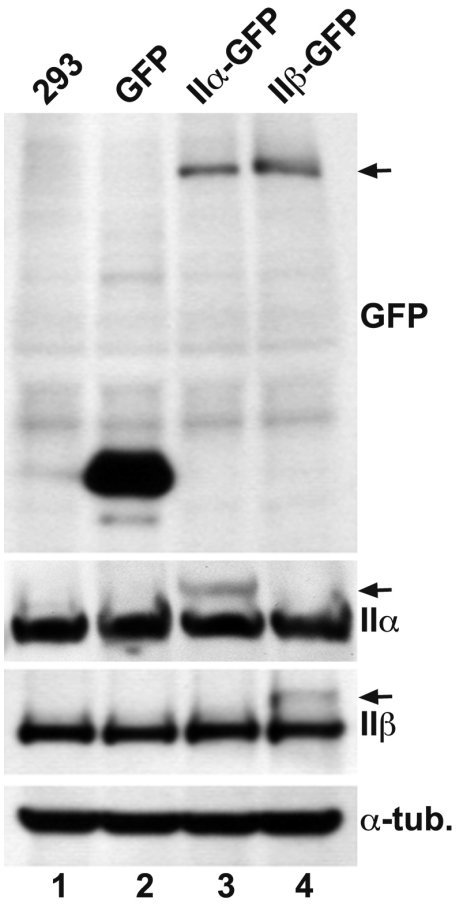

Figure 4.1: **Expression of topo II-GFP in HEK 293 cells.** Western analysis of untransfected 293 cells and cells expressing GFP, topo IIα-GFP, or topo IIβ–GFP. Blots were probed with GFP antibodies (*upper panel*), or antibodies against human topo IIα or topoIIβ *(middle panels*), or antibodies against α–tubulin serving as loading control (*lower panel*). Arrowheads on the right margin indicate positions of topo II-GFP chimera. Figure adapted from (Christensen et al., 2002b).

From comparison within each lane, it became evident that they were expressed at much lower levels than the endogenous proteins. Noteworthy, the amount of endogenous topo IIα and IIβ were the same in transfected and untransfected cells (compare lane 1 with lanes 3 and 4) demonstrating that a normal regulation of endogenous topo II expression is maintained in all stable cell lines.

In conclusion, the expression system is suitable for the stable expression of moderate levels of GFP-tagged topo II isoforms without affecting the normal cell morphology or cell-cycle-progression. In this context, it is of interest that heterologeous expression of topo IIα-GFP from a conventional vector (Mo et al., 1998; Mo and Beck, 1999) resulted in a transient doubling of the total amount of topo II in cell, but did not allow for establishing stable cell lines. In contrast, we achieved here by way of the bicistronic vector a balance between the expression of topo II (sufficiently low to not induce apoptosis) and the selection marker (high enough to allow growth in the presence of selection pressure).

4.2.2 *Topoisomerase IIα- and IIβ-GFP Chimera Have The Same Enzymatic Activity as Their Endogenous Counterparts.*

Since protein functions could be disrupted by fusion to GFP, we wanted to ascertain that the topo II-GFP chimera had the same activity as the endogenous enzymes.

A major role of topo II *in vivo* is to catalyse the double-stranded cleavage of DNA, allowing passage of a second DNA duplex through the break. This activity requires ATP and is necessary for separating catenated DNA dublexes found at the end of replication. The decatenation of DNA molecules is a topo II-specific reaction which can be used to assay topo II activity *in vitro* (Marini et al., 1980).

We separated endogenous and GFP-fused enzyme by immunoprecipitation in order to compare their *in vitro* DNA-decatenation activities in quantitative terms. GFP-directed immunoprecipitates contained GFP-fused and unfused topo II in equal proportions (Fig. 4.2, inserts, lanes 2 and 4), suggesting heterodimers of GFP-fused and endogenous topo II. That topo IIα-GFP and IIβ-GFP form dimers with their respective endogenous counterpart suggest that GFP does not disturb the formation of the topo II dimer. A quantitative determination of topo II-specific DNA decatenation activity in the immunopricipitates was achieved by serial dilution and subsequent assessment of kDNA-decatenation (Fig. 4.2). In the catenated form kDNA cannot enter an agarose gel, whereas single circles released by topo II from the catenated network will migrate into the gel. The specific decatenation activity is characterized

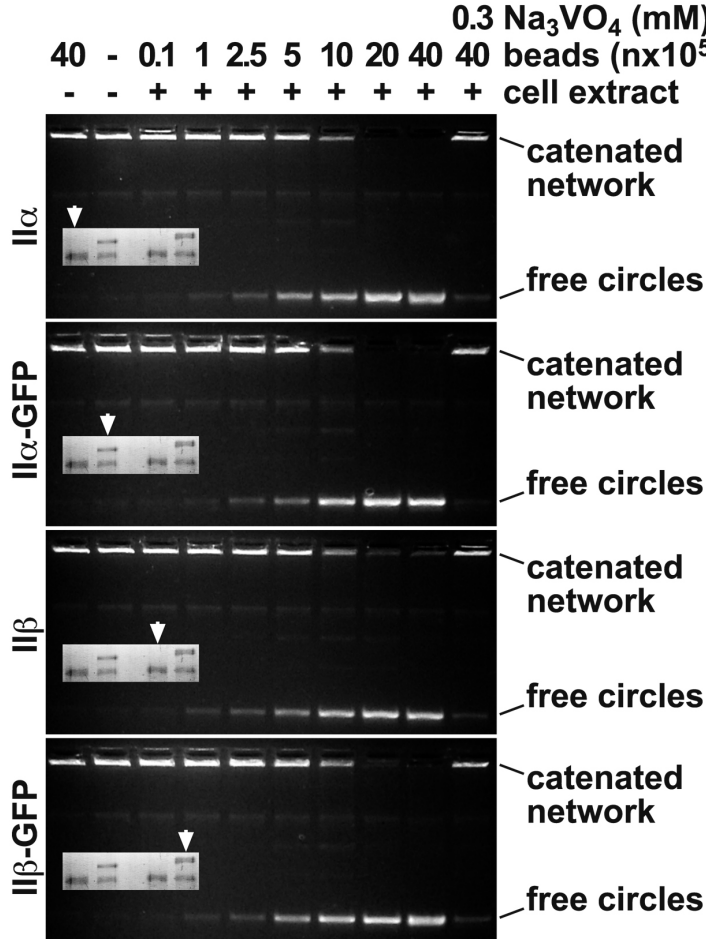

Figure 4.2: **DNA-decatenation activity of GFP-fused and endogenous topo II.** GFP-directed immunoprecipitates from cells expressing topo IIα-GFP or topo IIβ-GFP and immuno-precipitates from untransfected cells directed at topo IIa or IIb were subjected to serial dilution and assessed for kDNA-decatenation. SDS-PAGE of the immuno-precipitates is shown four times in the inserts with arrowheads identifying those lanes that correspond to the respective decatenation assay. Figure adapted from (Christensen et al., 2002b).

by the limiting dilution at which free circles are still to be seen. Upon comparing the DNA-decatenation activity of these mostly heterodimeric immunoprecipitates with similar amounts of endogenous enzymes obtained from untransfected 293 cells by topo II-directed immunoprecipitation (Fig. 4.2, inserts, lane 1 and 2, resp.), it appeared that topo IIα (Fig. 4.2, middle-top) and IIβ (Fig. 4.2, bottom) containing transgenic, GFP-fused subunits had the same activity as those composed entirely of unfused subunits encoded by the endogenous genes (Fig. 4.2, top and middle-bottom, resp.). Decatenation activity could in all cases be inhibited by the ATPase-inhibitor orthovanadate (outmost lanes on the right). Thus, fusion to GFP did not alter the catalytic properties of the enzymes.

To ascertain in addition that the GFP-fused enzymes were also active in the cells, we employed immuno-band depletion (Fig. 4.3), which is based on the fact that the transient, covalent complex between topo II and genomic DNA is stabilized by specific topo II poisons (Liu, 1989). As a consequence, topo II-specific signals are depleted in western analyses due to retention of covalent topo II·DNA complexes in the gel slots. Untransfected and transfected 293 cells were treated with the topo II-poison teniposide (VM 26), and subjected to immunoblotting using GFP-antibodies (Fig. 4.3, top). Obviously, the treatment depleted topo II-fused GFP bands from the blots (compare lanes 5 to 6 and 7 to 8). The extent of depletion was similar to that of endogenous topo II (data not shown). GFP alone (compare lanes 3 and 4 in top panel), or α-tubulin (bottom) was not depleted by VM 26, attesting to the specificity of the assay. These results confirmed that topo II-GFP chimera were as active as endogenous topo II because they formed the same amount of catalytic intermediates in the genome of the transfected cells.

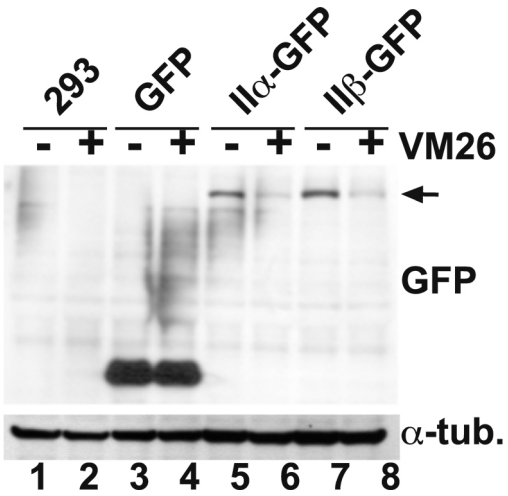

Figure 4.3: **Topo II immuno-band depletion assay**. The same cell lines as in Fig. 4.2 were cultured 1 h in medium containing 200 μM teniposide (+ *VM 26*) or in plain medium (- *VM 26*). Cells were then lysed, subjected to Western blotting and the blots were probed with GFP-antibodies (upper panel) or α-tubulin (lower panel). The position of topo II-GFP is indicated on the right margin by an arrow. Figure adapted from (Christensen et al., 2002b).

Transient expression has shown that fusion to GFP at the C-terminus does not abolish the ability of topo IIα to bind and cleave genomic DNA *in vivo* (Mo and Beck, 1999). However, such findings do not necessarily imply that biofluorescent topo II chimera are also able to provide all enzymatic functions required for regulated and complex processes like chromosome segregation. To judge the global functionality of topo II-GFP in mitosis, we tested complementation of topo II function in *S. cerevisiae* topo II-deletion strains. It has been shown that human topo IIα and IIβ are able to complement mitotic growth in *S. cerevisiae* (Jensen et al., 1996b), and that such an assay can be used to test for functionality of mutant

topo II versions in yeast (Jensen et al., 1996a). We have tested whether human topo IIα-GFP and IIβ-GFP rescue a yeast topo II deletion (Fig. 4.4). This assay demonstrated that GFP chimera of human topo IIα and topo IIβ were fully capable of supporting the mitotic growth of *Δtop2* yeast upon eradication of the endogenous salvage plasmid encoding *S.pombe* topo II (+5-FOA), whereas cell growth was abolished in the absence of an additional topo II construct. Apparently, complementation by GFP chimera was as good as by untagged topo II. Thus, the functionality of human topo II is fully preserved in the corresponding GFP chimera.

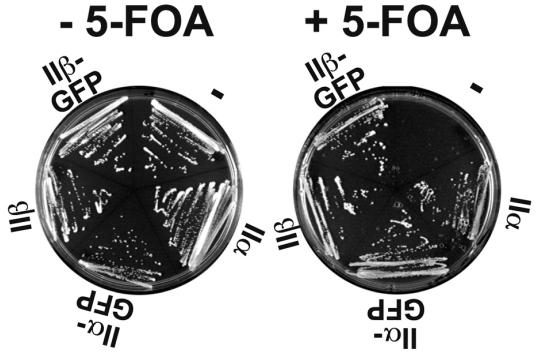

Fig 4.4: **Yeast complementation assay:** The *S. cerevisiae* strain BJ201 carrying a disrupted endogenous TOP2 gene which is substituted by the *S.pombe* Top2 gene on a URA3-based plasmid, was transformed with LEU2 plasmids carrying either the TPI-promoter alone (-) or TPI-promoted topo II cDNAs encoding topo IIα, topo IIα-GFP, topo IIβ, and topo IIβ-GFP, respectively. After growth on selective plates (without Leu), single clones were streaked on plates with 5-FOA (*right*) to select for clones that had lost the *S.pombe* topo II-expressing plasmid. Plates without 5-FOA served as a control (*left*). Mitotic growth of cells transformed with the topo II-expression plasmids on 5-FOA plates indicates that the essential topo II activity is likewise provided by GFP-tagged and untagged versions of human topo II isoforms. Figure adapted from (Christensen et al., 2002b).

4.2.3 Colocalization of GFP-Chimera with Endogenous Topoisomerase II

Although we show that the catalytic activity of topo II (Figs. 4.2 and 4.3) and the functionality of the enzyme in the cell (Fig. 4.4) is not compromised by a fusion to GFP, we cannot exclude that cellular targeting of the enzymes is affected. Therefore, we compared the distribution patterns of GFP-tagged topo II and the endogenous enzyme by indirect immunofluorescence.

The rational of this experiment is that the antibodies detect both endogenous and GFP fused topo II. By comparing the antibody- and the GFP-derived fluorescence we should be able to determine whether the GFP-tagged topo II colocalizes with the pattern of endogenous enzyme. Fig. 4.5 shows a typical outcome of such an experiment with examples of mitotic and of interphase cells labelled with isozyme specific topo II antibodies. Untransfected cells (Fig. 4.5, both panels, top) gave rise to antibody-derived signals only, whereas cells expressing topo IIα-GFP (Fig. 4.5, left, bottom) or topo IIβ-GFP (Fig. 4.5, right, bottom) also emitted GFP fluorescence. The distribution patterns of GFP fluorescence (specific for GFP fusion proteins only) and immunofluorescence (specific for fused and endogenous enzymes)

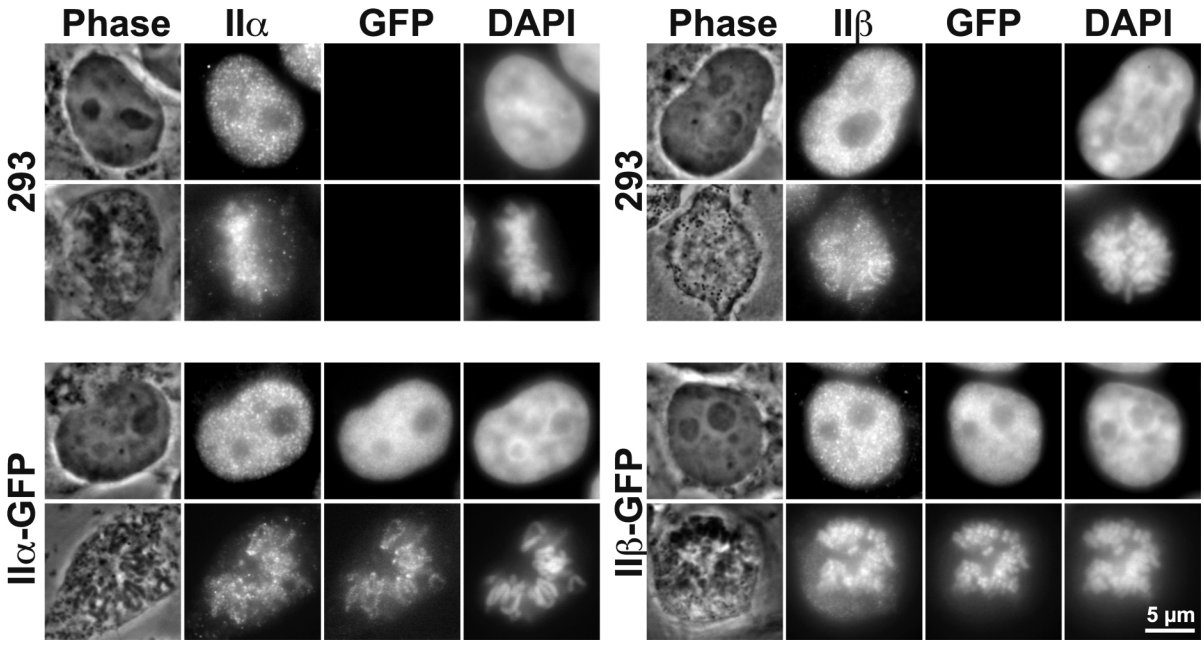

Figure 4.5: **Colocalization of GFP fusion proteins with endogenous topo IIα and IIβ by indirect immunofluorescence microscopy.** Untransfected 293 cells (top) and topo IIα- (bottom, left) or topo IIβ-GFP-expressing cells (bottom right) were grown on microscopic slides, PFA-fixed, permeabilized, and doublestained with isoform-specific topoII-antibodies (*columns IIα and IIβ, resp.*) and DAPI. Corresponding images of phase contrast (*Phase*) and green fluorescence (*GFP*) are shown. From each staining combination one representative cell in interphase (*upper image*) and pro-/metaphase (*lower image*) is shown. Figure adapted from (Christensen et al., 2002b).

were virtually identical, attesting to the fact that the GFP chimera colocalized with their endogenous counterparts. In addition, distribution patterns of topo II in non-transfected and in topo II-GFP-expressing cells were identical (Fig. 4.5, compare top and bottom portion), making it unlikely that the GFP chimera disrupted localization of the endogenous enzymes.

It should be noted that the patterns shown in Fig. 4.5 do not represent the true localization of topo IIα or topo IIβ in living cells, because these cells were fixed and permeabilized which is known to alter the cellular distribution of topo II in an unpredictable fashion (Chaly and Brown, 1996). In fact, when we applied different protocols for permeabilization and/or fixation, we also obtained patterns different from those shown in Fig. 4.5, but in each case antibody staining and GFP fluorescence were identical. The colocalization of topo II- and GFP-epitopes in fixed cells shows that the cellular targeting of topo II-GFP chimera is identical to that of the endogenous enzymes. Thus, the GFP-tagged enzymes should also reflect faithfully the localization of topo II in living cells.

4.2.4 Distribution of Topoisomerase IIα and IIβ in the Interphase Nucleus

Monitoring of topo IIα-GFP and topo IIβ-GFP in living cells by GFP-fluorescence revealed a very similar distribution of the isozymes in interphase nuclei (Fig. 4.6): Both were

exclusively in the nucleus, where they resided in the nucleoplasm and the nucleoli. The proper nuclear localization demonstrates that both topo II-GFP isoforms have a functional NLS, which indicates a proper folding of the two fusion proteins. The minor, nucleoplasmic subpopulation had a slightly uneven distribution. The major fraction of both isoforms accumulated in the nucleoli. It had a cloudy but otherwise unstructured distribution in the entire nucleolar space, which distinctively excluded small globular compartments, imposing as dark holes within the brightly fluorescent nucleolus (Fig. 4.6, insert, arrows). These globular compartments have not yet been unambiguously identified. It is, however, likely that they reflect an exclusion of topo II from the fibrillar centers.

