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Functional interactions of DNA topoisomerases with a human replication origin

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ABSTRACT

The mechanism by which certain DNA sequences are chosen to function as DNA replication origins, in metazoan genomes, is currently not understood. However, a comparison of the DNA loci identified as replicators so far points towards a role for DNA topology in this process. DNA topoisomerases are the modulators of DNA topology inside the cell, their activity being essential in all living organisms, nevertheless, the involvement of topoisomerases in metazoan DNA replication is poorly characterized. In this study, the role of topological modulation of the origin DNA was investigated by mapping the interaction of human topoisomerase I and II with a human origin. The lamin B2 DNA replication origin, located on human chromosome 19, interacts with the DNA topoisomerases I and II in a cell cycle modulated fashion. The topoisomerases interact *in vivo* with precise bonds ahead of the start sites of bidirectional replication, within the pre-replicative complex region. Topoisomerase I introduces two single stranded cleavages, on the origin upper and lower strand respectively, in M, early G1 and at late G1 - G1/S border, with topoisomerase II introducing also two single stranded cleavages, on the origin upper and lower strand respectively, in M and middle of G1 phase of the cell cycle. At the origin, topoisomerase II interacts with Orc2p during the assembly of the pre-replicative complex in the middle of G1 phase of the cell cycle. Furthermore, topoisomerase I interacts with Orc2p in late G1 - G1/S border as part of the origin initiation complex and inhibition of topoisomerase I activity abolishes origin firing. The two topoisomerases also compete for the same sites bound by the Orc2 protein, in different moments of the cell cycle. *In vitro*, human recombinant DNA topoisomerase I is able to distinguish the same sites on origin DNA as the *in vivo* cleaved ones, with some additional cuts on the upper strand. In contrast, human recombinant DNA topoisomerase II, alone, cannot introduce the same precise origin cleavages, but as part of an *in vitro* origin specific multi-protein complex it can recognize and cut exactly the same sites as *in vivo*. Thus, the two topoisomerases are members of the replicative complexes with DNA topology playing an important functional role for origin activation.

1. INTRODUCTION

1.1 DNA Topology and replication

In both prokaryotic and eukaryotic organisms DNA replication is the process upon which the cell relies to duplicate its DNA and ensure that at each cell division both daughter cells contain exactly the same genetic information. A process which deals not only with the problem of faithfully copying the entire genome in a considerably short time but also with managing the chromosomes, long polymers which require a higher or lower degree of packaging at any given moment.

When in 1953 James Watson and Francis Crick proposed their model for the structure of DNA in the *Nature* journal they had in fact guessed correctly the mechanism by which the double helix could be duplicated using the specificity of the base pairing. Melting the duplex allowed for semi-conservative DNA replication assuring that the two new DNA molecules were identical (Watson and Crick, 1953a). Nevertheless they also recognized a problem innate to this process: the DNA overwinding (Watson and Crick, 1953b). If DNA had a straight zipper-like structure denaturing the duplex would be easy enough, instead its helical conformation means trying to separate the two strands will create torsional stress outside the unwound region. They proposed the so-called 'speedometer model', where DNA molecules during replication can rotate around their axis much like a car speedometer.

Soon afterwards it was the turn of Max Delbrück to make the correct speculation that, in order to remove the stress of overwinding, DNA could be transiently broken, allowed to rotate around its axis and religated (Delbrück, 1954). Still it was only in 1971 that James Wang first described an enzyme which could catalyze this reaction, the *Escherichia coli* ω protein (later renamed topoisomerase I).

Topoisomerases are enzymes which can relax or supercoil DNA, decatenate chromosomes and catenate or decatenate circular plasmid DNA. All these reactions involve a single or double stranded cleavage of the substrate with concomitant covalent crosslink of the enzyme to the DNA. This feature has made topoisomerases targets for an ever growing collection of antimicrobial and anti-cancer drugs, which inhibit the

religation step of the catalytic cycle of the enzyme. These therapeutic agents act by 'poisoning' the enzyme, transforming it into a DNA-damaging agent due to the persistence of enzyme induced nicks or double stranded breaks.

Today we know that topoisomerases are enzymes essential in any organism, which deal with the topological requirements of not only DNA replication, but also transcription, repair, recombination and many other processes. Nevertheless many of the functions of these enzymes are not well characterized. Although DNA topoisomerases are absolutely required for DNA replication, a detailed characterization of which enzymes are involved in different steps or an investigation of the possible functional overlap between the many types of topoisomerases found in the cell is still to be described. Another interesting question is whether other enzymes might be able to replace DNA topoisomerases during this process.

The effectiveness of the aforementioned antimicrobial and anti-cancer drugs is thought to be replication-dependent: collision of a replication fork with a topoisomerase induced DNA break would generate a double stranded DNA break, a situation called 'replication run-off', which in turn would give rise to illegitimate DNA recombination and ultimately apoptosis if the DNA damage is extensive. A better understanding of the topoisomerase function in DNA replication would also help in developing better topoisomerase poisons.

Initiation of DNA replication is a complex process which requires the assembly of a large pre-replicative complex over a chromosomal region which will be used in S phase as an origin of DNA replication. The choice of DNA fragments which will function as replication origins is a poorly understood process. In metazoans, the few origins which have been characterized in detail exhibit no sequence similarity and, so far, no proteins have been discovered which bind specifically only to origin DNA. There are, however, a series of factors which seem to facilitate the ability of a region to function as an origin, like an open chromatin structure, bent DNA structures, gene promoters close-by, binding sites for sequence specific proteins or asymmetric AT-rich stretches. These clues hint at a role for DNA topology in establishing an origin of DNA replication. A particular DNA conformation could be the key to the high affinity binding of the first pre-replicative complex during G1. Furthermore, throughout the G1 phase of the cell cycle, the pre-

replicative complex undergoes a dynamic re-organisation process while bound on the DNA. Since topoisomerases are able to alter the topology of a DNA region, they could play a determining role in origin specification and regulation.

The present work concentrates on the role of human DNA topoisomerases I and II at a human origin of DNA replication: the lamin B2 origin. Currently the best characterized origin of DNA replication in humans, the lamin B2 offers a unique tool for the investigation of the importance of DNA topology regulation for origin function.

1.2 Reconciling the structure of DNA with its biological functions.

In order to understand how the cell deals with the complex process of faithful genome replication before mitosis, the way in which chromosomes are packed and maintained inside the nucleus should be considered. There is a huge discrepancy between the length of the chromosomes and the size of the nucleus and it is obvious that chromosomes are tightly packed inside the cell. Nevertheless, the possible need at any given moment for the production of a particular protein, in response to environmental or developmental cues (just to give one example of a process requiring chromosome decondensation), signifies the unpacking and transcription of a particular chromatin domain. Hence the need for a tightly regulated yet flexible DNA packing-unpacking regime inside the nucleus. DNA replication, transcription, recombination, mitosis and meiosis are all events which rely heavily on the ability of the cell to manage its genome.

1.2.1 The double helix

The genetic material inside all organisms is the deoxyribonucleic acid – DNA. It is a long double polymer made out of two chains of polynucleotides which run in an anti-parallel sense. A nucleotide is composed of one of four possible heterocyclic bases linked to a sugar which, in turn, is linked to a phosphate group. The polymerization reaction involves the formation of a phosphodiester bond between two sugar groups, with the base as a side chain. Figure 1.2.1.1 illustrates the building blocks of DNA.

The two polynucleotide chains are kept together by hydrogen bonds formed in between the heterocyclic bases, which are positioned, towards the inside of the DNA structure, roughly perpendicular on the backbone (the bases are stacked). Out of the four possible bases found in DNA: adenine, guanine, thymine and cytosine, the hydrogen bonding is possible only between an adenine and a thymine (2 hydrogen bonds per pair) or a guanine and a cytosine (three hydrogen bonds per pair). These normally weak hydrogen bond interactions give the strength of the polynucleotide-polynucleotide interaction by their sheer number. In fact, the longer the piece of DNA the harder it is to denature it, namely to physically detach one polynucleotide chain from its complement.

Because the base-base interaction in DNA is not random, the two polynucleotide polymers which bind each other are complimentary.

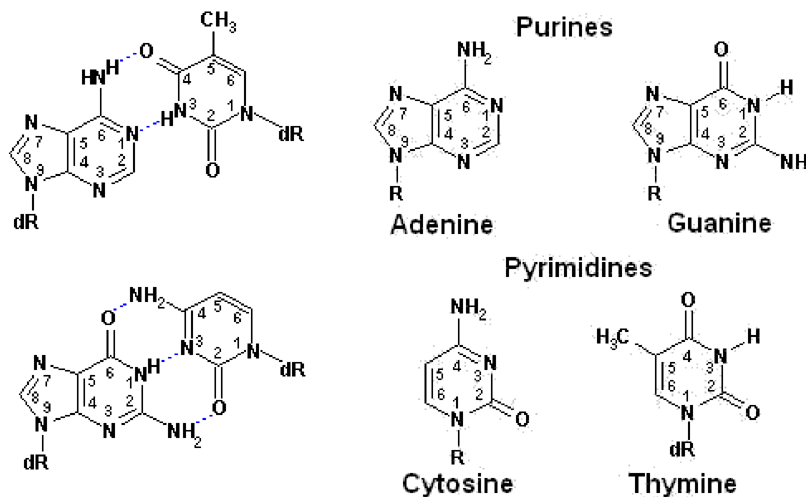


Figure 1.2.1.1 - The four heterocyclic bases found in DNA and the hydrogen bonding formed in double stranded DNA.

The polynucleotide chain has also a polarity stemming from the fact that, when linking the nucleotide groups, two different positions are used to form the bond: the 3' hydroxyl end of one sugar group with the phosphate found at the 5' end of another sugar group. As mentioned previously, the DNA is formed of two polynucleotide chains (also called DNA strands) which are anti-parallel. Furthermore, this double stranded structure was shown to adopt a right-handed helical conformation. This allows for the formation of a hydrophobic core, formed of the hydrogen bonded bases, and a hydrophilic exterior composed of the backbone sugar-phosphate groups. This helical structure derives from the fact that the building blocks of DNA are asymmetric (four possible nucleotides based on the four possible heterocyclic bases) and, as a result, the stacking of the hydrogen bonded bases is slightly staggered.

The overall conformation of the double helix depends not only on the sterical hindrance of the bonds within the molecule but also on the physical properties of the solvent. The classical DNA double helix structure proposed by Watson and Crick corresponds to B-form DNA which is thought to correspond to the majority of the cellular DNA. Nevertheless, two other types of DNA have also been described: the A-form and the Z-form. In the B-form DNA, the helix is right-handed and the bases are stacked almost perpendicularly on the backbone. The helicity of the molecule is not uniform and the DNA has a major groove (wide and deep) and a minor groove (narrow

and deep). The number of base-pairs per helical turn (also called the DNA helical repeat) was determined to be 10.5 and the diameter of the double helix was measured to be ~20Å (Feughelman et al., 1955).

In A-form DNA the helix is still right-handed but the bases are tilted to form angles much bigger or smaller than 90° with the sugar-phosphate backbone. This tilt results from a different conformation of the furanose ring of the sugar. The number of base-pairs per helical turn is 11 and the diameter of the double helix is ~26Å. As a result the major groove is narrow and deep, while the minor groove is wide and shallow (Feughelman et al., 1955).

In Z-form DNA the helix is, surprisingly, left-handed and the bases are alternatively perpendicular and tilted as in B-form and A-form DNA. This alternating pattern corresponds to the alternating conformation of the sugar, which is either C2'-*endo* (B-form) or C3'-*endo* (A-form). As a result the sugar-phosphate backbone has a zigzagged pattern, the major groove is completely flat while the minor groove is narrow and deep. The number of base-pairs per helical turn is 12 but the diameter of the double helix is much smaller, ~18Å. This particular form of DNA is typical of sequences composed of alternating GC tracts and is conditioned by the solvent composition (high-salt apparently promotes the formation of Z-form DNA) (Herbert and Rich, 1996).

The existence of A-form and Z-form DNA *in vivo* is still questioned but there is some evidence that at least in particular situations (e.g. gene activation, supercoiling) DNA may adopt a different conformation (Fairall et al., 1989, Singleton et al., 1982 and Liu et al., 2001) .

Being a flexible polymer DNA can be forced into a more compact or looser double helix, this property referring to the degree of supercoiling of a DNA molecule. In its relaxed state the B-form double helix has a helical repeat of 10.5 nucleotides. If a DNA molecule has this helical repeat for all its helices it is considered to be in its relaxed state (not supercoiled). If parts of the molecule or the whole of it has a higher or lower number of base-pairs per helical turn than this value, it is considered to be, respectively, negatively or positively supercoiled. Negative supercoiling leads eventually to the separation of the two DNA strands, while positive supercoiling tightens the helix and

ultimately makes it coil upon itself. This feature of DNA obviously stems from its helical nature and plays a central role in many aspects of DNA metabolism.

For a circular DNA molecule, or a linear one whose ends cannot rotate freely, a measure of the degree of DNA supercoiling can be obtained from the linking number. This value represents the number of double-helical turns the DNA molecule contains and it describes the topology of DNA. Two DNA molecules which differ only in the linking number are known as topological isomers or topoisomers. Therefore a change in DNA supercoiling implies a change in DNA topology.

All the characteristics here described for the double helix refer to biochemical and biophysical studies of DNA alone in different solutions. This is a good starting point for understanding the nature of DNA but is insufficient for appreciating its behavior in complex situations, as one would expect to find *in vivo*. Many factors affect the DNA conformation inside the cell, from the ionic strength, to the availability of different cations, to the interaction of the DNA with proteins. This is why it is important to understand the properties of the DNA molecule inside the living organism.

1.2.2 Nuclear architecture, chromatin and the scaffold.

In both prokaryotes and eukaryotes the chromosomes are packed within a much smaller space than the length of the extended DNA molecules. Just to give one example in eukaryotes the estimated packing ratio inside the interphase nucleus is that of 250 fold, while during mitosis it reaches 10000 fold. In spite of the almost one hundred years of research into how DNA is packed *in vivo*, the exact process by which this tremendous chromosome condensation is achieved remains poorly understood.

There is a striking difference between the way eukaryotes and prokaryotes deal with chromosomes inside the cell. Prokaryotic organisms often contain just one chromosome, for the most circular, which is much smaller in length compared to the eukaryotic counterpart. Inside the cell there is no specific membrane-bound structure (e.g. the nucleus in eukaryotes) which separates the genome from the rest of the cell, instead the chromosome can be detected by electron microscopy as an irregular dispersed structure called the nucleoid. There is evidence that proteins are associated with these

chromosomes for packing purposes but their exact role is not known. Still, the *E.coli* chromosome (~4.6kb of circular DNA) is compacted roughly 1000 fold in the cell.

Eukaryotes on the other hand, posses an array of chromosomes which are located inside a specific organelle, the nucleus. They also have more than one chromosomal set at least at one stage during their life cycle. The chromosomes are found in a packed state generally referred to as 'chromatin' and this term comprises all the different DNA packing stages from the least condensed one during interphase to the structures seen in mitosis. Chromatin inside the nucleus is usually of two types: euchromatin, a low-packing actively transcribed form, and heterochromatin, tightly packed regions which associate with a gene repression status.

The structure of chromatin is characterized well at its basic level of assembly but the mechanism concerning higher levels of packing remains strictly hypothetical. Eukaryotes have evolved a subset of nuclear proteins which associate specifically with DNA, called histones. These are small, highly basic proteins (rich in lysine and arginine) which are extremely well conserved across the whole eukaryotic domain. The histone family contains five members: H1, H2A, H2B, H3 and H4.

Histones H2A, H2B, H3 and H4 form an octameric subunit comprising 4 heterodimers: two H2A- H2B and two H3-H4. The DNA is wrapped 1.7 times around this octamer in a left-handed manner and the resulting structure is called a nucleosome, the smallest structural subunit of chromatin. Each histone heterodimer binds ~30bp of DNA and a nucleosome was found to contain 147bp of DNA. The nucleosome is a stable structure, with the ability to self assemble *in vitro*. The fifth histone, H1 binds on the outside of the nucleosome, possibly close to the entry/exit point of the DNA double strand, and has a stabilizing role for the DNA-histone octamer interaction. Together the nucleosome and histone H1 bind 167bp of DNA and the resulting structure is called a chromatosome.

The overall reduction in DNA length through the assembly of nucleosomes is only of ~7 fold and therefore highly insufficient for the requirements of the cell. A second step in DNA packaging involves the assembly of the 30nm fiber. Chromatosomes can be assembled onto DNA with a spacing of 0-80bp (this represents the length of the 'linker DNA'). In turn this structure can be further assembled into a solenoid by

compacting the chromatosomes into a tight left handed helix with a diameter of ~30nm. The exact way in which this compaction is achieved is still a matter of debate with several models proposed, nevertheless all models agree on a tight chromatosome assembly with the linker DNA completely folded inside the solenoid as a way of shortening the double helix.

It is thought that the majority of the DNA in interphase is in the solenoid state, with different regions being less packed. Actively transcribed regions still have nucleosomes but histone H1 is lost and higher order condensation is usually absent. In general, chromatin is a dynamic and highly regulated structure, with histone modifications altering the nucleosome stability and performing the so called ‘chromatin remodeling’.

Higher levels of chromatin condensation beyond the 30nm fiber are currently just at the speculation step with a range of scientific data pointing towards the assembly of the solenoid into a set of DNA loops attached to a nuclear scaffold. The chromosome size in mitosis makes it obvious that higher degrees of packing must exist and considering that the assembly of the basic unit of chromatin, the nucleosome, is based on protein-DNA interactions, it is logical to assume that further chromatin compaction is also protein dependent.

In a critical experiment it was shown that if the nuclei are isolated, histones are removed with 2M NaCl (along with the majority of the proteins) and DNA degradation is inhibited by inactivating nucleases, the DNA can be seen by electron microscopy as a halo surrounding the remnants of the nucleus (nucleoid). Surprisingly if this DNA preparation is treated with ethidium bromide, an intercalating dye which unwinds the double helix by inserting itself in between the bases, the DNA filaments become less diffused (McCready et al., 1979). This apparent paradox means that the local unwinding caused by the dye has in turn induced DNA supercoiling. This outcome is typical of circular DNA and is only possible in a linear DNA molecule if the ends are unable to freely rotate. Thus came the hypothesis that DNA inside the nucleus is organized into loops which are anchored to a nuclear structure.

Another experiment involving mitotic chromosomes reached the same conclusion using a completely different approach: using electron microscopy, metaphase

chromosomes depleted of histones show an electron-dense core, termed the nuclear scaffold, to which DNA loops are attached (Paulson and Laemmli, 1977). These loops are ~70μm in size and probably correspond to the next chromatin compaction level after the 30nm fiber. The nature of the nuclear structure which tethers the DNA loops (also called scaffold or matrix) remains an opened question. Problems stemming from the lack of methods for the isolation a 'bona fide' nuclear scaffold, with the high probability of many artifacts being present as a result of protein aggregation, made it impossible to progress further in this direction. If the existence of DNA loops is generally accepted, the nuclear structure isolating these loops is an incognita (Turner, 2001).

However this long struggle to characterize the nuclear scaffold has allowed the discovery of a series of important features of the nuclear architecture like the nuclear lamina. This proteinaceous network is formed of two families: A-type lamins (e.g. lamin A and C) and B-type lamins (e.g. lamin B). These proteins form a meshwork at the inner surface of the nuclear envelope and possibly also throughout the nucleoplasm (Hozak et al., 1995). The lamin family of proteins has not yet been found in lower eukaryotes or in plants but is conserved in metazoans. The role of the lamins is not well understood but protein-protein interactions show that they might bridge the gap between the nuclear envelope and chromatin binding proteins and could also function in mechanotransduction signaling inside the cell. Nevertheless, the nuclear lamina is unlikely to correspond to the nuclear scaffold since the high salt extraction method used to isolate chromosome loops removes the nucleoplasm lamin network.

In parallel, studies of scaffold proteins associated with metaphase chromosomes have led to the discovery of another important family of proteins, the SMC (Stability of Minichromosomes) family. The SMCs are proteins required for the correct chromosome condensation and segregation in yeast and homologues have been found in higher eukaryotes as well. They associate with chromosomes during mitosis and their similarity to motor proteins (e.g. kinesin) points towards a role in chromosome movement. Another component of the metaphase chromosome scaffold is topoisomerase II (Gasser et al., 1986). This enzyme is essential in eukaryotes due to its crucial role in chromosome decatenation and mitosis (Nitiss, 1998). If topoisomerase II is thought to be always

present in the nuclear scaffold, the SMC proteins are more likely to be associated with chromosome during mitosis only.

Out of all the proteins studied so far, topoisomerase II is probably the most likely 'bona fide' scaffold protein. It is considered the most abundant protein inside the scaffold and many searches of DNA scaffold/matrix attachment sites (SAR/MAR) were based on the affinity of topoisomerase II towards a piece of DNA (Razin et al., 1991). Yet, as an exact description of the scaffold (or safe method to isolate it) does not currently exist, all these data should be treated with a fair amount of skepticism.

However, if DNA loops exist, then they must be somehow kept in place, attached to a fairly rigid structure. If so, certain DNA regions will be indeed SARs/MARs. Whether these DNA regions are bound due to their sequence or at random, by simple charge interactions (as in the case of the histones), is not yet clear. The electron microscopy experiments described previously for both interphase and metaphase chromosomes suggest a very strong attachment of SARs/MARs to the scaffold, so why are these interactions so hard to reproduce *in vitro*?

A final piece of evidence in favor of DNA loops comes from chromosome staining techniques. The classic Giemsa chromosome staining, used for many years in karyotyping, reveals a species and chromosome-specific reproducible banding pattern of alternating dark bands (also called G-bands) and light bands. The opposite staining pattern (G-bands unstained and the light bands darkly stained) can be obtained by a reverse staining procedure (the dark colored bands resulting in this case are called R-bands). The crucial feature of these bands is that they can be obtained with minimum manipulation of the cells (as opposed to the lengthy and delicate procedures used to obtain the chromosomes and deplete histones) and that they seem to be reproducible with different staining techniques in both mitotic and meiotic chromosomes. Use of different dyes specific for AT-rich or GC-rich DNA has shown that G-bands correspond to chromatin domains containing very AT-rich regions, while R-bands correspond to chromatin domains containing very GC-rich regions. Based on the observation that G-bands have somewhat of a coiled structure in less condensed chromosomes, a new model was proposed for the organisation of DNA loops. Since AT-rich regions were found to be preferentially associated to nuclear scaffold preparations, the coiled structure of G-bands

would be a consequence of the high-density SARs/MARs nature of this DNA. Further condensation of these small loops would cause supercoiling. Instead the R-bands, GC-rich, would contain a much smaller number of SARs/MARs, resulting in much bigger DNA loops (Saitoh and Laemmli, 1994).

If DNA packing is an essential requirement for managing the eukaryotic chromosome complement, tight regulation of nuclear compartments is also necessary for efficient nuclear function. The organization of the interphase nucleus is not well characterized yet but some key features have emerged.

The most important nuclear compartment, the nucleolus, is the place of ribosomal DNA (rDNA) transcription and ribosome assembly. It contains a fibrillar centre, where both active and inactive rDNA genes are found, surrounded by a dense fibrillar component specialized in processing and assembly of rDNA, in turn surrounded by a granular component responsible for ribosome maturation (Shaw and Jordan, 1995). In humans five different pairs of chromosomes contain rDNA genes and all these loci cluster inside nucleoli.

Clustering seems to be a common mechanism by which different biological functions are regulated inside the nucleus. DNA loci used in the same process tend to cluster and form large foci. It is the case of active DNA replication (Nakamura et al., 1986). In mammalians centromeric heterochromatin tends to cluster in pericentric foci (Haaf and Schmid, 1991). Homology-dependent gene silencing which involves the down-regulation of a gene due to the presence of a transgene might function via clustering and physical interaction of the two genes (Fransz et al., 2002). DNA insulator elements which can block the effect of an enhancer or protect from a silencer are also thought to function by tethering a chromatin domain (possibly a DNA loop) to a particular structure (e.g. nuclear pore) (Blanton et al., 2003).

In addition, whole chromosomes seem to occupy individual territories inside the nucleus and rarely a chromosome will invade the territory of another in interphase. This is not a species specific organization, instead is more likely to reflect chromosome decondensation after mitosis. Still, chromosome movement is restricted inside the cell. One obvious reason is the sheer size of the DNA molecule. Quantitation of DNA movement in yeast has shown a difference between heterochromatic and euchromatic

loci, with the active chromatin domains moving more, presumably due to enzymatic events (Heun et al., 2001).

1.2.3 DNA structure and biological processes.

As mentioned in Section 2.1, supercoiling is a very important DNA feature and all nuclear processes involving DNA manipulations have to overcome the consequences arising from the helical nature of DNA. The chromosome is highly packed inside the cell and due to these topological constraints any form of supercoiling will affect heavily a chromatin domain (isolated by an anchored chromatin loop) without the possibility of distributing the strain over a much longer DNA molecule. Hence the need for topoisomerases, enzymes specialized in altering the topological state of DNA.

The chromatin organization inside the nucleus endows DNA with negative supercoiling. During nucleosome assembly positive supercoiling is accumulated on the DNA and is released by the action of topoisomerases. As a result when the nucleosome disassembles the DNA will be left negatively supercoiled. This ‘stored’ negative supercoiling can be used for different biological processes like DNA transcription and replication.

DNA replication initiation requires the formation of a bubble at the origin. This is obviously a process which requires a large amount of negative supercoiling to be introduced in the DNA in order to obtain the separation of the two strands, with resulting accumulation of positive supercoiling on both sides of the origin. Once the replication fork has been assembled and DNA synthesis has started the fork movement along the DNA will continue to unwind the two parental strands and even more positive supercoiling will accumulate ahead of the fork.

If the replication fork complex is able to rotate around the DNA axis (not a likely possibility due to the sheer size of the replisome), the positive supercoiling will be distributed both ahead and behind the fork. If this positive supercoiling is not removed the DNA will begin to coil upon itself much like an over-twisted rope. At the point of termination of DNA replication where two replication forks converge, due to the accumulated positive supercoiling, the two daughter molecules will be intertwines, i.e.

catenates are formed. Figures 1.2.3.1 and 1.2.3.2 illustrate the type of topological constraints encountered during DNA replication.

Nonetheless, all these considerations about DNA topology during replication are just logical assumptions, what truly happens inside the cell and how the various *in vivo* protein-DNA interactions help or complicate topological problems is unknown.

DNA transcription is another process which shares a few of the topological difficulties of DNA replication. The first step in transcription is also the separation of the two DNA strands in order to allow access of RNA polymerase to its template with resulting positive supercoiling in the surrounding region. Furthermore during active transcription it has been shown that the RNA polymerase (often coupled with ribosomes) does not rotate around the DNA axis and as a result positive supercoiling accumulates ahead of the enzyme, while negative supercoiling will build behind it (Figueroua and Bossi, 1988 and Tsao et al., 1989).

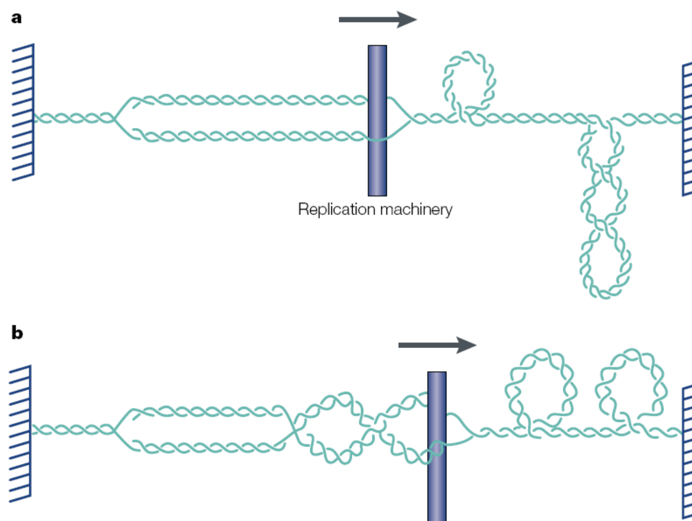


Figure 1.2.3.1 - Replication elongation creates an accumulation of positive supercoiling ahead of the replication fork (a). If the fork complex can rotate around the DNA axis the positive supercoiling will be distributed both ahead and behind the fork, with resulting formation of pre-catenates in the newly replicated region (b) (taken from Wang, 2002).

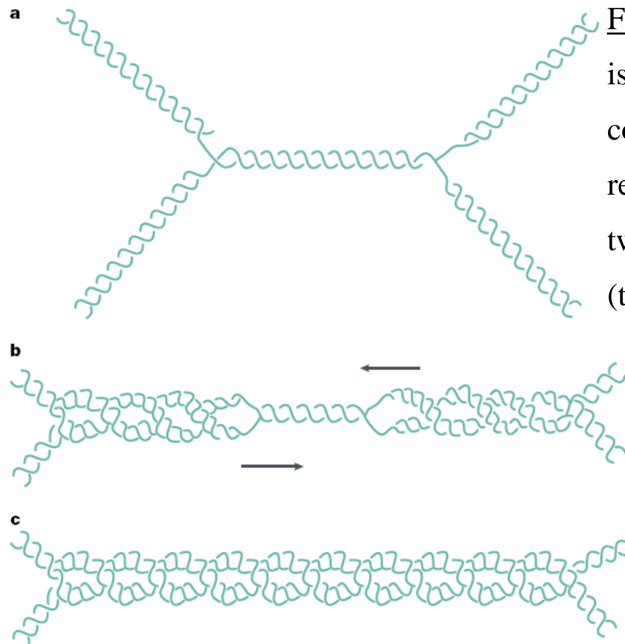


Figure 1.2.3.2 - Replication termination is the point were two replication forks converge. The positive supercoiling resulting from the advancement of the two forks results in catenated DNA (c) (taken from Wang, 2002).

1.3 DNA topoisomerases are modulators of DNA topology.

1.3.1 Classification of DNA topoisomerases.

In the cell, the only enzymes capable of modifying the degree of supercoiling of DNA (the so-called "linking number" of a DNA molecule) are the DNA topoisomerases. All topoisomerases identified so far regulate the degree of supercoiling of DNA via cleavage and formation of a transient covalent phosphor-tyrosine enzyme/DNA adduct. Moreover a particular subset of topoisomerases can also catenate/decatenate knot/unknot plasmid DNA. The catalytic cycle of DNA topoisomerases involves 4 steps:

1. binding of the DNA substrate.
2. cleavage of the DNA with concerted covalent attachment of the enzyme to the 3' or 5' DNA end via a nucleophilic attach of an enzyme tyrosine residue on a DNA phosphate.
3. rotation and/or strand passage of the substrate in order to remove/introduce positive or negative supercoiling or catenate/decatenate, knot/unknot DNA molecules.

4. religation of the gap via a reversal of the transesterification reaction and release of the DNA substrate.

An example of a topoisomerase cleavage reaction is given in Figure 1.3.1.1. Under physiological conditions the cleavage, rotation and religation are extremely quick and the covalent enzyme-DNA complex (also called the “cleavable complex”) is only a short lived intermediate. Nevertheless under specific conditions the equilibrium of the cleavage reaction can be shifted towards the cleavable complex state and the enzyme can be physically trapped on the DNA. Due to this particular property, the topoisomerases have long been recognized as potential DNA damaging agents and they are targets for a wide range of drugs. Currently topoisomerase inhibitors are used as antimicrobial and antitumor drugs.

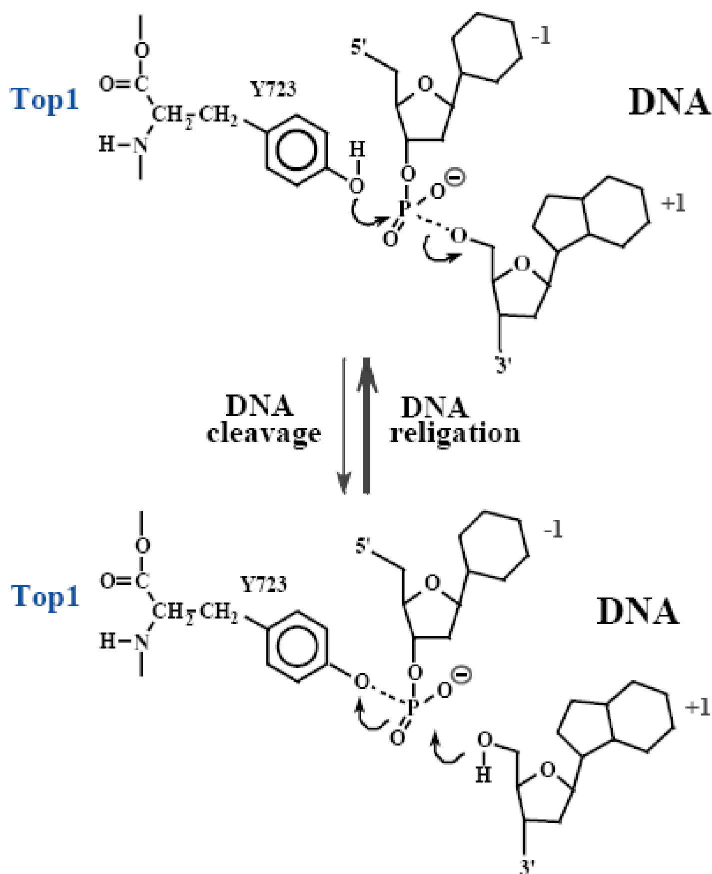


Figure 1.3.1.1 - Eukaryotic topoisomerase I is a type IB enzyme and becomes covalently attached to the 3’ end of its DNA substrate during the catalytic cycle.

Topoisomerases are usually specialized in one particular topological reaction (e.g. removing positive supercoiling as opposed to introducing it) and as a result it is common

to find more than one type of enzyme in each organism. At least one gene coding for a DNA topoisomerase has been identified in all organisms studied and it is generally assumed that topoisomerases are essential enzymes for cell growth due to their unique DNA decatenation activity (Champoux, 2001 and Wang, 2002). Based on the type of DNA cut they introduce, topoisomerases can fall into two classes: type I or type II. Type I enzymes can bind single or double stranded DNA and introduce single-stranded nicks, while type II cleave both DNA strands of a double helix. Further division within type I and type II into subtype A and subtype B is based on structural and catalytical properties. Table 3.1.1 illustrates the topoisomerase classification and enzyme properties.

Table 3.1.1 The topoisomerase classification.

Domain of life	Type	Structure	Notes
Topoisomerase Class **IA** The enzyme cleaves just one strand of the DNA substrate with concerted covalent attachment to the 5' end of the cut. The DNA substrate is always negatively supercoiled with the enzyme requiring an exposed single-stranded region for catalytical activity. The relaxation reaction does not go to completion (i.e. the DNA will still be negatively supercoiled at the end of the reaction). The enzyme is also able to catenate/decatenate and knot/unknot single stranded DNA circles or nicked duplex circles. The catalytical activity is dependent on Mg^{2+} ions.			
Archaea	Reverse DNA gyrase	Monomer	Reverse gyrase is the only enzyme capable of introducing positive supercoiling into its DNA substrate. All archaeal hyperthermophiles studied so far seem to have an active reverse gyrase, most probably important in neutralizing the effects of growth at high temperature.
	Topoisomerase III	Monomer	Relative sequence homology to the bacterial topo III. Found in

			few organisms so far.
Bacteria	Topoisomerase I	Monomer	Seems to be present in all bacterial genomes fully sequenced so far.
	Topoisomerase III	Monomer	Some bacteria lack this type of enzyme.
	Topoisomerase IIIβ	Monomer	Identified only in *Bacillus cereus*, has distinct featured from all other type IA enzymes.
	Reverse DNA gyrase	Monomer	This enzyme has only been reported in hyperthermophilic bacteria.
		Heterodimer	The only reverse gyrase with a heterodimeric structure was identified in the bacterial hyperthermophile *Methanopyrus kandleri*.
Eukarya Yeasts	Topoisomerase III	Monomer	*Saccharomyces cerevisiae* topo III is dispensable for cell growth while *Schizosaccharomyces pombe* topo III is essential.
Flies	Topoisomerase IIIα	Monomer	Essential during development in *Drosophila melanogaster.*
	Topoisomerase IIIβ	Monomer	Not essential in *D. melanogaster.*
Plants	None reported so far.		
Mammals	Topoisomerase IIIα	Monomer	Essential for embryogenesis in *Mus musculus*.
	Topoisomerase IIIβ	Monomer	Not essential in *M. musculus*.

