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Repair Of Uv-irradiated Plasmids In Escherichia Coli And Chinese Hamster Cells

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REPAIR OF UV-IRRADIATED PLASMIDS IN *Escherichia coli* AND CHINESE HAMSTER CELLS

by

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ABSTRACT

Nucleotide excision repair of DNA, demonstrated by the removal of cyclobutane pyrimidine dimers (CPDs), proceeds much more rapidly in genes undergoing transcription than in inactive genes and this rapid repair is confined to the strand being transcribed. The phenomenon is referred to as transcription-repair coupling and it has been demonstrated to occur in the chromosomes of mammalian cells, yeast cells and *Escherichia coli*. It was of interest to examine the repair characteristics of plasmids both in *E. coli* and Chinese hamster (CHO) cells. Because of the potential for manipulating the DNA damage in the plasmid, for choosing the genetic background of the cells and for varying the transcriptional efficiency of the plasmid, it was anticipated that studies with this model system would yield useful information about nucleotide excision repair. In the studies with *E. coli*, cells harbouring the plasmid were UV-irradiated while in the work with CHO cells, the plasmid was UV-irradiated and then transfected into the cells. The plasmid used in this study was pGA293. After being extracted form *E. coli* or CHO cells, it was cut with restriction endonucleases to generate domains of 2.4, 3.0, 3.5, and 5.5 Kbp and repair (removal of CPDs) was examined in these domains. The 3.5 Kbp domain contains the *β-lactamase* gene; the 3.0 Kbp domain contains the *β-galactosidase* gene; the 2.4 Kbp domain contains a portion of the *tet* gene.

Some of the findings obtained with the *E. coli* system were the following: The Rec A function is necessary for the efficient repair of pGA293 in *E. coli*.

Unexpectedly, both strands of the 3.5 Kbp and 2.4 Kbp domains of pGA293 were repaired with the same kinetics. An examination of the sequences of the designated non-transcribed strands revealed the presence of potential promoter sequences which left open the possibility that these strands were in fact being transcribed. The rates of repair of both strands of the 3.5 Kbp and 2.4 Kbp domains were slower than that reported for the transcribed strand of the *E. coli* chromosomal *lacZ* gene. However, there were 10 copies of the pGA293 genes but only 1 copy of the chromosomal gene, and this along with the greater number of CPDs in the plasmid DNA, could account for the slower rate of repair. These considerations made it likely that for both strands in the 3.5 Kbp and 2.4 Kbp domains transcription repair coupling was being observed. For the 3.0 Kbp domain, the repair rate for the non-transcribed strand was slower than that for the transcribed strand and was the same as the repair rate reported for the non-transcribed strand of the *E. coli* chromosomal *lacZ* gene. This suggests that the transcribed strand of the 3.0 Kbp domain was undergoing coupled repair while the other strand was not.

Using the CHO system, the following information was obtained: Expression of β-galactosidase from irradiated pGA293 recovered readily after transformation, indicating that repair had taken place. However, it was not possible to demonstrate removal of CPDs from the 3.0 Kbp domain of pGA293 which contains the transcribed *β-galactosidase* gene. This was the case for a number of transfection protocols and when using between 0.2 - 10 μg of plasmid per $2x10^6$ cells. In similar experiments, repair could not be

demonstrated in the 3.5 Kbp and 5.5 Kbp domains which are presumed not to be transcribed in CHO cells. Using amount of pGA293 between 0.2 and 5 μg for transfection, many plasmid molecules entered the nucleus. Only a small fraction of these were converted into minichromosomes which were detected as the supercoiled form of the plasmid. It is suggested that it is only the plasmid in the minichromosome form that is transcribed and repaired efficiently. This rare event makes it difficult to detect repair in the plasmid.

This thesis is dedicated to my mother

Kaiser Jehan Begum

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M.R. Baig

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ABBREVIATIONS

AAF	acetyl-2-aminofluorene
ADA	adenosine deaminase
AMP	ampicillin
AP	apurinic or apyrimidinic site
bp	base pair(s)
BrdUrd	bromodeoxyuridine
BSA	bovine serum albumin
CFE	cell free extract
CHO	Chinese hamster ovary
CPD	cyclobutane pyrimidine dimer
CS	Cockayne's syndrome
DEPC	diethylpyrocarbonate
DHRF	dihydrofolate reductase
DRP	damage recognition protein
dRpase	DNA deoxyribophosphodiesterase
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetate
ERCC*	excision repair cross-complementing rodent repair deficiency
ESS	endonuclease-sensitive site
FACC	Fanconi anaemia group C complementing

$FADH_2$	1,5 dihydroflavin adenine dinucleotide
FAPY	formamidopyrimidine
GPT	guanine phosphoribosyl transferase
HCFE	human cell free extract
Hepes	N (2-hyroxyethyl) piperazine-N-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
HPRT	hypoxanthine (guanine) phosphoribosyltransferase
HSSB	human single stranded binding protein
IDP	intracyclobutyl pyrimidine dimer DNA phosphodiesterase
IPTG	isopropyl β-D-thiogalactoside
LB	Luria broth
MFD	mutation frequency decline
MTHF	5,10 methenyl tetrahydrofolate
NER	nucleotide excision repair
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
Phr	photoreactivation
PRE	photoreactivating enzyme
PRP	photolyase regulatory protein
rDNA	ribosomal RNA genes
SDS	sodium dodecyl sulfate
SOD	superoxide dimutase

SV40	simian virus 40
TCA	trichloroacetic acid
TMP	thymidine monophosphate
TRCF	transcription repair coupling factor
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
TTD	trichothiodystrophy
USD	unscheduled DNA synthesis
UV	ultraviolet radiation
UV-DRP	UV-damaged DNA recognition protein
VSRP	very short repair pathway
XP	xeroderma pigmentosum
YCFE	yeast cell free extract

* A number of *ERCC* genes such as *ERCC1*, *ERCC2*, and *ERCC3*, capable of correcting nucleotide excision repair in the rodent cell lines, have been isolated using gene transfer strategies.

