Establishment and Characterisation of an in vitro Replication System with Human Cell Extracts

Diplomarbeit

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vorgelegt von
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1 Introduction

1.1 The Cell Cycle

For a cell to divide, and thus replicate, it is essential to proceed through the cell cycle. The cell cycle consists of four phases: G1-Phase ("gap"), S-Phase ("synthesis"), G2-Phase and M-Phase ("mitosis").

1.2 DNA Replication

Within the cell cycle, the DNA is replicated exactly once, so both daughter cells receive the same genetic information. This process is tightly controlled to guarantee the integrity of the resulting genomes. It involves licensing of the DNA and initiation of replication at a number of sites simultaneously. Replication proceeds in consecutive stretches along the chromosomes. Each of these so called Replicons (Hubermann and Riggs, 1968) own one origin of replication from which it springs. The origins are the starting points of the bidirectional replication forks (Hand, 1978).

1.3 Origins of DNA Replication

The replicon model (Jacob et al., 1963) describes that initiator proteins bind to a specific site on the chromatin (replicator) and initiate DNA replication. This model applies to the situation in prokaryotes and viruses. The eukaryotic origins that are best characterised are ARS (autonomously replicating sequence) in Saccharomyces cerevisiae. They can enable extra-chromosomal plasmids to replicate independently (Stinchcomb et al., 1979). The essential part of the ARS elements is called ACS (ARS consensus sequence), a conserved AT rich stretch of DNA. Together with the defined arrangement of A and
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B elements (Marahrens and Stillman, 1992) they form the binding site for the origin recognition complex (ORC) (Bell and Stillman, 1992) and serve as a platform for more initiation factors. This mechanism is in accordance with the replicon model. In contrast to the ARS, the origins of eukaryotes are very heterogeneous in size and structure, making their identification much harder. The replicators in *Saccharomyces pombe* for example are 500 - 1000 bp in length and contain several AT-rich stretches without any significant homology (Kelly and Brown, 2000). All attempts to identify characteristic autonomously replicating sequences in humans were in vain and it was found that any plasmid being larger than 15 kbp is replicated in a cell cycle dependent manner (Caddle and Calos, 1992; Krysan at al., 1993). Nevertheless, about 20 origins could be identified in mammals to date, showing almost no sequence homologies (DePamphilis, 1999; Gilbert, 2001). Though the existence of a putative consensus sequence was proposed recently (Price at al., 2003), the pattern seems to be very broad. Nevertheless some regions show similarities. As in prokaryotes AT rich stretches, serving as unwinding regions, surrounded by CpG-islands can be found. The CpG-islands are often associated with promoters of housekeeping genes, and interestingly most of the human origins were found to be situated within the promoter regions of actively transcribed genes (Keller et al., 2002; Ladenburger at al., 2002). This notion is supported by the finding that the less tightly coiled chromatin in the promoter region seems to play a role in the initiation events. Depending on the tightness of packing, promoter as well as origin activity can be blocked since the accessibility of a specific stretch of DNA for initiation factors is decreased (DePamphilis, 1997). Apart from that, components of the nuclear matrix are important for DNA replication and are probably involved in the initiation events.

In the group of Professor Knippers, University of Konstanz, an ORC binding site could be identified in the promoter region of the human *MCM4* gene using chromatin immuno precipitation (ChIP) analysis. The binding site is less than 1 kb in size and is situated between the two divergently transcribed genes *PRKDC* (catalytic subunit of the DNA dependent protein kinase) and *MCM4* (Ladenburger at al., 2002). Furthermore, nascent strand analysis revealed that this upstream promoter sequence acts as an origin of bidirectional DNA replication (see Figure 1.1).
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Figure 1.1: UPR ORC binding site.
Exons are depicted as black boxes with roman numbers. Diverging arrows indicate starting points and directions of transcription.

1.4 Components of the Pre-Replication Complex

Since every piece of DNA has to be replicated exactly once during the cell cycle, a marking of the the origins through appropriate factors is essential to distinguish between replicated and unreplicated DNA. Using cell fusion experiments it was proved that chromosomes are tagged by initiation factors in G1 phase to enable their replication in S phase (Rao and Johnson, 1970). This concept was refined in 1988 by Julian Blow and Ron Laskey who postulated a hypothetic ’replication licensing model’ (Rao and Johnson, 1970). Through the identification of potential initiation factors, it was possible to further specify the molecular mechanisms underlying this tightly regulated process (Diffl ey et al., 1994). The initiation of replication depends on at least 20 different factors that have to bind to the origin site in an orderly manner (Bell and Dutta, 2002; Blow and Hodgson, 2002; Ritzi and Knippers, 2000).

The first protein complex binding to origins in eukaryotes was discovered in S. cervisiae and was termed ’origin recognition complex’ (ORC) (Bell and Stillman, 1992). This complex, being composed of six different subunits (ScOrc1p-ScOrc6p), binds specifically to the ARS sites during G1 phase, thus recruiting more subunits like Cdt1 and Cde6. This triggers a binding of MCM proteins (MCM1-7p) to the chromatin and therefore the completion of the assembly of the pre-replication complex. The phosphorylation and
resulting change of conformation of the MCM complex results in a local unwinding of the DNA (Geraghty et al., 2000). After the recruitment of Cdc45 and the dissociation of Cdc6 and Cdt1 DNA replication is initiated through binding of RPA (replication protein A) and DNA polymerase α (primase) to the unwound DNA. During the following elongation process, the MCM protein complex slide along the DNA, leading the replication fork (Alexandrow et al., 2002; Aparicio et al., 1997; Schaarschmidt et al., 2002). At the same time the Orc proteins remain bound to the origin, forming the post replication complex. Since homologous replication factors could be identified in all examined species it seems likely that DNA replication is a conserved process. For an illustration see Figure 1.2.

Figure 1.2: **Model of the initiation of eukaryotic DNA replication.**

This simplified model summarises the processes involved in the initiation of DNA replication. In early G1 phase, Cdc6 and Cdt1 are recruited by the origin recognition complex (ORC) bound to the origin. This leads to the loading of Mcm-proteins onto the chromatin and thus to the completion of the pre-replication complex, that is activated by the Cdc7/Dbf4 kinase and CDKs. After the dissociation of Orc1p, Cdt1p and Cdc6p, Cdc45p is led to the replication complex, RPA and the DNA polymerase α-primase are loaded and the initiation of replication can take place.
1 Introduction

1.5 pEPI-1 - an Extrachromosomal Replicon

pEPI-1 is a vector which was constructed in the laboratory of Professor Lipps at the University of Witten/Herdecke, bearing a strong eukaryotic promoter and the S/MAR. The S/MAR (Scaffold/Matrix Attachment Region) of the 5’ region of the human interferon-β gene (Bode et al., 1992) was cloned into the MCS (multiple cloning site) of the pGFP-C1 plasmid obtained from Clontech (Figure 1.3). The resulting 6692 bp vector is able to replicate episomally in CHO cells in 5-10 copies over several hundred generations without transactivating viral factors and in the absence of selection pressure (Piechaczek et al., 1999). The continuous stability and constant number of the vector means it is replicated and segregated efficiently. It seems likely that pEPI-1 uses the cellular apparatus in order to replicate in parallel to the genome. It was shown by nuclear fractionating and FISH analysis that pEPI-1 is associated with metaphase chromosomes (Baiker et al., 2000) through interaction with the nuclear matrix protein SAF-A (scaffold attachment factor A) (Jenke et al., 2002). This interaction between the circular vector pEPI-1 and SAF-A contributes to the efficient propagation of the construct. pGFP-C1, a plasmid bearing the same elements as pEPI-1 except the S/MAR region is integrated into the host genome after transfection (Piechaczek et al., 1999). Furthermore, an active transcription into the S/MAR is required for autonomous replication (Stehle et al., 2003). On the other hand, the SV40 origin can be deleted without interfering with the vectors ability to replicate episomally (I.M Stehle and H.J. Lipps, unpublished data).

In our laboratory, pEPI-1 was used to investigate the binding behavior of eukaryotic replication initiation proteins in vivo. A recent study (Schaarschmidt et al., 2004) revealed that pEPI-1 replicates episomally in CHO and HeLa cells in an once-per-cell-cycle manner. By nascent DNA strand analysis it was shown that DNA replication is initiated at no particular site of the plasmid. This is in accordance with the finding observed by ChIP analysis and quantitative PCR that even though the binding and dissociation of ORC and MCM proteins takes place in a cell cycle dependent manner, there seems to be no preference for binding at a certain site on pEPI-1.

It was concluded that the specificity of ORC binding in the genome is determined not by sequence characteristics but rather by epigenetic factors like accessibility of the chro-
matin or interaction with components of the nuclear matrix.

