REPAIR OF UV-INDUCED DNA LESIONS IN RIBOSOMAL GENES OF YEAST S. cerevisiae

A dissertation submitted to the
Swiss Federal Institute of Technology Zürich (ETH Zürich)

For the degree of
Doctor of Natural Sciences

Presented by

ANDREAS MEIER
Dipl. Natw. ETH, ETH Zürich
Born December 18, 1972
Citizen of Uster, ZH

Accepted on the recommendation of

Prof. Dr. Fritz Thoma, examiner
Prof. Dr. Josef Jiricny, co-examiner
Prof. Dr. Ulrich Suter, co-examiner

Zürich, 2002

Hermann Hesse (Lektüre für Minuten)
ACKNOWLEDGEMENTS

Special thanks to Fritz Thoma - for supervising and supporting this thesis. For discussions, input of new ideas and providing me with time and space to develop my attitude for science.

I thank Profs. Josef Jiricny and Ulrich Suter for support and accepting to co-examine this thesis.

I acknowledge the fellowships of the Roche Research Foundation and the Janggen-Pöhn-Stiftung, which allowed me to finish my thesis.

Yeast mutant strains, which were very helpful for this work, were kindly provided by Prof. Masayasu Nomura.

Thanks to José Sogo, Ralf Wellinger and Martine Muller for long discussions, new ideas and experimental help with the rDNA experiments.

I especially appreciate the help and experimental assistance of Magda Livingstone-Zatchej, and Christoph Capiaghi, who contributed to this project in his semester work.

Thank you to Abdou Aboussekhra, Hélène Gaillard and Georg Schnappauf who 'survived' the time with me in E43 despite the discussions and chats.

A special thank to Bernhard Suter for nice scientific discussions.

Thanks to Elisabeth Enggist for background support and encouragement with the view of a microbiologist.

Thanks to all former and present members of the Fritz Thoma and José Sogo group - and also to all the people of the Institute of Cell Biology and the friends all around, which made the lab environment just great.
TABLE OF CONTENTS

Table of Contents ................................................................................................................. I
List of Figures ....................................................................................................................... V
List of Tables ......................................................................................................................... VII
Abbreviations ......................................................................................................................... IX

Summary ................................................................................................................................. 1
Zusammenfassung ..................................................................................................................... 5

1 General Introduction 9

1.1 Organisms, DNA Damage and Repair ........................................................................... 9
1.2 DNA Damage Induction by UV Light ............................................................................. 10
1.3 DNA Damage and Chromatin ....................................................................................... 11
  1.3.1 The Nucleosome ...................................................................................................... 12
  1.3.2 UV Damage Formation in Chromatin ...................................................................... 12
1.4 DNA Damage and Transcription .................................................................................... 13
  1.4.1 Eukaryotic Transcription ......................................................................................... 13
  1.4.2 UV Damage and Transcription Initiation ............................................................... 13
  1.4.3 UV Lesions Block Transcription Elongation ......................................................... 14
1.5 Repair of UV Photoproducts ......................................................................................... 16
  1.5.1 Photoreactivation (PR) ............................................................................................ 16
    1.5.1.1 The DNA Photolyase/Blue Light Photoreceptor Family .................................... 17
    1.5.1.2 The Molecular Mechanism of Photoreactivation ............................................... 19
    1.5.1.3 Photoreactivation in Yeast ............................................................................... 20
    1.5.1.4 Photoreactivation in Chromatin .................................................................... 20
    1.5.1.5 Photoreactivation and Transcription ............................................................... 21
  1.5.2 Nucleotide Excision Repair (NER) in Yeast ............................................................. 22
    1.5.2.1 Global-Genome Repair (GG-NER) .................................................................. 24
    1.5.2.2 Transcription-Coupled Repair (TC-NER) ....................................................... 25
    1.5.2.3 NER and Chromatin ....................................................................................... 27
  1.5.3 Interaction of Photoreactivation and NER ................................................................. 28
1.6 Ribosomal Genes ...................................................................................................... 28
  1.6.1 Chromatin Structure and Transcription of Ribosomal Genes .................................. 30
  1.6.2 Silencing of RNAP-II Transcription in the Nucleolus ......................................... 31
  1.6.3 The Ribosomal Intergenic Spacer (rDNA Spacer) ............................................... 32
  1.6.4 Transcription Initiation at the 35S rRNA Gene Promoter ....................................... 33
1.7 Repair of Ribosomal Genes ...................................................................................... 34
  1.7.1 Repair of UV Lesions in Ribosomal Genes .............................................................. 34
1.8 Aim of the Work ........................................................................................................... 35
# Table of Contents

2 CPD Repair of 35S rRNA Genes in Yeast 37

2.1 Summary ..................................................................................................................... 37
2.2 Introduction .................................................................................................................... 38
2.3 Results ........................................................................................................................... 40
  2.3.1 CPD Repair in Total rDNA of AMY3 (rad1A) and W303-1a (RAD1) ................. 40
  2.3.2 Accessibility of NheI to Ribosomal Genes after UV Damage and Repair ........... 42
  2.3.3 NheI Digestion of Nuclei Releases only Open and Active rDNA ................. 45
  2.3.4 CPD Repair in Transcriptionally Active and Inactive 35S Genes .................... 47
  2.3.5 CPD Repair in the GAL10 Locus ........................................................................ 51
  2.3.6 Damage Formation in the 35S gene .................................................................... 53
2.4 Discussion .................................................................................................................... 55

3 CPD Repair in the rDNA Spacer 61

3.1 Summary ..................................................................................................................... 61
3.2 Introduction .................................................................................................................... 62
3.3 Results ........................................................................................................................... 65
  3.3.1 Chromatin Structure of the rDNA Spacer ................................................................. 65
  3.3.2 CPD Repair by Photoreactivation and NER in the rDNA Spacer ....................... 68
3.4 Discussion .................................................................................................................... 71

4 Damage Formation and Photoreactivation of the 35S Promoter 73

4.1 Summary ..................................................................................................................... 73
4.2 Introduction .................................................................................................................... 74
4.3 Results ........................................................................................................................... 78
  4.3.1 UV Photofootprinting of the 35S Promoter .......................................................... 78
    4.3.1.1 UV Photofootprinting in Yeast Strains Defective in RNAP-I Transcription .......... 78
    4.3.1.2 UV Photofootprinting in Active and Inactive 35S Promoters ....................... 82
  4.3.2 Photoreactivation in Total, Active and Inactive 35S Promoters ....................... 87
4.4 Discussion .................................................................................................................... 94

5 Concluding Remarks 99

6 Materials and Methods 103

6.1 Yeast Strains and Cultures ........................................................................................... 103
6.2 UV Irradiation and Repair .......................................................................................... 104
  6.2.1 UV Irradiation and Repair of Yeast Cells .............................................................. 104
  6.2.2 UV Irradiation and Repair of DNA in vitro .......................................................... 105
6.3 Preparation of Yeast Crude Extracts and Digestion with NheI .................................. 105
6.4 Psoralen Crosslinking of Yeast Crude Extracts ......................................................... 106
6.5 Chromatin Mapping by Micrococcal Nuclease ......................................................... 106
### Table of Contents

6.6 DNA Purification ............................................................................................................ 107
   6.6.1 DNA Purification of Yeast Cells .............................................................................. 107
   6.6.2 DNA Purification of NheI Digested Yeast Extracts ................................................. 108
   6.6.3 DNA Purification of Psoralen-Crosslinked Yeast Extracts ....................................... 108

6.7 Fractionation of rDNA .................................................................................................. 108
   6.7.1 Separation of Active and Inactive 35S rRNA Genes .............................................. 108
   6.7.2 Separation of Active and Inactive 35S Promoters ................................................. 109

6.8 DNA Fragments and Radioactive Probes ..................................................................... 109
   6.8.1 Generation of Fragments by Yeast Whole Cell PCR .............................................. 109
   6.8.2 Radioactive ds DNA Probes by Random Priming ................................................. 112
   6.8.3 Radioactive ss DNA Probes by Primer Extension ................................................ 112

6.9 Neutral Agarose Gels .................................................................................................. 113
   6.9.1 Midi, Mini Agarose Gels ....................................................................................... 113
   6.9.2 Maxi Agarose Gels ............................................................................................... 114

6.10 Psoralen Gel Retardation Assay .................................................................................. 114

6.11 Mapping of CPDs by Indirect End-Labeling ............................................................ 114
   6.11.1 Restriction of Genomic DNA .............................................................................. 114
   6.11.2 T4-endonuclease V Assay ................................................................................ 115
   6.11.3 Alkaline Gel Electrophoresis ............................................................................ 115
   6.11.4 Alkaline Southern Blot and Hybridisation of Membranes .................................. 116

6.12 Mapping of CPDs by Primer Extension .................................................................... 116
   6.12.1 5' Labeling of Oligos ....................................................................................... 117
   6.12.2 Primer Extension and Sequencing Reactions ..................................................... 117
   6.12.3 5 % Acrylamide, 42 % Urea Sequencing Gel .................................................... 118

6.13 Creating and Processing PhosphorImager Files ....................................................... 119
   6.14 Analysis of PhosphorImager Files ........................................................................... 119
   6.14.1 CPD Repair in Whole DNA Fragments .............................................................. 119
   6.14.2 Repair of Regions in Agarose Gels .................................................................. 120
   6.14.3 Damage Formation and Repair of CPD Sites from High Resolution Experiments . 120
   6.14.4 Length Determination of DNA Fragments ....................................................... 121

7 References .................................................................................................................... 123

Curriculum Vitae ............................................................................................................. 145
LIST OF FIGURES

1 General Introduction

Figure 1-1: The Two Major Photoproducts Generated by UV Light ....................................................... 11
Figure 1-2: Photoreactivation (PR) ............................................................................................................. 16
Figure 1-3: Nucleotide Excision Repair (NER) in Yeast ........................................................................... 23
Figure 1-4: Schematic Representation of the rDNA Cluster in Yeast *S. cerevisiae* .................................. 29

2 CPD Repair of 35S rRNA Genes in Yeast

Figure 2-1: rDNA Repeat of Yeast *S. cerevisiae* .................................................................................. 40
Figure 2-2: CPD Repair by Photoreactivation and NER in Yeast rDNA .................................................. 41
Figure 2-3: Release of Active rRNA Genes by NheI ................................................................................. 43
Figure 2-4: Release of Active rRNA Genes after Damage Induction and Repair ....................................... 44
Figure 2-5: Psoralen Crosslinking of Yeast Nuclei and Psoralen Gel Retardation Assay ............................ 45
Figure 2-6: Accessibility of Psoralen to Yeast rDNA .............................................................................. 46
Figure 2-7: Photoreactivation in Active and Inactive rDNA of AMY3 (rad1Δ) ........................................... 48
Figure 2-8: NER and NER+PR in Active and Inactive rDNA of W303-1a (RAD1) ................................. 49
Figure 2-9: CPD Repair in Transcriptionally Active and Inactive rDNA .................................................. 50
Figure 2-10: Photoreactivation and NER of the GAL10 Gene of AMY3 and W303-1a .............................. 51
Figure 2-11: CPD Repair in the GAL10 Gene and in Total rDNA of *S. cerevisiae* .................................... 52
Figure 2-12: Formation of CPDs in 35S Genes of AMY3 and W303-1a ..................................................... 53

3 CPD Repair in the rDNA Spacer

Figure 3-1: rDNA Spacer of Yeast *S. cerevisiae* and its Chromatin Structure ......................................... 62
Figure 3-2: Chromatin and Photoreactivation in the rDNA Spacer of AMY3 (rad1Δ) ............................. 66
Figure 3-3: Chromatin, NER and Photoreactivation in the rDNA Spacer of W303-1a ............................. 67

4 Damage Formation and Photoreactivation of the 35S Promoter

Figure 4-1: The 35S Promoter of Yeast *S. cerevisiae* ............................................................................. 75
Figure 4-2: Photofootprinting in 35S Promoter of Yeast Strains Defective in RNAP-I 
  Transcription Factors .......................................................................................................................... 79
Figure 4-3: Pyrimidine Dimer Sites in the 35S Promoter of *S. cerevisiae* ............................................. 81
Figure 4-4: Damage Formation in 35S Promoter of Yeast Strains Defective in RNAP-I 
  Transcription ........................................................................................................................................ 83
Figure 4-5: Fractionation of Active and Inactive 35S Promoters ............................................................... 84
Figure 4-6: UV Photofootprint in Active and Inactive 35S Promoters of AMY3 (rad1Δ) ....................... 86
Figure 4-7: Photoreactivation in Total 35S Promoters of AMY3 (rad1Δ) .................................................. 88
Figure 4-8: Photoreactivation in Inactive 35S Promoters of AMY3 (rad1Δ) ............................................ 89
Figure 4-9: Photoreactivation in Active 35S Promoters of AMY3 (rad1Δ) .............................................. 90
Figure 4-10: CPD Repair by Photolyase in Total, Active and Inactive 35S Promoters ............................ 92
6 Materials and Methods

Figure 6-1: Fragments in rDNA Cluster of \textit{S. cerevisiae} .................................................................111
Figure 6-2: Fragment in GAL Gene Cluster of \textit{S. cerevisiae} ..............................................................111
Figure 6-3: Quantification of CPDs and CPD Repair in Whole DNA Fragments ......................................120
Figure 6-4: Quantification of CPD Repair in Regions of Agarose Gels .................................................121
Figure 6-5: Quantification of Site-Specific CPD Repair from Primer Extension Analysis ................122
LIST OF TABLES

1 General Introduction
Table 1-1: DNA Photolyase/Blue Light Photoreceptor Family ......................................................... 18
Table 1-2: Yeast and Human NER Proteins ......................................................................................... 22

2 CPD Repair of 35S rRNA Genes in Yeast
Table 2-1: Formation of CPDs in 35S rRNA Genes of W303-1a .......................................................... 54
Table 2-2: Formation of CPDs in 35S rRNA Genes of AMY3 .............................................................. 54

6 Materials and Methods
Table 6-1: Yeast Strains Used in this Study ..................................................................................... 103
Table 6-2: Reaction Mix for Yeast Whole Cell PCR ........................................................................... 110
Table 6-3: Oligos for Generation of Templates .................................................................................... 110
Table 6-4: DNA Fragments in rDNA Cluster and GAL10 Gene of S. cerevisiae .................................. 112
Table 6-5: Primer Extension Reaction for Generation of ss DNA Probes ........................................... 113
Table 6-6: Oligo for Primer Extension Analysis .................................................................................. 117
Table 6-7: Primer Extension Reaction for Mapping of UV Lesions .................................................... 117
Table 6-8: Sequencing Reactions ......................................................................................................... 118
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom, $10^{-10}$ metres</td>
</tr>
<tr>
<td>ADE</td>
<td>adenine</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomously replicating sequence</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine ribonucleotide triphosphate</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>CF</td>
<td>core factor of yeast RNAP-I transcription machinery</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CPD</td>
<td>$cis$-$syn$ cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>CRY</td>
<td>cryptochrome gene, coding for putative blue light photoreceptors</td>
</tr>
<tr>
<td>CS</td>
<td>Cockayne syndrome</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine ribonucleotide triphosphate</td>
</tr>
<tr>
<td>DI</td>
<td>dichloromethane-isoamylalcohol (24:1)</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded (DNA)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Eubacterium Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>f.c.</td>
<td>final concentration</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GG-NER</td>
<td>global-genome nucleotide excision repair subpathway</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine ribonucleotide triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>8-HDF</td>
<td>8-hydroxy-5-deazaflavin</td>
</tr>
<tr>
<td>HIS</td>
<td>histidine</td>
</tr>
<tr>
<td>KOAc</td>
<td>potassium acetate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>LEU</td>
<td>leucine</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milli ampère</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>m</td>
<td>milli, $10^{-3}$</td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methane sulfonate</td>
</tr>
<tr>
<td>MNase</td>
<td>micrococcal nuclease</td>
</tr>
<tr>
<td>MTHF</td>
<td>5,10-methenyltetrahydrofolate</td>
</tr>
<tr>
<td>µ</td>
<td>micro, $10^{-6}$</td>
</tr>
<tr>
<td>n</td>
<td>nano, $10^{-9}$</td>
</tr>
<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NEF</td>
<td>nucleotide excision repair factor</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NIB</td>
<td>nuclear isolation buffer</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NTS</td>
<td>non-transcribed DNA strand (coding strand)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
</tbody>
</table>
Abbreviations

ON          overnight
p           pico, $10^{-12}$
pH          potential hydrogenii, concentration of $[H_3O^+]$
PR          photoreactivation
rpm         revolutions per minute
RNA         ribo nucleic acid
RNAP        RNA polymerase
rRNA        ribosomal RNA
RT          room temperature
*S. cerevisiae*  budding yeast *Saccharomyces cerevisiae*
SDS         sodium dodecyl sulfate
ss          single-stranded (DNA)
T           thymine
T4endoV     T4-endonuclease V (from bacteriophage T4)
TBP         TATA-box binding protein
TC-NER      transcription-coupled nucleotide excision repair subpathway
TMP         trimethyl-psoralen
tRNA        transfer RNA
TS          transcribed DNA strand (template strand)
10T1E       10 mM Tris 1 mM EDTA
TRP         tryptophan
TTD         trichothiodystrophy
TTP         thymidine ribonucleotide triphosphate
U           units
UAF         upstream activating factor of yeast RNAP-I machinery
UAS         upstream activation sequence
UV          ultraviolet
(6-4) PP    pyrimidine-pyrimidone (6-4) photoproduct
V           volt
w/o         without
wt          wild-type
XC          xylene cyanol
XP          Xeroderma pigmentosum
YPD         yeast growth medium containing glucose
YPG         yeast growth medium containing galactose
SUMMARY

DNA lesions induced by the UV component of solar radiation block basic cellular functions such as transcription and replication. All organisms have molecular repair systems to remove UV lesions preventing cell death, mutations and cancer. Cyclobutane pyrimidine dimers, CPDs, and pyrimidine (6-4) pyrimidone photoproducts, (6-4) PPs, are the major classes of UV lesions. In many organisms, UV lesions are repaired by nucleotide excision repair (NER) and photoreactivation. NER is a complex pathway, which is fast in repair of transcribed strands of genes transcribed by RNA polymerase II (RNAP-II), because RNAP-II stalled at UV lesions promote repair (transcription-coupled NER, TC-NER). Photoreactivation is a one-enzyme pathway in which photolyase reverts the lesion in a light-dependent reaction. Photoreactivation is inhibited on transcribed strands of active RNAP-II genes, presumably by RNAP-II stalled at the lesion. In addition to the interference of repair with transcription, NER and photoreactivation are affected by the structural and dynamic properties of chromatin, the folding of eukaryotic DNA into nucleosomes and higher order chromatin structures.

The nucleolus is the factory of ribosome synthesis and harbours the multicopy ribosomal genes (rRNA genes, rDNA). In budding yeast, one rDNA repeat consists of the 35S rRNA gene, coding for the 35S rRNA precursor, and the intergenic spacer (rDNA spacer). The 35S gene is transcribed by a specialized RNA polymerase, RNAP-I. Only a fraction of the 100 to 200 tandemly-repeated rDNA copies are transcribed. Active genes are covered by RNAP-I and are free of nucleosomes. The other fraction is transcriptionally repressed and packaged into nucleosomes. NER in rDNA was studied in yeast and higher eukaryotes. While NER was slow in higher eukaryotes, repair was efficient in yeast. A contribution of transcription-coupled NER was suggested for repair of yeast RNAP-I genes. Photoreactivation in the nucleolus was never studied. The aim of this project was to investigate NER and photoreactivation and their
interaction with RNAP-I in ribosomal genes of yeast *S. cerevisiae*.

In the first part of this thesis, NER and photoreactivation were analyzed in the transcribed region of the 35S rRNA genes. Yeast strains AMY3 (*rad1Δ*), which is deficient in NER, and W303-1a (*RAD1*) were irradiated with UV light and incubated for repair. Active genes were released by digestion with restriction enzymes and separated from the inactive ones. Purified DNA was cut at CPDs by T4-endonucleaseV and analyzed by indirect end-labeling. We found that photoreactivation was more efficient than NER and is therefore the predominant pathway for CPD repair. Repair in rDNA was as efficient as in the nuclear *GAL10* gene. Thus, both pathways have unrestricted access to rDNA in the nucleolus. Photoreactivation of active genes was faster than in silenced genes, which is consistent with an open chromatin structure of active genes *in vivo*. The transcribed strands of active genes were preferentially repaired by NER, indicating transcription-coupled NER in RNAP-I transcribed genes. The inhibition of photoreactivation on the transcribed strand was mild compared to RNAP-II genes and suggests different properties of RNAP-I and RNAP-II stalled at DNA lesions.

The intergenic spacer (rDNA spacer) between two 35S genes contains the promoter of the 35S gene, a 5S rRNA gene, a potential origin of replication (rARS) and an enhancer. Photoreactivation was compared with chromatin analysis by nuclease digestion. Fast and slow photoreactivation correlated with nuclease accessibility, which indicated a modulation of photoreactivation by chromatin in the spacer region. A nuclease sensitive region upstream of the 35S promoter and four positioned nucleosomes were identified between the 5S gene and the 35S promoter. Modulation of photoreactivation between the 5S gene and the enhancer provided evidence for nucleosomes in this region, although no nuclease footprints were detected. As observed in the 35S transcribed region, photoreactivation was fast and NER was inefficient. CPDs in the 35S promoter region remained unrepaired by photoreactivation, NER and the combination of both pathways, indicating that RNAP-I transcription factors were bound to the promoter and inhibit repair. The 35S promoter consists of the core- and the upstream element, which bind the core factor (CF) and the upstream activating factor (UAF), respectively. In the last part of this study, the 35S promoter was analyzed to investigate the binding properties and stability of RNAP-I transcription factors. Yeast strains were irradiated with UV light. UV photofootprinting was analyzed at nucleotide resolution by primer extension. Photofootprinting in total rDNA revealed UV photofootprints in the upstream element (‘upstream footprint’) and the core element (‘core footprint’). Yeast strains mutated in essential subunits of the core factor (CF) lost the core footprint only, strains defective in the UAF lost both the core- and the upstream footprint. This is consistent with published data that CF binding requires UAF, while UAF binds the upstream element without CF.
To investigate 35S promoters of active and inactive genes, active and inactive promoters were fractionated by restriction enzyme digestion. Both 35S promoter fractions showed the core- and upstream footprint, suggesting that CF and UAF bind all promoters \textit{in vivo}, irrespective of whether the gene is transcribed or not.

To study the stability of the transcription complexes, irradiated cells were incubated for repair, active and inactive 35S promoters were fractionated and analyzed by primer extension. No repair by photolyase was observed in the core element of active promoters indicating that CF and UAF are stabilized when the gene is transcribed. On the other hand, photoreactivation was fast in inactive promoters, which suggests a lower stability of the complexes in promoters of genes that are not transcribed. The data imply that stabilization of CF and UAF is required for initiation of RNAP-I transcription.
ZUSAMMENFASSUNG


Wie bereits im transkribierten Bereich des 35S Gens beobachtet, zeigten die Resultate im rDNA Spacer schnelle Photoreaktivierung und langsamer NER. Überraschenderweise wurden UV Schäden im 35S Promotor weder durch Photoreaktivierung noch durch NER entfernt. Dies deutete darauf hin, dass RNAP-I Transkriptionsfaktoren an den 35S Promotor binden und so die
Reparatur verunmöglichen.
Der 35S rRNA Promotor besteht aus dem Core Element und dem Upstream Element. Der Core Faktor (CF) und der Upstream Aktivations Faktor (UAF) binden an die entsprechenden Elemente. Im letzten Teil dieser Arbeit wurde der 35S Promotor analysiert, um die Bindungseigenschaften und die Stabilität dieser Faktoren in vivo zu studieren. Hefezellen wurden mit UV Licht bestrahlter und die UV Schadensbildung wurde mittels der Primer Extension Technik mit Nukleotid-Auflösung analysiert (UV Photofootprinting). Die Resultate in total rDNA zeigten UV Footprints im Upstream-Element (Upstream Footprint) und im Core-Element (Core Footprint). Stämme, die in essentiellen Untereinheiten des CF mutiert sind, verloren den Core Footprint, Stämme mit defizientem UAF verloren sowohl den Core Footprint als auch den Upstream Footprint. Dies bestätigte publizierte Daten, dass die Bindung des CF von UAF abhängig ist. Im Gegensatz dazu bindet UAF das Upstream Element ohne CF.
1 GENERAL INTRODUCTION

1.1 ORGANISMS, DNA DAMAGE AND REPAIR

DNA, the carrier of the genetic information, is constantly challenged by a wide variety of damaging agents (Friedberg et al., 1995). First, environmental agents such as ultraviolet light (UV light), ionizing radiation and numerous genotoxic chemicals cause alterations in the DNA structure. Second, products of the normal cellular metabolism, such as base-base mismatches arising during DNA replication, or reactive oxygen species, compromise DNA integrity. Finally, DNA disintegrates spontaneously under physiological conditions. DNA damages lead to a transient arrest of DNA metabolism. They may block replication, chromosome segregation and transcription, which inactivates every gene containing damage on the transcribed strand.

Repair of DNA lesions is essential to prevent cell death, chromosome aberrations or mutations, which may enhance the risk of cancer, aging or diseases (reviewed in Hoeijmakers (2001)). DNA damages have acute effects on cell-cycle progression. Cells transiently stop the cell cycle by activation of special signal transduction pathways, the cell-cycle checkpoints (Lowndes and Murguia, 2000). The arrest allows cells to activate repair systems, which remove the DNA damage before the cells resume cell-cycle progression. All cells have a wide variety of repair systems, each specialized in removal of a distinct type of lesion (Hoeijmakers, 2001).

Alternatively, cells can tolerate DNA damages. Specialized DNA polymerases were discovered that are able to bypass lesions and therefore replicate their genome irrespective of complete repair, e.g. (Nelson et al., 1996; Masutani et al., 1999b; Matsuda et al., 2000; Ishikawa et al., 2001; Washington et al., 2001), reviewed in Livneh (2001); Wang (2001). However, translesion synthesis increases the risk of mutations (Goodman and Tippin, 2000).

In eukaryotic cells, the substrate for all DNA-dependent processes is not naked DNA, but chromatin (Van Holde, 1989). Since packaging of eukaryotic DNA into nucleosomes and higher
order chromatin structures restricts DNA accessibility, all DNA-dependent processes have to
deal with the structural and dynamic properties of chromatin (reviewed in Smerdon and Thoma
(1998); Thoma (1999)).
This work focuses on DNA damages introduced by UV light and the two repair systems required
for removal of these lesions: Nucleotide excision repair (NER) and photoreactivation (PR). In
particular, this study elucidates interactions of repair with transcription and chromatin structure
in ribosomal genes of budding yeast *S. cerevisiae*, which was used as a model system for a
eukaryotic cell.

1.2 DNA DAMAGE INDUCTION BY UV LIGHT

The ultraviolet (UV) radiation spectrum has been subdivided into three wavelength bands
designated UV-A (400 to 320 nm), UV-B (320 to 290 nm) and UV-C (290 to 100 nm). Solar
UV radiation consists mainly of UV-A and UV-B, since wavelength up to 300 nm are absorbed
by the stratospheric ozone layer. Therefore, the biologically-relevant wavelengths of UV light on
the Earth’s surface probably range between 300 and 400 nm. The UV component of solar
radiation is a major source of physical damage in DNA (Friedberg et al., 1995). Formation of
photolesions is highest at 254 nm, which is close to the absorption maximum of the DNA bases.
Identical lesions are induced at longer wavelength, although at lower rates.
Most UV lesions are formed at adjacent pyrimidine sites. The two major classes of stable
UV photoproducts are cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4)
pyrimidone photoproducts (6-4) PP (Fig. 1-1). CPDs are the most abundant lesions and are
induced four to five times more frequently than (6-4) PPs (Mitchell and Nairn, 1989). Besides
CPDs and (6-4) PPs, a number of rare other lesions were identified, which account for less than
1 % of total UV lesions, such as spore photoproducts, purine dimers, pyrimidine hydrates,
thymine glycols or DNA-protein crosslinks (Sage, 1993), reviewed in Friedberg et al. (1995).
In a CPD, two adjacent pyrimidines are covalently crosslinked by the formation of a cyclobutane
ring between their 5, 6 double bonds (Fig. 1-1 A). A CPD predominantly exists in the cis-syn
form in double-stranded B-form DNA. NMR and gel retardation studies revealed that a
cis-syn CPD induces a 7 to 9° bend in duplex DNA (Wang and Taylor, 1991; Kim et al., 1995)
(Fig. 1-1 B). The yield of CPD formation in DNA is influenced by the nucleotide composition
and the sequence context: CPDs are preferentially induced at TT and significantly less at CC
(Setlow, 1966; Setlow, 1968). CPD compared to (6-4) PP formation was enhanced by UV-B
irradiation when compared with UV-C (Mitchell et al., 1992). In addition, flanking bases affect
CPD formation (Gordon and Haseltine, 1980; Mitchell et al., 1992). CPDs are extraordinarily
stable to extremes of pH and temperature (Setlow, 1966), but can be enzymatically cleaved e.g. by using T4-endonuclease V (Gordon and Haseltine, 1980; Vassylyev et al., 1995).

In a (6-4) PP two adjacent pyrimidines are linked by a covalent single bond between positions 6 and 4 (Fig. 1-1 C). This photoproduct introduces a 44° kink in the DNA double helix (Kim et al., 1995) (Fig. 1-1 D). (6-4) PP are formed between TC, CC, less frequently at TT, but not at CT sites (Lippke et al., 1981). Irradiation of (6-4) PPs with 313 nm light leads to the formation of the Dewar isomer (Taylor et al., 1988), which is biologically relevant in sunlight carcinogenesis (Mitchell and Nairn, 1989). (6-4) PPs are sensitive to hot alkali and can be cleaved by piperidine (Franklin et al., 1982). (6-4) PPs were shown to be much more mutagenic than CPDs (e.g. (Zdzienicka et al., 1992; Gentil et al., 1996; Gentil et al., 1997).

1.3 DNA DAMAGE AND CHROMATIN

In eukaryotes, the substrate for UV damage formation and repair is chromatin. DNA is packaged by histone proteins into nucleosomes, chromatin fibres and higher order structures (Van Holde, 1989).
1.3.1 THE NUCLEOSOME

DNA in chromatin is organized in arrays of nucleosomes (Kornberg, 1977), which are connected by linker DNA. Two copies each of the histone proteins, H2A, H2B, H3 and H4, form the histone octamer. 146 bp of DNA are wrapped around the histone octamer in 1.65 left-handed helical turns. The X-ray crystal structure of the nucleosome core particle at 2.8 Å resolution (Luger et al., 1997) provided detailed insight in the arrangement of the histones and the DNA and the path of the DNA helix. The amino terminal tails of the histones pass over and between the DNA superhelix and provide contact sites for neighbouring particles or sites for posttranslational modifications (Luger et al., 1997). The path of the DNA superhelix has to be distorted in order to fit onto the octamer surface (Luger et al., 1997).

The position of a nucleosome on the DNA sequence is determined by the 'translational' and 'rotational' position (Simpson, 1991; Thoma, 1992). Translational positioning describes the location of the nucleosome on the DNA sequence, rotational positioning the orientation of the double helix with regard to the octamer surface. In vitro, nucleosome positioning is dominated by the bending properties of DNA sequences (reviewed in Widom (1998)). In vivo, positioning of a nucleosome depends on a combination of DNA sequence, DNA-binding proteins and chromatin folding (Thoma, 1992). Nucleosomes are dynamic structures. Their positions can be altered in vitro (Beard, 1978; Pennings et al., 1991; Flaus and Richmond, 1998; Whitehouse et al., 1999) and in vivo (Thoma, 1986) either by nucleosome mobility (sliding of histone octamers along the DNA sequence) or by local dissociation/reassembly at a new position (Yager et al., 1989). Nucleosomes can wobble within a few base pairs (Thoma et al., 1993; Tanaka et al., 1996), but generally there seems to be a preference to maintain the rotational setting, while translational setting is less strongly enforced (Pennings et al., 1991; Buttinelli et al., 1993).

1.3.2 UV DAMAGE FORMATION IN CHROMATIN

The structure and flexibility of DNA are major determinants of UV damage formation, because extensive rotation of the neighbouring pyrimidines from their usual B-form DNA conformation is required for the formation of CPDs and (6-4) PPs (Becker and Wang, 1989b).

In naked DNA, CPD formation is higher in flexible single-stranded DNA than in double-stranded DNA and rigid DNA structures interfere with photodimerization (Becker and Wang, 1989b; Becker and Wang, 1989a; Lyamichev et al., 1990; Lyamichev, 1991). Bending of DNA towards the minor groove reduces CPD formation (Pehrson and Cohen, 1992).

DNA binding proteins disturbing the B-form DNA structure, e.g. transcription factors, affect both yields and types of DNA damage formation (Becker and Wang, 1984; Selleck and Majors,
Bending of DNA in a nucleosome modulates CPD formation. In mixed sequence nucleosomes, the CPD distribution is modulated with a periodicity of 10.3 bp (Gale et al., 1987; Pehrson and Cohen, 1992), but not in linker DNA or in unfolded nucleosomes (Pehrson, 1989; Brown et al., 1993; Pehrson, 1995). However, the CPD pattern in individual nucleosomes with a defined sequence deviates substantially from the average damage formation in mixed sequence nucleosomes (Schieferstein and Thoma, 1996).

In contrast to CPDs, (6-4) PPs were almost randomly distributed throughout the nucleosome core domain (Gale and Smerdon, 1990), but (6-4) PP were found predominantly in linker DNA (Niggli and Cerutti, 1982). This phenomenon can be explained either by preferential (6-4) PP formation in the flexible linker or be the result of altered nucleosome mobility after (6-4) PP formation (Matsumoto et al., 1995). Since damage induced distortions alter bending properties of DNA, it is conceivable that nucleosomes can change their position as a result of damage induction. Bulky lesions and strong distortions might therefore end up in linker DNA. In vitro, UV irradiated nucleosomes were shown to alter their stability (Matsumoto et al., 1994; Matsumoto et al., 1995; Mann et al., 1997). However, direct evidence for damage-induced nucleosome mobility is missing.

