5 Discussion

5.1 Essential mitotic functions of topoisomerase IIα are not adopted by the β-form

DNA topoisomerase II, a multi-functional enzyme, is essential for cell survival. The functions of the enzyme in the DNA metabolism have been intensively investigated, particularly the requirement of DNA topoisomerase II during an active mitotic process. However, to study the biological functions of the enzyme *in vivo* is difficult. The expression of the enzyme can not be blocked without abrogating cell proliferation. The use of temperature sensitive mutants of DNA topoisomerase II in *Saccharomyces cerevisiea* and *Schizosaccharomyces pombe* has therefore been an important model to investigate the enzyme *in vivo*. From these studies it was found that the enzyme is needed for a proper segregation of sister chromatids at anaphase (Dinardo et al, 984; Uemura et al, 1987; Holm et al, 1989). However, during mitosis another aspect of the enzyme has been observed. Genetic and biochemical evidence shows that the enzyme may also be implicated in chromosome condensation. In *Schizosaccharomyces pombe* it was found that the chromosome condensation was blocked when grown at a restrictive temperature for topoisomerase II. As a consequence of an incomplete condensation an elevated non-disjunction was observed (Uemura et al, 1986; Uemura et al, 1987). In higher eukaryotes, viable mutants of topoisomerase II were lacking, so other approaches have been devised to investigate the role of the enzyme in chromosome condensation. One approach is to use mammalian cells, which are treated with topoisomerase II targeting drugs. A similar response has been seen in *Schizosaccharomyces pombe.* However, it may be a disadvantage to use drugs that trap topoisomerase II-DNA intermediates in which the DNA is cleaved. The damage of the cells may activate the cell cycle checkpoint, which arrests cells in G_2-

phase to allow time for DNA-repair before entering the mitosis (Roberge et al, 1990; Chen & Beck, 1995). It is therefore difficult to distinguish between a direct effect of a topoisomerase inactivation and an indirect effect from the consequence of activating the DNA damage checkpoint. Using catalytical inhibitors of topoisomerase II such as aclarubicin, merbarone and ICRF, which do not lead to double strand cleavage, it was found that they were unable to achieve complete condensation (Gorbsky, 1994; Anderson & Roberge, 1996; Ishida et al, 1996). In addition to these drug studies, biochemical approaches in mammalian cells have shown the requirement of the enzymes in this mitotic event. Chromatin incubated with mitotic extract from Xenopus egg or chicken cells was *in vitro* able to induce condensation in the chromosome (Adachi et al, 1991; Hirano et al, 1993). Depletion of topoisomerase II with antibodies against the enzyme resulted in an inability to condensate. A similar response has been reached by using extracts of mammalian cells (Wood & Earnshaw, 1990). However, the ability to condensate was re-established by adding purified enzyme to the immunodepleted extract, indicating that DNA topoisomerase II is required. These findings in yeast and higher eukaryotes indicate that DNA topoisomerase II must be present in order to template chromosome structure properly. The studies in higher eukaryotes did not distinguish between the two isoforms of DNA topoisomerase II. At the moment it is not known if IIα as well as β isoforms are involved in chromosome condensation. It is also not established whether the enzyme has to be physically present or whether its role is enzymatic. The exact functions of DNA topoisomerase IIα and β has not yet been clarified, but differences in cell cycle expression profiles indicate a unique function for each of the two isoforms. A number of observations for the IIα and β isoforms in the cell cycle progression have been done: The expression of mRNA of the IIα-isoform

peaks in late S-phase, whereas the expression of IIβ-isoform peaks early in S-phase (reviewed in Isaacs et al, 1998). The IIα-isoforms are up-regulated when the cell cycle is entered and reaches their maximum toward mitosis, whereas IIβ remains constant throughout the cell cycle (Woessner et al, 1991). The IIα-isoform is predominately found in proliferating cells, and is therefore a sensitive indication of the proliferative state of the cell (Heck and Earnshaw, 1986; Boege et al, 1995). The isoform IIβ is found in proliferating as well as in resting cells (Turley et al, 1997). The IIα-isoform remains bound to the chromatin during mitosis as seen previously in A431 cell (Meyer et al, 1996) and here in the parental cell line H69-WT, whereas the IIβ-isoform diffuses into the cytosol.

