Discussion

Accurate and timely replication of the metazoan genome involves the cell cycle regulated activation of DNA replication proteins upon discrete sites (origins) along the chromosomes. These proteins make up was is known as the prereplicative complex or pre-RC. The formation, activation and deactivation of the pre-RC is achieved through the ordered loading and unloading of an essential group of proteins whose interactions and activity are regulated by cell cycle kinases that alter the phosphorylation status of specific subunits. Once replication from an origin has begun, the pre-RC must be inactivated until the next round of DNA synthesis. The proteins that comprise the pre-RC are highly conserved across a wide range of species which suggests a common mechanism for the initiation of DNA replication (see figure 1).

Much of what is known about the initiation of DNA replication in eukaryotes has been discovered through an immense body of work carried out in *S. cerevisiae, X. laevis* and *D. melanogaster* (see introduction). These organisms have major advantages for studying the initiation process: conditional mutations and the presence of genetically defined, transposable origins of replication in *S. cerevisiae* and *D. melanogaster* and an *in vitro* DNA replication assay in *X. laevis.* These advantages do not exist in mammalian systems and a most commonly used tool for studying gene function, the knockout mouse model, offers no advantage for studying genes which are required before a single cell can divide. The use of knock-out models for mcm genes may be an option, but would be extremely labor intensive and involve highly sophisticated genetic knock-out/knock-in and transgenic strategies combined with chemical methods to keep gene expression on during developement and then turning it off in a tissue specific manner. The only published mouse knock-out model for a replication gene, cdc 45, is viable in the heterozygous state and presents no obvious defect in the initiation of DNA replication [64]. Cdc45 homozygous mutant mice die at

the earliest stages of embryogenesis. In another study, a hypomorphic knock-out of ORC2 in the HCT 116 human colon cancer cell line which resulted in a 90% decrease in ORC2 levels [23]. While G1 phase was prolonged, there was no apparent negative effect once cells entered S phase.

The human cervical carcinoma cell line, HeLa, has been used for a majority of research into the initiation of human DNA replication. Transfecting HeLa cells is a relatively easy process and has enabled the stable transgenic expression of *in vitro* mutated genes introduced into the genome. This has been of immense importance for elucidating the mechanisms of pre-RC formation *vis-à-vis* protein:protein and protein:DNA interactions. Even though it has been reported that the spatio-temporal localization of initiation proteins on chromatin is not altered between transformed and non-transformed cells [105], the study of cell cycle regulated control of pre-RC formation in a cell line with mutations in key cell cycle regulators which monitor and control the progression from G1 to S-phase may be problematic [106]. For example, in contrast to primary human cells, HeLa cells continue to proliferate in the absence of serum indicating an uncoupling of mitogenically driven S phase gene expression and DNA synthesis and removal of DNA replication genes in HeLa results in apoptosis.

Methods to succesfully introduce and express small interfering RNAs in primary, non-transformed cell lines have not been particularly successful with the majority of publications to date limited to transformed human cell lines [22, 107]. For example, RNAi has been successfully used to study DNA replication genes in *D. melanogaster* [57]. An alternative approach is the use of phosphorothioated antisense oligonucleotides which have been shown to efficiently block gene expression in transformed as well as non-transformed human cells [107, 108].

The work presented in this thesis involved the targeting of a vital DNA replication gene, mcm4, in primary human fibroblasts, IMR90 cells. Antisense mediated mcm4 gene inactivation blocked synthesis of MCM4 protein which resulted in the

degradation of non targeted MCM proteins. DNA synthesis was prevented and cells accumulated at the G1-S phase boundary and in what appears to be G2 phase when transfections were carried out in asynchronous cultures. Serum starved cultured restimulated in the absence of MCM4 failed to enter S phase. Flow cytometric analysis revealed no toxic side effects of the transfection reagent nor of the oligos themselves, in that no apoptosis was observed. Additionally, simple light microscopy of transfected primary human fibroblasts did not reveal any increase in the amount of floating necrotic cells. Lastly, controlled transfections altered neither the cell cycle distribution nor cellular proliferation as cultures grew to confluence with the same kinetics as untransfected cells. The novel finding presented in this thesis is the response to mcm4 gene inactivation which results in the degradation of non-antisense targeted MCM proteins. The data presented should provide a useful reference for future work in studying the formation of the eukaryotic pre-RC and potentially in the developement of therapies to halt oncogenic activation of unrestricted cell proliferation.

