Homology of DNA Repair Genes Between *Saccharomyces cerevisiae* and *Arabidopsis thaliana*

By

Brenda Chan

Department of Microbiology
The University of Manitoba
Winnipeg, Manitoba
Canada

A thesis submitted to the Faculty of Graduate Studies

of The University of Manitoba

in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

September 1996

ISBN 0-612-16111-0

Canada

Name _____

Dissertation Abstracts International and *Masters Abstracts International* are arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation or thesis. Enter the corresponding four-digit code in the spaces provided.

Molecular Biology

SUBJECT TERM

0307 UMI

SUBJECT CODE

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

COPYRIGHT PERMISSION

HOMOLOGY OF DNA REPAIR GENES BETWEEN *Saccharomyces cerevisiae* AND *Arabidopsis thaliana*

BY

BRENDA CHAN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

This work is dedicated to my mom and dad, Shea and Duck Chan, for always being very supportive and loving and for instilling in all of your children the confidence and strength to believe in ourselves. You have always taught us that as long as we persevere and try hard enough, we can accomplish anything.

Abstract

The identification of genes involved in DNA repair in the plant *Arabidopsis thaliana* was investigated. The first approach involved functional complementation of *Saccharomyces cerevisiae rad* mutants using an *Arabidopsis thaliana* cDNA library constructed in the λYES vector. The yeast strains KAM-1 (*rad1*), SX46 (*rad2*), LN116 (*rad3*) and WS8104-2B (*rad4*) were transformed with the cDNA expression library and screened for functional complementation by testing for UV sensitivity. No cDNA clones from this library were found to complement the yeast *rad* mutants tested. In addition, isogenic yeast *rad2*Δ and *rad4*Δ mutants were constructed for future work.

The second approach involved using yeast DNA repair genes as probes to identify *Arabidopsis* DNA genes with homology. The yeast *RAD2* gene was the only gene that showed homology to *Arabidopsis thaliana* genomic DNA, hybridizing to a 7.2 kb DNA fragment. An attempt was also made to identify *Arabidopsis thaliana* cDNA with *RAD2* homology. Fifty-three potential positive lambda clones were identified in an initial screening however, the cDNA plasmids isolated from the λYES clones did not show any homology to this probe. An explanation for these results is given.

Acknowledgments

My parents, my brother Jeff, my sister-in-law Karen and my sisters Pensy and Madge, for their constant encouragement, support and reassurances.

Dr. Bernard A. Kunz, for his patience, guidance, advice and financial support throughout the course of this study.

My committee members Dr. E.A. Worobec, Dr. R.D. Gietz and Dr. P.Y. Maeba, for their time, advice and guidance.

Dr. John D. Armstrong, for his advice, guidance and friendship.

Elizabeth Henson, for her friendship and encouragement.

Hazeline Roche, for her humour and friendship.

Andrew Christie, for his friendship and advice.

Douglas Barker, for his advice, assistance and unique sense of humour.

The summer student Rachel Suarez, for her assistance and friendship.

Dr. Karthikeyan Ramachandran, for his friendship and advice.

Theodore I. Kuschak, for his love, never ending patience, guidance, support, encouragement and help with my many computer problems.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	adenine
amp	ampicillin
C	cytosine
CPD	cyclobutane dimer
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
g, mg, ng, μg	gram, milligram, nanogram, microgram
h (rs)	hour (s)
kb	kilobase-pair
L, mL, μL	litre, millilitre, microlitre
M, mM, μM	molar, millimolar, micromolar
m, cm, nm	meter, centimeter, nanometer
min	minute
NaOH	sodium hydroxide
Na_2EDTA	disodium ethylenediaminetetraacetic acid

PEG	polyethylene glycol
pol	polymerase
RNA	ribonucleic acid
s	second
SDS	sodium dodecyl sulfate
tRNA	transfer RNA
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

1. Introduction

The environment that all life exist in poses continual threats to the genetic integrity of its organisms (Friedberg 1985). There are numerous agents, both natural and man made, that can cause alterations to the DNA. Without constant cellular monitoring and repair of these DNA alternations, all life on Earth would cease to exist (Friedberg 1985). Therefore, the survival of an organism depends on the presence and efficiency of various enzymatic mechanisms for DNA repair (Haynes and Kunz 1981). The field of DNA repair is very large and extensive however, for the purpose of this review the focus will be on some general DNA repair mechanisms and what is known about them. The processes of DNA repair have been extensively studied in the simple prokaryote *Escherichia coli* and are thus, best understood in this organism. As one begins to study these mechanism in more complex organisms such as *Saccharomyces cerevisiae* and in higher eukaryotes, the processes become less understood due to the complexity of these organisms' DNA. Another field of DNA repair that is relatively new is that of DNA repair in plants. Preliminary studies with plants have verified that DNA repair processes do exist (McLennan 1987; Quaite *et al.* 1994) however, how similar these processes are to those in prokaryotes and eukaryotes has yet to be determined.

The early studies in DNA repair utilized the natural and abundant mutagen ultraviolet radiation (Friedberg 1985; Friedberg *et al.* 1995). The deleterious affects of ultraviolet light on DNA has been known for some time and researchers have extensively studied DNA repair processes in organisms as a response to this type of DNA damage. For this reason this review focuses on the processes that are involved in repair of

ultraviolet-induced DNA damage.

DNA repair mechanisms can be classified into two categories: light repair and dark repair processes. The light repair DNA repair mechanism consists of the process known as enzymatic photoreactivation that utilizes the energy from visible light to directly reverse the damage caused by ultraviolet radiation (Cooke 1970; Rupert 1975). The dark repair processes consists of those that do not require light energy to operate and they repair the DNA damage that photoreactivation is unable to repair. These latter processes consist of daughter-strand gap repair, translesion synthesis and nucleotide excision repair (Friedberg 1985; Friedberg *et al.* 1995).

This review will discuss DNA repair mechanisms that have been discovered in both prokaryotes and eukaryotes. Pertinent genes involved in these processes will be mentioned as well. It will then discuss the DNA repair mechanisms that have been uncovered in plants to date along with the problems encountered in elucidating these processes in plants.

1.1 DNA Repair

Any damage to DNA that blocks transcription or replication would be lethal to a cell unless it can be repaired by cellular repair mechanisms. These repair processes have been well characterized in the bacterium *Escherichia coli.* However, less is known about repair of DNA damage in the yeast *Saccharomyces cerevisiae* and more complex eukaryotic cells. What is known about eukaryotic DNA repair has come mainly from the examination of repair-deficient mutants in yeast, cells from humans afflicted with xeroderma pigmentosum (XP) or Cockayne's syndrome (CS) as well as from cross

complementation studies between rodent and human cells (Armstrong 1993).

There are numerous DNA repair mechanisms within a cell that are able to deal with the various lesions that can arise. These mechanisms can be separated into two general classes: light repair and dark repair (Haynes and Kunz 1981). The dark repair processes can be further separated into three main types called: error-free repair, error-prone repair and recombinational repair (Game 1983).

1.1.1 Importance of Ultraviolet Radiation Studies

The investigation of ultraviolet (UV) radiation damage to DNA was the beginning of the study of the repair of DNA damage (Friedberg *et al.* 1995). In fact, the exposure of cells to UV radiation is probably the best studied and most extensively used model for exploring the biological consequences of DNA damage and its repair and tolerance (Friedberg *et al.* 1995). One of the many attributes of using UV radiation as a system for producing DNA damage is that UV radiation at a wavelength of 254 nm is readily available from an ordinary germicidal lamp and instrumentation for accurately measuring its intensity is commonplace. Also, UV radiation, in general, is highly biologically relevant as living organisms have had to deal with the genotoxic effects of solar UV radiation since the beginning of life on Earth.

UV wavelengths are separated into three ranges: 200-280 nm (UVC), 280-320 nm (UVB) and 320-400 nm (UVA) (Friedberg *et al.* 1995). Solar UV radiation consists mainly of UVA and UVB since most of the UVC is absorbed by the Earth's atmospheric ozone layer. However, with the shrinking of the ozone layer UVC may become an increasingly important DNA altering agent. As stated above, scientists tend to favour

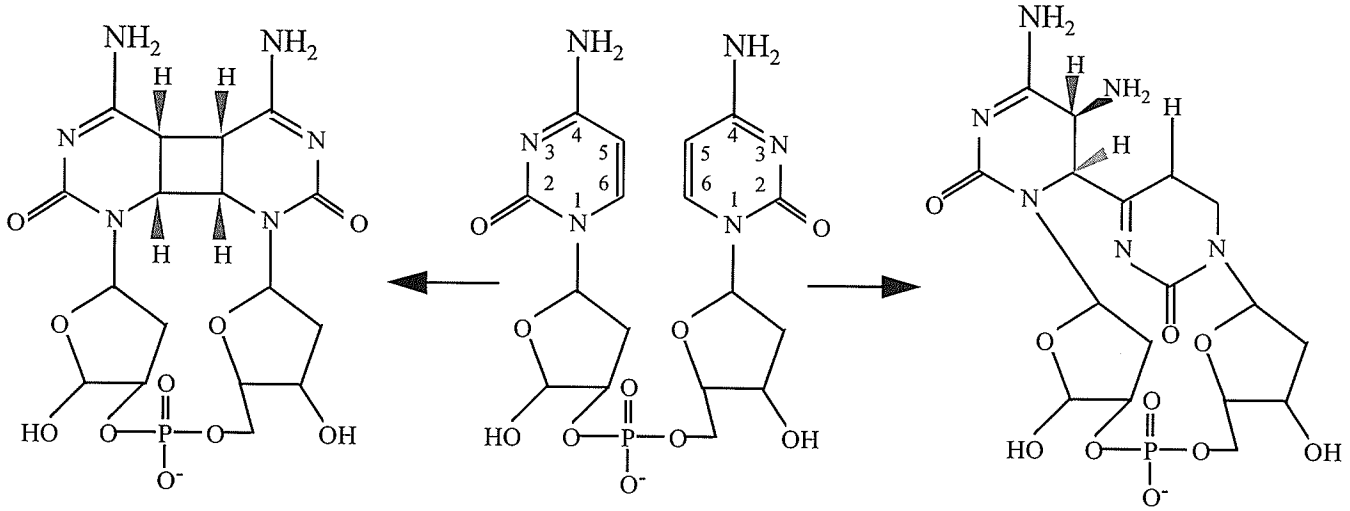
germicidal UVC for the study of DNA repair and mutagenesis since powerful monochromatic (254 nm) sources are readily available. Another reason for utilizing UVC in their studies is that DNA absorbs maximally at 260 nm. Therefore, these studies are based on the assumption that the results can be used as a model to comprehend the mutational mechanisms of the longer UV wavelengths that actually reach the Earth's surface (Friedberg *et al.* 1995). It is for this reason that a UV radiation wavelength of 254 nm is used throughout the experiments in this study.

It is generally believed that the biological effects of UV are the result of photoproducts formed in UV-irradiated DNA (Jagger 1985). This results primarily from the direct absorption of UV light by the DNA. There are two major classes of DNA photoproducts formed by irradiation with UVC light. They are 5-5,6-6 cyclobutane-type pyrimidine dimers (CPDs) (Freeman *et. al.* 1989) and pyrimidine [6-4] pyrimidone photoproducts ([6-4] photoproducts) (Mitchell and Nairn 1989). These two photoproducts are shown in Figure 1. There is evidence that both of these photoproducts play an important role in UV-induced killing and mutagenesis (Kunz and Glickman 1984; Mitchell *et al.* 1985; Wood 1985; Franklin and Haseltine 1986; Glickman *et al.* 1986; Protic-Sablijic *et al.* 1986; Bockrath *et al.* 1987; Armstrong 1993).

CPDs are formed between adjacent pyrimidines on a DNA strand after irradiation with UVC. The bases become covalently linked through their respective 5,6 double bonds to form a four-membered ring structure known as a cyclobutane dimer (Friedberg 1985). This dimer formation greatly distorts the DNA backbone since other adjacent nucleotides must then rotate considerably from their B-form DNA alignments. The result of this is an

Figure 1. Formation of cyclobutane dimers and [6-4] photoproducts.

C< >C cyclobutane dimers and C-C [6-4] photoproducts form from CC dinucleotide sequences upon irradiation of DNA with UV (modified from Friedberg 1985).



Cyclobutane dimer (C< >C)

CpC dipyrimidine

[6-4] photoproduct (C-C)

unwinding and kinking of the helix in the region of the dimer (Perlman *et al.* 1985; Husain *et al.* 1988). The overall effect of the dimer formation can vary. It may be noninstructive or partially noninstructive with regard to DNA replication (Armstrong 1993). It has been shown that dimers resulting from adjacent thymines still retain some of their coding capacity and are partially instructive compared to basic sites (Lawrence *et al.* 1990). Studies have also demonstrated that cyclobutane dimers can inhibit transcription as well as partially or completely block DNA replication *in vitro* (Setlow and Swenson 1963; Swenson and Setlow 1966; Villani *et al.* 1978; Morre and Strauss 1979; Protic-Sabljic and Kraemer 1985; Lawrence *et al.* 1990; Taylor and O'Day 1990). Semiconservative DNA replication is inhibited by cyclobutane dimers through their inhibition of chain elongation and is associated with a lack of both template-directed base insertion and with 3'→5' exonuclease editing of misinserted bases.

[6-4] photoproducts are formed through an azetidine ring intermediate that involves carbons 5 and 6 or a 5'-pyrimidine (pyr) and carbon 4 and the amino group of a 3'-pyr (Wang and Varghese 1967; Patrick and Rahn 1976; Franklin *et al.* 1982). Spontaneous breakage of the exocyclic group transfers the amino group to carbon 5 of the 5'-pyr, thus shifting the 3'-pyr 90^{o} relative to the 5'-pyr. Therefore, [6-4] photoproducts probably produce even more helical distortion in the structure of the DNA than the cyclobutane dimer (Franklin *et al.* 1985). The reason for this is due to the more significant rotation of the 3' base out of the plane of the DNA helix. It is predicted, based on the structure of the [6-4] photoproduct, that only the 3' base becomes non-coding whereas the 5' base retains some or even all of its coding properties (Rycena and Alderfer

1985). Therefore, the formation of [6-4] photoproducts is expected to profoundly alter the structure as well as the biological activity of a nucleic acid helix. It has also been shown that [6-4] photoproducts inhibits transcription (Protic-Sabljic and Kraemer 1986) as well as inhibit DNA replication in *E. coli* (LeClerc *et al.* 1991).

Cyclobutane dimers and [6-4] photoproducts form at sequence-specific sites and there are four possible sites. They are: TT, TC, CT and CC dipyrimidine sequences, written 5' to 3' (T = thymine; C = cytosine; A = adenine; G = guanine).

1.2 DNA Repair Mechanisms

1.2.1 Photoreactivation: Light Repair Mechanism

Enzymatic photoreactivation (EPR) is a process of DNA repair that involves the direct enzymatic cleavage of UV-induced DNA damage by the enzyme DNA photolyase (Cooke 1970; Rupert 1975) and it has been shown to occur both in *E. coli* as well as in yeast upon exposure of EPR-competent cells to visible light (for review of this process see Harm 1976). In both bacteria and yeast, EPR can only repair cyclobutane dimers and has no affect on [6-4] photoproducts due to its structure. Unlike cyclobutane dimers which contain 3' cytosines, [6-4] photoproducts cannot be reverted back to their original dipyrimidine complement by simple cleavage of covalent bonds (Friedberg 1985). The reversal of the cyclobutane dimer damage by this mechanism is achieved via a two-step process. The photolyase first binds to cyclobutane dimers in the DNA and upon absorption of light between 300 and 600 nm, the light energy is converted into chemical energy and used to break the cyclobutane ring which results in the restoration of the pyrimidines to their original configuration (Rupert 1962).

As for higher eukaryotes, studies have failed to show the presence of EPR in organisms more advanced than marsupials (Friedberg *et al.* 1995). However, there have been studies that seem to suggest the possible existence of DNA photolyase or photolyase-like proteins in mammalian cells (Sutherland 1974; Sutherland *et al.* 1974; Sutherland 1975). As well, research shows that there appears to exist a light-dependent loss of pyrimidine dimers from DNA in living cells in culture and in human skin (Sutherland and Oliver 1976; Sutherland *et al.* 1976 and Sutherland *et al.* 1980). In spite of this research there is no definitive evidence that proves that EPR exists in mammalian cells. In fact, a more recent study seems to contradict the above finding by failing to reveal the presence of EPR activity in human cells (Li *et al.* 1993).

1.2.2 Dark Repair Mechanisms In *E. coli*

In order to repair all of the other types of DNA damage that EPR is unable to repair, organisms have evolved other repair processes. Since these repair processes are not dependent on light energy they are collectively called the dark repair mechanisms and the types of DNA damage that they repair are generally referred to as DNA lesions. In the following sections discussing these processes in prokaryotes and eukaryotes not all of the genes involved are mentioned, only those relevant to this study are discussed.

1.2.2.1 Mechanisms of Tolerance of DNA Damage

Not all DNA damage can be repaired immediately. In order for the cell to survive, its replication and transcription machinery must somehow bypass the damage. This is achieved by processes collectively referred to as damage tolerance.

There are at least two mechanisms that were discovered in *E. coli,* whereby cells

can resume DNA synthesis on those templates that contain replication blocks and thus enhance their potential for survival. These two processes are more commonly known as daughter-strand gap repair (error-free post-replication repair) and translesion synthesis (error-prone post-replication repair). By definition, neither of these processes involves the physical removal of the initial lesion that is present in the DNA.

The predominant means of damage tolerance in *E. coli* is via daughter-strand gap repair and it involves a *recA*-dependent recombinational bypass mechanism (Rupp and Howard-Flanders 1968). In this process, DNA synthesis is halted at a DNA lesion, at which point there is a short delay before synthesis is reinitiated at a point further downstream of the blocking lesion. This results in a gap or discontinuity in the daughter strand (Sancar and Sancar 1988; Friedberg 1985). The RecA protein then polymerizes at the gap to form a nucleoprotein filament. This filament functions to promote homologous pairing and strand exchange with the undamaged sister chromatid. It is this strand exchange past the dimer that enables the closure of the gap on the daughter DNA strand (Dasgupta 1981; Friedberg *et al.* 1995). It is suggested that the nucleoprotein filament formed by the polymerization of the RecA protein promotes homologous pairing with the intact sister duplex where reciprocal strand exchange takes place. This strand exchange is believed to be partly facilitated by RecA polymerization from the free 3' terminus of the damaged duplex. The DNA polymerase is now able to use the complimentary strand of the intact duplex as a template and thereby able to replicate past the blocking lesion. During this replication period, the four DNA strands are intertwined and have two Holliday crossovers. Eventual resolution of these crossovers once the replication is

completed results in two uninterrupted duplexes (Sancar and Sancar 1988).

Translesion synthesis, the other mechanism of damage tolerance, also involves DNA synthesis past replication-blocking lesions. Unlike daughter-strand gap repair, this is a mutagenic process in which the replication block is bypassed by the DNA polymerase inserting one or more nucleotides directly opposite the lesion, and then continuing on to extending the replicating DNA chain (Friedberg *et al.* 1995). In *E. coli*, this process requires the induction of the SOS regulatory network, and is dependent on the *umuC*, *umuD* and *recA* gene products (Walker 1984; Echols and Goodman 1990). Alternate names for this repair mechanism is error-prone repair or SOS repair (Witkin 1976).

The SOS system was the first regulatory network induced by DNA damage to be recognized (Walker 1984). This system is regulated by two genes, *recA* and *lexA* (see review by Walker 1984). In an uninduced cell, the LexA protein acts as a repressor for a considerable number of unlinked genes, including *recA* and *lexA*. Upon induction of the SOS system, e.g. by the presence of DNA damage, the RecA protein, which is constitutively present in low levels, becomes activated and it stimulates the autocatalytic cleavage of LexA as well as stimulate other proteins. As the autocatalytic cleavage of LexA molecules continue, the concentration of LexA decreases so that various SOS genes, including the *recA* gene, are expressed at an increased level, and SOS responses mediated by these gene products occurs. As the cell recovers from the original DNA insult, e.g. by repair of the lesion, the SOS inducing signal is eliminated and the RecA molecules return to their inactive state. This decrease in active RecA molecules results in the gradual increase of LexA pools which, in turn, leads to the repression of the SOS

genes and a return of the cell to the uninduced state.

Translesion synthesis has been shown to be associated with both an increase in the processivity of DNA polymerase III (Echols and Goodman 1990), as well as a decrease in its proofreading activity (Woodgate *et al.* 1987; Shwartz *et al.* 1988). Due to its enhanced processivity the polymerase is now able to remain attached to the DNA and thus, can replicate past the blocking lesions. The decrease in its proofreading activity enables the polymerase to continue DNA synthesis after the incorporation of incorrect nucleotides opposite noncoding or miscoding lesions. Therefore, translesion synthesis allows the cell to replicate its DNA in spite of the presence of DNA lesions.

As stated previously, translesion synthesis requires the induction of the SOS system and is dependent on the *umuD*, *umuC* and *recA* gene products. The proteolytic active RecA protein stimulates the cleavage of UmuD which results in the formation of a shorter protein, UmuD'. This is the active form that is required for SOS mutagenesis (Burckhardt *et al.* 1988; Nohmi *et al.* 1988; Shinagawa *et al.* 1988). UmuC and UmuD' then interact and it is believed that RecA targets this UmuD'C complex to the site of a replication block. The binding of UmuD'C is hypothesized to function to inhibit the activity of the 3'→5' exonuclease activity of DNA pol III, and thereby promote translesion synthesis (Shwartz *et al.* 1988; Echols and Goodman 1990). The exact roles of the UmuD' and UmuC proteins in translesion synthesis and thus in UV mutagenesis are not fully understood (Friedberg *et al.* 1995).

1.2.2.2 Nucleotide Excision Repair

One of the most extensively studied and best understood DNA repair mechanisms

is nucleotide excision repair (NER). It is one of the most important error-free dark repair pathways for dealing with pyrimidine dimers and [6-4] photoproducts, as well as a wide range of other types of DNA damage. In its simplest form, this pathway consists of five steps: detection of damage, incision, excision, synthesis of the missing DNA, and ligation (Hoeijmakers 1991; Grossman and Thiagalingam 1993).

Excision repair in *E. coli* is controlled by the *uvr* system which consists of six structural genes, *urvA, uvrB, uvrC, uvrD, polA* and *lig* along with two regulatory genes, *recA* and *lexA* (Yeung *et al.* 1983; Sancar and Sancar 1988; Van Houten 1990). The process of nucleotide excision repair is initiated by the UvrA, UvrB and UvrC proteins that form a protein complex which incises the DNA on both sides of the damaged nucleotide (Sancar and Rupp 1983). The first incision is made at the 8th phosphodiester bond 5' to the damaged nucleotide and the second incision is made at the 4th or 5th phosphodiester bond 3' to the lesion (Lin and Sancar 1992). The 12-13 base damage-containing oligonucleotide is then released by helicase II, the *uvrD* gene product, along with DNA pol I, the product of *polA*. The resulting gap in the DNA is then filled by DNA pol I and sealed by the *lig* gene product, DNA ligase (Sancar and Sancar 1988; Selby and Sancar 1990). The UvrA protein is an ATPase and a DNA-binding protein and it is considered to be the damage recognition subunit of the excision nuclease (Orren and Sancar 1989). It was found that UvrA dimerizes and, in an ATP-dependent reaction, associates with UvrB to form a $(UvrA)_2(UvrB)_1$ complex (Orren and Sancar 1989). UvrB is then delivered onto the damaged DNA via this protein complex, guided by the affinity of UvrA for damaged DNA. Once UvrB has become bound to the damaged DNA UvrA

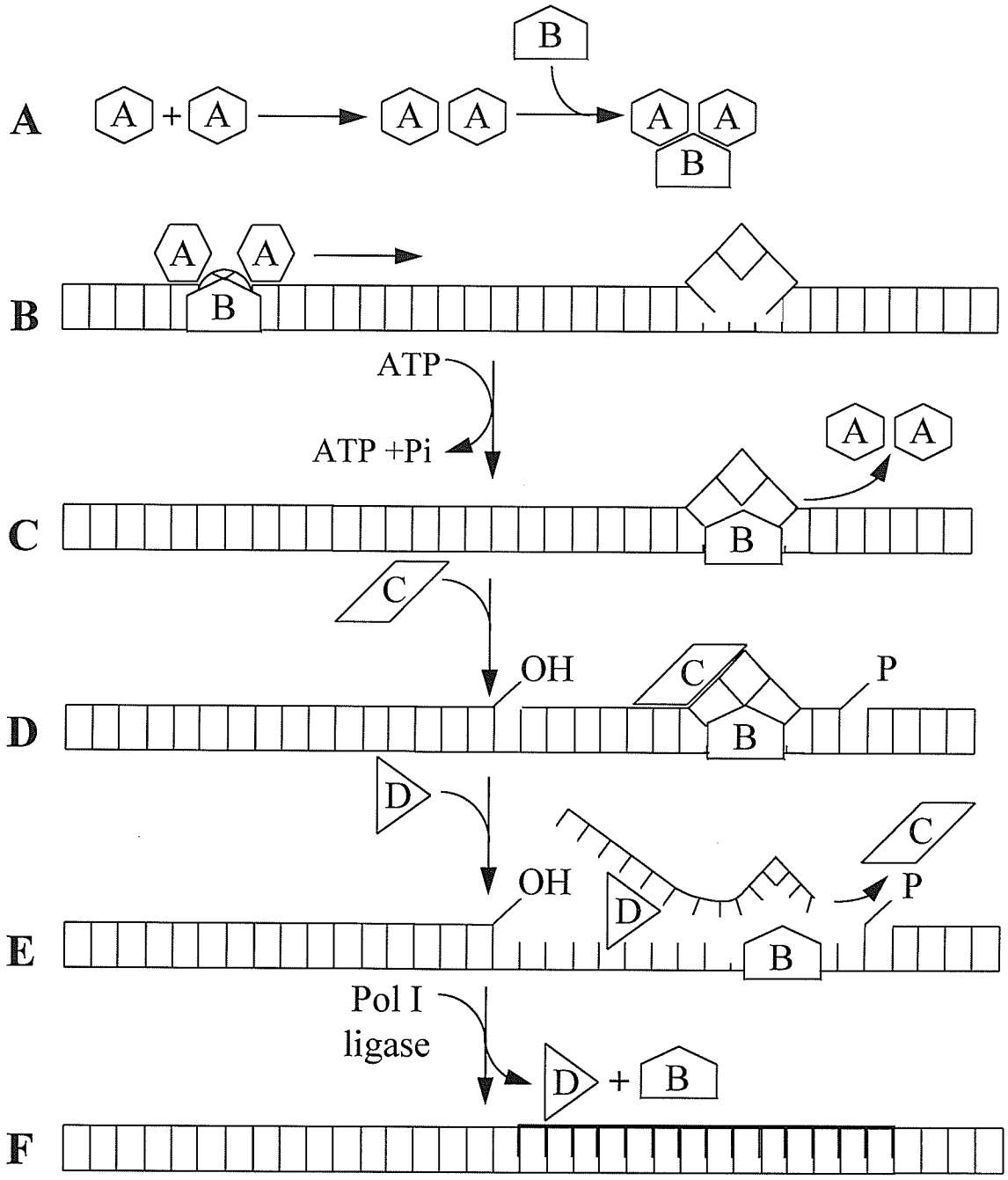
dissociates from the UvrB-DNA complex (Orren and Sancar 1989; Lin and Sancar 1992). UvrC then binds to this UvrB-DNA complex and this binding of UvrC forms the active nuclease which functions to incise the damaged DNA strand on either side of the blocking lesion (Orren and Sancar 1989; Lin and Sancar 1992). The 3' incision is made by UvrB, whereas the 5' incision is carried out by UvrC (Lin and Sancar 1992). UvrC and the damage-containing oligonucleotide is released by helicase II (Orren *et al.* 1992) and UvrB is released by DNA pol I which also functions to fill in the gap left by the departing oligonucleotide (Sibghat-Ullah *et al.* 1990). Figure 2 illustrates the process of excision repair in *E. coli*.