![Diagram of pEPI-1 vector map](image)

**Figure 1.3: Vector map of pEPI-1.**

The pEPI-1 vector (6692 bp) contains several functional elements. MCS = multiple cloning site; SV40 ori = SV40 origin of DNA replication, Neo/Kan = resistance cassettes, HSV TK poly A = Herpes simplex virus thymidine kinase-polyadenylation signal; pUC ori = pUC origin, pCMV = immediate early promoter of the human Cytomegalovirus, GFP = *green fluorescent protein* gene, S/MAR = S/MAR region of the 5’ region of the human interferon-β gene.

### 1.6 In vitro Replication Systems

#### 1.6.1 SV40 based System

In the mid 80s several groups established systems where *in vitro* replication of SV40 DNA (Ariga and Sugano, 1983; Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1987) or SV40 chromatin (Cheng and Kelly, 1989; Smith and Stillman, 1989; Stillman, 1986) was carried out. This was a great leap forward in identifying, purifying and characterising the proteins that drive replication.

The initiation of SV40 DNA replication results from the binding of the large T antigen (T-Ag) to the SV40 origin. T antigen is a multifunctional protein, carrying out a whole range of functions, DNA binding and unwinding (Stahl et al., 1986; Stahl and Knippers, 1987) among others. When ATP is present, a large oligomer of two T-Ag hexamers is
formed (Tsurimoto and Stillman, 1989), which unwinds the DNA strand around the origin but it is also needed for elongation of the replication (Borowiec et al., 1990; Stahl et al., 1985). The unwound double strand is then stabilised by the single strand binding protein RPA (Fairman and Stillman, 1988; Wobbe et al., 1987; Wold and Kelly, 1988). The actual DNA synthesis starts after a characteristic delay of 10 to 15 minutes (Stillman, 1989). Through interaction of the cellular polymerase α (primase) with the T-Ag and stimulation by RPA and RF-C, another replication factor (Tsurimoto and Stillman, 1989), and the stimulation of polymerase δ by T-Ag, RPA and PCNA (proliferating cell nuclear antigen), replication takes place. During this process, DNA polymerase δ is responsible for synthesis of the leading strand whereas DNA polymerase α synthesises the lagging strand (Stillman, 1992). RNaseH and a 5'-3'-exonuclease remove the RNA primers, the ligase links the Okazaki-fragments and the topoisomerase I and II relax the resulting torsional tension.

1.6.2 Non-Viral Systems

In order to get an origin specific one-compartment-system that allows the revelation of the initiation events at the origin, several cell-free replication systems with extracts of different eukaryotic cells where developed. In these in vitro replication assays, the proteins in the extracts are required to take over the functions of the T-Ag (see 1.6.1). For a long time, the doctrine was that for the regulation of replication, a nucleus-like structure is indispensable. It was shown that assays based on Xenopus egg extracts (Blow and Laskey, 1986; Gilbert et al., 1986), HeLa cell extracts (Krude et al., 1997) and Yeast extracts (Pasero et al., 1997) are replicating DNA in a cell cycle dependent manner when G1 phase nuclei are used as a substrate. When bare DNA is incubated with activated Xenopus egg extract, a nucleus-like structure is formed before replication occurs. The initiation seems to be totally sequence-independent (Blow and Laskey, 1986; Gilbert et al., 1986; Hyrien and Mechali, 1992; Mahbubani et al., 1992). What’s more, initiation of replication was achieved in nuclei from CHO cells specifically at the DHFR-origin (dehydrofolate reductase) by adding cytosolic extracts from Xenopus eggs (Wu et al., 1997). After removing the nuclear membrane, the efficiency of the replication was greatly reduced (Blow and Laskey, 1986; Newport, 1987; Pasero et al.,
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1997). One possible explanation for the importance of the nuclear membrane is that selective transport of specific factors is essential in order to provide an environment promoting replication (Leno and Laskey, 1991). The other possibility is that replication occurs at fixed locations within the nuclear matrix or scaffold. That would mean that replication machinery is fixed to subnuclear structures (Hozak et al., 1993; Hozak et al., 1993). Later studies revealed that in vitro replication does not depend on nuclear membranes although the cellular extracts used must be highly concentrated (Braguglia et al., 1998; Walter et al., 1997). However the initiation events are totally independent of the sequence.

The replication of plasmids with origin sequences from mammalian genomes in vitro using extracts from human cells has long been subject to intensive investigations. Initiation could be stimulated in recombinant plasmids containing Ors (origin rich sequences) from monkey cells by addition of HeLa S3 cell extracts. The replication started specifically at the Ors and was dependent on the activity of DNA polymerase α and/or δ (Pearson et al., 1991). This system was successfully used in numerous follow-up studies (Pearson et al., 1994; Pelletier et al., 1997; Pelletier et al., 1999; Todd et al., 1995; Zannis-Hadjopoulos et al., 1994) and to characterise of the Ku-antigen, an origin-specific antigen that is involved in DNA replication (Matheos et al., 2002). Another study shows that plasmids containing DNA from the human c-myc initiation zone (Leffak and James, 1989) are replicated when extracts from human embryonal kidney cells are added and that this replication starts specifically at the origin (Berberich et al., 1995). Some of the latter studies did not meet all requirements of a proper in vitro replication system. Namely, it was not distinguished clearly enough between nucleotide incorporation springing from repair and incorporation in the course of replication. The assay must depend on the activity of DNA polymerase δ and ε and especially pol α (primase). Secondly, a complete copy of the template strand has to be synthesised. At last the efficiency of the replication must depend on the cell cycle stage (Stillman, 1986) and the presence of ORC-complexes.

Another approach was to use dynamic molecular combing to investigate the changes in firing behavior of origins in Xenopus embryos during early development in vitro (Marheineke ET AL., 2004).
In the work proceeding my thesis, Jens Baltin established an \textit{in vitro} replication assay with extracts of HeLa cells and radiolabeled nucleotides in combination with DpnI digestion of the products to investigate the behavior of different constructs (Baltin, 2003). He found that neither the S/MAR elements, an \textit{in vivo} ORC binding site nor an active promoter is essential for replication \textit{in vitro}. The different plasmids where replicated in a sequence-independent manner but for unknown reasons pEPI-1 could not be replicated. What’s more, the products of the replication reaction that where replicated completely were always form II (nicked) and from III (cut) DNA.
2 Aims
After the characterisation of pEPI-1 and pEPI-1 derivates in vivo, these constructs should be characterised in vitro. An in vitro replication system with human cell extracts was established and the results of Jens Baltin’s work showed that the system was able to replicate template DNA regardless of its sequence. Nevertheless there were still open questions about exactly which processes are working behind the scenes. Before varying the conditions of the assay my aim was to learn the procedure and reproduce the results of the preceding work.

There are some requirements an in vitro replication system has to meet in order to serve as a model system of the processes in vivo. It was planned to perform a series of experiments in order to determine if the system complies with certain requirements. To gain another perspective on the range of products of the assay, I planned to use electron microscopy.

DNA topology was thought to be a central aspect of the processes in the assay so the next step should be to look at how the topology changes as the reaction proceeds and how the topology and integrity of the template DNA effects the products of the assay. An important part was to characterise the biochemical properties of the system used. Namely, to investigate whether the underlying mechanisms are the same ones initiating and carrying out replication in vivo.

If the situation in the in vitro assay resembles the situation in the living cell, the efficiency must depend on the cell cycle stage of the cells the protein extracts were prepared from. So it was planned to synchronise HeLa cells, extract the proteins from the nucleus and use them in the assay to drive the reaction.

What’s more, it should be clarified whether in vitro DNA replication depends on the presence of initiation proteins, like ORC and MCM. The approach was to deplete initiation factors from the extracts used and to look at the effect on the DNA in vitro replication. As a last experiment, it was planned to replace the factors depleted from the cell extracts with recombinantly expressed and purified ORC and MCM proteins.
3 Material

3.1 DNA

The pEPI-1 plasmid as well as the pGFP-C1 plasmid were kindly provided by the group of Prof. Lipps, Universitt Witten-Herdecke. The plasmids pUPR-EcoRI, pUPR-BglIII, pEX9-EcoRI and pEX9-BglII were the result of Jens Baltin’s work.

3.2 Compounds

If not indicated otherwise all compounds (purity p. A.) came from the following producers: Fluka, Gibco BRL, Merck, Pharmacia, Riedel-de Haen, Roche, Roth, Serva, Sigma and Quiagen. Radio labeled compounds were obtained from ICN Biomedicals.

3.3 Kits

The BCA protein assay kit used to determine protein concentrations was obtained from Pierce.

3.4 Enzymes

Restriction endonucleases were obtained from New England Biolabs and MBI Fermentas. RNaseH came from Roche and ProteinaseK from Roth.

3.5 Antibodies

The antibodies against Orc1, Orc2 and Mcm3 were purified by members of the group using antigen columns. IgG antibodies were obtained from Sigma. Horseradish peroxidase
linked secondary anti-rabbit antibodies were obtained from Jackson ImmunoResearch.

3.6 Cell Culture

Culture medium and fetal calf serum were obtained from Gibco, culture dishes from
Greiner. HeLa S3 cells came from Gibco.