Blocking mcm4 gene expression with antisense oligonucleotides

A panel of 8 phosphorothioated RNA:DNA:RNA antisense oligonucleotides called GeneBlocs (GBs) designed to target human Mcm4 mRNA, was transfected into asynchronously proliferating primary human fibroblasts, IMR90 cells, and downregulation of Mcm4 mRNA analysed by Real-time rtPCR. Two GBs (GB 22173 and GB2 2174) showed a strong downregulation of Mcm4 mRNA of up to 90% when compared to levels in untransfected cells. It was further shown that GB 22174 blocked the synthesis of MCM4 protein which was not detectable by immunoblotting 24 hours after transfection. Control transfection of an oligo containing 6 mismatched bases in relation to GB 22174 had no effect on MCM4 protein synthesis.

A single transfection of GB22174 was sufficient to obtain a sustained downregulation of mcm4 gene expression up to 120 hours post-transfection as

determined by immunoblotting (figure 9). As such, repeated transfections were not performed for any experiments. Binding of the GeneBloc to its target mRNA results in a DNA:RNA (GeneBloc central DNA portion: mRNA target) duplex which activates the RNase H pathway. Upon activation, RNase H degrades the targeted mRNA leaving the GeneBloc intact. Whether the released GeneBloc can catalyze further mRNA degradation is unclear, but the 5' and 3' abazic cap modifications of the oligos prevents their digestion by nucleases [97, 98]. Thus, the high transfection efficiency coupled with a presumable stable concentration of intracellular GeneBlocs results in a strong and specific down-regulation of target mRNA.

Antisense technology has been successful in blocking the expression of a number of genes involved in the formation of the pre-RC. Fujita et. al. have shown the inhibition of S phase entry of W138 cells, a primary human fibroblast, after mcm7 gene expression was blocked by antisense oligonucleotides [108]. Feng *et al.* have reported using phosphorothioated antisense oligonucleotides to block cdc6, cdc45 and mcm2 expression in normal and cancerous cells [107]. When any of these gene products were destroyed in cancer cells, the cells rapidly entered an abortive S phase resulting in apoptosis. Blocking the expression of these same genes in normal cells resulted in a G1 phase arrest with no obvious apoptotic activation. The differential response of transformed cells and primary cells in the absence of DNA replication genes could provide the basis of a future therapy against cancerous cell growth.

Of interest for the work presented in this thesis is the fact that throughout all the experiments, sustained depletion of MCM4 of up to 120 hours resulted in no detectable apoptotic response in IMR90 cells, underscoring the advantage of using nontransformed cell lines to obtain an accurate picture of cell cycle regulated control of pre-RC formation and DNA replication.

A time course experiment of GB 22174 transfected into IMR90 cells (figure 6, table3) revealed a specific kinetic of Mcm mRNA down regulation, not only of the targeted Mcm4, but of two non-targeted gene products, Mcm3 and Mcm5, both of which are essential for the formation of the pre-RC. Although the transfection efficiency of this particular experiment was not as high as the majority of other experiments, the levels of Mcm3, Mcm4 and Mcm5 mRNAs were downregulated over the course of 48 hours to 42%, 25% and 83% of the levels found in untransfected cells, respectively. The downregulation of Mcm3 and Mcm5 mRNAs parallel their disappearence at the protein level, with MCM3 protein disappearing before MCM5 protein. Interestingly, 15 hours post-transfection the levels of both Mcm3 and Mcm5 mRNAs were upregulated by 50% and 60%, respectively. Upregulation was not observed at the protein level at 15 hours after transfection (figure 11). After 48 hours Mcm3 mRNA was downregulated by 57% and Mcm 5 by 17%. Downregulation of mcm gene expression has been shown in HL60 cells in response to TPA mediated differentiation [48, 80]. In human fibroblasts starved of serum, Mcm mRNA levels are effectively abolished [81]. Whether the down regulation of non-targeted Mcm mRNA is a non specific can not be ruled out, but the mismatch control shows no down regulation. Additionally, the next best fit match for GB22174 would have 8 mismatched base pairings (see materials).