CHAPTER 1

HISTORICAL BACKGROUND AND INTRODUCTION

1.0 Effect of ultraviolet irradiation on *Escherichia coli*

1.1.0 Characterization of *Escherichia coli* DNA repair mutants

An ultraviolet light sensitive mutant, B_{s-1} derived from of *Escherichia coli B*, was discovered by Hill in 1958 when she was trying to isolate strains of *E. coli* that would be more resistant to the killing effect of ultraviolet-irradiation. This discovery was surprising to the radiobiologists because they were more concerned up to that time with the isolation of radiation-resistant strains (Hill, 1958). Later, *E. coli* K12 ultraviolet sensitive mutants containing mutations at three genetically distinct loci, *uvrA*, *uvrB*, and *uvrC* were isolated (Howard-Flanders and Theriot, 1962). These mutants are of historical importance in the repair field because their study and characterization led to the formulation of the major steps in DNA repair.

1.1.2 Discovery of Nucleotide Excision Repair

In 1964 two independent groups simultaneously reported that ultraviolet-sensitive *E. coli* K12 mutants uvrA and B_{s-1} could not excise the ultraviolet-induced cyclobutane pyrimidine dimers from DNA during a post-irradiation incubation. However, there was efficient excision of damaged nucleotides in most normal strains and by chemical analysis it was shown that the excision products were present in the acid-soluble fraction and disappeared from the acid-insoluble fraction during post-UV incubation. By using other mutants it

was established that two other genetic loci, *uvrB* and *uvrC* were also required for the excision of pyrimidine dimers from DNA. The double mutants of these genes were not dramatically different in their sensitivities to UV light compared with their single mutants. These findings gave rise to the concept that thymine dimers were excised from the DNA in the form of an oligonucleotide and that a multi-enzyme complex was involved (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964; van de Putte et al., 1965; Howard-Flanders et al., 1966; Howard-Flanders and Boyce, 1966). From these observations, it was established that *uvrA*, *uvrB*, and *uvrC* genes were essential for the excision of damaged nucleotides from DNA and studies with a cell free extract system supported this idea when it was found that all three gene products were required for the excision of ultraviolet-irradiated DNA; hence the term nucleotide excision repair was born (Shimada et al., 1968; Rupp and Howard-Flanders, 1968; Seeberg et al., 1976, 1978; Friedberg, 1985).

The *mfd* mutant, which was isolated and characterized by Witkin in 1966, was devoid of the mutation frequency decline phenomenon. Mutation frequency decline is a phenomenon in which an antimutation mechanism is operative in *E. coli* that depends on excision repair and is characterized by a rapid, irreversible loss of mutations during post-irradiation incubation. The loss-of-mutation-decline phenomenon affects only the mutation frequency and has no effect on survival. The discovery of the mutant helped to establish the relationship between repair and transcription (Witkin, 1966; Bridges and Munson, 1966; Witkin, 1969; Doudney, 1974; Selby and Sancar, 1993).

1.2 Effect of Ultraviolet Radiation on Biological Systems

The ozone layer in the upper atmosphere of the earth effectively shields the surface of our earth from ultraviolet radiation (Molina and Molina, 1986). A serious ultraviolet radiation threat has emerged with the depletion of the ozone layer in the atmosphere of our globe by man-made insults such as chlorofluorocarbons. The ultraviolet radiation range of UVA (320-400 nm) can induce the photoreactivation process as well as photodamage in living communities, but the amount of this radiation is independent of the concentration of atmospheric ozone. Thus, a change in the ozone level does not affect the radiation level in the UVA range. The ultraviolet radiation range of UVB (280-320 nm) is extremely harmful to living organisms because it damages DNA, and the level of UVB is dramatically increased with depletion of atmospheric ozone. The most injurious ultraviolet wavelength range, UVC (200-280 nm) is efficiently absorbed by our atmosphere before causing biological hazards on the surface of the earth (Caldwell et al., 1986; Anderson et al., 1991; Smith et al., 1992).

A variety of lesions are generated in DNA by UV-light including thymine glycols and interstrand cross links; however, cyclobutane pyrimidine dimers and pyrimidone (6-4) pyrimidine photoproducts are the most frequent lesions and both have been demonstrated to be mutagenic in *Escherichia coli* and mammalian cells (Friedberg, 1985; Schmid et al., 1982; Glickman et al., 1986; Drobetsky et al., 1987, 1989; Bourre et al., 1989; Mitchell and Nairn, 1989). The frequency of (6-4) photoproducts is about 10-30% of cyclobutane pyrimidine

dimers (Mitchell et al., 1990a).

1.3 Diverse Pathways for the Removal of DNA Damage

The DNA of organisms is subject to damage notably by UV- light but also by endogenous agents such as reactive oxygen species generated during normal metabolism (Demple and Amabile-Cuevas, 1991; Grollman and Moriya, 1993) or a multitude of exogenous insults that have evolved with man made exploitation of nature (Crutzen, 1992; Thompson, 1991; Lindahl, 1993). Nature has endowed living communities as simple as *Escherichia coli* or as complex as mammalian cells with a number of biochemical defensive systems to counteract this damage (Friedberg, 1985). These systems include enzymatic photoreactivation of pyrimidine dimers, base excision repair, inducible DNA repair, mismatch repair and nucleotide excision repair.

1.3.1 Enzymatic Photoreactivation of Pyrimidine Dimers

Photoreactivation was discovered serendipitously in the late 1940's by Albert Kelner in the course of his studies on the effect of UV-light radiation on *Streptomyces griseus*. He demonstrated that exposure of UV-irradiated fungi to visible light increased their survival rate over those kept in the dark. The term photoreactivation was coined by Renato Dulbecco working at the same time at Indiana University, when he discovered the reactivation of UV-light inactivated bacteriophage by visible light (Kelner, 1949; Dulbecco, 1949). An activity that catalyzed photoreactivation was discovered in *E. coli* extracts in 1958. When UV-irradiated DNA was incubated with bacterial extract in the presence of visible light, its biological activity returned (the biological activity

measured was transformation). The enzyme responsible for removing the UV-damage was called photoreactivating enzyme or DNA photolyase (Rupert et al., 1958; Rupert, 1960; Harm et al., 1968; Harm, 1980; Werbin and Madden, 1977).