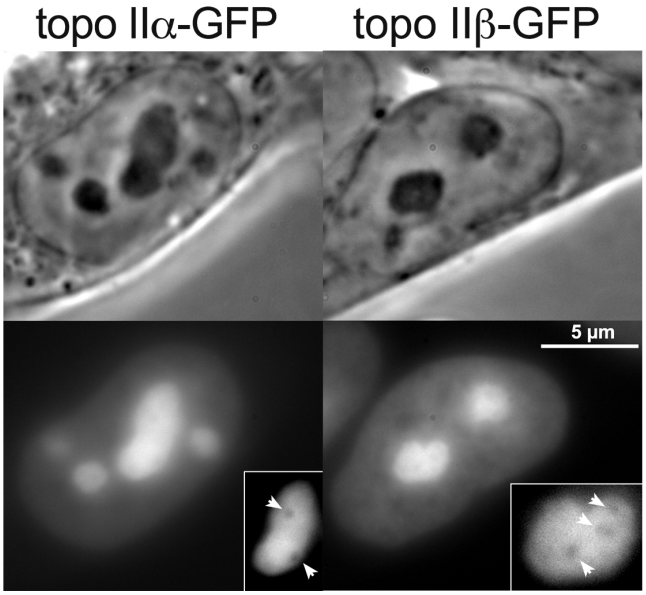

Figure 4.6: **Distribution of topo II-GFP in interphase nuclei.** Phase-contrast images (top) and corresponding green fluorescence (bottom) of living 293 cells expressing topo IIα-GFP (*left*) and topo IIβ-GFP (*right*). The insert shows images of selected nucleoli subjected to 2-fold magnification and contrast enhancement. Arrowheads indicate areas of decreased fluorescence intensity within the nucleoli. Figure adapted from (Christensen et al., 2002b).

The obvious discrepancy between the true cellular localization of topo II in interphase shown in Fig. 4.6 and the localization patterns obtained by indirect immunofluorescence in Fig 4.5 clearly supports my hesitations stated earlier with respect to immunofluorescent localization studies of topo II.

4.2.5 Isoform Specific Localization of Topoisomerase II in Mitosis

Fig. 4.7 shows time lapsed images of topo IIα-GFP (left) and topo IIβ-GFP (right) in cells proceeding from metaphase (0') to early G1-phase (90'). Topo IIα-GFP was mostly chromosome-associated from metaphase until telophase (0-10 min). The enzyme distinctively labelled chromosomal structures, whereas the mitotic cytosol contained only a minor fraction. In contrast, association of topo IIβ-GFP with chromosomes varied during mitosis. In metaphase (0 min) the enzyme was mostly in the cytosol. It started to associate with the chromosomes as soon as they were segregated (4 min) and continued to accumulate there until cytokinesis (18 min). From then on and throughout interphase the two isoforms behaved similarly. During nuclear reorganization in early G1-phase (18 – 90 min), they concentrated in small, light-dense structures (18 and 28 min), which later on (45 and 90 min) gradually

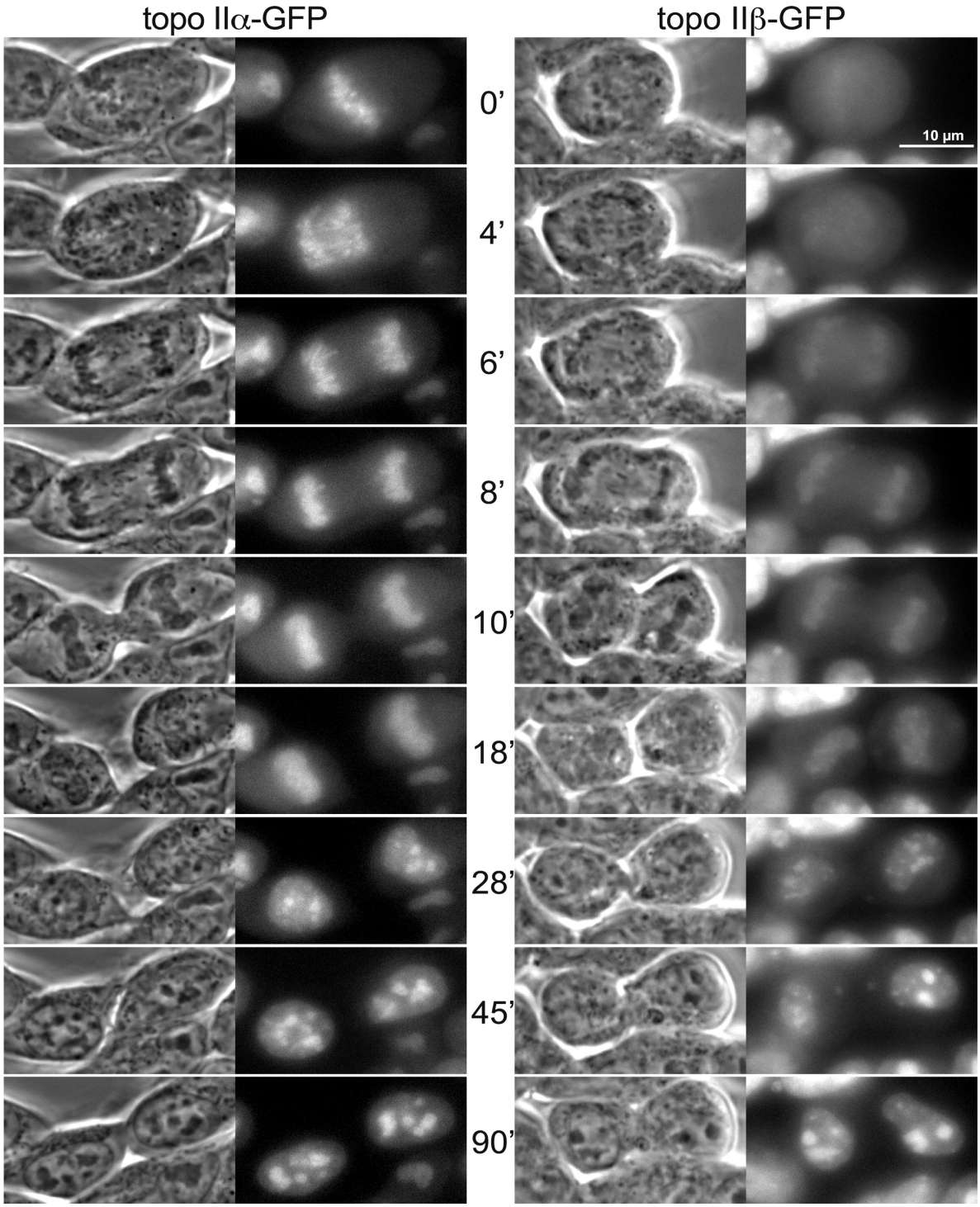

Fig 4.7: **Time-lapse imaging of topo II isoforms during mitosis.** Cells expressing either topo IIα-GFP (*left*) or topo IIβ-GFP (*right*) were grown on the microscope stage at 37 °C and imaged at subsequent time points as indicated. Phase-contrast images and corresponding green fluorescence of representative cells are shown during progression from metaphase to early G1-phase. Figure adapted from (Christensen et al., 2002b).

assembled into nucleoli. Numerous structures stained by topo II-GFP in the reforming nucleolus at late telophase are most likely connected to reforming nucleoli. Reformation of the nucleolus, often termed nucleologenesis, takes place in two stages. First, small

prenucleolar bodies (PNBs) are formed that contain various nucleolar proteins including fibrillarin, B23 and nucleolin (Bell et al., 1992; Jimenez-Garcia et al., 1994). When transcription is initiated, the PNBs associate with the other nucleolar components to form the complete nucleolus (Benavente et al., 1987). It is interesting to note that the structures stained by topo II at early G1-phase (e.g. 28 min) resemble those of established marker proteins of prenucleolar bodies (e.g. B23; (Olson et al., 2000)), suggesting that both topo II isozymes are present at PNBs and participate in postmitotic reassembly of nucleoli.

4.2.6 Accumulation of Topoisomerase IIα at the Centromeres is Lost Upon Sister Chromatid Separation

Previous studies have shown an upconcentration of topo IIα at the centromers during pro- and metaphase (Rattner et al., 1996; Sumner, 1996; Taagepera et al., 1993). However, due to the dense chromosome packing, we could not resolve the sub-chromosomal localization of topo IIα in metaphase plates of living cells (Fig. 4.7). To overcome this problem, metaphase chromosomes were spread out by gentle mechanical compression of the cells (Fig. 4.8), which revealed a centromeric accumulation of topo IIα-GFP in pro/metaphase (Fig. 4 8, left, arrowheads) in the still living cells. In anaphase, however, the enzyme appeared to be evenly distributed over the whole chromosome (Fig. 4.8, right). Taken together, these observations demonstrate that topo IIα is located at centromers during condensation but is released when the centromeric DNA is segregated at anaphase, which is in good agreement with the immunohistochemical findings described above and supports the idea that in metaphase (at the onset of chromatid segregation) topo IIα has its major role in separating the last catenates of centromeric DNA. The upconcentration of topo II at the centromere could be due to a requirement of a high enzymatic activity during the segregation of the centromeric heterochromatin. This could ensure that separation of the centromeric regions occurs

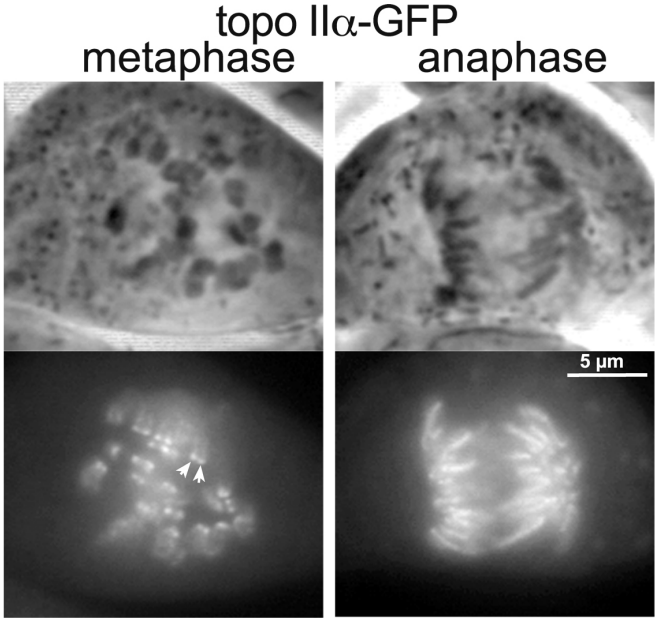

Fig 4.8: **Centromeric accumulation of topo IIα at metaphase is lost upon transition to anaphase.** Topo IIα-GFP-expressing cells were grown on cover slides, then flattened by placing the slide with adherent cells directly onto a glass slide, and imaged directly thereafter. Representative examples of cells at metaphase (*left*) and anaphase (*right*) are presented by phase contrast (*top*) and corresponding green fluorescence of GFP (*bottom*). Arrowheads indicate an example of centromeric accumulation of topo IIα, as characterized by two pairs of dots per chromosome. Figure adapted from (Christensen et al., 2002b).

simultaneously and fast in all chromosomes. In this context, it is also of interest to see here how fast the enzyme is lost from the centromere, considering the rapid progression of cells from metaphase to anaphase (comp. Fig. 4.7).

4.2.7 Topoisomerase IIβ Interacts with Metaphase Chromosomes, Too.

The data in Fig. 4.7 show that topo IIβ does not accumulate on chromosomes before the onset of anaphase. This could either mean that the enzyme is altogether excluded from the condensed chromatin in metaphase, or that a large cytoplasmic pool of topo IIβ obscures a smaller, chromosome-associated fraction. It is established that GFP alone is excluded from dense cellular structures such as the condensed chromosomes. To find out if this is also the case for topo IIβ, we compared by high-resolution confocal microscopy metaphases of cells expressing GFP alone and cells expressing topo IIβ-GFP (Fig. 4.9). The untagged, freely diffusible protein GFP was clearly excluded from densely packed metaphase chromosomes (Fig. 4.9, left), whereas this was not the case for topo IIβ-GFP (Fig. 4.9, right). Here, the

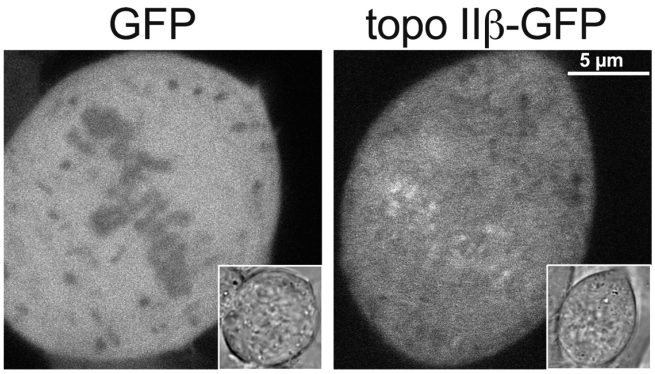

Figure 4.9: **Topo IIβ is not completely excluded from mitotic chromosomes.** A single high-resolution, confocal section of metaphase cells expressing either GFP alone (*left*) or topo IIβ-GFP (*right*) is shown (inserts: images of transmitted light of the same cells). Figure adapted from (Christensen et al., 2002b).

metaphase plate exhibited the same fluorescence intensity as the surrounding cytosol and was even highlighted by more intense speckles. It is likely that this represents an upconcentration of topo IIβ-GFP at centromers. As that may be, the pattern clearly argues against an exclusion of topo IIβ from metaphase chromosomes. It rather suggests that a minor fraction of the enzyme is chromosome-associated at this stage of mitosis.

However, it still remained unclear if the subfraction of topo IIβ at the metaphase chromosomes is a static fraction. Alternatively, the weak topo IIβ signal in metaphase plates could represent a dynamic equilibrium between a cytoplasmic and chromosomal population. Evidence for the latter assumption came from metaphase cells treated with the topo II-specific drug VM 26 (Fig. 4.10). VM 26 caused a rapid redistribution of both topo IIα-GFP (Fig. 4.10, top) and topo IIβ-GFP (bottom) from the cytosol to the chromosomes, indicating that the drug efficiently trapped both isozymes on chromosomal DNA. It is of interest that chromosomal accumulation induced by VM 26 was faster and more complete for topo IIα than for topo IIβ. In summary, these data indicate that during mitosis topo IIβ is able to engage in DNA-turnover, but the enzyme is clearly biased to the cytosolic state. In addition, the rapid

redistribution of both isoforms from the cytosol to the chromosomes after treatment with VM 26 suggests a dynamic interchange between the two populations thus identifying these enzymes as highly mobile constituents of the mitotic cell.

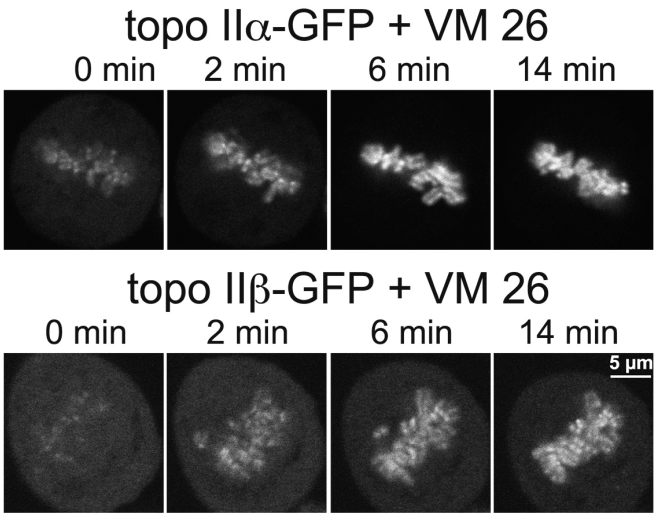

Figure 4.10: **Topo IIα and topo IIβ accumulate on mitotic chromosomes upon treatment with VM 26.** Cells expressing topo IIα-GFP (*top*) and IIβ-GFP (*bottom*) were imaged by confocal microscopy before (*0 min*) and at the indicated time points after the addition of 100 µM VM 26. Figure adapted from (Christensen et al., 2002b).