Whereas the levels of MCM proteins remain fairly constant throughout the cell cycle, mcm mRNA levels fluxuate with progression through the cell cycle and are in large part driven by E2F expression. Transcription of mcm genes peaks during G1 and steadily decreases as cells enter and traverse S- phase. Mitogenic signals result in the expression of a host of genes whose products are needed to traverse S-phase. MCM proteins are not only constant throughout the cell cycle, but found in abundance . Why would a cell need more of an already abundant protein? It may suggest a qualitative difference in "old" versus "new" proteins which may be the difference between phopshorylated and unphosphorylated MCM proteins. It could also be the case that assembly of new MCM

subcomplexes occurs only with newly synthesized MCMs whilst "older" or phosphorylated MCMs are prevented from reforming into new subcomplexes or are degraded. This is also supported by the half-life of MCM proteins which has been shown to be approximately 21 hours and thus would preclude a given set of MCMs from participating in a second round of DNA synthesis.

Blocking MCM4 synthesis destabilizes MCM complex subunits

The phosphorothioate antisense oligonucleotide GB 22174 was shown to downregulate mcm 4 gene expression in primary human fibroblast (IMR90 cells) to 10% of the levels found in untransfected cells. A mismatch control was shown to have little effect on mcm4 gene expression. These two GBs were used to study the effects on protein synthesis after their transfection into IMR90 cells. Figures 7 and 8 show that transfection of the control GB, MM, does not effect the synthesis of MCM proteins as their levels remain unaltered in comparison with untransfected cells. Twenty four hours after transfection with GB 22174, MCM4 and MCM2 proteins are not detected by immunoblotting. The level of MCM3 protein was also decreased, but still detectable and only after 48 hours were the levels approaching the lower detection limit. MCM5 protein levels at 24 hours post transfection were unaffected and gradually decreased to below detection after 72 hours (figures 8 and 9). None of the MCM proteins are detected 120 hours post transfection.

The mechanism of degradation of the nontargeted MCM subunits is unknown. One possibility is that the destabilization of the MCM2-4-6-7 complex by removing a subunit, MCM4, may trigger the proteolytic degradation of the remaining subunits, but an 8 hour treatment of IMR90 cells with 5 μM MG132 proteasome inhibitor (ref) 22 hours after transfection with GB 22174 did not prevent the disappearence of MCM2, MCM4 and MCM6. Thus the degradation of MCM proteins in these experiments is not achieved through proteolysis via the proteosome as is CDC6 and ORC2 [109]. Suprisingly, MCM7 levels remained

unchanged in GB 22174 transfected cells with or without MG132 treatment (data not shown, P. Debs and M. Gossen, manuscript in preparation) Thus MCM3, MCM5 and MCM7 are unchanged in IMR90 cells 30 hours post-transfection. In light of the data so far, this is an unexpected finding in that MCM7 has been found in an *in vitro* sub-complex with MCM 4-6-7 (± MCM2) [41] and was expected to be degraded with MCM2 and MCM6 in the absence of MCM4. Conversely, Tsuruga *et al.*(ref) showed that IP of HeLa whole cell extracts with MCM3 or MCM5 pulled down MCM7 and vice versa [41]. Thus, MCM7 may be sequestered when not involved in helicase activity and thus stabilized in the absence of MCM4. The question remains how the remaining subunits are degraded. The block in proliferation as effected by removal of MCM4 and as shown by removal of mitogenic stimulation, may turn off mcm gene expression and the rapid degradation of the remaining subunits may be just a function of protein half-life.

