Studies on the removal of human topoisomerase I cleavage complexes in vitro and the involvement of the tumor suppressor protein p53.

Dissertation

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Abbreviations

°C	Degrees Celsius
µg	Micro gram
µl	Micro liter
Å	Ångstrøm
aa	Aminoacids
Ab	Antibody
app.	Approximately
Asp	Aspartate
ATM	Ataxia telangiectasia protein M
ATP	Adenosine triphosphate
bp	Base pair
cpm	Counts per minute
CPT	Camptothecin
Da	Dalton
DMS	Dimethylsuberimidate
DMSO	Dimethyl sulphoxid
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DTT	Dithiotreitol
EDTA	Ethylendiamin-tetra-acetic acid
F	Phenylalanine
FPLC	Fast protein liquid chromatography
g	Gram or gravity
His	Histidine
hr.	Hour
HRP	Horse-radish-peroxidase
hrs.	Hours
htopo70	Human topoisomerase I 70 kDa truncation mutant
htopoI	Human topoisomerase I
ICT	In vivo crosslinked topoisomerase

k	Kilo
M	Molar
m	Murine
Mdm2	Mouse double minute 2 protein
mg	Milli gram
min	Minute
ml	Milli liter
mm	Milli meter
mM	Milli molar
NER	Nucleotide excision repair
ng	Nano gram
Ni	Nickel
NLS	Nuclear localisation signal
nt	Nucleotide
nts	Nucleotides
OL	Oligomer
p53	Tumor suppressor protein p53
PAGE	Polyacrylamide gel electrophoresis
PCNA	Proliferating cell nuclear antigen
PCV	Packed cell volume
PMSF	Phenylmethylsulfonylflourid
PVDF	Polyvinylidenfluorid
Rb	Retinoblastoma protein
rDNA	DNA stretch coding for ribosomal RNA.
Ref-1	Redox factor 1
RNA	Ribonucleic acid
RPA	Replication protein A
rpm	Rounds per minute
S	Serine
SDS	Sodium dodecylsulfate
Ser	Serine
SV40	Simian virus 40

TBE	Tris-borate-EDTA
Tdp1	Tyrosyl-DNA phosphodiesterase 1
TDP1	Gene encoding for the Tdp1 enzyme
TE	Tris-EDTA
topol	Topoisomerase I
Tyr	Tyrosine
U	Unit
UV	Ultra violet light
v/v	Volume to volume
vol.	Volume
W	Watt
w/v	Weight to volume
wt	Wildtype
XP-A	Xeroderma pigmentosum complementation group A
XPA	Xeroderma pigmentosum complementation group A protein
XP-D	Xeroderma pigmentosum complementation group D
XPD	Xeroderma pigmentosum complementation group D protein
Y	Tyrosine

1 Introduction

Topoisomerases are abundant enzymes that are involved in several important pathways, such as transcription and replication (Stewart *et al.* 1987, Yang *et al.* 1987, Snapka *et al.* 1988, Stewart *et al.* 1990) where the removal of positive supercoils is required for ongoing RNA and DNA synthesis. However, involvement in recombination and repair has also been suggested (Zhu and Schiestl 1996, Megonigal *et al.* 1997, Cheng *et al.* 1998, Nitiss and Wang 1988, Shcherbakova and Filatov 2000; Polanco *et al.* 2000).

1.1 Introduction to topoisomerase I

Topoisomerases are classified into two major groups, type I (A and B) and type II. Type II enzymes are the prokaryotic topoisomerase IV (Kato *et al.* 1990, 1992), DNA gyrase and the eukaryotic topoisomerase IIα and IIβ enzymes. They function as a homodimer and cleave both strands of the DNA thereby changing the linking number by two for every catalytic cycle. The catalytic activity is ATP-dependent. The incision results in a covalent linkage of the enzyme to the 5' end of each DNA strand and generates a double-strand break with a free 3'-OH group.

Type I A enzymes cover enzymes from various organisms: among others *Escherichia coli* topoisomerase I, yeast and human topoisomerase III (Andersen *et al.* 1996, Goulaouic *et al.* 1999). They are monomeric enzymes which cleave one DNA strand and change the linking number by one. During the catalytic cycle they become covalently attached to the 5' end of one strand and generate a nick with a free 3'-OH group. Type IB topoisomerases comprise the eukaryotic and poxvirus topoisomerase I (Andersen *et al.* 1996) and the prokaryotic (*Methanopyrus kandleri*) topoisomerase V (Slesarev *et al.* 1993). The human topoisomerase I exists both as a nuclear (htopoI) and a mitochondrial (htopoImt) enzyme (Zhang *et al.* 2001) which are encoded for by two separate nuclear genes. These topoisomerases also cleave one DNA strand and change the linking number by one without the use of ATP. However, in contrast to type I A and type II

they form a covalent complex with the 3' end of one DNA strand generating a nick with a free 5'-OH group.

1.1.1 Structure of htopoI

HtopoI consists of 765 aa (91 kDa) encoded by the TOP1 gene. Studies from limited proteolysis and crystal structure determination indicated that htopoI consists of four distinct domains: a N-terminal domain (aa 1 to 198), a core domain (aa 198 to 651), a linker domain (aa 651 to 696), and a C-terminal domain (aa 696 to 765) (Stewart *et al.* 1996, Stewart *et al.* 1997, Redinbo *et al.* 1998). The N-terminal domain is largely disordered, highly charged and contains very few hydrophobic residues (Redinbo *et al.* 1998). It contains four putative nuclear localization signals (NLS) which also have been shown to be responsible for the nuclear localization (Mo *et al.* 2000). Otherwise it is unclear what role the N-terminus plays, since it is dispensable for the catalytic activity (Stewart *et al.* 1997). The 54 kDa core domain is resistant to proteolysis. It contains the main DNA binding domain and, together with the C-terminal domain, forms a closed clamp around the DNA (Redinbo *et al.* 1998, Stewart *et al.* 1998). The core domain is coupled to the C-terminal domain by a 3 kDa short linker which has been found to be flexible and to change conformation upon binding and cleavage (Stewart *et al.* 1998, Redinbo *et al.* 1999). The C-terminal domain has a molecular weight of app. 10 kDa and contains the active site Tyr^{723} which is responsible for the formation of the phosphodiester bond to the 3' end of the cleavage site. The C-terminal domain is tightly associated with the core domain and remains associated with the core although the linker domain is cleaved. The minimal requirement for catalytic activity *in vitro* is only the core and C-terminal domains. They retain around 60 to 70% activity compared with wild-type. This was found by partial proteolysis investigations (Stewart *et al.* 1996) and by expression of each domain separately and mixing of the domains in a 1:1 ratio (Stewart *et al.* 1997).

The crystal structure of htopoI was published by Redinbo *et al.* (1998) and Stewart *et al.* (1998). The structure was solved of a partially reconstituted enzyme consisting of the core and the C-terminal domain (Redinbo *et al*. 1998) and also of htopo70, which only lacks the N-terminal domain (Stewart *et al.* 1998). The overall dimensions of the reconstituted enzyme were 70 Å x 60 Å x 60 Å (Redinbo *et al.*

1998). However, in this structure the linker domain is lacking. Stewart *et al.* (1998) published the crystal structure of htopo70 and found that it was very similar to the crystal of the reconstituted enzyme. Furthermore, in this crystal it was possible to identify the structure of the linker domain. The linker mainly consists of two long α-helices connected by a short turn in an antiparallel coiled-coil structure. This structure is very stable, but the position in respect to the core and C-terminal domains may vary considerably (Redinbo *et al.* 1999).

When bound to DNA the core and C-terminal domains form a closed clamp structure around the DNA with a diameter of the cavity of approximately 20 Å. The inner surface of this clamp has a high positive charge that mediates a strong binding to DNA. The enzyme contacts the DNA 4 bp upstream and 6 bp downstream of the cleavage site, with only a single base-specific contact one base upstream of the cleavage site on the scissile strand (Redinbo *et al.* 1998). At this site there is a preference for a thymidine, which also correlates with studies of preferred cleavage sites *in vitro* (Kjeldsen *et al.* 1988).

1.1.2 Catalytic mechanism of htopoI

On the basis of the information gained from the crystal structures a model for the catalytic cycle was set up by Stewart *et al.* (1998). This model is shown in a simplified version in Figure I. A. HtopoI is in an open clamp state, possibly by a hinge-bending within the core domain. B. HtopoI binds the DNA by closing the clamp completely and thus surrounds the DNA. As a result of these changes in conformation the active site is brought into the correct position for the nucleophilic attack on the DNA backbone. C. The nucleophilic attack takes place and the enzyme is covalently linked to the 3' end of the DNA. D. Subsequently, the controlled release of superhelical tension can occur during one or more cycles. E. Religation takes place and the covalent linkage is broken. F and G. The enzyme releases the DNA with reduced superhelicity and can start a new cycle.

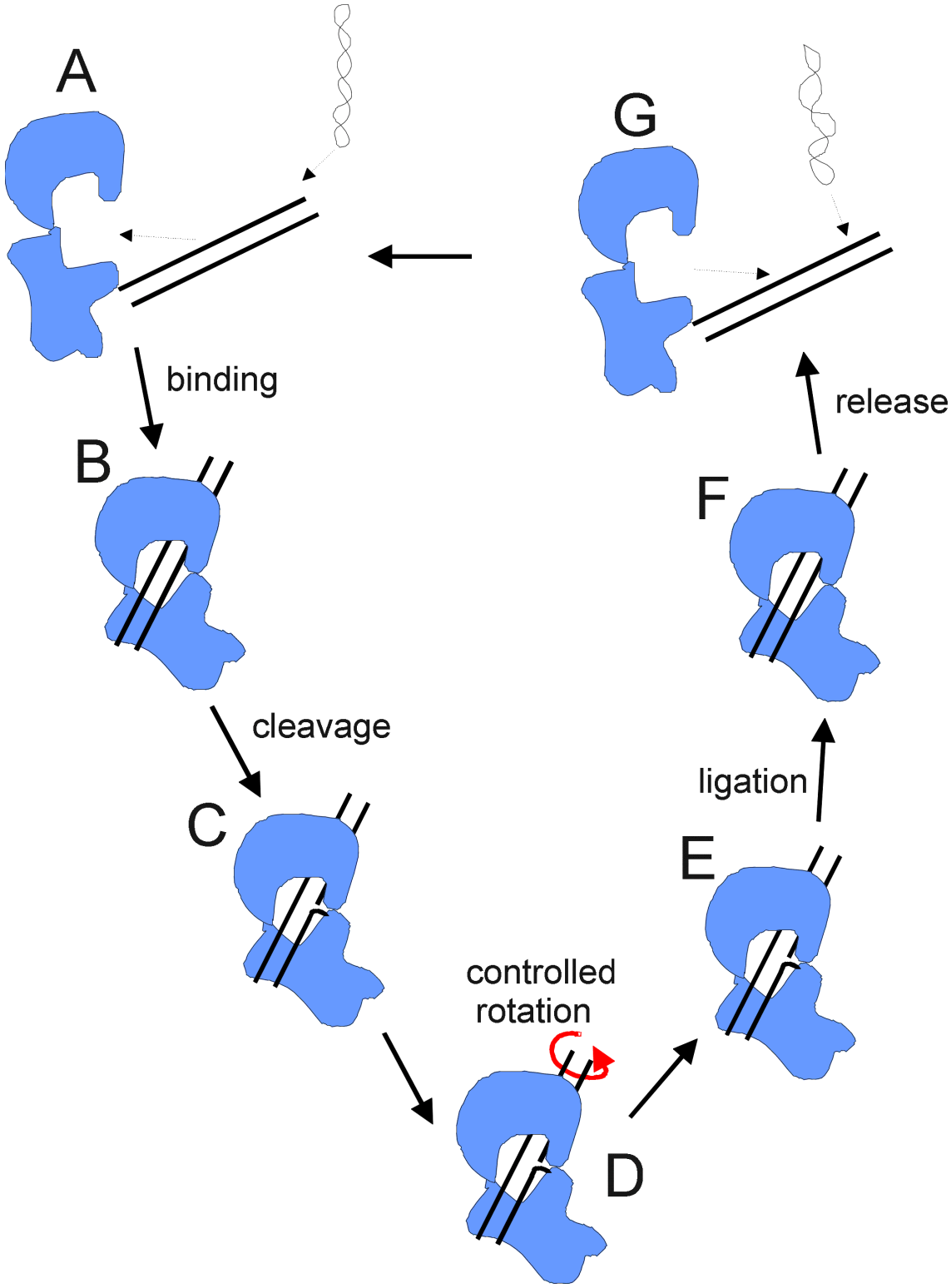

Figure I. Schematic representation of the catalytic cycle of htopoI.

See text for detail**.** Modified from Stewart *et al.* 1998.

1.1.3 Inhibitors of human topoisomerase I used in chemotherapy

In the treatment of several cancer types, such as ovarian cancer and colon cancer (Armand *et al.* 1995, O'Reilly *et al.* 1996, Creemers *et al.* 1996, Ten Bokkel Huinink *et al.* 1997), htopoI can be turned into a cellular poison to kill off the cancer cells. These clinical achievements are mainly due to the discovery of Wall *et al.* (1966). They found that the major anti-tumor alkaloid of the Chinese tree *Camptotheca acuminata* was an aromatic compound which they named camptothecin (CPT). The early clinical trials were aborted due to toxic side effects (Pommier, 1998). However, during the past years a whole family of camptothecin derivatives has been developed.

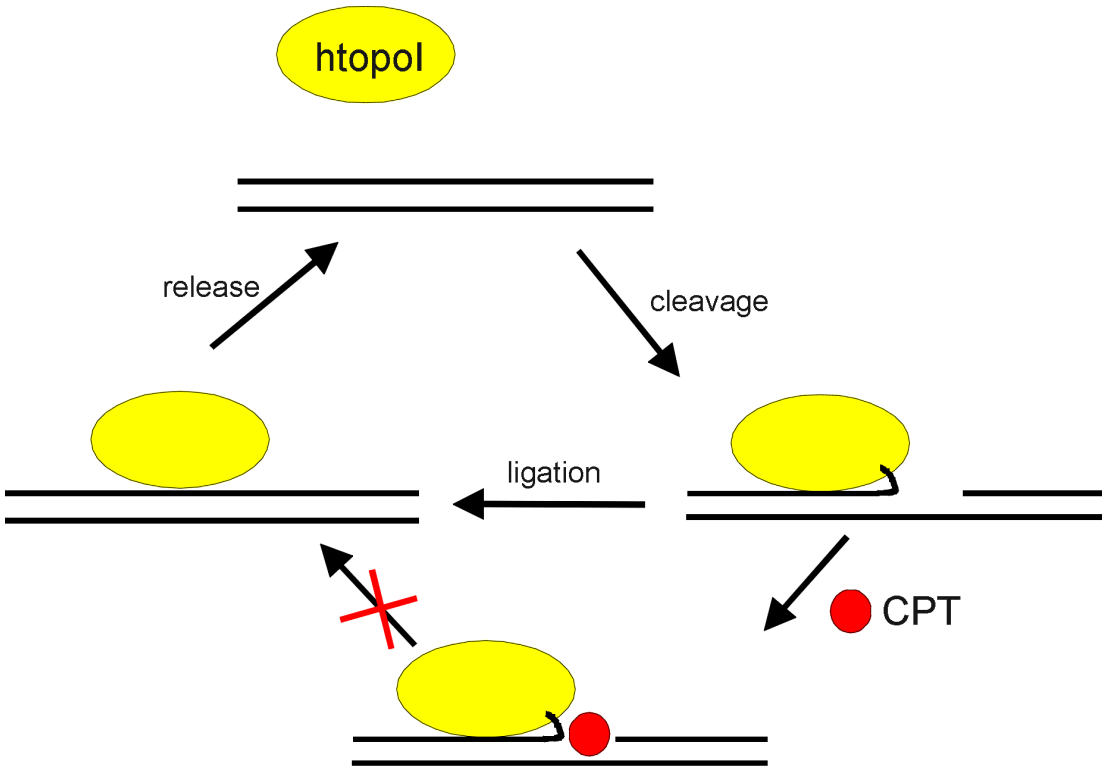

Figure II. Inhibitory function of CPT. HtopoI binds and cleaves the DNA and transiently forms a cleavage complex. This complex can be bound by CPT, which blocks the free 5'-OH necessary for religation from entering the active site and thereby causes a reversible stabilization of the cleavage complex.

Camptothecin drugs all function as specific topoisomerase I poisons. They cause the stabilization of the covalent state of the htopoI catalytic cycle where the enzyme is covalently bound to the DNA, which is also called the cleavage complex

(Figure II). This stabilization is reversible when the drug is removed from the solution. The stabilization of the protein-DNA complex generates a long lived single strand nick. In the normal catalytic cycle this nick occurs only transiently, but in the presence of CPT the nick is persistent. These persistent nicks have been shown to be converted into double-strand breaks. At the same time it has also been shown that the degree of double-strand breaks introduced correlates with cell death (Ryan *et al.* 1991, Squires *et al.* 1991, Squires *et al.* 1993). By treating cells with CPT and aphidicolin at the same time it was seen that no double-strand breaks were introduced and no cell death was observed (Ryan *et al.* 1991). Since aphidicolin inhibits replication, this experiment showed that the double-strand breaks were a result of replication forks colliding with a htopoI cleavage complex. The observation that the double-strand breaks correlate with cell death makes it highly likely that double-strand breaks rather than the covalent htopoI-DNA complexes alone are responsible for cell death caused by CPT.

1.2 Role of eukaryotic topoI

1.2.1 Knockout studies of topoI

Eukaryotic topoI is an essential enzyme which plays various roles during the cell cycle and development. It was found by genetic knock-outs in both *Drosophila melanogaster* and mouse that topoI is an essential enzyme. In *Drosophila melanogaster* topoI$^{-/-}$ larvae died at a very early stage (Zhang *et al.* 2000) which also holds for topoI$^{-/-}$ embryos of mice. These embryos could develop to the 4th or 8th cell stage, but failed to enter the 16th (Morham *et al.* 1996). In both organisms maternal topoI was present in the egg at the time of fertilization. When this stock of topoI had decreased to a critical level and no endogenous topoI was expressed the embryo died. The presence of maternal topoI in the egg underlines the important role of topoI during development. The importance of topoI is also deducible from its high degree of conservation during evolution. However, mammalian cancer cells in culture, which are resistant to CPT and its analogs, are able to survive although no topoI activity is detectable. This indicates that the

essential requirement for htopoI can be overcome in cancer cells (Mattern *et al.* 1991).

1.2.2 Involvement of topoI in transcription and replication

TopoI has been found to be linked to RNA polymerase II transcribed genes. This has primarily been found by treating cells with CPT and searching for introduced cleavages within particular genes. Gilmour and Elgin (1987) found topoI cleavage sites within the heat shock genes in cultured *Drosophila melanogaster* cells; a partial inhibition of transcription of the genes by CPT was also observed. Stewart and Schutz (1987) found in cultured rat cells that CPT-induced topoI cleavages were located within the tyrosine aminotransferase gene and that the degree of cleavage depended on the transcriptional activity. In addition they also found a repression of transcription by CPT. Finally, Stewart *et al.* (1990) found that the tightly regulated c-fos gene was cleaved by htopoI in the presence of CPT and active transcription. However, when transcription of the gene was shut off, cleavages were no longer detected. TopoI has also been found to be connected to RNA polymerase I transcription (Brill *et al.* 1987, Rose *et al.* 1988).

All of the aforementioned works show that topoI plays an important role in transcription, but what is its function? Tsao *et al.* (1989) found that transcription of a gene inserted into a plasmid could be inhibited by the addition of *E. coli* topoI *in vitro*. Prokaryotic topoI only removes negative supercoils. On a normal plasmid positive and negative supercoils can eliminate each other *in vitro* by rotation. When the negative supercoils are selectively removed, positive supercoils accumulate. Since this was found, it can be concluded that transcription introduces positive supercoils downstream of the polymerase and negative supercoils upstream of it. Tsao *et al.* (1989) and Gartenberg and Wang (1992) found that eukaryotic topoI is important for ensuring full length transcripts by removing the supercoils. Therefore topoI cleaves at distinct sites within the RNA polymerase II transcribed genes to ensure the formation of full length transcripts.

Besides the involvement in transcription topoI has been found to take part in DNA replication. TopoI was found to bind specifically to the Simian virus 40 (SV40) large T-antigen double hexamer *in vitro* (Gai *et al.* 2000) and to be directly involved in the removal of supercoils during SV40 replication *in vitro* (Tsao *et al.*

1998, Snapka 1986) as well as *in vivo* (Snapka *et al.* 1998). In addition it has been found to interact with the important replication factor PCNA (Loor *et al.* 1997). PCNA functions as a so called “sliding clamp” and plays a role in the processivity of DNA polymerase δ and ε.

Since topoI is involved in such important cellular activities it is of crucial importance. However, since it is an enzyme which transiently inserts a strand break it could be a potential threat to the cell if this transient state were to be prolonged. This can be achieved with chemotherapeutics as described above or when DNA damage is present in the genome.

1.2.3 Linkage between DNA damage and topoI

It has recently been demonstrated that various DNA lesions can increase the amount of htopoI reversible cleavage complexes (Kingma and Osheroff 1998). These lesions include abasic sites, oxidative damage, gaps, base mismatches, methylation, and UV-photoproducts (Lanza *et al.* 1996, Pourquier *et al.* 1997a, Pourquier *et al.* 1997b, Subramanian *et al.* 1998, Christiansen and Westergaard 1999, Pourquier *et al.* 1999, Pourquier *et al.* 2000, Pourquier *et al.* 2001). HtopoI recognizes these lesions, and a reversible covalent complex is formed. It is known that htopoI preferentially binds DNA which is bent due to supercoils or due to sequence dependent DNA structures (Krogh *et al.* 1991). It is thus an accepted hypothesis that htopoI does not recognize the damage itself, but rather the disturbance of the double helix which leads to bending. All the aforementioned mentioned lesions decrease the thermal stability of the DNA double helix and interfere with base paring which in turn causes bending of the DNA (reviewed in Kingma and Osheroff 1998, Pourquier and Pommier 2001). The tension of DNA can be released by an upstream cleavage by htopoI. The close proximity of the lesion prevents base paring of the 5’ hydroxyl carrying strand and thus inhibits the religation step. This leads to a stabilization of the cleavage complex (Figure III). This explanation holds for all the lesions mentioned above with the exception of oxidative damage, which was found by Pourquier *et al.* (1999) to stimulate DNA binding rather than inhibiting ligation. When present prior to DNA replication such complexes block fork migration and may initiate homologous or non-homologous

recombination (Nitiss and Wang 1988, Megonigal *et al.* 1997, Shcherbakova and Filatov 2000, Polanco *et al.* 2000). The stabilized cleavage complexes may also

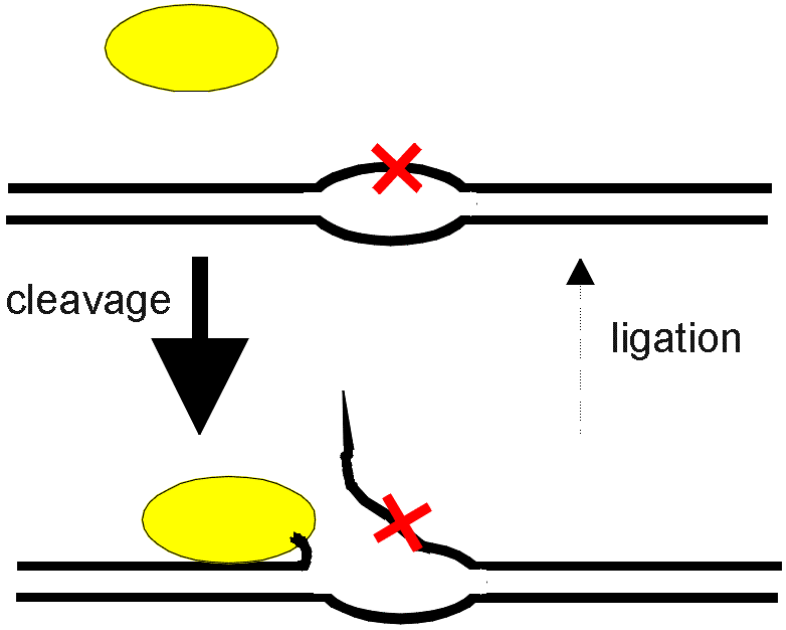

Figure III. Cleavage by htopoI at a DNA lesion. See text for details.

cause genome fragmentation, as has been shown for complexes formed in the presence of CPT (Ryan *et al.* 1991, Squires *et al.* 1991, Shcherbakova and Filatov 2000). Wu and Liu (1997) found that a collision with the transcription machinery can lead to the formation of irreversible complexes, which pose a great threat to the survival of the cell. The formation of such complexes in the vicinity of a DNA lesion have also been shown *in vivo* (Subramanian *et al.* 1998, Pourquier *et al.* 2000, Pourquier *et al.* 2001). It was suggested by Subramanian *et al.* (1998) that the formation of such complexes could be a step in the repair of DNA lesions by nucleotide excision repair. This was suggested because two nucleotide excision repair deficient cell lines (XP-A and XP-D) showed a decreased level of cleavage complex formation by htopoI on damaged DNA. However, this theory has not yet been confirmed. Rather, Frosina and Rossi (1992) found that htopoI cleavage complexes inhibited nucleotide excision repair *in vitro*. It is therefore possible that cleavage complexes are bulky lesions that need to be removed. A number of studies have been carried out to search for a repair mechanism for topoisomerase I-DNA complexes. Nash and co-workers (Yang *et al.* 1996; Pouliot *et al.* 1999) have identified a tyrosine-DNA phosphodiesterase (Tdp1) in *Saccharomyces*

cerevisiae, which can remove the cleavage complex at the bond between topoI and DNA *in vitro,* if the cleavage complex was in a denatured state. When the TDP1 gene was knocked out the cells became somewhat more sensitive to CPT treatment indicating that this enzyme may be involved in the repair of such a bulky lesion in yeast. Human DNA sequences which show homology to the identified yeast gene have also been identified (Pouliot *et al.* 1999, Interthal *et al.* 2001). However, it still remains unclear if this human enzyme is invoved in the repair of htopoI cleavage complexes *in vivo* and if so to what extent. Therefore, it is still unclear how htopoI cleavage complexes are repaired in human cells. This question thus remains an issue of further interest.