4.2.8 Mobility of Topo IIα and IIβ in Interphase Cells and the Effect of Topo II Poisons.

The dramatic changes in localization induced by VM 26 clearly demonstrated that topo IIα and IIβ are dynamic and highly mobile enzymes. To follow up on the mobility of topo IIα and topo IIβ in living cells, we employed photobleaching techniques. Fig. 4.11 summarizes a series of experiments, determining the mobility of topo IIα and IIβ in interphase nuclei *via* kinetics of fluorescence recovery after photobleaching (FRAP) (White and Stelzer, 1999). Topo II-linked GFP-fluorescence was bleached irreversibly in circular areas (Ø = 1 µm) by high-powered laser pulses and fluorescence recovery in the bleached spots as a consequence of topo II-GFP molecules moving in from unbleached areas was recorded over time. It is readily apparent from time-lapsed fluorescent images (Fig. 4.11, top) and quantitative plots of recovery kinetics (Fig. 4.11, bottom) that fluorescence recovery after photobleaching of a nucleoplasmic or a nucleolar area was in each compartment fast and complete for both topo II isoforms. This suggests that immobile molecules are virtually absent. Recovery kinetics of topo II-GFP were much faster than those of GFP-histone H3, a member of nucleosomal core proteins (Fig. 4.11, bottom, Ctrls.) known to be firmly immobilized on chromatin (Phair and Misteli, 2000). On the other hand, both topo II isozymes were by no means as freely diffusible as unfused GFP, which exhibited recovery kinetics even too fast to be recorded with our experimental settings (Fig. 4.11, bottom, Ctrls.). Moreover, recovery of topo IIα-GFP and topo IIβ-GFP was notably slower in the nucleoli ($t_{1/2}$ = 6.2 s and 10 s, respectively) than in the nucleoplasm ($t_{1/2}$ = 1.8 s and 3 s, respectively), suggesting less mobile enzyme subpopulations in the nucleoli. This and the slight difference in mobility between the isozymes (topo IIβ was somewhat slower) raised the question of what controls

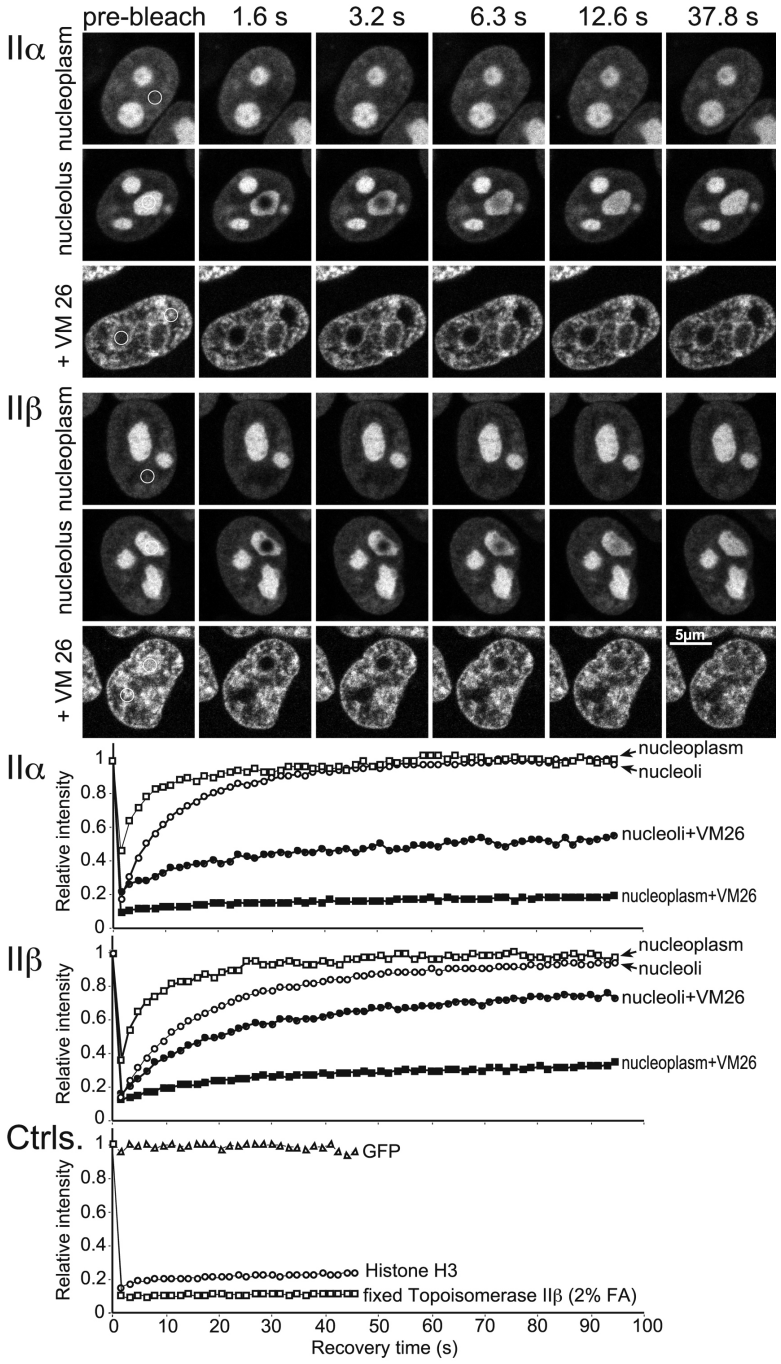

Figure 4.11: **FRAP analysis of topo II-GFP.** Cells expressing GFP chimera of topo IIα and topo IIβ were bleached for 1.8 s. Images were taken before bleaching and at the indicated time points after the end of the bleach pulse. The area to be bleached is indicated by a circle in the pre-bleach panels. In VM 26-treated cells a nucleolar or a nucleoplasmic region was bleached simultaneously in the same cell, whereas in untreated cells each compartment was bleached in separate cells. Corresponding quantitative data of fluorescence recovery kinetics are plotted below. Fluorescence intensities in the bleached region were measured and expressed as the relative recovery over time. Values represent means from at least 6 individual cells and 3 independent experiments. Standard deviations were in each case less than 5 % of the mean values (not displayed). FRAP kinetics of freely diffusible GFP, chromatin-bound histone H3-GFP and formaldehyde-fixed topo IIβ-GFP served as controls. Figure adapted from (Christensen et al., 2002b).

topo II mobility and which role DNA interactions play in this respect. We could address this issue experimentally, since covalent topo II·DNA intermediates are stabilized by specific agents such as VM 26 (compare Figs. 4.3 and 4.10). VM 26-treatment induced a profound redistribution of topo II in the nucleus, which was similar for both isoforms (Fig. 4.11, top panels, bottom rows). Nucleoli became largely depleted, and almost the entire enzyme pool concentrated at distinct sites in the nucleoplasm, forming there a granular pattern. FRAP-analysis revealed that topo II was clearly much less mobile at these granular nucleoplasmic sites. Notably, immobilization was less pronounced for topo IIβ, which still exhibited a slight ascension of the recovery curve, whereas topo IIα did not. This is in good agreement with previous pharmacological studies, showing that topo IIβ is less sensitive to VM 26 (Drake et al., 1989). In comparison, the small amount of topo IIα and IIβ remaining inside the nucleoli

after VM 26 treatment retained a much higher mobility and, here again, topo IIβ was more mobile (less affected by VM 26) than topo IIα. These data demonstrate for the first time directly that the vast majority of topo IIα and topo IIβ molecules present in a living cell becomes indeed trapped by VM 26 in its covalently DNA-bound intermediate state, which must be relieving for oncologists, relying on this mechanism of action for tumour therapy. These findings also suggest that nuclear sites where topo II normally accumulates (Fig. 4.6) do not necessarily represent sites where the enzymes are most actively engaged in DNA-catalysis. This is most evident in nucleoli where topo II concentration is highest (Fig. 4.6), enzyme mobility is lowest (Fig. 4.11, untreated cells), but entrapment by VM 26 is least efficient (Fig. 4.11, VM 26-treated cells).

It should be mentioned that we do not know if VM 26 gains equal access to nucleolar DNA and to nucleoplasmic DNA. However, it is known that VM 26 induces double stranded cleavage of rDNA inside the nucleoli (Govoni et al., 1995). Based on the assumption that VM 26 indeed has equal access to both compartments, we conclude that interactions with genomic DNA do not constitute the major determinant of topo II localization or mobility in interphase cells.

4.2.9 Topoisomerase II is Freely Moving Between Interphase Compartments.

Although the results in Fig 4.11 clearly demonstrate that topo II is highly mobile within each nuclear compartment (full recovery in nucleoli and nucleoplasm), they do not directly prove that topo II is moving between nuclear compartments in an unrestricted manner. The way to address this question is to bleach an area inside one nuclear compartment and to measure the fluorescence recovery in the bleached compartment (FRAP) and the loss of fluorescence in a second nuclear compartment (fluorescence loss in photobleaching, (FLIP)). In the case of free traffic between compartments the relative loss in intensity should be the same for the bleached and unbleached compartment after full recovery of the bleached compartment. The results of such a combined FRAP/FLIP analysis are shown in Fig. 4.12. First, an entire nucleolus was bleached in a circular area (Ø = 3 μm). Subsequently, we recorded by sequential imaging scans the fluorescence recovery of the bleached nucleolus and the loss of signal intensity in an unbleached nucleolus and in a nucleoplasmic area of the same cell. To enable a comparison of fluorescence recovery in the bleached nucleolus (FRAP) and of fluorescence loss in the selected reference areas (FLIP), quantitative data were plotted without the usual correction for overall loss of fluorescence due to the bleach pulse, giving rise to the slightly unusual drawings shown in Fig. 4.12. The plots for topo IIα-GFP (Fig. 4.12, top) and topo IIβ-GFP (Fig. 4.12, middle) show that in both cases the fluorescence of the bleached nucleolus recovered at the expense of the two neighbouring areas, which decreased by the same rate. The three curves rapidly reached equilibrium, and the equilibrium values were virtually the same, indicating that the mean fluorescence intensity of nucleoli, having completely recovered from bleaching, was identical to unbleached ones, having lost a corresponding amount of fluorescence over time. Moreover, unbleached nucleoli were

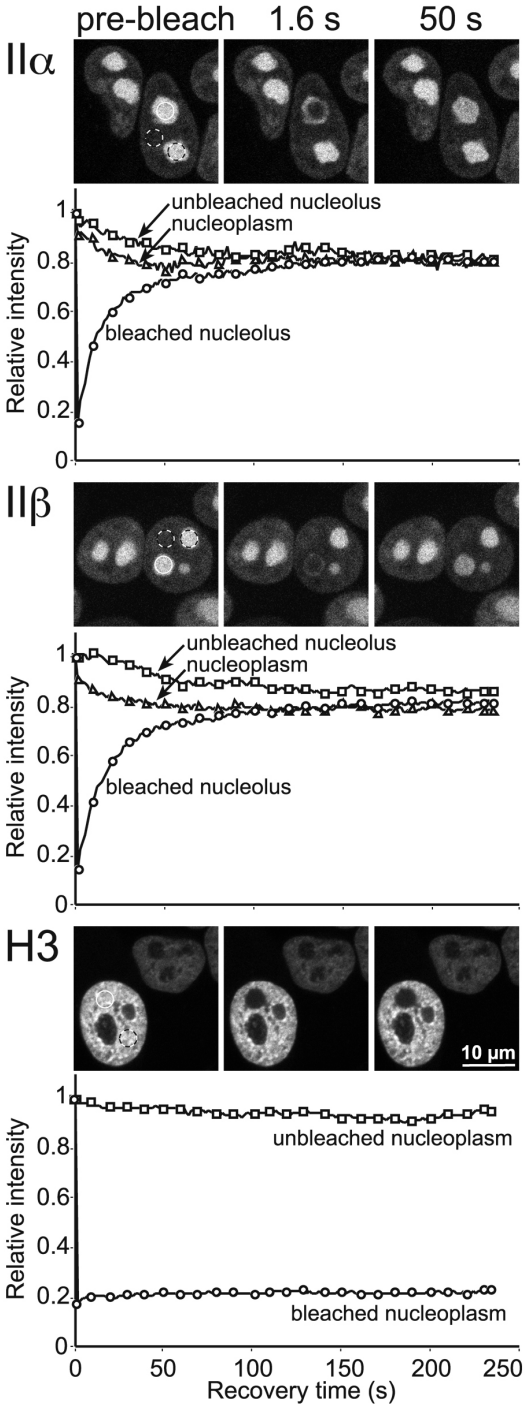

Figure 4.12: **Exchange between nucleoplasmic and nucleolar topo II-GFP.** Cells expressing GFP chimera of topo IIα, IIβ, and histone H3, respectively, were bleached and analyzed as in Fig. 5 with two exceptions: (i) A larger area (3 µm diameter; white circle) was bleached to achieve a substantial loss of total cellular fluorescence. (ii) The relative recovery over time in the bleach spot and the relative loss of fluorescence intensity in unbleached areas (black and white circles) is plotted below each image panel. Plotted data were not corrected for the overall loss of fluorescence induced by the bleach pulse (~20 %), to allow a quantitative comparison of signal loss in unbleached areas with signal gain in the bleached area. For greater clarity, only every tenth data point is marked by a symbol. Figure adapted from (Christensen et al., 2002b).

subjected to the same percentage loss of fluorescence intensity as the nucleoplasm. These data are in striking contrast to those obtained with the largely immobile GFP-histone H3 (Fig. 4.12, bottom), where fluorescence recovery in the bleach spot and fluorescence loss in the neighbourhood did not converge.

Thus, bleached topo II molecules (from the bleach spot) and unbleached ones (from the selected neighbouring areas) mixed completely within the time frame of the experiment, indicating that all of them could freely move between their respective nuclear compartments.

4.2.10 Immobile Fractions of Topoisomerase II do not Exist in Interphase Cells

Topo II has been suggested to be involved in building the so-called interphase nuclear matrix, an insoluble nuclear structure believed to play an architectural role in the interphase cell nucleus (Berrios et al., 1985). If topo II indeed is a component of such an immobile structure, at least a subfraction of the enzyme should be immobile, too. We have shown by FRAP that topo II is highly mobile. However on the basis of these data, we could not exclude the existence of an immobile fraction of topo II, since a small, immobile fraction could have been obscured by a much larger, mobile fraction. To exclude this possibility, we carried out conventional FLIP analysis (White and Stelzer, 1999).

Nucleoli labelled by topo IIα-GFP (top) or topo IIβ-GFP (bottom) were repeatedly bleached. It is readily apparent from time lapsed images (Fig. 4.13, top panels) and quantitative plots of loss of fluorescence (Fig. 4.13, bottom panels) that all topo II-GFP fluorescence was lost from the nucleoplasm as well as from other nucleoli, indicating that all fluorescent topo II molecules were eventually hit by a bleach pulse and therefore must be mobile.

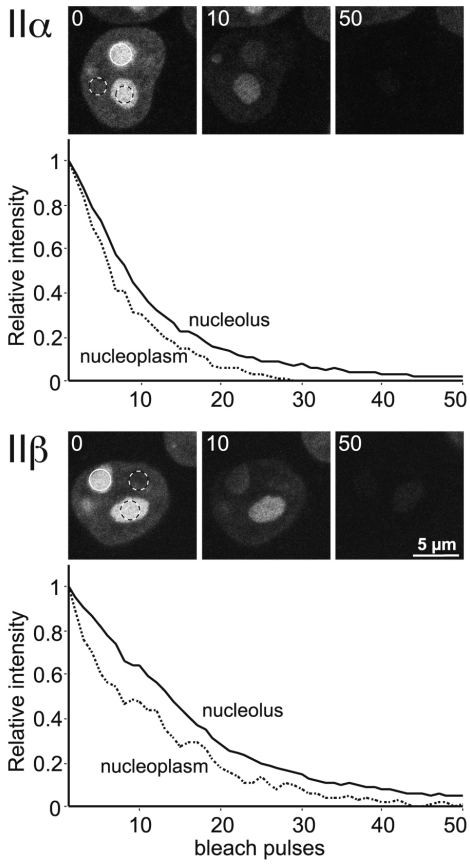

Figure 4.13: **FLIP analysis of interphase topo II-GFP.** Nucleoli of cells expressing topo IIα-GFP or topo IIβ-GFP were repeatedly bleached in a circular area (3 µm diameter; white circles). Cells were imaged before each new bleach pulse, and fluorescence intensities of neighboring nucleoli and nucleoplasmic areas (black and white circles) were determined. Almost all fluorescence was lost from both compartments after 50 bleach cycles, indicating an absence of an immobile fraction. Figure adapted from (Christensen et al., 2002b).

In summary, all these data (Fig 4.11-13) support the conclusion of a rapid and unrestricted exchange of fluorescent topo II molecules between individual nucleoli, and between the nucleoplasm and nucleoli. This interpretation is more in agreement with a recent concept proposing rapid and continuous diffusional movement of proteins between

nucleoplasm and nuclear compartments (Misteli, 2001) than with the older idea of topo II existing in separate cellular pools (Swedlow et al., 1993).

4.2.11 Mobility of Topo IIα at Mitosis

The unrestricted mobility of topo IIα and IIβ observed in interphase nuclei makes it unlikely that the enzymes play a structural role in building an interphase karyoskeleton. However, it is still possible that they participate in building the mitotic chromosome scaffold, which is the major structural role proposed for these enzymes (Adachi et al., 1991). To address this issue, we determined the mobility of topo IIα in chromosomes, using the same experimental strategy as in interphase cells (Fig. 4.14). These experiments were restricted to the α-isozyme, because chromosomal and cytosolic locations of topo IIβ-GFP could not be reliably discriminated (comp. Fig. 4.9) making mobility studies impossible.

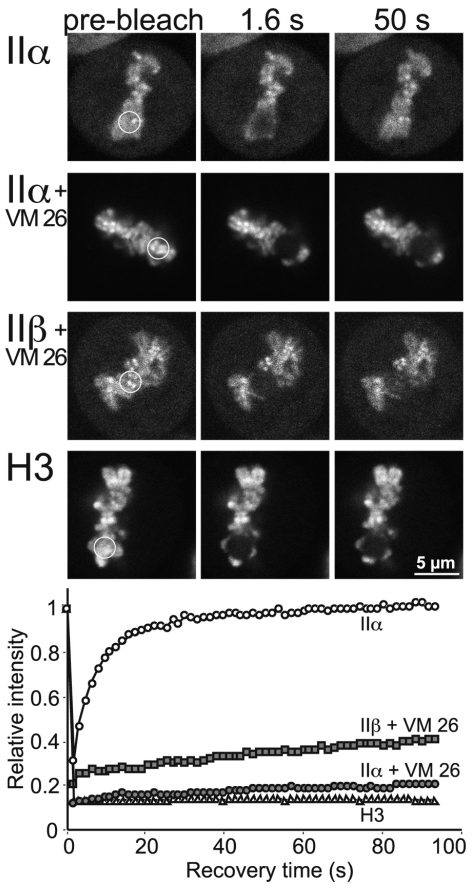

Figure 4.14: **FRAP experiments on mitotic chromosomes** FRAP (as described in Fig. 5) of metaphase chromosomes in cells expressing GFP chimera of topo IIα (with and without 100 µM VM 26), topo IIβ (VM 26-treated) or histone H3 after bleaching a circular area of 3 µm diameter. Bleaching of topo IIβ-GFP-labeled chromosomes not treated with VM 26 was not possible since they were obscured by large amounts of cytosolic topo IIβ-GFP (see Figs. 3 and 4 B). Figure adapted from (Christensen et al., 2002b).

Unexpectedly, fluorescence recovery of topo IIα-GFP (Fig. 4.14, upper panel) after photobleaching of a chromosomal area in a metaphase plate was fast ($t_{1/2}$ = 3.5 s) and complete. We obtained similar recovery kinetics with chromosomes of cells in pro-, ana- or telophase (data not shown). Moreover, the FLIP experiment shown in Fig. 4.15 demonstrates that all topo IIα-GFP fluorescence was lost from chromosomes and the cytoplasm of a given cell, when repeated bleach pulses were applied to a distinct chromosomal area. Comparable

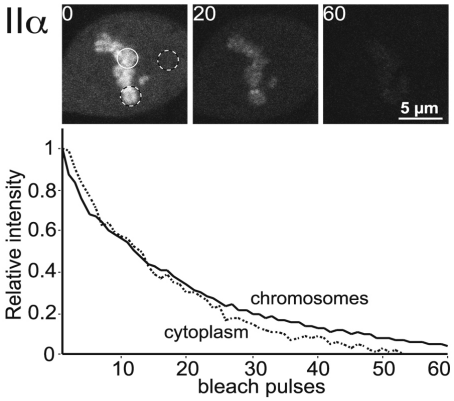

Figure 4.15: **FLIP analysis of mitotic topo IIα-GFP.** FLIP as described in Fig. 4.13 of a chromosomal and a cytoplasmic region (black and white circles) after bleaching of another chromosomal area (white circle) in a metaphase cell expressing topo IIα-GFP. Figure adapted from (Christensen et al., 2002b).

results were obtained when the cytosol was repeatedly bleached (not shown). In summary, these data indicate that topo IIα is mobile within the chromosome. An immobile fraction is virtually absent. The FLIP experiment (Fig. 4.15) also demonstrates unrestricted movements of the enzyme between chromosomes and the cytoplasm. To corroborate these controversial findings, we needed to prove that we would have detected an immobile fraction, if present. This was achieved in two ways. Firstly, we demonstrated immobility of GFP-histone H3 in mitotic chromosomes (Fig. 4.14, bottom panel). Secondly, we demonstrated immobility of topo IIα-GFP and topo IIβ-GFP trapped on chromosomal DNA by VM 26 (Fig. 4.14, middle panels). Both control experiments attest to the validity of our data regarding the unexpected degree of mobility of chromosomal topo IIα in the absence of VM 26.