Taken together with the results in figure 8 and 9, the destabilization of the MCM complex by removing MCM4 correlates with the subunit composition of isolated MCM sub-complexes and would most likely abolish any possible *in vivo* helicase activity of the MCM complex. The *in vivo* helicase activity of the MCM complex is thought to occur with chromatin bound heterohexameric holocomplexes containing MCM subunits 2 through 7. Such a complex has not been isolated and there is no *in vivo* data available to support its existence. The isolation of a MCM4-6-7 complex from both human and mouse systems which supports a helicase activity has been the basis for much of the biochemically proposed helicase activity. In support of this, crystallized archaeal MCM heterohexamer complexes and electron microscopy of the human MCM4-6-7 subcomplex have led to the same conclusion based on physical characteristics of hexameric ring formation of MCM proteins [73, 75].

After the MCM2-4-6 subunits are degraded, the MCM3-MCM5 subunits begins to disintegrate. As discussed above, Mcm3 and Mcm5 mRNA levels are not

downregulated as rapidly as Mcm4 and thus translation of remaining or newly synthesized mRNA may continue and new MCM3-MCM5 complexes could be reformed. Conversly, as the MCM3-MCM5 subunit would not be directly destabilzed in the absence of MCM4, the disappearence of MCM3-MCM5 may possibly be a result of a block in proliferation which results in an overall downregulation of DNA replication gene expression. This would also apply to all of the mcm genes over the time course in the experiments presented above.

The synthesis of MCM proteins is blocked in the absence of mcm4 gene expression

As has been shown with restimulated serum starved primary fibroblasts, mcm gene expression is capable of being reactivated after levels of Mcm mRNAs are depleated below detection limit of RNAse protection assays. One draw back with antisense oligonucleotides is the inability to remove them from cells to restimulate gene expression. Due to their stability and as a result of the block of mcm4 gene expression the halt in proliferation prevents dilution of the oligo through successive cell divisions.

To address the possibility that a feedback mechanism at the mRNA level may shut down mcm gene expression in response to targeted Mcm4 mRNA destruction, IMR90 cells were starved of serum for 3 days to clear the cell of Mcm mRNAs. After 3 days IMR90 cells cultured in the absence of mitogenic stimulation by serum withdrawal, neither MCM2 nor MCM4 are almost undetectable. The cells were then transfected with GB 22174 or the control MM and restimulated 24 hours later. As shown in Figure 12, MCM2 is not resynthesized in the absence of MCM4 after 40 hours of restimulation, whereas in control transfected cells, both MCM2 and MCM4 are resynthesized. Interestingly, the levels of MCM2 and MCM4 proteins in transfected cells are belowe those found after 4 days serum withdrawal indicating the strong block in mcm gene expression. The experiment was designed such that before restimulation, the antisense oligonucleotides would be present in the cell for 24

hours. It was reasoned that any residual mcm4 mRNA would be destroyed and thus increase the effect of absent MCM4 protein. However, as has been shown in Wiebusch [81], there is very little Mcm mRNA in serum starverd cells and it may be postulated that the repression of mcm2 gene expression is initiated at the mRNA level in response to a block in mcm4 gene expression. Detectable MCM2 and MCM4 was present at the time of transfection/restimulation and thus it cannot be ruled out that a lack of protein may in addition lead to decreased mRNA levels, triggering the downregulation of remaining mcm genes.

Chromatin Bound MCM proteins are solubilized in response to MCM4 degradation

Accurate duplication of the mammalian genome must be ensured not only through fidelity of DNA replication but by preventing over-replication which leads to genomic instability. Metazoans have evolved multiply layers of control which prevent anachronistic and inaccurate DNA replication. At the level of the initiation of DNA replication, cell cycle regulated kinases ensure that each of the components of the pre-RC are activated and inactivated at the appropriate time before the onset of and during S-phase. Inactivation of the MCM proteins by CDK/CyclinA mediated phosphorylation results in the disassembly of chromatin bound MCM subunits and thus prevents reinitiation from a given origin [88]. It remains unclear whether or not all complexes are inactivated as reports have shown the presence of MCM proteins travelling ahead of the replication fork [43, 71]. This implies a function during the elongation phase of replication and may require qualitatively different MCM complexes from those present during the formation of the pre-RC. Indeed, multiple phosphorylation sites have been reported for MCM2 and MCM4 subunits and shown to be differentially phosphorylated depending on the cell cycle stage [42, 51, 52, 101, 110]. Additionally, phosphorylation of MCM4 has been shown in response to check point activation during inhibition of DNA synthesis by hydroxyurea or UV irradiation in HeLa cells. In these experiments, MCM4 is released from the chromatin thus resulting in a block in synthesis [101].