1.2.4 Stimulation of htopoI by the tumor suppressor protein p53

Gobert *et al.* (1996) found that the activity of htopoI was stimulated by the tumor suppressor protein p53 *in vitro* and this was later confirmed by Albor *et al.* (1998). Albor *et al.* (1998) also found that mutant p53 lacking other activities still was able to stimulate htopoI *in vitro*. This was also found to be true *in vivo* (Gobert *et al.* 1999), and it was furthermore found that p53 was needed *in vivo* for the recognition of DNA lesions by htopoI (Mao *et al.* 2000). These findings have made it interesting to investigate further what the significance of these htopoI and p53 data could be. This was one of the goals in the work presented here.

1.3 Introduction to the tumor suppressor protein p53

One of the most frequently studied genes in the scientific literature is the p53 gene. The main reason for this interest is due to the observation that approximately 50% of the major human tumor forms contain mutations in this gene (Hollstein *et al.* 1994). This suggested that p53 could be involved in preventing cancer in one way or the other. In the late 1980's p53 was suggested to be a tumor suppressor (reviewed by Ko and Prives 1996). This was confirmed by the first p53 knock-out mice (Donehower *et al.* 1992).

Donehower *et al.* (1992) created a p53 knock-out mouse germ line by homologous recombination into exon 5 of the murine p53 gene in embryonic stem

cells. In this way chimeric mice heterozygous for the p53 gene were generated. These were crossed and in the litter a distribution of homozygous wild-type:heterozygous:homozygote p53 negative of 1:2:1 was observed showing that the hetero- as well as the homozygote embryos were viable. However, after 6 months a clear difference between the litter mates was observed. 74% of mice that were $p53^{-/-}$ had developed cancer within the first 6 months whereas 20% of the heterozygotes ($p53^{+/-}$) went on to develop cancer within 18 months after birth. Only 2% of the wild-type litter mates developed cancer after 14 months. In another study by Lebel *et al.* (2001) it was found that approximately 50% of p53 null mice developed cancer within 5 months which correlated very well with the results of Donehower and co-workers (1992). A lot of work demonstrates that p53 is a tumor suppressor protein and it has therefore been important to find out how and why this function is manifested. As mentioned above many scientists have been working on this problem for several decades and a long list of possible answers has been given. However, still many questions remain and the present thesis is also thought to address some of these, but this will be discussed in more detail later.

In order to give an overview of the functions which have been found for p53, different issues will be dealt with separately in the following sections. However, due to the enormous amount of information in this field only the issues which are most relevant to this thesis will be presented.

1.3.1 Regulation of p53

p53 has been found to be a tetramer and tetramerization is mediated through the aa 320-360 located in the C-terminal domain (Stürzbecher *et al.* 1992). This tetramer can function as a transcriptional activator of several genes involved in the regulation of the cell cycle and/or apoptosis. However, this activity is only useful upon genotoxic stress and it is therefore kept under tight control. One of the main mechanisms to regulate p53 appears to be mediated through the MDM2 protein. p53 physically forms a complex with MDM2 whereby it becomes inactivated as a transcription activator and is targeted for degradation (Oliner *et al.* 1993, Kubbutat *et al.* 1997). In addition, free p53 tetramer is able to transcriptionally activate the MDM2 gene in a negative feedback loop (Wu *et al.* 1993).

When a cell is exposed to genotoxic stress, however, the level of free p53 increases. Upon DNA damage p53 is phosphorylated at Ser15 (Siliciano *et al.* 1997) possibly by the ATM gene product (Siliciano *et al.* 1997, Banin *et al.* 1998). This leads to the dissociation of the MDM2/p53 complex (Shieh *et al.* 1997) and thereby to a stabilization of p53 and an increase in the concentration of p53. Once upregulated, p53 has been suggested to induce various pathways, which finally lead to cell cycle arrest and/or apoptosis, although many details of these pathways are still unclear.

1.3.2 p53 in cell cycle arrest

p53 has been shown to function as a transcriptional activator of several genes after genotoxic stress. One of the most well studied genes is the $p21^{WAF1}$ gene, which encodes a cyclin-dependent kinase inhibitor. $P21^{WAF1}$ prevents the phosphorylation of the Rb protein in complex with the growth factor E2F, and E2F can therefore not be released from the complex which leads to G1-arrest (reviewed by Albor and Kulesz-Martin 2000). $P21^{WAF1}$ has also been shown to inhibit PCNA-dependent DNA synthesis and thereby blocks ongoing DNA synthesis when the DNA is damaged (Waga *et al.* 1994, Li *et al.* 1994). The aforementioned negative feedback regulation of p53 (chapter 1.3.1) ensures that the induction of cell cycle arrest only takes place after genotoxic stress (either transiently or permanently).

The example mentioned above is one of the most studied pathways in which p53 can induce G1 arrest. There seem, however, also to be other functions of p53 besides the transactivator activity that is involved in the induction of cell cycle arrest. Jaks *et al.* (2001) found that when exposing cells to low concentrations of CPT there was a minimal increase in p53, that was however sufficient to activate the $p21^{WAF1}$ and MDM2 genes, although, surprisingly, cell cycle arrest was not induced. When higher concentrations of CPT were used, the p53 level rose several-fold and induced transcriptional activation of its target genes and induced cell cycle arrest. These data suggest that the transcriptional activation of $p21^{WAF1}$ alone is not sufficient to induce cell cycle arrest, but that the p53 concentration also has to exceed a certain threshold value.

1.3.3 p53 in apoptosis

Besides induction of a cell cycle arrest as a response to DNA damage p53 is believed also to induce apoptosis, if the stress signal is very strong or persistent. However, to a wide extent it is not known how this function is exerted by p53. The drug CPT can selectively kill cancer cells by inducing apoptosis. This induction of apoptosis is p53-dependent (Yang *et al.* 1996b, Li *et al.* 1998, Wang *et al.* 1999). Some studies have shown that the transactivation of the BAX gene is important for p53-dependent apoptosis (Zhan *et al.* 1994, Miyashita and Reed 1995). Bax can initiate apoptosis by binding to Bcl-2. Bcl-2 is bound to the cytoplasmic face of the mitochondrial outer membrane. Here it binds the caspase activator Apaf-1. When Bax binds to Bcl-2, Apaf-1 is released from the complex with Bcl-2 and in turn activates an apoptotic caspase pathway (Reviewed by Adams and Cory 1998). Bax may also induce apoptosis independently of Bcl-2 (Knudson and Korsmeyer 1997), possibly by forming pores in the mitochondrial membrane and thereby inactivating these, which eventually leads to cell death (reviewed by Green and Reed 1998). Despite this it has also been found that $bax^{-/-}$ mice are still able to undergo tissue specific apoptosis as a response to γ-irradiation. So Bax appears not to be absolutely essential for apoptosis (Brady *et al.* 1996).

Apparently there are also pathways by which p53 can induce apoptosis without a transactivation of genes. Haupt *et al.* (1995) found that HeLa cells with a mutant p53 lacking the ability to transactivate genes still could undergo apoptosis. This is supported by the observation that MDM2 can inhibit p53-dependent apoptosis in some cell lines (H1299), but not in others (HeLa). It therefore appears that there must be alternative pathways to p53 transactivation-dependent apoptosis.

Thus, to a large extent it still remains a puzzle how p53 induces apoptosis. The present thesis also tries to shed more light onto this issue.

1.4 Working hypothesis

The fact that htopoI can be trapped in the vicinity of various DNA lesions makes it very interesting to investigate the pathological significance of these complexes

and how cells deal with such a complex formation. It is known that CPT induced htopoI cleavage complexes are poisons as described above. In the presence of CPT, non-tumorgenic cells can survive the treatment by going into cell cycle arrest. When the drug is removed from the blood, the complexes vanish, most likely because the ligation function of htopoI is restored. However, in the case of cleavage complex formation at DNA lesions, the complexes cannot immediately be released and must pose a great threat to the cell. Despite the presence of these complexes the cells appear to survive, which makes it tempting to speculate that these complexes are repaired by an as yet unknown mechanism.

I previously investigated whether nucleotide excision repair (NER) could remove a htopoI cleavage complex in an *in vitro* assay. The result was that NER could not remove the lesion. We therefore wished to continue the search for a possible repair pathway using *in vitro* assays. In addition we were interested in investigating whether the tumor suppressor protein p53 could be involved in such a putative repair pathway, since the action of p53 had been shown to be connected to the action of htopoI *in vitro* as well as *in vivo*.

2 Materials

2.1 Oligonucleotides

All used oligonucleotides were synthesized by DNA technology, Aarhus, Denmark.

Name	**Sequence 5' to 3'**
OL1	AAAAAAAGACTTAGA
OL2	TTTTTTTTTTTTTTTTTTCTAAGTCTTTTTTTGCCTTCGCCCG GATCCCCGCCAAGCTTACCTGCCCTTTGGGCAGGTAAGCT TGGCGGGGATCCGGGCGAAGGC
OL3	AGAAAAAAAAAAAAAAAAAGGATCCCCGGAGTGAATTCG GCCCCTTTGGGGCCGAATTCACTCCGGGGATCC

2.2 Baculoviruses

Name	**Reference**
wt htopoI	Stewart *et al.* 1998
Y723F htopoI	A generous gift from Dr. Philippe Clertant, Nice, France
human p53	Bischoff *et al.* 1990
murine His p53	Wang *et al.* 1993
bovine p53	A generous gift from Dr. Hella Hartmann, IMB, Jena

2.3 Cell lines

Name	**Description**	
High Five™	Cell line from *Trichoplasia ni* 5B1-4	ITC Biotechnology, Heidelberg, Germany
Sf9	Cell line from *Spodoptera frugiperda* ovaries	ATCC-number: CRL-1711

2.4 Cell culture media

Name	Source
TC-100	Biowhittaker, Belgium
Fetal Calf Serum	GibcoBRL®

2.5 Commercial enzymes

Type	Specific activity	Source
T4 polynucleotide kinase	10,000 U/ml	New England BioLabs®
T4 DNA ligase	400,000 U/ml	New England BioLabs®
Proteinase K	5.0 DMC U/mg	Serva, Heidelberg
Trypsin	110 U/mg	Roche
Subtilisin	5 U/mg	Roche
Lysozyme	130,000 U/mg	Roche

2.6 Antibodies

The polyclonal rabbit-anti-htopoI antiserum was made by Bioscience, Göttingen from 500 µg purified htopoI with one primary injection and two booster shots.

The polyclonal sheep-anti-human p53 antibody Ab7 was purchased from Calbiochem.

3 Experimental Procedures

3.1 Preparation of recombinant protein

3.1.1 Recombinant wild-type and mutant Y723F human topoisomerase I

The htopoI expressed in *Saccharomyces cerevisiae* was a generous gift from Dr. Michael Lisby, Aarhus University, Denmark, and was purified by Ni-column and Heparin as described in Lisby *et al.* (1998). Storage was at -20°C in 5 mM Tris-HCl, 170 mM NaCl, 50% glycerol, pH 7.5, 0.5 mM DTT, 0.5 mM EDTA.

HtopoI expressed in the baculovirus system (both wild-type and mutant) was purified in the following way. The HiV insect cells were infected with the wild-type htopoI baculovirus clone or Y723F htopoI (when the tissue culture flasks were 3/4 full) for 48 hrs at 27°C. They were harvested by centrifugation at 220 g, and followingly redissolved in four packed cell volumes (PCV) *lysis buffer*. The cells were homogenized using a Dounce Teflon homogenizor (20 strokes) and afterwards four PCV *Sucrose/glycerol buffer* was added. 1 PCV of saturated ammonium sulfate (pH 7 at 4°C) was added drop wise and allowed to rest for 30 min on ice. The solution was spun for 3 hrs at 35000 rpm at 4°C using a Beckman SW40-Ti rotor. The pellet was discarded and 4 volumes (of supernatant) saturated ammonium sulfate was added to the supernatant and left on ice for 30 min The precipitate was spun down and redissolved in *Ni-wash buffer* (42x the vol of the ammonium sulfate pellet). The solution was loaded on a 1 ml pre-equilibrated Ni-NTA Agarose column (Qiagen) and subsequently washed with *Ni-wash buffer*. Topoisomerase I was eluted with 10 vol *Ni-elution buffer* and collected in fractions. The fractions were analyzed according to Bradford (1976) and by SDS-PAGE. The selected fractions were pooled and loaded on a pre-equilibrated 1 ml Resource Q column (Amersham Pharmacia) by the use of a FPLC (Amersham Pharmacia). HtopoI does not bind to the column and was collected. The selected fractions were stored at -20°C in *storage buffer*.

Lysis buffer: 10 mM Tris-HCl, pH 8.0 1 mM EDTA pH 8.0 5 mM DTT 1 mM PMSF 5 mM leupeptin 1% aprotinin	Ni-elution buffer: 30 mM Hepes-KOH, pH 7.9 150 mM NaCl 250 mM imidazole, pH 8.0 10% glycerol (v/v) 1 mM β-mercaptoethanol 1 mM PMSF
Sucrose/glycerol buffer: 50 mM Tris-HCl, pH 8.0 10 mM $MgCl_2$ 25% sucrose (w/v) 50% glycerol (v/v) 2 mM DTT	Storage buffer: 15 mM Hepes-KOH, pH 7.8 170 mM NaCl 0.5 mM DTT 0.5 mM EDTA pH 8.0 50% glycerol (v/v)
Ni-wash buffer: 30 mM Hepes-KOH, pH 7.9 150 mM NaCl 20 mM imidazole, pH 8.0 10% glycerol (v/v) 1 mM β-mercaptoethanol 1 mM PMSF	

3.1.2 Preparation of recombinant p53

Human wt, murine His-tagged, and bovine wt p53 were purified as follows. HiV insect cells (ITC Biotechnology, Heidelberg) were infected with either one of the above mentioned baculovirus clones (when the tissue culture flasks were covered to 3/4) for 48 hrs at 27°C, harvested by centrifugation, and resuspended in 4 pellet volumes *lysis buffer I* for the His-tagged murine p53. In the case of the untagged human and bovine p53, l*ysis buffer I* without imidazole was used. The cells were lyzed using a Dounce Teflon homogenizor, spun at 48000 g, and the crude extract was loaded on a pre-equilibrated column.

Murine His-p53 crude extract was loaded on a 1 ml Ni-NTA Agarose (Qiagen) pre-equilibrated in *wash buffer I*, washed, and eluted with *elution buffer I.*

The protein- rich fractions were identified according to Bradford (1976), pooled, and loaded on a 1 ml phosphocellulose column (P11, Whatmann) pre-equilibrated in *wash buffer II* using FPLC (Amersham Pharmacia). The column was washed, and eluted with *elution buffer II.* The fractions were analyzed according to Bradford (1976) and by 10% SDS-PAGE. The chosen fractions were dialyzed over-night against *storage buffer* and stored at 4°C.

For the purification of human and bovine wt p53 the crude extract was sucked on a 20 ml phosphocellulose column (P11, Whatmann) using vacuum, washed with *wash buffer II*, eluted with *elution buffer II*, and analyzed as above. The chosen fractions were pooled, adjusted to 0.5 M ammonium sulfate, loaded on a 20 ml HiLoad phenyl-sepharose column (Amersham Pharmacia) pre-equilibrated in *wash buffer III,* washed, eluted with *elution buffer III*, and examined as above. The chosen fractions were diluted 1:3 in *wash buffer IV* and loaded on a 1 ml HiTrap heparin-sepharose column (Amersham Pharmacia) pre-equilibrated in w*ash buffer IV*, washed, and eluted with a linear gradient from 0% to 50% *elution buffer IV.* The eluate was analyzed as above and diluted with *wash buffer II* to approximately 100 mM KPi. Thereafter, the protein was loaded on a 1 ml phosphocellulose column as described above, and eluted in a linear gradient from 0% to 100% *elution buffer II*. The final samples were dialyzed over-night against s*torage buffer* and stored at 4°C. The purified wt human p53 and bovine p53 was a generous gift from Dr. Hella Hartmann.

Lysis buffer I: 20 mM Tris-HCl pH 8 100 mM NaCl 20 mM imidazole 0.5% NP-40 1 mM DTT 1 mM PMSF 1 mM aprotinin 1 mM leupeptin	Wash buffer III: 50 mM KPi pH 7.8 0.5 M $(NH_4)_2SO_4$ 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol
Wash buffer I: 20 mM Tris-HCl pH 8 100 mM KCl 20 mM imidazole 2.5 mM β-mercaptoethanol	Elution buffer III: 50 mM KPi pH 7.8 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol
Elution buffer I: 20 mM Tris-HCl pH 8 100 mM KCl 250 mM imidazole 2.5 mM β-mercaptoethanol 1 mM PMSF	Wash buffer IV: 25 mM KPi pH 7.8 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol
Wash buffer II: 100 mM KPi pH 7.8 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol	Elution buffer IV: 800 mM KPi pH 7.8 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol
Elution buffer II: 300 mM KPi pH 7.8 1 mM EDTA pH 8.0 2 mM β-mercaptoethanol	Storage buffer: 20 mM Hepes-KOH pH 7.8 50 mM NaCl 0.1 mM EDTA pH 8.0 0.5 mM DTT 10% glycerol (v/v)

3.2 Purification of DNA substrate (L193s)

1200 pmol OL1 was labeled with 100 µCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia), while 1200 pmol OL2 was phosphorylated with non-radioactive ATP using 2 µl T4 polynucleotide kinase. OL1 and OL2 were precipitated, redissolved in deionized H_2O, and mixed with 1200 pmol OL3 in a total volume of 30 µl ligation buffer (as delivered by the supplier) with 3 µl T4 polynucleotide ligase and the mixture was incubated for 3 to 4 days at 4°C. To the ligation mixture 1 volume *sequencing loading buffer* was added, heated to 95°C for 5 min, and subsequently loaded on a 1 mm thick 7% denaturing polyacrylamide gel (SequaGel Sequencing System, National Diagnostics) 40x20 cm sequencing apparatus (Roth). The gel was run in TBE at 45 W (Power Pac 3000, BioRad) which resulted in a temperature of 50°C. The full length band was identified by 5 to 10 min exposure to an X-ray film (Hyperfilm™ MP, Amersham Pharmacia), excised, and eluted overnight (shaking) in 500 µl TE buffer at 37°C. The eluted oligonucleotide was precipitated by the addition of 1/10 vol 4 M NaCl and 2 vol absolute ethanol overnight at -20°C, spun at 18,000 g, redissolved, and phosphorylated with 300 µmol ATP using 1 µl T4 polynucleotide kinase for 30 min at 37°C. The full length oligonucleotide was precipitated (as described above) and redissolved in 30 µl TE. This oligonucleotide was called L193s. The function of this is described in the chapters 4.1 and 4.2.

2x Sequencing loading buffer: 80% (v/v) formamid 50 mM Tris-borate (pH 8.3) 1 mM EDTA pH 8.0 1% (w/v) Xylen cyanol 1% (w/v) Bromophenol blue	SequaGel Sequencing System: *Concentrate:* 25% (w/v) acrylamide 1.3% (w/v) methylene bisacrylamide 50% (w/v) urea (8.3M) *Dilluent:* 50% (w/v) urea *Buffer:* 50% (w/v) urea 10XTBE (1 M Tris, 1 M H_3BO_3, 20 mM EDTA pH 8.3)
TE: 10 mM Tris-HCl, pH 7.5 1 mM EDTA pH 8.0	

3.3 Detection of suicide cleavage by htopoI

3.3.1 Coomassie stained SDS-PAGE

500 ng htopoI was incubated with non-radioactive L193s (corresponding to approximately 30000, 60000, and 90000 cpm) for 20 min at 37°C in 50% *reaction buffer*. The reactions were stopped with *protein loading buffer*, then incubated for 5 min at 95°C and separated on a 7% SDS-PAGE. Subsequently the gel was stained with Coomassie – brilliant blue dye.

3.3.2 Western blot

500 ng recombinant baculovirus-expressed htopoI was incubated with non-radioactive L193s as described above. Following separation on a 7% SDS-PAGE the gel was transferred onto a PVDF membrane (Immobilon, Millipore) using a semi-dry transfer blot cell (BioRad) with *transfer buffer* for 1 hr at 3 mA/cm^2. Subsequently the membrane was blocked for 20 min in *CMF-PBS* with 5% skimmed milk. Thereafter the blot was incubated with the rabbit-anti-htopoI antiserum in a 1:2000 dilution in *CMF-PBS* with 5% skimmed milk over-night at 4°C. The blot was washed 3x5 min and 1x15 min in *CMF-PBS*, and thereafter incubated 1 hr with an anti-rabbit horse-radish-peroxidase coupled secondary antibody (1:3000 dilution, Amersham Pharmacia) in *CMF-PBS* with 5% skimmed

milk. The blot was washed 5x10 min in *CMF-PBS,* and developed by ECL by incubating it for 1 min in a 1:1 mixture of *solution A* and *solution B*. Subsequently the blot was exposed on an X-ray film for various periods of time and subsequently developed.