3.7 Markers

As a DNA marker a 1 kb latter from MBI-Fermentas was used. The LMW protein
marker came from Pharmacia (94, 67, 43, 30, 20 and 14 kDa).
4 Methods

4.1 DNA Working Techniques

4.1.1 Plasmid Preparation

To prepare large quantities of plasmid DNA the method of alkaline lysis was used. Positive clones were transferred into 5 ml LB medium containing the antibiotic to be selected against, shaken at 37° C overnight, transferred into 250 ml of the same medium and again shaken at 37° C overnight. The dense cell solution was centrifuged at 4° C and 2700 g for 15 min. The pellet was then dissolved in 20 ml GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8) and incubated on ice for 5 min. 40 ml alkaline SDS solution (0.2 m NaOH, 1 % SDS) was added with continuous stirring. After precipitation by addition of 30 ml salt solution (0.2 M NaOH, 1.8 M acidic acid) the solution was centrifuged at 4° C and 12000 g for 10 min. The supernatant containing the DNA was mixed with 0.6 x the volume isopropanol and centrifuged at 0° C and 10000 g for 15 min. The pellet was dissolved in 8 ml MOPS acetate (0.1 M Na acetate, 50 mM MOPS, 3-Morpholinopropanesulfonic acid pH 8) and the DNA was purified via CsCl gradient centrifugation.

4.1.2 CsCl Gradient Centrifugation

To purify plasmid DNA 8.2 g CsCl, 8 ml of DNA solution and 20 µl ethidium bromide was centrifuged at 18° C and 35000 rpm (T-50 Rotor) for 72 h. The resulting bands where identified under UV light and harvested with an injection needle. To remove the ethidium bromide the mixture was shaken out 10 to 15 times with the same volume of NaCl saturated isopropanol, followed by addition of 2 x the volume of TE and ethanol precipitation (Section 4.1.6). See also (Sambrook and Russel, 2001)
4.1.3 Agarose Gel Electrophoresis

DNA fragments were separated and analysed using agarose gel electrophoresis. By applying a marker besides the probes to be analysed, the length and/or form of the DNA fragments could be identified. 0.8 % agarose was dissolved in 0.5 x TBE (8.9 mM Tris, 8.9 mM Boric acid, 0.1 mM EDTA) by heating the mixture. After cooling to about 40° C it was casted into a form so after cooling to room temperature and solidifying a gel was formed. The probes where loaded into the slots after addition of loading buffer (0.1 % xylene cyanole, 0.1 % bromphenol blue, 30 % glycerine). Electrophoresis was carried out in 0.5 x TBE. See also (Sambrook and Russel, 2001)

4.1.4 Restriction Endonuclease Digest

1 Unit of the restriction endonuclease is able to digest 1 µg of DNA within 1 h. The enzymes where used according to the protocols provided with the kits.

4.1.5 Phenol Chloroform Extraction

The DNA samples containing protein were mixed with the same volume of 50 % phenol, 48 % chloroform and 2 % isoamyl alcohol. After vortexing for 30 s they were centrifuged at 10000 g for 5 min. From the resulting supernatant the DNA was precipitated with ethanol (Section 4.1.6). See also (Sambrook and Russel, 2001)

4.1.6 Ethanol Precipitation

To precipitate DNA from aqueous solutions the following was added: 2.5 x the volume of the sample 100 % ethanol, 0.5x the volume 7.5 M ammonium acetate and 20 µg glycogen. The mixture was left at −20° C for 30 min and centrifuged at 10000 g for 5 min. The resulting pellet was washed with 200 µl 70 % ethanol and centrifuged at 10000 g for 10 min. After thoroughly removing all liquid the pellets where dried at 60° C and then dissolved in TE to give the desired concentration. See also (Sambrook and Russel, 2001)
4.1.7 DNA Concentration Measurement

The concentration of DNA solutions was determined by photometry. The optical density (OD) was measured at a wavelength of $\lambda = 260 \text{ nm}$. An OD of 1 equals 50 $\mu\text{g/ml}$ double stranded DNA.

4.2 Cell Culture

4.2.1 Cultivation

HeLa-S3 cells where cultivated as a mono layer on coated culture dishes using DMEM with 5 % FCS. The condition were: $37^\circ \text{ C}$, 95 % humidity, 5 % CO$_2$. The medium contained 40 mg/ml penicillin and 80 mg/ml streptomycin. The cells were detached by a brief incubation with trypsin solution. The cells were transferred into medium, diluted according to the doubling time and the time until the next transfer.

4.2.2 Synchronisation

To synchronise HeLa cells in a certain stage of the cell cycle they were blocked by exposing them to 2.2 mM thymidine. After 15.5 h the cells were washed with PBS and released by transferring them into fresh medium. After another 9 h the second block (2.2 mM thymidine) was applied. Being blocked for 15 h the cells were all at the G1 to S transition border. At this point the cells were released again with fresh medium. 3 h after the release, the mid S phase extracts were prepared. 15.5 after release from the second block the G1 phase extracts were prepared.

4.2.3 FACS Analysis

Synchronised cells were rinsed three times using PBS with 5 mM EDTA and incubated in the same buffer over night at $8^\circ \text{ C}$. $4 \times 10^3$ cells were centrifuged at 600 g at $4^\circ \text{ C}$ for 5 min and the pellet was resuspended in 50 $\mu\text{l}$ PBS with 5 mM EDTA. Then 250 $\mu\text{l}$ staining solution ($1.4 \text{ mg/ml}$ propidium iodide, 5 mM EDTA and 1 % Triton-100 in PBS) and 1 U RNaseH was added. Propidium iodide intercalates between bases of DNA and thus gives an indication of the total amount of DNA present in the cell via
its characteristic fluorescence. The DNA content itself is an indicator of the current phase of the cell cycle. After 30 min on ice the propidium iodide content was measured using a flow cytometer (FACS Calibur, BD Bioscience). The data was analysed using the Cell-Quest software package (BD Bioscience).

4.3 Protein Working Techniques

4.3.1 SDS-PAGE

Protein samples were dissolved in Laemmli buffer (250 mM TRIS-HCl pH 6.8, 10 % SDS, 50 % glycerin, 0.4 % bromphenol blue, 12 % mercaptoethanol) and denatured by heating to 65°C for 10 min. The samples were then loaded on a SDS-polyacrylamide gel of appropriate percentage. To focus the samples an amperage of 16 mA was applied. After they were all level with each other the amperage was raised to 40 mA and kept for 2 h. The resulting gels were either stained with Coomassie (Section 4.3.2) or used for western blot (Section 4.3.3). See also (Laemmli, 1970)

4.3.2 Coomassie Stain

In order to visualise the proteins separated by SDS-PAGE (see 4.3.1) the gel was incubated for 30 min in Coomassie solution (40 % methanol, 10 % citric acid, 2 g/l Coomassie Blue) and then rinsed several times in destain solution (40 % methanol, 10 % citric acid) until the background vanished. See also (Bramhall et al., 1969)

4.3.3 Western Blot

A BioRad semidry blotting cell was used to transfer proteins from a gel onto a nitrocellulose membrane (Schleicher and Schuell BioScience). The membrane and the filters were soaked with transfer buffer (25 mM Tris, 192 mM glycine, 1.3 mM SDS, 20 % methanol). The amperage applied was 0.8 mA/cm². After the transfer the membrane was stained with Ponceau S, the marker lanes were traced, rinsed with TNT (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05 % Tween20) and blocked with Roti-Block. The membrane was then incubated with a TNT solution containing monoclonal antibodies (dilution 1:200-1:1000) for 1 h. The horseradish peroxidase-linked secondary antibodies
are applied 1:25000 in TNT containing 5 % nonfat dried milk. After 45 min of incubation
the membrane was rinsed three times with TNT and the proteins were detected according
to the "ECL Western Blotting System" protocol by Amersham. See also (Towbin et al.,
1979)

4.3.4 Preparation of Cell Extracts

The whole procedure was carried out on ice. 6-10 dishes with mono-layer HeLa cells
were washed twice with hypotonic buffer (20 mM Hepes pH 7.4, 5 mM KCl, 0.5 mM
MgCl₂, 0.1 mM DTT). All liquid was removed and the cells were scraped off using elastic
plastic scrapers. The volume was measured, 1 x protease inhibitor (Complete, Roche)
was added and the cells were left for 10 min on ice for swelling. After douncing the
cells 30 times in a 3 ml S-fit glass douncer they were left for 30 min on ice for elution.
The mixture was centrifuged at 10000 rpm for 10 min using a HB-4 rotor in a Sorvall
centrifuge. Then the samples were split, using the supernatant to prepare the soluble
proteins and the pellet to prepare the chromatin bound proteins.

To prepare soluble proteins, the supernatant was centrifuged at 40000 rpm for 1 h using
a TLA45-rotor in a Beckman ultra-centrifuge. The supernatant was split into aliquots
and stored at −70°C. Protein concentration was measured using a BCA Protein Assay
Kit (100000g supernatant).

