Multiple Mechanisms Regulate the Human Replication Factors - Replication Protein A and DNA Polymerase α- during DNA Replication and DNA Repair

Dissertation

Zur Erlangung des akademischen Grades
doctor rerum naturalium
(Dr. rer. nat)

Vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

Von
cand. scient. (Diplom Immunologie) Shuhua Chen

Geboren am 10.11.1969
in Wuhan, China

Table of Contents

Abbreviations

A	Adenosin
A	Ampere
APS	Ammonium persulfate
BSA	Bovin Serum Albumin
bp	Base pair
C	Cytosin
°C	degree
CdK	Cyclin-dependent Kinase
IP	Immunoprecipitation
Da	Dalton
DNA	Desoxyribonucleic acid
DAPI	4',6-Diamidino-2-phenylindole
ds	Double strand
DTT	Dithiotreitol
EDTA	Ethylendiamine tetraacetic acid
h	hour
k	kilo
l	liter
µ	micro
m	mili
m	meter
M	molar
MW	molecular weight
min	minute
n	nano
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PKC	protein-Kinase C
Pol	Polymerase
Pol-prim	DNA-polymerase-α-Primase

PP2A	protein phosphatase 2A
RNA	ribonucleic acid
RPA	replication protein A
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecylsulfate
sec	second
ss	single strand
T	Thymin
TE	Tris-EDTA solution
TEMED	Tetramethylendiamin
Tris	Tris-(hydroxymethyl)-aminomethane
UV	ultra violet
V	volt
Vol	volume
v/v	volume per volume
wt	wild type
w/v	weight per volume
XP	Xeroderma Pigmentosum

1 Introduction

1.1 Cell Cycle and DNA Replication

Duplication of eukaryotic cells and its regulation have been in the center of the biomedical research since more than hundred years. The model, which summarises the replication of the chromosomal DNA and finally the formation of two daughter cells, is called the eukaryotic cell cycle. The cell cycle can be divided into four phases. During S phase (S for synthesis), the cellular DNA of each cell is faithfully replicated which is an essential prerequisite for cell division. The phase, in which the cell splits into two, is called M phase of the cell cycle. G_1 phase (G for gap) is the time interval between the completion of M phase and the beginning of the next S phase. In contrast, G_2 phase is the interval between the end of S phase and the beginning of M phase.

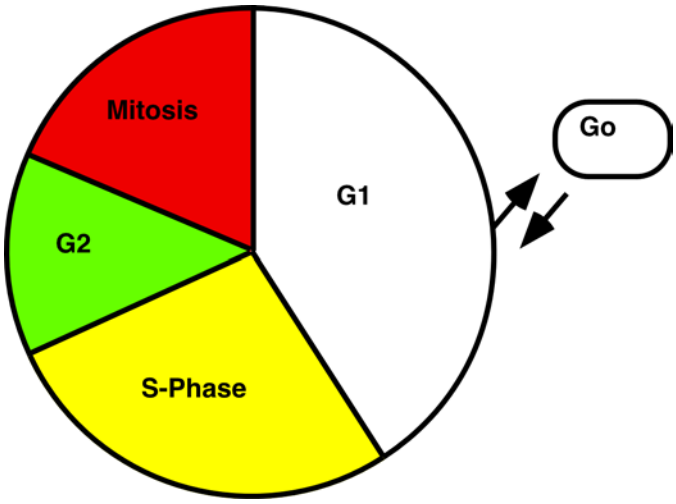


Figure 1.1 The Eukaryotic Cell Cycle (from Dr. H-P Nasheuer)

Eukaryotic DNA replication is tightly controlled and takes place during a restricted period of the cell cycle, the S phase (Donaldson and Blow, 1999; Stillman, 1996). The initiation of DNA replication requires a set of proteins to bind to a small region of DNA, the origin of DNA replication. In late mitosis to early G_1 phase these proteins assemble a complex which is called pre-replication complex (pre-RC). The pre-RC consists of the origin recognition complex (ORC), the cell division cycle protein 6 (Cdc6p), and the minichromosome maintainance protein complex (Mcm) (Takisawa and Kubota, 2000; Madine and Laskey, 2001). ORC is a sequence-specific DNA binding protein, which consists of 6 subunits (Orc 1-6). ORC binds and remains bound to origins during most or all of the cell cycle, and is a marker of replication origin.

Cdc6p associates with origins from late M phase until late G_1 phase of the next cell cycle. Human Cdc6p locates in nuclei during late M phase and G_1 phase, and moves to the cytoplasm in S phase (Saha *et al*., 1998). Cdc6p cooperates with ORC and Cdt7 to load the Mcm protein complex, which consists of the Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7 proteins. The Mcm2-7 complex is necessary not only for initiation but also for elongation of DNA synthesis. It is phosphorylated at the G_1-S phase transition. This phosphorylation probably causes a structural change of the Mcm complex which might allow the formation of Mcm4,6,7 complex and the binding of cell division cycle protein 45 (Cdc45p). The Mcm4,6,7 complex has DNA helicase activity *in vitro*. It can unwind dsDNA and load Cdc45p (Ishimi, 1997, 2000; You *et al*., 1999; Zhou and Stillman, 2000). Cdc45p is a critical factor for conversion from the pre-RC to the initiation complex (IC) at the transition from G_1 to S phase. IC consists of Mcm4,6,7 complex, Cdc45p, and replication protein A (RPA). The formation of Cdc45p, RPA and Mcm4,6,7 complex recruits DNA polymerase α-primase onto chromatin in S phase (Mimura and Takisawa, 1998).

DNA polymerase α-primase comprises two essential enzymes: a DNA-dependent RNA polymerase (also called primase) and DNA-dependent DNA polymerase. First the primase synthesises a 10-ribonucleotide-long RNA primer on the leading strand and later that of each Okazaki fragment on the lagging strand. Subsequently, a polymerase switch takes place and DNA polymerase α elongates this RNA primer by addition of deoxynucleotides to form an RNA-DNA primer with a size of about 40 nucleotides.

DNA polymerase α-primase complex is composed of four subunits: p180, p68, p58 and p48 (reviewed by Burgers, 1998; Hübscher *et al*., 2000; Nasheuer *et al*., 2002; Waga and Stillman, 1998). Their main structure is conserved among all eukaryotes (Wang 1996). The p48 subunit contains the catalytic center of primase activity. It can synthesise short RNA primers *in vitro* (Nasheuer and Grosse, 1988; Santocanale *et al*., 1993; Schneider *et al*., 1998). The p58 subunit has no enzymatic activity, but it stabilises the thermolabile p48 and supports the function of p48 (Copeland and Wang, 1993; Nasheuer *et al*., 1988; Santocanale *et al*., 1993; Schneider *et al*., 1998). p58 mediates the interaction between the p180 and p48 subunits, and might have a function in switching from primer synthesis to DNA elongation (Longhese *et al*., 1993). The p180 subunit has the DNA polymerase activity, but has no proofreading 3'-5' exonuclease activity (Kunkel *et al*., 1991). It is expressed from G_0/G_1 transition and

stays constant during cell cycle (Pearson *et al.*, 1991; Wahl *et al.*, 1988; Wang *et al.*, 1995). The p68 subunit, also called B subunit, has no enzymatic activity, but exhibits regulatory functions since it is phosphorylated in a cell cycle-dependent manner and its phosphorylation state is reported to be a consequence of DNA damage during S phase (Collins *et al.*, 1993; Foiani *et al.*, 1994,1995; Nasheuer *et al.*, 1991; Pellicioli *et al.*, 1999; Voitenleitner *et al.*, 1999). DNA polymerase α displays a low processivity and lacks an intrinsic 3'-5' exonuclease activity, it is more error-prone than other replicative DNA polymerases (Kunkel *et al.*, 1991; Maga *et al.*, 2001). However its association with the tumor suppressor protein p53 which has 3'-5' exonuclease activity might increase genome stability (Kühn *et al.*, 1999; Melle and Nasheuer, 2002).

In the following step after DNA polymerase α-primase has produced the short RNA-DNA primer, replication factor C (RFC) removes it from the newly synthesised RNA-DNA primer and loads the proliferating cell nuclear antigen (PCNA) in the primer. PCNA is a ring-shaped sliding clamp protein. RFC recognises the primed template junction and couples ATP hydrolysis to open and close PCNA around DNA (Naktinis *et al.*, 1996; Stukenberg *et al.*, 1991; Stillman,1994. It is a homotrimer of three 36kDa subunits, and assembles around the primed site. The ring formation and its interaction with DNA polymerase δ and ε allows the processive elongation of the primers (Liu *et al.*, 2000). PCNA increases the processivity of these DNA polymerases by preventing their premature dissociation from DNA.

Unlike DNA polymerase α-primase which only synthesises short stretches of DNA, DNA polymerases δ and ε can incorporate thousands of nucleotides into the newly synthesised DNA. In mammalian cells the DNA polymerase δ is a heterohexamer of 125 kDa, 68 Kda and 50 kDa and 12 kDa subunits. It is essential for both leading and lagging strand DNA synthesis during DNA replication in eukaryotes. The DNA polymerase ε probably also consists of 4 subunits. It is involved in the DNA replication of chromosomes in mammalian cells. The largest subunit is a catalytic subunit, which also has 3'-5' exonuclease activity, it enables DNA polymerase ε to play a role in base excision repair. DNA polymerase ε thus provides strand displacement synthesis in the long patch mode of repair (DeMott *et al.*, 1998). The current model of the initiation process of DNA replication is summarised in Figure 1.2.

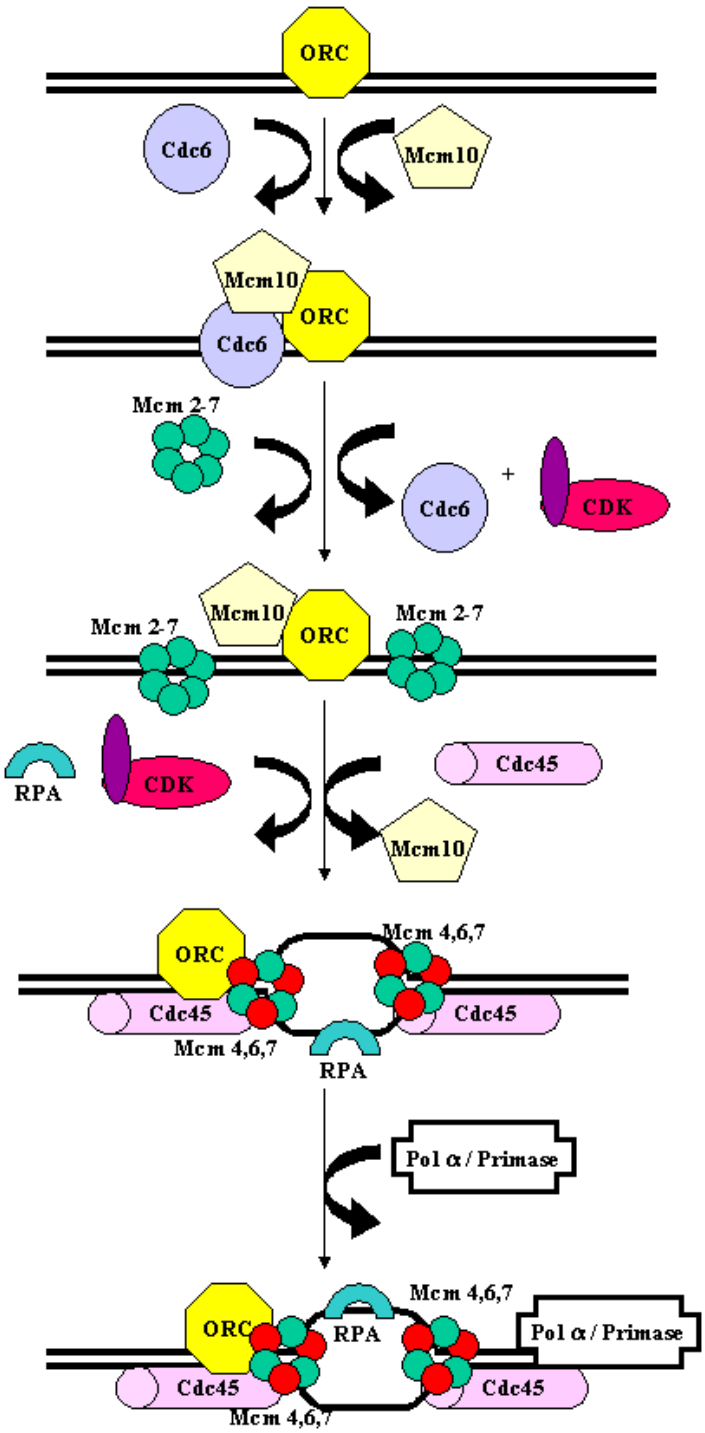


Figure 1.2 Model of Eukaryotic DNA Replication Initiation (from C. Bauerschmidt)

1.2 Replication Protein A

The prerequisite for DNA replication is that, double-stranded DNA (dsDNA) must be unwound to single-stranded DNA (ssDNA). This event is carried out by a co-orperation of helicases, Mcm4,6,7 complex, and replication protein A (RPA). ssDNA alone is not stable under physiological conditions. Therefore single-stranded DNA binding proteins (SSB) are required for stablising ssDNA.

Replication protein A (RPA), also named human single-stranded DNA binding protein (SSB) or replication factor A (RFA), is a heterotrimeric protein complex which consists of three subunits with molecular masses of 70, 32 and 14 kDa. RPA is highly conserved in its subunit structure, DNA binding activity and post translational modifications in all examined eukaryotes (Adachi & Laemmli, 1992; Atrazhev *et al.,* 1992; Brill and Stillman, 1989; Brown *et al.,* 1992; Mitsis *et al.,* 1993; Wobbe *et al.,* 1987; Wold and Kelly, 1988). The majority of RPA remains complexed under all examined conditions, but in some cell types, crude cell extracts may contain small amounts of p32 free from p70 (Loo *et al.*, 2000).

1.2.1 Functions of Replication Protein A

1.2.1.1 RPA in DNA Replication

The development of a cell-free SV40 DNA replication system, an extremely useful model for eukaryotic DNA replication *in vitro* which requires only one viral protein, the large T antigen, has led to the identification and characterisation of various essential cellular replication factors (Challberg and Kelly,1989; Hurwitz *et al.*, 1990; Kelly, 1988; Li and Kelly, 1984; Sogo *et al.,* 1986; Stillman 1989). RPA was first described as a factor required for SV40 DNA replication which stabilised ssDNA regions created during unwinding of dsDNA (Fairman and Stillman, 1988; Wobbe *et al.,* 1987; Wold and Kelly, 1988). It turned out later that RPA is a member of the DNA replication initiation complex and helps to form the replication fork. Its role in DNA replication elongation is discovered through indirect immunofluorescence in chinese hamster ovary (CHO) and HeLa cells showing that all three RPA subunits associate specifically with sites of ongoing DNA synthesis, similar to the replication protein PCNA (Dimitrova and Gilbert, 2000). In addition, RPA increases the fidelity of DNA replication (Carty *et al.,* 1992; 1993; Roberts *et al.,* 1993; Suzuki *et al.,* 1994), here it works as a ”fidelity clamp” for DNA polymerase α (Maga, *et al.*, 2001). RPA acts as an auxiliary factor for DNA polymerase α and plays a dual role: (i) it stabilises the DNA polymerase α/primer complex, thus acts as a polymerase clamp and (ii) it significantly reduces the

misincorporation rate of DNA polymerase α (Maga *et al.*, 2001). In addition, RPA modulates the strand displacement activity of DNA polymerase δ during Okazaki fragment processing (Maga, *et al.*, 2001)

1.2.1.2 RPA in DNA Repair

In addition to DNA replication, RPA has been shown to be essential in DNA repair. Since cellular DNA is continuously damaged by both environmental influences and spontaneous decay, such as depurination or deamination, repair of damaged DNA is essential to maintain the genetic integrity. Each eukaryotic organism has evolved its processes to repair DNA lesions. The major repair systems comprise mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and double-strand break (DSB) repair. RPA has an important role in NER, BER and MMR (Aboussekhra *et al.,* 1995; Baumann and West, 1997; Coverly *et al.,* 1991; 1992; DeMott *et al.,* 1998; Dianov *et al.,* 1999; Heyer *et al.,* 1990; Longhese *et al.,* 1994; Moore *et al.,* 1991; Otterlei *et al.,* 1999. Shivji *et al.,*1992; Stucki *et al.,* 1998). NER is important for repairing cyclobutane pyrimidine dimers resulting from DNA damage. In the early steps of the NER, RPA cooperates with the Xeroderma pigmentosum damage recognition protein A (XPA), which coordinates the NER process (He *et al.,*1995; Matsuda *et al.,* 1995).

RPA is also involved in post-replicative base excision together with major nuclear uracil-DNA glycosylase (UNG2) and PCNA to remove the incorporated uracil. Human RPA specifically stimulates the activity of human DNA ligase I, which is also a BER component, by approximately 15-fold, whereas other analysed single-stranded binding proteins could not substitute for RPA (Ranalli, *et al.*, 2002).

1.2.1.3 RPA in Recombination and Transcription

RPA is required in homologous recombination for stimulation of strand-exchange proteins of *Saccharomyces cerevisiae* and human cells (Heyer *et al.*, 1990; Moore *et al.*, 1991). In addition, Perrault *et al.*, (2001) discovered the effect of RPA on non-homologous end joining by using radiation-induced DNA-DSB and HeLa cell extracts as an enzyme source. Rejoining of DSB proceeded faster and to higher levels of completion when extra RPA was added. They further demonstrated that depletion of RPA from HeLa cell extracts reduced the rejoining activity. This reduction can be restored by addition of RPA. RPA has also connection to transcription since the function of RPA is modulated by direct binding of various transcription factors such as p53, VP16 and GAL4 and a direct role of RPA in transcriptional control has been

discussed (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan, 1993; Singh and Samson, 1995).

1.2.1.4 RPA in Checkpoint Control and Cellular Disorders

In G_1, S, and G_2 phases cells must decide whether to proceed to the next phase or pause to allow more time to prepare cellular content. These specific checkpoints are also involved in the control of repair mechanisms, transcription, telomere length, and apoptosis. There is a protein network for checkpoint control. RPA is part of this protein network. The RPA 70 subunit is required in the S-phase DNA damage checkpoint (Longhese *et al.,* 1996).

RPA is reported as being relevant to various human disorders, such as Werner Syndrome (WS) and Bloom Syndrome (BS). The genes responsible for WS and BS code WRN protein (WRNp) and BLM, respectively. Both WRNp and BLM belong to the RecQ DNA helicase family. WRNp forms distinct nuclear foci in response to DNA damaging agents, which overlap well with the foci of RPA and partially with foci of Rad 51p. RPA can also stimulate the helicase activity of WRNp. BLM co-localises transitionally with RPA when it enters the nucleolus (Opresko *et al.*, 2001; Sakamoto *et al.*, 2001; Sanz *et al.,* 2001). Bischof *et al.* (2001) reported that in normal human cells, RPA, hRad51p and BLM resemble ionising radiation-induced foci.

1.2.2 RPA-Protein Interactions

In the initiation of eukaryotic DNA replication E. coli SSB is unable to substitute for human RPA. This suggests that the activity of RPA requires specific protein-protein interactions (Dornreiter *et al.,* 1990; 1992; 1993; Gannon and Lane, 1987; Melendy and Stillman; 1993; Schneider *et al.,* 1992; Smale and Tijan, 1986). This view is supported by the observation that despite the conservation among species, only mammalian RPA can support SV40 replication *in vitro* (Brill and Stillman, 1991; Brown *et al.,* 1993; Collins and Kelly, 1991). It is well known that specific protein-protein interactions of replication factors are required during primosome assembly, during initiation of DNA replication and at the replication fork (Weisshart *et al.*, 2000), summarised by Hübscher *et al* (2000) and Nasheuer *et al* (2002).

1.2.2.1 RPA Interacts with DNA Replication Proteins

RPA has to interact with various proteins during cell cycle to perform its functions. Biochemical analysis has shown that RPA interacts with DNA polymerases α, δ and ε to synthesise DNA as well as with several DNA helicases to unwind long patches of nucleotides (Erdile *et al.,* 1991; Georgaki *et al.,* 1994; Haracska *et al.*, 2001; Kenny *et*

al., 1989; 1991; Lee *et al.,* 1989; 1990; 1991; Matsumoto *et al.,* 1990; Melendy and Stillman, 1993; Muramaki and Hurwitz, 1993; Seo *et al.,* 1991; Thömmes *et al.,* 1992; Tsurimoto and Stilllman, 1989; 1990). Recently It was reported that RPA binds to DNA polymerase η and ι (Washington *et al*., 2001). Human DNA polymerase η (hPolη) functions in the error-free replication of UV-damaged DNA, and mutations in hPolη cause cancer-prone syndrome. RPA, together with replication factor C and PCNA, stimulates the DNA synthetic activity of hPolη and hPolι and therefore increases the efficiency of nucleotide insertion.

1.2.2.2 RPA Interacts with DNA Repair Factors

The interaction of human RPA and XPA is essential for XPA-mediated NER activity, because the association of human RPA stimulates the ability of XPA to bind the damage sites (Saiji *et al*., 1996). XPA and XPG are required for damage recognition and excision. XPA interacts with both p32 and p70 and forms RPA-XPA complexes, which enhances the excision in the presence of damaged DNA-binding protein (DDB), or stabilises the complex of XPA-damaged DNA complex through protein-protein interactions. The XPA-RPA complex binds to damaged DNA with RPA mediating the contact and then recruits the XPG and ERCC1-XPF nucleases for excision of damaged sites (Hey *et al*., 2001; Li, *et al*., 1995; Wakasugi, *et al*., 2001; Wang, *et al*., 2000). Human RPA furthermore modulates the ability of these nucleases to cleave the damaged strands (Matsunaga *et al*., 1996; Evans *et al*., 1997). RPA itself can recognise damaged DNA with a low dissociation constant and a nearly 15-fold preference over undamaged DNA (He *et al*., 2001).

The function of RPA in BER depends on its binding to the residues aa 28 to 79 of human uracil-DNA glycosylase (UNG2), which has a homologous sequence to the RPA-binding region of XPA. Direct physical interactions of UNG2 with PCNA and RPA are shown (Otterlei *et al*., 1999). In addition, the human RPA subunit p32 binds to human Rad52p. Rad52p is an essential protein for DSB repair and binds to ssDNA to facilitate strand annealing. RPA-Rad52p binding promotes strand exchange of complementary ssDNA molecules (Shinohara *et al*., 1992; Sung, 1994; Mortensen *et al*., 1996).

1.2.2.3 RPA Interacts with Recombination, Transcription and Checkpoint Factors

Rad 51p is a DNA-dependent ATPase and has a central function in DNA recombination. RPA binds to Rad 51p through the residues aa 169 to 326 of p70, and

stimulates the strand transfer activity of Rad51p (Baumann and West, 1997; Golub *et al.*, 1998).

As mentioned above, RPA performs an influence on transcription by its interaction with transcriptional activators such as GAL4 and VP16. Human RPA also associates with EBNA1, the latent origin-binding protein of the Epstein-Barr virus (Zhang *et al.*, 1998) and E2 of bovine papillomavirus (BPV), a transcriptional activator and a BPV replication factor (Li *et al.*, 1993).

During checkpoint controls RPA can physically interact with nucleolin, a key factor in ribosome biogenesis, with high affinity. This binding prevents initiation of DNA replication after cell stress (Daniely and Borowiec, 2000).

1.2.3 The Structure of RPA

As mentioned above, RPA consists of three subunits p70, p32, and p14. The amino acid sequences of RPA show a high degree of homology in various species (Iftode, *et al.*, 1999), and some can even substitute for each other in certain reactions. However RPA also shows some species specificity. In specific processes such as SV40 DNA replication *in vitro,* mammalian RPA is essential and yeast RPA cannot replace it (Melendy and Stillman, 1993; Stadlbauer *et al.*, 1996). To study the structure and the modular composition of the RPA complex protease stability experiments were carried out. The p70 subunit of RPA was first cleaved by trypsin into two structural domains, a 18 KDa N-terminal domain and a 52 KDa C-terminal domain (Gomes, *et al.*, 1996). By deletion and structural analysis three functional domains of p70 were identified: an N-terminal domain (approximately residues 1-170), a central DNA binding domain (approximately residues 170-450) and a C-terminal domain (approximately residues 450-616, see Figure 1.3). The N-terminal domain of p70 is involved in physical interactions of RPA with proteins, such as DNA polymerase α-primase, T-antigen, p53 and various transcription factors. The central part of p70 is composed of two DNA-binding domains, DBD-A and DBD-B, which are essential for RPA binding to ssDNA. It has been reported that DBDs also bind to damaged DNA and are likely to bind to dsDNA. The C-terminal domain contains DBD-C and a Zinc finger. DBD-C is responsible for the formation of the RPA heterotrimeric complex (Gomes *et al.*, 1996, Gomes and Wold, 1995,1996, Kim, *et al.*, 1996; Lin *et al.*, 1996). DBDs have additional function like interaction with transcription factors and the SV40 large T antigen (Blackwell and Borowiec, 1994; Brill and Bestin-Shanower, 1998; Philipova *et al.*, 1996; Wold, 1997). Thus by its modular composition RPA can carry out various functions, such as DNA initiation, DNA recombination and DNA repair.

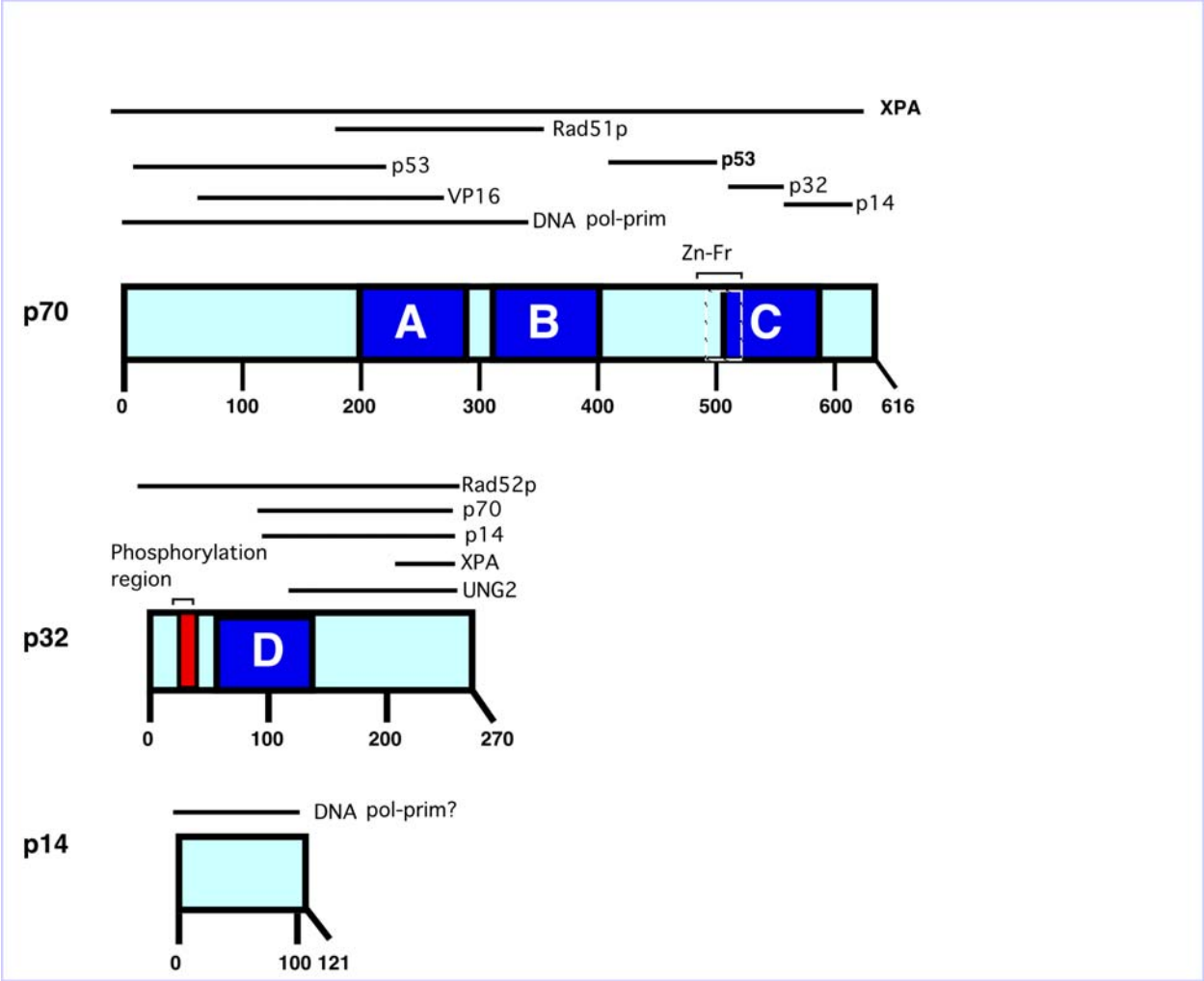


Figure 1.3: Modular Composition of RPA: DNA-binding Domains, Protein-interaction Sites and Phosphorylation Site, and Zn Finger (from Iftode et al, 1999)

p70 alone cannot support DNA replication, demonstrating that the p32 and p14 subunits are essential for RPA function. These two subunits form a soluble but ineffective complex. This RPA subcomplex has no detectable ssDNA binding activity and is not phosphorylated efficiently, although it contains various *in vivo*-phosphorylated sites. It interacts with SV40 T antigen only slightly better than nonspecific proteins. It is not able to support DNA replication. However, this subcomplex is believed to be essential for proper folding of the 70-kDa subunit and/or assembly of the RPA complex (Henricksen *et al.*, 1994; Stigger *et al.*, 1994). RPA p32 has three functional domains, an N-terminal domain, a DNA binding domain D (DBD-D) and a C-terminal domain. The N-terminal domain is required for p32 phosphorylation, whereas the DBD-D probably performs DNA binding and RPA complex assembly. The C-terminal domain is responsible for protein interaction. p32 interacts with proteins such as XPA, UNG2 and RAD52, each of which functions in a different repair pathway (Mer *et al.*, 2000; Nagelhus *et al.*, 1997). Although there is

little knowledge about p14, it is important for RPA complex association and stabilisation. It forms a stable p32-p14 subcomplex, and probably acts as a bridge between p70 and p32.