1.4 DNA DAMAGE AND TRANSCRIPTION

1.4.1 EUKARYOTIC TRANSCRIPTION

Eukaryotes have three RNA polymerases (RNAP) that catalyze transcription of nuclear genes (Zawel and Reinberg, 1995). Transcription of all three polymerases is tightly regulated to control cell growth and as response to environmental change (reviewed in Struhl (1995)). RNAP-II transcribes nuclear genes encoding the messenger RNAs and small nuclear RNAs. RNAP-II requires auxiliary transcription factors for correct initiation and elongation (Orphanides et al., 1996; Reinberg et al., 1998). RNAP-III is responsible for the transcription of small nuclear RNAs, the 5S rRNA and tRNAs (Zawel and Reinberg, 1995). Most transcripts of RNAP-III are very short. RNAP-I is specialized for transcription of the 35S precursor for all ribosomal RNAs except the 5S rRNA (for details, see Chapter 1.6).

1.4.2 UV DAMAGE AND TRANSCRIPTION INITIATION

Binding of transcription factors to their target sequences alters the capability of bases to form photoproducts in vivo. Several studies in E. coli, yeast and human cells report an influence of
UV damage formation with transcription factor binding (Becker and Wang, 1984; Selleck and Majors, 1987a; Pfeifer et al., 1992; Axelrod et al., 1993; Tornaletti and Pfeifer, 1995; Aboussekhra and Thoma, 1999). This suggests that this interference may affect the functional properties of the transcription complexes: Binding of transcription factors can be dramatically reduced by a CPD in the recognition sequence (Tommasi et al., 1996). UV damage inhibits binding of TFIIIA to 5S rDNA and irradiation of the TFIIIA/5S rDNA complex displaces TFIIIA (Liu et al., 1997). Furthermore, TBP selectively binds damaged DNA (Vichi et al., 1997; Coin et al., 1998), which suggests that damaged DNA can lure transcription factors away from their normal promoter sequences. In a recent study, a subunit of RNAP-II transcription factor TFIIID called TFTC was shown to bind preferentially UV damaged DNA (Brand et al., 2001). On the one hand, TFTC preferentially acetylated histone H3 in nucleosomes assembled on UV irradiated DNA and on the other, TFTC-mediated RNAP-II transcription was inhibited in damaged DNA (Brand et al., 2001). Thus, UV damaged DNA might affect transcription initiation.

1.4.3 UV Lesions Block Transcription Elongation

Inhibition of total RNA synthesis by UV irradiation is a well established phenomenon (Sauerbier and Hercules, 1978; Mayne and Lehmann, 1982). This inhibition is probably caused by RNA polymerases arrested at lesions on transcribed strands. Recent reports suggest that transcription initiation may also be involved (Rockx et al., 2000; Brand et al., 2001). UV lesions are a strong block for RNAP-II and RNAP-I on transcribed strands, but not on non-transcribed strands (Donahue et al., 1994; Selby et al., 1997; Tornaletti et al., 1997; Hara et al., 1999). In vitro, the ternary complex of the polymerase with the lesion is remarkably stable with a half life of about 20 hrs and 6 hrs for RNAP-II and RNAP-I, respectively (Selby et al., 1997; Hara et al., 1999). The footprint of RNAP-II on a CPD covers 35 to 40 bp asynchronously around the lesion (Selby et al., 1997; Tornaletti et al., 1999). The arrested complex with RNAP-II was competent to resume elongation, demonstrating that neither the polymerase nor the RNA product dissociates from the DNA template (Donahue et al., 1994).

What is the fate of blocked RNAP-II in living cells? In a yeast RNAP-II gene, the single repair enzyme photolyase was able to remove 80 % of all CPDs in two hours from transcribed strands, suggesting a half life of blocked RNAP-II of about one hour (Livingstone-Zatchej et al., 1997). Thus, RNA polymerases seem to dissociate much faster from a UV lesion in vivo than in vitro.

What are the molecular mechanisms of RNAP displacement in cells? The connection between NER and transcription suggested that NER proteins coupled with the RNAP-II machinery (reviewed in Citterio et al. (2000b), see Chapter 1.5.2.2) might be involved in this process.
However, evidence for displacement of stalled RNAP by NER proteins was not found. Recent studies indicate the involvement of proteins originally characterized as transcription factors in RNAP displacement (reviewed in Conaway and Conaway (1999)). In damaged and undamaged cells, a block or pausing of transcription elongation is a common phenomenon (Samkurashvili and Luse, 1996; Uptain et al., 1997). Transcription elongation factors are needed to overcome the block and resume transcription (Conaway and Conaway, 1999). Likewise, elongation factors may be needed to displace RNA polymerases blocked at UV lesions. Mutations in genes coding for elongation factors result in various human diseases and recent studies shed light on links between the RNAP-II elongation machinery and the pathways of DNA repair and recombination (reviewed in Conaway and Conaway (1999); Reines et al. (1999)).

Several candidate proteins may be involved in displacement of blocked RNA polymerases:

- Human CSB and its yeast homologue Rad26 have a dual function in transcription elongation (Conaway and Conaway, 1999) and transcription-coupled repair, the preferential repair of transcribed strands of active genes (see Chapter 1.5.2.2). The proteins are DNA-dependent ATPases (Guzder et al., 1996a; Selby and Sancar, 1997b) and were suggested to displace blocked RNA polymerases (Prakash and Prakash, 2000). However, direct evidence for such a role has not been demonstrated (Selby et al., 1997; Selby and Sancar, 1997a; Selby and Sancar, 1997b).

- Addition of transcription elongation factor SII to blocked RNAP-II in vitro results in increased repair of the lesion by the single repair enzyme photolyase and resumption of transcription, indicating that SII displaces RNAP-II, renders the CPD accessible for repair enzymes and allows resumption of transcription after repair (Donahue et al., 1994; Tornaletti et al., 1999). SII induces cleavage of the transcript and is not required for transcription-coupled NER (Verhage et al., 1997).

- Transcription release factor 2, found in D. melanogaster and humans, is a member of the SWI/SNF2 family and contains an ATPase activity (Xie and Price, 1997; Liu et al., 1998). It is required for RNAP-II elongation and termination and was shown to release both RNAP-I and RNAP-II stalled at CPDs in vitro (Hara et al., 1999).

In summary, elongating RNA polymerases are blocked at UV lesions in transcribed strands. In order to restore an intact gene, which can be further transcribed, RNAPs have to be displaced from the lesion, possibly by auxiliary transcription factors and/or repair proteins, before repair pathways can take care of the damaged site.
1.5 REPAIR OF UV PHOTOPRODUCTS

UV photoproducts are eliminated by three basic mechanisms: Nucleotide excision repair (NER), direct reversal by photoreactivation (PR) and base excision by UV specific endonucleases (e.g. T4-endonuclease V) (Friedberg et al., 1995). In yeast S. cerevisiae, CPDs and (6-4) PPs are repaired by NER and photoreactivation (Fig. 1-2 and Fig. 1-3).

1.5.1 PHOTOREACTIVATION (PR)

In the late 1940s, visible light was found to stimulate survival of UV irradiated strains of Streptomyces griseus (Kelner, 1949) (reviewed in Friedberg (1999); Sancar (2000b)). In the late 1950s, studies with extracts of E. coli and yeast S. cerevisiae demonstrated that this removal of pyrimidine dimers is an enzyme-catalyzed process (Rupert, 1958; Rupert, 1960). This light-dependent reversal of UV photoproducts was called photoreactivation (PR) (Friedberg et al., 1995) (Fig. 1-2). Photoreactivation was the first DNA repair pathway to be discovered. Enzymes that catalyze photoreactivation are referred to as DNA photolyases or photoreactivating enzymes (Friedberg et al., 1995). Photoreactivation is a two-step mechanism: First, a UV damage is recognized in a light independent way (dark reaction). Second, the covalently-linked pyrimidines are restored using the energy of photoreactivating blue light (300 - 500 nm) (enzyme-catalyzed monomerization). This light reaction is catalyzed by two non-covalently attached prosthetic groups, one of which is the catalytic cofactor 1,5-dihydroflavin adenine dinucleotide (FADH²), and the other a light harvesting cofactor (Sancar, 1994).

Figure 1-2: Photoreactivation (PR)

CPDs are repaired by photoreactivation (PR). The substrate for photoreactivation is damaged chromatin. Photoreactivation involves one single enzyme, CPD photolyase. In a light-independent first step, photolyase specifically binds to a CPD (dark reaction). Cleavage of the dimer requires blue light at photoreactivating wavelengths (300 to 500 nm, light reaction). After repair, the intact DNA dissociates from the enzyme. See text for details.
### 1.5.1.1 The DNA Photolyase/Blue Light Photoreceptor Family

Photolyase is a member of the DNA photolyase/blue light photoreceptor family. This protein family consists of three groups: CPD photolyases and (6-4) PP photolyases, which specifically remove CPDs and (6-4) PPs, respectively, and blue light photoreceptors (cryptochromes, CRY) (reviewed in Kanai et al. (1997); Todo (1999)). Tab. 1-1 lists an overview of the organisms known to contain DNA photolyases, (6-4) PP photolyase homologues or blue light photoreceptors. The CPD photolyase gene was first cloned in *E. coli* (Sancar and Rupert, 1978). Based on sequence homology, CPD photolyases are subdivided into class I and class II CPD photolyases with less than 20% sequence identity (Yasui et al., 1994).

Class I CPD photolyases were isolated from pro- and eukaryotic unicellular organisms and are further divided into two groups, containing either 5,10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazariboflavin (8-HDF) as the light harvesting cofactor. The *S. cerevisiae* photolyase is a MTHF class I enzyme.

Class II photolyases were found in eubacteria, archaeabacteria and higher eukaryotes, e.g. in goldfish *C. auratus* (Yasuhiira and Yasui, 1992), *D. melanogaster* (Todo et al., 1994) and the marsupial *M. domestica* (Kato et al., 1994). Besides these multicellular organisms, class II photolyases were also isolated from archaeabacteria (Kiener et al., 1989; Yasui et al., 1994) and eubacteria (O’Connor et al., 1996). Recently, a CPD class II photolyase that complements a photolyase-deficient *E. coli* strain, was found in fowlpox virus (Srinivasan et al., 2001). Furthermore, sequencing of the genomes of an entomopox virus, a rabbit fibroma virus and myxoma virus revealed putative class II photolyase genes, suggesting that enzymatic photoreactivation might be important for viruses (Afonso et al., 1999; Cameron et al., 1999; Willer et al., 1999; Afonso et al., 2000).

In the early 1990s, two important findings in the history of photolyase research were made: First, plant blue light photoreceptors from *A. thaliana* and *S. alba* were found to be structurally related to CPD photolyases, although cryptochromes function in signal transduction, not in DNA repair (Ahmad and Cashmore, 1993; Malhotra et al., 1995). Second, a new type of DNA photolyase, (6-4) PP photolyase was discovered in *D. melanogaster* (Todo et al., 1993). Genes for the (6-4) PP photolyase exhibit sequence similarity to CPD class I photolyases (Todo et al., 1996). Currently, the cDNAs of (6-4) PP photolyase have been cloned from *D. melanogaster* (Todo et al., 1996), *X. laevis* (Todo et al., 1997a) and *A. thaliana* (Nakajima et al., 1998). Additionally, (6-4) PP photolyase activity was detected in cultured goldfish cells (Uchida et al., 1997).

Are there photolyases in higher mammals and humans? The existence of photolyase in humans and other placental mammals has been a matter of debate (Ley, 1993). While several studies reported photoreactivation in human white blood cells and human skin (see e.g. (Roza et al.,
Table 1-1: DNA Photolyase/Blue Light Photoreceptor Family

<table>
<thead>
<tr>
<th>species</th>
<th>Kingdom</th>
<th>Cofactors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CPD Photolyases Class I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I / MTHF-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Sancar, 1985b; Sancar, 1985a)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Eubacterium</td>
<td>FAD, MTHF</td>
<td>(Sancar and Rupert, 1978)</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Eker et al., 1994)</td>
</tr>
<tr>
<td>Bacillus firmus</td>
<td>Eubacterium</td>
<td>FAD, MTHF</td>
<td>(Malhotra et al., 1994)</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>Eubacterium</td>
<td>FAD, MTHF</td>
<td>(Li and Sancar, 1991)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Eubacterium</td>
<td>FAD, MTHF</td>
<td>(Kim and Sundin, 2001)</td>
</tr>
<tr>
<td>Class I / 8-HDF-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anacystis nidulans</td>
<td>Eubacterium</td>
<td>FAD, 8-HDF</td>
<td>(Yasui et al., 1988)</td>
</tr>
<tr>
<td>Synechocystis sp.</td>
<td>Eubacterium</td>
<td>FAD, 8-HDF</td>
<td>(Ng et al., 2000)</td>
</tr>
<tr>
<td>Streptomyces griseus</td>
<td>Eubacterium</td>
<td>FAD, 8-HDF</td>
<td>(Kobayashi et al., 1989)</td>
</tr>
<tr>
<td>Halobacterium halobium</td>
<td>Archaebacterium</td>
<td>FAD, 8-HDF</td>
<td>(McCready, 1996)</td>
</tr>
<tr>
<td><strong>CPD Photolyases Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myxococcus xanthus</td>
<td>Eubacterium</td>
<td>FAD, 8-HDF</td>
<td>(O'Connor et al., 1996)</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum</td>
<td>Archaebacterium</td>
<td>FAD, 8-HDF</td>
<td>(Kiener et al., 1989; Yasui et al., 1994)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Petersen et al., 1999)</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Todo et al., 1994)</td>
</tr>
<tr>
<td>Carassius auratus (goldfish)</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Yasuhiro and Yasui, 1992)</td>
</tr>
<tr>
<td>Oryzias latipes (killifish)</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Yasui et al., 1994)</td>
</tr>
<tr>
<td>Scenedesmus acutus</td>
<td>Eukaryote</td>
<td>FAD, 8-HDF</td>
<td>(Jorns, 1990)</td>
</tr>
<tr>
<td>Monodelphis domestica</td>
<td>Eukaryote</td>
<td>FAD, 8-HDF</td>
<td>(Kato et al., 1994)</td>
</tr>
<tr>
<td>Potorous tridactylis (rat kangaroo)</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Yasui et al., 1994)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Ahmad et al., 1997; Kleiner et al., 1999)</td>
</tr>
<tr>
<td>Fowlpox virus</td>
<td>Viridae</td>
<td>FAD</td>
<td>(Srinivasan et al., 2001)</td>
</tr>
<tr>
<td><strong>(6-4) PP Photolyases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Todo et al., 1993)</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Todo et al., 1997a)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Nakajima et al., 1998)</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Uchida et al., 1997)</td>
</tr>
<tr>
<td>Crotalus atrox (rattlesnake)</td>
<td>Eukaryote</td>
<td>FAD</td>
<td>(Kim et al., 1996)</td>
</tr>
<tr>
<td><strong>Animal-, Plant Blue Light Photoreceptors / (6-4) PP Photolyase Homologues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Stanewsky et al., 1998; Selby and Sancar, 1999)</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Todo et al., 1996)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Kobayashi et al., 1998a)</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Kobayashi et al., 2000)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Malhotra et al., 1995)</td>
</tr>
<tr>
<td>Sinapis alba</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Batschauer, 1993; Malhotra et al., 1995)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>Eukaryote</td>
<td>FAD, MTHF</td>
<td>(Small et al., 1995)</td>
</tr>
</tbody>
</table>

1991; Sutherland and Bennett, 1995), other studies failed to detect photolyase activity in different human cell types (Li et al., 1993; Kato et al., 1994). Two cDNA clones from human as well as murine cells with high sequence homology to the *D. melanogaster* (6-4) PP photolyase
were identified (CRY1 and CRY2, see e.g. (Todo et al., 1996; van der Spek et al., 1996; Kobayashi et al., 1998a)). Both proteins were found to contain catalytic cofactors (Hsu et al., 1996), but lacked photolyase activity on the CPD and the (6-4) PP (Todo et al., 1997b) and are therefore not photolyases. Recent studies established their function as blue light photoreceptors (cryptochromes) regulating circadian rhythms (Thresher et al., 1998; Griffin et al., 1999; Kume et al., 1999; van der Horst et al., 1999; Sancar, 2000a).

Thus, photolyases occur in many pro- and eukaryotes. While some organisms such as *D. melanogaster* and *A. thaliana* have both CPD- and (6-4) PP photolyases, higher mammals including humans seem to have lost them during evolution.

### 1.5.1.2 The Molecular Mechanism of Photoreactivation

The molecular mechanism of photoreactivation has been extensively studied in the *E. coli* class I CPD photolyase (Kim and Sancar, 1993; Sancar, 1994). Photolyases contain two cofactors, FADH$^-$ as the catalytic cofactor and a second chromophore serving as a light-harvesting antenna. The second chromophore is either 5,10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazariboflavin (8-HDF), with absorption maxima of ~380 nm and ~440 nm, respectively (Sancar et al., 1987; Johnson et al., 1988). The energy is absorbed by MTHF or 8-HDF and transferred to the catalytic cofactor FADH$^-$. Splitting of the dimer is initiated by an electron transfer from the excited FADH$^-$ cofactor, donating an electron to the dimer. After the reversal of the dimer, the electron is transferred back to the FADH, regenerating catalytically active FADH$^-$ (reviewed in Hearst (1995)). Studies using time-resolved absorption spectroscopy suggests intraprotein radical transfer from excited FADH$^-$ to the dimer via several tryptophan close to the catalytic cofactor (Aubert et al., 2000).

Similar mechanisms exist in class II CPD photolyases (Aubert et al., 1999) and (6-4) PP photolyases (Hitomi et al., 1997; Zhao et al., 1997).

Detailed insight into the photoreactivation process was obtained from the crystal structure of the CPD photolyases from *E. coli* (Park et al., 1993; Park et al., 1995) and *A. nidulans* (Miki et al., 1993; Tamada et al., 1997), reviewed in Deisenhofer (2000). Both enzymes are class I photolyases. *E. coli* photolyase is of the MTHF type, while *A. nidulans* photolyase contains 8-HDF as the light-harvesting cofactor. The two structures are very similar. The FAD cofactor is accessible through a hole in the surface of the protein. Dimensions and polarity of the hole match those of a pyrimidine dinucleotide, suggesting that the dimer bases 'flip out' of the helix to fit into this pocket. This would allow the direct contact of the UV lesion and the catalytic cofactor. Although high-quality co-crystals of photolyase with its substrate are not yet available, modelling of the enzyme-substrate complexes of *S. cerevisiae* photolyase substantiates the
'dinucleotide flipping' model (Vande Berg and Sancar, 1998). Atomic force microscopy studies of DNA bound to *A. nidulans* photolyase reported an average bending angle of 35° of the damaged DNA (van Noort et al., 1999).

### 1.5.1.3 Photoreactivation in Yeast

The *S. cerevisiae* class I CPD photolyase is encoded by the *PHR1* gene (Schild et al., 1984; Sancar, 1985b). The enzyme has an overall sequence homology of 36.5 % to *E. coli* photolyase and contains MTHF as the light-harvesting cofactor (Sancar, 1985a; Sancar et al., 1987). Yeast *PHR1* and the *E. coli* photolyase gene can cross-complement mutant strains of both organisms that are deficient in photoreactivation (Langeveld et al., 1985; Sancar, 1985a). There are about 250 to 300 molecules of photolyase per yeast cell under constitutive conditions (Yasui and Laskowski, 1975). No indication for a (6-4) PP photolyase has been found.

The expression of the *PHR1* gene is induced by DNA damaging agents (reviewed in Sancar (2000b)). 5- to 10-fold induction was observed after treatment with UV light or alkylating agents and up to two-fold induction was measured after bleomycin treatment or heat shock at 37°C (Sebastian et al., 1990). Thus, it seems likely that *PHR1* expression is regulated by a global damage response pathway. Deletion and mutation analyses have shown three transcriptional regulatory elements (Sancar et al., 1995): The upstream activation sequence (UAS*PHR1*) is required for both basal level and damage-induced expression. The upstream repression sequence (URS*PHR1*) was identified as the binding site for a DNA damage responsive repressor called Prp (Sebastian and Sancar, 1991), and UES*PHR1* is required for an efficient damage response of *PHR1* expression. Ume6, Gis1 and Rph1 were shown to bind to the *PHR1* promoter and de-repress *PHR1* expression in a damage-responsive way (Sweet et al., 1997; Jang et al., 1999).

### 1.5.1.4 Photoreactivation in Chromatin

As nucleases or transcription factors, photolyase has to deal with chromatin as substrate in eukaryotic cells (see Chapter 1.3.1). Chromatin may interfere with photoreactivation at the level of damage recognition. CPD accessibility and repair in nucleosomes were tested *in vitro* using reconstituted nucleosomes as model substrates and two damage-specific enzymes, T4-endonuclease V (T4endoV) and *E. coli* DNA photolyase. T4endoV is a CPD specific enzyme isolated from bacteriophage T4 (Radany and Friedberg, 1980), which binds the lesion by a 'flip-out' mechanism similar to photolyase, and generates a single-strand cut 5’ from the CPD (Gordon and Haseltine, 1980; Vassylyev et al., 1995). T4endoV and photolyase were very efficient in naked DNA, but their activity was reduced dramatically on the surface of the reconstituted nucleosomes (Schierstein and Thoma, 1998; Kosmoski and Smerdon, 1999). Thus, packaging of DNA into nucleosomes restricts accessibility of repair enzymes to lesions.
In contrast to the severe inhibition on the nucleosome surface \textit{in vitro}, photolyase finds access to CPDs in nucleosomes \textit{in vivo} (reviewed in Thoma (1999)). In yeast minichromosomes, photolyase is fast in linker DNA and nuclease-sensitive regions such as promoters, 3’ ends of genes or ARS1 (Suter et al., 1997; Suter et al., 2000b). On the other hand, lesions that were mapped inside nucleosomes were removed more slowly (Suter et al., 1997). A detailed high resolution analysis of CPD repair in six nucleosomes covering the \textit{URA3} gene demonstrated slow repair of lesions around the dyad axis of nucleosomes and faster repair towards both ends (Suter and Thoma, unpublished). This modulation of repair \textit{in vivo} can be explained by mobility of nucleosomes along the DNA, which is consistent with the observation of multiple nucleosome positions in the \textit{URA3} gene (Tanaka et al., 1996). Alternatively, instability of nucleosomes by dissociation/reassembly with the help of chromatin remodelling factors or a partial unfolding of the DNA ends could enhance damage accessibility. Photolyase was proposed to serve as a model for damage recognition in chromatin. Since the tight modulation of photolyase accessibility to chromatin \textit{in vivo} matches the accessibility of micrococcal nuclease to chromatin \textit{in vitro}, photolyase is a molecular tool to measure DNA accessibility and structural and dynamic properties of chromatin in living cells (Livingstone-Zatchej et al., 1999; Suter et al., 1999), reviewed in Thoma (1999).

1.5.1.5 Photoreactivation and Transcription

RNA polymerases are blocked at UV lesions on transcribed strands of active genes, but they can bypass lesions on the non-transcribed strand (Selby and Sancar, 1990; Donahue et al., 1994). Are blocked RNA polymerases a barrier for photoreactivation? \textit{In vitro}, a blocked human RNAP-II was shown to inhibit access of photolyase to CPDs (Donahue et al., 1994). This inhibition of photoreactivation was reduced if the transcription elongation factor SII was present (Tomaletti et al., 1999).

\textit{In vivo}, the transcribed strands were repaired more slowly by photolyase than the non-transcribed strands of yeast genes transcribed by RNAP-II and RNAP-III. In inactive genes, both strands were repaired at similar rates (Livingstone-Zatchej et al., 1997; Suter et al., 1997; Aboussekhra and Thoma, 1998). This indicates that stalled RNAP-II and III protect lesions and inhibit repair by photolyase in living cells. However, despite the inhibition of photoreactivation compared to the non-transcribed strand, photoreactivation is efficient in two hours (Livingstone-Zatchej et al., 1997). Thus, blocked RNAPs must either fall off or step back from the lesion site.
1.5.2 NUCLEOTIDE EXCISION REPAIR (NER) IN YEAST

Besides photoreactivation, UV lesions can be removed by nucleotide excision repair (NER), a repair pathway that is conserved from prokaryotes to eukaryotes including humans. NER is a multistep mechanism involving about 30 proteins, which remove a wide class of DNA double helix distorting lesions, including CPDs and (6-4) PPs (reviewed in Sancar (1996a); de Laat et al. (1999); Wood (1999); Prakash and Prakash (2000)).

Detailed studies in yeast *S. cerevisiae* and human cells have elucidated the molecular mechanisms underlying NER in eukaryotes (reviewed in de Laat et al. (1999); Prakash and Prakash (2000)) (see Fig. 1-3).

In yeast, genes involved in NER belong to the *RAD3* epistasis group that can be subdivided into two classes (Prakash and Prakash, 2000). The essential genes *RAD1, RAD2, RAD3, RAD4, RAD10, RAD14* and *RAD25* confer a very high sensitivity to UV light and to other DNA damaging agents. *RAD7, RAD16* and *MMS19* cause a moderate degree of sensitivity. Homologues of all essential yeast proteins have been identified in humans (Tab. 1-2).

**Table 1-2: Yeast and Human NER Proteins**

<table>
<thead>
<tr>
<th><em>S. cerevisiae</em> gene</th>
<th>Human gene</th>
<th>Biochemical activities in yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RAD7</em></td>
<td>Not known</td>
<td>Rad7/Rad16 complex, a DNA-dependent ATPase, binds UV damaged DNA in an ATP dependent manner</td>
</tr>
<tr>
<td><em>RAD16</em></td>
<td>Not known</td>
<td></td>
</tr>
<tr>
<td><em>RAD14</em></td>
<td>XPA</td>
<td></td>
</tr>
<tr>
<td><em>RAD4</em></td>
<td>XPC</td>
<td></td>
</tr>
<tr>
<td><em>RAD23</em></td>
<td>HR23B</td>
<td></td>
</tr>
<tr>
<td><em>RAD3</em></td>
<td>XPD</td>
<td>5’ - 3’ DNA helicase</td>
</tr>
<tr>
<td><em>RAD25/SSL2</em></td>
<td>XPB</td>
<td>3’ - 5’ DNA helicase</td>
</tr>
<tr>
<td><em>SSL1</em></td>
<td>P44</td>
<td>-</td>
</tr>
<tr>
<td><em>TFB1</em></td>
<td>P62</td>
<td>-</td>
</tr>
<tr>
<td><em>TFB2</em></td>
<td>TFIH P52</td>
<td>-</td>
</tr>
<tr>
<td><em>TFB3</em></td>
<td>MAT1</td>
<td>-</td>
</tr>
<tr>
<td><em>TFB4</em></td>
<td>P34</td>
<td>-</td>
</tr>
<tr>
<td><em>Kin28</em></td>
<td>Cdk7</td>
<td>-</td>
</tr>
<tr>
<td><em>CCL1</em></td>
<td>Cyclin H</td>
<td>-</td>
</tr>
<tr>
<td><em>RAD2</em></td>
<td>XPG</td>
<td>Rad2 nuclease cuts damaged DNA on the 3’ side of the lesion</td>
</tr>
<tr>
<td><em>RAD1</em></td>
<td>XPF</td>
<td>Rad1/Rad10 nuclease cuts damaged DNA on the 5’ side of the lesion</td>
</tr>
<tr>
<td><em>RAD10</em></td>
<td>ERCC1</td>
<td></td>
</tr>
<tr>
<td><em>MMS19</em></td>
<td>Not known</td>
<td>-</td>
</tr>
<tr>
<td><em>RAD26</em></td>
<td>CSB</td>
<td>DNA-dependent ATPase</td>
</tr>
<tr>
<td><em>RAD28</em></td>
<td>CSA</td>
<td>-</td>
</tr>
<tr>
<td><em>RFA</em></td>
<td>RPA</td>
<td>ssDNA binding</td>
</tr>
</tbody>
</table>

a. adapted from de Laat et al. (1999); Prakash and Prakash (2000). See text for references.

NER is divided in two sub pathways, global-genome repair (GG-NER) and transcription-coupled repair (TC-NER) (reviewed in Tornaletti and Hanawalt (1999); Hoeijmakers (2001)).
NER is divided in two subpathways: Global-genome repair (GG-NER) refers to repair of the genome overall, transcription-coupled repair (TC-NER) removes lesions on transcribed strands of active genes. GG-NER involves damage recognition in chromatin possibly by Rad7/Rad16 and Rad4/Rad23, open complex formation and lesion verification by TFIH, Rad14, Rfa and double incision by the two endonucleases Rad2 and Rad10/Rad1. After removal of the oligo, the gap is filled by DNA repair synthesis and ligation. Chromatin is regenerated. In TC-NER, the damage is sensed by blocked RNA polymerase II, which is removed possibly by Rad26. All further steps as described in GG-NER. Scheme adapted from de Laat et al. (1999); Thoma (1999).
GG-NER refers to repair in non-transcribed parts of the genome, TC-NER refers to preferential repair of the transcribed strand of active genes, where RNA polymerase II stalled at a DNA lesion may serve as damage sensor and promote NER (Citterio et al., 2000b).

1.5.2.1 Global-Genome Repair (GG-NER)

Global genome repair (GG-NER) refers to repair in non-transcribed parts of the genome (de Laat et al., 1999) (Fig. 1-3).

The yeast Rad7/Rad16 was proposed to search chromatin for DNA damages (Guzder et al., 1997; Guzder et al., 1998b). The Rad4-Rad23 complex and its mammalian homologue, XPC-hHR23B, act as damage sensor as well (Guzder et al., 1998a; Jansen et al., 1998; Sugasawa et al., 1998). Next, the general transcription factor TFIIH containing the helicases Rad3 and Rad25/Ssl2 is recruited and mediates strand separation at the site of the lesion (Deschavanne and Harosh, 1993; Guzder et al., 1994). Rad14 (Guzder et al., 1993) and Rfa (RPA) verify and stabilize the opened DNA complex and position the endonuclease Rad2, which cuts the DNA 3’ from the lesion (Habraken et al., 1993), followed by 5’ incision by the Rad1/Rad10 complex (Tomkinson et al., 1993). After removal of the oligonucleotide containing the lesion, general replication factors fill in the remaining gap and close it.

It has been suggested that NER factors occur as a pre-assembled repairosome (Svejstrup et al., 1995). However, there is no good biochemical evidence, since most of the factors fail to remain associated even under relatively mild purification conditions (Guzder et al., 1996b). In yeast, the NER proteins are part of tightly-associated multisubunit complexes that can be purified intact (Prakash and Prakash, 2000). Interacting NER proteins have been termed nucleotide excision repair factors (NEFs) (Prakash and Prakash, 2000): Rad1/Rad10/Rad14 (NEF1), Rad4/Rad23 (NEF2), Rad2/TFIIH (NEF3) and Rad7/Rad16 (NEF4).

Since the Rad7/Rad16 homologue has not yet been identified in humans, the order of assembly of the human incision complex has been deduced in the absence of this factor. It has been suggested that XPC/hHR23B is the initial damage sensor (Sugasawa et al., 1998; Sugasawa et al., 2001; Volker et al., 2001). An alternative assembly pathway with XPA and RPA as the initial damage sensing factors was proposed (Wakasugi and Sancar, 1999; Wakasugi et al., 2001b). Thus, the order of assembly of the NER proteins is still controversial.

Unexpected links of NER to other mechanisms were reported: First, an interaction with the yeast proteasome was discovered. Rad23 has a ubiquitin-like domain (Watkins et al., 1993) that interacts with the proteasome (Schauber et al., 1998). This indicates a possible involvement of the proteasome in NER (Russell et al., 1999; Ortolan et al., 2000). Second, replication factor
ABF1 was shown to bind to Rad7/Rad16 and to be involved in NER \textit{in vitro} and \textit{in vivo} (Reed et al., 1999).

Defective NER in humans is associated with three human diseases: Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (reviewed in de Boer and Hoeijmakers (2000)). XP patients show photosensitivity and predisposition to skin cancer. Seven XP complementation groups have been identified, representing distinct NER genes XP-A to XP-G (Tab. 1-2). Patients of the XP-V complementation group have XP syndromes, but are defective in specialized polymerases involved in replicative bypass of lesions (Masutani et al., 1999a; Livneh, 2001; Wang, 2001). CS is associated with photosensitivity as well as severe neurological, developmental, and premature aging features, based on a specific defect in transcription-coupled repair. TTD shares many symptoms with Cockayne syndrome, but with the additional characteristics of brittle hair, nails and scaly skin (de Boer and Hoeijmakers, 2000).

**1.5.2.2 Transcription-Coupled Repair (TC-NER)**

Transcription-coupled NER (TC-NER) refers to the preferential repair of transcribed strands of active genes (reviewed e.g. in Friedberg (1996); Sancar (1996a); Wood (1996); Hanawalt (1998); Tornaletti and Hanawalt (1999); Citterio et al. (2000b)). Faster repair of the active \textit{DHFR} gene than in genome overall was observed in CHO cells (Bohr et al., 1985). Preferential repair of the transcribed strand over the non-transcribed strand was originally found in the mammalian \textit{DHFR} gene (Mellon et al., 1987) and later in \textit{E. coli} (Mellon and Hanawalt, 1989) and yeast (Smerdon and Thoma, 1990; Leadon and Lawrence, 1992; Sweder and Hanawalt, 1992; Livingstone-Zatchej et al., 1997). TC-NER was observed in eukaryotic genes transcribed by RNAP-II. No TC-NER was observed in RNAP-III transcribed genes of yeast and mammals (Dammann and Pfeifer, 1997; Aboussekhra and Thoma, 1998). Controversial results were found for RNAP-I transcribed genes: While repair in mammalian cells was not strand-specific (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995), repair in \textit{rad4}, \textit{rad7} and \textit{rad16} mutant yeast strains indicated strand-specific NER in RNAP-I genes (Verhage et al., 1996a) (for details, see Chapter 1.7.1).