1.2.3 Dark Repair Mechanisms In Eukaryotes

1.2.3.1 Mechanisms of Tolerance of DNA Damage In *S. cerevisiae*

The processes for the tolerance of UV-induced DNA damage in eukaryotes is poorly understood (Friedberg 1991). The mechanisms of SOS responses do not appear to be present in yeast (Ahne *et al.* 1992; Friedberg *et al.* 1995). However, there is some evidence for translesion synthesis and hence, error-prone repair in yeast and mammalian cells (Madzak and Sarasin 1991; Gibbs *et al.* 1993; Friedberg *et al.* 1995). The genes involved in translesion synthesis in yeast belong to the *RAD6* epistasis group (Friedberg 1988; Prakash *et al.* 1993; Friedberg *et al.* 1995). Genes are said to belong to the same epistasis group if the UV sensitivity of the double mutant is equivalent to that of the most sensitive of the two single mutants, whereas for genes belonging to different epistasis groups, the double mutant is much more sensitive than either single mutant (Haynes and Kunz 1981; Friedberg 1988; Prakash *et al.* 1993). Each epistasis group has been named

Figure 2. Nucleotide excision repair in *E. coli*. This illustration depicts the process of NER in the bacterium *E. coli*. In step A, two UvrA proteins dimerize and associates with UvrB to for the $(UvrA)_2(UvrB)_1$ complex. UvrB is then delivered onto the damaged DNA via this protein complex, guided by the affinity of UvrA for damaged DNA as is shown in step B. Step C shows the dissociation of the UvrA dimer from this protein complex once UvrB has become bound to the damaged DNA. In step D, UvrC binds to the UvrB-DNA complex and the binding of UvrC forms the active nuclease that functions to incise the damaged DNA strand on either side of the lesion. The UvrC and the lesion-containing oligonucleotide is then released from the DNA by the *UvrD* gene product as is shown in step E. In step F, the resultant gap is filled in by DNA pol I, which also functions to release UvrB from the DNA, and by ligase (modified from Bootsma 1993).


A
A + A
A A
B
A A
B
B
A A
B
ATP
ATP +Pi
A A
C
B
C
OH
C
P
D
B
D
OH
C
P
D
E
B
Pol I
ligase
D + B
F

for a prominent gene member of that group.

The genes in the *RAD6* epistasis group appear to be involved in both error-prone and error-free repair of UV-damaged DNA (Lawrence 1994). The *RAD18* gene is believed to be involved in an error-free repair branch of the complex pathway(s) that is controlled by the *RAD6* epistasis group (Friedberg *et al.* 1995). The exact function of this gene in the error-free repair pathway is not well known. The *RAD18* gene encodes a protein of 55.5 kDa (Chanet *et al.* 1988; Jones *et al.* 1988; Fabre *et al.* 1989) that has a nucleotide-binding domain as well as three regions that resemble zinc fingers (Chanet *et al.* 1988; Jones *et al.* 1988). It is believed that the Rad18 protein plays a role in DNA-binding and thus may be either directly involved in some aspect of DNA repair or functions indirectly as a transcription factor (Friedberg *et al.* 1995). This protein has been shown to bind to single-stranded DNA (Bailly *et al.* 1994) as well as to the *RAD6* protein, and it has been suggested that the DNA-binding activity of Rad18 may function to gather Rad6 protein onto sites of DNA damage. Since Rad6 has ubiquitin conjugating activity, it is postulated that the loading of Rad6 into sites of DNA damage may result in the modification of a protein or protein complex, e.g., a stalled DNA replication complex, such that synthesis through a DNA blocking lesion could then occur (Bailly *et al.* 1994).

Another gene belonging to the *RAD6* epistasis group is *REV2* (alternatively known as *RAD5*) and it is believed to function with *RAD18* in the error-free repair pathway (Johnson *et al.* 1992; Friedberg *et al.* 1995). The *REV2* gene encodes a 134 kDa protein and the amino acid sequence of this protein suggests that it has both DNA-binding and ATPase/DNA helicase activities (Johnson *et al.* 1992). The exact function of

the Rev2 protein is not known, but it has been suggested that it may act to increase the efficiency of the Rad18 protein in the error free repair pathway. It has been hypothesized that the way that translesion synthesis may occur in yeast is by a type of template switching mechanism of the DNA polymerase when it encounters a blocking lesion. What may occur is that when the DNA polymerase is blocked by a lesion, it bypasses this blockage by switching from the damaged template onto the undamaged strand of the sister chromatid. Here, it is then able to continue DNA synthesis by using the undamaged sister chromatid as the template. Once the polymerase has cleared the lesion, it then switches back onto the damaged template (Johnson *et al.* 1992). Therefore, it is postulated that Rev2 may function in translesion synthesis by assisting Rad18 in this template switching reaction or it could affect the polymerization step either by increasing the processivity of DNA polymerase or by unwinding the duplex DNA.

There are other *REV* genes in the *RAD6* epistasis group, but these genes are involved in an error-prone repair pathway. The *REV1* gene encodes a predicted protein of 112 kDa that contains an internal stretch of 152 residues which shares a 25% identity to the *E. coli* UmuC protein (Larimer *et al.* 1989). The exact function of the Rev1 protein is not known, but it is involved in damage tolerance and hence, error-prone repair in yeast and it may play a role similar to that of UmuC in *E. coli* (Friedberg *et al.* 1995). The *REV3* gene was first identified by isolating strains which exhibited reduced frequencies of UV mutagenesis (Lemontt 1971). This gene encodes a predicted protein of 173 kDa and the amino acid sequence has regions of homology with that of the Epstein-Barr virus DNA polymerase, Herpes Simplex virus DNA polymerase as well as general structural

similarities with mammalian DNA pol α and yeast DNA pol I (Morrison *et al.* 1989). Recent evidence has shown that the Rev3 protein combines with the Rev7 protein to form a complex that has DNA polymerase activity. This new polymerase, called DNA polymerase ζ was able to efficiently continue to replicate past a template thymine dimer (Nelson *et al.* 1996).

As for higher eukaryotes, it is still not known whether some form of damage tolerance occurs. Due to the complexity of the higher eukaryote genome damage tolerance processes have been difficult to define (Friedberg 1985; Friedberg *et al.* 1995). One study does seem to indicate that some form of translesion synthesis does take place, (Spivak and Hanawalt 1992) but the exact process has yet to be determined.

1.2.3.2 Recombination Repair In *S. cerevisiae*

Recombination repair is a postreplication repair process and therefore this mechanism is also referred to as postreplication repair. This repair pathway was discovered to be the only mechanism able to repair DNA double-strand breaks that results from such DNA damaging agents as ionizing radiation (Friedberg *et al.* 1995). It is believed that many different types of recombination are possible in yeast. These genetic exchanges between homologous DNA duplexes can be interchromosomal, *i.e.* between homologous or nonhomologous chromosomes which share a region of sequence homology. Alternatively, recombination could be intrachromosomal, where there is exchange of genetic material between sister chromatids of the same chromosome, or between reiterated sequences on the same DNA duplex. Also, it is possible that recombination could occur between yeast chromosomes and gapped or linearized

plasmids. These recombinational events could either be reciprocal or nonreciprocal with respect to the transfer of information between DNA molecules (Cooper and Kelly 1987). In yeast, most of the genes involved in this repair process belong to the *RAD52* epistasis group (Cooper and Kelly 1987). Since mutations in several *RAD52* group genes results in a slight sensitivity to UV, it has been suggested that recombination repair may also play a minor role in the post-replicative repair of UV-induced DNA damage (Kiefer 1987).

The *RAD50* gene is predicted to encode a protein of 153 kDa (Alani *et al.* 1989). The actual function of this gene is not known however, it has been suggested that it plays a role in the recognition and interaction of the homologous DNA molecules that would be required for a recombination event (Alani *et al.* 1989). The *RAD51* encodes a predicted protein of 43 kDa (Shinohara *et al.* 1992; Basile *et al.* 1992). This protein has ca. 27% homology with the RecA protein of *E. coli* (Shinohara *et al.* 1992; Basile *et al.* 1992) and has an ATP-dependent double-stranded and single-stranded DNA binding activity as well as an ATP-independent single-stranded DNA binding activity (Shinohara *et al.* 1992). The Rad51 protein also possesses a single-stranded DNA-dependent ATPase activity (Shinohara *et al.* 1992). Like the other proteins involved in recombination repair, the exact function of the Rad51 protein is unknown, but it has been shown to be able to bind to the Rad52 protein (Shinohara *et al.* 1992). The *RAD52* gene contains an open reading frame of 1,512 nucleotides which is predicted to encode a protein of 60 kDa with unknown function (Adzuma *et al.* 1984). It has been hypothesized that the combined proteins act in some aspect of recombination repair to convert double strand breaks into the next intermediate in recombination reactions (Shinohara *et al.* 1992). The *RAD54*

gene is a damage-inducible gene that has an open reading frame of 2,694 nucleotides. The amino acid sequence of the Rad54 protein implies that this protein possesses DNA helicase activity (Emery *et al.* 1991). Transcription of *RAD54* has been shown to be induced by both γ rays and UV radiation (Friedberg *et al.* 1995). Another yeast gene involved in recombination repair is the *RAD55* gene. Very little is known about this gene except that its protein has homology to Rad51 as well as to RecA of *E. coli* (Lovett 1994). Yeast strains with point mutations and deletion mutations in *RAD55* suggest that this gene is involved in DNA strand break repair at low temperatures since these mutants are sensitive to ionizing radiation at 23°C but not at 36°C (Lovett and Mortimer 1987).

1.2.3.3 Nucleotide Excision Repair In *S. cerevisiae*

Nucleotide excision repair (NER) in eukaryotes is more complex and not as well understood as it is in *E. coli*. In eukaryotes, no homologs of the UvrA, UvrB or UvrC proteins have been identified. In *Saccharomyces cerevisiae*, there are at least ten genetic loci that are members of the *RAD3* epistasis group and are involved in this repair pathway (Friedberg 1988; Friedberg *et al.* 1995). They include *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD7*, *RAD10*, *RAD14*, *RAD16*, *RAD23* and *RAD25* (Friedberg 1991; Prakash *et al.* 1993). Biochemical and phenotypical characterization of these genes revealed that *RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10* are essential for the incision step of NER (Friedberg 1991). Recent evidence has indicated that *RAD14* and *RAD25* could also be involved in these same steps (Prakash *et al.* 1993).

The *RAD1* is a nonessential gene that is involved in both nucleotide excision repair as well as intrachromosomal recombination involving repeated sequences (Schiestl

and Prakash 1988). This gene encodes a transcript of 3.1 kb that codes for a predicted protein of 110 kDa (Higgins *et al.* 1983b; Yang and Friedberg 1984) and was observed to be uninducible by DNA damage (Friedberg 1988). The *RAD10* gene, like the *RAD1* gene, is involved in both nucleotide excision repair as well as mitotic recombination events and is also haplo viable (Weiss and Friedberg 1985; Sung *et al.* 1992). This gene encodes a predicted protein of 23-24 kDa (Weiss and Friedberg 1985; Reynolds *et al.* 1985b). It was observed that the Rad10 protein binds DNA with a strong preference for single-stranded DNA (Sung *et al.* 1992).

Recent studies have shown that the *RAD1* and *RAD10* gene products interact to form a protein complex *in vitro*, as well as *in vivo*, which exhibits an endonucleolytic activity in the presence of Mg^{+2} (Bailly *et al.* 1992; Bardwell *et al.* 1993; Tomkinson *et al.* 1993; Siede *et al.* 1993; Sung *et al.* 1993a). It was subsequently observed that the Rad1/Rad10 protein complex produced 3'-hydroxyl and 5'-phosphate termini on both single-and double-stranded DNA molecules (Sung *et al.* 1993a). This observation lead to the hypothesis that the endonucleolytic activity by the Rad1/Rad10 complex functions in NER possibly, by initiating incision of damaged DNA (Tomkinson *et al.* 1993; Sung *et al.* 1993a). More recent studies have demonstrated that the endonuclease activity of the Rad1/Rad10 complex specifically recognizes the junction between double-stranded DNA and 3'-single-strand tails (Bardwell *et al.* 1993; Tomkinson *et al.* 1994). Therefore, it is believed that at sites of DNA damage there are Y-shaped duplex/single-strand DNA junctions located on either side of the lesion. On the 5' side of the damage, this Y-shaped junction is recognized by the Rad1/Rad10 complex and thus, it binds to this location.

This binding is believed to aid in its participation in DNA damage-specific incision of the damaged DNA molecule at this site during NER (Bardwell *et al.* 1993). In fact, it has recently been shown that the Rad1 protein is the catalytic subunit of the Rad1/Rad10 endonuclease (Habraken *et al.* 1994a).

The *RAD2* gene is a nonessential gene that codes for a 3.2 kb transcript which codes for a predicted protein of 111 kDa (Naumovski and Friedberg 1984; Higgins *et al.* 1984; Nicolet *et al.* 1985). This gene was shown to be inducible by DNA damage when steady-state levels of *RAD2* mRNA were observed to increase as a response to UV radiation (Madura and Prakash 1986; Siede *et al.* 1989). The C-terminal tail of the Rad2 polypeptide is rich in basic amino acids and this region was found to be important for the protein's function (Higgins *et al.* 1984; Friedberg 1988). It was observed that a deletion of the terminal 78 codons of the gene inactivated its ability to complement *rad2* mutants (Higgins *et al.* 1984). It was later discovered that the Rad2 protein has endonucleolytic activity with a specificity for single-stranded DNA (Habraken *et al.* 1993). In fact, the properties of this endonuclease are very similar to those of the Rad1/Rad10 protein complex mentioned previously in that it leaves 3' hydroxyl and 5' phosphate groups at sites of cleavage and that this cleavage activity requires the presence of Mg^{+2} (Habraken *et al.* 1993). It has been postulated that the Rad2 protein catalyzes damage-specific incisions on the 3' side of base damage, whereas the Rad1/Rad10 protein complex makes these incisions on the 5' side of the damage (Friedberg *et al.* 1995).

The *RAD4* gene is another gene involved in the incision step of nucleotide excision repair however, very little is known about this gene. The reason for this is that an

intact *RAD4* gene cannot be propagated in *E. coli* since its gene product is lethal to *E. coli* (Siede and Eckardt-Schupp 1986; Fleer *et al.* 1987). It was soon discovered that deliberate insertional mutagenesis of the wild-type *RAD4* gene would enable this gene to be propagated in *E. coli*, and that removal of this inserted DNA fragment restored normal *RAD4* function in yeast (Fleer *et al.* 1987b). By being able to temporarily disrupt *RAD4*, researchers were able to eventually clone and characterize this gene. The *RAD4* gene is a nonessential gene that is not inducible by DNA damage (Fleer *et al.* 1987a). It has an open reading frame of 2,262 bp that is believed to code for a protein of ca. 87 kDa (Gietz and Prakash 1988; Couto and Friedberg 1989).

The *RAD3* gene is the most well studied of all the genes involved in NER. This gene encodes a predicted protein of ca. 89.7 kDa (Naumovski *et al.* 1985; Reynolds *et al.* 1985a) that has both DNA helicase activity as well as ATPase activity (Sugino *et al.* 1986; Sung *et al.* 1987a; Sung *et al.* 1987b; Harosh *et al.* 1989). This latter activity requires the presence of not only the divalent cations Mg^{+2} or Mn^{+2}, but also single-stranded DNA. Double-stranded DNA does not support the Rad3 protein's ATP hydrolysis activity (Sung *et al.* 1987a). Conversely, the DNA helicase activity of this protein is dependent on the hydrolysis of ATP and it unwinds duplex DNA unidirectionally with 5'→3' polarity relative to the DNA strand to which it is bound (Sung *et al.* 1987b; Harosh *et al.* 1989). It was also discovered that the Rad3 protein has a DNA-RNA helicase activity that requires the hydrolysis of ATP as well (Bailly *et al.* 1991; Naegeli *et al.* 1992b). In this situation, the Rad3 protein was shown to translocate on the DNA strand of the DNA-RNA duplex while it displaced the RNA strand. It was

subsequently observed that the DNA helicase and ATPase activities of the Rad3 protein were inhibited by the presence of UV-induced photoproducts in duplex DNA (Harosh *et al.* 1989; Naegeli *et al.* 1992a). This inhibition was shown to be strand-specific in that it only occurred when the damage was on the DNA strand to which the Rad3 protein was bound and translocating. As a result of this inhibition, this protein was no longer able to continue translocating and so, it remained sequestered on the DNA at these sites of damage (Naegeli *et al.* 1992a). In fact, it has been shown that in the presence of UV-irradiated DNA, Rad3-DNA complexes are very stable with a greatly increased half-life (Naegeli *et al.* 1992a). Therefore, it has been suggested that this sequestering of the Rad3 protein at sites of base damage may result in the formation of a unique nucleoprotein structure, which is then recognized by other *RAD* gene products and is thereby generating a substrate for endonucleolytic cleavage (Naegeli *et al.* 1993). However, the protein's helicase activity is not involved in its DNA damage binding ability, since a mutant Rad3 protein, which lacks DNA helicase activity, was still able to bind to UV-damaged DNA with the same efficiency as the wild-type protein (Sung *et al.* 1994). It was shown that this binding to sites of DNA damage was dependent on both ATP and on the degree of negative superhelicity in the DNA. It has been suggested that the requirement of superhelicity may function to aid in targeting Rad3 to regions of DNA that are undergoing transcription, thereby resulting in the preferential repair of these regions (Sung *et al.* 1994).

The *RAD3* gene is a unique gene in that it is also as essential gene for viability in yeast (Naumovski and Friedberg 1983; Higgins *et al.* 1983a). This was shown by the fact

that diploid (*RAD3/rad3*) strains which were induced to undergo sporulation resulted in two viable and two inviable spores per tetrad (Naumovski and Friedberg 1983). This gene's essential function has just recently been elucidated. It has been discovered that the Rad3 protein is involved in the initiation of basal transcription by RNA polymerase II (Guzder *et al.* 1994; Bardwell *et al.* 1994). This protein is one of five subunits that make up the yeast transcription initiation factor b (now known as TFIIH) (Feaver *et al.* 1993). This initiation factor is itself part of a larger protein complex that is involved in the formation of a preinitiation trancription complex which binds to the promoters of genes in preparation for RNA polymerase II binding (Chalut *et al.* 1994; Friedberg *et al.* 1995). Recent evidence suggests that TFIIH may also play a role in NER since other excision repair proteins have been shown to associate with this complex *in vitro*. Rad2 and Rad4 proteins have been shown to bind to TFIIH however, unlike the Rad3 protein, they are not essential proteins so therefore, it is believed that they are not involved in transcription (Bardwell *et al.* 1994: Chalut *et al.* 1994). Exactly how TFIIH is involved in NER is unknown but it has been suggested that since it is involved in transcription its dual role may somehow play a function in the preferential NER of transcriptionally active versus silent areas of the genome (Bardwell *et al.* 1994).

1.2.3.4 Nucleotide Excision Repair in Mammalian Cells

Less is known about the mechanics of the NER process in mammalian cells. The indications with patients suffering from the genetic disease xeroderma pigmentosum, show that they are unable to carry out NER and as a result, these patients suffer from a number of deleterious phenotypes such as abnormal sensitivity to UV radiation, abnormal

pigmentation, predisposition to skin cancer and accelerated neurodegeneration (Hoeijmakers 1993). Some human genes have since been cloned that are involved in NER. Table 1 lists these genes along with their yeast homologs.

The human *ERCC1* cDNA has an open reading frame that encodes a polypeptide with a calculated molecular mass of ca. 32.5 kDa (van Duin *et al.* 1986). *ERCC1* has homologies to the *RAD10* gene from *S. cerevisiae* (van Duin *et al.* 1986). The human homolog of the yeast *RAD1* gene is *ERCC4* (also referred to as *XPF*) (Thompson *et al.* 1994). There are indications that the polypeptides encoded by the *ERCC1* and *ERCC4* genes interact to form a stable complex in living cells just as the Rad10 and Rad1 proteins do in yeast (Park and Sancar 1994).

The *XPD* gene (also known as *ERCC2*) has an open reading frame that encodes a polypeptide with a predicted mass of 86.9 kDa (Weber *et al.* 1990). The amino acid sequence of the XPD polypeptide revealed a 51% homology with that of the Rad3 protein (Weber *et al.* 1990). Experimental evidence has shown that the XPD protein is a DNA-dependent ATPase with DNA helicase activity and with properties very similar to those of Rad3 (Sung *et al.* 1993b). This seems to imply that the XPD protein functions in the same capacity in human NER as the Rad3 does in yeast. As stated above, the *RAD3* gene is involved in basal transcription by RNA polymerase II, however, it has yet to be shown that the XPD protein has a role in transcription in human cells.

The human homologue of *RAD2* is the *XPG* gene (also called *ERCC5*) (Scherly *et al.* 1993). This gene encodes a protein of 133 kDa and, like the Rad2 protein, exhibits a DNA endonucleolytic activity which generates 3' hydroxyl and 5' phosphate ends.

Table 1: Human DNA Repair Genes and Their Yeast Homologs (Friedberg *et al.* 1995)

S. cerevisiae gene	Human gene
RAD3	*XPD*
SSL2 (RAD25)	*XPB*
RAD2	*XPG*
RAD14	*XPA*
RAD4	*XPC*
RAD1	*ERCC4/?XPF*
RAD26	*CSB*
RAD10	*ERCC1*
RAD23	*HHR23A*
	HHR23B
TFB1	*p62*
SSL1	*p44*

However, unlike the Rad2 protein, its endonucleolytic activity is able to act on both single-stranded as well as on double-stranded DNA molecules, and it is able to use either Mg^{+2} or Mn^{+2} as its metal cofactor (Habraken *et al.* 1994b). It has recently been shown that the XPG protein makes structure-specific endonucleolytic incisions in a synthetic DNA structure. O'Donovan *et al.* (1994) showed that this protein recognizes duplex/5'-single-strand junctions in DNA and bound to these sites. At these sites, the XPG protein makes an incision 3' to this junction.

The *XPC* gene is the human homolog of the *RAD4* gene (Legerski and Peterson 1992; Henning *et al.* 1994). This gene is encodes a polypeptide of ca. 106 kDa (Friedberg *et al.* 1995). Like its yeast counterpart, little is known about the XPC protein in terms of its structure and function in NER in humans.

Research is still being done in order to fully understand DNA repair in yeast and mammals. Yet, as this work continues, a new area in DNA repair is starting to be explored. This relatively newer field is the study of DNA repair in plants.

1.3 DNA Repair in Plants

Plants and their biology have always been an interesting source of study, however, in the last few years there has been a refocusing of research on plants. This renewed interest in plants arose, in part, from the gene transfer technology that can be applied to crop plants to introduce novel genetic traits (Goldberg 1988). An area of research in plants with increased interest is that of DNA repair and this increased interest is for two main reasons (Velemínsky and Angelis 1990):

1. DNA repair may function as one of the defense mechanisms in the protection of

both wild and crop plants against environmental genotoxic agents to which most plants are continually being exposed, especially those plants that live in higher altitudes or in areas of great industrial activity.

2. Error-prone DNA repair is an important part of spontaneous mutation processes that affect the production of mutations which, in turn, determine the type and the frequency of beneficial or deleterious mutations in the breeding of crop plants.

For more than 30 years, induced mutagenesis by chemical and physical means has been extensively used as an adjunct to conventional plant breeding. The result of these mutagenic processes is the production and release of thousands of improved new varieties of plants for large scale cultivation (Evans 1989; Gasser and Fraley 1989; Velemínsky and Angelis 1990).

Plants are highly dependent on their environment and use extrinsic cues such as water, light and temperature to trigger many developmental events (Goldberg 1988). Unlike other organisms, plants are fixed organisms, unable to move away from hazardous agents that they may encounter. For this reason alone plants would be forced to encounter a wide range of deleterious agents, both natural as well as man made, in their environment. Therefore, in order to ensure their survival, plants have had to evolve mechanisms to neutralize or lessen the effects of these dangerous agents (Velemínsky and Angelis 1990). Some of the man-made agents includes: genotoxic pollution in the air, water and soil, which include pesticides and herbicides. Plants existing in higher altitudes have to deal with the more natural deleterious agents in the form of UV and ionizing radiation and cosmic rays. However, exposure to sunlight has increasingly become a

more dangerous agent for all plants on Earth in recent years as they may now be encountering more UV radiation than they are used to due to the rising level of harmful solar UV radiation reaching the Earth's surface as a result of the decline in the ozone level that surrounds the Earth (Chasan 1994).

1.3.1 *A. thaliana* as an Ideal Organism for Plant Molecular Biology

One of the more well studied plants in plant molecular biology is *Arabidopsis thaliana*. It is a weed with no food or economic value (Meyerowitz and Pruitt 1985). This plant is a member of the mustard family and is an ideal organism to use in plant genetic studies due to its small size (measuring about 30 cm), a small haploid genome, ca. $7x10^7$ nucleotide pairs, a haploid chromosome number of only 5 and a short generation time of five weeks (Leutwiler *et al.* 1984; Meyerowitz and Pruitt 1985; Pruitt and Meyerowitz 1986; Goldberg 1988; Meyerowitz 1989). *Arabidopsis* can produce more than 10,000 seeds per plant and its growth condition requirements are simple, only needing moist soil and fluorescent light for rapid growth (Meyerowitz and Pruitt 1985; Pruitt and Meyerowitz 1986). The flowers of *Arabidopsis* contain both anthers and pistils and thus enables self-fertilization which allows new mutations that arise to be made homozygous with minimal effort (Meyerowitz and Pruitt 1985). When required, cross-fertilization of *Arabidopsis* can be used. Cross-fertilization allows for both genetic mapping and for the generation of multiple mutant stocks. Mutagenesis is also easily carried out with this plant. This only requires soaking seeds in chemical mutagens or irradiation of seeds with γ radiation that have been presoaked in water. These mutagenized seeds can then be planted, grown to maturity and allowed to self-fertilize in order to produce seeds that are

homozygous for the new mutations (Meyerowitz and Pruitt 1985). The genome of this plant species contains little repetitive DNA, most of which is ribosomal RNA genes (Meyerowitz and Pruitt 1985; Pruitt and Meyerowitz 1986).

1.3.2 Physical Barriers to UV Radiation in Plants

As stated above, plants are immobile organisms and as such they cannot move so they are forced to deal with their environment. For this reason it only seems natural that they would have developed many physical protective mechanisms as their first line of defense against deleterious environmental agents. It has even been suggested that since plants are exposed to sunlight more than any other organism they may have evolved unique DNA repair mechanisms (Chasan 1994).

Plants, as their first line of defense, have thick cuticular waxes on their epidermis that function to filter out the harmful wavelengths from sunlight (Caldwell 1971 as stated in McLennan 1987). Their second and most important line of defense is the flavonoid pigments within plant cells. Flavonoids are a class of metabolites that are synthesized by vascular plants in response to a variety of environmental stimuli (Kootstra 1994). It is believed that these pigments play a crucial role in the protection of plant DNA from the harmful effects of UV irradiation by absorbing light in the UV region of the light spectrum. The induction of flavonoid production as well as their accumulation in plant tissues has been observed as a response to UV irradiation (Kootstra 1994; Lois 1994) and plants with decreased levels of flavonoids have showed increased sensitivity to DNA damage by UV irradiation (Li *et al.* 1993; Britt *et al.* 1993; Lois and Buchanan 1994). Even pollen contains flavonoids (Jackson 1987). Should genotoxic agents make it past

these physical defense mechanisms plants must also possess DNA repair mechanisms in order to survive.

1.3.3 DNA Damage in Plants

All plants start out as a seed. In this state the embryonic plant has very little or no metabolic activity and is therefore able to persist for long periods of time in a dormant state (Goldberg 1988). But, even as a seed, DNA lesions can appear spontaneously or become induced by mutagens that may be encountered by the embryo during this dormant period (Velemínsky and Angelis 1990). In order for the embryo to grow, these lesions must be repaired. The repair process in seeds occurs during the early phase of water uptake (imbibition) by the seed during germination when the metabolic processes begin (Velemínsky and Angelis 1990). This period of early germination, before the embryonic cells of the plant enter the S-phase, is important for the repair of spontaneous DNA lesions that have accumulated in the DNA (Osborne *et al.* 1984 as stated in Velemínsky and Angelis 1990). It is believed that this repair of spontaneous DNA lesions is an essential part of early germination in seeds, and that the lack of this repair in older seeds is connected with the loss of viability, germination and enhancement of chromosomal aberrations (Osborne *et al.* 1984 as stated in Velemínsky and Angelis 1990). It has been shown that apurinic/apyrimidinic sites and strand breaks can form in seeds during storage (Dandoy *et al.* 1987; Cheah and Osborne 1978) and that the older the seed becomes, the less viable it is due to the enhanced frequency of chromosomal aberrations (Dimitrov 1994).