1.2.4 Assembly of the RPA Trimer and its Physical Binding to ssDNA

The trimerisation of RPA is mediated by three domains DBD-C (on p70), DBD-D (on p32) and p14. p32 and p14 first form a stable p32-p14 subcomplex, then p70 binds to this complex (Bochkareva *et al.*, 2002). The conformation of the RPA complex is cell cycle-regulated and p32 acts as a marker for the assembly of RPA complex (Cardoso *et al.*, 1993; Murti *et al.*, 1996).

Human RPA binds to DNA with low cooperativity (Brill and Bastin-Shanower, 1998; Kim *et al.,* 1994; Kim and Wold, 1995). RPA associates with DNA in 3 different modes. First RPA binds to a stretch of 8-10 nucleotides (nt) in an unstable manner, in which DBD-D does not interact with ssDNA. The second mode is a 30nt binding mode. In this mode, DBD-A, -B, -C, and -D directly contact ssDNA. The third one is an intermediate 12-20nt binding mode, in which RPA binds to ssDNA with a higher affinity than in the 8-10nt mode, but DBD-C and -D do not contact with ssDNA (Bastin-Shanower and Brill, 2001; Blackwell and Borowiec, 1994; Gomes *et al.,* 1996, Lavrik *et al.*, 1998, 1999). These ssDNA binding modes reflect different requirements for DNA binding of RPA during the initial opening of duplex DNA and extensive unwinding.

1.2.5 Phosphorylation of RPA

The RPA protein undergoes phosphorylation in a cell cycle-specific manner on CdK consensus sites (Cdk is described later in section 1.3). One of the major RPA kinases present in human cell extracts was shown to be the Cyclin B-CdK1 complex (Din *et al.,* 1990; Dutta and Stillman, 1992). DNA-dependent protein kinase (DNA-PK) and ATM kinase can phosphorylate p32 during DNA replication *in vitro* and UV radiation as well. Evidence was presented and discussed that DNA-PK and ATM cooperate to phosphorylate RPA after DNA damage to redirect the functions of RPA from DNA replication to DNA repair (Brush *et al.,* 1994; Din *et al.*, 1990; Henricksen and Wold, 1994; Henricksen *et al.*, 1996; Pan *et al.*, 1994, Wang *et al.*, 2001). Research on *S. cerevisiae* showed that phosphorylation of p70 was required for the G_1/S and intra-S DNA damage checkpoints. This phosphorylation is dependent on the central checkpoint regulator Mec1p which is a homologue of human ATM and responds to

various forms of genotoxic stress including radiation and hydroxyurea treatment (Brush, 2000; Foiani *et al.*, 1997; Longhese *et al.*, 1996).

The phosphorylation of p32 happens *in vivo* at the G_1-S phase transition and occurs on DNA-bound RPA within the initiation complex (Din *et al.*, 1990; Fotedar and Roberts, 1992). Dephosphorylation of p32 occurs at mitosis, thereby resetting the cell cycle. This suggests a regulatory role of RPA in DNA replication on the basis of its cell cycle-dependent phosphorylation. Although protein kinases for phosphorylation of p32 are present at all stages of the cell cycle, p32 does not bind to the SV40 origin or become phosphorylated in extracts from G_1 cells. Therefore, cell cycle-dependent phosphorylation of p32 may be regulated by its binding to single-stranded DNA during replication initiation in S phase, but phosphorylation of p32 is a consequence of and not a prerequisite for binding to DNA to form the initiation complex at the SV40 origin. The phosphorylation of p32 takes place at the N-terminal 35 amino acids (Lee and Kim, 1995; Henricksen *et al.*, 1996). In addition, the human p32 subunit becomes hyperphosphorylated when cells are treated with UV and ionizing radiation (Liu and Weaver,1993; Carty *et al.*, 1994).

Furthermore, as determined recently the phosphorylation of p32 occurs not only in the mitotic cell cycle, but also during meiosis. The middle subunit of yeast RPA (p32) becomes phosphorylated in two discrete steps during meiosis. Primary p32 phosphorylation occurs early in meiotic progression and is independent of DNA replication and Mec1p. On the contrary, secondary p32 phosphorylation is activated upon initiation of recombination and requires Mec1p (Brush *et al*., 2001).

Fotedar and Roberts (1992) speculated that multiple phosphorylation of RPA p32 might be necessary to induce a conformational change which allows RPA to interact with other proteins such as DNA polymerase α-primase, SV40 T-Antigen, and therefore is involved in the initiation of DNA synthesis. This conformational change, in turn, as reported by Black (1996) and Gomes (1996) renders RPA a better substrate for phosphorylation.

1.3 Kinases Controlling the Cell Cycle

The eukaryotic cell cycle is controlled by the phosphorylation of specific proteins by special serin- and threonin-protein kinases, the Cyclin-dependent kinase (Cdk) complexes. The complex consists of two subunits, a positive regulatory subunit, the Cyclin and a catalytic Cdk subunit. Various Cyclins have been discovered whose expression is cell cycle-dependent. Cyclin E in humans is expressed in late G_1 phase,

Cyclin A in early S phase to G_2 phase, and Cyclin B in late S phase to G_2 phase. Their amounts are controlled by cell cycle phase-specific transcription and their destruction boxes at the N-termini which introduce their phase-specific, ubiquitin-dependent degradation. Cyclin-Cdk-complexes phosphorylate cell cycle proteins at definite time points, and therefore control the cell cycle. The proteins, which are phosphorylated by Cyclin-Cdk, change their activity and their subcellular localisation or are marked for ubiquitin proteolysis, so that each individual cell cycle phase and the transition from one phase to the next is temporally controlled.

The modulation of Cdks and Cyclins to DNA replication has been thoroughly researched. In the pre-RC formation, the kinase for Cdc6p is Cyclin A-Cdk2. Upon phosphorylation of Cdc6 by A-Cdk2, Cdc6 translocates from the nucleus to the cytoplasm. Dbf4-cdc7p (also named Dbf-dependent kinase, DDK) phosphorylates Mcm protein complexes to inhibit Mcm re-binding to DNA after they detach from origins. This process ensures that DNA is replicated only once per cell cycle. Cdc7p is the catalytic subunit and Dbf4p is the regulatory subunit. The activation of S phase Cdks leads to association of Cdc45p with chromatin. Cdc7p-Dbf4p is also required for efficient binding of Cdc45p onto chromatin. Both Cdc45p and RPA bind to Mcm2p at the G_1-S transition in a Cdk-dependent manner (Zou and Stillman, 2000). Cyclin E-Cdk2 and protein phosphatase 2A (PP2A) modulate DNA polymerase α-primase in G_1 phase. PP2A is a serine-threonine phosphatase. These two enzymes regulate cell cycle progression in a positive way, whereas the interaction of CyclinA-Cdk2 with DNA polymerase α-primase in S phase has positive as well as negative functions. The cell cycle-dependent phosphorylation pattern is complex since in human cells the p180 and p68 subunits are predominantly modified at G_2/M, in fission yeast the p180 subunit becomes phosphorylated in late S, whereas in budding yeast the p68 subunit is mainly phosphorylated at G_1/S phase (Bouvier *et al.*, 1993; Foiani *et al.*, 1995; Nasheuer *et al.*, 1991; Park *et al.*, 1995). Phosphorylation maps of human p180 and p68 suggest that a Cdk is probably responsible for the modification (Nasheuer *et al.*, 1991). Functional studies with purified recombinant human DNA polymerase α-primase show that phosphorylation of DNA polymerase α-primase by Cyclin A-Cdk1 and Cyclin A-Cdk2 strongly inhibits its ability to initiate DNA replication *in vitro* at the SV40 origin (Voitenleitner *et al.*, 1997,1999)

Kinases also play an important role in cell cycle checkpoints. The transition from G_1 to S phase and from G_2 phase to M phase in vertebrate cells is regulated by Cdks

(Johnston *et al.*, 1999; Nasmyth, 1996). Apart from Cdk family, members of the phosphoinositol-3-kinase (PI3K) family, ataxia telangiectasia and Rad-3-related(ATR) with ataxia telangiectasia-mutated (ATM) regulate DNA damage checkpoints, and are responsible for the cell cycle arrest in G_1 phase by inducing the cellular p53. p53 is a tumor suppressor protein and a transcription factor. The stabilised and activated p53 induces the expression of p21 to inhibit Cyclin E-Cdk2 activity and thus causes G_1 arrest.

Aim:

RPA and DNA polymerase α-primase are two central DNA replication proteins. RPA performs its function by RPA-DNA binding and RPA-protein interactions. It is known that the N- and C-terminus of p70 react differently with replication/repair proteins. Therefore, the antibodies 70B and RAC-1, which recognised the N- and C-terminus of p70, respectively, offered the possibility to study the biochemical properties of RPA and lead to a further view of the function of RPA in DNA replication and DNA repair. The questions, whether specific RPA subpopulations existed in human cells and whether they performed different activities, can be solved by comparing the behave of 70B and RAC-1 in different regards.

DNA polymerase α-primase binds to RPA and its function is controlled by its phosphorylation. The phosphorylation of DNA polymerase α-primase shows cell-cycle dependent. Therefore, the production of p68 phosphopeptide-specific antibody 858-PP1 should allow to clarify cell cycle-dependent phosphorylation of DNA polymerase α during cell cycle and after DNA damage *in vivo*.

2 Material and Methods

2.1 Material

2.1.1 Cell Lines

- HeLa S3 cell line: Adherent human, epithel-like cervix carcinoma cell line, obtained from Dr.H-P. Nasheuer. Cultured in DMEM medium with 10% FCS.
- CEM cell line: Suspension human T cell leukemia cell line, cultured in RPMI 1640 medium with 10% FCS.
-T98G cell line: Adherent human fibroblast cell line, cultured in RPMI 1640 medium with 10% FCS. It can be arrested at G0 phase by serum depletion. It was a gift from Dr. A. Schepers, Inst. f. clinic. Mol. Biology,München
- HEp2 cell line: Adherent epithelial-like human cell line, cultured in DMEM medium containing 10% FCS. It was from Dr. P. Hemmerich.

2.1.2 Antibodies

Primary Antibodies

- RAC-1: rat anti-human RPA p70 monoclonal antibody. Working concentration for western blotting was 1 μg/ml, for immunoprecipitation was 100μg/ml, for immunofluorescence was 40μg/ml.
- 70B: mouse anti-human RPA p70 monoclonal antibody (Kenny *et al*, 1990), it can detect the largest subunit (p70) of RPA with immunofluorescence and immmuno-precipitation. Working concentration for western blotting was 1 μg/ml, for immunoprecipitation was 100μg/ml, for immunofluorescence was 40μg/ml.
- 2CT25: mouse anti-polymerase-primase p180 subunit monoclonal antibody. Working concentration for western blotting was 1 μg/ml.
- p53: sheep polyclonal antisera from Oncogene Research Products (Ab-7). Diluted 1:3000 for western blotting.
- polyclonal anti-RPA serum: rabbit antiserum against RPA. Dilution ratio for western blotting, 1:1000
- p858: rabbit antiserum against mouse polymerase-primase p68 phosphorylated peptide I. Dilution ratio for western blotting, 1: 500
- p68: rabbit antiserum against mouse polymerase-primase p68, from Dr. H-P Nasheuer. Dilution ratio for western blotting, 1: 1000
- poly ε: mouse monoclonal antibodies against the catalytic subunit of human DNA polymerase ε (261 Kda) from Dr. J. Syvaoja, Finland. For western blotting, a mixture

of all antibodies: G1A, H3B and E24C was used, with a final concentration of 1 μg/ml of each.

- PDG: rat monoclonal antibody against the large subunit of DNA polymerase δ (125 KDa), from Dr. H-P. Nasheuer. Working concentration for western blotting was 1 μg/ml.
- PDK: rat monoclonal antibody against the small subunit of DNA polymerase δ (50 KDa), from Dr. H-P. Nasheuer. Working concentration for western blotting was 1 μg/ml.
-CBL 187: mouse monoclonal antibody against BrdU. Cymus Biotechnology LTD. UK. Working concentration for western blotting was 1 μg/ml.

Secondary Antibodies:

- AP-α-mouse: Alkaline Phosphatase-conjugated goat anti-mouse IgG antibody, Promega Biotech, Heidelberg. Dilution ratio for western blotting, 1: 7,500
- AP-α-rabbit: Alkaline Phosphatase-conjugated goat anti-rabbit IgG antibody, Promega Biotech, Heidelberg. Dilution ratio for western blotting, 1: 7,500
- AP-α-rat: Alkaline Phosphatase-conjugated goat anti-rat IgG antibody, Promega Biotech, Heidelberg. Dilution ratio for western blotting, 1: 10,000
- HRP-α-mouse: Horseradish Peroxidase-conjugated goat anti-mouse IgG antibody, Jackson ImmunoResearch Laboratories, Inc. West Grove, USA. Dilution ratio for western blotting, 1: 2,500
- HRP-α-rabbit: Horseradish Peroxidase-conjugated goat anti-rabbit IgG antibody, Jackson ImmunoResearch Laboratories, Inc. West Grove, USA Dilution ratio for western blotting, 1: 2,500
- HRP-α-rat: Horseradish Peroxidase-conjugated anti-rat IgG antibody, Jackson ImmunoResearch Laboratories, Inc. West Grove, USA. Dilution ratio for western blotting, 1:10,000
- HRP-goat-α-sheep: Horseradish peroxidase-conjugated goat anti-sheep IgG antibody.Sigma. Dilution ratio for western blotting, 1: 3,000
- DTAF (Dichlorotrizinyl Amino Fluorescein)-α-rat: purified F(ab')2-fragement DTAF-conjugated goat anti-rat antibody, Jackson ImmunoResearch Laboratories, Inc. West Grove, USA. Dilution ratio for immunofluorescence, 1: 100
- DTAF-α-rabbit: purified F(ab')2-fragement DTAF-conjugated goat anti-rat antibody,

Jackson ImmunoResearch Laboratories, Inc. West Grove, USA. Dilution ratio for immunofluorescence, 1: 100

- Rhodamin-α-mouse: purified F(ab')2-fragement Rhodamin-conjugated goat anti-mouse antibody, Jackson ImmunoResearch Laboratories, Inc. USA. Dilution ratio for immunofluorescence, 1: 200

- FITC-α-BrdU: Cymus Biotechnology LTD. UK. Dilution ratio for immunofluorescnece, 1: 200

2.1.3 Synthetic Peptide Representing p68

- peptide P1 of p68: NH2-VSTRSPHQLLSPSSF-COOH, from Dr. Nasheuer, synthesised by BioScience Pepsience, Göttingen, Germany

2.1.4 Protein-molecular weight marker

- prestained marker Life Technology ™ Karlsruhe
  205 kDa α_2-Macroglobulin
  130 kDa β-Galactosidase
  90 kDa Fructose-6-phosphate kinase
  64 kDa Pyruvate kinase
  53 kDa Fumarase
  37 kDa Lactic dehydrogenase
  32.6 kDa Triosephosphate isomerase

- 10 kDa ladder Life Technology ™ (GIBCO/BRL™) Karlsruhe
  10-120 kDa in each 10 kDa step, with additional 160 kDa and 220 kDa.

2.1.5 Instruments

Gilson Pipetman 2 μl, 20 μl, 200 μl, 1000 μl were from Abimed, Düsseldorf

Gel filtration Smart-System was from Amersham Pharmacia-Biotech, Freiburg

Phosphorimage Storm 860 with screens was from Molecular Dynamics

Beckman Centrifuge was from Beckman, München

Coulter Epics 4 color was from Beckman-Coulter Krefeld

Vortex Genie 2 was from Bender and Hohbein, München

Polyacrylamide gel electrophoresis clips was from Biometra ® biomedizinische Analytik, GmbH, Göttingen

Mini ProteanII™-Blot apparatus was from BioRad Laboratories GmbH, München

Microscope Televal Fluorecence microscope from Carl-Zeiss GmbH, Jena

Electrophoresis power supply E 443 and E 752 were from Consort, Wasserburg

Kodak Scientific Imaging Filming Biomax ™ MR was from Eastman Kodak Company Rocchester, New York, USA

Eppendorf labcentrifuge Centrifuge 5403 and Eppendorf Thermomixer 5436 were from Eppendorf Gerätebau, Netheler and Hinz GmbH, Hamburg

Forma Scientific-Biofreezer (-80°C) was from Forma Scientific, Inc., USA

Waterbath Haake DC1 was from Haake, Karlsruhe

Gel dryer SE 1150 was from Hoefer Scientific Instruments, San Francisco, USA

Balance PC 2000 was from Mettler, Bach

CO_2/ water incubator for cell culture was from NUAIRE^{TM}

Universal radiometer RM 3700 for measuring the dose of irradiation was from Polytec Waldbronn

Privileg Öko Refridgerator and deep freezer were from Privileg

Disposable yellow and blue tips and Pasteur-pipets were from Renner GmbH, Darmstadt

Fair balance MC1 research RC 210 P was from Sartorius AG, Göttingen

Schott-flasks and glass beakers were from Schott, Jena

2.1.6 Chemicals

All chemicals if not mentioned are in p.A. quality from Biomol, Hamburg; Fluka, Sigma-Aldrih GmbH, Deisenhofen; E. Merck AG, Darmstadt; Carl Roth GmbH &Co, Karlsruhe and Serva, Heidelberg.

Fetal calfserum (FCS) was from Biochrom KG, Berlin

BioRad Protein Assay for Bradford was from BioRad Laboratories GmbH, München,

BrdU was from Sigma Chemical CO. St. Louis USA

Polyacrylamide stock solution was from Biozym, Hess. Oldendorf

NBT was from Gerbu, Gaiberg

DTT was from ICN, Meckenheim

Bovine Albumin (BSA), Thymidine and 4', 6-Diamidino-2-phenylindole (DAPI) were from Sigma Chemical CO. St. Louis USA

Whatman 3MM paper was from Whatman Limited, Maidstone, England

Protein G Sepharose was from Phamacia Biotech AB, Sweden

PolyScreen PVDF Transfer Membrane was from NEN^{TM} Life Science Products, Inc. USA

2.2 Methods

2.2.1 Cell Culture

Different sorts of cells are cultured in different media according to their nutritional needs. Two types of media were generally used: DMEM and RPMI. FCS was supplemented for providing growth factors. Cell lines HEp2 and HeLa were cultured with DMEM medium supplemented with 10 % FCS in an incubator (NuAire^{TM} IR autoflow, Minnesota, USA) at 37°C and 10 % CO_2 with a humidity of 95 %. Cell lines T98G and CEM were cultured in RPMI medium supplemented with 10 % FCS in an incubator at 37°C and 5 % CO_2 with 95 % humidity.

2.2.2 Preparation of Crude Cell Extracts

Cells were lysed in the presence of detergent. With opitimal conditions, cellular proteins can be extracted from the cellular matrix maintaining their immunological and biochemical properties. Adherent cells were detached with 0.25 % Trypsin and 1 % EDTA/PBS and centrifuged at 240xg for 5 min. After the second wash with PBS, the supernatant was completely removed. Cell pellets were incubated in lysis buffer on ice for 30 min. Debris and nuclear structures were removed by 10,000xg at 4°C for 10 min. The lysates were then collected and protein concentration was measured according to Bradford (1976).

Lysis buffer

0.15 M TBS, pH 7.4, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1 % (v/v) NP40

TBS

150 mM NaCl, 3mM KCl, 25mM Tris-Cl, pH 7.4

2.2.3 Determination of Protein Concentration

Protein concentration was determined according to Bradford (1976). Several dilutions of protein standards (BSA) containing from 1 to 100 μg/ml were prepared. 0.1 ml of standard samples and appropriately diluted samples were placed in dry test tubes. 0.1 ml sample buffer was used as a negative control. 1.0 ml diluted dye reagent was added to each tube and mixed several times by gentle inversion. After 15 min, OD_{595} values versus reagent negative control were measured. OD_{595} versus concentration of standards was plotted. The protein of interest was calculated from the standard curve using the Microsoft Excel 5 software.

2.2.4 Preparation of Nuclear Protein

Nuclear protein preparation was performed according to Dignam *et al.,* (1983) with slight modifications. Cells were harvested by centrifugation at 800xg at 4°C for 10 min. in a Sorvall HB-rotor. The cells were resuspended in 10 volumes of Dulbecco's phosphate-buffered saline (without calcium and magnesium) at 4°C and centrifuged again as above. Nuclei were prepared as follows: The cells were resuspended in 5 pellet volumes of 0.3 M sucrose in buffer A, then lysed by 8-12 strokes with a B pestle in a Dounce glass homogenizer and 1-2 strokes in the presence of 0.3-0.4% Nonidet P-40. Completion of lysis was monitored using a phase-contrast microscope. The homogenate was then centrifuged at 1,200xg for 10 min and the pelleted nuclei were washed twice with 0.3 M sucrose in buffer A without NP-40. Nuclei were resuspended with an all-glass Dounce homogenizer (10 strokes) in 2.5 volumes of pelleted nuclei of resuspension buffer. The resuspended nuclei were stirred slowly for 30 min at 4°C followed by centrifugation for 60 min, 100,000xg using a Type 70 Ti rotor. After dialyzing the supernatant for 2-4 hours against 50 volumes of dialysis buffer, the extract was cleared by centrifugation at 25,000xg for 15 min, which removed precipitated material completely and partially removed lipids.

Buffer A

10 mM HEPES-KOH, pH 7.9
10 mM KCl
1.5 mM $MgCl_2$
0.1 mM EGTA
0.5 mM dithiothreitol (DTT)
0.5 mM PMSF
2 μm/ml each of leupeptin and pepstatin A

Resuspension Buffer

400 mM NaCl
10 mM HEPES-KOH, pH 7.9
1.5 mM $MgCl_2$
0.1 mM EGTA
0.5 mM DTT
5 % glycerol (w/v)
0.5 mM PMSF

Dialysis Buffer

20 mM HEPES-KOH, pH 7.9
75 mM NaCl
0.1 mM EDTA
0.5 mM DTT
20 % (w/v) glycerol
0.5 mM PMSF.

2.2.5 Immunofluorescence Microscopy

Using fluorochrome conjugated antibody, the immunofluorescence technique is useful for detecting cellular proteins without changing the antigenicity of protein, and directly showing the location of antigens. The cells were first fixed on glass slides and immunofluorescence was studied on these slides.

2.2.5.1 Preparation of Slides

For adherent cells, sterilized glass slides (Roth) were placed directly in cell culture dishes. The slides were harvested when cells were at 80% confluence, and they were treated as described in 2.2.5.2. For suspension cells, the cells were harvested and washed twice with PBS, resuspended in PBS, and then two volumes of methanol were added. The cells were placed on glass slides for 20 min at RT. After the cells were settled down on the slides, they were fixed by acetone or 4% paraformaldehyde.

Phosphate-buffered saline (PBS)

140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4

2.2.5.2 Immunofluorescence Microscopy

The samples were rinsed with PBS, then fixed with cold methanol for 5 min at -20°C, followed by cold acetone for 2 min at -20°C. Cells were rehydrated immediately by three washes in PBS, and permeabilised with permeabilising buffer for 15 min at room temperature, followed by a 15 min incubation in the blocking buffer. Antibodies were optimally diluted in dilution buffer. Slides were placed in a moist chamber and incubated for 60 min at room temperature. After 3 washes of 5 min each in PBS, fluorochrome-conjugated secondary antibodies were added for 60 min at room temperature. The cells were then washed as above, and mounted with mounting buffer (90% glycerol, 10% PBS), sealed with nail polish, and stored at 4°C in dark. Immunofluorescence microscopy was performed with a Zeiss Axiovert 135 microscope equipped with a Plan-Neofluar 100x1.30 oil-immersion objective. The photographs were taken with Sony 3 CCD Color Video Camera using the KS300 software.

Blocking Buffer

5 % BSA in PBS pH 7.4

Antibody Dilution Buffer

1% BSA, 0.1 %Triton X-100 in PBS, pH7.4

Permeabilising Buffer

0.5 % Triton X-100 in PBS pH 7.4

Mounting Buffer with DAPI

1 ml 0.1 M Tris-HCl, pH 8.4

9 g glycerol

1 μg/ml DAPI

2.2.6 Cell Synchronisation

Synchronised cells are required for defining regulatory mechanisms operative during specific periods of the cell cycle. Synchronisation of continously dividing cells can be effectively achieved by imposing metabolic blocks that meet several criteria. The cells can be arrested at a specific point in the cell cycle and later on permitted to progress through other stages of the cell cycle. This arrest must be rapidly reversible with minimal perturbation of cell proliferation.

2.2.6.1 Double Thymidine Block

The use of excess thymidine was the first widely accepted method for synchronising cells in the cell cycle and remains one of the most effective techniques. High concentrations of thymidine inhibits ribonucleotide reductase activity, and therefore inhibits DNA synthesis in S-phase cells by depleting the nucleotide precursor pools of dCTP. Cells in G_2, M and G_1 are not affected by excess thymidine treatment and continue to traverse the cell cycle until reaching the G_1-S phase boundary when the onset of DNA synthesis is inhibited. HeLa cells were synchronised in G_1/S phase by two consecutive thymidine (TdR) blocks (double TdR block) according to Bootsma *et al.,* (1964), with slight modifications. The cells were cultured in petri dishes at 37°C, 10 % CO_2 until they reached semiconfluency. TdR was added from a stock solution to a final concentration of 5 mM. The cells were incubated in TdR-containing medium for 16 h. The TdR-containing medium was then aspirated, and the cells were incubated with prewarmed PBS for 5 min to remove all traces of TdR, and incubated in prewarmed fresh medium. The cells were allowed to recover in fresh medium for 10 h. Afterwards TdR was added for the second time to a final concentration of 5 mM and incubated for another 16 h. TdR was removed as before and the cells were harvested at different time points. The distribution of cells between the different stages of the cell cycle was detemined by flow cytometry (see 2.2.7).

Thymidine Stock Solution (200 mM)

Thymidine (Sigma) 484 mg in 10 ml medium, filter-sterilised.

2.2.6.2 Serum Depletion

Cells can delay division by entering a specialised state. When proliferating cells in culture are deprived of serum, which contains growth factors, they stop growing, and enter into the so called G_0 phase. The T98G cells were washed with PBS and then

supplied with fresh RPMI medium with 0.5 % FCS, cultured for 120 h. The G_0-arrested cells were stimulated to re-enter the cell cycle by addition of complete medium containing 10% FCS, and harvested at the indicated time points, and stained with propidium iodide for flow cytometry and for additional purposes.

2.2.6.3 Nocodazole Synchronisation

Nocodazole (Sigma, Steinheim) synchronises cells at M-phase. Nocodazole binds to the microtubuli and prevents assembly of the spindle apparatus at the beginning of M-phase. This causes the arrest of the cells in M-phase. No other phases of the cell cycle are affected. Nocodazole was dissolved in DMSO as a stock solution with 10 mg/ml and stored at -20°C. To arrest cells, the final concentration of Nocodazole was adjusted to 1 ng/ml.

HeLa cells were cultured at a confluency of 60-70% and then supplied with Nocodazole for 16 h. The cells were harvested and checked with flow cytometry and used for the indicated experiments.

2.2.7 Flow Cytometry

The progression of cells through S phase and mitosis results in changes in cellular DNA content. The position of cells in the cell cycle can therefore be estimated on the basis of their DNA content measurement, stained by DAPI or propidium iodide (PI). DNA analysis of cells was carried out with slight modifications according to Cissman *et al,* (1973). The cells were harvested by treatment with trypsin plus 1% EDTA/PBS, centrifuged at 1,000g for 5 min. The cells were washed once with 5 ml PBS supplemented with 1% serum, and resuspended in 0.5 ml PBS. The cells were fixed with 5-10 ml ethanol at 4°C for 15 min, centrifuged at 1000xg for 5 min. The cell pellet was washed once with PBS supplemented with 1% serum and then stained by freshly prepared PI/RNase A solution (0.04 mg/ml PI, 0.25 mg/ml RNase in PBS) at 37°C for 30 min, protected from light. The cells were analysed with the flow cytometer Coulter Epics 4 color (Beckman-Coulter, Krefeld, Germany).

<u>50x Propidium Iodide Stock Solution</u>

2 mg/ml PI was dissolved in sodium citrate ($3.8x10^{-2}$ M, pH 7.0). The stock solution was stored at room temperature and protected from light by aluminium foil.

<u>40x RNase A</u>

10 mg/ml RNase A in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl solution. The solution was boiled for 15 min, and then cooled to room temperature. The final preparation was aliquoted and stored at -20°C

PI/RNase Solution

1/50 volume of the PI stock solution and 1/40 of RNase A stock solution were added to PBS^- supplemented with 1% serum.

2.2.8 Immunoprecipitation

2.2.8.1 Crosslinking of Antibodies to Protein G

The protein G Sepharose beads were equilibrated in TBS for 30 min, pelleted by centrifugation with 2,300xg for 3 min, and washed twice with TBS. The remaining protein G Sepharose was stored in TBS with 0.02% NaN_3 at 4°C.

The antibody was covalently attached to protein G Sepharose according to Schneider *et al.,* (1982), with some modifications. 10μl protein G Sepharose was first equilibrated in 10 wet volumes of borate buffer, followed by incubation with 50μg antibody for 30 min on a up-and-down rotating wheel. Afterwards protein G Sepharose was collected by centrifugation at 2,300xg for 3 min, and washed three times with wash buffer I, resuspended in binding buffer and incubated for 45 min. After the crosslinking, antibody-bound agarose was incubated in wash buffer II. After 3 washes with wash buffer I, the antibody-bound agarose was stored at 4°C in PBS with 0.1% NaN_3 .

Borate Buffer

0.1 M Borat, pH 8.2

Wash Buffer I

0.2 $NaHCO_3$

0.1 M Borat, pH 8.2

Binding Buffer

0.2 M $NaHCO_3$, pH 8.2

20 mM Dimethylpimelidat

Wash Buffer II

0.2 M $NaHCO_3$, pH 8.2

20 mM Tris, pH 8.2

20 mM ethanolamin

2.2.8.2 Immunoprecipitation of Proteins

The samples (cell crude extracts, proteins) were precleared with agarose CL4B at 4°C for 1 h on up-and-down rotating wheel. The supernatant was collected by centrifugation and added to the antibody covalently linked protein G Sepharose beads

for 2 to 3 h at 4°C on a rotating wheel. The sepharose beads were washed twice by centrifugation with 2,300xg for 5 min with the wash buffer, followed by the addition of loading buffer. Prior to loading on an SDS gel, the samples were boiled for 5 min in loading buffer.