What are the molecular mechanisms of TC-NER? In \textit{E. coli}, the \textit{mfd} gene product recruits the NER proteins to the stalled polymerase (Selby and Sancar, 1993). In eukaryotes, the NER reactions including the incision steps of NER have been reconstituted \textit{in vitro} (Aboussekhra et al., 1995). However, the TC-NER subpathway in eukaryotes is unclear, although key components were extensively characterized (Citterio et al., 2000b):

The general transcription factor TFIH (including the helicases Rad3/XPB and Rad25/XPD) is a nine subunit protein complex (Winkler et al., 1998), which plays a central role in the initiation of
RNAP-II transcription (Spangler et al., 2001), in NER (Feaver et al., 1993; Schaeffer et al., 1993; Drapkin et al., 1994) and possibly in cell cycle regulation (Feaver et al., 1994). In NER, TFIIH functions both in GG-NER and TC-NER (Feaver et al., 1993; Bardwell et al., 1994). TFIIH with an inactive XPD helicase is proficient in transcription initiation, but defective in TC-NER (Winkler et al., 2000). However, the fact that TFIIH is released after the first 30 nucleotides and subunits of TFIIH were not detected in isolated stalled elongation complexes (Zawel et al., 1995) argues against TFIIH as a direct link to TC-NER.

Mutations in CSA and CSB genes of Cockayne syndrome patients and mutations in the yeast homologue of CSB, RAD26, cause a specific defect in repair of the transcribed strand (Venema et al., 1990; Troelstra et al., 1992; van Gool et al., 1994; Tijsterman et al., 1997; Tijsterman and Brouwer, 1999). Yeast Rad26 was suggested to act as an elongation factor rendering transcription competent for TC-NER (Jansen et al., 2000). Spt4 was shown to modulate the requirement of Rad26 for TC-NER (Jansen et al., 2000). The yeast homologue of CSA, RAD28, is not required for repair of the transcribed strand (Bhatia et al., 1996). CSB is a member of the SNF2 family, has an ATPase and chromatin remodelling activity (Guzder et al., 1996a; Selby and Sancar, 1997b; Citterio et al., 2000a) and is associated with elongating RNAP-II and CSA (Henning et al., 1995; Iyer et al., 1996; van Gool et al., 1997). Recent studies suggest a role of CSB in phosphorylation and degradation of RNAP-II subunits after UV damage (Rockx et al., 2000; McKay et al., 2001).

Summarized in a model, RNAP-II stalled at a DNA lesion may serve as damage sensor and promote NER (Tornaletti and Hanawalt, 1999; Hoeijmakers, 2001). CSB/Rad26 bound to the elongating RNAP-II displaces the blocked RNA polymerase, recruits XPA and TFIIH and promotes the assembly of the repair complex. After excision and repair synthesis, RNAP-II resumes transcription.

As a new candidate involved in TC-NER, Xab2 was identified by its ability to bind XPA and was shown to interact in vitro with CSA, CSB and RNAP-II. Xab2 antibodies injected into fibroblasts inhibit transcription, TC-NER, but not GG-NER (Nakatsu et al., 2000). A yeast homologue was found, but not further characterized.

In addition to TC-NER of UV lesions, studies demonstrated preferential repair of elongation blocking lesions that are a substrate for base excision repair (BER), e.g. 8-oxoguanine (Cooper et al., 1997). Transcription-coupled BER requires several NER components, such as CSB, TFIIH, XPG, and likely CSA (Le Page et al., 2000), which implies that part of the NER machinery is borrowed for transcription-coupled BER.
1.5.2.3 NER and Chromatin

It was suggested that NER requires nucleosome remodelling activity for damage recognition and processing (Meijer and Smerdon, 1999; Moggs and Almouzni, 1999; Moggs et al., 2000). Chromatin remodelling activities involved in NER have been reported for Rad26 (Guzder et al., 1996a), ACF (Ura et al., 2001) and chromatin assembly factor I (CAF1) (Gaillard et al., 1996; Gaillard et al., 1997). However, chromatin remodelling might be involved not only in damage recognition, but also in regeneration of nucleosomes after DNA repair synthesis.

A fundamental problem of a GG-NER is to sense a wide variety of different substrates in chromatin (Batty and Wood, 2000). While blocked polymerases provide a signal for TC-NER and recruit (‘lure’) repair factors to the lesion site, damages in non-transcribed regions or on non-transcribed strands of active genes are not detected by RNA polymerases. Factors that participate in this early step of NER are the yeast Rad7/Rad16 complex and the human DDB.

The yeast Rad7/Rad16 was proposed to search chromatin for DNA damages (Guzder et al., 1998b). It is required for GG-NER, but not for TC-NER (Verhage et al., 1994; Mueller and Smerdon, 1995; Verhage et al., 1996b). Rad7 binds to Sir3, a protein involved in chromatin organization of silenced genes (Paetkau et al., 1994). Rad16 has a homology with Snf2, a protein of the SWI/SNF nucleosome remodelling complex (Schild et al., 1992). The Rad7/Rad16 complex binds UV-damaged DNA in an ATP-dependent manner (Guzder et al., 1998b; Guzder et al., 1999). This suggests a model, in which ATP hydrolysis promotes translocation of the complex on DNA in search of UV lesions (Guzder et al., 1998b; Guzder et al., 1999).

The human DNA damage binding protein DDB may be involved in the identification of lesions that are poorly recognized by the XPC-hHR23B complex (Hwang et al., 1999). Mutations in the gene of the p48 subunit of DDB were found in a subset of XP-E patients, which provided a link to the deficiency in global genomic repair of cyclobutane pyrimidine dimers (CPDs) in these cells (Rapic Otrin et al., 1998). DDB was shown to stimulate NER in vitro in concert with XPA and RPA (Wakasugi et al., 2001b) and may be involved in chromatin repair (Rapic Otrin et al., 1998; Wakasugi et al., 2001b). DDB accumulates at DNA damage sites immediately after UV irradiation and directly stimulates nucleotide excision repair in vitro (Wakasugi et al., 2001a).

What is known about NER at the nucleosome level? Nucleosomes were reported to inhibit NER in vitro (Wang et al., 1991; Hara et al., 2000; Liu and Smerdon, 2000). In vivo, GG-NER in inactive chromatin and on non-transcribed strands is modulated by nucleosomes (Smerdon and Thoma, 1990; Wellinger and Thoma, 1997; Tijsterman et al., 1999). NER is not only affected by nucleosomes, but also by other protein/DNA interactions (Tu et al., 1996; Aboussekhra and Thoma, 1998; Aboussekhra and Thoma, 1999; Suter et al., 2000b).
1.5.3 **Interaction of Photoreactivation and NER**

In yeast, photolyase is much faster than NER in repairing nucleosome-free regions, such as promoters and origins of replication, which identifies a role for photolyase in the regeneration of regulatory regions (Suter et al., 1997). In genes transcribed by RNAP-II, photolyase and NER serve complementary roles: They preferentially remove lesions in the non-transcribed and transcribed strands, respectively, and ensure the efficient repair of active genes (Livingstone-Zatchej et al., 1997). On the other hand, NER is indispensable for the removal of (6-4) PP and removes CPDs that are not accessible to photolyase.

Many organisms and tissues that are never exposed to sunlight express photolyase, suggesting a non-photoreactivating function for photolyase (Ozer et al., 1995). After UV irradiation, photolyase enhances the survival of *E. coli*, *S. cerevisiae* and *C. reinhardtii* in the dark, i.e. in the absence of photoreactivating light (Yamamoto et al., 1983; Sancar et al., 1984; Sancar and Smith, 1989; Vlcek et al., 1995), possibly by facilitating recognition of the dimer for NER enzymes. Photolyases of *E. coli* and yeast were shown to bind cisplatin intrastrand crosslinks and damages induced by alkylating agents (Fox et al., 1994; Ozer et al., 1995). Binding of photolyase to non-UV lesions stimulated NER in *E. coli* (Ozer et al., 1995). Surprisingly, photolyase bound to non-UV lesions inhibited NER in *S. cerevisiae* (Fox et al., 1994). Furthermore, UV sensitivity of *E. coli* cells was enhanced after transformation with photolyase genes from other organisms (Kobayashi et al., 1990). This suggests that the interaction of photolyase with different DNA lesions might affect NER of pro- and eukaryotes in a different way.

1.6 **Ribosomal Genes**

The nucleolus is a subcompartment of the nucleus, specialized in the biosynthesis of ribosomes, a dense, protein-crowded factory of transcription, RNA processing, ribosome assembly (Carmo-Fonseca et al., 2000) and possibly also cell cycle regulation (Cockell and Gasser, 1999b). The nucleolus is a membrane-less organelle within the nucleus, occupying approximately one third to one half of the nucleus in yeast *S. cerevisiae* (Melese and Xue, 1995). It harbours the clustered ribosomal RNA genes (rDNA) coding for the large ribosomal RNA transcripts (35S genes). RNA polymerase I (RNAP-I) is specialized in transcription of the 35S genes. The 35S rRNA precursor is subsequently processed into the ribosomal 25S, 18S and 5.8S RNAs (Venema and Tollervey, 1995; Venema and Tollervey, 1999). In yeast, the ribosomal genes are arranged in 100 to 200 copies of tandem arrays, which represent about 10% of the genome (Woolford, 1991; Chindamporn et al., 1993). 35S genes are flanked by the intergenic spacer (rDNA spacer, see...
Chapter 1.6.3), which contains an enhancer, the 5S rRNA gene, an origin of replication and the promoter for the 35S rRNA gene (Fig. 1-4).

100 to 200 copies of tandemly repeated rDNA repeats are located on chromosome XII in the nucleolus, representing about 10 % of the total yeast genome. RNA polymerase I (small grey circle with nascent rRNA) exclusively transcribes the 35S rRNA precursor. Only a fraction of the 35S rRNA genes is actively transcribed and free of nucleosomes. The other genes are silenced and packaged into nucleosomes (oval grey circles). The 35S rRNA genes are flanked by the intergenic spacer (rDNA spacer) containing an enhancer element (E), the 5S rRNA gene (5S) transcribed by RNAP-III in the opposite direction, the ribosomal origin of replication (ARS) and the 35S promoter (P). See text for details.
1.6.1 CHROMATIN STRUCTURE AND TRANSCRIPTION OF RIBOSOMAL GENES

Although rDNA is highly transcribed, accounting for up to 60% of total RNA synthesis in growing cells (Woolford, 1991), not all copies of rRNA genes are active at any given time (Warner, 1989). In exponentially-growing yeast cells, only about half of the rDNA repeats are transcribed, covered by elongating RNAP-I and free of nucleosomes (Dammann et al., 1993). The other fraction of the 35S genes is transcriptionally repressed (inactive) and maintains nucleosomes (Fig. 1-4). The rate of rRNA synthesis and the proportion of active and inactive genes is modulated in response to variations in environmental conditions in all organisms (Dammann et al., 1993; Grummt, 1999). Repressed and active rRNA genes in yeast are randomly interspersed (Dammann et al., 1995).

It is unknown how 35S genes are activated or repressed: First, transcription factors essential for RNAP-I transcription (e.g. the core factor CF or the upstream activating factor UAF) might regulate the fraction of active and inactive rDNA copies at the 35S rRNA gene promoter (see Chapter 4). Second, transcriptional regulation may include the ribosomal enhancer, which was shown to stimulate transcription of upstream and downstream flanking genes (Johnson and Warner, 1989; Morrow et al., 1993; Warner, 1998). However, the enhancer possibly increases transcription rates of active genes rather than changing the ratio of active and inactive genes (Banditt et al., 1999). Third, proteins so far not directly related to the 35S genes might control RNAP-I transcription: Reb1 was shown to bind the enhancer and ~200 bp upstream of the 35S rRNA gene promoter. It is essential for termination of transcription (Morrow et al., 1990; Reeder, 1998), but its involvement in regulation of RNAP-I transcription is unclear. Another candidate is Net1. This protein was shown to recruit the silencing factor Sir2 to the nucleolus (Straight et al., 1999) (see Chapter 1.6.2). It binds RNAP-I and was proposed to stimulate transcription (Shou et al., 2001). Thus, Net1 might be a key player involved in the regulation of active and inactive genes.

About 100 to 200 copies of the 5S rRNA gene are located in the rDNA spacer and transcribed by RNAP-III (Wolffe, 1991; Huang and Maraia, 2001). A study using different in vivo footprinting techniques determined the fraction of 5S genes associated with TFIIB to about 23 to 47% (Costanzo et al., 2001). This suggests that not all of the 5S genes are active at any given time, which stands in agreement with the regulation of transcription of the 35S genes.
1.6.2 **Silencing of RNAP-II Transcription in the Nucleolus**

Reporter genes integrated in the rDNA spacer or the 35S rRNA gene are frequently transcriptionally repressed (‘silenced’) (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; Cockell and Gasser, 1999a). A key player in this repression is Sir2 (Fritze et al., 1997; Smith and Boeke, 1997), a protein that was proposed to bind preferentially to the spacer region of the rDNA repeats in the nucleolus (Gotta et al., 1997). Sir2 homologues were found in pro- and eukaryotes (Brachmann et al., 1995; Sherman et al., 1999). Yeast Sir2p contains an ADP-ribosyltransferase activity that is essential for its silencing function (Tanny et al., 1999) and the yeast and mouse Sir2 proteins were shown to be NAD-dependent histone deacetylases, which deacetylate lysine-residues of histones H3 and H4 (Imai et al., 2000). Sir2 deletion resulted in increased psoralen crosslinking of the rDNA in vivo (Smith and Boeke, 1997) and increased accessibility of nucleases to chromatin in the rDNA spacer (Fritze et al., 1997) suggesting that Sir2 is required for its dense chromatin structure. The nucleolus competes with telomeres for the limited pool of Sir2 (Gotta et al., 1997; Smith et al., 1998) and mutations in RPD3 increased rDNA silencing by a Sir2-dependent mechanism (Smith et al., 1999). Sir2 represses not only RNAP-II transcription, but also mitotic and meiotic recombination between the rDNA genes (Gottlieb and Esposito, 1989).

Sir2 modulates chromatin structure in three different contexts: The silent (HM) mating-type loci, telomeres and rDNA (reviewed in Gartenberg, 2000; Gasser and Cockell, 2001; Moazed, 2001), (Rine and Herskowitz, 1987; Gottschling et al., 1990; Bryk et al., 1997). While silencing at the mating-type loci and telomeres is dependent on Sir2, Sir3 and Sir4 (reviewed in Gartenberg, 2000; Gasser and Cockell, 2001)), the role of Sir2 in rDNA silencing is Sir3/4 independent (Smith and Boeke, 1997). The silent mating type loci and the telomeres are localized at the nuclear periphery (Cockell and Gasser, 1999a), but it is not known, whether silenced rDNA is tethered to the nuclear periphery as well.

Interestingly, Sir2 was shown to extend longevity, providing a link to aging (Gottschling, 2000; Guarente, 2000). Is there a connection between rDNA silencing and aging? Repression of rDNA transcription was proposed to reflect a general protective mechanism preventing homologous recombination in the rDNA (Guarente, 2000). Recombination in the rDNA leads to variations of the rDNA copy number, referred to as expansion and contraction of the rDNA cluster (Pasero and Marilley, 1993; Gangloff et al., 1996; Kobayashi et al., 1998b; Oakes et al., 1999; Kobayashi et al., 2001). Aging yeast cells were shown to increase the number of rDNA copies (Sinclair and Guarente, 1997) by accumulation of extrachromosomal genes (extrachromosomal rDNA copies, ERCs), which accumulate as small circles (Kennedy et al., 1997; Sinclair and Guarente, 1997).
The selective repression of RNAP-II transcription and recombination in yeast rDNA by Sir2 depends on Net1p, a protein that recruits Sir2 to the nucleolus (Straight et al., 1999; Cuperus et al., 2000). Net1 (also known as Cfi1) was established as a key player involved in nucleolar structure and function. It not only recruits Sir2, but associates with the Cdc14 phosphatase and Nan1 to form RENT (‘regulator of nucleolar silencing and telophase exit’) (Cockell and Gasser, 1999b). This complex controls mitotic exit (Shou et al., 1999; Visintin et al., 1999), meiotic checkpoint control (San-Segundo and Roeder, 1999), nucleolar silencing (Straight et al., 1999; Cuperus et al., 2000) and localization of proteins implicated in pre-rRNA processing, methylation and ribosome assembly (Straight et al., 1999). It was reported that Net1 directly stimulates RNAP-I transcription \textit{in vivo} and \textit{in vitro} (Shou et al., 2001). Thus, Net1 is a key player in the nucleolus, which not only recruits Sir2 for establishing rDNA silencing, but is additionally involved in the regulation of transcription, cell-cycle and checkpoint control (reviewed in Garcia and Pillus (1999)).

Histone acetylation/deacetylation was proposed to contribute to repression of RNAP-II transcription in the rDNA cluster. Not only the histone deacetylase Sir2 might be involved, but also other silencing complexes such as SAS-I, CAF-I and Asf1 (Meijsing and Ehrenhofer-Murray, 2001).

### 1.6.3 The Ribosomal Intergenic Spacer (rDNA Spacer)

The rDNA spacer contains functional elements for regulation of transcription, replication and recombination of the rDNA cluster. The 317 bp ribosomal enhancer controls RNAP-I transcription initiation and termination (Lang and Reeder, 1993; Lang et al., 1994; Banditt et al., 1999) and contains HOT1, a DNA element involved in recombination (Keil and Roeder, 1984; Voelkel-Meiman et al., 1987). Furthermore, the enhancer contains the polar replication fork barrier, which selectively arrests the replication fork originating from the downstream ARS (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Gruber et al., 2000). The 5S gene coding for the 5S rRNA is transcribed by RNAP-III in the opposite direction (Venema and Tollervey, 1999). The ribosomal ARS serves as a potential origin of replication (Miller and Kowalski, 1993; Muller et al., 2000). During replication, less than one third of all rARSs are activated (Brewer and Fangman, 1988). The ~150 bp promoter of the 35S gene (35S promoter) is the transcription initiation site of RNA polymerase I (RNAP-I).

What is known about the chromatin structure of the rDNA spacer \textit{in vivo}? Evidence for irregularly-spaced nucleosomes at the 35S transcription start site was presented by Lohr (1983). Psoralen crosslinking experiments and electron microscopy studies demonstrated that the rDNA spacer is nucleosomal (Dammann et al., 1993). Non-nucleosomal and regularly packaged
enhancers were shown downstream of active and inactive 35S genes, respectively (Dammann et al., 1995). MNase footprinting- and psoralen crosslinking experiments using indirect end-labeling detected positioned nucleosomes between the 5S gene and 35S promoter and nucleosomes with multiple positions between the 3’ end of the 35S and the 5S gene (Fritze et al., 1997; Vogelauer et al., 1998; Lucchini et al., 2001). A nucleosome with multiple positions was reported to cover the entire 5S gene sequence (Buttinelli et al., 1993).

Several protein binding sites were identified. Reb1p and Abf1 were identified as enhancer binding proteins, and Reb1p has an additional binding site about 200 bp upstream of the transcription start of the 35S gene (Morrow et al., 1989; Kang et al., 1995). Footprinting studies mapped the RNAP-III transcription factor TFIIIB bound to the 5S promoter (Costanzo et al., 2001). The 35S rRNA promoter is associated with two multiprotein complexes containing RNAP-I specific transcription factors (reviewed in Nomura (1998), for details, see Chapter 4.2). Thus, the 35S promoter seems not to be packaged into nucleosomes.

Topoisomerase I is present in the nucleolus possibly to relax torsional stress during transcription and replication (Brill et al., 1987; Schultz et al., 1992) and suppress mitotic recombination (Christman et al., 1988). Cutting sites of topoisomerase I were mapped to the 35S rRNA gene promoter and the enhancer (Vogelauer and Camilloni, 1999).

### 1.6.4 Transcription Initiation at the 35S rRNA Gene Promoter

Promoter elements of the 35S gene and transcription factors required for RNAP-I transcription were studied in detail in *S. cerevisiae* (reviewed in Nomura (1998), for details see Chapter 4.2). In yeast *S. cerevisiae*, the 35S promoter consists of a core element and an upstream element. In vivo, the upstream activating factor UAF and TBP recruit the core factor CF, the Rrn3 protein and RNAP-I to the promoter (reviewed in Nomura (1998)). UAF is not only required for RNAP-I transcription, a second function is to prevent (‘silence’) transcription of rDNA by RNAP-II. Mutants with a defect in UAF give rise to variants able to grow by transcribing rDNA by RNAP-II (polymerase switch for growth, PSW) (Oakes et al., 1999; Vu et al., 1999; Siddiqi et al., 2001a). Mutants in the UAF subunit Uaf30 use mainly RNAP-I transcription, but RNAP-II transcription is not completely silenced (Siddiqi et al., 2001b).

Likewise, rDNA promoters of higher eukaryotes consist of two elements (reviewed in Paule (1998a)), a core element and an upstream element, which is called differently in some systems, e.g. upstream control element or UCE in the human system (Haltiner et al., 1986). Human and mouse rDNA transcription depends on activation factors, such as the upstream binding factor UBF and the selectivity factor SL1 (reviewed in Grummt (1998); Zomerdijk (1998)). Thus,
rDNA transcription seems to be conserved from lower to higher eukaryotes (Paule, 1998b). In higher eukaryotes, epigenetic mechanisms such as acetylation, methylation and phosphorylation of UBF or histones were shown to modulate the initiation of RNAP-I transcription (Chen and Pikaard, 1997; Pelletier et al., 2000; Hirschler-Laszkiewicz et al., 2001; Muth et al., 2001; Santoro and Grummt, 2001; Voit and Grummt, 2001). In yeast, phosphorylation of Rrn3 and RNAP-I were suggested to be required for transcription initiation (Peyroche et al., 2000; Fath et al., 2001).

1.7 Repair of Ribosomal Genes

Repair of DNA lesions in ribosomal genes is different in human, rodent and yeast cells. Efficient repair was shown for bleomycin-induced single-strand breaks in human fibroblasts (Fritz et al., 1996), MMS intrastrand adducts and cisplatin crosslinks in hamster cells (Stevnsner et al., 1993) and psoralen crosslinks in Tetrahymena thermophila (Fengquin et al., 1993). In contrast, monoadducts and interstrand crosslinks induced by psoralen were inefficiently repaired in rodent and human cells (Wauthier et al., 1990; Vos and Wauthier, 1991).

1.7.1 Repair of UV Lesions in Ribosomal Genes

Likewise, removal of UV lesions from rDNA by NER is controversial: CPD repair of total rDNA was absent in rodent cells and inefficient in human cells (Christians and Hanawalt, 1993; Stevnsner et al., 1993; Fritz and Smerdon, 1995; Balajee et al., 1999), possibly as a cause of limited access of NER enzymes to the nucleolus (Christians and Hanawalt, 1993; Balajee et al., 1999). Analysis of repair in XP cell lines and specific analysis of actively-transcribed rDNA suggested the lack of TC-NER in mammalian ribosomal genes (Christians and Hanawalt, 1994; Fritz and Smerdon, 1995). In contrast to CPDs, removal of (6-4) PP in hamster cells is comparable to repair in the DHFR gene (Balajee et al., 1999). While CPDs seem to be removed less efficiently than in the genome overall in mammalian cells, CPD repair is fast in rDNA of yeast S. cerevisiae (Verhage et al., 1996a). Preferential repair of the transcribed strand was detected in rad4, rad7 and rad16 mutant cells, which are deficient in GG-NER, indicating that TC-NER might exist in yeast. Since that study analyzed the total rDNA population and did not discriminate between actively-transcribed and silenced genes, the strand bias may not be related to transcription. A direct correlation between transcription and NER of active genes is still missing.

In contrast to NER, photoreactivation was never investigated in the nucleolus.
1.8 **AIM OF THE WORK**

Repair of UV lesions in ribosomal genes is still controversial (Chapter 1.7). It is not clear whether transcription-coupled NER exists in RNAP-I genes. The aim of this work was to study NER and photoreactivation in ribosomal genes of yeast *S. cerevisiae*. The following questions were addressed:

- How do repair enzymes access nucleolar chromatin?
- Are active and inactive rRNA genes differentially repaired?
- Is photoreactivation inhibited by stalled RNAP-I as observed in RNAP-II genes?
- Is there transcription-coupled NER in RNAP-I genes?
- How is the rDNA spacer repaired?

The approach was to expose yeast cultures to UV light. After allowing time for repair, active and inactive 35S genes were separated based on different accessibility to restriction enzymes (Müller et al., 2000). Damage formation and repair was analyzed in the different rDNA fractions at low resolution by indirect end-labeling (Smerdon and Thoma, 1990) or at nucleotide resolution using primer extension analysis (Wellinger and Thoma, 1997).

CPD repair in the 35S transcribed region was studied in Chapter 2. CPD repair in the RNAP-I transcribed region of actively transcribed or silenced 35S rRNA genes should provide further insights into transcription-coupled NER of rDNA. Repair by photolase was used as a tool to measure the stability of RNAP-I at CPDs on transcribed strands of active genes. In comparison with repair analyses in RNAP-II and RNAP-III genes, this project should complete our view on how repair enzymes access different subcompartments of the nucleus and how different eukaryotic RNA polymerases interact with damages and promote or inhibit repair.

In Chapter 3, repair of the rDNA spacer was studied at low resolution by indirect end-labeling to provide insights into repair of functional and structural elements required for high level transcription of ribosomal genes. UV light and photoreactivation were used to monitor the accessibility of chromatin in the rDNA spacer and should help identify nucleosome positions and nuclease-sensitive sites.

In Chapter 4, UV light and photoreactivation were used as a footprinting tool to assay *in vivo* binding properties of RNAP-I transcription factors. 35S rRNA gene promoters should be studied at nucleotide resolution. Analysis of 35S promoters might indicate, which factors are bound in active and inactive promoters, and reveal new insights into the stability of the transcription factors in living cells.
2 CPD REPAIR OF 35S rRNA GENES IN YEAST

2.1 SUMMARY

The nucleolus is a nuclear subcompartment containing the ribosomal RNA genes (rDNA) of which a fraction is transcribed by RNAP-I and the rest is silenced. Yeast S. cerevisiae was used to investigate how photoreactivation by photolyase (PR) and nucleotide excision repair (NER) process UV-induced cyclobutane pyrimidine dimers (CPDs) in the active and inactive fractions of rDNA. Yeast strains AMY3 (rad1Δ), which is deficient in NER, and W303-1a (RAD1) were irradiated with UV light and incubated for up to two hours for repair. Nuclei were isolated, active and inactive genes were separated by NheI digestion, and CPDs were measured in both fractions. We found that (i) photoreactivation was much more efficient than NER and therefore the predominant pathway for CPD repair; (ii) Repair in rDNA was as efficient as in the GAL10 gene. Thus, both pathways have unrestricted access to rDNA in the nucleolus; (iii) Active genes were slightly faster repaired by photoreactivation than silenced genes, which is evidence for an open chromatin structure of active genes in vivo; (iv) The transcribed strands of active genes were preferentially repaired by NER demonstrating transcription-coupled repair in RNAP-I transcribed genes; (v) There is no pronounced inhibition of photoreactivation on the transcribed strand, which stands in contrast to RNAP-II genes, and suggests different properties of RNAP-I and RNAP-II stalled at DNA lesions; (vi) Changes in the psoralen crosslinking and in the release of the active fraction by NheI suggest chromatin transitions following damage induction and repair; vii) Preferential CPD formation in inactive rDNA suggests that folding of rDNA in nucleosomes favours CPD formation.
2.2 **INTRODUCTION**

Nucleotide excision repair (NER) and photoreactivation (PR) are the major pathways to remove UV-induced DNA lesions, *cis-syn* cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4) PPs) (see Chapter 1.2).

Photoreactivation is a direct repair mechanism where a damage-specific enzyme (CPD photolyase) reverts CPDs in a light-dependent reaction (see Chapter 1.5.1). CPD photolyases were found in many prokaryotes and eukaryotes, including yeast, but not in humans (Chao, 1993; Li et al., 1993; Yasui et al., 1994; Friedberg et al., 1995; Sancar, 1996b; Todo, 1999; Sancar, 2000b). Photoreactivation in living yeast cells is modulated by chromatin structure and transcription (Thoma, 1999). Photolyase is fast in nucleosome-free regions and slow in nucleosomes (Suter et al., 1997; Suter et al., 2000a; Suter et al., 2000b). There is preferential repair of the non-transcribed strands in genes transcribed by RNAP-II and RNAP-III, whereas photoreactivation of the transcribed strands is partially inhibited by RNAP-II and RNAP-III blocked at CPDs (Livingstone-Zatcheij et al., 1997; Suter et al., 1997; Aboussekhra and Thoma, 1998).

NER is a multistep mechanism that removes a large range of DNA damages including CPDs and (6-4) PPs (see Chapter 1.5.2). NER is divided in two subpathways, global genome repair (GG-NER) and transcription-coupled repair (TC-NER) (Tornaletti and Hanawalt, 1999; Prakash and Prakash, 2000; Hoeijmakers, 2001). GG-NER refers to repair in non-transcribed parts of the genome. It is modulated by nucleosomes (Wellinger and Thoma, 1997; Tijsterman et al., 1999) and other protein/DNA interactions (Tu et al., 1996; Aboussekhra and Thoma, 1998; Aboussekhra and Thoma, 1999; Suter et al., 2000b). TC-NER preferentially repairs the transcribed strand, an observation that was originally made in mammalian and hamster cells (Mellon et al., 1987) and later in many more organisms including yeast (Smerdon and Thoma, 1990; Sweder and Hanawalt, 1992). RNAP stalled at a DNA lesion may serve as damage sensor and promote NER (Selby et al., 1997; Tornaletti and Hanawalt, 1999; Hoeijmakers, 2001). TC-NER was found in genes transcribed by RNAP-II, while genes transcribed by RNAP-III lack TC-NER in mammalian cells (Dammann and Pfeifer, 1997) and show a slight inhibition of NER in yeast (Aboussekhra and Thoma, 1998).

The nucleolus is a subcompartment of the nucleus specialized in biosynthesis of ribosomes, a dense, protein crowded factory of transcription, RNA-processing and ribosome assembly (Carmo-Fonseca et al., 2000), see Chapter 1.6). It harbours a cluster of 100 to 200 rRNA genes (rDNA) coding for the large ribosomal RNA transcripts (Venema and Tollervey, 1999). rDNA is transcribed by RNAP-I, but only a fraction of the genes is active. Active genes are free of
nucleosomes, while silenced genes maintain nucleosomes (Conconi et al., 1989; Dammann et al., 1993). In rDNA chromatin, restriction enzymes release transcriptionally-active rRNA genes, while silenced genes remain uncut (Conconi et al., 1989; Muller et al., 2000) (Fig. 2-3).

Repair of UV lesions in ribosomal genes is still not resolved. Removal of CPDs by NER was absent in rodent cells and inefficient in human cells (Christians and Hanawalt, 1993; Christians and Hanawalt, 1994; Fritz and Smerdon, 1995). (6-4) PP, however, were efficiently repaired (Balajee et al., 1999). NER of mammalian rDNA showed no strand bias and appears therefore not to be coupled to RNAP-I transcription (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995).

Repair of rDNA in *S. cerevisiae* is different. First, CPDs are efficiently removed. Since repair is absent in *rad1, rad2, rad3* and *rad14* mutants, CPDs are removed by NER. Second, experiments with *rad4, rad7* or *rad16* mutants, which compromise global genome NER, revealed preferential repair of the transcribed strand and suggested that TC-NER may exist in rDNA transcribed by RNAP-I (Verhage et al., 1996a). Since that study analyzed the total rDNA population and did not discriminate between actively-transcribed and silenced genes, it remains possible that the strand bias is not related to transcription. In contrast to NER, photoreactivation was never investigated in the nucleolus.

Here we investigated photoreactivation, NER and both pathways together in the active and silenced genes to address the following questions:

- What are the relative contributions of NER and photoreactivation to repair of UV lesions in the nucleolus?
- How do repair enzymes access DNA lesions in the nucleolus compared with other regions of the genome?
- Is RNAP-I blocked at a DNA lesion and does it inhibit photoreactivation?
- Is NER coupled to transcription by RNAP-I?
- Does UV irradiation and repair affect chromatin structure in rDNA?

Appropriate yeast strains were exposed to UV light and incubated allowing repair, the active rDNA was separated from the inactive genes by restriction digestion with *NheI* and repair was analyzed in both fractions. We found that photoreactivation is the predominant pathway for CPD repair in all situations. Moreover, we noticed that preferential repair of the transcribed strand by NER is restricted to the active rRNA genes, supporting transcription-coupled repair. In addition, we obtained evidence that active genes remain in an open conformation after damage formation and during repair.
2.3 **RESULTS**

2.3.1 **CPD REPAIR IN TOTAL rDNA OF AMY3 (rad1Δ) AND W303-1A (RAD1)**

Previous studies analyzed NER of total yeast rDNA without discrimination between active and inactive genes (Verhage et al., 1996a). Photoreactivation was never investigated in rDNA. We used the yeast strains AMY3 (rad1Δ) with an inactivated NER to analyze photoreactivation and W303-1a for analysis of NER and NER with photoreactivation. Cells were grown in YPD, irradiated in minimal medium with 150 J/m² and exposed to photoreactivating light for up to two hours at 24 to 26 °C in minimal medium supplemented with the appropriate amino acids. For NER, the cultures were incubated in the dark. DNA was isolated and cut with *Nhe*I, which generates a 4.7 kb fragment containing the non-transcribed spacer and the 5' end of the rRNA gene, and a 4.4 kb fragment of the 35S transcribed region (Fig. 2-1). To detect CPDs, the DNA was cut with T4-endonucleaseV at CPDs and the cutting sites in the transcribed region were displayed by indirect end-labeling using strand-specific probes.

A set of data is shown for AMY3 and W303-1a (Fig. 2-2 A,B). Non-irradiated DNA (-UV) and mock-treated DNA (T4-) reveal the intact restriction fragment (4.4 kb, top band). Treatment of damaged DNA with T4endoV generates a smear with some bands, which represent the distribution of pyrimidine clusters (T4+). The CPD patterns are different in the non-transcribed strand (NTS) and the transcribed strand (TS) demonstrating strand-specificity of the assay. The remaining top fragment represents the fraction of undamaged DNA. The initial damage was 0.17 ± 0.03 CPDs/kb on the non-transcribed strand and 0.2 ± 0.04 CPDs/kb on the transcribed strand. The difference reflects a difference in the pyrimidine distribution. According to the Poisson distribution, approximately 75% of the genes contain one or more CPDs per 35S gene (6.6 kb).