In summary, we show that topo II α is as mobile in mitotic cells as at interphase, thus excluding that it could be part of an immobile chromosomal scaffold.

4.2.12 Axial Localization of Topoisomerase II in Chromosomes?

Our data obviously conflict with the established view that topo IIα plays a structural role in building a static chromosome scaffold. This view stems in part from observations made by indirect immunofluorescence microscopy, demonstrating topo II along the longitudinal axes of chromosome arms (e.g. (Meyer et al., 1997; Sumner, 1996; Taagepera et al., 1993)). However, such axial patterns were not seen when rhodamine-labeled topo II was injected into living insect cells (Swedlow et al., 1993), and were not consistently seen with immunofluorescence microscopy (summarized in (Warburton and Earnshaw, 1997)). In the immunohistochemical experiments, a variety of reagents were used to swell the chromosomes and/or extract chromosomal proteins prior to fixation and immunostaining. It is quite possible that the extraction and swelling methods had numerous side effects both on chromosome structure and the protein composition as compared to native cellular chromosomes. The experimental system presented here, allows to monitor what actually happens to topo II

during chromosome spreading. Thus we were able to address the long-standing and controversial question about the axial localization of topo II in a finite manner.

The first objective was to determine the subchromosomal distribution of GFP-tagged topo IIα in living cells. Histone H3 is an integral part of the chromatin and can hence be assumed to cover the entire chromosome. It is therefore possible to determine the width of a chromosome by highly resolved confocal images of histone H3-GFP, instead of staining the DNA (e.g. with DAPI), as normally done in fixed specimen. Fig. 4.16 shows comparable images of topo IIα-GFP and GFP-histone H3 in single chromosomes of living cells (left). Topo IIα and histone H3 appeared to have a similar distribution, without any apparent axial enhancement. This was confirmed by a quantitative analysis of the distribution of fluorescence intensity in representative cross sections of the chromosomes (boxes). It shows that both proteins covered a similar chromosome diameter of 0,8 – 1 μm, and had a similar distribution (right). These data suggest that, in living cells, topo IIα is not confined to chromosome axes.

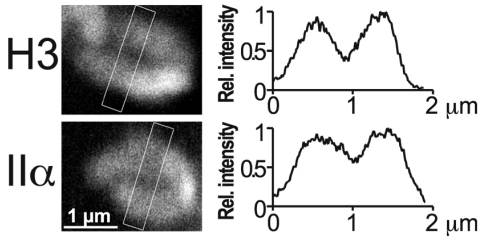

Figure 4.16: **Chromosomal distribution of topo IIα.** Single chromosomes in living metaphase cells expressing topo IIα-GFP or GFP-histone H3 were imaged by high resolution confocal microscopy (left). Distribution of fluorescence intensity was quantified in the boxed cross sections of the chromosomes. Mean fluorescence intensity of pixel columns across the short diameter of the boxes was normalized to maximal values, and plotted against corresponding distances measured across the long diameter of the boxes (right). Figure adapted from (Christensen et al., 2002b).

Our next step was to investigate how topo II-GFP responds to hypotonic treatment, usually the first step in preparation of chromosomes. We exposed cells at metaphase to hypotonic swelling, while they were under the microscope. Thus, we could monitor the effects of the procedure on topo in the cell. Swelling was performed with 75 mM KCl for 2 min, which is a standard procedure for preparing chromosomal spreads. Fig. 4.17 A shows the result of such an experiment with cells expressing topo IIα-GFP (left) or topo IIβ-GFP (right). As expected the cells increased in volume (Fig. 4.17 A, top, compare 0 and 2 min). Unexpectedly, the metaphase plate was completely disrupted by the treatment and, most notably, the entire cellular complement of topo IIα and IIβ became associated with the chromosomes (Fig. 4.17 A, bottom, compare 0 and 2 min). This redistribution closely resembles the effect of VM 26 (comp. Fig. 4.10), suggesting that hypotonic treatment disrupts the catalytic cycle of topo II in such a way that all enzyme molecules are rapidly (within 2 min) trapped in the DNA-bound state. In this context it is worth mentioning that *in vitro* DNA binding of topo II is also favoured by lowering the salt concentration.

To investigate the distribution of topo II in spread chromosomes, we employed a method to generate spreads of minimally processed, native chromosomes, omitting fixation methods. Cells were swollen in hypotonic solution, spotted on microscope glass slides, and spread chromosomes were then washed and mounted in an isotonic "cytoskeleton" buffer. When such native chromosomes were inspected immediately after spreading (Fig. 4.17 B, top panel), both GFP-tagged topo II isoforms (GFP) appeared to be distributed over the whole length and width of the chromosome arms as defined by the DNA stain (DAPI). However, distribution was not homogeneous any more. Topo IIα-GFP and IIβ-GFP were now concentrated in a central part of the chromosomes (most evident for topo IIα-GFP), although they were not altogether excluded from the chromosomal periphery. This demonstrates that spreading of chromosomes in some cases may induce an apparent axial localization of topo II. It should also be noted, that chromosome spreading enhanced accumulation of topo IIα at the centromers (arrows), whereas this was less evident for topo IIβ-GFP.

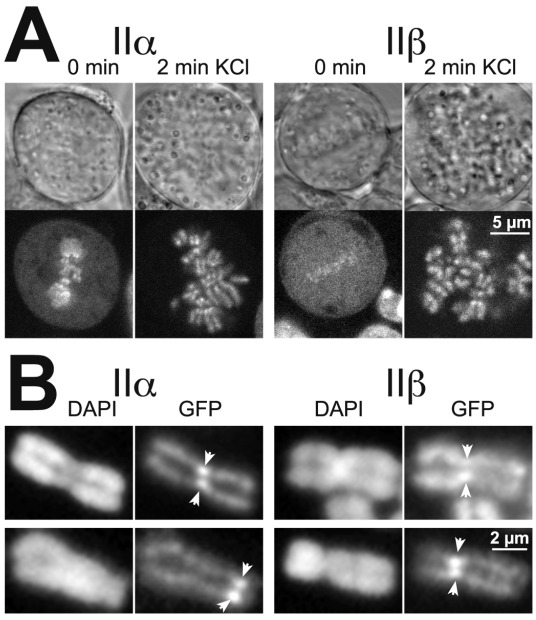

Figure 4.17: (A) **Effect of hypotonic treatment on topo II distribution in metaphase**. Two cells expressing topo IIα-GFP or topo IIβ-GFP were imaged by confocal microscopy before (*0 min*) and 2 min after exchanging the medium for KCl solution (75 mM). Images of transmitted light are shown above a corresponding confocal section showing green fluorescence of the same cell. Note the substantial increase in size of KCl-treated cells. (B) **Subchromosomal distribution and stability of topo II isoforms in unfixed, spread chromosomes.** Cells expressing topo IIα-GFP or topo IIβ-GFP were harvested, collected by centrifugation, swollen in hypotonic solution, and spotted on glass slides without fixation. Spread chromosomes were mounted in DAPI-containing CSK-buffer and imaged either immediately (upper panel) or 2 h later (lower panel). Arrowheads mark the position of centromer pairs. Figure adapted from (Christensen et al., 2002b).

When the same specimen were inspected after incubation at ambient temperature for 2 h (Fig. 4.17 B, bottom), the overall intensity of topo II-GFP-specific signals decreased and the pattern changed. Both isoforms were now clearly confined to the central axis of chromosome arms, delineating a structured helical pattern that closely resembled numerous published immunohistochemical images. In addition, centromeric accumulation became also evident with topo IIβ-GFP.

In summary, these observations demonstrate that the localization of topo II in chromosome spreads differs notably from the situation in living cells. Apparently, hypotonic treatment of cells and spreading of chromosomes triggers a series of events eventually generating an axial pattern of topo II: First, the whole complement of the enzymes relocates

rapidly from the cytosol to the chromosomes. Then, a loosely bound fraction of topo II is gradually lost over time from the outer region of the chromosome arms. This unveils another fraction bound to the centromer and the chromosomal core in a more stable manner. We envisage that during immunohistochemical staining procedures topo II can easily be lost from the chromosomal periphery, thus leading to the impression that it is confined to the chromosome axis. This would also explain why in some cases both patterns – accumulation in the axes and homogeneous distribution over the whole chromosome arms – have been detected within the same sample (Sumner, 1996).

4.3 Cellular Localization and Mobility of Human DNA Topoisomerase I

We transfected 293 cells with pMC-EGFPP-N, pMC-EGFP-TIP and pMC-EGFP-TI^{Tyr723Phe}P, bearing in the first cistron GFP alone or hybrid genes of topo I or topo I^{723Phe} fused at their N-termini to GFP, and in the second cistron the selection marker *pac*. Stable cell lines expressing either GFP alone, GFP-topo I or GFP-topo I^{723Phe} were then generated by selection of the transfected cells in the presence of 0.35 μg/ml puromycin. The three cell lines were maintained as mixed populations and not separated into individual clones. Less puromycin-resistant cell clones emerged from transfection with GFP-topo I than from transfection with the GFP gene alone, although the difference was smaller than for topo II. Cell lines supporting constitutive expression of GFP-topo I or GFP-topo I^{723Phe} had growth rates and morphologies identical to untransfected cells or cells expressing GFP alone. This suggested a *quasi* physiological expression of the GFP-tagged topo I suitable for fluorescence studies in living cells, provided that the fusion proteins were active and colocalized with their endogenous counterparts. Control experiments addressing these issues are addressed in the two following sections.

4.3.1 Expression of Active GFP-Topoisomerase I in 293 Cell

To determine expression levels, we performed western blots of cell lines expressing GFP-topo I (Fig. 4.18, lane 3) or GFP-topo I^{723Phe} (Fig. 4.18, lane 5). Untransfected cells (Fig. 4.18, lane 1) served as control. When these western blots were probed with GFP-antibodies (Fig. 4.18, top), GFP-topo I, and GFP-topo I^{723Phe} were readily detected in transfected cells as single protein bands (lanes 3 and 5, respectively) not apparent in untransfected cells (lane 1), or cells expressing GFP alone (data not shown). Thus, rearrangements of the chimeric gene could be excluded and green fluorescence in the cell lines could be unambiguously assigned to full-length GFP-topo I, or GFP-topo I^{723Phe}. The expression level of GFP-topo I was

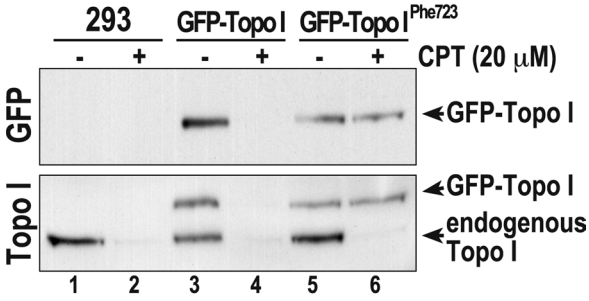

Figure 4.18: **Expression and immuno-band depletion of GFP-topo I and GFP-topo I^{723Phe} in HEK 293 cells.** Immunoblotting of 293 cells not transfected (*lanes 1 and 2*), expressing GFP-topo I (*lanes 3 and 4*), or expressing GFP-topo I^{Phe723} (*lanes 5 and 6*). Blots were probed with antibodies against GFP (*top*) or human topo I (*bottom*). The positions of GFP-linked and endogenous topo I are indicated on the right margin. Cells in lanes 2, 4, and 6 were cultured with CpT (20 mM, 20 min) prior to analysis. Figure adapted from (Christensen et al., 2002a).

approximately 2 fold higher than that of GFP-topo I^{723Phe}.

When western blots were probed with topo I antibodies (Fig. 4.18, bottom), topo I chimeras appeared as additional bands of slower migration (lanes 3 and 5). In untransfected 293 cells the additional band was absent (lane 1). In transfected cells, GFP-topo I had a similar intensity as endogenous topo I (lane 3), whereas the GFP-topo I^{723Phe} were less expressed than endogenous topo I (lane 5). It is interesting to note that expression of GFP-topo I was accommodated by a slight reduction in expression of endogenous topo I (Fig. 4.18 A, bottom, lane 3). Thus, the GFP-tagged species was not overexpressed and the overall expression of topo I was apparently the same as in untransfected cells.

The activity of GFP-topo I was tested by immuno-band depletion (Liu, 1989). Before immunoblotting, cells were treated with camptothecin (CpT) which stabilizes covalent DNA·topo I intermediates inherent in the enzyme's catalytic cycle. Since these intermediates are too large to enter the gel, the active fraction of the enzyme becomes depleted from the blots. Apparently, CpT caused an effective depletion of both endogenous and GFP-linked topo I within the same cell sample (Fig. 4.18, lower panel, compare lanes 3 and 4). In the case of cells expressing the active site mutant GFP-topo I^{phe723} only endogenous topo I was depleted, whereas GFP-topo I^{phe723} was unaffected (compare lanes 5 and 6). Finally, to ascertain that GFP-topo I possessed the same specific activity as its endogenous counterpart, we investigated the dose response to camptothecin. Fig. 4.19 shows that it was similar for GFP-tagged and endogenous topo I.

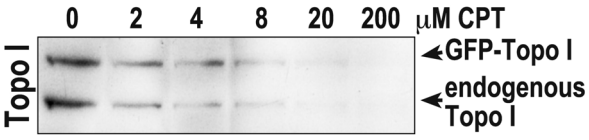

Fig 4.19: **Camptothecin dose response.** Cells expressing GFP-topo I were cultured for 20 min with various concentrations of camptothecin and subjected to immunoblotting with antibodies against topo I. Figure adapted from (Christensen et al., 2002a).

In summary these data demonstrate that we achieved expression of full length GFP-topo I and GFP-topo I^{phe723}. GFP-topo I has the same activity as endogenous topo I whereas the active site mutant GFP-topo I^{phe723} is catalytically inactive.

4.3.2 Colocalization of Biofluorescent and Endogenous Topoisomerase I

Considering finally that fusion to GFP might disrupt the cellular targeting of topo I, we performed indirect immunofluorescence (same rational as for topo II, see section 4.2.3). Fig. 4.20 shows a typical outcome of such an experiment, where untransfected cells (left double column) and cells expressing GFP-topo I (right double column) were stained with topo I specific antibodies, and the fluorescent patterns obtained with GFP-antibodies (top), topo I-antibodies (middle), and DAPI (bottom) are compared within individual cells. Untransfected

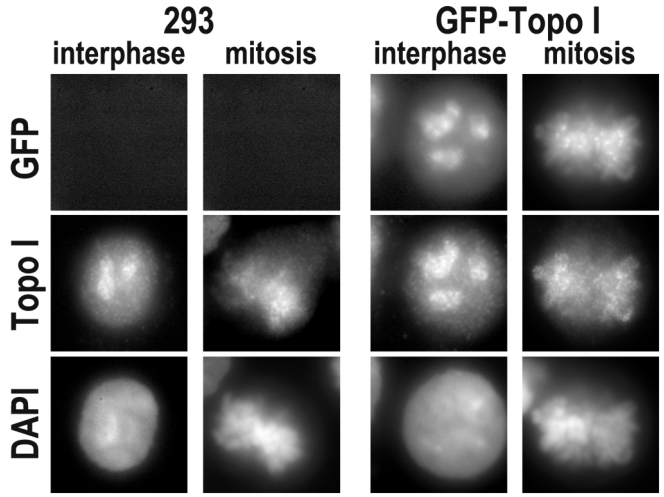

Figure 4.20: **Colocalization of GFP-topo I with endogenous topo I.** Untransfected 293 cells (*left double column*) and GFP-topo I (*right double column*) were grown on microscopic slides, PFA-fixed, permeabilized, and double stained with topo I-antibodies *(middle)* and DAPI (*bottom*). The top row shows corresponding images of GFP fluorescence (*GFP*). Each double column shows representative cells in interphase (*left*) and pro-/metaphase (*right*). Figure adapted from (Christensen et al., 2002a).

cells gave rise to antibody-derived signals only, whereas cells expressing GFP-topo I also emitted GFP fluorescence. The patterns of GFP fluorescence and immunofluorescence were virtually the same, confirming colocalization of the GFP-signal with endogenous topo I. Moreover, immunostaining patterns of untransfected and transfected cells were similar (Fig. 4.20, compare left and right) excluding that transgenic expression of GFP-topo I disrupted localization of the endogenous enzyme. Thus, the transgenic cell lines had fully integrated the GFP-chimera into their cellular pool of topo I and could be used to study the behaviour of the enzyme.

4.3.3 Distribution of Topoisomerase I in the Interphase Nucleus

Fig. 4.21 shows interphase nuclei of cells expressing GFP-topo I, or GFP-topo I^{723Phe}. Both enzymes were exclusively located in the nucleus and resided in the nucleoplasm and the nucleoli. Normal topo I accumulated in the nucleoli, whereas the active site mutant did not. Moreover, the nucleoplasmic pattern of normal topo I appeared to be more homogeneous that

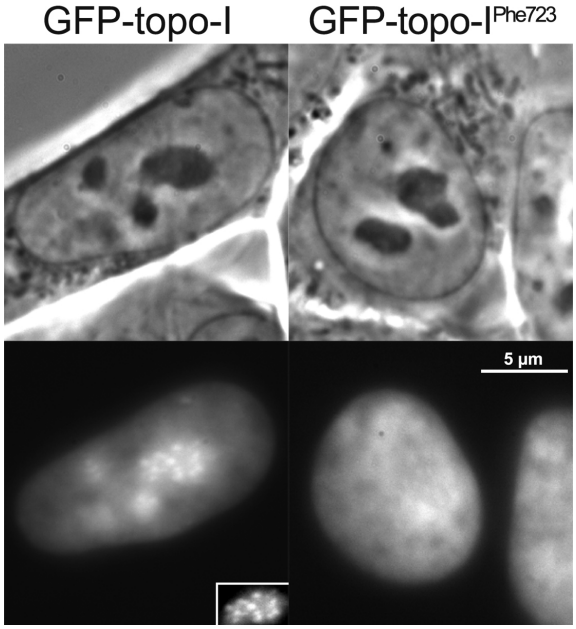

Figure 4.21: **Distribution of GFP-topo I and GFP-topo I^{723Phe} in interphase nuclei.** Phase-contrast images (top) and corresponding green fluorescence (bottom) of living 293 cells expressing GFP-topo I (*left*) and GFP-topo I^{723Phe} (*right*). The insert shows image of nucleoli subjected to contrast enhancement.

that of the active site mutant. These differences will later be explained by differences in mobility between GFP-topo I and GFP-topo I^{723Phe} (refer to section 4.3.7).