Of much importance for the maintenance of the stability of the plant genome is

the ability of germinating pollen grains to repair DNA damage that may have been induced during their dry state (Jackson 1987). In this state, pollen can be exposed to many environmental mutagens which could lead to considerable damage to its DNA. Therefore, the pollen would need to repair any DNA damage that had developed during its dehydrated state (Jackson 1987; Velemínsky and Angelis 1990). The dehydrated state of the pollen seems to pose the most threat to the DNA since dehydration can lead to other harmful events. It has been suggested that dehydrated DNA has an increased sensitivity to damage by solar UV irradiation. It is believed that thymines in dehydrated DNA are converted to 5-thyminyl-5,6-dihydrothymine by UV irradiation (Jackson 1987) which can only be repaired via a dark repair mechanism. Other potential dangers to the pollen gamete DNA comes from heavy metals and alkaloids due to man-made chemicals, as well as other naturally occurring carcinogens and poisons that may be encountered from both insect secretions and from the admixture with other pollens in the insect pollen load (Jackson 1987).

1.3.4 Removal of DNA Damage In Plants

It only makes sense that plants should have some form of DNA repair processes in order to remove DNA damage. These mechanisms have yet to be elucidated. The reasons for the difficulty in discovering the DNA repair systems in plants is that plants have physical defense mechanisms against hazardous environmental agents (as mentioned above). However, there is evidence that some form of DNA repair does occur in plants. Studies to date have indicated that plants possess both photoreactivation as well as excision repair systems for the repair of UV-induced cyclobutane-type pyrimidine dimers

(McLennan 1987; Quaite *et al.* 1994). Clear evidence for the biological effects of photoreactivation in plants has been hindered by the obvious detrimental effects of growing plants in the dark (Britt 1995). However, photoreactivation has been demonstrated in UV-irradiated cells of *Nicotiana tabacum* (Trosko and Mansour 1968 as stated in McLennan 1987), *Ginkgo biloba* (Trosko and Mansour 1969b as stated in McLennan 1987) and *Caucus carota* (Howland 1975), in seedlings of *Lathyrus sativus* and alfalfa (Soifer and Tsieminis 1974, 1977a as stated in McLennan 1987; Quaite *et al.* 1994), in intact plants of *Wolffia microscopia* and *Spirodela polyrhiza* (Delgani *et al.* 1980), and in pollen of *Petunia hybrida* (Jackson and Linskens 1979; Jackson 1987). This mechanism has also been recently shown to occur in *Arabidopsis thaliana* and appears to be inducible upon irradiation with UV-B (Pang and Hays 1991). In addition, a recent study has indicated the presence of a light-dependent repair pathway that eliminates the UV-induced [6-4] photoproduct in the organism *Arabidopsis thaliana* (Chen *et al.* 1994). [6-4] photoproducts are usually eliminated via a dark repair pathway and the only other evidence for the existence of a photolyase specific for [6-4] photoproducts comes from *Drosophila* cell extracts (Todo *et al.* 1993; Kim *et al.* 1994). Such an enzyme has not been found in other eukaryotes or in any prokaryote to date. Therefore, it appears that *Arabidopsis* (and possibly other plants) has the ability to photoreactivate both of the major UV-induced DNA damage photoproducts (Britt 1995). Plants also possess chloroplasts which contain their own DNA. It seems that chloroplast DNA also have their own DNA repair systems that are distinct and separate from those of nuclear DNA. Photoreactivation has been observed in both nuclear as well as in chloroplast DNA from

the green alga, *Chlamydomanas reinhardtii* (Small 1987). These mechanisms use different enzymes and are under different nuclear control from one another (Small 1987).

It has been suggested that photoreactivation may not be universal within the plant kingdom (McLennan 1987) and that the relative role that photoreactivation plays in those plants that use this mechanism may differ among plant species (Quaite *et al.* 1994). Plants whose natural habitat is shade might not have the ability to photoreactivate, or if they do possess this mechanism it is used less extensively. On the other hand, those plants whose preferred environment contains a lot of sunlight, may have this mechanism and use it preferentially over the dark repair mechanisms (Quaite *et al.* 1994).

Less is known about the dark repair mechanisms in plants. Excision repair of pyrimidine dimers from a UV-irradiated plant has been demonstrated in the grass pea *Lathyrus sativus* (Soifer and Tsieminis 1974, 1977a, b as stated in McLennan 1987), in alfalfa seedlings (Quaite *et al.* 1994), in nuclear DNA of *Chlamydomonas reinhardtii* (Small 1987) and is presumed to function in the chloroplast DNA of this organism as well. There are also indications that excision repair occurs in UV-irradiated pollen of *Petunia hybrida* (Jackson and Linskens 1978; Jackson and Linskens 1979). To date there is no clear evidence for the existence of other dark repair mechanisms in plants, *i.e.* recombination repair and damage tolerance. It is of interest to note that a mutagenic damage tolerance repair pathway may exist in plants as indicated by the discovery of an *Arabidopsis thaliana* mutant that exhibits phenotypic properties similar to those of yeast mutants in the *RAD6* epistasis group (Harlow *et al.* 1994). Like the *RAD6* yeast mutants, this *Arabidopsis* mutant, called *uvh1*, exhibits hypersensitivity to both UV light and

ionizing radiation.

There are some indications that plants may have inducible repair systems, one for dealing with alkylating agents and another for dealing with nonalkylating agents. The exact molecular mechanisms of these inducible systems have yet to be determined, but preliminary evidence shows that this system, once activated by harmful agents, protects plants from subsequent damage by these agents (Heindorff *et al.* 1987).

1.3.5 Isolation of DNA Repair Genes and/or Proteins From Plants

Even though very little is known about the exact steps involved in the dark repair processes in plants, some DNA repair enzymes have been isolated from a number of different plants which include: photolyase from maize pollen and from several types of bean (McLennan 1987); uracil-DNA glycosylases from wheat germs, onion roots, and carrot cell suspensions (Gutierrez 1987); AP-endonucleases from leaves and embryos of beans and barley (Thibodeau and Verly 1976; Svachulová *et al.* 1978 as stated in Velemínsky and Angelis 1990) and endonucleases specific for UV-induced pyrimidine dimers from carrot cells (McLennan and Eastwood 1986). This latter endonuclease has been found to be similar to that of the uvrABC endonuclease of *E. coli* with regards to both its size and its activity with respect to the type of dimer it excises (McLennan and Eastwood 1986).

Recently, a ubiquitin carrier protein has been isolated from both wheat germ and *Arabidopsis thaliana* (Sullivan and Vierstra 1991). This protein has been shown to have high amino acid sequence similarity to the Rad6 protein of yeast. In the case of the wheat germ ubiquitin carrier protein, it has been shown that it is structurally and functionally

similar the yeast Rad6 repair protein (Sullivan and Vierstra 1989).

Since the *Arabidopsis* plant is such an easy plant to work with (see section 1.3.1) many genes that are believed to be involved in DNA repair have been isolated from this plant. Four *Arabidopsis* cDNAs have been isolated that partially complement dark repair deficiencies in *E. coli* (Pang *et al.* 1992, 1993a). These cDNAs were designated *DRT100*, *DRT101*, *DRT102* and *DRT103*. Two other *Arabidopsis* cDNAs have been discovered by Pang *et al.* (1993b) that were designated *DRT111* and *DRT112*. These cDNAs were observed to increase resistance to UV light as well as to several chemical DNA-damaging agents in *E. coli* strains that were deficient in recombination intermediate resolution activities, *i.e.* unable to resolve Holliday junctions formed during homologous recombination events (Pang *et al.* 1993b). The activities of *DRT101* and *DRT102* were found to be specific for UV light damage and they complemented both $UvrB^-$ and $UvrC^-$ *E. coli* phenotypes (Pang *et al.* 1993a). It was also discovered that the Drt101 protein exhibited some short blocks of amino acid sequence homologous to that of Rad10 as well as the human repair protein homolog ERCC1 (Pang *et al.* 1993a). *DRT100* was observed to partially complement the $RecA^-$ phenotype in *E. coli* (Pang *et al.* 1992). The exact function of *DRT103* was not discovered, however its amino acid sequence exhibited a small degree of similarity to the sequences of microbial photolyases (Pang *et al.* 1993a). More recently, Santerre and Britt (1994) have cloned a 3-methlyadenine-DNA glycosylase from *Arabidopsis thaliana*. This gene was observed to complement the methyl methanesulfonate-sensitive phenotype of *E. coli* and its amino acid sequence showed significant homology to that of the rat 3-methyladenine-DNA glycosylase.

1.4 Purpose of This Study

The aim of this study was to isolate and characterize a gene involved in nucleotide excision repair in *Arabidopsis thaliana*. The approach was to transform mutant yeast strains defective in nucleotide excision repair with plasmids containing *Arabidopsis thaliana* cDNAs and attempt to isolate a complementing cDNA. In addition, genomic DNA from *Arabidopsis thaliana* was probed with various yeast DNA repair genes with the hope of detecting an *Arabidopsis* DNA repair gene with DNA homology. Any yeast DNA repair genes that bound to the genomic DNA from *Arabidopsis* was then used to probe an *Arabidopsis* cDNA library with the hope of isolating a cDNA that is an homologous *Arabidopsis* DNA repair gene.

2 MATERIALS AND METHODS

2.1 Chemicals and Media Components

All chemicals used were analytical reagent grade and were obtained commercially from Fisher Scientific, Mallinckrodt, Sigma, or Bethesda Research Laboratories (BRL). Media components such as yeast extract, tryptone, peptone, yeast nitrogen base w/o amino acids and agar were purchased from Canlab.

2.2 Yeast and Bacterial Strains

The complete genotypes of all strains used in this study are given in Table 2. Construction of MKP-o and KAM-1 (*rad1Δ::LEU2*) have been described by Pierce *et al.* 1987 and Kunz *et al.* 1990 respectively.

2.3 Plasmids and Phage Vectors

Table 3 lists the numerous plasmids and phage vectors used throughout this study.

2.4 *Arabidopsis thaliana* cDNA Library

The *Arabidopsis thaliana* cDNAs that were used in this study were carried on a unique vector called λYES (see Table 3). λYES (Figure 3) is a multifunctional vector having an insert capability of 8.4 kb that is capable of replicating as a lambda phage and as a plasmid lysogen in *E. coli,* and contains a plasmid component that when excised can replicate as a centromere plasmid in yeast (Elledge *et al.* 1991). The cDNA-containing plasmid component of the vector can be excised from the lambda phage by site-specific recombination between the *lox* sites in the vector using the Cre protein. The cDNAs are inserted in both direction. In one, they are expressed from the *E. coli lacI* promoter and in

Table 2. Yeast and Bacterial Strains

Strain	Genotype	Reference/Source
Yeast Strains		
MKP-o	*MATa, can1-100, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901*	Pierce *et al.* 1987
KAM-1	as for MKP-o but *rad1Δ::LEU2*	Kunz *et al.* 1990a
SX46	*MATa, rad2, ade2, ura3-52, trp1-289, his3-832*	E.C. Friedberg
BCRS2-51	as for MKP-o but *rad2Δ::URA3*	this study
LN116	*MATα, rad3-2, ade2-101, his3Δ, lys2-1, trp1Δ, ura3-52*	E.C. Friedberg
WS3-1	as for MKP-o but *rad3-1::TRP1*	W. Siede
WS8104-2B	*MATα, rad4-4, ade2-1, ura3-52, trp1-289*	Fleer *et al.* 1987
BCRAD4Δ	as for MKP-o but *rad4Δ::hisG*	this study
334	*MATα, pep4-3, prbl-112, ura 3-52, leu2-3,112, reg1-501, gal1*	Hovland *et al.* 1989

Table 2. Continued

Strain	Genotype	Reference/Source
Bacteria Strains		
JF1754	*Δlac, gal, metB, leuB, hisB436, hsdR*	Pierce *et al.* 1987
LE392	*hsdR514, supE44, supF58, lacY1* or *Δ(lacIZY)6, galK2, galT22, metB1, trpR55, mcrA*	N. Crawford
LE392/pMC9	LE392 containing pMC9	Elledge *et al.* 1991
JM107	*endA1, gyr96, thi, hsdR17, supE44, relA1, Δ(lac-proAB), (F', traD36, proAB', lacI^{q}ZΔM15)*	Elledge *et al.* 1991
BNN132	JM107 lysogenized with λKC that expresses *cre*, *neo* and *kan*	Elledge *et al.* 1991

Table 3. Plasmids and Phage Vectors Used In This Study

Name	Relevant Genotype	Reference/Source
Plasmids		
pNF1000	*RAD1*, *URA3*[a], *bla*[b]	Yang and Friedberg 1984, Reynolds *et. al.* 1987
pNF2005	*RAD2*, *URA3*, *bla*	Nicolet *et al.* 1985
pDG255	*RAD3*, *URA3*, *bla*	R.D. Gietz
pDG7	*RAD4*, *URA3*, *bla*	Gietz and Prakash 1988/ R.D. Gietz
pNF102	*RAD10*, *URA3*, *bla*	Reynolds *et al.* 1985b, Weiss and Friedberg 1985
pFF11.56	*RAD18*, *URA3*, *bla*, *ARS1*[c], *CEN4*[d]	Fabre *et al.* 1989
pNKY74	*RAD50*, *URA3*, *bla*	Alani *et al.* 1989
YEp13RAD51-23	*RAD51*, *LEU2*[a], *bla*	Calderone *et al.* 1983, Shinohara *et al.* 1992
pSM13	*RAD52*, *bla*	B. A. Kunz (personal communication)
YCpRAD52-111	*RAD52*, *LEU2*, *bla*, *lacZ*	Adzuma *et al.* 1984
YEp13RAD54-216A	*RAD54*, *LEU2*, *bla*	Emery *et al.* 1991, Calderone *et al.* 1983
YEp13RAD55-13C	*RAD55*, *LEU2*, *bla*	Lovett and Mortimer 1987, Calderone *et.al.* 1983
pFL41	*REV1*, *URA3*, *bla*, *ARS1*, *CEN4*	Larimer *et al.* 1989
pWS301/C1	*REV2*, *URA3*, *bla*, *ARS1*, *CEN4*	Ahne *et al.* 1992
pJA6	*REV3*, *URA3*, *bla*, *ARS1*, *CEN4*	Morrison *et al.* 1989

Table 3. Continued

Name	Genotype	Reference/Source
pBL304	*Pol III, LEU2, bla*	Boulet *et al.* 1989
pKM55	*rad2Δ::URA3, lacI, lacZ,bla*	K. Madura/ R.D. Gietz
pDG38	*rad4Δ::URA3, lacI, lacZ, bla, hisG*	Gietz and Prakash 1988/ R.D. Gietz
pMC9	*bla, lacI*	Elledge *et al.* 1991
pNKY51	*bla, URA3, hisG*	Alani *et al.* 1987
Phage		
λYES cDNA library	*URA3, bla, ARS1,CEN4*	Elledge *et al.* 1991; N. Crawford
λYES	*URA3, bla, ARS1,CEN4*	Elledge *et al.* 1991/R.D. Gietz

[a] Selectable markers in yeast

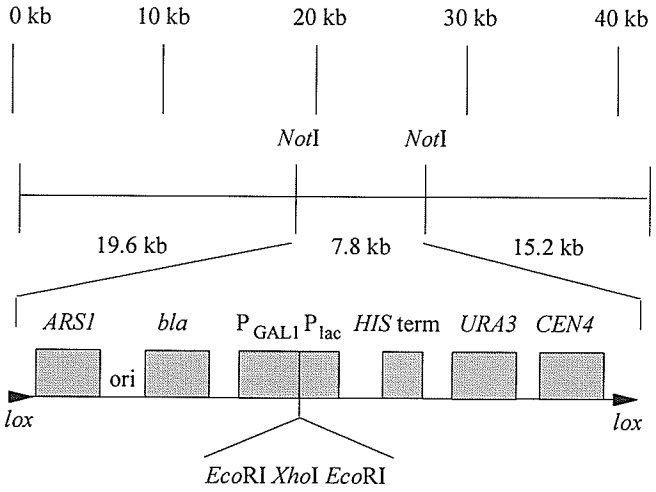
[b] β-lactamase gene conferring ampicillin resistance and used as a selectable marker in *E. coli.*

[c] Yeast replication origin

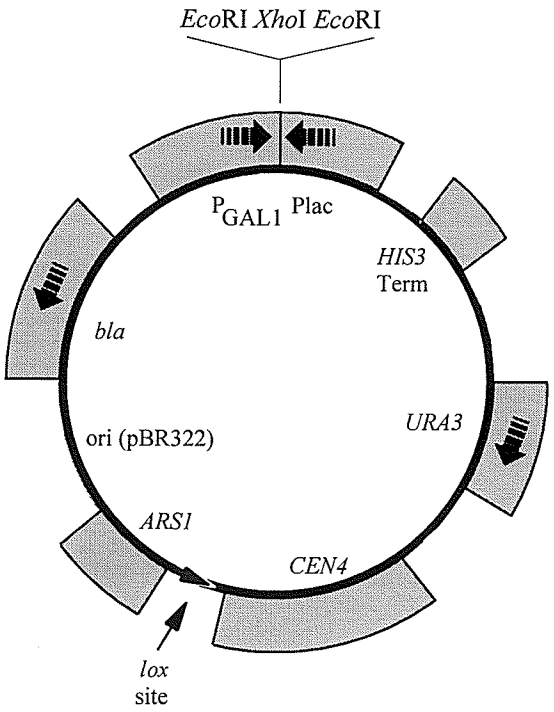
[d] Yeast centromere sequence

Figure 3. λYES Vector. The figure illustrates the λYES vector that was used to carry the *Arabidopsis thaliana* cDNAs. The linear phage vector is shown in A with the plasmid component of this vector shown in detail underneath, between the *Not*I restriction sites. Upon infection into a bacterial strain that produce the Cre protein, the plasmid portion of this vector is excised via site-specific recombination involving the Cre protein and the *lox* sites in the vector. The excised vector is shown in B. The cDNAs are located non-directionally at the *Xho*I site in between the *GAL1* and the *lac* promoters (modified from Elledge *et al.* 1991).

A


0 kb
10 kb
20 kb
30 kb
40 kb
NotI
NotI
19.6 kb
7.8 kb
15.2 kb
ARS1
bla
P GAL1 P lac
HIS term
URA3
CEN4
ori
lox
lox
EcoRI XhoI EcoRI

B


EcoRI XhoI EcoRI
P GAL1 Plac
HIS3
Term
bla
ori (pBR322)
URA3
ARS1
CEN4
lox
site

the other they are expressed from the yeast *GAL1* promoter. The selectable markers are *bla* (encoding the enzyme β-lactamase) in *E. coli* and *URA3* (encoding orotidine-5'-phosphate decarboxylase) in yeast. The cDNAs are inserted into a *Xho*I site flanked by *Eco*RI sites. The *Xho*I sites should be regenerated during the cloning process which allows for the isolation of the insert by digestion with this enzyme. However, sometimes these *Xho*I sites are not present and the insert can then be excised by *Eco*RI.

2.5 Media

2.5.1 Yeast Growth Media

A. SD (Synthetic Dextrose) (Sherman *et al.* 1983):
per litre: 40 g dextrose
6.7 g Bacto yeast nitrogen base w/o amino acids (Difco)

Necessary nutrients were added at the concentrations suggested by Sherman *et al.* (1983). When required, galactose was substituted for dextrose (same concentration) and the medium was then designated SG (synthetic galactose). Medium lacking uracil was designated SD-ura and SG-ura.

B. YPD (Yeast Peptone Dextrose) (Sherman *et al.* 1983)
per litre: 20 g dextrose
20 g Bacto peptone (Difco)
10 g Bacto yeast extract (Difco)

For yeast transformation, 10 mg adenine sulfate was added per litre of YPD and the medium was designated YPDA.

2.5.2 *E. coli* Growth Medium

C. YT (Yeast Tryptone) (Miller 1972)
per litre: 8 g Bacto tryptone (Difco)
5 g Bacto yeast extract (Difco)
5 g sodium chloride

When required, ampicillin (100 mg/L) was added after autoclaving and cooling of the medium to 45°C. The medium was then designated YT+amp. Medium containing twice the concentration of the YT components was designated 2x YT and was used for bacterial transformation.

D. LB (Luria Broth) (Sambrook *et al.* 1989)

per litre: 10 g Bacto tryptone (Difco)
10 g Bacto yeast extract (Difco)
5 g sodium chloride

When required, ampicillin (100 mg/L) was added after autoclaving and cooling of the medium to 45°C. The medium was then designated LB+amp.

E. For solid media, 20 g/L agar was added.

F. Terrific Broth (Sambrook *et al.* 1989)

per 900 mL: 12 g Bacto tryptone (Difco)
24 g Bacto yeast extract (Difco)
4 mL glycerol

0.17 M KH_2PO_4, 0.72 M K_2HPO_4:

per 100 mL: 2.31 g KH_2PO_4
12.54 g K_2HPO_4

After sterilization by autoclaving and allowing the broth and the solution to cool to 60°C, the 100 mL of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 was added to the broth.

2.6 DNA Isolation

2.6.1 Large Scale Yeast DNA Preparation

SCE buffer: 770 mM sorbitol
100 mM sodium citrate
60 mM Na_2EDTA, pH 8.0

Zymolyase: Zymolyase 100,000 (Seikagaku Kogyo) was dissolved at a concentration of 6 mg/mL in SCE buffer.

TE buffer: 50 mM Tris, pH 7.5
20 mM Na_2EDTA, pH 8.0

RNase: 1 mg/mL RNase was dissolved in 5 mM Tris (pH 8.0), 4,000 U/mL RNase T1 was added and the mixture was heated to 100°C for 10 min and cooled slowly to room temperature. Aliquots (100 μL) were stored at -20°C.

Potassium acetate: 5 M potassium acetate was adjusted to pH 4.8 with glacial acetic acid.

Total yeast DNA used for Southern dried gel analysis was isolated by a modification of a procedure described by Sherman *et al.* (1983). Yeast was grown in 50 mL YPD to stationary phase (1-2 x 10^8 cells/mL) and the cells were pelleted by centrifugation (3,020 x g, 5 min, room temperature) and washed with and resuspended in 3 mL SCE buffer. Zymolyase (250 μL) was added and the cell suspension was incubated for 45 min at 37°C with gentle shaking. The resulting spheroplasts were pelleted by centrifugation (755 x g, 5 min, room temperature) and resuspended in 5 mL TE buffer. Sodium dodecyl sulfate (SDS, 500 μL, 10%, w/v) was added. The contents were mixed gently by inversion and the tube was incubated for 30 min at 65°C. Potassium acetate, 1.5 mL, 5M, was added and the suspension was chilled on ice for 30 min. The precipitate was pelleted by centrifugation (34,800 x g, 20 min, 4°C), the supernatant was transferred to a fresh tube, and 14 mL of ice-cold ethanol (95%) was added. The precipitated nucleic acids were pelleted by centrifugation (3,020 x g, 5 min, 4°C), and the pellet was dried and dissolved in 3 mL TE buffer. RNase (150 μL) was added and the suspension was incubated for 30 min at 37°C with shaking. Then, 3 mL isopropanol (room temperature) was added, the solution was mixed and the DNA pelleted by centrifugation (1,085 x g, 4 min, 4°C). The DNA pellet was dried, dissolved in 300 μL TE buffer, transferred to a 1.5 mL microfuge tube and stored at -20°C.

2.6.2 Rapid Alkaline Procedure for Plasmid DNA Isolation From *E. coli* I

GTE buffer: 50 mM glucose
25 mM Tris, pH 8.0
10 mM Na_2EDTA, pH 8.0

NaOH/SDS:	200 mM NaOH 1% (w/v) SDS prepared just prior to use.
Ammonium acetate:	7.5 M ammonium acetate was dissolved in water while being heated to 65°C and was titrated with glacial acetic acid to achieve a pH of 5.8.
TE buffer:	See section 2.6.1

To quickly isolate a large number of plasmid DNA samples, a modification of the procedure of Morelle (1989) was used. Bacteria were grown overnight in 5 mL YT+amp at 37°C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 1 mL of GTE buffer and transferred into a microfuge tube. Cells were pelleted by centrifugation (Brinkman 5415C, 30 sec., room temperature), and resuspended in 190 µL GTE buffer. The cell suspension was mixed gently and then transferred to ice. The NaOH/SDS solution (400 µL) was added drop-wise and the mixture incubated on ice for 5 min, after which the ammonium acetate (300 µL) was added drop-wise and the mixture incubated on ice for 10 min. The precipitate was pelleted by centrifugation (Brinkman 5415C, 30 min, 4°C) and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated twice more, but the centrifugation times were reduced to 20 min. Then, 500 µL isopropanol (room temperature) was added, the contents were mixed by inversion and the tube was held at room temperature for 10 min. Following centrifugation (Brinkman 5415C, 5 min, room temperature) the nucleic acid pellet was washed with 70% ice-cold ethanol, dried under vacuum, and dissolved in 70 µL TE buffer. DNA samples were stored at -20°C.

2.6.3 Rapid Alkaline Procedure for Plasmid DNA Isolation From *E. coli* II

GTE buffer:	See Section 2.6.2
NaOH/SDS:	See Section 2.6.2
Neutralizing solution:	1.25 M potassium acetate was dissolved in water and titrated with glacial acetic acid to achieve a pH of 5.2.

Partway through these studies a more rapid procedure was used for isolating plasmid DNA that was not going to be sequenced (Birnboim and Doly 1979 as modified by K. Ramachandran, personal communication). Bacteria were grown overnight in 5 mL YT+amp at 37°C with shaking and the cells were collected by centrifugation (1,850 x g, 10 min, room temperature), resuspended in 1 mL GTE buffer and transferred to a microfuge tube. Cells were centrifuged again (Brinkman 5415C, 30 sec, room temperature) and resuspended in 200 μL GTE buffer. Lysis solution (200 μL) was added to the microfuge tube and the contents were thoroughly mixed by inversion. Neutralizing solution (200 μL) was then added and once again the contents were mixed by inverting the tube several times. The precipitate was pelleted by centrifugation (Brinkman 5415C, 10 min, room temperature) and the supernatant was transferred to a fresh microfuge tube. This step was repeated once or twice more until either there was no longer a pellet or the pellet was very small. Then, isopropanol was added (500 μL, room temperature) the contents of the tube were mixed by inversion, and the tube was incubated at room temperature for 10 min. The nucleic acid was pelleted by centrifugation (Brinkman 5415C, 2 min, room temperature) washed with 1 mL ice cold 70% ethanol and dried under vacuum. It was finally resuspended in 50 μL TE buffer and stored at -20°C.