Reaction buffer: 25 mM Hepes-KOH, 7.9 100 mM KCl 12 mM $MgCl_2$ 17% (v/v) glycerol 1 mM EDTA pH 8.0 1 mM DTT	Solution A: 100 mM Tris-HCl pH 8.5 0.4 mM p-coumaric acid 2.5 mM 5-amino-2,3-dihydro-1,4-phthalazinedione, luminol
Transfer buffer: 10% methanol 96 mM glycine 10 mM Tris-base	Solution B: 100 mM Tris-HCl pH 8.5 0.02% H_2O_2
CMF-PBS: 167 mM NaCl 20 mM Na_2HPO_4 2 mM KH_2PO_4 0.05% (v/v) Tween 20	5xProtein loading buffer: 330 mM Tris-HCl pH 6.8 5% SDS 713 mM β-mercaptoethanol 2.5% (w/v) bromophenol blue 50% (v/v) glycerol

3.4 Titration with htopoI using L193s as substrate

30,000 to 40,000 cpm L193s (as measured with a Packard 1900 TR scintillator) were incubated with increasing amounts of yeast-expressed htopoI (as indicated in the text) for 40 min at 37°C in 20 µl with 50% *reaction buffer* (concentration in the reaction mixture: 12.5 mM Hepes-KOH pH 7.9, 6 mM $MgCl_2$, 50 mM KCl, 42.5 mM NaCl, 21% glycerol, 0.5 mM EDTA pH 8.0, 1 mM DTT). The reaction was stopped by the addition of 0.4% SDS and 125 µg/ml proteinase K, and further incubated at 37°C for 60 min In some cases each aliquot was split into two whereafter one fraction was incubated with protease and the other was not. The

DNA molecules were precipitated by 3 vol 0.6 M LiCl in absolute ethanol. After 30 min on ice the mixture was centrifuged at 18,000 g. The pellet was redissolved in sequence loading buffer, heated to 95°C for 5 min and analyzed on a 0.4 mm 20% denaturing polyacrylamide gel (see chapter 3.2). The gels were analyzed using a phosphorimager (Molecular Dynamics, Storm 860) and quantified using the software ImageQuant (Molecular Dynamics).

3.5 Comparison of proteinase K- and trypsin digestion

30,000 to 40,000 cpm L193s was incubated with 0 or 500 ng baculovirus-expressed htopoI in a 10 µl reaction volume in 50% *reaction buffer* (see chapter 3.4). The reactions were stopped by the addition of a final concentration of 0.4% SDS and either no protease or 125 µg/ml proteinase K, or by the addition of 80 µg/ml trypsin (no SDS). The samples were subsequently incubated for 60 min at 37°C, whereafter the DNA was precipitated by ethanol/LiCl (see chapter 3.4) and analyzed on a 14% denaturing polyacrylamide gel and analyzed as above (see chapter 3.4), but using a 14% denaturing polyacrylamide gel.

3.6 Investigating the effect of camptothecin

30,000 to 40,000 cpm L193s was incubated with 0 or 500 ng baculovirus-expressed htopoI in a 13 µl reaction volume in 50% *reaction buffer* for 5 min at 37°C. Subsequently, 2 µl DMSO or CPT dissolved in DMSO was added to the concentrations indicated and incubated for an additional 15 min at 37°C. The reactions were stopped as described above (see chapter 3.4). A sample was taken for SDS-PAGE analysis prior to protease digestion. The analysis was performed as described above (see chapter 3.4), but using a 14% denaturing polyacrylamide gel.

3.7 Influence of the mutant Y723F htopoI on the double cleavage of htopoI

30,000 to 40,000 cpm L193s was or was not incubated with 500 ng htopoI, and with the indicated amounts of mutant Y723F htopoI in 50% *reaction buffer* (see chapter 3.4) (concentration in the reaction mixture: 15.5 mM Hepes-KOH pH 7.9, 50 mM KCl, 34 mM NaCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 18.5% glycerol) in 20 µl for 40 min at 37°C. The reaction was stopped with a final concentration of 0.7% SDS. An aliquot of 2 µl was taken from each sample and analyzed on a 7.5% SDS-PAGE followed by quantification using a phosphorimager and the ImageQuant software (Storm 860, Molecular Dynamics, Amersham Pharmacia). The remainder of each sample was incubated with 1 mg/ml proteinase K for 30 min at 37°C, and treated and analyzed as described in chapter 3.4, however analyzed on a 14% denaturing polyacrylamide gel.

3.8 Limited proteolysis of htopoI with subtilisin

Two µg of baculovirus-expressed htopoI (4 µl) was incubated with TE (pH 7.5) or various concentrations of subtilisin (as indicated in the text) diluted in TE (pH 7.5) in a total volume of 10 µl (6 mM Hepes, pH 7.9, 6 mM Tris-HCl, pH 7.5, 68 mM NaCl, and 20% glycerol) for 20 min at 25°C. The reaction was stopped by 5 mM PMSF and the mixture was put on ice. One µg htopoI was analyzed by SDS-PAGE and Coomassie-staining. The rest was incubated with 30,000 to 40,000 cpm L193s in a reaction volume of 15 µl in 50% *reaction buffer* (see chapter 3.4) (13 mM Hepes-KOH, pH 7.9, 30 mM NaCl, 43 mM KCl, 5 mM $MgCl_2$, and 16% glycerol) for 40 min at 37°C. The reactions were stopped with 0.4% SDS and a sample was taken for analysis on a 7.5% SDS-PAGE. The rest was incubated with proteinase K and analyzed as described above.

3.9 Preparation of competent *E. coli* and transformation

The *Escherichia coli* strain BL21 DE3 (Stratagene®) was cultured in *LB-medium* and made competent according to the $CaCl_2$ method described by Mandel and Higa (1970).
The transformation with pUC 19 was performed according to Sambrook *et al.* (1989).

LB-medium:

10 g/l Bacto-Tryptone
5 g/l Bacto yeast extract
10 g/l NaCl
pH 7 (adjusted with NaOH)

3.10 Purification of supercoiled pUC 19

This procedure differs from standard plasmid purification protocols because for this work it was very important to have ONLY supercoiled DNA with as few lesions as possible. This is not achievable through standard procedures.

Following transformation, a single bacterial colony was picked and grown in 500 ml LB medium (see chapter 3.9) shaking over-night at 37°C. The culture was harvested by 15 min centrifugation at 3000 g at 4°C. The pellet was resuspended in 100 ml cold *STE buffer* and spun as before. Followingly, the pellet was redissolved in 20 ml cold *sucrose buffer* and subsequently devided into two 50 ml tubes on ice. In each tube 1.7 ml *lysozyme solution* was added, mixed gently, put on ice for 5 min, 1.7 ml 0.5 M EDTA was added, mixed gently, rested on ice for 5 min, and finally 17 ml *lysis buffer* was added, mixed gently and left on ice for 15 min After this procedure the solution should be highly viscous. The slurry was spun at 48,000 g at 4°C for 30 min The complete supernatant was poured through a filter and loaded on a pre-equilibrated NucleoBond® PC 2000 column (Macherey-Nagel) and the plasmid was purified according to protocol of the supplier.

The eluted plasmid still contained nicked, linear, and catenated forms. In order to get only supercoiled plasmids a native linear sucrose gradient was produced. A 10 ml linear gradient sucrose gradient was made from 5% and 25% *sucrose solutions*. Up to 100 µg purified plasmid in TE buffer (max. 1 ml) was carefully loaded on top of the gradient and spun at 30,000 rpm at 4°C for approximately 16 hrs (SW 40 rotor, Beckmann). The gradient was fractionated by inserting a needle through the bottom of the tube and collected in approximately 20 fractions. A 7 µl sample was taken from each fraction, mixed with agarose loading buffer and analyzed on a 0.8% agarose gel in TBE, and then stained with ethidium bromide. The fractions containing only supercoiled plasmid were pooled, 3 vol absolute ethanol was added, put at -20°C over-night and spun at 48,000 g at 4°C for 30 min The pellet was redissolved in TE (pH 7.5) and the concentration was adjusted to 250 ng/µl.

STE buffer: 0.1 M NaCl 10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0	Lysis buffer: 50 mM Tris-HCl pH 8.0 62.5 mM EDTA pH 8.0 0.5% Triton X-100
Sucrose buffer: 25% (w/v) sucrose 50 mM Tris-HCl pH 8.0 0.1 mg /ml RNase A	Sucrose solution: 5 or 25% (w/v) sucrose 25 mM Tris-HCl pH 7.5 1 M NaCl 5 mM EDTA pH8.0
Lysozyme buffer: 10 mM Tris-HCl pH 8.0 20 mg/ml lysozyme	6 x Agarose gel loading buffer: 0.25% (w/v) Bromophenol blue 0.25% (w/v) xylene cyanol 30% (v/v) glycerol

3.11 Influence of p53 on htopoI relaxation activity

Recombinant htopoI (as indicated) was incubated with 1 µg supercoiled pUC 19 plasmid (purified as described in chapter 3.10), and the indicated amount of recombinant human, bovine, or murine his-tagged p53 in 50% *reaction buffer* (see

chapter 3.4) (19 mM Hepes-KOH pH 7.9, 50 mM KCl, 30 mM NaCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 16% glycerol, and 15 µg/ml BSA) in 20 µl for 5 min at 37°C or 30°C (as indicated). The reactions were stopped by *agarose gel loading buffer* and subsequently incubating for 5 min at 95°C followed by placement on ice. The plasmids were analyzed on a 0.8% agarose gel in TBE and thereafter stained with ethidium bromide.

3.12 Protease digestion of htopoI for Far-Western analysis

Thirty µg htopoI was incubated with 3.5 µg/ml subtilisin in TE (pH 8.0) in a final volume of 150 µl for 20 min at room temperature (25°C), and stopped with 5 mM PMSF.

3.13 Far-Western analysis

Eleven µg of baculovirus-expressed htopoI and 11 µg subtilisin treated htopoI (as described above) were incubated in *sample buffer* for 5 min at room temperature. 11 µg of BSA was treated the same way. Proteins were then separated by SDS-PAGE as indicated. The gel was incubated for 1 h in *renaturation buffer* and transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell) in *transfer buffer.* Membranes were blocked in *TBS/T buffer* and 5% skimmed milk powder for 1 h at room temperature, washed two times for 5 min in *TBS/T buffer*, incubated 1 h at room temperature with 12 µg/ml murine His-p53 or BSA in *TBS/T buffer*, washed four times for 15 min with *TBS/T buffer*, and incubated overnight with the p53 primary polyclonal sheep antibody Ab-7 (Oncogene) (1:3000 dilution) at 4 °C in *TBS/T buffer* with 5% milk powder. Membranes were washed four times for 15 min in *TBS/T buffer*, incubated 1 h at room temperature with HRP-conjugated anti-sheep secondary antibody (Sigma, 1:3000) in TBS/T with 5% milk powder, and detected by ECL (Amersham Pharmacia Biotech) according to the supplier's instructions. The Far-Western analysis was kindly performed by Dr. Hella Hartmann.

The identification of the N-terminus of the core domain was done as follows: HtopoI was digested as described in chapter 3.12. The digest was separated on a 7.5% SDS-PAGE and blotted on a PVDF membrane (Millipore). The blot was stained with Coomassie stained the band of interest was cut out. This membrane piece was subsequently subjected to Edman degradation (Applied Biosystems 494 Protein Sequencer) to find the N-terminal sequence. The N-terminal sequencing was kindly performed by Dr. Bernhard Schlott and Anita Willitzer.

Sample buffer: 2.5% SDS 2.5 mM Tris-HCl pH 8.0 100 mM DTT 10% glycerol 0.05% Pyronin T1	Transfer buffer: 10 mM $NaHCO_3$ 3 mM Na_2CO_3
Renaturation buffer: 50 mM Tris-HCl pH 7.5 20% glycerol	TBS/T buffer: 10 mM Tris-HCl pH 8.0 150 mM NaCl 0.1% Tween 20

3.14 Influence of p53 on the htopoI double cleavage reaction

For the titration experiments with murine His-tagged p53, 400 ng of htopoI was incubated with murine his-tagged p53 as indicated for 10 min at 37°C in 20 µl of 50% *reaction buffer*. Ten µM CPT or 2 µl DMSO were added 2 min after the reactions were started. The mixture was treated and analyzed as described in chapter 3.4, but using a 14% denaturing polyacrylamide gel.

4 Results

4.1 Sequence, two dimensional structure, and function of the DNA substrates

In order to investigate whether a htopoI cleavage complex could be removed *in vitro* it was crucial to induce a cleavage complex at one particular location on a DNA substrate. To do this a previously published method by Svejstrup *et al.* (1991) was utilized. This method is based on a small DNA substrate which efficiently traps covalent htopoI/DNA intermediates. A so called suicide substrate, L193s, was designed, which efficiently trapped a htopoI cleavage complex at only one position, according to the method of Svejstrup *et al.* (1990), but in another design. The complete sequence and two dimensional structure of the DNA substrate is depicted in Figure 1A. L193s consisted of a 193 nts long oligonucleotide that folded back onto itself, thereby forming a 92 bp double-stranded region with a 3 nts long loop at both ends. In the center of the substrate was a 3 nts long 5' overhang at a nick. The sequences of the last 3 nts at each end of the oligonucleotide were identical. Furthermore, a specific 16 nts long htopoI binding and cleavage sequence (Busk *et al.* 1987, Stevnsner *et al.* 1989) was included in such a way that the nick was located 3 nts 3' to the htopoI cleavage site. Cleavage by htopoI at this site became „suicidal" due to the presence of a 5' phosphate next to the cleavage site (see also Figure 1B). Thus, the presence of the 5' phosphate prevented ligation and thereby blocked the release of htopoI after the cleavage reaction had taken place. This stable complex was designated as "cleavage complex".

Correct substrate folding was verified by the existence of BamH I cleavage sites at the positions indicated in Figure 1A (data not shown). Through BamH I cleavages it was also verified that the suicide cleavage of htopoI took place at the correct position (data not shown).

The utilized recombinant htopoI preparations were either expressed in yeast or by the baculovirus expression system and purified to near homogeneity (Figure 2A and B).

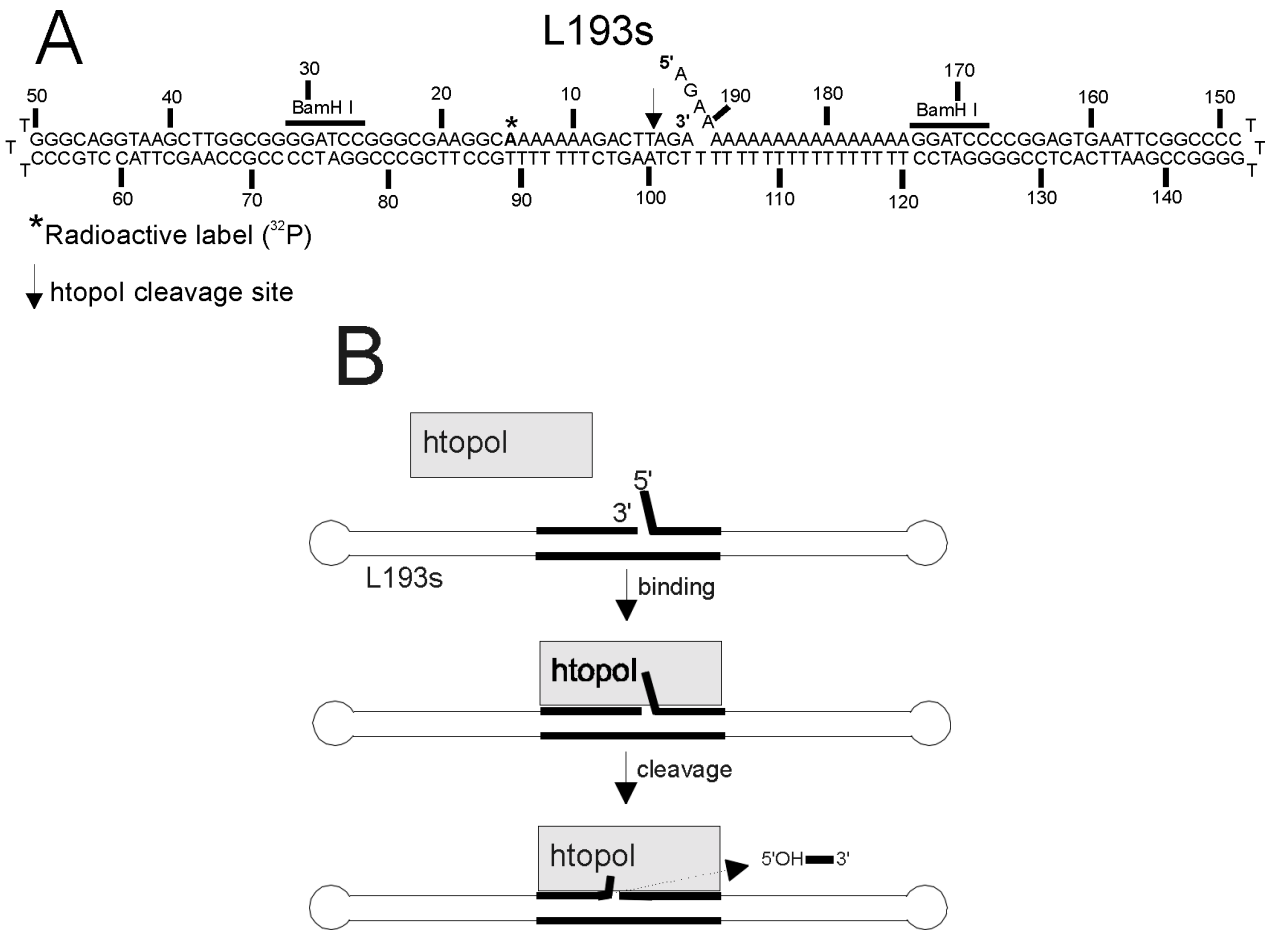

Figure 1. Two dimensional structure and complete sequence of the substrate L193s and schematic outline of the function. A) The htopoI cleavage site is indicated with an arrow. The radioactive label (^{32}P) is located on nucleotide 15 (indicated with an asterisk and the base shown in boldface). BamH I restriction sites are indicated. See text for further details. B) HtopoI binds to the hexadecamer binding sequence (bold lines) and cleaves the upper strand forming a covalent phosphotyrosine-linkage with the 4th nt (numbering as in Fig. 1A) and thus produces an oligomer consisting of 3 bases. The overhang is identical with those 3 bases, therefore, when the trimer is liberated by htopoI, the 5' end can hybridize to the complementary strand and close the gap. Since the 5' end is phosphorylated, htopoI is irreversibly trapped.

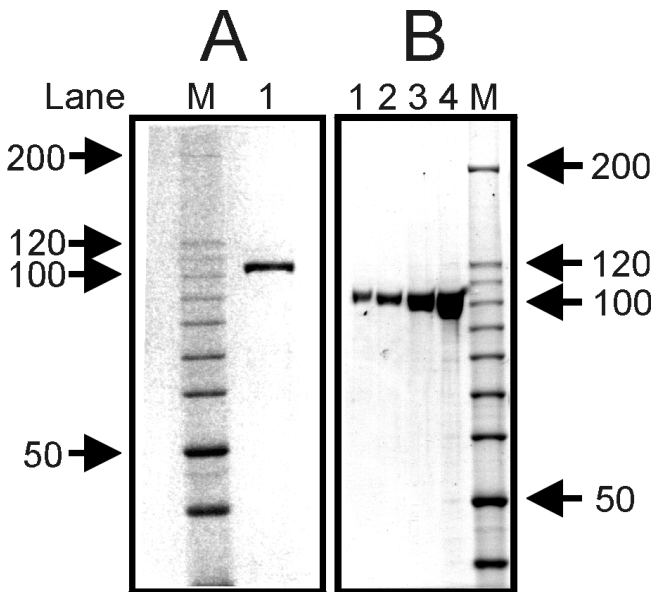

Figure 2. Coomassie staining of yeast and baculovirus-expressed recombinant htopoI. Coomassie staining of a 7.5% SDS-PAGE. A) Recombinant htopoI expressed in *S. cerevisiae*, lane 1, ≈ 3µg htopoI. B) Recombinant htopoI expressed by baculoviruses, lane 1, 200 ng; lane 2, 400 ng; lane 3, 1000 ng; lane 4, 1500 ng. Marker is a 10 kDa ladder and the sizes of some of the bands are indicated in kDa.

4.2 Detection of htopoI suicide cleavage on L193s

In order to verify that htopoI did indeed cleave L193s suicidally, reactions with htopoI and L193s were analyzed by SDS-PAGE. As seen in Figure 3A a shifted protein band can be detected with increasing L193s concentration (compare lane 1 with lanes 2 to 4). Since the htopoI preparation was highly pure (see lane 1) this shift is likely due to the suicide cleavage and attachment of L193s, which changes the mobility of htopoI dramatically. However, to show that the shifted protein band indeed was htopoI the reactionproducts were also analyzed by Western-blotting. From Figure 3B it can be seen that the shifted protein band in Figure 3A truly was htopoI which had cleaved L193s. This shows that the substrate works as intended. In addition it was possible to estimate the ratio between L193s and htopoI. When considering the results in Figure 3A and B it can be estimated that the molar ratio between htopoI and L193s was approximately 10:1 respectively (Figure 3A and B

lane 2). In general, this was the ratio between htopoI and L193s in the following experiments.

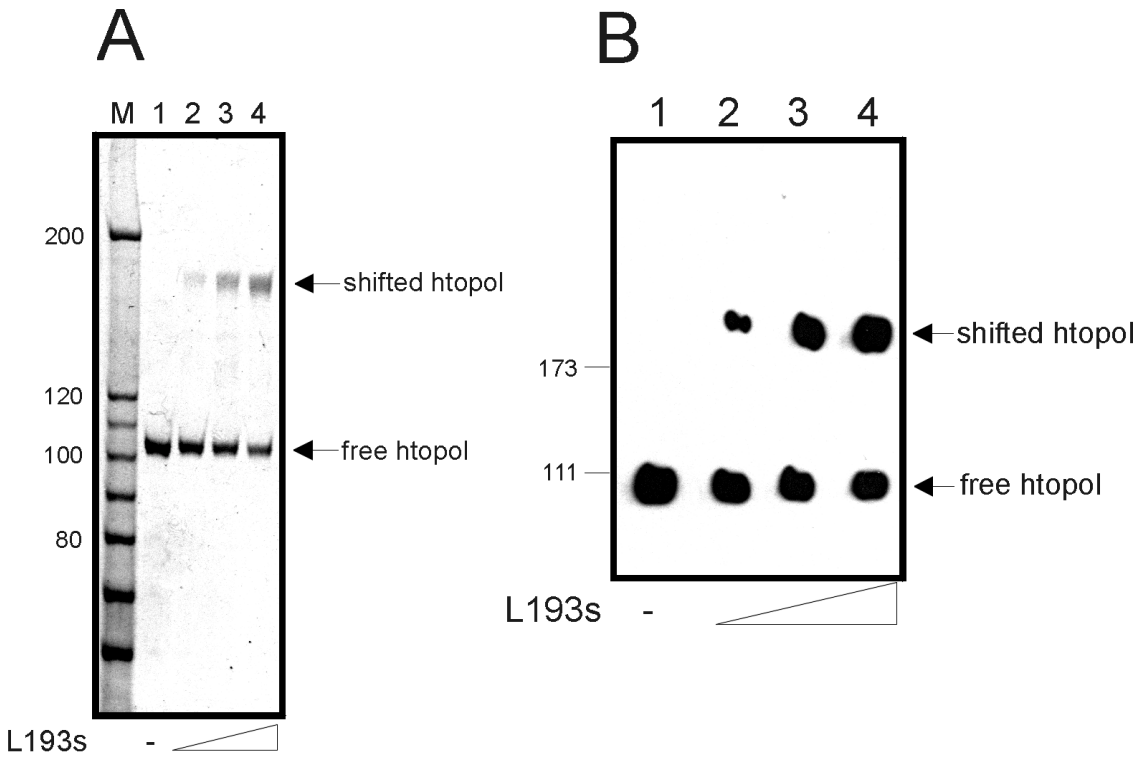

Figure 3. HtopoI cleaves L193s suicidally. A. Coomassie staining of a 7% SDS-PAGE. Lane 1, 1 µg htopoI (baculo-virus expressed), 2, 1 µg htopoI + 2 µl L193s, 3, + 4 µl L193s, 4, + 6 µl L193s. B. Western blot of a 7% SDS-PAGE as in A using a polyclonal rabbit-anti-htopoI antibody. The mobility of a prestained marker is indicated (kDa). The conditions for the reaction shown in lane 2 correspond to the ratio between htopoI and radioactively labeled L193s in the forthcoming figures. See text for further details.