To prepare chromatin bound proteins the pellet was dissolved in 5 ml hypotonic buffer
(see above) and centrifuged at 10000 rpm for 10 min using a HB-4 rotor in a Sorvall
centrifuge. The supernatant was removed and the pellet was dissolved in a minimal
volume of high salt buffer (20 mM Hepes pH 7.4, 5 mM KCl, 0.5 mM MgCl₂, 500 mM
Na-acetate, 1x Complete). After 90 min on ice the samples were centrifuged at 83000 rpm
for 1 h using a TLA100.2-rotor in a Beckman ultra-centrifuge (300000g supernatant).
The supernatant was split into aliquots and stored at −70°C. Protein concentration
was measured using a BCA Protein Assay Kit.

4.3.5 Immuno Precipitation

Chromatin bound protein extracts were incubated with antibodies, high salt buffer (Sec-
Section 4.4) and Complete for 1 h on ice (input). Then 20 µl of equilibrated 50 % Protein
A Sepharose solution (Amersham) were added and rolled at 8° C for 45 min. After spinning down the mixture the supernatant was put aside (flow-through). The precipitate was washed three times with high salt buffer and liquid was removed thoroughly with a pasteur pipette that has been drawn razor-thin over a flame. To release the proteins from the antibodies, elution buffer (2 % SDS, 5 % mercaptoethanol) was added and after 15 min of incubation at 37° C and spinning, the protein was in the supernatant (eluate). See also (Harlow and Lane, 1988) and (Towbin et al., 1979)

### 4.4 In vitro Replication Assay

The procedure consists of several steps: Pre-incubation, replication reaction, stop of reaction, followed by extraction, precipitation and digestion of DNA using endonucleases. Pre-incubation took place on ice for 20 min in a total volume of 35 µl. 16 µg chromatin bound protein was mixed with 2.85 mM ATP, high salt buffer (20 mM Hepes pH 7.4, 5 mM KCl, 0.5 mM MgCl, 500 mM K-acetate) to get a final salt concentration of 80 mM, 4x10¹⁰ plasmid copies (equals 160 ng of pUPR-BglII (7.2 kb)) as a template, 1 µl Complete (50x) and water.

Replication reaction took place at 37° C for 60 min in a total volume of 50 µl. In addition to the components of the pre-incubation was added: 45 µg extract of soluble nuclear protein, 32 mM creatin phosphate, 0.6 µg/µl creatin kinase, 24 mM K-acetate, 64 µM CTP, GTP, UTP respectively, 80 µM dGTP, dCTP, dTTP respectively, 30 µM dATP, 10 µCi α[³²P]dATP and water.

Replication was stopped by adding 90 µl stop-mix (60 mM EDTA, 2 % SDS), followed by an incubation at 60° C for 10 min.

The next steps were proteinase K digestion (0.22 µg/µl for 1 h at 37° C), phenol chloroform extraction (Section 4.1.5) and ethanol precipitation (Section 4.1.6).

The pellet was dissolved in 20 µl TE, optionally digested (Section 4.1.4) with DpnI for 1 h at 37° C and separated via agarose gel electrophoresis (Section 4.1.3) for 16 h at 30 V.
4.5 Electron Microscopy

An *in vitro* replication assay was carried out as described (selection 4.4) but without stopping and purification. The samples were incubated in fixer (2 mM TEA-HCl pH 7.5, 0.1 % glutaraldehyde) at 37° C for 20 min and were then purified by gel filtration using Biogel A5m spin columns (mash size 100-200, Biorad). Fixed samples were spread using benzylalkyldimethylammonium chloride. Rotary shadowing was done with tungsten at an angle of 8° and controlled with a quartz crystal. The thickness of the tungston layer was adjusted to 5 nm by the frequency change of the oscillating quartz crystal (500 Hz). For negative staining, samples were directly absorbed for 1 min to air-flow discharge-treated (15 s, 7 Pa, 110 V, 20-40 mA) carbon-coated grids and stained with 5 % uranyl acetate for 15-20 min. Excess uranyl acetate was removed by blotting, and the grids were air dried. Tilt series were done with a Phillips 400-T transmission electron microscope.
5 Results

5.1 Different Plasmids in the in vitro Replication Assay

5.1.1 Different Plasmids are Replicated

The first part of my work was to learn the in vitro replication assay, which was established in the laboratory of Prof. Knippers, and to reproduce the results of Jens Baltin’s diploma thesis. Being the very basis of the thesis the in vitro replication assay was expected to replicate DNA plasmids bearing certain features (see Section 3.1) in a way resembling the situation in the living cell.

Basically DNA-Plasmids were incubated with protein extracts from HeLa cells, components for energy regeneration and radio-labeled nucleotides were added, creating suitable conditions for the reaction. After the reaction, the products of the assay were purified by removing protein and increasing DNA concentration. In some experiments, the resulting DNA was digested using DpnI to cut all DNA but the products of complete replication. The samples were separated using agarose gel electrophoresis, the gels were then dried and used for autoradiography. After one day of exposure, bands of radioactive DNA were visible on the film. By comparing the strength of the bands, the efficiency of the replication reaction under different condition could be compared.

The DNA that served as a template for the reaction was mainly in form I (see input lanes, Figure 5.1). After in vitro replication, most of the DNA visible in the ethidium bromide stain was present in form II. Due to its smaller size, the two forms of pGFP-C1 run lower than the other plasmids. The total amount of DNA was diminished and a considerable signal could be seen in the slots. This signal was observed whenever the products of the assay are not digested with restriction endonucleases. One explanation is the existence of large DNA - protein aggregates (see Figure 5.4). At the bottom of
the gel, some 'blurry' areas are located that are due to RNA being present in the cell extracts.

The autoradiography shows where α[32P]dATP has been incorporated. There are several possible processes that involve incorporation of nucleotides. On the one hand there is the replication reaction that is the subject of the investigation. Replication can either be completed resulting in two new and intact plasmids or stall. This process gives rise to a wide range of products from plasmids with some radiolabeled nucleotides to plasmids with partially unwound stretches and catenae consisting of two or more plasmids. This replication intermediates can be found above form II since they are more bulky than the relaxed form.

On the other hand there is repair, a process giving an unspecific background smear. A substrate for repair could be incompletely replicated plasmids.

There are three clearly distinguishable bands representing the three forms of DNA: Form I (supercoiled), form II (relaxed, nicked) and form III (linear, cut). Between form I and form II a ladder of topoisomers can be found. These forms are products of the consecutive relaxation of the tightly supercoiled template DNA by topoisomerases present in the extracts. (For a more detailed description of the relaxation processes see Section 5.3.3)

In order to exclude products of more unspecific processes, the assay was followed by a digestion with the endonuclease DpnI. What was left were the plasmids that have been replicated completely (Figure 5.2). (For a detailed explanation of the differentiation between products by different digests see Section 5.3.5.) In the ethidium bromide stain there was no detectable signal at the height of the plasmid. This is due to the fact that the plasmids present are too few. There is also no signal in the slots. What can be observed are low running bands that represent the digestion products of the DpnI sensitive DNA.

The total amount of DNA equals the one before digestion. On the autoradiography these bands can be found again. This means that there was incorporation of the radiolabeled nucleotide into DNA that was not completely replicated. There is also a signal in the slots. This was overcome by slightly changing the protocol in later experiments. Form II and form III represent the products of one complete round of replication. It should be kept in mind that the amount detected by autoradiography is minute compared to the
one visible in the ethidium bromide stain. Therefore the proportion of template been completely replicated is estimated to be around 1%.

Figure 5.1: **Undigested products of the *in vitro* replication assay.**
The assay was carried out as described in Section 4.4. M=Marker (1 kb ladder), EtBr=ethidium bromide stain of 0.8% agarose gel, AR=autoradiography (exposition time 24h at −70°C)
Figure 5.2: **Dpn I digested products of the in vitro replication assay.**
The assay was carried out as described in Section 4.4. Products were digested with DpnI. M=Marker (1 kb ladder), EtBr=ethidium bromide stain of 0.8% agarose gel, AR=autoradiopraphy (exposition time 24h at −70° C).
5.1.2 pEPI-1 Plasmid is Replicated

In contrast to earlier observations, the pEPI-1 plasmid is replicated. This was observed after the plasmid was prepared anew using CsCl Gradient Centrifugation. Figure 5.3 shows the difference between replication efficiency of the two preparations.

In the ethidium bromide stain, both sides look similar: Input is mainly in form I, undigested products of the assay are present in form II mainly and their amount is reduced. The lanes with DpnI treated products show digestions products only. Differences can be observed in the autoradiography: The old preparation gives only a weak signal where the products are undigested, the lane with the digested products shows digestion products only. In contrast replication products of the newly prepared plasmids are clearly visible in the autoradiography before (form I, II and III) and after digestion with DpnI (form II and III).