To summarize, these studies indicate that the α-isoform is predominantly involved in cell cycling, particularly mitosis, whereas the function of the β- isoform is at this moment not fully understood. To gain further insight into the functions of IIα and β in mitosis *in vivo*, it is necessary to distinguish between the two forms. It turned out that an etoposide resistant small cell lung cancer cell line (H69-VP) was optimal for these studies due to a homozygotic deletion in the NLS sequence of the DNA topoisomerase IIα gene. A consequence of this deletion is that the topoisomerase IIα is lacking in the nucleus at the onset of mitosis. Here, I found that in interphase the cell line expresses the mutant IIα-isoform mostly in the cytoplasma, whereas the wild type IIβ isoform appears mostly in the nucleus. The lack of nuclear topoisomerase IIα did not stimulate upregulation of either topoisomerase II isoforms compared with the parental cell line H69-WT. Moreover, the mutant IIα isoforms was full length with a molecular weight of 170 kDa. Although the enzyme was excluded from the nucleus, it showed similar activity as compared to the parental cell line. In mitotic cells the isoform IIβ was not recruited to

the condensating chromosome to compensate for the lack of the IIα-isoform. However, these cells were able to proliferate due to the ability of the cytosolic topoisomerase IIα to enter the chromatin under the breakdown of the nucleus envelope at the onset of mitosis. Interestingly, an abortive type of mitosis was also observed. In a small percent of the mitotic cells both isoforms were absent at the chromatin. As a consequence of this an incomplete formation of the chromosomes was observed and these cells were not viable. The mitotic functions are predominantly performed by the isoform IIα, whereas the activity of the IIβ isoform seems not to be required. This is in agreement with previous indications. Furthermore, the IIβ isoform, which was present and active, was not able to adopt the mitotic functions of the IIα-form. This is in contrast to yeast cells, which do not distinguish between the two isoforms at mitosis and are obviously capable of utilizing both forms functionally. However, there might be important differences in the mitotic events between yeast and higher eukaryotes.

5.2 Role of topoisomerase IIα in chromosome condensation and disjunction

To obtain a successful cell division, mammalian cells must rely on the ability of topoisomerase IIα to bind to the chromatin. Here, the chromosomal organization of topoisomerase IIα for the H69-WT is assumed to reflect a normal situation. A more chaotic organization of the chromosome has been seen in some of the mitotic cells in H69-VP. In these cells segmented DNA spheroids were formed instead of chromosomes. This abnormality has also been observed when AT-rich regions at the base of radial chromatin loops were blocked with synthetic multiple AT-hook proteins (Strick & Lämmli, 1995). It is generally accepted that topoisomerase IIα has a strong preference for binding and

cleaving in AT-rich regions in the scaffold (Sander & Hsieh, 1983; Gasser & Lämmli, 1986). Topoisomerase IIβ, however, is associated with GC-rich regions in hypersensitive regions (Drake et al, 1989). Based on this information, the α- but not the β-isoenzyme is suggested to be selectively inhibited by AT-rich oligonucleotides. To sum up, if a sufficient amount of IIα isoform is not present at the onset of mitosis spherical chromatid balls will be created instead of a proper organization of the chromosome. However, DNA-sequence specificity alone does not explain why in mammalian cells only the α- and not the β-isoenzyme templates chromosome condense, because otherwise isoenzyme-specificity of this process would also be seen in yeast. It has been suggested that additional mechanisms must be involved in the selective targeting of the α-isoenzyme to the chromosomal scaffold. As mentioned in the introduction, topoisomerase II might interact with other proteins, i.e. condensins, which represent the most abundant structural component in the mitotic chromosome (Bhat et al, 1996; Hirano et al, 1997). In *Drosophila* a link between topoisomerase II and BARREN has been found (Kimura & Hirano, 1997). BARREN is homologous to a component in 13S condensin, a multisubunit protein complex, which is also found in yeast and *Xenopus* (reviewed in Yanagida, 1998). Mutations in BARREN, however, do not affect the chromosome condensation. Instead the separation of the chromosome arm is blocked (Kimura & Hirano, 1997). This implies that topoisomerase II is not tightly associated with BARREN in the mitotic event of condensation. The wild type of topoisomerase II is either sufficient to drive the formation of the chromosome or the enzyme interacts with other proteins than condensins to obtain a successful chromosome structure. Other studies have also been challenging the co-operation of topoisomerase II with condensin complexes. Recently, it has been reported in *Xenopu*s that the 13S

condensin complex condensate the chromosome independently of the presence of topoisomerase II. Interestingly, it is found that topoisomerase I co-operate with the complex. Briefly, *in vitro* the 13S condensin complex can renature DNA by winding up single-stranded DNA into a double helix. The activity of topoisomerase I is required, perhaps to relieve potential torsional stresses associated with condensation (Kimura et al, 1998). This new and unexpected function of topoisomerase I has also been observed in a double top1 trf4-ts mutant in *Saccharomyces cerevisiea.* Both topoisomerase I (top1) and the topoisomerase I-related protein (trf4) are found non-essential in yeast. However, it turned out that top1 in combination with Trf4 seemed to establish and maintain chromosome condensation in the rDNA (Castano et al, 1996). According to these studies, topoisomerase I and condensins co-operates in this particular event, whereas topoisomerase II seems to play a minor role. However, the data presented here suggests that the lack of topoisomerase IIα in the nucleus had a significant effect on chromatin condensation. It should be noted that the cell line H69-VP did not have a mutated DNA topoisomerase I. Separate roles for topoisomerase IIα and condensins in condensation of the chromosomes have been suggested. Topoisomerase II is limited to an early stage in condensation, perhaps a resolution of tangled strands, whereas the condensins may contribute a high order coiling of condensed chromosome, and are therefore needed late in the process (Castano et al, 1996).