4.3 Titration with human topoisomerase I

To investigate whether htopoI could cleave L193s at positions other than at the suicide cleavage site, L193s was incubated with increasing amounts of yeast-expressed htopoI (Figure 4) (a generous gift from Michael Lisby, Aarhus University, Denmark). The reactions were stopped by the addition of 0.4% SDS, and htopoI was digested with proteinase K. With increasing concentrations of

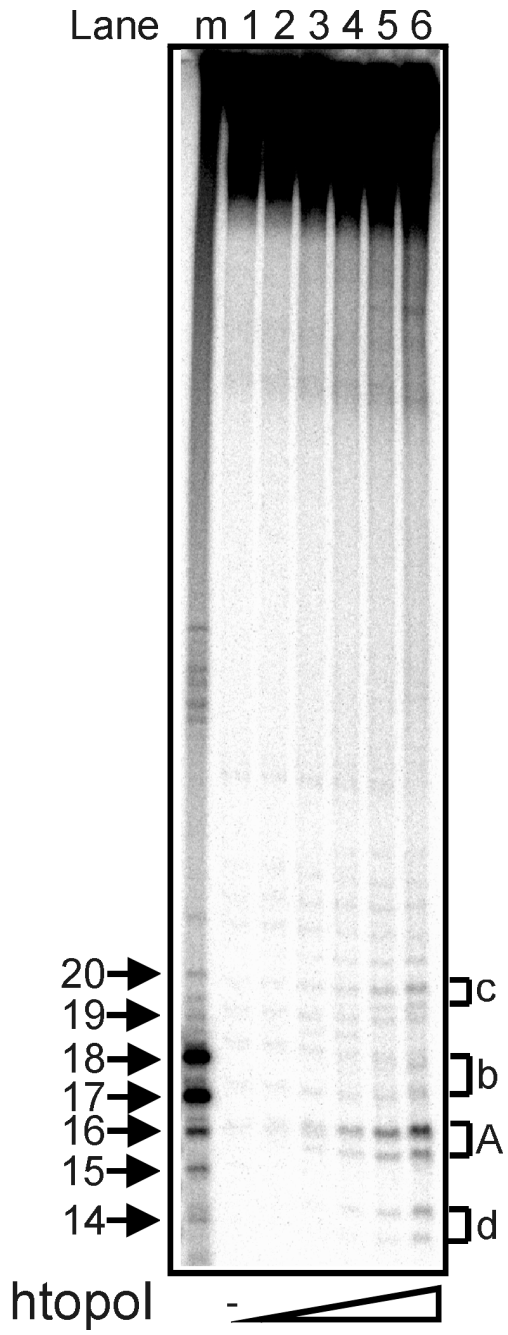

Figure 4. Titration with recombinant yeast expressed htopoI. Autoradiogram of a 20% sequencing gel. L193s was incubated with: lane 1, no htopoI, lane 2, 125 ng, lane 3, 250 ng, lane 4, 500 ng, lane 5, 750 ng, and lane 6, 1250 ng htopoI for 40 min at 37°C. In all samples the buffer conditions were identical. Thereafter all reactions were stopped by the addition of 0.4% SDS and the protein was digested with proteinase K. Letters ”A”, ”b”, ”c”, and ”d” indicate the double bands discussed in the text.

topoI a cleavage pattern consisting of 4 double bands occurred (lanes 2 to 6).The double bands were designated by the letters A, b, c, and d, where A represented the most predominant cleavage site. None of the bands migrated to a position that precisely fitted the marker; instead they had an intermediate mobility. Since highly pure recombinant htopoI was used (Figure 2A, lane 1) we assumed that these bands were due to additional htopoI cleavages of substrates that already had been cleaved suicidally.

4.4 DNA fragments are protein linked

In order to investigate further, if the observed bands represented DNA fragments with proteinase K resistant peptides bound to the 3' end, three parallel experiments with varying concentrations of yeast-expressed htopoI were performed. The DNA from the four double bands should still enter the gel without proteinase K digestion if the DNA fragments had no covalently attached protein moieties. In contrast, if htopoI was attached to the 3' end, the DNA would no longer be able to enter the gel, but remain in the slot.

As shown in Figure 5 (lanes 3, 5 and 7), none of the four double bands were seen when the proteinase K digest was omitted, indicating that a large molecule was bound to the DNA fragments. The cleavage frequency depended on the amount of htopoI added (Figure 5, compare lanes 2 and 4), indicating that htopoI indeed was responsible for the formation of the double bands. Furthermore, since four double bands were produced, htopoI must have cleaved the substrate at several distinct positions just upstream from the radioactively labeled nucleotide. It is well known that a high concentration of salt shifts the equilibrium of htopoI from the cleaving to the non-bound state. Thus, if cleavage was due to htopoI, it should be possible to reverse the cleavage reactions by the addition of high concentrations of salt. Therefore, one reaction was stopped with 300 mM NaCl and incubated for another 10 min at 37°C prior to the addition of SDS. The reaction mixture was then divided into two halves, and one portion was treated with proteinase K (Figure 5, lane 8) whereas the other aliquot was kept untreated (lane 9). Salt treatment provoked a clear reduction of the band intensities (compare lanes 6 and 8). For the bands A and c product formation was

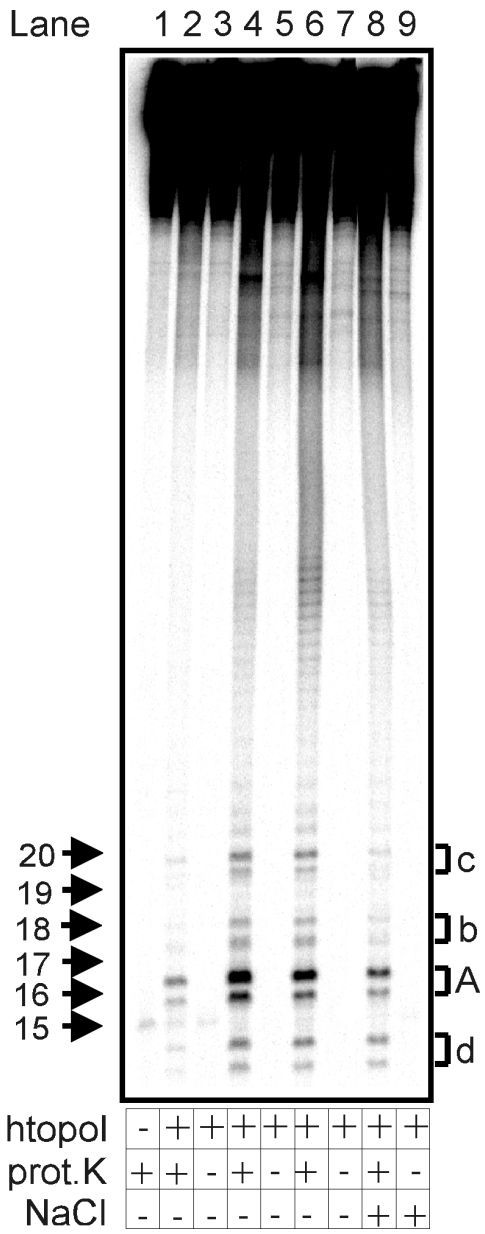

Figure 5. **DNA fragments are protein linked.** Autoradiogram of a 20% sequencing gel. L193s was incubated with: lane 1, no htopoI, lanes 2 and 3, 250 ng, lanes 4 and 5, 750 ng, and lanes 6 to 9, 1250 ng yeast expressed htopoI for 40 min at 37°C. For each concentration of htopoI used, the reaction mixtures were split into two aliquots and stopped by the addition of SDS (e.g. lanes 2 and 3). The samples shown in lanes 1, 2, 4, and 6 were incubated with proteinase K. The samples shown in lanes 8 and 9 were stopped by the addition of 300 mM NaCl for 10 min at 37°C, divided into two aliquots; the one shown in lane 8 was incubated with proteinase K. Lettering is the same as in Figure 4.

diminished by 60 to 70%, while for the bands b and d a reduction of about 40% was observed. When the samples were incubated for longer than 40 to 60 min after htopoI had been added, the intensities of the cleavage products A, b, c, and d declined, but did not completely disappear (data not shown). A similar effect was seen with the salt reversal shown in Figure 5, lane 8. This strongly suggests that htopoI was responsible for these cleavage reactions, since a putative contaminating endonuclease would be expected to produce an accumulating number of cuts with increasing incubation time.

4.5 Position of the second cleavage site on L193s

The results from Figure 4 and 5 were further supported by comparing digests performed with either proteinase K (Figure 6, lanes 1 and 2), trypsin (lane 3), or

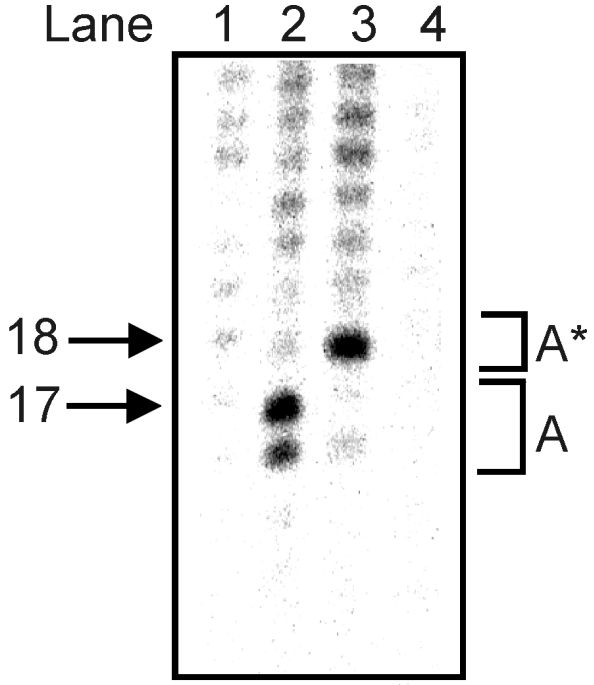

Figure 6. Position of the second cleavage. Autoradiogram of a 14% sequencing gel. L193s was incubated with: lane 1, no htopoI; lanes 2 to 4, 500 ng htopoI for 40 min at 37°C. The reactions shown in lanes 1, 2, and 4 were stopped by the addition of SDS and subsequently aliquots 1 and 2 were incubated with proteinase K. The reaction shown in lane 3 was stopped by the addition of trypsin. Lane 4, no protease was added.

without protease treatment (lane 4). In these experiments only band A was clearly seen, whereas the cleavage sites b, c, and d were too weak to be detected.

Trypsin produced a different digestion pattern (Figure 6, compare lanes 2 and 3). Thus, the double bands produced by proteinase K treatment turned to a single band when trypsin digestion was employed instead. It has previously been shown that a complete digest of DNA-bound htopoI with trypsin leaves seven amino acids bound to the DNA (Christiansen and Westergaard 1999, Svejstrup *et al.* 1990). This supports the results from Figure 5 where cleavage by a second htopoI only occurred on substrates that already contained a suicidally attached htopoI molecule. The cleavage product, named A*, represented the same cleavage site as the proteinase K double band A. This shows that proteinase K digestion yielded a protease-resistant peptide with two different lengths most likely corresponding to 5 and 6 amino acids, respectively.

For the results shown in Figure 6 baculovirus-expressed htopoI was used. The fact that the cleavage sites from Figure 4 and 5 were reproduced with a highly purified htopoI from another source (Figure 2B) strongly supported the view that htopoI was responsible for the cleavage reaction. In order to identify the precise location of the cleavage sites we relied on the experimental finding that trypsin cleavage of htopoI covalently bound to DNA generates a mobility shift of 5 nts in a 14% sequencing gel (Christiansen and Westergaard 1999, Svejstrup *et al.* 1990). Since A* had a mobility of 18 nts, the covalently bound DNA fragment must have been app. 13 nts long. Thus, the incision was estimated to have occurred 13 nts upstream from the suicidally bound htopoI. Comparable mobility shifts were also observed for the bands b, c, and d (data not shown). Therefore, it can be approximated that these bands resulted from cleavages that took place 15, 17, and 11 nts respectively upstream from the covalently attached htopoI. Since the radioactive label was placed 12 nts upstream from the suicidal cleavage site, band d may also have resulted from a fraction of the suicidally cleaved substrates that were not cleaved at position 3, but instead at position 5 (see Figure 1A for numbering). It is well known that the majority of htopoI incisions take place at position 3, but a minor fraction occur at position 5 in the htopoI recognition sequence which is included in our substrate, L193s (Christiansen *et al.* 1993, Christiansen and Westergaard 1994).

4.6 The second cleavage reaction is sensitive to CPT treatment

To further support the view that the cleavage reaction was caused by a htopoI, L193s and htopoI were incubated in the presence of the topoisomerase I specific

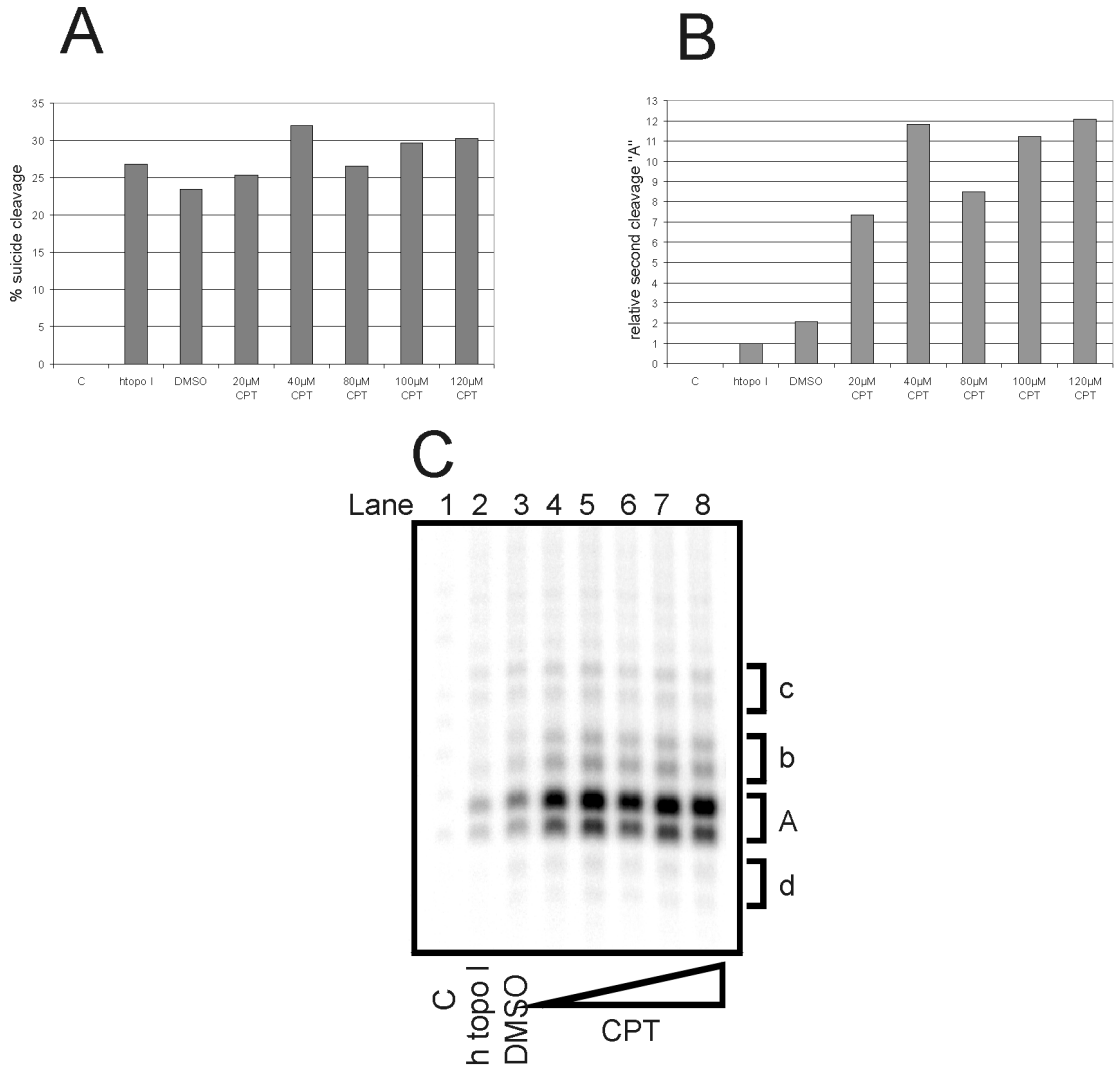

Figure 7. CPT sensitivity of the second cleavage. 500 ng baculovirus-expressed htopoI was incubated with the suicide substrate in the presence of DMSO or CPT as indicated. ”C” is without htopoI; ”htopoI” is htopoI under standard conditions. A) The graphics represents a quantification (as described in chapter 3.4) of the percentage of suicide cleavage in regard to the total substrate used (see Experimental Procedures). B) The graphics is a quantification of the relative effect of DMSO and CPT on the second cleavage site ”A”. C) Autoradiogram of a 14% sequencing gel showing the results quantified in B. The results shown are representative for three independent experiments.

drug CPT. A titration with CPT was performed and the influence on the suicide cleavage as well as the cleavage complex specific incisions were tested. CPT showed no effect on the suicide cleavage (Figure 7A). This, however, is not surprising since the suicide substrate has the same effect as CPT, namely the inhibition of the religation step. Therefore, CPT cannot block ligation at the site of suicide cleavage since religation is already inhibited. In Figure 7B and C the effect of CPT on the second cleavage reaction is shown. In the presence of CPT up to app. 12-fold stimulation was observed. However, since DMSO alone also showed a app. two fold stimulation, the stimulatory effect of CPT may have been at least six fold. The autoradiogram to Figure 7B indicates that CPT also had a stimulatory effect on the cleavage sites b and c and to a smaller extent on d (Figure 7C). These alternate cleavage reactions were rather rare events compared to the cleavage at position A. In general the double cleavage A had a maximum efficiency of 12% in the absence of CPT when only taking the suicidally cleaved DNA into consideration. In the presence of CPT this could be increased up to a maximum of 35%.

4.7 Active site mutant Y723F htopoI can compete with htopoI double cleavage

The data shown above bring strong proof that a second htopoI can cleave specifically upstream from a htopoI cleavage complex. To be absolutely sure that the second incision is due to an additional htopoI enzyme a titration was done with purified mutant htopoI. This mutant carries an amino acid exchange of the active site Tyr^{723} to Phe. Tyr^{723} is responsible for the covalent linkage to the DNA backbone, thus the mutant Y723F htopoI has the same functions concerning protein interactions, DNA binding etc., but is unable to cleave the DNA. It was speculated that this mutant htopoI should be able to compete with the second htopoI cleavage step in a 1:1 ratio, meaning that equal amounts of mutant and wt htopoI should inhibit the reaction by approximately 50%.

As a control the influence of Y723F htopoI on the suicide cleavage was tested (Figure 8A). As it can be seen, the mutant htopoI had no inhibitory effect on

the suicide cleavage. This would also not be expected since the suicide cleavage is an irreversible reaction. The double cleavage, however, was significantly

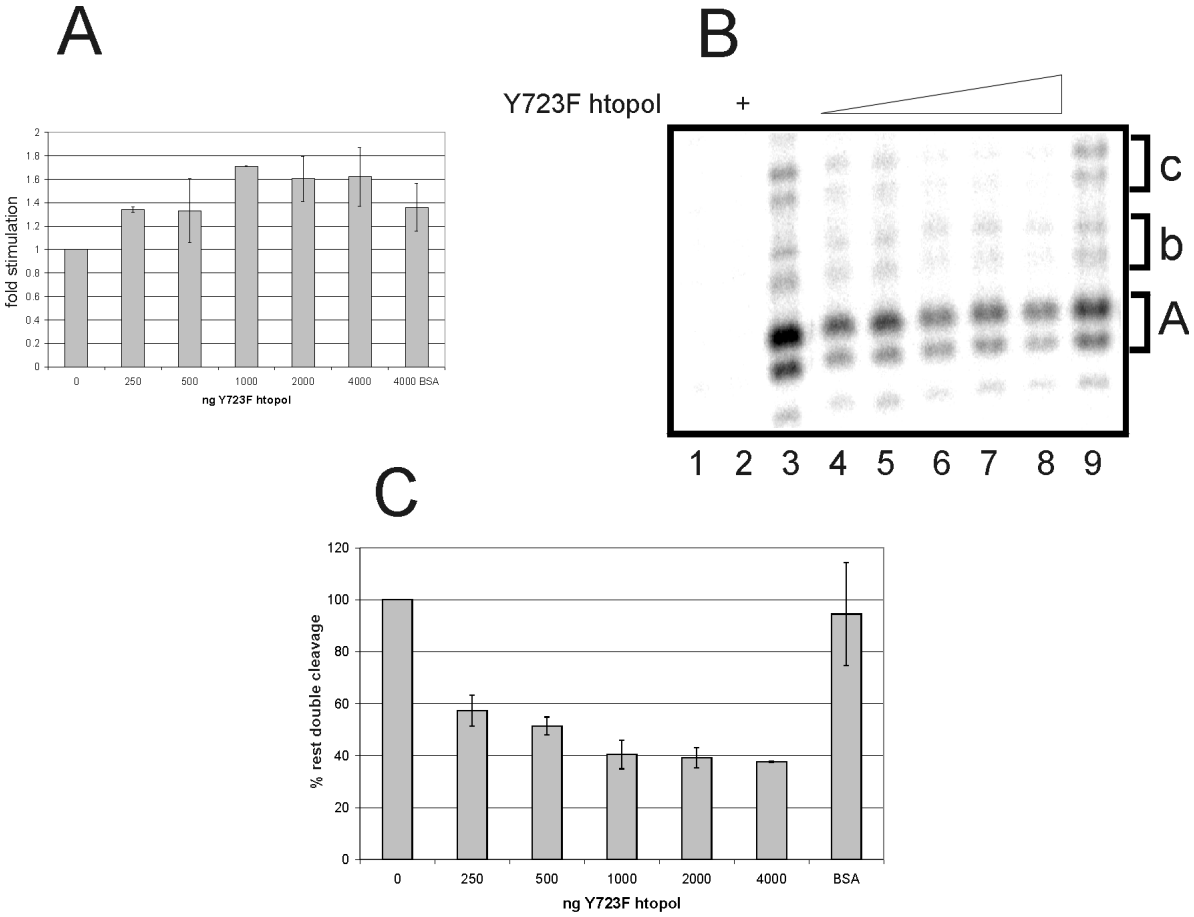

Figure 8. The mutant Y723F htopoI can compete with the htopoI double cleavage. Wt htopoI was incubated with L193s and mutant Y723F htopoI as described in Experimental Procedures. A) Shows the influence of mutant Y723F htopoI on the suicide cleavage of htopoI in an average from 3 to 5 experiments. B) Autoradiogram of a 14% sequence gel. Lane 1, no htopoI, lane 2, 500 ng Y723F, lane 3, 500 ng htopoI, lanes 4 to 9, 500ng htopoI and 250, 500, 1000, 2000, 4000 ng Y723F htopoI, and 4000 ng BSA respectively C) Comparison of double cleavage „A“ from 3 to 5 experiments as shown in B. A, b, and c represent the htopoI double cleavage as shown in Figure 4.

inhibited by the titration with the active-site mutant (Figure 8B, compare lane 3 with lanes 4 to 8). With increasing concentrations of Y723F htopoI the double cleavage was inhibited, however not completely. This is particular clear to see in Figure 8C. As expected about 50% inhibition is seen when the ratio between wt

and mutant htopoI is 1:1 (Figure 8C, 500ng Y723F htopoI). However, with increasing concentration of the mutant enzyme a minimum is reached at about 40% residual double cleavage activity. In Figure 5 the same degree of remaining double cleavage was detected when the reactions were stopped with high salt prior to protease digestion. This suggest that around 40% of the second cleavage complex topoisomerases are not able to ligate. This may be caused by a release of the suicide htopoI attached to the 13 nt long oligomer, thus making a ligation by the second htopoI impossible.