Figure 5.3: Products of the in vitro replication assay with pEPI-1.
The assay was carried out as described in Section 4.4. Products were digested with DpnI where indicated. M=1kb Marker, I=input, white bands on black background=ethidium bromide stain of 0.8% agarose gel, black bands on white background=autoradiography (exposition time 24h at \(-70^\circ\) C).
5.2 Electron Microscopic Characterisation

5.2.1 Replication Bubbles and Aggregates are Visible

To ensure that the process observed in Section 5.1.1 is replication and to investigate possible causes of the strong signals in the slots I carried out electron microscopic analysis of the products of the in vitro replication assay. The replication reaction was stopped after 10 min at 37° C and the products were prepared for EM analysis. Circular DNA plasmids were found containing replication bubbles (Figure 5.4, A). This form represents only a very small fraction of the total DNA found, whereas most were in the form of small fragments, rings without bubbles or bunches (not shown).

The majority of the products were replication intermediates. Extensive aggregates of DNA were found on EM pictures (Figure 5.4, B). They accounted for a majority of the DNA. At the center of these interconnected bunches, proteins-centers were located, tying up around 10 DNA strands each.

Figure 5.4: In vitro replication assay products in EM.

pUPR-BglII DNA was used in the in vitro replication assay as described in Section 5.1.1. The replication reaction was stopped after 10 min. The products were analysed using electron microscopy (Section 4.5). A=Plasmid with replication bubbles, arrows indicate replication forks. B=DNA-protein aggregates.
5.3 DNA Characterisation

5.3.1 Repair is not triggered by Lesions of the Input DNA

In order to ensure that the DNA used as an input in the in vitro assay was intact it was incubated with Klenow fragment and radio-labeled nucleotides. The Klenow fragment of the DNA polymerase I from E. coli adds complementary nucleotides to single stranded DNA when ATP is present. Digested DNA with sticky ends was used as a control. The ethidium bromide stain showed that the undigested DNA was mainly form I, with a small portion being form II DNA. Digested DNA was form III only.

The autoradiography showed no signal for the undigested DNA, but a strong band for the DNA with sticky ends, indicating incorporation of radio labeled nucleotides.

Figure 5.5: Klenow reaction.
Input DNA in the in vitro assay (Form I and II) and digested DNA (Form III, sticky ends) were incubated with Klenov fragment, dNTPs and $\alpha^{[32P]}dATP$ under suitable conditions. EtBr=ethidium bromide stain of 0.8% agarose gel, AR=autoradiography (exposition time 1 h at $-70^\circ$ C).
5.3.2 Replication Takes Place over a Long Stretch of Time

The in vitro replication assay protocol allows the replication reaction to work for 1 h. I was interested in when the products were actually synthesised, therefore I performed a time course of assays with replication times from 5 to 120 min.

The ethidium bromide stain in Figure 5.6 shows that the input DNA was mainly in form I, a small proportion in form II whereas the products of the in vitro replication were nearly all form II. The amount of input and products of the assay were comparable. The autoradiography showed no signal for the input but significant signals increasing in strength with time from 5 min to 120 min. The biggest portion was form II, a smaller portion form III, their ratio was constant.

![Image of agarose gel and autoradiogram showing DNA forms I, II, and III over time](image)

Figure 5.6: Undigested product of the in vitro replication assay accumulate over a long stretch of time.

The assay was carried out as described in Section 5.1.1. The replication reaction was stopped after different stretches of time. The purified DNA samples were run on an 0.8% agarose gel. The gel was stained with ethidium bromide (EthBr), dried and used for autoradiography (AR, exposition time 24 h at −70° C). M=marker (1 kb ladder).
5 Results

The ethidium bromide stain of the digested products of the assay in Figure 5.7 shows comparable amounts of digestion products only. The autoradiography shows bands at the height of form II and form III, increasing in strength with time from 5 min to 120 min. The strength of the background increased in the same manner.

**Figure 5.7:** Dpn I-digested product of the *in vitro* replication assay accumulate over a long stretch of time.

The assay was carried out as described in Section 5.1.1. The replication reaction was stopped after different stretches of time. The purified DNA samples were digested with DpnI and run on an 0.8% agarose gel. The gel was stained with ethidium bromide (EthBr), dried and used for autoradiography (AR, exposition time 24 h at −70° C). M=marker (1 kb ladder).
5 Results

5.3.3 Cell Extracts alter the Template

During the in vitro replication assay (Section 5.1.1) the form I input DNA was transformed into form II DNA. To investigate the timeframe and conditions under which this transition takes place, I performed a time series experiment with different protein extracts at different temperatures (reflecting pre-incubation and replication reaction) and varying incubation times.

![Figure 5.8: Time series of template incubated with protein extracts.](image)

Input DNA (form I) was incubated with protein extract from asynchronously growing cells. Reflecting the conditions used in the in vitro replication assay (Section 4.4) the DNA was incubated with the chromatin bound protein extracts on ice, whereas the incubation with the chromatin bound and soluble protein extracts took place at 37°C. Products were loaded on a 0.8% agarose gel and stained with ethidium bromide.

The ethidium bromide stain showed that input was mainly form I, a small portion was form II.

Where incubated with chromatin bound protein, a consecutive transformation from form I to form II occurred over time. After 2 minutes of incubation a major part of the DNA was present in form II, whereas almost none was form I. Between the two forms, a ladder of topoisomers was visible. After 15 min the DNA was virtually completely transformed to form II.
5 Results

Incubation with chromatin bound and soluble protein leads to the complete transformation of the templates into form II within 2 min.
5.3.4 There is a Preference for a Certain Form of DNA as Input

A recent publication (Remus, et al., 2004) revealed that in Drosophila ORC binding efficiency largely depends on DNA topology. The authors found that form I DNA is strongly preferred. Consequently it was interesting to investigate if the replication efficiency in the in vitro replication assay also depended on the topology of the input DNA.

In the SybrGold stain (Figure 5.9), the input lanes showed form I input (a mixture of form I and form II DNA), form II input and form III input. Form II DNA was obtained by treating form I DNA with Topoisomerase I and form III DNA by cutting form I DNA blunt with SmaI. The lanes of the SybrGold stain with the digested products of the in vitro replication assay showed only the DpnI digestion products.

In the autoradiography form II and form III replication products could be found where the input DNA was in form I. The form II and form III input did not result in radiolabeled DpnI resistant plasmids. The lower portion of the autoradiography showed DpnI digestion products in all lanes. In both stains there was no visible difference between samples that have were pre-incubated and those with no pre-incubation.
Figure 5.9: *In vitro* replication assay with different forms of template. Form II DNA was obtained by treating form I DNA with Topoisomerase I and form III DNA by cutting form I DNA blunt with Smal. SybrGold=SyberGold stain of 0.8% agarose gel, AR=autoradiography (exposition time 24 h at −70° C), M=marker (1 kb ladder).
5.3.5 Different Products can be Separated

Since the plasmids were isolated from dam-positive \textit{E.coli} strains, the input DNA was methylated at the adenosine residue in the sequence GATC. Nucleotides incorporated during the replication reaction were not methylated. There is a set of restriction endonucleases being sensitive to methylation. With the help of these specificities, it was possible to exclude products of certain processes while leaving others untouched. Figure 5.10 shows specificity for different products and Table 5.1 shows how the enzymes were used.

![Diagram showing different methylation patterns and restriction endonucleases](image-url)

Figure 5.10: \textit{Overview of restriction endonucleases used.}
The illustration shows which methylation patterns are generated by which processes and which endonucleases were used to digest the plasmids.
### Results

<table>
<thead>
<tr>
<th>Left after digestion</th>
<th>Nuclease</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DpnI</td>
</tr>
<tr>
<td>Replication products</td>
<td>X</td>
</tr>
<tr>
<td>Replication products of first round</td>
<td>X</td>
</tr>
<tr>
<td>Template and replication products of first round</td>
<td>X</td>
</tr>
<tr>
<td>Nothing</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: **Usage of restriction endonucleases.**

In Figure 5.11 the ethidium bromide stain showed the input being mainly form I with a small portion form II. Undigested and DpnI digested replication products contained the same amounts of DNA, mainly form II with a small fraction form III. The lanes loaded with products digested with DpnI and Sau3A showed only digestion products. The amount of DNA on all lanes was comparable. The autoradiography of the untreated and MboI digested samples showed strong bands for form II and less stronger ones for form III. There were strong signals in the slots fading into a smear. The DpnI digested samples showed clear signals of similar intensity for form II and form III as well as digestion products. Double digest with DpnI and MboI gave similar results (not shown). The Sau3A digested sample consisted only of digestion products but contained no form II or III DNA.
5 Results

Figure 5.11: **Products of the in vitro replication assay digested with different restriction endonucleases.**

The assay was carried out as described in Section 4.4. The Products were digested with different restriction endonucleases (as indicated). M=marker (1 kb ladder), EtBr=ethidium bromide stain of 0.8% agarose gel, AR=autoradiopraphy (exposition time 24h at −70°C).
5.4 Biochemical Characterisation

After investigating different aspects of the DNA in the \textit{in vitro} replication assay, I turned to the biochemical characteristics. It was to be proofed that the DpnI-resistant DNA found after the replication reaction was actually a product of the same proteins carrying out the replication reaction \textit{in vivo}. Therefore I tried different inhibitions targeting potential actors and compared the amount of products to the undisturbed reaction.