2.6.4 Large Scale Preparation of Plasmid DNA

STE buffer:	100 mM sodium chloride 10 mM Tris HCl, pH 8.0 1 mM Na_2EDTA, pH 8.0
GTE buffer:	See section 2.6.2
Lysozyme	25 mg/mL dissolved in GTE buffer (Boehringer Manheim).
NaOH/SDS:	See section 2.6.2
Potassium acetate:	See section 2.6.1
Ammonium acetate:	10 mM ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C. The final pH of 7.8.
PEG:	1.3% (w/v) polyethylene $glycol_{8000}$ (Sigma) was dissolved in 1.6 M sodium chloride solution and filter-sterilized just prior to use.
TE buffer	See section 2.6.1
RNase	See section 2.6.1

Double-stranded plasmid DNA used for probes was prepared by a scaled up version of the alkaline procedure by Birnboim and Doly (1979). Bacterial cultures were grown overnight in 5 mL YT+amp at 37°C with shaking. Two mL of overnight culture was transferred to 500 mL YT+amp and grown overnight at 37°C with shaking. Cells were pelleted by centrifugation (2,000 x g, 15 min, 4°C), resuspended in 100 mL of ice-cold STE buffer, pelleted again, and resuspended in 18 mL GTE buffer. Lysozyme (2 mL) was added and the cell suspension incubated at room temperature for 10 min and then chilled on ice for 10 min. The NaOH/SDS (40 mL) was added and the mixture was

incubated on ice for 10 min. Potassium acetate (20 mL) was added and the contents were mixed by inversion and incubated on ice for 10 min. The precipitate was then pelleted by centrifugation (5,000 x g, 20 min, 4°C) and the supernatant filtered through 5 double layers of cheesecloth and transferred to a fresh centrifuge tube. The nucleic acids were precipitated by adding 50 mL of isopropanol (room temperature) and incubating at room temperature for 15 min. The precipitate was pelleted by centrifugation (4,080 x g, 15 min, room temperature), washed with 70% ethanol (room temperature), air dried and dissolved in 3 mL TE buffer. The RNA was removed by precipitation with 3 mL ice-cold 5 M lithium chloride followed by centrifugation (12,100 x g, 4°C). The supernatant was transferred to a fresh tube and 6 mL of isopropanol (room temperature) was added. The tube was incubated at room temperature for 10 min and the resulting precipitate was pelleted by centrifugation (12,100 x g, 10 min, room temperature). The pellet was washed with 70% ethanol (room temperature), dried under vacuum, dissolved in 500 μL TE buffer and transferred to a microfuge tube. The RNase (4 μL) was added and the tube was incubated at 37°C for 30 min. The DNA was precipitated with the PEG solution, pelleted by centrifugation (Brinkman 5415C, 5 min, 4°C), the supernatant was discarded and the pellet was dissolved in 570 μL TE buffer. The nucleic acids were extracted once with 570 μL TE-saturated phenol, once with 540 μL TE-saturated phenol-chloroform (1:1) and once with 500 μL TE-saturated chloroform. After each extraction, the samples were centrifuged (Brinkman 5415C, 2 min, room temperature) and the upper aqueous layer was transferred to a fresh microfuge tube. Following the last extraction, one-third the volume of ammonium acetate and twice the volume of 95% ethanol were added, the

contents were mixed by inversion and incubated at room temperature for 10 min. The precipitate was pelleted by centrifugation (Brinkman 5415C, 5 min, 4°C). The pellet was washed with 1 mL 70% ice-cold ethanol, dried under vacuum and dissolved in 500 µL TE buffer. DNA samples were stored at -20°C.

2.6.5 Isolation of Genomic DNA from *Arabidopsis thaliana*

Extraction buffer:	100 mM Tris pH 8.0 50 mM Na_2EDTA 500 mM sodium chloride 10 mM β-mercaptoethanol
Potassium acetate:	See Section 2.6.1
Sodium acetate:	3 M sodium acetate was titrated to pH 4.8 with glacial acetic acid.
TE-X buffer:	50 mM Tris pH 8.0 10 mM Na_2EDTA pH 8.0
RNase:	See Section 2.6.1
TE buffer:	See Section 2.6.1

Total genomic DNA from *Arabidopsis thaliana* that was used for Southern dried gel analysis was isolated by a modification of a protocol described by Dellaporta *et al.* (1983). 0.8 g of plant tissue was frozen in liquid nitrogen and ground to a fine powder. Equal quantities of this powder were transferred to a 1.5 mL microfuge tube containing 485 µL of extraction buffer and 35 µL of SDS (20%, w/v). Care was taken to keep the tissue frozen before it was placed into the extraction buffer. All materials used for handling and grinding of the plant tissue were precooled with liquid nitrogen. After the ground tissue was placed into the microfuge tube containing the extraction buffer, the tube was vigorously mixed on a vortex for 1 min and placed at 65°C for 10 min. The potassium acetate (161 µL) was added and the solution mixed well by shaking

vigorously. The microfuge tube was incubated on ice for 20 min, then centrifuged (Brinkman 5415C, 15 min, 4°C) and the supernatant transferred to a fresh tube. This was repeated once with the centrifugation time decreased to 5 min. Ice cold isopropanol (325μL) was added, the contents of the tube mixed well by inversion and the tube incubated at -20°C for 30 min. The tube was then centrifuged (Brinkman 5415C,1.5 min, 4°C) and the nucleic acid pellet was dried under vacuum. The pellet was dissolved in 700 μL of TE-X buffer and 10 μL of RNase was added. The tube was incubated at 37°C for 30 min. The nucleic acids were then extracted with 700 μL of TE-X-saturated phenol, and 700 μL of TE-X-saturated chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated by the addition of 75 μL of sodium acetate and 500 μL of isopropanol (room temperature). The microfuge tubes were placed at -20°C for 30 min and the precipitate was collected by centrifugation (Brinkman 5415C, 3 min, 4°C) and dried under vacuum. The DNA was dissolved in 100 μL of TE buffer and stored at -20°C.

2.6.6 Preparation of Bacterial Nucleic Acids (RNA plus DNA)

STE buffer:	See section 2.6.4
NaOH/SDS:	See section 2.6.2
Potassium acetate:	See section 2.6.1

Bacterial RNA plus DNA was prepared for use as a carrier in yeast transformations by modifying the latter half of the protocol described in section 2.6.4 Nucleic acids were prepared through the first isopropanol precipitation and 70% ethanol wash. Following the wash, the pellet was dried, dissolved in 6 mL TE buffer and transferred by pipette to a 30 mL sterile Corex tube. The nucleic acids were then

extracted once with TE-saturated phenol, once with 6 mL TE-saturated phenol:chloroform (1:1) and once with 6 mL TE-saturated chloroform. After each extraction, approximately 5 mL of the top aqueous layer was transferred to a sterile 15 mL Corex tube. Next, 6 mL of isopropanol was added to precipitate the nucleic acids. The contents of the tube were mixed well by inversion and the tube was incubated at room temperature for 10 min. The nucleic acids were then pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C), washed with 1 mL ice-cold 70% ethanol, dried under vacuum, dissolved in 500 µL TE buffer and stored at -20°C.

2.7 Transformation Procedures

2.7.1 Bacterial Transformation I

Buffer A: 100 mM sodium chloride
5 mM Tris, pH 7.5
5 mM $MgCl_2$

Buffer B: 100 mM $CaCl_2$
5 mM Tris, pH 7.5
5 mM $MgCl_2$

Bacterial cells were transformed using a modification (Pierce *et al.* 1987) of the calcium chloride procedure of Mandel and Higa (1970). *E.coli* strains were grown overnight in 5 mL YT. The cells were then diluted 1:100 in YT (40 mL of YT for every 10 transformations), grown for 1 h 45 min at 37°C with shaking ($O.D._{600}$ = 0.6). The culture was then incubated on ice for 10 min. The cells were collected by centrifugation (3,020 x g, 10 min, 4°C) and, for every 40 mL of culture, the pellet was washed and resuspended in 10 mL of buffer A and then incubated on ice for 20 min. Next, the cells were collected by centrifugation (3,202 x g, 10 min, 4°C) and for every 40 mL of original

culture, resuspended in 10 mL of buffer B, incubated on ice for 1 h, pelleted by centrifugation (3,020 x g, 10 min, 4°C) and resuspended in 2 mL of the same solution. For each transformation, 200 µL of the cell suspension was transferred to a sterile microfuge tube containing ca. 0.5-1.0 µg plasmid DNA that was isolated previously (see sections 2.6.2 or 2.6.3). The tube was incubated on ice for 1 h, heat shocked at 42°C for 2 min and immediately placed back on ice for 2 min. 2x YT (200 µL) was added to each tube which was then incubated at 37°C for 1 h. The cell suspensions were then plated on YT+amp medium and incubated overnight at 37°C.

2.7.2 Bacterial Transformation II

Transformation LB:

per litre:		
	10 g	Bacto tryptone (Difco)
	5 g	Bacto yeast extract (Difco)
	10 g	sodium chloride
	10 mM	$MgSO_4 \cdot 7H_2O$
	0.2% (v/v)	glucose

Storage LB:

per 100 mL:		
	1 g	Bacto tryptone (Difco)
	0.5 g	Bacto yeast extract (Difco)
	1 g	sodium chloride
	36 mL	glycerol
	12 g	polyethylene $glycol_{8000}$ (Sigma)
	1.2 mM	$MgSO_4 \cdot 7H_2O$

Partway through the experiments the transformation procedure of Nishimura *et al.* (1990) was used. *E. coli* strains were grown overnight at 37°C with shaking in 5 mL of LB broth. The cells were then diluted 1:100 in Transformation LB (50 mL of Transformation LB for every 30 transformations), grown for 1 h 45 min at 37°C with shaking ($O.D._{460}$= 0.3-0.4) and then chilled on ice for 10 min. The cells were then

collected by centrifugation (3,020 x g, 10 min, 4°C) and, for every 50 mL of culture, the cells were gently resuspended in 0.5 mL of ice cold Transformation LB. Once the cells were fully resuspended, 2.5 mL of ice cold Storage LB was added to every 50 mL culture and the contents were gently mixed. At this point, the competent cells could either be used immediately for transformation or they could be stored in 100 µL aliquots at -60°C for future transformations. To use these frozen competent cells, they were first allowed to slowly thaw on ice. For each transformation, a 100 µL aliquot of the cell suspension was added to an ice cold sterile microfuge tube containing ca. 0.5-1.0 µg of plasmid DNA. The DNA and cell suspension was mixed well by inversion and placed on ice for 30 min. Following incubation on ice the microfuge tube was heated at 42°C for 2 min and then chilled on ice for 2 min. Transformation LB (300 µL, room temperature) was added to each tube, which was then placed at 37°C for 1 h. The cell-DNA mixtures were then plated onto LB+amp medium and incubated overnight at 37°C.

2.7.3 Yeast Transformation

TE buffer:	Section 2.6.1
PEG:	44% (w/v) polyethylene $glycol_{3350}$ (Sigma) was dissolved in 100 mM lithium acetate and filter-sterilized just prior to use.
Lithium acetate:	100 mM dissolved in TE buffer.

Yeast cells were transformed using the lithium acetate procedure described by Ito *et al.* (1983) as modified by Schiestl and Gietz (1989) and Gietz *et al.* (1992). YPDA (100 mL) was inoculated with stationary phase yeast cells and the culture was incubated with shaking at 30°C overnight until the cell titre reached 1 x 10^7 cells/mL as determined

using a Coulter counter. The culture was centrifuged (4,080 x g, 5 min, room temperature) to pellet the cells. The cell pellet was washed twice in 5 mL TE buffer and resuspended in 1 mL lithium acetate. The suspension was then incubated at room temperature for 5 min. For each transformation, a 100 μL aliquot of cell suspension was transferred to a sterile 13 mm plastic tube (Sarstedt). The DNA to be transformed (1 μg) was added to the tube and 20 μL of bacterial nucleic acids (3.5 μg/μL) (see section 2.6.6) was added as a carrier and the contents of the tube were mixed gently by tapping. PEG (440 μL) was added and the tube was incubated at room temperature for 30 min. Dimethyl sulfoxide (final concentration 10% v/v) was added and the tube was heated to 42°C for 15 min. The cells were then pelleted by centrifugation (1,850 x g, 5 min, room temperature) and washed twice with sterile water and resuspended in 1 mL of the same. Aliquots (0.2 mL) of the cell suspension were plated on appropriately supplemented minimal medium to select for transformants and the plates were incubated at 30°C for up to 6 days. Individual transformants were purified by streaking single colonies onto the same medium and incubating the plates for 2-3 days at 30°C. The phenotypes of the transformants were then characterized by testing for growth on appropriately supplemented media.

2.8 Lambda Library Screening

2.8.1 Lambda Plaque Plating

SM Buffer:

per litre:
- 5.8 g sodium chloride
- 2 g $MgSO_4 \cdot 7H_2O$
- 50 mM Tris (pH 7.5)
- 0.1 g gelatin

The buffer was adjusted to pH 7.5 with 10N NaOH.

LB top agarose:

per litre: 10 g Bacto tryptone (Difco)
10 g Bacto yeast extract (Difco)
5 g sodium chloride

The above ingredients were dissolved and agarose was added so that the final concentration was 0.7% (w/v). The mixture was heated to boiling to dissolve the agarose and was aliquoted prior to sterilization by autoclaving.

LE392/pMC9 was inoculated into 5 mL LB+amp and grown overnight at 37°C, without shaking, until the titre reached ca. $1x10^8$ cells/mL. The following morning, the cells were vigorously shaken at 37°C for 30 min to ensure that all the cells were in log phase. The cells were then collected by centrifugation (1,850 x g, 10 min, room temperature) and resuspended in 2.5 mL 10 mM $MgSO_4$. The lambda library was titred to be approximately $1x10^8$ pfu/mL. The library was diluted with SM Buffer which would result in approximately 2,000-6,000 plaques per 150 mm diameter plate for the primary screening and 200-300 plaques and 50-100 plaques per 100 mm diameter plate for the secondary and tertiary screenings respectively. For the primary screening, 600 μL of cells were added to 50 μL of diluted phage in a microfuge tube, whereas for the secondary screening, 100 μL of cells were added to 100 μL of diluted phage. The cell-phage solution was mixed gently and placed at 37°C for 15 min. Following the 15 min incubation, the cell-phage solution was added to LB top agarose (10 mL for the large plates and 5 mL for the smaller plates) and poured onto LB+amp medium. After the top agarose had solidified the plates were then incubated upside down at 37°C for 8-10 h. The plates were then placed at 4°C for at least 6 h prior to plaque lifts.

2.8.2 Lambda Plaque Lifts

20x SSC:	3 M sodium chloride 0.3 M sodium citrate Solution was adjusted to pH 7.0 with 10 N NaOH.
Denaturing solution:	1.5 M sodium chloride 0.5 M NaOH
Neutralizing solution:	1.5 M sodium chloride 0.5 M TrisHCL, pH 7.2 0.001 M Na_2EDTA, pH 8.0

For lambda plaque hybridizations using DNA probes, lifts were done of the freshly formed plaques. The 132 mm diameter nylon membrane was used for the primary screening and the 82 mm diameter nylon membrane was used for the secondary and tertiary screenings. The membranes were first marked by cutting out three asymmetric notches before being placed on the plaque-containing top agarose. The location of the notches were transferred to the plate by marking the agar with a sterile needle at the apex of each of the notches. This was necessary to ensure proper orientation of the plaques and the positive plaques could then be identified after hybridization. After approximately 1 min, the membrane was removed and placed, plaque side up, on a sheet of 3MM Whatman chromatography paper soaked with denaturing solution. A replica membrane was made for each plate but this second membrane was left on the agar plate for 4 min prior to its removal onto the denaturing solution soaked chromatography paper. The membranes were incubated for 7 min then transferred to another sheet of chromatography paper soaked with neutralizing solution and incubated for 3 min. They were then transferred to another sheet of chromatography paper soaked in neutralizing solution for an additional 3 more min after which the filters were washed briefly in 2x SSC. The

membranes were then placed on dry paper towels and allowed to air dry. The DNA was crosslinked by placing the membranes plaque side down on a standard UV transilluminator (254 nm) for 3 min to covalently link the DNA onto the membrane. After UV irridiation of the membranes, they were wrapped in one layer of Saran Wrap (Dupont) and stored under vacuum at room temperature until they were used for hybridization.

2.8.3 Lambda Plaque Hybridizations

Salmon sperm DNA: Salmon sperm DNA (Sigma) was dissolved in water (5 mg/mL) and then pressed through a fine syringe several times to shear the DNA. Aliquots were stored at -20°C. The salmon sperm DNA was heated at 100°C for 10 min then cooled in ice-water prior to use in order to denature the DNA.

Prehybridization Solution:
6x SSC
5x Denhardt's solution
0.5% (w/v) SDS
0.02 mg/mL salmon sperm DNA (See section 2.10.2)
Prepared just prior to use and heated to 65°C.

Solution I:
2x SSC

Solution II:
2x SSC
0.1% (w/v) SDS

Solution III:
0.1x SSC
0.1% (w/v) SDS

Plaque lifts of the lambda library was screened following the procedures in the Amersham International instruction manual. Duplicate filters from each plate were placed in a glass hybridization bottle (Bio/Can Scientific), 35 mm x 150 mm for the 82 mm diameter membranes and 35 mm x 300 mm for the 132 mm diameter membranes. When

necessary, nylon mesh was used to prevent membrane overlap. The prehybridization solution (20 mL or 30 mL for the small or large glass bottles respectively) was added and the bottle was sealed and incubated at 65°C in a Bio/Can Scientific Turbo Speed Rotary Hybridization Oven. After 10 min, the bottle was opened to release the built-up pressure, closed, and incubated in the oven for at least 5 h at 65°C. The radio-labelled DNA probe (100μL) was added and the bottle was sealed and incubated overnight at 65°C. The membranes were then subjected to the following washes:

1. 40 mL of solution I for 15 min at 65°C (twice).
2. 40 mL of solution II for 30 min at 65°C.
3. 40 mL of solution III for 15 min (washed once or twice depending on the background count).

The membranes were then individually wrapped in a single layer of Saran Wrap (Dupont) while still wet and exposed to Kodak XAR-5 film with an intensifying screen at -60°C (the exposure time varied according to the radioactive intensity of the membranes).

2.8.4 Lambda Plaque Rescue

Positive plaques were isolated by using sterile Pasteur pipettes. A plug of agar containing the positive plaque was isolated by placing the Pasteur pipette overtop of the positive plaque and gently pushing down through the agar to the bottom of the plate. The agar plug was then placed in a 1.5 mL microfuge tube under 500 μL of SM buffer containing 30 μL of chloroform to inhibit growth of any remaining bacteria. The microfuge tube was stored at 4°C for at least 24 h to allow the phage to diffuse into the buffer. This was then used for either further screenings or for infection into the bacterial

strain used to promote the excision of the plasmid component of the library containing the *Arabidopsis* cDNA.

2.8.5 Excision of Plasmid λYES From Positive Plaques and Plasmid Isolation

The plasmid containing part of the λYES vector was excised using the protocol described by Elledge *et al.* (1991). BNN132 was inoculated into 5 mL of LB and grown overnight without shaking at 30°C until the titre reached ca. 1 x 10^8 cells/mL. The following morning the culture was vigorously shaken at 30°C for 30 min to ensure the cells were in log phase. The cells were then collected by centrifugation (1,850 x g, 10 min, room temperature) and resuspended in 2.5 mL of 10 mM $MgCl_2$. Phage dilutions ranging from 1 x 10^{-1} to 1 x 10^{-5} were made using SM buffer. 100 μL of each dilution was added to 100 μL of cells in a 1.5 mL microfuge tube and the solution mixed by inverting several times. This mixture was then incubated at 30°C, without shaking, for 30 min. LB broth (200 μL) was then added and the microfuge tubes were further incubated at 30°C for 1 hour with gentle shaking. These cells were then plated onto 100 mm LB+amp (50 μg/mL) plates containing 0.1% glucose and incubated overnight at 37°C. Ampicillin resistant colonies were then inoculated into 5 mL terrific broth with ampicillin (50 μg/mL) and grown to stationary phase at 37°C with shaking. The excised plasmid was then isolated using a modification of the alkaline method described in section 2.6.2. To ensure lysis of the bacteria the cells were resuspended in 180 μL of GTE buffer (rather than 190 μL as stated in section 2.6.2) and 10 μL of lysozyme (see section 2.6.4) was added. The contents were then gently mixed by inversion 2-3 times and the tubes were

held at room temperature for 5 min prior to proceeding with the rest of the alkaline isolation procedure.

2.9 Preparation of DNA Fragments for Strain Construction and Hybridizations

2.9.1 Restriction Digests-General Procedure

Enzyme digest:	1x restriction enzyme buffer (10x buffer supplied by BRL) 1 μg DNA 50 units of enzyme per μg of DNA
Stop buffer:	50 mM Na_2EDTA 50% (w/v) sucrose 0.1% (w/v) bromophenol blue Stored at 4°C.
λ DNA	*Hind*III digested λ DNA, 500 mg/mL (BRL). The DNA was stored at 4°C and heated at 65°C for 5 min before use.
Loening's buffer:	400 mM Tris 200 mM sodium acetate 10 mM Na_2EDTA Solution was adjusted to pH 8.0 with glacial acetic acid.

DNA fragments used as probes for Southern dried gel or plaque hybridizations were obtained by purification from agarose gel electrophoresis after restriction enzyme-digestion. Plasmid DNA was digested with the appropriate enzyme for 2.5 h at 37°C, after which stop buffer (1/4 of the reaction volume) was added to terminate the reaction. The resulting DNA fragments were separated by agarose gel electrophoresis (0.7% (w/v) agarose (BRL) dissolved in Loening's buffer), for 18 h at 1 Volt/cm. The gel was stained with ethidium bromide (0.5 μg/mL) for 15 min and then destained for 20 min in distilled water.

2.9.2 Isolation of DNA Fragments

TE buffer: See section 2.6.1

Neutralizing solution: See section 2.6.3

DNA fragments were isolated from agarose gels by a freeze-squeeze method of Thuring *et al.* (1975). DNA fragments to be isolated were visualized on a UV transilluminator. Segments of the gels containing the desired DNA were cut out and transferred to microfuge tubes (500 μg/tube) and frozen at -20°C. The tubes were centrifuged (Brinkman 5415C, 1.5 h, 4°C) and the supernatant was transferred to a fresh microfuge tube. 1 mL of 2-butanol (room temperature) were added and the contents was mixed by inversion to reduce the amount of water to ca. 50%. The bottom aqueous layer was transferred to a fresh microfuge tube and extracted once with 200 μL TE-saturated chloroform to remove residual 2-butanol. To precipitate the DNA, 2.5 volumes ice-cold 95% ethanol were added, the contents of the tube were mixed by inversion, and the tube was incubated at -20°C overnight. Following centrifugation (Brinkman 5415C, 15 min, 4°C), the nucleic acid pellet was washed with ice-cold 70% ethanol, dried under vacuum and dissolved in TE buffer. DNA samples were stored at -20°C.

Partway through this study, another extraction method as described by Heery *et. al.* (1990) was used to purify the DNA fragments from the agarose gels. Glass wool was first coated with dimethyldichlorosilane prior to its use. This involved soaking the glass wool with enough dimethyldichlorosilane so that it was adequately covered. The glass wool was left to soak for 10-15 min, with occasional stirring, to ensure that all the glass wool was covered by the dimethyldichlorosilane. The solvent was removed and the glass

wool was washed several times with double distilled water. This silanised glass wool was now ready for use and was stored under double distilled water.

A small amount of silanised glass wool (ca. 2 mm in thickness) was packed into the bottom of a 0.5 mL microfuge tube with a 21 gauge needle hole in the bottom. This tube was then placed inside a decapitated 1.5 mL microfuge tube and the both tubes were centrifuged (Brinkman 5415C, 20 min, room temperature) to remove any loose bits of glass wool as well as any remaining liquid. The 0.5 mL microfuge tube was then placed into a fresh decapitated 1.5 mL microfuge tube and the agarose gel piece containing the DNA fragment to be isolated placed inside it. The microfuge tubes were then centrifuged (Brinkman 5415C, 30 min, room temperature) and the liquid was collected from the 1.5 mL microfuge into a fresh 1.5 mL microfuge tube. This centrifugation was repeated until no more liquid could be further extracted from the gel slice. The pooled liquid containing the DNA was either used immediately or concentrated by precipitation. To precipitate the DNA 1/3 the volume of neutralizing solution and 2.5 total volume of isopropanol (room temperature) was added to the collected liquid. The tube was then incubated at room temperature for 5 min. Following centrifugation (Brinkman 5415C, 2 min, room temperature) the DNA pellet was washed with ice-cold 70% ethanol, dried under vacuum and dissolved in 15-20 μL TE buffer. The DNA was then stored at -20°C.

2.9.3 Ligations Performed For Strain Constructions

Ligation of purified DNA fragment to plasmid vector DNA was performed at a molar ration of 3:1, insert to vector. The total amount of DNA in a ligation reaction never exceeded 1 μg.

2.9.3.1 Blunt Ending of Fragments For Ligation

10x Klenow Buffer:	0.5 M Tris, pH 7.6 0.1 M $MgCl_2$
Blunt Ending Reaction Mix:	1 mM dGTP (10 mM stock) 1 mM dCTP (10 mM stock) 1 mM TTP (10 mM stock) 1 mM dATP (10 mM stock) 1 x buffer (10x Klenow Buffer) 1 µl Klenow polymerase (3u/µl)

The treatment of DNA with Klenow polymerase to fill in the 5' overhang was performed in a total volume of 20 µL. The DNA fragments to be treated (ca. 1 µg) was gently mixed with the Blunt Ending Reaction Mix in a 1.5 mL microfuge tube. The tube was incubated at room temperature for 30 min. Klenow was then inactivated by placing the microfuge tube at 55°C for 20 min. These DNA fragments were now ready to be used for blunt end ligation.

2.9.3.2 Alkaline Phosphatase Reaction

Vector DNA was treated with alkaline phosphatase to remove the 5' phosphate residues which inhibits self ligation. The vector DNA was placed in a 1.5 mL microfuge tube and 1 µL (5-10 units) of calf intestinal phosphatase (CIP, Boehringer Manheim) was added along with enough GTE buffer so that the total volume was 19 µL. The contents were mixed and the tube was placed at 37°C for 15 min. Another 1 µL (5-10 units) of CIP was added to the reaction which was then placed at 55°C for 45 min. The CIP was then inactivated by placing the tube at 75°C for 20 min to denature the enzyme. The vector was then used for ligation.

2.9.3.3 Ligation Reaction

Ligation reactions were performed according to the instructions provided by BRL. Vector and insert DNA were combined with the ligase buffer (5 x, BRL) and the ligase (T4 ligase, BRL). The reaction was incubated at 15°C overnight. The ligation mixture was then transformed into the *E. coli* strain JF1754 as described in section 2.7.1 and 2.7.2.

2.10 DNA Hybridizations With Dried Agarose Gels

2.10.1 Agarose Gel Electrophoresis

Enzyme digest:	1x reaction buffer 3 or 1x reaction buffer 2 (10x buffer 3 or 2 supplied by BRL). 5.0 μg DNA 100 units of *Bam* HI (BRL) per μg of DNA
Stop buffer:	See section 2.9.1
λ DNA:	See section 2.9.1
Loening's buffer:	See section 2.9.1

Genomic DNA either from *S. cerevisiae* or *A. thaliana* was digested with *Bam*HI for 2.5 h. The reaction was terminated with stop buffer (1/4 of the total reaction volume) and the samples analyzed on 0.7% agarose gel electrophoresis (agarose (BRL) dissolved in Loening's buffer) at 1 V/cm (a λ DNA size standard was included). The gel was stained with ethidium bromide (0.5 μg/mL) for 10 min, destained for 20 min in distilled water and a picture was taken for later sizing of the bands. It was then soaked for 1 h each in 0.5 M NaOH and in 1 M Tris (pH 8.0) at room temperature with slow shaking. The gel was then sandwiched between 3MM Whatman chromatography paper and a single layer of Saran Wrap (Dupont), vacuum-dried at 60°C for 1 h (Tsao *et al.* 1983) and stored at

room temperature.

2.10.2 Hybridization Procedure

20x SSC:	See Section 2.8.2
50x Denhardt's solution:	10 mg/mL ficoll 10 mg/mL polyvinylpyrrolidone 10 mg/mL bovine serum albumin (BRL) Stored at -20° C.
Prehybridization solution:	6x SSC 5x Denhardt's solution 0.5% (w/v) SDS 0.2 mg/mL salmon sperm DNA (see section 2.8.3) Prepared just prior to use and heated to the desired temperature.
Hybridization solution:	10 mM Na_2EDTA 6x SSC 5x Denhardt's solution 0.5% (w/v) SDS Prepared just prior to use and heated to the desired temperature.
Solution I:	2x SSC 0.5% (w/v) SDS
Solution II:	2x SSC 0.1% (w/v) SDS
Solution III:	0.1x SSC 0.5% (w/v) SDS

DNA hybridization was carried out in agarose gels according to a modification of the procedures of Tsao *et al.* (1983), and Sambrook *et al.* (1989). *A. thaliana* genomic DNA was hybridized at the less stringent temperatures of 55°C and 42°C. All other direct DNA hybridizations in agarose gels were carried out at the temperature of 65°C. The dried gel was floated paper-side down on 2x SSC for 30 sec to allow the paper backing to be gently peeled off. The gel was then rolled into a tight spiral, using a nylon mesh to prevent overlap, and placed into a 35 mm x 150 mm glass hybridization bottle (Bio/Can

Scientific). The prehybridization solution (20 mL) was added and the bottle was sealed and incubated at the appropriate temperature in a Bio/Can Scientific Turbo Speed Rotary Hybridization Oven. The bottle top was opened after 10 min to release the pressure build up, the lid was retightened and the bottle was further incubated for 2-4 h. The prehybridization solution was replaced with hybridization solution (20 mL), the radio-labelled DNA probe (100 μL) was added and the bottle was sealed and incubated overnight at the specific temperature. The hybridization solution was removed from the bottle and the following washes were performed:

1. 50 mL of solution I for 5 min at room temperature (twice).