4.8 Limited digest with subtilisin does not affect the suicide cleavage, but eliminates the second cleavage step

The short distance from the suicidal cleavage site to the cleavage sites A, b, c, and d suggests that the two htopoI molecules were in extreme proximity of each other on the DNA substrate. Therefore, we wanted to investigate if protein interactions between the two enzymes were possibly taking place. To investigate this we performed a limited proteolysis of htopoI with subtilisin. Stewart *et al.* (1996) published that limited proteolysis of htopoI with subtilisin removed the N-terminal domain by specifically cleaving in the linker region so that the core and the COOH-terminal domain were liberated, which however still remained associated with each other and maintained catalytic activity. Later Ireton *et al.* (2000) generated a mutant htopoI that lacked the N-terminal domain and parts of the linker domain. After purifying this enzyme both monomers and dimers were present. Based on these findings a model for the dimerization was set up and a ”domain swapping” interaction of the dimer was suggested. According to this model the core domain of one enzyme binds to the C-terminal domain of the other and vise versa. We therefore tested whether such a model could explain our results. HtopoI was digested with subtilisin as described. The reaction was followed by SDS-PAGE and Coomassie-staining (Figure 9A). A minor fraction of htopoI was converted to a 80 kDa form (lanes 2 and 3) while the major fraction existed as a 60 kDa fragment (lanes 2 and 3, mobility of about 63kDa) (Figure

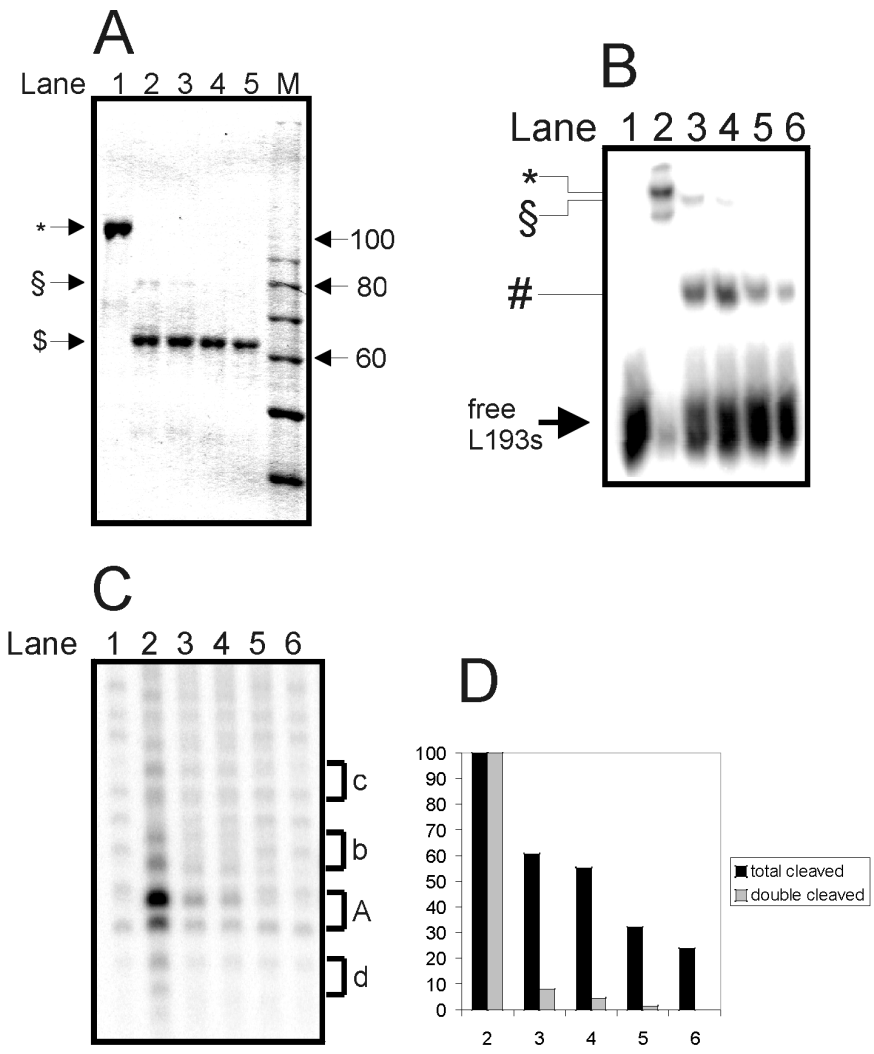

Figure 9. Limited digest of baculovirus-expressed htopoI with subtilisin. A) Coomassie staining of a 7.5% SDS-PAGE showing the partial digest of htopoI by subtilisin. Two µg htopoI was incubated with 0, 20, 40, 60, and 120 ng subtilisin for 20 min at room temperature and 800 ng htopoI of each reaction was loaded in lanes 1 to 5 respectively. *, full length htopoI; §, htopoI lacking the N-terminus; $, core domain lacking the N- and C-terminus; M, 10 kDa protein ladder. B) Autoradiogram of a 7.5% SDS-PAGE. L193s was incubated with: Load as in A. *, represents the shift caused by suicide cleavage of full length htopoI; §, is caused by the suicide cleavage of htopoI missing the N-terminus (80 kDa); #, is caused by the suicide cleavage of the C-terminal domain and the core domain, however only the C-terminal domain is covalently attached to the substrate. C) Autoradiogram of a 14% sequencing gel. The same numbering is used as in B. D) The results from B and C were quantified and depicted in a column chart. The numbering on the X-axis represents the same as in B and C. Black bars represent the relative

degree of suicide cleavage and quantify the results shown in B; gray bars represent the relative degree of second cleavage site ”A” and quantify the results shown in C. The results shown are representative for 3 independent experiments.

9A). With increasing concentrations of subtilisin the 80 kDa form was completely converted to the 60 kDa product (lane 4 and 5). From Stewart *et al.* (1996) it was known that the 80 kDa fragment had lost the N-terminal domain while the 60 kDa fragment had lost both the N- and C-terminal domain. The C-terminus ran out of the gel as well as the fragments of the N-terminal domain. L193s was incubated with these different digestion products and the amount of suicide cleavage was detected. The bandshift caused by the suicide cleavage reaction changed drastically after digestion with subtilisin (compare lanes 2 and 3 of Figure 9B). The bandshift caused by undigested htopoI (marked with ”*”) is shown in lane 2. The upper band in lane 3 represents the bandshift caused by the 80 kDa fragment (marked with ”§”) which had a slightly faster mobility than the full length htopoI. The lower migrating band (marked with ”#”) is due to the attachment of the approximately 10 kDa C-terminal domain of htopoI (containing the active site tyrosine) which associates with the 60 kDa fragment under native conditions. From Figure 9B it can be concluded that the 60 kDa core domain and the 10 kDa C-terminal domain retain enough activity to support an effective suicide cleavage reaction although this was slightly less effective than that caused by the full length enzyme. In Figure 9C the ability of these htopoI fragments to perform the double cleavage reaction is shown. When comparing lane 2 with lane 3 a dramatic effect can be seen. Despite the fact that the digested htopoI still retained the ability to perform the suicide cleavage (Figure 9B, lanes 3 to 5) the ability to carry out the double cleavage reaction was lost (Figure 9C, lanes 3 to 6). In Figure 9C lanes 3 and 4 a residual activity of the cleavage product A was still detectable, but this was most likely caused by a small fraction of 80 kDa htopoI in these reactions. The data from Figs. 9B and C are presented in a diagram form in Figure 9D. Although the degraded forms of htopoI as shown in lanes 2 and 3 retained around 60% of the suicide cleavage activity the ability to perform the double cleavage reaction was reduced to less than 10% or even totally lost. This indicates that a protein-protein interaction is necessary for the double cleavage to occur and that

the two htopoI enzymes may dimerize on the DNA mediated through the core and C-terminal domain. When only the 80 kDa fragment was produced by subtilisin digestion this had no effect on the suicide or double cleavage (data not shown). The latter result rules out that the N-terminus was involved in the dimerization reaction.

4.9 Midway status

Before going on with the second part of the thesis the results from the aforementioned experiments are summarized in Figure 10. In Figures 4 and 5 it

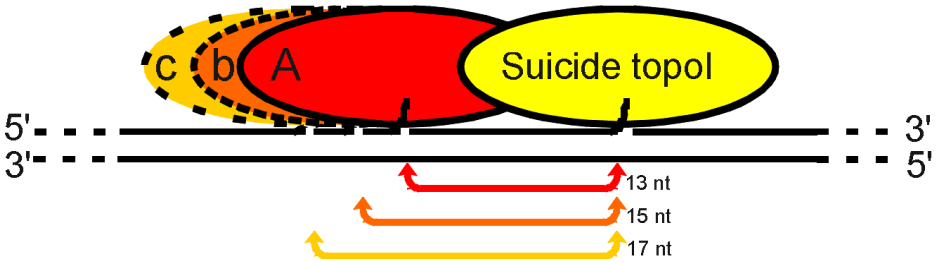

Figure 10. Schematic representation of results. The htopoI double cleavage complex arise after a stable cleavage complex (suicide topoI) has formed. This is subsequently recognized by an additional htopoI molecule (A, b, or c) which cleaves the DNA at the indicated distance to the other cleavage site and a double cleavage complex is formed. The cleavage "d" is left out for reason of simplicity.

was shown that immediately upstream from a htopoI cleavage complex additional incisions took place. From Figure 6 it was found that these incisions occured primarily 13 nts upstream from the cleavage complex, but also to a smaller extent at distances of 11, 15, and 17 nts. It was shown in Figures 7 and 8 that these incisions were done by additional htopoI molecules on DNA substrates which contained htopoI cleavage complexes. In Figure 9 it was indicated that these incisions, which are called „double cleavages", may be mediated through protein-protein interactions and therefore forming a complex (double cleavage complex). Knowing all this it was now interesting to investigate if the tumor suppressor protein p53 would be involved in this novel activity of htopoI, since p53 was shown to regulate htopoI *in vitro* and *in vivo* (as described in paragraph 1.2.4).

4.10 p53 stimulates the relaxation activity of htopoI *in vitro*

It was previously shown by Gobert *et al.* (1996 and 1999) and Albor *et al.* (1998) that human and murine p53 stimulate the htopoI mediated relaxation of supercoiled plasmid *in vitro*. As shown in Figure 11A these results were confirmed and also show that there was a clear concentration dependent effect of the stimulation by p53 (compare lane 2 with lanes 3 to 6). BSA showed no stimulatory effect (lane 7) and the murine p53 preparation contained no detectable topoisomerase I activity (lane 8). For this experiment very low amounts of htopoI (1 and 3 ng) were used in order to see the stimulation clearly. If more htopoI was

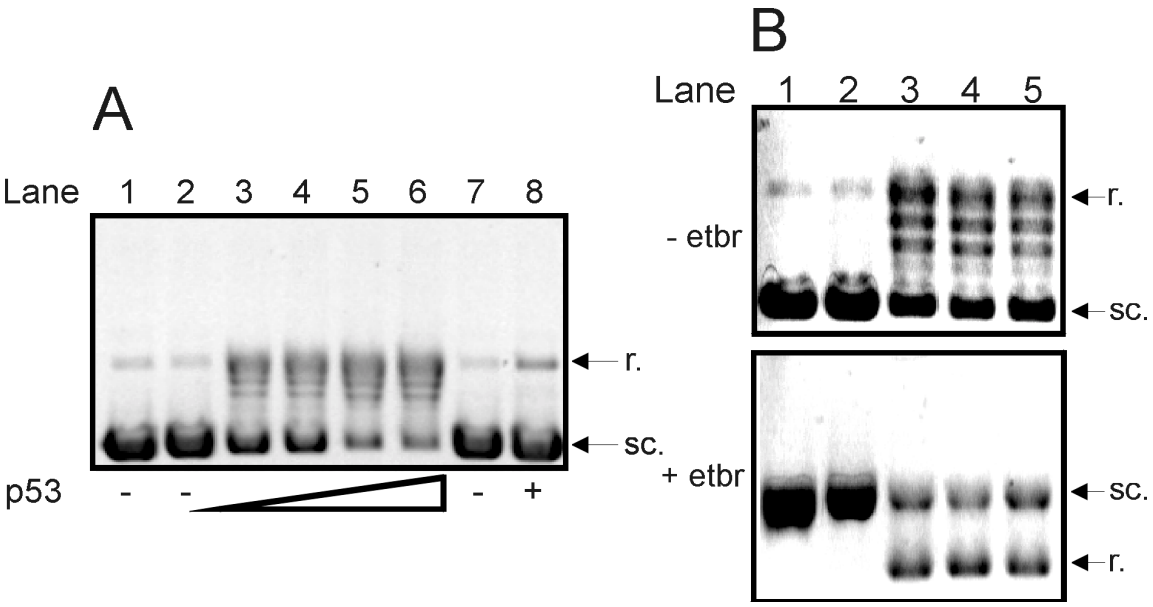

Figure 11. Human wt, bovine wt, and murine His-p53 stimulate the relaxation activity of htopoI equally well. A 0.8% agarose gel run in the absence (- etbr) (A and B) or presence of 0.5 μg/ml ethidiumbromide (+ etbr) (B). A) 3 ng htopoI was incubated with or without murine His-p53 for 5 min at 37°C. Lane 1, no htopoI, 2, htopoI, lane 3 to 7, htopoI and 50, 75, 140, 210 ng mHisp53, and 210 ng BSA respectively, lane 8, 140 ng mHisp53. B) 1 ng htopoI was incubated with or without 300 ng p53 for 5 min at 30°C. Lane 1, no htopoI, 2, htopoI, lanes 3 to 5, htopoI and hp53, bp53, and mHisp53. r., relaxed, sc., supercoiled.

used the turn-over of the substrate was so fast that no stimulation could be detected.

Previously it was shown that human (Gobert *et al.* 1996 and 1999) or murine p53 (Albor *et al.* 1998) could stimulate the activity of htopoI *in vitro*. However, since it was of importance for the further investigations, p53 from three different species were compared for their ability to stimulate htopoI activity *in vitro*. Recombinant human, bovine, and murine His-p53 were tested and by comparing lanes 3 to 5 (Figure 11B) it is seen that p53 from the three different species was able to stimulate equally well. This shows that there is a high degree of conservation for the ability to stimulate topoisomerase I activity among p53 from the three species tested.

The similar behavior of p53 from different species may be due to the high degree of conservation of the sequence sufficient for htopoI activation namely aa 302-320 for the human p53 (Gobert *et al.* 1999). Through a sequence homology comparison it was found that this region had a high degree of homology. The bovine p53 had 90% sequence identity and 95% sequence similarity and the murine 70% identity and 85% similarity to the human p53 sequence in this region (comparison was done using the alignment tool Clustal W; www2.ebi.ac.uk/clustalw).

4.11 Murine His-p53 binds specifically to the core domain of htopoI

As mentioned, Gobert *et al.* (1999) published that a p53 peptide was sufficient to stimulate the activity of htopoI. However, it was not known which part of htopoI bound to p53. We therefore performed a partial digest of htopoI using subtilisin (Figure 12) and investigated by Far Western analysis to which domain of htopoI p53 bound. The partial digest was performed under conditions where the N-terminus was removed and the C-terminus and the core domain were separated, as published by Stewart *et al.* (1996) (Figure 12A lane 2, 12B lane 1). The band marked with an „a“ represents the full-length htopoI, „b“ the core domain, and the fragment marked with „c“ represents the C-terminal part (Figure 12A and B). When comparing the Coomassie stained gel with the Far-Western blot of Figure 12A a clear interaction between full-length htopoI and p53 can be detected (lane

3) whereas no interaction with BSA was seen (lane 1). In lane 3 (Figure 12A) degradation products of the N-terminus can be detected just below the full-length band. This is frequently seen in a htopoI preparation since the N-terminus is highly protease-sensitive.

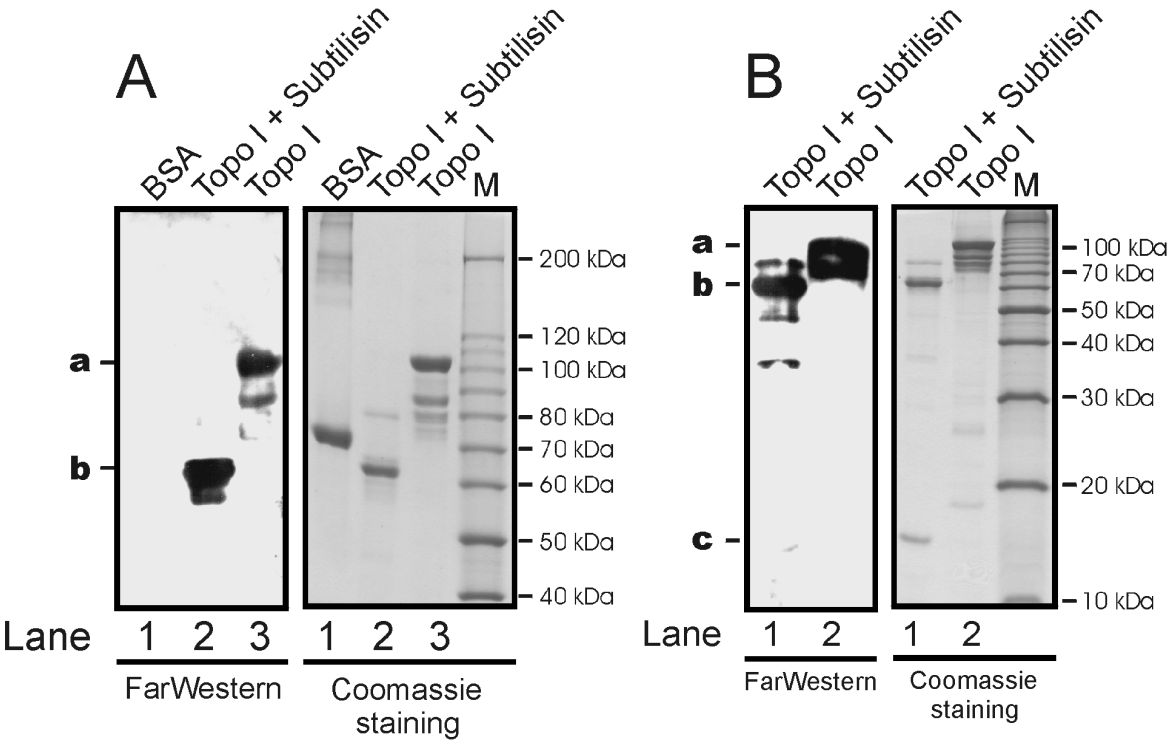

Figure 12. p53 binds to the core domain of htopoI. A) 7.5% SDS-PAGE. Far-Western and Coomassie staining as indicated Lane 1, BSA, lane 2, htopoI + 12 µg/ml subtilisin, lane 3, htopoI B) 13.75% SDS-PAGE Far-Western and Coomassie staining as indicated. Lane 1, htopoI + 12 µg/ml subtilisin, lane 2, htopoI. Far-Western was made with murine His-p53 in solution. Detection was done by p53 antibody (Ab-7) and ECL detection as described in Experimental Procedures. M, marker, a, full length htopoI, b, core domain, c, C-terminal domain.

In Figure 12A lane 2 it is shown that this interaction was mediated solely through the core domain of htopoI since the C-terminus showed no interaction (Figure 12B lane 1) and the N-terminus was removed. Through N-terminal sequencing I found that the core domain seen in Figure 12A lane 2 and Figure 12B lane 1 started at Asp156. Far-Western analysis using human or bovine p53 also showed a clear interaction with htopoI (data not shown). As a further control it was verified that the p53 antibody did not bind unspecifically to htopoI (data not shown).

4.12 Murine His-p53 stimulates the double cleavage activity of htopoI *in vitro*

Since others and I have shown that p53 stimulates the activity of and forms a physical complex with htopoI (as shown above) it was interesting to investigate if p53 also was able to influence the htopoI double cleavage reaction. Thus, a titration with murine His-p53 was performed in the presence or absence of CPT and tested for any effect on the suicide or double cleavage. Figure 13A shows an autoradiogram of a SDS-PAGE from which it can be seen that p53 had no significant effect on the suicide cleavage with or without CPT (compare lane 3 with lanes 4 to 8 and lane 12 with lanes 13 to 17). However, this was not the case when the same reactions shown in Figure 13A were tested for the influence on the second cleavage step. In Figure 13B a clear increase in the double cleavage by htopoI was detected with increasing p53 concentrations (compare lane 3 with lanes 4 to 8). From Figure 13B it can also be seen that addition of CPT showed an additive effect in the presence of p53. This suggests that the mechanism by which p53 stimulates the htopoI double cleavage reaction is different from the stimulatory action of CPT. There are two possible ways for p53 to stimulate the htopoI double cleavage: First, stimulation can be achieved by increasing the half-life of each complex. This would be the same effect as it is the case with CPT (see chapter 1.1.3). Thus, p53 would not be expected to have any significant effect in the presence of CPT. Second, stimulation may occur by an increased formation of the htopoI double cleavage complex. Here, it would be expected that an incubation with p53 and CPT would result in an additive effect. Figure 13B (lanes 10 to 18) shows such an effect when p53 was titrated to htopoI in the presence of CPT. When comparing lane 3 with lane 12 (Figure 13B) an about 2.5-fold effect of CPT on htopoI alone can be detected. A 2.5-fold effect of CPT was also found in the presence of p53 (Figure 13B, compare lane 7 with lane 16). The maximum effect of p53 alone was approximately four fold (Figure 13B, compare lane 3 with lane 7), however, when CPT was present as well their joined effect was approximately 10 fold. BSA showed no effect in the presence or absence of CPT (Figure 13B, compare lane 3 with lane 9 and lane 12 with lane 18). This strongly

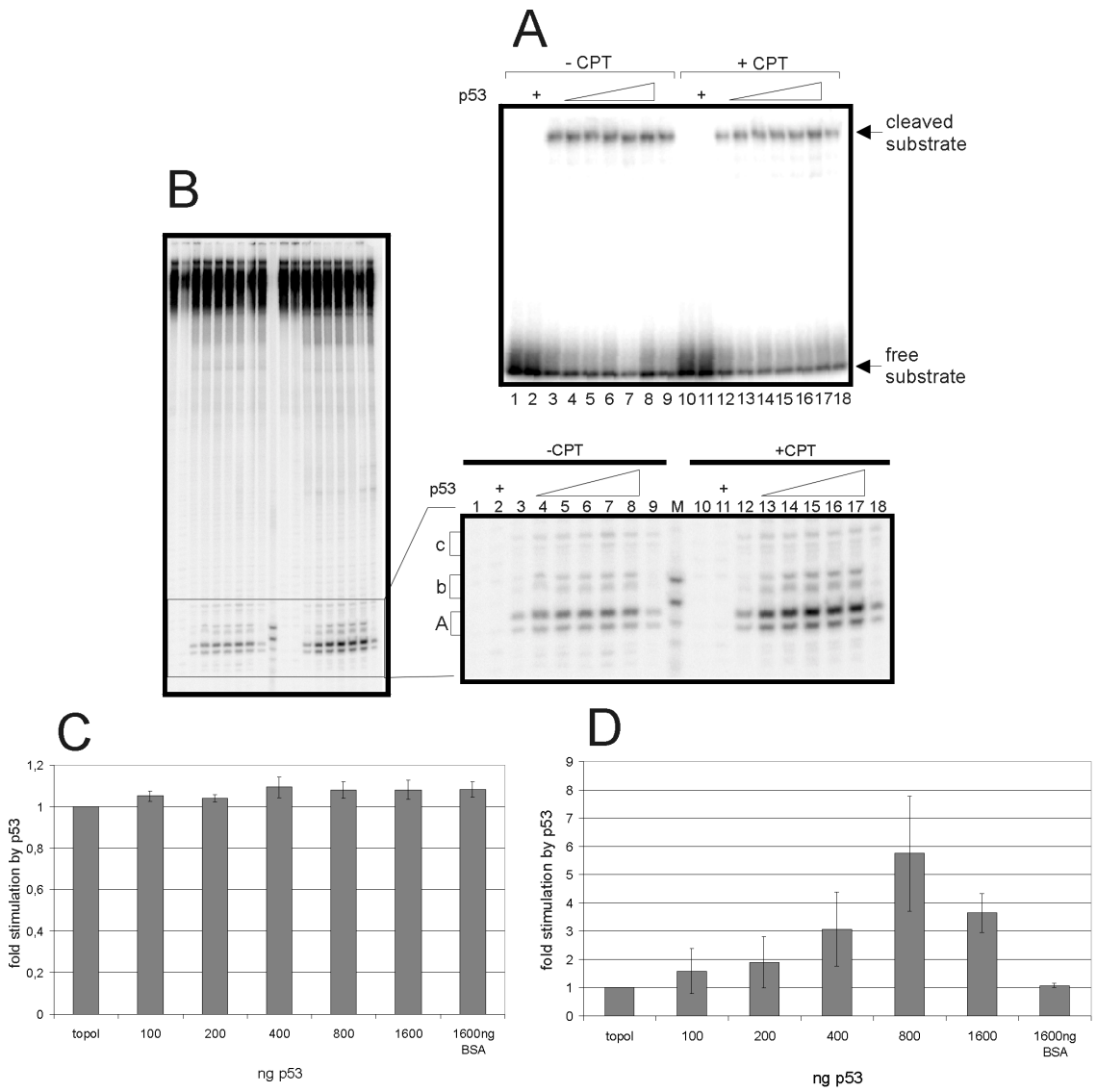

Figure 13. Murine His-p53 stimulates the double cleavage of htopoI. A) Autoradiogram of a 7.5% SDS-PAGE showing the degree of suicide cleaved substrate in the presence or absence of p53 and CPT as described in Experimental Proceedures. Lane 1, no htopoI, lane 2, 800 ng murine His-p53, lane 3, 400 ng htopoI, lanes 4 to 9, 400 ng htopoI and 100, 200, 400, 800, 1600 ng murine His-p53, and 1600 ng BSA respectively. Lanes 10 to 18 the same as lanes 1 to 9 but in the presence of 10 μM CPT. B) Autoradiogram of 14% sequence gel. Lanes 1 to 18 as in A. C) Comparison of the influence of murine His-p53 on the suicide cleavage from 3 to 5 experiments as shown in Figure 13A. D) Comparison of the influence of mHisp53 on the htopoI double cleavage from 3 to 5 experiments as shown in Figure 13B.

indicates that the stimulatory effect of p53 and CPT are additive. Thus, the data suggest that p53 stimulates the double cleavage by inducing the formation of more double cleavage complexes without increasing their half-life. Figure 13C shows a schematic representation of the influence of p53 on the suicide cleavage and clearly shows that there was no significant effect. In Fig 13D the average stimulation of the double cleavage A by p53 from three experiments is shown. p53 can stimulate the double cleavage by up to six fold, whereas BSA had no effect. It can also be seen that an optimum was reached at around 800 ng p53, whereafter the stimulation declined. This optimum was reached at a molar ratio of 1:4 between htopoI and p53 monomer. From previous studies it is known that p53 preferentially forms a tetramer (Stürzbecher *et al.* 1992; Friedman *et al.* 1992). So, since a clear optimum was found when htopoI and the p53-tetramer were in a 1:1 ratio, this may indicate that tetrameric p53 is needed for an optimum stimulation.