Wash Buffer

TBS, pH 7.4, 0.25% NP-40, 100 μg/ml PMSF, 1μg/ml Leupeptin, 1 μg/ml Aprotinin

2.2.8.3 Co-immunoprecipitation

Cell crude extracts contain a variety of proteins. Some proteins form complexes in the cells, and are not separated after cell lysis. When a protein is specifically precipitated by an antibody, the other proteins are co-precipitated as well. These protein-antibody complexes can be analysed by western blotting using various antibodies recognising those other proteins. Crude extracts were immunoprecipitated with one antibody, usually with RAC-1 or 70B. The immunoprecipitates were transferred to PVDF membrane, and detected with another antibody by western analysis. All the immunoprecipitation steps were the same as described in 2.2.8.2, and western blotting steps were described in section 2.2.12.

2.2.9 Chromatin Preparation

The chromatin contains histones as well as various nonhistone proteins. To prepare chromatin proteins, HeLa cells were washed three times with ice-cold EDTA solution (0.5 mM, pH 8.0), and then incubated in 40 ml of this EDTA solution for 40 min at 4°C. The cells were harvested by centrifugation at 600xg for 5 min. Cell pellets were washed again with the EDTA solution, and suspended in 4 ml of 0.5 mM EDTA/0.5% NP-40 solution. The suspension was layered on top of a 20-ml sucrose cushion and centrifuged for 10 min at 2,700xg (Rotina 48R rotor). The pellets were suspended in the EDTA solution and again centrifuged through a sucrose cushion as above to remove all the NP-40 traces. The final pellets were suspended in the EDTA solution. The concentration of proteins on chromatin was measured by determining the OD_{260}. OD_{260}=1.0 were equivalent to ca. 50μg chromatin-bound proteins per 1 ml. 100 μg of proteins were used for one test. The samples were incubated at 65°C for 1 h in the presence of 2 % SDS to detach proteins from DNA. The volume of each sample was adjusted to 500μl with water, followed by the addition of 500μl methanol and 100μl of chloroform to denature proteins. The samples were vortexed for 30 sec, centrifuged at 15,000xg for 2 min. The denatured protein phase was collected and supplemented

with an additional 500 µl of methanol, vortexed and centrifuged again. The denatured protein pellet was resuspended in 2% SDS solution, incubated at 80°C for 30 min. The samples were supplied with loading buffer (without SDS) prior to loading in SDS-PAGE gel.

Sucrose Cushion Solution: 100 mM sucrose, 0.25 mM Tris, pH 8.0

2.2.10 Crosslinking

Formaldehyde is a tight ($2A^0$) crosslinking agent that efficiently produces both protein-nucleic acid and protein-protein crosslinks *in vivo*. Its carbon atom acts as a nucleophilic centre. Amino and imino groups of amino acids (lysines, arginines and histidines) and of DNA (primarily adenines and cytosines) readily react with formaldehyde leading to the formation of a Schiff base, which can further react with a second amino group and condense to give the final DNA-protein or protein-protein complex (McGhee and Hippel, 1975). A key advantage of the use of formaldehyde as a crosslinking agent is that the crosslinks are fully reversible by rehydration (Orlando, 2000).

2.2.10.1 Preparation of Crosslinked Proteins

The crosslinking of proteins was performed according to Frank and Frank (1997). The cells in petri dishes were washed twice with warm PBS, aspirated to near dryness, and incubated in 20 ml DMEM (without serum) containing 1% formaldehyde (freshly added from 37% stock solution) for 15 min at room temperature. The crosslinked cells were rinsed thoroughly with two washes of cold PBS. The cells were scraped off in 10 ml of PBS with a rubber spatula, and centrifuged for 5 min at 750xg in a Sorvall SS34 rotor. Cells were resuspended in 10 ml of RSB, homogenised by 15 strokes in a chilled Dounce homogenizer. The nuclei were collected by centrifugation for 8 min, 750xg and washed twice with RSB. The majority of unbound proteins were extracted in buffer E. This extraction step was tolerated by crosslinked nuclei (in contrast to non-crosslinked control nuclei that were lysed and formed a highly viscous solution). After extraction, the nuclei were pelleted by centrifugation for 8 min, 750xg.

2.2.10.2 Sonication

The pelleted nuclei were suspended in 0.5 ml TBS with 0.5 mM EDTA, then sonicated by Branson Sonicator 450 machine with microtip at constant power. The samples were kept on ice-salt-ethanol in a glass beaker and the microtip was appropriately immersed at all times. The output control was set to 8 and the samples were

sonicated using 10 sec. bursts. This treatment was repeated six times with 30 sec. intervals. The samples were checked by proteinase K digestion and the DNA size was visualised under UV light after agarose electrophoresis. These nuclear pellets were sonicated until the DNA had an average size of between 0.5 and 1.0 kb (Orlando *et al,*, 1997).

2.2.10.3 Immunoprecipitation with Crosslinked Samples

The antibodies had been crosslinked to protein G before samples were added (see section 2.2.8.1). The sonicated samples were incubated with antibody-protein G beads in RIPA buffer for 2 to 3 h at 4°C in a rotor. After three washes in wash buffer (2,300xg at 4°C for 5 min), pelleted beads were boiled in 95°C for 30 min in SDS loading buffer prior to SDS-PAGE and then detected by western blotting. The long time boiling is to reverse the crosslinking reaction, so that all crosslinked proteins can be separated.

RIPA buffer
150 mM NaCl
20 mM Tris-Cl, pH 8.8
1mM EDTA
1% Triton 100
0.1% SDS
0.5% Deoxycholic Acid (DOT)

Buffer E
10 mM Tris-HCl, pH8.0
10 mM $Na_2S_2O_5$
1 M NaCl
0.1% NP-40
1 mM EDTA-KOH
0.5 mM PMSF, pH 8.0

RSB buffer
10 mM Tris-HCl, pH8.0
10 mM NaCl
3 mM $MgCl_2$, pH 8.0

2.2.11 Separation of Proteins by Polyacrylamide Gel Elelectrophoresis

In the denaturing SDS polyacrylamide gel elelectrophoresis (PAGE) the proteins are denatured and dissociated into their individual polypeptides in general. The denatured polypeptides efficiently bind to SDS and become negatively charged. The SDS migrates in the polyacrylamide gels according to the size of polypeptides. And the polypeptides are separated according to their molecular masses.

2.2.11.1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out with Biometra-Minigel apparatus (Göttingen) using gels with a size of 80mmx70mmx1mm. The buffer system was prepared according to Laemmli (1970). The stacking gel contained 0.1% (w/v) SDS, 4 % (w/v) acrylamide, 130 mM TrisHCl pH 6.75, 0.09% (w/v) APS and 0.3% (v/v) TEMED; the separating gel contained 0.1% (w/v) SDS, 10% (w/v) acrylamide, 380 mM TrisHCl pH 8.8, 0.05% (w/v) APS and 0.12 % (v/v) TEMED.

4xSDS Loading Buffer	1x Laemmli Running Buffer
40 mM Tris/HCl, pH 6.75	25 mM Tris/HCl, pH 8.3
4% SDS (w/v)	193 mM Glycin
10 % 2-β-Mercaptoethanol	0.1 % SDS (w/v)
40 % Glycerin (w/v)	
0.002% Bromphenole blue	

2.2.11.2 Coomassie^R Brilliant Blue Staining of Protein

After PAGE, proteins were visualised by Coomassie^R Brilliant blue staining (its sensitivity is approximately 25-50 ng protein per band).The proteins were stained and fixed by incubating in Coomassie blue solution for 30 min with agitation, followed by incubation in destaining solution, until protein bands were visible on a colourless background. For storage the gels were incubated in gel drying solution (20% ethanol, 10% glycerin in water) for 30 min and then put in between 2 sheets of cellophane (Roth, Karlsruhe) for air drying.

2.2.11.3 Silver Staining of proteins

Silver staining was performed according to Gottieb and Chavko (1987), to detect small amounts of protein (its sensitivity is detecting 2-5 ng protein per band).

After SDS-PAGE, the gel was incubated for 4 h at 4°C in the fixing solution, followed by incubation in 30 % ethanol (2x 10 min) and 1 min in sodiumsulfate-pentahydrate (0.3 g/l). After 3x 20 sec wash with H_2O, the gel was incubated in staining solution for 20 min in the dark with gentle agitation. The gel was once more washed with water, and the protein bands were developed with developing solution. The staining was stopped by incubating in stop solution. The gel was dried as described above.

Fixation Solution	Staining Buffer
100 ml methanol	200 mg silver nitrate
24 ml acetic acid	75µl 37 % formaldehyde

100 µl 37% formaldehyde
in 76 ml water

in 100 ml water

Develope Solution:
6 g sodium carbonate,
2.5 ml $Na_2S_2O_3.5H_2O$,1.2 mM
50 µl 37% Formaldehyde
in 100 ml water

Stop Solution
50% methanol,
12% acetic acid
in water

2.2.12 Western Blotting

Polypeptides which were separated by the SDS-PAGE can be transfered to solide supports, such as nitrocellulose membranes and PVDF membranes. The proteins on membranes can be probed with their specific antibodies.

2.2.12.1 Protein Transfer and Immunoreaction

PVDF membrane was prewet in 100% methanol for 15 seconds prior to protein transfer. The membrane was equilibrated for at least 5 min in transfer buffer. The proteins were transferred to membrane with 3mA per cm² of membrane surface for one hour. Afterwards, non-specific binding sites were blocked by immersing the membrane in blocking buffer and twice washed with TBST for 10 min. The primary antibody was optimally diluted in antibody dilution buffer, and the membrane was incubated in primary antibody solution for 1 h at room temperature or overnight at 4°C. The membrane was washed three times, each time for 15 min, optimally diluted secondary antibody was applied for 1 h incubation at room temperature, the membrane was then washed as described above. The specific protein was visualised either by ECL or colorimeric detection, depending on the type of the secondary antibody.

2.2.12.2 Detection of Western Blot

2.2.12.2.1 Luminescence-based Detection (ECL)

Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence the excitation is effected by a chemical reaction. One system is horseradish peroxidase (HRP) and the hydrogen peroxide-catalysed oxidation of luminol in alkaline conditions. The luminol is then in an excited state and decays to ground state via a light emitting pathway. The maximum light emission is at a wavelength detectable by films.

2.2.12.2.2 Colormetric Detection

The blot membrane was incubated in 20 ml AP-buffer containing 66μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution and 132 μl nitro blue tetrazolium (NBT) stock solution, protected from light with aluminium foil. The substrate BCIP/NBT was converted in situ into a dense blue compound by the immunolocalised alkaline phosphatase (AP). When the desired signal strength was obtained, the staining solution was washed out twice by PBS to stop the reaction.

2.2.12.3 Stripping and Reprobing

The membrane was submerged in stripping buffer and incubated at 50°C for 30 min with occasional agitation. The membrane was washed twice with TBST and blocked by immersing in 5% blocking reagent for 1 h at room temperature. The immunodetection was performed as western blotting.

TBS
50 mM Tris base
150 mM NaCl, pH 7.5

AP Buffer
100 mM NaCl
5 mM $MgCl_2$
100 mM Tris, pH 9.0

Transfer Buffer
39 mM glycine
48 mM Tris base, pH 7.4
0.037% SDS
20% methanol

TBST Buffer 10x
250 mM Tris/HCl, pH 7.4
1.37 M NaCl
27 mM KCl
0.5% (v/v) Tween-20

Blocking Buffer
5% fat-free milk powder (g/ml) in TBST

Antibody Dilution buffer
5% FCS in TBST

BCIP Stock Solution
0.5 g NBT in 10 ml of 70% dimethylformamide

NBT Stock Solution
0.5 g of BCIP disodium salt in 10 ml of 100% dimethylformamide

HRP-ECL working solutions

Solution1
2.5 mM Luminol

Solution2
5.4 mM H_2O_2

100 mM Tris-HCl, pH 8.5
400 mM Paracoumaric acid

100mM Tris-HCl, pH 8.5

AP-ECL Dilution Buffer
0.1M diethanolamine
1mM $MgCl_2$, pH 9.7

Stripping Buffer
100 mM 2-mercaptoethanol
2% sodium dodecyl sulphate
62.5 mM Tris-HCl , pH 6.7

2.2.13 BrdU Incorporation and Detection

DNA synthesis was monitored by pulse labelling the culture 20 min with the thymidine analoge 5-bromo-2'-deoxyuridine (0.1 mM BrdU, Sigma) prior to fixation. The cells were incubated with 50nM BrdU for 15 min in a CO_2 incubator, and then washed twice with PBS. The cells were fixed for 10 min in acetone at -20°C. After rehydration in PBS, cellular DNA was denatured by incubation in 70% formamide for 5 min at 72°C followed by passing through a series of ethanol incubations (70%, 80%, 95% at 0°C, 5 min each) to remove the formamide. The denaturing of DNA is important for antibody accessibility. The cells were blocked and permeabilised as previously described. BrdU was visualised by immunofluorescence with antibodies recognising BrdU (Dianova).

2.2.14 DNA Damage

2.2.14.1 UV-A Irradiation

$5x10^7$ cells were harvested and washed twice with PBS. The cells were resuspended in 300µl PBS followed by irradiation with UV-A at 365 nm (±10) nm with a total dose of 500 kJ/m^2 carried out at 0°C for 30 min. The dose of irradiation was controlled by a universal radiometer RM3700 with the Rkt-10-cal sensor head.

2.2.14.2 UV-C irradiation

The cells were cultured in a 135mm petri dish. The culture medium was removed and 3ml PBS was added. The petri dish was placed under UV-C light with a total dose of 50J/m2. Immediately after the irradiation, the cells were recovered in complete medium at indicated time points.

2.2.15 Metabolic Radiolabelling of Proteins

Methionine is an essential amino acid for human cells. When cultured in the medium which contains ^{35}S-labelled methionine cells can incorporate it into newly synthesised

proteins. Adherent cells were cultured in petri dishes and twice washed with warm PBS, and cultured in methionine-free medium plus 10 % dialysed FCS and ^{35}S-Met (Amersham Biotech) for different periods. The amount of incubation medium was minimised to just cover the surface of culture in order to increase the concentration of radioactivity. At the indicated time points, medium was removed and cells were lysed for 30 min on ice with lysis buffer. The lysed cells were harvested from petri dish by a spatula, then centrifuged at 10,000xg at 4°C for 10 min to remove nuclear structures. The crude extracts were immunoprecipitated with RAC-1 and 70B. The protein-antibody-proteinG complex were boiled at 95°C for 5 min and then were loaded on SDS-PAGE. The protein bands on the gel were stained with Coomasse brilliant blue. The gel was then placed between wet 3 MM Whatman filter paper and Saran Wrap, and dried on a gel dryer with 65°C heating. When the gel was dry, it was exposed onto Kodak Biomax film and developed.

2.2.16 Elutriation

The centrifugal elutriation can separate large numbers of cells on the basis of their size. Usually, cells are smaller in G_1 phase, and increase their size during the cell cycle until cell division. Therefore, elutriation can separate cells into sequential cell cycle phase populations of relatively high purity without the use of drugs or inhibitors (Lindahl, 1986). The cells were cultured to the concentration of 5-8x10^5 per ml and centrifuged at 600xg for 5 min at RT and resuspended in 20 ml elutriation buffer. Before starting elutriation, the flow rate of the pump was measured and all the conditions were optimised. The resuspended cells were loaded and 10 fractions were collected. 10^6 cells of each fraction were fixed for flow cytometry, the remaining was used to produce crude extracts.

Elutriation buffer 1x PBS, pH 7.4, 3 mM EDTA, 0.1 % Glucose, 1 % FCS

3 Results

3.1 Characterisation of Two Subpopulations of RPA in Human Cells

RPA was initially discovered as a single-stranded DNA binding protein. Since then its functions have been investigated in great detail by biochemical, genetic, cellular and molecular biological means. To gain a further view of RPA *in vivo*, the new set of monoclonal antibodies, RAC-1, RAC-3, and RAC-4 were established in collaboration with Dr. Kremmer (München). The antibody RAC-1 recognised the C-terminus of RPA p70 aa 441 to aa 525. The antibody RAC-3 interacted with the region of residues 441 to 525; whereas, the antibody RAC-4 bound to aa residues 525-616 (Figure 3.1). The binding region of RAC-1 to p70, namely aa 441-525 is part of the DBD-C (DNA binding domain C) and includes the putative Zn-finger of p70, which is involved in the regulation of DNA binding. DBD-C, together with the central DNA-binding domain of subunit p32 (DBD-D) and the entire p14 subunit, form the RPA trimerisation core (Bastin-Shanower, 1998; Bochkareva *et al.*, 2000; 2002; Wold 1997). These three antibodies show similar properties in immunofluorescence and immunoprecipitation. Due to its high affinity, RAC-1 was selected for further experiments.

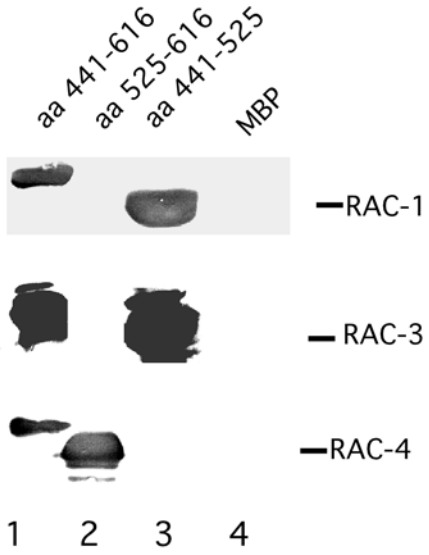


Figure 3.1: Determining the Recognition Sites of the Newly Established RAC Antibodies
Regions of RPA p70 (aa 441-525, aa 525-616, aa 441-616) were fused to maltose binding protein (MBP). 10μg of peptide MBP-aa 441-616 (lane 1), MBP-aa 525-616 (lane 2) and MBP-aa 441-616 (lane 3) as well as MBP (lane 4) were applied to SDS-PAGE and western blotting. The primary antibodies for western blotting were RAC-1 (upper), RAC-3 (middle), and RAC-4 (lower), respectively. The secondary antibody for western blotting was AP-conjugated anti-rat antibody. The membranes were developed by NBT/BCIP method.

3.1.1 Immunostaining of Two Subpopulations of RPA

To investigate RPA *in vivo*, the binding of these two antibodies RAC-1 and 70B (Kenny *et al*, 1990) to cellular RPA was compared by immunofluorescence techniques in cycling HeLa cells. The antibodies 70B and RAC-1 showed significant differences in their staining patterns. 70B revealed nuclei with discrete foci, concentrated on the central part of the nucleus, whereas RAC-1 stained nuclei dispersively, and no obvious foci were determined. The merged images did not show efficient colocalisation. Especially in some cells (one of these is indicated by the arrow at right), 70B staining is reduced in comparison to the other cells, whereas RAC-1 staining in this cell is about average. To the contrary, in some cells such as the one marked by the arrow in the upper left corner, 70B staining was dominant (Figure 3.2). Although RAC-1 and 70B recognise the same RPA subunit, each antibody binds to different regions of this polypeptide. Immunofluorescence is a method to reflect the status of cellular proteins *in vivo* and *in situ*. These immunofluorescence results were reproduced by different fixation methods, such as formaldehyde fixation and 0.5% Triton X-100 extraction prior to methanol/aceton fixation. These findings suggested that two subpopulations of RPA might exist in the nucleus and be recognised by RAC-1 and 70B.

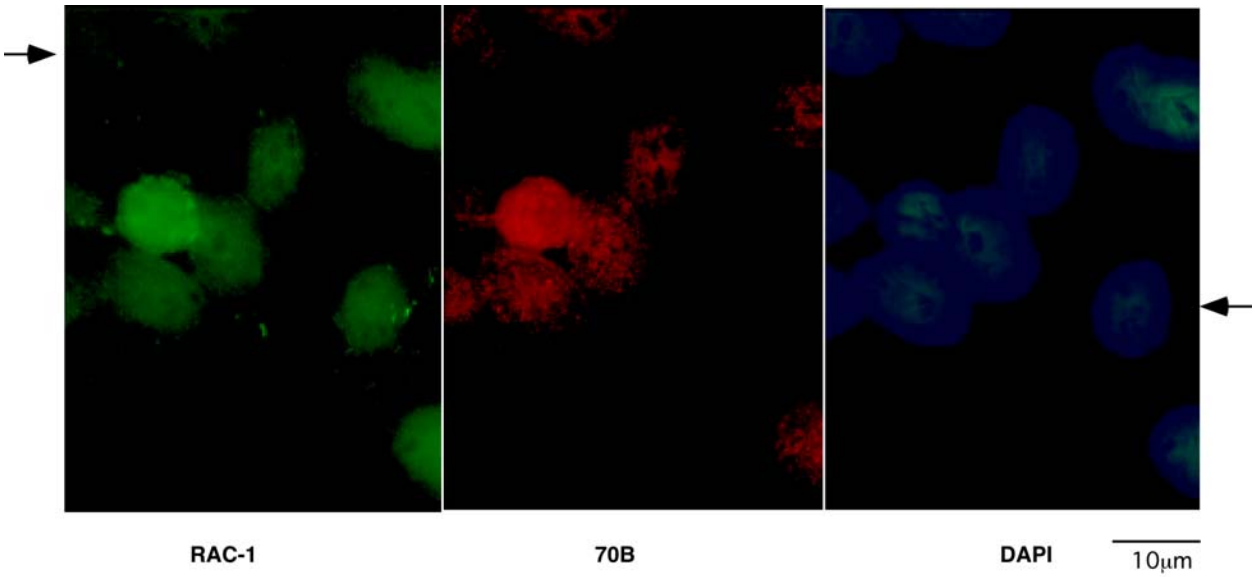


Figure 3.2: Recognition of RPA by RAC-1 and 70B HeLa Cells were grown on glass slides and then fixed by methanol/aceton. The slides were rinsed in PBS and blocked in 5% BSA, follwed by immunostaining with 70B (mouse) and RAC-1 (rat) antibody. The secondary antibodies were Rhodamine-conjugated anti-mouse and DTAF-conjugated anti-rat antibody. Nuclei were stained by DAPI in blue.

3.1.2 **Different p32 Phosphorylation Forms in the Two RPA Subpopulations**

In order to characterise these two RPA forms, their cellular distribution was analysed. Identical amounts of proteins in crude extracts of HeLa cells were examined for RPA by immunoprecipitation with RAC-1 and 70B. In parallel, purified RPA was used as a standard to compare the affinity of the antibodies. The quantification of western blotting using the ImageQuant software showed that the intensity of purified RPA precipitated by RAC-1 was about 80% of that of 70B. In contrary, RAC-1 recognised RPA in crude extracts were about one third of that recognised by 70B. Therefore, RPA in crude extracts was more readily detected by 70B than by RAC-1 (Figure 3.3). Because RPA exists in cells as a stable heterotrimer containing p70, p32 and p14, p32 and p14 could be co-immunoprecipitated with p70 by antibodies against p70, and p32 was also detected by polyclonal anti-RPA serum on western blotting. Furthermore, the middle subunit p32 exists in various phosphorylation forms: hypophosphorylated, low and highly phosphorylated (Brill and Stillman, 1989; Treuner, *et al*., 1999; Wold and Kelly, 1988). Indeed p32 co-immunoprecipitated with RAC-1

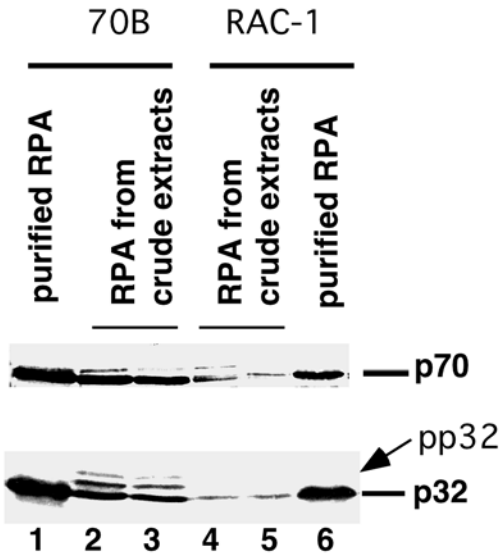


Figure 3.3: Immunoprecipitation of RPA in Human Cells with RAC-1 and 70B
5 μg purified RPA (lanes 1, 6) or 100 μg crude extracts of HeLa cells (lanes 2-5) were incubated with monoclonal antibodies 70B (lanes 1-3) and RAC-1 (lanes 4-6) to immunoprecipitate RPA, followed by western blotting. p70 and p32 were detected by polyclonal rabbit anti-RPA antibody. The secondary antibody for western blotting was AP-conjugated goat anti-rabbit antibody. The blots were developed with BCIP/NBT. p70 and p32 are indicated by bars and the arrow marks the phosphorylated p32 (pp32).

and 70B, but with differences in the phosphorylation forms. The phosphorylated and hyperphosphorylated forms of p32 were coprecipitated with 70B. However, only non-

or low phosphorylated p32 was coprecipitated with RAC-1 (Figure 3.3). These results are consistent with the idea that two subpopulations of RPA exist in mammalian cells, which can be identified by RAC-1 and 70B. Therefore, they were named RAC-1-RPA and 70B-RPA.

3.1.3 Cellular Distribution of RAC-1-RPA and 70B-RPA

Since the level of 70B-RPA in cells is higher than that of RAC-1-RPA, the amounts of crude extracts were increased for immunoprecipitation with RAC-1 in the later experiment for better comparison (detailed method see section 2.2.8). After increasing the amount of cell extracts by a factor of three, RAC-1 immunoprecipitated about the same amount of RPA as 70B with less crude extracts, and the same intensity of RPA p70 was detected in western blotting (Figure 3.4A, lanes 5 and 6). To characterise these RPA subpopulations it was important to determine whether the RAC-1-RPA and 70B-RPA were distributed equally or not. Cellular proteins were fractionated into cytosolic and nuclear proteins (detailed method see section 2.2.4), and these proteins were used for immunoprecipitation. Despite the increase of protein amounts in the immunoprecipiation experiments with RAC-1 (see above), the intensity of RAC-1-RPA was much less than that of 70B-RPA in the cytosol or in the high ionic strength (400mM NaCl) extracted nuclei (Figure 3.4A, lanes 1 and 3 compare with lanes 2 and 4). To localise the main portion of RAC-1-RPA, the pellet of salt-extracted nuclear protein was treated with NP-40 (1%). RAC-1 detected more RPA in the nuclear extract treated with NP-40 than in the cytosolic extract or the nuclear extacts without NP-40 treatment. In parallel, 70B precipitated similar amounts of RPA in the cytosolic extracts and in the nuclear extracts with or without NP-40 treatment (Figure 3.4B). The salt-only extraction of nuclei contains the free form of RPA and the RPA binding loosely to nuclear structure, whereas 1% NP-40 treated nuclear extract contains RPA tightly binding to the nucleus. These experiments strongly suggested that RAC-1-RPA and 70B-RPA are distributed differently in cell compartments. 70B-RPA was present in the cytosol and nucleoplasm, and was bound tightly as well as loosely to nuclear structures. On the contrary, RAC-1-RPA was found predominantly in the nucleus and tightly bound to nuclear structures, and can be extracted with detergent and high ionic strength (400mM NaCl was used, compared to crude extracts with 150mM NaCl).