*Figure 2-1: rDNA Repeat of Yeast S. cerevisiae*

Schematic representation of a 9.1 kb rDNA repeat containing the 35S ribosomal RNA gene (35S) with the 35S promoter (P) and the spacer with the enhancer (E), the 5S rRNA gene (5S) and the ribosomal origin of replication (A). 4.7 kb and 4.4 kb are restriction fragments generated by *Nhe*I. Black bar: rDNA probe used for hybridization.
Figure 2-2: CPD Repair by Photoreactivation and NER in Yeast rDNA

PR in AMY3 (rad1Δ) is shown in A, NER and NER + PR in W303-1a (RAD1) in B, for the transcribed (TS) and the non-transcribed strand (NTS). Cells were grown in glucose, irradiated with UV light (150 J/m², UV+), exposed to photoreactivating light (PR) for 7, 15, 30, 60 or 120 min. or kept in the dark for 15 to 120 min. to allow NER. DNA was extracted and cut with NheI. DNA was cut at CPDs with T4endoV (T4+) or mock treated (T4-), fractionated by alkaline agarose gel electrophoresis, blotted and hybridized with strand-specific probes for the 4.4 kb fragment containing part of the 35S gene (see Fig. 2-1). Repair curves of AMY3 (rad1Δ) in C and in W303-1a (RAD1) in D; displayed as averages of three independent UV experiments and at least two gels per UV experiment. Filled symbols, TS; open symbols, NTS. Circles, PR; diamonds, NER in AMY3; squares, NER + PR; triangles, NER in W303-1a. Marker lanes (M) represent multiples of 256 bp.
Photoreactivation is the Predominant Pathway to Remove CPDs from the Nucleolus

Repair of CPDs is visualized by a time-dependent decrease of CPDs and an increase of the intact restriction fragment (Fig. 2-2 A,B). CPD repair was quantified and the average of three independent experiments is displayed (Fig. 2-2 C,D). AMY3 (rad1Δ) reveals efficient repair by photolyase on both strands. About 50% of the lesions are removed by exposing cells to photoreactivating light for 15 minutes and more than 80% of the lesions are removed in two hours. No repair is observed when cells are kept in the dark demonstrating that the NER pathway was inactivated.

Repair in the NER proficient strain W303-1a shows slow removal of CPDs by NER when cells are incubated in the dark. A substantial fraction of lesions (about 60%) remains unrepaired after two hours. In contrast, exposure of those cells to photoreactivating light shows that the combination of NER and photoreactivation very efficiently removes CPDs. 50% of the lesions are repaired in less than 15 minutes. This experiment demonstrates that both pathways repair rDNA in the nucleolus, but photoreactivation is the predominant pathway to remove CPDs from rDNA.

A Strand Bias of NER Indicates Transcription-Coupled Repair in rDNA

It was previously reported that the transcribed strand (TS) of total rDNA was slightly faster repaired than the non-transcribed strand (NTS), suggesting transcription-coupled NER (Verhage et al., 1996a). Consistent with that report, we observe preferential repair of the transcribed strand by NER (Fig. 2-2 D). The difference between the two strands is small compared with the strand bias observed in genes transcribed by RNAP-II (Livingstone-Zatchej et al., 1997). This might reflect either a small number of active genes in the population, or transcription-coupled repair might be less pronounced compared with RNAP-II transcribed genes. To address this topic, repair in active and inactive fractions was investigated separately.

2.3.2 ACCESSIBILITY OF NheI TO RIBOSOMAL GENES AFTER UV DAMAGE AND REPAIR

Psoralen crosslinking and nuclease digestion studies have established that actively-transcribed rDNA is devoid of nucleosomes, while inactive genes are packaged in nucleosomes (Dammann et al., 1993; Dammann et al., 1995; Lucchini and Sogo, 1998). It was further shown that restriction enzymes such as NheI efficiently cut in active rRNA genes of yeast, but not in the inactive nucleosomal genes (Muller et al., 2000). This approach was used to purify DNA fragments of active genes for repair analysis: Yeast cells were UV irradiated and incubated
Figure 2-3: Release of Active rRNA Genes by NheI

Each 9.1 kb rDNA repeat contains two recognition sites for the restriction enzyme NheI. In yeast nuclei, transcriptionally active genes are free of nucleosomes, covered with elongating RNAP-I (grey circles) and can be cut with NheI resulting in the release of a 4.4 kb chromatin fragment. Inactive genes are packaged into nucleosomes (open circles) and are resistant to NheI cleavage (Muller et al., 2000). Arrows indicate NheI restriction sites. Black bars represent rDNA probe.

for repair as described in Chapter 2.3.1. A nuclear extract was prepared using glass beads and digested with NheI, which liberates a 4.4 kb fragment of active rDNA (Fig. 2-3). The DNA of digested nuclei was purified, fractionated in neutral agarose gels and the rDNA fragments were identified after blotting using an rDNA probe, which hybridized to the 4.4 kb NheI fragment (Fig. 2-4 A,B). The DNA originating from inactive genes, which were resistant to NheI digestion, shows up as long DNA fragments (> 9.1 kb; inactive fraction of rDNA). The 9.1 kb fragment represents a partial digest of two adjacent active genes and was not further used for repair analysis. The fraction of active genes released by NheI, is represented by the 4.4 kb band.

Altered Chromatin Accessibility Induced by UV Irradiation and Repair

The fraction of active genes in yeast is sensitive to growth conditions (Dammann et al., 1993; Grummt, 1999). If UV lesions block transcription elongation of RNAP-I, one might expect that inactivation could lead to a reformation of nucleosomes in rDNA and make it less accessible to restriction enzymes. We therefore measured the fraction of 4.4 kb fragments released by NheI during the repair experiments. In unirradiated cells, this fraction was about 20 % (Fig. 2-4 C, D, -UV). After UV irradiation (+UV), the fraction decreased indicating that UV irradiated chromatin became less accessible to the restriction endonuclease (0 min repair). The decrease was only about 25 %, although about 75 % of the genes received at least one transcription-blocking CPD in the transcribed strand. This result illustrates that damage induction in transcribed genes altered the structure of some genes, but was not sufficient to generate a chromatin structure, which was as resistant to nuclease digestion as inactive nucleosomal rDNA.
In W303-1a, which is proficient in NER and photoreactivation, the fraction of released genes increased with increasing repair times in the presence and absence of photoreactivating light (Fig. 2-4 D). The effects were small, but they were observed in all three independent experiments. Thus, the increased release suggests that a fraction of rRNA genes opens chromatin as a consequence of repair.

In the NER-deficient AMY3 cells, no increase of the released fraction was observed during exposure to photoreactivating light (Fig. 2-4 C). Since yeast photolyase in contrast to NER does not repair (6-4) PPs, the increased accessibility to Nhel might depend on removal of these photoproducts.

**Figure 2-4: Release of Active rRNA Genes after Damage Induction and Repair**

A yeast nuclear extract was digested with Nhel, genomic DNA was fractionated on an agarose gel and the fragments were identified by Southern blotting using an rDNA probe (see Fig. 2-1). AMY3 is shown in A and W303-1a in B. The 4.4 kb fragment represents the released active rRNA gene copies, the 9.1 kb fragments are partial digests of two adjacent active genes. Fragments longer than 9.1 kb correspond to inactive genes. Uncut genomic DNA and Nhel-cut DNA is shown in A in lanes 1 and 2, respectively. The fraction of 4.4 kb fragments released by Nhel is shown for AMY3, C and W303-1a, D as the average of three independent experiments for each strain.
2.3.3  \textbf{NheI Digestion of Nuclei Releases only Open and Active rDNA}

To demonstrate that liberated genes originate from transcriptionally active genes, we used psoralen crosslinking and gel retardation assay (Fig. 2-5). Active genes with an open chromatin structure bind more psoralen than nucleosomal inactive genes, which leads to different gel retardation of the crosslinked active DNA (slow migration, s-band) and inactive DNA (fast migration, f-band) (Conconi et al., 1989; Dammann et al., 1993) (Fig. 2-5).

![Diagram of psoralen crosslinking and gel retardation assay](image)

\textit{Figure 2-5: Psoralen Crosslinking of Yeast Nuclei and Psoralen Gel Retardation Assay}

\textit{NheI}-digested nuclei (dashed circle) are crosslinked with trimethyl psoralen (\textbf{X}). Actively transcribed rRNA genes are free of nucleosomes, covered with elongating RNAP-I (small grey circles with nascent RNA) and are heavily crosslinked. Inactive genes are packed into nucleosomes (big grey circles) and are only slightly crosslinked between two nucleosomes. Purified DNA was cut to completion with \textit{NheI} (lane 3) or mock treated (lane 2), fractionated on agarose gels and identified using an rDNA probe (Fig. 2-1). Non-crosslinked, \textit{NheI}-cut DNA is shown in lane 1. In \textit{NheI}-cut nuclei (lane 2), uncut rRNA genes appear as long fragments whereas the \textit{NheI}-cut genes are heavily crosslinked (s-band) and migrate slowly. Redigestion with \textit{NheI} (lane 3) results in the appearance of the fast-migrating band (f-band). This indicates that active genes (s-band) are released by \textit{NheI} in nuclei, while inactive genes (f-band) remain uncut. See text for details and references. Adapted from (Lucchini and Sogo, 1998).

This approach was used to characterize the chromatin structure of the released fragments in \textit{NheI}-digested nuclei (Fig. 2-4). \textit{NheI}-digested nuclei were heavily crosslinked with trimethyl-psoralen. The purified DNA was cut with \textit{NheI} to completion or mock treated, fractionated on neutral agarose gels and, after blotting, the fragments were identified using an rDNA probe, which hybridizes to the 4.4 kb \textit{NheI} fragment (Fig. 2-1). A set of data for AMY3 (\textit{rad1}A) and for W303-1a (\textit{RAD1}) is shown in Fig. 2-6 A and B, respectively.
Lanes 3 in AMY3 and W303-1a show crosslinked DNA isolated from NheI-treated nuclei and redigested to completion with NheI. Two bands (s, f) are observed at 4.4 kb that are shifted with respect to uncrosslinked DNA (lanes 1). The intensities of both bands illustrate that about 40 % of total rDNA was in an open and active conformation (s-band), while the major fraction (about 60 %) was inactive and packaged into nucleosomes (f-band). Dammann et al. (1993) found that the fraction of active genes in yeast is sensitive to growth conditions. About 40 % active rRNA genes were active in exponentially-growing cells in complex medium, while about 30 % active genes were found in minimal medium. Cells in this work were grown in complex YPD medium.

**Figure 2-6: Accessibility of Psoralen to Yeast rDNA**

A AMY3 (rad1Δ). B W303-1a (RAD1). Nuclei were digested with NheI and crosslinked with trimethyl-psoralen. The DNA was analyzed by agarose gel electrophoresis and Southern blotting using the rDNA probe (Fig. 2-1) (even lanes). An aliquot of DNA was cut to completion with NheI (odd lanes except lane 1) prior to analysis. Unirradiated cells (-UV), after damage induction (+UV) and after repair for 7 to 120 minutes. A, PR, B, NER and NER+PR. Non-nucleosomal DNA of active genes migrates slowly (s-band), nucleosomal DNA of silenced genes migrates fast (f-band). Non crosslinked 4.4 and 9.1 kb rDNA fragments in lanes 1.
Thus, our results of 40% active genes correspond to the published results (Dammann et al., 1993). Lanes 2 show DNA fragments crosslinked after NheI digestion of nuclei. Only the s-band is visible at 4.4 kb, demonstrating that only DNA of active genes was released by NheI digestion. The released active rDNA (lanes 2) represents about 65% of total active rDNA (s-band in lanes 3). Since some 25% of active rDNA is found in the partial digests (9.1 kb band in lanes 2), we estimate that the inactive rDNA (>9.1 kb) contains less than 10% of the active genes.

Lanes 4 to 17 in Fig. 2-6 A and lanes 4 to 25 in Fig. 2-6 B shows rDNA after damage formation and repair. Odd lanes show the total fraction of active (s) and inactive (f) rDNA. Active rDNA remains a minor fraction throughout the experiment. Even lanes show the products of the NheI-digested nuclei. No f-bands were detected, which demonstrates that only active and no inactive rDNA was released by NheI digestion.

Fig. 2-6 reveals some subtle differences in psoralen crosslinking, which might suggest that the chromatin structure changes after damage induction and during repair. First, while DNA released by NheI from non-irradiated cells (-UV) showed a heavily crosslinked band typical for active genes (lanes 2), the DNA released from irradiated cells (+UV) revealed a broad band reflecting a heterogeneous population of crosslinked material (lanes 4). This indicates that a fraction of the genes is less accessible to psoralen and might have been partially refolded in nucleosomes.

However, it is important to realize that UV irradiation did not result in a complete reformation of nucleosomes on transcribed genes. Second, psoralen crosslinking at different repair times indicates that the slow band characteristic for open genes is regenerated to some extent during repair. This is observed for photoreactivation alone (Fig. 2-6 A, even lanes 6 - 14), NER (Fig. 2-6 B, even lanes 6 - 14) and NER with photoreactivation (Fig. 2-6 B, even lanes 16 - 24), but not in the absence of repair (Fig. 2-6 A, lane 16). Thus, the psoralen crosslinking data imply that structural transitions occur in chromatin as a consequence of UV irradiation and repair.

### 2.3.4 CPD Repair in Transcriptionally Active and Inactive 35S Genes

For analysis of DNA damage and repair, the 4.4 kb fragment of the active fraction and the longer fragments of inactive rDNA (>9.1 kb) were purified from preparative low melting agarose gels (as shown in Fig. 2-4), redigested to completion with NheI, and analyzed as described in Chapter 2.3.1. The active fraction contains only active (open) genes (see above), while the inactive fraction may still contain DNA of a few active genes. Fig. 2-7 and Fig. 2-8 show representative sets of data for AMY3 and W303-1a, respectively, and the quantifications are presented in Fig. 2-9.
Photoreactivation is the Predominant Pathway of CPD Repair in Active and Inactive rRNA Genes

Analysis of the non-transcribed strand, which is not affected by stalled RNAPs, revealed that the active fraction was faster repaired by photolyase than the silenced genes or total rDNA. Inactive rDNA was repaired more slowly by photolyase than the active fraction. This suggests that chromatin structure remains open during repair in active genes, but is more compact in inactive genes, which may explain the difference in repair rates.

**Enhanced Repair in Active Genes Implies that Chromatin Structure Remains Open During Repair**

Analysis of the non-transcribed strand, which is not affected by stalled RNAPs, revealed that the active fraction was faster repaired by photolyase than the silenced genes or total rDNA. In
15 minutes, photolyase alone removed about 68 ± 14 % of CPDs from the active genes and 45 ± 7 % from the silenced genes (Fig. 2-9). NER and photoreactivation together also remove about 66 ± 9 % and 52 ± 3 % from active and inactive genes, respectively, which demonstrates that the contribution of NER to CPD repair is minimal in the first few minutes. The difference in repair of both fractions by NER alone is less pronounced: In two hours, NER removes 46 ± 6 % of the CPDs in the active fraction (non-transcribed strand) and 40 ± 8 % in the silenced fraction. Thus, the repair results provide direct in vivo evidence that the active genes are in an open conformation in living cells and remain preferentially accessible after damage induction and
CPD repair results are shown for the transcriptionally active fraction (A, B) and the inactive fraction (C, D) of AMY3 and W303-1a. PR: Photoreactivation; NER: Nucleotide excision repair. Open symbols: non-transcribed strands; filled symbols: transcribed strands. The curves are averages of three independent UV experiments and at least two gels per UV experiment.

during repair. In contrast, repair of the inactive genes is partially inhibited, presumably due to their packaging into nucleosomes.

A Strand Bias in Repair of Active Genes is Consistent with TC-NER and an Inhibition of Photolyase by RNAP-I

NER shows preferential repair of the transcribed strand in the active fraction. No strand bias was observed in the inactive fraction (Fig. 2-9 B, D). This is an indication that RNAP-I promotes repair of CPDs by NER in the transcribed strands, a phenomenon called transcription-coupled repair, TC-NER. On the other hand, photoreactivation is slightly slower in the transcribed strand and again, this strand bias was not measured in the inactive fractions (Fig. 2-9 A, C), suggesting that RNAP-I might inhibit CPD repair by photolyase. The strand bias of photoreactivation and NER was small, but it was reproducibly observed in all three independent experiments. The error bars (Fig. 2-9) obtained by averaging the individual data points of all independent experiments.
overlap, since the absolute repair values were slightly different in the different UV experiments, but the relative values (e.g. NTS vs TS) were not changed. Summarizing the results of fractionated rDNA (Fig. 2-7, Fig. 2-8 and Fig. 2-9) and total rDNA (Fig. 2-2), we conclude that there is a contribution of transcription-coupled repair by NER to DNA repair of rDNA and that photolyase is inhibited by RNAP-I.

2.3.5 CPD Repair in the GAL10 Locus

Having observed efficient repair in the nucleolus, we investigated how nucleolar repair compares with repair of a genomic locus outside of the nucleolus. We therefore analyzed photoreactivation (AMY3) and NER (W303-1a) of the nuclear GAL10 gene. The GAL10 gene was chosen, since it

---

**Figure 2-10: Photoreactivation and NER of the GAL10 Gene of AMY3 and W303-1a**

A Indicated are the GAL7, GAL1 and GAL10 genes, relevant restriction sites, the DNA segment used for generation of strand-specific probes (black bar) and a size marker (open box).

B Photoreactivation of GAL10 gene in AMY3 (rad1Δ) on transcribed (TS) and non-transcribed strand (NTS).

C NER of GAL10 gene in W303-1a (RADJ) on TS and NTS. Experiments were done as for rDNA repair in glucose where the GAL10 gene is repressed and packaged into nucleosomes (Cavalli and Thoma, 1993). Repair was analyzed in the 1.6 kb *EcoRI / SalI* fragment by indirect end labeling as described in Chapter 2.3.1.

---
is repressed and packaged into nucleosomes, when cells are grown in glucose (Cavalli and Thoma, 1993).

**Unrestricted Access of Repair Enzymes to the Nucleolus**

UV experiments and repair analysis were done as described in Chapter 2.3.1 and CPD repair was measured in the 1.6 kb SalI/EcoRI fragment containing the major part of the nuclear GAL10 gene (Livingstone-Zatchej et al., 1997). A representative example of photoreactivation of GAL10 is shown in Fig. 2-10 B, of NER in Fig. 2-10 C. Repair is visualized by a time-dependent decrease of T4endoV cutting sites at CPD clusters (Fig. 2-10 B, C; +T4 lanes 4 to 14). It is apparent that photoreactivation in AMY3 (rad1Δ) was efficient in 120 min on both strands (Fig. 2-10 B, lanes 14). No repair was observed in the dark (Fig. 2-10 B, lanes 16). In contrast, NER was slow in 120 min (Fig. 2-10 C). Most of the CPD sites were still present after 120 min of NER (lanes 12). Thus, photoreactivation is the predominant pathway for CPD repair in the GAL10 gene. These results confirm the results published by Livingstone-Zatchej et al. (1997).

![Figure 2-11: CPD Repair in the GAL10 Gene and in Total rDNA of S. cerevisiae](image)

**Figure 2-11: CPD Repair in the GAL10 Gene and in Total rDNA of S. cerevisiae**

CPD repair results are shown for photoreactivation, A and NER, B in AMY3 and W303-1a, respectively. Comparison of repair in the nuclear GAL10 gene and total rDNA. Open symbols: non-transcribed strands; filled symbols: transcribed strands. The curves are averages of one independent UV experiment and at least two gels per UV experiment.

Quantified CPD repair of the GAL10 gene compared with repair of rDNA is presented in Fig. 2-11. Photoreactivation of rDNA is slightly faster than photoreactivation of GAL10 and there is no dramatic difference in NER. Thus, the yeast nucleolus does not play an inhibitory role with respect to the accessibility of repair proteins. In contrast, rDNA might be in general more accessible than extranucleolar chromatin.
2.3.6 Damage Formation in the 35S Gene

The number of CPDs per 4.4 kb NheI fragment of the 35S gene was quantified in all repair experiments and all repair samples according to the Poisson distribution (Bohr et al., 1985). The CPDs in DNA of irradiated, but unrepaired cells represents the initial damage induced by the UV dose of 150 J/m². A comparison of the initial damage in total, active and inactive rDNA on both the transcribed and non-transcribed strands is displayed in Fig. 2-12 for strains W303-1a and AMY3.

Two tendencies were observed. First, CPD formation was always higher in the transcribed strands (TS, grey bars) compared with the non-transcribed strands (NTS, white bars). This was observed in all rDNA fractions. Although the standard deviations overlap in the active rDNA fractions of W303-1a and AMY3 as well as in total rDNA of W303-1a, the strand bias is obvious. One explanation for this observation provides the fact that 31% more adjacent pyrimidine bases are present in the DNA sequence of the transcribed strand compared to the non-transcribed strand (1376 and 1046 neighbouring pyrimidines on the transcribed and non-transcribed strand, respectively). Thus, the preferential damage induction in the transcribed strand might reflect a difference in pyrimidine distribution.

Second, slightly more CPDs were formed in inactive rDNA than in active rDNA fractions. This was observed for both transcribed (TS) and non-transcribed strand (NTS). In total rDNA,

![Figure 2-12: Formation of CPDs in 35S Genes of AMY3 and W303-1a](image-url)
containing active and inactive genes, average CPD formation was lower than in inactive and higher than in active rDNA, except on the non-transcribed strand in AMY3.

**Table 2-1: Formation of CPDs\(^a\) in 35S rRNA Genes of W303-1a**

<table>
<thead>
<tr>
<th>UV Experiment</th>
<th>Total rDNA TS(^b)</th>
<th>Active rDNA TS</th>
<th>Inactive rDNA TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC 2</td>
<td>0.16</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>CC 3</td>
<td>0.19</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>CC 4</td>
<td>0.17</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.17</strong></td>
<td><strong>0.15</strong></td>
<td><strong>0.19</strong></td>
</tr>
<tr>
<td>st. deviation</td>
<td>0.016</td>
<td>0.013</td>
<td>0.008</td>
</tr>
</tbody>
</table>

\(\text{a. CPDs per kb ss DNA in the 4.4 kb NheI fragment located in the 35S rRNA gene}\)
\(\text{b. TS: transcribed strand (bottom strand)}\)
\(\text{c. NTS: non-transcribed strand (top strand)}\)

**Table 2-2: Formation of CPDs\(^a\) in 35S rRNA Genes of AMY3**

<table>
<thead>
<tr>
<th>UV Experiment</th>
<th>Total rDNA TS(^b)</th>
<th>Active rDNA TS</th>
<th>Inactive rDNA TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM pol I 2</td>
<td>0.23</td>
<td>0.21</td>
<td>0.24</td>
</tr>
<tr>
<td>AM pol I 4</td>
<td>0.22</td>
<td>0.19</td>
<td>0.23</td>
</tr>
<tr>
<td>AM pol I 5</td>
<td>0.20</td>
<td>0.18</td>
<td>0.24</td>
</tr>
<tr>
<td>AM pol I 7</td>
<td>0.24</td>
<td>0.21</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.23</strong></td>
<td><strong>0.21</strong></td>
<td><strong>0.24</strong></td>
</tr>
<tr>
<td>st. deviation</td>
<td>0.013</td>
<td>0.013</td>
<td>0.012</td>
</tr>
</tbody>
</table>

\(\text{a. CPDs per kb ss DNA in the 4.4 kb NheI fragment located in the 35S rRNA gene}\)
\(\text{b. TS: transcribed strand (bottom strand)}\)
\(\text{c. NTS: non-transcribed strand (top strand)}\)

The observed differences between rDNA fractions are small. Overlapping standard deviations of the initial damage (e.g. of the transcribed strand (grey bars) of the rDNA fractions in AMY3) question their significance. Tab. 2-1 and Tab. 2-2 show CPD formation in individual repair experiments of W303-1a and AMY3, respectively. The bias between inactive and active genes was observed in all individual experiments in two different yeast strains. However, the difference varied from about 50 % (CC 3) to about 10 % (AM pol I 7).

The bias between the fractions was more pronounced in the transcribed strands than in the non-transcribed strands. Since inactive rDNA is packaged into nucleosomes and active rDNA is in an open chromatin structure (Dammann et al., 1993), the results might indicate that packaging of DNA sequences into different chromatin structures might affect the formation of CPDs.
2.4 DISCUSSION

Photoreactivation is the Predominant CPD Repair Pathway in the Nucleolus

Yeast *S. cerevisiae* has two mechanisms to repair UV-induced DNA lesions, photoreactivation and NER (Sancar, 2000b). Photoreactivation was shown to be more efficient than NER and appeared to be the predominant pathway for CPD repair, while NER is required to remove non-CPD lesions (Livingstone-Zatchej et al., 1997; Suter et al., 1997; Aboussekhra and Thoma, 1998; Thoma, 1999). Here, we show that photoreactivation is also the predominant pathway for CPD repair in the nucleolus, in the active and silenced rRNA genes. Thus, when yeast cells are exposed to DNA damaging sunlight, photolyase is ready to remove the major lesions, the CPDs, from most regions of the genome.

Photoreactivation and NER Repair rDNA as Efficiently as the Inactive Nuclear GAL10 Gene

In yeast, the nucleolus is a morphologically distinct compartment, which covers about a third of the nucleus, contains fibrillar centres, dense fibrillar component and granular components similar to those of higher eukaryotes, and appears morphologically more compact than the rest of the nucleus (Leger-Silvestre et al., 1999; Carmo-Fonseca et al., 2000). We have shown that NER repairs rDNA as efficiently as the inactive GAL10 gene that is located on chromosome II outside of the nucleolus, and that photoreactivation is even faster (Fig. 2-11). Thus, despite the compartmentalization, the components of both pathways find unrestricted access to the nucleolar chromatin. It is interesting to note that CPDs are inefficiently removed by NER in human and hamster cells (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995), but strand breaks in human cells, and (6-4) PPs, intrastrand adducts and interstrand crosslinks in rodent cells are more efficiently repaired (Vos and Wauthier, 1991; Stevnsner et al., 1993; Fritz et al., 1996; Balajee et al., 1999). Thus, DNA repair in nucleoli appears not to be directly related to nucleolar compartmentalization, but rather to the specific properties of damage recognition and processing by the different repair pathways.

Chromatin Structure of Active and Inactive Genes

Active rRNA genes are depleted of nucleosomes, while inactive genes are packaged into nucleosomes (Dammann et al., 1993; Lucchini and Sogo, 1998; Muller et al., 2000). Photolyase is an enzyme that strongly discriminates between nucleosomes and nucleosome-free DNA, both *in vitro* and in yeast and therefore can be used as a tool to test whether DNA is packaged into nucleosomes *in vivo* (Suter et al., 1997; Suter et al., 2000a; Suter et al., 2000b); Thoma unpublished results). Here, we found that the inactive rDNA was repaired by photolyase as fast
as the GAL10 gene, which is packaged in nucleosomes when cells are grown in glucose (Cavalli, 1994). Thus, the photoreactivation data are consistent with a nucleosomal conformation of inactive rRNA genes. Moreover, photoreactivation of the non-transcribed strand of the active rDNA was much faster than nucleosomal DNA (68 % CPDs removed in 15 min compared with 45 % and 50 % in the inactive rDNA and GAL10, respectively). This is direct evidence obtained in vivo, that rDNA of inactive genes is in a nucleosomal conformation, while the active rRNA genes are in an open, non-nucleosomal conformation. Moreover, the same data demonstrate that this open conformation is maintained after damage induction.

**Damage and Repair Dependent Structural Transitions in Chromatin**

Yeast cells can modulate the proportion of active and inactive rRNA gene copies in response to variations in environmental conditions, which suggests that yeast can regulate rRNA synthesis by varying the number of active gene copies (Dammann et al., 1993; Grummt, 1999). Since UV damage blocks RNA polymerases, it is conceivable that inactivation of transcription by UV lesions might promote an alteration of chromatin structure by regeneration of nucleosomes (Thoma, 1999). Psoralen crosslinking in mouse cells showed that the fraction of open (active) rDNA remained constant during repair and there was no indication of chromatin rearrangements following UV damage formation (Fritz and Smerdon, 1995). Here, two observations indicate that UV damage formation caused alterations in chromatin accessibility. UV irradiation resulted in a reduction of *Nhe*I-released chromatin fragments and psoralen crosslinking revealed a broad band in the released fraction consistent with a heterogeneous chromatin population. Thus, a fraction of the active rDNA underwent a structural change, which affected *Nhe*I and psoralen accessibility. However, UV irradiation was not sufficient to convert all the active rDNA into an inaccessible (nucleosomal) state. This interpretation is further substantiated by the observation that photoreactivation was much faster in the active fraction than in inactive nucleosomal DNA. We therefore conclude that only a fraction of active genes was remodeled into a less accessible state. We do not yet know the structural basis for this transition. It is possible that nucleosomes form at random on the damaged genes, but there are not enough histones available to package the whole gene. Alternatively, it is possible that nucleosomes form only downstream of the blocked polymerase, while the upstream region is still challenged by reinitiation and transcription. Direct analysis of rRNA synthesis after UV irradiation and repair using run-on experiments would contribute to a better understanding of the DNA damage dependent structural transitions in chromatin.

An additional observation related to chromatin structure was that the fraction of released genes recovered with increasing NER times, but not with photoreactivation in the absence of NER.
Since yeast photolyase in contrast to NER cannot repair 6-4PPs, it is possible that removal of (6-4) PPs is a limiting process. (6-4) PPs are generated in lower yields than CPDs, but they are removed faster (Tijsterman et al., 1999). One interpretation is that a fraction of rRNA genes resumes transcription elongation after the damage is removed and regenerates an open chromatin structure. Alternatively, the enhanced release of rDNA during NER could be an indirect effect of removal of DNA lesions from other parts of the genome. Only cells proficient in the repair of (6-4) PPs in transcription-blocked genes might be able to resume transcription and a normal metabolism including the activation of rRNA genes.

**Interactions of Photoreactivation and NER with RNAP-I**

Previous studies have reported a strand bias of NER in bulk rDNA of yeast (Verhage et al., 1996a). Here, we confirm this result and further demonstrate that the strand bias is due to preferential repair of the transcribed strand in the active fraction. This is evidence that TC-NER exists in genes transcribed by RNAP-I. Further evidence for transcription-coupled NER in yeast RNAP-I genes was presented by Conconi et al. (2002). Rad26 is involved in TC-NER in RNAP-II genes (van Gool et al., 1994), but the strand bias in RNAP-I repair is independent of Rad26 (Verhage et al., 1996a). Thus, the molecular mechanisms for TC-NER are different for RNAP-I and RNAP-II genes.

*In vitro* experiments established that human RNAP-I and RNAP-II are firmly blocked at CPDs on transcribed strands, RNAP-I having a shorter half life at a lesion than RNAP-II of about 6 and 20 hours, respectively (Donahue et al., 1994; Hara et al., 1999). While blocked polymerases can serve as a signal for NER, they inhibit access to photolyase (Livingstone-Zatchej et al., 1997). Here we find that photolyase is only slightly inhibited in the transcribed strand of active rRNA genes, which is in contrast to a strong inhibition observed in the GAL10- and the URA3 genes transcribed by RNAP-II (Livingstone-Zatchej et al., 1997; Suter et al., 1997). Apparently, photolyase has almost normal access to lesions in transcribed strands of active rDNA. Different transcription rates are unlikely to explain this result, since both the GAL10 and rRNA genes are heavily transcribed (Cavalli and Thoma, 1993; Cavalli et al., 1996). Therefore, RNAP-I seems to dissociate from CPDs shortly after blockage. In agreement with the *in vitro* data, our experiments suggest differential stability of RNAP-I and -II blocked at CPDs. Furthermore, the short persistence of RNAP-I at a lesion might reduce the chance of recruitment of factors required for TC-NER and explain the weak strand bias of NER in active RNAP-I genes.

A rapid dissociation *per se* would render the UV lesion accessible for repair enzymes. Rad26 was suggested to displace RNA polymerases from UV lesions on transcribed strands (Selby et
al., 1997; Selby and Sancar, 1997b; Prakash and Prakash, 2000). Thus, the low stability of RNAP-I would provide an explanation why Rad26 is dispensable for TC-NER in RNAP-I genes (Verhage et al., 1996a).

**CPD Formation in Active and Inactive Genes**

It is well established that distortion of the DNA double helix affects damage formation (see Chapter 1.3.2). Since a CPD generates a 7 - 9° bend (Wang and Taylor, 1991) and a (6-4) PP a 44° kink (Kim et al., 1995). DNA binding proteins that alter the regular structure of the double helix and generate bending of the DNA (Becker and Wang, 1984; Selleck and Majors, 1987b) may promote or inhibit formation of UV lesions (Pfeifer et al., 1992; Tornaletti and Pfeifer, 1995; Aboussekhra and Thoma, 1999).

Here, CPD formation was analyzed in active and inactive rDNA in living cells. Since transcriptionally-active, nucleosome-free genes and inactive, nucleosome-packaged genes were irradiated simultaneously in cells, CPD formation in the same 35S DNA sequence, either in compact or open chromatin was monitored and compared.

First, we show a preference for CPD formation in the transcribed strand compared to the non-transcribed strand. The DNA sequence of the 35S gene could provide an explanation for this observation: 31 % more adjacent pyrimidine bases (TTs, TCs, CTs or CCs) are present in the transcribed strand compared to the non-transcribed strand of the 4.4 kb NheI fragment of the 35S rRNA gene (1376 and 1046 neighbouring pyrimidines on the transcribed and non-transcribed strand, respectively). Thus, preferential damage induction in the transcribed strand might reflect a difference in pyrimidine distribution.

Second, higher CPD yields were induced in inactive as compared to active rDNA. The differences between the rDNA fractions are small, but they were observed in all UV experiments (Tab. 2-1 and Tab. 2-2). Inactive rDNA is packaged into nucleosomes, while active rDNA is covered by transcribing RNAP-I and is free of nucleosomes (Dammann et al., 1993). Thus, the preferential CPD formation in inactive rDNA might reflect either an effect of the nucleosomes in inactive rDNA or an effect of the transcribing RNA polymerases I in active genes.

Does packaging of DNA into nucleosomes affect CPD formation? DNA wrapped around histone proteins is heavily bent (Luger et al., 1997). This may lead to increased or decreased yields of CPDs. CPD formation was shown to be higher in the single-stranded 5S DNA sequence compared to double-stranded DNA, since flexible DNA structures may favour the formation of a lesion (Becker and Wang, 1989b). Thus, the flexible DNA helix in open rRNA genes might promote CPD formation, whereas the rigid DNA on nucleosomal inactive rDNA might decrease CPD yields.
Formation of (6-4) PPs and CPDs differed with respect to core and linker DNA. Whereas a similar yield of CPDs was found in nucleosomal core and linker DNA, (6-4) PPs occurred with a 6-fold higher frequency per nucleotide in linker DNA (Niggli and Cerutti, 1982; Mitchell et al., 1990). CPD formation in the nucleosome itself was studied in core particles isolated from irradiated human cells. The relative CPD distribution was modulated with a period of 10.3 bp (Gale et al., 1987).