Here, I found that the isoform IIα needs to be physically present and bound to the chromatin with a high affinity in order for condensation and sharpening of the chromosome. Actually, previous studies in A431 cells showed that during mitosis only a fraction of topoisomerase IIα was involved in catalytic DNA-turnover. The catalytically active fraction is not tightly chromatin-bound (Meyer et al, 1996). It should also be noted

that in mitosis multimerization of topoisomerase IIα stimulated through hyperphosphorylation has been reported. These protein-protein interactions are supposed to be required for the compaction of DNA in mitotic cells, although the activity of the enzyme is decreased (Vassetzky et al, 1994). Thus, the chromosome condensation and shaping are assumed to involve high-affinity binding of topoisomerase IIα to the chromosomal scaffold, whereas catalytic DNA-topoisomerisation at these places seems to be dispensable for the condensation process. In contrast, activity of topoisomerase II is clearly required for chromosome-disjunction because treatment of mammalian cells with catalytic inhibitors of topoisomerase II causes non-disjunction, asymmetrical cell division, and polyploidity (Gorbsky, 1994; Anderson & Roberge, 1996; Ishida et al, 1996). Here, in H69-VP giant polyploid nuclei were observed. It is reasonable to assume that these abnormalities are due to the lack of topoisomerase IIα in the nuclei. Topoisomerase IIα is indeed required after the chromosome condensation, and an essential and unique role of topoisomerase IIα in chromosome segregation has been suggested.

However, the presence of topoisomerase IIα activity *per se* seems to be insufficient for supporting these functions because after the breakdown of the nuclear envelope, topoisomerase IIα should have access to the chromatin of H69-VP cells in an active form and in similar amounts as in wild type H69 cells. However, the cytosolic enzyme apparently fails to form a high-affinity chromatin bond. Thus, it appears that the enzyme does not interact with all its putative substrates by free diffusion and random collision.

5.3 DNA-topoisomerase IIα-like protein is associated with centrosomes

Topoisomerase II is indeed required in a number of independent processes in the DNA metabolism. Particularly the IIα-isoform plays an impressive role in cell division. In the absence of topoisomerase IIα the attempt to carry out mitosis in a human cell line ends up fatally with a failure in chromosome condensation eventually leading to non-disjunction of the chromatin. More mysterious is the function of topoisomerase IIα in the centrosome. In order to understand the role of topoisomerase IIα the centrosome will be described briefly. The centrosome is a unique organizer of microtubules, which is a major component of the eukaryotic cytoskeletal. Involvement of this organelle and its associated microtubules is found in many important cellular functions. It includes organization and movement of cytoplasmic organelles and determination of cell shape and polarity. Moreover, the centrosome also plays a role during mitosis by replacing the microtubule network with the spindle apparatus (reviewed in Kellogg et al, 1994; Zimmerman et al, 1999). In interphase, the centrosome lies close to the nucleus and is composed of a pair of centrioles surrounded by an amorphous matrix, termed pericentriolar material from which microtubules emanate. The centriole is a pair of cylinders built from nine triplet microtubules, which consist of tubulin (Bodo & Gull, 1996). Less is known about the structure of the pericentriolar material. However, it has been observed that pericentrin and γ-tubulin are organized into a highly ordered lattice structure within the pericentriolar material (Dictenberg et al, 1998). Although the centrosome was described by Theodor Boveri over 100 years ago (Boveri, 1901), only little progress has been made in understanding the structure. It is believed that the organelle nucleates the growth and distribution of microtubules, which

interact with other cytoplasma components and therefore play a central role in the organization of the interior of the cell. Microtubules are also important in the formation of the mitotic spindle, which organizes a proper distribution of the chromosomes as well as the cytoplasma. The duplication of the centrosomes is briefly described: the centriole pair move away from each other early in the S-phase. Subsequently, the centrioles duplicate, which means that a new centriole is formed next to the original centriole. Finally, the new pair of centrioles migrate to opposite sites to the pole in the mitotic spindles (Kochanski & Borisay, 1990). Indeed the structure of the organelle changes continually during the cell cycle, because the centrosome can be seen as a dot next to the nucleus, whereas in mitosis cells centrosomes are present at the poles of the spindle apparatus.