4.3.4 Topoisomerase I Accumulates at the Fibrillar Centers of the Nucleolus

The most distinct feature of normal GFP-topo I was its nucleolar accumulation and, more precisely, its accumulation in small globular substructures inside the nucleoli (Fig 4.21, insert). Since topo IIα-GFP and IIβ-GFP were excluded from similar nucleolar substructures (compare insert, figs. 4.6 and 4.21), might be that diverse nucleolar functions of type I and type II topoisomerases correlate to the association with different nucleolar subcompartments. Currently, three structurally distinguishable constituents of the nucleolus are described: the fibrillar centers (FC), the dense fibrillar component (DFC), and the granular component (GC). A model, though not shared by all investigators, proposes that the FC contains rDNA, RNA polymerase I, and associated transcription factors, and that transcription of rDNA occurs mostly at the boundary between the FC and DFC (rewired in (Lamond and Earnshaw, 1998; Shaw and Jordan, 1995)). Taking into account the undisputed role of topo I in rDNA-transcription (Muller et al., 1985), it seems feasible to assume that the substructure, at which topo I accumulates inside nucleoli, might be the FC.

To follow up on this hypothesis, we counterstained cells expressing GFP-topo I with antibodies against RNA polymerase I, which delineates the FC of mammalian nucleoli (Gilbert et al., 1995; Scheer and Rose, 1984). As shown in Fig. 4.22, a similar nucleolar substructure was delineated by antibodies against RNA polymerase I (middle) and by the fluorescence of GFP-topo I (left). High-resolution images of single nucleoli (insert) show that the two signals indeed colocalized. It should furthermore be noted that these images obtained in fixed cells faithfully reflected the *in vivo*-situation, because the fluorescent pattern of topo I-GFP was similar in the nucleoli of fixed and living cells (compare Fig. 4.22, left with Fig. 4.21, bottom, insert). Thus, it can be concluded that in a living cell nucleus topo I accumulates in the fibrillar centers of the nucleolus. This structure is actually the most distinct working place of the enzyme at interphase.

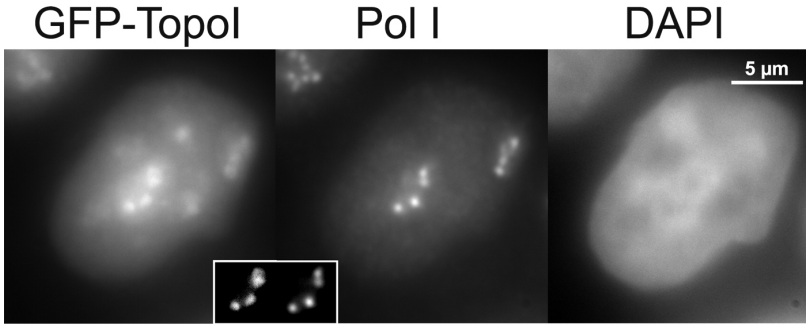

Fig 4.22: **Colocalization of GFP-Topo I with RNA polymerase I.** GFP-Topo I expressing cells were grown on microscope slides, PFA-fixed, permeabelized, and doublestained with specific RNA polymerase I antibody (middle), and DAPI (right). Left image shows corresponding GFP fluorescence (*GFP-Topo I*). The inserts show images of nucleoli subjected to contrast enhancement.

4.3.5 Topoisomerase I is Chromosome-Associated throughout Mitosis

Fig. 4.23 shows time lapsed epifluorescence microscopy of a cell expressing GFP-topo I, as it goes through mitosis. Monitoring begins in late G2-phase/early prophase (0 min), where GFP-topo I was concentrated still in the nucleoli. As the cell moved into prophase (4 min), nucleoli disappeared and a granular fluorescent pattern emerged indicating association of GFP-topo I with the condensing chromatin from early on. The enzyme stayed chromosome bound until telophase (12 – 48 min). Finally, during G1-phase (77 min – 3 h) it accumulated again in the reforming nucleoli and was otherwise distributed in the nucleoplasm in a uniform manner.

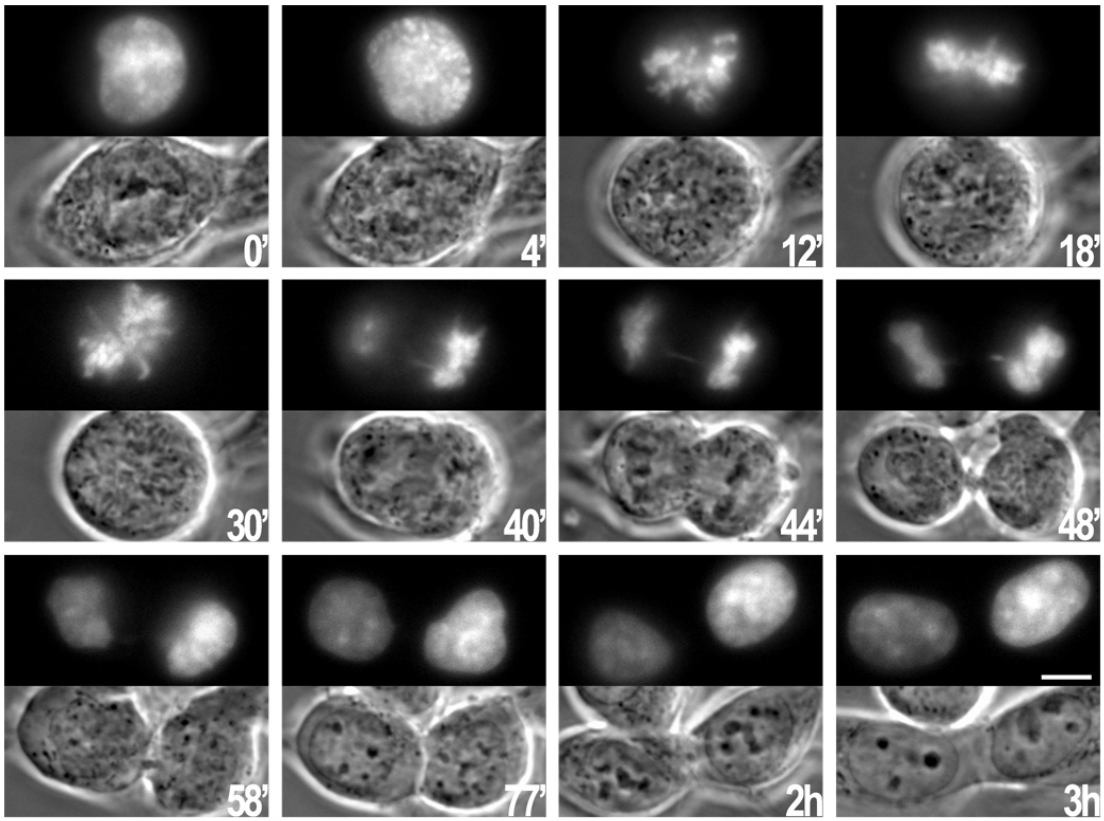

Figure 4.23: **Time-lapsed imaging of a GFP-topo I expressing cell proceeding from late G2- to early G1-Phase.** Cells expressing GFP-topo I were cultured at 37 °C under an inverted epifluorescence microscope. A cell in late G2-Phase was selected and imaged at the indicated time points by phase contrast (*top*) and green fluorescence (*bottom*) until it reached G1-phase. Figure adapted from (Christensen et al., 2002a).

Clearly GFP-topo I was closely associated with chromosomes throughout the mitotic cell division. We did not observe an accumualtion of topo I at the centromeric regions, at any time of the mitotic cycle. This is in clear contrast to topo II and again emphasizes the specific function of the type II enzyme at the centromers. It should also be noted that the active site mutant was also closely associated with mitotic chromosomes (not shown), although it is

catalytically inactive. This finding is consistent with biochemical data showing that the active site mutant possesses DNA binding properties similar to those of the wild type enzyme (Madden et al., 1995). Thus, GFP-topo I and GFP-topo I^{phe723} had the same localization in mitotic cells, but a different one in the interphase nuclei.

4.3.6 Mobility of Topo I in Interphase

The mobility of topo I in the living cell nucleus was studied by photobleaching. To determine FRAP kinetics, cells expressing GFP-topo I were cultured under a confocal laser scanning microscope and GFP-fluorescence was irreversibly bleached by high-powered laser pulses in circular areas of the nucleoplasm or the nucleoli, respectively (Fig 4.24). Subsequently, fluorescence recovery in the bleached spots as a consequence of other GFP-topo I molecules moving in from unbleached areas was recorded over time by sequential imaging scans (Fig. 4.24). As a control for a freely diffusible protein we used cells expressing GFP alone, which exhibited FRAP kinetics too fast for recording with our experimental settings (Fig. 4.24, insert). As a control for immobile proteins we used cells expressing GFP-histone H3 (Fig. 4.24, insert) known to be firmly chromatin-bound (Kimura and Cook, 2001). FRAP of GFP-topo I was complete after 30 s in nucleoplasm and nucleoli, indicating that the enzyme is entirely mobile in both compartments (as opposed to histone H3). However, GFP-topo I was clearly much slower than GFP alone, suggesting that the enzyme is not freely diffusible. Moreover, fluorescence recovery of GFP-topo I in the nucleoli was slower than in

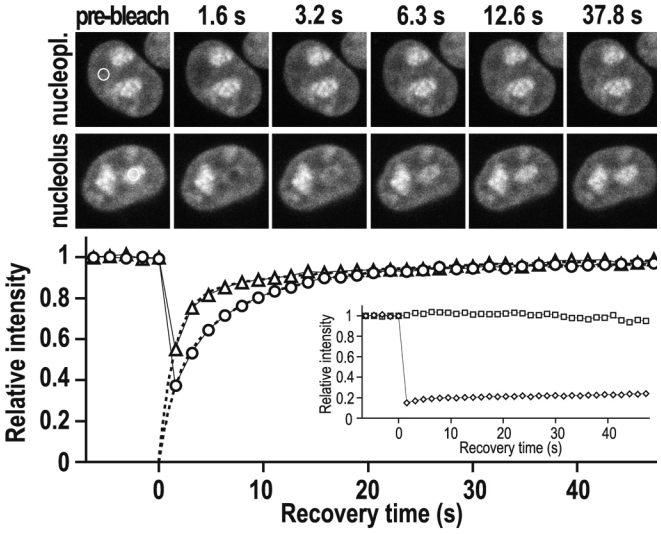

Figure 4.24: **FRAP analysis of living cells expressing GFP-topoisomerase I.** Circular areas (Ø = 2 μm) of the nucleoplasm (*top*) or a nucleolus (*bottom*) of interphase nuclei were bleached. Consecutive images at 1.6 s time intervals were taken before and at the indicated time points after the bleach pulse. Areas to be bleached are indicated by a circle in the pre-bleach panels. Corresponding quantitative data of FRAP kinetics are plotted below. Fluorescence intensities in the bleached nucleoplasmic (△) or nucleolar (○) region were measured and expressed as the relative recovery over time after the bleach pulse (at 0 s). Mean values from at least 6 individual cells and 3 independent experiments are shown. Standard deviations were in each case less than 5 % of the mean values (not displayed). Hatched lines represent the results of nonlinear regression analyses of the data. The insert shows FRAP kinetics obtained with cells expressing unfused GFP (□), or GFP-histone H3 (◇). Figure adapted from (Christensen et al., 2002a).

the nucleoplasm, demonstrating that the enzyme's mobility at the two locations was restrained to a different extent. Nonlinear regression of these data (hatched lines in Fig. 4.24) indicated with significance ($p<0.0001$) that in both cases two different mobility states of the fluorescent enzyme contributed to the apparent FRAP kinetics. A major portion appeared to be moving fast ($t_{1/2} = 1.1 \pm 0.1$ s and 1.9 ± 0.2 s for nucleoplasm and nucleoli, resp.), whereas a minor portion was moving much slower ($t_{1/2} = 14.3 \pm 2.3$ s and 12.5 ± 1.4 s for nucleoplasm and nucleoli, resp.). The slow portion amounted to 28 ± 3 % in nucleoli as opposed to only 16 ± 2 % in nucleoplasm. This difference explains, why overall mobility of GFP-topo I in the nucleoli was about 2-fold less than in the nucleoplasm. Thus GFP-topo I appears to be moving slow in areas where it is upconcentrated

Rapid fluorescence recovery in nucleoli and nucleoplasm, as observed here with GFP-topo I, suggests free and unrestricted exchange of the protein between nuclear compartments. We corroborated this notion by the FLIP approach (White and Stelzer, 1999). These experiments are summarized in Fig. 4.25. When one nucleolus was repeatedly bleached, all

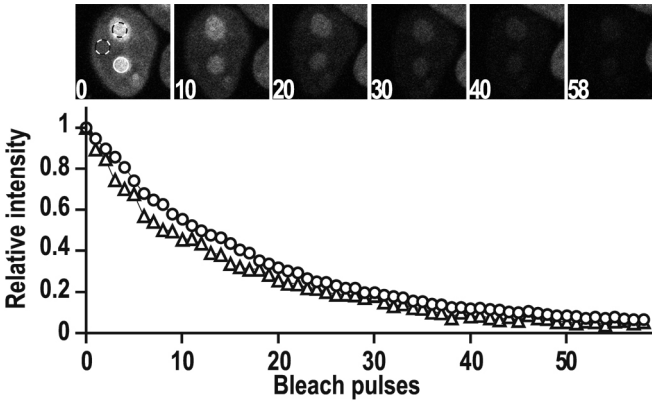

Figure 4.25: **FLIP analysis of cells expressing GFP-topoisomerase I**. A circular area (Ø = 3 µm; *white circle*) of a nucleolus was repeatedly bleached. Cells were imaged before each new bleach pulse (selected images are shown in the top). Fluorescence intensities of neighboring nucleoli and nucleoplasm (*black and white circles*, resp.) were determined, and plotted below (△, nucleoplasm; ○, nucleolus). Figure adapted from (Christensen et al., 2002a).

topo I-linked GFP-fluorescence was eventually lost from other nucleoli of the same nucleus and also from the surrounding nucleoplasm. Similar results were obtained, when repeated bleaching was applied to the nucleoplasm (not shown). These data imply that fluorescent topo I molecules originally localized in both compartments were eventually hit by bleach pulses aimed to only one of them, thus demonstrating a rapid, continuous, and unrestricted traffic of all enzyme molecules between individual nucleoli, and between nucleoplasm and nucleoli.

4.3.7 Correlation between Mobility and Localization of Topoisomerase I

The apparent link between localization and mobility of GFP-topo I suggested to us that the different localization of GFP-topo I and GFP-topo I^{phe723} in the interphase nucleus might also be reflected by a difference in mobility of the two enzyme variants. This notion is strongly supported by the altered mobility of the active site tyrosine mutant of topo I.

In contrast to the normal enzyme, FRAP-kinetics of GFP-topo I^{phe723} were virtually the same in nucleoli and nucleoplasm (Fig. 4.26, compare circles and open triangles), indicating

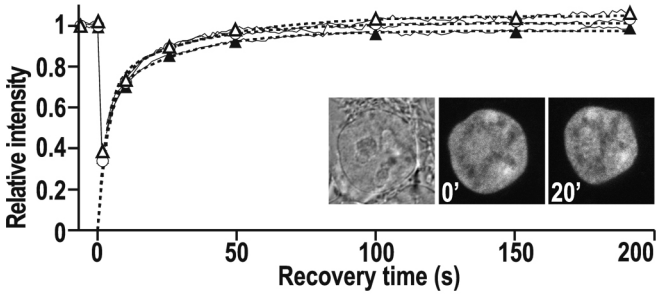

Figure 4.26: **The impact of camptothecin on localization and mobility of GFP-topo I^{Phe723}.** FRAP analysis in nucleoli (○) and in the nucleoplasm (△) of untreated cells, and in the nucleoplasm of cells treated with camptothecin (▲; 20 μM, 20 min). The insert shows representative images of an individual cell expressing GFP-topo I^{Phe723} before (0 min) and 20 min after addition of 20 μM camptothecin. Figure adapted from (Christensen et al., 2002a).

that the inactive enzyme had the same mobility in both compartments. This may explain, why it did not accumulate in nucleoli. Interestingly, FRAP-kinetics of topo I^{phe723} were in general slower ($t_{1/2}$ = 2.5 ± 0.2 s and 22.4 ± 2.2 s for fast and slow subpopulations in both compartments, resp.) than those of the active enzyme. We can only speculate at the reason for this phenomenon. Taking into account current concepts of the catalytic cycle of topo I (Fig. 1.17), it can be envisaged that DNA-catalysis (cleavage, strand rotation, religation) somehow facilitates the subsequent detachment of the enzyme from its DNA binding site. Since both proteins are able to interact with DNA, but only GFP-topo I performs DNA catalysis, such a model may explain the different interphase localization and mobility of topo I^{phe723}.

4.3.8 The Impact of Camptothecin on Topoisomerase I Mobility

In the light of these findings, we were curious how mobility and distribution of topo I would be influenced by camptothecin, which stabilizes covalent enzyme·DNA complexes. For this purpose, we cultured cells expressing GFP-topo I under a confocal laser scanning microscope and added camptothecin to the culture medium, while taking serial confocal scans every 3.1 seconds (Fig. 4.27 A). The first image (0 s) recorded immediately before adding camptothecin shows the normal nucleolar accumulation of topo I. Subsequent images demonstrate a very rapid redistribution of topo I from the nucleoli to the nucleoplasm. After 20 - 30 seconds nucleoli were largely reduced in fluorescence and most of the enzyme was localized in radial substructures within the nucleoplasm. The time scale of this phenomenon seemed to be independent of the CpT concentration, since depletion of the nucleoli took place within the same time frame (30 seconds) when CpT was added at concentrations ranging from 2 to 200μM CpT. Only at the lowest concentration of CpT tested (0,01 μM) we did not observe any nucleolar depletion, whereas a partial depletion was observed at 0,1 μM and an almost complete depletion at concentrations of 1μM and higher (not shown). With all effective concentrations the full effect was achieved within 30 seconds. Thus, mechanisms acting beyond the time frame of 30 seconds should not play a role in CpT induced nucleolar depletion of topo I.