<table>
<tr><td colspan="4">Topoisomerase Class IB
The enzyme cleaves just one strand of the DNA substrate with concerted covalent attachment to the 3’ end of the cut. The enzyme can relax positively or negatively supercoiled DNA substrates and the relaxation reaction goes to completion (i.e. the DNA will be in a relaxed state at the end of the reaction). The catalytical activity is not dependent on metal ions.</td></tr>
<tr><td>Archaea</td><td>None reported so far.</td><td></td><td></td></tr>
<tr><td>Bacteria</td><td>Topoisomerase V</td><td>Monomer</td><td>Present only in Methanopyrus kandleri and structurally distinct from all other topoisomerases.</td></tr>
<tr><td>Eukarya

Yeasts</td><td>Topoisomerase I</td><td>Monomer</td><td>Not essential for growth.</td></tr>
<tr><td>Flies</td><td>Topoisomerase I</td><td>Monomer</td><td>Essential in D. melanogaster.</td></tr>
<tr><td>Plants</td><td>Topoisomerase I</td><td>Monomer</td><td>Essential in Arabidopsis thaliana. The Pisum sativum enzyme also exhibits reverse gyrase activity.</td></tr>
<tr><td>Mammals</td><td>Topoisomerase I</td><td>Monomer</td><td>Essential in M. musculus.</td></tr>
<tr><td colspan="4">Topoisomerase Class IIA
The enzyme cleaves both strands of the DNA substrate (with a 4bp stagger) with concerted covalent attachment to the two 5’ ends of the cut. The enzyme has the ability to bind and pass another DNA double stranded region or molecule through the gap resulting from the DNA cleavage. This process results in DNA relaxation, knotting/unknotting or catenation/decatenation events. The catalytical activity is dependent on Mg^{2+} ions and ATP. However, it should be noted that different members of this class have very different efficiencies for relaxation, knotting/unknotting or catenation/decatenation reactions.</td></tr>
<tr><td>Archaea</td><td>DNA gyrase</td><td>Heterotetramer</td><td>Not very common in archaebacteria.</td></tr>
<tr><td rowspan="2">Bacteria</td><td>DNA gyrase</td><td>Heterotetramer</td><td>Found in all bacteria studied so far.</td></tr>
<tr><td>Topoisomerase IV</td><td>Heterotetramer</td><td>Not found in all bacteria.</td></tr>
</table>

Eukarya Yeasts	Topoisomerase II	Homodimer	Essential for growth.
Flies	Topoisomerase II	Homodimer	Essential in *D. melanogaster*.
Plants	Topoisomerase II	Homodimer	
Mammals	Topoisomerase IIα	Homodimer	In *M. musculus* essential for early embryogenesis.
	Topoisomerase IIβ	Homodimer	In *M. musculus* essential for late embryogenesis.
Topoisomerase Class **IIB** The only member of this class of topoisomerases, topoisomerase VI, has the same catalytical properties of class IIA enzymes but shows very little sequence similarity with them. Topo VI is a A_2B_2 heterotetramer and the A subunit is homologous to the yeast Spo11 protein involved in meiotic recombination.			
Archaea	Topoisomerase VI	Heterotetramer	Found in all archaebacteria studies so far.
Bacteria	None reported so far.		
Eukarya Yeasts	None reported so far.		
Flies	None reported so far.		
Plants	Topoisomerase VI	Heterotetramer	Essential for endoreduplication in *A. thaliana*.
Mammals	None reported so far.		

As mentioned before DNA topoisomerases are encoded in the genome of every genetic organism. Because topoisomerases participate in every aspect of DNA metabolism it is not surprising that during evolution they have acquired specialization for a particular topological reaction. At the same time prokaryotic and eukaryotic

topoisomerases seem to have evolved divergently and their characteristics show less similarities between these two taxonomical domains than expected.

In prokaryotes the first topoisomerase discovered was the *E.coli* ω protein, now known as topoisomerase I (Wang, 1971). This is a 97 kDa monomer which exhibits a type IA catalytical reaction. The eukaryotic topoisomerase I (which has a reasonably conserved sequence from yeast to man) is a ~100 kDa monomer with a type IB catalysis. It should be noted that these enzymes were both called topoisomerase I assuming that they were orthologs and performed the same function within the cell. Nevertheless they have been shown not only to have no structural or sequence similarities but also to differ in the range of topological actions they can perform. Just to give an example eukaryotic topo I can relax both positive and negative supercoiling while *E.coli* topo I cannot relax positive supercoils.

Type I topoisomerases act as monomers, contain only one catalytic tyrosine residues (as they introduce single stranded breaks) and do not normally require an energy cofactor (i.e. ATP). Type IA enzymes can relax only negative supercoils, require a single-stranded stretch in the DNA substrate, become covalently attached to the 5' end of the DNA cut and cannot function in the absence of Mg^{2+} ions. Members of this class include prokaryotic topoisomerase I, prokaryotic and eukaryotic topoisomerase III and prokaryotic reverse gyrase. Apart from removing negative supercoiling, type IA enzymes can also perform knotting/unknotting and catenation/decatenation reactions. James Wang has postulated in his latest review about topoisomerases that "the minimal requirement for DNA topoisomerases in living organisms is probably one type IA and one type II enzyme" (Wang, 2002).

E.coli topo I is efficient in removing negative supercoils but inefficient as a DNA decatenase. It plays a critical role in maintaining the optimum DNA supercoiling for all cellular functions and its role is particularly important during transcription (Drolet et al., 1995). A topo I enzyme seems to be present in all bacteria investigated so far, however this enzyme is absent from archaebacteria (Champoux, 2001). *E.coli* topo III is related to the *E.coli* topo I and able to perform the same relaxation reaction in the presence of negative supercoiling. Nonetheless, it requires that the DNA substrate should be hypernegatively supercoiled and it is much more efficient in catenation/decatenation

reactions than topo I (DiGate and Marians, 1988 and Hiasa et al., 1994). Topo III is thought to be involved in the elongation and termination/decatenation events of DNA replication even though these roles would overlap with those of the *E.coli* type II topoisomerases (Hiasa and Marians, 1994 (b) and Hiasa et al., 1994). More evidence for this apparent functional redundancy comes from the fact that a number of other bacteria lack a topo III homologue (Champoux, 2001).

Interestingly, in the bacterium *Bacillus cereus* two topo III enzymes have been identified, designated topo IIIα and topo IIIβ (Li et al., 2006). The *B.cereus* topo IIIα has the same catalytical properties as the *E.coli* topo III, while topo IIIβ can only partially relax negative supercoils and lacks decatenase activity. Furthermore, topo IIIβ cannot compensate for the lack of *E.coli* topo III *in vivo* (Li et al., 2006). The *B.cereus* topo IIIβ seems to be distinct from all other type IA topoisomerases and it remains to be seen if it performs an essential function *in vivo*.

An archaeal topo III enzyme, related to the bacterial topo III, has also been reported for the hyperthermophilic archaeon *Sulfolobus solfataricus* (Dai et al., 2003).

Eukaryotic topoisomerases III have been described in yeast, *Drosophila*, mouse and humans (Wallis et al., 1989, Wilson et al., 2000, Seki et al., 1998 and Hanai et al., 1996). They have all the main features of the bacterial topo III, they can relax only negative supercoiling, need a single stranded stretch of DNA and Mg^{2+} ions in order to function. Even though *Saccharomyces cerevisiae* topo III is not absolutely required for cell growth, mutants for the gene exhibit slow growth, increased mitotic recombination and a defect in meiosis resulting in failed sporulation (Gangloff et al., 1999). Instead, deletion of the topo III enzyme is lethal in the yeast *Schizosaccharomyces pombe* and its presence is essential for accurate nuclear division (Maftahi et al., 1999 and Goodwin et al., 1999). Higher eukaryotes have two topo III isoforms: α and β. In *Drosophila* topo IIIα is essential during development, while topo IIIβ appears to be dispensable for viability in spite of having a peak of expression during the first 6h of embryogenesis (Wilson et al., 2000 and Plank et al., 2005). Knockout mice for topo IIIα die early during embryogenesis, while topo IIIβ knockouts have a reduced life-span (Li and Wang, 1998 and Kwan and Wang, 2001). Topo III seems to interact with DNA helicases of the RecQ family at least in yeast and humans and possibly functions in the same processes which

require these helicases (Bennett et al., 2000, Wu et al., 2000 and Shimamoto et al., 2000). Topo IIIα together with BLM, Bloom's syndrome helicase, seem to be involved in sister chromatid dissolution in mitosis (Seki et al., 2006). Interestingly, *E.coli* topo III also interacts with *E.coli* RecQ helicase (Harmon et al., 1999).

Reverse gyrase is an enzyme found only in hyperthermophilic organisms including archaea and eubacteria and is the only type IA enzyme which requires ATP as a cofactor. The role of reverse gyrase is that of introducing positive supercoiling in a DNA substrate (Declais et al., 2001). Considering the special type of environment in which hyperthermophilic organisms grow in, it has been postulated that this enzyme helps protect the genome from the DNA denaturing effect of the extreme temperature (Charbonnier and Forterre, 1994).

It is worth mentioning that genes coding for type IA topoisomerases were also found in several plasmids from Gram-positive and Gram-negative bacteria (Champoux, 2001). Interestingly in one case it has been reported that initiation of plasmid replication by DNA polymerase I creates a substrate specific for a plasmid encoded topoisomerase (Bidnenko et al., 1998).

If type IA enzymes are found in every organism, type IB are typical of eukaryotes with just one exception, topoisomerase V, which is present in a hyperthermophilic bacterium, *Methanopyrus kandleri*.

Type IB topoisomerases can relax both positive and negative supercoiling, become covalently attached to the 3' end of the DNA cut and do not require any type of metal ion or energy cofactor (Pommier et al., 1998). Their action is inhibited by single-stranded DNA in contrast to type IA enzymes (Been and Champoux, 1984). In contrast to type IA enzymes, they are not capable of catenation/decatenation or knotting/unknotting reactions. Eukaryotic topoisomerase I, the vaccinia virus topoisomerase and *Methanopyrus kandleri* topoisomerase V are type IB enzymes.

A topo I has been reported in all eukaryotic model genetic organisms including yeasts, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, wheat germ, mouse and human (Goto and Wang, 1985, Uemura et al., 1987a, Kim et al., 1996, Hsieh et al 1992, Pandit et al., 1996, Dynan et al., 1981, Koiwai et al., 1993 and Kunze et al., 1989). In the yeasts *S.cerevisiae* and *S.pombe* topo I is not essential for growth, while in

higher eukaryotes, like *Drosophila* and mouse, absence of this enzyme is lethal (Thrash et al., 1984, Uemura and Yanagida, 1984, Lee et al., 1993 and Morham et al., 1996). Topo I is involved in many aspects of DNA metabolism including DNA replication, transcription, damage repair and possibly chromosome condensation in mitosis (Pommier et al., 1998). Along with the nuclear topoisomerase I, a mitochondrial topo I was identified in *Xenopus*, calf thymus and humans (Brun et al., 1981, Lazarus et al., 1987 and Zhang et al., 2001).

Unexpectedly, a topo I enzyme isolated from *Pisum sativum* (pea) showed, besides the typical type IB catalytic reaction, the ability to introduce positive supercoiling in the presence of Mg^{2+} ions, a property of reverse gyrases found only in hyperthermophiles (Reddy et al., 1998). This is a unique feature, not detected so far in any other eukaryotic topo I.

Until recently it was thought that type IB enzymes were exclusive to eukaryotes, considering the complete absence of these enzymes in classic model prokaryotes (e.g. *E.coli*, *Bacillus subtilis*). In spite of this, the bacterium *M.kandleri*, a hyperthermophilic methanogen, contains in its complement of topoisomerases a type IB enzyme, named topoisomerase V (Slesarev et al., 1993). Considering that topo V is dissimilar structurally from all other topoisomerases there it has been proposed that it should be classified apart from other enzymes as type IC (Forterre, 2006). Topo V can relax both positive and negative supercoiling and becomes covalently attached to the 3' end of the DNA cut, still, the exact role of this enzyme is not currently understood, since other hyperthermophilic bacteria do not seem to require one. *M.kandleri* is also unusual in the fact that it has a novel heterodimeric reverse gyrase, while all the previously discovered reverse gyrases are monomeric (Krah et al., 1996).

The vaccinia virus (poxvirus) is one of the few viruses known to encode its own topoisomerase (Bauer et al., 1977). Coded by the E6 gene, this topoisomerase is a lot smaller compared to the eukaryotic type IB enzymes and was shown to be essential for viral proliferation in cell culture (Shchelkunov et al., 1993).

Type II topoisomerases differ from type I enzymes in that, when bound to the double helix, they introduce double stranded cleavages (usually four base pair staggered). They can be homodimers or heterotetramers, contain two catalytic tyrosine residues (as

they introduce double stranded breaks) and become covalently attached to the 5' end of the DNA break. They can also catenate or decatenate (knot or unknot) DNA molecules, by passing an intact DNA molecule through the double stranded break created, this strand passing activity involving ATP hydrolysis. All organisms seem to require a type II topoisomerase since these are the only type of enzymes capable of decatenating double stranded DNA molecules (i.e. newly replicated DNA) (Champoux, 2001 and Wang, 2002). Type II enzymes are also divided into two subcategories, A and B, a distinction based on structural characteristics. The recent discovery of an atypical topoisomerase in the genome of the archebacterium *Sulfolobus shibatae*, named topoisomerase VI, has prompted the classification of this type II enzyme apart from all others, as type IIB (Bergerat et al., 1997).

The type IIA enzymes are DNA gyrase and topoisomerase IV, found in eubacteria and topoisomerase II, found only in eukaryotes. DNA gyrase was first identified in *E.coli* as an enzyme capable of transforming a relaxed DNA molecule into a negative supercoiled one (Gellert et al., 1976). Indeed DNA gyrase, a heterotetramer, is the only enzyme known so far able to introduce negative supercoiling. This property plays an essential role in the initiation of DNA replication at the *E.coli* oriC (Fairweather et al., 1980, Filutowicz, 1980 and Baker and Kornberg, 1988). DNA gyrase can also relax negatively or positively supercoiled DNA but in a much more inefficient manner, while its decatenation activity is almost absent *in vivo* (Levine et al., 1998). In the cell this enzyme has a role in maintaining the overall DNA superhelicity, facilitating replication elongation and transcription and possibly decatenation of chromosomes after DNA replication (Levine et al., 1998). Even though DNA gyrases are found predominantly in bacteria, there have been several reports of archaebacteria which also posses this type of enzyme (Gadelle et al., 2003). Recently, a genome analysis in the model plant *A.thaliana* revealed four putative gyrase genes and knockout studies show that all four genes are essential for plant development (Wall et al., 2004). Furthermore, cloning of the *A.thaliana* putative gyrase genes in *E.coli* gyrase temperature-sensitive strains lead to phenotype rescue (Wall et al., 2004). However, detailed biochemical analysis of the gene products is still missing for the time being.

Topoisomerase IV is the other main type II enzyme found in eubacteria. Topo IV is also a heterotetramer and a potent decatenase responsible for decatenating daughter chromosomes at the end of DNA replication. Like DNA gyrase, this enzyme can also relax negatively or positively supercoiled DNA but at a much slower rate than decatenation and its roles in the cell include maintaining the overall DNA superhelicity, facilitating replication elongation and transcription (Champoux, 2001).

Topoisomerase II acts as a homodimer and it is the only type II topoisomerase found in eukaryotes (except for the newly discovered topo VI in plants). When the work concerning topoisomerases was just at the beginning, the identification of a type II enzyme in eukaryotes led to the logical assumption that its topological activity would be very similar to the prokaryotic type II enzymes. As it was discovered later, eukaryotic topoisomerase II, in spite of being homologous to both DNA gyrase and topo IV, has a behavior more similar to topo IV.

Topo II can relax both negative and positive supercoiled DNA but it cannot perform the opposite reaction of introducing either type of supercoiling. This characteristic leaves the puzzling question of whether there is a yet undiscovered enzyme that can function as a gyrase or reverse gyrase in eukaryotes or if during evolution eukaryotes have lost the need for this topological reaction. In addition to the DNA relaxation activity, topo II is able to decatenate DNA, an enzymatic reaction essential in de-tangling sister chromatids at the end of DNA replication. Topo II action requires ATP and Mg^{2+} ions (Champoux, 2001). The topo II decatenation reaction is illustrated in figure 1.3.1.2.

In the yeasts *S.cerevisiae* and *S.pombe* topo II is absolutely required for the decatenation of daughter chromosomes and chromosome condensation before cell division (diNardo et al., 1984, Holm et al., 1985 and Uemura et al., 1987b). In *Drosophila* topo II is essential for anaphase sister chromatid separation and does have a partial role in chromosome condensation (Chang et al., 2003). Higher eukaryotes (with the exception of *Drosophila*) have two isoforms of this enzyme: topo IIα and topo IIβ (Champoux, 2001). The two isozymes have a different expression pattern: topo IIα is preferentially expressed in proliferating cells while topo IIβ apparently has the same level of expression in all cell types (Hsiang et al., 1988 and Turley et al., 1997). Topo IIα

seems to correspond to the yeast and Drosophila topo II, being required for chromosome decatenation and condensation, while topo IIβ is not required for cell division (Nitiss, 1998). Knockout mice for topo IIα die at the 4- or 8-cell stage of embryonic development, while knockout mice for the topo IIβ isoform die shortly before birth and show abnormal neural development (Akimitsu et al., 2003a and Yang et al., 2000). So in spite of the fact that topo IIβ is not essential for cell growth, it plays an important role during development. Furthermore, both murine embryos and HeLa cells lacking topo IIα, show an abnormal nuclear structure and go into apoptosis (Akimitsu et al., 2003b).

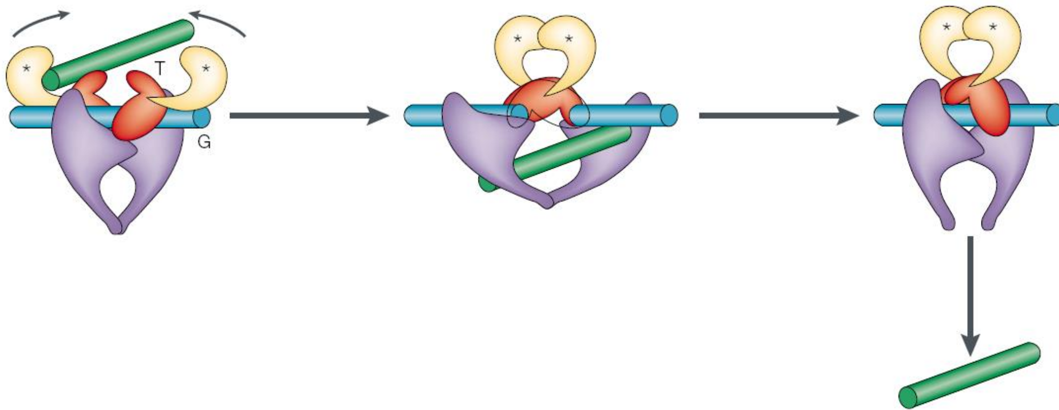


Figure 1.3.1.2 - Topoisomerase II can pass a second DNA double helix through a induced DNA double strand break. The green DNA segment (T) represents the intact double helix, while the blue DNA segment (G) represents the cleaved double helix. The asterisks represent ATP binding sites (taken from Wang, 2002).

Apart from these critical functions, topo IIα and topo IIβ probably share with topo I a functional redundancy, since both type of topoisomerases seem to have a role in the elongation step of DNA replication, DNA transcription and genome stability (Nitiss, 1998).

Aside from the topoisomerases described so far, a type IIA enzyme was also found in the genome of three viruses: bacteriophage T4, African swine fever virus and paramecium *Bursaria chlorella* virus (Liu et al., 1979, Garcia-Beato et al., 1992 and Lavrukhin et al., 2000).

As mentioned before, the discovery of a novel type II topoisomerase in Archaea has provoked the split of type II enzyme into two subcategories: A and B. This was due

to the fact that the *S.shibatae* topoisomerase VI, a heterotetrameric A_2B_2 enzyme, was not homologous to any other topoisomerase, but one of its subunits (subunit A) showed homology to Spo11p, an enzyme involved in meiotic recombination (Malone et al., 1991 and Bergerat et al., 1997). It seems that Spo11 cleaves the DNA in order to produce the double stranded break required for meiotic recombination initiation. Furthermore it was shown that Spo11 is covalently attached to the DNA substrate much like a regular topoisomerase (Keeney et al., 1997). It remains to be seen if indeed Spo11 and its homologues in higher eukaryotes can function indeed as topoisomerases.

Topoisomerase VI is also found in plants, as revealed from the sequencing of the *A.thaliana* genome. Since plants are normally polyploid it is thought that this enzyme plays a role in endoreduplication of the genome, a common event in plant development (Sugimoto-Shirasu et al., 2002).

1.3.2 Human DNA topoisomerases and their roles in the cell.

The human topoisomerase complement contains 6 enzymes: a nuclear topoisomerase I (topo I), a mitochondrial topoisomerase I (topo Imt), two topoisomerases II, α and β, and two topoisomerases III, α and β (Kunze et al., 1989, Zhang et al., 2001, Tsai-Pflugfelder et al., 1988, Austin et al., 1993, Hanai et al., 1996 and Ng et al., 1999). Table 1.3.2.1 presents the human topoisomerases and their key features.

Table 1.3.2.1 Human nuclear topoisomerases.

Name (Class)	Catalytical activity	Homologues	Function
Topo I IB	Cleaves just one DNA strand and becomes covalently crosslinked to the 3’ end of the DNA cut. Relaxes both	All eukaryotic topo I.	• DNA transcription • DNA repair • Apoptosis • ? DNA replication

	positive and negative supercoiling. Cannot catenate/decatenate, knot/unknot DNA molecules. Cannot introduce negative or positive supercoiling.		elongation.
Topo IIα IIA	Cleaves both DNA strands with a 4bp stagger and becomes covalently crosslinked to the 5' end of the DNA cut. Relaxes both positive and negative supercoils. Catenates/decatenates, knots/unknots DNA molecules. Cannot introduce negative or positive supercoiling.	Yeasts and *Drosophila* topo II and topo IIα from higher eukaryotes.	• Nuclear scaffold. • Chromosome condensation. • Chromosome segregation. • DNA replication termination (decatenation). • ? DNA replication elongation.
Topo IIβ IIA	Cleaves both DNA strands with a 4bp stagger and becomes covalently crosslinked to the 5' end of the DNA cut. Relaxes both positive and negative supercoils. Catenates/decatenates, knots/unknots DNA molecules. Cannot introduce negative or positive supercoiling.	Yeasts and *Drosophila* topo II and topo IIβ from higher eukaryotes.	• Signal dependent transcriptional activation • ? Mitosis. • ? DNA replication.

Topo IIIα IA	Cleaves just one DNA strand and becomes covalently crosslinked to the 5' end of the DNA cut. Relaxes only negative supercoiling and requires a region of single stranded DNA. The catenation/decatenation, knotting/unknotting activity has not been tested yet. Cannot introduce negative or positive supercoiling.	Prokaryotic and yeast topo III, *Drosophila* and mouse topo IIIα.	• ?
Topo IIIβ IA	No biochemical characterization is available yet.	Prokaryotic and yeast topo III, *Drosophila* and mouse topo IIIβ.	• ?

The mitochondrial topoisomerase I is 601 amino acids (aa) long and highly homologous to the nuclear topoisomerase I. Topo Imt is a type IB topoisomerase and requires Ca^{2+} or Mg^{2+} and alkaline pH for optimum activity (Zhang et al., 2001).

Human nuclear topo I is 765aa long, has a predicted molecular weight of 91kDa and presents four structural domains: a N-terminal domain, non-essential for catalytical activity but involved in protein-protein interactions, a highly-conserved core domain which is in contact with DNA, a positively charged linker domain and a conserved C-terminal domain (Stewart et al., 1996 and Champoux, 2001). It has a type IB catalysis with the active tyrosine (Tyr^{732}) present in the C-terminal domain. As all type IB

enzymes it can relax both positively and negatively supercoiled DNA and shows higher affinity towards these two substrates than towards relaxed DNA (Zechiedrich and Osheroff, 1990 and Madden et al., 1995).

In vivo topo I activity is thought to be regulated by a series of post-translational modifications including phosphorylation (Cardellini and Durban, 1993). p53 can stimulate the topo I DNA relaxing activity when bound to it (Albor et al., 1998). p53 can also stimulate homologous recombination (HR) in a manner strictly depending on topoisomerase I, while binding of poly[adenosine diphosphate (ADP)–ribose] polymerase–1 (PARP-1), an enzyme involved in DNA damage detection and repair, to topo I antagonizes this HR stimulation (Baumann et al., 2006).

Topo I helps remove the supercoiling which accumulates during transcriptional elongation (Zhang et al., 1988). It also has a role in activating DNA transcription, but this is apparently unrelated to its catalytical activity, suggesting that topo I might be part of protein complexes which function as transcription activators (Kretzschmar et al., 1993).

Topo I also has a second catalytical function as a kinase (via its C-terminal domain) and phosphorylates members of the SR family of RNA splicing factors (Rossi et al., 1996 and Rossi et al., 1998).

In the presence of different types of DNA damage or anti-cancer drugs the catalytic cycle of topo I can be altered (by shifting the equilibrium towards the cleavage reaction or inhibiting the relegation step) and as a result topo I can remain trapped on the DNA as part of the cleavable complex (Lanza et al., 1996 and Pommier et al., 1998). However there is a mechanism for removing the topo I cleavable complex which involves partial enzymatic digestion of both topo I and the DNA substrate followed by cleavage of the tyrosyl-DNA phosphodiester linkage by the tyrosyl–DNA phosphodiesterase (Tdp1). For efficient hydrolysis of the cleavable complex, the Tdp1 requires that the topo I should be degraded to a short polypeptide and that the DNA should contain a single stranded region so that the 3’ end phosphotyrosyl bond is exposed (Debethune et al., 2002).

Topo I also seems to play a role in apoptosis since high numbers of cleavage complexes associate with apoptotic cells (Søe et al., 2004). It has been shown that *in vitro* a topo I cleavage complex can attract a second topo I molecule that introduces another cut in the close proximity upstream from the original cleavage complex (Søe et al., 2001).

This double topo I cleavage reaction might have a role in recombination-mediated DNA repair (Søe et al., 2001). Furthermore *in vitro* p53 can stimulate the formation of these double topo I cleavages (Søe et al., 2002).

Human cells contain two topoisomerase II isoforms: topo IIα and topo IIβ. The α isozyme is 1531aa long with a molecular weight of 170kDa, whereas the β isozyme is 1625aa long and has a molecular weight of 180kDa (Tsai-Pflugfelder et al., 1988 and Austin et al., 1993). Even though these two isoforms are extremely similar they are encoded by two different genes: 17q21-22 for topo IIα and 3p24 for topo IIβ. Both enzymes generally function as homodimers but topo IIαβ active heterodimers have been detected *in vivo* (Biersack et al., 1996). Both topo IIα and topo IIβ seem to have the same enzymatic properties with topo IIβ being more processive at DNA relaxation (Drake et al., 1989). They both also show increased affinity for supercoiled, bent and Z-form DNA (Austin et al., 1995, Zechiedrich and Osheroff, 1990 and Bechert et al., 1994).

As all type II topoisomerases, they can relax positively or negatively supercoiled DNA but cannot introduce either type of supercoiling into DNA. They cleave double stranded DNA with a 4bp stagger and during this reaction they become covalently attached to the 5' of the DNA molecule. They are also the only enzymes inside the cell able to decatenate DNA in a process where ATP binding triggers a conformational adjustment that facilitates the transport of a second DNA segment through the DNA break (Champoux, 2001).

Topo IIα and topo IIβ have different expression levels during the cell cycle: for topo IIα the mRNA abundance peaks in late S phase and during mitosis, while topo IIβ is constantly expressed throughout the cell cycle (Goswami et al., 1996 and Isaacs et al., 1998). At the same time, topo IIα has high levels of expression in proliferating cells while topo IIβ is apparently equally expressed in all cell types (Hsiang et al., 1988 and Turley et al., 1997).

Early studies on topoisomerase II (which presumably were looking at the topo IIα isoform) showed that it is part of the nuclear scaffold and is evenly distributed along the axial core of metaphase chromosomes (Adolphs et al., 1977 and Gasser et al., 1986). The main function of topo II in human cells seems to concern chromosome condensation, but

the specific role of the two isoforms is not yet clear. It is obvious that lack of both topo IIα and topo IIβ severely impairs chromosome condensation and proper mitotic segregation (Sakaguchi and Kikuchi, 2004). However, whether the two enzymes are able to substitute one another in this task remains an open question. In one report topo IIα depletion alone led to failed chromosome segregation, while experiments performed in a different lab showed that both topo IIα and topo IIβ need to be absent for failed mitosis (Carpenter and Porter, 2004 and Sakaguchi and Kikuchi, 2004).

Recently it has been proposed that topo IIα performs its specific function during G2/M as part of a multisubunit complex, called the toposome (Lee et al., 2004). This large protein complex contains, besides the topo IIα enzyme, two ATPase/helicase proteins (RNA helicase A and RHII/Gu), one serine/threonine protein kinase (SRPK1), one HMG protein (SSRP1), and two pre-mRNA splicing factors (PRP8 and hnRNP C). A possible role for the assembly the toposome is stimulation of topo II decatenation activity (Lee et al., 2004).

Topo IIβ is also detected as part of a specific multi-protein complex involved in transcriptional activation of genes subjected to ligand or signal-dependent regulation (Ju et al., 2006). A topo IIβ induced DNA double-stranded break leads to the activation of poly[adenosine diphosphate (ADP)–ribose] polymerase–1 (PARP-1), an enzyme involved in DNA damage detection and repair, which in turn poly(ADP-ribosyl)ates histone H1 and de-represses a specific chromatin locus. Therefore PARP-1 and topoIIβ, along with DNA-PK/Ku86/Ku70 form a complex responsible for transcriptional activation in response to specific signals (Ju et al., 2006). This new topoIIβ function correlates well with previous reports concerning its association with chromatin remodeling complexes (LeRoy et al., 2000)

There is substantial evidence that both topo II α and β are *in vitro* substrates for casein kinase II and protein kinase C, while topo IIα can also be phosphorylated by protein kinase A (Wells et al., 1994, Watt and Hickson, 1994 and Isaacs et al., 1998). In mitosis both isozymes seem to be hyperphosphorylated but the exact role of this modification is not well understood (Kimura et al., 1994 and Kimura et al., 1996). Also,

both human topo II α and β can substitute the *S.cerevisiae* topo II enzyme in chromosome condensation and recombination (Jensen et al., 1996).

Human cells contain two topoisomerase III isoforms: topo IIIα and topo IIIβ. The topo IIIα gene, found on chromosome 17p11.2-12, codes for two variants of 1001aa and 976aa respectively of this enzyme, due to the presence of two alternative start codons (Hanai et al., 1996). The topo IIIβ gene on chromosome 22q11-12 encodes three alternatively spliced transcripts to give rise to three protein variants, 862aa 730aa and 707aa long (Kawasaki et al., 1997 and Ng et al., 1999).

Characterization of the topo IIIα enzyme shows that it is a classical type IA topoisomerase which is able to cleave negatively supercoiled DNA in the presence of a single-stranded region and requires Mg^{2+} ions for catalytical activity (Goulaouic et al., 1999). Even though the same type of characterization is not yet available for topo IIIβ, the high-homology between the two enzymes and the fact that topo IIIβ expression in yeast cells was shown to rescue at least in part the *S.cerevisiae top3*Δ phenotype strongly indicate that also topo IIIβ is a classical IA topoisomerase (Ng et al., 1999).

Topo IIIα interacts with BLM and its supercoiling relaxation activity is stimulated by it, while both topo IIIα and IIIβ interact with the RecQ5β helicase (Wu and Hickson, 2002 and Shimamoto et al., 2000). Ataxia telangiectasia (AT) is a recessive disorder which shares some of the features of Bloom's syndrome and knock down of topo IIIα protein levels or overexpression of a truncated form of the enzyme has been shown to partially rescue the AT phenotype in cell culture (Fritz et al., 1997). The topo IIIα interaction with the Bloom's syndrome helicase *in vivo* might be required in solving a recombination intermediate containing a double Holliday junction (HJ) (Wu and Hickson, 2003).

Topo IIIα is present both in the nucleus and mitochondria, while topo IIIβ is exclusively nuclear, moreover topo IIIα seems to be concentrated in the nucleoli, possibly required for rDNA metabolism (Lin et al., 2000 and Wang et al., 2002). There seems to be a difference in the levels of topo IIIα enzyme in cycling versus non-cycling cells, with apparent high-levels of proteolytic cleavage of the isoform in resting cells (Lin et al., 2000). Topo IIIα was also reported to associate with telomeres, while topo IIIβ

associates with metaphase chromosomes (Kobayashi and Hanai, 2001 and Tsai et al., 2006).

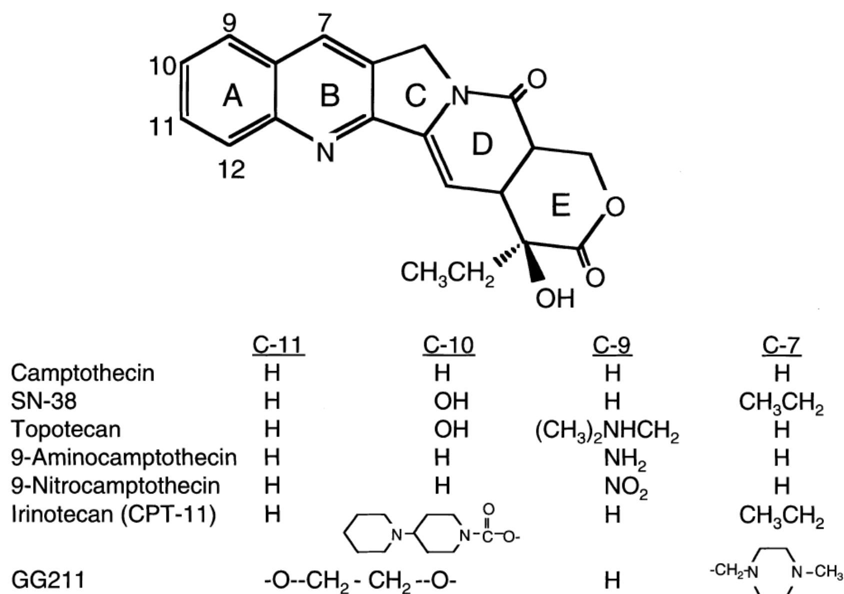
1.3.3 Topoisomerase inhibitors.

DNA topoisomerase inhibitors are drugs which either poison the enzyme, by inhibiting the religation step and transforming the enzyme in a DNA damaging agent, or they inhibit the DNA relaxation or decatenation activity and therefore mimic a null mutation. These agents are currently used in the treatment of bacterial infections and also represent one of the major classes of anti-cancer drugs used in the clinic. Topoisomerase inhibitors have been discovered only for type IB and type II enzyme, no report of type IA inhibitors has been published so far.

The first eukaryotic topo I inhibitor identified was camptothecin (CPT) an alkaloid with potent anti-tumor activity from the tree *Camptotheca acuminate* (Wall et al., 1966 and Hsiang et al., 1985). Camptothecins (CPT and its derivatives) are five-member ring alkaloids which inhibit specifically the religation step in the catalytic cycle of topo I (Hsiang et al., 1985). They do not interact with either the DNA or the enzyme alone but form a ternary complex by binding at the topo I - DNA interface and blocking a specific conformational state of the enzyme – DNA complex (namely the cleavage complex) resulting in a special case of noncompetitive inhibition called interfacial inhibition (Pommier and Cherfils, 2005). As a result of this inhibition the enzyme is left covalently attached to the 3’ end of the DNA substrate and in addition its DNA relaxation activity is inhibited. Figure 1.3.3.1 illustrates CPT and a few derivatives.