The interactions between DNA photolyase and substrate occur 3-4 nucleotides on each side of the dimer and catalyzes the cyclobutane ring splitting upon absorption of visible light energy (Friedberg, 1985; Sancar and Sancar, 1988; Sancar, 1990). The sugar-phosphate backbone is a key structural requirement for efficient binding of the photolyase to its substrate (Kim and Sancar, 1991). It has also been demonstrated that DNA photolyase can monomerize cyclobutane pyrimidine photoproducts with a cleaved intradimer phosphodiester linkage in polynucleotides but does so at a rate six to seven fold slower than for intact dimers (Weinfeld and Paterson, 1988; Liuzzi and Paterson, 1992). Genes encoding *photolyases* from various species have been cloned and characterized and it has been shown that there are structural and functional domains conserved among them (Sancar and Sancar, 1984a; Sancar et al., 1984b; Sancar et al., 1985; Yasui and Langeveld, 1985; Yasui et al., 1988; Sancar, 1990; Takao et al., 1989). Photolyases possess at least three chromophores which are responsible for facilitating the photoreactivation. (Eker et al., 1988; Johnson et al., 1988; Kim et al., 1992a,b,c).

In addition to mediating photoreactivation, photolyases are also involved in interacting with nucleotide excision repair. It has been suggested that photolyases increase the efficiency of nucleotide excision repair (Yamamoto, et.

al., 1983; Boyd and Harris, 1987; Sancar and Smith, 1989). A protein called a photolyase regulatory protein (PRP) which is detectable during normal growth also disappeared after UV irradiation. The binding site for PRP is upstream from the *photolyase-I* gene, and deletion of this sequence increased the basal level of expression of the *photolyase-I* gene (Sebastian, et. al., 1990; Sebastian and Sancar, 1991).

1.4 Base Excision Repair

Potentially, genetically harmful methylated bases are generated naturally by a reaction with S-adenosylmethionine, and experimentally, by exposing the cell to a methylating agent such as N-nitrosourea. Both in *E. coli* and human cells, a distinct mechanism known as base excision repair has evolved to repair the modified bases. In this process, the modified bases are excised by DNA glycosylases generating apurinic sites (AP sites). AP sites are then repaired by the stepwise reactions of AP endonuclease, exonuclease, DNA polymerase and DNA ligase (Lindhal et al., 1988; Friedberg, 1985; Sancar and Sancar, 1988). Much insight into the base excision repair process has been gained by purification and cloning of the enzymes involved. (Pierre and Laval, 1986; Karen and Lindhal, 1980; Riazuddin, et. al., 1987; Blaisdell and Warner, 1983; Sakumi and Sekiguchi, 1989).

1.5 Inducible DNA Repair of Damage by Alkylating Agents

The inducible *ada* gene of *E. coli* is responsible for encoding a 39 KDa protein that shows methyl transfer activity for O^6- methylguanine, O^4-methylthymine and methylphosphotriesters on a double stranded DNA (Nakabeppu et al., 1985; Teo et al., 1984; McCarthy et al., 1984). The

methyltransferase activity becomes irreversibly inactivated by accepting the methyl group onto cysteine residue 69 in a direct, error free, damage-reversal process (Lindahl et al., 1988) The Ada protein has two domains containing separate transfer activites for O^6-methylguanine and for methylphosphotriesters. The expression of the *ada* gene is controlled at the transciptional level and its product binds to its promoter after methylation at cysteine residue 69 (Nakabeppu et al., 1985; Sedgewick et al., 1987). Similar methyl transferases for repairing O^6-methylguanine in DNA have been demonstrated in other organisms such as *Drosophila*, fish, and human cells (Green and Deutsch, 1983; Nakatsuru et al., 1987; Day et al., 1987).

1.6 Mismatch Repair

Mismatches are generated in DNA during replication, and they can also arise spontaneously through hydrolytic deamination of 5-methylcytosine and during recombination of nonidentical DNA fragments. The repair of mismatches relies on the two following factors - mismatch recognition and strand discrimination. Mismatch recognition is required to target the repair enzymes to the sites of errors. In *E. coli* base-base mispair recognition is mediated by a protein of 97 kDa known as Mut S. This protein recognizes the mispairing of purine/pyrimidine and purine/purine types. The Mut L protein has been demonstrated to stabilize the Mut S/mismatch DNA complex. The strand discrimination for the repair of mismatches generated during replication is achieved in favor of the parental DNA strand by a methylation-directed pathway that uses the undermethylated adenine residue of a d(GATC) site of the nascent DNA as a discriminatory signal. The repair is initiated by the Mut H protein,

which incises the newly replicated DNA duplex 5′ from the undermethylated adenine of the d(GATC). The repair synthesis of this type is carried out by DNA pol III holoenzyme. Other organisms use different types of discriminatory signal (Modrich, 1987 and 1991; Jircny, 1991).

The hydrolytic deamination of 5-methylcytosine generates G/T type mispairs which are repaired by the very short repair pathway (VSP) which is characterized by short patches of repair synthesis. The repair is initiated by incising the DNA heteroduplex 5′ from the mispaired thymine by the *VSR* gene product which is an endonuclease of 18 kDa molecular weight. The incision is mismatch-dependent and strand-specific. This enzyme has been purified and characterized as a DNA mismatch endonuclease (Lieb, 1985; Hennecke et al., 1991). In *E. coli*, G/A is another mismatch generated during replication which does not require the discriminatory signal. This mismatch is repaired by the Mut Y protein which is a DNA glycosylase and removes the mismatched adenine (Lu and Chang, 1988; Au et al., 1989).

It has been demonstrated by using SV40 heteroduplexes that higher eucaryotes are capable of repairing all known mismatches *in vivo* (Brown and Jiricny, 1987, 1988). It has also been shown that HeLa nuclear extracts possess a protein that binds specifically to oligonucleotides containing G/T mismatches. The repair of G/T mismatches on a synthetic substrate under *in vitro* conditions has been demonstrated and repair synthesis has been shown to be mediated by DNA polymerase ß (Wiebauer and Jiricny, 1989, 1990).

1.7.0 Nucleotide Excision Repair

1.7.1 Induction of SOS Response

In *E. coli*, the enzymes responsible for repairing DNA are present normally in small amounts, but when cellular DNA is damaged the repair genes are induced by the so called SOS system and DNA repair enzymes are produced in large amounts to meet the challenge posed by the damaged DNA. The SOS system in *E. coli* was first postulated by Defais in 1971 and further developed by Radman (Defais et al., 1971; Radman, 1974, 1975). There are at least 20 genes which are induced in the SOS system by DNA-damaging agents. From the point of view of this thesis, the important members of this group of induced genes are *uvrA*, *uvrB*, *uvrD*, *rec A* and *ssb*. The model of the SOS regulatory system is schematically depicted in Figure 1.1A (Little and Mount, 1982; Walker, 1987; Kenyon and Walker, 1980; Walker, 1984).