The existence of two immunologically distinct subpopulations was confirmed by fractional immunoprecipitation (Figure 3.4C and D). Because high amounts of RPA

were present in NP-40-treated nuclear extracts, and detected by both RAC-1 and 70B,

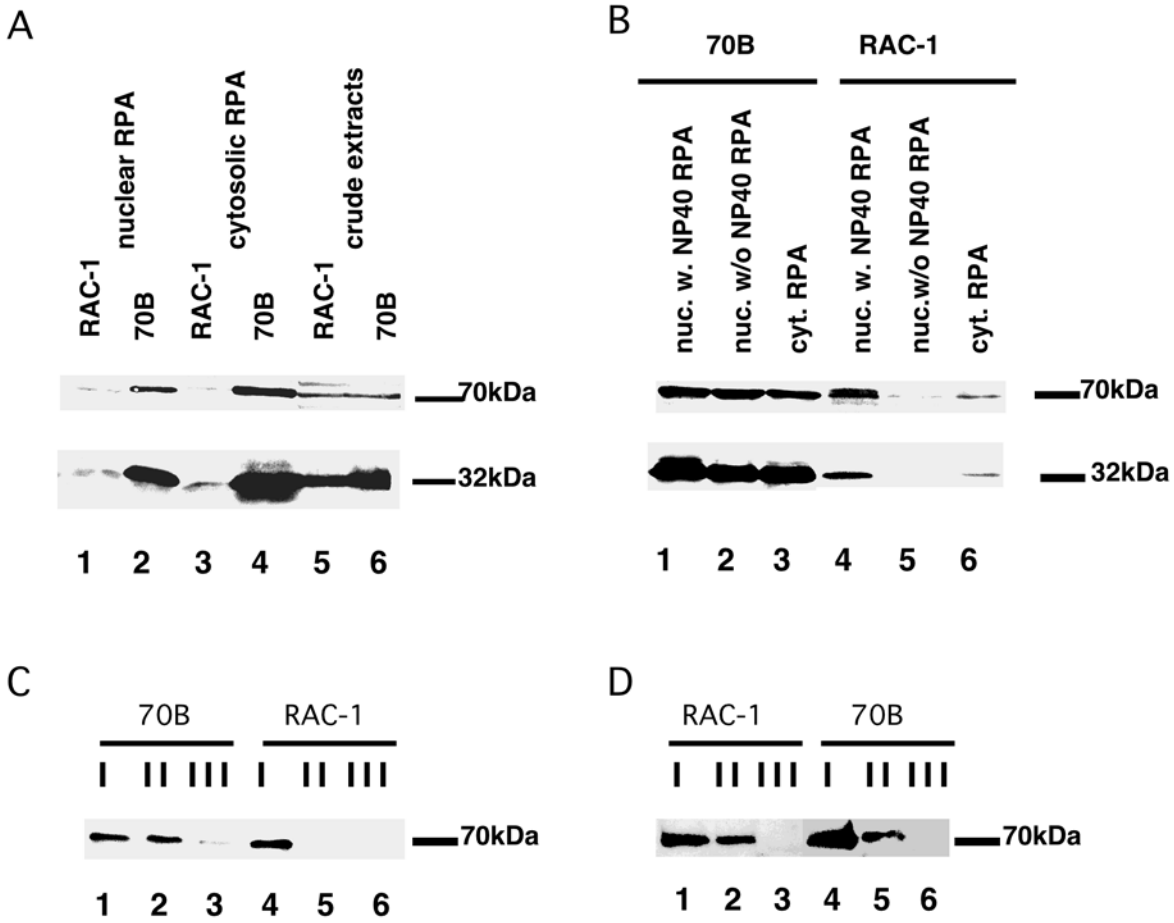


Figure 3.4: Cellular Distribution of RAC-1 and 70B Recognised RPA Forms
(A) Nuclear proteins (lanes 1 and 2), cytosolic proteins (lanes 3 and 4) and crude extracts (lanes 5 and 6) of HeLa cells were precipitated by the antibodies RAC-1 (lanes 1, 3, 5) and 70B (lanes 2, 4, 6). The amounts of crude extracts used in the RAC-1 (600 μg) precipitation were three times more than those in the case of the 70B (200 μg). **(B)** 200 μg nuclear proteins (lanes 1, 2, 4, 5) together with cytosolic proteins (lanes 3 and 6) were tested by 70B (lanes 1-3) and RAC-1 (lanes 4-6) with immunoprecipitation and followed by western blotting. Nuclear proteins extracted from nuclear structure without NP-40 was indicated with nuc. w/o NP40 (lanes 2 and 5); nuclear proteins from second treatment of nuclei in the presence of 1% NP-40 was nuc. NP40 (lanes 1 and 4) The immunoprecipitated RPA subunits were detected by western blotting , the primary antibody for western blotting was polyclonal anti-human RPA antiserum, the secondary antibody was AP-conjugated goat anti-rabbit antibody. The immunoblot was developed with NBT/BCIP. p70 and p32 are indicated by bars.
(C) and (D) 200 μg proteins in the nuclear extracts of HeLa cells treated with NP-40 were immuno-precipitated three times with 70B (**C**) or RAC-1 (**D**) (lanes 1 to 3), followed by three rounds of immunoprecipitation with RAC-1(**C**) and 70B (**D**) (lanes 4 to 6). RPA was determined by western blotting analysis with polyclonal anti-RPA serum as primary antibody. The secondary antibody was HRP-conjugated goat anti-rabbit antibody, and the blots were developed with ECL.

these NP-40 extracts were chosen for fractional assays in order to test whether RAC-1

and 70B recognised different forms of RPA or not. 70B efficiently precipiated RPA in the first two rounds, as determined by p70 immunodetection. But with the third round of immunoprecipitation with 70B, only minor amounts of RPA were detected (Figure 3.4C, lanes 1-3). Nevertheless in the next round of immunoprecipitation, when the antibody RAC-1 was used, RPA was again precipitated (Figure 3.4C, lane 4). However, after the fourth round of precipitation, RPA was no longer detectable with RAC-1 (Figure 3.4C, lanes 5 and 6). And vice versa in Figure 3.4D, when RAC-1 was used for the first three rounds of immunoprecipitation, followed by three rounds immunoprecipitation of 70B.

3.2 Characterisation of RAC-1-RPA and 70B-RPA in Cell Cycle

3.2.1 From Early S Phase until G_2 Phase in HeLa Cells

Since there were two subpopulations of RPA in human cells, whose cellular distribution and cell cycle staining were different (section 3.1), these results raised the question how these RPA subpopulations behave during the cell cycle. To address this question, synchronisation experiments were carried out. HeLa cells were TdR-blocked and then released and collected at the indicated time points (detailed method see section 2.2.6.1). One portion of these cells was stained with propidium iodide to determine their cell cycle distribution. The remaining was used for additional experiments, such as preparation of chromatin protein, production of crude extracts, immunofluorescence and chromatin crosslinking experiments. Most cells were arrested by double TdR block in early S phase with a DNA content close to the content of G_1 cells (Figure 3.5), since a significant fraction of them was capable to incorporate BrdU (Figure 3.21). The arrested cells were released from the cell cycle block in TdR-free medium and synchronously went through the cell cycle for at least 12 h. During the first three hours, cells were in early S phase. After 5-6 hours, most cells reached mid S phase, whereas 9 hours after recovery, cells synchronously went into G_2 phase. 12 hours after recovery, cells reproducibly started another cell cycle with a high degree of synchronisity (Figure 3.5).

The synchronised cells were first tested for their level of chromatin-bound RPA (chromatin protein preparation see section 2.2.9). The amount of chromatin-bound RPA increased obviously before cells went into mid S phase, and gradually decreased from mid S phase until G_2/M phase, with its minimum level at 9 hours after recovery.12 hours after recovery from TdR block, when most

cells started the next cell cycle, chromatin-bound RPA slightly increased in

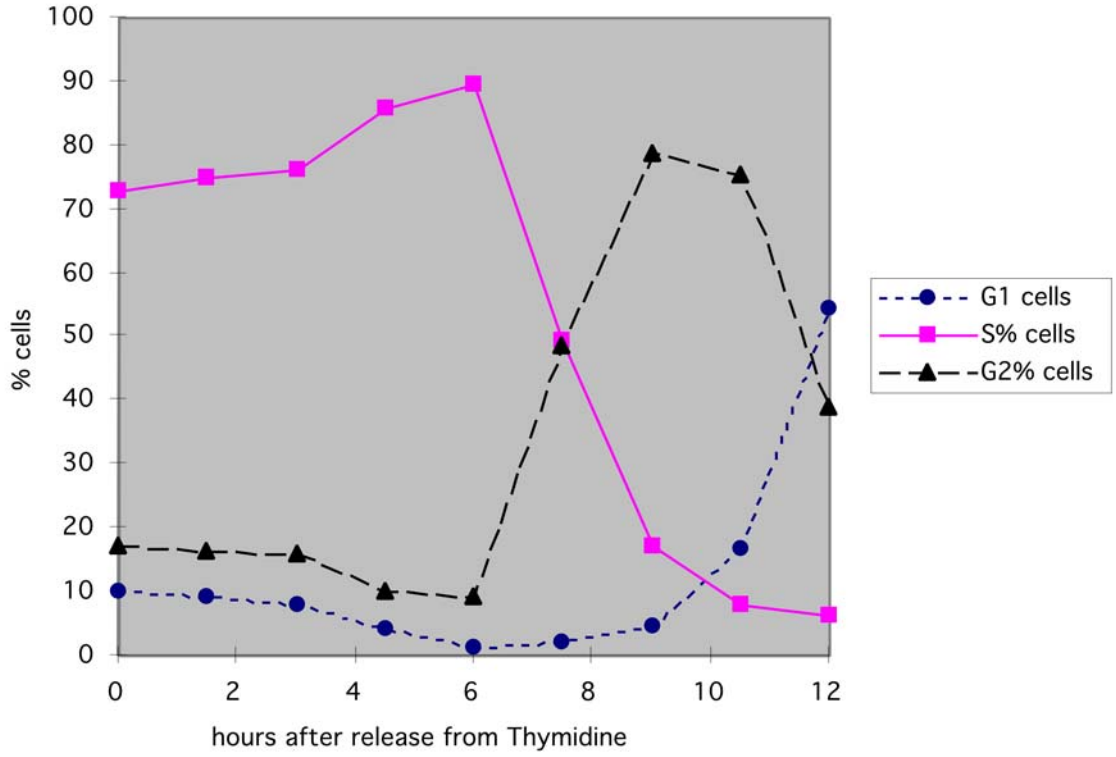


Figure 3.5: Profile of Thymidine Synchronisation HeLa cells were synchronised by double TdR block, released by removing TdR and supplying fresh medium. Cells were harvested at the indicated time points (0, 1.5, 3, 4.5, 6, 7.5, 9, 10.5 and 12 hours) after release, stained by propidium iodide, checked by flow cytometry. The X-axis shows time points after release. Y-axis shows precentage of cells which are in G_1, S, G_2 phase. Filled circles indicate cells in G_1 phase, grey squares indicate S phase and filled triangles indicate G_2 phase cells.

comparision to cells in G_2/M phase, but still less than that in mid S phase (Figure 3.6). Similar experiments using RAC-1 showed no difference. Chromatin proteins were already denatured by incubation in 2% SDS solution before they were detached from DNA, therefore, it is reasonable to show no difference between 70B and RAC-1, which recognise the same polypeptide in western blotting.

In addition, chromatin was prepared under nearly salt free condition, under which it was very viscous. Once salt was added to the chromatin preparation, the proteins were precipitated and were no longer soluble. Therefore, chromatin proteins were not directly suitable for immunoprecipitation. To determine the physiological DNA binding

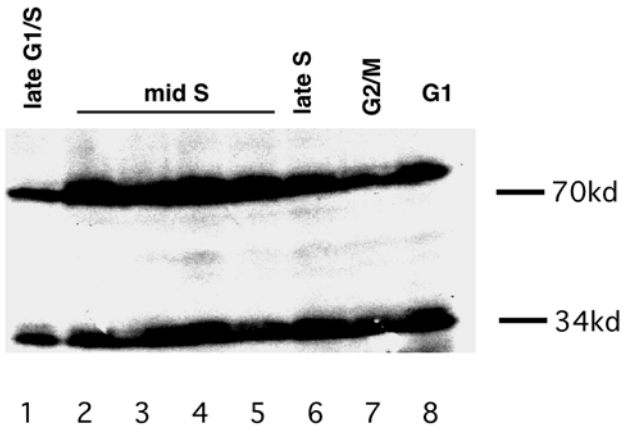


Figure 3.6: Chromatin-binding RPA during Cell Cycle. HeLa cells were synchronised and released at the indicated time points. Chromatin was prepared from these cells and chromatin proteins were separated from the nuclear matrix by incubation with 2% SDS, followed by methanol/ chloroform extraction. Chromatin proteins were applied to SDS-PAGE and detected by western blotting. The primary antibody of western blotting was 70B, the secondary antibody was HRP-conjugated anti-mouse antibody, blot was developed by ECL. lanes 1 to 8, chromatin prepared from cells 0h, 1.5h, 3h, 4.5h, 6h, 7.5h, 9h, 12h, respectively, after release from TdR block.

status of RAC-1-RPA and 70B-RPA, chromatin crosslinking assays using formaldehyde were performed.

Formaldehyde is a tight (2Å) crosslinking agent that efficiently produces both protein-nucleic acid and protein-protein crosslinks *in vivo*. Formaldehyde is a highly reactive dipolar compound in which the carbon atom acts as a nucleophilic center. Amino and imino groups of amino acids (lysines, arginines and histidines) and of DNA (primarily adenines and cytosines) readily react with formaldehyde leading to the formation of Schiff bases, which can further react with a second amino group and condense to give the final DNA-protein complex (McGhee and Hippel, 1957). A key advantage of the use of formaldehyde as a crosslinking agent is that the crosslinks are fully reversible by rehydration (Orlando, 2000).

The TdR arrested and then synchronously released cells were crosslinked *in vivo* at the indicated time points (detailed method see 2.2.10). Proteins, which bound to and were therefore crosslinked to nuclear structures, were retained in the nucleus during the washing and sonicating procedure. When crosslinked samples were used for immunoprecipitation experiments, RIPA buffer was used to ensure the solubility of chromatin and the accessibility of proteins. In order to avoid loss of antibodies during RIPA buffer treatment, the antibodies were covalently coupled to protein G sepharose before precipitations were performed. The immunoprecipitates were boiled for 30 minutes prior to analysis by SDS-PAGE in order to reverse the formaldehyde

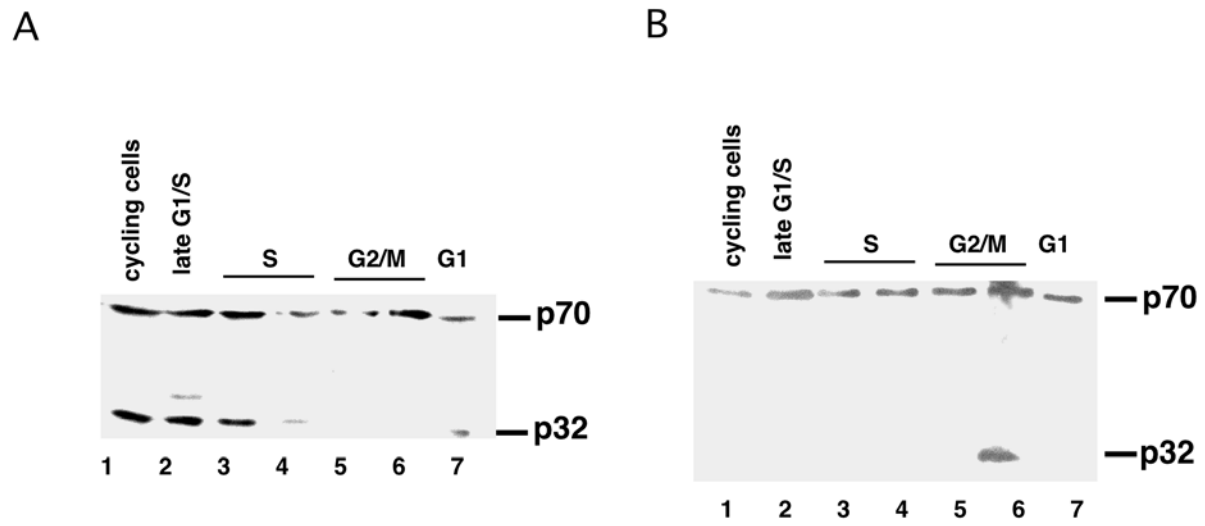


Figure 3.7: p32 Differentially Crosslinks to p70 in RAC-1-RPA and 70B-RPA TdR synchronised and released HeLa cells were crosslinked *in vivo* by 1% formaldehyde. Crosslinked cells were broken, nuclear structures were pelleted and sonified to a size of about 1kb. The concentration of DNA-binding proteins was measured by OD260. The equivalent OD260 amount of proteins was applied for immunoprecipitation using RAC-1 **(A)** and 70B **(B)** in RIPA buffer, followed by western blotting. The primary antibody for western blotting was polyclonal anti-RPA antibody, the secondary antibody was HRP-conjugated goat anti-rabbit antibody, the blots were developed by ECL. Molecular masses are indicated by lines. lane 1, cycling cells; lane 2, cells in consecutive TdR blocks; lanes 3 to 7, cells for 3 h, 6 h , 9 h, 10.5 h, and 12 h, respectively, after release from TdR block.

crosslinking reaction and separate crosslinked proteins so that they can be individually detected.

p70 in RAC-1-RPA was evenly crosslinked to nuclear structures in the early S phase. It decreased in the mid and late S phase (Figure 3.7A, lanes 4 and 5). p70 increased in G_2/M phase and slightly dropped in the G_1 phase of next cell cycle (Figure 3.7A, lanes 6 and 7). However, p32 crosslinked to RAC-1-RPA p70 subunit during S phase (Figure 3.7A, lanes 1 to 4) and G_1 phase (Figure 3.7A, lane 7), but not in G_2 phase. The amount of chromatin crosslinked p70 in 70B-RPA remained constant from S phase until G_1 phase in the next cell cycle. Nevertheless the p32 subunit crosslinked to nuclear structure-binding p70 was found in varying amounts during cell cycle, it crosslinked to p70 in G_2/M phase, but not from G_1 phase (Figure 3.7B, lane 7) to mid S phase (Figure 3.7B, lanes 2 to 4).

RPA levels are reported to remain constant before and after DNA replication (Din *et al*., 1990; Virshup *et al.,* 1990). In order to learn more about the amounts of RAC-1-RPA and 70B-RPA in cell cycle, crude extracts of cells in different cell cycle phases were analysed by immunoprecipitation with 70B or RAC-1.

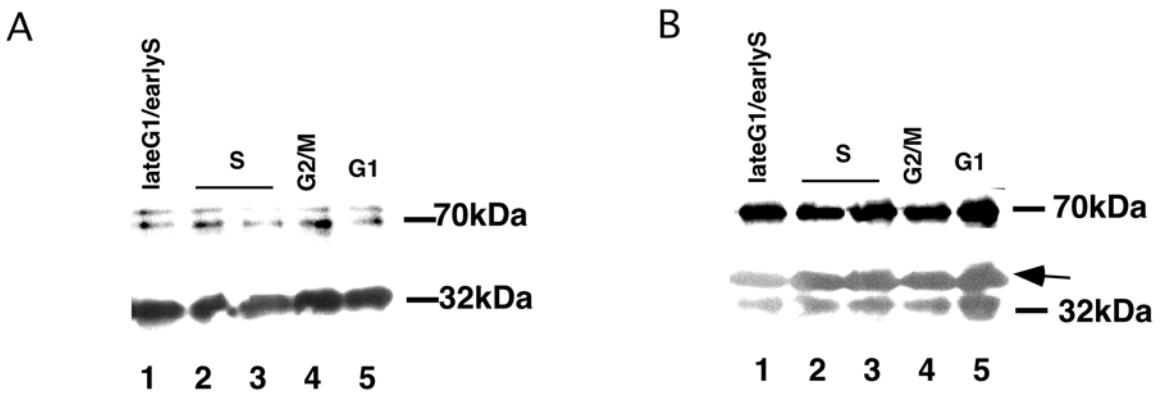


Figure 3.8: RAC-1-RPA and 70B-RPA during Cell Cycle 100 μg proteins in crude extracts of TdR-blocked and released HeLa cells (lanes 1-5) were immunoprecipitated by RAC-1 (**A**) or 70B (**B**), visualised by western blotting. The primary antibody was polyclonal anti-human RPA antiserum. The secondary antibody of western blotting was HRP-conjugated anti-rabbit antibody. The blots were developed by ECL. RPA subunits are indicated by their molecular masses. The arrow indicates phosphorylated p32. lanes 1 to 5, cells were released from TdR block for 0h, 3h, 6h, 9h, and 12h, respectively.

The amount of p32 did not show a cell cycle difference in RAC-1-RPA, nor in 70B-RPA. Furthermore, the phosphorylated p32 was co-immunoprecipitated with 70B-RPA, but not with RAC-1-RPA, thus confirming the results shown in Figure 3 and 4. Nevertheless, p70 did not show a quantitative difference in the 70B-RPA complex from early S until G_2/M phase. In RAC-1-RPA, however, the level of p70 slightly dropped in mid S phase (Figure 3.8, lane 3, cell were at 6 hours after recovery). This is consistent with the observation in Figure 3.7.

The localisation of RPA was determined using immunofluorescence. In early S phase, both 70B and RAC-1 showed a dispersive staining. In mid S phase, however, 70B revealed RPA as foci or clusters, which were detectable until late S phase, but gradually decreased. In contrary, when cells were immunostained with RAC-1, the change in the distribution of RPA was not as obvious as with 70B. When cells were in G_2 phase, the staining of RAC-1 and 70B became dispersive again as in early S phase (Figure 3.9). It is generally believed that initiation complexes (among them RPA) form foci at the replication origin, but RAC-1-RPA did not show foci in the whole cell cycle. This again illustrates that 70B-RPA, but not RAC-1-RPA, directly takes part in DNA replication initiation. The different staining patterns also hint at different functions of these two RPA subpopulations.

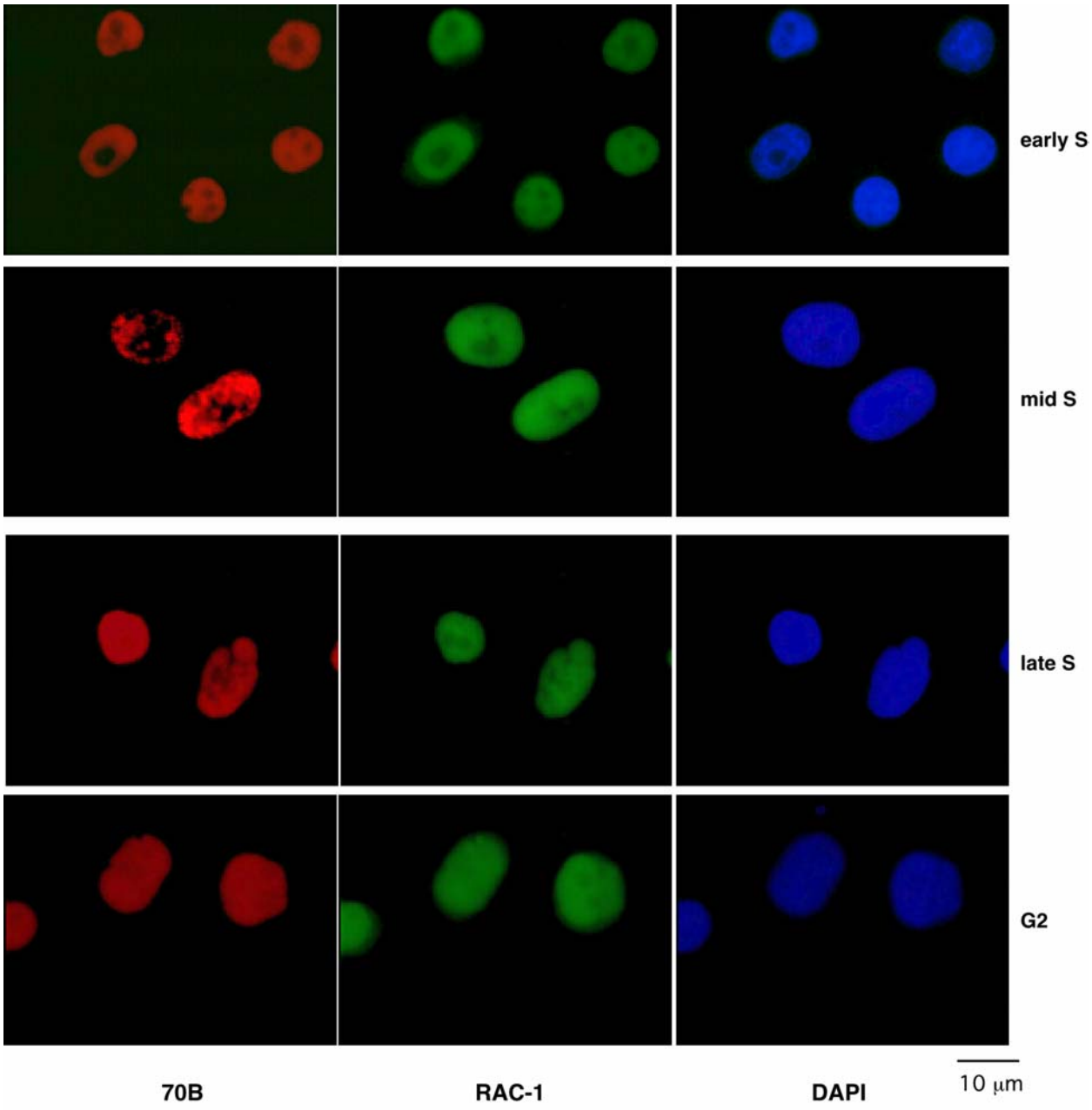


Figure 3.9: RPA Subpopulations in Cell Cycle. HeLa cells were synchronised and released from double TdR block, and harvested at early S, mid S, late S and G2 phase (0h, 3h, 6h, and 9h, respectively, after release from TdR block). Immunofluorescence experiments were carried out with these cells. The cells were double stained with 70B (mouse) and RAC-1 (rat). The secondary antibodies were Rhodamine-conjugated anti-mouse antibody (red) and DTAF-conjugated anti-rat antibody (green). The nuclei were indicated by DAPI in blue.

3.2.2 Synchronisation of T98G Cells by Serum Depletion

HeLa cells cannot be arrested in G_0 phase. In order to obtain a knowledge of the RAC-1-RPA and 70B-RPA in proliferating as well as nonproliferating cells the cell line T98G was chosen to study RPA in dependency of growth stimuli because these cells can be arrested in G_0 phase by serum depletion (detailed method see section 2.2.6.2). Conditions for G_0 arrest were

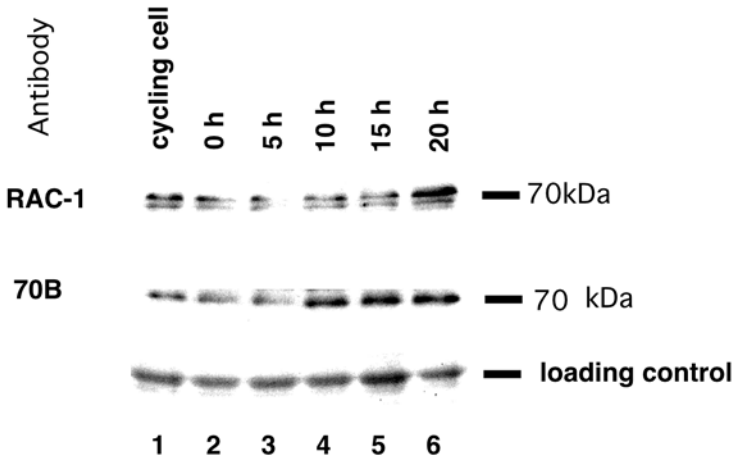


Figure 3.10: The Amount of RPA Increases from G_0 to S phase. T98G cells were arrested at G_0 phase by serum depletion, followed by release in complete medium at the indicated time points: 0 h, 5 h, 10 h, 15 h, and 20h (lanes 2 to 6, respectively). The crude extracts were prepared from these cells, and 50 μg proteins in crude extracts were detected by western blotting with RAC-1. The secondary antibody of western blotting was HRP-conjugated anti-rat antibody. The blot was developed by ECL (upper panel). The same membrane was stripped and reprobed by the 70B antibody. The secondary antibody was HRP-conjugated anti-mouse antibody. The membrane was developed by ECL (middle panel). As loading control, identical amounts of protein of each crude extracts were loaded in parallel onto another SDS gel. After proteins were separated, the gel was stained with Coomassie brillian bluesolution and destained with destaining solution (lower panel).

optimised in several pilot experiments using different incubation times in medium with a low FCS concentration. After the addition of medium with 10% FCS the arrested T98G cells reversibly enter the cell cycle at early G_1 phase and then synchronously pass through cell cycle. This procedure is called serum stimulation in the following text. Compared to TdR synchronisation, serum depletion has the advantage of showing protein synthesis in the early stages of the cell cycle. As the addition of serum stimulates quiescent cells to grow, the proteins involved in cell proliferation will be synthesised *de novo*. Therefore, proliferation-dependent RPA synthesis in T98G cells can be studied by immunological techniques. The minor amounts of RPA were detected in G_0 cells and in cells 5 hours after serum addition. Afterwards the level of RPA started to increase (Figure 3.10, lane 4).

As described before, the RPA trimer is required for DNA replication, DNA repair and recombination through interaction with other proteins. As demonstrated above (in section 3.1), two RPA subpopulations existed in human cells. These two forms of RPA most likely performed different functions in cell cycle. These data and speculations led to an investigation of RPA interacting with other proteins, and metabolic protein labelling experiments were performed. As speculated, both RAC-1-RPA and 70B-RPA interacted with various proteins, and formed complexes during immunoprecipitation with p70. These proteins were visualised in Figure 3.11.

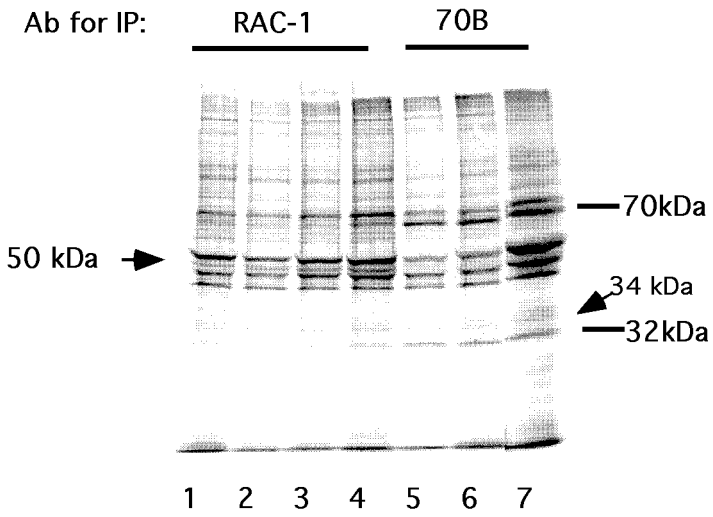


Figure 3.11: Metabolic Labelling of RAC-1-RPA and 70B-RPA Subpopulations T98G cells were arrested in G_0 phase by culturing in 0.5% FCS containing medium for 120 h, followed by culturing in complete medium for 5 h. The cells then were labelled by ^{35}S-Met (30 μCi per sample) by incubation in methionine-free RPMI medium containing 10% dialysed FCS. The labelled cells were harvested at different time points. Crude extracts were made from these cells and immunoprecipitated by RAC-1 (lanes 1 to 4) and 70B (lanes 5 to 7). After SDS-PAGE, the gel was dried and exposed to Kodak-Biomax film. Labelling time: 1 h (lanes1 and 5), 2 h (lanes 2 and 6); 4 h (lane 3); 5 h (lanes 4 and 7). Molecular masses are indicated by bars and arrows

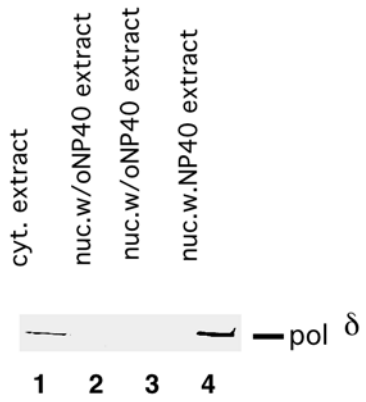


Figure 3.12: DNA Polymerase δ is Extracted by NP-40 in Nuclei 200 μg of nuclear extract of HeLa cells without NP-40 (lanes 2 and 3) and with NP-40 (lane 4) together with cytosolic extract (lane 1) were immunoprecipitated by PDG mono-clonal antibody, which recognises the large subunit of DNA polymerase δ, and followed by western blotting. The primary antibodies were the mixture of several PDG monoclonal antibodies, the secondary antibody was AP-conjuagted goat anti-rat antibody, the blot was developed by NBT/BCIP method. Nuclear proteins extracted from nuclear structure without NP-40 was indicated with nuc. w/o NP40; nuclear proteins from second treatment of nuclei in the presence of 1% NP-40 was nuc. w. NP40.