Currently two CPT derivatives are used in cancer therapy: hycamtin (Topotecan®) and CPT-11 (Camptosar®). Topotecan is effective in the treatment of advanced colorectal carcinomas while Camptosar is prescribed to patients with ovarian and small-cell lung cancers (Pommier and Cherfils, 2005). In spite of their excellent anti-cancer activity CPT and its derivatives have tree main disadvantages *in vivo*: they are toxic, the topo I inhibition is reversible within minutes upon drug removal and at physiological pH they reach quickly equilibrium with their carboxylate derivate which is

inactive (Pommier et al., 1998). Currently the family of CPT derivatives is ever expanding in the search for more potent drugs with less side effects.



	C-11	C-10	C-9	C-7
Camptothecin	H	H	H	H
SN-38	H	OH	H	CH_3CH_2
Topotecan	H	OH	$(CH_3)_2NHCH_2$	H
9-Aminocamptothecin	H	H	NH_2	H
9-Nitrocamptothecin	H	H	NO_2	H
Irinotecan (CPT-11)	H	N- N-C(O)-O-	H	CH_3CH_2
GG211	$-O--CH_2 - CH_2--O-$		H	$-CH_2-N$ $N-CH_3$

Figure 1.3.3.1 - CPT and its derivatives (Taken from Takimoto et al., 1998).

Other topo I inhibitors of the religation reaction include a series of DNA intercalating agents like benzoanthracenes (e.g. saintopin and derivatives), benzophenanthridines and protoberberines, indolocarbazoles (e.g. NB-506 and KT6006), intoplicine and anthracyclines (e.g.morpholinodoxorubicin and aclacinomycin). Some of these compounds are not specific for topo I and have been shown to inhibit topo II also (e.g. intoplicine) (Pommier et al., 1998).

It is worth mentioning that a series of endogenous and exogenous DNA damaging agents also lead to the freezing of the topo I – DNA cleavage complex. Amongst these the most common are ultraviolet (UV) irradiation, alkylating and intercalating agents (Lanza et al., 1996).

A range of drugs capable of binding or intercalating DNA such as doxorubicin, aclacinomycin A and naphthacenecarboxamide antibiotics have been reported to inhibit the topo I forward cleavage reaction, but their specificity towards topo I is questionable (Pommier et al., 1998).

Epipodophyllotoxins are synthetic derivatives of podophyllotoxins, naturally occurring lignants found in plants, particularly in the genus *Podophyllum* but found also in other genera. They inhibit specifically the religation step in the catalytic cycle of eukaryotic topoisomerase II (Minocha and Long,

1984). They are also thought to be interfacial inhibitors and form ternary complexes at the topo II - DNA interface (Pommier and Cherfils, 2005).

In contrast to topo I, the stabilized topo II – DNA covalent complex consists of the enzyme being covalently bound to the 5’ end of the break. 4’-demethylepipodophyllotoxin-4-(4,6-O-ethylidene-beta-D-glucopyranoside) (also known as etoposide or VP16) and 4’-demethylepipodophyllotoxin-4-(4,6-O-thenylidene-beta-D-glucopyranoside (also known as teniposide or VM26) are two of the most used epipodophyllotoxin inhibitors. Figure 1.3.3.2 illustrates the structure of etoposide and teniposide.

Etoposide is a widely used anticancer drug in the therapy of testicular cancer, small cell lung cancer, lymphomas, Ewing's sarcoma, Kaposi's sarcoma, ovarian cancer (Damayanthi and Lown, 1998). Teniposide is used in poor prognosis acute lymphoblastic leukemia (Damayanthi and Lown, 1998).

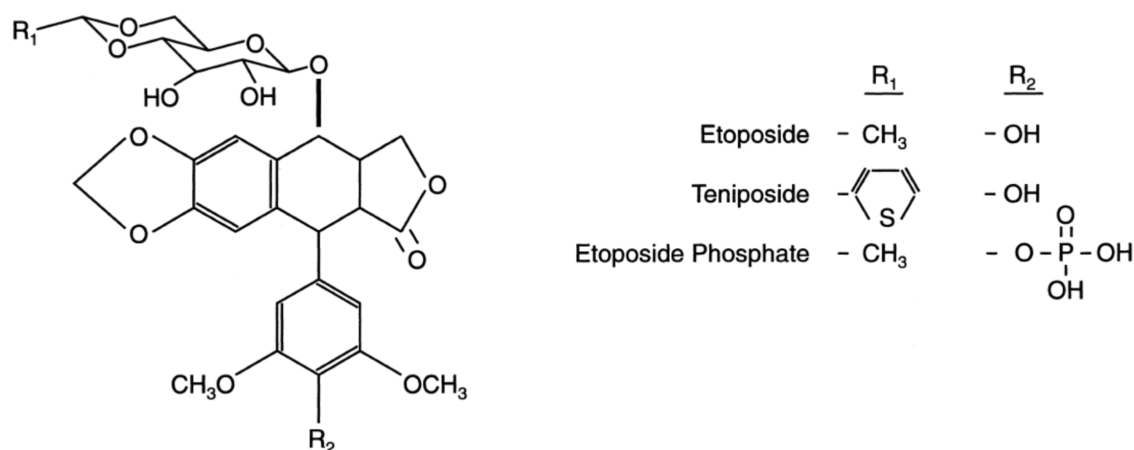


Figure 1.3.3.2 - The epipodophyllotoxins (Taken from Hande, 1998).

Anthracycline antibiotics are another group of topoisomerase II inhibitors used in cancer therapy, originally isolated from fermentation products of *Streptomyces peucetus*. They have a range of effects *in vivo* apart from topo II inhibition, which include a DNA intercalating effect, inhibition of helicase activity and induce free-radical formation. Nevertheless the main tumor-cell killing effect is thought to be due to topo II poisoning. There are four anthracyclines currently in clinical use: doxorubicin (lymphomas, breast cancer, sarcomas, Kaposi's sarcoma and leukemias), daunorubicin (acute lymphocytic leukemia), idarubicin (acute myelogenous leukemia) and epirubicin (lymphomas, breast

cancer, sarcomas, Kaposi's sarcoma and leukemias) (Hortobagyi, 1997). Figure 1.3.3.3 illustrates the anthracycline antibiotics in clinical use.

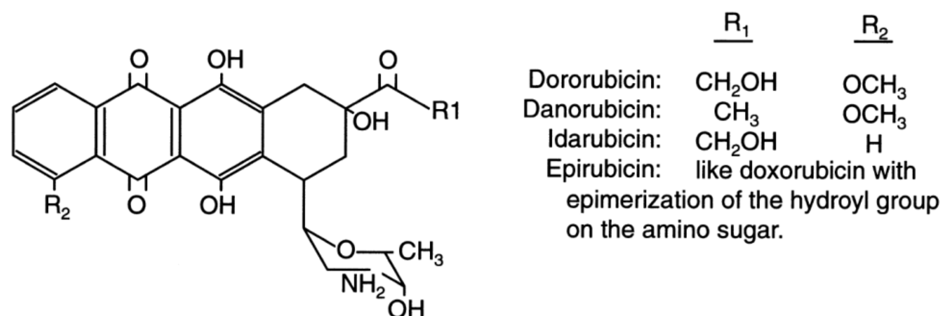


Figure 1.3.3.3 - The anthracycline antibiotics in clinical use (Taken from Hande, 1998).

Mitoxantrone is the only anthracenedione topo II inhibitor currently in clinical use. It is a compound similar to the anthracycline antibiotics, but it has a reduced ability to form free-radicals, and therefore reduced toxicity. It is used in the treatment of breast cancer, prostate cancer, leukemia (myelocytic) and lymphoma (Hande, 1998). Figure 1.3.3.4 illustrates the structure of mitoxantrone.

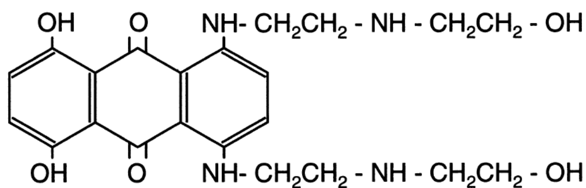


Figure 1.3.3.4 - The structure of mitoxantrone (Taken from Hande, 1998).

Similar to topo I, topo II cleavage is also induced by certain types of DNA damage but, in contrast to topo I, UV irradiation does not trap the enzyme cleavable complex on the DNA (Kingma et al., 1997 and Rosenstein et al., 1997).

The quinolones nalidixic acid and its derivatives, norfloxacin, enoxacin, lomefloxacin, ciprofloxacin, ofloxacin, levofloxacin, and sparfloxacin are very potent antibiotics used against Gram-positive and Gram-negative bacteria (Hooper, 1998). Their primary mode of action is inhibition of bacterial DNA gyrase and topoisomerase IV, much like epipodophyllotoxins inhibit eukaryotic topoisomerase II (Kreuzer and Cozzarelli, 1979).

Coumarins are a different class of antibiotics which inhibit DNA gyrase by competitive inhibition of ATP binding, and include novobiocin, coumermycin A1, and chlorobiocin (Lewis et al., 1996).

1.3.4 Topoisomerases in DNA replication.

As a process DNA replication can be divided into three steps: initiation, elongation and termination. During initiation the pre-replicative complex assembled at the origin of DNA replication gets converted into two replication forks which will start to replicate the two parental DNA strands in opposite directions. This initiation step of DNA synthesis requires the origin DNA be melted.

In elongation the two fork complexes continue to unwind and replicate simultaneously both parental DNA strands. The DNA unwinding performed by the fork leads to the accumulation of positive supercoiling (see figure 1.2.3.1). When two replication forks, from adjacent replicons, converge and finish replicating a DNA segment, termination occurs. At this stage the two daughter double helices will be intertwined in a structure called a catenate (see figure 1.2.3.2).

As mentioned previously topoisomerases are enzymes essential for dealing with the topological constraints of DNA during replication. In an overly simplified view they are needed to solve three topological problems in replication: the unwinding of the origin, removal of supercoiling during elongation and decatenation of sister chromatids at termination.

Table 1.3.4.1 illustrates the current knowledge about the topoisomerases involved in DNA replication.

Table 1.3.4.1 Topoisomerases involved in DNA replication.

Model system	Initiation	Elongation	Termination (decatenation)
Bacteria - *E. coli*	*In vivo* - DNA gyrase. *In vitro* - DNA gyrase or topo IV.	*In vivo* - DNA gyrase. *In vitro* - DNA gyrase, topo III or topo IV.	*In vivo* - topo IV. *In vitro* - topo IV or topo III.
Yeast - *S. cerevisiae*	*In vivo* - none. *In vitro* - none.	*In vivo* - topo I, topo II and/or topo III. *In vitro* - topo I.	*In vivo* - topo II. *In vitro* - ?.
Viruses - SV40	*In vitro* - topo I.	*In vitro* - topo I ot topo II.	*In vitro* - topo II.
Human	?	? topo I or topo II.	*In vivo* - topo II.

DNA replication has been characterized in detail in bacteria. In *E.coli* the initiation of DNA replication at *oriC* requires origin binding by the dnaA protein which induces a local melting of the duplex. Further binding of the dnaB (helicase) and dnaC leads to the formation of the pre-priming complex. In turn DNA gyrase, the only known topoisomerase able to introduce negative supercoiling, enlarges the melted region to allow RNA priming and subsequent fork movement (Gellert et al., 1976 and Sekimizu et al., 1988). Both *in vivo* and *in vitro* initiation of DNA synthesis seems to require the activity of DNA gyrase (Fairweather et al., 1980, Filutowicz, 1980 and Baker and Kornberg, 1988). Nevertheless, is seems that, *in vitro*, topoisomerase IV can substitute to some extent DNA gyrase in the initiation event (Smelkova and Marians, 2001). This is unusual considering that topo IV cannot introduce negative supercoiling and its DNA relaxation activity is quite poor (topo IV is a potent decatenase).

Gyrase has a particular mode of action: approximately 140bp of DNA is wrapped around the enzyme in a right-handed manner and the negative supercoiling is introduced by an intramolecular strand passage mechanism, in which the DNA wrapped around the enzyme is cleaved and another region of the same molecule is passed through the double-stranded break (Liu and Wang, 1978a, Liu and Wang, 1978b and Ullsperger and Cozzarelli, 1996). DNA gyrase prefers relaxed and positively supercoiled DNA as a substrate but the exact mechanism by which it is recruited at the origin in a timely fashion is unknown (Higgins and Cozzarelli, 1982).

In vitro DNA elongation in *E.coli* can be supported by either gyrase, topo III or topo IV and presumably *in vivo* all these enzymes contribute to DNA relaxation to different extents (Hiasa and Marians, 1994a and Hiasa and Marians, 1994b).

The enzyme responsible for *in vivo* decatenation of replicating daughter DNA molecules at the termination step of DNA replication is topoisomerase IV (Adams et al., 1992 and Zechiedrich and Cozzarelli, 1995). Nonetheless *in vitro* both topo IV and topoisomerase III can perform this action (Hiasa et al., 1994). This finding is interesting since topo III does not introduce double stranded breaks in the DNA, therefore it acts before the replication is complete or can take advantage of an already present single-stranded nick.

In eukaryotes DNA replication is a much more complex process, which involves many levels of regulation including origin decision, timing of origin firing and inhibition of origin re-firing. All these features relate to the high complexity genomes which characterize eukaryotes. Even though many basic

features of eukaryotic DNA replication are currently known a thorough understanding of the process is still lacking.

The most detailed understanding of the role of DNA topoisomerases in DNA replication has come from yeast experiments. Initiation of DNA replication does not seem to require any topoisomerase. Double mutants of topo I and topo II can still synthesize short DNA molecules, even though their DNA replication is severely impaired (Kim and Wang, 1989 and Brill et al., 1987). Topoisomerase III might act at the initiation step to facilitate the residual DNA replication observed but this is highly unlikely.

Cells containing mutated topo I or topo II can essentially complete one round of DNA replication. Nevertheless topo I inactivation leads to delays in chain elongation in early S-phase, while cells lacking topo II cannot decatenate the sister chromatids at the end of DNA replication and have broken chromosomes in mitosis (Brill et al., 1987 and Kim and Wang, 1989). This indicates that both topo I and topo II can relieve supercoiling during elongation but topo II is the enzyme responsible for DNA decatenation.

Interestingly, in a yeast *in vitro* system, efficient plasmid replication was dependent on topo I activity and not topo II suggesting that at least in this case topo II is unable to support replication elongation (Mitkova et al., 2005).

Topoisomerase III mutants in *S.cerevisiae* exhibit a slow growth phenotype suggesting that this enzyme might also be involved in replication elongation (Gangloff et al., 1999). This potential role might be connected with the reported interaction of topo III with helicases, a known component of replication forks (Bennett et al., 2000). Instead, the topo III enzyme is essential in the yeast *S.pombe* and its presence is required for accurate DNA replication and chromosome segregation (Maftahi et al., 1999 and Oh et al., 2002).

In human cells there is some evidence that topo IIα interacts preferentially with nascent DNA (Qiu et al., 1996). Topoisomerase I and II could be isolated as part of a large protein complex called the synthesome which includes DNA polymerases alpha, delta and epsilon, proliferating cell nuclear antigen, replication factor A, replication factor C, flap endonuclease 1 and DNA ligase I, all members of replication forks (Jiang et al., 2002 and Sandoval et al., 2005).

Another way to investigate the requirement of DNA replication for topoisomerases in human cells has been by the study of viruses since they normally use the host cell proteins for replication.

Replication of simian virus 40 (SV40) DNA relies on the sequential binding of two hexamers of T antigen (a helicase) to the SV40 origin followed by the recruitment of DNA polymerase/αprimase, replication protein A (RPA) and topoisomerase I (Simmonds et al., 2004). Nucleolin seems to associate with T antigen hexamers and topo I at the replication fork to form a DNA unwinding and relaxation complex, which is essential for initiation of viral replication (Seinsoth et al., 2003). Instead, topo II can replace topo I during elongation but is absolutely required for decatenation of daughter chromatids at the end of SV40 replication (Yang et al., 1987 and Ishimi et al., 1995).

Herpes simplex virus 1 (HSV-1) replication-dependent recombination requires the assembly of a replication fork that involves the viral replication proteins, including the polymerase catalytic subunit (UL30), processivity factor (UL42), helicase-primase core enzyme (UL5/UL52), loading factor (UL8), SSB (ICP8), and a relaxing enzyme e.g. eukaryotic topoisomerase I. (Nimonkar and Boehmer, 2003). In addition inhibition of topo II activity prevents efficient replication of both HSV-1 and HSV-2 (Nishiyama et al., 1987 and Hammarsten et al., 1996).

Both topoisomerases I and II are also involved in the replication of Epstein-Barr virus and adenovirus, while topo I might have a role in parvovirus replication (Schaak et al., 1990, Gu and Rhode, 1991 and Kawanishi, 1993).

To conclude there is a lot of indirect evidence that both topo I and topo II are involved in human DNA replication, but their exact role is far from characterised.

1.4 The human lamin B2 origin - a tool for studying the role of DNA topology in DNA replication.

The human origin of DNA replication located close to the lamin B2 gene on chromosome 19p13.3 is currently the best characterized human origin. This origin is positioned at the 3' end of the lamin B2 gene and close to the promoter of the housekeeping gene TIMM13 (Giacca et al., 1994).

It is an early firing origin which has been shown to be active in a variety of normal and cancer cell-lines and to be covered by a proliferation-dependent footprint which varies dynamically with the cell-cycle (Dimitrova et al., 1996 and Abdurashidova et al., 1998). This footprint covers the very end of the lamin B2 transcribed region along with a part of the downstream intergenic spacer. The structure of the footprint is asymmetric on the two complementary strands with a larger area protected on the lower strand and this region is protected in both G1 and S phases of the cell cycle but not in M (Abdurashidova et al., 1998).

The start-site of bi-directional DNA replication has been mapped with nucleotide resolution and it shows that the DNA synthesis begins with a 4 nucleotide overlap in the footprinted region (Abdurashidova et al., 1999).

Furthermore, *in-vivo* studies have shown that the hOrc1, hOrc2, hCdc6 and hMcm3 proteins, known members of eukaryotic pre-replicative complexes (pre-RC), are in direct contact with the origin sequence at different moments of the cell cycle (Abdurashidova et al., 2003).

The origin recognition complex (ORC) is a six subunit protein complex (Orc1-6) which marks in G1 genomic loci which will be used as replication origins in S phase (Mendez and Stillman, 2003). Cell division cycle 6 (CDC6) is a member of the AAA+ ATPases protein family which is essential in the formation of pre-RC as it helps with the binding of the mini-chromosome maintenance (MCM) proteins (Mendez and Stillman, 2000). MCM 2-7 proteins form a hexameric molecule which is thought to act as a DNA helicase at the replication fork (Labib and Diffley, 2001).

Nucleotide-level investigation, of the lower strand only, shows the position bound by the hOrc1, hOrc2 and hCdc6 proteins at the origin, throughout the cell cycle (Abdurashidova et al., 2003). In M phase, in good agreement with the lack of a protein footprint at the origin, none of these proteins were found on the DNA (Abdurashidova et al., 1998). In the middle of G1 Orc1, Orc2 and Cdc6 are located at the lamin B2 origin as part of the pre-replicative complex. In the middle of S, after the origin has fired, only the Orc2 protein is still bound on the DNA, moreover its position has been shifted closer to the start-site (Abdurashidova et al., 2003). Figure 1.4.1 illustrates the current known facts about the lamin B2 origin.

Considering the lack of knowledge about the involvement of human DNA topoisomerases in the initiation step of DNA synthesis, the lamin B2 origin represents the perfect tool for investigating the importance of topology and topology modifying enzymes at the origin.

Both human topo I and topo II are targets of anti-cancer drugs which offer a specific method for topo - DNA crosslinking in the cell. If indeed these enzymes interact with the lamin B2 origin, their position on the DNA would be easily identifiable. Data on the SV40 replication origin hints at a possible interaction between a topoisomerase (topo I), a helicase (T-antigen) and DNA polymerase alpha/primase at the origin (Simmons et al., 2004). Furthermore, the stabilizing effect of nucleolin on the topo I - T-antigen complex suggests a helicase-topoisomerase association at the SV40 replication fork (Seinsoth et al., 2003). Such interactions would also be beneficial for human origins of DNA replication and their replication forks.

Topo II, on the other hand, is considered a 'bona fide' member of the nuclear scaffold and it has been shown previously that a topo II cutting-site, considered a DNA loop anchorage site, is located within 1kb of the origin (Lagarkova et al., 1998). There is a lot of controversy regarding whether origins of DNA replication are indeed anchored to the nuclear matrix or not and a detailed analysis of the topo II interaction with the origin promises to shed more light on this topic.

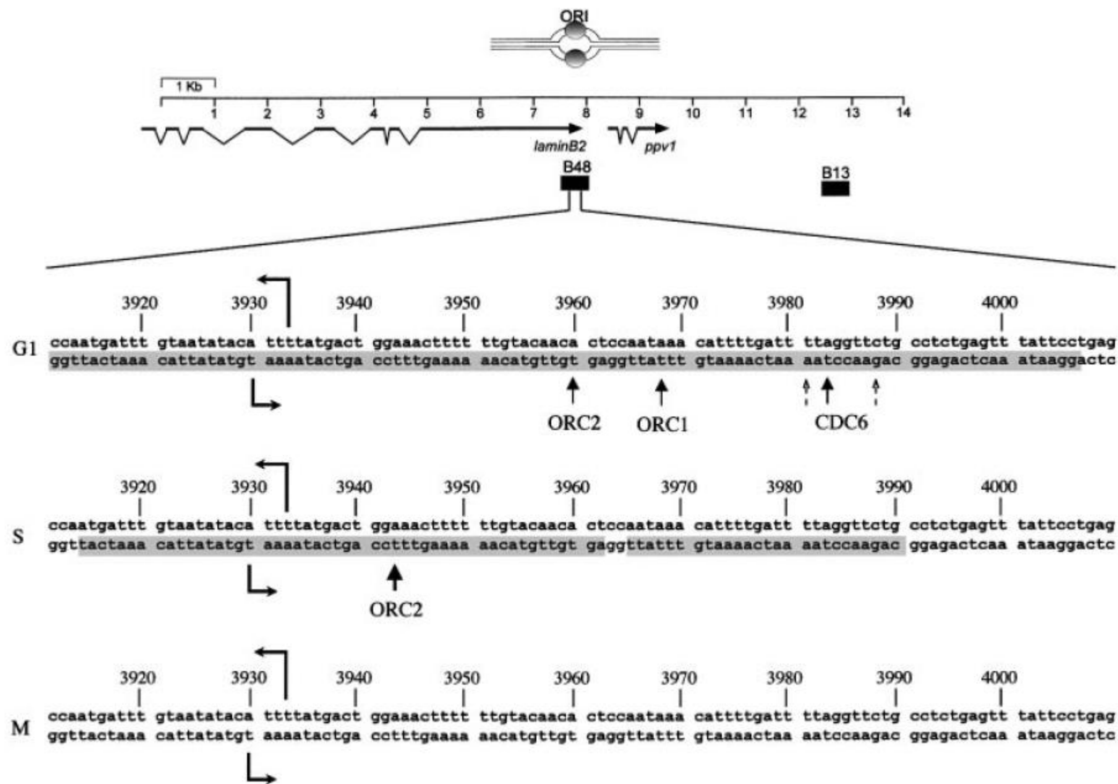


Figure 1.4.1 The lamin B2 origin of DNA replication. The genomic region containing the origin is shown in the upper part, lamin B2 and ppv1 (now called TIMM13) are the two genes found close by. B48 and B13 correspond respectively to the origin and non-origin region used for competitive PCR analysis. The highlighted sequences correspond to the protein footprint at the origin (shown for the lower strand only). The arrows indicate the position of the origin bound proteins with respect to the start-sites, footprint and phase of the cell cycle (taken from Abdurashidova et al., 2003).

2. MATERIALS AND METHODS

2.1 Cell culture and synchronization.

Adherent HeLa cells (ATCC Number: CCL-2) were cultured in D-MEM/F-12 (1:1) with GlutaMAX™ I (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 50µg/ml gentamicin kept in a 5% CO2 atmosphere at 37°C. Exponentially growing cells were arrested in M phase by incubation with nocodazole (Sigma) 50 ng/ml final concentration. Mitotic cells were recovered by mechanical shake-off 16 hours later, washed from nocodazole and replated in nocodazole-free medium. The start of G1 phase was considered when the cells were plated in complete medium. At different points of the G1 phase unattached cells were washed away and only those cells still firmly attached were used. To arrest cells in late G1 - G1/S border M cells were released in complete medium for 5 hours and then were incubated with either mimosine (Sigma), 5mM final concentration, for 24 hours or aphidicolin (Sigma), 5µg/ml, for 16 hours. The mimosine synchronized cells will be referred to as late G1 - G1/S border, while aphidicolin synchronized cells will be considered G1/S border-early S. Cells synchronized with aphidicolin were then washed three times with complete medium and released for different lengths of time to give different S phase populations. The start of S phase was considered after the third wash in complete medium. Cell cycle progression was followed by flow-cytometric analysis shown in figure 2.1.1.

2.2 Mapping of topoisomerase I *in vivo*.

For each experiment ~ 10^7 HeLa cells were incubated with 1µM CPT (Sigma) or 10µM gimatecan in complete medium for 1 minute, washed twice with warm PBS containing the same amount of drug to avoid reversal of cleavable complexes and lysed in 1.5ml buffer A (250mM Tris pH8, 25mM EDTA, 5mM NaCl, 0.5% SDS, 800µg/ml Proteinase K) supplemented with 1µM CPT. Gimatecan was synthesized according to published protocols by Sotir Zahariev (Dallavalle et al., 2001 and Wadkins et al., 2004).

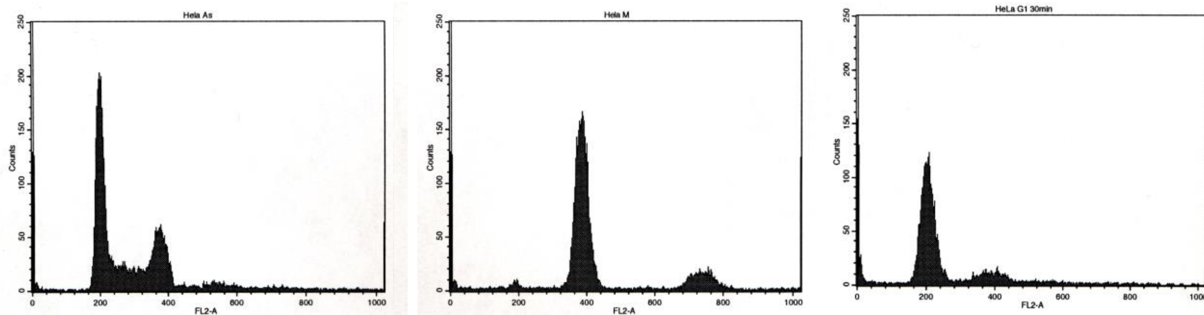
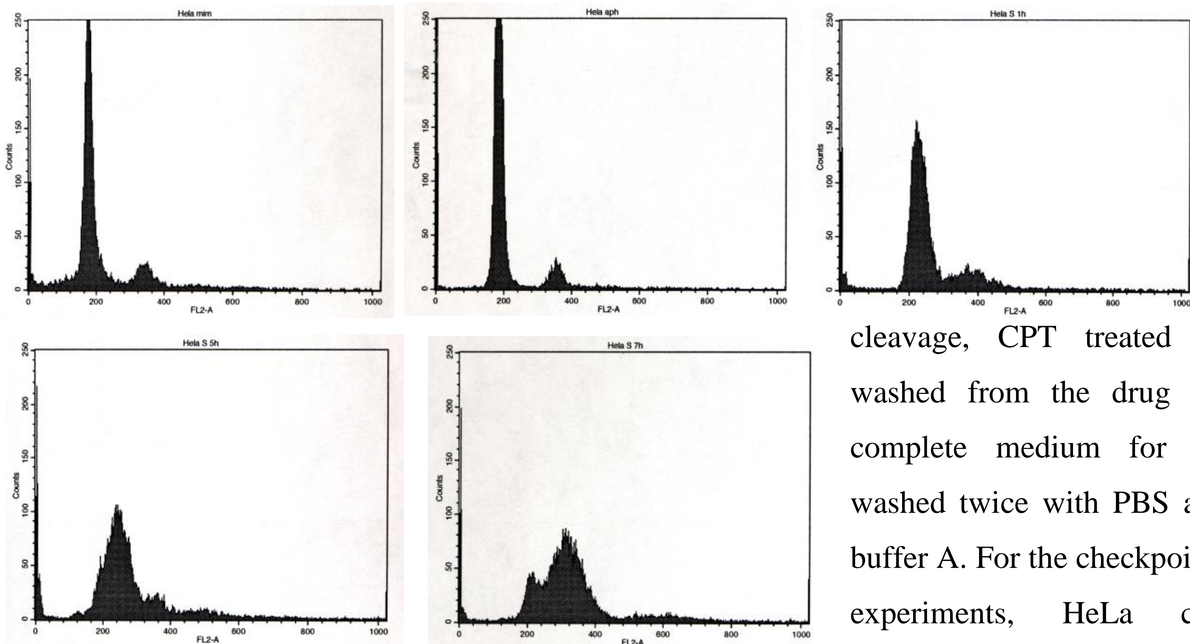


Figure 2.1.1 - FACS analysis of HeLa cells synchronized throughout the cell cycle. Starting from top left asynchronous, M, G1 30 minutes, G1 1 hour, G1 5 hours, G1 7 hours, late G1 – G1/S border (mimosine), G1/S - early S (aphidicolin), S 1hour, S 5 hours, S 7 hours.



To check reversal of the drug induced cleavage, CPT treated cells were washed from the drug and left in complete medium for 5 minutes, washed twice with PBS and lysed in buffer A. For the checkpoint inhibition experiments, HeLa cells were incubated simultaneously with 1μM CPT and 5mM caffeine (Sigma) for 5 minutes, the cells were then washed with PBS supplemented with 1μM CPT and immediately lysed in lysis buffer A containing 1μM CPT. Cell lysates were incubated overnight at 37°C, total genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and resuspended in 20μl TE buffer pH7.5 (1mM EDTA, 10mM Tris-HCl pH7.5). The free 5' ends of the DNA were phosphorylated by incubation with 2μl T4 polynucleotide kinase (10U/μl) (New England Biolabs) in 10X T4 polynucleotide kinase buffer, containing 1mM ATP, for 1 hour at 37°C. The DNA was re-purified by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The topo I cleavage sites were then detected by LMPCR analysis. The primer sets used for both LM and TDPCR are shown in table 2.2.1 and the procedure for LMPCR has been described in detail elsewhere (Dimitrova et al., 1996 Abdurashidova et al., 2000 and Mueller et al., 2001).

Table 2.2.1 – Position of the sequence specific primers used for LM and TDPCR analysis at and around the lamin B2 origin. The numbering corresponds to the GeneBank accession number M94363.		
Name	Sequence	Genomic position
A	A1 5' TTACCTACACGAGCTACCCGTGGTT 3' A2 5' TGGTGCGACTCCGCGGAAGA 3' A3 5' TCCGCGGAAGAGGGAGGCCCTGAGTT 3'	4385-4409 4368-4389 4353-4379
B	B1 5' GGCTAGTGTAGCTAGTGTAAACAGGACC 3' B2 5' GTAAACAGGACCCAGGCGATGCATG 3' B3 5' CAGGACCCAGGCGATGCATGGGACCCT 3'	4091-4118 4107-4131 4112-4138
C	C1 5' TCGCATCACGTGACGAAGAGTCAGC 3' C2 5' GAGTCAGCTTGTGCAACAGCGTCGG 3' C2 5' GCTTGTGCAACAGCGTCGGAGGCTCAC 3'	4179-4203 4162-4189 4154-4180
D	D1 5' GTCACAGCACAACCTGCAAAAACGG 3' D2 5' CAAAAACGGAGCTGGGCTGCAGCTG 3' D3 5' GGGCTGCAGCTGGGGCTGGCATGGAC 3'	3795-3821 3813-3837 3826-3851
E	E1 5' GGGGTGGAGGGATCTTTCTTAGACA 3' E2 5' GACATCCGCTTCATTAGGGCAGAGGCC 3' E3 5' TCATTAGGGCAGAGGCCCGGCTCGAGC 3'	4049-4074 4026-4052 4016-4042
F	F1 5' GAGTCCCTCAGATCTTTAACAAAGAACTGC 3' F2 5' AACTGCCGCGTGCAGGCTTCAGACC 3' F3 5' CGCGTGCAGGCTTCAGACCAACCCCAGCCA 3'	3712-3742 3693-3718 3682-3712
G	G1 5' TGCACAGCGCCAGGTTAACGC 3' G2 5' CAGGTTAACGCTGAAGCCTGCCCCG 3' G3 5' CGGTTTTTAAGAAGATGCATGCCTAGCGTGTTC 3'	3573-3594 3584-3609 3593-3900

In brief, 4µl total genomic DNA was heat denatured and annealed to 0.1pmol of primer 1 for 30 minutes, at 60°C, in 10µl final volume of 1x Vent polymerase buffer. Primer extension was performed in 20µl final volume in the presence of 0.5mM dNTPs and 1U of Vent polymerase exo(-) (New

England Biolabs), for 5 minutes at 60°C, 10 seconds at 65°C, 10 seconds at 70°C and elongated 10 minutes at 76°C. The ligation reaction contained the 20µl of the primer extension reaction to which 23µl PEG-40, 5µl 10x T4 DNA ligase buffer and 2µl T4 DNA ligase (400U/µl) (New England Biolabs) were added. The ligation was performed overnight at 4°C. After the ligation step, samples were purified by phenol extraction and ethanol precipitation and PCR amplification was performed in 1x Vent buffer, in the presence of 10pmol of primer 2, 10pmol of a primer complimentary to the asymmetric linker, 0.2mM dNTPs and 4mM $MgSO_4$, with 1U of Vent polymerase in a final volume of 50µl. The amplification conditions were: 3 minutes denaturation at 95°C, 18 cycles of 1 minute denaturation, 2 minutes annealing and 3 minutes extension at 76°C, with 5 seconds further extension added at each cycle, and a final extension step of 7 minutes at 76°C. 20µl of amplified product was used for a final hot extension reaction with 3µl 10X Vent buffer, 0.6µl $MgSO_4$ (100mM), 1µl dNTPs (10mM each), 4µl of primer 3 labeled with ^{33}P-γATP (2 pmols), 1.15µl H_2O and 0.25µl Vent Exo- (2U/µl) (New England Biolabs). The hot extension program included a first denaturation step of 5 minutes, then 5 cycles of 1 minute denaturation, 2 minutes annealing and 5 minutes of polymerase extension at 76°C followed by a final elongation step at 76°C for 5 minutes. The hot extension product was then phenol/chloroform/isoamyl alcohol treated, precipitated with ethanol and resuspended in a sample buffer containing formamide. Before loading on a 7% acrylamide 6M urea sequencing gel the sample was denatured for 3 minutes at 95°C and then cooled in ice. The gel was run at 70W for 1h, dried and autoradiographed using a Kodak Biomax MR film.

2.3 Mapping of topoisomerase II *in vivo*.

For each experiment ~ 10^7 HeLa cells were incubated with 10nM VP16 (Sigma) in complete medium for 1 minute, washed twice with PBS containing the same amount of drug to avoid reversal of cleavable complexes and lysed in 1.5ml buffer A supplemented with 10nM VP16. To check reversal of drug induced cleavage, treated cells were washed from the drug and left in complete medium for 5 minutes, washed twice with PBS and lysed in buffer A. Cell lysates were incubated overnight at 37°C, total genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and resuspended in 20µl TE buffer pH7.5. The topo II cleavage sites were detected by TDPCR analysis and the position of the primers used is shown in table 2.2.1. The TDPCR procedure has been described in detail elsewhere (Komura and Riggs, 1998).