5.4.1 The Presence of an Energy Regeneration System is Essential

In general, DNA polymerase reactions require energy. To verify if our system involving this reaction also depends on the supply of ATP, I performed \textit{in vitro} replication assays without certain parts of the energy providing system.

The autoradiography of the undigested products of the assay in Figure 5.12 showed a strong signal for form I and form II DNA where creatin kinase (CK) was present. The situation does not depend on the presence of ATP. Whereas, where both ATP and creatin kinase were skipped, no incorporation of radiolabeled nucleotides could be observed.

![Image](image.png)

Figure 5.12: Undigested products of the \textit{in vitro} replication assay with and without creatin kinase (CK) or ATP.

The assay was performed as described in Section 4.4. The addition of creatin kinase and ATP was skipped where indicated. Products were loaded on a 0.8% agarose gel. The dried gel was used for autoradiography (exposition time 24h at $-70^\circ C$).
5 Results

The autoradiography of the DpnI digested products of the assay (Figure 5.13) showed strong bands for form I and form II DNA where ATP was present in the pre-incubation but did not show a significant signal when ATP was not added to the pre-incubation, regardless of its presence in the replication reaction. Radio-labeled digestion products were visible for all samples.

![Figure 5.13: DpnI digested products of the in vitro replication assay with and without ATP.](image)

The assay was performed as described in Section 4.4 and the products were digested using DpnI. The addition of ATP was skipped or shifted where indicated. Products were loaded on a 0.8% agarose gel. The dried gel was used for autoradiography (exposition time 24h at −70° C).
5 Results

5.4.2 The Presence of Nucleotides (NTPs/dNTPs) is Essential

Nucleotides are essential for the DNA polymerase reaction. Ribonucleotides (NTPs) are the substrate of the primase (DNA polymerase α) and are the building blocks of the primers that are the starting-point of the elongation process. Deoxyribonucleotides (dNTPs) are the building blocks of the DNA and are the substrate of the DNA polymerase δ. They are needed for the elongation process.

A system involving replication must depend on both NTPs and dNTPs for that purpose. I tested the effect of omitting these components of the in vitro replication assay.

The autoradiography of the products (Figure 5.14) showed bands for form I, II and III DNA. Where either NTPs or dNTPs were omitted, no significant signal could be observed.

![Image of autoradiography](image)

**Figure 5.14:** In vitro Replication Assay with and without Nucleotides (NTPs/dNTPs).

The assay was performed as described in Section 4.4 but with NTPs or dNTPs omitted where indicated. Products were run on a 0.8% agarose gel which was dried and used for autoradiography (exposition time 24h at −70° C).
5.4.3 Replication is Inhibited by Aphidicolin

Aphidicolin (an tetracyclic diterpene) is a specific inhibitor of DNA polymerase α and polymerase δ, which are responsible for priming and replication, respectively. A system involving replication must be sensitive to aphidicolin as shown before (Braguglia et al., 1998), so I tested its effect on the *in vitro* replication assay.

The ethidium bromide stain (Figure 5.15, EtBr) showed that the input DNA was mainly form I, a small portion form II DNA. The undigested products of the assay were present mainly in form II with a small fraction in form III. The lane of the sample with 30 μl/ml aphidicolin added gave a signal at the height of form I DNA. The digested products of the assay were only visible as DpnI digestion products. The control sample without DNA did not give any signal.

The autoradiography (Figure 5.15, AR) of the DpnI digested sample without aphidicolin showed a strong signal in the slot, for form II, III and intermediates. With increasing aphidicolin concentration, the intermediates vanished and the buildup in the slots became less prominent. The control sample without DNA did not give any signal.

Within the DpnI digested samples the one without addition of aphidicolin showed the characteristic bands of DpnI resistant replication products (form II and III), whereas the samples with 5 and 30 μl/ml aphidicolin did not show those bands. In all the lanes DpnI digestion products were visible. The sample without DNA did not give any signal.
Figure 5.15: **In vitro replication assay with and without aphidicolin.**

The *in vitro* replication assay was performed according to Section 4.4 but with varying concentrations of aphidicolin (0 g/ml, 5g/ml and 30g/ml) added. As a control the assay was performed without DNA (- DNA). Undigested and DpnI digested products were run on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The dried gel was used for autoradiography (AR, exposition time 24h at −70° C). M=Marker (1 kb ladder)
5 Results

5.4.4 Replication is Not Inhibited by α-Amanitin

α-Amanitin is a strong inhibitor of the eukaryotic RNA polymerase. To ensure that the products observed in the in vitro replication assay are not due to RNA polymerase activity, an assay with α-amanitin had to be performed.

The ethidium bromide stain (Figure 5.16, EtBr) showed that the input was mainly form I, with a small portion being form II DNA. The lanes with DpnI digested products of the assay showed only digestion products.

The autoradiography (Figure 5.16, AR) showed signals of similar intensity for form II and form III for the products of both assays, with and without α-Amanitin.

Figure 5.16: DpnI digested products of an in vitro replication assay with and without α-Amanitin.

The assay was performed according to Section 4.4 but with 50 μg/ml α-Amanitin added where indicated. Products were run on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The dried gel was used for autoradiography (AR, exposition time 24h at −70° C). M=Marker (1 kb ladder)
5 Results

5.4.5 Replication is Not Inhibited by ddNTPs

At concentrations up to 500 µM Dideoxy-nucleotides (ddNTPs) are inhibitors of the polymerase β which is responsible for repair. Higher concentration also inhibits the activity of polymerase δ which is responsible for replication (Kornberg and Baker, 1992).

To test whether the in vitro replication assay is impaired by ddNTPs, an assay without and with increasing concentrations of ddNTPs added, topping 500 µM, was performed. The ethidium bromide stain (Figure 5.17, EtBr) showed that the input was mainly form I, with a small portion being form II DNA. The lanes with DpnI digested products of the assay showed only digestion products.

The autoradiography (Figure 5.17, AR) showed characteristic signals for form II and form III for the products of all assays, regardless of the amount of ddNTPs added.

Figure 5.17: DpnI digested Products of an in vitro replication assay with and without ddNTPs.

The assay was performed according to Section 4.4 but with 100, 200 and 400 µM ddNTPs added where indicated. Products were digested with DpnI, run on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The dried gel was used for autoradiography (AR, exposition time 24h at −70°C). M=Marker (1 kb ladder), I=Input.
5 Results

5.4.6 Replication is Not Inhibited by RNaseH

The replication in the living cell is thought to start by unwinding of DNA and synthesis of RNA primers. Since the template DNA in the *in vitro* replication assay was isolated from bacteria, it might contain RNA primers right from the start (Braguglia et al., 1998). Replication by elongation of these existing primers would not resemble the natural situation where primers are synthesised de novo.

To investigate whether the *in vitro* replication reaction is independent from possible primers, I performed assays with RNaseH digested template DNA. Untreated DNA served as a control.

In the ethidium bromide stain (Figure 5.18, EtBr) untreated as well as RNaseH digested input DNA was mainly form I with a small portion being form II. The lanes with DpnI digested products of both samples showed only digestion products.

The autoradiography (Figure 5.18, AR) showed characteristic signals for form II and form III for the products of both assays, with untreated and RNaseH digested template DNA.
Figure 5.18: DpnI digested products of an *in vitro* replication assay with untreated and RNaseH digested template.

The assay was performed according to Section 4.4 but the template DNA was digested with RNaseH at 37° C for 1h where indicated. Products were run on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The dried gel was used for autoradiography (AR, exposition time 24h at −70° C). M=Marker (1 kb ladder)
5 Results

5.4.7 Soluble as well as Chromatin-Bound Protein Extract is Essential

In living cells, some replication factors are constantly bound to the DNA whereas others bind at certain stages of the cell cycle. In order to achieve a behavior resembling the natural process \textit{in vitro}, extracts of chromatin bound as well as extracts of soluble proteins were incubated with the protein-free template DNA (see 4.4).

To investigate whether both protein extracts are essential I performed assays with one of the extracts omitted. The omitted extracts were replaced with the corresponding buffers in order to provide the same assay conditions.

In the ethidium bromide stain (Figure 5.19, EtBr) the input was mainly form I with a small portion of form II DNA. The products of the assay were only visible as DpnI digestion products.