Even in the repressed state, SOS genes are transcribed at a significant level. The operator regions of SOS genes have considerable homology with one another and their expression is inhibited differentially by binding of Lex A protein to consensus sequences because the strength of Lex A binding to these different operators varies considerably (Walker, 1984). The SOS gene network is controlled by Rec A and Lex A proteins. The central event in response to DNA damage is the induction of SOS genes by activated Rec A protein through its binding to ssDNA and ATP, which in turn promotes the cleavage of Lex A repressor at an Ala-Gly peptide located near the middle of the protein (Walker, 1987). The cleavage of Lex A can also be observed in absence of Rec A protein and this autodigestion is either inhibited or increased in certain mutants. It is believed that Rec A facilitates autodigestion rather than acting directly as a protease. Whatever the mechanism of Lex A cleavage, the central idea is the

same. It must be cleaved to increase the expression of the SOS genes (Little, 1984; Smith et al., 1991; Roland et al., 1992).

E. coli contains 500 RecA protein molecules per cell in normally growing cells and this level is increased to about 500,000 molecules per cell in response to DNA damage. This little versatile protein contains 352 amino acids, possesses binding sites for single stranded DNA, double stranded DNA, ATP and for repressor molecules (Dicapua and Koller, 1987). In order to induce the SOS induction signal after UV-irradiation there is a requirement for DNA replication such that DNA replication gaps are created at a dimer site. This situation generates single stranded DNA which is the site of RecA activation. The completion of repair is reflected in the reappearance of Lex A in *E. coli* accompanied by loss of the SOS inducing signal (Sassanfar and Roberts, 1990; Tessman and Peterson, 1985).

1.7.2 Protein Products of *uvrA*, *uvrB*, and *uvrC* Genes

The *uvrA*, *uvrB*, and *uvrC* genes have been cloned, sequenced and recombinant proteins have been overproduced (Sancar et al., 1981a, b, c; Sancar and Sancar, 1988; Backendorf et al., 1986; Thomas et al., 1985; Reardon and Sancar, 1991). The *uvrA* gene size is 2820 bp and encodes a protein of molecular mass 103.8 kDa. This gene is constituted of a single operon and is under SOS regulation. The level of uvrA is increased from about 25 molecules per cell to 250 molecules per cell, under SOS induction. The uvrA protein is a dimer under physiological conditions with two zinc finger DNA-binding motifs and ATPase activity (Orren and Sancar, 1989; Husain et al;, 1986; Selby and Sancar, 1990a; Van Houten, 1990; Hoeijmaker, 1993a).

The *uvrB* gene, which is 2019 bp long and is transcribed from a constitutive and an inducible promoter, encodes a monomeric form of protein of molecular mass 78 kDa. The constitutive cellular level of this protein is about 10 fold higher than uvrA and is further increased about 4 fold upon SOS induction. The uvrB protein is not a DNA binding protein but is loaded onto damaged DNA by uvrA protein (Sancar et al., 1982; Arikan et al., 1986; Markham et al., 1984; Orren and Sancar, 1989; Lin and Sancar, 1992a). The *uvrC* gene is 1930 bp long and encodes a protein with a molecular mass of 68.5 kDa. This gene is transcribed at a relatively low level and is not induced by the SOS response (Sancar and Sancar, 1988; Foster and Strike, 1988). The uvrC protein does not associate with uvrA and uvrB but has a binding affinity for a complex of DNA and uvrB protein bound at the damage site that is generated after dissociation of uvrA protein from the preincision complex (Lin and Sancar, 1991, 1992a; Selby and Sancar, 1990a).

1.7.3 Mechanism of Excision Reaction by ABC Excinuclease

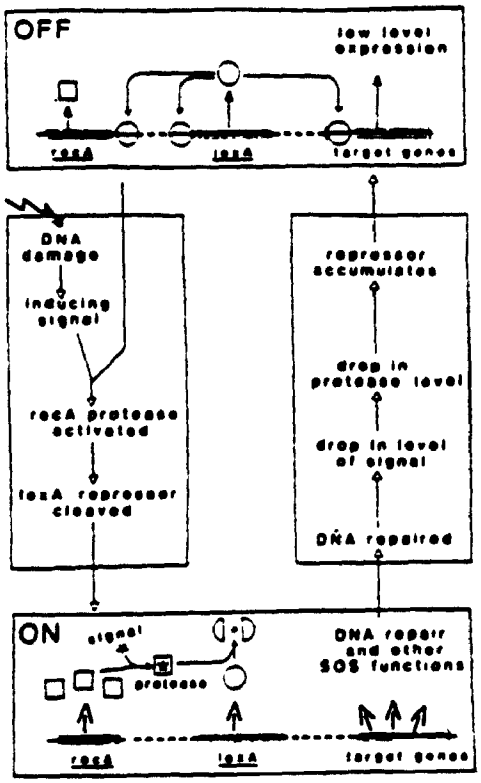
The molecular mechanism of nucleotide excision repair in *E. coli* is schematically depicted in Figure 1.1B. DNA damage is first recognized by a recognition complex which is generated in an ATP dependent reaction wherein a uvrA dimer associates with one molecule of uvrB. It is believed that DNA becomes either denatured or kinked upon binding of the recognition complex at the damaged site in the process of nucleotide excision repair (Orren and Sancar, 1989, 1990; Lin et al., 1992, Lin and Sancar, 1992a). At this stage uvrA dimer dissociates from the uvrB-DNA complex. This new complex is not only very stable but also constitutes a highly specific site for uvrC protein. It has

FIGURE 1.1A A MODEL FOR THE SOS REGULATORY SYSTEM IN *ESCHERICHIA coli.*

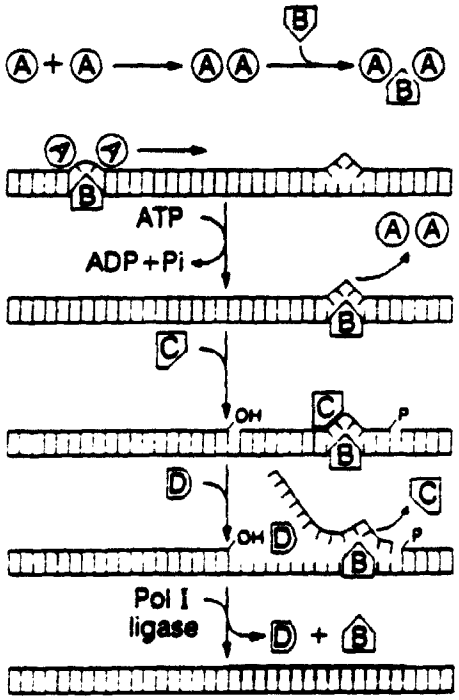
LexA is the repressor of at least 20 genes on the *E. coli* chromosome. A SOS inducing signal is generated after DNA damage. LexA repressor is cleaved leading to derepression of the SOS regulator. The induced SOS state is transient and cell division is inhibited in this state. As DNA damage is repaired, the inducing signal disappears. LexA repressor starts accumulating in cells to repress the SOS genes. (This model was adapted from Little and Mount, 1982.)