The level of RAC-1-RPA was low in G_0 and early G_1 phases, and increased 10 hours after serum stimulation. On the contrary, the amount of 70B-RPA did not increase from 7 hours until 10 hours after serum stimulation. When RAC-1-RPA reached its high amount at 10 hour after serum addition (Figure 3.11, Figure 3.7), cells synthesised as much RAC-1-RPA as 70B-RPA. By comparision of Coomassie-stained

protein bands and radioactivity exposed bands, one band was found, which existed in RAC-1-RPA but not 70B-RPA, with an apparent molecular mass of 50 kDa. It might be a subunit of DNA polymerase δ. Both p70 and p32 subunits showed stronger signals in 70B-RPA than in RAC-1-RPA. The pp32 form was detectable in 70B-RPA after serum addition but not in RAC-1-RPA (Figure 3.11). Several proteins were coprecipitated with 70B-RPA, but not with RAC-1-RPA (the lower parts of lane 7, Figure 3.11). Due to a lack of positive controls, they could not yet be identified. These findings suggested that RAC-1- and 70B-RPA interacted with different proteins. Figure 3.11 provided hints of DNA polymerase δ interaction with RAC-1-RPA, most of which bound to nuclear structure tightly. To confirm my finding, nuclear extracts with and without NP-40 treatment (Figure 3.12, lanes 3 and 4, respectively) as well as the cytosolic extracts (Figure 3.12, lanes 1 and 2) were investigated. DNA polymerase δ was detected in cytoplasm, but a significant amount of DNA polymerase δ existed in nucleus, when nuclei were extracted by NP-40.

Because of difficulties in directly identifying proteins detected by metabolic labelling, co-immunoprecipitation experiments were performed. Some cellular proteins which tightly bind to RPA can be co-immunoprecipitated by anti-RPA antibodies and then visualised by specific antibodies. Although co-immunoprecipitation is not as sensitive as metabolic labelling, with the help of high affinity monoclonal antibodies, several proteins of interest can be tested. It also shows interaction between two proteins.

The amount of 70B-RPA, as determined by the intensity of p70 did not change significantly in G_0 and S phase cells. In contrast to 70B-RPA, RPA immunoprecipitated with RAC-1 was low in G_0 and early G_1 cells, but increased in the late G_1 phase (Figure 3.13, lanes 2 to 4), and slightly dropped just before cells went into S phase (Figure 3.13, lanes 5 to 7).

In order to compare different associations of RPA with DNA replication and repair proteins in G_0 to S phase, the co-immunoprecipitated proteins were analysed by various antibodies. The tumor suppressor protein p53 was associated with 70B-RPA in G_0 phase, a slight increase of the associating p53 was seen after serum stimulation, whereas p53 bound to RAC-1-RPA increased several folds in S phase compared to G_0 phase (Figure 3.13A, and B). In addition the complex formation of RPA with DNA polymerases was investigated. Low amounts of DNA polymerase α shown by the presence of the largest subunit p180 coprecipitated with RAC-1-RPA complex in late

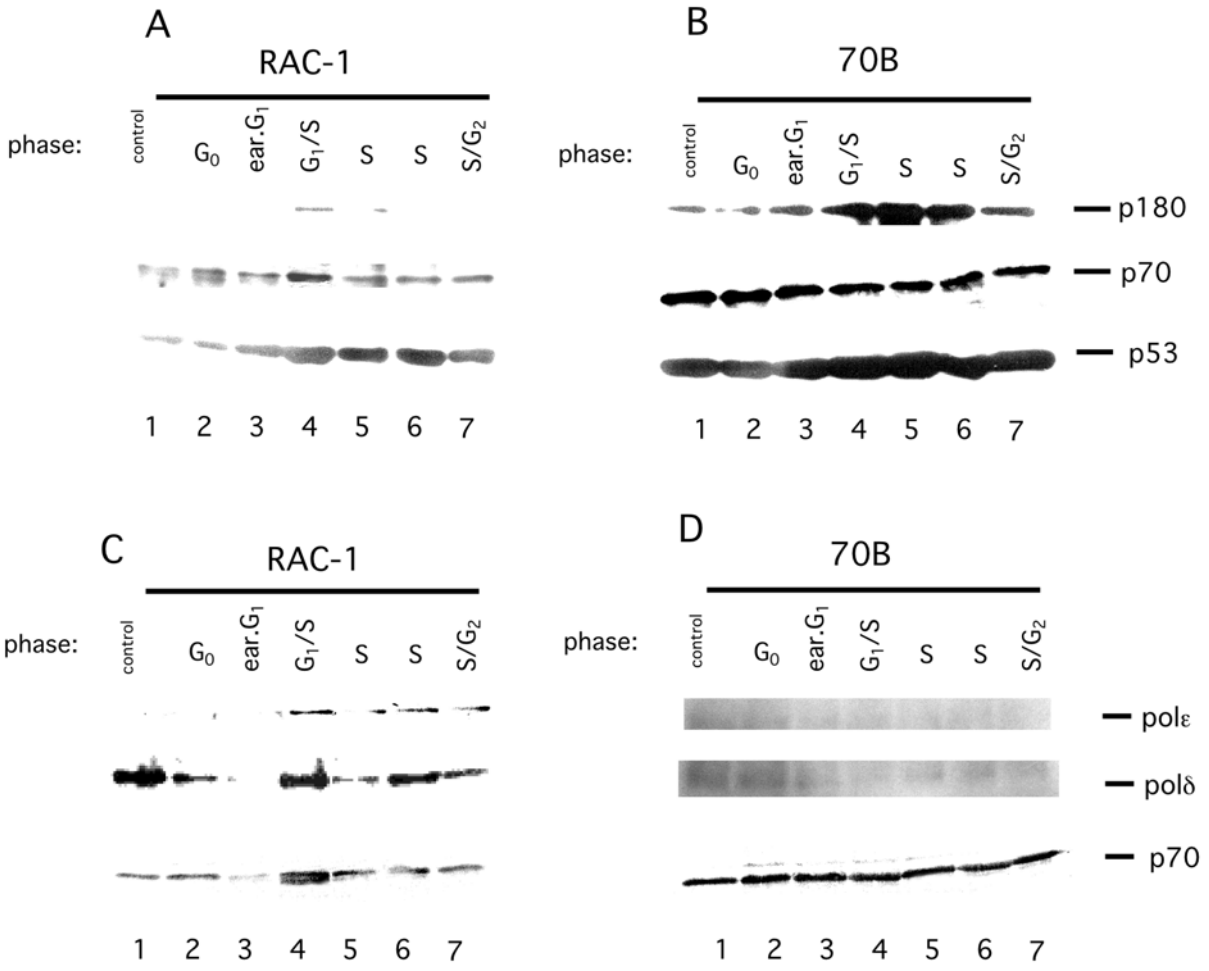


Figure 3.13: RAC-1-RPA and 70B-RPA Interact with Different Proteins T98G cells were synchronised by serum depletion. Cells were harvested at the indicated time points after the addition of complete medium and crude extracts were prepared for immunoprecipitation. Immunoprecipitation were carried out either with RAC-1 (**A, C**) or with 70B (**B, D**). The proteins which coimmuno-precipitated with RPA complex were detected by western blotting. The same membranes were stripped and reprobed with anti-p180 (murine) and anti-p53 (goat) antibodies (**A, B**), anti-DNA polymerase ε (murine) and DNA polymerase δ antibodies (rat) (**C, D**).In addition, all membranes were incubated with poly-anti-RPA serum (rabbit) as the primary antibody, the secondary antibodies were HRP-conjugated goat anti-mouse, HRP-conjugated sheep anti-goat, HRP-conjugated goat anti-rat, HRP-conjugated goat anti-rabbit, respectively. The proteins were detected by ECL method. Cells were harvest at 0 h, 5 h, 10 h, 15 h, 20 h, 25 h (lanes 2 to 7, respectively) after complete medium was added. Lane 1 are normal cycling cells as control. Lanes 2 to 7 are relative to the cells at G_0, early G_1, G_1/S, S, S, and S/G_2 phases, respectively.

G_1 and early S phase (Figure 3.13A, lanes 4 and 5), but the enzyme complex detached from the RAC-1-RPA later in cell cycle (Figure 3.13A, lanes 6 and 7). In contrast to this result, high amounts of DNA polymerase α bound to 70B-RPA in G_0 phase. Its binding to 70B-RPA increased during G_1 and S phase, but dropped slightly in G_2 and M phase (25h, Figure 3.13B, lane 7). The DNA polymerases δ and ε behaved differently from DNA polymerase α and they exclusively coprecipitated with

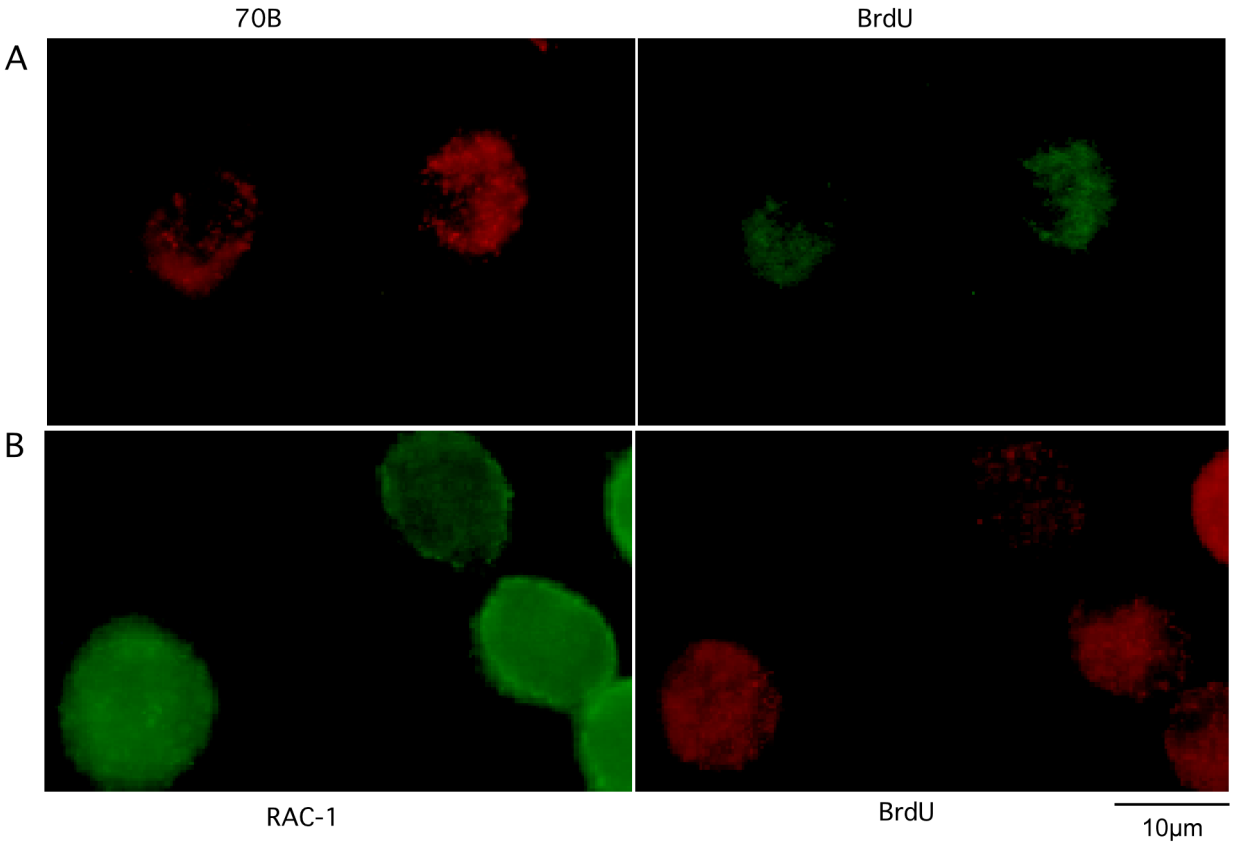


Figure 3.14: Colocalisation Patterns of RAC-1-RPA and 70B-RPA with BrdU
Logarithmically growing HeLa cells were double stained with RAC-1 and BrdU **(A)** as well as 70B and BrdU (**B**). In the RAC-1/BrdU double staining, RAC-1 was in green and BrdU was in red; in the 70B/BrdU double staining, 70B was in red and BrdU was in green.

RAC-1-RPA, but not with 70B-RPA at any time of the cell cycle (Figure 3.13C, and D). This observation was in line with experiments using metabolic labelling (Figure 3.11). These data confirmed the hypothesis that the two RPA subpopulations interacted with different proteins: the RAC-1-RPA trimer interacted with p53, DNA polymerases δ and ε. In addition, RAC-1-RPA contained non-phosphorylated p32, whereas the 70B-RPA trimer contained phosphorylated p32. 70B-RPA interacted with p53 and DNA polymerase α. Their functional difference might lie in their different associations with DNA replication and DNA repair proteins.

3.3 Relationship between the RPA Subpopulations and DNA Synthesis

70B-RPA coprecipitated with DNA polymerase α, which is responsible for the initiation step of DNA replication, whereas RAC-1-RPA coprecipitated with DNA polymerase δ and ε, which are essential for the elongation step of DNA replication (Figure 3.13C and 13D). The association of RAC-1- and 70B-RPA with different DNA polymerases raised the question of whether the subpopulations colocalised with the areas of ongoing DNA synthesis. RAC-1-RPA and 70B-RPA might form DNA initiation and

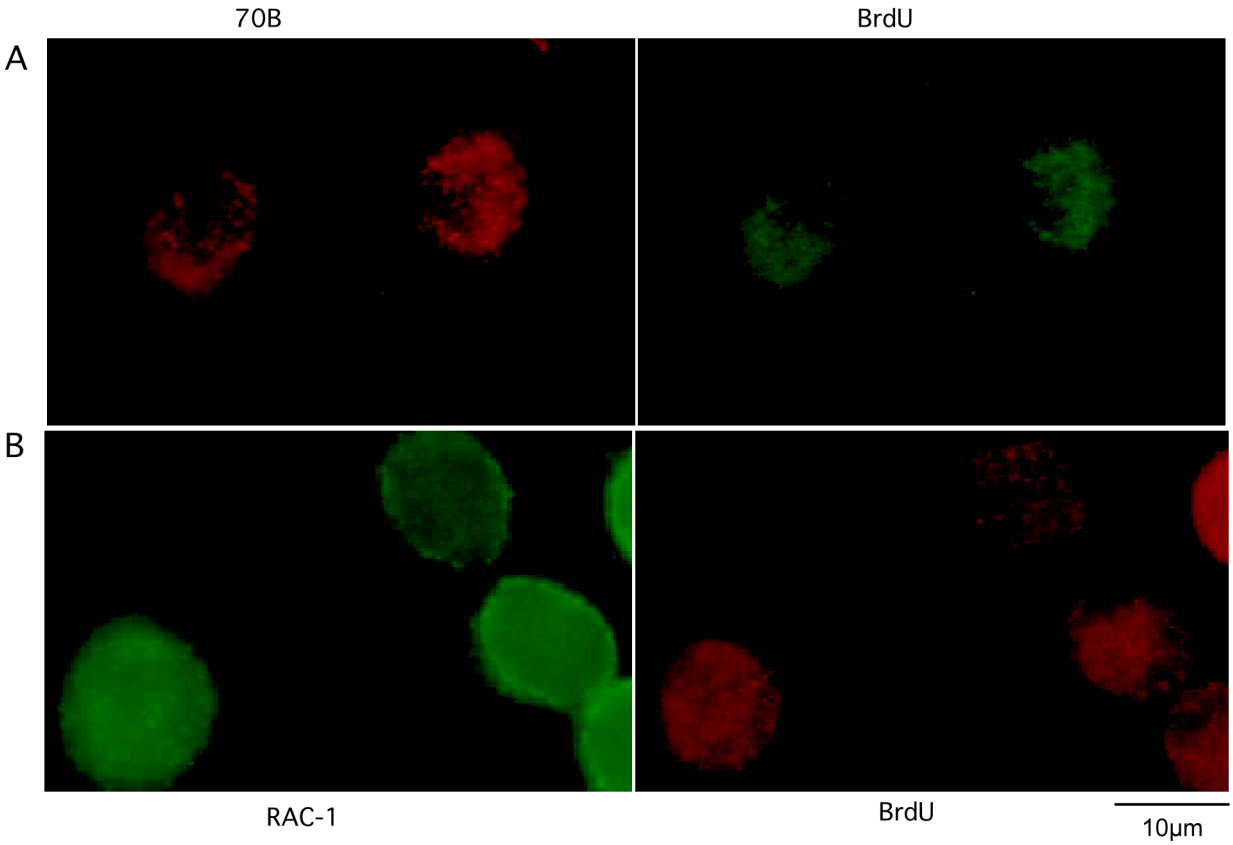


Figure 3.14: Colocalisation Patterns of RAC-1-RPA and 70B-RPA with BrdU
Logarithmically growing HeLa cells were double stained with RAC-1 and BrdU **(A)** as well as 70B and BrdU (**B**). In the RAC-1/BrdU double staining, RAC-1 was in green and BrdU was in red; in the 70B/BrdU double staining, 70B was in red and BrdU was in green.

RAC-1-RPA, but not with 70B-RPA at any time of the cell cycle (Figure 3.13C, and D). This observation was in line with experiments using metabolic labelling (Figure 3.11). These data confirmed the hypothesis that the two RPA subpopulations interacted with different proteins: the RAC-1-RPA trimer interacted with p53, DNA polymerases δ and ε. In addition, RAC-1-RPA contained non-phosphorylated p32, whereas the 70B-RPA trimer contained phosphorylated p32. 70B-RPA interacted with p53 and DNA polymerase α. Their functional difference might lie in their different associations with DNA replication and DNA repair proteins.

3.3 Relationship between the RPA Subpopulations and DNA Synthesis

70B-RPA coprecipitated with DNA polymerase α, which is responsible for the initiation step of DNA replication, whereas RAC-1-RPA coprecipitated with DNA polymerase δ and ε, which are essential for the elongation step of DNA replication (Figure 3.13C and 13D). The association of RAC-1- and 70B-RPA with different DNA polymerases raised the question of whether the subpopulations colocalised with the areas of ongoing DNA synthesis. RAC-1-RPA and 70B-RPA might form DNA initiation and

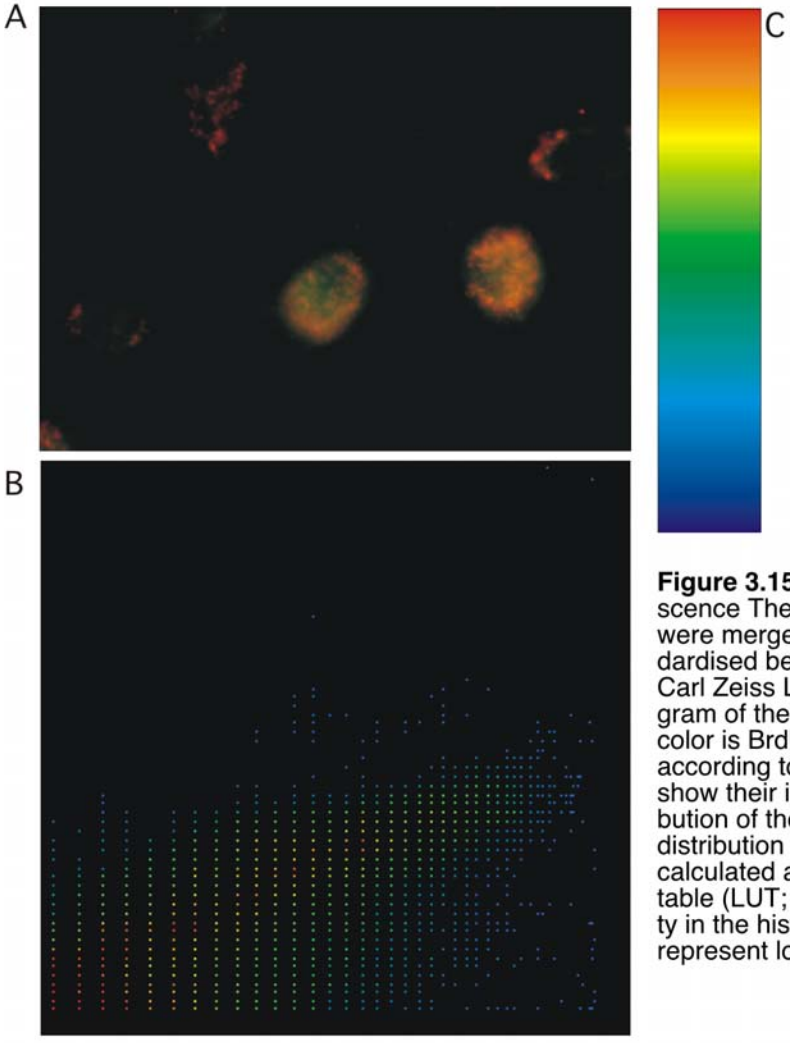


Figure 3.15: Analysis of crosstalk in Fluorescence The two figures of Figure 3.14A and B were merged (A) and their intensity was standardised before these images were loaded in the Carl Zeiss LSM software 2.4. The intensity histogram of the red color is 70B staining and green color is BrdU staining. The values were calculated according to the pixel amount of each pixel, to show their intensity distribution. The intensity distribution of the red chanel was plotted against the distribution of the green one. The difference was calculated and presented according to the lookup table (LUT; C). Red dots represent highest similarity in the histogram distribution, whereas blue dots represent lowest similarity (**B).**

elongation complexes separately. In order to address this question, immunofluorescence experiments were performed with BrdU-labelled cells to study whether these RPA forms are associated with DNA replication or have other functions. The newly synthesised DNA as determined by BrdU staining was clustered in nuclei. The staining patterns of RAC-1 and 70B for RPA were the same as shown in Figure 3.2A. The 70B-RPA staining merged well with BrdU staining, suggesting that most 70B-RPA colocalised with DNA replication forks (Figure 3.14A). However, RAC-1-RPA had a more extensive staining within the cells than BrdU and did not cluster, suggesting that RAC-1-RPA might have functions in addition to direct involvment in DNA synthesis (Figure 3.14B). The BrdU and 70B-RPA stainings colocalised well, to control the possibility that whether one part of the staining was from the fluorescence of the second fluorophore which could pass through its filter, the two images were analysed by the Carl Zeiss LSM software 2.4. The staining intensity in these two images showed a large difference, as plotted in Figure 3.15B, with dominant blue and

green dots, and a few red dots. This analysis showed that the pixel values and intensities of the single color planes in Figure 3.14A and 14B are from their own staining, not from crosstalk of the other fluorophore.

3.4 The Two RPA Subpopulations in DNA Repair

As RAC-1-RPA but not 70B-RPA co-immunoprecipitated with DNA polymerase ε, which provides strand displacement synthesis and gap filling in DNA repair, the function of RPA in DNA repair was investigated.

Ultraviolet light (UV light) with a wavelength of less than 400 nm can damage tissues and cells. The UV spectrum can be divided into three ranges according to their biological interference: First, the very short-wavelength radiation called the UV-C region (190-280 nm). The following spectral region ranges from 280-320 nm, named UV-B. UV-A light, with a wavelength of 320-400 nm, contains even less energy than UV-B light. The radiation energy absorbed by biological macromolecules in cells and tissues is transferred to other cellular compounds or even to cellular oxygen. The resulting radicals are able to react with biological macromolecules. The reaction of oxygen-radicals with DNA leads to single-stranded breaks when radicals break the DNA backbone by re-esterification.

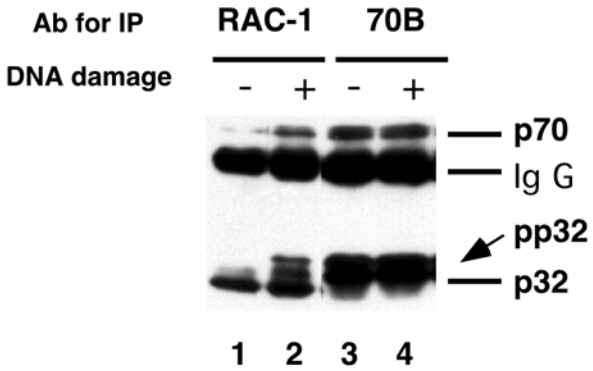


Figure 3.16: RAC-1-RPA Increases during DNA Repair Cycling T98G cells were harvested with trypsin/EDTA/PBS treatment. Half of the cells were irradiated by UV-A for 30min. Irradiated cells were cultured in complete medium for 5 h after irradiation. Crude extracts were made from irradiated (lane 2 and 4) and non-irradiated cells (lane 1 and 3), and the same amount of protein was applied for immunoprecipitation with RAC-1 antibody (lane 1 and 2) and 70B antibody (lane 3 and 4). Immunoprecipitates were then detected by SDS-PAGE followed by western blotting, polyclonal anti-hsRPA antiserum as the primary antibody, the secondary antibody was HRP-conjugated goat anti-rabbit antibody. The membrane was developed by ECL.

In proliferating T98G cells, the amount of RAC-1-RPA increased after DNA damage by UV-A light, moreover, its p32 subunit was partially phosphorylated (Figure 3.16, comparing lanes 2 to 1). However, no difference was observed for 70B-RPA, neither its amount, nor its phosphorylation forms changed (Figure 3.16, comparing lanes 4 to

3). It has been previously reported that upon UV irradiation, the amount of RPA increases and its p32 subunit is phosphorylated within 4 to 8 hours after irradiation (Carty *et al*., 1994). The data presented here confirmed previous reports, and indicated that RAC-1-RPA, but not 70B-RPA, was strongly induced after DNA damage, and that phosphorylated p32 was associated with RAC-1-RPA in DNA repair events (Figure 3.16, comparing lane 2 to 1).

3.5 Phosphorylation of DNA Polymerase α-primase in Living Cells

As decribed above, 70B-RPA might perform its function through interaction with DNA polymerase α-primase. DNA polymerase α-primase is the only DNA polymerase which can start DNA replication *de novo*. It consists of 4 subunits: p180, p68, p58, and p48. Its phosphorylation status, and specially that of p68, is important for cell cycle regulation.

3.5.1 Detection of Phosphorylated p68 by Phosphopeptide-specific Antibodies

The p68 subunit of DNA polymerase α-primase is phosphorylated in a cell-cycle dependent manner (Bouvier *et al*, 1993, Foiani *et al*,1995; Nasheuer *et al*, 1991; Park *et al*, 1995). These studies using various organisms and methods gave conflicting reports regarding the time course of these modifications within the cell cycle. Therefore, phosphopeptide-specific antibodies were established to examine the phosphorylation status of human DNA polymerase α-primase *in vivo* and *in vitro*. The peptide P1 (P1: aa 137 to 151 of p68) contains two conserved motifs, serines S141 and S147, which are phosphorylated by Cyclin A-Cdk2 *in vivo* and *in vitro* (Rehfuess, 1998; Voitenleitner *et al*, 1999). The antibody 858-PPI was produced by immunisation with the phosphorylated P1 (PP1). The properties of 858-PP1 antibody have been tested especially with its affinity and specificity in eukaryotic cells. To confirm that 858-PP1 antibody only recognised phophorylated p68, but not non-phosphorylated p68, a peptide competition assay was performed**.** The 858-PP1 antibody recognised a polypeptide with an apparent molecular mass of about 68 kDa in cycling cells (Figure 3.17, lane 1) as well as in purified DNA polymerase α-primase (A. Schneider, personal communication). The recognition of 858-PP1 could be completely abolished by low levels of phosphorylated peptide PP1 (Figure 3.17, lane 4). When 858-PP1 was preincubated with non-phosphorylated peptide P1 before it was applied for western blotting, its affinity to phosphorylated p68 was not affected (Figure 3.17, lane 7). The 858-PP1 antibody distinguished phosphorylated p68 from non-phosphorylated p68

and thus provided the opportunity to study p68 phosphorylation by various techniques such as western blotting and immunofluorescence.

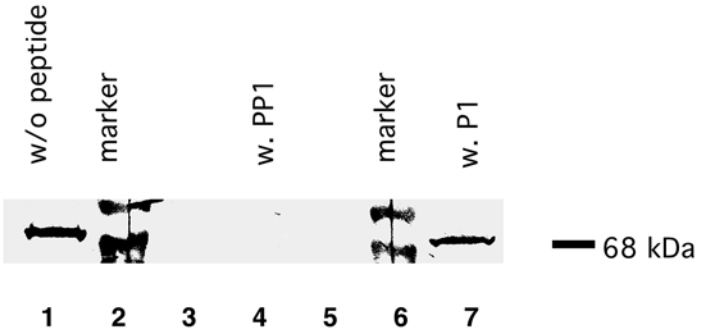


Figure 3.17: Phosphorylated Peptide PP1 Inhibits the Recognition of 858-PP1 Antibody to p68. 100 mg of HeLa cell crude extract (lanes 1, 4 and 7) and 10 mg of BSA (lanes 3 and 5, as negative control) were loaded on an SDS-gel as three regions separated by prestained marker (lanes 2 and 6). The proteins were blotted onto the PVDF membrane. The membrane was cut into three parts according to the prestaining marker, and p68 was detected separately by pre-treated 858-PP1 antibodies as primary antibodies: 858-PP1 (lane 1), 858-PP1 pre-incubated with phosphorylated peptide P1(lanes 3, 4 and 5), 858-PP1 pre-incubated with un-phosphorylated peptide P1 (lane 7), the secondary antibody for detection was AP-conjugated goat anti-rabbit antibody. The blots were developed by ECL method. The pre-incuabtion of 858-PP1 with peptides took place at RT for 30 min prior to using for western blotting.

3.5.2 Nuclear Localisation of P1-phosphorylated p68 in Late G_1 and S Phase but not in Mitotic Cells

Phosphorylation influences the nuclear transport and the subcellular localisation of proteins (Petersen *et al*, 1999; Pines, 1999). As p68 is required for the nuclear transport of p180 (Mizuno *et al*, 1999), it was interesting to know whether the p68 phosphorylation changes cellular distribution of the polypeptide by using the antibody 858-PP1. Nucleus and cytoplasm of the fixed cyling cells were determined by phase contrast microscopy (Figure 3.18). To investigate the localisation of DNA polymerase α-primase the p180-specific antibody 2CT25 was used in parallel. p180 was readily detectable and all examined cells were stained (Figure 3.18A and B).