The autoradiography (Figure 5.19, AR) of the assay where both protein extracts are present showed the characteristic bands of form II and form III replication products. The lanes of the assay where either one of the extracts was omitted did not show any DpnI resistant replication products whereas all lanes showed similar amount of DpnI digestion products.
Figure 5.19: **In vitro replication assay with and without protein extracts.**

The assay was performed as described in Section 4.4 but with extract of chromatin bound protein or extract of soluble protein replaced with the corresponding buffer where indicated. Products were run on a 0.8% agarose gel and stained with ethidium bromide (EtBr). The dried gel was used for autoradiography (AR, exposition time 24h at −70°C). I=Input DNA, M=Marker (1 kb ladder).
5 Results

5.5 Sensitivity to Cell Cycle Stages

5.5.1 Protein Extracts from Synchronised HeLa Cells

As the cell cycle proceeds, different proteins bind to and are released from the origins of replication. Thus, at different phases a different set of replication factors can be found on chromatin. If the processes in the *in vitro* replication assay resemble the way replication takes place in the living cell, the efficiency of the replication reaction in the assay should depend on the cell cycle stage of the cells the protein extracts where prepared from. HeLa cells were synchronised using a double thymidine-block and prepared extracts of chromatin bound and soluble proteins from cells in the G1 and S phase, measured the protein concentration, ran a commassie gel and performed western blotting. Synchronisation was verified by analysis of DNA content in a flow cytometer (see Figure 5.20, A).

The histogram of the FACS (Flow Cytometer/Cell Sorter) analysis of the cells in G1 phase showed a spike at the absorption characteristic for haploid cells. For cells in S phase, a peak in the middle between haploid and tetraploid was found.

The Commassie stain (Figure 5.20, B) showed similar concentrations for extracts of chromatin bound and soluble proteins from both phases. The patterns of bands from the different phases where identical while varying considerably between chromatin bound and soluble proteins.

The western blot analysis (Figure 5.20, C) showed the following picture: Orc1 was present in large amounts in the chromatin bound extract of the G1 phase, but in lower amounts in the S phase and was absent in the soluble fraction. Orc2 was only found in the extracts of chromatin bound protein at same levels at both phases. Mcm3 was present on the same level in both fractions at both phases.
5 Results

Figure 5.20: **FACS analysis, Commassie stain and western blots of extracts from synchronised cells.**

Cells where synchronised using double thymidine-block and prepared as described in Section 4.2.2 and Section 4.3.4, respectively. FACS analysis (A) of isolated nuclei was performed as described in Section 4.2.3. Samples were separated via SDS-PAGE and Commassie stain (B) was performed as described in Section 4.3.2. Western blot analysis (C) was performed as described in Section 4.3.3 using antibodies against Orc1, Mcm3 and Orc2 as indicated.
5.5.2 The Efficiency of the Replication Depends on the Cell Cycle Stage

The efficiency of the *in vitro* replication assay should depend on the cell cycle phases of the cells the protein extracts used were prepared from (as described in Section 5.5.1). To test this I performed an assay using extracts of soluble and chromatin bound proteins coming from cells in G1 and S phase, respectively.

The SybrGold stain (Figure 5.21) showed that the input was mainly form I, with a small portion being form II DNA. The lanes with DpnI digested products of the assay showed minor amounts of form II and III as well as digestion products.

The autoradiography (Figure 5.21, AR) showed characteristic signals for form II and form III for the products of both assays where extracts of chromatin bound protein from S phase were used, regardless of the phase the chromatin bound protein was prepared from. The lanes with products of the assays where extracts of chromatin bound protein were used did not show bands of DpnI resistant replication products, neither with S phase nor with G1 phase extracts of soluble protein.
Figure 5.21: *In vitro* replication assay with extracts from different cell cycle phases.

The assay was performed according to Section 4.4 but with cell extracts from synchronised cells (Section 5.5.1) as indicated. Products were run on a 0.8% agarose gel and stained with SybrGold. The dried gel was used for autoradiography (AR, exposition time 24h at $-70\,^\circ\mathrm{C}$). M=Marker (1 kb ladder), I=Input.
5 Results

5.6 Sensitivity to Depletion of Orc Proteins

5.6.1 Depletion of Orc-Proteins from Cell Extracts

Orc1 and Orc2 are essential parts of the origin recognition complex (ORC) which plays a vital role in the initiation of DNA replication. If the *in vitro* replication assay resembles the natural process in this respect it should be possible to disrupt the reaction by removing these proteins.

To test this, I depleted Orc1 and Orc2 from the S phase extracts of chromatin bound protein by immuno precipitation. The soluble protein fraction was left untreated since it did not contain any of these proteins (see Section 5.5.1).

The western blot (Figure 5.22) showed that Orc1 (A, Input) and Orc2 (B, Input) were present in the extracts. This is in accordance with the findings in Section 5.5.1. After precipitation with Orc1 and Orc2 antibodies respectively, no protein was left in the flow-through whereas a significant amount was found in the eluate (A and B, Flow-through and Eluate).

After precipitation with IgG antibodies (Figure 5.22, C) a significant amount of protein could be found in the flow-through whereas the eluate was virtually protein free.
5 Results

Figure 5.22: **Western Blots of depleted S phase extracts.**

Extract of chromatin bound protein (see Section 4.3.4) from cells in S phase (see Section 4.2.2) was used for immuno precipitation as described in Section 4.3.5 with antibodies against Orc1 (A), Orc2 (B) and IgG (C). The input, flow-through and eluate were then analysed by western blotting (see Section 4.3.3).
5 Results

5.6.2 ORC-Proteins are Essential for in vitro DNA Replication

To address the fundamental and essential role of Orc-proteins for the in vitro replication reaction, I used the flow-through of the immuno precipitation to perform an in vitro replication assay.

The SybrGreen stain (Figure 5.23) showed that the input was mainly form I, with a smaller portion being form II DNA. The lane where the digested products of the assay with Orc2 depleted extract was run showed bands for form I and II. The remaining lanes with DpnI digested products of the assay showed only digestion products and very weak bands for form I, II and III respectively, if any.

The autoradiography (Figure 5.23, AR) showed signals of similar intensity for form II and form III for the products of the assays with extracts where IgG antibodies and beads, only beads and only buffer was added respectively. The bands on the lane with the products from the assay without any incubation showed the same intensity.

The lanes loaded with the products of the assays carried out using extract with Orc1 or Orc2 depleted did not show any bands at the height corresponding to DpnI resistant replication products. Nevertheless all lanes showed strong signals for DpnI digestion products.
Figure 5.23: In vitro Replication Assay with Extracts after Depletion of Orc1 and Orc2.

The assay was carried out as described in 4.4. The extracts of the chromatin bound protein fraction from S phase HeLa cells used were treated as indicated: Orc1=immuno precipitation with Orc1 antibodies, Orc2=immuno precipitation with Orc2 antibodies, IgG=immuno precipitation with IgG antibodies, No AB=incubation with beads and buffer, No Beads=incubation with buffer only, No incubation=regular assay. Products were run on a 0.8% agarose gel and stained with SybrGreen. The dried gel was used for autoradiography (AR, exposition time 24h at −70°C). M=Marker (1 kb ladder). I=Input.
5.6.3 No Rescue after Depletion of Orc Proteins

In the depletion experiments, removal of Orc Proteins from the cell extracts prevented replication \textit{in vitro}. To investigate if the Orc proteins are the only essential factors missing, I performed an assay where recombinantly expressed Orc-complexes (Wollschied, 2004) were added to the Orc1-depleted cell extracts (see Section 5.6.1).

The results of this experiments were inconsistent. On the one hand, the samples where Orc-complexes were added after depletion showed replication activity whereas negative-control samples with water and depleted cell extracts showed no activity, but on the other, negative-control samples with buffer and depleted cell extracts showed replication activity.
6 Discussion

My work was a continuation of the work of Daniel Schaarschmidt (Schaarschmidt, 2004) and Jens Baltin (Baltin, 2003). They were able to show that semi-conservative replication is possible in vitro using human cell extracts, regardless of the presence of an in vivo or SV40 origin, or an S/MAR. The relative inefficient replication when compared to the SV40 based system may be due to the fact that replication occurs only once per template and not exponentially. Another fact to consider is that the system is highly deluted when compared to the nucleus.

After learning the procedure of the in vitro replication assay, I was able to reproduce the results from previous work in our laboratory. The undigested products of the standard assay were comparable in composition regardless of the plasmid used as a template. From the pattern of DNA with incorporation of radiolabeled nucleotides, it can be concluded that the supercoiled DNA used as a template is relaxed consecutively during the reaction. A majority remains in the totally relaxed form whereas a small portion is cut. Besides this clearly defined forms, an undefined smear can be seen, resulting from a whole palette of products ranging from DNA with attached proteins to replication intermediates like catenae of multiple plasmids in different stages of replication and aggregates of proteins. These products are likely to be due to incomplete replication, repair or a combination of both. An example of incomplete replication can be found in the electron microscopy picture showing a plasmid with two replication bubbles, spanning half of its length. The most prominent signal that could not be assigned to a specific process were the ones in the slots. Since their composition was unknown, they were suspected to distort the ratio of the products that were clearly distinguishable. This notion was supported by the electron microscopy pictures which showed extensive clusters of DNA and proteins. To tackle this problem, the protocol was slightly modified and it was possible to dissolve
the aggregates in later experiments. When the products were treated with DpnI in order to digest all but the plasmids that have been replicated completely, what remained was the signals in the slots as mentioned above and two bands corresponding to form II and form III DNA. The strength of these two bands were looked at as an indication of how efficiently the replication reaction worked and were used to compare different conditions in later assays. The strength of signals given by the digestion products reflect the extend of repair. This was comparable for all plasmids.