2. 50 mL of solution II for 15 min at room temperature (twice).

3. 50 mL of solution III for 2 h, 1 h, and 0.5 h at the hybridization temperature.

The gel was then transferred to 3MM Chr Whatman chromatography paper, air-dried, covered with a single layer of Saran Wrap and exposed to Kodak XAR-5. Depending on the radioactive signal from the dried gel, an intensifying screen was sometimes used. In these cases the film, along with the screen, was incubated at -60°C.

2.10.3 DNA Probe Production With Random Primers

The Random Primer Buffer Mixture, dNTPs, stop buffer, and DNA polymerase were supplied by BRL with the Random Primer Labelling Kit.

Random Primer Buffer Mixture:

0.6	M	HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethane sulfonic acid])
0.17	M	Tris-HCl
17	mM	$MgCl_2$
33	mM	β-mercaptoethanol
1.33	mg/mL	bovine serum albumin
18 OD_{260}	units/mL	oligodeoxyribonucleic acid primers (hexamer fraction), pH 6.8

dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA.
dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA.
dTTP solution: 0.5 mM dTTP in 3 mM Tris-HCl, pH 7.0, 0.2 mM Na_2EDTA.

Stop buffer: 0.2 mM Na_2EDTA, pH 7.5

Reaction mix: 15 μL Random Primer Buffer Mixture
2 μL dCTP
2 μL dGTP
2 μL dTTP
50 μCi [^{32}P] dATP
Each reaction was prepared in a final volume of 49 μL.

DNA polymerase: 5 U/μL DNA polymerase I Klenow fragment from BRL. Stored at -20°C.

[^{32}P] dATP: 3,000 Ci/mmol α-[^{32}P] dATP (DuPont). Stored at -60°C.

[^{32}P]-labeled probes for hybridization were prepared by using a random primer labelling kit (BRL) according to a modification of the protocol supplied by the manufacturer. 25 ng of DNA was denatured in 5-20 μL of distilled water in a sterile screw cap microfuge tube by heating in a boiling water bath for 5 min and then immediately cooled in an ice-water bath. The Reaction Mix, as listed above, was added to the tube on ice and the contents of the tube were mixed by gentle tapping. DNA polymerase (1 μL) was then added and the tube was incubated at 25°C for 2 h. The reaction was stopped by adding 5 μL stop buffer. Successively, 5 μL yeast tRNA (20 mg/mL, Boehringer Mannheim), 7 μL $MgCl_2$ (100 mM), 7 μL sodium acetate (3 M, pH 4.8) and 200 μL ice-cold ethanol (95%) were added. The tube was incubated at -60°C for 1 h and the precipitate was pelleted by centrifugation (Brinkman 5415C, 15 min, 4°C). The supernatant was removed with a Pasteur pipette and 1 mL ice-cold ethanol (70%)

was added, incubated at -60°C for 1 h and centrifuged (Brinkman 5415C, 15 min, 4°C). The supernatant was removed with a Pasteur pipette and the pellet was dissolved in 100 μL sterile water. The tube was heated at 100°C for 10 min then transferred to ice.

2.11 Testing Yeast Strains For UV Sensitivity

Yeast strains were tested for UV sensitivity by irradiating with UVC from a Sylvania G30T8 bulb giving >98% of its output at 254 nm (manufacturer's specifications). The incident dose rate was set to 0.5 $Jm^{-1}s^{-2}$ as measured by an Ultraviolet Products UVX-radiometer fitted with a UVX-25 short-range sensor. To set the incident dose rate, two layers of nylon window screen were used to reduce the incident dose rate to 0.5 Jm^{-1} s^{-2} without affecting the quality of the UV. The nylon screen diminished light intensity by the same fraction at all wavelengths from 250-450 nm (determined with a Milton Roy 3000 spectrophotometer). After irradiation, cultures were incubated at 30°C in the dark to prevent photoreactivation.

Yeast strains to be tested were inoculated in the appropriate omission medium and grown to stationary phase (1-2 x 10^7 cells/mL, as determined by Coulter Counter) at 30°C with shaking. For spot testing which gave a quick analysis of a strains UV sensitivity cultures were diluted down to 1 x 10^3 cells/mL with sterile water and 10 μL was spotted onto the appropriate media. Plates were allowed to dry and were irradiated with the lids off. Following incubation, in the dark, at 30°C for 6-7 days, the plates were observed to see if there were any UV-resistant colonies. For survival curves, the yeast cultures were diluted with sterile water such that their concentration ranged from 1 x 10^3 cells/mL to 1 x 10^6 cells/mL. 0.2 mL of the various dilutions were plated onto the appropriate media

and once again the plates were allowed to dry prior to irradiation. The UV doses that were used were 0 J/m , 2.5 J/m , 5.0 J/m , and 10 J/m. Table 4 shows the concentrations of cells that were plated for each UV dose that was used. Following incubation in the dark at 30°C for 6-7 days, the plates were observed, the number of colonies present per dilution plated per dosage used was noted and survival was assessed.

2.12 Functional Complementation

Plasmids excised from the λYES cDNA library were isolated and transformed into the yeast repair-deficient strains KAM-1 (*rad1*), SX24 (*rad2*), LN116 (*rad3*) and WS8104-2B (*rad4*). These yeast strains each contain an *ade2* mutation that causes the colonies to turn red on medium with limiting adenine. Transformants that arose on SD-ura plates were streaked onto a fresh SD-ura plate, allowed to grow for 2 days and then replica-plated, in duplicate, onto 2 SD-ura as well as onto 2 SG-ura plates. Replica-plating onto galactose medium allows the induction of the *A. thaliana* cDNA from the *GAL1* promoter. Prior to the irradiation, the replica plates were first incubated at 30°C for 1 hr to allow the transformants to begin growing on the galactose medium. The replica plates, one SD-ura and one SG-ura were irradiated with a dosage of UV between 20-40 J/m and incubated in the dark. The other dextrose and galactose replica plates were not irradiated. All the plates were then incubated at 30°C for 5 days. The irradiated plates were observed everyday under minimal lighting conditions to determine if any growth had occurred on the SG-ura plates in comparison to the SD-ura plates.

Table 4. The Range of Cell Concentrations Used For Each UV Dose In Determining the Yeast Survival Curves

UVC Dose (J/m)	Cell Concentration (cells/mL)
0.0	1×10^2
2.5	1×10^2, 1×10^3
5.0	1×10^3, 1×10^4
10.0	1×10^4, 1×10^5

2.13 Assay For Stability of Inactivation of *URA3*

To enable the isolation of a stable Ura$^-$ version of the *rad2::URA3* deletion/disruption mutant, the *rad2::URA3* 5-fluoro-orotic acid resistant (FOA^R) mutants were tested for stability of the inactivation of their *URA3* gene. The FOA^R isolates were inoculated YPDA medium (5 mL) and grown at 30°C with shaking until they reached a titre of ca. 1 x 10^7 cells/mL (as determined by Coulter count). The cells were then collected by centrifugation (1,850 x g, 10 min, room temperature) and resuspended in 5 mL of sterile water. The cells were pelleted and washed once more with sterile water to ensure that all of the rich medium had been removed. Again the cells were pelleted and finally resuspended in 5 mL of sterile water. 10 µL of the cell suspension was plated onto SD-ura medium and 10 µL of a 10^4 diluted culture was plated onto SD medium supplemented with all of the required amino acids. These plates were then incubated at 30°C for 5 days. Those isolates that did not produce a large number of Ura$^+$ colonies but did produce colonies on the SD medium were thought to have a stable inactivation of the *URA3* gene and were collected from the SD medium plate for further analysis.

3 Results and Discussion

3.1 Functional Complementation

The identification of a cDNA from *Arabidopsis thaliana* that complemented yeast DNA repair mutants was the initial approach used to identify plant DNA repair genes. This approach seemed viable since the literature showed that other labs have been able to clone genes involved in a variety of repair and nonrepair pathways from *Arabidopsis* by complementation (Anderson *et al.* 1992; Nasr *et al.* 1994; Pang *et al.* 1993a, 1993b; Santerre and Britt 1994; Sentenac *et al.* 1992; Smith *et al.* 1994). The mutant yeast strains KAM-1 (*rad1*), SX46 (*rad2*), LN116 (*rad3*), and WS8104-2B (*rad4*) were transformed with a plasmid library containing *Arabidopsis thaliana* cDNAs cloned behind the inducible yeast *GAL1* promoter (Elledge *et al.* 1991). Each of these yeast strains have an *ade2* mutation which causes them to turn red on medium with limiting concentrations of adenine. The resultant transformants should express the plant cDNA on medium containing galactose and if a plant DNA repair protein can function in yeast, it should be able to complement the defect in specific yeast *rad* mutants. The transformants that arose on SD-ura medium were streaked onto a fresh SD-ura plate, allowed to grow for 2 days and then replica-plated, in duplicate onto two SD-ura plates and onto two SG-ura plates. Replica-plating onto the latter medium was done to induce expression of the *A. thaliana* cDNA. One of the SD-ura and SG-ura replica plates were each then irradiated with a UV dose ranging from 20 J/m^2 to 40 J/m^2 and incubated, in the dark to prevent photoreactivation, at 30°C for 5 days. This UV dose range was used in order to ensure that survivors that arose would be due to an increase in UV resistance caused by the

expression of the *Arabidopsis* cDNA. Prior to UV irradiation, the replicas were allowed to grow at 30°C for 1 hr to allow the transformants to induce the *GAL1* promoter. The unirradiated SD-ura and SG-ura plates were also incubated at 30°C for 5 days. The unirradiated galactose plate functioned as a control to see if there would be any inhibition of growth of the replicas by the expression of the cDNA. The irradiated plates were observed everyday under minimal lighting conditions to determine if any growth had occurred on the SG-ura plates in comparison to the SD-ura plates.

For each of the yeast strains transformed with the cDNA library, no UV resistant colonies arose on either the SD-ura or the SG-ura plates. It was noted that the unirradiated SG-ura plates did not support growth of the pink coloured colonies which grew on the SD-ura plates. However, the red colonies were found to grow on both types of media. Table 5 shows the proportion of transformants that were red and pink for each yeast strain. The majority of the transformants were pink and unable to grow on galactose medium.

The presence of the pink colonies on the SD-ura medium, which were unable to grow on the SG-ura medium, was an unexpected finding. The initial possible explanation for the above results was that perhaps the expression of some of the cDNAs could somehow be interfering with the utilization of galactose as a carbon source by the yeast. To determine if this could be the explanation, plasmid λYES was isolated from these pink *rad* transformants and put into the yeast strain 334. This strain has a *reg1-501* mutation and is therefore unable to metabolize galactose in the presence of dextrose. However, this *reg1-501* strain is still able to use galactose as a gratuitous inducer of the *GAL1* promoter

Table 5. Proportion of Red and Pink λYES Transformants That Were Picked and Replica-plated onto Galactose-Containing Medium.

Transformant	Red Colonies	Pink Colonies
KAM1BCλ	32	735
SX46BCλ	154	1000
LN116BCλ	237	2089
WS8104BCλ	54	1274

in λYES (Hovland *et al.* 1989). The resulting transformants were then replica-plated onto SD plates that contained differing amounts of galactose. The galactose levels ranged from 0% to 4% (w/v), with the balance of the carbon being made up of dextrose in concentrations ranging from 4% to 0% (w/v). This ensured that the concentration of carbon source in the medium totalled 4% (w/v). Full growth of the 334 replicas was observed on the plates which contained low levels of galactose, thereby showing that the yeast cells containing a λYES plasmid were able to utilize galactose. The 334 replicas were unable to grow on those plates containing high galactose concentrations. The failure of the 334 transformants to grow this medium is probably due to the fact that it contained concentrations of dextrose too low to support yeast growth. Another possible explanation for the lack of growth of the pink transformants on the SG-ura medium is that expression of the *Arabidopsis* cDNA in these transformants on the SG-ura medium resulted in a protein which interfered with some other aspect of yeast metabolism and resulted in cell death. However, this explanation does not account for the pink colouration of the transformants on the SD-ura medium, since there would be no expression of the *Arabidopsis* cDNA on this medium. It was also suggested that perhaps these pink replicas were the result of the yeast transformation protocol (see section 2.7.3) and not due to the expression of the *Arabidopsis* cDNAs. To determine if this explanation was correct, the four yeast strains were transformed according to the protocol, but no plasmid was actually delivered into the yeast strains. These cells were plated on dextrose medium and incubated at 30°C for 5 days. Only red cells grew on the dextrose medium. Therefore, the pink yeast transformants that were unable to grow on galactose medium did not result

from the transformation protocol itself, but were due to the expression of the *Arabidopsis* cDNAs.

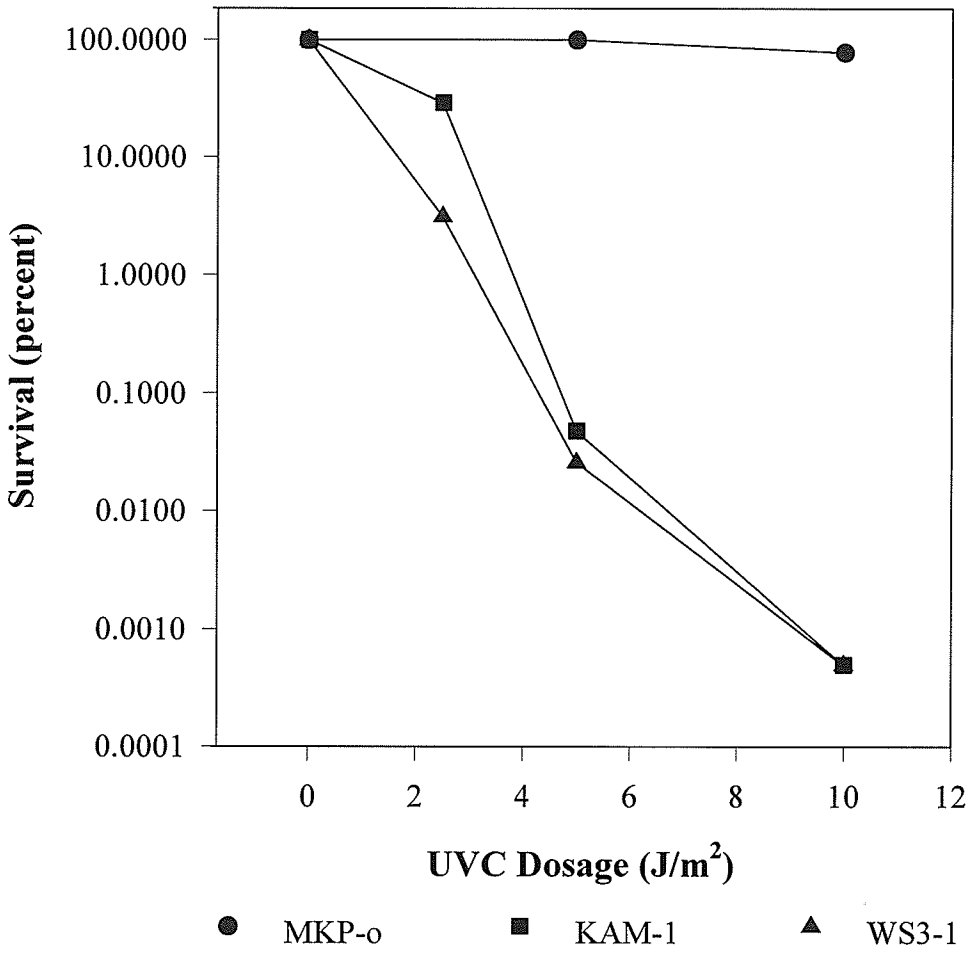
It was noted that the *rad* yeast strains used in the initial functional complementation attempt were not isogenic. Therefore, before attempting this approach a second time, isogenic *rad* strains should be used. This would ensure that any phenotype identified in these strains would not be due to genetic differences between strains. Isogenic *rad1* (KAM-1) and *rad3* (WS3-1) strains were provided by E.C. Friedberg and W. Siede, respectively. Survival curves were determined for each strain to determine the UV dosage required to kill all of the UV-sensitive transformants. The survival curves for each strain as well as for the wild-type strain MKP-o are shown in Figure 4. Two isogenic *rad* mutant strains remained to be constructed; the *rad2*Δ and the *rad4*Δ. An attempt at constructing these two strains was made in this study.

3.2 Construction of the *rad2* and *rad4* Yeast Strains

Isogenic yeast *rad* deletion strains were constructed to eliminate any results from the functional complementation study that were due to genetic differences between the strains. The *rad1* and *rad3* strains had been previously constructed (see Table 2) therefore, the only strains that still needed to be made were *rad2* and *rad4*.

In order to construct the *rad2* yeast strain, the plasmid pKM55 (Table 3; provided by R.D. Gietz) was used to disrupt the yeast chromosomal copy of *RAD2.* This was to be achieved by the insertion of the *URA3* gene contained in pKM55 into the chromosomal copy of *RAD2*. The plasmid pKM55 was constructed by Kiran Madura as follows: a 6.5 kb yeast *Xho*I-*Hind*III DNA fragment containing *RAD2* was cloned into the *Sal*I-*Hind*III

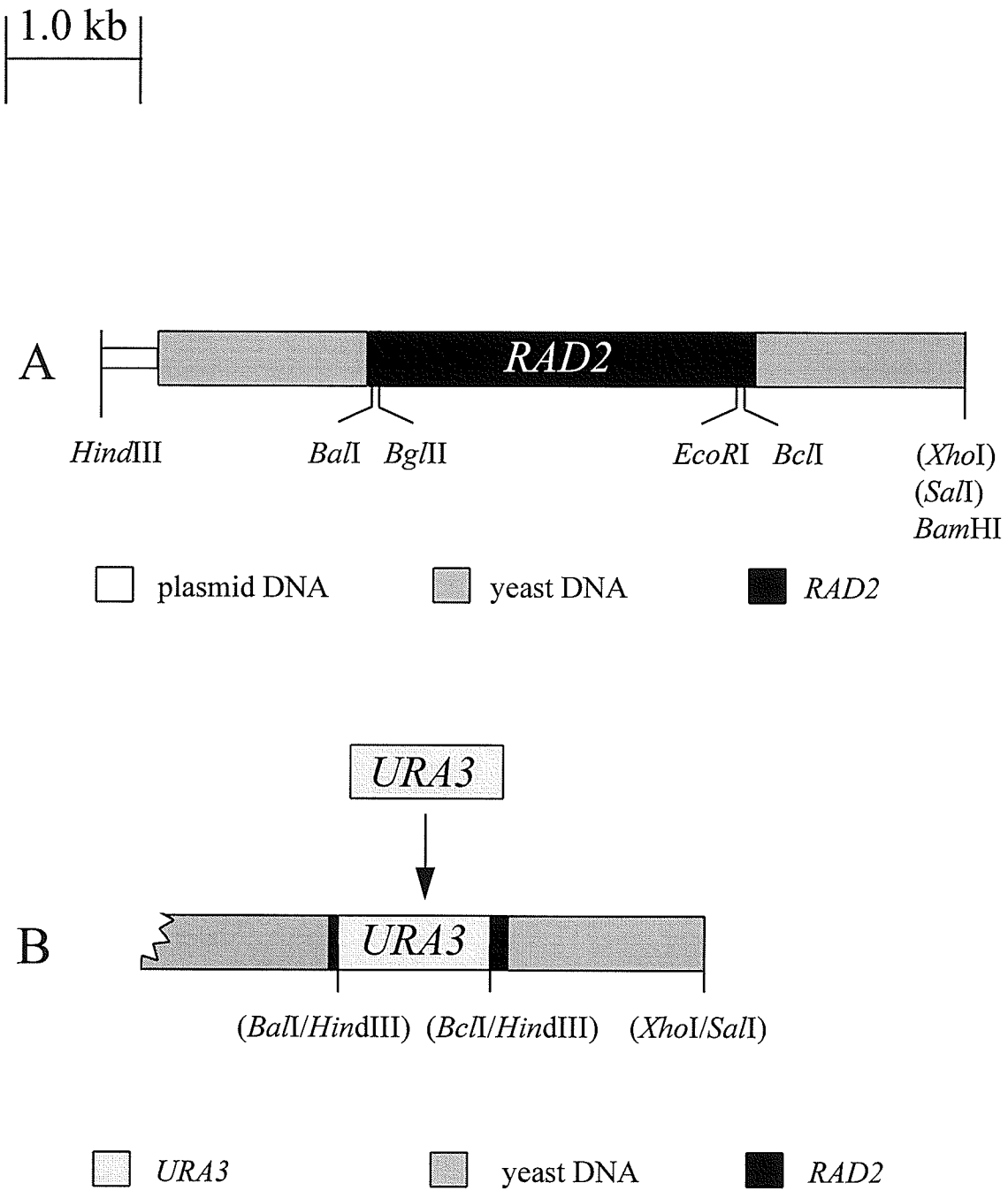
Figure 4. Survival curves for yeast strains KAM-1 (*rad1*), WS3-1 (*rad3*) and MKP-o (*RAD*$^+$). Survival curves to determine the UV sensitivity of the strains KAM-1, WS3-1 and MKP-o were produced. The log of percent survival is plotted against the dose of UV in J/m^2.


100.0000
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
Survival (percent)
0
2
4
6
8
10
12
UVC Dosage (J/m2)
MKP-o
KAM-1
WS3-1

sites of pUC9. A 1.1 kb *Hind*III yeast DNA fragment carrying the *URA3* gene was then blunt end ligated between the *Bcl*I-*Bal*I sites inside of *RAD2,* resulting in an internal deletion/disruption. Figure 5 illustrates the structure of the ORF of the *RAD2* gene and the disruption cassette. The plasmid pKM55 was then digested with *Bam*HI and *Hin*dIII to release the 9.4 kb *rad2*Δ disruption cassette and transformed (Gietz *et. al.* 1992) into MKP-o (*RAD*$^{+}$). The Ura^{+} transformants that arose on SD-ura medium were tested for UV sensitivity to ensure that the yeast chromosomal copy of *RAD2* had been disrupted by the integration of the *URA3* gene. However, in order to maintain selection for the λYES plasmid in this new *rad2* strain, the *URA3* gene would have to be inactivated. This was achieved by growing a UV-sensitive *rad2* Ura^{+} transformant on medium containing 5-fluoro-orotic acid (FOA (Sigma)). This medium selects for Ura^{-} colonies as a result of spontaneous inactivation of the *URA3* gene. The Ura^{-} colonies isolated from the FOA plates were then tested for stability of the inactivation of the *URA3* gene (see section 2.13). Those colonies that had a stable inactivation of the *URA3* gene were further analyzed by retesting for UV sensitivity. Unfortunately, all of the Ura^{-} colonies isolated and irradiated were no longer sensitive to UV radiation. They had reverted to the UV resistant phenotype.

The results of the *rad2* construction were not expected. The UV-sensitive *rad2*Δ transformants became UV resistant after growing on medium containing FOA. In theory, growth on the medium containing FOA should have produced *rad2*Δ colonies with an inactive *URA3* gene due to spontaneous mutation. However, the FOA^{R} *rad2*Δ colonies all reverted to the UV-resistant phenotype. The plasmid pKM55 that was used in the

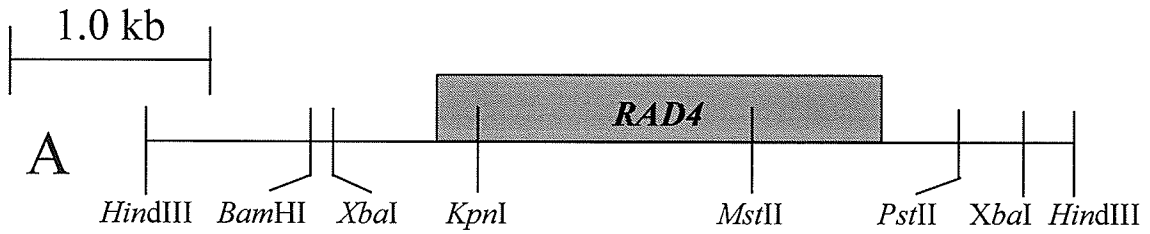
Figure 5. Construction of pKM55. The figure illustrates the construction of pKM55 that was used to disrupt the yeast chromosomal copy of *RAD2*. The section of yeast DNA that contains *RAD2* along with some flanking plasmid DNA is shown in A. The blunt end ligation of the *Hind*III fragment containing *URA3* into the *Bal*I-*Bcl*I sites within *RAD2* is illustrated in B. The *RAD2* ORF has been mostly deleted by the insertion of *URA3*.

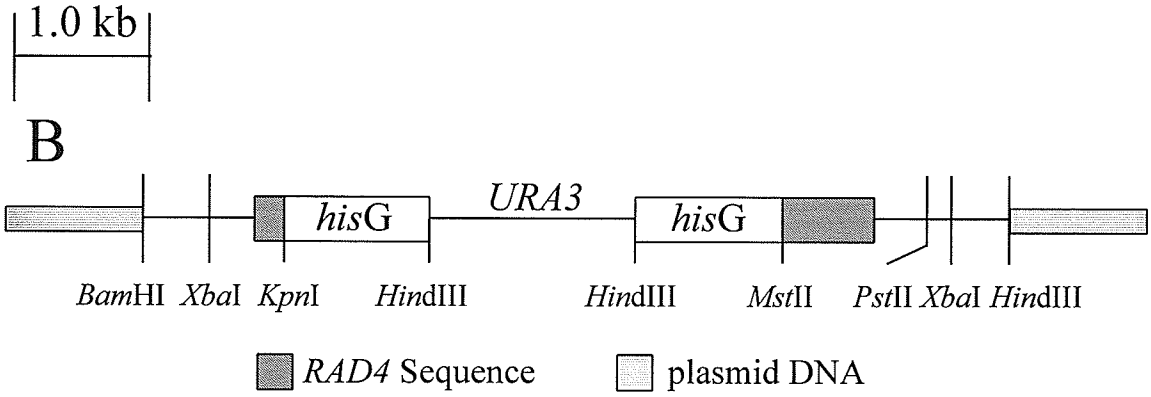

1.0 kb
A
RAD2
HindIII
BalI
BglII
EcoRI
BclI
(XhoI)
(SalI)
BamHI
plasmid DNA
yeast DNA
RAD2
URA3
B
URA3
(BalI/HindIII)
(BclI/HindIII)
(XhoI/SalI)
URA3
yeast DNA
RAD2

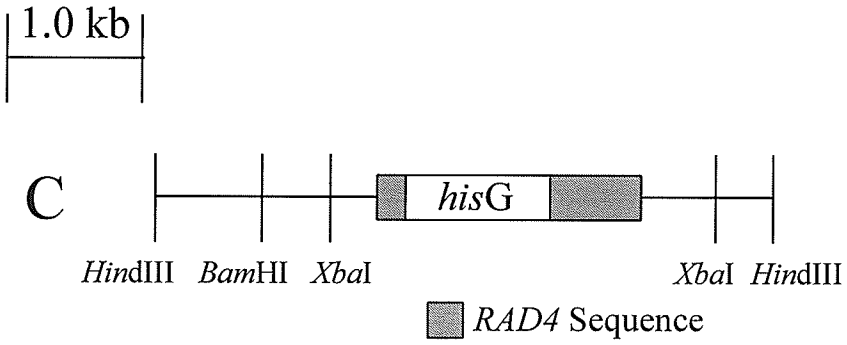
construction of the *rad2* strain was digested with *Bam*HI and *Hin*dIII prior to its transformation into MKP-o. This double digestion released a 9.4 kb DNA fragment containing *URA3* flanked by *RAD2* sequences. Subsequently, it was realized that the *Bam*HI and *Hin*dIII sites in pKM55 utilized to digest pKM55 for the integration of the *rad2*Δ*::URA3* deletion/disruption allele into the chromosomal location were located within the multicloning site of the pUC9 portion of the plasmid. A probable explanation of the above results suggests that this 9.4 kb fragment from pKM55 integrated into the chromosomal location of *RAD2* resulting in a *rad2* UV-sensitive phenotype. However, this type of integration event is not stable and has a naturally high reversion rate to give rise to the *RAD2*, UV-resistant phenotype. Therefore, growing the UV-sensitive *rad2* colonies on FOA selected for these naturally occurring revertants (R.D. Gietz, personal communication). This hypothesis could be tested by DNA hybridization of the UV-sensitive *rad2* colonies following transformation with pKM55, as well as with the UV-resistant FOA^R colonies, which are assumed to result due looping out of the inserted pKM55 fragment. Probing with the *RAD2* gene would determine if such an integration event had occurred. After the transformation with pKM55, the *RAD2* probe should bind to a genomic DNA fragment from the *rad2*Δ colonies that is ca. 9.4 kb larger than the genomic DNA fragment from the control *RAD2* DNA. However, with the genomic DNA from the UV-resistant FOA^R colonies, this same probe should bind to a DNA fragment that is the same size as the control DNA, suggesting that the 9.4 kb *Bam*HI-*Hin*dIII fragment from pKM55 had looped out of the genome to leave an intact chromosomal copy of *RAD2*.