FIGURE 1.1B A MODEL FOR THE NUCLEOTIDE EXCISION REPAIR MECHANISM IN *ESCHERICHIA coli*

ABC Excinulease initiates incision process when $(uvrA)_2(uvrB)$ complex is targetted to a lesion site, then uvrA dissociates from uvrB damaged DNA complex. uvrC interacts with bound uvrB and this uvrBC heterodimer is responsible for a dual incision in the damaged strand. Excision of the damage-containing oligonucleotide and turnover of uvrB and uvrC are dependent on the concerted action of uvrD and DNA polymerase I. This ABC excinuclease pathway repairs a wide range of DNA damages produced by many genotoxicants. (This model was adapted from Lin and Sancar, 1992a.)


OFF
low level expression
recA
lexA
target genes
DNA damage
inducing signal
recA protease activated
lexA repressor cleaved
repressor accumulates
drop in protease level
drop in level of signal
DNA repaired
ON
signal
protease
DNA repair and other SOS functions
recA
lexA
target genes

A


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

B

been demonstrated that both uvrB and uvrC possess nuclease activity in this new complex. uvrC cuts on the 5′ side of the lesion while uvrB cuts on the 3′ side of the lesion. The damaged nucleotide within a 12-13 bp fragment is released along with uvrC by helicase II. It is DNA polymerase I that releases uvrB and fills the gap (Lin et al., 1992; Lin and Sancar, 1992a,b; Orren et al., 1992; Sibghat-ullah et al., 1990). In a standard reaction, there is cleavage of the 8th phosphodiester bond 5′ and the 4th or 5th phosphodiester bond 3′ to the adduct generating a 12-13 bp fragment. The incision on the 5′ side is fixed while a variable incision is observed at the 4th or 5th phosphodiester bond on the 3′ side of the adduct (Sancar and Rupp, 1983). It has also been reported that incisions at the lesion site are salt and pH dependent (Sancar and Sancar, 1988).

1.7.4 Damage Recognition

ABC excinuclease has the remarkable ability to work on DNA lesions of different molecular natures and structures such as produced by UV light, mitomycin C, psoralen and therapeutic drugs that bend DNA (Husain et al., 1988; Lambert et al., 1989; Dombert et al., 1989; Selby and Sancar, 1991a). O^6-methylguanine lesions which do not generate any appreciable distortion to the sugar phosphate backbone are also repaired by ABC excinuclease, but inefficiently (Voigt et al., 1989). It has also been demonstrated that ABC excinuclease can repair apurinic/apyrimidinic sites and thymine glycols which were thought to be exclusively repaired by the base excision repair process (Lin and Sancar, 1989). However, naturally bent DNA, phosphotriesters, monointercalators such as ethidium bromide and chloroquine, do not serve as

substrates for ABC excinclease (Selby and Sancar, 1990a, 1991a). It has also been demonstrated that patterns of incision are sequence dependent, lesion dependent and incision could be at the 6th phosphodiester bond on both sides of the lesion (Myles et al., 1987).

1.8.0 A Comparison of Nucleotide Excision Repair in *E. coli* and Human Cells

The mechanistic details of nucleotide excision repair in mammalian cells are not well understood but analysis of mutants has revealed the multiple components involved and indicated the operation of an even more complex mechanism than in *E. coli*. It is of interest to compare these repair systems because the lessons learned with one system can be applied to the understanding of the other.

1.8.1 Size of the Excised Fragment

The *E. coli* ABC excision nuclease excises DNA CPDs as a short oligonucleotide by incising the 8th phosphodiester bond 5′ and 5th phosphodiester bond 3′ to the damaged nucleotide (Sancar and Rupp, 1983). Human cells remove the CPDs from DNA as a short oligonucleotide 27-29 nucleotides in length by incising the 22-24th phosphodiester bond 5′ and the 5th phosphodiester bond 3′ to the damaged nucleotide. (Huang et al., 1992).

1.8.2 Nucleotide Excision Repair in *E. coli* and Human Cells is Performed by a Multisubunit Complex

The demonstration of seven natural human nuclueotide excision repair deficient mutants, XPA through XPG, suggests the existence of a multisubunit repair complex that is involved in the human excision repair process (Cleaver and Kraemer, 1989). The degree of severity of the defect varies from one group XP patient to another which shows that some genes are dispensable to a

certain extent whereas others are vital, as is also observed in *E. coli*, wherein uvrABC mutants are repair defective and *uvrD* mutants are dispensable to some degree (Cleaver and Kraemer, 1989; Boyce and Howard-Flander, 1964; Kuemmerle and Masker, 1980).

1.8.3 There is Coupling Between Transcription and Repair in *E. coli* and Human Cells

Preferential repair of cyclobutane pyridimine dimers in transcriptionally active genes as well as in the transcribed strand have been demonstrated for human cells as well as *E. coli*. This shows that in these two evolutionary distant organisms excision repair processes are conserved. The similarity is extended by the finding that the human *ERCC6* protein is implicated in preferential repair and appears to be the homolog of the bacterial mfd protein. In addition, the human *ERCC3* gene product has been shown to be involved both in transcription and repair processes (Troelstra et al., 1992b; Selby and Sancar, 1993; Schaeffer et al., 1993).