A question that remained after Jens Baltin’s work was why pEPI-1 would not replicate. After preparing the DNA anew from bacteria, the replication went as efficient as with the other plasmids. Thus, it seems likely that the reaction was prevented by impurities. The large proportion of repair was not immediately explainable so the question arose if the input DNA may be nicked or does even have single stranded stretches. These lesions would have made it a substrate of repair. This was proofed wrong using Klenow reaction since there was no incorporation of nucleotides.

The replication reaction was always allowed to proceed for 60 min, but nothing was known about the actual timeframe. As it was thought to be rather short it came as a surprise that it didn’t top out even after 120 min. Notably, the DpnI-resistant and DpnI-sensitive fractions of the products increased to the same degree over time. The stable ratio shows that the two processes act in parallel. The reason for the slow progression could be the fact that the factors have to meet by diffusion in this diluted system in order to carry out the reactions.

To further investigate the fate of the DNA during the assay, I analysed the samples with greater spacial and temporal resolution than before and found that under the assay conditions, all of the supercoiled input DNA is transformed into relaxed DNA within a matter of minutes. Since supercoiled DNA is strongly preferred over relaxed DNA by the replication process, it seems likely that this transformation is the reason for the relatively inefficient replication in the assay when compared to SV40 based systems.

To confirm this notion, I was able to show that literally no complete replication occurred when relaxed or cut DNA was used as an input. Unfortunately the system did not respond to topoisomerase I inhibitors added in order to stabilise the supercoiled template. Identifying and specifically inhibiting the factors responsible for the relaxation
could boost the efficiency of the *in vitro* replication assay and could even render the radiolabeling obsolete.

One difference between the conditions *in vitro* and the situation in the living human cell is that the DNA serving as a template in the assay was methylated by the bacteria used to amplify it. In eukaryotic cells methylation of DNA is not a signal used for replication, and the effect on the accessibility for replication proteins is thought to be inhibiting (Harvey and Newport, 2003). Nevertheless it serves as an excellent marker for the state of replication of a plasmid. Using different methylation-sensitive restriction-endonucleases I was able to separate products of repair and products of replication and thereby show that the background is due to material that is inaccessible to digestion and that the DNA found in the slots is either unchanged input DNA or partially modified DNA with the modified parts being masked by proteins.

Another important aspect of my investigation was to identify the biochemical actors involved in the assay. I was able to show that the demand for energy can be met with the ATP present in the extracts being available to bridge between the creatin kinase and the ATP hydrolizing proteins carrying out repair. By contrast, the replication processes require a larger amount of ATP to be present specifically during pre-incubation. A possible explanation is that there are two processes taking place at the same time, competing for ATP but with different affinities. If the low-affinity-process is the binding of ORC or another process promoting replication and the high-affinity-process is a reaction hindering initiation or replication, possibly the one been responsible for relaxation of the template, then a shortage of ATP would penalise the replication reaction. Biochemical properties or concentration differences might be responsible for the difference in affinity. The temperature of the samples when ATP is added does not affect replication efficiency. A possible approach would be to systematically vary the ATP concentration, exceeding the levels used to date.

Replication in eukaryotes is carried out by polymerase α using NTPs for primer synthesis and polymerase δ using dNTPs for elongation of the newly synthesised DNA strand. As I could demonstrate the reaction *in vitro* requires both substrates and is inhibited by compounds known to specifically disrupt the polymerase reactions. In this respect it resembles the situation *in vivo*. An interesting finding is that while the replication
products totally disappeared when inhibiting replication, the replication intermediates and DNA-protein clusters were also diminished. A possible explanation is that, without inhibitor been present, a large number of replication forks stall and therefore stimulate repair processes. When inhibitor is added replication does not start in the first place so the plasmids never become a target for repair. The stalling itself may be due to a disproportion or lack of factors carrying out or stimulating replication. Varying the ratio of chromatin bound and soluble protein would be a simple but quite crude approach. Speaking of the protein extracts, both of them are essential for replication and a smear appears when DNA was incubated with chromatin-bound protein only, indicating DNA binding. This is in accordance with the model stating that factors bind to and leave the DNA as the cell proceeds through the cell cycle. It can also be excluded that primers are synthesised by RNA polymerase or are present in the extracts. I was also able to support the idea that DpnI digests all products of repair and that the remaining signals spring from replication.

The results from the experiments involving protein extracts from synchronised cells show that chromatin-bound protein from S-Phase cells is essential for replication. Since Orc1 which is involved in the initiation process is virtually not present in these extract, the efficiency of the replication does not seem to be limited by initiation. Rather the elongation process seems to be limiting. This would be in accordance with the idea of stalling replication forks.

Orc1 might not be the limiting factor but it is proofed to be essential for replication. The same applies to Orc2. This was indicated by the complete inhibition of replication by depletion of these factors from the cell extracts. But this has to be taken with a grain of salt since it is not clear exactly which components are removed by the specific antibodies. It seems feasible that all proteins associated with Orc1 and Orc2 were at least diminished during the procedure.

In order to test this, I performed rescue experiments in which I tried to replace the depleted proteins with recombinantly expressed protein complexes. There are many possible reasons why it did not work, but the simplest is that the depletion removed a bigger set of proteins than was added to rescue. Apart from that it is not clear if the purified complexes are able to promote replication at all.
6 Discussion

The degree of characterisation of this *in vitro* replication system with human cell extracts is comparable to systems using yeast nuclear extracts (Braguglia et al., 1998). The behavior of these systems seem to be similar despite the difference of the processes *in vivo*. One advantage of the system presented is that it works with separate extracts for chromatin-bound and soluble nuclear proteins and thus offers greater flexibility.

Nevertheless there are still some experiments that could be done in order to further characterise the system. At first, the rescue experiments should be redone and the experimental condition improved. To compliment the experiments using DpnI-resistance as an indication for complete replication, CsCl gradient centrifugation after incorporation of BrdU could be used to quantify the different stages of replication. The conformations of DNA that occur during the reaction could be investigated in greater detail by 2D gel electrophoresis.
7 Summary

In the work presented, I was able to characterise several aspects of an in vitro DNA replication system with human cell extracts.

I could confirm that plasmids without special sequence characteristics are replicated by the system and that the replication of each template takes place only once, resembling the way genomic DNA is replicated in vivo. The occurrence of different kinds of replication intermediates was shown by electron microscopy, and the fate of the template DNA during the reaction was clarified. I also demonstrated that only a specific form of DNA can serve as substrate and that the products of the reaction can be separated by differential digest in a reasonable manner. Furthermore I was able to proof that the factors responsible for replication in vitro are the the same ones driving the reaction in the cell. It could also be demonstrated that the efficiency of the reaction depends on the cell cycle stage of the cells the protein extracts were prepared from. At last the reaction could be inhibited by depletion of ORC proteins, although the inhibition could not be reverted by the addition of recombinantly expressed ORC complexes.

In conclusion this work is a contribution towards the complete characterisation of an in vitro replication assay as a model for the replication of the genome in human cells.
A Zusammenfassung


Die Produkte der Reaktion wurden durch differenziellen Verdau voneinander getrennt. Desweiteren konnte ich beweisen, dass die Faktoren, die für die Replikation in vitro verantwortlich sind, die selben sind, die in der Zelle diese Aufgabe erfüllen. Ebenso wurde gezeigt, dass die Effizienz der Reaktion davon abhängt, in welcher Phase des Zellzyklus sich die Zellen zum Zeitpunkt der Präparation der Proteinextrakte befinden.

Abschließend konnte die Reaktion durch die Depletion von ORC-Proteinen aus den Zellextrakten inhibiert werden, wohinge gen das Aufheben der Inhibition durch die Zugabe rekombinant exprimierter ORC-Komplexe nicht gelang.

Die Arbeit hat so einen Beitrag zu der Charakterisierung eines In vitro-DNA-Replikations-Systems als Modell für die Genomreplikation in menschlichen Zellen geleistet.
B Abbreviations
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARS</td>
<td>Autonomously Replicating Sequences</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>bp</td>
<td>Base Pairs</td>
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<td>Dideoxyribonucleotide phosphate</td>
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<td>Acceleration of Gravity</td>
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<td>Minichromosome Maintenance</td>
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<td>OD</td>
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<td>ORC</td>
<td>Origin Recognition Complex</td>
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<td>TRIS</td>
<td>Tris Base, Tris (hydroxymethyl)-aminomethane</td>
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