5 Discussion

The results presented in this dissertation have brought about new insights into the possible roles of htopoI as well as of the tumor suppressor protein p53. Using a htopoI suicide substrate in a novel design, it was shown that a htopoI cleavage complex can attract an additional htopoI molecule to perform a nearby cleavage, which here was called a htopoI double cleavage. The distance between the two cleavage sites was estimated to be primarily 13 nts and to a smaller extent 11, 15, or 17 nts. This additional cleavage was in part reversible as it could be inhibited up to 60% by competion with the active site mutant Y723F htopoI. The small distance between the two htopoI molecules gave rise to the speculation that the additional cleavage could be mediated by direct protein-protein contacts. Through partial digests with subtilisin a direct protein-protein interaction could be shown. Thus the htopoI molecules formed an unstable complex on this substrate. This complex was called a htopoI double cleavage complex. Data from the literature stating that the tumor suppressor protein p53 can stimulate htopoI *in vitro* (Gobert *et al.* 1996, Albor *et al.* 1998, Gobert *et al.* 1999) were confirmed. As a new finding, it was directly shown that human, murine and bovine p53 stimulate htopoI equally well. From the literature it is known that this stimulation is mediated by direct protein-protein interactions (Gobert *et al.* 1996, Albor *et al.* 1998). In the present work it is shown that p53 interacts with the core domain of htopoI. Finally it is shown that the p53 tetramer specifically stimulates the formation of the htopoI double cleavage complex *in vitro*.

In the following sections, a model and various scenarios will be presented and discussed. These serve to explain the data summarized above, and to suggest possibilities for possible physiological or pathological functions of these complexes.

5.1 Model for htopoI double cleavage complex formation

This study demonstrates that recombinant htopoI expressed in *S. cerevisiae* or by baculovirus is able to recognize a htopoI cleavage complex and cleave predominantly 13 nts upstream from this complex (see Figure 10). This was

shown by a strong sensitivity of the second cleavage step to CPT and by the fact that it could be competed for by the active-site mutant Y723F htopoI but not by BSA. The recognition of the cleavage complex is likely to be mediated by a dimer formation between the two htopoI molecules on the DNA substrate as indicated by the results shown in Figure 9.

In the literature one example showing that a mutant htopoI can form a dimer has been previously reported. Ireton *et al.* (2000) observed that a mutant htopoI (topo70ΔL) missing the N-terminus and having a shortened linker domain could form dimers in solution. This dimer eluted much later from a Mono S column compared with the monomeric mutant htopoI. It retained full activity and the dimer was even more active than the monomer. Furthermore, the mutant was more sensitive towards CPT than was the monomer. Based on limited digestions of htopoI70ΔL with subtilisin a model was suggested according to which the C-terminus of one enzyme can bind to the core of another enzyme and vice versa. This phenomenon was called ”domain swapping”. It was suggested that this dimer could form due to the shortened linker domain. This could affect the position of the C-terminal domain with respect to the core domain. Thus, the interaction between these two domains may be weakened considerably. This in turn could result in the domain swapping mentioned above. Since it was found that the cleavage of the linker region had a dramatic effect on the double cleavage reaction, but only a minor influence on the suicide cleavage (see Figure 9), this indicates that for the detection of the double cleavage it is essential that the core and C-terminal domains are attached. This phenomenon could be explained by the following model (Figure 14).

A htopoI enzyme binds and cleaves DNA suicidally. Thus, it is blocked in the catalytic cycle and becomes ”frozen” in the cleavage conformation. This conformation was shown by Stewart *et al.* (1996) to be different from the non-bound state especially in the linker region which connects the core to the C-terminal domain. It is therefore possible that the interaction between the C-terminal and the core domains is less strong than that in the the non-bound state. This may then open the possibility that the core domain of a second htopoI can interact with the C-terminus of the ”frozen” htopoI and simultaneously cleave the DNA, thereby generating the second cleavage through the formation of an

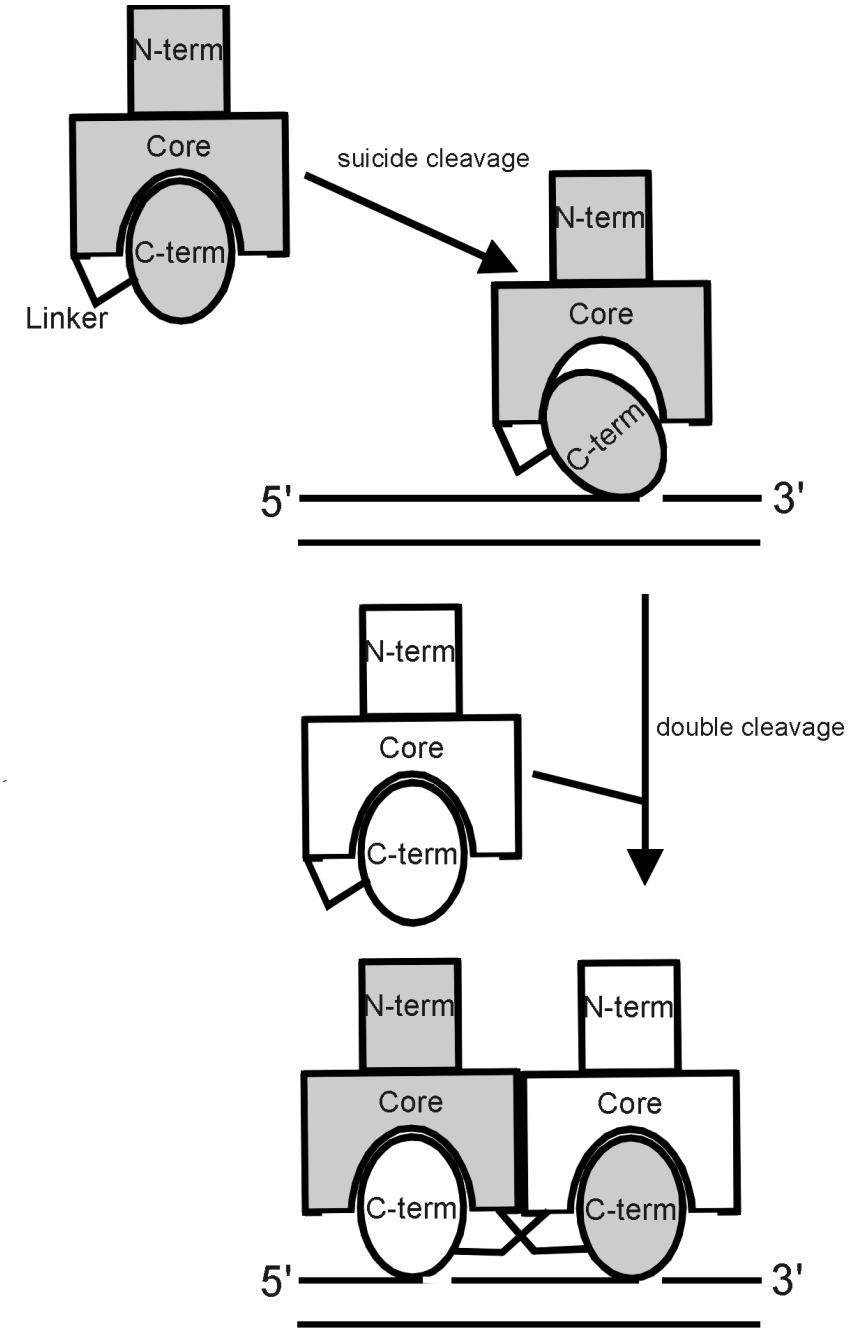

Figure 14. Model for htopoI double cleavage complex. See text for further details.

unstable dimer. However, if the linker of htopoI is broken the second htopoI enzyme may still cleave upstream of the ”frozen” htopoI and also ”swap” core domains, but since the linker is broken there is no longer a protein contact that holds the second htopoI in place. This can now religate and diffuse away. Thus, the second cleavage reaction can no longer be detected. Although hypothetical, this model is nevertheless supported by the direct protein-protein interactions

presented in Figure 9, and by the very close proximity between the two cleavage sites. The distance of 13 nts corresponds to approximately 44 Å. Redinbo *et al.* (1998) and Stewart *et al.* (1998) published the crystal structure of a reconstituted htopoI bound or complexed to DNA. They found that htopoI contacted DNA 4 bp upstream and 6 bp downstream of the cleavage site in a htopoI recognition sequence similar to the one used here. When bound to DNA htopoI covered 60 Å along the DNA. Since the 44 Å we found is considerably less than the 60 Å published for the crystal structure, not only direct protein-protein interactions between the two adjacent htopoI molecules are highly likely, but also some compaction is required possibly by domain swapping.

5.2 Could htopoI double cleavage complexes form *in vivo*?

The presence of htopoI dimers *in vivo* was recently suggested by Mao *et al.* (2000). MCF-7 cells were irradiated with UV-light, and dimethyl suberimidate (DMS) was subsequently added to the media in order to crosslink proteins. The chromosomal DNA was then extracted, bound to a filter, and probed by antibodies for htopoI that was bound covalently to the DNA. Using this technique the authors found that there was a significant increase in the amount of htopoI bound covalently to the UV irradiated DNA when DMS was added. This was interpreted as a possible dimer formation between two htopoI molecules which occurred only when the DNA was irradiated with UV light (Mao *et al.* 2000).

Lesions such as abasic sites, UV-photoproducts, and oxidative DNA damage are preferentially bound by htopoI and all these lesions lead to a stabilization of the enzyme in the cleaved state (Lanza *et al.*1996, Mao *et al.* 2000, Subramanian *et al.* 1998, Pourquier *et al.* 1999). This situation is mimicked by our suicide substrate. Thus, a recognition of the ”frozen” htopoI as proposed for our *in vitro* system may also be true for htopoI ”frozen” *in vivo* in the vicinity of the kinds of DNA damage mentioned above. Such a mechanism would also ensure that a htopoI double cleavage complex does not form in the absence of damage, since the cleaved state during a normal catalytic cycle of htopoI would be too short-lived to be recognized by another htopoI molecule. I have also found that htopoI double cleavage complexes could be observed when suicide substrate and recombinant htopoI were added to whole cell extracts. The double cleavage reaction was even

stimulated up to 4-fold (data not shown). This was done under reaction conditions where the added recombinant htopoI made up only 0.3% of the total protein mass in the reaction mixture. This has not yet been investigated further. However, it does support the assumption that htopoI double cleavage complexes also can form *in vivo*.

5.3 Repair through recombination?

When a htopoI enzyme recognizes a DNA lesion and forms a cleavage complex, it is in principle able to perform the religation step and diffuse away. However, religation and diffusion are much slower on damaged DNA. The cleavage complex may also be converted into an irreversible lesion (Tsao *et al.* 1993, Wu an Liu 1997) which effectively blocks transcription and replication. Several groups have searched for a mechanism that would repair such a lesion (Yang *et al.* 1996, Sastry and Ross 1998, Pouliot *et al.* 1999, Pouliot *et al.* 2001, Interthal *et al.* 2001), but up to now little is known about how such lesions are removed in human cells.

Several groups have shown that htopoI may be involved in recombination events. Recombination *in vitro* by eukaryotic topoI was shown by Shuman (1992), Christiansen and Westergaard (1994), as well as Pourquier *et al.* (1997a). They found that topoI, when stabilized in a covalent cleavage complex, was able to accept a 5'-OH from a foreign DNA strand, that could hybridize downstream from the cleavage complex. This was supported by a study which compared eukaryotic topoI to known recombinases. Cheng *et al.* (1998) found that eukaryotic topoI was not related to recombinases such as the Cre-recombinase family in their amino acid sequence but upon comparing the structures they found that there were great similarities in the catalytic domains of these enzymes. They also share common mechanistical features, namely that they both form covalent complexes with the 3'-end of the DNA. By sequencing known recombination sites and looking for preferred topoI cleavage sites it was found by Zhu and Schiestl (1996) that, when topoI was overexpressed in *Saccharomyces cereviciae,* increased recombination was observed. The sites of recombination were sequenced and it was found that 60% of these sites contained preferred topoI cleavage sites immediately 5' to the

recombination site. Some viruses, which need recombination events either to integrate into the host genome or to form closed circular viral DNA, have been found to involve topoI at least *in vitro* (Wang and Rogler 1991, Pourquier *et al.* 1999). Finally, the most convincing evidence for the involvement of topoI cleavage complexes in recombination was published by Megonigal *et al.* (1997). They had generated a topoI mutant in yeast which could ligate, but much more slowly than the wt enzyme. When expressing this enzyme in yeast they found that at high levels of expression it was lethal, but when expressed at sub-lethal levels it gave rise to a great increase in rDNA recombination.

All of the aforementioned results strongly suggest that htopoI could be involved in DNA recombination *in vivo*. It is thus tempting to speculate that formation of an unstable htopoI double cleavage complex could be the initiation of a repair event via recombination where the second htopoI enzyme is used as a ligase.

What could such a repair pathway look like? In Figure 5 and 8 it was found that approximately 40% of the cleavages could not be reversed by addition of high salt or by mutant htopoI. These 40% may very well represent double cleavages where the first cleavage complex (suicide complex) was released, since the weak protein-protein interactions could no longer hold the suicide htopoI with the attached oligomer in place. Once it has diffused away it makes a reversal of the second cleavage impossible. In this situation a gap is formed which may permit a 5'-OH containing DNA strand to hybridize and start a homologous recombination event using the htopoI molecule as a ligase. This scenario is shown in Figure 15. A htopoI cleaves in the vicinity of a DNA lesion and is frozen in the cleavage confirmation (damage recog. htopoI, red). A „repair" htopoI (green) recognizes this complex through a protein-protein interaction, and cleaves just upstream of it. The large damage recognition htopoI is bound to a very short DNA fragment. When also taking the rather weak protein-protein interactions into consideration, it could be released and thereby leave a gap in front of the repair htopoI which is now trapped. Now, a foreign 5'-OH containing DNA strand could enter and hybridize to

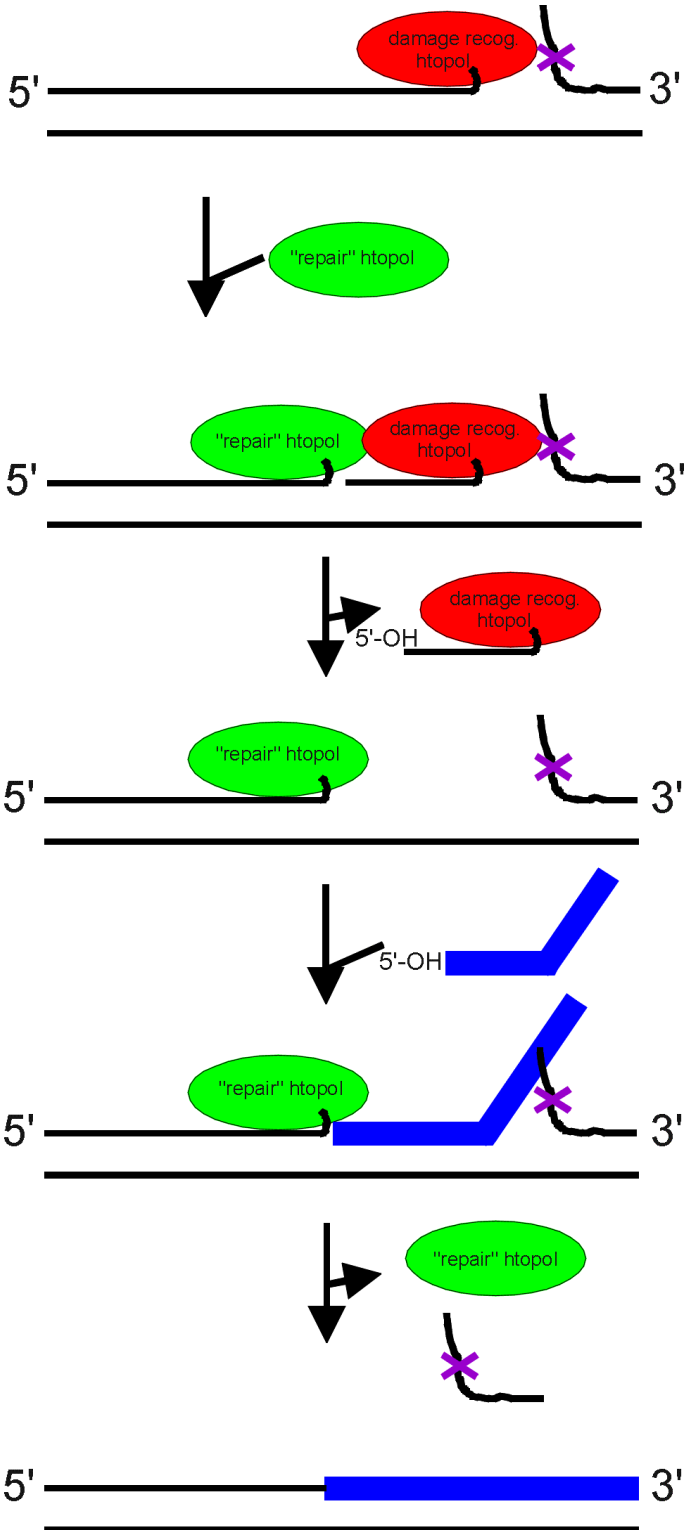

Figure 15. Hypothetical recombination repair of htopol cleavage complexes. A DNA lesion is shown with a purple cross. The incoming foreign DNA strand is marked in thick blue to distinguish it from the original strand. See text for further details.

the gap. Then a strand transfer reaction could take place where the DNA strand containing the damage is completely or partly released. This would also ensure the removal of the original DNA lesion. The repair htopoI could then function as a ligase and thereby release itself from the DNA. A gap resulting in this way could subsequently be filled up by DNA replication.

The scenario described above is presently being tested in a Masters degree project. Data from this project confirm that the „repair“ htopoI can indeed ligate a foreign DNA strand which can hybridize to the predicted gap, thus confirming the scheme suggested above.

5.4 The tumor suppressor protein p53 interacts with the core domain of htopoI

We have shown that human, bovine, as well as murine p53 stimulate the relaxation activity of htopoI equally well (Figure 11). In addition it was shown through limited digests of htopoI with subtilisin and subsequent Far-Western analysis using p53 in solution that p53 interacts solely with the core domain of htopoI (Figure 12). The core domain is responsible for binding the DNA and undergoes conformational changes when binding and cleaving the DNA, especially within the active site (Redinbo *et al.* 1998, Stewart *et al.* 1998). Since p53 was found to interact with this domain it may also explain why the activity of htopoI is dramatically increased upon binding of p53. It is possible that p53 induces conformational changes within the core domain which causes the enzyme to increase its catalytic activity. However, what the more precise mechanism for the stimulation is and with which part of the core domain the interaction takes place needs further investigation. Investigations are going on in our laboratory to answer these questions. The strong direct interaction between htopoI and p53 makes it very interesting to investigate what role this interaction could play in the cell. This is discussed in detail in the next chapter.

5.5 Could htopoI function as a p53-dependent switch between repair and apoptosis?

As shown in Figures 12 and 13 it was not only found that p53 interacts with the core domain of htopoI, but also that it has a significant stimulatory effect on the formation of double cleavage complexes. p53 stimulated complex formation on average by a factor of six. p53 mainly exists as a tetramer in solution (Stürzbecher *et al.* 1992, Friedman *et al.* 1992). In agreement with this it was observed here that the optimum for p53 stimulation was achieved when htopoI and p53 monomer were in a molar ratio of 1:4 respectively (Figure 13). Since complex formation between p53 and htopoI could be demonstrated (see Figure 12), it is highly likely that this stimulation was mediated by direct interactions of p53 with htopoI and not by interactions mediated by the DNA. By investigating the effect of CPT with or without p53 it was found that CPT and p53 had additive effects (Figure 13). Since CPT efficiently stabilizes htopoI cleavage complexes, it would be expected that p53 would not be able to further stimulate complex formation if it also stabilized the double cleavage complex. It was therefore more likely that p53 did not stabilize the double cleavage complexes, but rather stimulated complex formation by for instance increasing the affinity.

However, could this observation have any significance *in vivo*? Based on all of the results presented here and those from the literature, I propose that this might very well be the case. Since p53 has been found to be involved in DNA repair and apoptosis, and since the lethality of CPT induced htopoI cleavage complexes depend on p53 (Yang *et al.* 1996b, Li *et al.* 1998), it is tempting to speculate that p53 and htopoI may help to decide between DNA repair and apoptosis after genotoxic stress. This is shown in Figure 16.