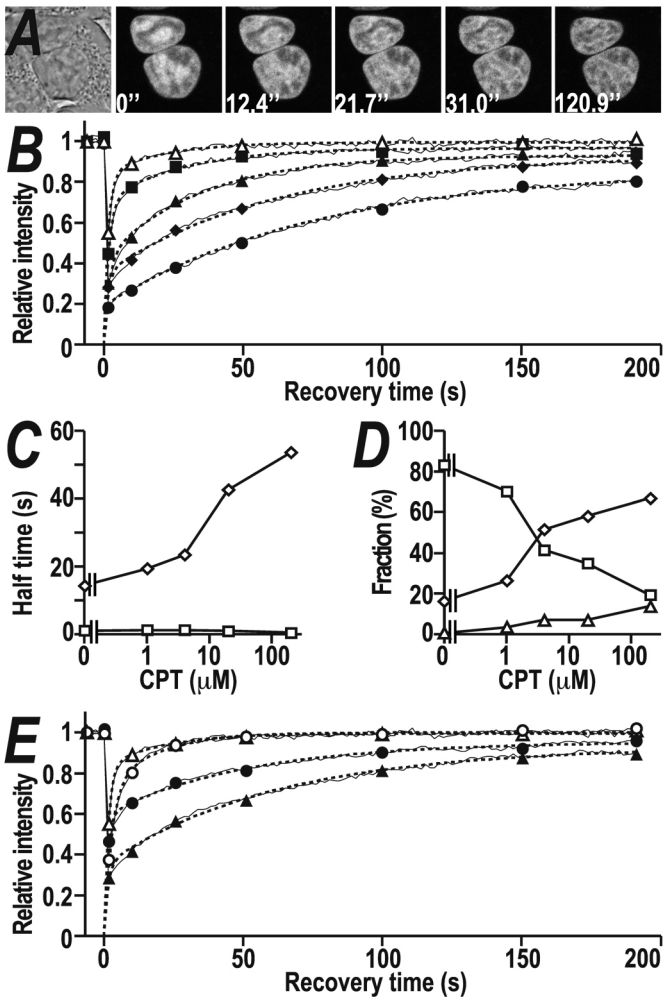

Figure 4.27: **The impact of camptothecin on localization and mobility of GFP-topoisomerase I.** (A) Time-lapsed confocal images of a cell exposed to camptothecin. Cells expressing GFP-topoisomerase I were cultured at 37 °C under the microscope and confocal midsection scans of green fluorescence were taken every 3.1 seconds. Representative images of an individual cell are shown before (0 s) and at the indicated time points after addition of 20 μM camptothecin. The complete sequence can be viewed in the supplemental QuickTime movie (TopI/CPT.mov). (B) FRAP analysis (comp. Fig. 2 B) of GFP-topoisomerase I in the nucleoplasm of cells exposed to increasing concentrations of camptothecin (△, control; ■, 1 μM; ▲, 4 μM; ◆, 20 μM; ●, 200 μM). For clarity, data points are displayed by a connecting line, and only selected time points are marked by a symbol. Hatched lines represent the results of nonlinear regression analyses of the data. (C) Half times of slow (◇) and fast (□) fractions of GFP - topoisomerase I derived from nonlinear regression of the data in (B) are plotted over the log molar concentration of camptothecin. (D) Percentages of slow (◇), fast (□), and immobile (△) fractions of GFP-topoisomerase I derived from nonlinear regression of the data in (B) are plotted over the log molar concentration of camptothecin. (E) Comparative FRAP analysis of GFP-topoisomerase I in nucleoli (○ and ●) and nucleoplasm (△ and ▲) of untreated cells (open symbols) and cells treated with camptothecin (20 mM, 20 min; closed symbols). Data acquisition and plotting was done as in (B). Figure adapted from (Christensen et al., 2002a).

Although, the cellular distribution of GFP-topo I was equally effected within a broad range of CpT concentrations, there must be a concentration dependent effect on GFP-topo I based on the fact that the cellular toxicity of CpT varies greatly within this range. This is also illustrated by the correlation between extent of immuno band-depletion and CpT concentration shown in Fig. 4.19. Since CpT stabilizes covalent enzyme DNA complexes, one would expect a concentration-dependent response in the mobility of GFP-topo I. To test whether such a relationship exists, we determined FRAP-kinetics in the nucleoplasm of cells treated for 20 min with various concentrations of camptothecin (Fig. 4.27 B). Evidently, fluorescence recovery of GFP-topo I became increasingly slower with increasing doses of CpT administered to the cells.

The existence of two states of different mobility in untreated cells (Fig. 4.24) raised the question if CpT preferentially targeted one of these states. To investigate this, we

subjected the recovery curves of GFP-topo I in Fig. 4.27 B to nonlinear regression analysis (hatched lines). Again the contribution of two different mobility states of the fluorescent enzyme to each of the curves was significantly ($p<0.0001$). Upon plotting the half times against the log molar concentration of CpT (Fig. 4.27 C), it became apparent that the drug acted preferentially on the slow fraction of topo I (◇). This fraction was retarded by CpT in a dose dependent manner, becoming slower and slower as CpT concentration increased. In contrast, recovery half times of the fast fraction (□) were not significantly altered by the drug (Fig. 4.27 C). Upon plotting of the relative proportions (Fig. 4.27 D) of slow (◇), fast (□) and immobile (△) enzyme fractions against the log molar concentration of CpT, it becomes apparent that, an increasing proportion of the enzyme was recruited to the slow state, whereas only an insignificant portion actually became immobile (Fig. 4.27 D, compare ◇, □ and △). These findings suggest that CpT recruits GFP-topo I to the slow state, which becomes increasingly slower as the dose is increased.

A comparison of FRAP-kinetics determined in nucleoli and nucleoplasm of camptothecin treated cells (Fig. 4.27 E), showed that in the nucleoli topo I was retarded to a much lesser extent than in the nucleoplasm, suggesting that camptothecin acted preferentially on topo I in the nucleoplasm. As a consequence, the enzyme was moving much faster in the nucleolus than in the nucleoplasm of a camptothecin treated cell, whereas in an untreated cell it was the other way around (Fig. 4.27 C, comp. open and closed symbols). Thus, nucleolar accumulation and camptothecin- induced nucleolar delocalization of topo I seem to be driven by differences in mobility.

Support of this notion was gained from the behaviour of the catalytically inactive mutant topo I^{phe723}. CpT does not induce covalent GFP-topo I^{phe723} DNA complexes (Fig 4.18) and FRAP-kinetics of GFP-topo I^{phe723} were virtually the same with and without camptothecin (Fig 4.26, compare open and closed triangles), attesting to the fact that mobility of the inactive enzyme was the same in both compartments and not affected by exposure to CpT at concentrations as high as 20 μM. Consequently, topo I^{phe723} did not delocalize from nucleoli upon exposure to camptothecin (Fig. 4.26, insert, compare 0 with 20 min). This was also true for higher CpT concentrations (data not shown). These findings confirm, that catalytically inactive topo I is not targeted by CpT and that CpT-induced relocations of topo I are a direct consequence of mobility changes.

4.4 Its Non-conserved N-terminal Domain Anchors Topoisomerase I at Fibrillar Centers of Nucleoli and Nucleolar Organizer Regions of Mitotic Chromosomes

The nonconserved N-terminal region of eukaryotic topo I is considered to be dispensable for catalytic activity *in vitro* but essential *in vivo* (Alsner et al., 1992; Stewart et al., 1996b; Stewart et al., 1998). Until now, the biological role of the N-terminal region (first 210 amino acids) has mainly been credited to 5 putative NLS sequences contained therein (Alsner et al., 1992). We describe here that this domain plays an essential role in directing topo I to nucleolar substructures and fixing the enzyme at these structures during the entire cell-cycle. These data were obtained from 293 cells expressing biofluorescent chimera of (i) full-length topo I (GFP-TopoI), (ii) the C-terminal domain of human topo I (GFP-TopoI-C: amino acids 210-765, supplemented with an artificial NLS sequence of SV40 NLS at the N-terminus), and the N terminal domain (GFP-TopoI-N: amino acids 1-210 from human topo I), all fused at their N-termini to GFP.

4.4.1 Expression of Chimeras of GFP with N- and C-terminal Domains of Topoisomerase I

To verify that GFP fusion proteins of expected size were expressed, we subjected lysates of stably expressing cell lines to immunoblotting. Use of a GFP-specific antibody (Fig 4.28) detected full length GFP-topo I (lane 1), GFP-topo I-C (lane 2) or GFP-Topo I-N (lane 3) as single protein bands of expected size in transfected cells, which were not apparent in untransfected cells or cells expressing GFP alone (data not shown). Thus, we could exclude rearrangements of the chimeric genes, and ascertain that green fluorescence of the cells was due to expression of the intended proteins. Interestingly, the expression level of the two deletion mutants was higher than that of the full length GFP-topo I.

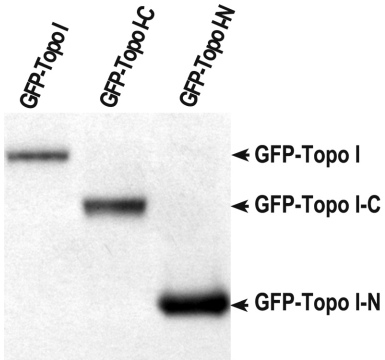

Fig 4.28: Expression of GFP-Topo I, GFP-Topo I-C and GFP-Topo I-N. Immunoblotting of 293 cells expressing GFP-Topo I (lane 1), GFP-Topo I-C (lane 2), or GFP-Topo I-N (lane 3). Blot was probed with antibodies against GFP. The position of GFP-linked topo I are indicated on the right margin.

4.4.2 The N-Terminal Domain Directs Topo I to Fibrillar Centers of Nucleoli

The subcellular distribution of GFP-topo I, GFP-topo I-N and GFP-topo I-C in interphase cells was examined by fluorescence microscopy. Fig 4.29 shows representative examples. Apparently, adding an SV40 NLS to the C-terminal domain was sufficient to achieve a mostly nuclear localization of this domain that does not contain an endogenous NLS (fig 4.29 right), whereas GFP-topo I (Fig 4.29, left) and GFP-topo I-N (Fig 4.29, middle) were exclusively located in the nucleus. All three GFP-chimeras showed a similar homogenous distribution in the nucleoplasm and accumulated in the nucleoli. However, distribution within the nucleoli differed (Fig 4.29, inserts). As shown above (section 4.3.4) the full-length enzyme showed an accumulation in the fibrillar centers (FCs) of nucleoli. This feature was even more pronounced in cells expressing only the N-terminus, whereas the C-terminus did not accumulate in FCs. Instead, it was evenly distributed through the entire nucleolar space (Fig 4.29, compare inserts). This suggested that nucleolar accumulation of topo I is a general phenomenon, whereas the specific recruitment to FCs is mediated by the non-conserved N-terminal domain. In an attempt to discriminate a defined targeting signal within the N-terminal domain, we constructed GFP fusions of various smaller fragments of this domain. To our surprise, none of these fusion proteins accumulated at the FC, when expressed in the cells (data not shown). Thus, the entire N-terminus seems to be required to direct topo I to FCs.

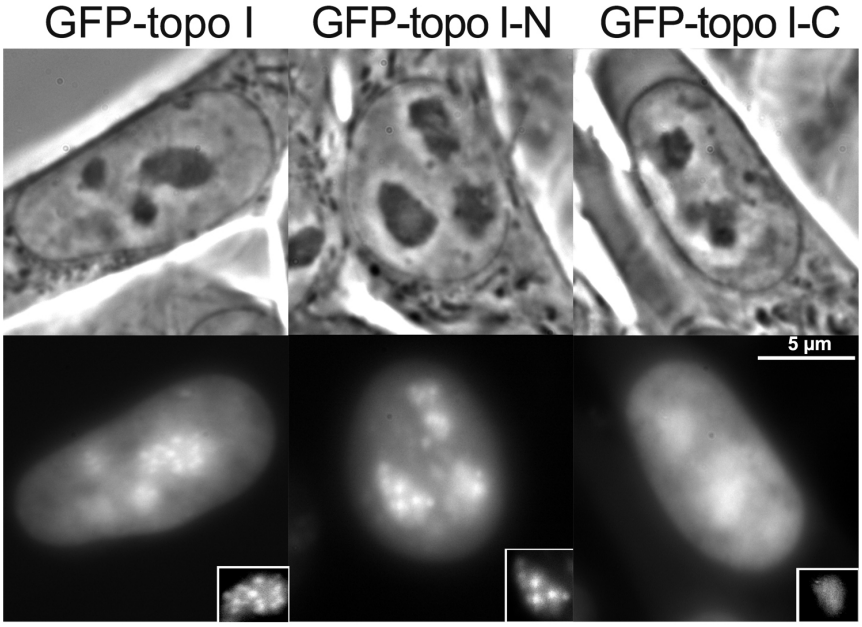

Figure 4.29: **Distribution of GFP-topo I, GFP-topo I-N, and GFP-topo I-C in interphase nuclei.** Phase-contrast images (top) and corresponding green fluorescence (bottom) of living 293 cells expressing GFP-topo I (*left*), GFP-topo I-N *(middle)* and GFP-topo I-C (*right*). The insert shows images of nucleoli subjected to contrast enhancement.

4.4.3 On Mitotic Chromosomes, The N-Terminal Domain Allocates Topoisomerase I to Nucleolar Organizer Regions

Next, we examined the distribution of the N- and C-terminal domains on metaphase chromosomes (Fig. 4.30). GFP-topo I (Fig 4.30, left) and GFP-topo I-C (Fig 4.30, right) were closely associated with metaphase chromosomes. This was expected since the C-terminus contains the core domain responsible for DNA binding of topo I (Madden et al., 1995). In contrast, large parts of GFP-topo I-N (Fig 4.30, middle) resided in the mitotic cytoplasm. However, the protein was not entirely excluded from the condensed mitotic chromosomes, and we consistently observed small globular aggregations of this domain within in the metaphase planes. This observation was a puzzling finding, because the N-terminus is not considered to be involved in DNA-interactions.

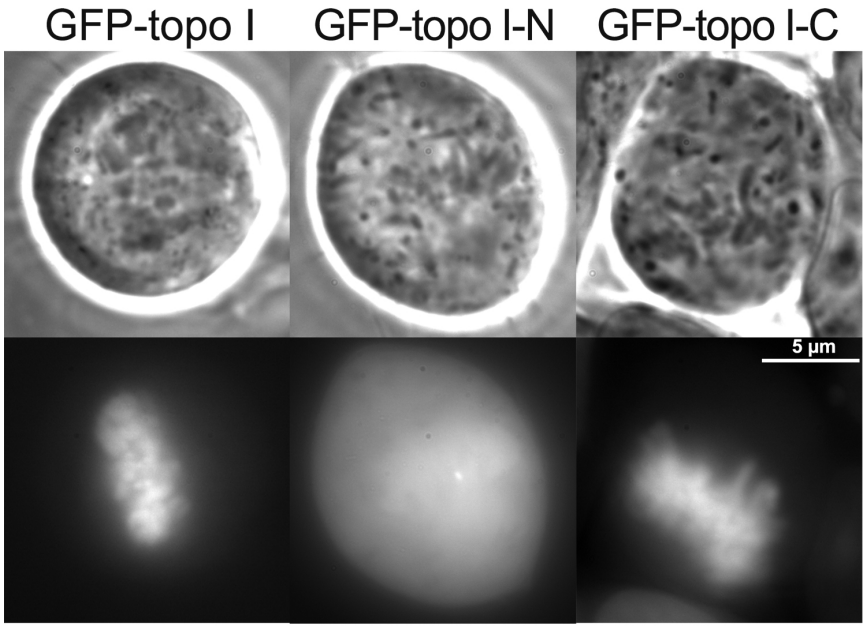

Figure 4.30: **Distribution of GFP-topo I, GFP-topo I-N, and GFP-topo I-C in metaphase cells.** Phase-contrast images (top) and corresponding green fluorescence (bottom) of living 293 cells expressing GFP-topo I (*left*), GFP-topo I-N *(middle)* and GFP-topo I-C (*right*).

To obtain a more detailed picture of the distribution of the N-terminus of topo I in chromosomes, we performed high-resolution confocal microscopy of a metaphase cell expressing GFP-topo I-N. Fig 4.31. shows 6 consecutive confocal planes of such a cell (A-F). It is apparent, that the metaphase plane itself has the same fluorescence intensity as the surrounding cytosol, indicating again that GFP-topo I-N is not excluded from the mitotic

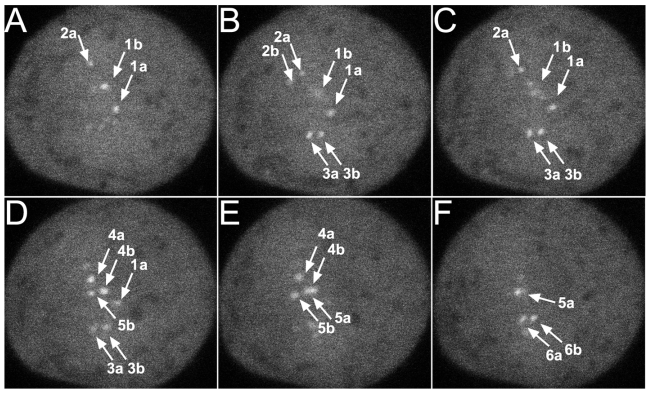

Figure 4.31: **GFP-topo I-N is concentrated in paired dots on metaphase chromosomes.** Cells expressing GFP-topo I-N were imaged by confocal microscopy. 6 consecutive confocal sections *(A-F)* are shown. Arrows indicate positions of paired dots, and such pairs are indicated by numbers (e.g., 1a and 1b)

chromosomes. Moreover, intense speckles could clearly be detected within the metaphase plate (Fig. 4.31, arrows), and it appeared as if these dots were paired (Fig. 4.31, numbered arrows). This is most apparent for the dots 3a and 3b (Fig. 4.31, compare panels B, C, and D).