The results presented here provide *in vivo* evidence that the chromatin context of a gene might affect damage formation. It remains to be demonstrated whether nucleosomes or transcribing RNA polymerases account for the differential CPD yields.
3 CPD REPAIR IN THE rDNA SPACER

3.1 SUMMARY

In the yeast *S. cerevisiae*, two adjacent transcription units coding for the 35S precursor of the ribosomal RNA are separated by the intergenic spacer (rDNA spacer). This region contains the promoter of the 35S gene, the 5S rRNA gene, a potential origin of replication (rARS) and an enhancer, which is required for regulation of transcription and replication of rDNA. Photoreactivation (PR) and nucleotide excision repair (NER) were investigated in the rDNA spacer. Strain AMY3 (*rad1Δ*), deficient in NER, and the NER-proficient strain W303-1a (*RAD1*) were irradiated with UV light and incubated for up to two hours for repair under photoreactivating light or in the dark. Strand-specific repair of CPDs was analyzed in the total population of rDNA spacers and compared with chromatin.

CPDs in the 35S promoter region remained unrepaired by photoreactivation, NER and by the combination of photoreactivation and NER, indicating that RNAP-I transcription factors bound to the promoter inhibit repair.

Comparison of micrococcal nuclease footprints and photoreactivation revealed a modulation of photoreactivation by chromatin. One positioned nucleosome downstream of the rARS, three positioned nucleosomes between the rARS and the 5S gene and nucleosomes between the 5S gene and the enhancer were detected. MNase cutting and slow photoreactivation in the rARS indicated that no nucleosome, but possibly replication factors are located on the rARS. The spacer region flanking the 35S promoter was rapidly photoreactivated, which indicates an open chromatin structure.
3.2 INTRODUCTION

In the yeast *S. cerevisiae*, the genes coding for the 35S rRNA precursor are organized in one cluster of about 100 to 200 copies of tandem repeats on chromosome XII (Woolford, 1991; Chindamporn et al., 1993; Venema and Tollervey, 1999). The transcription units are separated by the non-transcribed rDNA spacer (Fig. 3-1).

---

**Figure 3-1:** *rDNA Spacer of Yeast S. cerevisiae and its Chromatin Structure*

A Two adjacent 35S rRNA genes are separated by the ribosomal intergenic spacer (rDNA spacer). The rDNA spacer is 2.5 kb in size and contains an enhancer element (E), the 5S rRNA gene (5S), the ribosomal origin of replication (ARS) and the 35S promoter (P) (see text for details). The restriction enzyme *ClaI* has recognition sites at the 3’ end of the upstream 35S rRNA genes. *NdeI* cuts at the 5’ end of the downstream 35S gene. The probe detecting the 3.9 kb *ClaI / NdeI* fragment containing the rDNA spacer is indicated (black bar). B Schematic representation of the chromatin structure of the spacer. MNase-exposed sites in chromatin are indicated by arrowheads. Map units corresponding to the +1 transcription start site of the 35S gene are indicated from studies of Vogelauer et al. (1998) and Cavalli (1994). Chromatin regions of 150 to 210 bp, protected against MNase cleavage represent positioned nucleosomes (white ovals). The proposed five nucleosomes (N1 to N5) are indicated.

The rDNA spacer contains an enhancer, the 5S gene, the ribosomal ARS and the promoter for the 35S gene. The 317 bp ribosomal enhancer regulates RNAP-I transcription (Lang and Reeder,
1993; Lang et al., 1994; Banditt et al., 1999), recombination (Keil and Roeder, 1984; Voelkel-Meiman et al., 1987) and contains the polar replication fork barrier (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Gruber et al., 2000). The 5S gene coding for the 5S rRNA is transcribed by RNAP-III in the opposite direction (Venema and Tollervoey, 1999). The ribosomal ARS (rARS) serves as a potential origin of replication (Miller and Kowalski, 1993; Muller et al., 2000). The ~160 bp promoter of the 35S gene is the transcription initiation site of RNA polymerase I (RNAP-I).

In vivo, the rDNA spacer is packaged into chromatin (Dammann et al., 1993; Fritze et al., 1997; Vogelauer et al., 1998; Lucchini et al., 2001). Psoralen crosslinking experiments and electron microscopy studies demonstrated that the rDNA spacer is nucleosomal (Dammann et al., 1993). Non-nucleosomal and nucleosomal enhancer were observed downstream of active and inactive 35S genes, respectively (Dammann et al., 1995). A study using MNase footprinting and indirect end-labeling detected five positioned nucleosomes between the 5S gene and 35S promoter (nucleosomes N1 to N5, see Fig. 3-1 B) and nucleosomes with multiple positions between the 3’ end of the 35S and the 5S gene (Vogelauer et al., 1998). A similar observation was made previously in our laboratory with analyses of a single copy rDNA spacer (Cavalli, 1994). In addition, the studies detected nuclease-sensitive regions downstream from the enhancer and upstream of the 35S promoter, indicating a more open chromatin structure at these sites (Morrow et al., 1989; Vogelauer et al., 1998). A nucleosome with multiple positions seems to cover the 5S gene (Buttinelli et al., 1993). Transcription and footprinting studies in vivo and in vitro in the 35S promoter suggest binding of two multiprotein complexes containing RNAP-I specific transcription factors (Vogelauer et al., 1998; Bordi et al., 2001), reviewed in Nomura (1998). Thus, the 35S promoter seems not to be packaged into nucleosomes (Chapter 4.2).

In yeast, nucleotide excision repair (NER) and photoreactivation (PR) are the major pathways that remove UV-induced DNA lesions, cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4) PPs) (Chapter 1.5). Repair of UV lesions in the rDNA spacer was never investigated. Although repair was measured in rDNA of yeast, rodent and human cells (e.g. (Christians and Hanawalt, 1993; Fritz and Smerdon, 1995; Verhage et al., 1996a)), all studies focused on repair of the coding region of the 35S rRNA gene. This is surprising, since UV lesions not only block transcription, but may also affect binding of transcription factors to DNA (Pfeifer et al., 1992; Tornaletti and Pfeifer, 1995; Aboussekhra and Thoma, 1999). Thus, DNA damage in functional elements of the rDNA spacer could affect RNAP-I transcription and replication. Furthermore, the DNA sequence of the enhancer and its flanking regions contain several very long stretches of poly(dA.dT) sequences (T-tracts). They adopt an unusual straight and rigid structure in vitro (Nelson et al., 1987). T-tracts are involved in
transcriptional regulation of yeast genes (Struhl, 1985; Iyer and Struhl, 1995) and may interfere with the formation and arrangement of nucleosomes (Prunell, 1982; Losa et al., 1990; Schieferstein and Thoma, 1996). Since formation of DNA lesions depends on the DNA sequence and structure (Becker and Wang, 1984; Becker and Wang, 1989b), T-tracts generate a characteristic pattern of CPDs upon irradiation (Lyamichev, 1991). It was shown that T-tracts exist \textit{in vivo} and can be efficiently repaired (Suter et al., 2000a).

Here we study repair of UV lesions in the rDNA spacer of yeast by photoreactivation and NER. We show a modulation of photoreactivation by the chromatin structure of the rDNA spacer. NER is less heterogeneous than photoreactivation and inefficient. Enhanced damage formation and inefficient repair in the 35S promoter suggest that RNAP-I transcription factors remain bound to the promoter sequences and inhibit access to repair enzymes.
3.3 RESULTS

3.3.1 CHROMATIN STRUCTURE OF THE rDNA SPACER

Chromatin structure was analyzed by micrococcal nuclease digestion and indirect end-labeling. Yeast strain UCC510 was grown in glucose and a crude nuclear extract was prepared using zymolyase. The nuclear extract and purified genomic DNA were digested with micrococcal nuclease (MNase) (experiment done by M. Livingstone and R. dePril). The DNA was cut with Clal and Ndel, which generate a 3.9 kb fragment containing the rDNA spacer (Fig. 3-1). The DNA was separated on an alkaline agarose gel, blotted on a nylon membrane and hybridized with strand-specific probes abutting the Ndel site (Fig. 3-1). MNase-cut DNA and chromatin (CHR) are shown in lanes 1 and 2, respectively in panels A and B of Fig. 3-2 and Fig. 3-3.

MNase sites that are cut in DNA but protected in chromatin represent footprints. MNase-cuts in chromatin indicated nuclease-sensitive regions or linkers between nucleosomes. Footprints were observed between the 35S promoter and the ribosomal enhancer (Fig. 3-2 A, lanes 1 and 2). MNase cutting sites in chromatin were mapped and their positions are shown with respect to the 35S transcription start site (arrowheads in Fig. 3-2 E). N1 represents a footprint from -300 to -502. N3 mapped from -630 to -837, N4 from -837 to -1007 and N5 from -1007 to -1183. These four footprints are about 170 to 200 bp and were interpreted as positioned nucleosomes. A footprint was also observed between nucleosome N1 and N3 (Fig. 3-2 A, lane 2). The protected region at the rARS was only 128 bp and may therefore be too small for accommodating a site (top of the gel) to the Ndel site (bottom of gel). DNA of unirradiated cells is shown in lane 3 (-UV). The white bar defines the rDNA spacer from the 3’ end of the 35S gene to the 35S promoter, the asterix the CPD site in the 35S promoter quantified in C and D, respectively. White triangles mark fast-repaired CPD sites, black triangles slowly-repaired sites. The black circle depicts a MNase cutting site flanking the rARS, which is repaired slowly. Marker lanes (M) represent multiples of 256 bp. Arrowheads (lanes 10) mark the position of long poly (dA,dT) stretches. C Quantified CPD repair in the top and bottom strand of the rDNA spacer (black curves). Bars represent the standard deviation of four gels of one UV experiment. Dotted curves represent photoreactivation of the 4.4 kb Nhel fragment of inactive 35S genes (adapted from Fig. 2-9 C) D The bottom strand of the 35S promoter. Circles show photoreactivation, squares NER in AMY3. Bars represent the standard deviation of four gels of one UV experiment. E Schematic drawing of the chromatin structure and photoreactivation of the rDNA spacer. Symbols as in Fig. 3-1 B. MNase sites are averages of four gels of one chromatin experiment. Note that an additional nucleosome (?) was suggested covering the ARS (Fig. 3-1 B), although the footprint is only 128 bp long. Sites of enhanced MNase cutting (arrows) in chromatin and their position with respect to the transcription start site are indicated. White and black triangles show the position of fast- and slowly-repaired CPD sites, respectively, on the top and the bottom strand (see panels A and B).
Figure 3-2: Chromatin and Photoreactivation in the rDNA Spacer of AMY3 (rad1Δ)

A top strand, B bottom strand (transcribed strand of 35S gene). Cleavage sites for MNase in naked DNA (DNA, lane 1) and in chromatin (CHR, lane 2) of yeast strain UCC510. A schematic map indicating the structural elements and proposed nucleosomes (white ovals) is shown next to lane 1. For photoreactivation, AMY3 cells were grown in glucose, irradiated with UV light (150 J/m², +UV) and exposed to photoreactivating light (PR) for 7 to 120 minutes (lanes 5 to 9) or incubated in the dark (lane 10). DNA was cut with Clal and NdeI, cut at CPDs with T4endoV (lanes 3-10), fractionated on alkaline agarose gels, blotted and hybridized with strand-specific probes for the 3.9 kb Clal / NdeI fragment (see Fig. 3-1 A). CPDs are displayed from the Clal
conventional nucleosome. Alternatively, factors of the origin of replication complex may cover the rARS (Bell and Stillman, 1992).

A different MNase pattern in chromatin and DNA was observed between the enhancer and the 5S gene, but no clear footprints of nucleosomes. This indicates that nucleosomes might be

Figure 3-3: Chromatin, NER and Photoreactivation in the rDNA Spacer of W303-1a

A, top strand, B, bottom strand. Chromatin and repair analysis was done as described in Fig. 3-2. Cleavage sites for MNase in naked DNA (DNA, lane 1) and in chromatin (CHR, lane 2) of yeast strain UCC510. DNA of unirradiated cells is shown in lanes 3 and damaged DNA in lanes 4. Photoreactivation + NER is shown in lanes 5 to 9, NER in the dark in lanes 10 to 13. CPDs are displayed from the ClaI site (top of the gel) to the Ndel site (bottom of gel). Marker lanes (M) represent multiples of 256 bp. A schematic interpretation of the chromatin structure is shown (lane 1). Symbols as described in Fig. 3-2. The white bar depicts rDNA spacer from the 3' end of the 35S gene to the 35S promoter, the asterix the CPD site on the bottom strand quantified in C and D, respectively. C Quantified CPD repair in the top and bottom strands of the rDNA spacer (black curves). The average of two gels of one UV experiment is shown. The dotted curves show repair of the 4.4 kb NheI fragment of inactive 35S genes (adapted from Fig. 2-9 D). D CPD repair of the bottom strand of the 35S promoter. Rectangles show photoreactivation + NER, triangles NER. The curves are averages of two gels of one UV experiment.
present, but not positioned (overlapping white ovals in Fig. 3-2 A). Vogelauer et al. (1998) and Cavalli (1994) reported five positioned nucleosomes N1 to N5 between the 35S promoter and the 5S gene, and nucleosomes with multiple positions in the rDNA spacer upstream the 5S gene (summarized in Fig. 3-1 B). Thus, our results confirm the positioned nucleosomes N1, N3 to N5. Whether the footprint at the rARS (Fig. 3-1 B) reflects a nucleosome, a modified nucleosome or an alternative complex remains to be investigated.

### 3.3.2 CPD Repair by Photoreactivation and NER in the rDNA Spacer

We used the yeast strains AMY3 (radΔ) with inactivated NER to analyze photoreactivation and W303-1a for analysis of NER and NER with photoreactivation. Cells were grown in YPD, irradiated in minimal medium with 150 J/m² and incubated for repair for up to two hours at 24 to 26°C in minimal medium supplemented with the appropriate amino acids. For NER, the cultures were incubated in the dark. For photoreactivation, cells were exposed to photoreactivating light.

DNA was purified and cut with ClaI and NdeI, which generates a 3.9 kb fragment containing the rDNA spacer (Fig. 3-1). The DNA was cut with T4-endonucleaseV at CPDs and the cutting sites were displayed by indirect end-labeling using strand-specific probes abutting the NdeI site (Fig. 3-1).

A set of data is shown for AMY3 and W303-1a in Fig. 3-2 and Fig. 3-3, respectively. Non-irradiated DNA (-UV) reveals the intact restriction fragment (3.9 kb, top band). Treatment of damaged DNA (+UV) with T4endoV generates a smear with bands, which represent the distribution of pyrimidine clusters. The CPD patterns are different in the top and bottom strands demonstrating strand-specificity of the assay. The top fragment represents the fraction of undamaged DNA. The initial damage was 0.21 CPDs/kb on the top strand and 0.27 CPDs/kb on the bottom strand.

**Photoreactivation is the Predominant Pathway to Repair CPDs in the rDNA Spacer**

Repair of CPDs is visualized by a time-dependent decrease of the CPD sites and an increase of the intact restriction fragment (Fig. 3-2 and Fig. 3-3 in panels A, B). Generally, the CPD bands are efficiently removed by photoreactivation alone (Fig. 3-2, lanes 4 to 9) as well as by NER with photoreactivation (Fig. 3-3, lanes 4 to 9). NER (Fig. 3-3, lanes 10 to 13) shows no obvious removal of CPDs. The results demonstrate the predominant role of photoreactivation in removal of CPDs in the rDNA spacer region.

**Slow Repair in the rDNA Spacer as Observed in Inactive 35S Genes**

How does repair of the rDNA spacer compare to repair in the 35S transcribed region
CPD repair was quantified in the rDNA spacer region defined from the 3' end of the 35S gene to the 5' end of the 35S promoter (Fig. 3-2 and Fig. 3-3, marked by the white bar). Repair curves for AMY3 (PR) and W303-1a (PR+NER, NER alone) are displayed in panels C of Fig. 3-2 and Fig. 3-3, respectively. In AMY3 (rad1Δ), both strands were repaired efficiently (Fig. 3-2 C). After 30 minutes, 55 and 47% of the lesions were removed from the top and bottom strands, respectively. More than 80% of the lesions were repaired in two hours. In the NER-proficient strain W303-1a (Fig. 3-3 C), only about 20% of the lesions were removed in two hours. W303-1a cells exposed to photoreactivating light removed CPDs as efficiently as photoreactivation alone in AMY3 (rad1Δ), indicating that the contribution of NER is minimal. Comparison of photoreactivation in the rDNA spacer (Fig. 3-2 C) and the 35S transcribed region reveals that photoreactivation in the rDNA spacer is slightly slower than in inactive 35S genes (dotted curves in Fig. 3-2, adapted from Chapter 2.3.4). The same was observed for NER + photoreactivation and NER alone (Fig. 3-3 C; black curves: rDNA spacer; dotted: inactive 35S genes). Inactive 35S genes were shown to be packaged into nucleosomes (Dammann et al., 1993) and slow repair of the inactive 35S genes was interpreted by the inhibitory role of nucleosomes (Chapter 2.3.4). The rDNA spacer region is, except for the small 5S rRNA gene, not transcribed and packaged into nucleosomes (Chapter 3.3.1). Thus, the slow repair in vivo is evidence for a compact chromatin structure in the rDNA spacer.

**Inefficient Repair in the 35S Promoter**

Visual inspection of individual CPD sites in Fig. 3-2 A shows fast photoreactivation at the 5' end, the 3' end of the 35S gene and at the enhancer. All CPDs were removed in less than 60 minutes. This correlates to the fast repair observed in the 35S transcribed region of total rDNA (Fig. 2-2). CPDs in the nucleosomal rDNA spacer region were more slowly repaired. CPDs located in the bottom strand of the 35S promoter were poorly repaired by photoreactivation and NER (asterix in Fig. 3-2 B and Fig. 3-3 B). Quantification of this CPD site (Panels D in Fig. 3-2 and Fig. 3-3) visualizes the severe inhibition of repair: About 20% of the lesions were removed in two hours by photoreactivation. NER alone removed less than 10%. Both NER and photoreactivation together repaired only about 40% of the lesions. Strikingly, CPDs only a few base pairs upstream of this site were removed within minutes (Fig. 3-2 B, white triangle lane 4 in 35S-P). RNAP-I specific transcription factors were shown to interact with the promoter sequence in vitro (Keener et al., 1998) and in vivo (Bordi et al., 2001). This observation indicates that RNAP-I specific transcription factors cover the CPDs and deny access of the repair enzymes to the lesions in the promoter.
Photoreactivation of the rDNA Spacer is Modulated by Chromatin Structure

A close inspection of photoreactivation in the rDNA spacer region (Fig. 3-2 A, B) reveals a repair heterogeneity at different CPD sites. Generally, CPDs that correlate with footprints of nucleosomes are slowly repaired (black triangles, N1 to N5). CPDs that correlate with MNase-cutting sites in chromatin are rapidly repaired (white triangles). This is consistent with the study of Suter et al. (1997).

At nucleosomes N3 to N5, CPD bands correlate with footprints of nucleosomes.

N1 is a *bona fide* nucleosome. It is characterized by a strong footprint (Fig. 3-2 A, lane 2) and two slowly-repaired CPD sites (black triangles, top and bottom strand) inside the footprint. Two CPD sites, which are faster repaired (white triangles), correspond to MNase cutting sites.

In the N2-rARS region, one CPD site (bottom strand) maps in the footprint and is slowly repaired and hence might be in a nucleosome. Another CPD site (top strand), however, mapped in the MNase cutting site, but remained resistant to photoreactivation (black circle). Thus, proteins bound in this region might inhibit photoreactivation but not MNase. Slowly-repaired sites were also found in ARS1 (Suter et al., 2000b). Thus, it is possible that proteins involved in initiation of replication (Bell and Stillman, 1992) might bind the rARS and inhibit repair.

The region between the 35S promoter and the nucleosome N1 is a sensitive to MNase and rapidly repaired on both strands. This indicates that this region is free of nucleosomes and accessible to proteins.

A modulation of photoreactivation between the enhancer and the 5S gene provides evidence for nucleosomes in this region, although no MNase footprints were observed. The T_{18}-stretch on the top strand in this nucleosomal region (Fig. 3-2 A, black arrow T_{18}) was inefficiently repaired. Other CPD sites including the three t-tracts (T_{5,6,16}, T_{20}, T_{29}) were removed in two hours.
3.4 DISCUSSION

Predominant Role of Photoreactivation in the rDNA Spacer

We found that photoreactivation was much more efficient in CPD removal from the rDNA spacer than NER. This was observed before in the 35S gene (Chapter 2), the nuclear GAL10 gene (Livingstone-Zatchej et al., 1997), in yeast minichromosomes (Suter et al., 1997; Wellinger and Thoma, 1997) and an RNAP-III gene (Aboussekhra and Thoma, 1998). The results presented in this chapter extend the predominant role of photoreactivation in CPD repair to the rDNA spacer region of yeast.

Inefficient Repair in the 35S Promoter

Lesions in the 35S promoter were poorly repaired. Photoreactivation alone removed only about 20% in two hours, NER and photoreactivation about 40%. RNAP-I specific transcription factors were shown to interact with the promoter sequence in vitro (Keener et al., 1998) and in vivo (Bordi et al., 2001). This suggests that RNAP-I transcription factors may inhibit CPD repair in the 35S promoter. The repair kinetic of photoreactivation (Fig. 3-2 D) reveal repair of 20% of the lesions in the first 30 minutes and a plateau up to two hours. These repair kinetics suggest the existence of two populations of 35S promoters: A minor fraction, which is ’open’ to photolyase, and a major fraction which restricts access to the repair enzyme. It was shown that only a fraction of all 35S genes are actively transcribed in exponentially-growing yeast cells, while the other genes are transcriptionally inactive (Dammann et al., 1993). Either fraction of the 35S promoters might be accessible or inaccessible to photolyase. Thus, differential binding properties of RNAP-I transcription factors in active and inactive 35S promoters might account for these repair kinetics.

Could damaged 35S promoters affect transcription of 35S genes? 0.27 CPDs/kb were generated on average on the bottom strand and the promoter is about 160 bp long. Therefore, there are only about 0.04 CPDs per promoter, which leaves the major fraction of the 100 to 200 copies of 35S promoters per cell intact. It is therefore unlikely that damaged promoters affect RNAP-I transcription.

Chromatin Structure of the rDNA Spacer

Vogelauer et al. (1998) reported nucleosomes with multiple positions between the enhancer and the 5S gene and five positioned nucleosomes between the 5S gene and the 35S promoter. A similar observation was made by analysis of a single copy rDNA spacer (Cavalli, 1994). Here we identified four MNase footprints of 170 - 200 bp, which support the existence of four nucleosomes between the 35S gene promoter and the 5S gene. Furthermore, slow
photoreactivation confirms the presence of nucleosomes. No clear nucleosome footprints were observed between the 5S gene and the enhancer. However, a modulation of photoreactivation indicates that nucleosomes are present, but not positioned. Slow repair in nucleosomes and fast repair in linkers is characteristic for nucleosomes (Suter et al., 1997). Thus, the photoreactivation data support a nucleosomal structure of the rDNA spacer.

The MNase footprint around the rARS was only 128 bp, which is too small for accommodating a nucleosome (Tanaka et al., 1996). Further evidence against a nucleosome on the rARS was found in the photoreactivation experiments: A CPD site mapped at a MNase cutting site and remained resistant to photoreactivation (Fig. 3-2, black circle). Since photolyase would remove the CPD site, if located in linker DNA, it is more likely that other proteins than histones are bound in the rARS, which inhibit photoreactivation but not access to MNase. Yeast ARS1 was shown to be associated with proteins of the origin of replication complex (ORC) (Bell and Stillman, 1992). Photoreactivation in ARS1 was modulated, but generally fast despite the interaction of protein complexes with ARS1 (Suter et al., 2000b). Thus, more detailed investigations would be necessary to characterize the proteins bound to the ribosomal ARS.

**Silencing in the rDNA Spacer**

Transcription of RNAP-II genes integrated in the rDNA spacer is silenced (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997). The repression of RNAP-II transcription is dependent on Sir2. Smith and Boeke (1997) and Fritze et al. (1997) reported a lower accessibility of the rDNA spacer in wt cells for the intercalating drug psoralen or MNase compared to mutants defective in SIR2. This indicated that the chromatin structure of the rDNA spacer is silenced in wt cells. Here we report that photoreactivation is slightly slower in the rDNA spacer than in inactive 35S genes, which are packaged into nucleosomes. This may indicate a chromatin structure that is even more inaccessible than inactive, nucleosomal 35S genes. Analysis of photoreactivation in sir2Δ cells would be an approach to test the influence of Sir2 in the establishment of silenced chromatin in the rDNA spacer.
4 Damage Formation and Photoreactivation of the 35S Promoter

4.1 Summary

The nucleolus is a nuclear subcompartment containing the multicopy ribosomal RNA genes (rDNA, 35S genes). The 35S genes are transcribed by RNAP-I. However, only a fraction of the 100 to 200 copies of the genes is active at any given time, while transcription of the other fraction is repressed. The ratio of active versus inactive genes is dependent on the growth conditions. The 35S promoter consists of the core and the upstream element. The upstream activating factor (UAF) recruits the core factor (CF), Rrn3 and RNAP-I with help from TBP to form the initiation complex. To study the interaction of UAF and CF with active and inactive 35S promoters in vivo, strains were irradiated with UV light and incubated for up to two hours for repair. 35S promoters of active and inactive genes were fractionated by restriction enzyme digestion. UV damage formation (photofootprinting) and repair by photoreactivation (PR) were analyzed at nucleotide resolution in active and inactive promoters.

The results show UV photofootprints in the upstream element (‘upstream footprint’) and the core element (‘core footprint’). Yeast strains defective in the CF lost the core footprint, strains defective in the UAF lost both the core- and the upstream footprint. This indicates that CF binding is dependent on the CF as well as on the UAF, while UAF binds the upstream element without CF. In wt cells, both core- and upstream footprint were observed in promoters of active and inactive 35S genes, suggesting that CF and UAF bind all promoters in vivo, irrespective of whether the gene is transcribed or not. Inhibition of photoreactivation in the core element of active promoters indicates that CF and UAF are stabilized when the gene is transcribed. Partial inhibition of photoreactivation in inactive promoters suggests a lower stability of the complexes.
4.2 INTRODUCTION

The nucleolus, a subcompartment of the nucleus, harbours the cluster of 100 - 200 ribosomal RNA genes (rDNA, 35S genes) coding for the large ribosomal RNA transcripts (Carmo-Fonseca et al., 2000) (Chapter 1.6). For transcription of the 35S genes, eukaryotic cells use a specialized RNA polymerase (RNAP-I), which is conserved from lower to higher eukaryotes (Paule, 1998b).

Transcription initiation by RNAP-I was studied in detail in *S. cerevisiae* (reviewed in Nomura (1998)) (Fig. 4-1). The 35S promoter consists of two elements, the upstream element (UE) and the core element (core) (Nomura, 1998). *In vitro* reconstitution experiments of RNAP-I transcription identified a set of essential transcription factors (Keener et al., 1998): Basal transcription requires the core element, RNAP-I, Rrn3 and the core factor (CF), which consists of three subunits Rrn6, Rrn7 and Rrn11 (Keys et al., 1994; Lin et al., 1996). Rrn3 was shown to interact with RNAP- and possibly with the Rrn6 subunit of the CF (Peyroche et al., 2000). High level transcription requires a template containing both the core and the upstream elements and in addition the TATA-binding protein (TBP) and the upstream activating factor (UAF) (Steffan et al., 1996; Keener et al., 1998). UAF is a multisubunit complex containing Rrn5, Rrn9, Rrn10 and Uaf30p and binds to the upstream element and stimulates transcription *in vitro* (Keys et al., 1996; Keener et al., 1997). *In vivo*, UAF is thought to bind the upstream element and recruit the CF with the help of TBP (Steffan et al., 1996; Steffan et al., 1998). DNaseI footprinting studies are consistent with this hierarchy of assembly (Bordi et al., 2001). Histones H3 and H4 were co-purified with UAF and might be required for stabilization of UAF *in vivo* (Keener et al., 1997). Tagged H4 was used to affinity-purify UAF (Keener et al., 1997).

A second function of UAF is to prevent transcription of rDNA by RNAP-II (called 'silencing' of RNAP-II transcription). Mutant strains with a defect in *RRN5, RRN9 or RRN10* accumulate cells able to grow by synthesizing rRNA by RNAP-II (polymerase switch for growth, PSW) (Oakes et al., 1999; Vu et al., 1999; Siddiqi et al., 2001a). Mutants in the Uaf30 subunit use mainly RNAP-I transcription, but RNAP-II transcription is not completely silenced (Siddiqi et al., 2001b). Thus, UAF is not only required for recruitment of CF and RNAP-I.

The role of TBP is controversial. While TBP was shown to stimulate RNAP-I transcription in an UAF-dependent manner *in vitro* and *in vivo*, serving as a bridge between UAF and CF (Steffan et al., 1996; Steffan et al., 1998), stimulation of CF- directed RNAP-I transcription by TBP *per se* is a matter of debate (Aprikian et al., 2000; Siddiqi et al., 2001a).

RNAP-II transcription of reporter genes integrated in the rDNA locus are frequently silenced (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997) (Chapter 1.6.2). This repression is dependent on Sir2, but also other silencing complexes such as SAS-I, CAF-I and Asf1 may be
involved, possibly via acetylation/deacetylation of histones (Meijsing and Ehrenhofer-Murray, 2001). Low levels of Sir2p in the nucleolus reduce rDNA silencing (Smith et al., 1998). Yeast Sir2 is a NAD-dependent histone deacetylase (Imai et al., 2000) involved in chromatin silencing, metabolism and aging (Guarente, 2000). rDNA silencing by Sir2 requires an essential nucleolar
protein, Net1, which recruits Sir2 to the nucleolus (Straight et al., 1999). Net1 associates with the Sir2, Cdc14 and Nan1 to form RENT (‘regulator of nucleolar silencing and telophase exit’). This complex controls mitotic exit (Shou et al., 1999; Visintin et al., 1999), meiotic checkpoint control (San-Segundo and Roeder, 1999), nucleolar silencing (Straight et al., 1999; Cuperus et al., 2000) and localization of proteins implicated in pre-rRNA processing, methylation and ribosome assembly (Straight et al., 1999). It was suggested that Net1 directly stimulates RNAP-I transcription in vivo and in vitro (Shou et al., 2001). Thus, Net1 is a key player in the nucleolus, which not only recruits Sir2, but is additionally involved in regulation of transcription, cell-cycle and checkpoint control (reviewed in Garcia and Pillus (1999)).

Although rDNA is highly transcribed by RNAP-I, accounting for approximately 60 % of total RNA synthesis in growing cells (Woolford, 1991), not all copies of rRNA genes are active at any given time (Warner, 1989). In exponentially-growing yeast cells only about half of the rDNA repeats are transcribed, covered by elongating RNAP-I and free of nucleosomes. The other half of the 35S genes are transcriptionally repressed and maintain nucleosomes (Dammann et al., 1993). The rate of rRNA synthesis and the ratio of active and inactive genes is dependent on the growth conditions (Dammann et al., 1993; Grummt, 1999) and the ‘silenced’ and active rRNA genes are randomly interspersed (Dammann et al., 1995).

What renders a 35S gene competent for transcription initiation? What are the regulatory mechanisms controlling the ratio of active to inactive 35S rRNA genes? Regulation could occur at the level of the 35S promoter involving RNAP-I transcription factors. DNaseI footprinting studies analyzed a mixed population of both active and inactive promoters and did not discriminate between the two fractions (Vogelauer et al., 1998; Bordi et al., 2001). Thus, it is not known whether CF and UAF are bound to promoters of both active and repressed 35S genes in vivo (‘active’ and ‘inactive’ promoters). Furthermore, posttranslational modifications of RNAP-I transcription factors or histones might regulate the transcriptional activity of a 35S gene. In higher eukaryotes, acetylation/deacetylation of the RNAP-I transcription factor UBF or histones was shown to modulate RNAP-I transcription initiation (Pelletier et al., 2000; Hirschler-Laszkiewicz et al., 2001; Muth et al., 2001; Voit and Grummt, 2001). In yeast, phosphorylation of RNAP-I and Rrn3 was shown to be required for transcription initiation (Peyroche et al., 2000; Fath et al., 2001). Furthermore, Net1 was shown to bind directly to RNAP-I and was suggested to stimulate RNAP-I transcription in vivo and in vitro (Shou et al., 2001). In addition, Net1 recruits Sir2, a histone deacetylase to the nucleolus (Straight et al., 1999). Loss of Sir2 function increased the ratio of actively-transcribed to silenced rDNA copies (Smith and Boeke, 1997). However, a direct involvement of Sir2 in RNAP-I transcription initiation is unclear.
CPD and (6-4) PP are the major DNA lesions generated by UV light. Analysis of CPD repair by NER and photoreactivation in the rDNA spacer revealed inefficient repair in the 35S promoter (Chapter 3.3.2).