When a cell is exposed to endogenous or exogenous DNA damaging agents DNA lesions are formed and the p53 level is up-regulated in response to it (see chapter 1.3.1). Once up-regulated, p53 could activate htopoI to form cleavage complexes in the vicinity of DNA lesions. If present in abundance, these bulky complexes could induce apoptosis (discussed in detail later). If, however, only a few cleavage complexes have formed, these could signal that the damage level is low and that repair should be initiated. The lesions could then be repaired

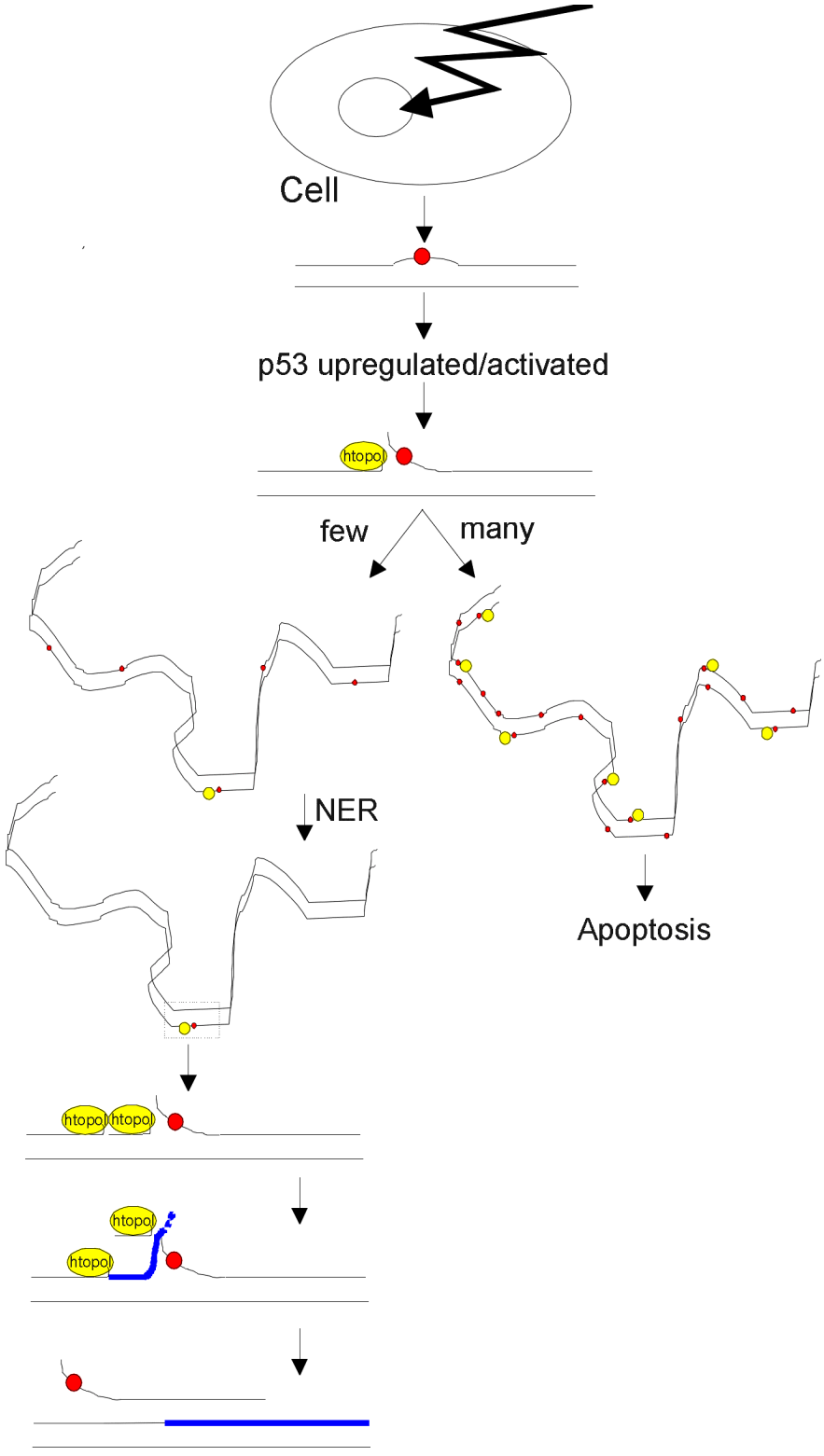

Figure 16. Suggested "switch"-function of p53 and htopoI between repair and apoptosis. The cell is damaged by for example UV light. The resulting lesions are represented by red circles. HtopoI is shown as yellow circles. The incoming strand in recombination is shown in blue. See text for further details.

by the characterized repair mechanisms such as NER, base excision repair, and mismatch repair. However, the htopoI cleavage complex must also be removed. This could be done by the recombination repair suggested above (Figure 15). Once all lesions are removed, p53 is no longer up-regulated and the cell may enter the cell cycle again.

Subramanian *et al.* (1998) presented a model, which suggested that htopoI form cleavage complexes at DNA lesions, because it is directly involved in the repair of the lesion. They suggested this on the basis of investigations where they found for the first time that htopoI formed cleavage complexes on UV damaged DNA *in vivo*. The complex formation was reduced in XP-A cells and not above background level in XP-D cells. XP-A cells carry mutations in the XPA protein, which is involved in damage recognition in NER. XP-D cells carry mutations in the XPD protein, which is a 5' to 3' helicase involved in NER. Both cell lines are highly sensitive to UV-irradiation. Based on these results they suggested a model in which htopoI is directly involved in the repair of the lesion possibly by relieving tension in the DNA around the lesion. However, this would imply that htopoI cleavage complexes should form at every lesion and this is unlikely to happen. Jones *et al.* (1991), Frosina and Rossi (1992), Stevnsner and Bohr (1993), and Thielman *et al.* (1993) all found that CPT had no or only a weak negative influence on NER *in vitro* and *in vivo*. This either suggests that there is no significant role of htopoI in NER either *in vitro* or *in vivo* or that in up to 18% of the cases (Jones *et al.* 1991, Thielman *et al.* 1993) a htopoI cleavage complex is located so close to a lesion that it hampers its recognition/repair (Frosina and Rossi 1992). This is further supported by the fact that BER as well as NER can be reconstituted *in vitro* with purified proteins without the addition of htopoI (Aboussekhra *et al.* 1995, Kubota *et al.* 1996). I therefore do not find the model presented by Subramanian *et al.* (1998) convincing. Rather, I find it more likely that a small fraction of the lesions are recognized by htopoI in a p53-dependent manner and that if these complexes exceed a threshold, p53-dependent apoptosis is induced in a similar fashion to cancer cells treated with CPT (Yang *et al.* 1996b, Li *et al.* 1998). When few htopoI cleavage complexes form, these may be removed by the formation of a double cleavage complex as suggested above (Figure 16).

How could such a repair through recombination take place? Rad51 has been shown to be an important factor for homologous recombination. It is homologous to the *E. coli* RecA, which builds nucleoprotein filaments on ss-DNA and searches for homologous sequences (reviewed by Shinohara and Ogawa 1999). p53 has been found to interact with Rad51 *in vitro* as well as *in vivo*. Through this interaction p53 was found to inhibit Rad51 mediated recombination (Stürzbecher *et al.* 1996). However, this interaction could be released by the SV40 T-antigen, which also interacts with p53, and thereby reactivated Rad51. It was speculated that this could ensure that Rad51 only would initiate recombination events when needed and that p53 would function as a regulator of recombination through Rad51 (Stürzbecher *et al.* 1996). What is presented in Figure 16 also implies homologous recombination. Hence, Rad51 also may play a role in such a putative recombination reaction. Thus, it could be speculated that p53 (bound to Rad51) binds to htopoI following DNA damage and induces the formation of htopoI double cleavage complexes. Thereby it releases Rad51 and Rad51 can participate in repair through recombination as suggested in Figures 15 and 16.

In Figure 16 it was suggested that many htopoI cleavage complexes lead to p53 dependent apoptosis. However, it remains puzzling how p53 induces cell death (see chapter 1.3.3). It was recently published that the multifunctional protein Ref-1 plays an important role in the p53-dependent cell cycle arrest after CPT treatment. Furthermore, it was found that Ref-1 was important in p53-dependent apoptosis (Gaiddon *et al.* 1999). This was concluded since antisense Ref-1 mRNA lowered the sensitivity to CPT treatment by 2.5 fold *in vivo*. It is therefore possible that Ref-1 is involved in the p53-dependent induction of apoptosis in the presence of htopoI cleavage complexes.

When discussing the induction of apoptosis as presented in Figure 16 it may be important to distinguish between htopoI bound p53 and non-bound p53. As suggested in Figure 16 p53 is induced/activated after DNA damage. This could be facilitated through N-terminal phosphorylation of p53 by the ATM gene product (Siliciano *et al.* 1997, Banin *et al.* 1998,), which prevents MDM2 dependent degradation of p53. This induction of the p53 level may only be limited and serve to activate htopoI cleavages at DNA lesions. The p53 associated htopoI complexes could, however, initiate a stronger damage signal than for example a

pyrimidine dimer alone. Such a bulky lesion may be recognized better than a small pyrimidine dimer by proteins such as the ATM gene product and/or DNA dependent protein kinase (DNA-PK). These in turn may cause a further release of MDM2 bound p53 in a feedback loop. This newly released p53 may in turn activate genes such as BAX and $p21^{WAF1}$ dependening on the amount of p53 released from MDM2. In the case of small amounts, p53 may induce transcription of genes such as $p21^{WAF1}$ to allow repair. If a large amount of p53 is released from MDM2, genes involved in apoptosis, such as BAX, may be activated. In agreement with this Jaks *et al.* (2001) found that when few htopoI cleavage complexes were induced by CPT transcriptional activation of $p21^{WAF1}$ took place, but no cell cycle arrest or apoptosis. Only when more htopoI cleavage complexes were introduced this also led to cell cycle arrest.

As suggested above DNA-PK could sense the htopoI cleavage complex and thereby activate a p53 dependent response. p53 and the replication protein A (RPA) have been found to form a complex (Li and Botchan 1993, Dutta *et al.* 1993). Abramova *et al.* (1997) observed that when cells are UV-irradiated this complex is disrupted. The release of p53 from RPA was found to be essential for activation of DNA repair. Later Shao *et al.* (1999) found that when human cells were treated with CPT RPA was phosphorylated by DNA-PK. Kohn *et al.* (2000) suggested a model where RPA becomes phosphorylated by DNA-PK after DNA damage and this causes the release of p53 which in turn can activate downstream pathways. It has been suggested that ATM could be an alternative to DNA-PK as the sensor of p53 induced htopoI cleavage complexes. Piret *et al.* (1999) found that the ATM gene product was essential for the response to CPT treatment. This indicates that ATM participates in a pathway which senses the level of htopoI cleavage complexes

In conclusion the possibilities discussed above suggest that factors such as Ref-1, ATM, and DNA-PK can play important roles in sensing the p53 induced htopoI cleavage complexes and to induce p53 dependent DNA repair or apoptosis.

In the above a possible role for p53 induced htopoI cleavage complexes in apoptosis or repair was discussed, and also how the htopoI cleavage complexes could be removed by recombination through the formation of a htopoI double

cleavage complex. It is, however, also possible that there may exist alternative repair pathways for removal of htopoI cleavage complexes in human cells. Nash and co workers (Yang *et al.* 1996, Pouliot *et al.* 1999, Pouliot *et al.* 2001) have identified an enzyme in *Saccharomyces cereviciae*, which they named Tdp1. Tdp1 can cleave a htopoI cleavage complex at the bond between Tyr^{723} and the DNA backbone. A human Tdp1 homologue has been identified and cloned (Interthal *et al.* 2001). Tdp1 can only remove the covalently linked topoI enzyme if the intact topoI is digested by a protease which leaves a small peptide rest covalently attached to the DNA. However, Tdp1 is very inefficient when this peptide rest is situated on double stranded DNA. Pouliot *et al.* (2001) found when a topoI peptide was located at a blunt end Tdp1 efficiently removed the peptide rest. They therefore suggested that Tdp1 cannot repair topoI cleavage complexes on intact DNA, but only when double-strand breaks, due to DNA replication, have been produced. That Tdp1 most likely is involved in repair of topoI cleavage complexes in yeast was found in knockout studies (Pouliot *et al.* 1999 and 2001). A role of the human TDP1 gene has not yet been shown. The knocking out of the TDP1 gene in yeast resulted in an increased sensitivity to CPT. However, this increase was small, and therefore indicates that there must be alternative pathways.

Pouliot *et al.* (1999 and 2001) found that a Rad9-dependent pathway seemed more important than the Tdp1 pathway. Rad9 is a kinase involved in a checkpoint response pathway in yeast. After DNA damage Rad9 associates with Mec1 (another kinase) at a DNA lesion. Once this complex has formed, Mec1 phosphorylates Rad9 and the complex dissociates. In return Rad9 phosphorylates Rad53 (another kinase) (Gilbert *et al.* 2001). Rad53 activates pathways, which lead to cell cycle arrest and allow DNA repair (Pellicioli *et al.* 1999, Neecke *et al.* 1999).

The Rad9 dependent repair pathway suggested by Pouliot *et al.* (2001) could be the functional analog to the p53-dependent repair pathway discussed here since there are significant similarities. p53 is activated after DNA damage through phosphorylation, as it also is the case of Rad9 and Rad53. The phosphorylation of p53 is also mediated by a “damage-sensor” such as ATM, which could be the equivalent to the Mec1 kinase. Furthermore, the activation of both p53 and Rad53 lead to activation of a cell cycle arrest or apoptosis. If these pathways truly are

analogues it could mean that the scenario suggested in Figure 16 is an alternative to a possible Tdp1-dependent repair pathway.

5.6 Can htopoI double cleavage formation in part explain genomic instability in cells with mutated p53?

p53 exists in a mutant form in about 50% of all tumors (as mentioned previously). All of these mutations lead to a loss of wild-type activity, but some also result in a so-called “gain of function”. Gain of function describes p53 mutations that still retain some p53 activities, although the transactivation role has been lost. This means that they can still bind to various protein factors and are also up-regulated, but fail to induce cell cycle arrest or apoptosis due to the inactivation of the DNA binding domain. If p53 mutations are located on one allele, it can be speculated that enough wt p53 tetramers could form to retain a normal p53 response. However, it has been found that mutant p53 can interact with wt p53 to form tetramers in a dominant-negative fashion. Through this complex formation the wt p53 is influenced by the mutant p53 in such a way that wt activity is lost. Such cells show genomic instability, exemplified by increased recombination events (Mekeel *et al.* 1997, reviewed by Murphy and Rosen 2000, Albor and Kulesz-Martin 2000). p53 mutants have been found to show “gain of function” activities concerning stimulation of htopoI. Gobert *et al.* (1999) showed that a p53 mutant (R273H) was constantly associated with and stimulated htopoI *in vivo,* whereas wt p53 only stimulated in a brief period following cellular stress. In addition, Albor *et al.* (1998) found that two other p53 mutants N239S and R245S also could stimulate htopoI *in vitro*. These mutants have lost their ability to induce cell cycle arrest/apoptosis, but still retain their htopoI stimulating activity. Therefore, with the model suggested above it can be predicted that in a cell with such mutant p53, DNA damage such as UV irradiation could lead to genomic instability. This could be due to the loss of the regulatory function of p53 in such cells (Cao *et al.* 1997; Sigal and Rotter 2000) and therefore the htopoI mediated recombination events (see Figures 15 and 16) therefore could go out of control and lead to cancer as suggested by Albor and Kulesz-Martin (2000).

5.7 Future perspectives

The results presented above are part of a relatively new advancement in the investigations on the role of p53 and htopoI in cancer development, DNA repair, and apoptosis. At present there are only few publications on this issue, but it is receiving increasing attention in recent reviews (e.g. Albor and Kulesz-Martin (2000) and Kohn *et al.* (2000)). This field opens up new possibilities to learn more about how p53 can function as a tumor suppressor and in mutated form as a promoter of cancer. In this context it could therefore be fruitful to follow up the ideas presented in Figure 15 and 16.

As mentioned previously there are current investigations in our laboratory showing that the recombination model presented in Figure 15 indeed does take place *in vitro*. These investigations will also include studies on the influence of p53 on this observed htopoI-mediated recombination *in vitro*. These are important investigations since they could shed more light on the question whether this observed recombination could be a p53 regulated repair pathway. This pathway, however, can go out of control in cells with “gain of function” p53 mutants and cause genomic instability.

At present, experiments have just started in our laboratory to investigate whether htopoI double cleavage complexes also form *in vivo*. Furthermore, we are trying to verify whether the model suggested in Figure 16 is correct. For both purposes a so called ICT assay will be used which can detect the formation of htopoI cleavage complexes on DNA *in vivo*. The method was first published by Subramanian *et al.* (1998). In this assay cells are treated with UV light or other DNA damaging agents. Subsequently at different time points after introduction of damage the DNA is extracted and the protein denatured in order to trap htopoI cleavage complexes on the DNA. Western-blotting is used to detect the htopoI signal on the DNA. It is hoped that this technique can be used to confirm the hypothetical pathway presented in Figure 16. Cells will be exposed to various intensities of UV-C irradiation and allowed to recover for various periods of time. Using the ICT-assay the occurrence of htopoI cleavage complexes can be detected. At low UV-C dosage it would be expected that the cleavage complexes will disappear in time due to repair. However, at high exposures the scenario

presented in Figure 16 would predict that the cleavage complexes would be persistant. Simultaniously the degree of apotosis will be tested at various timepoints and irradiation intensities. In this way it may be possible to show a correlation between the occurrence of htopoI cleavage complexes and the onset of apoptosis. This would then suggest that the persistancy of htopoI cleavage complexes correlate with apoptosis and thereby support the in Figure 16 presented scenario. By using different cell-lines with various p53 status the possible coordinated action of p53 and htopoI in this putative process could be investigated.

Furthermore it will be investigated if htopoI double cleavage complexes form *in vivo*. The ICT assay will for this pupose be modified slightly. Various cell lines will be exposed to UV-C irradiation and allowed to recover for various periods of time. Subsequently, the cells will be treated with a protein-protein croslinker (dimethyl suberimate) and lyzed. The DNA is blotted and Western-blot analysis performed. If a stronger htopoI signal is evident after treatment with the crosslinker, it would suggest that htopoI double cleavage complexes also form *in vivo*. Such a result would confirm the data of Mao *et al*. (2000). I will try to extend these data by investigating the putative htopoI complexes formation under various conditions. In parallel to this investigation the crosslinking of p53 to htopoI will also be investigated. I especially wish to test under what conditions p53 forms a complex with htopoI. In this respect it is of particular interest to investigate if the interaction with htopoI in a cell line with mutant p53 shows another pattern than it is the case in a cell line with wt p53. In particular this would serve to support the assumption that the association between htopoI and mutant p53 could lead to genomic instability as discussed in chapter 5.6.

6 Summary

HtopoI is an essential enzyme in higher eukaryotes. It has been shown to be involved in transcription, replication as well as in recombination. Recently a substantial number of publications have shown that htopoI can cleave in the vicinity of various DNA lesions and thereby form cleavage complexes. This has been demonstrated *in vitro* as well as *in vivo*. HtopoI cleavage complexes have been shown to be lethal in cancer cells when induced by the chemotherapeutic agent CPT and its analogs. It was therefore highly interesting to investigate which function the induction of htopoI cleavage complexes on damaged DNA may play. In the literature it was suggested that these complexes might be involved in a repair of the DNA lesions. However, in the major part of the literature it seems to become more and more accepted that these cleavage complexes should rather be regarded as bulky lesions. Therefore it has been attempted to find out how such complexes can be repaired in the cell. However, there is at present no clear picture of how this occurs in human cells. The present thesis deals with this topic.

In the present thesis this issue was investigated by the use of a novel suicide substrate. Such substrates trap a htopoI cleavage complex irreversibly at one position on the substrate. Using this substrate with a radioactive label at position 15 from the 3' end it was found that *in vitro* an additional htopoI molecule, could recognize a stable suicide cleavage complex through protein-protein interactions. The distance between the two cleavage sites was estimated to be primarily 13 nts, but with minor cleavage sites at distances of 11, 15, and 17 nts as well. The interaction was found to be fairly unstable since the active site mutant htopoI could compete with wt molecules for the interaction. This complex was called a htopoI double cleavage complex. From the literature it is known that the tumor suppressor protein p53 interacts with and stimulates the activity of htopoI *in vitro* as well as *in vivo*. In the present thesis it was shown, that this interaction was mediated through the core domain of htopoI. This was found by Far-Western analysis where the domains of htopoI were separated through partial digests by subtilisin. *In vitro* the interaction between htopoI and p53 was found to stimulate the formation of double cleavage complexes about six-fold by an as yet

unidentified mechanism. It was however found that p53 most likely stimulates the formation of the double cleavage complexes and not the stability of the complex.

Since htopoI cleavage complexes also form *in vivo* at DNA lesions a model is proposed that is based on the results presented here and data from the literature. It suggests that the double cleavage complex formation could be the first step in a repair of the htopoI cleavage complex through a p53-dependent recombination pathway. This however would only take place if few cleavage complexes have formed. If high amounts of complexes exist this could lead to apoptosis. It is also speculated that this could help to explain the phenotype of genomic instability observed in several p53 mutant cells.

7 Zusammenfassung

HTopoI ist ein essentielles Enzym in höheren Eukaryoten für das es eine Beteiligung an der Transkription, Replikation und Rekombination nachgewiesen worden ist. Zahlreiche aktuelle Publikationen zeigen, dass hTopoI in der Nähe von verschiedenen DNA-Schäden schneidet und so einen kovalenten Schnittkomplex bildet. Dies wird sowohl *in vitro* als auch *in vivo* gefunden. Die von dem Chemotherapeutikum CPT und seinen Derivaten hervorgerufene hTopoI-Schnittkomplexe sind für Tumorzellen letal. Aus diesem Grund war es von Interesse, die Funktion des hTopoI-Schnittkomplexes auf geschädigter DNA zu klären.

In der Literatur wird vorgeschlagen, dass hTopoI-Schnittkomplexe eine Mitwirkung der hTopoI an der DNA-Reparatur repräsentieren könnten. In der Mehrheit der Publikationen setzt sich aber die Meinung durch, dass der hTopoI-Schnittkomplex als ein großes DNA-Schadensereignis gelten kann. Aus diesem Grunde gibt es Bemühungen, die möglichen Wege, die eine Zelle zur Reparatur solcher DNA-Schäden einschlagen kann, aufzuklären. Zur Zeit existiert bezüglich humaner Zellen auf diese Fragestellung noch keine klare Antwort.

Diese Fragestellung wurde in der vorliegenden Arbeit, mittels eines neu konstruierten Suizidsubstrats, untersucht. Solche Substrate „fangen“ hTopoI-Schnittkomplexe irreversibel an einer Position ein. Mit einem an Position 15 vom 3‘ Ende radioaktiv markierten Suizidsubstrat konnte *in vitro* gezeigt werden, dass ein stabiler Schnittkomplex von einem zweiten hTopoI Molekül mittels Protein-Protein Interaktionen erkannt werden kann. Der Abstand zwischen den beiden Schnittstellen wurde überwiegend auf 13, seltener auch auf 11, 15 und 17 Nukleotide gemessen. Die Interaktion war relativ instabil, da eine Mutante des aktiven Zentrums von hTopoI die Interaktion zwischen den beiden Wildtyp Enzymen austitrieren konnte. Dieser Komplex wurde im Folgenden als ein Doppel-Schnittkomplex bezeichnet.

In der Literatur wurde beschrieben, dass das Tumorsuppressor Protein p53 mit hTopoI interagiert und so die Aktivität von HtopoI *in vitro* und *in vivo* stimuliert. In dieser Arbeit konnte gezeigt werden, dass die Interaktion von der core-Domäne der hTopoI vermittelt wird. Dies wurde mit einem Far-Western Verfahren

nachgewiesen, wobei die Domänen der hTopoI durch einem partiellen Verdau mit Subtilisin voneinander getrennt wurden. Die Interaktion zwischen hTopoI und p53 stimulierte mittels eines unbekannten Mechanismus den hTopoI-Doppel-Schnittkomplex *in vitro* um das 6-fache. Dabei stimuliert p53 wahrscheinlich die Bildung eines solchen Komplexes und erhöht nicht dessen Stabilität.

Da hTopoI-Schnittkomplexe, wie bereits erwähnt, auch *in vivo* an geschädigter DNA gefunden werden, wurde unter Berücksichtigung der hier präsentierten Ergebnisse und der Literatur dieses Fachgebiets ein Model entwickelt. Dies beschreibt die Bildung eines Doppel-Schnittkomplexes als ersten Schritt in einer vom hTopoI-Schnittkomplex ausgeführten DNA-Reparatur, die außerdem einen p53-abhängigen Rekombinationsweg umfasst. Dieser Vorgang würde aber nur ablaufen, wenn relativ sich wenige Komplexe bilden; die Bildung vieler Komplexe würde zur Apoptose führen. Abschließend wird spekuliert, dass dies möglicherweise den bei vielen Zellen mit mutiertem p53 auftretenden Phänotyp der genomischen Instabilität erklären könnte.

8 References

Abramova NA, Russell J, Botchan M, Li R. (1997) Interaction between replication protein A and p53 is disrupted after UV damage in a DNA repair-dependent manner. *Proc Natl Acad Sci U S A.* **94**, 7186-91.

Adams JM, Cory S. (1998) The Bcl-2 protein family: arbiters of cell survival. *Science*. **281**, 1322-6.