This pairing of GFP-topo I-N labelled dots suggested that they might be attached to related structures of sister chromatids. If the latter was the case, each pair of dots should be separated in anaphase and equally distributed into the two daughter cells. This hypothesis was tested by acquisition of confocal images of a cell expressing GFP-topo I-N as it proceeds under the microscope from meta- (0 min) to early G1-phase (45 min) (fig 4.32). For each time point four corresponding confocal sections are shown (rows). In metaphase (Fig 4.32, 0 min) paired dots were clearly detectable. However, as the cell proceeded to anaphase (12-17 min) the dots were separated and distributed into the two daughter plates (Fig 4.32, see arrows in

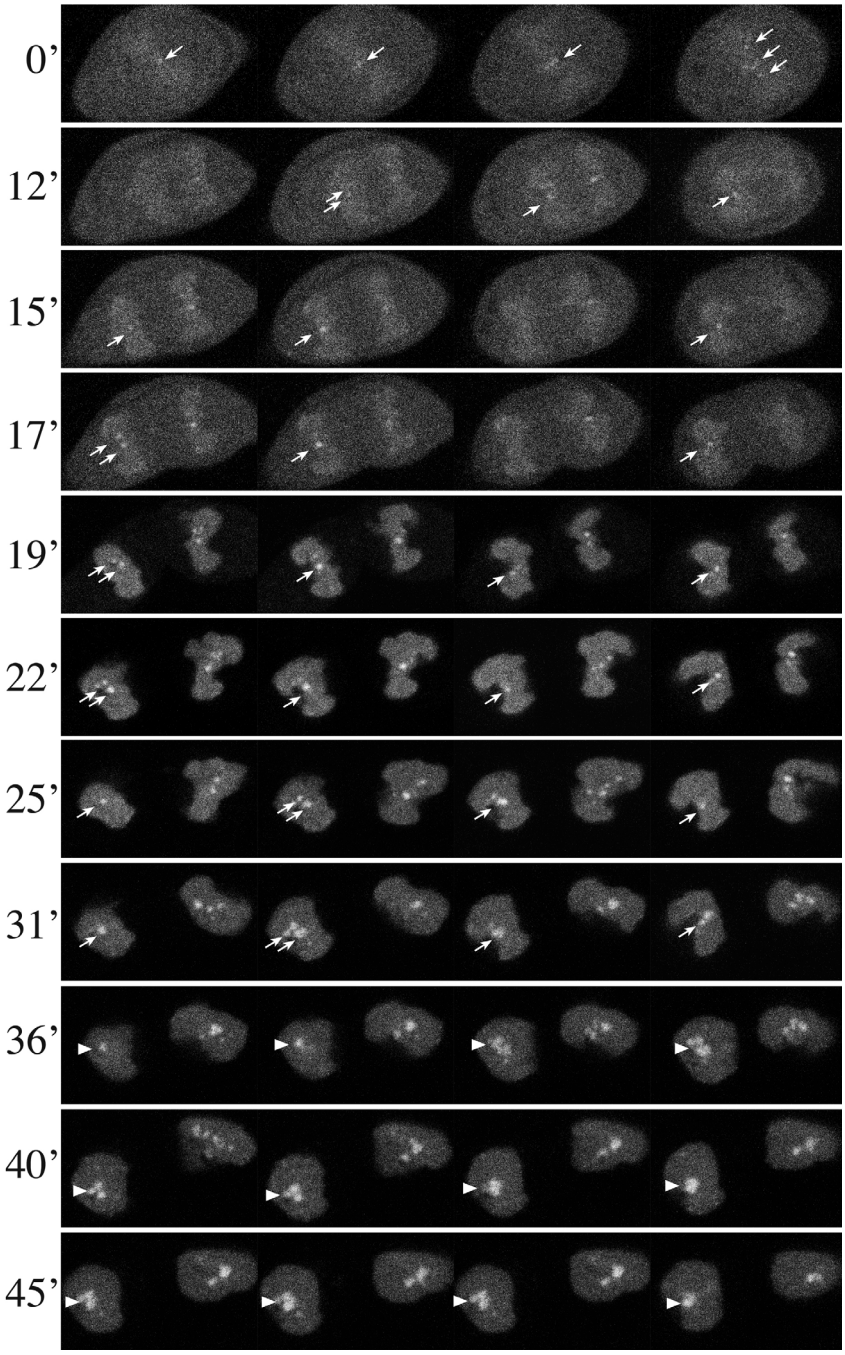

Fig 4.32: **Confocal time-lapsed imaging of a GFP-topo I-N-expressing cell proceeding from meta- to early G1-Phase.** Cells expressing GFP-topo I-N were cultured at 37 °C under the microscope. A cell in metaphase was selected and confocal sections were obtained at the indicated time points until it reached G1-phase. 4 selected confocal sections are shown for each time point. Arrowheads indicate areas of increased fluorescence. For clarity arrows from ana- (12') until telophase (31') are only shown for the left daughter cell. Reforming nucleoli in G1-phase (36'-45') are indicated by arrowheads.

left daughter cell). The total number of dots appeared to be the same in the two anaphase plates. These observations are consistent with an association of the dots with corresponding structures of sister chromatids. In early telophase, the entire population of GFP-topo I-N rapidly relocated to the reforming nucleus (19 min). As the cells further proceeded into telophase, the dots increased in size and intensity and moved into close vicinity of each other (19-31 min). In late telophase / early G1 phase, they finally converged to a single larger structure (Fig 4.32, 36-45 min, arrowheads), which in transmitted light could be identified as the nucleoli (not shown).

In summary, these data (Fig 4.31 and 4.32) indicate that the N-terminus plays a role in directing topo I to defined sites of metaphase chromosomes, of which there exists a limited number (less than one per chromosome). Since at interphase topo I is heavily involved in rDNA transcription and prominently located in FCs (Fig 4.22), we speculated that the distinct chromosomal accumulation of the enzyme might also be related to rDNA, a view supported by the observation that these structures merged with pre-nucleolar bodies and nucleoli in early G1.

During mitosis, the major ribosomal genes in humans are located in five chromosomal locations called nucleolar organizer regions (NOR) (Mikelsaar et al., 1977a; Mikelsaar et al., 1977b). In interphase, the NORs are located in nucleolar FCs and are bound to the machinery for rRNA-transcription (i.e. polymerase I and UBF). It has been suggested that during cell division most of these components remain associated with NORs, thus forming defined protein attachment sites on condensed chromosomes (Chan et al., 1991; Scheer and Rose, 1984). Earlier immunohistochemical observations suggested that topo I resides at NORs of mitotic chromosomes (Suja et al., 1997). The data in Figs. 4.31 and 4.32 suggest that topo I indeed was bound to such NORs *in vivo* and that this binding (like the association of the enzyme with FCs of interphase nucleoli) is mediated by the N-terminus of the enzyme. This notion was corroborated by counterstaining against RNA polymerase I, which is an established marker of NORs. Fig 4.33 shows a cell at anaphase (see DNA stain at bottom) expressing GFP-topo I-N and labelled with an antibody specific for RNA polymerase I. Upon

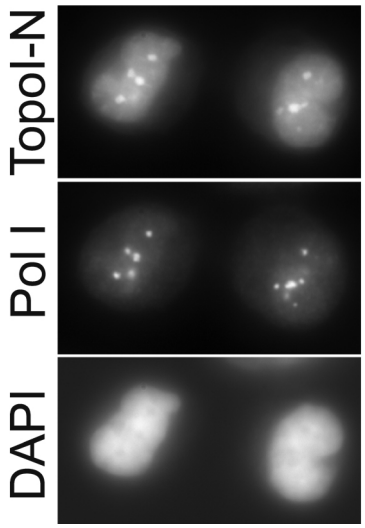

Fig 4.33: **Colocalization of GFP-Topo I-N with RNA polymerasee I.** GFP-Topo I-N expressing cells were grown on microscope slides, PFA-fixed, permeabilzed, and doublestained with specific RNA polymerase I antibody (middle) and DAPI (right). Top image show corresponding GFP fluorescence (GFP-Topo I-N).

comparing topo I-specific GFP-fluorescence (top) and RNA polymerase I specific immunofluorescence (middle), it becomes apparent that both proteins colocalize. Thus, we concluded that the N-terminus directs topo I to NORs and mediates its binding to this structure throughout the entire cell-cycle. It should be noted that our data add significantly to previous immunohistochemical data in as much as they show that in a living cell.

5. Discussion

In this work, an experimental model is presented enabling the study of DNA topoisomerases in living cells. Thus, we could monitor the localization of these enzymes in interphase and during the cell-cycle, and we could measure the mobility of topoisomerases at various locations in the nucleus of a living cell. These investigations allowed us to address and in some cases clarify several issues regarding the biological functions of mammalian topoisomerases, which, up to now, have been a subject of intense debate.

Firstly, we could address the question, whether the two isoforms of mammalian topo II have specific functions, and, if so, what these might be.

Secondly, we could investigate whether topo IIα and/or topo IIβ are mobile or not, and thus, whether they are likely to partake in the building of a fixed nuclear matrix/chromosomal scaffold.

Thirdly, we could address questions regarding the role that topoisomerases I and II might play in the nucleolus, and the related issue of the association of topo I with the machinery of rDNA-transcription.

Finally, we could for the first time observe in a direct manner the primary cellular effects of substances stabilizing covalent catalytic DNA-intermediates of topoisomerases (topoisomerase poisons). This last issue is of considerable interest to clinical oncology, because poisons of topo I and II are frequently used in the clinic for tumour chemotherapy.

5.1 Topoisomerase I and II are Entirely Mobile Nuclear Proteins.

5.1.1 Mobility of Topoisomerase I and its Implications

We found that topo I is entirely mobile within the cell nucleus, which is in clear contradiction to previous speculations of many sorts suggesting a compartmentalization of several distinct enzyme pools (Danks et al., 1996; Mo et al., 2000b; Mo et al., 2002; Muller et al., 1985). The enzyme is clearly not compartmentalized in the nucleus of a living cell. Despite its apparent accumulation in nucleoli, it is in continuous flux between nucleoli and the nucleoplasm and this finding conforms to a recent concept proposing that nuclear compartments are generated by a binding equilibrium of entirely mobile proteins (Misteli, 2001). Photobleaching techniques have shown that only a few components of the nucleus are immobile, i.e. the chromatin itself, chromatin-bound core histones, and structural components building the nuclear lamina (Moir et al., 2000), whereas most other nuclear proteins move rapidly throughout the nucleus. Moreover, the components of nuclear compartments, e.g. nucleoli, seem to be in continuous flux between the compartment and the nucleoplasm (Misteli, 2001). In keeping with this, we show here that topo I is not restricted to the nucleoli but imposes as a nucleolar protein, because it is moving more slowly in nucleoli than in the

nucleoplasm. This concept of a stringent correlation between mobility and localization is strongly supported by our data obtained with the inactive mutant topo I^{Phe723}, which has the same mobility in both compartments and is therefore more evenly distributed between them.

A comparison of FRAP kinetics shows that GFP-tagged topo I moves slower than GFP alone. While movements of GFP are solely governed by free diffusion, topo I is obviously restricted in its mobility by additional factors. It has been proposed that these must be less mobile nuclear components (Misteli, 2001). However, the nature of such components – the chromatin, a putative karyoskeleton, or both - is an unsolved issue (Misteli, 2001; Shopland and Lawrence, 2000). Such an attenuated mobility is a common feature of many nuclear proteins that are mobile (Misteli, 2001). However, we show here in addition that in each nuclear compartment topo I is divided into a slow and a fast fraction. We do not know whether these fractions are inter-convertible, but the most plausible interpretation is that the enzyme switches between an *off state*, where it is more or less freely diffusible ($t_{1/2}$ = 1 – 2 s), and an *on state*, where it's mobility is about 10-fold reduced ($t_{1/2}$ = 12 – 14 s). Since topo I is continuously scanning the entire nuclear space, interactions of the enzyme with processes and places where its activity is required must implicitly be transient. It stands to reason that such interactions will involve less mobile nuclear components, such as chromatin, nuclear matrix, multi protein complexes etc., and, therefore, slow down the enzyme (Misteli, 2001; Shopland et al., 2001). Thus, the slow fraction (the *on state*) most likely represents topo I engaged in processes, whereas the fast fraction (the *off state*) represents topo I moving between processes. This interpretation of our data is in good agreement with a "stop-and-go" model recently suggested for the interaction of histone H1 with chromatin (Hendzel et al., 1999; Misteli et al., 2000).

5.1.2 Mobility of Topoisomerase II and its Implications

We show here, that the two isoforms of human topo II isoforms also belong to the class of entirely mobile proteins, and it is reasonable to assume that their distribution between nucleoli and nucleoplasm is governed by the same principles as that of topo I. As that may be, it is beyond doubt that the entire pools of the type II enzymes are mobile and exchange rapidly between nuclear compartments in interphase and between chromosomes and the cytoplasm in mitosis. This came as a surprise, considering the general opinion on the dual role of topo II as an enzyme and as a structural protein forming a mitotic scaffold or interphase nuclear matrix (Adachi et al., 1991; Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al., 1986). Our observation that at each stage of the cell-cycle bleached areas were rapidly and completely replenished with fluorescent topo II molecules, makes it unlikely that even a subpopulation of topo II could be an integral component of immobile structures such as a putative karyoskeleton or chromosome scaffold. However, we cannot rule out that topo II transiently associates with such structures. In fact, the colocalization of topo II with the chromosomal core often reported by others and our own observations on the distribution of

topo II-GFP in native chromosome spreads even suggests a tight (but dynamic) interaction of topo II with something like a chromosomal scaffold.

5.2 Topoisomerase I and II at the Nucleolus

During interphase, the most prominent feature of topo I and II is their accumulation in nucleoli. Closer inspection shows that topo IIα and IIβ have the same subnucleolar distribution, whereas topo I is clearly different in this respect. Thus, topo IIα and IIβ seem to have similar nucleolar functions, whereas a different set of functions is attended to by topo I.

5.2.1 Putative Nucleolar role of Topoisomerases IIα and β

Genetic studies in yeast suggest that type II topoisomerases are required for the organization of the rDNA genes, as a cofactor of RNA polymerase I, and for the topological organization of the nucleolar chromatin (Hirano et al., 1989). They are also believed to be required in the nucleoli to relax DNA supercoils generated during the transcription of rDNA (Christman et al., 1988). These functions imply that the enzyme must be present at the nucleolus, a proposal supported by the observation that topo II poisons induce double stranded cleavage of rDNA (Govoni et al., 1994). While these studies indicate that type II topoisomerases in general must be present at the nucleolus, they do not answer the question, if both of the two mammalian isoforms are working there, or just one.

Isoform specific nucleolar functions have been suggested by immunohistochemical localization studies of mammalian topo IIα and IIβ, proposing that one isoform is present in nucleoli, whereas the other is not. One study claimed that topo IIβ is the isoform responsible for organization and regulation of nucleolar rDNA (Zini et al., 1992; Zini et al., 1994). Another study proposed the opposite (Meyer et al., 1997). Here, we were able to settle the argument by showing that in a living cell nucleus both isoforms accumulate in the nucleoli. Actually, they do so to such an extent that both of them impose as mainly nucleolar proteins. This finding argues against specific functions of topo II isoforms in the nucleoli, a view also supported by complementation studies in yeast, indicating that both human isoforms can suppress intra-chromosomal excision recombination of the rDNA locus in yeast, which is a hall-mark of Δtopo II mutants (Jensen et al., 1996b).

We clearly demonstrate, that fixation and permeabilization of cells in the course of immunostaining changes the nucleolar localization of topo II (see fig. 4.5). Similar observations have been made in Drosophila, where topo II was excluded from the nucleoli of fixed cells (Berrios et al., 1985), whereas the enzyme was present in the nucleoli of living cells (Swedlow et al., 1993). In the light of these observations it becomes overwhelmingly plausible that the previous controversy regarding the nucleolar localization of topo II must be due to artefacts introduced by the immunostaining procedure. Our data and the previous study of topo II microinjected into drosophila embryos (Swedlow et al., 1993) show that the

association of topo II with nucleolar structures is quite delicate and can easily perturbed. We show furthermore, that topo IIα and IIβ are getting depleted from nucleoli upon exposure to topo II poisons. This indicates that their nucleolar accumulation is not entirely dependent on DNA-interactions, but rather seems to be effected by an altered mobility due to protein-protein interactions. Considering, on the other hand, the dense packing of topo IIα and IIβ in the nucleoli of living cells, and considering that among numerous topo II-interacting proteins described in the literature, none is a nucleolar protein, it could also be imagined that polymerization of topo II (Vassetzky et al., 1994) might create a transient, less mobile structure thus contributing to accumulation of the enzymes in nucleoli. Both mechanisms are suitable to explain why the association of topo IIα and β with nucleoli is so fragile and easily perturbed.

Our data allow some speculations about the biological significance of topo IIα and topo IIβ at the nucleolus. Firstly, we observed that the enzymes are excluded from small globular structures inside the nucleoli. We have not yet been able to devise an immunstaining procedure preserving this pattern in such a way that a comparison with other markers would be possible. Thus, the identity of the globular structures remains unsolved. However, based on their appearance, we believe that these globules, from which topo IIα and IIβ are excluded, are most likely fibrillar centers. This would mean that topo IIα and IIβ do not play a prominent role in rDNA-transcription, which is in clear contrast to topo I, as will be discussed later.

Along this reasoning the question arises of why topo IIα and topo IIβ accumulate in nucleoli to such an extent that in a living interphase nucleus they impose as proteins that are almost exclusively based at the nucleolus.

One possible explanation would be that nucleoli serve as storage places for enzyme molecules that should not be active in the chromatin at a given time. Thus, dosage of topo IIα and topo IIβ in the extra-nucleolar chromatin might be regulated by nucleolar accumulation and release. Four considerations add feasibility to this concept: (i) The margin of cellular tolerance to topo II levels is extremely narrow, making it likely that a cell needs a rapid mechanism for adapting the level of topo II active in the chromatin. (ii) Nucleolar accumulation of topo IIα and IIβ seems mainly not to involve catalytic DNA-interactions, making it likely that this portion of the enzyme is neutral with respect to the genomic DNA. (iii) Nucleolar and nucleoplasmic pools of the enzyme are rapidly interchanging, and thus suited for rapid regulational processes. (iv) At any given time, the nucleoli contain more topo II than can plausibly be required for the organization of the topological organization of the nucleolar chromatin, which is small in comparison to the entire genome.

On the other hand, our data might indicate that topo IIα and IIβ play some role in nucleolar structure, which is independent of DNA. Actually, the enzymes could be involved in the early steps of nucleologenesis. We consistently observed that at late telophase both isoforms concentrated at multiple light dense structures, which are most likely pre-nucleolar bodies (Bell et al., 1992), because they converge to nucleoli, as daughter nuclei reform and cells progress into G1-phase.

5.2.2 Tight Association of Topoisomerase I with FCs and NORs Throughout the Cell-cycle.

In the literature, nucleolar localisation of topo I is much less controversial than that of topo II (Baker et al., 1995; Bharti et al., 1996; Danks et al., 1996; Meyer et al., 1997; Mo et al., 2000b; Muller et al., 1985; Wadkins et al., 1998). Moreover, genetic studies in yeast show that the enzyme must have a prominent function in the maintenance and the use of the rRNA-genes, because rDNA-recombination increases in the absence of topo I (Gangloff et al., 1994). Therefore, we found it most puzzling that in several recent studies (Mo et al., 2000a; Mo et al., 2000b; Mo et al., 2002), only 15% of cells transiently expressing GFP-topo I showed nucleolar accumulation of the biofluorescent enzyme, whereas the rest showed a globular aggregation in the nucleoplasm and the cytosol. Here, we show clearly that biofluorescent topo I constitutively expressed at normal levels and in an active form accumulates to a major part in the nucleoli and does not at all form nucleoplasmic or cytosolic globules. Thus, our data are in good agreement with the older literature, whereas conflicting observations more recently made with transient expression, are most probably due to an aberrant cellular placement of topo I not properly folded, and/or to the observation of cells gone into apoptosis due to overexpression of the enzyme.

With our more physiological expression system we could even distinguish the subnucleolar distribution of topo I. We demonstrate that the enzyme is highly accumulated in the FC-subcompartment of the nucleolus, where it co-localizes with RNA polymerase I (Gilbert et al., 1995; Scheer and Rose, 1984). Actually, these two proteins are known to copurify (Rose et al., 1988). Thus, our data fit the long standing concept that rDNA-transcription is a focus of topo I function, and that the enzyme is most probably a component of the RNA-polymerase holoenzyme. Interestingly, we show here that this specific sub-function is under the direction of the non-conserved N-terminus of topo I, a domain that distinguishes the full length mammalian enzyme from a minimal variant encoded by vaccinia virus (Shuman and Moss, 1987), and from the distantly related tyrosin recombinases (Grainge and Jayaram, 1999). Studies in *Drosophila* have shown that this N-terminal domain can target topo I to sites that are transcriptionally active (Shaiu and Hsieh, 1998), whereas it seems to contribute very little (if anything at all) to the enzyme's activity *in vitro* (Lisby et al., 2001). We show here quite clearly that the main function of the N-terminus is, to associate topo I with nucleolar FCs. When expressed alone, the N-terminus localizes almost exclusively to FCs of interphase nucleoli. Some of it even stays bound to corresponding loci on mitotic chromosomes (i.e. the NOR), and it colocolizes with RNA-polymerase I at both locations. These data are supported by older immunohistochemical studies also demonstrating topo I at the NORs of mitotic chromosomes (Guldner et al., 1986; Suja et al., 1997). They suggest that at all times a certain fraction of topo I is integrated into the RNA-polymerase I holoenzyme by virtue of its N-terminus. One is tempted to speculate that in this way the enzyme remains associated with the rDNA during the entire cell-cycle.