Typically 4µl total genomic DNA was mixed with 3µl 10X Vent buffer, 1µl $MgSO_4$ (100mM), 1µl dNTPs (10mM each), 0.6µl primer 1 (10pmol/µl), 1µl Vent Exo- (2U/µl) and H_2O up to 30µl total volume. The extension mix was incubated at 95°C for 5 minutes then at 95°C for 1 minute, 60°C for 2 minutes, 76°C for 7 minutes, cycled 10 times, with a final incubation at 76°C for 3 minutes. The extension products were then ethanol precipitated and resuspended in 10µl 0.1x TE pH 7.4 to which 4µl 5x TdT buffer, 4µl rGTP (10mM) (Promega), 1.33µl H_2O and 0.67µl TdT (15U/µl) (Invitrogen) was added. The ribotailing reaction was incubated at 37°C for 15 minutes after which the DNA was ethanol precipitated again and resuspended in 20µl H_2O. For the ligation reaction 23µl PEG-40, 5µl 10x T4 DNA ligase buffer and 2µl T4 DNA ligase (400U/µl) (New England Biolabs) were added to the DNA and the sample was incubated overnight at 4°C. After the ligation product was purified by phenol extraction and ethanol precipitation the PCR amplification and the hot extension were performed as for the LMPCR (see above).

For immunoprecipitation of topo II-DNA covalent complexes, HeLa cells incubated with 10nM VP16 were washed twice with PBS containing 10nM VP16 and lysed with a solution of 1% sarkosyl in TE pH 8 (10mM Tris-HCl pH8, 1mM EDTA). The lysates were layered on top of a CsCl solution, 1.5 g/ml density and the samples were centrifuged at 70000rpm for 5 hours at 20ºC in a Beckman L8-70M Ultracentrifuge in order to separate the bulk DNA and protein-DNA complexes (bottom fraction) from free proteins (top fraction). The cellular DNA was collected, washed twice in TE buffer pH8 and twice in restriction enzyme buffer. The DNA was incubated with 100U of BstNI restriction enzyme for one hour at 37°C, washed three times in TE buffer pH8 and dissolved in ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris HCl pH8.1). Chromatin immunoprecipitation using a goat polyclonal anti-topoisomerase II antibody (Santa Cruz) was performed using the Chromatin immunoprecipitation assay kit (Upstate Biotechnology) as described by the manufacturer. The immunopurified DNA was isolated by proteinase K treatment, phenol extraction and ethanol precipitation and subjected to TDPCR analysis as described above.

2.4 UV laser photo-footprinting.

Asynchronous HeLa cells growing in 5cm diameter Petri dishes were treated for 1 minute with either 1µM CPT or 10nM VP16, washed with PBS containing the same drug concentration and UV laser irradiated at two wavelengths, obtained by frequency conversion of infrared femtosecond pulses

produced by a Ti:sapphire system (Russman et al., 1998). Second and third harmonic light pulses at 400 and 266nm, of duration 120fs and 200fs, pulse energy of 25 and 40μJ, respectively, were produced in two consecutive BBO crystals and expanded to a diameter of about 60mm to allow simultaneous irradiation of the surface of the dish. For comparison, HeLa cells not treated with the drugs were irradiated in the same conditions. The cells were immediately lysed in lysis buffer A after irradiation, the DNA isolation and TDPCR were performed as described above.

2.5 BrdU labeling.

HeLa cells synchronized with mimosine were treated with 1μM CPT in complete medium containing mimosine for 30 minutes, washed from mimosine in complete medium containing 1μM CPT and pulsed with 50μM BrdU (Sigma) in the presence of CPT for 30 minutes. The cells were washed first from BrdU with complete medium supplemented with 1μM CPT, after were washed from the drug and left in complete medium for 10 minutes. As a control, late G1 - G1/S synchronized cells were incubated with BrdU for 30 minutes in the presence or absence of mimosine, washed and released in complete medium for 10 minutes. Nascent DNA was isolated as described below (section 2.6).

2.6 Nascent DNA isolation and competitive PCR.

HeLa cells were collected by scraping, resuspended in PBS containing 10% glycerol and lysed for 10 minutes in the wells of a 1.2% alkaline agarose gel immersed in alkaline running buffer: 50mM NaOH, 1mM EDTA. The gel was run for 16 hours at 2V/cm and the nascent DNA of size 0.6-1 kb was isolated from the gel using a Qiagen Gel Extraction kit. BrdU-enriched sequences were immunoprecipitated using anti-BrdU antibody (Abcam) as described by the manufacturer. The isolated nascent DNA was analyzed by competitive PCR. The quantification of the abundance of the origin (B48) and non-origin (B13) DNA fragments was performed as described previously (Diviacco et al., 1992). The primers used for the B48 amplification are: B48 Dx 5'GACTGGAAACTTTTTTGTAC3', B48 Sx 5' TAGCTACACTAGCCAGTGACCTTTTTCC 3'. The B48 amplification reaction contained 5μl 10x Taq polymerase buffer with 15mM $MgCl_2$, 2.7μl 25mM $MgCl_2$, 1μl (10pmol) of each primer, 1μl dNTPs (10mM each), 0.25μl Taq polymerase (2U/μl) (Promega), 5μl nascent DNA, 5μl competitor DNA and H_2O up to 50μl. The primers used for the B13 amplification are: B13 Dx 5'

GCCAGCTGGGTGGTGATAGA 3’, B13 Sx 5’ CCTCAGAACCCAGCTGTGGA 3’. The B13 amplification reaction contained 5µl 10x Taq polymerase buffer, 3µl 25mM $MgCl_2$, 1µl (10pmol) of each primer, 1µl dNTPs (10mM each), 0.25µl Taq polymerase (2U/µl) (Promega), 5µl nascent DNA, 5µl competitor DNA and H_2O up to 50µl. The amplification conditions for both B48 and B13 were: 95°C 5 minutes, (95°C 30 seconds, 56°C 30 seconds, 72°C 30 seconds) cycled 35 times, 72°C 5 minutes.

2.7 Trichostatin A (TSA) treatment.

Asynchronous growing HeLa cells were incubated for 1 or 4 hours with 100ng/ml TSA (Sigma) in complete medium, then incubated with 1µM CPT or 10nM VP16 in complete medium for 1 minute, washed twice with PBS supplemented with the same drug concentration and lysed in buffer A containing the same amount of drug. The topo I and topo II covalent complexes were detected by LMPCR or TDPCR analysis, respectively, as described above (sections 2.2 and 2.3).

2.8 *In vivo* crosslinking

2.8.1 Topoisomerase I.

HeLa cells synchronized at the late G1 - G1/S border were crosslinked with 1% formaldehyde (Merck) in PBS for 5 minutes. The reaction was stopped by adding a cold solution of 125mM glycine in PBS, cells were washed twice in cold PBS and incubated, for 10 minutes on ice, with RSB buffer (10mM Tris-HCl pH 8.0, 10mM NaCl, 3mM $MgCl_2$) followed by an addition of an equal volume of RSB buffer containing 0.2% NP-40 for another 10 minutes on ice. Nuclei were pelleted by centrifugation at 3000rpm for 10 minutes at 4°C. After centrifugation, the nuclei were lysed in ChIP lysis buffer and samples were sonicated on ice, five times for 10 seconds, using a Cellai Soniprep 150 on full power (amplitude = 24-26 microns). Immunoprecipitations were performed using rabbit polyclonal anti topoisomerase I antibodies (Abcam) and mouse monoclonal anti-Orc2p antibodies (Stressgen) with the aid of the Chromatin immunoprecipitation assay kit as described by the manufacturer. Competitive PCR analysis was performed as described above (section 2.6).

HeLa cells synchronized at the late G1 - G1/S border were incubated first with 1µM CPT for 1 minute and immediately after subjected to protein-protein cross-linking with DSP 100µg/ml (Lomant's reagent, Pierce) still in the presence of 1µM CPT. The cells were then washed twice with PBS containing 1µM CPT, lysed with a solution of 1% sarkosyl in TE pH8 and the lysates were layered on top of a CsCl solution, 1.5 g/ml density. The samples were centrifuged at 70000rpm for 5 hours at 20ºC in a Beckman L8-70M Ultracentrifuge in order to separate the bulk DNA and protein-DNA complexes (bottom fraction) from free proteins (top fraction). The cellular DNA was collected, washed twice in TE buffer pH8 and twice in restriction enzyme buffer. The DNA was incubated with 100U of BstNI restriction enzyme for one hour at 37°C, washed three times in TE buffer pH8 and dissolved in ChIP lysis buffer. Chromatin immunoprecipitation using rabbit polyclonal anti topoisomerase I antibody (Abcam) and mouse monoclonal anti-Orc2p (Stressgen) was performed using the Chromatin immunoprecipitation assay kit as described by the manufacturer. The immunopurified DNA was isolated, following proteinase K treatment, by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. This DNA was used as a substrate for a simultaneous PCR amplification of two DNA fragments using 3 sequence specific primers: D1, E1 and T1 (for D1, E1 sequence and genomic position see table 2.2.1, primer T1 5' GGTTCTGCCTCTGAGTTTATTCCTGAGG 3', genomic position 3984-4011, corresponding to GeneBank accession number M94363). The PCR reaction contained 5µl 10x Taq polymerase buffer, 4µl 25mM $MgCl_2$, 1µl (10pmol) D1, 1µl (10pmol) E1, 1µl (10pmol) T1, 1µl dNTPs (10mM each), 0.25µl Taq polymerase (2U/µl) (Promega), 5µl immunopurified DNA and H_2O up to 50µl. The amplification conditions used were the following: 95°C 5 minutes, (95°C 30 seconds, 60°C 30 seconds, 72°C 30 seconds) cycled 35 times, 72°C 3 minutes.

2.8.2 Topoisomerase II.

HeLa cells synchronized in the middle of G1 were incubated first with 10nM VP16 for 1-10 minutes and immediately after subjected to protein-protein cross-linking with DSP as in the above experiment. Chromatin immunoprecipitation using goat polyclonal anti-topoisomerase II antibody (Santa Cruz) and mouse monoclonal anti-Orc2p antibody (Stressgen) was performed using the CHIP

assay kit as described above. Immunoprecipitated DNA was purified and analyzed by TDPCR as described in section 2.3.

2.9 *In vitro* DMS treatment.

Asynchronous growing HeLa cells were lysed in buffer A and incubated overnight at 37°C. Total genomic DNA was isolated by phenol extraction and ethanol precipitation and resuspended in 20μl H_2O. The DNA was incubated for 5 minutes with DMS (Sigma) 1% final concentration at room temperature. The reaction was stopped with 2 volumes of 1.5M sodium acetate, 1M β-mercaptoethanol, the DNA was precipitated with ethanol and the pellet was resuspended in 100μl piperidine (Sigma) and incubated at 95°C for 30 minutes. The sample was then frozen in dry ice and lyophilized in a Heto DNA Plus speedvac. The resulting pellet was resuspended in 50μl H_2O, frozen in dry ice and lyophilized again three times. Finally the DNA was resuspended in 50μl H_2O and used for LMPCR amplification as described above (section 2.2).

2.10 Western blot analyses

In order to check antibody specificity, Western blots were performed after immunoprecipitation with the CHIP assay kit. The IP products was run on a 10% SDS-PAGE, the proteins transferred to a Optitran BA-S 85 reinforced nitrocellulose membrane (Schleicher & Schuell) using a Hoefer Semiphor (Amersham) system according to the manufacturer's instructions. The membrane was blocked overnight at 4°C with SuperBlock® Blocking Buffer in PBS (Pierce). The membrane was then incubated with the primary antibody, diluted in PBS, for 1 hour, followed by 6 washes in PBS (1.5 hours in total). The secondary antibody, diluted in PBS, was added for another 30 minutes and the 6 washes with PBS were repeated. The SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce) was used for signal detection. The antibody dilutions were as follows: topo I and topo II primary 1:10000, secondary (Pierce) 1:20000, Orc2 primary 1:20000, secondary (Pierce) 1:7000.

2.11 Mapping of topo I *in vitro*.

A 216 bp PCR fragment containing the lamin B2 origin was amplified using primers D3 and E3 (see table 2.2.1). The PCR reaction mixture contained 5µl 10x Taq polymerase buffer with 15mM $MgCl_2$, 4µl 25mM $MgCl_2$, 1µl (10pmol) of each primer, 1µl dNTPs (10mM each), 5µl genomic DNA (100ng/µl), 0.25µl Taq polymerase (2U/µl) (Promega) and H_2O up to 50µl. The amplification product was then purified by PAGE, the DNA was extracted overnight from the gel by incubation in H_2O at 37°C, precipitated with ethanol and resuspended in H_2O. 20ng of origin fragment were incubated with 1U of human recombinant topo I (Sigma) in 10mM Tris pH7.4, 50mM NaCl, 5mM MgCl2, 0.1mM DTT, 100 µg/ml BSA for 15 minutes at room temperature and with 1µM CPT, 10µM CPT-7[CH2-Tris] or 10µM gimatecan for another 5 minutes. CPT-7[CH2-Tris] and gimatecan were synthesized according to published protocols by Sotir Zahariev (Dallavalle et al., 2001 and Wadkins et al., 2004). The reaction was stopped by addition of SDS 2% final concentration. Topo I was digested with 200µg/ml Proteinase K overnight, the DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and resuspended in 20µl of water. The topo I cleavage was mapped by the hot extension reaction (the final step in LM and TDPCR, see section 2.2)

2.12 Mapping of topo II *in vitro*.

The D3/E3 PCR fragment, 20ng (see above), was incubated first with 5U-25U of human recombinant topo II (USB) in 10mM Tris pH7.9, 50mM NaCl, 50mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 15µg/ml BSA, 1mM ATP for 10 minutes at room temperature and 5 minutes more with VP16, 100µM final concentration. The reaction was stopped with SDS 2% final concentration. Topo II was digested overnight with 200µg/ml Proteinase K, the DNA purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and hot extension was performed as above.

2.13 *In vitro* complex formation.

Dignam HeLa 0.4M nuclear extract 50µg (Cilbiotech) was incubated in 22.5mM HEPES pH7, 1mM Tris pH7.5, 0.2mM EDTA, 5mM $MgCl_2$, 20mM KCl, 1mM ATP, 1mM DTT, 8% glycerol with 20µg of each competitor: poly (dA-dT)-poly(dA-dT) and poly(dG-dC)-poly(dG-dC) (Amersham) at room temperature for 20 minutes. In the case of Trichostatin A treatment, the mixture was incubated in

the presence of 100ng/ml TSA for 1 hour at room temperature instead of 20 minutes. Afterwards, 20ng of origin DNA was added for another 30 minutes. The reaction was stopped with SDS 2% final concentration or EDTA 50mM final concentration. Alternatively the complex was treated for a further 5 minutes with 100µM VP16, then the reaction was stopped with SDS 2% final concentration. Proteins were digested overnight with 200µg/ml Proteinase K while the DNA purification and hot extension were performed as above (section 2.7).

For mapping the border of the *in vitro* complex, 50U λ exonuclease (New England Biolabs) were added to the preformed complex, incubated at 37°C for 3 hours and then stopped with 2% SDS final concentration. The DNA was then purified and subjected to hot extension as described above.

For the topo II immunoprecipitation experiment, the pre-formed protein complex on origin DNA was treated with 100µM VP16 for 5 minutes and the reaction was stopped by the addition of an equal volume of 2x lysis buffer for ChIP (2%SDS, 20mM EDTA, 100mM Tris HCl pH8.1). Chromatin immunoprecipitation using a goat polyclonal anti-topoisomerase II antibody (Santa Cruz) was performed using the Chromatin immunoprecipitation assay kit as described by the manufacturer. The immunopurified DNA was isolated by proteinase K treatment, phenol extraction and ethanol precipitation and subjected to TDPCR analysis as described above (section 2.3).

2.14 Construction of mutated PCR fragments.

Primers used to obtain mutated D3/E3 PCR fragments 1, 2 and 3:

Mut1A: 5' CAGCGCCAGGCCAATGATTTGTAATATACATTTTATGACTGG 3',
Mut1B: 5' GAACACGCTAGGCATGCATCTTC 3',
Mut2A: 5' CAGCGCCAGGGTACAACACTCCAATAAACATTTTGATTTTAGG 3',
Mut2B: 5' CCAGTCATAAAATGTATATTACAAATCATTGG 3',
Mut3A: 5' CTCCAATAAACATTTTGATTTTAGGTTCTGCCTCTG 3',
Mut3B: 5' GCGCTGACAAAAAAGTTTCCAGTCATAAATGTATATTAC 3'

The PCR reaction mixture contained: 5µl 10x Vent Exo- buffer, 1.5µl 100mM $MgSO_4$, 1µl (10pmol) of each primer, 1µl dNTPs (10mM each), 5µl D3/E3 PCR product (0.1ng/µl), 0.5µl Vent Exo- (2U/µl) and H_2O up to 50µl.

The amplification conditions are as follows. For primers Mut1A/E3bio: 95°C 5 minutes, (95°C 30 seconds, 60°C 30 seconds, 76°C 1 minute) cycled 10 times, (95°C 30 seconds, 72°C 30 seconds, 76°C 1 minute) cycled 25 times, 76°C 5 minutes. For primers Mut1B/D3bio: 95°C 5 minutes, (95°C 30 seconds, 70°C 30 seconds, 76°C 1 minute) cycled 35 times, 76°C 5 minutes. For primers Mut2A/E3bio: same as Mut1A/E3bio. For primers Mut2B/D1bio: 95°C 5 minutes, (95°C 30 seconds, 60°C 30 seconds, 76°C 1 minute) cycled 35 times, 76°C 5 minute. For primers Mut3A/E3bio: same as Mut1A/E3bio. For primers Mut3B/D3bio: same as Mut1B/D3bio.

For each mutation two PCR fragment were amplified using Vent Exo- as a polymerase in order to obtain blunt ends, each fragment having one of the 5'-ends blocked with a biotin tag. The fragments were purified by PAGE and subsequently on Streptavidin Paramagnetic Beads (Promega) according to the manufacturer's instructions. The fragments were detached from the beads by boiling at 95°C for 5 minutes in 0.1% SDS, reannealed in a PCR machine, precipitated with ethanol and resuspended in 10µl H_2O. The two fragments were incubated together overnight at 4°C in a 50µl ligation mixture containing: 23µl PEG-40, 10µl of each purified PCR fragment, 5µl 10x T4 DNA ligase buffer and 2µl T4 DNA ligase (400U/µl) (New England Biolabs). The ligation products were purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and used for PCR amplification using the D3/E3 primers.

3. RESULTS

3.1 Mapping of the interaction of topoisomerases I and II with the lamin B2 origin in asynchronous cells.

3.1.1 Topoisomerase I.

In order to detect a possible interaction of topoisomerase I with the lamin B2 origin of DNA replication, the anti-cancer drug camptothecin was used. CPT is a topo I specific poison which reversibly inhibits the religation step in the catalytic cycle of topo I, leaving the enzyme covalently trapped on the DNA. The free 5'end of the DNA nick, introduced by topo I, can be mapped by LMPCR amplification, thus revealing the topo I cleavage position with nucleotide resolution. A schematic diagram of the method used to map the topo I-DNA cleavage complexes is shown in figure 3.1.1.1.

Asynchronous growing HeLa cells at 60-70% confluence were incubated for 1 minute in complete medium supplemented with 1μM CPT, then washed twice with warm PBS containing 1μM CPT. All liquid was removed and the cells were lysed in a lysis buffer supplemented with 1μM CPT to avoid reversal of the cleavage. Total cellular DNA was isolated, after proteinase K treatment of the lysate, by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and resuspended in 20μl of H_2O. The free 5' ends of the DNA were phosphorylated with T4 polynucleotide kinase and ATP in order to make them amenable to LMPCR amplification and the DNA was reprecipitated and resuspended in 20μl of 0.1x TE buffer pH 7.4. 4μl of DNA were used to perform the LMPCR amplification for the detection of the CPT stabilized topo I induced cleavable complex and the results are shown in figure 3.1.1.2.

In the presence of CPT two topo I induced single stranded breaks are found, on the upper and lower strand respectively, in the origin area covered by the replicative complexes. The exact position of the cuts is between nucleotides 3890 and 3891 on the upper strand and between nucleotides 3956 and 3957 on the lower strand.

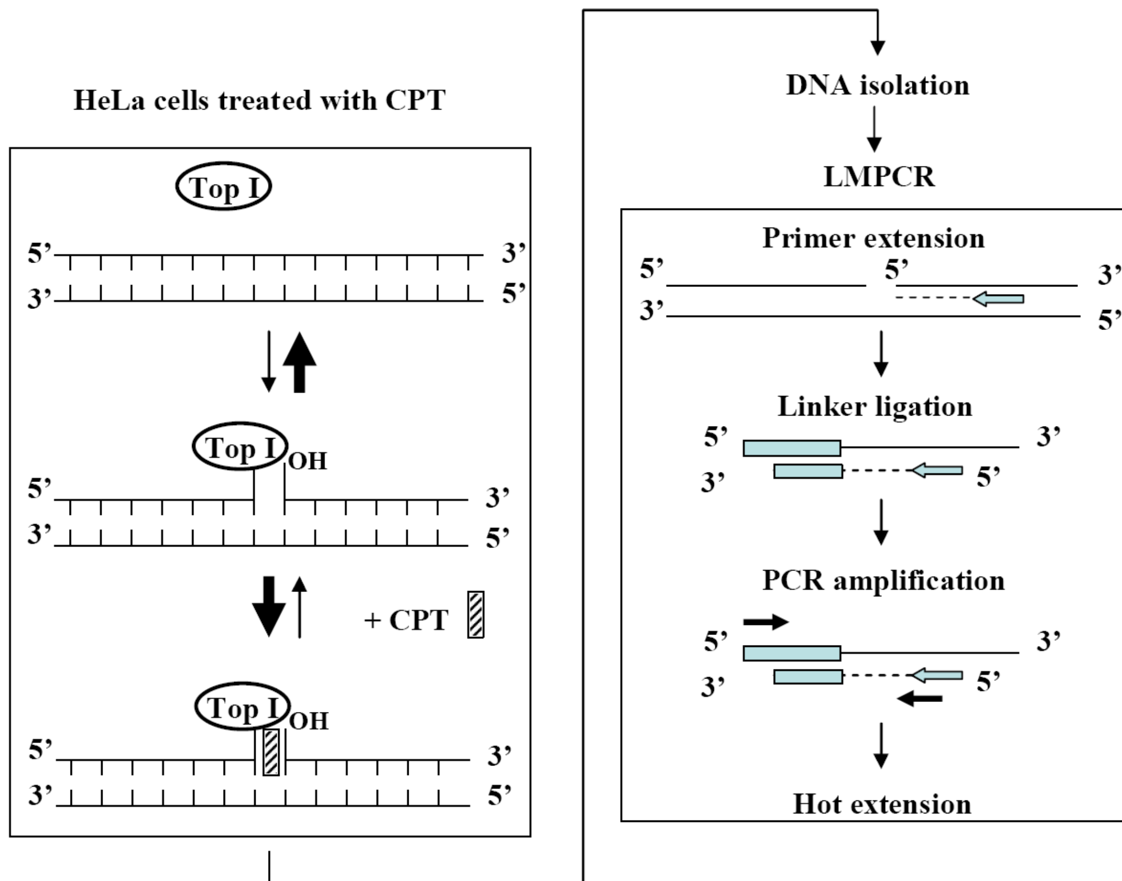


Figure 3.1.1.1 - Mapping of the topoisomerase I-DNA cleavage complexes – experimental outline. HeLa cells were treated with 1μM CPT for one minute in order to stabilize the topo I-DNA covalent complexes (left panel). The cells were then lysed, the total genomic DNA was isolated and used for ligation mediated PCR (LMPCR) analysis (right panel). In order to perform LMPCR analysis, the 5' end of the DNA must be phosphorylated, therefore the genomic DNA was subjected first to an *in vitro* phosphorylation reaction. As a first step in LMPCR a sequence specific primer was annealed to the region of interest and extended in order to obtain a double stranded DNA blunt end, which, in turn, was ligated to an asymmetric linker. The next step was a PCR amplification of the substrate using a second sequence specific primer and a primer complimentary to the linker sequence. As a final step, a third sequence specific primer, radioactively labeled, is used for hot extension and the product is then visualized, following sequencing gel electrophoresis, by autoradiography.

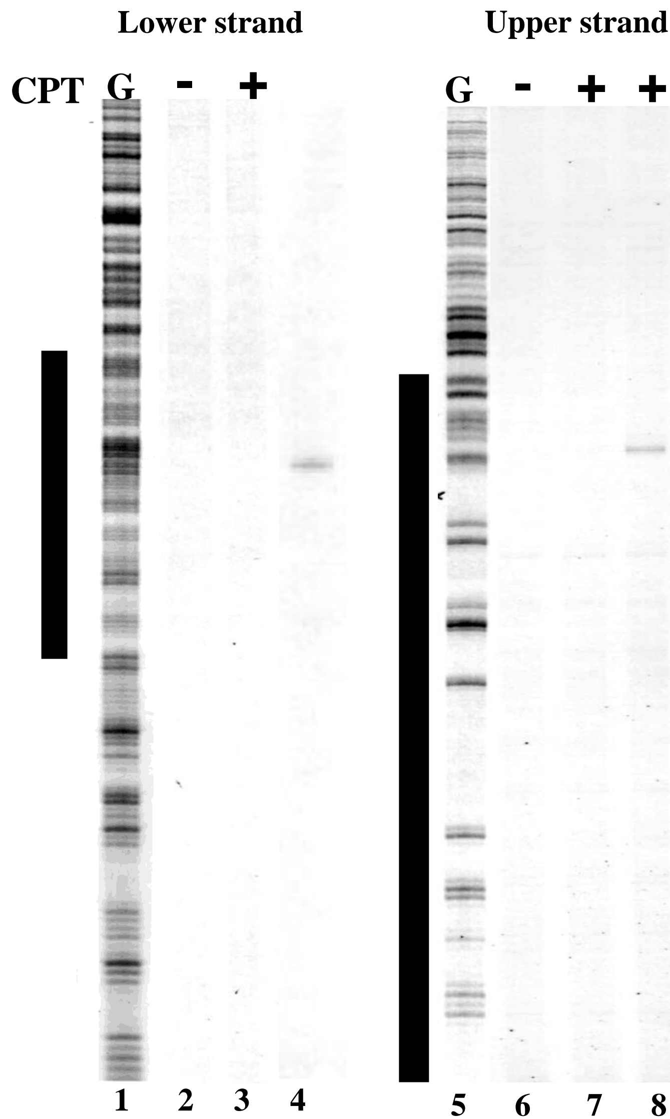


Figure 3.1.1.2 - In asynchronous HeLa cells topoisomerase I cleaves the region of the lamin B2 origin which is covered by the replicative complexes. On both the upper and lower strand, respectively, one LMPCR stop corresponding to one CPT stabilized topo I cleavage complex can be detected. These stops correspond to the DNA 5' end arisen from the topo I action at the origin. The black bars indicate the origin area covered by a protein footprint in asynchronous HeLa cells. Lanes 4,8 – cells treated with 1μM CPT for one minute. Lanes 3,7 - cells treated with 1μM CPT for one minute, washed from the drug and released for 5 minutes in complete medium. Lanes 2, 6 – cells treated with DMSO only. G – *In vitro* DMS treated genomic DNA.

3881 3891 3901 3911 3921
Topo I
GATGCATGCC TAGCGTGTTC TTT TTT TT TT CCAATGATTT GTAATATACA
CTACGTACGG ATCGCACAAG AAAAAAAAAA GGTTACTAAA CATTATATGT

Figure 3.1.1.3 - The position of the topoisomerase I cleavage sites at the lamin B2 origin in asynchronous HeLa cells.

These cleavages lie very close to the position of the start-sites of bi-directional DNA synthesis, which correspond to nucleotide 3990 for the lower strand and 3993 for the upper strand, and are located on the strands template for leading strand synthesis. Figure 3.1.1.3 illustrates the position of the topo I cutting sites in relationship with the position of the start-sites of DNA synthesis.

These breaks are not detected when the cells are only treated with DMSO, the solvent used for CPT. Furthermore, when the cells are treated in the same manner with gimatecan, a CPT derivative which has a different electronic structure, the same cleavage can be detected, by LMPCR analysis, on the lower strand (figure 3.1.1.4). This proves that the position of the topo I cut is determined by the enzyme and is independent of the drug used to stabilize the cleavage complex.

The CPT frozen topo I cleavable complexes are quickly and fully reversible. Upon drug removal and incubation of the cells for 5 minutes in complete medium both the upper and lower strand cuts disappear when LMPCR analysis of the origin area is performed (see figure 3.1.1.2).

Nonetheless, CPT is a very efficient anti-cancer drug and its cytotoxicity is mainly due to the formation of replication dependent double stranded breaks (DSBs) when a replication fork collides with a stabilized topo I-DNA cleavable complex (Hsiang et al., 1989 and Ryan et al., 1991). Also, these DSBs lead to the phosphorylation of histone H2AX which in turn induces the recruitment of the Mre11-Rad50-Nbs1 (MRN) complex involved in the repair of DNA damage (Furuta et al., 2003).

Therefore CPT probably activates a variety of checkpoints inside the cell by its action on topo I and, even though the time of CPT treatment used for the above experiments was very short (only one minute) at a low drug concentration of 1μM, there still a possibility that the single stranded breaks detected by LMPCR are due to a side effect of the CPT treatment. In order to rule out a possible disruption of the interaction of the replicative complex with the origin upon CPT treatment, a photofootprinting analysis of the origin region was performed in the presence and absence of CPT.

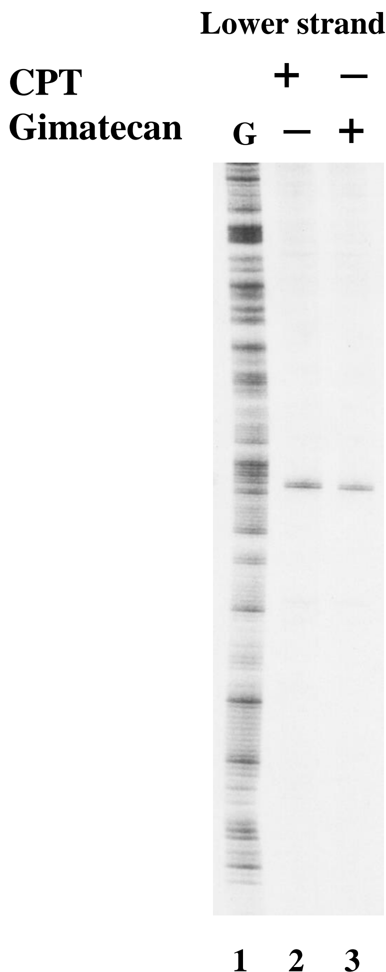


Figure 3.1.1.4 - The effect of gimatecan on the formation of the *in vivo* topo I cleavable complex. The position of the topo I cutting site is not influenced by the drug used to stabilize the topo I-DNA covalent complex. Lane 2 – cells treated with 1μM CPT. Lane 3 – cells treated with 10μM gimatecan. G – *In vitro* DMS treated genomic DNA.

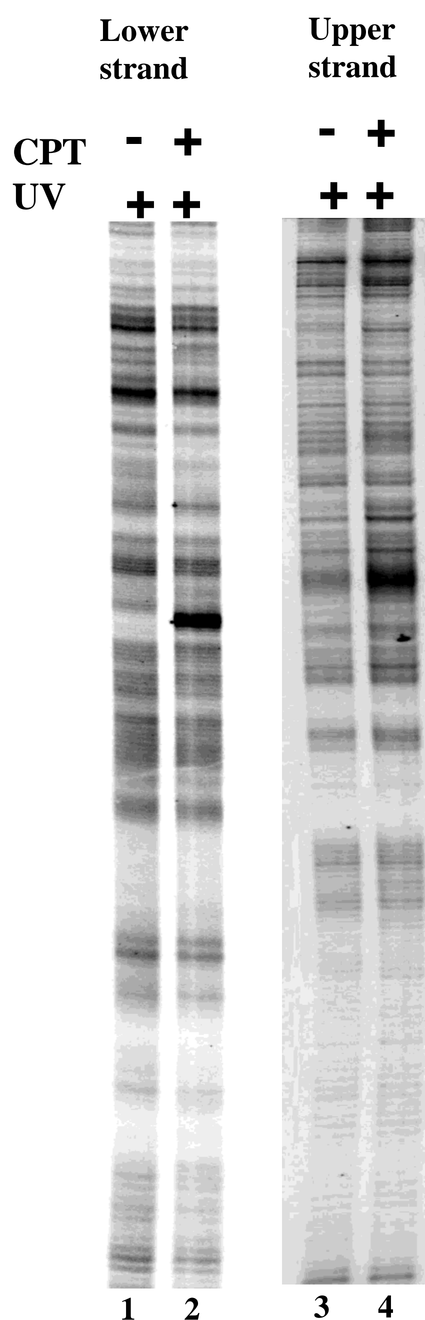


Photo-footprinting is an alternative method to chemical footprinting which has the advantage of highlighting both the protein-DNA interactions as well as the DNA conformation within a region. Asynchronous growing HeLa cells treated or not with 1μM CPT, as in the above experiments, were washed with PBS supplemented or not with 1μM CPT and, after removal of all liquid, they were irradiated directly in the Petri dishes with one femtosecond pulse of two wavelengths (266nm and 400nm) coming from a femtosecond UV laser source. The cells were lysed immediately after irradiation, the DNA was isolated as before and TDPCR analysis was performed (see figure 3.1.2.1 for an outline of the TDPCR procedure). The resulting pattern of DNA damage detected at the origin (the footprint) represents a combination of UV DNA damage and protein-DNA crosslinks and gives an overall image of the DNA conformation in this region. The results are presented in figure 3.1.1.5.

Figure 3.1.1.5 - Photofootprinting analysis of the lamin B2 origin area in asynchronous cells treated or not treated with CPT. The band which appears after CPT treatment corresponds to the topo I-DNA cleavage complex previously mapped.

A comparison of the overall footprint in the cells treated or not with CPT reveals no significant change over a region of ~500bp containing the origin, with the exception of a prominent band which appears after CPT treatment on both upper and lower strand. This band corresponds to the previously detected LMPCR stop arisen from the inhibition of the topo I religation step and the consequent stabilization of the DNA single stranded break.

Therefore treatment with CPT does not alter the existing protein-DNA interactions at the origin in a significant manner.

3.1.2 Topoisomerase II.

For the detection of an interaction of topoisomerase II with the lamin B2 origin of DNA replication, another anti-cancer drug was used, etoposide or VP16, a poison specific for topo II. Similar

to CPT, VP16 reversibly inhibits the religation step in the topo II catalytic cycle, leaving the enzyme covalently trapped on the DNA. In contrast to topo I, topo II becomes covalently attached to the 5'end of the DNA, and, as a consequence, it is impossible to use LMPCR amplification for the detection of this type of cleavage. To circumvent this problem, TDPCR was used, a procedure very similar to LMPCR which is insensitive to the state of the 5' end of the DNA. A schematic diagram of the method used to map the topo I-DNA cleavage complexes is shown in figure 3.1.2.1.

Asynchronous growing HeLa cells were incubated for 1 minute in complete medium supplemented with 10nM VP16 and then washed twice with PBS containing 10nM VP16. All liquid was removed and the cells were lysed in a lysis buffer supplemented with 10nM VP16. Total cellular DNA was isolated, after proteinase K treatment of the lysate, by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and resuspended in 20μl of 0.1x TE buffer pH 7.4. 4μl of DNA were used to perform the TDPCR amplification for the detection of the topo II DNA cleavages. The results are shown in figure 3.1.2.2.