For the construction of the *rad4* strain, the plasmid pDG38 (Gietz and Prakash 1988) was used for disrupting the yeast chromosomal copy of *RAD4*. The plasmid pDG38 was constructed as follows: the yeast *Bam*HI-*Hind*III DNA fragment containing *RAD4* was cloned between the *Bam*HI-*Hind*III sites of pUC9. The 3.8 kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* cassette from pNKY51 (see Table 3) was then blunt end ligated between the *Mst*II-*Kpn*I sites of *RAD4*. Plasmid pDG38 was then digested with *Xba*I to release the 6.1 kb *rad4* flanking sequences carrying the *hisG-URA3-hisG* cassette. The digested DNA was then transformed (Gietz *et. al.* 1992) into MKP-o. Ura^+ transformants that emerged were streaked onto SD-ura medium, tested for UV sensitivity, and further analyzed by hybridization to determine the structure of the *rad4Δ::hisG-URA3-hisG* allele, using the *RAD4* gene as a probe. Figure 6 illustrates the structure of pDG38 as well as the resulting yeast *rad4Δ* deletion/disruption allele. In order to utilize the *URA3* selection on the λYES plasmid the *URA3* gene in the disruption cassette in the *rad4Δ* gene would have to inactivated. In this case, the loss of the *URA3* gene function was accomplished by growing the UV-sensitive *rad4* cells on medium containing FOA. The *URA3* gene can be lost by recombination between *hisG* repeats (Alani *et. al.* 1987), leaving a single *hisG* remaining in the *rad4Δ* gene. The FOA^R/Ura^- colonies that were isolated were retested for UV sensitivity and analyzed for the presence of the remaining *hisG* gene in the yeast chromosomal copy of *RAD4* by hybridization, using the *RAD4* gene as a probe. Figure 7A shows the results of the hybridization performed to determine the structure of the *rad4Δ::hisG-URA3-hisG* allele. Genomic DNA from MKP-o and 5 UV-sensitive Ura^+ transformants were digested with *Hin*dIII and probed. The

Figure 6. Construction of pDG38. The figure illustrates the structure of the *RAD4* gene, the plasmid pDG38 and the disrupted yeast chromosomal copy of *rad4.* A *Hind*III segment of the yeast chromosome containing *RAD4* is shown in A. The *Bam*HI-*Hind*III fragment containing *RAD4* was cloned into pUC9. The *Bam*HI-*Bgl*II *hisG-URA3-hisG* cassette was cloned into the *Mst*II-*Kpn*I sites of *RAD4* as is shown in B. The structure of the *rad4:hisG* deletion/disruption allele is shown in C. The *URA3* gene is lost due to recombination between *hisG* direct repeats.


1.0 kb
A
RAD4
HindIII
BamHI
XbaI
KpnI
MstII
PstII
XbaI
HindIII


1.0 kb
B
hisG
URA3
hisG
BamHI
XbaI
KpnI
HindIII
HindIII
MstII
PstII
XbaI
HindIII
RAD4 Sequence
plasmid DNA


1.0 kb
C
hisG
HindIII
BamHI
XbaI
XbaI
HindIII
RAD4 Sequence

chromosomal *RAD4* gene is carried on a 4.7 kb *Hin*dIII fragment as shown by the binding of the probe to such a fragment with the genomic DNA from MKP-o in lane 1. The *URA3* gene in the *rad4Δ:hisG-URA3-hisG* cassette is flanked on both sides by a *Hin*dIII site. Therefore, digestion of the 5 UV-sensitive Ura^+ transformants with *Hin*dIII gave rise to two DNA fragments of ca. 3.0 kb, each containing a *hisG* gene flanked by the remaining *RAD4* sequences. Lanes 2-6 in Figure 7A, show such a result with the *rad4* transformants. Figure 7B shows the results of hybridization analysis with the *rad4Δ* FOA^R colonies. The *RAD4* probe binds to a 4.7 kb *Hin*dIII fragment with the genomic DNA from MKP-o (lane 1). However, with the *rad4* strains in lanes 2-6, the probe now binds to a 4.5 kb fragment, showing the loss of the *URA3* gene as well as a single *hisG* repeat from the chromosomal copy of *RAD4*. This *rad4* yeast strain was then named BCRAD4Δ.

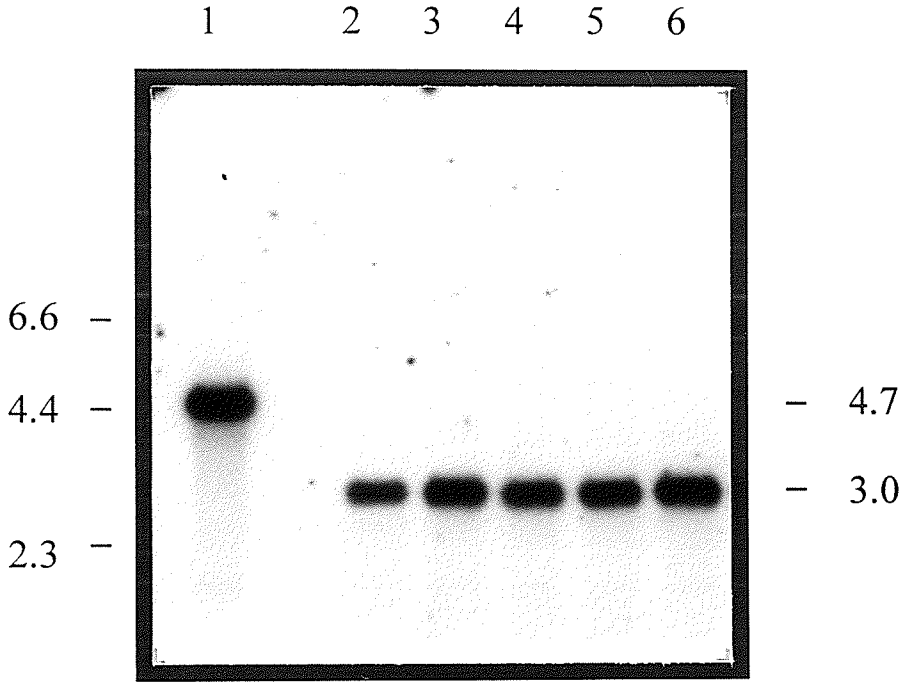
3.3 Restriction Digests For the Isolation of Yeast DNA Repair Genes

Figures 8, 9, and 10 show the various yeast DNA repair gene fragments used as probes in an attempt to identify homologous genes in *Arabidopsis*. The detailed descriptions of how the DNA fragments to be used as probes were obtained are listed below:

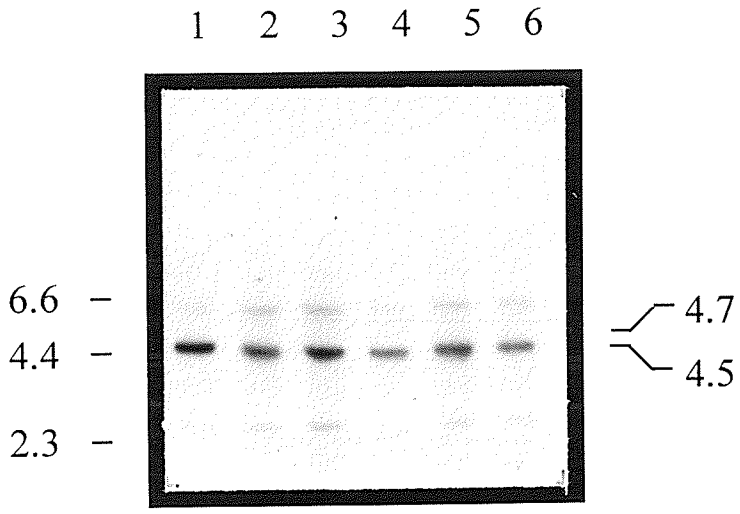
The plasmid pNF1000, carrying *RAD1*, was digested with *Sst*I, resulting in the formation of 2.62 kb and 14.94 kb DNA fragments (Yang and Friedberg 1984; Reynolds *et al.* 1987). The larger fragment, containing *RAD1*, was isolated and further digested with *Bcl*I. This 3.4 kb *Bcl*I-*Sst*I fragment was isolated and further digested by *Kpn*I, which cuts the fragment approximately in half, and both halves were used for

Figure 7. Hybridization analysis of *rad4* constructs BCRAD4Δ. The genomic DNA of the wild-type yeast strain MKP-o and the *rad4*Δ constructs were digested with *Hind*III. In both A and B photographs, MKP-o is located in lane 1 and the *rad4*Δ constructs are located in lanes 2 to 6. The entire *RAD4* gene was used as a probe. In A, the integration of the *rad4*Δ*:hisG-URA3-hisG* cassette is shown by the presence of 3.0 kb fragment with the *rad4*Δ constructs. The *RAD4* allele gave a a 4.7 kb fragment in the MKP-o DNA. The loss of *URA3* and a *hisG* is shown in B. Once again, the *RAD4* probe binds to a 4.7 kb fragment in MKP-o (lane 1) whereas the *rad4*Δ*:hisG* allele produces a 4.5 kb fragment in the strain BCRAD4Δ (lanes 2-6).

A



B



hybridizations since the 3.4 kb fragment was too large to be adequately labelled by random primers. See Figure 8A.

The plasmid pNF2005, containing *RAD2*, was digested with *Sal*I and the 4.5 kb fragment containing *RAD2* was isolated (Nicolet *et al.* 1985). The flanking noncoding regions were then removed by digesting the 4.5 kb *Sal*I fragment with *Msl*I and *Kpn*I, leaving a 2.8 kb piece that contained most of *RAD2*. See Figure 8B.

The plasmid pDG255, kindly provided by R.D. Gietz, was used to isolate the *RAD3* gene. It was digested with *Hae*III and the 2.17 kb and the 0.74 kb DNA fragments were isolated from this digestion (Reynolds *et al.* 1985a). These two pieces contained most of the *RAD3* gene. See Figure 8C.

The *RAD4*-containing plasmid pDG7 was first linearized with *Sph*I and then digested with *Pst*I (Gietz and Prakash 1988). This resulted in three DNA fragments of 3.14 kb, 2.63 kb and 1.62 kb. The *RAD4* gene was contained on the 2.63 kb fragment which was used as a probe. See Figure 8D.

The plasmid pNF102, containing the *RAD10* gene, was digested with *Pvu*II which resulted in a 7.1 kb and a 3.0 kb DNA fragment (Reynolds *et al.* 1985b). The 7.1 kb piece was isolated and further digested with *Nhe*I, which gave a 1.28 kb DNA fragment that contained the *RAD10* gene. See Figure 8E.

Plasmid pFF11.56 that contains the *RAD18* gene was digested with *Cla*I which resulted in DNA fragments of 5.26 kb and 7.85 kb (Fabre *et al.* 1989). The 5.26 kb fragment contained the *RAD18* gene and was isolated. This fragment was further digested with *Eco*RI, to remove some of the flanking noncoding sequences, resulting in a 1.6 kb

and a 2.4 kb fragment, both containing parts of *RAD18*. The 2.4 kb fragment was isolated and further digested with *Stu*I giving a 1.9 kb and 0.54 kb DNA fragments which contain part of *RAD18*. The 1.6 kb *Eco*RI and the 0.54 kb *Eco*RI-*Stu*I fragments were then used for hybridizations. See Figure 9A.

The plasmid pNKY74, carrying *RAD50*, was digested with *Hin*dIII, which resulted in a 2.9 kb and 7.0 kb DNA fragments (Alani *et al.* 1989). The 7.0 kb fragment was isolated and further digested with *Sal*I to give a 3.0 kb and 4.0 kb DNA fragments which contain the *RAD50* gene. This 4.0 kb *Hin*dIII-*Sal*I fragment was isolated and further digested by *Bgl*II prior to radio-labelling. See Figure 9B.

The plasmid YEp13-RAD51-23 was used to isolate the *RAD51* gene. It was digested with *Bam*HI and the 3.7 kb piece containing the *RAD51* gene was isolated (Calderone *et al.* 1983; Shinohara *et al.* 1992). This *Bam*HI fragment was further digested with *Pst*I to remove the 3' 1.6 kb noncoding region giving rise to a 2.1 kb fragment which was used as a probe. See Figure 9C.

Two plasmids were used to isolate the *RAD52* gene, pSM13 and YCpR52-111. pSM13 was digested with *Hpa*II resulting in a 1.2 kb fragment which contains the *RAD52* (Figure 9Di; B.A. Kunz, personal communication). This DNA fragment was found not to contain the entire *RAD52* gene, so plasmid YCpR52-111 was used to isolate a 3.3 kb *Sal*I fragment. This DNA fragment contained the entire *RAD52* gene (Adzuma *et al.* 1984). This *Sal*I fragment was digested with *Hpa*II and *Sph*I which resulted in a 1.5 kb fragment containing the *RAD52* gene (Figure 9Dii). Both *RAD52* probes were used in the hybridization analyses.

The plasmid YEp13RAD54-216A, carrying *RAD54*, was digested with *Eco*RI and the 4.2 kb fragment was isolated (Emery *et al.* 1991). This fragment was then partially digested with *Bgl*II and the 2.7 kb *Eco*RI-*Bgl*II fragment containing the entire *RAD54* gene was isolated. See Figure 9E.

The plasmid YEp13RAD55-13C, containing *RAD55*, was digested with *Hin*dIII (Calderone *et al.* 1983; Lovett and Mortimer 1987). From the five fragments generated, a 1.8 kb fragment containing the *RAD55* gene was isolated. See Figure 10A.

REV1 was contained on the plasmid pFL41. It was digested with *Pst*I to give a 3.2 kb and a 10.1 kb fragment (Larimer *et al.* 1989). The 3.2 kb fragment was isolated and digested with *Hin*dIII prior to radio-labelling by random primers. The left 0.4 kb 5' end of the *REV1* gene was missing. See Figure 10B.

The plasmid pWS301/C1 was used to isolate the *REV2* gene (Ahne *et al.* 1992). It was digested with *Nco*I and a 2.7 kb fragment containing *REV2* was isolated and used to probe for homology. See Figure 10C.

The plasmid pJA6 (Morrison *et al.* 1989), containing the *REV3* gene, was digested with *Xba*I and the 4.6 kb fragment carrying *REV3* was isolated. Prior to radio-labelling with random primers, this fragment was digested with *Hin*dIII, which cuts it approximately in half. See Figure 10D

The *Pol III*-containing plasmid pBL304 was linearized with *Sal*I and then digested with *Hin*dIII (Boulet *et al.* 1989). This resulted in a 3.7 kb fragment containing the yeast *Pol III* gene. Prior to radio-labelling this fragment was digested into three smaller fragments with *Eco*RI. See Figure 10E.

Figure 8. Diagrams of gene fragments containing *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10*. This illustration shows the various gene fragments that were used to probe the *Arabidopsis thaliana* genomic DNA for the presence of homologs of these yeast genes.

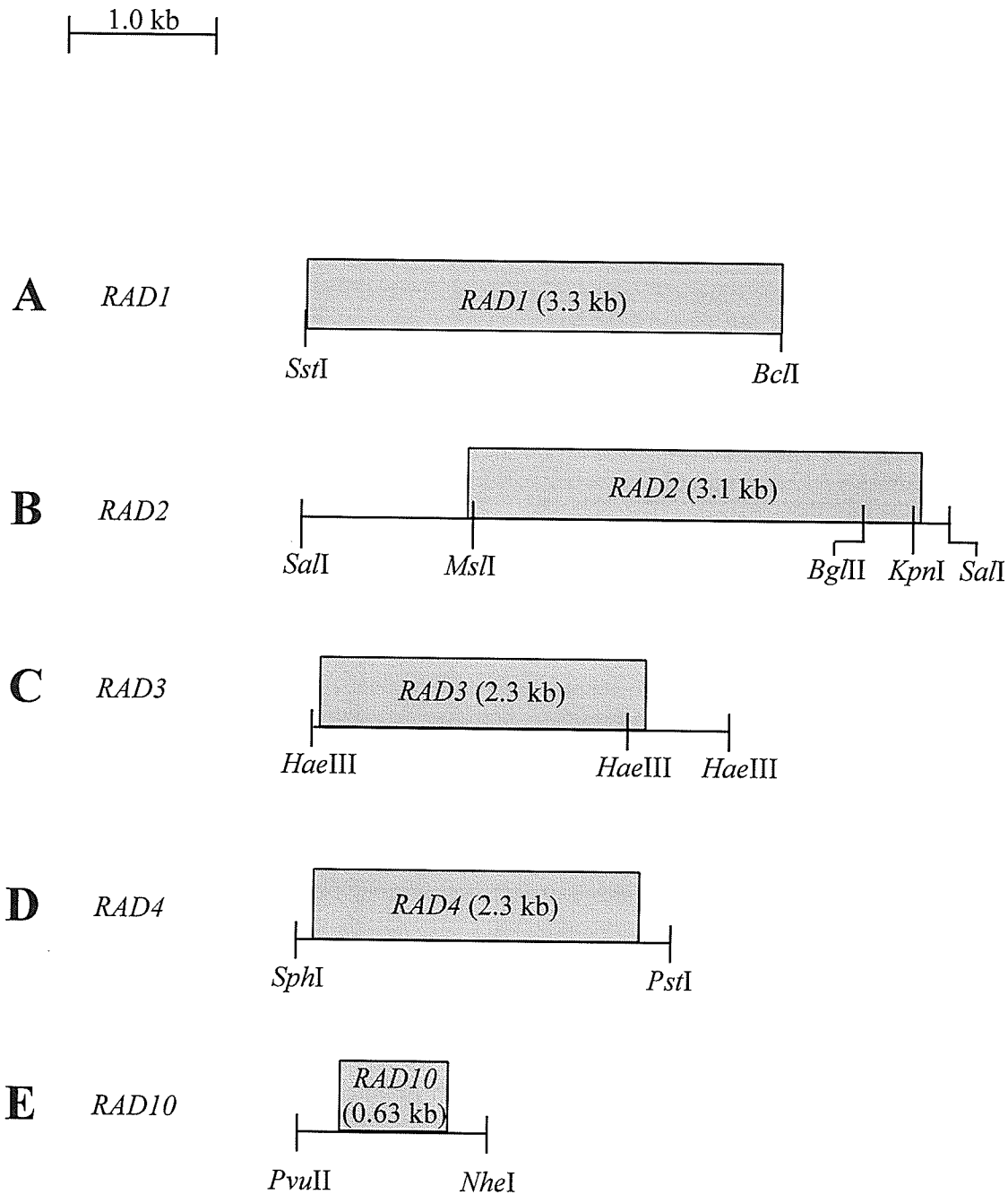

1.0 kb
A
RAD1
RAD1 (3.3 kb)
SstI
BclI
B
RAD2
RAD2 (3.1 kb)
SalI
MslI
BglII
KpnI
SalI
C
RAD3
RAD3 (2.3 kb)
HaeIII
HaeIII
HaeIII
D
RAD4
RAD4 (2.3 kb)
SphI
PstI
E
RAD10
RAD10
(0.63 kb)
PvuII
NheI

Figure 9. Diagrams of gene fragments that contained *RAD18, RAD50, RAD51, RAD52,* and *RAD54.* These various gene fragments were used to probe the *Arabidopsis thaliana* genomic DNA for the presence of the homologs of these yeast genes.

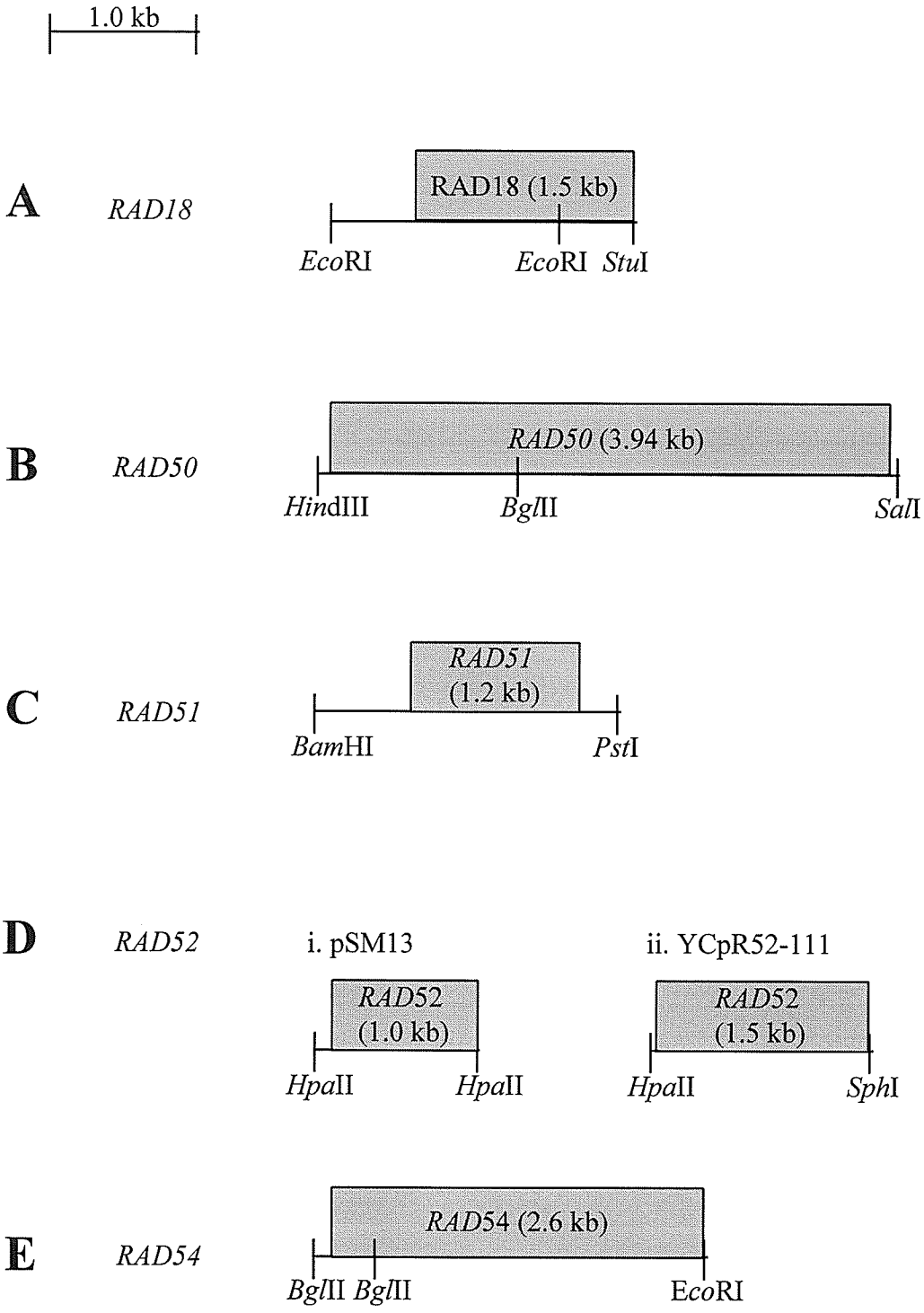
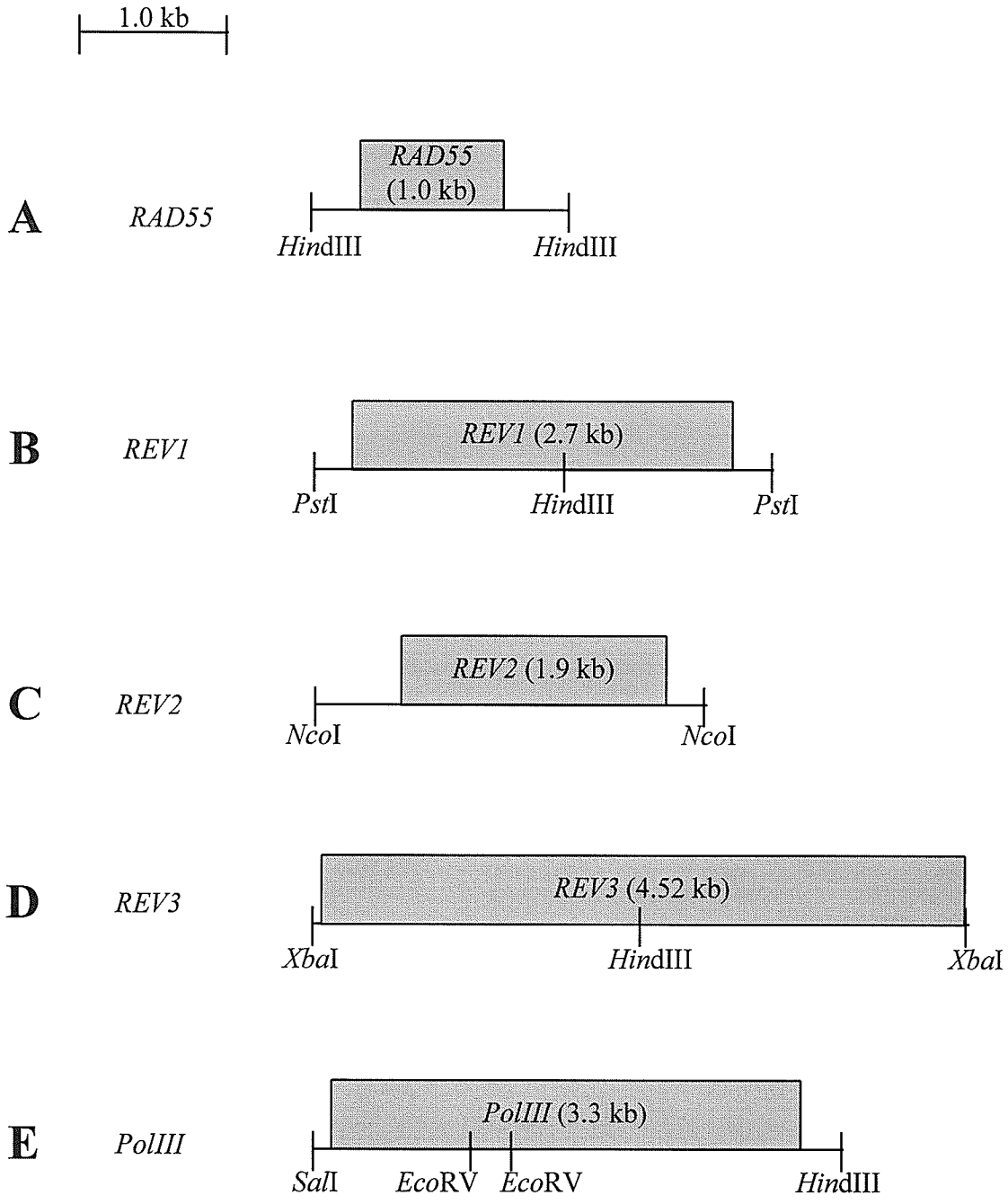

1.0 kb
A
RAD18
RAD18 (1.5 kb)
EcoRI
EcoRI StuI
B
RAD50
RAD50 (3.94 kb)
HindIII
BglII
SalI
C
RAD51
RAD51 (1.2 kb)
BamHI
PstI
D
RAD52
i. pSM13
RAD52 (1.0 kb)
HpaII
HpaII
ii. YCpR52-111
RAD52 (1.5 kb)
HpaII
SphI
E
RAD54
RAD54 (2.6 kb)
BglII BglII
EcoRI

Figure 10. Diagrams of gene fragments containing *RAD55, REV1, REV2, REV3,* and *PolIII*. These gene fragments were used to probe the *Arabidopsis thaliana* genomic DNA for the presence of the homologs of these yeast genes.