1.8.4 Broad Specificity and Mechanism of Recognition in *E.coli* and Human Cells

E. coli ABC excinuclease and human cells are both capable of repairing a broad range of lesions such as alkylation damage, psoralen damage and crosslinks in addition to those caused by UV-light (Bohr, 1991; Lin and Sancar, 1989, 1992a). Moreover, certain overlaps in repair systems of both organisms have also been shown. O^6 methylguanine lesions are repaired not only by O^6 alkylguaninetransferase but also by nucleotide excision repair. In fact, it has been demonstrated that the most efficient repair of O^6 alkyguanine is dependent on O^6 alkyguanine DNA alkyltransferase and nucleotide excision

repair (Voigt et al., 1989; Bronstein et al., 1992). The photoreactivating enzymes have been demonstrated to stimulate excision repair in *E. coli* (Sancar and Smith, 1989). XPE protein binds selectively to (6-4) photoproducts and it is suggested that this protein is the human homolog of photolyase and facilitates the assembly of excision repair enzymes (Patterson and Chu, 1989; Treiber et al., 1992).

1.8.5 Similarities in Repair Enzymes

The human repair protein, ERCCI, resembles uvrC and part of uvrA. There are other enzymes such as AP endonucleases which have been characterized both in *E. coli* and human cells (Doetsch and Cunningham, 1990). A recently characterized protein which has AP endonuclease activity as well as AP-I activating activity has been found in mammalian cells and a similar repair protein has also reported in *E. coli* (Xanthoudakis et al., 1992; Demple et al., 1991).

1.8.6 ATP is Required for Both *E. coli* and Human Excision Nuclease

The ABC exinuclease cuts the damaged DNA strand on each side of the lesion in a non-concerted manner which means that 3′ incision by *uvrB* precedes the 5′ incision by *uvrC*. Another component of the nucleotide excision repair complex is DNA helicase II (*uvrD* gene product) which is a DNA dependent ATPase (Lin and Sancar, 1992a; Husain et al., 1985; Caron et al., 1985). The requirement for ATP hydrolysis in the operation of the human excision nuclease has been demonstrated using human cell extracts (Wood et al., 1988; Sibghat-ullah et al., 1989; Nichols and Sancar, 1992; Shivji et al., 1992; Svoboda et al., 1993).

1.8.7 Helicases May be Involved in *E. coli* and Human Nucleotide Excision Repair

The predicted amino acid sequence of *E. coli* Mfd protein has revealed that there is a helicase motif near the central region. It has been demonstrated *in vitro* that this protein targets repair enzymes and stimulates the repair of the transcribed strand only when transcription is taking place (Selby and Sancar, 1993). Similarly, helicase motifs have been predicted in the human repair genes, *ERCC6*, (Troelstra et al., 1992b), *ERCC2* and *ERCC3*. Moreover, *ERCC3* encodes a protein that is also part of a transcription factor. It has been speculated that the presumed helicase activity may be directly or indirectly involved in both nucleotide excision repair and transcription (Weber et al., 1990; Weeda et al., 1990a,b; Schaeffer et al., 1993).

1.9.0 Intragenomic Heterogeneity of DNA Repair

The evidence for intragenomic heterogeneity of DNA repair at the gene level was first documented through repair measurements on the transcriptionally active, multicopy *DHFR* locus and on the genome overall in cultured CHO cells. It was found that repair occurred much more rapidly in the transcriptionally active *DHFR* locus than in the genome overall (Bohr et al., 1985; Mellon et al., 1986; Bohr et al., 1986a,b; Smith and Mellon, 1990). The repair efficiencies of human and mouse fibroblasts in culture are very different. The human cells remove most of the cyclobutane pyrimidine dimers within 24 hours after UV-irradiation, while rodent cells remove less than 20% of the dimers after 24 hours. However, the most puzzling phenomenon is that human and mouse cells nevertheless display similar UV survival characteristics. The explanation proposed for this rodent paradox is that preferential repair of essential genes

is responsible for the high survival in spite of the low overall repair efficiency. Therefore, survival of CHO cells could be a function of the repair of essential genes rather than that of overall genomic repair efficiency. The DNA domains that are preferentially repaired provide us with the opportunity to define the regions of the genome which are essential for survival (Smith and Mellon, 1990; Hanawalt, 1991; Mullenders et al., 1991).

Preferential repair has been observed in a number of other transcriptionally active genes such as the *C-abl* gene in mouse cells, hamster *HMG CoA reductase*, human *ADA* and *HPRT* genes, and active *metallothionein* genes. The transcriptionally active alpha mating type locus in *S. cerevisiae* also is preferentially repaired. By contrast, the inactive *c-mos* gene of the mouse is very poorly repaired (Madhani et al., 1986; Leadon and Snowden, 1988; Leadon and Lawrence, 1991; Venema et al., 1992; Vrieling et al., 1991; Terleth et al., 1989). The preferential repair of transcriptionally active genes so far reported takes place in genes that are transcribed by RNA polymerase II. Another class of housekeeping genes, the *ribosomal RNA* genes, are transcribed by RNA polymerase I, and it has been shown that both in CHO and human cells, psoralen photoadducts and UV-induced cyclobutane pyrimidine dimers (CPDs) are repaired less efficiently than in the *DHFR* gene. Therefore it has been concluded that there is no transcription-coupled repair and that repair is impeded by the nucleolar compartmentalization of rDNA (Smith and Mellon, 1990; Vos and Wauthier, 1991; Christians and Hanawalt, 1993).

1.9.1 The Boundaries of Preferential Repair

In the hamster cells, preferential repair of cyclobutane pyrimidine dimers

located in the *metallothionein* gene was observed in a region much larger than the size of the gene. Similarly, the hamster *DHFR* gene has a size of about 30 Kbp but repair of UV-damage in this gene was extended in a "DNA repair domain" with a size of 60-80 Kbp. Maximal DNA repair efficiency was at the 5′ end of the gene and the repair efficiency decreased in both 3′ and 5′ directions until reaching the level of the overall genome at the ends of the domain. The size of this proposed "DNA repair domain" correlates well to the reported sizes of higher order structure domain or loops in chromatin (Bohr et al., 1986b; Okumoto and Bohr, 1987; Mullenders et al., 1988).

1.9.2 Preferential Repair is Coupled to Transcription

When strand specific riboprobes were used to analyze repair in individual strands of the transcriptionally active *DHFR* gene in CHO cells, it was found that rapid repair was confined to the transcribed strand of the gene. The cyclobutane pyrimidine dimers were completely removed in the transcribed strand within 8 hours while only 5 - 12% were removed in the non-transcribed strand. The rate of repair of the cyclobutane pyrimidine dimers in the transcribed strand of the *DHFR* gene in CHO cells was just like that of the transcribed strand of the *DHFR* gene of human cells, whereas the non-transcribed strand was repaired slowly and resembled repair in the human genome as a whole (Mellon et al., 1987; Smith and Mellon, 1990). Repair deficient CHO cells that had been transfected with the human *ERCC1* gene showed the strand specific characteristics of the wild type CHO cells. Repair deficient CHO cells in which repair was restored by the expression of T4 endonuclease V did not manifest strand specific bias for the repair of CPDs. In

this situation, the repair endonuclease acts independently of transcription (Hanawalt et al., 1989). The finding that gene specific repair was highly selective for the transcribed DNA strands in transcriptionally active genes provided the notion that repair may be directly coupled to transcription and it was proposed that an uncoupled repair complex may be responsible for the lower rate of repair in silent genomic DNA.