Merging pictures showed that the DNA polymerase α-primase and phosphorylated p68 were exclusively nuclear. When the cells were arrested in G_2/M phase of the cell cycle by Nocodazole, their p180 level was still high, but the level of phosphorylated p68 was too low to be detected by immunofluorescence using 858-PP1 (Figure 3.18B)

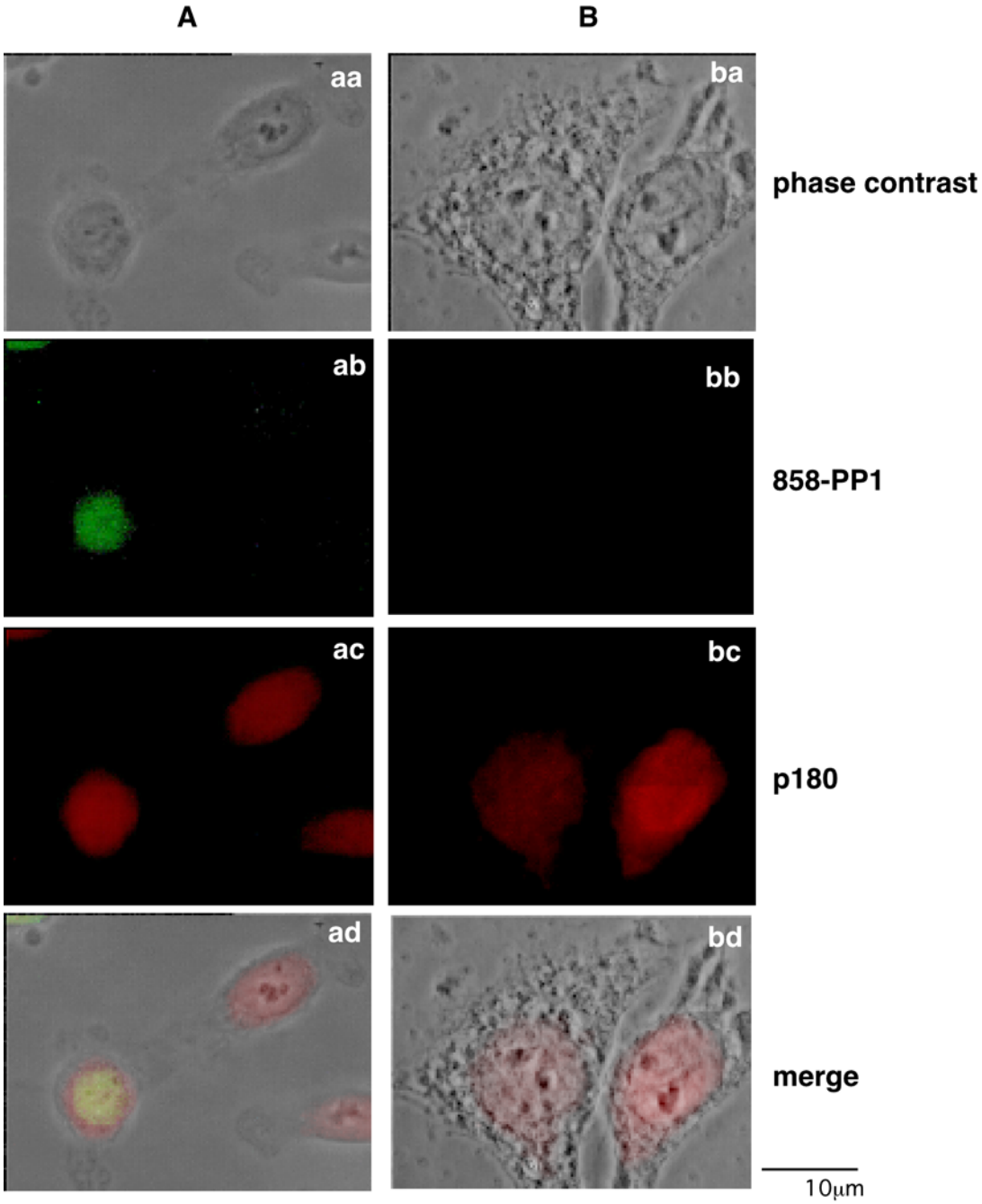


Figure 3.18: Phosphorylated p68 is Nuclear. Logarithmically growing HeLa (panel A) or HeLa cells grown for 16h in the presence of Nocodazole (panel B) were fixed and double immunostained with the antibody 858-PP1 and 2CT25 (Dornreiter, 1991). aa and ba: phase contrast pitctures; ab and bb: antibody 858-PP1 determined with FITC-conjugated 2nd antibody in green; ac and bc: p180-specific mouse antibody determined with Rhodamine Red-conjugated 2nd antibody in red. ad: merging pictures of aa, ab and ac ; bd: merging pictures of ba and bc.

Peptide P1 of p68 is Phosphorylated in Late G_1/Early S Phase

T98G cells were arrested at G_0 phase by serum depletion. Flow cytometry and BrdU incorporation experiments indicated that cells were at late G_1/early S phase about 10 hours after serum addition. The phosphorylation level of p68 was low when cells were

in G_1 phase, but significantly increased at late G_1/early S phase (Figure 3.19, upper

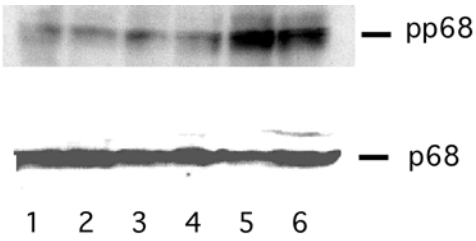
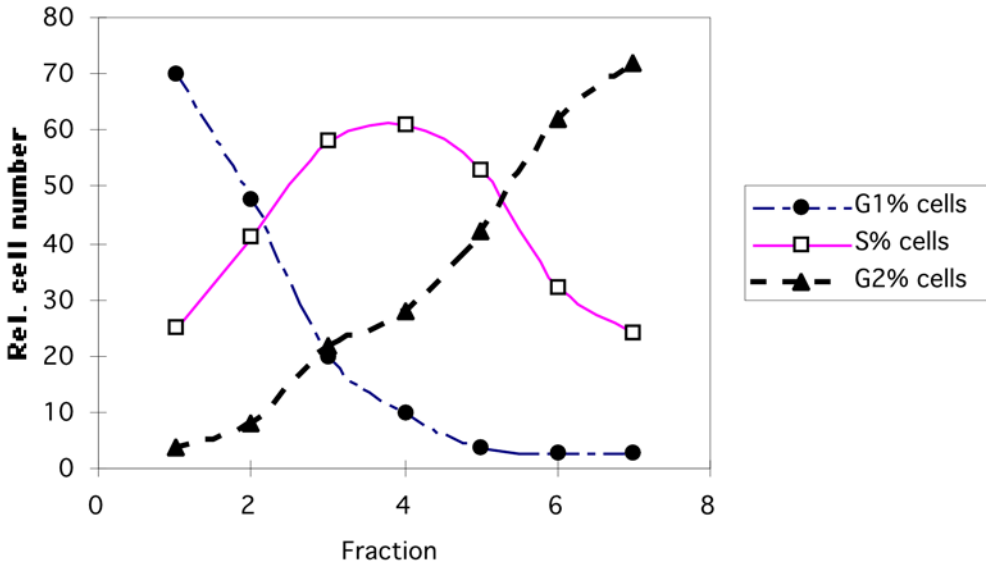


Figure 3.19: p68 is Phosphorylated just prior to S Phase. T98G cells were arrested at G_0 phase by serum depletion, then stimulated by complete medium containing 10% FCS. The cells were harvested at indicated time points. Crude extracts were made from these cells. The 100 μg protein in crude extracts were applied for SDS-PAGE. The membranes were probed first with 858-PP1 as primary antibody (upper panels), and HRP-conjugated anti-rabbit antibody as secondary antibody. After developed by ECL, the membranes were stripped and reprobed by anti-full-length p68 as the primary antibody, and HRP-conjuagted anti-rabbit antibody as the secondary antibody, followed by development by ECL (lower panels). lanes 1 to 6: 5 h, 6 h, 7 h, 8 h, 9 h, and 10 h after serum stimulation, respectively.

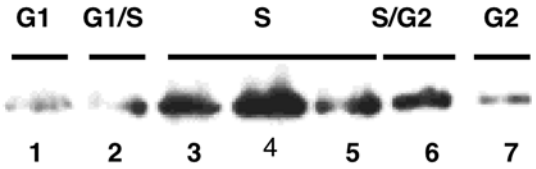
panel). Meanwhile, the total level of p68 stayed constant from G_1 to S phase (Figure 3.19, lower panel).

Another experiment was performed in a system which does not interfere with the cellular metabolism, the elutriation centrifugation (its principle described in section 2.2.16). The CEM cells were fractionated to 7 fractions by increasing centrifuge speeds. The cell cycle distributions of these fractions were determined by flow cytometry on the basis of the DNA content of cells. This DNA analysis revealed that cells in fraction 1 were highly enriched in G_1 phase of the cell cycle, and that fractions 3 and 4 contained about 60% S phase cells. The fraction 7 had a high level of G_2 phase cells (Figure 3.20A). Identical amounts of proteins from crude extracts of these fractions were subjected to western blotting. The degree of p68 phosphorylation was determined using the 858-PP1 antibody (Figure 3.20B). In comparison, the level of p68 in the same crude extracts was monitored by stripping the same blot after detection of phosphorylated p68, and reprobing with an antiserum recognising full length p68 (Figure 3.20C). The p68-peptide P1 was sightly phosphorylated in early G_1 phase but was considerably modified in late G_1 to early S phase (Figure 3.20, lanes 1 to 3). The phosphorylation level was high during S phase and S/G_2 transition (Figure 3.20, lanes 2 to 6) and decreased in G_2 (Figure 3.20B, lane 7, summarised in 3.20D). In contrast to the phosphorylation level, the total cellular level of p68 varied only slightly during the cell cycle (Figure 3.20C).

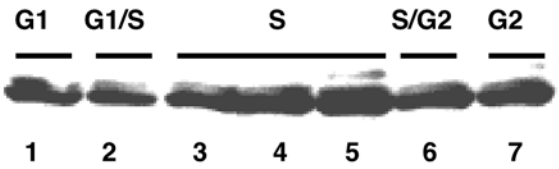
A


Rel. cell number
Fraction
G1% cells
S% cells
G2% cells

B


G1
G1/S
S
S/G2
G2
1
2
3
4
5
6
7

C


G1
G1/S
S
S/G2
G2
1
2
3
4
5
6
7

D

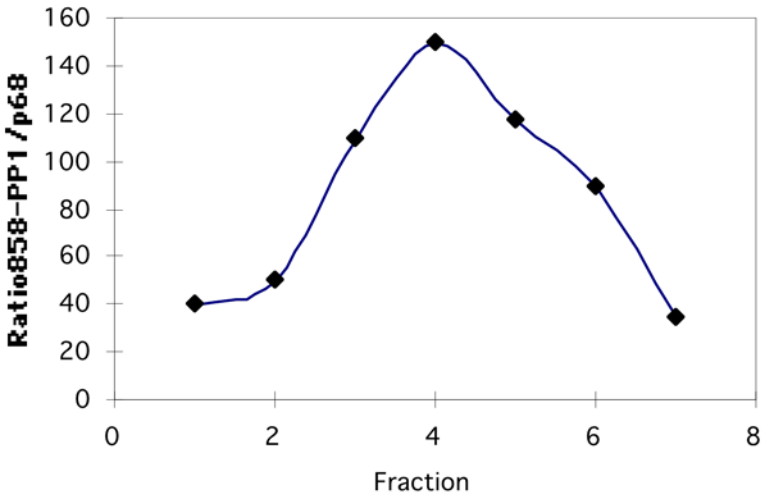

Ratio858-PP1/p68
Fraction

Figure 3.20: The Peptide PP1 of p68 is Phosphorylated in Late G_1 to Early S, S and Early G_2 phase. (A) CEM cells were fractionated by elutriation centrifugation. Cell cycle distribution of each fraction was detected by flow cytometry. **(B)** and **(C)** After determination of protein concentration, 50 μg of crude extracts of cells in different cell cycle were analysed by SDS-PAGE and western blotting using antibodies which were either specific for pp68 (purified antibody 858-PP1; **panel B**), or directed against full length p68 (**panel C**), respectively, on the same blot. First, the membrane was incubated with antibody 858-PP1 and detected by ECL according to the manufacture's protocol (Amersham-Phamacia-Biotech). For reprobing in panel C, the membrane was stripped to remove all antibodies and was detected by antibody against p68 with ECL. **(D)** Signals of panel B and C were scanned and the quantified by using the Image Quant programm. The signal ratio of pp68 to p68 was calculated for each fraction and is presented in arbitrarly units.

The increase of p68 phosphorylation started at S phase, when DNA replication starts. To understand the relationship between p68 phosphorylation and DNA synthesis, cells in different phases (early S, mid S, later S and G_2 phase) of the cell cycle were checked for p68 phosphorylation and BrdU incorporation. In early S phase, phosphorylation of p68 occurred prior to BrdU incorporation (Figure 3.21, upper panel), whereas in mid S phase the incorporation of BrdU and the phosphorylation of p68 were strong and detectable in each cell. When in G_2 phase, the p68 phosphorylation as well as BrdU incorporation decreased (Figure 3.21, lower panels). This is further evidence to suggest that p68 did get phosphorylated in the early S phase. Furthermore, the phosphorylation of p68 in S phase is not homogenous, i.e. not all cells phosphorylate p68 before S phase.

With three different mammalian cell lines and different synchronisation methods, the above results indicated the same tendency of p68 subunit in cell cycle. Nocodazole synchronisation was selected because it led to a higher percentage of G_2/M cells than the TdR block-and-release method. Serum depletion gave a good view of the period from G_0 to S. This period of the cell cycle could not be reached with other synchronisation methods, as only serum depleted cells were in this G_0 phase. Elutriation provided a confirmation of the other synchronisation methods. It separated the cells according to their size. The identity of the fractions were later confirmed by FACS analysis. Although the fractions were not absolutely pure of containing only cells at a certain phase, the advantage of this method was that it permitted to study cells in each phase of the cell cycle coming from the same history and growing condition.

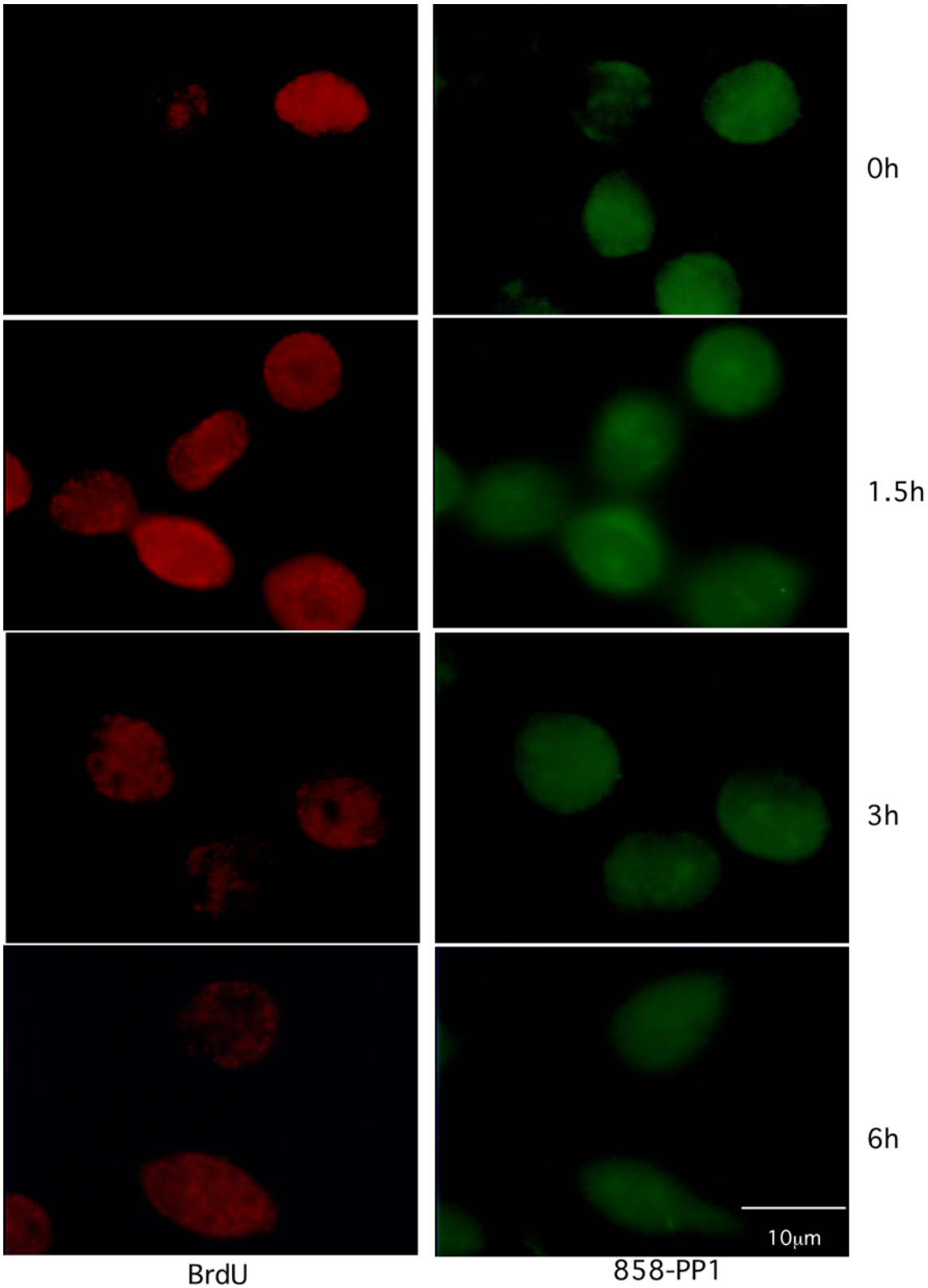


Figure 3.21: Phosphorylation of p68 Takes Place Earlier than BrdU Incoporation. HeLa cells grown on glass slides were synchronised by TdR and released in TdR-free medium. Before harvested at the indicated time points (0h, 1.5h, 3h, 6h, respectively), the cells were incubated in the medium supplied with 50 μM BrdU for 15 min, followed by fixation with methanol/acetone. The DNA was denatured by formamide and the cells were double stained with anti-BrdU (mouse) and 858-PP1 (rabbit) antibodies. The secondary antibodies were Rhodamine-conjugated anti-mouse and FITC-conjugated anti-rabbit antibodies.

3.5.3 Phosphorylation of p68 is Downregulated after DNA Damage

DNA polymerase α-primase has been reported to play a role in DNA repair (Pellicioli *et al*, 1999; Voitenleitner *et al*, 1999). To test the level of phosphorylated p68 after DNA damage, HEp2 cells were UV-C irradiated and the variation of p68 phosphorylation was determined. Immediately after irradiation, the phosphorylation level of p68 significantly dropped compared to untreated control cells (Figure 3.22, compare lanes 1 and 2). Although the phosphorylated p68 increased its amount from 0 hour until 1.5 hours after recovery, it was still lower than in normal cells. Similar to Figure 3.21, the full length p68 was stably detected before and after irradiation (Figure 3.22, lower panel).

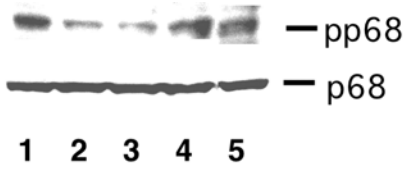


Figure 3.22: Phosphorylation of p68 is Downregulated during DNA Repair HEp2 cells were irradiated by UV-C light with 50 J/m$^{2.}$ Cells were cultured in normal medium for indicated time periods after irradiation. Crude extracts were made, and 50 μg proteins for each crude extract were applied for western blotting. The blot was first probed with 858-PP1 antibody as primary antibody, and HRP-conjugated goat anti-rabbit antibody as secondary antibody, followed by ECL development. The blot was stripped and re-probed with anti-full length p68 as primary antibody, HRP-conjugated anti-rabbit antibody as secondary antibody, the blot was developed by ECL. lane 1: normal cells; lanes 2 to 5, 0 h; 1h;1.5 h; 6 h after irradiation, respectively.

4 Discussion

Eukaryotic DNA metabolism is highly organised and tightly regulated (Wolffe, 1998). Replication protein A, RPA, is an essential protein involved in various processes of the DNA metabolism such as DNA replication, DNA repair, and DNA recombination (Iftode *et al.,* 1999; Nasheuer *et al.,* 2002; Wold 1997). In addition, RPA associates with transcription factors suggesting that RPA has central functions in the nucleus (Dutta *et al.,* 1993; He *et al.,* 1993). The discovery that 70B-RPA is expressed even in G_0 phase suggests that living cells always contain RPA, therefore RPA is important for viability of cells (Figure 3.13). There are contradictory reports about whether p70 of RPA binds to chromatin after replication or not. Several reports claimed that p70 is dissociated from chromosome after replication (Adachi and Laemmli, 1992. Cardoso *et al.,* 1993; Murti *et al.,* 1996). However, Dimitrova and Gilbert (2000) recently demonstrated that RPA is observed in association with chromatin in G_2/M phase. The studies presented here confirm the latter finding by using *in vivo* crosslinking assays (Figure 3.7) and chromatin bound protein assays (Figure 3.6), which show that RPA associates with nuclear structures from G_1 until G_2 phase and that the association is as stable in G_2/M phase as it is in S phase.

4.1 The RAC-1-RPA and 70B-RPA Subpopulations

Immunofluorescence experiments with RAC-1 and 70B antibodies suggest that two RPA subpopulations exist in human cells (Figure 3.2 and 3.9). The variation in their immunostaining patterns and dominance indicates that in human cells some RPA molecules present epitopes specific for RAC-1, whereas others present epitopes for 70B. One subpopulation in nuclear extracts is still detectable when the other one is depleted by its specific antibody (Figure 3.4 C and D). Furthermore, most of RAC-1-RPA is immobilised on the nuclear matrix, whereas a significant amount of 70B-RPA is loosely restrained within nuclear structures. These findings suggest 70B-RPA is involved in some events other than RAC-1-RPA.

The cellular levels of RAC-1-RPA and 70B-RPA change differentially during the cell cycle. The level of RAC-1-RPA does not stay as constant from early G_1 to S phase as that of 70B-RPA does (Figure 3.13). These results demonstrate RAC-1-RPA is induced in response to DNA replication, whereas p70 of 70B-RPA is stably expressed. As will be discussed below, 70B-RPA does play an important role in the initiation of DNA replication. This would suggest the hypothesis that 70B-RPA does not change its

conformation before and after DNA replication, but could change its localisation in and out of nuclei. In Figure 3.2 some cells showed only 70B staining, but very weak RAC-1 staining. These cells are most likely in early G_1 phase when the amount of RAC-1-RPA is much lower than that of 70B-RPA.

The nuclear binding properties of the two RPA subpopulations are different, both p32 and p70 of RAC-1-RPA are crosslinked as a complex with the nuclear matrix in G_1 until S phase, whereas p32 of 70B-RPA crosslinked to p70 in G_2 phase (Figure 3.7). Furthermore, the 70B-RPA trimer contains p32 in various phosphorylation states, whereas the RAC-1-RPA trimer has non- or hypophosphorylated p32 (Figures 3.3 and 3.8). This is further evidence that RAC-1-RPA and 70B-RPA are two different protein complexes with distinct quarternary structures.

Because RPA performs its functions through protein-protein interactions, the different protein interaction patterns of RAC-1-RPA and 70B-RPA (Figure 3.13) suggest their roles in different DNA metabolism events, which are mediated by the proteins they interact with.

4.2 RAC-1-RPA and 70B-RPA have Different Cellular Functions

The existence of two forms of RPA raises the question of whether RAC-1-RPA and 70B-RPA have different functions in eukaryotic DNA metabolism and whether the two forms differ in their activities.

4.2.1 Functions of RAC-1-RPA

RAC-1-RPA is induced after serum stimulation or after DNA damage. In addition, RAC-1-RPA interacts with DNA polymerases δ and ε, which carry out processive DNA synthesis during DNA elongation in DNA replication and gap filling after DNA damage (Figures 3.13 and 3.16). These findings suggest that RAC-1-RPA might function in the elongation stage of DNA replication and in DNA repair. The importance of these findings is underlined by recent reports that a significant amount of DNA polymerase δ, which plays a role in DNA elongation, tightly binds to nuclear structures and interacts with the TREX2 3'-5' exonuclease. The TREX2 3'-5' exonuclease acts as a proofreading exonuclease for DNA polymerases during DNA replication, repair and recombination (Shevelev *et al.,* 2002). Considering that RAC-1-RPA can be co-isolated with DNA polymerase δ, and that their association is cell cycle-dependent (Figure 3.13), the function of RAC-1-RPA for DNA replication elongation and for DNA repair is confirmed.

To test whether RAC-1-RPA is directly associated with DNA synthesis or not, immunofluorescence and BrdU incorporation experiments were performed using the RAC-1 antibody. In contrast to 70B, the RAC-1 antibody shows a more dispersive staining throughout the cell cycle (Figure 3.9). The BrdU incorporation studies revealed that only a fraction of RAC-1-RPA colocalises with BrdU (Figure 3.14), suggesting that these molecules are directly involved in DNA synthesis during the elongation of DNA replication, where RPA is required to stabilise the unwound DNA. The rest may have other functions, such as loading factors for DNA polymerases δ and ε prior to their recruitment onto replication forks. A possible order of events related to RAC-1-RPA in DNA replication elongation could be that replication factors such as DNA polymerases δ and ε bind to nuclear structures either via or together with the hypophosphorylated RPA (RAC-1-RPA). Since RPA specifically interacts with 3'-ends of newly synthesised primers, it could mediate the initial loading of DNA polymerase δ and ε to the primers and the sliding clamp PCNA (Lavrik *et al.,* 1998; Mass *et al.,* 1998; Waga and Stillman, 1998). After DNA polymerases have bound to these DNA structures, the RPA-DNA polymerase δ/ε complex dissociates. This view is consistent with the ordered loading of DNA polymerase δ investigated in a cell-free system where RPA and DNA polymerase δ form a complex but RFC disassembles the DNA polymerase δ/RPA complex (Yuzhakov *et al.,* 1999).

Alternatively, the remaining of RAC-1-RPA, which does not colocalise with BrdU incorporation sites, might be part of a larger complex immobilised on chromatin and sequester DNA metabolic proteins such as p53, DNA polymerase δ, DNA polymerase ε and also other replication as well as repair factors to the nucleus.

4.2.2 Functions of 70B-RPA

Prior to the onset of DNA replication RPA has been described to accumulate in brightly stained dots, which are called pre-replication centers. The staining becomes dispersive as DNA replication proceeds (Adachi and Laemmli, 1992; Dimitrova and Gilbert, 2000; Yan and Newport, 1995). 70B-RPA accumulates such dots from early to mid S phase but switches to dispersive staining in late S phase (Figure 3.9). 70B-RPA behaves very much like the above description. The bright dots of 70B immunostaining indicates that most of 70B-RPA is localised in these so called pre-replication centers. Furthermore, 70B-RPA colocalises with BrdU (Figure 3.14) and binds to DNA polymerase α during S phase but does not associate with DNA polymerases

δ and ε (Figure 3.13). These results suggest that 70B-RPA is involved in the initiation of leading strand DNA replication as well as in the priming reaction of the Okazaki fragments on the lagging strand.

Only low amounts of DNA polymerase α interact with 70B-RPA in G_0 phase and this increases in late G_1 phase just prior to the beginning of S phase. The RPA-DNA polymerase α interaction does not occur throughout the cell cycle, suggesting that the interaction is tightly controlled in time. The increased interaction of RPA and DNA polymerase α matches the model of DNA replication. In the DNA replication model, RPA first binds to chromatin before DNA polymerase α-primase is recruited to chromatin. The conclusion that chromatin associates with RPA prior to DNA polymerase α is supported by an *in vitro* study, which demonstrates that the aggregation of RPA around pre-replication centers takes place prior to the initiation of DNA replication and even occurs before the formation of the nucleus (Adachi and Laemmli, 1994). These data are in line with other reports that RPA is localised in foci prior to the initiation of replication and remains there during DNA synthesis (Cardoso *et al*, 1993; Yan and Newport, 1995).

The 70B-RPA complex also co-immunoprecipitates significant amounts of the tumor suppressor p53 in G_0 phase (Figure 3.13). p53 induces apoptosis and is responsible for G_1 phase arrest. One of the apoptosis pathways can be triggered by withdrawal of essential growth factors (B.Lewin, 2000). When T98G cells are cultured in 0.5% FCS-containing medium for 120 hours, some of them enter apoptosis with small fragmented nuclei. p53 could play an important role in this apoptosis pathway and might be also involved in the G_0 phase arrest. The interaction between 70B-RPA and p53 in G_0 cells is a strong hint at the function of 70B-RPA on apoptosis and G_0 arrest.

4.3 Phosphorylation of p32 in 70B-RPA

p32 is phosphorylated in a cell cycle-dependent manner upon binding to ssDNA and in stressed cells (Wold MS, 1997; Treuner *et al.,* 1999). The phosphorylation of p32 is a consequence of and not a prerequisite for its binding to ssDNA to form the initiation complex at the SV40 origin (Fotedar and Roberts, 1992). This notion adequately explains that only the phosphorylated p32 form is detected in chromatin bound RPA (Figure 3.6 and Betteraey in Universität Tübingen pers. comm.).

The binding of human RPA to ssDNA makes RPA a better substrate for phosphorylation, and both RPA p70 and p32 are involved (Black *et al*, 1996, Gomes *et al*, 1996). Using *in vitro* experiments, Fotedar and Roberts (1992) showed that RPA p32 from initiation reaction of DNA replication is phosphorylated to a greater extent, however, p32 is much less phosphorylated when taking part in the elongation reaction. This is consistent with the observations and hypothesis presented here regarding 70B-RPA, which is part of the initiation complex for DNA replication and contains phosphorylated p32. Nevertheless, no phosphorylated p32 in RAC-1-RPA, which is involved in elongation of DNA replication, is detected. These results suggest that RAC-1-RPA might not be the only RPA subpopulation involved in DNA replication elongation.