Topo II cleaves the origin, inside the area covered by the replicative complexes, in a manner similar to topo I: two single stranded cuts are observed, on the upper and lower strand respectively, close to the start-sites of DNA replication. The precise position of these cleavages is between nucleotides 3914 and 3915 on the upper strand and between nucleotides 3940 and 3941 on the lower strand, on the strands template for leading strand synthesis. Figure 3.1.2.3 illustrates the position of the topo II cleavage sites with respect to the start-sites of DNA synthesis.

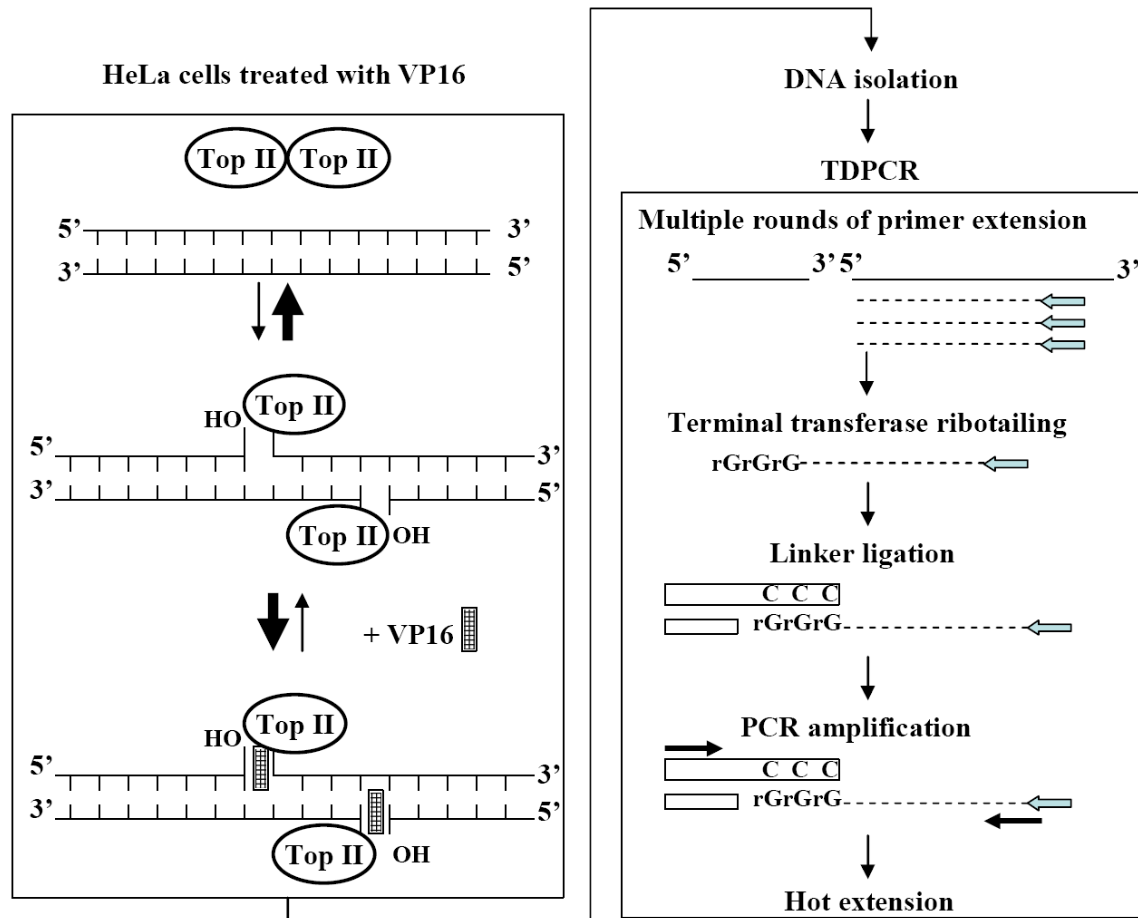


Figure 3.1.2.1 - Mapping of the topoisomerase II-DNA cleavage complexes – experimental outline. HeLa cells were treated with 10nM VP16 for one minute in order to stabilize the topo II-DNA covalent complexes (left panel). The cells were then lysed, the total genomic DNA was isolated and used for terminal transferase dependent PCR (TDPCR) analysis (right panel). As a first step a sequence specific primer was annealed to the region of interest and multiple rounds of polymerase extension were performed. The extension products, thus obtained, were subjected to 3'end ribotailing using terminal transferase (TdT) and riboG. This ribonucleotide is not the preferred substrate for TdT and under determined conditions the enzyme is able to add only 3 nucleotides (on average). The three riboGs are complimentary to an overhand of three C residues contained in the asymmetric linker, which aides the linker ligation reaction. The next step involves PCR amplification of the substrate using a second sequence specific primer and a primer complimentary to the linker sequence. As a final step, a third sequence specific primer, radioactively labeled, is used for hot extension and the product is then visualized, following sequencing gel electrophoresis, by autoradiography.

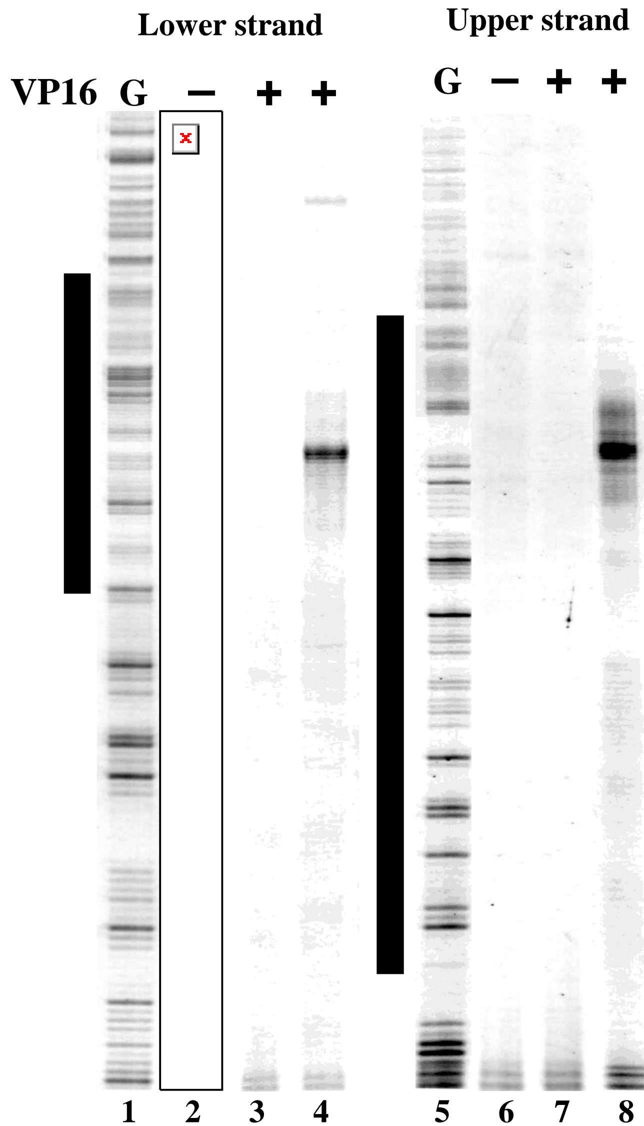


Figure 3.1.2.2 - In asynchronous HeLa cells topoisomerase II cleaves the region of the lamin B2 origin which is covered by the replicative complexes. On both the upper and lower strand, respectively, one TDPCR stop corresponding to one VP16 stabilized topo II cleavage complex can be detected. These stops correspond to the DNA 5' end arisen from the topo II action at the origin. The black bars indicate the origin area covered by a protein footprint in asynchronous HeLa cells. Lanes 4,8 – cells treated with 10nM VP16 for one minute. Lanes 3,7 - cells treated with 10nM VP16 for one minute, washed from the drug and released for 5 minutes in complete medium. Lanes 2, 6 – cells treated with DMSO only. G – *In vitro* DMS treated genomic DNA.

3881 3891 3901 3911 3921 3931
Topo
GATGCATGCC TAGCGTGTTC TTT TTT TT TT CCAATGATTT GTAATATACA TTTT
CTACGTACGG ATCGCACAAG AAAAAAAAAA GGTTACTAAA CATTATATGT AAAA

Figure 3.1.2.3 - The position of the topoisomerase II cleavage sites at the lamin B2 origin in asynchronous HeLa cells.

As expected these cleavages are not detected when the cells are treated with DMSO in the absence of the topo II poison. Furthermore the VP16 stabilised topo II cleavage complexes are fully reversible; if the cells are washed from the drug and incubated in complete medium for 5 minutes no cleavage can be detected by TDPCR analysis on either origin upper or lower strands.

In order to ensure that the treatment with VP16 has not disturbed the pre-existing protein-DNA interactions in the origin area, a photofootprinting analysis was performed in the presence and absence of the anti-cancer drug. As in the case of the photofootprinting controls for CPT treatment, the cells were treated or not with VP16, washed with PBS with or without drug addition and, after complete

removal of all liquid, they were irradiated directly on the Petri dishes with one femtosecond pulse of two wavelengths (266nm and 400nm) coming from a femtosecond UV laser source. The results are displayed in figure 3.1.2.4.

Comparison of the footprinting pattern obtained by TDPCR analysis of a 500bp region containing the lamin B2 origin shows no significant difference, on both upper and lower strands, between the VP16 treated or untreated samples, with the exception of one major band present after drug treatment. This band corresponds to the VP16 frozen topo II cleavage-complex previously mapped.

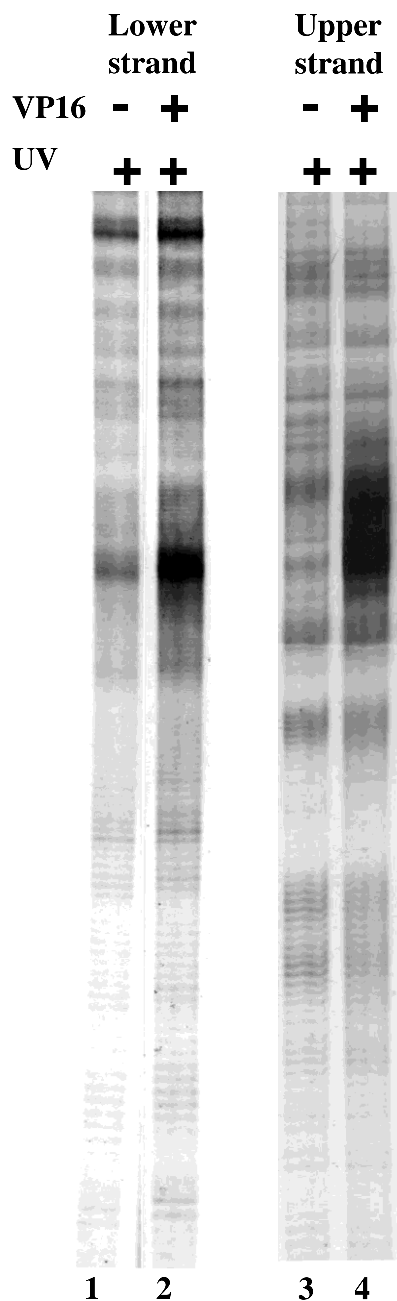


Figure 3.1.2.4 - Photofootprinting analysis of the lamin B2 origin area in asynchronous cells treated or not treated with VP16. The band which appears after VP16 treatment corresponds to the topo II-DNA cleavage complex previously mapped.

3.2 Mapping of the interaction of topoisomerases I and II with the lamin B2 origin in synchronized cells.

3.2.1 Topoisomerase I.

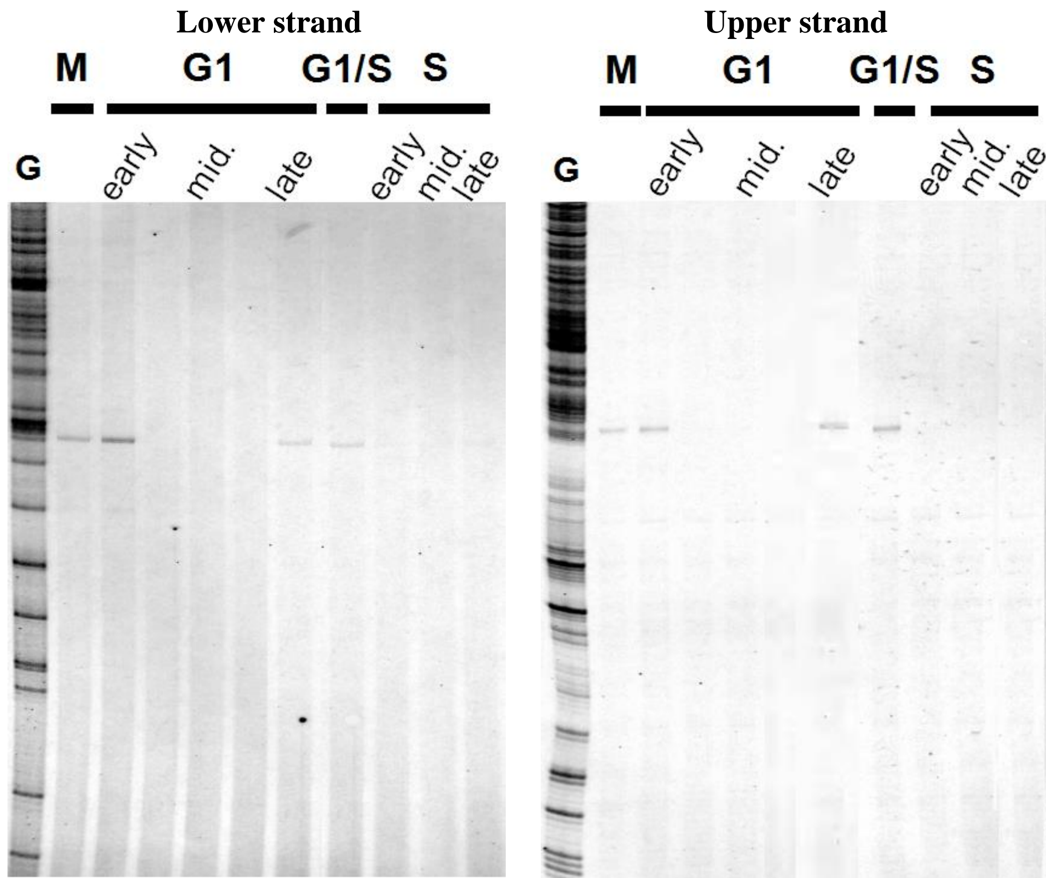
In order to establish in which phases of the cell cycle topoisomerase I interacts with the origin, HeLa cells were synchronized in M, G1, G1/S and S phases of the cell cycle, treated with 1μM CPT for one minute and lysed as for the experiments involving asynchronous cells. For cell synchronization three different drugs were used: nocodazole in order to obtain M phase cells, a drug which inhibits microtubule polymerization, mimosine for late G1 - G1/S border cells, a strong iron chelator which is thought to inhibit ribonucleotide reductase and therefore lower intracellular dNTP pools, and aphidicolin for G1/S border - early S cells, a DNA polymerase inhibitor. The DNA was isolated and LMPCR was performed for the upper and lower strand of the origin region using the same primers

which allowed the detection of the topo I cutting sites in asynchronous HeLa cells. The results are shown in figure 3.2.1.1.

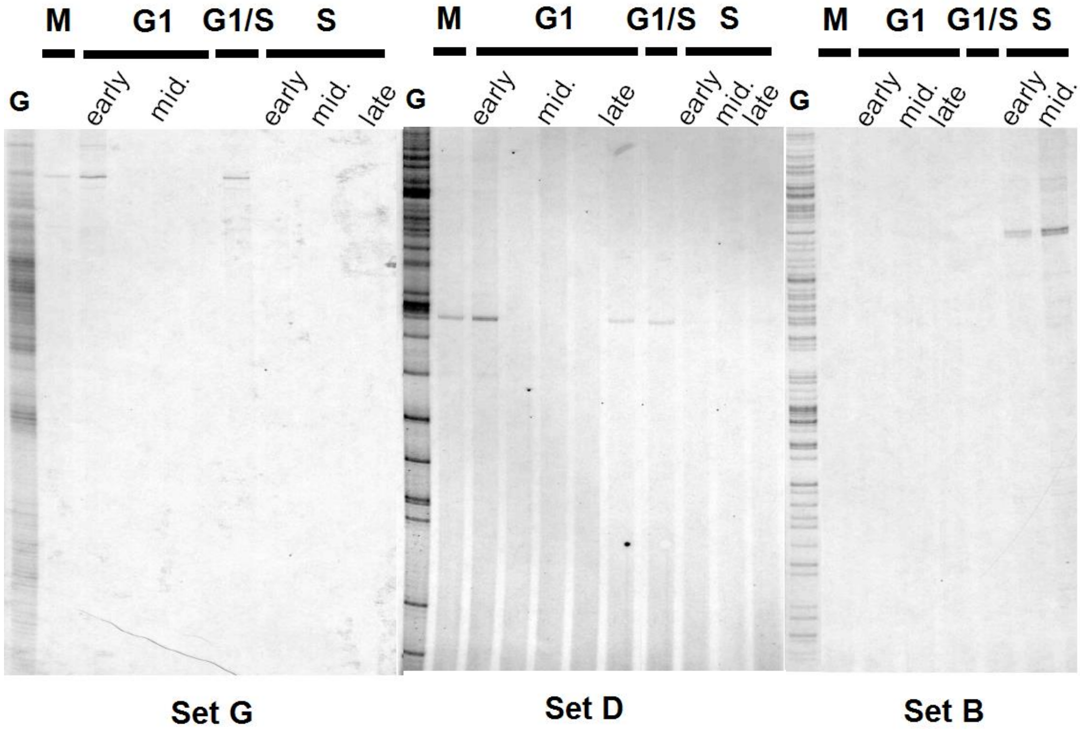
In synchronized cells topo I introduces single-stranded nicks in both the origin upper and lower strand in M phase, very early G1 and late G1 - G1/S border. The position of these cleavages coincides with the position of the two cleavages mapped in asynchronous growing HeLa cells.

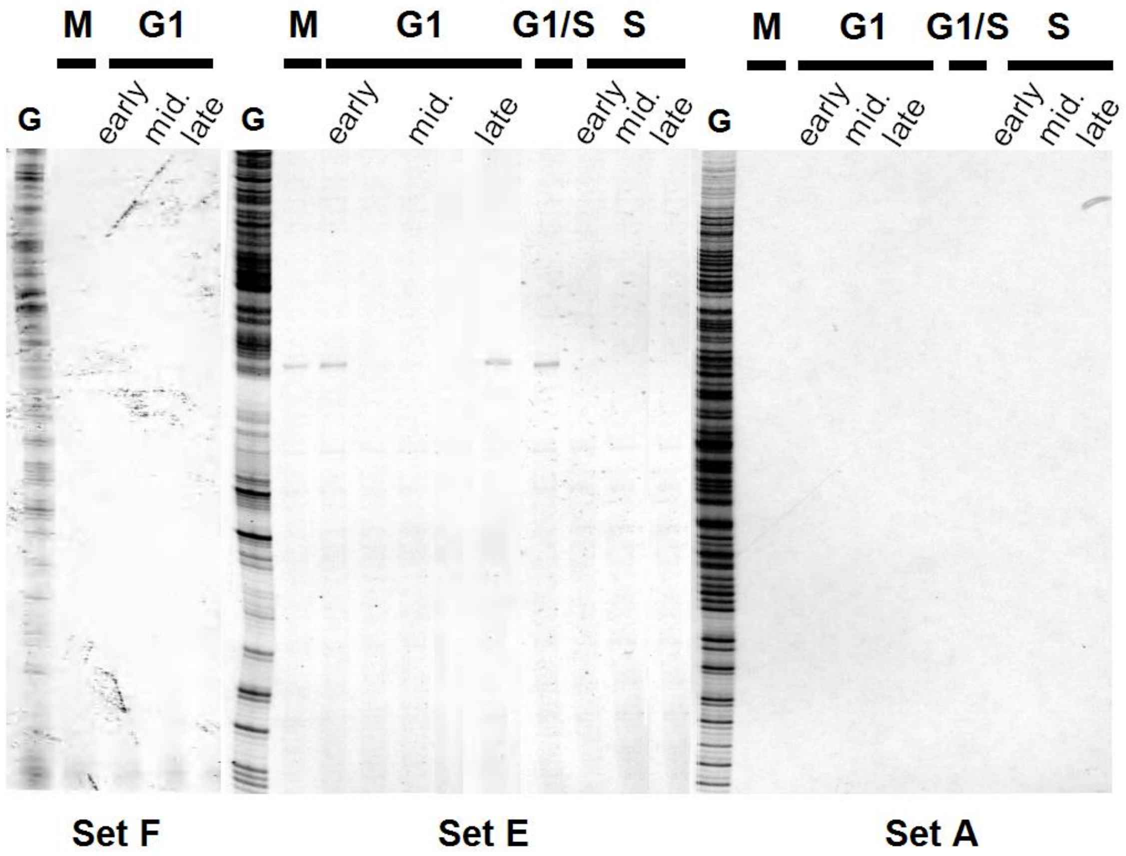
With the aim of further characterizing the dynamic relationship of topoisomerase I with the origin, the surrounding region was also analyzed by LMPCR. Different primer sets were used to explore both the upper and lower strand, to the left and to the right of the origin, for an extended analysis of ~1500bp (for the position of the primers see figure 3.2.1.2). Since human topo I seems to be involved in many aspects of DNA metabolism which might not be necessarily connected with DNA replication, this supplementary LMPCR analysis in synchronized HeLa cells provides a better picture of the topological requirements for topo I activity in this region.

The results, illustrated in figure 3.2.1.2, show that topo I function appears to concentrate at the origin since in the region immediately adjacent the enzyme does not cleave the DNA. There is, however, one exception to this observation, namely a topo I cutting site, exclusively in the middle of S phase, approximately in the region of nucleotides 4335 to 4340, close to the promoter of the TIMM13 housekeeping gene. It should be noted that the lamin B2 origin is an early firing origin with the peak of nascent DNA enrichment (as analyzed by competitive PCR) detectable in the first minutes of S phase. The TIMM13 promoter is cleaved by topo I after at least 1h of S, at which point the region would have already been replicated. In view of the fact that topo I is actively involved in transcription, this cleavage is very likely connected with the transcriptional regulation of the TIMM13 gene.



<u>Figure 3.2.1.1</u> - Interaction of DNA topoisomerase I with the lamin B2 origin in synchronized HeLa cells. Lanes 2, 13 - M phase. Lanes 3, 14 - 30 minutes of G1. Lanes 4, 15 - 1 hour of G1. Lanes 5, 16 - 5 hours of G1. Lanes 6, 17 - 7 hours of G1. Lanes 7, 18 - late G1 - G1/S border (mimosine). Lanes 8, 19 - G1/S border - early S (aphidicolin). Lanes 9, 20 - 1 hour of S. Lanes 10, 21 - 5 hour of S. Lanes 11, 22 - 7 hour of S. G – *In vitro* DMS treated genomic DNA.


M
G1
G1/S
S
G
early
mid.
early
mid.
late
M
G1
G1/S
S
G
early
mid.
late
early
mid.
late
M
G1
G1/S
S
G
early
mid.
late
early
mid.
Set G
Set D
Set B


M
G1
G
early
mid.
late
M
G1
G1/S
S
G
early
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early
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G1
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early
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early
mid.
late
Set F
Set E
Set A

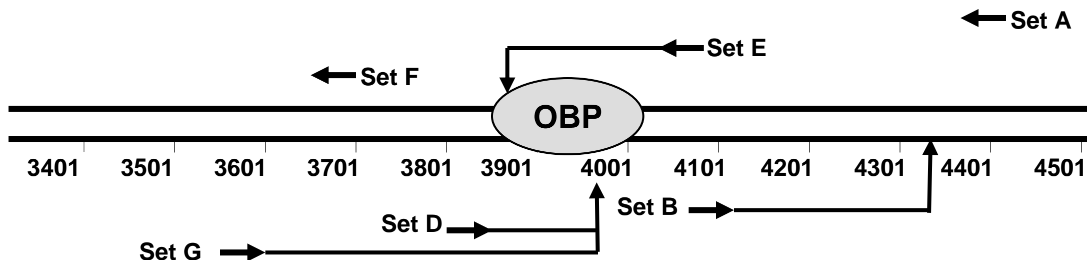


Figure 3.2.1.2 - Interaction of DNA topoisomerase I with the 1.5kb region surrounding the lamin B2 origin in synchronized HeLa cells. Primer sets D and E are the primers used to analyze the origin area (see figure 3.2.1.1). Primer set B allows the detection of a topo I cleavage site, on the lower strand, in the vicinity of the TIMM13 gene promoter. G – *In vitro* DMS treated genomic DNA.

3.2.2 Topoisomerase II.

An analysis of the behavior of topoisomerase II in synchronized cells has also revealed a dynamic interaction of this enzyme with the origin. HeLa cells were synchronized in M, G1, G1/S and S phases of the cell cycle, treated with 10nM VP16 for one minute, washed, lysed and the isolated DNA was analyzed by TDPCR as in the case of asynchronous cells. The results are shown in figure 3.2.2.1.

The upper and lower strand topo II cutting sites mapped at the origin in asynchronous growing cells were found to be present only in M phase and in the middle of G1. An additional TDPCR analysis was performed in the case of topo II as well, with the region surrounding the origin being investigated and the results reported in figure 3.2.2.2. For this analysis the same primers were used as in the case of topo I and a map with their position is found in figure 3.2.1.2.

As in the case of topo I, the topo II activity appears to be concentrated at the origin with no cleavages being present outside this region apart from one cleavage site on the lower strand, between nucleotides 3727 and 3728. This position is over 200bp away from the origin topo II cutting sites and, it seems that, contrary to the origin cuts, this particular topo II-DNA interaction is constant throughout the cell cycle. Due to the difference in the behaviour of the two topo II molecules, at and outside of the origin, during the cell cycle, a logical supposition is that the two enzymes belong to two different nuclear topo II subsets involved in different processes.

In M phase both topo I and topo II seem to be in contact with the origin DNA, however, it is not known if this interaction occurs simultaneously on the same DNA molecules.

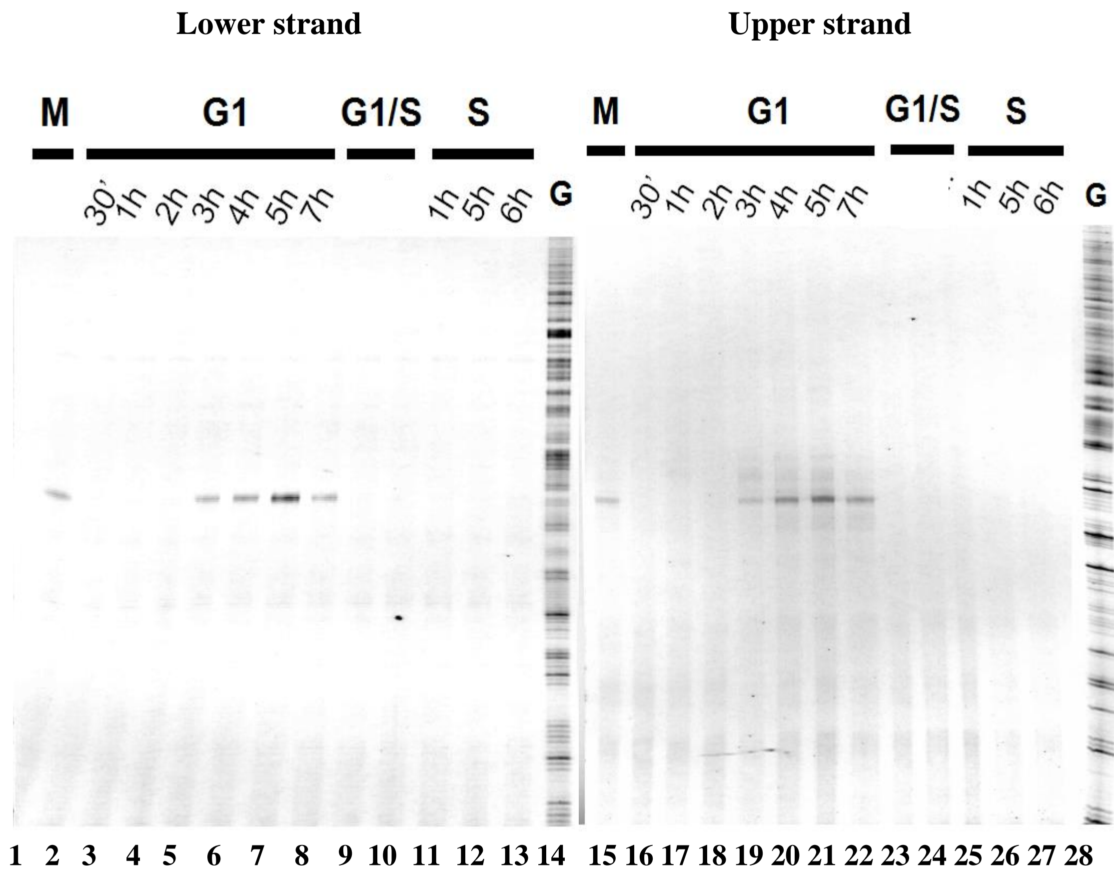
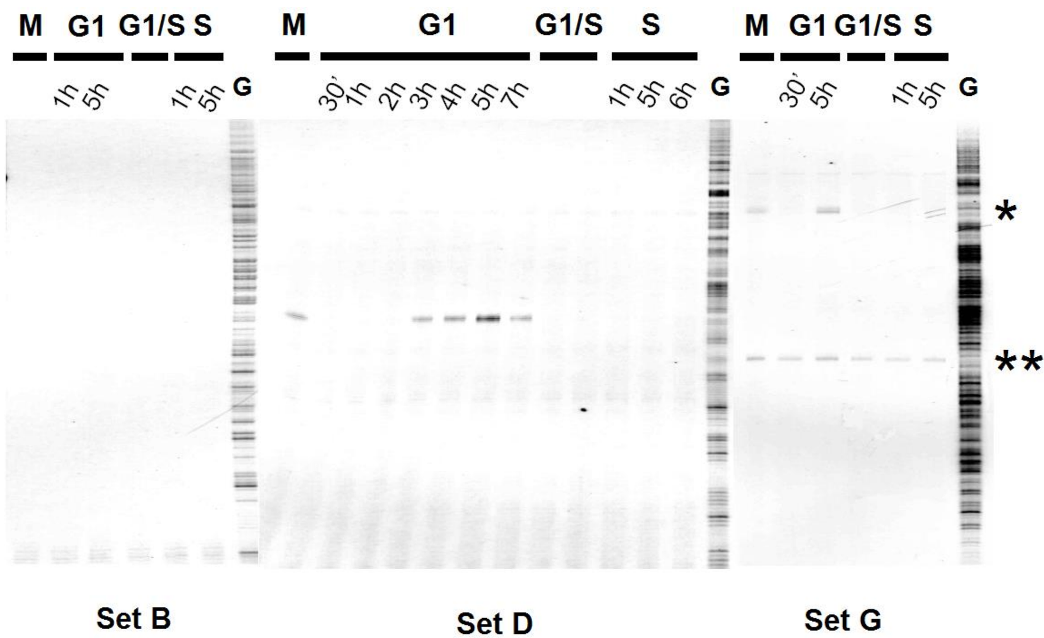


Figure 3.2.2.1 - Interaction of DNA topoisomerase II with the lamin B2 origin in synchronized HeLa cells. Topo II introduces single stranded cuts on both the origin lower and upper strands in M phase (lanes 1 and 15) and from 3 up to 7 hours of G1 with a peak in 5 hours of G1 (lanes 5-8 and 19-22). G – *In vitro* DMS treated genomic DNA.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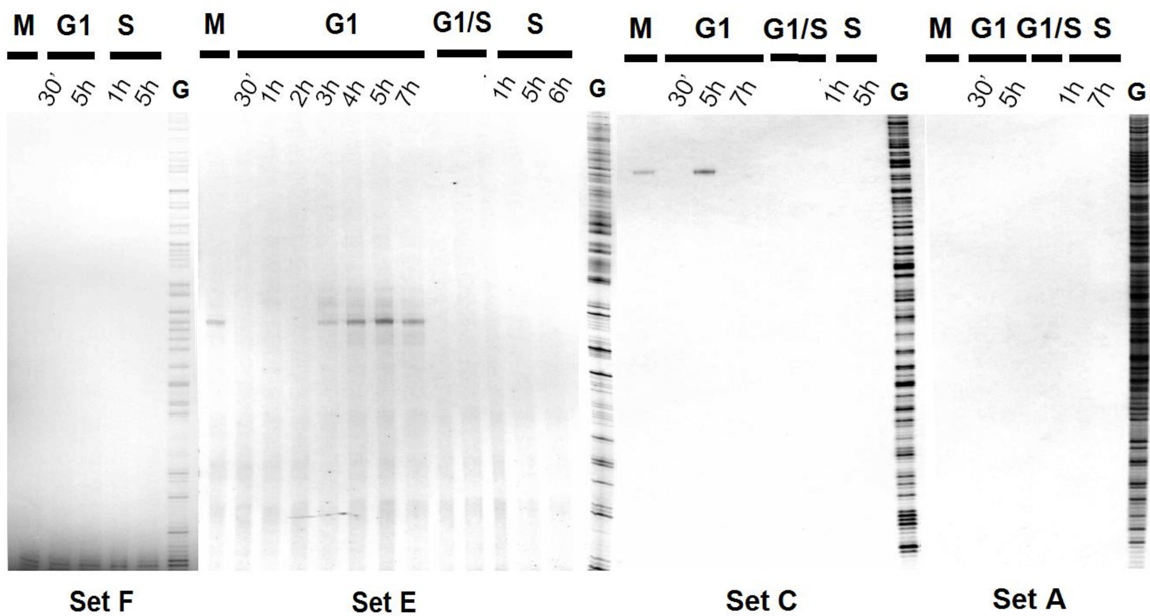
1h 5h 1h 5h G
Set B
M G1 G1/S S
30' 1h 2h 3h 4h 5h 7h 1h 5h 6h G
Set D
M G1 G1/S S
30' 5h 1h 5h G
*
**
Set G


M G1 S
30' 5h 1h 5h G
Set F
M G1 G1/S S
30' 1h 2h 3h 4h 5h 7h 1h 5h 6h G
Set E
M G1 G1/S S
30' 5h 7h 1h 5h G
Set C
M G1 G1/S S
30' 5h 1h 7h G
Set A

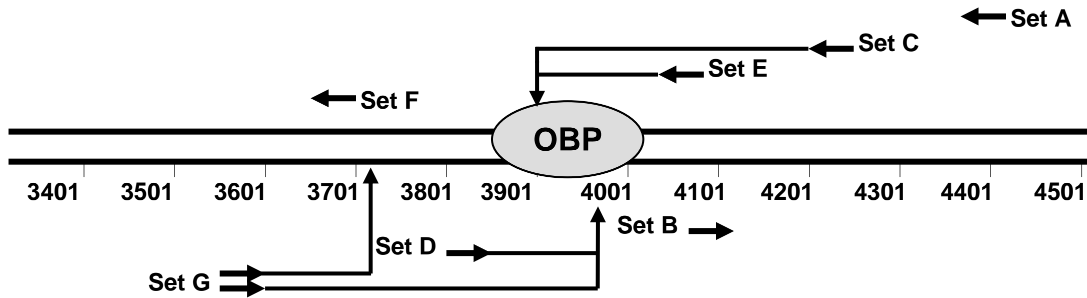


Figure 3.2.2.2 - Interaction of DNA topoisomerase II with the 1.5kb region surrounding the lamin B2 origin in synchronized HeLa cells. Primer sets D and E are the primers used to analyze the origin area in asynchronous cells. Primer set G allows the detection, on the lower strand, of the same origin topo II cleavage site as primer set D (*) along with a second topo II cleavage site far away from the origin (**). G – *In vitro* DMS treated genomic DNA.