Mammalian MCM proteins are found exclusively in the nucleus which is in contrast to *S. cerevisae* where they fluctuate in location throughout the cell cycle. Phosphorylation of MCM2 and MCM4 has been extensively characterized in HeLa cells, Hamster and *Xenopus* [52, 100, 111]. During the transition from G1 to S phase, hypophosphorylated MCM proteins are loaded onto potential origins. As S phase proceeds, MCM 2, MCM 4 and MCM 7 proteins are increasingly hyperphosphorylated by CDK/Cyclin A, resulting in their solubilization and translocation from chromatin into the nucleoplasm (R). Extracts of G2 nuclei show little chromatin bound MCMs and proteins isolated from M phase extracts are hyperphosphorylated .

The MCM localization in asynchronously proliferating IMR90 cells was investigated by immunoblotting of fractionated cell extracts. Figure x shows the immunoblotting results. The majority of MCM2, MCM4, MCM6 and MCM7 are found in the soluble fraction with a substantially smaller amount bound to chromatin (insoluble fraction). MCM2 in the insoluble fraction is exclusively hypophosphorylated (upper band) while soluble MCM2 is both hypo- and hyperphosphorylated (see also [51]). Phosphoisoforms of MCM4 are difficult to detect in figures 10 and 11 as they migrate relatively close to each other. However two different bands are distinguishable in the insoluble fraction and may be the diffentially phosphorylated chromatin bound isoforms of MCM4 as shown in [52]. MCM3 and MCM5 subunits are found at almost equal ratios in both fractions. Flow cytometry shows the majority of asynchronous IMR90 cells in the G1 phase and would seem to agree with the level of MCM bound proteins. Isolating pure populations of very early S phase cells by elutriation showed increase in chromatin bound MCM3, MCM4 and MCM5 in HeLa cells [109]. Beyond the initial firing of DNA synthesis, MCMs are rapidly dislocated from the chromatin, incontrast to ORC2 which is found exclusively bound to chromatin.

The effect of MCM4 degradation on MCM localization was investigated in GB 22174 transfected asynchronous IMR90 cells. Figure X shows the immunoblot results. MCM3 and MCM5 degradation is not significant at these early time points. Compared to untreated or control transfected cells, soluble MCM4 is rapidly degraded after 15 hours treatment with GB 22174. After 30 hours, the insoluble fraction has translocated into the soluble fraction. The same is observed for MCM2. Thus, a block in mcm4 gene expression results in the solubilization of MCM 4 containing chromatin bound subcomplexes. The degradation of MCM proteins occurs rapidly in response to targeted Mcm 4 mRNA destruction and the abundance of MCM proteins does not seem to be sufficient to maintain subcomplex formation for longer than 30 hours after transfection. This suggests that a constant synthesis of MCM proteins is required to maintain MCM complex formation and that "older" MCM proteins are prevented from forming new complexes.

Inactivation of the MCM complex stops proliferation and prevents completion of S phase

The decision to synthesize DNA and divide is achieved through the expression of S phase genes in response to mitogenic stimulation coupled with the genetic program of a individual cells. Cell cycle regulators activate key transcription factors, such as E2F, which drive the expression of S phase genes. These include including several mcm genes and as such their expression is under intimate control of the cell cycle. The loading of hypophosphorylated MCM proteins onto potential origins followed by the activation of the pre-RC by CDC7/DBF4 drives DNA synthesis and traversal through S phase. Prior to and during DNA synthesis, MCM proteins become phosphorylated and are dissociated from chromatin. MCM proteins have been reported to travel ahead of the replication fork and may implicate a role for the MCM complex during elongation. As cells enter G2, the majority of MCM proteins are hyperphosphorylated and not bound to chromatin. Late in M phase and during

G1 hypophosphorylated MCM proteins begin to form on potential origins in preparation for another round of DNA synthesis.