When nucleotide excision repair was examined in the induced *lacZ* gene of the lactose operon of ultraviolet-irradiated *E. coli*, it was found that repair of the transcribed strand was much more efficient than that in the non-transcribed strand (Mellon and Hanawalt, 1989). The dependence on transcription of strand selectivity in repair has been shown in *S. cerevisiae*. When the rate of excision repair of UV-induced damage was monitored in the active *URA3* gene carried on a minichromosome, it was found that the transcribed strand was repaired more rapidly than the non-transcribed strand (Smerdon and Thoma, 1990). The transcription rates of different genes were compared with their repair rates and it was observed that the rate of repair was proportional to the rate of transcription up to a point beyond which the rate of repair did not increase (Bedoyan et al., 1992).

1.9.3 Rapid Repair of Pyrimidine (6-4) Pyrimidone Photoproducts

The pyrimidine (6-4) pyrimidone photoproducts are believed to be cytotoxic, mutagenic, and carcinogenic (Glickman et al., 1986; Protic-Sabljc and Kraemer, 1985; Leclere et al., 1991). The repair rate of (6-4) photoproducts has been quantified by using different approaches such as radioimmunoassays, specific nicking of dimers by alkali, and a combined photolyase-ABC

excinuclease assay. When repair kinetics of (6-4) photoproducts were measured in UV-irradiated mammalian cells by using an immunological assay, it was found that repair of (6-4) photoproducts was much faster than the repair of cyclobutane pyrimidine dimers. A similar finding was obtained when repair of (6-4) photoproducts was quantified by an alkali nicking method (Mitchell et al., 1982, 1985b). These similar results confirmed the validity of techniques, and established the fact of fast excision repair directed toward (6-4) photoproducts. An additional significant finding was that there was very little repair of (6-4) photoproducts observed in cells from XP complementation group A (Mitchell et al., 1990b).

When repair of (6-4) photoproducts in the *DHFR* gene of CHO cells was monitored by first utilizing DNA photolyase to revert all pyrimidine dimers and subsequently the ABC excinuclease to cleave at (6-4) photoproducts sites, the following observations were made. The formation of (6-4) photoproducts was not only directly proportional to UV dose but also they were repaired more rapidly than cyclobutane pyrimidine dimers (Thomas et al., 1989). Finally, it was demonstrated that human cell free extracts were capable of repairing UV-irradiated plasmids that contained only (6-4) photoproducts (Wood, 1989).

1.9.4 Repair of DNA Damage where there is a Lack of Strand Specificity

Repair of UV-light induced (6-4) photoproducts, unlike that of cyclobutane pyrimidine dimers, displayed no strand selectivity in the transcriptionally active *DHFR* gene (May et al., 1993). There are other lesions such as 4NQO adducts where no strand bias in repair has been reported (Snyderwine and Bohr, 1992). Similarly, it has been shown that repair of aminofluorene adducts in coding and

non-coding sequences of the *DHFR* domain was found to be without strand bias (Tang et al., 1989).

1.9.5 Preferential Repair of Other Lesions

Cisplatin generates interstrand and intrastrand lesions and the most common form of cisplatin-induced damage is the intrastrand adduct which was shown to be preferentially removed from the transcribed strand of the hamster *DHFR* gene. The cisplantin interstrand cross links were removed with equal efficiency from both strands (Jones et al., 1991; May et al., 1993). Psoralen plus UVA light creates two types of lesions, monoadducts and cross links. It has been demonstrated that psoralen cross links are repaired much faster in the *DHFR* gene than monoadducts (Vos and Hanawalt, 1987). Damage caused by the alkylating agent, nitrogen mustard, was shown to be preferentially repaired in the hamster *DHFR* gene and there was a slight bias toward repair in the transcribed strand while dimethyl sulfate showed no strand bias in repair (Wasserman et al., 1990; May et al., 1993).

1.9.6 *E. coli* Transcription-Repair Coupling Factor

The understanding of the molecular mechanism of transcription-repair coupling started to develop when it was demonstrated in an *in vitro* system , that repair is inhibited at the damage site where RNA polymerase was stalled. A transcription-repair coupling factor (TRCF) is responsible for targeting the repair enzyme on the damaged site of the transcribed gene. This notion received experimental support with the purification of a coupling factor which turned out to be missing in the *mfd* mutant (Selby and Sancar, 1990b, 1991b,c, 1993; Bockrath et al., 1987).

On the basis of these observations, the role of the coupling factor in *mfd* mutants was examined, and it was found that in these mutants there were more damage induced mutations in transcribed genes (Kunala et al., 1992; Oller et al., 1992). The role of the coupling factor in preferential repair became clear with the cloning of the *Mfd* gene and a demonstration of interactions of mfd protein with transcription and repair proteins. The Mfd protein is 1148 amino acids long with a molecular weight of 130 KDa. The predicted amino acid sequence suggests that a putative helicase motif is conserved near the central region. It also has sequence homology with uvrB protein at the amino terminus and a leucine zipper motif at the C terminus. The salient biochemical characteristics of mfd protein are that it displaces *E. coli* RNA polymerase but not T7 RNA polymerase from the damage site. Moreover, it does not facilitate transcription by RNA polymerase through lesions or cleave the truncated RNA transcripts nor have any effect on formation of the initiation complex.

This protein does not interact with uvrA or uvrB *per se* but associates with the uvrA_2B complex. The current model to explain the participation of Mfd protein in preferential repair has been defined as follows. A ternary complex of RNA polymerase-RNA-DNA at the damaged site is a preferred site for this protein. After binding, mfd protein dislodges *E. coli* RNA polymerase and the truncated RNA transcript. The bound Mfd protein at the damaged site facilitates the binding of the uvrA_2B complex. The uvrA protein is released at this stage and the uvrC protein is bound to the uvrB-DNA complex. The bound uvrBC complex then makes a double incision at the damage site (Selby and Sancar, 1993).