3.3 Topoisomerase – ORC interactions at the lamin B2 origin.

3.3.1 Topoisomerase I.

Given the presence of the topoisomerase I enzyme at the origin at late G1 - G1/S border, a crucial moment for replicon initiation, it is appealing to assume that this interaction has a role in origin firing. Nonetheless the mere presence of a protein in this region does not automatically imply a role in DNA replication. In order to verify that indeed topo I is involved in origin regulation an interaction with other members of the pre-initiation complex should be proven.

The hOrc2p, member of the human origin recognition complex, has been shown to interact with the origin in both G1 and S phases of the cell cycle and its position on the DNA (only on the lower strand) was mapped with nucleotide resolution (Abdurashidova et al., 2003). In the middle of G1, Orc2 is bound at the origin on the lower strand at nucleotide 3960 (as detected by UV laser DNA-protein crosslinking), while in S phase its position on the DNA is shifted 17bp towards the start-sites, at nucleotide 3943 (see figure 1.4.1 in introduction for a map of the known protein-DNA interactions at the lamin B2 origin).

The exact position of Orc2 in late G1 or at the G1/S border is not known but it is logical to assume that the protein is still in contact with the DNA, as the 6-subunit ORC was shown to be an essential element for origin activity (Mendez and Stillman, 2003). To confirm this assumption, a ChIP assay was performed in cells synchronized in late G1 - G1/S border using anti-Orc2 antibodies.

HeLa cells synchronized in M phase were subjected to a second block using mimosine, a plant amino-acid which has been shown to block human cells shortly before initiation of S phase (Krude, 1999). Due to the lack of a precise estimation of the G1 time-point at which these cells are blocked, the synchronization with mimosine will be referred to as late G1 - G1/S border.

The synchronized cells were crosslinked with formaldehyde for 5 minutes, lysed, the DNA was sonicated and immunoprecipitation was performed with either anti-Orc2 or anti-topo I antibodies (as a positive control). The immunopurified protein-DNA covalent complexes were decrosslinked by heating for 5 minutes at 95°C, the proteins were digested with proteinase K and the DNA was isolated by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. In order to asses the abundance of origin and non-origin DNA, competitive PCR amplification was carried out for the B48

(origin) and B13 (non-origin) region. The relative abundance of B48 and B13 PCR products is shown in figure 3.3.1.1.

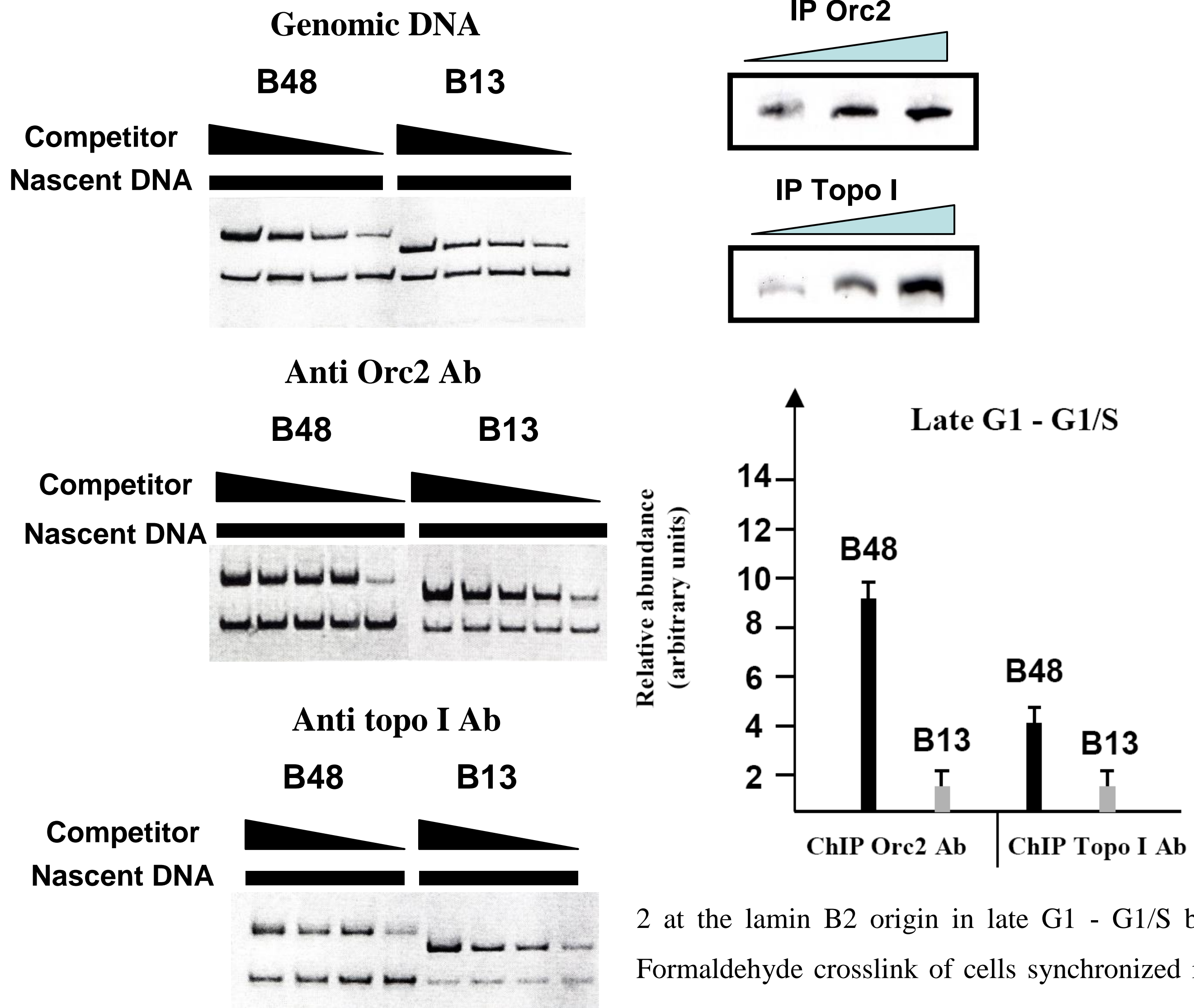


Figure 3.3.1.1 - Presence of Orc2 at the lamin B2 origin in late G1 - G1/S border. Formaldehyde crosslink of cells synchronized in late G1 - G1/S border, followed by ChIP using anti topo I or anti Orc2 antibodies and competitive PCR amplification of origin (B48) and non-origin (B13) DNA fragments demonstrates the presence of topo I (as a control) and Orc2 at the origin in this phase of the cell cycle. Western blot of increasing amounts of immunoprecipitate obtained with anti-Orc2 and anti-topo I antibodies is shown in the upper right panels.

As expected both ChIP assays for hOrc2p and htopo I showed an enrichment in origin fragments after competitive PCR amplification. The difference between the B48:B13 ratio in the case of Orc2 and topo I immunoprecipitation could represent the antibody affinity towards the antigen and

no stoichiometric estimation of the Orc2/topo I ratio on the DNA can be inferred from this experiment. It should be noted that the procedure involved no treatment with a topo I poison and therefore it reinforces the conclusion that CPT does not direct the topo I-DNA interaction but only interferes with the catalytic cycle of the enzyme.

Thus the Orc2p is indeed present on the DNA in late G1 - G1/S border and its precise position is most likely at one of the two binding sites previously mapped. Considering that on the lower strand the topo I cutting site overlaps one of these Orc2 binding sites and is close to the other (topo I cleaves nucleotides 3956/3957, while the two previously mapped Orc2 binding sites are at positions 3943 and 3960), the question arises whether these two proteins are interacting.

In order to confirm this hypothesis an elaborate experiment was performed aimed at showing that Orc2 and topo I are interacting concomitantly with the topo I cleavage of the origin DNA. An outline of this experiment is shown in figure 3.3.1.2. The nature of the topo I cleavage with the enzyme being covalently bound to the 3'end of the DNA makes it impossible to perform LM or TDPCR amplification following immunoprecipitation of the topo I cleavable complex. Both these techniques can only map the 5' end of the DNA break which will be lost during IP as it is not crosslinked to the enzyme. In order to circumvent this problem an alternative PCR amplification was devised based on the knowledge of the positions of the topo I cutting sites at the origin.

In a first instance the procedure was validated by a simple topo I immunoprecipitation experiment, performed after CPT treatment, followed by PCR amplification of the origin region. HeLa cells synchronized in late G1 - G1/S border with mimosine were treated for one minute with 1μM CPT, washed twice with PBS containing 1μM CPT, lysed with a solution of 1% sarkosyl in TE and loaded on top of a CsCl solution (1.5 g/ml density). The samples were centrifuged at 70000rpm for 5 hours, allowing the separation of the bulk DNA and protein-DNA complexes (bottom fraction) from free proteins (top fraction). The DNA fraction was collected and the origin region was liberated by restriction enzyme digestion. Anti-topo I antibodies were used to pull down the topo I-DNA cleavable complexes, the protein was digested with proteinase K and the DNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation.

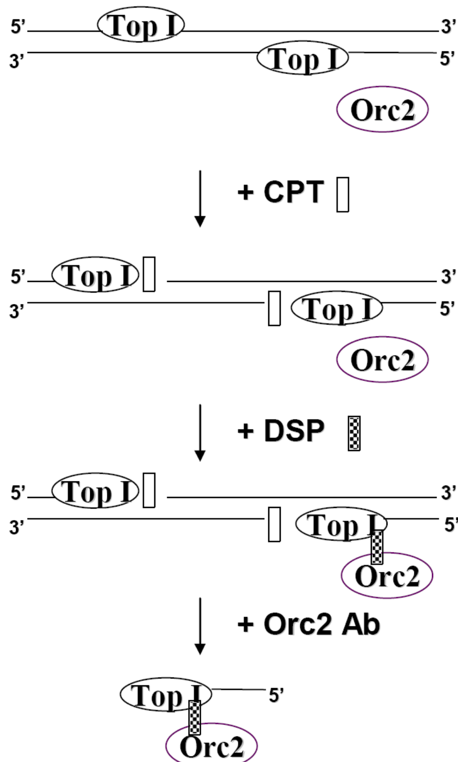
PCR amplification of the origin region was performed using three sequence specific primers chosen to amplify, simultaneously, a long (276bp) and a short (88bp) PCR fragment. The long PCR fragment contains both upper and lower strand topo I cleavage sites and is not amplifiable upon CPT treatment, while the short one is positioned adjacent and to the right of the lower strand topo I cutting

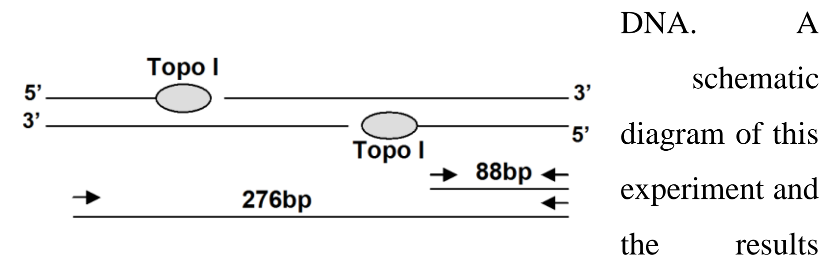
site. This makes it so that, upon CPT treatment and topo I IP, the substrate for the short PCR fragment is immunopurified, since it has the topo I enzyme covalently bound on its 3' end.

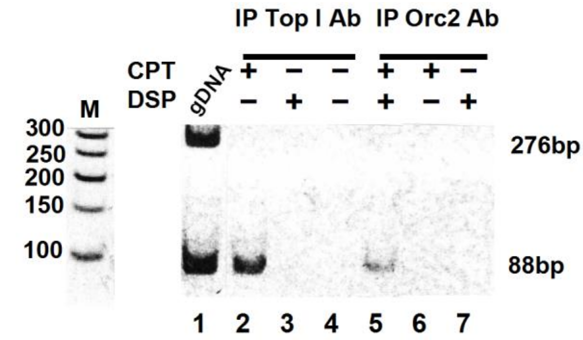
The results of this experiment are reported in figure 3.3.1.2 and, as can be observed, if in the absence of CPT both PCR fragments are amplified in an equal manner, upon CPT treatment only the short fragment is detected after PCR amplification. Needless to say this experiment brings further proof that the DNA single stranded breaks detected after CPT treatment correspond indeed to topo I action.

Having developed a tool for the detection of the topo I DNA substrate immunopurified with the aid of anti-topo I antibodies, the possible interaction of Orc2 with topo I at the origin was investigated. HeLa cells synchronized in late G1 - G1/S border were treated for one minute with 1μM CPT then treated for 5 minutes with dithiobis-(succinidylproprionate) (DSP or Lomant's reagent), a protein-protein crosslinking agent, still in the presence of CPT. The cells were washed twice with PBS containing 1μM CPT, lysed and loaded on top of a CsCl solution (1.5 g/ml density). The samples were centrifuged at 70000rpm for 5 hours, the DNA fraction was collected and the origin region was liberated by restriction enzyme digestion of the DNA. Immunoprecipitation was performed with either anti-topo I or anti-Orc2 antibodies, the proteins were digested with proteinase K, the DNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and PCR amplification of the long and short fragment was carried out.

If the Orc2p is in close proximity of the topo I enzyme the two will be crosslinked to each other by DSP. Having already treated the cells with CPT means that topo I is covalently bound to the DNA, therefore IP with an anti-Orc2 antibody will pull down Orc2 linked to topo I which in turn is linked to the DNA. If Orc2 is not in close proximity to topo I they will not be crosslinked together and the immunoprecipitate obtained using the anti-Orc2 antibody will not contain any DNA. A schematic diagram of this experiment and the results obtained are shown in figure 3.3.1.2.







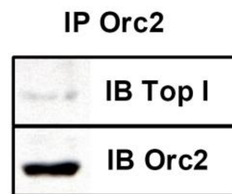


Figure 3.3.1.2 - Interaction of topoisomerase I with Orc2p at the lamin B2 origin in late G1 - G1/S border. PCR amplification of the origin region (gDNA – genomic DNA) was performed using three sequence specific primers (see the top right diagram for the position of the primers) in order to obtain a long (276bp) and a short (88bp) PCR fragment (lane 1). Due to the position of the primers, the long PCR fragment can be obtained only in the absence of CPT treatment, while the short product is insensitive to CPT action. Immunoprecipitation with anti topo I antibodies following CPT treatment of cells synchronized in late G1 - G1/S border allows the simultaneous purification of the DNA substrate used in the amplification of the short PCR fragment (lane 2). In the absence of CPT treatment, IP with anti topo I antibodies does not result in concomitant origin DNA purification (lane 4). CPT treatment coupled with DSP protein-protein crosslinking, in cells synchronized in late G1 - G1/S border, followed by IP with anti Orc2p antibodies, leads to the simultaneous purification of the same DNA substrate used in the amplification of the short PCR fragment (lanes 5). Topo I co-precipitates with Orc2 also in the absence of DSP crosslinking. Immunoprecipitation of Orc2p from HeLa nuclear extracts also pulls down topo I (lower right panel).

The Orc2 and topo I proteins are in fact interacting at the lamin B2 origin, IP with an anti-Orc2 antibody pulls down DNA which can be used to amplify the short PCR fragment but not the long one. Notably treatment of the cells with either CPT or DSP does not result in origin DNA immunopurification by the anti-Orc2 antibody.

These results prove that topo I is a member of the origin binding complex at late G1 - G1/S border and that it interacts directly with the Orc2 member of the human ORC.

3.3.2 Topoisomerase II.

Having established that topo I is a protein partner of Orc2 in the origin pre-replicative complex, the possibility that topo II might also interact with Orc2 was explored. As shown by the experiments

with synchronized cells, topo II cleaves the origin region in the middle of G1, a time point for which the Orc2 position on the DNA (at least on the lower strand) is known. The topo II lower strand cleavage site is only 19 nucleotides away from the Orc2 binding site (topo II cleaves nucleotides 3940/3941, while Orc2 binds position 3960). Taking into account that topo II is a large enzyme (the molecular weight of topo IIα is 170kDa while that of topo IIβ is 180kDa) it is conceivable to assume that the two proteins are almost in contact if bound to the same DNA molecule.

In order to prove this deduction, an experiment similar to the topo I-Orc2 crosslinking assay was used. The property of topo II of becoming covalently bound to the 5'end of the DNA break during its catalytic cycle means that TDPCR analysis can be used also after immunoprecipitation with an anti-topo II antibody (the 5'end of the DNA will be immunopurified along with the topo II protein covalently attached to it).

HeLa cells treated with 10nM VP16 for 1 minute were washed twice with PBS containing 10nM VP16, lysed and loaded on top of a CsCl solution (1.5 g/ml density). The samples were centrifuged at 70000rpm for 5 hours, the DNA fraction was collected and the origin region was liberated by restriction enzyme digestion of the DNA. Immunoprecipitation was performed with anti-topo IIα antibodies, the proteins were digested with proteinase K, the DNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and analyzed by TDPCR. As can be observed in figure 3.3.2.1, the DNA immunopurified with the anti-topo IIα antibody, shows on the origin lower strand a break at the same position as the previously mapped topo II lower strand cutting site. This is further proof that the topo II enzyme is indeed responsible for the DNA breaks detected after VP16 treatment.

HeLa cells synchronized in the middle of G1 were treated for 1 minute with 10nM VP16 and then treated for 5 minutes with DSP in the presence of VP16, washed twice with PBS containing 10nM VP16, lysed and loaded on top of a CsCl solution (1.5 g/ml density). The samples were centrifuged at 70000rpm for 5 hours, the DNA fraction was collected and the origin region was liberated by restriction enzyme digestion of the DNA. Immunoprecipitation was performed with either anti-topo IIα or anti-Orc2 antibodies, the proteins were digested with proteinase K, the DNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation and TDPCR analysis was performed with a primer set which allows the detection of both origin and non-origin topo II molecules (see section 3.2.2). The results of this experiment are reported in figure 3.3.2.2.

In the middle of G1 topo II and Orc2 are in close proximity at the lamin B2 origin, seeing that they can be crosslinked by DSP. Upon combined treatment with VP16 and DSP, immunoprecipitation with anti-Orc2 antibodies pulls down a DNA fragment which contains a break at the same position as the topo II lower strand cleavage site. Notably the same anti-Orc2 antibody is not able to immunoprecipitate a DNA fragment which contains a break corresponding to the non-origin topo II cleavage site. This difference in protein partners is an additional piece of evidence towards the hypothesis that the origin and non-origin bound topo II molecules perform different functions on the DNA (see section 3.2.2).

In brief, in the middle of G1, topo II is an ORC protein partner via its interaction with Orc2 and is a certified member of the origin pre-replication complex.

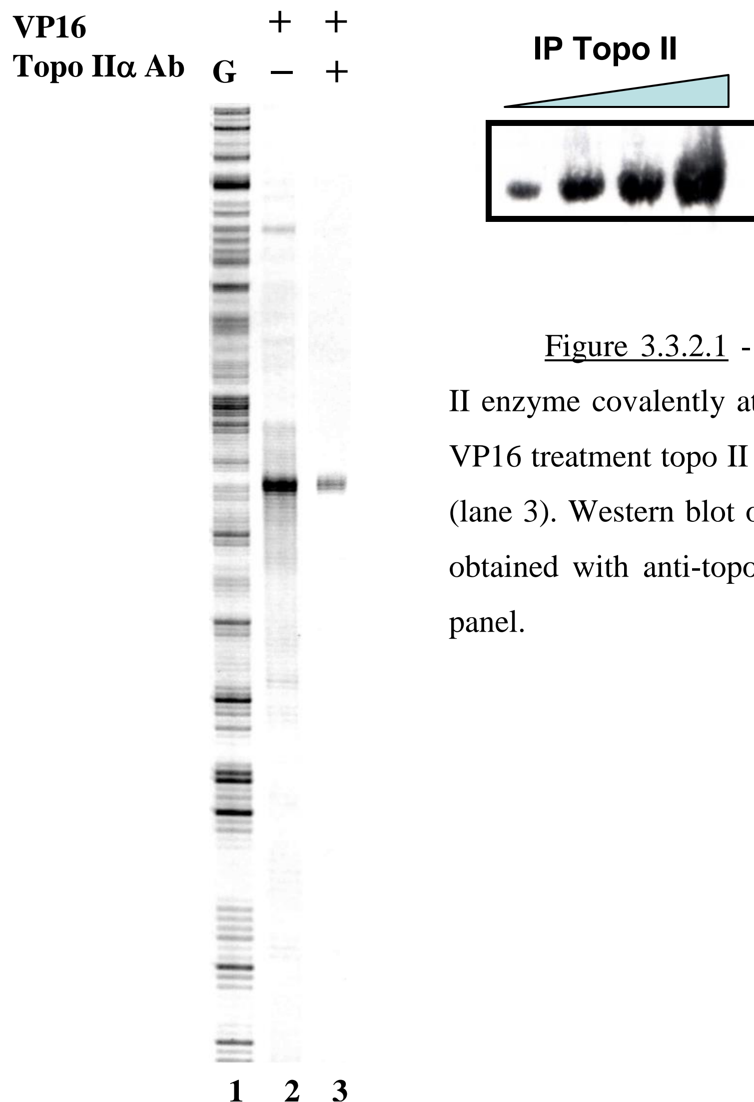
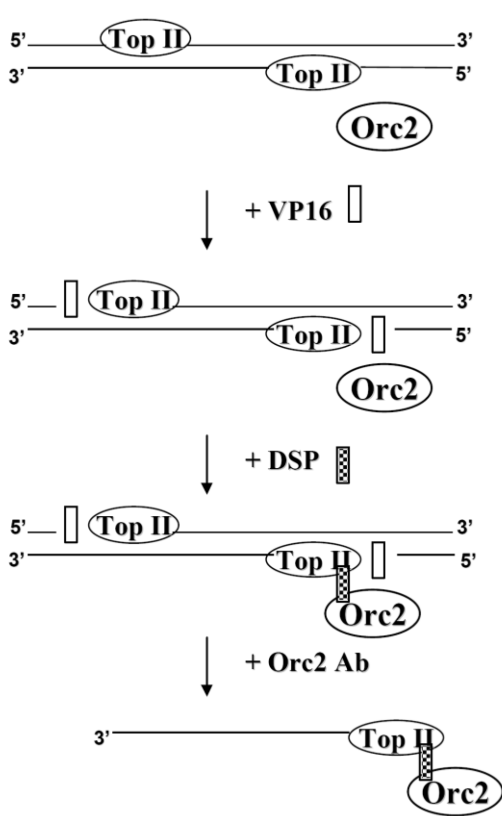
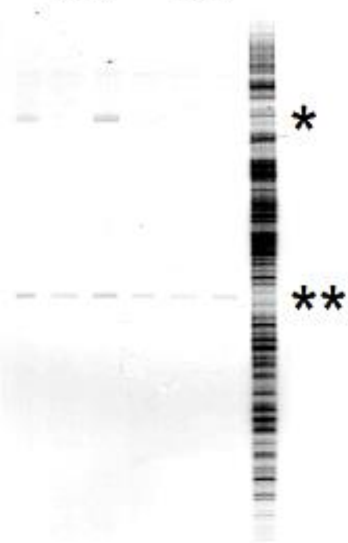


Figure 3.3.2.1 - Immunoprecipitation of the topoisomerase II enzyme covalently attached to the lamin B2 origin DNA. Upon VP16 treatment topo II is covalently crosslinked to the origin DNA (lane 3). Western blot of increasing amounts of immunoprecipitate obtained with anti-topo II antibodies is shown in the upper right panel.





1 2 3 4

Figure 3.3.2.2 - Interaction of topoisomerase II with Orc2p at the B2 origin in the middle of G1. VP16 treatment coupled with DSP protein-protein crosslinking, in cells synchronized in the middle of G1, followed by immunoprecipitation with anti Orc2p antibodies, leads to the simultaneous purification of a DNA fragment which contains a break at the same position as the lower strand topo II cleavage (lane 10). The topo II molecule bound throughout the cell cycle outside of the origin (lanes 1-6) does not interact with Orc2. * - lower strand topo II cleavage site at the origin. ** - lower strand topo II cleavage site outside of the origin region. G – *In vitro* DMS treated genomic DNA.

3.4 The role of topoisomerase I in lamin B2 origin activation.

What is the role of topoisomerase I as a member of the origin binding complex at late G1 - G1/S border? Certainly right before origin firing modulation of DNA topology must be very important, but does topo I play a crucial role in this process? One way to investigate this issue would be to asses origin activity in the presence of CPT, having thus blocked the topo I activity. If the topo I relaxation activity represents an essential step in origin firing, then CPT treatment should abolish DNA synthesis. If instead topo I function is not absolutely required for initiation of DNA synthesis, then the presence of the blocked topo I molecules on the DNA will lead to the formation of double stranded breaks by a process called ‘replication run-off’.

Pommier and colleagues have shown that upon CPT treatment of human colon carcinoma HT29 cells they could detect the conversion of topoisomerase I cleavage complexes into replication mediated DNA double-strand breaks (Strumberg et al., 2000). This process was only detected on the leading strand of DNA synthesis and was due to the collision of a replication fork with a persistent single stranded break corresponding to the CPT stabilized topo I-DNA cleavable complex. They also observed that the extension of the nascent DNA strand occurred all the way to the DNA break leading to a double-stranded DNA blunt end, which could be in turn used as a substrate directly for the ligation step in LMPCR.

LMPCR is a technique devised for mapping DNA breaks within a known region and differs from a normal PCR amplification in the fact that only one end of the DNA amplified is known. PCR amplification is achieved by using a sequence specific primer complimentary to the region analysed and a second primer complimentary to a linker molecule ligated to the DNA substrate which is being analyzed.

When performing a LMPCR analysis in order to detect the 5’ end of the DNA break corresponding to a topo I covalent complex (the enzyme is attached covalently to the 3’ end of the nick), the first step involves the annealing of a sequence specific primer and polymerase extension up to the cleavage in order to obtain a double stranded DNA blunt end. This is in turn ligated to an asymmetric linker and PCR amplification is performed.

The double-stranded DNA blunt end arisen from the replication run-off process is also amenable to ligation to the asymmetric linker and, thus, there is no need to perform the initial step of LMPCR, the extension from the sequence specific primer. This replication run-off property of

generating double stranded breaks can be exploited to distinguish between origin firing and non-firing upon CPT treatment. Figure 3.4.1 illustrates this concept.

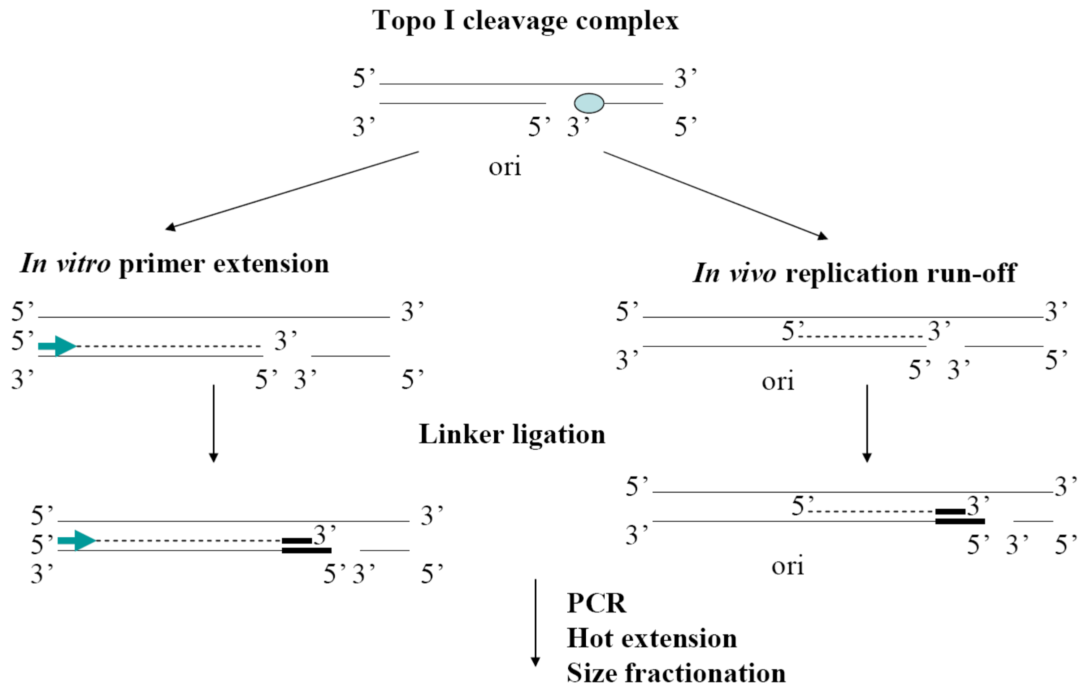


Figure 3.4.1 - The use of LMPCR in distinguishing between single and double stranded DNA breaks at the origin. The use of primer extension as a first step in LMPCR (left diagram) allows the formation of a double stranded blunt end which in turn allows the mapping if the 5’ end of the topo I single stranded cut. Upon origin firing (right diagram), the collision of the replication fork with the topo I single stranded cleavage results in the formation of a double stranded blunt end, removing therefore the need of the primer extension reaction.

HeLa cells were synchronized by mimosine treatment in late G1 - G1/S border, washed from mimosine and released into S phase in the presence of 1μM CPT for up to 30 minutes. The cells were then lysed, the proteins were digested with proteinase K and the DNA purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. LMPCR analysis was performed, for both the upper and lower strand of the origin region, with or without the first step (namely the primer extension) in order to detect the corresponding DNA single or double stranded breaks derived from the topo I-DNA covalent complexes. The results are displayed in figure 3.4.2.

The CPT stabilized topo I induced DNA nicks are not repaired up to 30 minutes if the drug is not removed from the cell medium. Both the origin upper and lower strand topo I DNA cleavages can

still be detected by regular LMPCR analysis after 30 minutes of cell incubation with CPT, and no difference in the intensity of the LMPCR stops is visible. This proves that DNA replication from the lamin B2 origin is severely impaired upon continuous CPT treatment, given the presence of persistent DNA breaks very close to the start-sites of DNA synthesis and on the strands template for leading strand synthesis.

If the first step of LMPCR amplification is omitted (namely the primer extension) and the DNA substrate is subjected directly to the linker ligation step, no LMPCR stops are visible for the origin area. This indicates that, in this area, there are no double stranded DNA blunt ends which can be ligated to the LMPCR asymmetric linker. Considering that DNA synthesis might have been aborted before reaching the topo I nick, thus creating a staggered double stranded break, the same experiment was also performed including an initial polymerase end filling reaction in order to obtain the blunt end needed for the ligation step. Also in this case no LMPCR stops could be detected in the origin area confirming the fact that there is no region of double stranded DNA at the origin.

As a control the same experiment was performed also in the presence of caffeine, an inhibitor of checkpoint response, since 30 minutes of CPT treatment might activate DNA replication checkpoints inside the cell and therefore inhibit replicon firing (Sarkaria et al., 1999). Even in this case no double stranded DNA molecules could be detected at the origin.

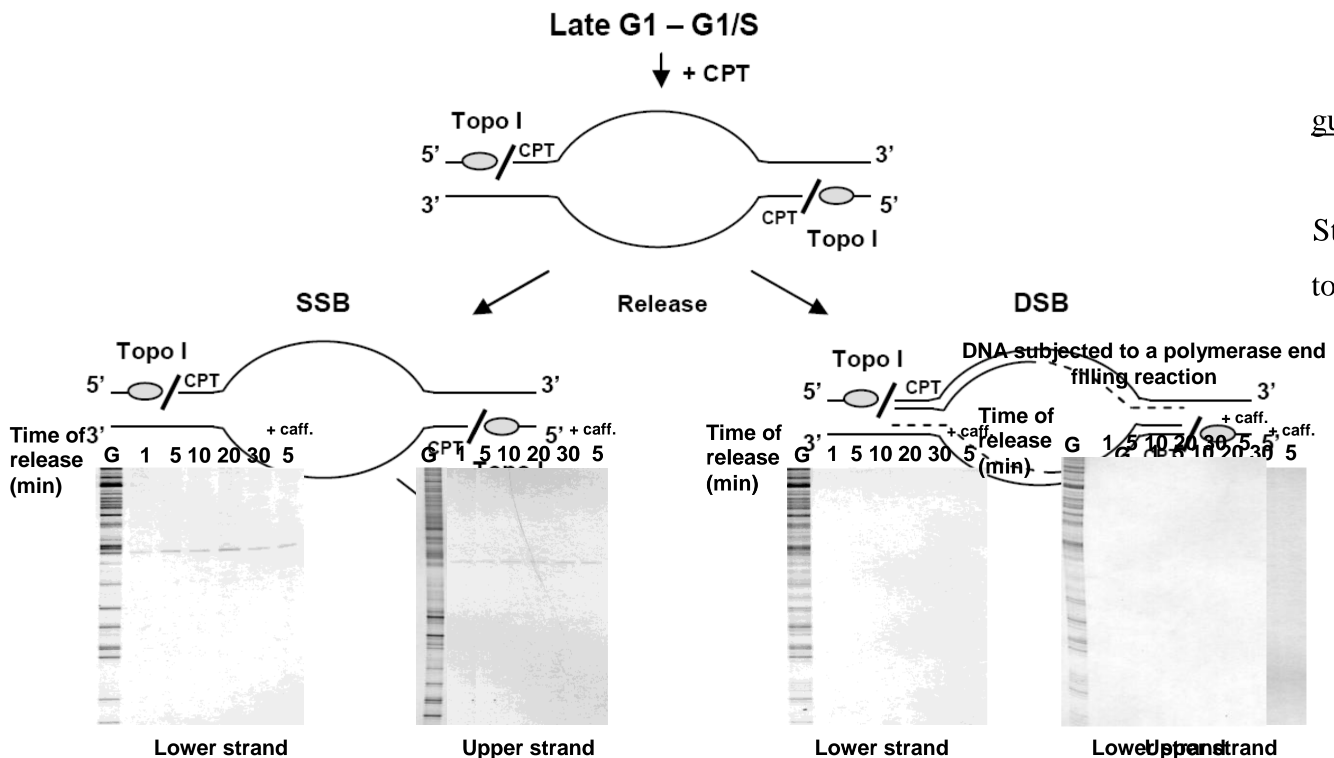


Figure 3.4.2 - Stabilized topo I

cleavage complexes at the lamin B2 origin do not lead to replication run-off. Cells synchronized in late G1 - G1/S border and released up to 30 minutes into S phase, in the presence of CPT, exhibit a single stranded DNA break (SSB) at the lamin B2 origin due to the persistence of the topo I-DNA covalent complexes (left side lower and upper strand gels). These topo I induced single stranded breaks are not transformed into double stranded breaks (DSB) by 'replication run-off' (top right side lower and upper strand gels). The single stranded breaks are detected by standard LMPCR analysis, while the double stranded breaks are mapped by performing an LMPCR analysis without the primer extension reaction step. In the event that the 'replication run-off' process did not yield double stranded DNA blunt ends but recessed ones, a polymerase end filling reaction was performed before linker ligation (bottom right side lower strand gel). The same results are obtained also when the cell cycle checkpoint activation is inhibited by caffeine treatment.

This allows the conclusion that, in the presence of CPT, release of the cells in S phase is not accompanied by the firing of the lamin B2 origin. Nonetheless the method used in this experiment is based on the purification of a very short piece of double stranded DNA (the topo I cleavage sites are located only 41 and 26 nucleotides away from the upper and lower strand start-sites respectively). The possibility of melting these short double stranded molecules during the DNA purification step and, as a result, losing the substrate for the ligation reaction cannot be excluded.

Consequently another method was used to validate these results. This experiment is based on the fact that if DNA synthesis is initiated in the presence of CPT then the nascent DNA can be labeled with bromodeoxyuridine (BrdU). However if the DNA synthesis is blocked no BrdU incorporation is possible.