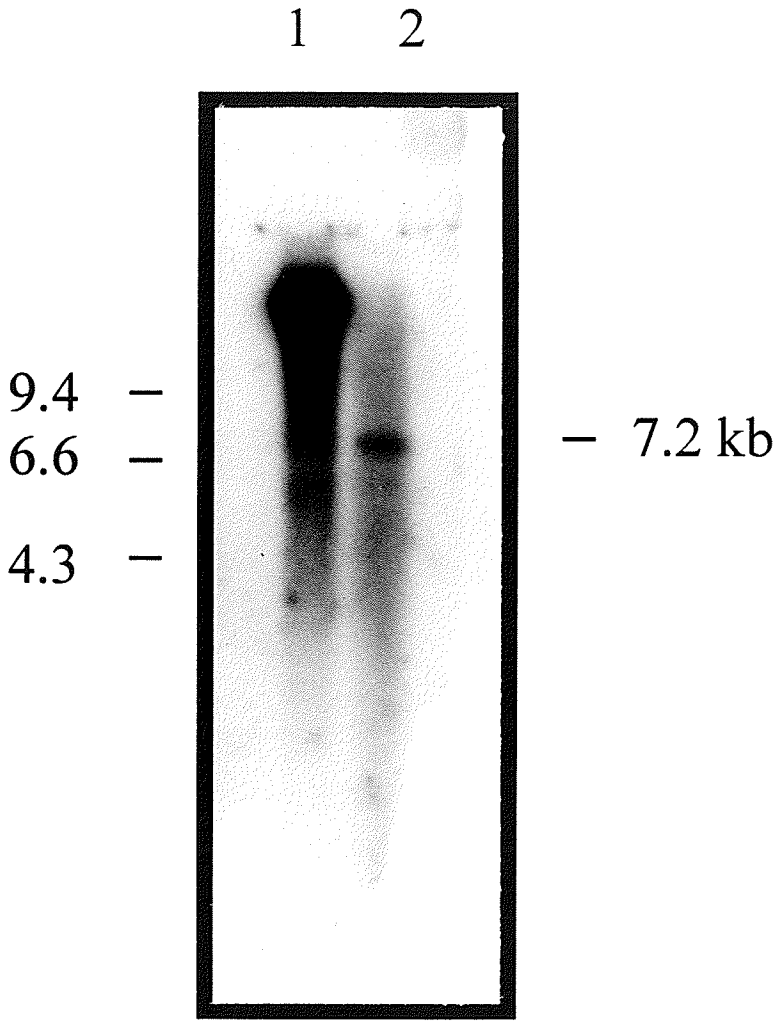

1.0 kb
A
RAD55
RAD55 (1.0 kb)
HindIII
HindIII
B
REV1
REV1 (2.7 kb)
PstI
HindIII
PstI
C
REV2
REV2 (1.9 kb)
NcoI
NcoI
D
REV3
REV3 (4.52 kb)
XbaI
HindIII
XbaI
E
PolIII
PolIII (3.3 kb)
SalI
EcoRV
EcoRV
HindIII

3.3.1 Probing *Arabidopsis thaliana* Genomic DNA

An alternate approach to the identification of plant DNA repair genes is to look for DNA homology to existing yeast DNA repair genes. Genomic DNA isolated from *Arabidopsis* and from the yeast strain MKP-o were digested with *Bam*HI and electrophoresed on agarose gels (0.7% (w/v)). The DNA was probed for homologous plant DNA repair genes with homology to 15 different yeast DNA repair genes used as probes. Section 3.3 describes how the DNA fragments that were used as probes were isolated and shows the actual fragments that were used for the Southern hybridizations. In the first attempt using this approach, a hybridization temperature of 55°C was used. None of the probes had homology to the *Arabidopsis* genomic DNA under the condition used, they only hybridized to the genomic DNA of MKP-o that was used as a control. This was likely due to the hybridization temperature of 55°C being too stringent.

The probes were hybridized a second time to the plant DNA, but at the less stringent hybridization temperature of 42°C. Figure 11 shows the binding of the yeast *RAD2* gene to the *Arabidopsis* genomic DNA in lane 2. A 7.2 kb band was visible in the *A. thaliana* genomic DNA when probed with the yeast *RAD2* gene. The control DNA, *Bam*HI-digested genomic DNA from MKP-o, is located in lane 1 and clearly shows the hybridization of the yeast *RAD2* gene to a DNA fragment of ca. 24 kb. This result suggests that there may a DNA repair gene in *Arabidopsis thaliana* that is homologous to the yeast *RAD2* gene. None of the other probes hybridized detectably to the *Arabidopsis* genomic DNA. This could be due to the hybridization conditions still being too stringent. The yeast *RAD2* may share more extended homology at the DNA level than the other

Figure11. Hybridization results showing the binding of *RAD2* to *A. thaliana* genomic DNA. Yeast and plant genomic DNA were digested with *Bam*HI. The yeast strain MKP-o was used as a control and is located in lane1. The *Arabidopsis* DNA is located in lane 2. The photograph clearly shows the binding of the yeast *RAD2* gene to a 7.2 kb fragment with the plant DNA whereas it binds to a fragment of approximately 24 kb in MKP-o.


1
2
9.4
6.6
4.3
7.2 kb

genes, such that hybridization at 42°C was possible. It is possible that if the other yeast probes were tested at a further reduced stringency, such as 40°C, some homology to these genes may be uncovered.

3.4 Probing an *Arabidopsis thaliana* cDNA Library

Due to the identification of the 7.2 kb *Arabidopsis thaliana* genomic DNA fragment with homology to the yeast *RAD2* gene, an attempt was made to identify an *Arabidopsis thaliana* cDNA with *RAD2* homology. Once such a cDNA was isolated, it could then be cloned, characterized, and used for another attempt at functional complementation of repair defective yeast strains. The *Arabidopsis* cDNA library, carried on the vector called λYES, was constructed by Elledge *et al.* (1991) and was obtained from N. Crawford (B.A. Kunz, personal communication). The λYES vector is a unique vector, as described in section 2.4, and the plasmid component can be propagated in both *E. coli* and yeast, which makes it an ideal vector for the production of cDNA expression libraries. Some *Arabidopsis* genes have already been cloned from this library by other labs (Pang *et al.* 1992, 1993a, 1993b; Santerre and Britt 1994). The plasmid component of the λYES can be automatically excised from the lambda phage backbone by site-specific recombination between flanking *lox* sites in the vector using the Cre protein (Elledge et al. 1991).

The cDNA library was probed with the yeast *RAD2* gene. In the primary screening of the library approximately 44,700 phage plaques were screened. Of those, 53 positive plaques were identified and isolated. The primary, secondary, and tertiary screenings of the plaques were performed at a temperature of 65°C, which represents

stringent hybridization conditions. This stringent hybridization temperature was used to lower the occurrence of false positive phage plaques from binding to the probe and thus, being isolated for further analysis (D. Barker, personal communication). The plasmid carrying the *Arabidopsis* cDNAs were rescued from these 53 phage plaques by infecting into the *E. coli* strain BNN132 (Table 2) which expresses the Cre protein that stimulates the excision of the plasmid at the *lox* recombination sites. The plasmids were purified and the cDNA inserts were released from the plasmid backbone by digestion with either *Xho*I or *Eco*RI. The cDNAs were analyzed on agarose gels (0.7% (w/v)) and ranged in size from ca. 0.4 kb to 2.3 kb. These cDNAs were then analyzed by restriction digestion to determine how many unique cDNAs had been isolated, with the extent of similarity being based on restriction digest patterns. A total of nine cDNAs were eliminated from further analysis. In four of these cases, restriction digest of the isolated λYES plasmids by *Xho*I or *Eco*RI failed to release a cDNA insert. The plasmid backbones of three of the plasmids were not the correct size of 7.8 kb. Therefore, it was believed that the cDNA no longer consisted of only *Arabidopsis* sequence in these plasmids. Restriction digest analysis of two of the clones revealed that they were identical to another plasmid and were therefore eliminated. The remaining 44 cDNA clones were analyzed by hybridization, again using the yeast *RAD2* gene as a probe, to ensure that these isolated cDNAs had homology to the *RAD2* gene. However, the *RAD2* gene failed to show homology to these 44 cDNAs when retested. Evidently these plasmids that were isolated did not carry homology to the yeast *RAD2* gene. Perhaps the original λYES positive phage plaques contained some homology to the probe which was lost during plasmid excision.

4 Future Work

The results from this study suggest that there is future work that can be done to isolate DNA repair genes from the plant *Arabidopsis thaliana*. The *Arabidopsis* genomic DNA should be probed again with the other remaining 14 yeast DNA repair genes however, a lower hybridization temperature should be used, e.g. a temperature of 40°C. Perhaps other yeast genes will hybridize to the *Arabidopsis* genomic DNA at this less stringent temperature. As well, another attempt should be made to identify a homologous *Arabidopsis* cDNA repair gene from a cDNA library using the yeast *RAD2* gene as well as with the other yeast DNA repair genes as probes. However, the quality of the library should be assured either by purchasing it from a reputable company or by making it yourself.

Should any *Arabidopsis* cDNAs be isolated from the above library, they should then be characterized. This characterization could then be used to determine if the proteins encoded by these cDNAs are similar in any way to other DNA repair proteins that have so far been isolated from both prokaryotes and eukaryotes. The isolated *Arabidopsis* cDNAs could also be used to reattempt the functional complementation study using repair-defective isogenic yeast strains to identify any functionally complementing cDNAs. These isogenic yeast strains could also be used to determine why most of the yeast transformants failed to grow on the SG-ura medium during the first attempt at this method.

Another avenue of research would be to clone the 7.2 kb DNA fragment from the *Arabidopsis* genomic DNA that shared some homology with the yeast RAD2 gene. This

fragment could then be characterized to determine if there are any similarities that exist with other known DNA repair genes in prokaryotes and eukaryotes.

As stated previously, there is a great deal of future work that could be done to continue this project. The work that was done during the course of this study was just preliminary in the isolation of a plant DNA repair gene.

5 Concluding Remarks

The aim of this study was to isolate and characterize a DNA repair gene from the plant *Arabidopsis thaliana*. The first approach used was to functionally complement DNA repair-defective yeast mutant strains with plasmids carrying *Arabidopsis thaliana* cDNAs. However, this approach proved unsuccessful due to the majority of the yeast transformants displaying the inability to grow on SG-ura medium. These pink colonies were shown to be a result of the plasmids that were transformed into the host yeast strains. As well, it was thought that perhaps the genetic variability in the strains that was used could also be a factor for these strange results. Therefore, it was decided that isogenic *rad1, rad2, rad3* and *rad4* strains would be required before trying this functional complementation method again. By using isogenic strains for the functional complementation study any results that occurred from the expression of the *Arabidopsis thaliana* cDNA would actually be due to the cDNA and not to genetic differences between the strains. Isogenic *rad1* and *rad3* strains had already been constructed and provided by two other research labs thus, all that remained to be done was to make the *rad2* and *rad4* strains.

An attempt at constructing these isogenic strains was made during the course of this study. The *rad4*Δ strain was constructed which was named BCRAD4Δ. A UV sensitive *rad2*Δ strain was constructed however, this strain could not be made Ura^- without reverting to the UV-resistant phenotype. It was necessary to make these *rad* strains Ura^- in order to allow for the maintenance of the λYES plasmid which uses *URA3* as the selectable marker in yeast. Another attempt at making this *rad2*Δ strain should be

made. However, a different *rad2* deletion allele should be used. Possibly one that is *rad2::Leu2*. Alternatively, the *rad2*Δ could be reconstructed with the use of the *hisG-URA3-hisG* disruption cassette as was the case in the construction of the *rad4*Δ strain.

In addition to the functional complementation study, genomic DNA from *Arabidopsis thaliana* was probed with 15 different yeast DNA repair genes. These yeast genes were members of the three epistasis groups, *RAD3*, *RAD6* and *RAD52* that are involved in nucleotide excision repair, recombinational repair and postreplication repair. This approach was taken with the belief that homology may exist between the genes of different species that are involved in the same essential functions. Probing the genomic DNA of *Arabidopsis* with these 15 yeast genes was done twice. A 7.2 kb DNA fragment was identified with the yeast *RAD2* gene as the probe. This suggests that there may be a *RAD2* homology in *A. thaliana*. However, the *RAD2* probe was not successful in identifying a cDNA with homology. Perhaps this *RAD2* probe should be used to clone the 7.2 kb *Arabidopsis* DNA fragment from the genomic DNA to determine if this fragment does, in fact, encode a *RAD2* gene homolog.

Plants should have DNA repair systems similar to that of other eukaryotes. The fact that the yeast *RAD2* gene bound to the *Arabidopsis thaliana* genomic DNA gives some hope that these plant genes are homologous to those in yeast. This study showed that further work can be done in order to isolate these genes and some suggestions for such type of work was previously given.

References

Adzuma, K., T. Ogawa and H. Ogawa, 1984 Primary structure of the *RAD52* gene in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **4**: 2735-2744.

Ahne, F., M. Baur and F. Eckardt-Schupp, 1992 The *REV2* gene of *Saccharomyces cerevisiae*: cloning and sequencing. Current Genetics **21**: 1-6.

Alani, E.L., L. Cao and N. Kleckner, 1987 A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics **116**: 541-564.

Alani, E., S. Subbiah and N. Kleckner, 1989 The yeast *RAD50* gene encodes a predicted 153-kD protein containing a purine nucleotide-binding domain and two large heptad-repeat regions. Genetics **122**: 47-57.

Anderson, J.A., S.S. Huprikar, L.V. Kochian, W.J. Lucas and R.F. Gaber, 1992 Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. (U.S.A.) **89**: 3736-3740.

Armstrong, J.D., 1993 *Mutational specificity of ultraviolet light in Saccharomyces cerevisiae*, Ph. D. thesis. University of Manitoba, Winnipeg.

Bailly, V., J. Lamb, P. Sung, S. Prakash and L. Prakash, 1994 Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites. Genes Dev. **8**: 811-820.

Bailly, V., P. Sung, L. Prakash and S. Prakash, 1991 DNA•RNA helicase activity of RAD3 protein of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. (U.S.A.) **88**: 9712-9716.

Bailly, V., C.H. Sommers, P. Sung, L. Prakash and S. Prakash, 1992 Specific complex formation between proteins encoded by the yeast DNA repair and recombination genes *RAD1* and *RAD10*. Proc. Natl. Acad. Sci. (U.S.A.) **89**: 8273-8277.

Bardwell, A.J., L. Bardwell, D.K. Johnson and E.C. Friedberg, 1993 Yeast DNA recombination and repair proteins Rad1 and Rad10 constitute a complex in vivo mediated by localized hydrophobic domains. Mol. Microbiol. **8**: 1177-1188.

Bardwell, A.J., L. Bardwell, N. Iyer, J.Q. Svejstrup, W.J. Feaver, R.D. Kornberg and E.C. Friedberg, 1994 Yeast nucleotide excision repair proteins Rad2 and Rad4 interact with RNA polymerase II basal transcription factor b (TFIIH). Mol. Cell. Biol. **14**: 3569-3576.

Bardwell, L., A.J. Bardwell, W.J. Feaver, J.Q. Svejstrup, R.D. Kornberg and E.C. Friedberg, 1994 Yeast RAD3 protein binds directly to both SSL2 and SSl1 proteins: implications for the structure and function of transcription/repair factor b. Proc. Natl. Acad. Sci. (U.S.A.) **91**: 3926-3930.

Basile, G., M. Aker and R.K. Mortimer, 1992 Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene *RAD51*. Mol. Cell. Biol. **12**: 3235-3246.

Birnboim, H.C. and J. Doly, 1979 A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7**: 1513-1523.

Bockrath, R., M. Ruiz-Rubio and B.A. Bridges, 1987 Specificity of mutation by UV light and delayed photoreversal in *umuC*-defective *Escherichia coli* K-12: a targeting intermediate at pyrimidine dimers. J. Bacteriol. **169**: 1410-1416.

Bootsma, D., 1993 The genetic defect in DNA repair deficiency syndromes. Eur. J. Cancer. **29A**: 1482-1488.

Boulet, A., M. Simon, G. Faye, G.A. Bauer and P.M.J. Burgers, 1989 Structure and function of the *Saccharomyces cerevisiae CDC2* gene encoding the large subunit of DNA polymerase III. EMBO J. **8**: 1849-1854.

Britt, A.B., J.-J. Chen, D. Wykoff and D. Mitchell, 1993 A UV-sensitive mutant of *Arabidopsis* defective in the repair of pyrimidine-pyrimidone (6-4) dimers. Science **261**: 1571-1574.

Britt, A.B., 1995 Repair of DNA damage induced by ultraviolet radiation. Plant Physiol. **108**: 891-896.

Burckhardt, S.E., R. Woodgate, R.H. Scheuermann and H. Echols, 1988 The UmuD protein of *E. coli*: overproduction, purification and cleavage by RecA. Proc. Natl. Acad. Sci. (U.S.A.) **85**: 1811-1815.

Calderon, I.L., C.R. Contopoulou and R.K. Mortimer, 1983 Isolation and characterization of yeast DNA repair genes. I. Isolation of plasmids that complement the mutations *rad50-1*, *rad51-1*, *rad54-3* and *rad55-3*. Current Genetics **7**: 93-100.

Chalut, C., V. Moncollin and J.M. Egly, 1994 Transcription by RNA polymerase II: a process linked to DNA repair. Bioessays **16**: 651-655.

Chanet, R., N. Magana-Schwencke and F. Fabre, 1988 Potential DNA-binding domains in the *RAD18* gene product of *Saccharomyces cerevisiae*. Gene **74**: 543-547.

Chasan, R., 1994 A ray of light on DNA repair. The Plant Cell **6**: 159-161.

Cheah, K.S.E. and D.J. Osborne, 1978 DNA lesions occur with loss of viability in embryos of aging rye seeds. Nature **272**: 593-599.

Chen, J.-J., D.L. Mitchell and A.B. Britt, 1994 A light-dependent pathway for the elimination of UV-induced pyrimidine (6-4) pyrimidone photoproducts in *Arabidopsis*. The Plant Cell **6**: 1311-1317.

Cooke, J.S., 1970 Photoreactivation in animal cells. Photophysiology **5**: 191-233.

Cooper, A.J. and S.L. Kelly, 1987 DNA repair and mutagenesis in *Saccharomyces cerevisiae*. In: *Enzyme Induction, Mutagen Activation and Carcinogen Testing in Yeast*, edited by A. Wiseman. Ellis Horwood, London.

Couto, L.B. and E.C. Friedberg, 1989 Nucleotide sequence of the wild-type *RAD4* gene of *Saccharomyces cerevisiae* and characterization of mutant *rad4* alleles. J. Bacteriol. **171**: 1862-1869.

Dandoy, E., R. Schyns, R. Deltour and W.G. Verly, 1987 Appearance and repair of apurinic/apyrimidinic sites in DNA during early germination of *Zea mays*. Mutation Res. **181**: 57-60.

Dasgupta, C., A.M. Wu, R. Kahn, R.P. Cunningham and C.M. Radding, 1981 Concerted strand exchange and formation of Holliday structures by *E. coli* RecA protein. Cell **25**: 507-516.

Delgani, N., E. Ben-Hur and E. Riklis, 1980 DNA damage and repair: induction and removal of thymine dimers in ultraviolet light irradiated intact water plants. Photochem. Photobiol. **31**: 31-36.

Dellaporta, S.L., J. Wood and J.B. Hicks, 1983 A plant DNA minipreparation. Plant Mol. Biol. Rep. **1**: 19-21.

Dimitrov, B.D., 1994 Types of chromosomal aberrations induced by seed aging in *Crepis capillaris*. Environ. Mol. Mutag. **23**: 318-322.

Echols, H. and M.F. Goodman, 1990 Mutation induced by DNA damage: a many protein affair. Mutat. Res. **236**: 301-311.

Elledge, S.J., J.T. Mulligan, S.W. Ramer, M. Spottswood and R.W. Davis, 1991 λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc. Natl. Acad. Sci. (U.S.A.) **88**: 1731-1735.

Emery, H.S., D. Schild, D.E. Kellogg and R.K. Mortimer, 1991 Sequence of *RAD54*, a *Saccharomyces cerevisiae* gene involved in recombination and repair. Gene **104**: 103-106.

Evans, D.A., 1989 Somaclonal variation-genetic basis and breeding applications. Trends In Genetics **5**: 46-50.

Fabre, F., N. Magana-Schwencke and R. Chanet, 1989 Isolation of the *RAD18* gene of *Saccharomyces cerevisiae* and construction of *rad18* deletion mutants. Mol. Gen. Genet. 215: 425-430.

Feaver, W.J., J.Q. Svejstrup, L. Bardwell, A.J. Bardwell, S. Buratowski, K.D. Gulyas, T.F. Donahue, E.C. Friedberg and R.D. Kornberg, 1993 Dual roles of a multiprotein complex from *S. cerevisiae* in transcription and DNA repair. Cell **75**: 1379-1387.

Fleer, R., C.M. Nicolet, G.A. Pure and E.C. Friedberg, 1987a *RAD4* gene of *Saccharomyces cerevisiae*: molecular cloning and partial characterization of a gene that is inactivated in *Escherichia coli*. Mol. Cell. Biol. **7**: 1180-1192.

Fleer, R., W. Siede and E.C. Friedberg, 1987b Mutational inactivation of the *Saccharomyces cerevisiae RAD4* gene in *Escherichia coli*. J. Bacteriol. **169**: 4884-4892.

Franklin, W.A., K.M. Lo and W.A. Haseltine, 1982 Alkaline lability of flourescent photoproducts produced in ultraviolet light-irradiated DNA. J. Biol. Chem. **257**: 13535-13543.

Franklin, W.A., P.W. Doetsch and W.A. Haseltine, 1985 Structural determination of the ultraviolet light induced thymine-cytosine pyrimidine-pyrimidone [6-4] photoproduct. Nucl. Acids Res. **13**: 5317-5325.

Franklin, W.A. and W.A. Haseltine, 1986 The role of the [6-4] photoproduct in ultraviolet light-induced transition mutations in *E. coli*. Nucl. Acids Res. **13**: 5317-5325.

Freeman, S.E., H. Hacham, R.W. Gange, D.J. Maytum, J. Sutherland and B.M. Sutherland, 1989. Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated *in situ* with ultraviolet light. Proc. Natl. Acad. Sci. (U. S. A.) **86**: 5606-5609.

Friedberg, E.C., 1985 *DNA Repair*. W. H. Freeman and Co., San Francisco.

Friedberg, E.C., 1988 Deoxyribonucleic acid repair in the yeast *Saccharomyces cerevisiae*. Microbiol. Rev. **52**: 70-102.

Friedberg, E.C., 1991 Eukaryotic DNA repair: glimpses through the yeast *Saccharomyces cerevisiae*. Bioessays **13**: 295-302.

Friedberg, E.C., G.C. Walker, and W. Siede, 1995 *DNA Repair and Mutagenesis*. American Society for Microbiology, Washington.

Game, J.C., 1983 Radiation-sensitive mutants and repair in yeast, p. 109-137. In: *Yeast Genetics Fundamental and Applied Aspects*, edited by J.F.T. Spencer, D.M. Spencer and A.R.W. Smith. Springer-Verlag, New York.

Gasser, C.S. and R.T. Fraley, 1989 Genetically engineering plants for crop improvement. Science **244**: 1293-1299.

Gibbs, P.E.M., B.J. Kilbey, S.K. Banerjee and C.W. Lawrence, 1993 The frequency and accuracy of replication past a thymine-thymine cyclobutane dimer are very different in *Saccharomyces cerevisiae* and *Escherichia coli*. J. Bacteriol. **175**: 2607-2612.

Gietz, R.D. and S. Prakash, 1988 Cloning and nucleotide sequence analysis of the *Saccharomyces cerevisiae RAD4* gene required for excision repair of UV-damaged DNA. Gene **74**: 535-541.

Gietz, R.D., A. St. Jean, R.A. Woods and R.H. Schiestl, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. **20**: 1425.

Glickman, B.W., R.M. Schaaper, W.A. Haseltine, R.L. Dunn and D.E. Brash, 1986 The C-C [6-4] UV photoproduct is mutagenic in *Escherichia coli*. Proc. Natl. Acad. Sci. (U. S. A.) **83**: 6945-6949.

Goldberg, R.B., 1988 Plants: novel developmental processes. Science **240**:1460-1467.

Guzder, S.N., H. Qiu, C.H. Sommers, P. Sung, L. Prakash and S. Prakash, 1994 DNA repair gene *RAD3* of *S. cerevisiae* is essential for transcription by RNA polymerase II. Nature **367**: 91-94.

Grossman, L. and S. Thiagalingam, 1993 Nucleotide excision repair, a tracking mechanism in search of damage. J. Biol. Chem. **268**:16871-16874.

Gutierrez, C., 1987 Excision repair of uracil in higher plant cells: uracil-DNA glycosylase and sister-chromatid exchanges. Mutation Res. **181**: 111-126.

Habraken, Y., P. Sung, L. Prakash and S. Prakash, 1993 Yeast excision repair gene *RAD2* encodes a single-stranded DNA endonuclease. Nature 366: 365-368.

Habraken, Y., P. Sung, L. Prakash and S. Prakash, 1994a Holliday junction cleavage by yeast Rad1 protein. Nature **371**: 531-534.

Habraken, Y., P. Sung, L. Prakash and S. Prakash, 1994b Human xeroderma pigmentosum group G gene encodes a DNA endonuclease. Nucl. Acids Res. **22**: 3312-3316.

Harlow, G.R., M.E. Jenkins. T.S. Pittalwals and D.W. Mount, 1994 Isolation of *uvh1*, an *Arabidopsis* mutant hypersensitive to ultraviolet light and ionizing radiation. The Plant Cell **6**: 227-235.

Harm, H., 1976 Repair of UV-irradiated biological systems: photoreactivation. p. 219-262. In: *Photochemistry and Photobiology of Nucleic Acids*, edited by S.Y. Wang. Volume II, Academic Press, New York.

Harosh, I., L. Naumovski and E.C. Friedberg, 1989 Purification and characterization of Rad3 ATPase/DNA helicase from *Saccharomyces cerevisiae*. J. Biol. Chem. **34**: 20532-20539.

Haynes, R.H. and B.A. Kunz, 1981 DNA repair and mutagenesis in yeast, p. 371-414. In: *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*, edited by J. N. Strathern, E. W. Jones and J. R. Broach, Cold Spring Harbor Laboratory, Cold Spring Harbor.

Heery, D.M., F. Gannon and R. Powell, 1990 A simple method for subcloning DNA fragments from gel slices. Trends In Genetics **6**: 173.

Heindorff, K., R. Rieger, I. Schubert, A. Michaelis and O. Aurich, 1987 Clastogenic adaptation of plant cells-reduction of the yield of clastogen-induced chromatid aberrations by various pretreatment procedures. Mutation Res. **181**: 157-171.

Henning, K.A., C. Peterson, R. Legerski and E.C. Friedberg, 1994 Cloning the *Drosophila* homolog of the xeroderma pigmentosum complementation group C gene reveals homology between the predicted human and *Drosophila* polypeptides and that encoded by the yeast *RAD4* gene. Nucl. Acids Res. **22**: 257-261.

Higgins, D.R., S. Prakash, P. Reynolds, R. Polakowska, S. Weber and L. Prakash, 1983a Isolation and characterization of the *RAD3* gene of *Saccharomyces cerevisiae* and inviability of *rad3* deletion mutants. Proc. Natl. Acad. Sci. (U.S.A.) **80**: 5680-5684.

Higgins, D.R., S. Prakash, P. Reynolds and L. Prakash, 1983b Molecular cloning and characterization of the *RAD1* gene of *Saccharomyces cerevisiae*. Gene **26**: 119-126.

Higgins, D.R., L. Prakash, P. Reynolds and S. Prakash, 1984 Isolation and characterization of the *RAD2* gene of *Saccharomyces cerevisiae*. Gene **30**: 121-128.