However, the precise localization of transcriptionally active rDNA within the nucleolus is still open to discussion. Some authors suggest that rDNA transcription occurs largely at the boundaries between the FC and the DFC, whereas the FC is believed to serve as a storage place for inactive RNA polymerase I and other transcription factors (Stanek et al., 2001). In controverse to this, other studies suggest that the FC is the place, where the transcriptionally active rDNA is confined (Mais and Scheer, 2001). This controversy provides us with two alternative interpretations of our data. One is that topo I at the FC is a storage form, and the other is that topo I at the FC is heavily involved in the relaxation of supercoils generated during transcription of rDNA. The second model is supported by the published observation that ongoing transcription is crucial for the nucleolar localization of topo I (Mao et al., 2002). The inverse should be the case, if the enzyme was stored there in an inactive form. The second model is also more appealing from a teleological point of view. The rRNA genes are the most heavily transcribed genes in the cell, and it is sense making that a substantial portion of the machinery required for this process including topo I should stay associated with the sites where these genes are. The second model is however somewhat disfavoured by our observation that CpT treatment causes a rapid depletion of topo I from the nucleoli. If topo I was heavily engaged in DNA catalysis at the FC, then it should be fixed there by CpT, and not become depleted. However, we do not know if the nucleoli are as accessible to CpT as the nucleoplasm.

5.3 Specific Roles of Topoisomerases IIα and β during Cell Division

While the *in vivo*-morphology of topo IIα and IIβ is very similar at interphase, the two isozymes differ significantly from each other, when it comes to their localization during the mitotic cycle. We find that in metaphase topo IIβ is scarcely chromosome-associated. This fits with the recent observation that disruption of the *TOP2β* gene has little effect on early embryonal development in mouse (Yang et al., 2000) and with our previous finding that in a mammalian cell topo IIβ cannot substitute topo IIα during mitosis (Grue et al., 1998). It seems now quite clear that topo IIβ is not required for mitotic cell division. Yet, in a living cell topo IIβ is not fully excluded from metaphase chromosomes, and it retains to some extent the ability to engage in catalytic DNA-turnover in the chromosome. This might be sufficient to support mitotic topo II functions in yeast (Jensen et al., 1996b), but not in mammals (Grue et al., 1998), and thus explain discrepancies between yeast complementation studies and mammalian knock out-approaches. In keeping with this, only topo IIα accumulates at the centromers. This feature seems to be restricted to those stages of the cell-cycle, where the two chromatids are still linked together at the centromeric region. Such findings were also hinted at by previous immunohistochemical studies (Rattner et al., 1996; Sumner, 1996; Taagepera et al., 1993). They suggest that topo IIα accumulates at centromers until the centromeric DNA is segregated at anaphase and that it is released immediately thereafter. This phenomenon most probably reflects a requirement of a high topo II activity during the segregation of the

centromeric heterochromatin ensuring simultaneous and fast separation of the centromeric regions of all chromosomes upon initiation of sister chromatid-separation.

5.4 Immobilisation of Topoisomerases by Topoisomerase Poisons.

Inhibitors of DNA topo I and II play an important role in anti-cancer chemotherapy. Actually, there are very few therapy protocols currently in use that do not employ a topoisomerase-targeted drug at one stage or another. Most important in this respect are those compounds that inhibit the second transesterification step of the catalytic cycle and thus prolong the half-life of the covalent catalytic DNA-intermediate. As reviewed in sections 1.2.4 and 1.3.4 of the introduction, a host of biochemical data indicates this mechanism of action. However, until now direct evidence has been lacking that shows these drugs to actually immobilize topoisomerases on the genomic DNA. It has not been possible to access the primary *in vivo*-effect of these drugs in quantitative terms. It has not been possible to investigate, where in the genome it actually happens. It has not been possible to measure the extent to which the half-life of the covalent intermediates is prolonged. And it has not been possible to determine how much of the cellular complement of the enzymes is actually affected, and how this will influence the general disposition of the enzymes in the cell nucleus. The data presented here, allowed us to address most of these issues.

First of all, we show here for the first time that topoisomerase poisons actually render topoisomerases less mobile in the genome of a living cell, and that, given a sufficient dose, most if not all of the enzyme molecules present in the cell nucleus are affected. Secondly, we show that these drugs target topoisomerases preferentially in the nucleoplasm, whereas the enzyme is much less affected in the nucleolus. Thus, they induce a relocation of the enzyme from the nucleolus to the nucleoplasm. Thirdly, we show that drugs targeting topo I act in a manner distinctively different from drugs targeting topo II. Topo II is simply immobilized as long as the drug is present, whereas in the case of topo I a more complex situation is encountered, which was investigated in more detail.

5.4.1 The Impact of CpT on the Mobility of Topoisomerase I

CpT is known to bind and stabilize the covalent topo I·DNA intermediate (Hertzberg et al., 1989). At least in living cells, the drug does not immobilize topo I completely, which fits some previous biochemical data (Straub et al., 1997) showing that topo I·DNA intermediates stabilized by camptothecin have a comparatively short half life (< 1 min). In keeping with this, the drug attenuates the mobility of the enzyme in a gradual manner. Apparently, CpT attenuates preferentially the slow fraction of topo I, and it does so preferentially in the nucleoplasm. This supports the notion of this fraction being the one engaged in DNA-turnover, thus fitting the *on/off state*-model suggested in 5.1.1. Why does topo I disappear so rapidly from its favoured nucleolar residence when camptothecin is

present? It has been proposed that nucleolar delocalization is triggered by conjugation of the enzyme with small ubiquitin-like modifiers (Mo et al., 2002). However, this process is operating on a minute scale (Mao et al., 2000a), whereas we show here that nucleolar delocalization occurs within seconds. Moreover, it is unlikely that an entirely mobile protein like topo I should require specific signals in order to move from one place in the nucleus to another. As elaborated in section 5.1.1, topo I is prone to accumulate in the nucleoli, because here it is moving more slowly than in the nucleoplasm. In the presence of CpT, however, the situation is rapidly reversed: Now, the enzyme is moving more slowly in the nucleoplasm than in the nucleoli. Accordingly, it accumulates in the nucleoplasm and delocalizes from nucleoli, although it is still freely exchanging between the two compartments. Thus, nucleolar delocalization of topo I upon exposure to camptothecin seems to be a plausible epiphenomenon of the enzyme's nuclear traffic and not a reflection of some specific cellular response to the drug. In keeping, topo I^{Phe723}, which cannot be stabilized in covalent DNA-complexes by camptothecin, does not delocalize from nucleoli upon exposure to the drug, as should be the case, if nucleolar delocalization was due to some kind of a coordinated stress reaction and not a direct consequence of the altered mobility of the enzyme. Although our data strongly argues against a direct role of sumoylation in nucleolar delocalization this process may very well be involved in later steps of cellular response to CpT.

5.4.2 Mobility of Topoisomerases as a Means to Characterize Topoisomerase-directed Drugs

Mobility measurements of bio-fluorescent topoisomerases expressed in mammalian cells provide a powerful tool for the study of topoisomerase directed anti-cancer agents. For example it is possible to assess by this means the isoform-selectivity of established topo II poisons. We show here that the podophyllotoxin VM 26 has a certain preference for the α-form of topo II. It is not known yet how other classes of established topo II poisons (e.g. anthracyclins or aminoacridines) behave in this respect. Recently, new compounds have been discovered, which are claimed to be selective poisons of topo IIβ (Barthelmes et al., 2001; Gao et al., 1999). The scope of tumours targeted by these new compounds differs markedly from that targeted by established topo II poisons. Thus, it is conceivable that isoform selectivity determines the efficacy of topo II poisons against various tumour types and should be taken into account, when applying these drugs in the clinic. The experimental system described here would allow such an assessment in a very rapid and precise manner. It could moreover been used to screen substance libraries for new topo II poisons and again the focus could be put on the identification of isoform-selective substances, e.g. ones that are specific for the α-form.

With topo I poisons the situation of drug development is even more open. Camptothecins constitute so far the only class of substances that are indeed specific poisons of topo I. Some camptothecin derivatives have just entered clinical use as tumour therapeutics. We show here that camptothecin immobilises topo I to a lesser extent and much

more transiently than VM 26 immobilises topo II. This explains, why camptothecins have to be administered as a continuous dose in order to be clinically efficient (Slichenmyer et al., 1993). Continuous administration provokes a number of extremely unpleasant side effects (e.g. nausea) that would be much less, if the drug could be given as a bolus. Thus, it would be desirable to find a topo I poison that immobilises topo I more permanently, and again we provide here the ideal tool to find and characterise such a drug.

Finally, it has been proposed that dual drugs poisoning at the same time type I and type II topoisomerases, would be the ideal cancer therapeutics. So far such substances have not been found and we propose that they could be found if our experimental system was used for screening.

6. Summary

The first goal of this study was to develop cell lines with a stable expression of bio-fluorescent topo II and topo I. This was successfully achieved using a bicistronic vector system. Control experiments showed that proteins of expected size were expressed, and that GFP-tagged topos I, IIα, and IIβ were active in the cells and fully integrated in the endogenous pools of the enzymes. These cell-lines provided a novel tool for investigating the cell biology of human DNA topoisomerases.

Our most important finding was, that both types of mammalian topoisomerases are entirely mobile proteins that are in continuous and rapid flux between all compartments of the nucleus and between the cytososl and the chromosomes of mitotic cells. This was particularly surprising with regard to topo II, which is considered to be a structural component of the nuclear matrix and the chromosome scaffold. We must conclude that if this was the case, then these architectural structures appear to be much more dynamic than believed until now. In this context it should also be mentioned, that the alignment of topo II with the central axes of the chromosome arms, which has until now been considered a hall-mark of the enzyme's association with the chromosomal scaffold, is not seen *in vivo* and can be demonstrated to be to some extent an artefact of immunohistochemistry. Furthermore, we show that the two isoforms of topo II (α and β) have a different localisation during mitotic cell division, supporting the general concept that topo II functions at mitosis are exclusively assigned to the α-form, whereas at interphase the two isoenzymes work in concert.

Despite unrestricted mobility within the entire nuclear space, topoisomerases I and II impose as mostly nucleolar proteins. We show that this is due to the fact that in the nucleoli they are moving slower than in the nucleoplasm. The decreased nucleolar mobility cannot be due to DNA-interactions, because compounds that fix topoisomerases to the DNA deplete them from the nucleoli. Interestingly, the subnucleolar distribution of topoisomerases I and II was complementary. The type II enzymes filled the entire nucleolar space, but excluded the fibrial centers, whereas topo I accumulated at the fibrial centers, an allocation directed by the enzyme's N-terminus. During mitosis, it also mediates association with the nucleolar organising regions of the acrocentric chromosomes. Thus, topo I stays associated with the rDNA during the entire cell-cycle and consistently colocalizes there with RNA-polymerase I.

Finally, we show that certain cancer drugs believed to act by stabilising covalent catalytic DNA-intermediates of topoisomerases, do indeed immobilize the enzymes in living cells. Interestingly, these drugs do not target topoisomerases in the nucleoli but only in the nucleoplasm.

7. Zusammenfassung

Diese Arbeit hatte zunächst zum Ziel, humane Zelllinien zu etablieren, in denen GFP-Chimären der humanen DNS-Topoiosmerasen I, IIα und IIβ stabil und constitutiv exprimiert werden. Dies wurde mit Hilfe eines bicistronischen Expressionsvektors erreicht, der eine *quasi* physiologische Expression der GFP-Chimären in humanen Zellen ermöglichte. Wir zeigen, dass die chimärischen Proteine die erwartete Größe haben, aktiv sind, und vollständig in die jeweiligen endogenen Enzympopulationen integriert werden. Damit halten wir ein ideales Werkzeug zur Untersuchung der Zellbiologie der Topoisomerasen in Händen.

Der wichtigste Befund dieser Arbeit ist, dass sowohl Topoisomerase I, als auch die beiden Typ II-Enzyme innerhalb des Zellkerns vollständig mobil sind und zwischen Nukleoplasma und Nukleolen, sowie zwischen Zytosol und Chromosmen mitotischer Zellen ständig austauschen. Dieser Befund ist insbesondere hinsichtlich der Topoisomerase II erstaunlich, da man bisher davon ausgeht, dass dieses Enzym eine zentrale Rolle bei der Konstituierung der Kernmatrix bzw. des Chromosomengerüstes spielt, was mit einem vollständig und rasch beweglichen Protein unvereinbar scheint. Falls Topoisomerase II tatsächlich eine solche Rolle spielen sollte, müssen die Kernmatrix und das Chromosomengerüst sehr viel dynamischere Strukturen sein, als bisher angenommen. Es sei in diesem Zusammenhang auch darauf hingewiesen, dass wir in der lebenden Zelle keine Konzentrierung der Topoisomerase entlang der zentralen Längsachsen der Chromosmenarme gesehen haben, was bisher als Markenzeichen von deren Gerüstfunktion galt. Vielmehr konnten wir zeigen, dass eine solche axiale Anordnung überwiegend ein Artefakt immunhistologischer Methoden darstellt. Wir zeigen weiterhin, dass die beiden Isoformen der Topoisomerase II (α und β) während der Zellteilung unterschiedlich lokalisiert sind, was mit dem generellen Konzept zusammenpasst, dass nur die α-Form mitotische Funktionen wahrnimmt, während die beiden Isoenzyme in der Interphase konzertiert arbeiten.

Ungeachtet ihrer unbegrenzten Mobilität innerhalb des gesamten Zellkerns, imponieren Topoiosmerase I und II in der lebende Zelle als überwiegend nukleoläre Proteine, was damit zusammenhängt, dass sie sich hier etwas langsamer bewegen, als im Nukleoplasma. Diese relative Abbremsung kann nicht auf DNS-Interaktionen beruhen, weil chemische Susbtanzen, die Topoisomerasen an der DNS fixieren, eine Entleerung der Nukleolen bewirken. Interessanterweise ist die subnukleoläre Verteilung von Topoisomerase I und II komplementär. Die Typ II- Enzyme füllen den gesamten nukleolären Raum aus, sparen aber die fibrillären Zentren aus, während das Typ I- Enzym fast ausschließlich an den fibrillären Zentren zu finden ist. Diese Assoziation wird durch den N-Terminus des Enzyms vermittelt, der darüber hinaus während der Mitose auch die Bindung an die nukelolären Organisationszentren der akrozentrischen Chromosomen bewirkt. So bleibt Topoisomerase I über den gesamten Zellzyklus hinweg mit der rDNS physisch verbunden und kolokalisiert hier mit der RNS-Polymerase I.

Schließlich konnten wir zeigen, das bestimmte Tumnortherapeutika, von denen man annimt, dass sie über eine Stabilisierung der kovalenten katalytischen DNS-Intermediate der

Topoiosmerasen wirken, diese Enzyme in der lebenden Zelle tatsächlich immobil machen. Interssanterweise treffen diese Substanzen überwiegend im Nukleolplasma und nicht in den Nukleolen.

8. List of Abbreviations

5-FOA	5-fluoroorotic acid
2-AP	2-aminopurine
ACF	ATP-dependent chromatin assembly and remodeling factor
AMPPNP	Adenosine 5'-[β,γ-imido]triphosphate (a non-hydrolysable ATP analogue)
ATP	Adenosine triphosphate
CAP	Catabolite activator protein
CHRAC	Chromatin accessibility complex
CpT	Camptothecin
DAPI	4, 6-diamidino-2-phenylindole
DFC	Dense fibrillar component
IRES	Internal ribosome entry site
EtBr	Ethidium bromide
EM	Electron microscopy
FC	Fibrillar center
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GAM	Goat anti-mouse
GAR	Goat anti-rabbit
GC	Granular component
GFP	Green fluorescent protein
G-segment	Gate segment (the DNA segment to be cleaved and opened to form a gate)
Gyr A	A-subunit of DNA gyrase
Gyr B	B-subunit of DNA gyrase
HTH	Helix turn helix
kb	Kilo base
kDa	Kilo dalton
kDNA	Crithidia fasciculata kinetoplast DNA
LK	Linkage number
MCS	Multiple cloning site
NLS	Nuclear localization signal
NORs	Nucleolar organizer regions
NURF	Nucleosome remodeling factor
pac	Pyromycin-N-acetyltransferase (puromycin resistance)
PARP	Poly(ADP-ribose)polymerase
Pat 1	Protein associated with topoII
PCR	Polymerase chain reaction
PFA	Para formaldehyde
PKC	Protein kinase C
PK CK2	Casein kinase II
PNBs	Prenucleolar bodies

Rb	Retinoblastoma protein
S/MAR	Scaffold/matrix attached region
SDS	Sodium dodecyl sulfate
Sc2	Scaffold protein 2
Sgs 1	Slow growth suppresser
SUMOs	Small ubiquitin-like modifiers
T2C	Structure of the DNA-closed gyrase fragment
T2O	Original topoII DNA-open conformation of the yeast 92 kDa fragment
TDP1	Tyrosine-DNA phosphodiesterase
T-segment	Transport-segment (the DNA segment transported through the gate in the G-segment)
Topo II	DNA topoisomerase type IIA
Topo I	DNA topoisomerase type IB
TOP2A	Gene encoding topoIIα
TOP2B	Gene encoding topoIIβ
TPI	Triose phosphate isomerase
Tw	Twist
T-X-100	Triton X-100
VM 26	Teniposide
Wr	Writhe
Wt	Wild type

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10. List of publications

1. Mielke, C., M.O. Christensen, O. Westergaard, J. Bode, C.J. Benham, and M. Breindl. 2002. Multiple collagen I gene regulatory elements have sites of stress-induced DNA duplex destabilization and nuclear scaffold/matrix association potential. *J Cell Biochem*. 84:484-496.

2. Christensen, M.O., M.K. Larsen, H.U. Barthelmes, R. Hock, C.L. Andersen, E. Kjeldsen, B.R. Knudsen, O. Westergaard, F. Boege, and C. Mielke. 2002. Dynamics of human DNA topoisomerases IIalpha and IIbeta in living cells. *J Cell Biol*. 157:31-44.

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4. Christensen, M.O., H.U. Barthelmes, F. Boege, and C. Mielke. 2002. The N-terminal domain anchors human topoisomerase I at fibrillar centers of nucleoli and nucleolar organizer regions of mitotic chromosomes. *J. Biol. Chem. In press*.

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