HeLa cells synchronized by mimosine treatment in late G1 - G1/S border were washed from mimosine and released into S phase in the presence of BrdU and 1μM CPT for up to 30 minutes. Subsequently the cells were washed from both CPT and BrdU and released in complete medium for up to 10 minutes. This release step enables origin firing, since the topo I covalent complexes have the property of being completely reversible within a short time after drug removal. The cells were then collected, resuspended in PBS containing 10% glycerol and lysed in the wells of a 1.2% alkaline agarose gel immersed in alkaline running buffer. The gel was run overnight and the short nascent DNA was isolated from the gel. Competitive PCR analysis was performed in order asses the abundance of

origin (B48) and non-origin (B13) DNA before and after immunoprecipitation with anti-BrdU antibodies. The results are shown in figure 3.4.3.

Origin firing during the CPT block leads to BrdU incorporation into the nascent DNA, but if the DNA synthesis starts only after reversal of the topo I-DNA covalent complexes, no BrdU incorporation is possible. Competitive PCR analysis of the abundance of origin and non-origin DNA shows that, even though the origin DNA is enriched after CPT treatment, there is no incorporation of BrdU in this sample. Therefore the origin has fired only after the reversal of the topo I cleavage complexes. This brings further proof to the initial observation that inhibition of topo I activity by CPT treatment effectively inhibits the initiation step of DNA replication at the lamin B2 origin.

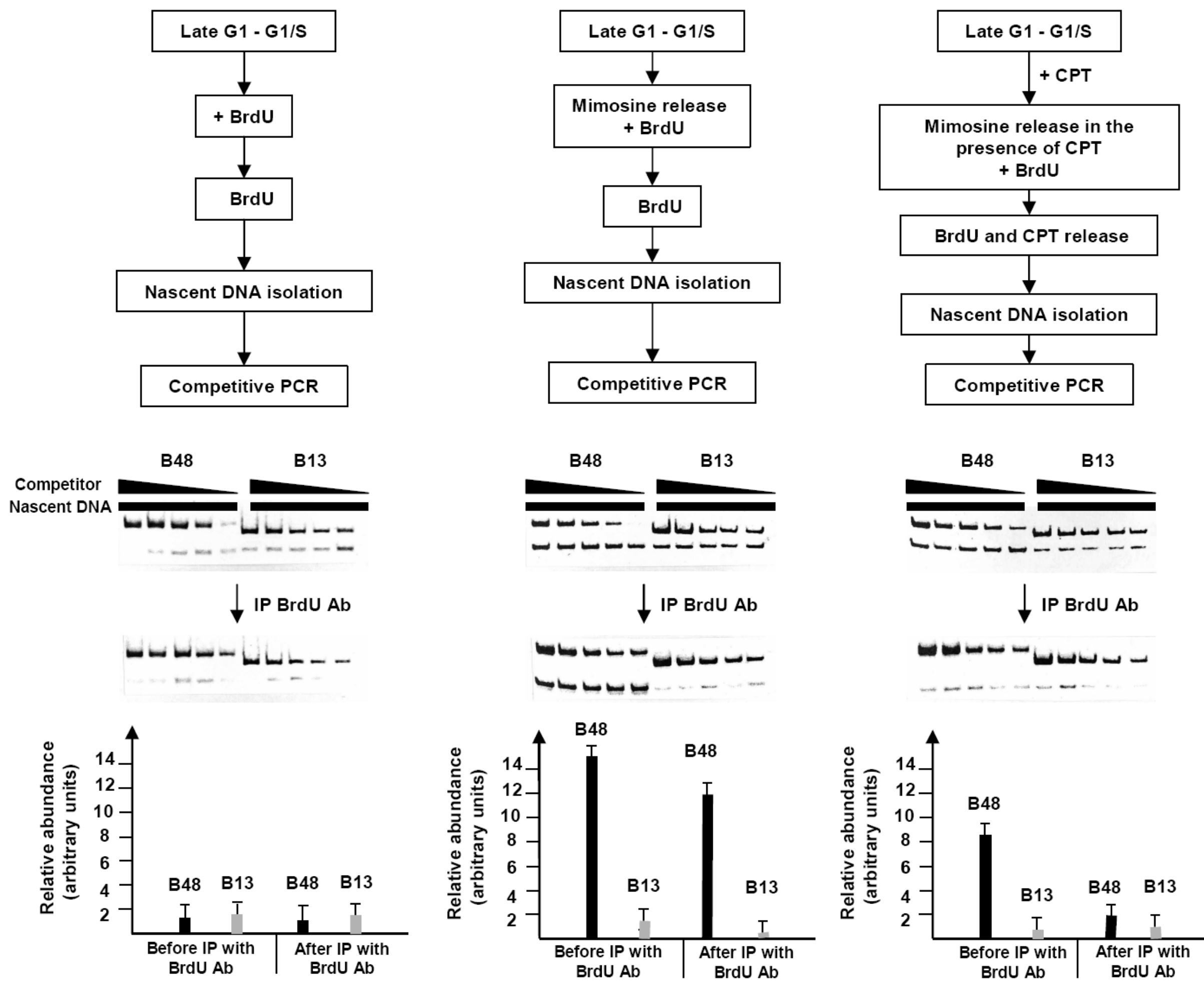


Figure 3.4.3 - Inhibition of replicon activation following stabilization of topo I. Left panel - negative control. Mimosine does not allow entry into S phase. Middle panel -positive control. Upon mimosine removal cells enter S phase. Right panel - upon release into S phase in the presence of CPT the lamin B2 origin does not fire. However after CPT removal the lamin B2 origin is able to fire.

3.5 Mapping of the interaction of topoisomerases I with the lamin B2 origin *in vitro*.

Type IB DNA topoisomerases have very degenerate cleavage consensus sequences (Capranico and Binaschi, 1998). However, considering the observed specific topoisomerase I interaction with the origin sequence *in vivo*, the affinity of the enzyme for this region was investigated in a series of *in vitro* reactions.

A 216bp DNA fragment containing the lamin B2 origin of DNA replication was incubated with human recombinant topoisomerase I, at room temperature, for 15 minutes followed by the addition of CPT (1μM final concentration) for a further 5 minutes incubation. The reaction was stopped by the addition of a strong denaturant (SDS), the DNA isolated by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and the topo I induced DNA breaks were mapped by hot extension. The results are shown in figure 3.5.1.

Surprisingly the origin fragment does not exhibit a random cleavage pattern in the presence of topo I but displays only 5 specific cutting sites: four located on the upper strand (positions 3880-3881, 3890-3891, 3897-3898 and 3924-3925) and only one located on the lower strand (position 3956-3957). Out of these, one of the four upper strand sites and the lower strand cleavage site are identical to the ones found *in vivo*. Most remarkably, on the lower strand, in a piece of DNA long 216bp, topo I is able to cleave just one site. This indicates that the enzyme has the ability to recognize specifically this region.

As mentioned in section 3.1.1, the *in vivo* use of gimatecan instead of CPT does not affect the position of the topo I cleavage site on the lower strand. This is also true for experiments performed *in vitro*. When either gimatecan or CPT-7[CH2-Tris] (another CPT derivative) were used instead of CPT in order to stabilize the topo I-DNA covalent complex, the same topo I cutting site was detected on the lower strand (see figure 3.5.2). This reinforces the idea that the affinity of the enzyme for the region is the main factor which determines the choice of the cleaved bond. This is particularly important in the view of the fact that in the absence of a topo I poison no cleavages are detected at the origin. However, incubation of the DNA fragment with CPT in the absence of topo I does not result in DNA breakage (see figure 3.5.1), in good agreement with reports which emphasize the affinity of this drug for the topo I-DNA complex and not for either the enzyme or DNA alone (Pommier and Cherfils, 2005).

Figure 3.5.1 - Interaction of human recombinant DNA topoisomerase I with the lamin B2 origin *in vitro*. In the presence of 1μM CPT topo I recognizes discrete sites in a DNA fragment containing the lamin B2 origin. G – *In vitro* DMS treated genomic DNA. * – position of topo I cleavages present also *in vivo*.

Figure 3.5.2 - The effect of gimatecan and CPT-7[CH2-Tris] on the *in vitro* formation of topoisomerase I cleavable complexes. The drug used to stabilize the topo I-DNA covalent complex has no influence on the position

of the topo I cleavage. Lane 1 - 1μM CPT. Lanes 2-4 – 1, 10, 50μM CPT-7[CH2-Tris]. Lanes 7-9 – 1, 10, 50μM gimatecan. G – *In vitro* DMS treated genomic DNA. * – position of topo I cleavages present also *in vivo*.

In spite of the high affinity which topo I displays for the lamin B2 origin DNA *in vitro*, the five single stranded cutting sites mapped for the enzyme do not show a consensus DNA sequence. At least three of the upper strand cleavage sites lie very close to one another (~8-10 nucleotides apart) and are most probably found on different DNA molecules. Furthermore, if the 6 nucleotides which contain the cleavage site are taken into account, all five cutting sites have unrelated sequences: GAA/GAT, GCC/TAG, GTG/TTC, TAA/TAT for the upper strand and GTT/GTA for the lower strand. This striking dissimilarity points towards a three dimensional DNA conformation recognition process, with apparent lack of sequence preference of the topo I enzyme.

To substantiate this hypothesis three mutated DNA fragments were constructed and the ability of topo I to cleave these substrates was assessed. The results of this experiment can be found in figure 3.5.3, while a map with all the mutated sequences and the corresponding cleavages is shown in figure 3.5.4.

In a first instance a stretch of 10 Ts (upper strand position 3901-3910) was replaced by a GC-rich sequence: CAGCGCCAGG (mutation 1). This long T stretch is thought to be involved *in vitro* in the formation of a triplex structure at the origin (Kusic et al., 2005). Certainly the weak interactions between the two DNA strands in this region would give rise to a particular DNA conformation, which might influence the topo I DNA recognition. However, when the topo I cleavage assay was performed in the presence of this mutated fragment only the two cleavage sites close to the mutated region were lost (positions 3890-3891 and 3897-3898), with all the other cutting sites remaining unaltered.

A second mutated DNA fragment involved the replacement of a 10 nucleotide sequence close to the lower strand topo I cleavage site. The AT-rich sequence AAACTTTTTT (upper strand position 3943-3953), was replaced with the same GC-rich sequence CAGCGCCAGG (mutation 2). This change is also based on the supposition that the weak A-T hydrogen bonding in this region is important for the conformation adopted by the DNA fragment *in vitro*. Interestingly, in this instance, two of the topo I

cleavages are drastically reduced: the nearby lower strand cleavage (position 3956-3957) and one of the upper strand cleavages (position 3890-3891) situated far away from the mutated region.

Finally, in a third mutated fragment, 6 nucleotides comprising the topo I lower strand cleavage site were changed from the original sequence of ACAACA (upper strand position 3955-3960) to the following GC-rich sequence: CAGCGC. This corresponds to a topo I cleavage site mutation from GTT/GTA to GTCGCG. In this case the lower strand topo I cutting site is completely abolished, while none of the upper strand cleavage sites are affected.

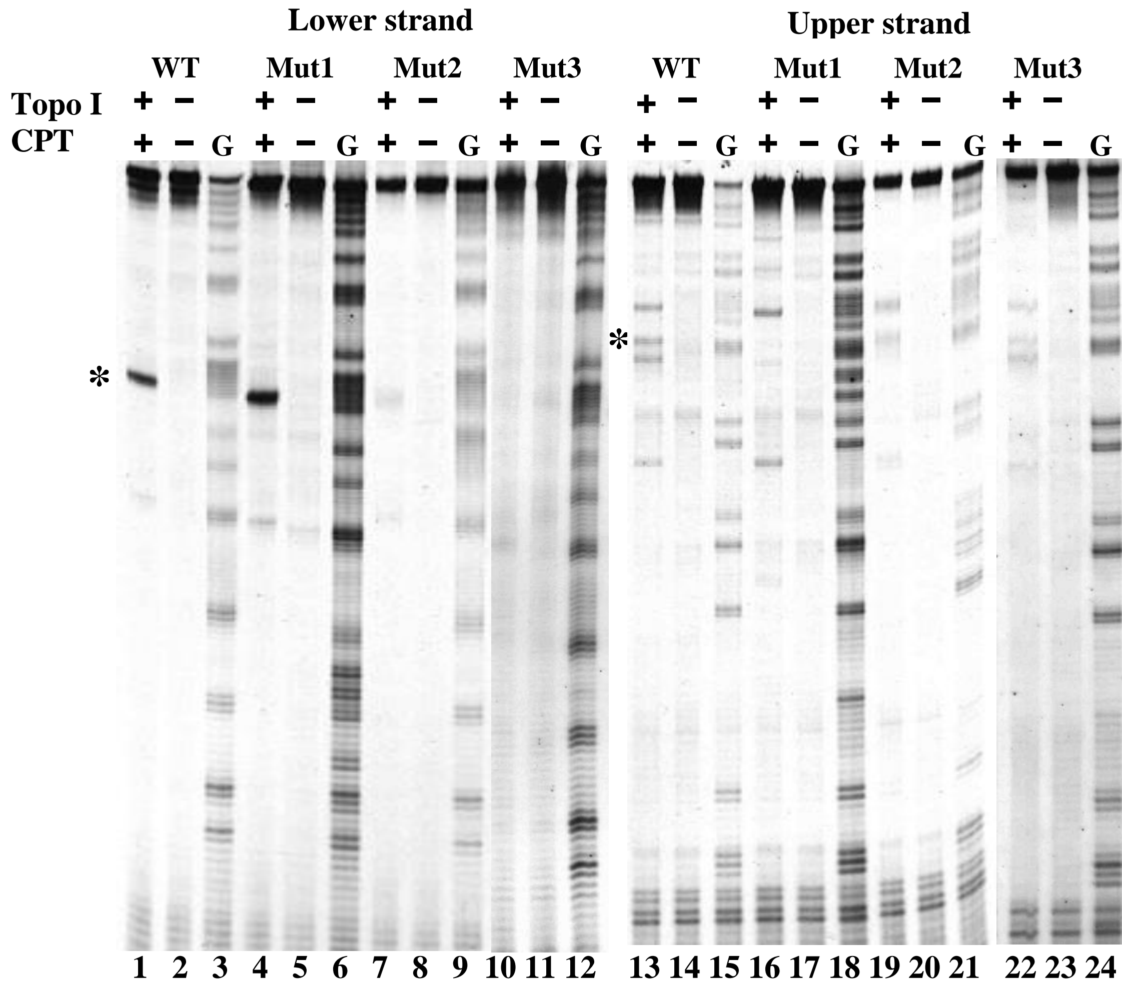


Figure 3.5.3 – The pattern of *in vitro* topo I cleavage sites in normal and mutated lamin B2 DNA fragments. WT – normal, Mut1 – mutation 1, Mut2 – mutation 2, Mut3 – mutation 3 (see figure 3.5.4 for sequences). G – *In vitro* DMS treated genomic DNA. * – position of topo I cleavages present also *in vivo*.

In conclusion topo I has a clear affinity for certain regions of the lamin B2 origin in spite of a lack of sequence consensus at the cleavage sites. The effect of DNA mutations seems to be restricted to the surrounding region with the observation that the lower strand cleavage site is sensitive to the primary DNA sequence.

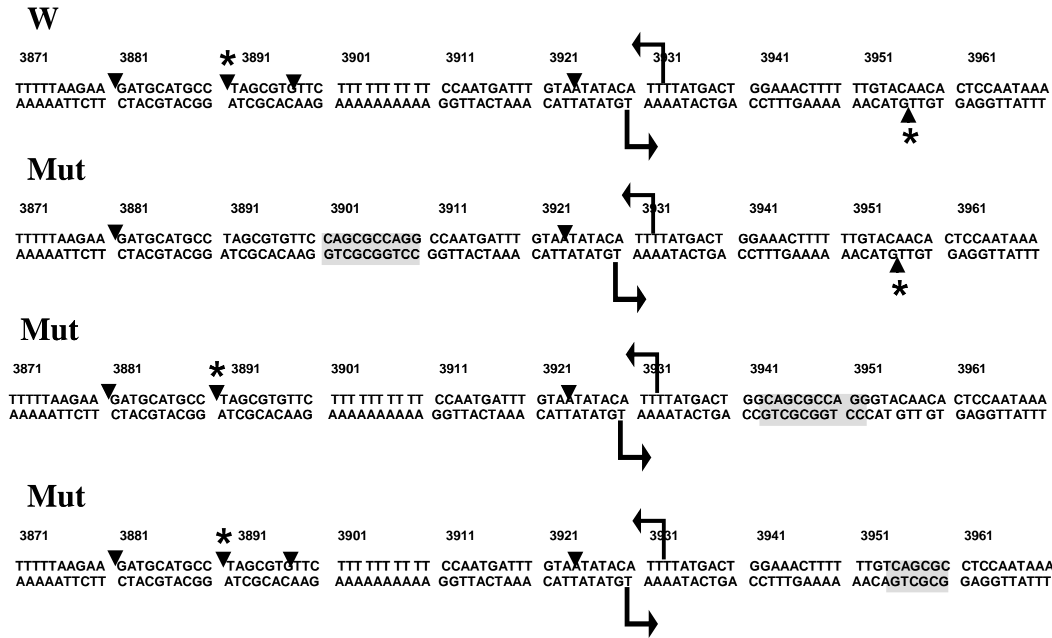


Figure 3.5.4 - Map of the normal and mutated lamin B2 origin fragments. The mutated regions are highlighted. The position of the *in vitro* topo I-cleavable complexes are indicated by filled triangles. * – position of topo I cleavages present also *in vivo*.

3.6 Mapping of the interaction of topoisomerases II with the lamin B2 origin *in vitro*.

Human topoisomerases II (both α and β isoforms) share the topoisomerase I property of having a degenerate cleavage consensus (Capranico and Binaschi, 1998). When an *in vitro* cleavage assay was performed, using the same 216bp lamin B2 DNA fragment, the human recombinant topo IIα enzyme and VP16 as a poison, a random cleavage pattern of origin DNA was detected (see figure 3.6.1). This observation allowed the conclusion that, in contrast to topo I, topo IIα is not able to recognize specifically the lamin B2 origin fragment *in vitro*.

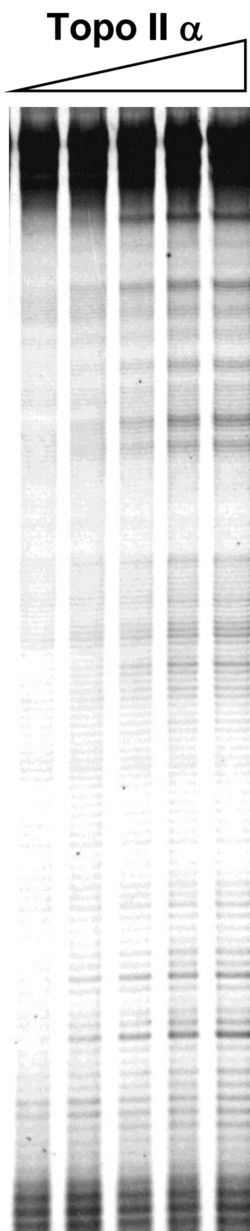


Figure 3.6.1 - Interaction of human recombinant DNA topoisomerase II with the lamin B2 origin *in vitro*. In the presence of VP16 topo II introduces random cuts in a DNA fragment containing the lamin B2 origin.

Nevertheless, the precise *in vivo* localisation of the topo II enzyme on origin DNA might be influenced by other nuclear proteins. To further investigate

this possibility an *in vitro* assay was developed based on the assembly of a specific multi-protein complex on origin DNA.

A Dignam HeLa nuclear extract, obtained from asynchronously growing cells, was pre-incubated at room temperature with 100-fold excess of competitor DNA, poly(dA-dT)-poly(dA-dT) and poly(dG-dC)-poly(dG-dC), for 20 minutes, followed by a 30-minute incubation with the 216bp lamin B2 origin fragment (radioactively labeled). The whole reaction mix was then loaded on a 5% non-denaturing acrylamide gel, in a bandshift assay, in order to verify the formation of a protein complex on the origin DNA (illustrated in figure 3.6.2).

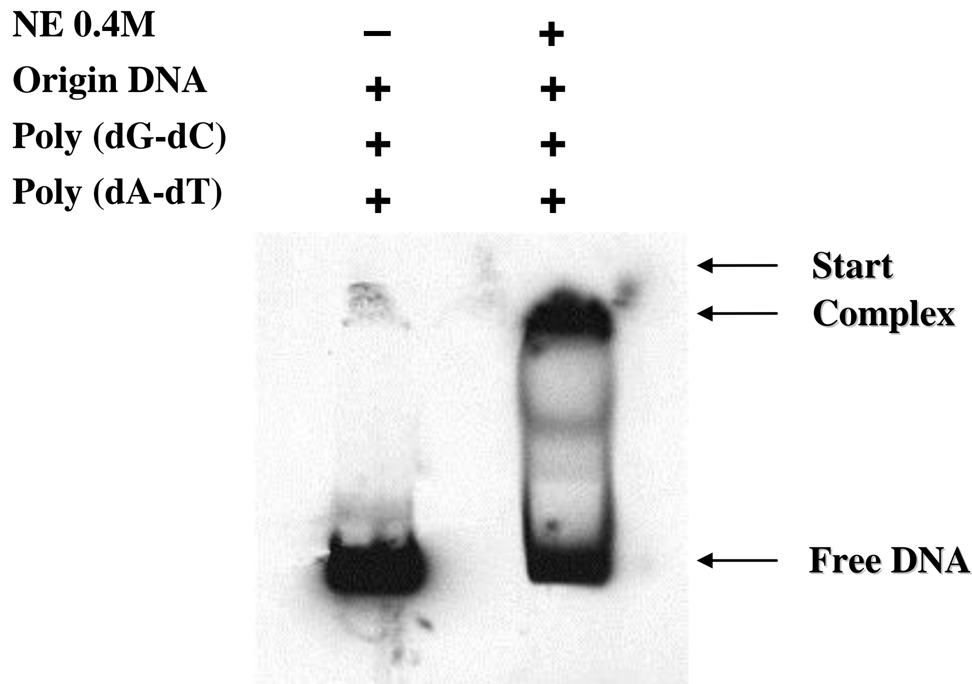


Figure 3.6.2 - Nuclear proteins form a specific *in vitro* complex on a DNA fragment containing the lamin B2 origin. The bandshift assay allows the detection of a retarded band (corresponding to part of the origin DNA) in the presence of the 0.4M nuclear extract.

After establishing a reproducible complex formation reaction, the incubation with the origin DNA was followed by the addition of the topo II poison VP16 (100μM final concentration) for a further 5 minutes and the reaction was stopped by the addition of detergent (SDS). The DNA was purified by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol

precipitation. The topo II induced DNA breaks were mapped by hot extension and the results are presented in figure 3.6.3.

Remarkably in the presence of the Dignam nuclear extract the origin DNA exhibits two single stranded breaks, on the upper and lower strand respectively, which share the position of the *in vivo* mapped topo II cleavage sites.

The *in vitro* origin complex occupies a precise region, as shown by λ exonuclease protection assay. The pre-formed protein-DNA complex was subjected to λ exonuclease digestion in order to detect its left and right borders. The DNA was further purified by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and the position of the upper strand left and lower strand right borders was mapped by hot extension. The results are shown in figure 3.6.4.

The region covered by the *in vitro* origin complex is similar to the region covered *in vivo* by the G1 pre-replication complex (as mapped by DMS footprinting). The upper strand left border is positioned at nucleotide 3851 and the lower strand right border is positioned at nucleotide 4007. The *in vivo* DMS footprint in the middle of G1 covers a similar region with the same lower strand border but with a much reduced area protected on the upper strand (see figure 3.6.4). This is in good agreement with the fact that the Dignam nuclear extract used to build the *in vitro* complex comes from asynchronous growing cells, the majority of which are in the G1 phase of the cell cycle.

An immunoprecipitation experiment using an anti-topo IIα antibody was able to pull down the topo II enzyme covalently linked to the origin DNA, following the formation of the *in vitro* protein complex (see figure 3.6.3). The Dignam nuclear extract, pre-incubated with 100-fold excess of competitor DNA for 20 minutes, was incubated for another 30 minutes with the origin fragment and an additional 5 minutes with VP16. Anti-topo IIα antibodies allowed the purification of the enzyme covalently bound to the 5' end of the DNA break, the DNA was further purified by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and the position of the DNA breaks was mapped by TDPCR analysis.

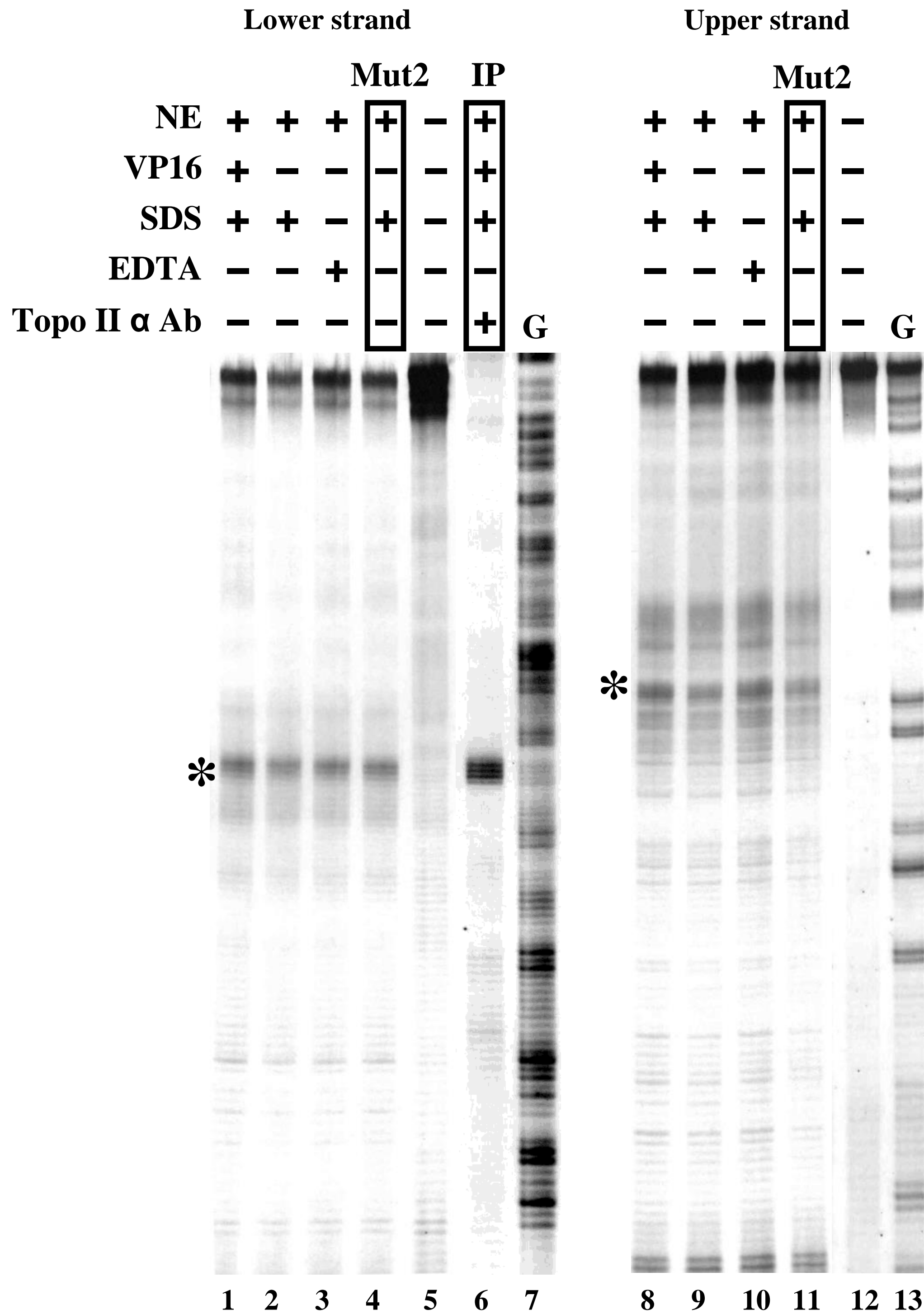
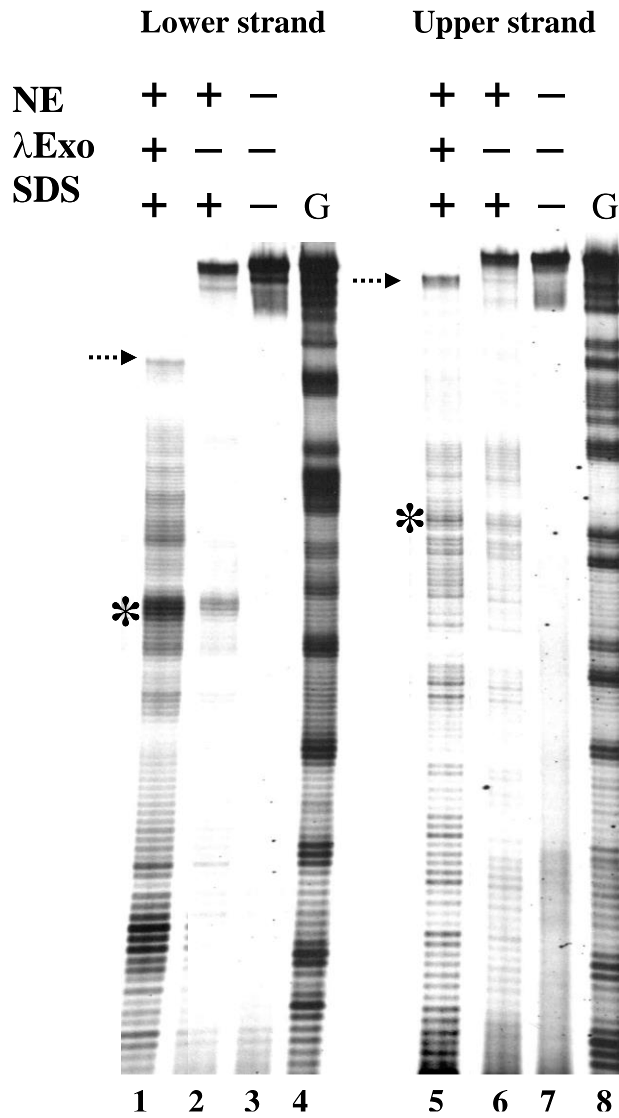


Figure 3.6.3 - Interaction of DNA topoisomerase II with the lamin B2 origin *in vitro*, as part of an origin specific protein complex. Topo II is helped by other nuclear proteins in recognizing specifically the lamin B2 origin DNA. The topo II – DNA covalent complex can be stabilized by VP16, SDS or EDTA treatment. Lanes 4, 11 – mutated origin fragment (mutation 2, see figure 3.5.4 for

sequence). Lane 6 - immunoprecipitation with anti-topo IIα antibodies confirms the presence of the topo II enzyme on the DNA. * – position of topo I cleavages present also *in vivo*. G – *In vitro* DMS treated genomic DNA.



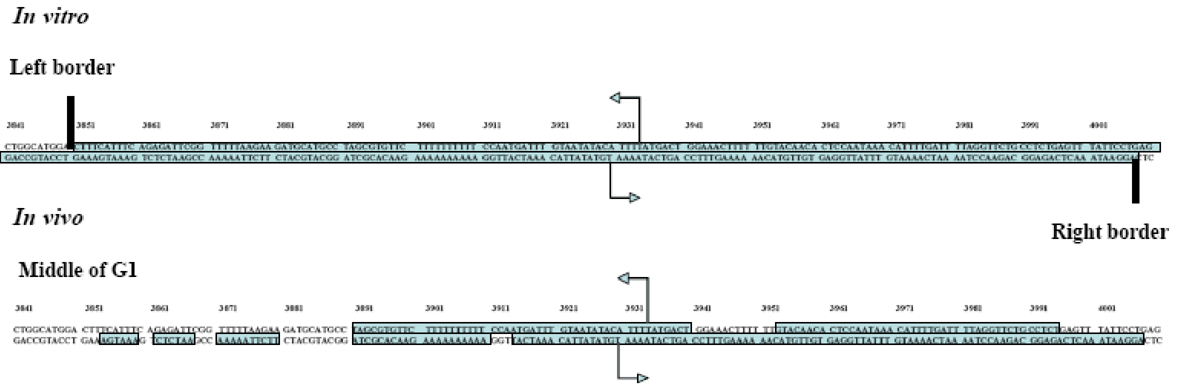


Figure 3.6.4 - Comparison between the position of the *in vitro* origin complex and the *in vivo* pre-replicative complex. The upper strand left border and the lower strand right border (dashed arrows) were mapped for the *in vitro* origin specific complex by λ exonuclease protection assay (lanes 1 and 5). The sequence covered by the *in vitro* formed complex is similar to the region covered *in vivo* by the origin binding complex in the middle of G1, as previously mapped by DMS footprinting (Abdurashidova et al., 1998). G – *In vitro* DMS treated genomic DNA. * – position of topo II cleavages present *in vivo* and *in vitro*.

The DNA single stranded breaks obtained after the incubation of the origin fragment with the HeLa nuclear extract are indeed due to topo IIα action.

Furthermore, these two breaks are not dependent upon VP16 treatment, as indicated in figure 3.6.3. Treatment with a strong denaturant, namely SDS, is sufficient for stabilizing these DNA nicks. Addition of a chelating agent, EDTA, is also sufficient to stabilize the topo I-DNA covalent complex. These results indicate that the topo II cleavage reaction is sensitive to both denaturing conditions and Mg^{2+} availability. They also highlight the fact that VP16 has no influence over position of the topo II cleavage sites.

The fact that topo II does not recognize by itself the lamin B2 origin DNA is also supported by evidence that, when a mutated origin fragment (mutation 2, see section 3.5 and figure 3.5.4) is used to build the *in vitro* origin complex, the position of the topo II cleavages is not changed. Mutation 2 is located just 2 nucleotides away from the topo II lower strand cleavage site but it does not affect the ability of the enzyme to cleave the DNA (figure 3.6.3).

Therefore, *in vitro*, human recombinant topo IIα does not recognize specifically the lamin B2 origin, but as part of a protein complex with other nuclear proteins is able to cleave the same sites on DNA as *in vivo*.

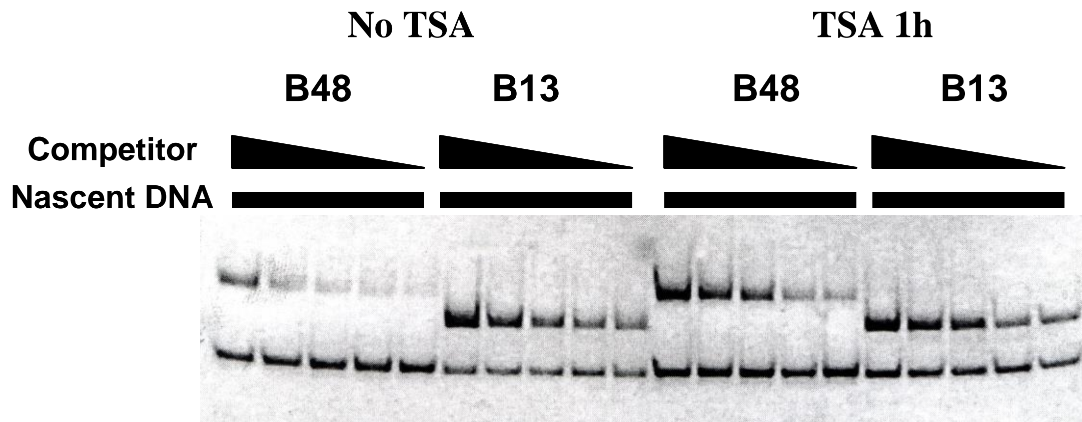
3.7 The effect of the histone deacetylase inhibitor trichostatin A on the topoisomerase I and II cleavages at the lamin B2 origin.