Hoeijmakers, J.H.J., 1991 How relevant is the *Escherichia coli* UvrABC model for excision repair in eukaryotes? J. Cell Sci. **100**: 687-691.

Hoeijmakers, J.H.J., 1993 Nucleotide excision repair II: from yeast to mammals. Trends In Genetics. **9**: 211-217.

Hovland, P., J. Flick, M. Johnston and R.A. Sclafani, 1989 Galactose as a gratuitous inducer of *GAL* gene expression in yeasts grown on glucose. Gene. **83**: 57-64.

Howland, G.P., 1975 Dark repair of ultraviolet-induced pyrimidine dimers in the DNA of wild carrot protoplasts. Nature **254**: 160-161.

Husain, I., J. Griffith and A. Sancar, 1988 Thymine dimers bend DNA. Proc. Natl. Acad. Sci. (U. S. A.) **85**: 2258-2562.

Ito, H., Y. Fukada, K. Murata and A. Kimura, 1983 Transformation of intact yeast cells treated with alkali. J. Bacteriol. **153**: 163-168.

Jackson, J.F. and H.F. Linskens, 1978 Evidence for DNA repair after ultraviolet irradiation of *Petunia hybrida* pollen. Mol. Gen. Genet. **161**: 117-120.

Jackson, J.F. and H.F. Linkens, 1979 Pollen DNA repair after treatment with the mutagens 4-nitroquinoline-1-oxide, ultraviolet and near-ultraviolet irradiation and boron dependence of repair. Mol. Gen. Genet. **176**: 11-16.

Jackson, J.F., 1987 DNA repair in pollen. A review. Mutation Res. **181**: 17-29.

Jagger, J., 1985 Multicellular systems, p. 113-137. In: *Solar-UV Actions on Living cells*. Praeger, New York.

Johnson, R.E., S.T. Handerson, T.D. Petes, S. Prakash, M. Bankmann and L. Prakash, 1992 *Saccharomyces cerevisiae RAD5*-encoded DNA repair protein contains DNA helicase and zinc-binding sequence motifs and affects the stability of simple repetitive sequences in the genome. Mol. Cell. Biol. **12**: 3807-3818.

Jones, J.S., S. Weber and L. Prakash, 1988 The *Saccharomyces cerevisiae RAD18* gene encodes a protein that contains potential zinc finger domains for nucleic acid binding and a putative nucleotide binding sequence. Nucl. Acids Res. **16**: 7119-7131.

Kiefer, J., 1987 UV responses of the temperature-conditional *rad54* mutant of the yeast *Saccharomyces cerevisiae*. Mutation Res. **191**: 9-12.

Kim, S.-T., K. Malhotra, C.A. Smith, J.-S. Taylor and A. Sancar, 1994 Characterization of [6-4] photoproduct DNA photolyase. J. Biol. Chem. **269**: 8535-8540.

Kootstra, A., 1994 Protection from UV-B-induced DNA damage by flavonoids. Plant Mol. Biol. **26**: 771-774.

Kunz, B.A. and B.W. Glickman, 1984 The role of pyrimidine dimers as premutagenic lesions: a study of targeted vs. untargeted mutagenesis in the *lacI* gene of *Escherichia coli*. Genetics **106:** 347-364.

Kunz, B.A., L. Kohalmi, X. Kang and K.A. Magnusson, 1990 Specificity of the mutator effect caused by disruption of the *RAD1* excision repair gene of *Saccharomyces cerevisiae*. J. Bacteriol. **172**: 3009-3014.

Larimer, F.W., J.R. Perry and A.A. Hardigree, 1989 The *REV1* gene of *Saccharomyces cerevisiae*: isolation, sequence, and functional analysis. J. Bacteriol. **171**: 230-237.

Lawrence, C.W., S.K. Banerjee, A. Borden and J.E. LeClerc, 1990. T-T cyclobutane dimers are misinstructive rather than non-instructive mutagenic lesions, Mol. Gen. Genet. **222**: 166-168.

Lawrence, C., 1994 The *RAD6* DNA repair pathway in *Saccharomyces cerevisiae*: what does it do, and how does it work. Bioessays **16**: 253-258.

LeClerc, J.E., A. Borden and C.W. Lawrence, 1991 The thymine-thymine pyrimidine-pyrimidone [6-4] ultraviolet light photoproduct is highly mutagenic and specifically induces 3' thymine-to-cytosine transitions in *Escherichia coli*. Proc. Natl. Acad. Sci. (U. S. A.) **88**: 9685-9689.

Legerski, R. and C. Peterson, 1992 Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature **359**: 70-73.

Lemontt, J.F., 1971 Pathways of ultraviolet mutability in *Saccharomyces cerevisiae.* I. Some properties of double mutants involving *uvs9* and *rev*. Mutat. Res. **13**: 311-317.

Leutwiler, L.S., B.R. Hough-Evans and E.M. Meyerowitz, 1984 The DNA of *Arabidopsis thaliana*. Mol. Gen. Genet. **194**: 15-23.

Li, J., T.-M. Ou-Lee, R. Raba, R.G. Amundson and R.L. Last, 1993 *Arabidopsis* flavonoid mutants are hypersenstitive to UV-B irradiation. The Plant Cell **5**: 171-179.

Li, Y.F., S.T. Kim and A. Sancar, 1993 Evidence for lack of DNA photoreactivating enzyme in humans. Proc. Natl. Acad. Sci. (U. S. A.) **90**: 4389-4393.

Lin, J-J. and A. Sancar, 1992 (A)BC excinuclease: the *Escherichia coli* nucleotide excision repair enzyme. Mol. Microbiol. **6**: 2219-2224.

Lois, R., 1994 Accumulation of UV-absorbing flavonoids induced by UV-B radiation in *Arabidopsis thaliana* L. I. Mechanisms of UV-resistance in *Arabidopsis*. Planta **194**: 498-503.

Lois, R. and B.B. Buchanan, 1994 Severe sensitivity to ultraviolet radiation in an *Arabidopsis* mutant deficient in flavonoid accumulation. II. Mechanisms of UV-resistance in *Arabidopsis*. Planta **194**: 504-509.

Lovett, S.T. and R.K. Mortimer, 1987 Characterization of null mutants of the *RAD55* gene of *Saccharomyces cerevisiae*: effects of temperature, osmotic strength and mating type. Genetics **116**: 547-553.

Lovett, S.T., 1994 Sequence of the *RAD55* gene of *Saccharomyces cerevisiae*: similarity of *RAD55* to prokaryotic RecA and other RecA-like proteins. Gene **142**: 103-106.

Madura, K. and S. Prakash, 1986 Nucleotide sequence, transcript mapping and regulation of the *RAD2* gene of *Saccharomyces cerevisiae*. J. Bacteriol. **166**: 914-923.

Madzak, C. and A. Sarasin, 1991 Mutation spectrum following transfection of ultraviolet-irradiated single-stranded or double-stranded shuttle vector DNA into monkey cells. J. Mol. Biol. **218**: 667-673.

Mandel, M. and A. Higa, 1970 Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53**: 159-162.

McLennan, A.G. and A.C. Eastwood, 1986 An endonuclease activity from suspension cultures of *Daucus carota* which acts upon pyrimidine dimers. Plant Sci. **46**: 151-157.

McLennan, A.G., 1987 The repair of ultraviolet light-induced DNA damage in plant cells. Mutation Res. **181**: 1-7.

Meyerowitz,E.M. and R.E. Pruitt, 1985 *Arabidopsis thaliana* and plant molecular genetics. Science **229**: 1214-1218.

Meyerowitz, E.M., 1989 *Arabidopsis*, a useful weed. Cell **56**: 263-269.

Miller, J.H., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mitchell, D.L., C.A. Haipek and J.M. Clarkson, 1985 [6-4] photoproducts are removed from the DNA of UV-irradiated mammalian cells more efficiently than cyclobutane dimers Mutation Res. **143**: 109-112.

Mitchell, D.L. and R.S. Nairn, 1989 The biology of the [6-4] photoproduct. Photochem. Photobiol. **49**: 805-819.

Moore, P. and B.S. Strauss, 1979 Sites of inhibition of *in vitro* DNA synthesis in carcinogen- and UV-treated øX174 DNA. Nature **278**: 664.

Morelle, G., 1989 A plasmid extraction procedure on a miniprep scale. Focus **11**: 1-8.

Morrison, A., R.B. Christensen, J. Alley, A.K. Beck, E.G. Berntine, J.F. Lemontt and C.W. Lawrence, 1989 *REV3*, a *Saccharomyces cerevisiae* gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. J. Bacteriol. **171**: 5659-5667.

Naegeli, H., L. Bardwell and E.C. Friedberg, 1992a The DNA helicase and adenosine triphosphatase activities of yeast Rad3 protein are inhibited by DNA damage. J. Biol. Chem. **267**: 392-398.

Naegeli, H., L. Bardwell, I. Harosh and E.C. Friedberg, 1992b Substrate specificity of the Rad3 ATPase/DNA helicase of *Saccharomyces cerevisiae* and binding of Rad3 protein to nucleic acids. J. Biol. Chem. **267**: 7839-7844.

Naegeli, H., L. Bardwell and E.C. Friedberg, 1993 Inhibition of Rad3 DNA helicase activity by DNA adducts and abasic sites: implications for the role of a DNA helicase in damage-specific incision of DNA. Biochem. **32**: 613-621.

Nasr, F., N. Bertauche, M.-E. Dufour, M. Minet and F. Lacroute, 1994 Heterospecific cloning of *Arabidopsis thaliana* cDNAs by direct complementation of pyrimidine auxotrophic mutants of *Saccharomyces cerevisiae*. I. Cloning and sequence analysis of two cDNAs catalyzing the second, fifth and sixth steps of the *de novo* pyrimidine biosynthesis pathway. Mol. Gen. Genet. **244**: 23-32.

Naumovski, L. and E.C. Friedberg, 1983 A DNA repair gene required for the incision of damaged DNA is essential for viability in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. (U.S.A.) **80**: 4818-4821.

Naumovski, L. and E.C. Friedberg, 1984 *Saccharomyces cerevisiae RAD2* gene: isolation, subcloning and partial characterization. Mol. Cell. Biol. **4**: 290-295.

Naumovski, L., G. Chu, P. Berg and E.C. Friedberg, 1985 *RAD3* gene of *Saccharomyces cerevisiae*: nucleotide sequence of wild-type and mutant alleles, transcript mapping and aspects of gene regulation. Mol. Cell. Biol. **5**: 17-26.

Nelson, J.R., C.W. Lawrence and D.C. Hinkle, 1996 Thymine-thymine dimer bypass by yeast DNA polymerase ζ. Science **272**: 1646-1649.

Nicolet, C.M., J.M. Chenevert and E.C. Friedberg, 1985 The *RAD2* gene of *Saccharomyces cerevisiae*: nucleotide sequence and transcript mapping. Gene **36**: 225-234.

Nishimura, A., M. Morita, Y. Nishimura and Y. Sugino, 1990 A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. Nucl. Acids Res. **18**: 6169.

Nohmi, T., J.R. Battista. L.A. Dodson and G.C. Walker, 1988 RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and post-translational activation. Proc. Natl. Acad. Sci. (U.S.A.) **85**: 1816-1820.

O'Donovan, A., A.A. Davies, J.G. Moggs, S.C. West and R. Wood, 1994 XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. Nature **371**: 432-435.

Orren, D.K. and A. Sancar, 1989 The (A)BC excinuclease of *Escherichia coli* has only the UvrB and UvrC subunits in the incision complex. Proc. Natl. Acad. Sci. (U.S.A.) **86**: 5237-5241.

Orren, D.K., C.P. Selby, J.E. Hearst and A. Sancar, 1992 Post-incision steps of nucleotide excision repair in *Escherichia coli*. Disassembly of the UvrBC-DNA complex by helicase II and DNA polymerase I. J. Biol. Chem. **267**: 780-788.

Pang, Q. and J.B. Hays, 1991 UV-B-inducible and temperature-sensitive photoreactivation of cyclobutane pyrimidine dimers in *Arabidopsis thaliana*. Plant Physiol. **95**: 536-543.

Pang, Q., J.B. Hays and I. Rajagopal, 1992 A plant cDNA that partially complements *Escherichia coli recA* mutations predicts a polypeptide not strongly homologous to RecA proteins. Proc. Natl. Acad. Sci. (U.S.A.) **89**: 8073-8077.

Pang, Q., J.B. Hays, I. Rajagopal and T.S. Schaefer, 1993a Selection of *Arabidopsis* cDNAs that partially correct phenotypes of *Escherichia coli* DNA-damage-sensitive mutants and analysis of two plant cDNAs that appear to express UV-specific dark repair activities. Plant Mol. Biol. **22**: 411-426.

Pang, Q., J.B. Hays and I. Rajagopal, 1993b Two cDNAs from the plant *Arabidopsis thaliana* that partially restore recombination proficiency and DNA-damage resistance to *E. coli* mutants lacking recombination-intermediate-resolution activities. Nucl. Acids Res. **21**: 1647-1653.

Park, C.H. and A. Sancar, 1994 Formation of a ternary complex between human XPA, ERCC1, and ERCC4 (XPF) excision repair proteins. Proc. Natl. Acad. Sci. (U.S.A.) **91**: 5017-5021.

Patrick, M.H. and R.O. Rahn, 1976 Photochemistry of DNA and polynucleotides: photoproducts. p. 35-95. In: *Photochemistry and Photobiology of Nucleic Acids*, edited by S.Y. Wang. Academic Press, New York.

Pearlman, D.A., S.R. Holbrooke, D.H. Pirkle and S.H. Kim, 1985. Molecular models for DNA damage by photoreaction. Science **227**: 1304-1378.

Pierce, M.K., C.N. Giroux and B.A. Kunz, 1987 Development of a yeast system to assay mutational specificity. Mutation Res. **182**: 65-74.

Prakash, S., P. Sung and L. Prakash, 1993 DNA repair genes and proteins of *Saccharomyces cerevisiae*. Annu. Rev. Genet. **27**: 33-70.

Protic-Sabljic, M. and K.H. Kraemer, 1985 One pyrimidine dimer inactivates expression of a transfected gene in xeroderma pigmentosum cells. Proc. Natl. Acad. Sci. (U. S. A.) **82**: 6622-6626.

Protic-Sabljic, M. and K.H. Kraemer, 1986 Reduced repair of non-dimer photoproducts in a gene transfected into xeroderma pigmentosum cells. Photochem. Photobiol. **43**: 509-513.

Protic-Sabljic, M., N. Tuteja, P.J. Munson, J. Hauser, K.H. Kraemer and K. Dixon, 1986 UV light-induced cyclobutane dimers are mutagenic in mammalian cells. Mol. Cell Biol. **6**: 3349-3356.

Pruitt, R.E. and E.M. Meyerowitz, 1986 Characterization of the genome of *Arabidopsis thaliana*. J. Mol. Biol. **187**: 169-183.

Quaite, F.C., S. Takayanage, J. Ruffini, J.C. Sutherland and B.M. Sutherland, 1994 DNA damage levels determine cyclobutyl pyrimidine dimer repair mechanisms in alfalfa seedlings. The Plant Cell **6**: 1635-1641.

Reynolds, P., D.R. Higgins, L. Prakash and S. Prakash, 1985a The nucleotide sequence for the *RAD3* gene of *Saccharomyces cerevisiae*: a potential adenine nucleotide binding amino acid sequence and a nonessential acidic carboxyl terminal region. Nucleic Acids Res. **13**: 2357-2372.

Reynolds, P., L. Prakash, D. Dumais, G. Perozzi and S. Prakash, 1985b Nucleotide sequence of the *RAD10* gene of *Saccharomyces cerevisiae*. EMBO J. **4**: 3549-3552.

Reynolds, P., L. Prakash and S. Prakash, 1987 Nucleotide sequence and functional analysis of the *RAD1* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**: 1012-1020.

Rupert, C.S., 1962 Photoenzymatic repair of ultraviolet damage in DNA: II. formation of the enzyme-substrate complex. J. Gen. Physiol. **45**: 725-741.

Rupert, C.S., 1975 Enzymatic photoreactivation: overview. Part A. In: *Molecular Mechanisms for Repair of DNA*, edited by P.C. Hanawalt and R.B. Setlow. Plenum, New York, 73.

Rupp, W.D. and P. Howard-Flanders, 1968 Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet radiation. J. Mol. Biol. **31**: 291-304.

Rycena, R.E. and J.L Alderfer, 1985 UV irradiation of nucleic acids: formation, purification and solution conformational analysis of the "6-4 lesion" of dTpdT. Nucl. Acids Res. **13**: 5949-5463.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989 *Molecular Cloning, A Laboratory Manual. Second Edition.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sancar, A. and W.D. Rupp, 1983 A novel repair enzyme: UvrABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region. Cell **33**: 249-260.

Sancar, A. and G.B. Sancar, 1988 DNA repair enzymes. Ann. Rev. Biochem. **57**: 29-67.

Santerre, A. and A.B. Britt, 1994 Cloning of a 3-methyladenine-DNA glycosylase from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. (U.S.A.) **91**: 2240-2244.

Scherly, D., T. Nouspikel, J. Corlet, C. Ucla, A. Bairoch and S.G. Clarkson, 1993 Complementation of the DNA repair defects in xeroderma pigmentosum group G cells by a human cDNA related to yeast *RAD2*. Nature **363**: 182-185.

Schiestl, R.H. and S. Prakash, 1988 *RAD1*, an excision repair gene of *Saccharomyces cerevisiae*, is also involved in recombination. Mol. Cell. Biol. **8**: 3619-3626.

Schiestl, R.H. and R.D. Gietz, 1989 High efficiency transformation of intact yeast cells using single-stranded nucleic acids as carrier. Current Genet. **16**: 339-346.

Selby, C.P. and A. Sancar, 1990 Molecular mechanisms of DNA repair inhibition by caffeine. Proc. Natl. Acad. Sci. (U.S.A.) **87**: 3522-3525.

Sentenac, H., N. Bonneaud, M. Minet, F. Lacroute, J.-M. Salmon, F. Gaymard and C. Grignon, 1992 Cloning and expression in yeast of a plant potassium ion transport system. Science **256**: 663-665.

Setlow, R.B. and P.A. Swenson, 1963. Thymine dimers are inhibition of DNA synthesis by ultraviolet irradiation of cells. Science **142**: 1464.

Sherman, F., G.R. Fink and J.B. Hicks, 1983 *Methods in Yeast Genetics-Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Shinagawa, H., H. Iwasaki, T. Kato and A. Nakata, 1988 RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. Proc. Natl. Acad. Sci. (U.S.A.) **85**: 1806-1810.

Shinohara, A., H. Ogawa and T. Ogawa, 1992 Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell **69**: 457-470.

Shwartz, H., O. Shavitt and Z. Livneh, 1988 The role of exonucleolytic processing and polymerase-DNA association in bypass of lesions during replication *in vitro*. J. Biol. Chem. **263**: 18277-18285.

Sibghat-Ullah, A. Sancar and J.E. Hearst, 1990 The repair patch of *E. coli* (A)BC excinuclease. Nucl. Acids Res. **18**: 5051-5053.

Seide, W. and F. Eckardt-Schupp, 1986 DNA repair genes of *Saccharomyces cerevisiae*: complementing *rad4* and *rev2* mutations by plasmids which cannot be propagated in *Escherichia coli*. Curr. Genet. **11**: 205-210.

Siede, W., G.W. Robinson, D. Kalainov, T. Malley and E.C. Friedberg, 1989 Regulation of the *RAD2* gene of *Saccharomyces cerevisiae*. Mol. Microbiol. **3**: 1697-1707.

Siede, W., A.S. Friedberg and E.C. Friedberg, 1993 Evidence that the Rad1 and Rad10 proteins of *Saccharomyces cerevisiae* participate as a complex in nucleotide excision repair of UV radiation damage. J. Bacteriol. **175**: 6345-6347.

Small, G.D., 1987 Repair systems for nuclear and chloroplast DNA in *Chlamydomonas reinhardtii*. Mutation Res. **181**: 31-35.

Smith, A.G., M.A. Santana, A.D.M. Wallace-Cook, J.M. Roper and R. Labbe-Bois, 1994 Isolation of a cDNA encoding chloroplast ferrochelatase from *Arabidopsis thaliana* by functional complementation of a yeast mutant. J. Biol. Chem. **269**: 13405-13413.

Spivak, G and P.C. Hanawalt, 1992 Translesion DNA synthesis in the dihydrofolate reductase domain of UV-irradiated CHO cells. Biochemistry **31**: 6794-6800.

Sugino, A., B.H. Ryu, T. Sugino, L. Naumovski and E.C. Friedberg, 1986 A new DNA-dependent ATPase which stimulates yeast DNA poylmerase I and has DNA-unwinding activity. J. Biol. Chem. **261**: 11744-11750.

Sullivan, M.L. and R.D. Vierstra, 1989 A ubiquitin carrier protein from wheat germ is structurally and functionally similar to the yeast DNA repair enzyme encoded by *RAD6*. Proc. Natl. Acad. Sci. (U.S.A.) **86**: 9861-9865.

Sullivan, M.L. and R.D. Vierstra, 1991 Cloning of a 16-kDa ubiquitin carrier protein from wheat and *Arabidopsis thaliana*. J. Biol. Chem. **266**: 23878-23885.

Sung, P., L. Prakash, S. Weber and S. Prakash, 1987a The *RAD3* gene of *Saccharomyces cerevisiae* encodes a DNA-dependent ATPase. Proc. Natl. Acad. Sci. (U.S.A.) **84**: 6045-6049.

Sung, P., L. Prakash, S.W. Matson and S. Prakash, 1987b RAD3 protein of *Saccharomyces cerevisiae* is a DNA helicase. Proc. Natl. Acad. Sci. (U.S.A.) **84**: 8951-8955.

Sung, P., L. Prakash and S. Prakash, 1992 Renaturation of DNA catalyzed by yeast DNA repair and recombination protein RAD10. Nature **355**: 743-745.

Sung, P., P. Reynolds, L. Prakash and S. Prakash, 1993a Purification and characterization of the *Saccharomyces cerevisiae* RAD1/RAD10 endonuclease. J. Biol. Chem. **268**: 26391-26399.

Sung, P., V. Bailly, C. Weber, L.H. Thompson, L. Prakash and S. Prakash, 1993b Human xeroderma pigmentosum group D gene encodes a DNA helicase. Nature **365**: 852-855.

Sung, P., J.F. Watkins, L. Prakash and S. Prakash, 1994 Negative superhelicity promotes ATP-dependent binding of yeast RAD3 protein to ultraviolet-damaged DNA. J. Biol. Chem. **269**: 8303-8308.

Sutherland, B.M., 1974 Photoreactivating enzyme from human leukocytes. Nature **289**: 109-112.

Sutherland, B.M., P. Runge and J.C. Sutherland, 1974 DNA photoreactivation enzyme from placental mammals. Origins and characteristics. Biochemistry **13**: 4710-4715.

Sutherland, B.M., 1975 The human leukocyte photoreactivating enzyme, p. 107-113. In: *Molecular Mechanisms for Repair of DNA*, edited by P.C. Hanawalt and R.B. Setlow. Plenum Plublishing Corp., New York.

Sutherland, B.M. and R. Oliver, 1976 Culture conditions affect photoreactivating enzyme levels in human fibroblasts. Biochim. Biophys. Acta **442**: 358-367.

Sutherland, B.M., R. Oliver, C.O. Fuselier and J.C. Sutherland, 1976 Photoreactivation of pyrimidine dimers in the DNA of normal and xeroderma pigmentosum cells. Biochemistry **15**: 402-406.

Sutherland, B.M., L.C. Harber and I.E. Kochevar, 1980 Pyrimidine dimer formation and repair in human skin. Cancer Research. **40**: 3181-3185.

Swenson, P.A. and R.B. Setlow, 1966 Effects of ultraviolet radiation on macromolecular synthesis in *E. coli*. J. Mol. Biol. **15**: 201.

Taylor, J.S. and C.L. O'Day, 1990 Cis-syn thymine dimers are not absolute blocks to replication by DNA polymerase I of *Escherichia coli in vitro*. Biochemistry **29**: 1624-1632.

Thibodeau, L. and W.G. Verly, 1976 Endonuclease for apurinic sites in plants. FEBS Letters **69**: 183-185.

Thuring, R.W.J., J.P.M. Sanders and P. Borst, 1975 A freeze-squeeze method for recovering DNA from agarose gels. Anal. Biochem. **66**: 213-220.

Thompson, L.H., K.W. Brookman, C.A. Weber, E.P. Salazar, J.T. Reardon, A. Sancar, Z. Deng and M.J. Siciliano, 1994 Molecular cloning of the human nucleotide excision-repair gene *ERCC4*. Proc. Natl. Acad. Sci. (U.S.A.) **91**: 6855-6859.

Todo, T., H. Takemori, H. Ryo, M. Ihara, T. Matsunaga, O. Nikaido, K. Sato and T. Nomura, 1993 A new photoreactivating enzyme that specifically repairs ultraviolet light-induced (6-4) photoproducts. Nature **361**: 371-374.

Tomkinson, A.E., A.J. Bardwell, L. Bardwell, N.J. Tappe and E.C. Friedberg, 1993 Yeast DNA repair and recombination proteins Rad1 and Rad10 constitute a single-stranded DNA endonuclease. Nature **362**: 860-862.

Tomkinson. A.E., A.J. Bardwell, N. Trappe, W. Ramos and E.C. Friedberg, 1994 Purification of Rad1 protein from *Saccharomyces cerevisiae* and further characterization of the Rad1/Rad10 endonuclease complex. Biochemistry **33**: 5305-5311.

Tsao, S.G.G, C.F. Brunk and R.E. Pearlman, 1983 Hybridization of nucleic acids directly in agarose gels. Anal. Biochem. **131**: 365-372

van Duin, M., J. de Wit, H. Odijk, A. Westerveld, A. Yasui, M.H.M. Koken, J.H.J. Hoeijmakers and D. Bootsma, 1986 Molecular characterization of the human excision repair gene *ERCC-1*: cDNA cloning and amino acid homology with the yeast DNA repair gene *RAD10*. Cell **44**: 913-923.

Van Houten, B., 1990 Nucleotide excision repair in *Escherichia coli*. Microbiol. Rev. **54**: 18-51.

Velemínsky, J. and K.J. Angelis, 1990 DNA repair in higher plants. In: *Mutation and the Environment*. pp. 195-203. Wiley-Liss Inc.

Villani, G., S. Boiteux and M. Radman, 1978. Mechanisms of ultraviolet induced mutagenesis: extent and fidelity of *in vitro* DNA synthesis on irradiated templates. Proc. Natl. Acad. Sci. (U. S. A.) **75**: 3037.

Walker, G.C., 1984 Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiological Reviews **48**: 60-93.

Wang, S.Y. and A.J. Varghese, 1967 Cytosine-thymine addition product from DNA irradiated with ultraviolet light. Biochem. Biophys. Res. Comm. **29**: 543-549.

Weber, C.A., E.P. Salazer, S.A. Stewart and L.H. Thompson, 1990 *ERCC2*: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast *RAD3*. EMBO J. **9**: 1437-1447.

Weiss, W.A. and E.C. Friedberg, 1985 Molecular cloning and characterization of the yeast *RAD10* gene and expressing of RAD10 protein in *E. coli*. EMBO J. **4**: 1575-1582.

Witkin, E.M., 1976 Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. Bacteriol. Rev. **40**: 869-907.

Wood, R.D., 1985 Pyrimidine dimers are not the principle pre-mutagenic lesion induced in lambda phage DNA by ultraviolet light. J. Mol. Bio. **184**: 577-585.

Woodgate, R., B.A. Bridges, G. Herrera and M. Blance, 1987 Mutagenic DNA repair in *Escherichia coli* XIII. Proofreading exonuclease of DNA polymerase III holoenzyme. Proc. Natl. Acad. Sci. (U.S.A.) **81**: 7747-7751.

Yang, E. and E.C. Friedberg, 1984 Molecular cloning and nucleotide sequence analysis of the *Saccharomyces cerevisiae RAD1* gene. Mol. Cell. Biol. **4**: 2161-2169.

Yeung, A.T., W.B. Mattes, E.Y. Oh and L. Grossman, 1983 Enzymatic properties of purified *Escherichia coli* uvrABC proteins. Proc. Natl. Acad. Sci. (U.S.A.) **80**: 6157-6161.