The mcm genes were first described in yeast mutants which did not support replication of extrachromasomal plasmids containing transposable yeast origins (autonomously replicating sequences, ARS), failed to synthesize DNA and accumulating at the beginning of S phase [6, 38]. The *S. cerevisiae* mcm 2 through 7 degron mutants constructed by Labib *et al*, showed that once MCM proteins were loaded onto origins and the mutant yeast placed at the restrictive temperature, DNA synthesis was blocked [43]. Thus showing the requirement of MCM proteins not only for licensing of origins, but also for elongation. In human cells, antisense mediated downregulation of mcm 7 gene expression in serum starved W138 cells restimulated 2 hours after transfection inhibited S phase entry [108].

It was shown that in response to MCM complex down regulation, the proliferatve capacity and synthesis of DNA is drastically reduced. The significant reduction of incorporated BrdU 2 days after transfection confirmed the block of DNA synthesis (figure 17). Additionally, when serum starved cells were restimulated in the absence of MCM, the cells did not enter S phase and exhibited little BrdU incorporation (figure 12,b). Over the course of 96 hours, IRM90 cells in the absence of MCM go through a single population doubling (PDL) as compared to approximately 3 PDLs in untransfected or mismatch transfected cells. The cell cycle distribution in response to targeted mcm4 gene inactivation was investigated by flow cytometry of asynchronously proliferating IMR90 cells transfected with GB 22174 or the control GeneBloc, MM. The data shows that 3 days post transfection, in response to targeted destruction of MCM 4 which, as shown above, results in a degradation of non targeted MCM proteins, primary human cells accumulate at the G1-S phase boundary (figures 15 and 16) and do not incorporate BrdU, a measure of DNA synthesis (figure 17). After 7 days an apparent shift further into S phase is observed and is most likely mitochondrial

DNA replication which continues in the absence of cellular DNA synthesis and is not targeted by the antisense oligonucletides (Dr. Aloys Schepers, personal communication). This type of shift into S phase in the absence of cellular DNA synthesis is also present in the degron mcm mutants, but not discussed.

Further inspection of the flow cytometry experiments reveals an accumulation of cells in what appears to the end of S phase and G2 phase. As these experiments were carried out in asynchronous cultures, it is likely that the cells proliferate until MCM protein levels fall below a critical threshold, stalling at whichever phase this may occur. This is supported by experiments in *Xenopus* extracts which suggested that ~ 10 MCM complexes per origin are required to support rapid DNA synthesis [102]. When depleted to ~ 1 MCM complex per origin, DNA synthesis continues but a drastically reduced rate, mirroring mcm mutants in *S. pombe*.

An accumulation of cells in G2/M phase peak is observed in GB 22174 transfected cells. That this peak does not represent an accumulation of cells in mitosis was determined by flow cytometry of MPM2 immunoreacted cells after transfection. Anti-MPM2 recognizes surface proteins exclusively phosphorylated during mitosis [104]. IMR90 cells missing MCM in these experiments are not reactive for MPM2. Additionally, Hoechst staining of DNA revealed no accumulation of GB 22174 transfected IMR90 cell in mitosis (figure 20). Studies in HeLa have reported in addition to a decrease in DNA synthesis, a defect in chromatin condensation in the absence of Orc6 [109]. In *Drosophilia* depletion of Mcm10, Cdc45, Mcm2, Mcm5, and Orc2 results in aberrant chromosome condensation [57]. And in *Xenopus*, it has been shown that the MCM proteins are loaded onto chromatin at the end of mitosis [69, 95].