Here, we performed a high resolution UV-footprinting approach (Becker and Wang, 1984; Selleck and Majors, 1987a; Axelrod and Majors, 1989; Tornaletti and Pfeifer, 1995) to detect binding of UAF and CF in 35S promoters of active and inactive genes. A photofootprint was observed in the core element ('core footprint’) and the upstream element ('upstream footprint’). Yeast strains mutated in RNAP-I transcription factors revealed that the core footprint is dependent on the CF as well as on the UAF, while the upstream footprint is dependent on UAF only. The footprints were observed both in promoters of active and inactive 35S genes, suggesting that CF and UAF bind all promoters in vivo, irrespective of whether the gene is transcribed or not. Photoreactivation was analyzed in active and inactive 35S promoters. Fast and slow photoreactivation were measured in inactive and active promoters, respectively. This indicates differential stability of the bound factors in active promoters and inactive promoters.
4.3 RESULTS

4.3.1 UV PHOTOFOOTPRINTING OF THE 35S PROMOTER

4.3.1.1 UV Photofootprinting in Yeast Strains Defective in RNAP-I Transcription

CPD repair analysis in the rDNA spacer by NER and photoreactivation revealed enhanced damage induction and inhibition of repair in the 35S promoter (Chapter 3.3.2). To test an involvement of RNAP-I transcription factors, UV photofootprinting was performed in strains defective in RNAP-I transcription factors or RNAP-I. The strain NOY408-1a (rpa135Δ) is defective in a subunit of RNAP-I. NOY567 (rrn6Δ), NOY558 (rrn7Δ) and NOY730 (rrn11Δ) are defective in the factors, which bind as a complex to the core element (Lalo et al., 1996). NOY699 (rrn5Δ), NOY703 (rrn9Δ) and NOY704 (rrn10Δ) lack a subunit of the upstream activating factor (UAF) (Keys et al., 1996). NOY604 (rrn3Δ) lacks an essential transcription factor binding to RNAP-I (Yamamoto et al., 1996). All mutant strains are defective in RNAP-I transcription but grow in galactose medium due to a helper plasmid carrying the 35S gene under the control of the GAL7 promoter (Nomura, 1998).

The RNAP-I mutants, their host strain NOY556 (carrying the helper plasmid only) and W303-1a (RAD1) were grown in YPG medium and irradiated in selective medium containing galactose with 150 J/m². Purified DNA was digested with AvaII and used for primer extension analysis (Wellinger and Thoma, 1997): An end-labeled primer was annealed to the bottom strand about 280 bp upstream of the transcription initiation site (Fig. 4-1 B) and was extended by Taq polymerase towards the transcription start site of the 35S gene. Taq polymerase elongation is efficiently blocked at CPDs and (6-4) PPs and frequently stops one nucleotide 3’ from a damaged dipyrimidine, producing radiolabeled fragments of different sizes, which indicate the pyrimidine dimer positions (Wellinger and Thoma, 1996; Wellinger and Thoma, 1997). Primer extension products of the photofootprinting analysis are shown in Fig. 4-2.

Unirradiated DNA (lane 8) shows the top fragment and no stops. DNA irradiated in vitro (lane 6) and DNA of irradiated cells (lanes 9 and 10) reveal numerous stops. Comparison with the transcription is functional in W303-1a (lane 9) and NOY556 (lane 10). Damaged NOY556 DNA as in lane 10, treated with E. coli photolyase in lane 11. NOY408-1a (rpa135Δ) in lane 12 is defective in RNAP-I. NOY604 (rrn3Δ), NOY567 (rrn6Δ), NOY558 (rrn7Δ), NOY730 (rrn11Δ), NOY699 (rrn5Δ), NOY703 (rrn9Δ) and NOY704 (rrn10Δ) are mutated in one of the RNAP-I transcription factors (lanes 13 - 19). Quantified damage clusters and their position with respect to the transcription initiation site (+1) are indicated by black boxes. A schematic representation of the 35S promoter region indicating functional elements is adapted from Keener et al. (1998).
Figure 4-2: Photofootprinting in 35S Promoter of Yeast Strains Defective in RNAP-I Transcription Factors

Primer extension products of the bottom strand using the primer AMrDNA-N, which anneals about 280 bp upstream of the 35S transcription initiation site (see Fig. 4-1). The lanes represent: dideoxy sequencing reactions A, G, C and T (lanes 1 - 4). DNA damaged in vitro with 80 J/m² in 50 % DMSO in lane 5 (test for T-tract structures) or 10 mM Tris, 1 mM EDTA, pH 8.0 (lane 6). Damaged DNA as in lane 6, treated with E. coli photolyase to remove CPDs and to display (6-4) PPs and other non-CPD lesions in lane 7. DNA of non-irradiated W303-1a cells (lane 8) and DNA of different strains irradiated with 150 J/m² (chromatin) in lanes 9 - 19. RNAP-I
sequencing lanes (lanes 1 - 4) shows that stops correlate to dipyrimidines and pyrimidine clusters. A UV dose of 150 J/m² generates on average 0.27 CPDs per kb in the rDNA spacer (Chapter 3.3.2), which corresponds to about 0.04 CPDs per 35S promoters (160 bp). The intensity of the bands corresponds to the frequency of pyrimidine dimer formation at particular sites. To detect non-CPDs (predominantly (6-4) PPs), irradiated DNA was treated with *E. coli* photolyase (lanes 7 and 11). The remaining stops are weak, demonstrating that CPDs are the major photoproducts in DNA and chromatin. The pyrimidine site at position -102 shows pronounced formation of non-CPD lesions in chromatin (lane 11), but not in DNA (lane 7). This represents a photofootprint in chromatin.

Comparison of damage formation in chromatin of wt strains (lanes 9 and 10) and RNAP-I transcription mutants (lane 12 to 19) reveals three distinct groups of damage patterns: i) Irradiated chromatin of wt cells (lanes 9 and 10). ii) Mutants in RNAP-I, Rrn3 and CF proteins (lanes 12 to 16) and iii) the three UAF mutants (lanes 17 to 19).

**UV Photofootprints in the Core Element and the Upstream Element**

A close view of the 35S promoter region of irradiated DNA, NOY556 (wt), the CF-mutant NOY730 (*rrn11Δ*) and the UAF-mutant NOY699 (*rrn5Δ*) is shown in Fig. 4-3. Relative pyrimidine dimer yields are visualized by PhosphorImager-scans. Comparison of wt chromatin and irradiated DNA shows enhanced dimer formation in chromatin at positions -26, -102 and -119. Fewer dimers than in DNA are formed at -56, -62 and -83. *In vitro* repair of CPDs by *E. coli* photolyase (Fig. 4-2, lane 11) did not remove the dimers at position -102 and suggests that these dimers represent (6-4) PPs. The altered damage pattern in wt chromatin from -26 to -119 represents the UV photofootprint of chromatin in the 35S promoter.

**Core Factor (CF) Binding Requires the Upstream Activating Factor (UAF)**

Analysis of the CF-mutant NOY730 (*rrn11Δ*) shows that the footprint in the core element (-26) is absent (Fig. 4-3). The damage pattern at -26 is similar to that seen in DNA. Thus, this footprint is dependent on a functional CF and is therefore called ‘core footprint’. The chromatin footprint in the upstream element (from -56 to -119) is present.

A lack of the core footprint similar to the *rrn11Δ* strain is observed in the other CF-mutants NOY567 (*rrn6Δ*) and NOY558 (*rrn7Δ*), as well as in NOY604 (*rrn3Δ*) and the RNAP-I mutant strain NOY408-1a (*rpa135Δ*) (Fig. 4-2, lanes 12 to 16). Thus, binding of the CF requires not only a functional CF, but Rrn3 and RNAP-I as well.

The strain defective in UAF (NOY699 (*rrn5Δ*)) lacks the chromatin footprint at positions -56 to -119 (Fig. 4-3). Thus, the footprint from -56 to -119 is called ‘upstream footprint’. The upstream footprint is also absent in NOY703 (*rrn9Δ*) and NOY704 (*rrn10Δ*) (Fig. 4-2, lanes 18 and 19).
4 - Damage Formation and Photoreactivation of the 35S Promoter

Damage formation in the UAF mutants is similar in chromatin and in DNA. The results show that the footprint can be assigned to binding of UAF. Furthermore, UAF components Rrn5, Rrn9 and Rrn10 are required for binding of UAF to the 35S promoter.

The UAF mutants not only lack the upstream footprint, but also the core footprint, although the CF proteins are intact (Fig. 4-3). This suggests that UAF binding is required for subsequent assembly of the core factor. This binding hierarchy is consistent with in vivo transcription studies (Steffan et al., 1996; Steffan et al., 1998) and Dsase footprinting experiments (Bordi et al., 2001), which proposed UAF as being necessary for CF binding and assembly of the RNAP-I initiation complex.

Figure 4-3: Pyrimidine Dimer Sites in the 35S Promoter of S. cerevisiae

Pyrimidine dimer patterns of the 35S promoter region of irradiated DNA and chromatin of the strains NOY556 (wt), the CF-mutant NOY730 (rrn11Δ) and the UAF-mutant NOY699 (rrn5Δ). DNA was irradiated with 80 J/m², chromatin with 150 J/m². The purified DNA was analyzed as described in Fig. 4-2. A schematic map of the upstream element, the core element and the position of dimer sites relative to the transcription start site (+1) is displayed. A comparison of relative pyrimidine dimer yields is shown (PhosphorImager-scans). Dashed lanes depict dimer positions with marked differences in damage formation (see text for details).
Quantification of Pyrimidine Dimer Yields in Yeast Mutants Defective in RNAP-I Transcription

Damage sites marked with black bars (Fig. 4-2) were quantified using a PhosphorImager. Relative pyrimidine dimer yields (% of total damages) in each strain are shown in Fig. 4-4. The quantified results confirm visual inspections of the gels and the scans in Fig. 4-3. Comparison of DNA and the yeast strains revealed three distinct groups of damage patterns in chromatin: i) The damage pattern of wt cells (NOY556, W303-1a), characterized by the peak at -26 (core element) and the altered damage pattern in the upstream element (-56 to -119). ii) The patterns of the CF-mutants (NOY567, NOY58, NOY730), NOY604 ($rrn3\Delta$) and NOY408-1a ($rpa135\Delta$) lost the -26 peak, but showed the upstream footprint (-56 to -119). iii) The UAF-mutants (NOY699, NOY703, NOY704) displayed a damage pattern similar to that of irradiated DNA.

Detailed inspection of damage yields at position -26 revealed that CPD yields were two-fold higher in wt chromatin than in DNA (16 % and 8 %, respectively). About 10 % were formed in NOY408-1a ($rpa135\Delta$) and NOY604 ($rrn3\Delta$) and about 8 to 9 % in CF-mutants, UAF-mutants and NOY604 ($rrn3\Delta$). Thus, damage yields in all mutant strains were similar to those in DNA. This indicates that CF was absent in all mutant strains defective in RNAP-I, Rrn3, the CF or UAF.

Comparison of the upstream element of UAF mutants and DNA revealed similar damage yields. This was obvious at positions -56, -83, -102 and -119. Thus, the quantification confirmed that UAF was absent in UAF-mutants.

In addition, the footprinting experiments in mutants defective in RNAP-I transcription factors demonstrate that UV photofootprinting allows to detect binding or absence of the CF and the UAF in vivo.

4.3.1.2 UV Photofootprinting in Active and Inactive 35S Promoters

In yeast, only a fraction of the 35S genes is actively-transcribed, while the other fraction is repressed (Dammann et al., 1993). Thus, the UV photofootprint in total wt chromatin might represent an average of two footprints of active and inactive 35S promoters. We were interested to know whether CF and UAF bind promoters of active and inactive 35S genes and therefore analyzed pyrimidine dimer formation in active and inactive 35S promoters.

The strain AMY3 ($rad1\Delta$, derived from W303-1a) was grown in full medium containing glucose and irradiated with UV light and a dose of 150 J/m$^2$. A yeast nuclear extract was prepared using glass beads and digested with NheI (Fig. 4-5 B-1) to release active genes and the rDNA spacer between two active genes (Muller et al., 2000). DNA was purified and cut with NarI (Fig. 4-5 B-3), resulting in four different species of rDNA spacer fragments: Spacer with downstream active
Figure 4-4: Damage Formation in 35S Promoter of Yeast Strains Defective in RNAP-I Transcription

Quantification of pyrimidine dimer yields in the 35S promoter of DNA and in cells irradiated with 80 J/m² and 150 J/m², respectively. Cells were grown in full medium containing galactose and irradiated in selective galactose medium. RNAP-I transcription is functional in NOY556 and W303-1a. NOY408-1a (rpa135Δ) is defective in RNAP-I. NOY604 (rrn3Δ), NOY567 (rrn6Δ), NOY558 (rrn7Δ), NOY730 (rrn11Δ), NOY699 (rrn5Δ), NOY703 (rrn9Δ) and NOY704 (rrn10Δ) are mutated in one of the RNAP-I transcription factors. The sum of all quantified yields was set to 100% damage. Pyrimidine dimers (%) represent the relative damage yields at individual sites (black bars, Fig. 4-2). The position with respect to the transcription start site is indicated. Sites located in the core element (Core) or upstream element (UE) are indicated. Data are derived from one irradiation experiment per strain and 10 gels (DNA), four gels (W303-1a and NOY408-1a) or two gels. Standard deviations depict the average of at least three gels.
genes ('active' promoters, open circles) are 4.7 kb and 5.5 kb in length, depending on whether the upstream gene was active or inactive. rDNA spacer fragments containing inactive promoters (black circles) resulted in 8.4 kb and 9.1 kb fragments, with upstream active and inactive genes, respectively. The DNA was fractionated on agarose gels (Fig. 4-5 B-4) and the active promoter fragments (4.7 kb, 5.5 kb) were separated from the inactive ones (8.4 kb and 9.1 kb) by gel purification. The DNA was redigested with *Ava*II (Fig. 4-1 B) and the active and inactive 35S promoters were analyzed by primer extension.
**Similar Core and Upstream Footprint in Active and Inactive 35S Promoters**

Primer extension analysis of irradiated DNA and chromatin of either total, active or inactive 35S promoters is shown in Fig. 4-6 A. PhosphorImager-scans revealed the relative dimer yields in the footprint region (as in Fig. 4-3). Comparison of DNA and total 35S promoters of strain AMY3 (*rad1Δ*) revealed the chromatin footprint in the upstream- and the core element of the 35S promoters. The footprint is similar to the chromatin footprint observed in NOY556 and W303-1a (Fig. 4-3), which were grown in galactose medium. This demonstrates that the chromatin footprint does not change when cells are grown in galactose or glucose and that both CF and UAF are bound to the promoters of AMY3.

Both active and inactive promoters showed a similar pyrimidine dimer pattern as all 35S promoters. As in all 35S promoters, there was enhanced damage formation at positions -26, -102 and -119. Reduced damage formation was observed at -56, -62 and -83. Thus, there was no obvious difference between the two promoter fractions. This suggests that CF and UAF bind both active and inactive promoters *in vivo*, irrespective of the transcriptional activity of their downstream 35S rRNA genes. It was shown in Chapter 4.3.1.1 that RNAP-I and Rrn3 are essential for CF binding. Thus, both RNAP-I and Rrn3 have to be present in both active and inactive promoters.

As an alternative explanation for the similar UV photofootprint in active and inactive promoters one might argue that the fractionation of the 35S promoters was inefficient: However, psoralen crosslinking experiments (Chapter 2.3.3) demonstrated that *Nhe*I released only the active genes. Some 35 % of the active genes might have been partially *Nhe*I-cut (Chapter 2.3.3). After digestion of the purified DNA with *Nar*I (Fig. 4-5 B-3), about half of the partially-cut active genes (about 15 to 20 % of all active promoters) might have been co-purified with inactive promoters. Thus, the active promoter fraction may have been pure, while the inactive promoter fraction might have contained about 15 to 20 % of active 35S promoters.

**Quantification of Pyrimidine Dimer Yields in Active and Inactive 35S Promoters**

Quantification of the relative pyrimidine dimer yields in total, active and inactive 35S promoters is shown in Fig. 4-6. The quantified results confirm the visual inspection of the gel and the scans in Fig. 4-3. A closer inspection reveals subtle differences in the core footprint. At position -26, more dimers are formed in active promoters compared with the inactive fraction (17 % and 13 %, respectively). The differences were small, but reproduced in all sets of experiments (four and two gels for active and inactive promoters, respectively). The results may indicate that CF binding in active promoters is tight, while inactive promoters might be partially free of CF. Examination of the pyrimidine clusters in the 35S transcribed region (positions +26 and +5)
**Figure 4-6: UV Photofootprint in Active and Inactive 35S Promoters of AMY3 (rad1Δ)**

A Pyrimidine dimer formation of 35S promoters of irradiated DNA, total 35S promoters (35S-P), active and inactive 35S promoters of AMY3 (rad1Δ). DNA was irradiated with 80 J/m². Cells were grown in YPD and irradiated with 150 J/m². The purified DNA was analyzed as described in Fig. 4-2. The star marks an unspecific Taq polymerase stop probably due to nicking during restriction digest. A schematic map of the upstream element, the core element and the position of dimer sites relative to the transcription start site (+1) is displayed. A comparison of relative pyrimidine dimer yields is shown (PhosphorImager-scans). Dashed lanes depict dimer positions with marked differences in damage formation in DNA and chromatin. B Quantification of relative pyrimidine dimer yields. Pyrimidine dimers (%) represent the relative damage yields at individual sites (black bars in A). The position with respect to the transcription start site is indicated. Sites located in the core- (Core) or upstream element (UE) are indicated. Data are derived from one irradiation experiment and 10 gels (DNA), 4 gels (Active Promoters) or 2 gels (Total and Inactive Promoters). Standard deviations are indicated for DNA and active 35S promoters.
shows that more lesions were formed in repressed genes compared with active genes (position +26: 16 % and 10 %, Position +5: 8.5 % and 6 %, respectively). The effects were very small, but reproducible in four and two gels for active and inactive promoters, respectively. This suggests that repressed genes are more susceptible to pyrimidine dimer induction than actively-transcribed ones. CPD induction in the 4.4 kb *Nhe*I fragment of the 35S gene was found to be higher in inactive genes than in active genes, in both AMY3 (*rad1*Δ) and W303-1a (see Chapter 2.3.6, Fig. 2-12). Thus, the results of two pyrimidine clusters close to the transcription start site are consistent with the CPD formation in a whole DNA fragment.

### 4.3.2 Photoreactivation in Total, Active and Inactive 35S Promoters

Having observed that UAF and CF were bound to 35S promoters of both active and inactive genes, and that CPD repair was slow in the whole promoter region (see Chapter 3.3.2), we were interested in the repair of CPDs in active and inactive 35S promoters at high resolution. The strain AMY3 (*rad1*Δ) with inactivated NER was grown in YPD, irradiated in minimal medium with 150 J/m² and exposed to photoreactivating light for up to two hours at 24 to 26°C. For analysis of CPD repair in total 35S promoters, purified DNA was digested with *Ava*II and analyzed by primer extension. An analysis of photoreactivation (PR) in total 35S promoters is shown in Fig. 4-7. To analyze repair in active and inactive 35S promoters, promoter fractions of active and inactive promoters were prepared (Chapter 4.3.1.2). The DNA was analyzed by primer extension. An example of photoreactivation in inactive 35S promoters is shown in Fig. 4-8, photoreactivation in active promoters in Fig. 4-9.

DNA irradiated *in vitro* and photoreactivated with *E. coli* photolyase (lanes 7) indicated that most of the pyrimidine dimers were CPDs. DNA of irradiated chromatin and photoreactivated *in vitro* with *E. coli* photolyase revealed the preferential formation of (6-4) PPs at position -102 (as seen in Fig. 4-2).

DNA of irradiated cells (chromatin) is shown in lanes 10. Lanes 11 to 15 show photoreactivation for 7, 15, 30, 60 and 120 minutes. No repair was observed in cells incubated for 120 minutes in the dark (lanes 16), which demonstrates that AMY3 is defective in NER.

(Fig. 4-7), continued

150 J/m² (chromatin, lanes 9 - 16); photoreactivated for 7 - 120 min (lanes 11 - 15) or incubated in the dark for 120 min (lane 16). Damaged DNA as in lane 10, but treated with *E. coli* photolyase in lane 9. Damage sites used for quantification of CPD repair and their position with respect to the transcription initiation site (+1) are indicated by black boxes. A schematic representation of the 35S promoter region indicating functional elements is adapted from Keener et al. (1998).
Primer extension products of the bottom strand using the primer AMrDNA-N, which anneals about 280 bp upstream of the 35S transcription initiation site (see Fig. 4-1). The lanes represent dideoxy sequencing reactions A, G, C and T (lanes 1 - 4); DNA damaged in vitro with 80 J/m² in 50 % DMSO (lane 5) or 10 mM Tris, 1 mM EDTA, pH 8.0 (lane 6); damaged DNA as in lane 6, treated with *E. coli* photolyase to remove CPDs and to display (6-4) PPs and other non-CPD lesions in lane 7; DNA of non-irradiated cells (lane 8); DNA of cells irradiated with
Figure 4-8: Photoreactivation in Inactive 35S Promoters of AMY3 (rad1Δ)

Primer extension products of the bottom strand using the primer AMrDNA-N, which anneals about 280 bp upstream of the 35S transcription initiation site (see Fig. 4-1 B). The lanes represent dideoxy sequencing reactions A, G, C and T (lanes 1 - 4). Primer extension reactions in lanes 5 to 16, symbols and schemes as described in Fig. 4-7. Stars mark unspecific Taq polymerase stops probably due to nicking during restriction digest. Quantification of CPD repair of sites marked by black boxes is shown in Fig. 4-10.
Figure 4-9: Photoreactivation in Active 35S Promoters of AMY3 (rad1Δ)

Primer extension products of the bottom strand using the primer AMrDNA-N, which anneals about 280 bp upstream of the 35S transcription initiation site (see Fig. 4-1). The lanes represent: dideoxy sequencing reactions A, G, C and T (lanes 1 - 4). Primer extension reactions in lanes 5 to 16, symbols and schemes as described in Fig. 4-7. Stars mark unspecific Taq polymerase stops probably due to nicking during restriction digest. Quantification of CPD repair of sites marked by black boxes is shown in Fig. 4-10.
CPD repair of pyrimidine dimer clusters (Fig. 4-7, black bars) was quantified using a PhosphorImager. The corresponding signal of the pyrimidine cluster in the *in vitro* photoreactivated lane 9 was subtracted from the damage signal in lanes 10 to 15 to subtract non-CPD lesions. Repair of the CPDs in lane 10 was set to 0 %. CPD repair curves of total, active and inactive 35S promoters are shown in Fig. 4-10.

**Inhibition of Photoreactivation in the Core Element of Active 35S Promoters**

Repair is visualized by a time-dependent decrease in the intensities of the different bands with increasing repair time (lanes 11 - 15). CPDs in the core element of total 35S promoters appeared to be poorly repaired (Fig. 4-7, positions -26, -32 and -40). This result is consistent with the analysis of CPD repair of the rDNA spacer at low resolution by photoreactivation and NER, where an inhibition of repair could be mapped to the 35S promoter region (Chapter 3.3.2). This inhibition of photoreactivation in the core is evidence *in vivo* that the CF remains bound to the core element after damage induction and shields lesions from repair. CPD repair curves of total 35S promoters (Fig. 4-10, Total) substantiated the visual observation. About 10 to 20 % of the lesions were repaired after two hours (positions -26, -32 and -40).

Comparison of photoreactivation in the core element of active and inactive promoters (lanes 10 to 15 in Fig. 4-8 and Fig. 4-9) revealed that repair was absent in active promoters (no repair visible after 120 min.). Quantification of CPD repair (Fig. 4-10, Active, positions -26, -32, -40) confirmed that repair was absent (0 % in two hours). Thus, the CF bound to active promoters apparently restricts access of photolyase to CPDs for at least two hours after damage induction. This result indicates that the interaction of CF with the core element is stable in active promoters. In contrast, the core element of inactive promoters was partially repaired (Fig. 4-8). About 50 % of the lesions were removed within two hours (Fig. 4-10). Thus, photolyase has access to at least a fraction of the damage, which suggests that the complex of the CF with the core element may be less stable after damage induction than in active promoters.

**Slow Photoreactivation in the Upstream Element of Active 35S Promoters**

Comparison of CPD repair of different damage sites in the upstream element of active and inactive promoters (Fig. 4-10, Active and Inactive) revealed in general fast repair in inactive and slow repair in active promoters. This is obvious at positions -77, -102, -115 and -129. Thus, UAF inhibits access of photolyase to lesions predominantly in active promoters, while inactive promoters are better accessible for photolyase. This indicates that binding of UAF in active promoters is more stable than in inactive promoters.
Figure 4-10: CPD Repair by Photolyase in Total, Active and Inactive 35S Promoters

A A schematic representation of the 35S promoter region indicating functional elements (adapted from Keener et al. (1998)). The position of the damage clusters is depicted by black boxes and the positions with respect to the transcription initiation site of the 35S rRNA gene. B Quantified CPD repair from 0 to 100 % in up to two hours is shown for total 35S promoters (promoters of inactive and active 35S genes, Total, see Fig. 4-7) and purified inactive- and active 35S promoters (Inactive, Active, see Fig. 4-8 and Fig. 4-9, respectively). Repair curves are averages of two gels (Total, Inactive) or three gels (Active) of one repair experiment.
**CPD Repair in Total 35S Promoters is Slower than in Inactive- and Faster than in Active 35S Promoters**

Comparison of repair curves of different sites in total 35S promoters with the corresponding sites in active and inactive fractions shows that in all sites, repair of total was faster than in the active fraction, but slower than in inactive fraction. This observation suggests that analysis of all 35S promoters indeed reflected a mixed population of two fractions of promoters and in addition indicates that the fractionation of active and inactive 35S promoters allowed to separate two structurally different types of 35S promoters.
4.4 DISCUSSION

A Chromatin Footprint in the 35S Promoter

In *S. cerevisiae*, the combination of both NER and photoreactivation efficiently repairs UV lesions. Analysis of the rDNA spacer revealed inefficient repair of the 35S promoter by both NER and photoreactivation (Chapter 3.3.2). Here we show that these protected lesions are located in the core element. Comparison of pyrimidine dimer patterns of DNA and chromatin revealed a UV photofootprint in the upstream and the core element of the 35S promoter.

Pyrimidine dimer formation in mutants, which are defective in RNAP-I transcription factors, revealed two classes of footprints:

i) Mutant strains defective in the CF proteins Rrn6, Rrn7, Rrn11 as well as Rrn3 and RNAP-I lost the footprint in the core element (core footprint). Our UV experiments map the core footprint from about -26 to -40. Two previous studies mapped the boundaries of the core and the upstream element: *In vitro* transcription studies defined the core element from +5 to -38 (Keener et al., 1998), DNaseI footprinting experiments revealed a footprint in this region from +1 to -70 (Vogelauer et al., 1998) (Fig. 4-1 A). Our results suggest that binding of the CF exceed the mapped position -38 towards the upstream element.

ii) Mutant strains defective in the UAF proteins Rrn5, Rrn9 and Rrn10 lack the upstream footprint. Thus, binding of UAF is required for the upstream footprint. We map UAF footprints to the region of -56 to -119. The published studies mapped the upstream element from -60 to -155 (Keener et al., 1998) and from -70 to -154 (Vogelauer et al., 1998) (Fig. 4-1 A). Thus, our results suggest that the downstream border of the UAF binding site ranges at least to position -56. The lack of UV footprints upstream of -119 does not exclude that promoter regions up to about -150 are necessary for binding of UAF.

UAF is Required for Binding of the CF

The core footprint was dependent on the CF subunits Rrn6, Rrn7, Rrn11 and Rrn3 and RNAP-I. This suggests that Rrn3 and RNAP-I are required for binding of the CF. The absence of the CF did not affect the footprint of UAF, which suggests that CF binding is not required for association of UAF with the upstream element.

UAF mutants lack both the upstream footprint and the core footprint. This suggests that UAF is required for the recruitment of CF to the core element. *In vitro* transcription studies established that the CF is recruited by UAF and TBP (Steffan et al., 1996). In addition, interaction of TATA-binding protein with UAF was required for activated transcription of rDNA by RNAP-I *in vivo* (Steffan et al., 1998). DNaseI footprinting in total yeast 35S promoters observed a footprint in
the core element, which was dependent on UAF (Bordi et al., 2001). Thus, our results stand in agreement with the transcription and DNaseI footprinting studies and confirm the binding hierarchy of the RNAP-I transcription factors in living cells.

In addition, our results demonstrate that binding of CF and UAF results in a characteristic UV photofootprint. Thus, irradiation of cells and analysis of damage formation is a useful tool to monitor the presence or absence of the factors in vivo.

**Fractionation of Active and Inactive 35S Promoters**

Since only a fraction of all rDNA copies is active in growing cells, we wondered whether binding of CF and UAF is different in promoters of actively-transcribed and repressed genes (‘active’ and ‘inactive’ promoters). Active and inactive 35S promoters were fractionated by restriction enzyme digestions and gel purification. Two lines of evidence indicate that the 35S promoter fractions indeed contain two structurally different promoters: First, the release of active genes by \(N\)hel was shown to be specific for active genes (Muller et al., 2000; Meier et al., 2002). Thus, the active promoter fraction contains no inactive promoters. Quantification of \(N\)hel-cut genes (not shown) revealed that about 35% of active genes were partially \(N\)hel-cut. After redigestion of the purified DNA with \(N\)arl, about half of the partially-cut active genes might appear in the active promoter fraction. Thus, we estimate that not more than about 20% of all active promoters might be co-purified with inactive ones. Since the majority of all genes (about 65%) were inactive in our experimental conditions and only about 35% of the genes active (Meier et al., 2002), the contamination of the inactive fraction with active promoters might be small. Second, the photoreactivation experiments presented here show a clear difference in repair of active and inactive promoters. Thus, we conclude that our fractionation protocol results in a pure active promoter fraction and an inactive fraction, which might contain a minor fraction of active promoters.

**CF, UAF, Rrn3 and RNAP-I Bind Both Active and Inactive 35S Promoters in vivo**

Both core and upstream footprints were found in active and inactive promoter fractions. This indicates that CF and UAF are bound to promoters of all genes, irrespective of whether they are transcribed or not. Since Rrn3 and RNAP-I are required for the core footprint (see above), we suggest that Rrn3 and RNAP-I are bound to active and inactive promoters as well. The observation that both complexes were bound to promoters in the inactive fraction was unexpected, since CF and UAF in concert with Rrn3 and RNAP-I are sufficient to initiate transcription in vitro (Keener et al., 1998). The presence of UAF, CF, Rrn3 and RNAP-I in inactive 35S promoters implies that transcription initiation does not solely depend on these core transcription factors. Additional initiation factors might be required in vivo.
As alternative explanations, the complexes in inactive promoters might be destabilized and
nevertheless produce a UV photofootprint. Indeed, subtle differences in the core footprint of
active and inactive promoters indicate that the interaction of CF with inactive promoters might
be less stable.
Furthermore, the observation of the footprint in both active and inactive promoters may indicate
that the fractionation was not complete. As argued above, the active promoter fraction is pure,
since the liberation of active genes by NheI never releases inactive genes (Muller et al., 2000).
The inactive promoter fraction might contain about 20% of all active promoters (see above).
CPD repair in active and inactive promoters revealed marked differences between the two
fractions. This is consistent with two fractions, which indeed represent distinct populations of
promoters.

**Differential Stability of CF and UAF in Active and Inactive Promoters**

Since analysis of damage formation reveals the state of bound factors at the time-point of
irradiation but does not allow to monitor the stability during a time-period, we analyzed
photoreactivation in active and inactive promoters for up to two hours after irradiation. The
repair enzyme photolyase was shown to be an *in vivo* tool to monitor accessibility of chromatin
in living cells (Suter et al., 1997; Aboussekhra and Thoma, 1999; Livingstone-Zatchej et al.,
1999). Photolyase removes CPDs within minutes in open sequences such as nuclease-sensitive
regions or the URA3 promoter (Suter et al., 1997; Aboussekhra and Thoma, 1999). Thus, a
temporal dissociation of the CF in the range of minutes would be sufficient for photolyase to
remove at least a part of the lesions.
Photoreactivation in the core element is inhibited in active promoters at three positions. The
inhibition of photoreactivation in the core element strongly suggests that CF is tightly bound. In
inactive promoters, photoreactivation removed about 50% of the CPD clusters in the core
element. Thus, photolyase has at least partial access to the core element in the inactive fraction.
This can be interpreted as a lower stability of the CF on inactive promoters. However, 50% of
the lesions remained unrepaired. This indicates that inactive promoters are not fully accessible
for photolyase and is consistent with the observation of the core footprint in inactive promoters,
which suggested the presence of the CF (see above). Alternatively, the partial inhibition might be
a result of a fraction of active promoters, which were co-purified with the inactive fraction. As
observed in the core element, CPD repair is inhibited in the UAF of active promoters, but not in
inactive promoters. By analogy to the CF, this indicates that binding of UAF to the upstream
element is more stable in active than in inactive promoters.
The results can be interpreted and summarized as follows: In active promoters, UAF binds the
upstream element and recruits the CF, which is dependent on Rrn3 and RNAP-I (and possibly TBP; (Keener et al., 1998)). The CF and UAF are bound to the core element resulting in the core footprint and an inhibition of photoreactivation. Inactive promoters bind UAF and CF as well. The factors are not stabilized and may dissociate. As a consequence, photoreactivation is inefficient and removes only part of the lesions.

Using UV photofootprinting, no differences were observed between active and inactive promoters. In contrast, photoreactivation revealed differential stability of CF and UAF in the two fractions. Thus, UV photofootprinting allowed to study the CF and UAF at the time-point of irradiation, while photoreactivation served as a more sensitive tool to compare the stability of transcription factors during a time-period of two hours.

*In vitro* transcription experiments using immobilized templates suggested that UAF is permanently bound to the template while RNAP-I, Rrn3 as well as CF dissociate after transcription initiation (Aprikian et al., 2001). These results stand in contrast to our interpretation, which favours a model where CF is tightly bound to active core promoters. The lack of additional stabilizing transcription factors in the *in vitro* assay could explain the discrepancy with the *in vivo* results presented here.

What are the consequences of the slow CPD repair for RNAP-I transcription? Since the 35S promoters are short (about 160 bp), the chance that a major fraction of the 100 to 200 35S promoters of a cell is damaged is very small. A few damaged promoters are unlikely to affect the level of rRNA transcripts. Furthermore, damaged promoters do not inhibit the binding of the RNAP-I transcription factors. Therefore, transcription may still initiate despite a damage in the promoter.

**Activation and Repression of RNAP-I Transcription Initiation**

What are the molecular mechanisms of the repression of 35S genes? How are UAF and CF stabilized in active genes? There is only speculation about candidate factor(s) or mechanisms: TBP was shown to be required for stable association of CF *in vivo* and *in vitro* (Steffan et al., 1996; Steffan et al., 1998). Since TBP is required for the recruitment of CF, and CF is present in both active and inactive promoters, it is unlikely that TBP is present only in active and absent in inactive promoters.