The behaviour of the two RPA subpopulations is reminiscent of DNA polymerase α, which also exists in distinct forms in human cells. The hypophosphorylated DNA polymerase α is loaded in G_1 phase onto chromatin and associates with Cyclin E-Cdk2, protein phosphatase 2A, and Mcm2p. However, during S phase this DNA polymerase α subpopulation does not colocalise with newly synthesised DNA as determined by BrdU incorporation. The phosphorylation of DNA polymerase α by Cyclin A-dependent kinases interferes with its binding to replication initiation factors and probably regulates its origin-dependent initiation activity. Similarly, modification of RPA might modulate its functions and its interactions with DNA polymerases.

4.4 RAC-1-RPA is Involved in DNA Repair

During DNA repair damaged nucleotides have to be removed and a large number of DNA gaps are generated, which have to be filled to sustain the stability of genome. DNA polymerases δ and ε are responsible for DNA gap filling (Hübscher *et al.*, 2000). RPA is also involved in the gap-filling reaction, along with PCNA, RFC, DNA polymerase δ and ε (Aboussekhra *et al.*, 1995; DeMott, *et al.*, 1998). It has been reported that after DNA damage proteins which are important for DNA repair, such as RPA, are induced (Carty *et al.,* 1994). Figure 3.16 demonstrates that the increase in RPA during DNA repair is due to an increased levels of RAC-1-RPA. RAC-1-RPA participates in DNA repair probably through its interaction with DNA polymerases δ and ε. Importantly in UV-A-irradiated cells the p32 subunit of RAC-1-RPA is phosphorylated (Figure 3.16). This is not necessarily in conflict with the above observation that RAC-1-RPA contains the nonphosphorylated p32 form, because these experiments were performed with the cells in different growth conditions: normal

cycling cells and irradiated cells. p32 was reported to be phosphorylated increasingly in DNA repair 4-8 hours after DNA damage (Carty *et al.,* 1994; Liu *et al.,* 1993). When cells repair their DNA, the p32-specific kinases (ATM, ATR, and DNA-PK, but not Cdks) are highly active, and therefore these kinases might phosphorylate not only the p32 subunit which binds to DNA but also those in the RAC-1-RPA trimer. The results with stressed cells confirm that RAC-1-RPA has a function in DNA repair events.

4.5 Interaction of p32 and p70 with Nuclear Structures

The p70 and p32 subunits shown on crosslinking assays (Figure 3.7) are those which are crosslinked to nuclear structure and form a crosslinked DNA-p70-p32 ternary complex. The prerequisite for producing this crosslinked complex is a closed association of the right amino and imino groups of amino acids and of DNA (details see Methods and Results sections). Without a direct contact of the right amino and imino groups of p70, of p32, and of DNA, they cannot be crosslinked even if they are spatially closed to each other.

Before DNA replication occurs, 70B-RPA binds to DNA with a binding mode to dsDNA which allows the contacting amino and imino groups to be crosslinked. In S phase, where DNA replication happens, the DNA binding mode of 70B-RPA switches to a different mode, thereby it undergoes an extensive conformational change in which both p70 and p32 are engaged (Bochkareva *et al*., 2002). Because of this conformational change, the contact of p70, p32 and DNA might be changed as well and the prerequisite for forming a crosslinked DNA-p70-p32 ternary complex is not fulfilled, and p32 cannot be crosslinked to p70. Contrary to 70B-RPA, RAC-1-RPA does not initiate DNA replication, but is involved in the elongation stage of DNA replication through its interaction with DNA polymerases δ and ε. The protein-RPA interaction in S phase might enable p32 and p70 to make a correct contact with DNA to form a crosslinked complex. After S phase these interacting proteins are no longer required and may therefore detach from RAC-1-RPA. This could lead to p32 changing its binding mode to p70 and DNA. Consequently p32 may not be crosslinked to p70 or DNA as the critical amino and imino groups do not contact each other any more. There might be an alternative explanation regarding RAC-1-RPA. One function of RAC-1-RPA could be sequestering replication/repair proteins in nuclei. In S phase, the sequestering proteins would be released to perform their functions on DNA replication. After S phase when they have finished their task, these proteins would be sequestered by RAC-1-RPA and might interfere with the critical connection of p70 and

p32 to each other and also to DNA, and thus crosslinked p70-p32-DNA complex cannot be not formed.

4.6 Conformational Models of RAC-1-RPA and 70B-RPA

Based on biochemical and immunological results, there are several possible interpretations for the different conformations of RAC-1-RPA and 70B-RPA.

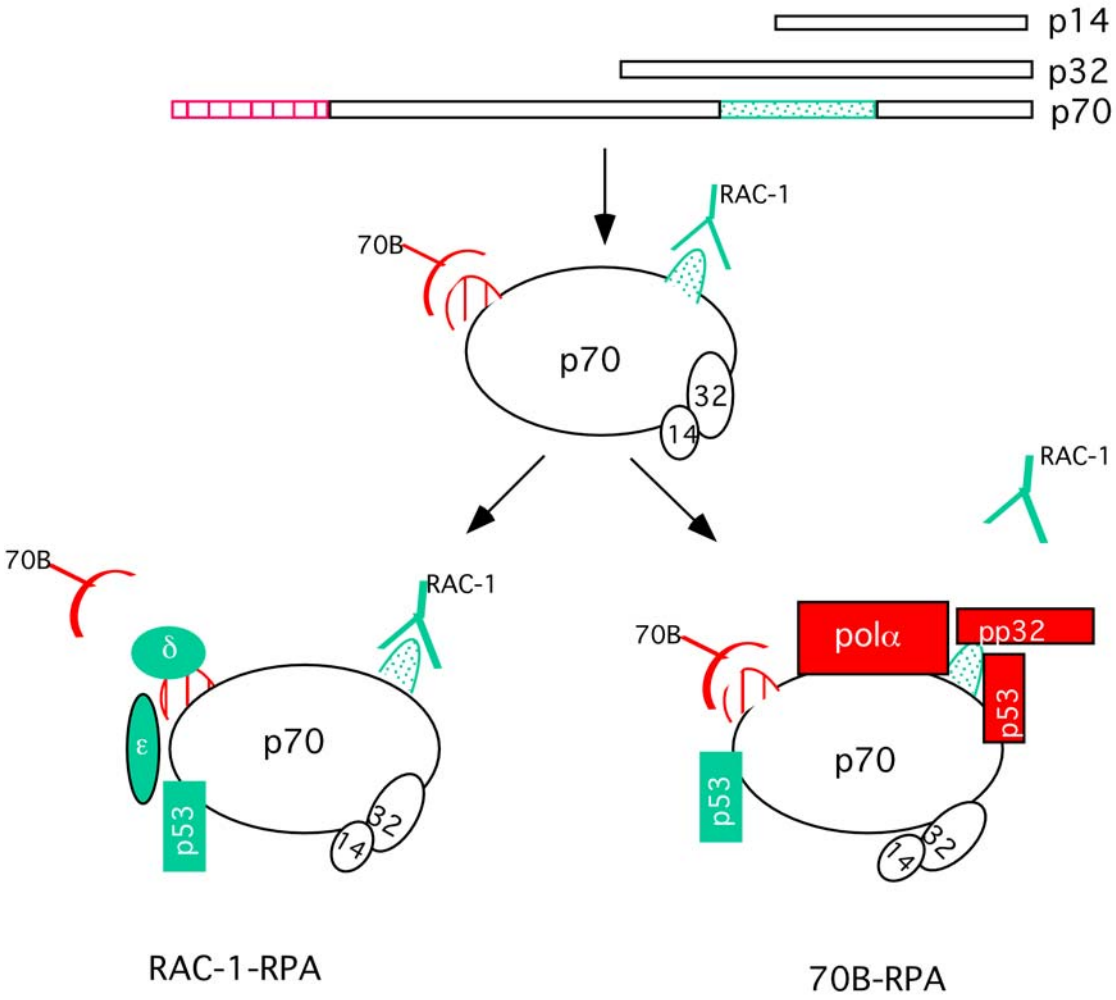


Figure 4.1 Protein-protein Interaction Model of RAC-1-RPA and 70B-RPA

First, there might be a RPA pool in the cell. Epitopes for both RAC-1 and 70B are presented on the surface of RPA. In a certain cellular environment, RPA interacts with different proteins, and the protein-RPA interactions may mask some epitopes on RPA. For instance, DNA polymerases δ and ε interact with the N terminus of p70, covering 70B-recognising sites, but the epitopes for RAC-1 are unoccupied and available for RAC-1 binding. The phosphorylated p32 of 70B-RPA binds to the C terminus of p70, blocking the RAC-1 recognition sites and therefore this subpopulation of RPA is only

detected by 70B (Figure 4.1). This model explains the co-immunoprecipitation of DNA polymerases δ and ε with RAC-1-RPA and of phosphorylated p32 with 70B-RPA.

It has been reported from *in vitro* experiments that DNA polymerase α-primase binds to the N terminus of p70. According to this protein-protein interaction model, however, this binding might not affect the epitopes for 70B, because DNA polymerase α can still bind to and be co-isolated with 70B-RPA. p53 is reported to have two interaction regions with p70 (Iftode *et al.,* 1999), one is round 1-200 amino acid residues at the N terminus of p70. This binding does not influence the binding of 70B to p70, therefore a large amount of p53 co-precipitated with 70B. The other binding region is at 400-500 amino acid residues at the C terminus of p70 and partially overlaps the residues recognised by RAC-1-RPA, and therefore only a smaller amount of p53 co-precipitates with RAC-1.

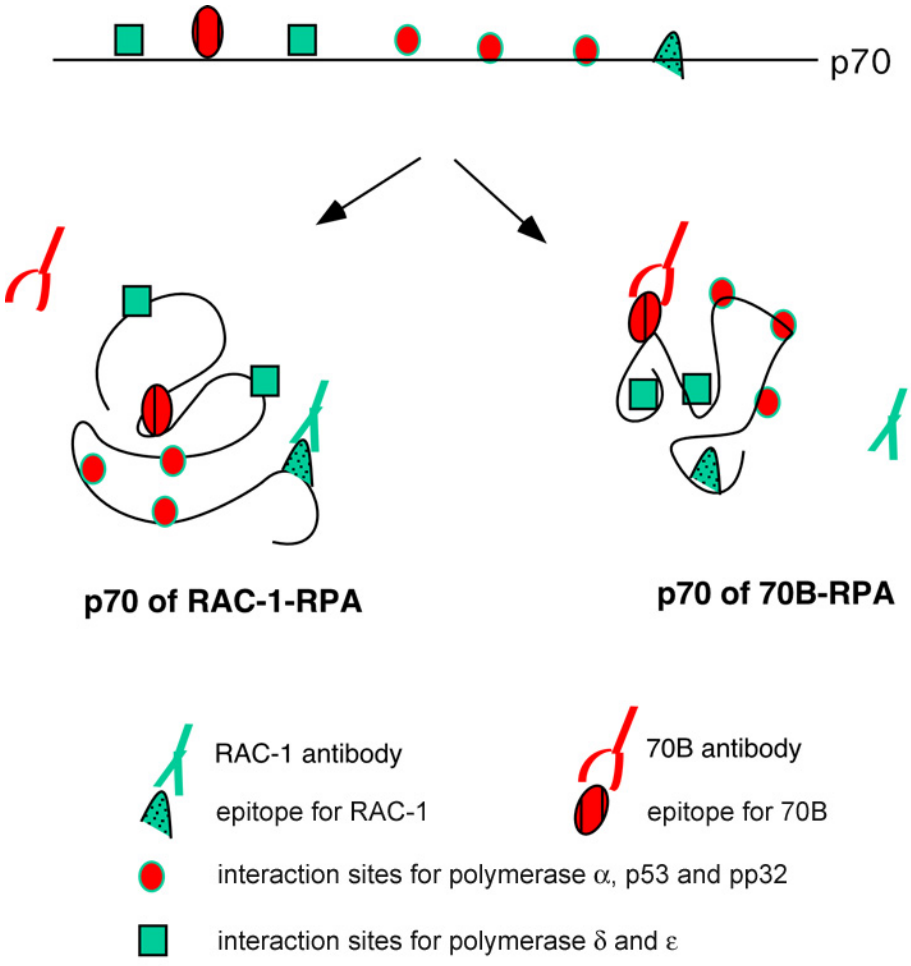


Figure 4.2 Structural Model of p70 in RAC-1-RPA and 70B-RPA

In an alternative model, it could be speculated that the RPA trimer, and in particular the p70 subunit exists in different structures in human cells. As the peptides used for

generating the antibodies RAC-1 and 70B are from the same p70 primary sequence, it can be argued that their differences in recognition patterns is defined by variation of the tertiary structure of p70 (Figure 4.2). These structural differences originally determine which epitopes and interaction sites for specific proteins are available in each subpopulation. According to this hypothesis, DNA polymerases δ and ε do not form a complex with 70B-RPA, because the regions, which are necessary for the binding of these two polymerases to p70, are buried inside this conformation. Similarly, such a conformational difference might be the reason that phosphorylated p32 preferentially associateds with 70B-RPA rather than with RAC-1-RPA.

4.7 The Phosphorylation of p68 is Cell Cycle-dependent

Genetic and biochemical experiments showed that cellular Cdks have positive and negative influences on cellular DNA replication (Dutta and Bell, 1997; Johnston *et al*, 1999; Lee and Bell, 2000; Takisawa *et al*, 2000). The cell-free initiation system of SV40 DNA replication showed that four conserved Cdk phosphorylation sites (S141, S147, S152 and T156) within the T antigen binding site of p68 (aa 1-239) are required to stimulate and inhibit the initiation of DNA replication, where Cdks and PP2A mediate these modifications (Dehde *et al.,* 2001; Schub *et al*., 2001; Voitenleitner *et al*., 1997; 1999).

As the phosphorylation of DNA polymerase α-primase controls DNA replication, it is a central element in the regulation of the cell cycle. The phosphorylation-specific antibody 858-PP1 can discriminate the phosphorylated and unphosphorylated forms of p68 (Figure 3.17). Although the cellular level of p180 and p68 is constant, the phosphorylation of peptide P1 in p68 changes in a cell cycle-dependent manner. The peptide P1 is phosphorylated early in the cell cycle just at the transition from G_1 to S phase (Figures 3.19, 3.20, and 3.21). These findings are in line with results from *S. cerevisiae* that the B subunit of DNA polymerase α–primase exbihits a phosphorylation-dependent shift early in S phase (Foaini *et al*., 1999). In addition, in a cell-free system using nuclei and extracts from cells which are synchronised in different phases of the cell cycle, the addition of the purified kinases Cyclin A-Cdk2 and Cyclin E-Cdk2, which are responsible for the phosphorylation of p68 *in vitro,* stimulated DNA replication in G_1 nuclei (Krude *et al.,* 1997; Voitenleitner *et al.,* 1999).

However, these phosphorylation data are in contrast to an earlier report (Nasheuer *et al*., 1991) that p68 is predominately phosphorylated in G_2/M phase. The reason for the apparent contradition probably lies in the different experimental procedures. The

experiments carried out by Nasheuer *et al* (1991) are based on radioactive labelling which requires turnover of phosphate and are tested with immunoprecipitation method which takes longer than western blotting. Furthermore, the labelling with radioactive phosphate represents total phosphorylation of p68, but 858-PP1 only recognises two (S141 and S147) of the phopshorylated sites on p68. It is presumed that the S141 and S147 sites are probably phosphorylated in S phase and can be rapidly dephosphorylated *in vitro*. Therefore, this phosphorylation cannot be detected after immunoprecipitation is performed. The other Cdk phosphorylation sites, however, might be stably phosphorylated in M phase and can be detected by radioactive phosphate labelling, but not by 858-PP1. Thus 858-PP1 does not reveal phosphorylated p68 in M phase, but *in vivo* labelling of phosphorylated peptide does.

4.8 The Phosphorylation of p68 is Downregulated after DNA Damage

The phosphorylation of p68 is prevented while DNA is damaged. The discovery that the phosphorylation level of p68 in irradiated cells is lower than in unstressed cells (Figure 3.22) is line with other results from yeast (Pellicioli *et al.,* 1999). In *S. cerevisiae*, the phosphorylation of B subunit (p68 in yeast) was prevented by Rad 53, an essential protein kinase required for cell cycle arrest. Rad 53 controls B subunit phosphorylation in response to genotoxic treatment by negatively regulating the corresponding kinases. These data, together with the observation presented here would support an assumption that negative regulation of p68 phosphorylation is not an initial element but a consequence of cell cycle arrest at DNA damage checkpoints.

5 Summary

The replication protein A (RPA) specifically binds to single-stranded DNA and consists of three subunits p70, p32, and p14. RPA has multiple functions in the cellular DNA metabolism. To study its role in DNA replication and DNA repair of human cells a new monoclonal antibody RAC-1 recognising the C terminus of p70 was compared with the monoclonal antibody 70B which recognises the N terminus of p70 (Kenny *et al.,* 1990). By using these two antibodies evidence is presented that at least two immunologically distinct subpopulations of RPA exist in human cells, RAC-1-RPA and 70B-RPA. They differ in their immunostaining patterns, in their phosphorylation states, in their cellular distribution, in their colocalisation with BrdU, and in their induction after DNA damage. RAC-1-RPA interacts with DNA polymerases δ and ε whereas 70B-RPA preferentially binds to DNA polymerase α. These findings suggest that 70B-RPA has a function during the initiation of DNA replication whereas RAC-1-RPA plays other roles in the elongation stage of DNA replication and in DNA repair.

DNA polymerase α-primase is the only DNA polymerase which can synthesise DNA *de novo*. Its p68 subunit has no enzymatic functions but is involved in the control of the initiation of DNA replication. Using an antibody specific for phosphorylated p68, it was shown in this thesis that the phosphorylation of p68 is cell cycle-dependent. Furthermore, it was shown that the level of phosphorylated p68 but not that of total p68 is downregulated after DNA damage.

6 Zusammenfassung

Das Replikationsprotein A (RPA) bindet spezifisch an einzelsträngige DNA und besteht aus den drei Untereinheiten p70, p32 und p14. RPA hat vielfältige Funktionen im DNA-Haushalt der Zelle. Um die Rolle von RPA in der DNA-Replikation und der DNA-Reparatur menschlicher Zellen zu untersuchen, wurde ein neuer monoklonaler Antikörper RAC-1 gegen den C-Terminus von RPA hergestellt, und dessen Eigenschaften mit denen des Antikörpers 70B verglichen, der den N-Terminus von RPA erkennt. Durch den Vergleich dieser beiden Antikörper konnte gezeigt werden, dass in menschlichen Zellen mindestens zwei immunologisch unterschiedliche Subpopulationen von RPA existieren (RAC-1-RPA und 70B-RPA). Die Subpopulationen unterscheiden sich in ihrem spezifischen Färbemuster, in ihrem Phosphorylierungszustand, in ihrer Kolokalisierung mit BrdU und in ihrer Induktion nach DNA-Schäden. RAC-1-RPA interagiert mit DNA-Polymerase δ and ε, während 70B-RPA vorzugsweise an DNA-Polymerase α bindet. Diese Ergebnisse deuten darauf hin, dass 70B-RPA eine Rolle während der Initiation der DNA-Replikation spielt und RAC-1-RPA eine andere Rolle im Elongationsstadium der DNA-Replikation und bei der DNA-Reparatur spielt.

DNA-Polymerase-α-Primase ist die einzige DNA-Polymerase, die die *de novo* DNA-Synthese initiieren kann. Die p68-Untereinheit der DNA-Polymerase-α-Primase besitzt selbst keine enzymatische Funktion, sondern ist für die Kontrolle der Initiationsschritte der DNA-Replikation verantwortlich. Mittels eines phosphopeptidespezifischen Antikörpers für p68, 858-PP1, wird in der vorliegenden Arbeit gezeigt, dass die Phosphorylierung von p68 abhängig vom Zellzyklus ist. Außerdem konnte gezeigt werden, dass nach DNA-Schäden der Phosporylierungsgrad von p68 bei gleichbleibender Gesamtmenge abnimmt.

7 References

Aboussekhra, A., Biggerstaff, M., Shivji, M.K., Vilpo, J.A., Moncollin, V., Protic, M., Hübscher, U., Egly, J.M. and Wood, R.D. (1995). Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell 80: 859-68.

Adachi, Y. and Laemmli, U.K. (1992). Identification of nuclear pre-replication centers poised for DNA synthesis in *Xenopus* egg extracts: Immunolocalization study of replication protein A. J. Cell Biol. 119: 1-15.

Adachi Y. and Laemmli U.K. (1994). Study of the cell cycle-dependent assembly of the DNA prereplication centres in *Xenopus* egg extracts. EMBO J. 13:4153-64.

Atrazhev, A., Zhang, S. and Grosse, F. (1992). Single-stranded DNA binding protein from calf-thymus: purification, properties and stimulation of the homologous DNA polymerase alpha-primase complex. Eur. J. Biochem. 210: 855-65.

Bastin-Shanower, S. A. and Brill, S. J. (2001). Functional analysis of the four DNA binding domains of replication protein A. The role of RPA2 in ssDNA binding. J. Biol. Chem. 276(39): 36446-53.

Baumann, P. and West, S. C. (1997). The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A. EMBO J. 16: 5198-206.

Bischof, O., Kim. S.H., Irving J., Beresten S., Ellis, N.A., Campisi J. (2001). Regulation and localization of the Bloom syndrome protein in the response to DNA damage. J Cell Biol. 153:367-80.

Blackwell, L.J. and Borowiec, J.A. (1994). Human replication protein A binds single-stranded DNA in two distinct complexes. Mol. Cell. Biol. 14: 3993-4001.

Bochkareva E, Korolev S, Bochkarev A. (2000). The role for zinc in replication protein A. J Biol Chem. 275(35):27332-8.

Bochkareva, E., Korolev, S., Lees-Miller, S. P., and Bochkarev, A. (2002). Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA. EMBO J. 21(7): 1855-63.

Bouvier, D., De Recondo, A. M., and Baldacci, G. (1993). In vivo phosphorylation, mitotic behavior, and nuclear binding of the catalytic subunit of DNA polymerase alpha in fission yeast. Exp. Cell Res. 207(1): 41-7.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248-54.

Brill S.J. and Bastin-Shanower S. (1998). Identification and characterization of the fourth single-stranded-DNA binding domain of replication protein A. Mol Cell Biol. 18(12).: 7225-34.

Brill, S.J. and Stillman, B. (1989). Yeast replication factor-A functions in the unwinding of the SV40 origin of DNA replication. Nature 342: 92-5.

Brown, G.W., Melendy, T. and Ray, D.S. (1992). Conservation of structure and function of DNA replication protein A in trypanosomatid *Crithidia fasciculata*. Proc. Natl. Acad. Sci. USA 89: 10227-31.

Brush G.S., Clifford D.M., Marimco S.M. and Bartrand A.J. (2001). Replication protein A is sequentially phosphorylated during meiosis. Nucleic Acids Res. 29 (23): 4808-17.

Brush G.S., Kelly T.J. and Stillman B. (1995). Identification of eukaryotic DNA replication proteins using simian virus 40 *in vitro* replication system. Methods Enzymol. 262: 522-48.

Brush, G.S. and Kelly, T.J. (2000). Phosphorylation of the replication protein A large subunit in the *Saccharomyces cerevisiae* checkpoint response. Nucleic Acids Res. 28(19): 3725-32.

Brush, G.S., Sonderson, C.W. and Kelly, T.J. (1994). The DNA-activated protein kinase is required for the phosphorylation of replication protein A during simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA 91: 12520-4.

Burgers, P. M. (1998). Eukaryotic DNA polymerases in DNA replication and DNA repair. Chromosoma 107(4): 218-27.

Cardoso, M.C., Leonhardt, H. and Nadal-Ginard, B. (1993). Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. Cell. 74(6): 979-92.

Carty, M.P., Hauser, J., Levine, A.S. and Dixon, K. (1993). Replication and mutagenesis of UV-damaged DNA templates in human and monkey cell extracts. Mol. Cell. Biol. 13: 5333-542.

Carty, M.P., Levine, A.S. and Dixon, K. (1992). HeLa cell single-stranded DNA binding protein increases the accuracy of DNA synthesis by polymerase alpha *in vitro*. Mut. Res. 274: 29-43.

Carty, M.P., Zernik-Kobak, M., McGrath, S. and Dixon, K. (1994). UV light-induced DNA synthesis arrest in HeLa cells is associated with changes in phosphorylation of human single-stranded DNA-binding protein.EMBO J. 13: 2114-23.

Challberg, M. D. and Kelly, T. J. (1989). Animal virus DNA replication. Annu. Rev. Biochem. 58:671-717.

Collins, K. L., Russo, A. A., Tseng, B. Y., and Kelly, T. J. (1993). The role of the 70 kDa subunit of human DNA polymerase alpha in DNA replication. EMBO J. 12(12): 4555-66.

Collins, K.L. and Kelly, T.J. (1991). The effects of T antigen and replication protein A on the initiation of DNA synthesis by DNA polymerase alpha-primase. Mol. Cell. Biol. 11: 2108-15.

Copeland, W. C. and Wang, T. S. (1993). Enzymatic characterization of the individual mammalian primase subunits reveals a biphasic mechanism for initiation of DNA replication. J. Biol. Chem. 268(35): 26179-89.

Coverly, D., Kenny, M.K., Lane, D.P. and Wood, R.D. (1992). A role for human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair. Nucl. Acid. Res. 20: 3873-80.

Coverly, D., Kenny, M.K., Munn, M., Rupp, W.D., Lane, D.P. and Wood, R.D. (1991). Requirement for the replication protein SSB in human DNA excision repair. Nature 349: 538-41.

Daniely, Y. and Borowiec, J. A. (2000). Formation of a complex between nucleolin and replication protein A after cell stress prevents initiation of DNA replication. J. Cell Biol. 149(4): 799-810.

Dehde, S., Rohaly, G., Schub, O., Nasheuer, H.P., Bohn, W., Chemnitz, J., Deppert, W. and Dornreiter, I. (2001). Two immunologically distinct human DNA polymerase alpha-primase subpopulations are involved in cellular DNA replication. Mol. Cell. Biol. 21(7): 2581-93.

DeMott, M. S., Zigman, S. and Bambara, R. A. (1998). Replication protein A stimulates long patch DNA base excision repair. J. Biol. Chem. 273: 27492-8.

Dianov, G.L., Jensen, B.R., Kenny, M.K. and Bohr, V.A. (1999). Replication protein A stimulates proliferating cell nuclear antigen-dependent repair of abasic sites in DNA by human cell extracts. Biochemistry. 38(34): 11021-5.

Dimitrova, D.S. and Gilbert, D.M. (2000). Stability and nuclear distribution of mammalian replication protein A heterotrimeric complex Exp. Cell. Res. 254 (2): 321-7.

Din, S.-U., Brill, S.J., Fairman, M.P. and Stillman, B. (1990). Cell cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. Genes Dev. 4: 968-77.

Donaldson, A.D. and Blow, J.J. (1999). The regulation of replication origin activation. Curr Opin Genet Dev. 9(1):62-8.

Dornreiter, I., Copeland, W.C. and Wang S.-F. (1993). Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase alpha with large T antigen. Mol. Cell. Biol. 13: 809-20.

Dornreiter, I., Erdile, L.F., Gilbert, I.U., von Wikler, D., Kelly, T.J. and Fanning, E. (1992). Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen. EMBO J. 11: 769-76.

Dornreiter, I., Höss, A., Arthur, A.K. and Fanning, E. (1990). SV40 T antigen binds directly to the large subunit of DNA polymerase alpha. EMBO J. 9: 3329-36.

Dutta, A. and Bell, S. P. (1997). Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell Dev. Biol. 13:293-332

Dutta, A. and Stillman, B. (1992). Cdc2 family kinases phosphorylate human cell DNA replication factor, RPA and activate DNA replication. EMBO J. 11: 2189-99.

Dutta, A., Ruppert, J.M., Aster, J.C. and Winchester, E. (1993). Inhibition of DNA replication factor RPA by p53. Nature 365: 79-82.

Erdile, L.F., Heyer, W.-D., Kolodner, R. and Kelly, T.J. (1991). Characterization of a cDNA encoding the 70-kDa single-stranded DNA-binding subunit of human replication protein A and the role of the protein in DNA replication. J. Biol. Chem. 266: 12090-6.

Evans, E., Moggs, J. G., Hwang, J. R., Egly, J. M., and Wood, R. D. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J. 16(21): 6559-73.

Fairman, M.P. and Stillman, B. (1988). Cellular factors required for multiple stages of SV40 replication *in vitro*. EMBO J. 7: 1211-8.

Foiani, M., Ferrari, M., Liberi, G., Lopes, M., Lucca, C., Marini, F., Pellicioli, A., Falconi, M. M., and Plevani, P. (1998). S-phase DNA damage checkpoint in budding yeast. Biol. Chem. 379(8-9): 1019-23.

Foiani, M., Liberi, G., Lucchini, G., and Plevani, P. (1995). Cell cycle-dependent phosphorylation and dephosphorylation of the yeast DNA polymerase alpha-primase B subunit. Mol. Cell Biol. 15(2): 883-91.

Foiani, M., Lucchini, G., and Plevani, P. (1997). The DNA polymerase alpha-primase complex couples DNA replication, cell-cycle progression and DNA-damage response. Trends Biochem. Sci. 22(11): 424-7.

Foiani, M., Marini, F., Gamba, D., Lucchini, G., and Plevani, P. (1994). The B subunit of the DNA polymerase alpha-primase complex in Saccharomyces cerevisiae executes an essential function at the initial stage of DNA replication. Mol. Cell Biol. 14(2): 923-33.

Fotedar, R. and Roberts, J.M. (1992). Cell cycle regulated phosphorylation of RPA-32 occurs within the initiation complex. EMBO J. 11(6): 2177-87.

Gannon, J.V. and Lane, D.P. (1987). p53 and DNA polymerase alpha compete for binding to SV40 T antigen. Nature 329: 456-8.

Georgaki, A., Tuteja, N., Sturzenegger, B. and Hübscher, U. (1994). Calf thymus DNA helicase F, a replication protein A copurifying enzyme. Nucl. Acid. Res. 22: 1128-34.