Thus the conclusion from these experiments is that cells faced with a continuous decline in MCM synthesis continue to proliferate until MCM proteins are exhausted. As the majority of cells are in G1 phase, the largest block occurs at

the G1-S phase boundary and is further evidence of the requirement MCM proteins for entrance into S-phase. Their seems to be very little increase in the amount of cells at what would be considered the half way point through S-phase, but interestingly there is a significant increase of cells at the S-G2 phase boundary and may suggest a block in the initiation of late firing origins in cells with have a significant portion of the genome already replicated. As mentioned previously, the block in the G2-M phase peak does not seem to be a mitotic arrest and would thus have to be considered a late S-G2 phase arrest. The serum withdrawal experiment as presented shows a clear block of the entry into S-phase in the absence of MCM proteins, but nothing of the potential effects during S-phase traversal. Future work will seek to address these possibilities by utilizing non-chemical methods to synchronize or isolate cells in early S-phase and late S-phase before and after transfection of Mcm4 antisense Genebloc.

MCM proteins in human cells: future prospects

The importance of MCM2 through 7 proteins for DNA replication has been shown in the literature and through the data presented in this work. From the simplest single cell eukaryote to the most advanced metazoan, the absence of any MCM protein is lethal. In spite of a large body of work the function of MCM proteins remains elusive. They are proposed to perform the unwinding of DNA ahead of bidirectional replication forks and thus function as a DNA helicase. Structural studies of the archaeal hexameric MCM complex and electron microscopic analysis reveals ring-like structures which is suggestive of a protein which may form a type of clamp upon DNA, a feature that would be expected for a DNA helicase. Unfortunately, to date there is no *in vivo* data to support a MCM complex helicase activity. It has been suggested that while the MCM complexes may indeed form a clamp around DNA, they serves mainly to attach or anchor the DNA to the nuclear matrix. In this case, the DNA is pulled through the MCM complexes and remains in the absence of solid *in vivo* data, speculative. The developement of a mammalian *in vitro* replication assay would go a long way in clarifying the role of MCM proteins *in vivo*. Conversely, *ex vivo* experiments may never be able to clarify their role in replication. The MCM complex may well be a helicase, but only *in vivo* and extraction from the nuclear milieu may destroy its helicase activity.

It is however clear that MCM proteins are needed for DNA synthesis and that they represent a convergence point for a number of cell cycle regulators which when mutated or inactivated result in unrestrained cellular proliferation as is the case with cancer. Regardless of the the type of cancer and its etiology, the final result is DNA synthesis and uncontrolled cellular proliferation. MCM proteins are increasingly used in the diagnostics of various cancers and more often than not, the simple presence of detectable MCM proteins is a postive marker for cancerous cell growth. Examples include diagnosis of genito-urinary tract cancer

by detection of MCM5 protein in urine sediments , the upregulation of MCM7 by N-Myc in neuroblastomas and the detection of MCM2 expression in cervical cancer [85, 86, 112]. Thus, targeting MCM proteins may offer a potential therapy in the treatment of cancer. Indeed the removal of DNA replication proteins in transformed cells result in abortive S-phase and apoptosis and if a general feature of all cancerous cells, would be a first step [106, 107]. It is however, in my opinion, necessary to establish a clearer picture of the process of the initiation of DNA replication in primary human cell lines with unperturbed cell cycle regulatory pathway components.

Targeting gene expression in living organisms remains a challenging protocol. One possibility is the use of phosphorothioated and other modified antisense oligonucleotide drugs. The only FDA approved antisense drug, Vitravene (Isis Pharmaceuticals) targets the cytomegalovirus IE2 gene and is used to treat CMV related retinitis. A second antisense drug, Genasense (Genta, USA), which targets Bcl2 is in clinical phase III and is being used in conjuction with chemotherapy, which the antisense potentiates in the treatment of metastatic melanomas [113]. Several other antisense based drugs are in various stages of clinical trials. Regardless of the technology used, be it antisense, RNAi, gene therapy or pharmaceutical, it is clear that targeting MCM proteins presents a strong potential for preventing cancer cell growth.