In the literature it has been reported that several proteins associate with the centrosome. This includes a number of regulatory proteins such as kinases (Lacey et al, 1999), phosphates, and proteins of degradation pathways (Kimura et al, 1999; Zimmerman et al, 1999). Here, a centrosomal protein was recognized by three distinct epitopes of human topoisomerase IIα, located at opposite ends of topoisomerase IIα. It was not surprising that topoisomerase IIα might be associated with the centrosome according to the immunofluorescence data seen in previous observations (Meyer et al, 1996), and here in the staining pattern for the lung cancer cell lines. However, the centrosomal protein seems not to be identical to the normal nuclear topoisomerase IIα. It turned out that the centrosomal protein was observed in quiescent lymphocytes. According to the literature and according to my findings from immunofluorescence and immunoblotting, the expression of normal topoisomerase IIα is barely detectable in resting cells. Another difference between the centrosomal protein and the nuclear topoisomerase II has been observed.

The centrosomal protein does not show any topoisomerase II activity. This may be an indication that the protein associated with the centrosomes might have other functions in the cells compared to the normal topoisomerase II. Moreover, a biochemical analysis of isolated centrosomes supports the presence of the topoisomerase IIα-like protein at the centrosomes. It was found that the mobility of the protein in SDS-geleelectrophoresis suggests a molecular weight of 205 kDa, whereas normal topoisomerase II is 170 kDa. It is still unclear which additional mechanisms give rise to a changed electrophoretic mobility, but it could not be accounted for by alternative splicing or by phosphorylation of the normal enzyme. A problem could have arisen if I had been dealing with an electrophoretic artefact due to cross-reaction of the antibodies. The antibodies were totally differently raised in different species against different types of antigens. Furthermore, the antibodies recognize the topoisomerase IIα either at the extreme NH_2 terminal or at different residues in the COOH-domain. Thus, it is unlikely that a cross-reaction will occur. The centrosomal protein might be a subform of topoisomerase IIα that has undergone an as yet unknown covalent modification. This modification is probably responsible for the shift of the electrophoretic position and eventually influences the activity of the enzyme. At present no such subform of topoisomerase IIα has been described.

It seems that topoisomerase IIα is endowed with different functions in human cells. According to the literature and data presented in the first part of this thesis, the normal nuclear topoisomerase IIα was found essential in mitosis. Here, I can only speculate what kind of behaviour the topoisomerase IIα-like protein shows at the centrosome. Several cell cycle related proteins are associated with centrosomes, perhaps to ensure a successful cell division (Compton & Cleveland, 1993; Hsu & White, 1998). It is notable that a precise duplication and segregation of the

centrosome is required for a normal division of the cell. Before the onset of cell division the duplication of the centrosome should be completed. This organelle is believed to play a role in organizing the poles of the spindle, which is important to achieve an equal distribution of the chromosomes. The possible mechanism for co-ordinating these events has yet not been found. Several numbers of centrosomal proteins are found involved in the G_2/M checkpoint (Brown et al, 1994; Pietromonaco et al, 1996) but only a cyclin/cdk2 complex is needed in the early stage in duplication of the centrosome (Lacey et al, 1999). This complex is, moreover, supposed to couple DNA replication and the duplication of the centrosome. No link between cyclin/cdk2 and other centrosomal proteins has been published.

However, a protein termed 14-3-3 is found localized at the centrosome as well as the mitotic spindle (Pietromonaco et al, 1996). Recently, it has been observed that the protein binds to topoisomerase IIα *in vitro*. The group claims that the 14-3-3 protein recruits topoisomerase IIα to the centrosome and thus involves the enzyme in co-ordination of the centrosome duplication with the normal genomic cell division (Giesler et al, 1999). It is not likely that either the normal nuclear topoisomerase IIα or the centrosomal protein is associated with the 14-3-3 protein. In mitotic cells the distribution of nuclear topoisomerase IIα is found along the axis of the chromosome. It is neither found in the spindle apparatus nor at the poles of this structure. Furthermore, the centrosomal protein is not only restricted to cycling cells but also appears in resting cells, which indicates that it is probably not recruited to the centrosome during mitosis. According to the results the expression of the centrosomal protein seems to be constant in all stages of the cell cycle. Finally, it should be noted that the protein may be inactive, so the function of this protein is extended beyond the DNA turnover. Based on this information

the topoisomerase IIα-like protein may not be involved in any biological function but stored at the centrosome. It could be meaningful for resting cells, which are devoid of topoisomerase IIα to have such a spare fraction. According to the data presented in the first part of this thesis the mitotic function of topoisomerase IIα are not adopted by topoisomerase IIβ. However, the centrosomal proteins might adopt the function and a mitotic catastrophe in the cells could therefore be prevented.