Albor A and Kulesz-Martin M. (2000). *DNA Alterations in Cancer*. Ehrlich M (ed). Eaton Publishing: Natrick, MA., pp. 409-422.

Albor A, Kaku S, Kulesz-Martin M. (1998) Wild-type and mutant forms of p53 activate human topoisomerase I: a possible mechanism for gain of function in mutants. *Cancer Res*. **58**, 2091-4.

Andersen AH., Bendixen C, and Westergaard O. (1996*)* *DNA Replication in Eukaryotic Cells*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Armand JP, Ducreux M, Mahjoubi M, Abigerges D, Bugat R, Chabot G, Herait P, de Forni M, Rougier P. (1995) CPT-11 (irinotecan) in the treatment of colorectal cancer*. Eur J Cancer.* **31A**,1283-7.

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*. **281**, 1674-7.

Bischoff JR, Friedman PN, Marshak DR, Prives C, and Beach D. (1990) Human p53 is phosphorylated by p60-cdc-2 and cyclin B-cdc-2. *Proc. Natl. Acad. Sci. USA.* **87**, 4766-70.

Bradford MM. (1976) *Anal. Biochem*. **72**, 248-254.

Brady HJ, Salomons GS, Bobeldijk RC, Berns AJ. (1996) T cells from baxalpha transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53 gene product. *EMBO J.* **15**, 1221-30.

Brill SJ, DiNardo S, Voelkel-Meiman K, Sternglanz R. (1987) Need for DNA topoisomerase as a swivel for DNA replication and transcription of ribosomal RNA. *Nature* **326**, 414-16.

Brun G, Vannier P, Scovassi I, Callen JC. (1981) DNA topoisomerase I from mitochondria of Xenopus laevis oocytes. *Eur J Biochem.* **118**, 407-15.

Busk H, Thomsen B, Bonven BJ, Kjeldsen E, Nielsen OF., Westergaard, O. (1987) Preferential relaxation of supercoiled DNA containing a hexadecameric recognition sequence for topoisomerase I. *Nature* **327**, 638-640.

Cao Y, Gao Q, Wazer DE, Band V. (1997) Abrogation of wild-type p53-mediated transactivation is insufficient for mutant p53-induced immortalization of normal human mammary epithelial cells. *Cancer Res.* **57**, 5584-89.

Castora FJ, Kelly WG. (1986) ATP inhibits nuclear and mitochondrial type I topoisomerases from human leukemia cells. *Proc Natl Acad Sci U S A.* 83, 1680-4.

Champoux JJ, McConaughy BL. (1976) Purification and characterization of the DNA untwisting enzyme from rat liver. *Biochemistry.* **15**,4638-42.

Cheng C, Kussie P, Pavletic N, and Shuman S. (1998) Conservation of structure and mechanism between eukaryotic topoisomerase I and site-specific recombinases. Cel**l 92**, 841-850.

Christiansen K and Westergaard O. (1994) Characterization of intra- and intermolecular DNA ligation mediated by eukaryotic topoisomerase I. Role of bipartite DNA interaction in the ligation process. *J. Biol. Chem.* **269,** 721- 729.

Christiansen K and Westergaard O. (1999) Mapping of eukaryotic DNA topoisomerase I catalyzed cleavage without concomitant religation in the vicinity of DNA structural anomalies. *Biochim. Biophys. Acta* **1489**, 249-262.

Christiansen K, Svejstrup ABD, Andersen AH, and Westergaard O. (1993) Eukaryotic topoisomerase I-mediated cleavage requires bipartite DNA interaction. Cleavage of DNA substrates containing strand interruptions implicates a role for topoisomerase I in illegitimate recombination. *J. Biol. Chem.* **268**, 9690-9701.

Creemers GJ, Bolis G, Gore M, Scarfone G, Lacave AJ, Guastalla JP, Despax R, Favalli G, Kreinberg R, Van Belle S, Hudson I, Verweij J, Ten Bokkel Huinink WW. (1996) Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study. *J Clin Oncol.* **14**, 3056-61.

Desai SD, Liu LF, Vazquez-Abad D, and D'Arpa P. (1997) Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J. Biol. Chem.* **272**, 24159-24164.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-21.

Dutta A, Ruppert JM, Aster JC, Winchester E. (1993) Inhibition of DNA replication factor RPA by p53. *Nature.* **365**, 79-82.

Frosina G, Rossi O. (1992) Effect of topoisomerase poisoning by antitumor drugs VM 26, fostriecin and camptothecin on DNA repair replication by mammalian cell extracts. *Carcinogenesis* **13**, 1371-7.

Gai D, Roy R, Wu C, Simmons DT. (2000) Topoisomerase I associates specifically with simian virus 40 large-T-antigen double hexamer-origin complexes. *J Virol.* **74**, 5224-32.

Gaiddon C, Moorthy NC, Prives C. (1999) Ref-1 regulates the transactivation and pro-apoptotic functions of p53 *in vivo*. *EMBO J.* **18**, 5609-21.

Gartenberg MR and Wang JC. (1992) Positive supercoiling of DNA greatly diminishes mRNA synthesis in yeast. *Proc Natl Acad Sci U S A* **89**, 11461-5.

Gilbert CS, Green CM, Lowndes NF. (2001) Budding Yeast Rad9 Is an ATP-Dependent Rad53 Activating Machine. *Mol Cell.* **8**, 129-36.

Gilmour DS, Elgin SC. (1987) Localization of specific topoisomerase I interactions within the transcribed region of active heat shock genes by using the inhibitor camptothecin. *Mol Cell Biol.* **7**, 141-8.

Gobert C, Bracco L, Rossi F, Olivier M, Tazi J, Lavelle F, Larsen AK, Riou JF. (1996) Modulation of DNA topoisomerase I activity by p53. *Biochemistry* **35**, 5778-86.

Gobert C, Skladanowski A, Larsen AK. (1999) The interaction between p53 and DNA topoisomerase I is regulated differently in cells with wild-type and mutant p53. *Proc Natl Acad Sci U S A.* **96**, 10355-60.

Goulaouic H, Roulon T, Flamand O, Grondard L, Lavelle F, Riou JF. (1999) Purification and characterization of human DNA topoisomerase IIIalpha. *Nucleic Acids Res.* **27**, 2443-50.

Green DR and Reed JC. (1998) Mitochondria and apoptosis. *Science*. **281**, 1309-12.

Haupt Y, Barak Y, Oren M. (1996) Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.* **15**, 1596-606.

Haupt Y, Rowan S, Shaulian E, Vousden KH, Oren M. (1995) Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev.* **9**, 2170-83.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R, Harris CC. (1994) Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res.* **22**, 3551-5.

Interthal H, Pouliot JJ, Champoux JJ. (2001) The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. *Proc Natl Acad Sci U S A.* **98**, 12009-14.

Ireton GC, Stewart L, Parker LH, Champoux JJ. (2000) Expression of human topoisomerase I with a partial deletion of the linker region yields monomeric and dimeric enzymes that respond differently to camptothecin. *J. Biol. Chem.* **275**, 25820-25830.

Jaks V, Joers A, Kristjuhan A, Maimets T. (2001) p53 protein accumulation in addition to the transactivation activity is required for p53-dependent cell cycle arrest aftertreatment of cells with camptothecin. *Oncogene.* **20**, 1212-9.

Kato J, Nishimura Y, Imamura R, Niki H, Hiraga S, Suzuki H. (1990) New topoisomerase essential for chromosome segregation in E. coli. *Cell.* **63**, 393-404.

Kato J, Suzuki H, Ikeda H. (1992) Purification and characterization of DNA topoisomerase IV in Escherichia coli. *J Biol Chem.* **267**, 25676-84.

Kingma PS and Osheroff N. (1998) The response of eukaryotic topoisomerases to DNA damage. *Biochim. Biophys. Acta* **1400**, 223-232.

Kjeldsen E, Mollerup S, Thomsen B, Bonven BJ, Bolund L, Westergaard O. (1988) Sequence-dependent effect of camptothecin on human topoisomerase I DNA cleavage. *J. Mol. Biol.* **202**, 333-342.

Knudson CM, Korsmeyer SJ. (1997) Bcl-2 and Bax function independently to regulate cell death. *Nat Genet.* 16, 358-63.

Ko LJ, Prives C (1996) p53: puzzle and paradigm. *Genes Dev.* **10**, 1054-72.

Kohn KW, Sharo RG, Pommier Y. (2000) How do drug-induced topoisomerase I-DNA lesions signal to the molecular interaction network that regulates cell cycle checkpoints, DNA replication, and DNA repair? *Cell Biochem Biophys.* **33**, 175-80.

Krogh S, Mortensen UH, Westergaard O, Bonven BJ. (1991) Eukaryotic topoisomerase I-DNA interaction is stabilized by helix curvature. *Nucleic Acids Res.* **19**, 1235-41.

Kubbutat MH, Jones SN, Vousden KH. (1997) Regulation of p53 stability by Mdm2. *Nature.* **387**, 299-303.

Lanza A, Tornaletti S, Rodolfo C, Scanavini MC, Pedrini AM. (1996) Human DNA topoisomerase I-mediated cleavages stimulated by ultraviolet light-induced DNA damage. *J. Biol. Chem.* **271**, 6978-6986.

Lazarus GM, Henrich JP, Kelly WG, Schmitz SA, Castora FJ. (1987) Purification and characterization of a type I DNA topoisomerase from calf thymus mitochondria. *Biochemistry.* **26**, 6195-203.

Lebel M, Cardiff RD, Leder P. (2001) Tumorigenic effect of nonfunctional p53 or p21 in mice mutant in the Werner syndrome helicase. *Cancer Res.* **61**, 1816-9.

Li G, Tang L, Zhou X, Tron V, Ho V. (1998) Chemotherapy-induced apoptosis in melanoma cells is p53-dependent. *Melanoma Res.* **8**, 17-23.

Li R, Botchan MR. (1993) The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. *Cell.* **73,** 1207-21.

Li R, Waga S, Hannon GJ, Beach D, Stillman B. (1994) Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature.* **371**, 534-7.

Lisby M, Krogh BO, Boege F, Westergaard O, and Knudsen BR. (1998) Camptothecins inhibit the utilization of hydrogen peroxide in the ligation step of topoisomerase I catalysis. B*iochemistry* **37**, 10815-10827.

Liu LF, Miller KG. (1981) Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. *Proc Natl Acad Sci U S A.* **78**, 3487-91.

Loor G, Zhang SJ, Zhang P, Toomey NL, Lee MY. (1997) Identification of DNA replication and cell cycle proteins that interact with PCNA. *Nucleic Acids Res.* **25**, 5041-6.

Mandel M, Higa A. (1970) Calcium-dependent bacteriophage DNA infection. *J Mol Biol.* **53**, 159-62.

Mao Y, Okada S, Chang LS, Muller MT. (2000) p53 dependence of topoisomerase I recruitment *in vivo*. *Cancer Res.* **60**, 4538-43.

Mattern MR, Hofmann GA, McCabe FL, Johnson RK. (1991) Synergistic cell killing by ionizing radiation and topoisomerase I inhibitor topotecan (SK&F 104864). *Cancer Res.* **51**, 5813-6.

Megonigal MD, Fertala J, Bjornsti M-A. (1997) Alterations in the catalytic activity of yeast DNA topoisomerase I result in cell cycle arrest and cell death. *J. Biol. Chem.* **272**, 12801-12808.

Mekeel KL, Tang W, Kachnic LA, Luo CM, DeFrank JS, Powell SN. (1997) Inactivation of p53 results in high rates of homologous recombination. *Oncogene.* **14,** 1847-57.

Merino A, Madden KR, Lane WS, Champoux JJ, Reinberg D. (1993) DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* **365**, 227-32.

Miyashita T and Reed JC. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell.* **80**, 293-9.

Mo YY, Wang C, Beck WT. (2000) A novel nuclear localization signal in human DNA topoisomerase I. *J Biol Chem.* **275**, 41107-13.

Morham SG, Kluckman KD, Voulomanos N, Smithies O. (1996) Targeted disruption of the mouse topoisomerase I gene by camptothecin selection. *Mol Cell Biol.* **16**, 6804-9.

Mosner J, Mummenbrauer T, Bauer C, Sczakiel G, Grosse F, Deppert W. (1995) Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.* **14**, 4442-9.

Murphy KL, Rosen JM (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer.*Oncogene*. **19**, 1045-51.

Neecke H, Lucchini G, Longhese MP. (1999) Cell cycle progression in the presence of irreparable DNA damage is controlled by a Mec1- and Rad53-dependent checkpoint in budding yeast. *EMBO J.* **18**, 4485-97.

Nitiss J, Wang JC (1988) DNA topoisomerase-targeting antitumor drugs can be studied in yeast. *Proc Natl Acad Sci U S A* **85**, 7501-5.

O'Reilly S, Rowinsky EK, Slichenmyer W, Donehower RC, Forastiere AA, Ettinger DS, Chen TL, Sartorius S, Grochow LB. (1996) Phase I and pharmacologic study of topotecan in patients with impaired renal function. *J Clin Oncol*. **14**, 3062-73.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. (1993) Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature.* **362**, 857-60.

Pellicioli A, Lucca C, Liberi G, Marini F, Lopes M, Plevani P, Romano A, Di Fiore PP, Foiani M. (1999) Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. *EMBO J.* **18**, 6561-72.

Piret B, Schoonbroodt S, Piette J. (1999) The ATM protein is required for sustained activation of NF-kappaB following DNA damage*. Oncogene.* **18**, 2261-71.

Polanco C, Gonzalez AI, Dover GA. (2000) Patterns of variation in the intergenic spacers of ribosomal DNA in Drosophila melanogaster support a model for genetic exchanges during X-Y pairing. *Genetics* **155**, 1221-9.

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. (1997) A model for p53-induced apoptosis. *Nature.* **389**, 300-5.

Pommier Y. (1998) Diversity of DNA topoisomerases I and inhibitors. *Biochimie.* **80**, 255-70.

Pouliot JJ, Robertson CA, Nash HA (2001) Pathways for repair of topoisomerase I covalent complexes in Saccharomyces cerevisiae. *Genes Cells* **6**, 677-87.

Pouliot JJ, Yao KC, Robertson CA, and Nash HA. (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. *Science* **286**, 552-555.

Pourquier P and Pommier Y. (2001) Topoisomerase I-mediated DNA damage. *Adv Cancer Res.* **80**, 189-216.

Pourquier P, Takebayashi Y, Urasaki Y, Gioffre C, Kohlhagen G, Pommier Y. (2000) Induction of topoisomerase I cleavage complexes by 1-beta -D-arabinofuranosylcytosine (ara-C) *in vitro* and in ara-C-treated cells. *Proc Natl Acad Sci U S A.* **97**, 1885-90.

Pourquier P, Waltman JL, Urasaki Y, Loktionova NA, Pegg AE, Nitiss JL, Pommier Y (2001) Topoisomerase I-mediated cytotoxicity of N-methyl-N'-nitro-N-nitrosoguanidine: trapping of topoisomerase I by the O6-methylguanine. *Cancer Res.* **61**, 53-8.

Pourquier P, Jensen AD, Gong SS, Pommier Y, Rogler CE. (1999) Human DNA topoisomerase I-mediated cleavage and recombination of duck hepatitis B virus DNA *in vitro*. *Nucleic Acids Res.* **27**, 1919-1925.

Pourquier P, Pilon AA, Kohlhagen G, Mazumder A, Sharma A, Pommier Y. (1997a) Trapping of mammalian topoisomerase I and recombinations induced by damaged DNA containing nicks or gaps. Importance of DNA end phosphorylation and camptothecin effects. *J. Biol. Chem.* **272**, 26441-26447.

Pourquier P, Ueng L-M, Fertala J, Wang D, Park H-J, Essigman JM, Bjornsti M- A, Pommier Y. (1999) Induction of reversible complexes between eukaryotic DNA topoisomerase I and DNA-containing oxidative base damages 7, 8-dihydro-8-oxoguanine and 5-hydroxycytosine. *J. Biol. Chem*. **274**, 8516-8523.

Pourquier P, Ueng L-M, Kohlhagen G, Mazumder A, Gupta M, Kohn KW, Pommier Y. (1997b) Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. *J. Biol. Chem.* **272**, 7792-7796.

Redinbo MR, Stewart L, Champoux JJ, Hol WG. (1999) Structural flexibility in human topoisomerase I revealed in multiple non-isomorphous crystal structures. *J Mol Biol.* **292**, 685-96.

Redinbo MR, Stewart L, Kuhn P, Champoux JJ, Hol WGJ. (1998) Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science* **279**, 1504-1513.

Rose KM, Szopa J, HanF-S, Cheng YC, Richter A, Scheer U. (1988) Association of DNA topoisomerase I and RNA polymerase I: a possible role for topoisomerase I in ribosomal gene transcription. *Chromosoma* **96**, 411-16.

Ryan AJ, Squires S, Strutt HL, Johnson RT. (1991) Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double-strand breaks in replicating DNA. *Nucleic Acids Res.* **19**, 3295-3300.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2. Edition. 1989, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Sastry S and Ross BM. (1998*)* Mechanisms for the processing of a frozen topoisomerase-DNA conjugate by human cell-free extracts. *J. Biol. Chem.* **273**, 9942-9950.

Shao RG, Cao CX, Zhang H, Kohn KW, Wold MS, Pommier Y. (1999) Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. *EMBO J.* **18**, 1397-406.

Shcherbakova OG and Filatov MV. (2000) Camptothecin enhances random integration of transfected DNA into the genome of mammalian cells. *Biochim. Biophys. Acta* **1495**, 1-3.

Shieh SY, Ikeda M, Taya Y, Prives C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell.* **91**, 325-34.

Shinohara A and Ogawa T. (1999) Rad51/RecA protein families and the associated proteins in eukaryotes. *Mutat Res*. **435**, 13-21.

Sigal A and Rotter V. (2000) Oncogenic mutations of the p53 tumor supressor: The demons of the guardian of the genome. *Cancer Res.* **60**, 6788-93.

Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.* **11**, 3471-81.

Slesarev AI, Stetter KO, Lake JA, Gellert M, Krah R, Kozyavkin SA. (1993) DNA topoisomerase V is a relative of eukaryotic topoisomerase I from a hyperthermophilic prokaryote. *Nature.* **364**, 735-7.

Snapka RM. (1986) Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replication. *Mol Cell Biol.* **6**, 4221-7.

Snapka RM, Powelson MA, Strayer JM. (1988) Swiveling and decatenation of replicating simian virus 40 genomes *in vivo*. *Mol. Cell. Biol.* **8**, 515-521.

Squires S, Ryan AJ, Strutt HL, Smith PJ, Johnson RT. (1991) Deoxyguanosine enhances the cytotoxicity of the topoisomerase I inhibitor camptothecin by reducing the repair of double-strand breaks induced in replicating DNA. *J. Cell Sci.* **100**, 883-893.

Squires S, Ryan AJ, Strutt HL, Johnson, R. T. (1993) Hypersensitivity of cockayne's syndrome cells to camptothecin is associated with the generation of abnormally high levels of double-strand breaks in nacscent DNA. *Cancer Res.* **53**, 2012-2019.

Stevnsner T, Mortensen UH, Westergaard O, Bonven BJ. (1989) Interactions between eukaryotic DNA topoisomerase I and a specific binding sequence. *J. Biol. Chem.* **264**, 10110-10113.

Stewart L, Ireton GC, Champoux JJ. (1997) Reconstitution of human topoisomerase I by fragment complementation. *J Mol Biol.* **269**, 355-72.

Stewart AF and Schutz G. (1987) Camptothecin-induced *in vivo* topoisomerase I cleavages in the transcriptionally active tyrosine aminotransferase gene. *Cell* **50**, 109-1117.

Stewart AF, Herrera RE, Nordheim A. (1990) Rapid induction of c-fos transcription reveals quantitative linkage of RNA polymerase II and DNA topoisomerase I enzyme activities. *Cell* **60**, 141-149.

Stewart L, Ireton GC, Champoux JJ. (1996) The domain organization of human topoisomerase I. *J. Biol. Chem.* **271**, 7602-7608.

Stewart L, Redinbo MR, Qiu X, Hol WGJ, Champoux JJ. (1998) A model for the mechanism of human topoisomerase I. *Science* **279**, 1534-1541.

Stürzbecher HW, Brain R, Addison C, Rudge K, Remm M, Grimaldi M, Keenan E, Jenkins JR. (1992) A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization. *Oncogene.* **7**, 1513-23.

Stürzbecher HW, Donzelmann B, Henning W, Knippschild U, Buchhop S.(1996) p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. *EMBO J* **15**, 1992-2002.

Subramanian D, Rosenstein BS, and Muller MT. (1998) Ultraviolet-induced DNA damage stimulates topoisomerase I-DNA complex formation *in vivo*: possible relationship with DNA repair. *Cancer Research* **58**, 976-984.

Svejstrup JQ, Christiansen K, Andersen AH, Lund K, Westergaard O. (1990) Minimal DNA duplex requirements for topoisomerase I-mediated cleavage *in vitro*. *J. Biol. Chem.* **265**, 12529-12535.

ten Bokkel Huinink W, Gore M, Carmichael J, Gordon A, Malfetano J, Hudson I, Broom C, Scarabelli C, Davidson N, Spanczynski M, Bolis G, Malmstrom H, Coleman R, Fields SC, Heron JF. (1997) Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer. *J Clin Oncol.* **15**, 2183-93.

Tsao YP, Wu HY, Liu LF. (1989) Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. *Cell* **56,** 111-8.

Tsao Y-P, Russo A, Nyamuswa G, Silber R, Liu LF. (1993) Interaction between replication forks and topoisomerase I-DNA cleavable complexes: studies in a cell-free SV40 DNA replication system. *Cancer Research* **53**, 5908-5914.

Waga S, Hannon GJ, Beach D, Stillman B. (1994) The p21 inhibitor of cyclin-dependen kinases controls DNA replication by interaction with PCNA. *Nature.* **369**, 574-8.

Wall ME, Wani MC, Cooke CE, Palmer KH, McPhail AT, Slim GA. (1966) *J. Am. Chem. Soc.* **88**, 3888-3890.

Wang H, Zeng X, Oliver P, Le LP, Chen J, Chen L, Zhou W, Agrawal S, Zhang R. (1999) MDM2 oncogene as a target for cancer therapy: An antisense approach. *Int J Oncol.* **15**, 653-60.

Wang Y, Reed M, Wang P, Stenger JE, Mayr G, Anderson ME, Schwedes JF, Tegtmeyer P (1993) *Genes Dev.* **7**, 2575-86.

Wu X, Bayle JH, Olson D, Levine AJ. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7**, 1126-32.

Wu J. and Liu LF. (1997) Processing of topoisomerase I cleavable complexes into DNA damage by transcription. *Nucleic Acids Res.* **25**, 4181-4186.

Yang B, Eshleman JR, Berger NA, Markowitz SD. (1996b) Wild-type p53 protein potentiates cytotoxicity of therapeutic agents in human colon cancer cells. *Clin Cancer Res.* **2**, 1649-57.

Yang L, Wold MS, Li JJ, Kelly, TJ, Liu LF. (1987) Roles of DNA topoisomerases in simian virus 40 DNA replication *in vitro*. *Proc. Natl. Acad. Sci.* **84**, 950-954.

Yang S-W, Burgin AB, Huizenga BN, Robertson CA, Yao KC, and Nash HA. (1996) A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. *Proc. Natl. Acad. Sci.* **93**, 11534-11539.

Zhan Q, Fan S, Bae I, Guillouf C, Liebermann DA, O'Connor PM, Fornace AJ Jr. (1994) Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene.* **9**, 3743-51.

Zhang CX, Chen AD, Gettel NJ, Hsieh TS. (2000) Essential functions of DNA topoisomerase I in Drosophila melanogaster. *Dev Biol.* **222**, 27-40.

Zhu J and Schiestl RH. (1996) Topoisomerase I involvement in illegitimate recombination in Saccharomyces cerevisiae. *Mol. Cell. Biol*., 1805-1812.

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Scientific talk

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Selbständigkeitserklärung

Hiermit versichere ich, die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt und keine anderen als die angegebenen Quellen und hilfsmittel verwendet zu haben.
Ferner versichere ich, daß ich diese Dissertation noch an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

Jena, den 5. November 2001

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