Attractive candidates involved in regulation of RNAP-I transcription are Net1 and Sir2: Net1 is a key player in the nucleolus involved in rDNA silencing, regulation of transcription, cell-cycle and checkpoint control (reviewed in Garcia and Pillus (1999)). Net1 interacts with RNAP-I and was proposed to stimulate RNAP-I transcription *in vivo* and *in vitro* (Shou et al., 2001). In addition, Net1 recruits Sir2 to the nucleolus (Straight et al., 1999). Sir2 is a histone deacetylase,
which deacetylates lysine-residues of histones H3 and H4 (Imai et al., 2000). Sir2 is required for repression of nucleolar RNAP-II transcription (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997). Deletion of SIR2 was shown to increase the fraction of actively-transcribed genes (Smith and Boeke, 1997). However, a direct involvement of Sir2 in RNAP-I transcription initiation is unknown. How could Sir2 regulate the transcriptional activity of 35S genes? In higher eukaryotes, acetylation/deacetylation of the RNAP-I transcription factor UBF or histones was shown to modulate RNAP-I transcription (Pelletier et al., 2000; Hirschler-Laszkiewicz et al., 2001; Voit and Grummt, 2001). Furthermore, acetylation of subunits of the RNAP-I transcription factor SL1 enhanced binding to the rDNA promoter (Muth et al., 2001). In yeast, by analogy to higher eukaryotes, UAF in active promoters might be acetylated and thus stabilized. Sir2 might repress transcription initiation by destabilization of RNAP-I transcription factors via deacetylation of UAF. Alternatively, Sir2 might repress RNAP-I transcription via deacetylation of lysine-residues of histones H3 and H4. Indeed, H3 and H4 were shown to interact with UAF and were suggested to stabilize the complex in vivo (Keener et al., 1997). It may be speculated that only deacetylated histones H3 and H4 stabilize UAF. More detailed investigations will be required to clarify the role of Sir2 in regulation of RNAP-I transcription initiation.

In addition, the phosphorylation state of RNAP-I and Rrn3 was shown to be critical for transcription initiation (Peyroche et al., 2000; Fath et al., 2001). Thus, multiple mechanisms might be involved in the activation and repression of 35S genes in living cells.
5 CONCLUDING REMARKS

Repair of UV Lesions in the Nucleolus

Repair of UV lesions in ribosomal DNA is controversial: CPD repair by nucleotide excision repair (NER) was absent in rodent cells and inefficient in human cells (Christians and Hanawalt, 1993; Stevnsner et al., 1993; Fritz and Smerdon, 1995; Balajee et al., 1999). It was therefore suggested that the nucleolus might restrict access of repair enzymes to the compartmentalized rDNA (Christians and Hanawalt, 1993; Balajee et al., 1999). In contrast to CPDs, removal of (6-4) PPs in hamster cells was efficient in rDNA (Balajee et al., 1999), although both types of lesions are substrates for NER. CPD repair of rDNA was efficient in budding yeast *S. cerevisiae* (Verhage et al., 1996a).

*S. cerevisiae* and many other organisms have two different repair pathways, NER and photoreactivation, to remove UV-induced CPDs from the genome. In this study we analyzed photoreactivation and NER in the yeast nucleolus and show that photoreactivation is the predominant pathway for CPD removal from the nucleolus. Repair of both repair pathways is as fast in the nucleolus as in the nuclear *GAL10* gene. Thus, the yeast nucleolus seems not to be a barrier for repair enzymes. The inefficient repair by NER in higher eukaryotes might rather be a problem of damage recognition.

Transcription-Coupled NER in RNA Polymerase I Genes of Yeast

Photoreactivation was established as the predominant pathway for CPD repair under saturating light conditions (Livingstone-Zatchej et al., 1997; Suter et al., 1997). Elongating RNA polymerases are blocked at UV lesions on transcribed strands and inhibit photoreactivation. The slow repair on transcribed strands is matched by fast NER, which is coupled to the transcription machinery (Transcription-coupled NER, TC-NER), a phenomenon attributed to RNAP-II genes. Although the *mfd* gene product of *E. coli* was identified as the coupling factor between
transcription and repair in prokaryotes (Selby and Sancar, 1993), and factors such as the yeast Rad26, XAB2 and TFIIH were shown to be involved in TC-NER in eukaryotes, the detailed coupling mechanism is still unknown. TC-NER was not found in RNAP-III genes, (Dammann and Pfeifer, 1997; Aboussekhra and Thoma, 1998) and RNAP-I genes of higher mammals (Fritz and Smerdon, 1995).

Using *S. cerevisiae*, we demonstrate preferential NER of transcribed strands in active ribosomal genes of yeast, while no strand bias is observed in repressed genes. Thus, we extend the phenomenon of TC-NER to genes transcribed by RNAP-I. Our results are consistent with recent results published by Conconi et al. (2002).

At the moment, the phenomenon of transcription-coupled repair is known for the two RNA polymerases I and II and in addition for two types of damage (UV- and oxidative lesions) (Citterio et al., 2000b; Conconi et al., 2002). It is fascinating that TC-NER exists in RNAP-I and RNAP-II genes, but obviously not in RNAP-III genes (Dammann and Pfeifer, 1997; Aboussekhra and Thoma, 1998). Since RNAP-I and RNAP-II transcription machineries use different polymerases and transcription factors (Paule and White, 2000), the question arises whether the molecular coupling mechanisms with NER are different in both classes or whether a common subset of proteins is used.

**Stability of Blocked RNA Polymerases at Lesions**

Photoreactivation is inhibited on transcribed strands of active RNAP-II genes of yeast (Livingstone-Zatchej et al., 1997), probably caused by stalled RNAP-II which inhibits access of photolyase to the lesion site. Interestingly, we find only a slight inhibition of photoreactivation in active RNAP-I genes, despite the high frequency of transcription of ribosomal genes (Grummt, 1999). Thus, RNAP-I seems not to be a strong barrier for the repair enzyme. This indicates differential stability of blocked RNAP-I and RNAP-II at UV lesions. RNAP-I could be released either by dissociation or step back on the template to make the UV lesion accessible for repair (Hara et al., 1999). Additional transcription elongation factors may be involved in this process. Rad26, the mammalian homologue of CSB, is required for TC-NER in RNAP-II genes (Tijsterman and Brouwer, 1999), but it is dispensable for repair in RNAP-I genes (Verhage et al., 1996a). Rad26 was proposed to function in rendering transcription competent for TC-NER in RNAP-II genes (Jansen et al., 2000), possibly by displacing the blocked polymerase (Prakash and Prakash, 2000). Our indication for a lower stability of RNAP-I at a damage would provide an explanation why Rad26 is dispensable in rDNA repair. However, it cannot be excluded that unknown accessory RNAP-I transcription factors displace the stalled RNAP-I instead of Rad26.
Transcription Initiation in RNAP-I Genes

Only a fraction of the 100 to 200 rDNA copies is transcribed at any given time, while the other copies are repressed (Dammann et al., 1993; Grummt, 1999). We show that the basic transcription factors (core factor CF and upstream activating factor UAF), which may initiate transcription \textit{in vitro} (Keener et al., 1998), bind both active and inactive promoters \textit{in vivo}, irrespective of whether the downstream gene is active or repressed. Inhibition of photoreactivation in active promoters indicates a tight interaction of the factors when the gene is transcribed. Reduced inhibition in inactive promoters suggests a less stable interaction of the transcription factors with promoters which do not initiate rRNA synthesis.

These findings beg several questions: Why are inactive promoters, which associate with the basic RNAP-I transcription factors, not able to initiate transcription? Is there need for additional, so far unknown transcription factor(s) to stabilize the initiation complex? Which proteins might be involved in regulation of activation and repression of rDNA copies?

Net1 was suggested as a key player in regulation of transcription, cell cycle- and checkpoint control of the nucleolus (Shou et al., 2001), reviewed in Garcia and Pillus (1999). Future transcription studies will shed light on the influence of Net1 on transcription initiation.

A topic not addressed in this study is the role of Sir2, a protein required for silencing of nucleolar RNAP-II transcription (reviewed in Gartenberg (2000)), in regulation of RNAP-I transcription. Sir2 was suggested to silence the chromatin structure in the rDNA spacer (Fritze et al., 1997; Smith and Boeke, 1997). The protein was shown to be recruited to the nucleolus by Net1 (Straight et al., 1999). Sir2 is a NAD-dependent histone deacetylase (Imai et al., 2000). In higher eukaryotes, deacetylation of the RNAP-I transcription factor UBF or histones was shown to repress RNAP-I transcription (Pelletier et al., 2000; Hirschler-Laszkiewicz et al., 2001; Voit and Grummt, 2001). In yeast, by analogy to higher eukaryotes, Sir2 might repress transcription initiation \textit{via} deacetylation of UAF or histones H3 and H4. More detailed investigations will be required to clarify the role of Sir2 in regulation of RNAP-I transcription initiation.

Sir2 is involved in silencing of telomeres (Gottschling et al., 1990) and the yeast mating type loci (Rine and Herskowitz, 1987). It was shown that silenced telomeres and HM loci are found in a zone near the yeast nuclear envelope (Cockell and Gasser, 1999a). Since the nucleolus is a subnuclear compartment at the nuclear periphery as well (Carmo-Fonseca et al., 2000), the question arises whether repressed rDNA copies might be tethered to the nuclear envelope. Future experiments will elucidate the involvement of the nuclear environment in regulation of rDNA transcription.
6  MATERIALS AND METHODS

6.1  YEAST STRAINS AND CULTURES

*S. cerevisiae* W303-1a was kindly provided by Dr. R. Sternglanz. AMY3 was generated by deletion of part of the RAD1 gene in W303-1a using a gene blaster construct (pR1.6, kindly provided by Dr. L. Prakash). RAD1 knock-out was checked by PCR (Georg Schnappauf, personal communication). AMY3 exhibits a strong UV sensitivity typical for *rad1Δ* strains (Semesterwork Andreas Meier 1995/96, ETH Zürich). Strains NOY556, NOY604, NOY699, NOY567, NOY558, NOY703, NOY704 and NOY730 were kindly provided by Dr Masayasu Nomura. NOY408-1a was kindly provided by Dr J. Sogo. The 'NOY-strains' except NOY556 (wt) are defective in a transcription factor for the RNAP-I machinery. All mutant strains are defective in RNAP-I transcription but grow in galactose medium due to a helper plasmid (pNOY102 or pNOY103) carrying the 35S gene under the control of the *GAL7* promoter (Nomura, 1998). Yeast strain UCC510 was provided by Dr K. Struhl. Strains used in this study, mating type and growth markers are listed in Tab. 6-1.

*Table 6-1: Yeast Strains Used in this Study*

<table>
<thead>
<tr>
<th>Name</th>
<th>Mat</th>
<th>Markers</th>
<th>Plasmid</th>
<th>Growth Medium</th>
<th>Ref, Origin,</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1a</td>
<td>a</td>
<td>ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100</td>
<td>YPD(^d) or YPG(^e)</td>
<td></td>
<td>(Livingstone-Zatchej et al., 1997)</td>
</tr>
<tr>
<td>AMY3</td>
<td>a</td>
<td>ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112,100, <em>rad1Δ:URA3</em></td>
<td>YPD</td>
<td></td>
<td>(Oakes et al., 1999)</td>
</tr>
<tr>
<td>NOY556</td>
<td>a</td>
<td>ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, pNOY103(^d)</td>
<td>YPG</td>
<td></td>
<td>(Yamamoto et al., 1996)</td>
</tr>
<tr>
<td>NOY604</td>
<td>alpha</td>
<td>ade2-1, ura3-1, his3-11, trp1-1, leu2-3,112, can1-100, <em>rrn3Δ:HIS3</em></td>
<td>YPG</td>
<td></td>
<td>(Keys et al., 1996)</td>
</tr>
<tr>
<td>NOY699</td>
<td>alpha</td>
<td>ade2-1, ura3-1, his3-11, trp1-1, leu2-3,112, can1-100, <em>rrn5Δ:LEU2</em></td>
<td>YPG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Yeast cells were grown at 30°C in complete medium containing glucose (YPD; 1% bacto yeast extract, 2% bacto peptone, 2% dextrose) or galactose (YPG; 1% bacto yeast extract, 2% bacto peptone, 2% galactose) (Sherman et al., 1986) to a density of about 0.5 x 10^7 cells/ml.

### UV IRRADIATION AND REPAIR

#### UV IRRADIATION AND REPAIR OF YEAST CELLS

Cells, grown in 3 to 6 l of complete medium to a density of about 0.5 x 10^7 cells/ml, were harvested (10 min at 6000 rpm, 4°C, GS3 rotor) and resuspended in minimal medium without amino acids (SD or SG; 0.67% Yeast Nitrogen Base w/o aa, 2% dextrose or 2% galactose) (Sherman et al., 1986) to about 3 x 10^7 cells/ml. 250 ml aliquots were transferred to plastic trays to form 4 mm layers and irradiated at room temperature at an average flux of 4.7 J/m^2 sec (measured with a radiometer (UVX-25, UVP Inc. San Gabriel, CA)) with 150 J/m^2 of UV light (predominantly 254 nm) generated by germicidal lamps (Sylvania, Type G15 T8). After irradiation, the cells were pooled and the medium was supplemented with the appropriate amino acids (f.c. 2 mg/ml for adenine, histidine, tryptophan, 3 mg/ml for leucine) and uracil (f.c. 2 mg/ml).
To allow photoreactivation, 300 ml to 750 ml samples (5 to 12 mm suspension layers) of the irradiated cells were placed in plastic trays (22 cm x 31 cm) on a metal cooling plate (cooled to 20°C) and exposed to photoreactivating light (Sylvania, Type F15 T8/BLB bulbs, peak emission at 375 nm) at an average flux of about 13 J/m²/sec (measured with a radiometer (UVX-36, UVP Inc. San Gabriel, CA)). NER samples were incubated at room temperature (24°C to 26 ºC) in the dark. Repair samples were collected at different repair times and chilled on ice (100 ml samples in experiments with subsequent fractionation of rDNA, 250 ml samples in all other experiments). All further steps until proteinase K digestion of lysed cells (see Chapter 6.6.2) were performed in yellow safety light (Sylvania GE Gold Fluorescent Light, F36WT8, wavelength ≥525 nm).

6.2.2 UV IRRADIATION AND REPAIR OF DNA in vitro

About 500 ng of purified DNA (as described in Chapter 6.6.1) was dissolved either in 40 µl of 10T1E pH 8.0 or in 40 µl of 50 % DMSO and was pipetted on a coverslip wrapped with parafilm. The DNA was irradiated at room temperature at an average flux of 5.0 J/m² sec at a dose of 80 J/m² of UV light (predominantly 254 nm) generated by germicidal lamps (Sylvania, Type G15 T8). \textit{In vitro} irradiated DNA was purified using the QIAEX II protocol (QIAGEN, 1997). The purified DNA was resuspended in 30 µl 10T1E pH 8.0.

About 300 ng of irradiated DNA in 10T1E pH 8.0 was adjusted with 10T1E pH 8.0 to a total volume of 100 µl. 0.5 µg of \textit{E. coli} photolyase (Becton Dickinson, Cat.No. 40031K, diluted to 0.5 µg/µl in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 10 mM DTT, 50 % glycerol) were added. The suspension was transferred to an inverted Eppendorf™ cap, placed on ice and exposed to photoreactivating light (Sylvania, Type F15 T8/BLB bulbs, peak emission at 375 nm) for 60 min at a flux of about 24 J/m² sec. \textit{In vitro} photoreactivated DNA was purified using the QIAEX II protocol (QIAGEN, 1997). The purified DNA was resuspended in 30 µl 10T1E pH 8.0.

6.3 PREPARATION OF YEAST CRUDE EXTRACTS AND DIGESTION WITH NheI

All steps were performed in yellow safety light (Sylvania GE Gold Fluorescent). Yeast crude extracts were prepared according to Muller et al. (2000): 100 ml cells (3 x 10⁷ cells/ml) were harvested by centrifugation (10 min at 6000 rpm, 4°C, GSA rotor), washed once with cold water and resuspended in 2 ml nuclear isolation buffer (NIB; 17 % glycerol, 50 mM MOPS, 150 mM potassium acetate, 2 mM MgCl₂, 0.5 mM spermidine, 0.15 mM spermine, all adjusted to pH 7.2). The suspension was vortexed with 2 ml of glass beads (Ø 0.5 mm, acid-washed and
equilibrated in nuclear isolation buffer) for 30 sec at full speed and chilled on ice for 30 sec. About 15 - 20 cycles of vortexing/chilling were performed, until about 90 % of the cells were broken (checked under light microscope for loss of contrast of broken cells). Crude extracts of 3 x 10^9 cells were pelleted (10 min, 4°C, 4000 rpm, Heraeus Table Top (4500 x g)), resuspended in 2 ml 1x restriction buffer (AGS Buffer Violet, Heidelberg, Germany: 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 100 µg/ml BSA, pH 7.9) and split in two Eppendorf™ tubes. 160 U NheI (AGS) were added per aliquot of 1.5 x 10^9 spheroplasts and incubated at 37°C for 1 hr to release the active ribosomal genes (Muller et al., 2000). Aliquots were used for psoralen crosslinking (see Chapter 6.4) or DNA purification (see Chapter 6.6.2).

NER activity in the extract at 37°C was checked as described in Chapter 6.11.2 ff, but no repair activity was detected after 1 hr incubation of the extract at 37°C (data not shown).

### 6.4 Psoralen Crosslinking of Yeast Crude Extracts

After digestion of yeast crude extracts of 2 to 4 x 10^8 cells with NheI (see Chapter 6.3), the digested extract was centrifuged (8 min. at 4000 rpm, 4°C, Heraeus table top (3400 x g)) and resuspended in 300 µl nuclear isolation buffer (NIB; 17 % glycerol, 50 mM MOPS, 150 mM potassium acetate, 2 mM MgCl_2, 0.5 mM spermidine, 0.15 mM spermine, adjusted to pH 7.2). The suspension was transferred to wells of a multiwell-dish (Ø 1.6 cm) and placed on ice. 15 µl of TMP (4,5′,8-trimethylpsoralen, SIGMA, 200 µg/ml in EtOH) were added. After 5 min of incubation in the dark, the cells were irradiated on ice for 15 min using Sylvania, Type F15 T8/BLB bulbs (peak emission at 375 nm) at an average flux of about 24 J/m^2 sec. The psoralen addition/irradiation-procedure was repeated four times. The crosslinked nuclear extract was pelleted (2 min at 13000 rpm, RT, Heraeus microfuge) and resuspended in 400 µl TNE (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mM NaCl) and used for DNA purification (see Chapter 6.6.3).

### 6.5 Chromatin Mapping by Micrococcal Nuclease

The experiment was done according to Tanaka et al. (1996): Yeast cells (UCC510) were grown at 30°C in 3 l of YPD (1 % bacto yeast extract, 2 % bacto peptone, 2 % dextrose) to an OD_{600} of about 1.0. The cells were harvested, spheroplasted using zymolyase (Seikagaku Kogyo Co., Ltd. (Bio-Science Products AG), 120493), broken with the help of a Dounce homogenizer and a crude nuclear extract was prepared. Half of the crude nuclear extract was used for DNA purification. Purified DNA and the crude nuclear extract were digested in parallel with micrococcal nuclease
(MNase, Roche Diagnostics). 0.6 to 6 U of MNase were used for digestion of the purified DNA, 15 to 210 U of MNase for digestion of the crude nuclear extract. DNA was purified by phenol extractions as described by Tanaka et al. (1996), cut with ClaI and NdeI (see Chapter 6.11.1) and analyzed by indirect end-labeling as described in Chapter 6.11.3 and Chapter 6.11.4.

6.6 DNA PURIFICATION

6.6.1 DNA PURIFICATION OF YEAST CELLS

Lysis of yeast cells and DNA purification was done as described in QIAGEN (1999). All steps until proteinase K digestion of lysed cells were done under yellow safety light (Sylvania GE Gold Fluorescent) to prevent photoreactivation. 250 ml yeast cells (3 x 10^7 cells/ml) were pelleted by centrifugation (10 min at 6000 rpm, 4°C, GS3 rotor) and washed once with 16 ml cold 10T1E pH 8.0, pelleted as above and resuspended in 12 ml of QIAGEN yeast lysis buffer Y1 (1 M sorbitol, 100 mM EDTA pH 8.0, 14 mM β-mercaptoethanol) and transferred to yellow cap centrifugation tubes. Cells were lysed by addition of 1000 U zymolyase (Seikagaku Kogyo Co., Ltd. (Bio-Science Products AG), 120493, dissolved in H2O to a concentration of 1000 U/ml) and incubation at 30°C for 30 - 60 min. Spheroplasting was monitored by light microscopy (loss of contrast of lysed cells) and OD₆₀₀ measurement of lysed and intact cells in 1 % SDS (OD₆₀₀ of spheroplasted cells decreases more than 90 % compared to intact cells). Spheroplasts (from 7.5 x 10⁹ cells) were pelleted by centrifugation (10 min at 5900 rpm, 4°C, SA-600 rotor) and resuspended in 15 ml of QIAGEN general lysis buffer G2 (800 mM GuHCl, 30 mM EDTA pH 8.0, 30 mM Tris-HCl pH 8.0, 5 % Tween-20, 0.5 % Triton X-100, all adjusted to pH 8.0). RNase A (Roche Cat.No 109 169, dissolved in 10 mM Tris-HCl pH 7.5, 15 mM NaCl to 10 mg/ml) was added to a f.c. of 200 µg/ml and incubated at 30°C for 30 min. Proteinase K (Roche, Cat.No. 745 723, dissolved in 50 mM Tris-HCl pH 8.0 to 10 mg/ml) was added to a f.c. of 300 µg/ml and incubated for 2 hrs at 50°C and ON at RT. The cellular debris was pelleted by centrifugation (10 min at 6000 rpm, 4°C, SA-600 rotor). The clear supernatant was loaded on a QIAGEN Genomic-tip 500/G, which was equilibrated with 10 ml QIAGEN Equilibration Buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % EtOH, 0.15 % Triton X-100, adjusted to pH 7.0). After two wash steps with each 15 ml QIAGEN Wash Buffer QC (1 M NaCl, 50 mM MOPS, 15 % EtOH, adjusted to pH 7.0), the DNA was eluted with 15 ml of QIAGEN Elution Buffer QF prewarmed to 50°C (1.25 M NaCl, 50 mM Tris Base, 15 % EtOH, adjusted to pH 8.5) and precipitated by adding 0.7 volumes of isopropanol. The DNA was pelleted by centrifugation (30 min at 10000 rpm, 4°C, HB-4 rotor), washed with 4 ml cold 70 % EtOH and centrifuged for 15 min as above. The pellet was air dried for 10 min and resuspended in 800 µl 10T1E pH 8.0.
6.6.2 DNA PURIFICATION OF \textit{NheI} DIGESTED YEAST EXTRACTS

DNA was purified as described in QIAGEN (1999). All steps until proteinase K digestion were done under yellow safety light (Sylvania GE Gold Fluorescent Light, F36WT8, wavelength $\geq 525$ nm) to prevent photoreactivation. A \textit{NheI} digested yeast nuclear extract (from $3 \times 10^9$ cells) was pelleted (10 min at 13000 rpm, 30$^\circ$C, Heraeus microfuge), resuspended in 2 ml QIAGEN general lysis buffer G2. RNase A (Roche Cat.No 109 169, dissolved in 10 mM Tris-HCl pH 7.5, 15 mM NaCl to 10 mg/ml) was added to a f.c. of 250 µg/ml and incubated for 30 min at 30$^\circ$C. Proteinase K (Roche, Cat.No. 745 723, dissolved in 50 mM Tris-HCl pH 8.0 to 10 mg/ml) was added to a f.c. of about 500 µg/ml and incubated for 2 hrs at 50$^\circ$C and ON at RT. The cellular debris was pelleted by centrifugation (10 min at 6500 rpm, 4$^\circ$C, HB-4). 2 ml of QIAGEN Equilibration Buffer QBT (750 mM NaCl, 50 mM MOPS, 15 % EtOH, 0.15 % Triton X-100, adjusted to pH 7.0) were added to the supernatant and loaded on a QIAGEN Genomic-tip 500/G. Column purification was done as described in Chapter 6.6.1. The DNA was resuspended in 200 µl 10T1E pH 8.0.

6.6.3 DNA PURIFICATION OF PSORALEN-CROSSLINKED YEAST EXTRACTS

\textit{NheI} digested and psoralen-crosslinked yeast extracts (of about 2 to 4 x $10^8$ cells, see Chapter 6.4) in 400 µl TNE (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA) was used for DNA purification: 40 µl 16.5 % Sarcosyl (SIGMA, N-Lauroyl-Sarcosine Sodium Salt, Cat.No. L-5125) and 30 µl Proteinase K (10 mg/ml) were added and incubated for 2.5 hrs at 50$^\circ$C and ON at RT. After centrifugation for 5 min at 5000 rpm (Heraeus Microfuge), the supernatant was extracted with phenol, phenol-DI and DI. The DNA was precipitated with 2.5 volumes of 100 % EtOH, washed with 70 % EtOH$_\text{cold}$, resuspended in 300 µl TNE and digested with 10 µl RNase A (10 mg/ml) for 20 min at 37$^\circ$C. After extraction with phenol, phenol-DI and DI, the DNA was precipitated as above and resuspended in 50 µl 10T0.5E pH 7.5. The DNA was digested with \textit{BamHI} or \textit{NheI} and used for psoralen gel retardation assay (see Chapter 6.10).

6.7 FRACTIONATION OF RDNA

6.7.1 SEPARATION OF ACTIVE AND INACTIVE 35S rRNA GENES

60 µl of purified genomic DNA (About 5 µg) from \textit{NheI}-digested crude extracts (see Chapter 6.6.2) was separated on 0.8 % SeaPlaque low melting agarose (FMC BioProducts,
6.7.1 Separation of Active and Inactive rDNAs

Cat.No. 50100) midi gels in 1 x TBE (90 mM Tris, 90 mM boric acid, 20 mM EDTA pH 8.0, according to Maniatis et al. (1989)) and 0.5 µg/ml EtBr for 5 hrs with 90 V at 4°C, until the xylene cyanol dye was about 1 cm away from the end of the gel. Marker lanes (λ/HindIII) were cut off and marked under UV light with paper ticks. Cut off marker and sample lanes were adjusted and DNA fragments corresponding to active (4.4 kb), inactive (>9.1 kb) and partially cut active rDNA (9.1 kb) were cut out without exposure to UV light in order to prevent UV damage formation. The fractions were purified using the Promega AgarACE™ protocol (Promega, 1995): Gel slices (0.3 to 1.5 g) were melted 8 min at 72°C. The melted agarose was cooled for 5 min to 43°C in a waterbath and digested with ≥1.2 U of AgarAce (Promega, 0.3 U/µl, Cat. No. M174A) per 1 g melted agarose for 3 to 4 hrs. The samples were remelted and incubated with AgarAce as above. The DNA was precipitated with 2.5 volumes of cold 100% EtOH and 0.1 volume of 3 M NaOAc pH 4.8. After 1 hr incubation at -20°C, the DNA was centrifuged (30 min at 13000 rpm, 4°C, Heraeus Microfuge) and washed with cold 70% EtOH. The pellet was air dried for 10 min and resuspended in 100 - 200 µl 10T1E pH 8.0. All fractions were digested to completion with NheI (Roche) and repurified by extraction with phenol-DI, DI and EtOH precipitation (as above). The fractionation and DNA recovery were tested by agarose gel electrophoresis and hybridisation for the 4.4 kb NheI fragment of the yeast rDNA with the probe AMribAM (see Tab. 6-4).

6.7.2 Separation of Active and Inactive 35S Promoters

About 4 µg of purified genomic DNA from NheI-digested crude extracts (see Chapter 6.6.2) were digested with NarI (NEB, 4 U/µl). About 3 µg of the digested DNA were separated on 0.8% low melting agarose gels (as described in Chapter 6.7.1). Fragments containing active and inactive 35S promoters were excised and purified using the Promega AgarACE™ protocol (Promega, 1995) as described in Chapter 6.7.1. A schematic representation of the fractionation procedure is displayed in Fig. 4-5. To check for correct fractionation, the fractions were analyzed on agarose midi gels, blotted on nylon membranes and hybridised with AMribbLM (see Tab. 6-4) to detect fragments containing the 35S promoter (as described in Chapter 6.9.1 ff).

6.8 DNA Fragments and Radioactive Probes

6.8.1 Generation of Fragments by Yeast Whole Cell PCR

DNA fragments used for generation of ss or ds DNA probes were amplified from cells by yeast whole cell PCR (protocol obtained from S. te Heesen (1994), ETH Zürich): A loopful of yeast cells (from a master plate) were heated in a PCR tube for 1 min in the microwave and
snap-cooled on ice. 48 µl of the following PCR mix were added:

**Table 6-2: Reaction Mix for Yeast Whole Cell PCR**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Compound</th>
<th>final concentration</th>
<th>Reference, origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>10 x PCR buffer</td>
<td>10 mM Tris-HCl pH 7.5, 50 mM KCl, 0.08 % Nonidet P40</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>4 µl</td>
<td>MgCl₂, 25 mM</td>
<td>2 mM</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>1.7 µl</td>
<td>Glycerol, 87 %</td>
<td>3 %</td>
<td>Fluka</td>
</tr>
<tr>
<td>1 µl</td>
<td>dNTPs, 2.5 mM</td>
<td>50 µM</td>
<td>Pharmacia Biotech, 27-2035-01</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>BSA, 20 mg/ml</td>
<td>0.2 mg/ml</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>0.25 µl</td>
<td>Taq Polymerase, 4U/µl</td>
<td>1 U</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>25.55 µl</td>
<td>H₂O sterile</td>
<td></td>
<td>Millipore</td>
</tr>
<tr>
<td>5 µl</td>
<td>Primer A, 1 pmol/µl</td>
<td>5 pmol</td>
<td>desalted, Microsynth</td>
</tr>
<tr>
<td>5 µl</td>
<td>Primer B, 1 pmol/µl</td>
<td>5 pmol</td>
<td>desalted, Microsynth</td>
</tr>
<tr>
<td>48 µl</td>
<td>total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR mix was overlaid with mineral oil and cycled in a OmniGene HybAid PCR machine. After 10 min denaturation at 94°C, 30 PCR cycles (denaturation at 94°C for 1 min, annealing at 48 or 50°C for 1 min., extension at 72°C for 1 min.) were performed, followed by a final extension step at 72°C for 10 min. PCR products were checked for correct length on 1.5 % agarose gels and purified: Fragments # 1 - 4 located in the rDNA locus (see Fig. 6-1) were gelpurified using (QIAGEN, 1997), fragments # 5 and 6 (rDNA locus) and fragment # 7 (GAL10) were twice column-purified using a PCR purification kit (Roche, 1999).

Oligos (Microsynth, Switzerland) used for generation of the probes are listed in Tab. 6-3. The DNA sequence was retrieved from [http://genome-www.stanford.edu/Saccharomyces/](http://genome-www.stanford.edu/Saccharomyces/); (Johnston et al., 1997). The locations of the fragments are shown in Fig. 6-1 (rDNA cluster) and Fig. 6-2 (GAL10 gene). Fragment details are listed in Tab. 6-4.

**Table 6-3: Oligos for Generation of Templates**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' - 3')⁹</th>
<th>Length (nt)</th>
<th>Tₘ b</th>
<th>Position c</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMrDNA-A</td>
<td>GTACGGATCCGGACTATCGGTTTCAAGC</td>
<td>28</td>
<td>57.6°C</td>
<td>4593 top</td>
</tr>
<tr>
<td>AMrDNA-B</td>
<td>GTCCGAATTCAGTTCTCTTAATGACCAAGT</td>
<td>31</td>
<td>56.7°C</td>
<td>4922 bot</td>
</tr>
<tr>
<td>AMrDNA-C</td>
<td>GTACGGATCCGGATGATGGTCGCAAGGCT</td>
<td>29</td>
<td>62.3°C</td>
<td>4319 top</td>
</tr>
<tr>
<td>AMrDNA-D</td>
<td>GTCCGAATTCTGTCGTCTGTTATCGCAAT</td>
<td>30</td>
<td>58.4°C</td>
<td>4503 bot</td>
</tr>
<tr>
<td>AMrDNA-E</td>
<td>GTATGGATCCGGCTTGGGTGCTTGGTCTGG</td>
<td>28</td>
<td>64.5°C</td>
<td>9005 top</td>
</tr>
<tr>
<td>AMrDNA-F</td>
<td>GTTGAATATGAGGCAGGCTTCGTCGC</td>
<td>27</td>
<td>66.8°C</td>
<td>28 bot</td>
</tr>
<tr>
<td>AMrDNA-G</td>
<td>GTACGGATCCGGCTTCTCCTATCATACCG</td>
<td>29</td>
<td>60.2°C</td>
<td>8636 top</td>
</tr>
<tr>
<td>AMrDNA-H</td>
<td>GTCCGAATTCTATGGGTCAGCGACGCC</td>
<td>29</td>
<td>62.3°C</td>
<td>8940 bot</td>
</tr>
<tr>
<td>AMrDNA-I</td>
<td>GTACGGATCCGAGCTAGAGGTTGCGAGAGCAGAGC</td>
<td>29</td>
<td>62.3°C</td>
<td>8541 top</td>
</tr>
</tbody>
</table>
Table 6-3: Oligos for Generation of Templates

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' - 3')a</th>
<th>Length (nt)</th>
<th>Tm b</th>
<th>Position c</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMrDNA-K</td>
<td>GTCCGAATTCCGAATGAACTGTTCCTCTCG</td>
<td>30</td>
<td>60.4°C</td>
<td>8766 bot</td>
</tr>
<tr>
<td>AMrDNA-L</td>
<td>GTACGGATCCGTGCTATGGTATGGTGACG</td>
<td>29</td>
<td>60.2°C</td>
<td>2985 top</td>
</tr>
<tr>
<td>AMrDNA-M</td>
<td>GTCCGAATTTCACTACTGGCAGGATCAACC</td>
<td>29</td>
<td>60.2°C</td>
<td>3196 bot</td>
</tr>
<tr>
<td>GAL10.KM-L</td>
<td>CCGCACCAGTACATGCTAGATAATGA</td>
<td>28</td>
<td>66.1°C</td>
<td>626 bot</td>
</tr>
<tr>
<td>GAL10.KM-U</td>
<td>CGCACCATTTCTCCGTACCAATAG</td>
<td>28</td>
<td>67.5°C</td>
<td>455 top</td>
</tr>
</tbody>
</table>

a. Sequences from *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/).