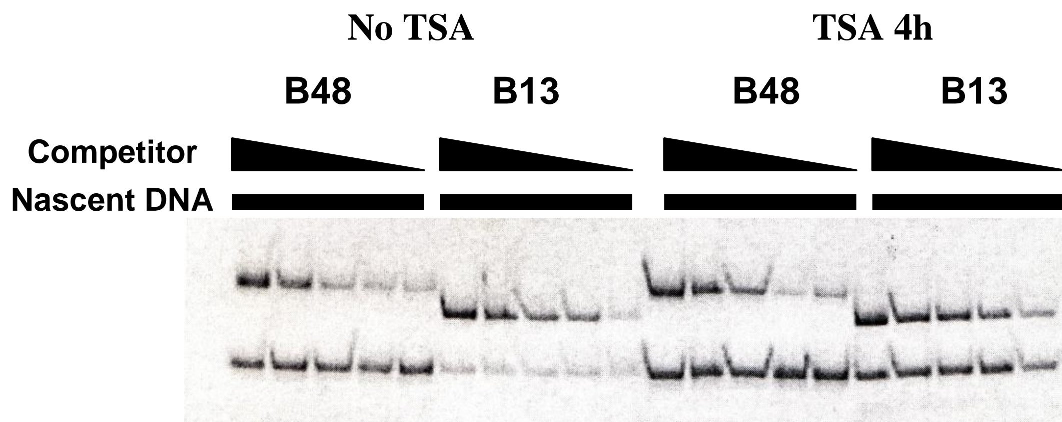
Trichostatin A (TSA) is a histone deacetylase inhibitor which induces chromatin remodeling by raising the levels of histone acetylation. In HeLa cells treated with TSA the lamin B2 origin of DNA replication shows a relocalisation of start-sites of DNA synthesis (Kemp et al., 2005). Over a region of 2kb the peak nascent DNA abundance is shifted from the usual position in order to give a dispersed region of initiation.

Indeed HeLa cells treated for a short time with TSA show a reduced lamin B2 origin activity. Asynchronous growing HeLa cells were treated for 1-4h with 100ng/ml TSA, collected and resuspended in PBS with 10% glycerol and lysed in the wells of a 1.2% alkaline agarose gel. After an overnight run the short nascent DNA was isolated from the gel and competitive PCR analysis was performed in order to asses the abundance of origin (B48) and non-origin (B13) DNA fragments. The results are shown in figure 3.7.1.

After one hour treatment with TSA the lamin B2 origin shows half of the nascent DNA enrichment compared to untreated controls. After four hours of TSA treatment the origin nascent DNA abundance is further reduced to a quarter of normal levels, with a B48:B13 ratio of roughly 2:1. This proves that even a short TSA incubation leads to the accumulation of acetylated histones and possibly other proteins with dramatic effects on origin function.

This strong effect is accompanied by a disruption of the interaction of both topoisomerases I and II with the origin. Asynchronous growing HeLa cells were treated for 1-4h with TSA followed by treatment with either 1μM CPT or 10nM VP16 in order to freeze, respectively, the topo I or topo II covalent complexes. The cells were then lysed, the DNA was isolated by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and the topo I or topo II cleavages were mapped by LM or TDPCR respectively. The results are illustrated in figure 3.7.2 for topo I and in figure 3.7.3 for topo II.





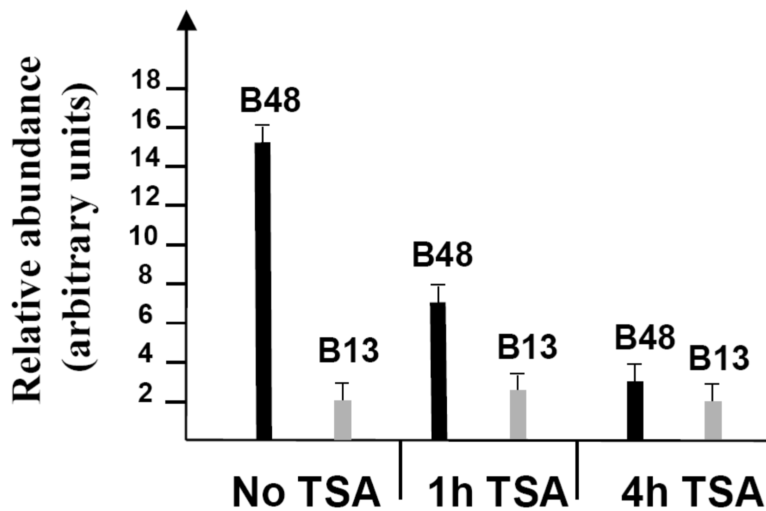
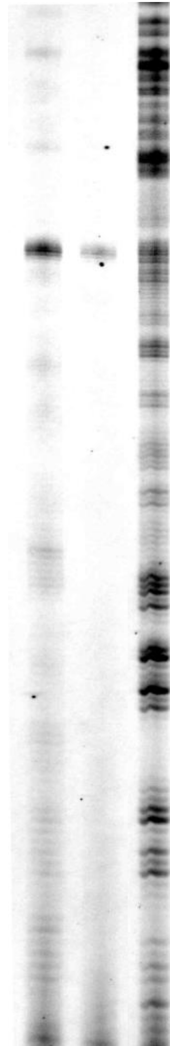


Figure 3.7.1 - Inhibition of the lamin B2 origin in the presence of the histone deacetylase inhibitor trichostatin A. The abundance of nascent DNA for origin (B48) and non-origin (B13) regions was measured by competitive PCR analysis in cells treated or not with TSA.



<u>Figure 3.7.2</u> - Effect of trichostatin A on the interaction of topoisomerase I with the lamin B2 origin *in vivo*. G – *In vitro* DMS treated genomic DNA.

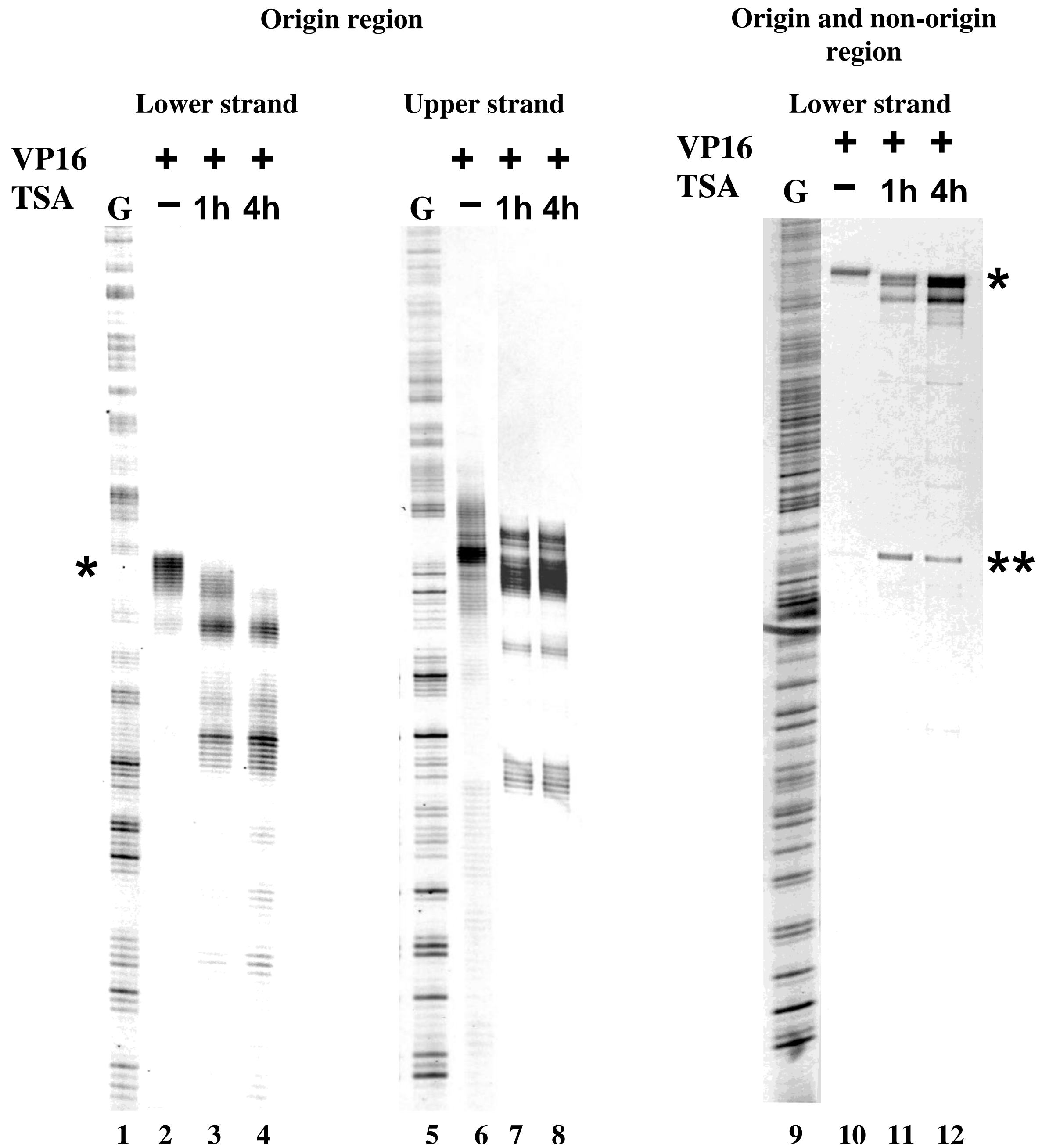


Figure 3.7.3 - Effect of trichostatin A on the interaction of topoisomerase II with the lamin B2 origin *in vivo*. Treatment of HeLa cells with TSA leads to the redistribution of the topo II cleavage sites at the origin (lanes 3,4 and 7,8) but not of the topo II cleavage site located far away from the origin (lanes 11,12). * - lower strand topo II cleavage site at the origin. ** - lower strand topo II cleavage site outside of the origin region. G – *In vitro* DMS treated genomic DNA.

Upon TSA treatment the topo I lower strand interaction with the origin is reduced as demonstrated by the faint LMPCR stop. However the topo II covalent complexes at the origin become relocated following TSA treatment. Both the upper and lower strand topo II cutting sites show a dispersed pattern of multiple cleavages over a large area. Furthermore, a comparison of the behavior of the origin and non-origin bound topo II molecules shows a distinct difference. If the origin cleavage sites show a dispersive pattern, the non-origin topo II cutting site is unaffected by the TSA treatment (the intensity of the TDPCR seems to become even stronger). This is yet further proof towards the idea that these two topo II molecules play very different roles on the DNA.

Considering the strong effect of TSA on the topoisomerase II behavior at the origin, the *in vitro* origin complex assay was also performed in the presence of TSA. The Dignam nuclear extract, to which TSA was added up to a final concentration of 100ng/ml, was pre-incubated with 100-fold excess of competitor DNA for 20 minutes, then incubated for another 30 minutes with the origin fragment and topo II covalent complexes were stabilized by denaturation with SDS. The position of the topo II cleavages was mapped by hot extension and the results are shown in figure 3.7.4.

In this case no difference in the topo II cleavage pattern could be detected, indicating that TSA does not interact directly with the topo II enzyme but influences its behavior *in vivo* via modifications of other proteins.

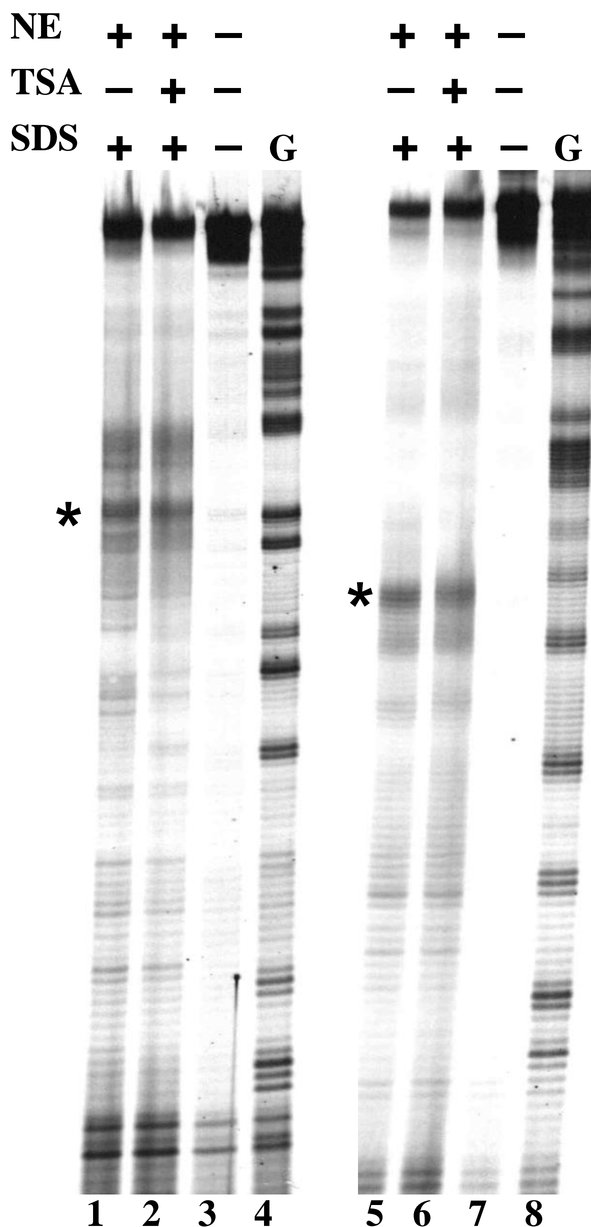


Figure 3.7.4 - Effect of trichostatin A on the interaction of topoisomerase II with the lamin B2 origin *in vitro*. The addition of TSA to the *in vitro* complex formation reaction does not influence the position of the topo II cleavage sites at the origin

(lanes 2 and 6). * – position of topo II cleavages present also *in vivo*. G – *In vitro* DMS treated genomic DNA.

4. DISCUSSION

DNA topoisomerases are crucial enzymes for all living cells; they are the only proteins capable of modulating DNA topology in response to any DNA metabolical process. The wide range of effective anti-cancer agents which function by disrupting the catalytical cycle of topoisomerases is indicative of the uniqueness of these enzymes. It is not, thus, surprising that interfering with their biological action leads to cell death. Still, detailed information about their exact role, regulation and functional redundancy lacks in almost all model genetic systems.

In humans there are six topoisomerase enzymes encoded in the nuclear genome, two type IB (topo I and topo Imt), two type II (topo IIα and topo IIβ) and two type IA (topo IIIα and topo IIIβ) proteins. Three of these enzymes, topo I, topo IIα and topo IIβ, have been involved in a large number of studies as targets for anti-cancer drugs. However, most of these experiments have focused on acquiring knowledge about how to efficiently kill cancer cells, rather than on understanding why the topoisomerase-DNA covalent complexes are cytotoxic. A detailed characterization of the roles of topoisomerases inside the cell is needed in order to determine the most effective drug target.

DNA replication in humans is a highly regulated and complex process, which involves the use of many apparently unrelated DNA sequences as origins of initiation of DNA replication. These sequences are chosen during the G1 phase of the cell cycle by the binding of pre-replicative complexes, which undergo dynamic reorganizations all through the length of G1. At the G1/S border, for all the sequences within the genome which will function as origins, a timing of replication initiation has already been assigned and only a subset of these (early origins) will fire immediately after entry into S phase. The rest (middle and late origins) will fire at different points in S phase in an orderly manner. The mechanism by which replication origins are chosen in the genome or the way in which it is established at which time point in S phase the origin will fire it is still not understood.

In human cells, currently, there are very few origins well characterized and they show no sequence similarity between them. There seem to be many factors which influence the location and activity of the origin: the nuclear localization, an open chromatin structure, promoter regions for house-keeping genes, the presence of binding sequences for specific protein factors, of bent DNA structures, of asymmetric AT-rich stretches (DePamphilis, 2006). Nevertheless there is no doubt that origins of DNA replication exist, they initiate DNA synthesis reproducibly from the same start-sites at every cell cycle, they function in different cell types and they also function when transferred to other genomic

loci, even in different organisms. The origin recognition complex (ORC), the structure which marks all replication origins and which is needed for the sequential assembly of the full pre-replicative complex, has no sequence specificity. In view of all these observations the question whether DNA topology is also a determining factor in the choice of sequences to be used as origins needs to be considered.

In the present study the interaction of both human DNA topoisomerase I and II with the lamin B2 origin of DNA replication was investigated. The method applied to this purpose involved the use of two anti-cancer drugs, CPT and VP16, which are, respectively, enzyme-specific poisons for topo I and topo II. These drugs inhibit the religation step in the catalytic cycle of the enzymes leaving them covalently crosslinked to the DNA substrate. In this way the transient single (topo I) or double (topo II) stranded brakes introduced in the DNA are stabilized and can be mapped with the aid of LMPCR (topo I) or TDPCR (topo II). One important observation should be made regarding the mapping of the interaction of topoisomerase I and II with the origin, namely that the method used allows the detection of these enzymes only when they cleave the DNA. Should either topo I or topo II be present in the region analyzed without actively cleaving the DNA, they would be invisible in this study. However, lack of catalytical activity of either enzyme would point towards a structural role, most probably unrelated to topological regulation of the DNA.

Treatment of asynchronous HeLa cells with the topo I poison CPT, revealed two single stranded cleavage sites, on the upper and lower strand respectively, well inside the origin region protected by the replicative complexes. These sites are located close and almost in a symmetrical manner with respect to the start-sites of DNA replication, on the templates for leading strand synthesis. Analysis of HeLa cells synchronized throughout the cell-cycle revealed that topo I interacts with both strands of the origin in M phase, very early G1 and late G1 towards the G1/S border.

Unexpectedly treatment of asynchronous HeLa cells with VP16 also revealed two single stranded cuts, on the upper and lower strand respectively, close to the topo I sites but positioned closer to the start-site. These topo II cuts are also found inside the region protected by the replicative complexes, on the strands template for leading strand synthesis and are rather symmetrical with respect to the start-sites. However synchronization of HeLa cells showed that in contrast to topo I, topo II cleaves the origin only in M phase and in the middle of G1.

This is not the first report of a topo II single stranded cut, in spite of the fact that the double stranded 4bp staggered cleavage is considered to be a hallmark of type II topoisomerases (Liu et al., 1983, Muller et al., 1988 and Wong and Hsu, 1990). The reason for these *in vivo* single stranded breaks

is not obvious, nor is the existence of *in vivo* double stranded cleavages for the human enzyme. Remarkably, topo II can introduce the same single stranded cuts as part of an *in vitro* protein complex bound to origin DNA. These breaks are indeed due to topo II, as highlighted by the immunoprecipitation experiment both *in vivo* and *in vitro*. Furthermore, *in vitro*, the same cleavages can be stabilized by treatment with SDS or EDTA instead of VP16. This indicates that the single stranded nicks introduced by the enzyme are not conditioned by the anti-cancer drug used.

Following the characterization of the interaction of the two topoisomerases with the origin (namely a region of ~500bp) the surrounding area (500bp to the left and to the right) was also analyzed. For topo I no other cuts were observed with the exception of one cleavage positioned close to the TIMM13 promoter, a housekeeping gene located to the right with respect of the lamin B2 origin. Nevertheless this cleavage was observed only in S phase and may be related to the transcription of this gene.

In the case of topo II just one other lower strand cleavage was observed, also single-stranded, in a region located over 200bp away and to the left of the origin. In contrast with the lower strand topo II cleavage close to the start-site, this particular cut is constant throughout the cell-cycle. There are three strong indications towards the idea that the origin and non-origin bound topo II molecules are part of different enzyme subsets and perform different actions on the DNA. First of all they behave differently throughout the cell cycle, they have different protein partners when bound to DNA (the origin topo II interacts with the hOrc2p in the middle of G1, while the non-origin bound topo II enzyme does not) and finally they show different sensitivity upon TSA treatment (the topo II origin cleavage becomes redistributed, while the one outside remains unaffected).

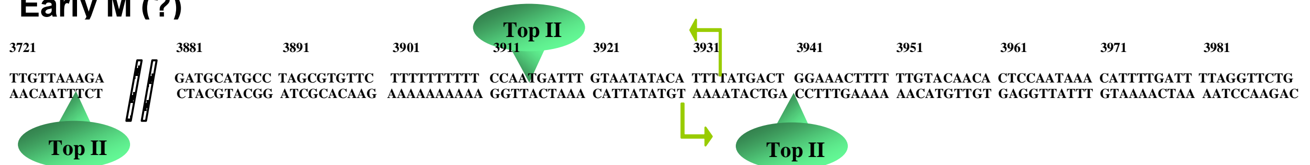
Since topo II is a known member of the nuclear scaffold it is tempting to hypothesize that this cleavage far away from the origin might represent a scaffold attachment site. In the past many experiments aimed at mapping the position of scaffold attachment sites were based on drugs which specifically poisoned the topo II enzyme (Razin et al., 1991). Using this technique the lamin B2 origin itself was shown to be located close to a scaffold attachment region with a resolution of 2kb (Lagarkova et al., 1998). This theory would explain the behavior of this cleavage after TSA treatment: the nuclear scaffold is a structure insensitive to chromatin remodeling. Nevertheless further evidence is needed to substantiate this hypothesis.

Previously, the positions of hOrc1p, hOrc2p and hCdc6p at the lamin B2 origin were mapped with nucleotide resolution for the M, middle of G1 and middle of S phases of the cell-cycle. Together

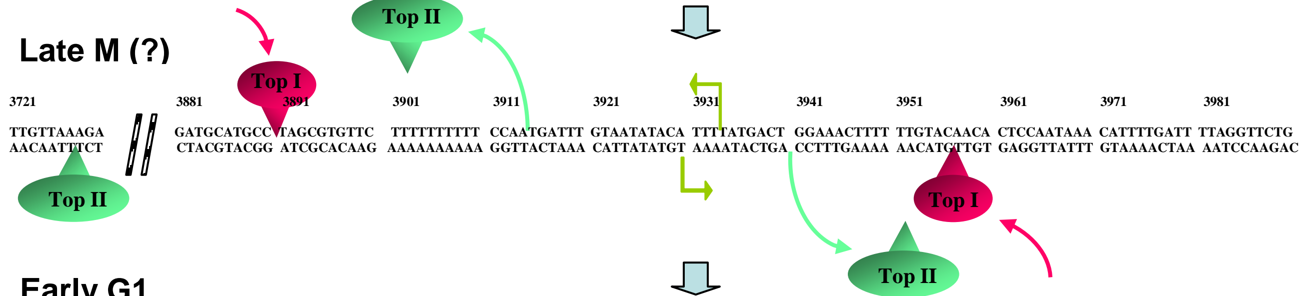
with the data obtained for topo I and topo II a new picture of the dynamic interaction of the origin binding proteins with the DNA emerges. Figure 4.1 illustrates the current knowledge of the protein-DNA interactions at the human lamin B2 origin in different phases of the cell cycle.

In mitosis the only proteins in contact with the origin are topo I and topo II as indicated by the lack of a DMS footprint in this area (Abdurashidova et al., 1998). Topoisomerase II is a known member of the protein scaffold of metaphase chromosomes and lack of both topo IIα and topo IIβ isoforms in the cell severely impairs chromosome condensation and proper mitotic segregation (Gasser et al., 1986 and Sakaguchi and Kikuchi, 2004). This allows the speculation that topo II might be present at the origin in the first part of M, concurrent with DNA condensation.

Early M (?)

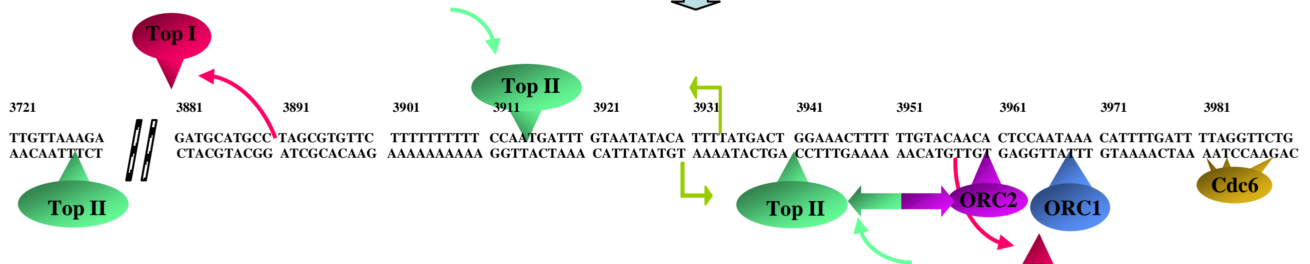


Late M (?)

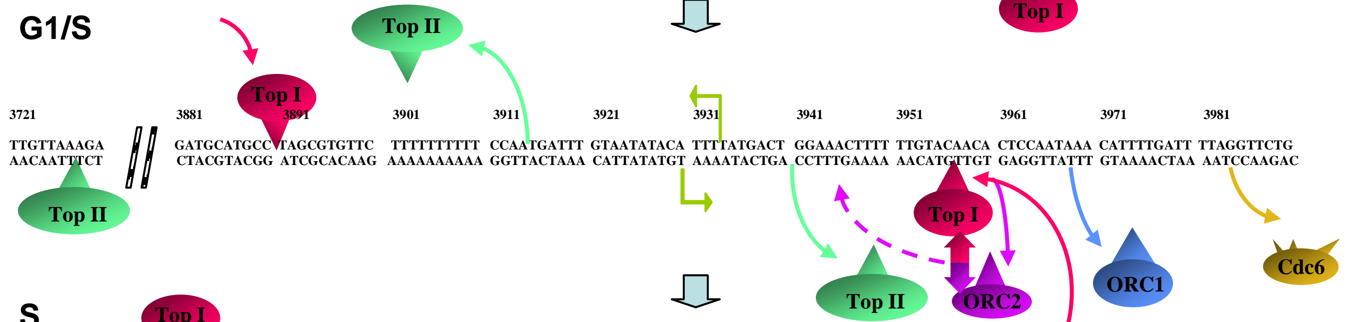


Early G1

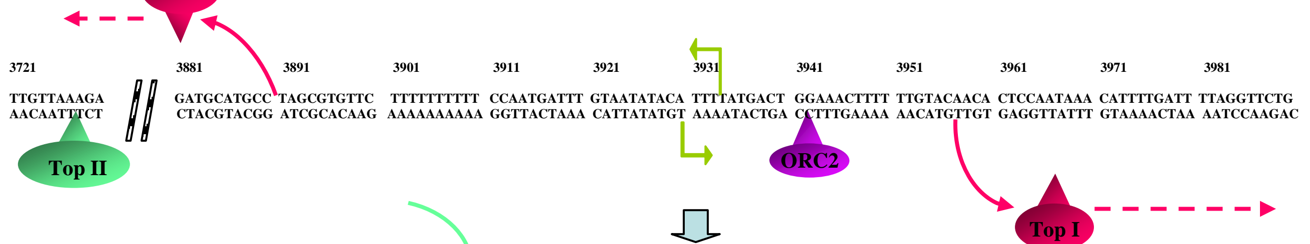
Middle of G1



G1/S



S



Early M (?)

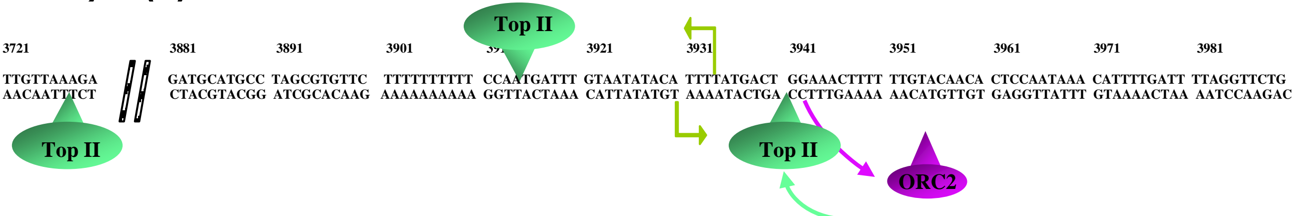


Figure 4.1 - Summary of the mapped protein-DNA and protein-protein interactions at the lamin B2 origin of DNA replication. The position of Orc1p, Orc2p and Cdc6p on the lower strand was previously mapped (Abdurashidova et al., 2003).

Topoisomerase I cleaves the origin DNA not only in M phase but also in the first half hour of G1. This piece of evidence would be in agreement with a possible role of topo I towards the end of mitosis, when it could contribute towards unpacking of chromosomes into their interphase relaxed state. Immediately entering G1 topo I might help with the first binding of the ORC to DNA. It is not known when the first binding of the origin recognition complex takes place at the lamin B2 origin. Interestingly, metazoan ORC has no sequence specificity but recent experiments in *Drosophila melanogaster* have showed that DmORC seems to prefer negatively supercoiled DNA (Remus et al., 2004). If this were true also for hORC, then the presence of topo I might be connected with preparing the origin DNA for ORC binding. Furthermore considering that so far topoisomerase I is the only member of the replicative complexes which has a high affinity for the origin region *in vitro*, this enzyme might be itself a factor important for origin positioning.

In the middle of G1 we find that topo II is again bound at the origin, on both the upper and lower strand, along with Orc2p, Orc1p and Cdc6p. The lower strand topo II molecule and Orc2p are interacting at the origin, which makes topo II an ORC protein partner in the middle of G1.

As the cell enters the late G1 phase towards the G1/S border, topo II is no longer detectable in the origin area while the topo I enzyme has returned to this region. The exact position of Orc2 at this time is unknown but protein-protein cross-linking experiments have shown that topo I and Orc2 are interacting, making topo I an ORC protein partner at the origin, shortly before replicon initiation.

In the middle of S phase, after the origin has fired, the only protein still in contact with the DNA is Orc2, its binding site shifted 17 nucleotides towards the start-sites. Finally, as the cell enters again M phase Orc2 disassociates from the origin and topo I and topo II come again in contact with this region.

The nucleotide resolution mapping of the protein-DNA interactions at the lamin B2 origin has highlighted a dynamic competition between topo I, topo II and Orc2 proteins, throughout the cell cycle, for the same DNA binding sites.

The Orc2 binding site in the middle of G1 overlaps with the lower strand topo I cleavage site (only 3 nucleotides difference). In good agreement with this observation topo I cannot be detected at

the origin at this point in the cell cycle. The S phase Orc2 binding site coincides with the lower strand topo II cleavage site (one nucleotide difference). Again no binding of topo II in the origin area is observed in this phase of the cell cycle. Furthermore, in mitosis when both topo I and topo II interact with the origin (but not with the same DNA molecule), ChIP analysis showed that Orc2 is not bound to the DNA (Abdurashidova et al., 2003).

Therefore both topo I and topo II are not only protein partners for Orc2 at certain points during the cell cycle, but compete with this protein for the same binding sites. A possible explanation for this competition is the constant need for the modulation of origin DNA topology in order to maintain and/or strengthen the ORC-DNA binding. Another plausible hypothesis is that topo I and topo II are, together with ORC, part of a large multi-protein complex which is always in contact with the origin. Nevertheless the proteins which come into direct contact with the DNA change according to the needs of the DNA metabolism and cell cycle program.

The proteins which address this specific topo I, topo II and ORC interaction with the origin are, most probably, still to be identified. Both ORC and topo II need to be directed to this region by other proteins, while the topo I affinity towards the origin area is, in all probability, not sufficient for dictating the behaviour of a replicative complex. Furthermore topo I is missing from the origin region for the most part of the G1 phase of the cell cycle.

Topo I, however, seems to play a crucial role in replicon firing at the G1/S border. As shown in section 3.4, inhibition of the DNA relaxation activity of topo I by CPT treatment inhibits the initiation of DNA replication at the lamin B2 origin. This is a rather surprising result considering the generally accepted mechanism of CPT cytotoxicity: collision of a replication fork with a CPT stabilized topo I induced DNA break generates 'replication run-off', namely the formation of double-stranded breaks. If the CPT treatment occurs during S phase when DNA synthesis has already started then this outcome is likely. However if the cell is still in G1 when CPT is added do all origins still fire? Inhibition of at least a part of the origins of DNA replication would lead to a slower S phase, possibly an early mitosis when not all the DNA has been replicated yet and daughter cells with severe chromosome abnormalities.

Unfortunately the role of topo II for origin activation is not easy to investigate. The topo II-origin DNA interaction observed in the middle of G1 is too far time-wise from the initiation of DNA synthesis, an inhibition of the enzyme with VP16 for hours will activate a variety of checkpoint systems, making it impossible to distinguish between an inhibition of DNA synthesis as a primary or secondary effect of drug treatment.

The experiments involving the histone deacetylase inhibitor Trichostatin A do hint towards a correlation between topo II action and origin regulation. In asynchronous growing HeLa cells treatment with TSA leads to a redistribution of the origin topo II cleavages concurrent with a decrease in origin activity. However it is obvious from both *in vivo* and *in vitro* experimental data that origin bound topo II functions only as part of a complex with other proteins. Hence the topo II cleavage redistribution seen after TSA treatment is possibly connected to an accumulation of the acetylated form of members of the pre-replicative complex. This type of modification would probably lead to proteins losing their affinity for DNA and disassembly of the origin bound complex.

Therefore both topoisomerase I and topoisomerase II are involved in origin regulation with topoisomerase I playing an essential role for the initiation of DNA replication.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Both human topoisomerase I and topoisomerase II are new members of the lamin B2 origin pre-replicative complex. Topoisomerase I introduces a single stranded cut in both origin upper and lower strand, positioned 42 and 27 nucleotides away from the start sites respectively, on the strands template for leading strand synthesis. These two cleavages are present at the origin in M phase, very early G1 and late G1 towards the G1/S border. Topo I interacts with the human origin recognition complex via Orc2p immediately before origin activation and inhibition of its full catalytical activity at this moment of the cell cycle inhibits replicon initiation. *In vitro* human recombinant topo I shows a clear affinity for the lamin B2 origin by interacting with the same residues as *in vivo* on both the upper and lower strand. However the enzyme is able to cut 3 other sites on the upper strand and no consensus sequence was obtained by comparing all of the *in vitro* binding sites.

Topoisomerase II also introduces a single stranded break in both the upper and lower strand of the origin DNA, positioned 19 and 11 nucleotides away from the start sites respectively, on the strands template for leading strand synthesis. These two cuts are present at the origin in M phase and middle of G1. Topo II is also a human origin recognition complex protein partner, via its interaction with hOrc2p, in the middle of G1. *In vitro* human recombinant topo II is unable to specifically recognise the origin DNA by itself, but as part of an *in vitro* origin specific protein complex it cleaves exactly the same sites as *in vivo*.

Both topo I and topo II are competing *in vivo* with the Orc2p for the same binding sites. Topo I shares the Orc2 G1 binding site, while topo II the Orc2 S phase binding site.

In the future the topoisomerase cell cycle specific interaction with the origin can be exploited in order to identify new members of the origin binding complex. Protein-protein crosslinking coupled with a topoisomerase block would enable the detection of other topoisomerase interactors on origin DNA. The *in vitro* origin specific protein complex can also be used for the same purpose. An added advantage of *in vitro* work is that of being able to identify new proteins by mass spectrometry. Considering the fact that topo II cannot recognise by itself the origin DNA, the protein partners which direct it at the origin could be determined.

The question whether these two topoisomerases are interacting also with other origins of DNA replication should be taken into account. If that were the case, a new method for origin mapping might be established, based on cell cycle specific topo I-Orc2 and topo II-Orc2 interactions. Furthermore

considering the positions of the topo I and topo II cleavages with respect to the start-sites of DNA synthesis at the lamin B2 origin, the mapping of the start-sites for new origins would be reduced to the analysis of a ~100bp region.

The possible relationship of the lamin B2 origin with the nuclear scaffold can also be explored by studying the topo II cleavage site found ~200bp away and to the left of the start-sites. This high-resolution analysis of the topo II interaction with an origin and the surrounding region represents an unique opportunity for probing the hypothesis that origins of DNA replication represent scaffold anchoring sites, since, at present, topo II is considered the only 'bona fide' nuclear scaffold member.

The data reported here concerning the effect of Trichostatin A on origin function can be used as a starting point for investigating the nucleosome position in this region and the connection between chromatin remodeling and origin position and function.

Finally, the topoisomerase I site specific cleavages detected *in vitro* on a DNA fragment containing the origin can be developed into a topo I inhibitor or poison assay. The efficiency of new anti-cancer agents in freezing the topo I-DNA covalent complex or in inhibiting the DNA cleavages can be assessed in this manner. This method would be much more accurate that measuring the levels of topo I depletion from nuclear extracts following drug cell treatment.

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