Golub, E. I., Gupta, R. C., Haaf, T., Wold, M. S., and Radding, C. M. (1998). Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. Nucleic Acids Res. 26(23): 5388-93.

Gomes, X. V. and Wold, M. S. (1995). Structural analysis of human replication protein A. Mapping functional domains of the 70-kDa subunit. J. Biol. Chem. 270(9): 4534-43.

Gomes, X. V. and Wold, M. S. (1996). Functional domains of the 70-kilodalton subunit of human replication protein A. Biochemistry 35(32): 10558-68.

Gomes, X.V., Henricksen, L.A. and Wold, M.S. (1996). Proteolytic mapping of human replication protein A: Evidence for multiple structural domains and a conformational change upon interaction with single-stranded DNA. Biochemistry 35: 5586-95.

Gottlieb M. and Chavko, M. (1987). Silver staining of native and denatured eucaryotic DNA in agarose gels. Anal Biochem. 165(1):33-7.

Haracska, L., Johnson, R.E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L. and Prakash, S. (2001). Physical and functional interactions of human DNA polymerase eta with PCNA. Mol. Cell Biol. 21 (21): 7199-206.

Haracska, L., Kondratick, C. M., Unk, I., Prakash, S., and Prakash, L. (2001). Interaction with PCNA is essential for yeast DNA polymerase eta function. Mol. Cell 8(2): 407-15.

Haracska, L., Unk, I., Johnson, R. E., Johansson, E., Burgers, P. M., Prakash, S., and Prakash, L. (2001). Roles of yeast DNA polymerases delta and zeta and of Rev1 in the bypass of abasic sites. Genes Dev. 15(8): 945-54.

Haracska, L., Johnson, R.E., Unk, I., Phillips, B., Hurwitz, J. Prakash, L., and Prakash, S. (2001). Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA. Proc. Natl. Acad. Sci. USA, 98(25): 14256-61.

He, Z., Brinton, B.T., Greenblatt, J., Hassell, J.A. and Ingles, C.J. (1993). The transactivator proteins VP16 and GAL4 bind replication factor A. Cell 73: 1223-32.

He, Z., Henricksen, L.A., Wold, M.S. and Ingles, C.J. (1995). RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. Nature 374: 566-9.

Henricksen, L.A. and Wold, M.S. (1994). Replication protein A mutants lacking phosphorylation sites for p34^{cdc2} kinase support DNA replication. J. Biol. Chem. 269: 24203-8.

Henricksen, L.A., Carter, T., Dutta, A. and Wold, M.S. (1996). Phosphorylation of human replication protein A by the DNA-dependent protein kinase is involved in the modulation of DNA replication. Nucl. Acid. Res. 24: 3107-12.

Hey, T., Lipps, G. and Krauss, G. (2001). Binding of XPA and RPA to damaged DNA investigated by fluorescence anisotropy. Biochemistry 40(9): 2901-10.

Heyer, W.-D., Rao, M.R.S., Erdile, L.F., Kelly, T.J. and Kolodner, R. (1990). An essential *Saccharomyces cervisiae* single stranded DNA binding protein is homologous to the large subunit of human RP-A. EMBO J. 9: 2321-9.

Hübscher, U., Nasheuer, H.P. and Syvaoja, J.E. (2000). Eukaryotic DNA polymerases, a growing family. Trends Biochem. Sci. 25(3): 143-7.

Hurwitz, J., Dean, F. B., Kwong, A. D., and Lee, S. H. (1990). The in vitro replication of DNA containing the SV40 origin. J. Biol. Chem. 265(30): 18043-6.

Iftode, C., Daniely, Y., and Borowiec, J. A. (1999). Replication protein A (RPA): the eukaryotic SSB. Crit Rev. Biochem. Mol. Biol. 34(3): 141-80.

Ishimi Y, Komamura-Kohno Y, You Z, Omori A, Kitagawa M (2000). Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2. J Biol Chem.26;275(21):16235-41

Ishimi Y. (1997) A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J. Biol. Chem. 26;272(39):24508-13.

Johnson, R. E., Washington, M. T., Prakash, S., and Prakash, L. (2000). Fidelity of human DNA polymerase eta. J. Biol. Chem. 275(11): 7447-50.

Johnston, L. H., Masai, H., and Sugino, A. (1999). First the CDKs, now the DDKs. Trends Cell Biol. 9(7): 249-52.

Kelly, T. J. (1988). SV40 DNA replication. J. Biol. Chem. 263(34): 17889-92.

Kenny, M.K., Lee, S.H. and Hurwitz, J. (1989). Multiple functions of human single-stranded DNA binding protein in Simian virus 40 DNA replication: single-strand stabilization and stimulation of DNA polymerase alpha and delta. Proc. Natl. Acad. Sci. (USA) 86: 9757-61.

Kenny, M.K., Schlegel, U., Furneaux, H. and Hurwitz, J. (1990). The role of human single-stranded DNA binding protein and its individual subunits in Simian virus 40 DNA replication. J. Biol. Chem. 265 (13): 7693-700.

Kim, C. and Wold, M.S. (1995). Recombinant human replication protein A binds to polynucleotides with low cooperativity. Biochemistry 34: 2058-64.

Kim, C., Paulus, B.F. and Wold, M.S. (1994). Interactions of human replication protein A with oligonucleotides. Biochemistry 33: 14197-206.

Kim, D.-K., Stigger, E. and Lee, S.-H. (1996). Role of the 70-kDa Subunit of human Replication protein A (I): Single-stranded DNA binding activity, but not polymerase stimulatory activity, is required for DNA replication. J. Biol. Chem. 25: 15124-9.

Krude, T., Jackman, M., Pines, J. and Laskey, R.A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. Cell 88(1): 109-19.

Kuhn, C., Muller, F., Melle, C., Nasheuer, H.P., Janus, F., Deppert, W. and Grosse, F. (1999). Surface plasmon resonance measurements reveal stable complex formation between p53 and DNA polymerase alpha. Oncogene 18(3): 769-74.

Kunkel, T. A., Roberts, J. D., and Sugino, A. (1991). The fidelity of DNA synthesis by the catalytic subunit of yeast DNA polymerase alpha alone and with accessory proteins. Mutat. Res. 250(1-2): 175-82.

Lavrik, O. I., Kolpashchikov, D. M., Nasheuer, H. P., Weisshart, K., and Favre, A. (1998). Alternative conformations of human replication protein A are detected by crosslinks with primers carrying a photoreactive group at the 3'-end. FEBS Lett. 441(2): 186-90.

Lavrik, O. I., Kolpashchikov, D. M., Weisshart, K., Nasheuer, H. P., Khodyreva, S. N., and Favre, A. (1999). RPA subunit arrangement near the 3'-end of the primer is modulated by the length of the template strand and cooperative protein interactions. Nucleic Acids Res. 27(21): 4235-40.

Lee, D. G. and Bell, S. P. (2000). ATPase switches controlling DNA replication initiation. Curr. Opin. Cell Biol. 12(3): 280-5.

Lee, S. H. and Hurwitz, J. (1990). Mechanism of elongation of primed DNA by DNA polymerase delta, proliferating cell nuclear antigen, and activator 1. Proc. Natl. Acad. Sci. U. S. A 87(15): 5672-6.

Lee, S. H. and Kim, D. K. (1995). The role of the 34-kDa subunit of human replication protein A in simian virus 40 DNA replication in vitro. J. Biol. Chem. 270(21): 12801-7.

Lee, S.-H., Eki, T. and Hurwitz, J. (1989). Synthesis of DNA containing the simian virus 40 origin of replication by the combined action of DNA polymerases alpha and delta. Proc. Natl. Acad. Sci. USA 86: 7361-5.

Lee, S.-H., Pan, Z.-Q., Kwong, A.D., Burgers, P.M.J. and Hurwitz, J. (1991). Synthesis of DNA by DNA polymerase epsilon *in vitro*. J. Biol. Chem. 266: 22707-17.

Lewin B. (2000) Gene VII Oxford University Press p867

Li, J.J. and Kelly, T.J. (1985). Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. Mol Cell Biol. 5(6):1238-46.

Li, L., Lu, X., Peterson, C.A. and Legerski, R.J. (1995). An interaction between the DNA repair factor XPA and replication protein A appears essential for nucleotide excision repair. Mol. Cell. Biol. 15: 5396-402.

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

Lindahl P.E. (1986). One counterstreaming centrifugation in the separation of cells and cell fragments. Biochem. Biophys. Acta. 21: 411-415

Liu, L., Mo, J., Rodriguez-Belmonte, E. M., and Lee, M. Y. (2000). Identification of a fourth subunit of mammalian DNA polymerase delta. J. Biol. Chem. 275(25): 18739-44.

Liu, V.F. and Weaver, D.T. (1993). The ionizing radiation induced replication protein A phosphorylation response differs between ataxia telangiectasia and normal human cells. Mol. Cell. Biol. 13: 7222-31.

Longhese, M. P., Jovine, L., Plevani, P., and Lucchini, G. (1993). Conditional mutations in the yeast DNA primase genes affect different aspects of DNA metabolism and interactions in the DNA polymerase alpha-primase complex. Genetics 133(2): 183-91.

Longhese, M.P., Necke, H., Paciotti, V., Lucchini, G. and Plevani, P. (1996). The 70 kDa subunit of replication protein A is required for the G1/S and intra-S DNA damage checkpoints in budding yeast. Nucl. Acid. Res. 24: 3533-3537.

Longhese, M.P., Plevani, P. and Lucchini, G. (1994). Replication factor A is required *in vivo* for DNA replication, repair, and recombination. Mol. Cell. Biol. 14: 7884-90.

Loo, Y.M. and Melendy, T. (2000). The majority of human replication protein A remains complexed throughout the cell cycle. Nucleic Acids Res. 28 (17): 3354-60.

Madine M, Laskey R. (2001) Geminin bans replication licence. Nat Cell Biol. 3(2):E49-50

Maga, G., Frouin, I., Spadari, S. and Hübscher, U. (2001). Replication protein A as a „fidelity clamp“ for DNA polymerase alpha. J. Bio. Chem. 276 (21): 18235-42.

Mass, G., Nethanel, T., and Kaufmann, G. (1998). The middle subunit of replication protein A contacts growing RNA-DNA primers in replicating simian virus 40 chromosomes. Mol. Cell Biol. 18(11): 6399-407.

Matsuda, T., Saijo, M., Kuraoka, I., Kobayashi, T., Nakatsu, Y., Nagai, A., Enjoji, T., Masutani, C., Sugasawa, K., Hanaoka, F., Yasui, A. and Tanaka, K. (1995). DNA repair protein XPA binds replication protein A (RPA). J. Biol. Chem. 270: 4152-7.

Matsumoto, T., Eki, T. and Hurwitz, J. (1990). Studies on the initiation and elongation reactions in the simian virus 40 DNA replication system. Proc. Natl. Acad. Sci. USA 87: 9712-6.

Matsunaga, T., Park, C. H., Bessho, T., Mu, D., and Sancar, A. (1996). Replication protein A confers structure-specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA repair excision nuclease. J. Biol. Chem. 271(19): 11047-50.

McGhee, J.D. and von Hippel, P.H. (1975). Formaldehyde as a probe of DNA structure. II. Reaction with endocyclic imino groups of DNA bases. Biochemistry 14(6):1297-303.

Melendy, T. and Stillman, B. (1993). An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. J. Biol. Chem. 268: 3389-95.

Meller, C. and Nasheuer, H.P. (2002). Physical and functional interactions of the tumor suppressor protein p53 and DNA polymerase alpha-primase. Nucleic Acids Res 30(7): 1493-9.

Mer, G., Bochkarev, A., Gupta, R., Bochkareva, E., Frappier, L., Ingles, C.J., Edwards, A.M. and Chazin, W.J. (2000). Structural basis for the recognition of DNA repair proteins UNG2, XPA, and RAD52 by replication factor RPA. Cell 103 (3): 449-56.

Mimura S, Takisawa H(1998) Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk.EMBO J;17(19):5699-707.

Mitsis, P.G., Kowalczykowski, S.C. and Lehman, I.R. (1993). A single-stranded DNA binding protein from Drosophila melanogaster: characterization of the heterotrimeric protein and its interaction with single-stranded DNA. Biochemistry 32: 5257-66.

Mizuno, T., Yamagishi, K., Miyazawa, H. and Hanaoka, F., (1999) Molecular architecture of the mouse DNA polymerase alpha-primase complex. Mol. Cell. Biol. (11):7886-96.

Moore, S.P., Erdile, L., Kelly, T.J. and Fishel, R. (1991). The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A. Proc. Natl. Acad. Sci. USA 88: 9067-71.

Mortensen, U. H., Bendixen, C., Sunjevaric, I., and Rothstein, R. (1996). DNA strand annealing is promoted by the yeast Rad52 protein. Proc. Natl. Acad. Sci. U. S. A 93(20): 10729-34.

Muramaki, Y. and Hurwitz, J. (1993). Functional interactions between SV 40 T antigen and other replication proteins at the replication fork. J. Biol. Chem. 268: 11008-17.

Murti, K.G., He, D.C., Brinkley, B.R., Scott, R. and Lee, S.H. (1996). Dynamics of human replication protein A subunit distribution and partitioning in the cell cycle. Exp. Cell. Res. 223(2): 279-89.

Nagelhus, T. A., Haug, T., Singh, K. K., Keshav, K. F., Skorpen, F., Otterlei, M., Bharati, S., Lindmo, T., Benichou, S., Benarous, R., and Krokan, H. E. (1997). A sequence in the N-terminal region of human uracil-DNA glycosylase with homology to XPA interacts with the C-terminal part of the 34-kDa subunit of replication protein A. J. Biol. Chem. 272(10): 6561-6.

Naktinis, V., Turner, J., and O'Donnell, M. (1996). A molecular switch in a replication machine defined by an internal competition for protein rings. Cell 84(1): 137-45.

Nasheuer, H. P. and Grosse, F. (1988). DNA polymerase alpha-primase from calf thymus. Determination of the polypeptide responsible for primase activity. J. Biol. Chem. 263(18): 8981-8.

Nasheuer, H.P., Moore, A., Wahl, A.F. and Wang, T.S. (1991). Cell cycle-dependent phosphorylation of human DNA polymerase alpha. J. Biol. Chem. 266(12): 7893-903.

Nasheuer, H.P., Smith, R., Bauerschmidt, C., Grosse, F. and Weisshart, K. (2002). Initiation of Eukaryotic DNA Replication: Regulation and Mechanisms Progress in Nucleic Acid Research and Molecular Biology, 27: 41-83.

Nasmyth, K. (1996). Viewpoint: putting the cell cycle in order. Science 274(5293): 1643-5.

Opresko, P.L., Laine, J.P., Brosh, R.M., Seidman, M.M. and Bohr, V.A. (2001). Coordinate action of the helicase and 3' to 5' exonuclease of Werner syndrome protein. J. Biol. Chem. 276(48): 44677-87.

Orlando, V. (2000). Mapping chromosomal proteins *in vivo* by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem. Sci. 25(3): 99-104.

Orlando, V., Strutt, H. and Paro, R. (1997). Analysis of chromatin structure by in vivo formaldehyde cross-linking. Methods (2):205-14.

Otterlei, M., Warbrick, E., Nagelhus, T.A., Haug, T., Slupphaug, G., Akbari, M., Aas, P.A., Steinsbekk, K., Bakke, O. and Krokan H.E. (1999). Post-replication base excision repair in replication foci. EMBO J. 18(13): 3834-44.

Pan, Z.-Q., Amin, A.A., Gibbs, E., Niu, H. and Hurwitz, J. (1994). Phosphorylation of the p34 subunit of human single-stranded-DNA-binding protein in cyclinA-activated G_1 extracts is catalyzed by cdk-cyclin A complex and DNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA 91: 8343-7.

Park, H., Davis, R., and Wang, T. S. (1995). Studies of Schizosaccharomyces pombe DNA polymerase alpha at different stages of the cell cycle. Nucleic Acids Res. 23(21): 4337-44.

Pearson, B.E., Nasheuer, H.P., Wang, T.S. (1991). Human DNA polymerase alpha gene: sequences controlling expression in cycling and serum-stimulated cells. Mol. Cell. Biol. 11(4): 2081-95.

Pellicioli, A., Lucca, C., Liberi, G., Marini, F., Lopes, M., Plevani, P., Romano, A., Di Fiore, P.P. and Foiani, M. (1999). Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J. 18(22):6561-72.

Pellicioli, A., Lucca, C., Liberi, G., Marini, F., Lopes, M., Plevani, P., Romano, A., Di Fiore, P.P. and Foiani, M. (1999). Activation of Rad 53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J, 18(22): 6561-72.

Perrault, R., Cheong, N., Wang, H., Wang, H. and Iliakis, G. (2001). RPA facilitates rejoining of DNA double-strand breaks in an *in vitro* assay utilizing genomic DNA as substrate. Int. J. Radiat. Biol. 77(5): 593-607.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J. and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. EMBO J. 18(2):396-410.

Philipova, D., Mullen, J.R., Maniar, H.S., Lu, J., Gu, C. and Brill, S.J. (1996). A hierarchy of SSB protomers in replication protein A. Genes & Dev. 10: 2222-33.

Pines, J. (1999). Four-dimensional control of the cell cycle. Nat. Cell Biol. (3):E73-9.

Ranalli, T.A., DeMott, M.S. and Bambara, R.A. (2002). Mechanism underlying replication protein a stimulation of DNA ligase I. J. Biol. Chem. 277(3): 1719-27.

Roberts, J.D., Nguyen, D. and Kunkel, T.A. (1993). Frameshift fidelity during replication of double-stranded DNA in HeLa cell extracts. Biochemistry 32: 4083-9.

Saha P, Chen J, Thome KC, Lawlis SJ, Hou ZH, Hendricks M, Parvin JD, Dutta A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase.Mol Cell Biol. 18(5):2758-67.

Saijo, M., Kuraoka, I., Masutani, C., Hanaoka, F., and Tanaka, K. (1996). Sequential binding of DNA repair proteins RPA and ERCC1 to XPA in vitro. Nucleic Acids Res. 24(23): 4719-24.

Sakamoto, S., Nishikawa, K., Heo, S.J., Goto, M., Furuichi, Y. and Shimamoto, A. (2001). Werner helicase relocates into nuclear foci in response to DNA damaging agents and co-localizes with RPA and Rad51. Genes Cells 6 (5): 421-30.

Santocanale, C., Foiani, M., Lucchini, G., and Plevani, P. (1993). The isolated 48,000-dalton subunit of yeast DNA primase is sufficient for RNA primer synthesis. J. Biol. Chem. 268(2): 1343-8.

Santocanale, C., Neecke, H., Longhese, M.P., Lucchini, G. and Plevani, P. (1995). Mutations in the gene encoding the 34 kDa subunit of yeast replication protein A cause defective S phase progression. J. Mol. Biol. 254: 595-607.

Sanz, M.M., Proytcheva, M., Ellis, N.A., Holloman, W.K. and German, J. (2000). BLM, the Bloom's syndrome protein, varies during the cell cycle in its amount, distribution, and co-localization with other nuclear proteins. Cytogenet Cell Genet. 91(1-4):217-23.

Schneider, A., Smith, R. W., Kautz, A. R., Weisshart, K., Grosse, F., and Nasheuer, H. P. (1998). Primase activity of human DNA polymerase alpha-primase. Divalent cations stabilize the enzyme activity of the p48 subunit. J. Biol. Chem. 273(34): 21608-15.

Schneider, C., Newman, R.A., Sutherland, D.R., Asser, U. and Greaves, M.F. (1982). A one-step purification of membrane proteins using a high efficiency immunomatrix. J Biol Chem. 257(18):10766-9.

Schneider, C., Weißhart, K., Guarino, L.A., Dornreiter, I. and Fanning, E. (1994). Species-specific functional interactions of DNA polymerase alpha-primase with Simian virus 40 (SV40) T antigen require SV40 origin DNA. Mol. Cell. Biol. 14: 3176-85.

Schub, O., Rohaly, G., Smith, R. W., Schneider, A., Dehde, S., Dornreiter, I., and Nasheuer, H. P. (2001). Multiple phosphorylation sites of DNA polymerase alpha-primase cooperate to regulate the initiation of DNA replication in vitro. J. Biol. Chem. 276(41): 38076-83.

Seo, Y.-S., Lee, S.-H. and Hurwitz, J. (1991). Isolation of a DNA helicase from HeLa cells requiring the multisubunit human single-stranded DNA-binding protein for activity. J. Biol. Chem. 266: 13161-70.

Shevelev, I.V. Ramadan, K. and Hübscher, U. (2002). The TREX2 3' 5' exonucleases physically interacts with DNA polymerase delta and increases its accuracy. The Scientific World Journal 2:1-7

Shinohara, A., Ogawa, H., and Ogawa, T. (1992). Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. Cell 69(3): 457-70.

Shivji, K. K., Kenny, M. K., and Wood, R. D. (1992). Proliferating cell nuclear antigen is required for DNA excision repair. Cell 69(2): 367-74.

Singh, K.K. and Samson, L. (1995). Replication protein A binds to regulatory elements in yeast DNA repair and DNA metabolism genes. Proc. Natl. Acad. Sci. USA 92: 4907-11.

Smale, S.T. and Tijan, R. (1986). T-antigen-DNA polymerase alpha complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. 6: 4077-87.

Sogo, J. M., Stahl, H., Koller, T., and Knippers, R. (1986). Structure of replicating simian virus 40 minichromosomes. The replication fork, core histone segregation and terminal structures. J. Mol. Biol. 189(1): 189-204.

Stadlbauer, F., Voitenleitner, C., Bruckner, A., Fanning, E., and Nasheuer, H. P. (1996). Species-specific replication of simian virus 40 DNA in vitro requires the p180 subunit of human DNA polymerase alpha-primase. Mol. Cell Biol. 16(1): 94-104.

Stigger, E., Dean, F. B., Hurwitz, J., and Lee, S. H. (1994). Reconstitution of functional human single-stranded DNA-binding protein from individual subunits expressed by recombinant baculoviruses. Proc. Natl. Acad. Sci. U. S. A 91(2): 579-83.

Stillman, B. (1989). Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. 5:197-245. 197-245.

Stillman, B. (1994). Smart machines at the DNA replication fork. Cell 78(5): 725-8.

Stillman, B. (1996) Cell cycle control of DNA replication. Science 74(5293):1659-64.

Stucki, M., Pascucci, B., Parlanti, E., Fortini, P., Wilson, S.H., Hübscher, U. and Dogliotti, E. (1998). Mammalian base excision repair by DNA polymerases delta and epsilon. Oncogene. 17(7): 835-43.

Stukenberg, P. T., Studwell-Vaughan, P. S., and O'Donnell, M. (1991). Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. J. Biol. Chem. 266(17): 11328-34.

Sung, P. (1994). Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. Science 265(5176): 1241-3.

Suzuki, M., Izuta, S. and Yoshida, S. (1994). DNA polymerase alpha overcomes an error-prone site in the presence of replication protein-A. J. Biol. Chem. 269: 10025-8.

Takisawa, H., Mimura, S., and Kubota, Y. (2000). Eukaryotic DNA replication: from pre-replication complex to initiation complex. Curr. Opin. Cell Biol. 12(6): 690-6.

Thömmes, P., Ferrari, E., Jesseberger, R. and Hübscher, U. (1992). Four different DNA helicases from calf thymus. J. Biol. Chem. 267: 6063-73.

Thommes, P., Kubota, Y., Takisawa, H., and Blow, J. J. (1997). The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. EMBO J. 16(11): 3312-9.

Treuner, K., Eckerich, C. and Knippers, R. (1998). Chromatin association of replication protein A. J. Biol. Chem. 273: 31744-50.

Treuner, K., Findeisen, M., Strausfeld, U., Knippers, R. (1999). Phosphorylation of replication protein A middle subunit (RPA32) leads to a disassembly of the RPA heterotrimer. J. Biol. Chem. 274(22): 15556-61.

Tsurimoto, T. and Stillman, B. (1989). Multiple replication factors augment DNA synthesis by two eukaryotic DNA polymerases, alpha and delta. EMBO J. 8: 3883-9.

Tsurimoto, T., Melendy, T. and Stillman, B. (1990). Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 replication origin. Nature 346: 534-9.

Voitenleitner, C., Fanning, E. and Nasheuer, H.P. (1997). Phosphorylation of DNA polymerase alpha-primase by cyclin A-dependent kinases regulates initiation of DNA replication in vitro. Oncogene 14(13): 1611-5.

Voitenleitner, C., Rehfuess, C., Hilmes, M., O'Rear, L., Liao, P.C., Gage, D.A., Ott, R., Nasheuer, H.P. and Fanning, E. (1999). Cell cycle-dependent regulation of human DNA polymerase alpha-primase activity by phosphorylation. Mol. Cell. Biol. 19(1): 646-56.

Waga, S. and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. Annu. Rev. Biochem. 67:721-51. 721-51.

Wahl, A. F., Geis, A. M., Spain, B. H., Wong, S. W., Korn, D., and Wang, T. S. (1988). Gene expression of human DNA polymerase alpha during cell proliferation and the cell cycle. Mol. Cell Biol. 8(11): 5016-25.

Wakasugi, M., Shimizu, M., Morioka, H., Linn, S., Nikaido, O. and Matsunaga, T. (2001). Damaged DNA-binding protein DDB stimulates the excision of cyclobutane pyrimidine dimers *in vitro* in concert with XPA and replication protein A. J. Biol. Chem., 276 (18): 15434-40.

Wang, H., Guan, J., Wang, H., Perrault, A.R., Wang, Y. and Iliakis, G. (2001). Replication protein A2 phosphorylation after DNA damdage by the coordinated action of ataxia telangiectasia-mutated and DNA-dependent protein kinase. Cancer Res. 61(23): 8554-63.

Wang, J., Yu, P., Lin, T. C., Konigsberg, W. H., and Steitz, T. A. (1996). Crystal structures of an NH2-terminal fragment of T4 DNA polymerase and its complexes with single-stranded DNA and with divalent metal ions. Biochemistry 35(25): 8110-9.

Wang, M., Mahrenholz, A. and Lee, S.H. (2000). RPA stabilizes the XPA-damaged DNA complex through protein-protein interaction. Biochemistry 39(21): 6433-9.

Wang, T. S., Copeland, W. C., Rogge, L., and Dong, Q. (1995). Purification of mammalian DNA polymerases: DNA polymerase alpha. Methods Enzymol. 262:77-84. 77-84.

Washington, M. T., Johnson, R. E., Prakash, S., and Prakash, L. (2001). Mismatch extension ability of yeast and human DNA polymerase eta. J. Biol. Chem.19;276(3): 2263-6.

Weisshart, K., Forster, H., Kremmer, E., Schlott, B., Grosse, F., and Nasheuer, H. P. (2000). Protein-protein interactions of the primase subunits p58 and p48 with simian virus 40 T antigen are required for efficient primer synthesis in a cell-free system. J. Biol. Chem. 275(23): 17328-37.

Wobbe, C.R., Dean, F.B., Murakami, Y., Borowiec, J.A., Bullock, P. and Hurwitz, J. (1987). In vitro replication of DNA containing either the SV40 or the polyoma origin. Philos Trans R Soc Lond B Biol Sci. 317(1187):439-53.

Wobbe, C.R., Weissbach, L., Borowiec, J.A., Dean, F.B., Murakami, Y., Bullock, P. and Hurwitz, J. (1987). Replication of simian virus 40 origin-containing DNA *in vitro* with purified proteins. Proc. Natl. Acad. Sci. USA 84: 1834-8.

Wold, M. (1997). RPA: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu. Rev. Biochem. 66: 61-92.

Wold, M. S. and Kelly, T. (1988). Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc Natl Acad Sci U S A. 85(8): 2523-7.

Wolffe, A. P. (1998). When more is less. Nat. Genet. 18(1): 5-6.

Yan, H. and Newport, J. (1995). An analysis of the regulation of DNA synthesis by cdk2, Cip1, and licensing factor. J. Cell Biol. 129: 1-15.

You, Z., Komamura, Y., Ishimi, Y.(1999). Biochemical analysis of the intrinsic Mcm4-Mcm6-mcm7 DNA helicase activity. Mol Cell Biol. 19(12):8003-15.

Yuzhakov, A., Kelman, Z., Hurwitz, J., and O'Donnell, M. (1999). Multiple competition reactions for RPA order the assembly of the DNA polymerase delta holoenzyme. EMBO J. 18(21): 6189-99.

Zhang, D., Frappier, L., Gibbs, E., Hurwitz, J., and O'Donnell, M. (1998). Human RPA (hSSB) interacts with EBNA1, the latent origin binding protein of Epstein-Barr virus. Nucleic Acids Res. 26(2): 631-7.

Zou, L. and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol. Cell Biol. 20(9): 3086-96.

Acknowledgements

First of all I wish to express my deep gratitude to Dr. Heinz-Peter Nasheuer for his numerous discussions on these projects and my thesis writing. His always- ready-to-help attitude facilitated my work very much.

I am very grateful to Professor Frank Große for providing a good work atmosphere and supervision of my project.

My warm thanks to A. Schneider and J.Fuchs for their full support in and out of the lab. Many thanks to A. Rapp and K. Søe for introducing me irradiation experiments, and to Dr. P. Hemmerich for initial support. Many people in the Department of Biochemistry provided me valuable help with my projects and with my German studies. I feel grateful to them.

My life in Germany would not have been the same without Prof. Winkler's family in Meckenheim, I am very grateful to them for much encouragement, entertainment, sympathy and concrete as well as moral help.

Most of all, I thank my family: parents, sisters and children. My parents and my sisters supported me with their full love and effort to take care of my children during their non-mother period.

Selbständigkeitserklärung

I hereby declare that this thesis was composed by myself and that the work described is my own, unless otherwise stated.

Hiermit versichere ich, die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt und keine anderen als die angegebenen Quellen und hilfsmittel verwendet zu haben.

Ferner versichere ich, daß ich diese Dissertation noch an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

Jena, den 3. June 2002

Shuhua Chen

Publication:

Shuhua Chen, Xiangming Li, Shan Wu and Shanhua Chen (1997) Development of a Bispecific McAb against TNF and HRP. J. of Cellular and Molecular Immunology. (Chinese Edition), 13(2):51-53

Gutachter (Referees):
Professor Stephan Diekmann
Professor Heinz-Peter Nasheuer
Professor Juhani Syväoja

Verteidigung (Defence) on:
06, January, 2003