Nucleotides in italic: 10 bp tag with either the *Eco* RI or *Bam* HI restriction site.

b. Nucleotides for the restriction tag not included. Melting temperature acc. to http://www.microsynth.ch/

\[ T_m (°C) = 81.5 + 0.41 (G % + C %) + 16.6 \log (J^+) - 500 / \text{L} \]

\( (J^+) = \text{Concentration of monovalent cation in buffer, 0.1 M was used} \)

L = Length of oligo in bp

c. Position defines 3' end of oligo in sequence.

Oligo in rDNA sequence: Map unit 1 corresponds to the 5' end from the rDNA spacer. Primers with sequence of top strand (top) were used for generation of probes for the bottom strand (transcribed strand of 35S gene). Primers with sequence of bottom strand (bot) are used for generation of probes for the top strand (non transcribed strand of the 35S gene).

Oligo in *GAL10* gene: map unit 1 corresponds to the 3' end from the *GAL10* gene. Primer with sequence of top strand (top) was used for generation of a probe for the bot strand (non transcribed strand), the primer with sequence of the bottom strand (bot) for generation of a probe for the top strand (transcribed strand).

---

**Figure 6-1: Fragments in rDNA Cluster of *S. cerevisiae***

Schematic map of an rDNA repeat on chromosome XII. A 35S rRNA gene (35S), the rDNA spacer containing an enhancer (E), the 5S rRNA gene (5S), an ARS (A) and the 35S Promoter (P) and part of the upstream 35S rRNA gene are shown. Black bars represent the fragments generated by yeast whole cell PCR. Arrows indicate relevant restriction sites abutting the generated fragments. Details about the numbered fragments are listed in Tab. 6-4.

---

**Figure 6-2: Fragment in GAL Gene Cluster of *S. cerevisiae***

Schematic map of the yeast GAL gene cluster on chromosome II. The *GAL1*, *GAL10* and *GAL7* genes are shown. Arrows indicate relevant restriction sites. The black bar represents the fragment generated by yeast whole cell PCR. Details about fragment # 7 are listed in Tab. 6-4.
6.8.2  RADIOACTIVE DS DNA PROBES BY RANDOM PRIMING

ds DNA probes were generated by the random priming method using an 'oligo labeling kit' ((Amersham, 2000), Cat. No. 27-9250-01): 25 - 50 ng of the purified DNA fragment were adjusted with H₂O sterile to a total volume of 35 µl. After heat denaturation (5 min at 96°C, snap-cooling on ice), 10 µl of reagent mix (buffered aqueous solution containing dATP, dGTP, dTTP, random hexadeoxyribonucleotides), 4 µl of (32P)-dCTP (Hartmann Analytics, Cat. No. SCP-205; 3000 Ci/mmol; 10 µCi/µl) and 1 µl Klenow enzyme (buffered glycerol solution, 5 - 10 U/µl) were added. The labeling was performed at 37°C for 30 - 60 min. The volume of the reaction mix was adjusted with 10T1E pH 8.0 to a total of 100 µl. Unincorporated nucleotides were removed using a Sephadex™ G-50 quick spin column (in 10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl (Boehringer, 1999)). A typical labeling reaction resulted in a probe activity of 1.5 - 4 x 10⁷ cpm (determined by scintillation counting of 1 µl of the probe using a Cerenkov protocol).

6.8.3  RADIOACTIVE SS DNA PROBES BY PRIMER EXTENSION

Single-stranded DNA probes were generated by linear amplification of DNA fragments in the presence of (32P)-dCTP. DNA templates were amplified by yeast whole cell PCR and purified as described in Chapter 6.8.1. Primer extension reactions were prepared for both top and bottom
strand as follows:

**Table 6-5: Primer Extension Reaction for Generation of ss DNA Probes**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Compound</th>
<th>final concentration</th>
<th>Reference, origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>DNA fragment, ~1 ng/µl</td>
<td>2 - 5 ng, 0.02 - 0.04 pmol(^a)</td>
<td>in 10T1E pH 8.0</td>
</tr>
<tr>
<td>5 µl</td>
<td>Primer A or B, 2 pmol/µl</td>
<td>10 pmol</td>
<td>desalted, in H(_2)O, Microsynth</td>
</tr>
<tr>
<td>4 µl</td>
<td>10 x PCR buffer</td>
<td>10 mM Tris-HCl pH 7.5, 50 mM KCl, 0.08 % Nonidet P40</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>4 µl</td>
<td>MgCl(_2), 25 mM</td>
<td>3 mM</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>10 µl</td>
<td>dA/dT/dGTP mix (0.1 mM each)</td>
<td>25 µM</td>
<td>Pharmacia Biotech, 27-2035-01</td>
</tr>
<tr>
<td>5 µl</td>
<td>Taq Polymerase, 0.2U/µl</td>
<td>1 Unit</td>
<td>Fermentas, EP0072</td>
</tr>
<tr>
<td>4 µl</td>
<td>H(_2)O sterile</td>
<td></td>
<td>Millipore</td>
</tr>
<tr>
<td>3 µl</td>
<td>α(^23)P-dCTP</td>
<td>30 µCi, 10 pmol</td>
<td>SCP-205, Hartmann Analytics</td>
</tr>
<tr>
<td>40 µl</td>
<td>total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Ø 330 g/mol dNTP: Example for a 280 bp ds DNA fragment: 2 x 280 nucleotides
560 nucleotides x 330 g/mol dNTP = 184 800 g/mol = 184.8 ng/pmol
5 ng correspond to 0.027 pmol

The PCR mix was overlaid with mineral oil and cycled in a Perkin Elmer PCR thermocycler. 15 cycles (denaturation at 94°C for 45 sec, annealing at 53 - 60°C\(^1\) for 5 min., extension at 72°C for 3 min.) were performed. The volume of the reaction mix was adjusted with 60 µl 10T1E pH 8.0 to a total of 100 µl. Unincorporated nucleotides were removed using a Sephadex\(^TM\) G-50 quick spin column (in 10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl (Boehringer, 1999)). A typical labeling reaction resulted in a probe activity of 1.5 to 3 x 10\(^7\) cpm (determined by scintillation counting of 1 µl of the probe using a Cerenkov protocol). Strand specificity of the probes was checked with a mock primer extension reaction where the template, but no oligo was added. After purification, less than 10 % of the signal of the probe was detected in the mock reaction.

### 6.9 Neutral Agarose Gels

#### 6.9.1 Midi, Mini Agarose Gels

0.8 % - 1.5 % agarose (Gibco BRL, ultra pure) gels were run in 1 x TBE (90 mM Tris, 90 mM boric acid, 20 mM EDTA pH 8.0, according to Maniatis et al. (1989)) and 0.5 µg/ml EtBr. DNA samples were mixed with TBE sample buffer (f.c. 0.01 % bromophenolblue, 0.01 % xylene

---

\(^1\) Annealing temperature = Calculated T\(_m\) of the oligo with the lower melting temp. (see Tab. 6-3) - 5°C.
cyanol, 6 % glycerol, 1 x TBE according to Maniatis et al. (1989)). Midi (14.5 x 10 cm) and Mini gels (6 x 10 cm) were run at 100 V at RT for 1 to 3 hours. Gels were photographed using a UVI tec CCD camera and thermoprint. For further hybridisation, gels were soaked for 8 min in 0.25 N HCl, for 5 min in 0.4 N NaOH and used for alkaline blotting (see Chapter 6.11.4).

6.9.2 Maxi Agarose Gels

1 % agarose (Gibco BRL, ultra pure) maxi gels (20 cm x 25 cm) were run in 1 x TAE (40 mM Tris pH 7.6, 0.114 % acetic acid, 1 mM EDTA; according to Maniatis et al. (1989)) with 0.5 µg/ml EtBr. DNA samples were mixed with TBE sample buffer (f.c. 0.01 % bromophenolblue, 0.01 % xylene cyanol, 6 % glycerol, 1 x TBE according to Maniatis et al. (1989)). Maxi gels were run at 64 V (about 45 mA) for 20 hours, buffer exchanging with help of a peristaltic pump. After the run, the gel was soaked for 10 min in 0.25 N HCl, for 5 min in 0.4 N NaOH and used for alkaline blotting (see Chapter 6.11.4).

6.10 Psoralen Gel Retardation Assay

Phenol purified DNA from a *Nhe*I-cut and psoralen-crosslinked yeast nuclear extract (see Chapter 6.6.3) was used for the psoralen gel retardation assay. 10 µl of the purified DNA (in 10 mM Tris, 0.5 mM EDTA pH 7.5) were digested either with 20 U of *Nhe*I (Roche, 10 U/µl) to completion or with 20 U of *Bam*HI (Roche, 10 U/µl, no cutting sites in rDNA cluster) for 2 hours at 37°C. Completion of the restriction digest was checked by gel electrophoresis, blotting on nylon membranes and hybridisation for the 4.4 kb *Nhe*I fragment using AMribAB as a probe (see Tab. 6-4). 1 % or 1.2 % agarose gels (Gibco BRL, ultra pure; 250 ml in 20 x 25 cm trays) were run in 1 x TAE (40 mM Tris pH 7.6, 0.114 % acetic acid, 1 mM EDTA; according to Maniatis et al. (1989)) without EtBr ON at 64 V (about 68 mA) for 20 hours (1 % gels) or 30 hours (1.2 % gels) with buffer exchange using a peristaltic pump. After the gel run, the psoralen DNA in the gel was de-crosslinked under 254 nm light (Sylvania, Type G15 T8 lamps, about 30 J/m² sec) for 12 min. The DNA was depurinated in 1 l of 0.25 N HCl for 12 min, the gel was soaked for 5 min in 0.4 N NaOH and used for alkaline Southern blotting (see Chapter 6.11.4).

6.11 Mapping of CPDs by Indirect End-Labeling

6.11.1 Restriction of Genomic DNA

QIAGEN purified genomic DNA (from Chapter 6.6.2) was used for digestion and subsequent indirect end-labeling. For analysis of the 35S rRNA gene, DNA was cut with *Nhe*I (10 U/µl;
Roche) in SuRE/Cut Buffer M (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.5 (at 37°C). For analysis of the rDNA spacer, DNA was cut with *Nde*I (10 U/µl; Roche) and *Cla*I (10 U/µl; Roche) in SuRE/Cut buffer H (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.5 (at 37°C)). For analysis of the 35S promoter, DNA was cut with *Ava*II (10 U/µl; New England Biolabs) in NEB buffer 4 (50 mM KOAc, 20 mM TrisAc, 10 mM MgAc, 1 mM DTT, pH 7.9 at 25°C). For analysis of the *GAL10* gene, DNA was cut with *Eco*RI (10 U/µl; Roche) and *Sal*I (10 U/µl; Roche) in SuRE/Cut buffer H (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.5 (at 37°C)). All digestions were performed at 37°C for 2 hours. DNA was subsequently repurified by extraction with phenol-DI, DI and EtOH precipitation and resuspended in 10T1E pH 8.0. DNA was checked on 0.8 % agarose gels, Southern blotted and hybridised with the fragment of choice (as described in Chapter 6.11.4) to test completeness of the digest and to quantify DNA concentrations.

6.11.2 T4-ENDONUCLEASE V ASSAY

For mapping CPDs by indirect end-labeling in rDNA, 12 µl of the *Nhe*I digested DNA (40 - 100 ng in 10T1E pH 8.0) were incubated with 2 µl T4endoV (1 U/µl, diluted in H₂O from 20 U/µl, Epicentre Technologies, Cat.No TE661K) in 50 mM Tris, 5 mM EDTA pH 7.5 in a total volume of 32 µl at 37°C for 2 hours. Additional 1 U T4endoV was added after 1 hour to all samples. Mock DNA samples were treated in parallel in the same volume of reaction buffer lacking the enzyme. For mapping CPDs in the *GAL10* gene, 12 µl of DNA (about 1.5 µg in 10T1E pH 8.0) were digested with 5 + 2 U of T4endoV as described above. The digestion was stopped and the DNA denatured for 10 - 30 min by the addition of 9 µl of freshly-prepared 5 x alkaline loading buffer (f.c. 50 mM NaOH, 1 mM EDTA pH 8.0, 2.5 % Ficoll-400, traces of bromocresol green (Maniatis et al., 1989), but with only traces of bromocresol green, because it interferes with DNA Southern transfer and hybridisation).

6.11.3 ALKALINE GEL ELECTROPHORESIS

For strand separation, alkaline agarose gels (20 x 25 cm, Gibco BRL) were prepared according to Maniatis et al. (1989) with modifications: 1.5 % Agarose (ultrapure, Gibco BRL) was melted in 250 ml 50 mM NaCl, 1 mM EDTA pH 8.0. Polymerized gels were soaked 2 to 3 times for each 1 hour in alkaline running buffer (50 mM NaOH, 1 mM EDTA pH 8.0). The gels were transferred to 4°C, covered with 2 l of alkaline running buffer and left to cool down for 1 hour. 20 µl of denatured DNA samples (from Chapter 6.11.2) were loaded per slot. 10 µl of 5 x alkaline loading buffer (250 mM NaOH, 5 mM EDTA pH 8.0, 12.5 % Ficoll-400, ≥0.2 % bromocresol green (Maniatis et al., 1989)) were loaded in empty slots flanking the DNA samples.
(as a distance marker to control the gel run). DNA was allowed to enter the gel at 56 V (about 300 mA per gel) for 15 - 30 min. Gel run was performed ON with constant 250 mA per gel and buffer exchange in both directions, using a peristaltic pump and magnetic stirrers. Voltage increased from 40 - 44 V at the beginning to 70 - 80 V at the end of the gel run after 13 - 15 hours, the bromocresol green dye at about 14 - 15 cm.

### 6.11.4 Alkaline Southern Blot and Hybridisation of Membranes

After the gel electrophoresis, the agarose gel was laid upside-down onto two 3MM Whatman filter papers (pre-soaked in 0.4 N NaOH). A BIORAD Zeta GT nylon membrane (BIO-RAD, Cat.No. 162-0196, presoaked in H$_2$O$_{Millipore}$) was laid on the gel and covered with six pre-wetted 3MM Whatman papers. A stack of paper towels was put on the top. All was covered with a glass-plate. Alkaline transfer was allowed to proceed for 8 - 20 hours. The wet paper towels were replaced every 2 - 4 times. The membrane was neutralized two times 20 min. in 2 x SSC (3 M NaCl; tri-Na citrate, 2 x H$_2$O, pH 7.0) and dried between two Whatman 3MM papers at 80°C for 60 min.

Hybridisation with radiolabeled probes was done according to BIO-RAD (1997) with modifications: The BIORAD Zeta GT membrane was prehybridized for 10 - 30 min rotating at 65°C in a hybridisation cylinder (ø 4 cm) with 15 ml of prewarmed Zeta hybridisation solution (0.25 M Na$_2$HPO$_4$ pH 7.2, 7 % SDS, 65°C), supplemented with 50 µg/ml tRNA (SIGMA, Cat. No. R-1753; Type XX from *E. coli*, strain W). The prehybridisation solution was replaced by 15 ml fresh prewarmed Zeta hybridisation solution. 20 - 50 µl of the radioactively labeled ss DNA probe were heat denaturated (5 min. boiling, 5 min. on ice) and added to the hybridisation solution. Hybridisation was done rotating at 65°C for at least 3 hours or ON. The membrane was transferred to a plastic tray and washed twice for 20 min with prewarmed Zeta wash solution I (40 mM Na$_2$HPO$_4$ pH 7.2, 5 % SDS) and three times for 20 min with prewarmed Zeta wash solution II (40 mM Na$_2$HPO$_4$ pH 7.2, 1 % SDS). The membrane was dried between 3MM Whatman papers, wrapped in Saran Wrap and exposed on Fuji RX-100 autoradiography film at -80°C or on Kodak PhosphorImager screens at RT.

### 6.12 Mapping of CPDs by Primer Extension

Primer extension was done according to the protocol of R. Wellinger (Wellinger and Thoma, 1996) with modifications, using the QIAGEN *Taq* polymerase and buffer system (QIAGEN, Cat.No. 201 203).
### 6.12.1 5’ LABELING OF OLIGOS

**Table 6-6: Oligo for Primer Extension Analysis**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ - 3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Length (nt)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Position&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmrDNA-N</td>
<td>GTATGTTTTGTATGTTCCCGG</td>
<td>22</td>
<td>60.8°C</td>
<td>-266 top</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ - 3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Length (nt)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Position&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMrDNA-N</td>
<td>GTATGTTTTGTATGTTCCCGG</td>
<td>22</td>
<td>60.8°C</td>
<td>-266 top</td>
</tr>
</tbody>
</table>

- Melting temperature acc. to [http://www.microsynth.ch/](http://www.microsynth.ch/)
  \[ T_m (^{°}C) = 81.5 + 0.41 (G % + C %) + 16.6 \log (J^+) - 500 / L \]
  \( J^+ \) = Concentration of monovalent cation in buffer, 0.1 M was used
  \( L \) = Length of oligo in bp
- Position defines 3’ end of oligo in sequence of top strand: Map unit 1 corresponds to the +1 transcription start site of the 35S rRNA gene.

PAGE-purified oligos (Tab. 6-6) were labeled at the 5’ end using T4-polynucleotide kinase and (γ-32P) ATP: 10 µl primer solution (1 pmol/µl in H2O) were heat denatured at 94°C for 2 min and snap-cooled on ice. 8.5 µl 10T1E pH 8.0, 3 µl 10 x T4 PNK buffer (f.c. 70 mM Tris-HCl pH 7.6, 10 mM MgCl2, 5 mM DTT, New England Biolabs), 7.5 µl (γ-32P) ATP (15 pmol; Hartmann Analytics, Cat. No. SCP-401; >5000 Ci/mmol; 10 µCi/µl) and 1 µl T4 polynucleotide kinase (10 U/µl, New England Biolabs, Cat.No. M0201S) were added to a final volume of 30 µl. End-labeling was performed at 37°C for 30 - 60 min. The volume of the reaction mix was adjusted with 70 µl 10T1E pH 8.0 to a total of 100 µl and unincorporated nucleotides were removed using a Sephadex™ G-50 quick spin column (in 10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl (Boehringer, 1999)). A typical labeling reaction resulted in a probe activity of 2 - 5 x 10<sup>7</sup> cpm (determined by scintillation counting of 1 µl of the probe using a Cerenkov protocol).

### 6.12.2 PRIMER EXTENSION AND SEQUENCING REACTIONS

For primer extension, DNA was cut to completion with *Ava*II (as described in Chapter 6.11.1), purified using QIAEX II protocol (QIAGEN, 1997) and resuspended in 30 µl 10T1E pH 8.0. 30 - 40 ng of DNA samples were adjusted with H2O to a total volume of 10 µl. Primer extension reaction mix w/o *Taq* polymerase were prepared as follows:

**Table 6-7: Primer Extension Reaction for Mapping of UV Lesions**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Compound</th>
<th>final concentration</th>
<th>Reference, origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µl</td>
<td>DNA, <em>Ava</em>II cut</td>
<td>30 - 40 ng</td>
<td>QIAEX II purified</td>
</tr>
<tr>
<td>1.2 µl</td>
<td>dNTPs, 5 mM each</td>
<td>0.15 µM</td>
<td>Pharmacia Biotech, 27-2035-01</td>
</tr>
<tr>
<td>2 µl</td>
<td>DMSO 100 %</td>
<td>5 %</td>
<td>Fluka, 41 641</td>
</tr>
<tr>
<td>4 µl</td>
<td>10 x <em>Taq</em> PCR buffer</td>
<td>Tris HCl, KCl, (NH₄)SO₄, 15 mM MgCl₂, pH 8.7</td>
<td>QIAGEN, 201 203</td>
</tr>
<tr>
<td>18 µl</td>
<td>(γ-32P) ATP end-labeled primer</td>
<td>0.5 - 0.7 pmol</td>
<td>Chapter 6.12.1</td>
</tr>
<tr>
<td>35.2 µl</td>
<td>total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sequencing reactions (chain termination method in the presence of dideoxy NTPs using Taq DNA polymerase) were prepared in parallel. 10 - 15 ng of AvaII cut, QIAEX II purified DNA were used per sequencing reaction:

**Table 6-8: Sequencing Reactions**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Compound</th>
<th>final concentration</th>
<th>Reference, origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µl</td>
<td>DNA</td>
<td>10 - 15 ng</td>
<td></td>
</tr>
<tr>
<td>1.2 µl</td>
<td>ddNTP mix (ddT, ddC, ddG or ddA)²</td>
<td>79-90 µM dd/dNTP</td>
<td>Pharmacia Biotech, 27-2035-01</td>
</tr>
<tr>
<td>2 µl</td>
<td>DMSO 100 %</td>
<td>5 %</td>
<td>Fluka, 41 641</td>
</tr>
<tr>
<td>4 µl</td>
<td>10 x Taq PCR buffer</td>
<td>Tris HCl, KCl, (NH₄)SO₄, 15 mM MgCl₂, pH 8.7</td>
<td>QIAGEN, 201 203</td>
</tr>
<tr>
<td>18 µl</td>
<td>(γ-³²P) ATP end-labeled primer</td>
<td>0.5 - 0.7 pmol</td>
<td>Chapter 6.12.1</td>
</tr>
<tr>
<td>35.2 µl</td>
<td>total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. ddNTP to NTP ratio: 20:1 for adenine (f.c. 0.079 mM dd/dATP), 10:1 for cytosine (f.c. 0.082 mM dd/dCPT), 5:1 for guanine (f.c. 0.09 mM dd/dGTP), 10:1 for thymine (f.c. 0.082 mM dd/dTTP)

Primer extension and sequencing reactions were denatured for 10 min. at 95°C in a Perkin Elmer thermocycler and immediately put on ice. After addition of 5 µl Taq DNA polymerase (5 U/µl, diluted in H₂O to 0.2 U/µl, QIAGEN, Cat.No. 201 203), the tube was briefly centrifuged and the mix overlaid with two drops of mineral oil. Cycling was done in a Perkin Elmer thermocycler overnight. 15 PCR cycles (denaturation at 94°C for 45 sec, annealing at 58°C for 4 min 30 sec, extension at 72°C for 3 min) were performed. The aqueous phase was transferred to a new tube. DNA was precipitated by the addition of 0.1 volume of 3 M NaOAc pH 4.8 and 4 volumes of 100 % EtOHcold for 15 min on dry ice. After centrifugation (30 min at 13000 rpm, 4°C, Heraeus microfuge), the supernatant was discarded and the pellet was dried in a Speedvac™ and resuspended in 8 µl of sequencing buffer (80 % formamide, 10 mM NaOH, 1 mM EDTA pH 8.0, 0.1 % xylene cyanol, 0.1 % bromophenol blue).

### 6.12.3 5 % ACRYLAMIDE, 42 % UREA SEQUENCING GEL

Electrophoresis was done in 0.35 mm thick, 33.5 cm x 39.5 cm sequencing gels (Gibco BRL, Life Technologies, Model S2) with 5 % (w/v) acrylamide (Appligene, BIAC1902, Acrylamide-Bis-Acrylamide 19:1 mixture, 40 % w/v stock) in the presence of 42 % urea and 1 x TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA pH 8.0). Gels were prerun for 1 - 2 hours at 2000 V / 50 mA. 2 - 5 µl of heat denaturated samples (2 min at 95°C, snap-cooled on ice) were loaded per lane. Gel run was done at 2000 V / 50 mA for about 1 hr 45 min (running distance of xylene cyanol: 29 cm). The gel was dried on Whatman 3MM (with an underlying Whatman DE81 paper) for 2 hours at 80°C and exposed on Fuji RX-100 autoradiography film at -80°C or
on Kodak PhosphorImager screens at RT.

6.13 Creating and Processing PhosphorImager Files

Radioactive membranes were dried for 5 min between Whatman 3MM papers and wrapped in Saran Wrap. Exposures on Kodak PhosphorImager screens were done at RT for about 1 to 5 days. Similar exposure time was chosen for exposure on X-ray films at -80°C. PhosphorImager screens were scanned with a Molecular Dynamics PhosphorImager Storm 820. The plates were mounted on the scanner under dimmed light and scanned with the Molecular Dynamics Scanner Software 'Scanner Control' (build 2 or 3). 16bit TIFF files with a resolution of 100 µm pixel size were generated. Rotation and cropping of images was done with the Molecular Dynamics Software 'Image Quant Tools' (version 1.0, build 31). Signal quantifications were done with the Molecular Dynamics Software 'Image Quant' (version 1.2 for Apple Macintosh, build 039). For image processing in Adobe Photoshop, 16bit PhosphorImager files were opened in Image Quant v1.2, grey/color settings were adjusted and the gels were exported as 8bit TIFF file.

6.14 Analysis of PhosphorImager Files

PhosphorImager files were quantified using Molecular Dynamics ImageQuant software (version 1.2 for Apple Macintosh). For subtraction of membrane or gel background signal, a background box was placed at a region containing no DNA signal (typically next to gel lanes or above gel slots). The average pixel value of this background box was subtracted from all areas in ImageQuant using the 'Object Average’ command. For further calculations, ImageQuant volume reports were transferred to Microsoft Excel 98. Graphical representations were generated in Kaleidagraph (version 3.0.5, Abelbeck Software) and processed in Canvas (version 6.01, Deneba Systems, Inc. 1998).

6.14.1 CPD Repair in Whole DNA Fragments

UV-irradiated DNA was digested with T4endoV or mock-treated. The products were separated on agarose gels, blotted and hybridized. An example is shown in Fig. 6-3. Rectangles depict areas that were quantified. The loading correction factor f was calculated from the total volumes of each lane ($V_{tot} (-T4)$ and $V_{tot} (+T4)$). The intact restriction fragment IRF was quantified by dividing the signal in the top band of the +T4 lane ($V_{top} (+T4)$) by the signal in the top band of the -T4 lane ($V_{top} (-T4)$) and multiplication with f. The average number of CPDs per fragment
was calculated according to the Poisson distribution (Mellon et al., 1987) as the negative natural logarithm of the IRF. CPD repair was calculated by normalizing the number of CPDs per fragment of all repair samples to the initial damage sample, which was set to 0 % repair.

### 6.14.2 Repair of Regions in Agarose Gels

Repair of distinct regions from indirect end-labeling experiments was calculated of the rDNA spacer including the 35S promoter. The gene regions and CPD sites were mapped using Kodak Digital Science™ 1D Image Analysis Software (version 3.0) (see Chapter 6.14.4). An example is shown in Fig. 6-4. First, the normalised CPD content of the +T4 lane was calculated as the signal in the region of interest ($V_{dam,+T4}$) divided by the signal of the whole lane ($V_{tot,+UV}$). Second, the normalised signal of the corresponding -T4 lane was subtracted as lane background, resulting in the Net CPDs C of the region of interest. For quantification of CPD repair, Net CPDs C of the initial damage sample were set to 0 % repair and CPD repair was calculated by normalizing the Net CPDs C of all repair samples to the initial damage sample.

### 6.14.3 Damage Formation and Repair of CPD Sites from High Resolution Experiments

DNA damage formation and CPD repair was calculated from primer extension analysis. CPD sites were identified by comparison with the sequencing lanes adjacent to primer extension lanes.
6 - Materials and Methods

An example is shown in Fig. 6-5. For quantification of pyrimidine dimer formation, the signal at pyrimidine sites \(V_{\text{dam}, +UV}\) was divided by the signal of the whole lane \(V_{\text{tot}, +UV}\). The signal arising from unspecific Taq polymerase stops was calculated in the -UV lane as described for the +UV lane and subtracted from the normalized damage in the +UV lane, resulting in the fraction of molecules that contain a pyrimidine dimer at a defined site (Net Damage D).

For CPD repair analysis, the signal at pyrimidine sites \(V_{\text{dam}, +UV}\) was divided by the signal of the whole lane \(V_{\text{tot}, +UV}\). Second, the signal arising from unspecific Taq polymerase stops or stops at (6-4)PP and other photoproducts was calculated in the +PL lane as described for the +UV lane and subtracted from the normalized damage in the +UV lane, resulting in the fraction of molecules that contain a CPD at a defined site (net CPDs C). Net CPDs C of the initial damage sample were set to 0 % and CPD repair was measured as a decrease in CPDs.

### 6.14.4 LENGTH DETERMINATION OF DNA FRAGMENTS

For identification of CPD sites in indirect end-labeling experiments and for determination of fragment length in gels, the 1D Analysis Software (version 3.0) of Kodak Digital Science™ was used. EtBr gels were photographed using a UVI tec CCD camera and saved as TIFF file. PhosphorImager files were opened in ImageQuant and, after grey/color adjustment, exported as 8bit TIFF file. The file size was reduced in Adobe Photoshop to less than 1 MB and the files were opened in the Kodak 1D Analysis Software. After identification (command ‘find lanes’) and

\[
\text{Net CPDs (C)} = \frac{V_{\text{dam} (+T4)}}{V_{\text{tot} (+T4)}} - \frac{V_{\text{dam} (-T4)}}{V_{\text{tot} (-T4)}}
\]

\[
\text{CPD Repair} = 1 - \frac{C(t)}{C(t=0)}
\]

**Figure 6-4: Quantification of CPD Repair in Regions of Agarose Gels**

UV-irradiated DNA was digested with T4endoV (+T4) or mock-treated (-T4). The products were separated on an alkaline agarose gel, blotted and hybridized. The PhosphorImager files were quantified using Molecular Dynamics ImageQuant software. Rectangles depict areas that were quantified. \(V_{\text{dam}}\) depicts the signal of the damage region of interest in the -T4 and +T4 lane, respectively. \(V_{\text{tot}}\) corresponds to the signal of the whole lane. For subtraction of membrane background signal, the average pixel value of a background box (bg) was subtracted from all areas. Formulae are shown for quantification of the net CPDs C of the +T4 lane and CPD repair (see text for details).
manual adjustment of the gel lanes, 'smiling' of gel lanes was marked using isomolecular weight lanes. The marker lanes were selected and corresponding fragment sizes were assigned. Bands were identified using the command 'find bands' and manual adjustments. Resulting fragment sizes were exported to Microsoft Excel 98 and used for further calculations.

Figure 6-5: Quantification of Site-Specific CPD Repair from Primer Extension Analysis

Primer extension products of non-irradiated DNA (-UV), irradiated DNA (+UV) and *in vitro*-photoreactivated DNA (+photolyase, PL), separated on a 5 % acrylamide gel are shown. PhosphorImager files were quantified using Molecular Dynamics ImageQuant software. Red rectangles depict areas that were quantified. $V_{\text{dam}}$ depicts the signal of the damaged site of interest in the -UV, +PL and +UV lane. $V_{\text{tot}}$ corresponds to the signal of the whole lane. For subtraction of gel background signal, the average pixel value of a background box (bg) was subtracted from all areas. Formulae are shown for quantification of the Net Damage D, Net CPDs C of the +UV lane and CPD repair (see text for details).

$$\text{Net Damage (D)} = \frac{V_{\text{dam}} (+UV)}{V_{\text{tot}} (+UV)} - \frac{V_{\text{dam}} (-UV)}{V_{\text{tot}} (-UV)}$$

$$\text{Net CPDs (C)} = \frac{V_{\text{dam}} (+UV)}{V_{\text{tot}} (+UV)} - \frac{V_{\text{dam}} (+PL)}{V_{\text{tot}} (+PL)}$$

$$\text{CPD Repair} = 1 - \frac{C_t}{C_{t=0}}$$
References


Cavalli, G. (1994). Transcription dependent chromatin transitions in the yeast Saccharomyces cerevisiae, ETH Zürich, CH-8093 Zürich. Diss. ETH No 10965


Sancar, G. B. (1985b). Sequence of the *Saccharomyces cerevisiae* PHR1 gene and homology of the PHR1 photolyase to *E. coli* photolyase, Nucleic Acids Res 13, 8231-8246.


Smith, J. S., Brachmann, C. B., Pillus, L., and Boeke, J. D. (1998). Distribution of a limited Sir2 protein pool regulates the strength of yeast rDNA silencing and is modulated by Sir4p, Genetics 149, 1205-1219.


CURRICULUM VITAE

Personal Data
Name: Meier Andreas
Date of birth: December 18, 1972
Nationality: CH
Present position: Ph.D. student
Present address: Institute of Cell Biology
ETH Hönggerberg, CH-8093 Zürich, Switzerland
http://www.cell.biol.ethz.ch
Telephone No: ++41-1-633 33 43
Fax No: ++41-1-633 10 69
e-mail: andreas.meier@cell.biol.ethz.ch / a.meier@gmx.ch

Professional Data
High school: Alte Kantonsschule Aarau
Matura Typus B: 1992
University: ETH Zürich, Studium in Biologie
Diplom als Naturwissenschaftler mit Titel
‘Dipl. Natw. ETH’: 1997
PhD thesis, ETH Zürich, Institute of Cell Biology
Fellowship Roche Research Foundation: 2000-2001
Fellowship Janggen-Pöhn-Stiftung: 2000-2001
ETH Zürich, Didaktischer Ausweis in Biologie: 1996 -
Publications


Oral Presentations

- RRR Club (Repair, Recombination, Replication), F. Thoma, Zürich 1997
- RRR Club, F. Thoma, J. Sogo, Zürich 1999
- 10. Swiss Workshop on Genetic Recombination & DNA Repair, P. Schär, Les Diablerets 1999
- Swiss Yeast Meeting, M. Hall, P. Philippsen, Basel 2000
- RRR Club (Repair, Recombination, Replication), F. Thoma, J. Sogo, Zürich 2001

Poster Presentations

- ASM Conference on DNA Repair and Mutagenesis, G. Walker, E. Friedberg, S. Wallace, Hilton Head, South Carolina, USA 1999
- D-BIOL Symposium ETH Zurich, K. Wüthrich, Davos 2000
- 1st ELSO and USGEB meeting, K. Simons, Geneva, Switzerland 2000
- Swiss Yeast Meeting, P. Linder, Geneva 2001

Additional Education

- Swiss Yeast Meeting 1996, S. te Heesen, ETH Zürich 1996
- D-BIOL Symposium ETH Zurich, K. Wüthrich, Davos 1998
- Cambridge Certificate in Advanced English, T. Meuter, ETH Zürich, 1998-1999
- Kurs ‘Präsentationen beurteilen’, W. Wellstein, Center for Teaching and Learning, ETH Zürich 1999
- Kurs ’Exploratives Lernen’, Dr. V. Steiner, ETH Tools, ETH Zürich 2001