1.10.0 Human Cell Extracts to Dissect Nucleotide Excision Repair

An *in vitro* nucleotide excision repair assay was developed and extensively used to characterize the various aspects of human nucleotide excision repair. The assay is based on the incorporation of exogenously supplied radiolabelled nucleotides into the damaged plasmid DNA by human cell free extracts. Enzymes present in human cell extracts can perform all essential reactions involved in excision repair such as recognition of the damage induced by UV-light, psoralens and platinating agent, exclusive incision at the site of lesions, excision of the damaged oligomer, patch filling by DNA polymerase(s) and patch ligation (Wood et al., 1988; Sibghat-ullah et al., 1989; Wood, 1989; Hansson et al., 1989). The human cell extracts prepared from XP cell lines are deficient in performing excision repair in this *in vitro* system (Wood et al., 1988; Hansson et al., 1990). With normal cell extracts, an incubation time of two hours is necessary and only a small fraction of the damage is repaired. However, by exploiting this versatile system, the following aspects of nucleotide excision repair have been characterized.

1.10.1 Repair was Stimulated by HSSB

Human single strand binding protein (HSSB) consists of three polypeptides of relative molecular weights of 70 kDa, 34 kDa and 13 kDa, and can stimulate the activity of DNA polymerases including polymerase ε (Kenny et al., 1989, 1990). HSSB is required for DNA replication. When anti HSSB antibodies were added to *in vitro* replication systems for SV40, replication was inhibited (Keeny, 1990). When different anti-HSSB antibodies were added to human cell extracts the repair synthesis was reduced to background levels.

The inhibitory effect could be reversed by addition of pure HSSB to the assay mixture. *E. coli* SSB could not reverse the inhibitory effect of anti-HSSB antibodies (Coverley et al., 1991, 1992). The lack of cross-species complementation indicates that a specific protein-protein interaction is responsible for this phenomenon which is also supported by the finding of a stimulatory effect of HSSB on mammalian DNA polymerase(s) (Kenny et al., 1989; Tsurimoto and Stillman, 1989).

1.10.2 Patch Size and Nature of Excised Oligomer

Nucleotide excision repair in UV irradiated mammalian cells was reported to generate repair patches with a size in the range of 10-30 nucleotides as measured by the buoyant density shift method (Edenberg and Hanawalt, 1972; Hanawalt et al., 1979; Th'ng and Walker, 1986). It has been demonstrated that most of the cyclobutane pyrimidine dimers were released from the UV-irradiated DNA as short oligonucleotides which were retained at least 24 hours inside the cells and could be recovered in a TCA-soluble fraction (LaBelle and Linn, 1982). *In vitro* studies with cell free extracts also indicated that a patch size of about 30 nucleotides was formed (Coverley et al.,1991; Shivji et al.,1992; Huang et al.,1992.)

In spite of all this progress, the precise border around the damaged nucleotide remained undelineated. However, when *in vitro* excision repair assay was aided with phosphothioate chemistry and site specific targeting of adducts on a synthetic substrate, it was successfully demonstrated that cyclobutane thymine dimers were excised from substrate by human cell free extracts, in a fashion analogous to that in *E. coli*. In this cell free extract

system, the use of deoxynucleoside 5′ (α thio) triphosphates results in uniquely labelled repair patches containing phosphorothioate linkages. The 3′ border of the repair patch was delineated by the use of exonuclease III. The phosphorothioate linkage is resistant to exonuclease III. On this basis it was found that the fourth phosphodiester bond 3′ to the pyrimidine dimer constitutes the 3′ border. Phosphorothioate linkages are sensitive to cleavage by iodine. On this basis it was determined that the repair patch extends 21-24 phosphodiester bonds 5′ to the pyrimidine dimer. Moreover, an excised fragment of the same patch size was also detected. This approach demonstrated that asymmetric patches are generated by human excision nucleases by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the dimer. By this study, the precise borders of repair patches were for the first time delineated which corresponded very well with *in vivo* studies (Huang et al., 1992; Edenberg and Hanawalt, 1972; Cleaver et al., 1991).

Cyclobutane thymine dimers are also excised from damaged plasmid DNA by the same double incision pattern in *Xenopus laevis* oocytes (Svoboda et al., 1993). In another study using human cell extracts only one incision at the fourth phosphodiester bond 3′ to the pyrimidine dimer was observed on a linear DNA containing a single pyrimidine dimer. This incision was Mg^{2+} dependent and ATP independent. Although the incision at the 3′ border of the damaged nucleotide corresponded to other reports (Huang et al., 1992), the lack of incision at the 5′ border was suggested to be linked to the limited length of the substrate (Tateishi et al., 1993). The incision at 3′ to the dimer was also

very weak and abnormal in cells extracts prepared from XP complementation groups C, D and G (Tateishi et al., 1993).

Weinfeld et al., 1986 reported that the excision fragments isolated from UV-irradiated human cells and analyzed by ion-exchange HPLC were heterogeneous in size. Most of the excised fragments had an average size of 3.7 nucleotides in contrast to the size of up to 30 nucleotides that one could expect from the repair patch size measurement. This small, heterogeneous size was suggested to be a result of nonspecific nucleolytic degradation of excised oligonucleotides. It was also shown that photoreversal of cyclobutane pyrimidine dimers in the olgionucleotides released thymidine and thymidine monophosphate. This finding indicates that the phosphodiester bond between the dimerized nucleotide had been cleaved. The basic postulate was that breakage of the phosphodiester linkage between cyclobutane dimers might be an early reaction in excision repair pathways (Weinfeld et al., 1986). Subsequently, an intracyclobutyl pyrimidine dimer DNA phosphodiesterase (IDP) was found that catalyzed an intradimer backbone-nicking reaction. A protein of molecular mass 52 KDa with associated IDP has been partially purified from a variety of cells such as lymphocytes, liver and cultured fibroblasts. Huang et al., 1992 have recently suggested that in cells cleavage of the intradimer phosphodiester bond occurs only after excision of a 27-29 nucleotide oligomer. The experimental evidence in support of this was that photoreversal of a cyclobutane pyrimidine dimer containing oligomer did not change the migration of a 29 nucleotide oligomer (Liuzzi et al., 1989; Paterson et al., 1992; Huang et al., 1992).

1.10.3 Patch Size in Base Excision Repair

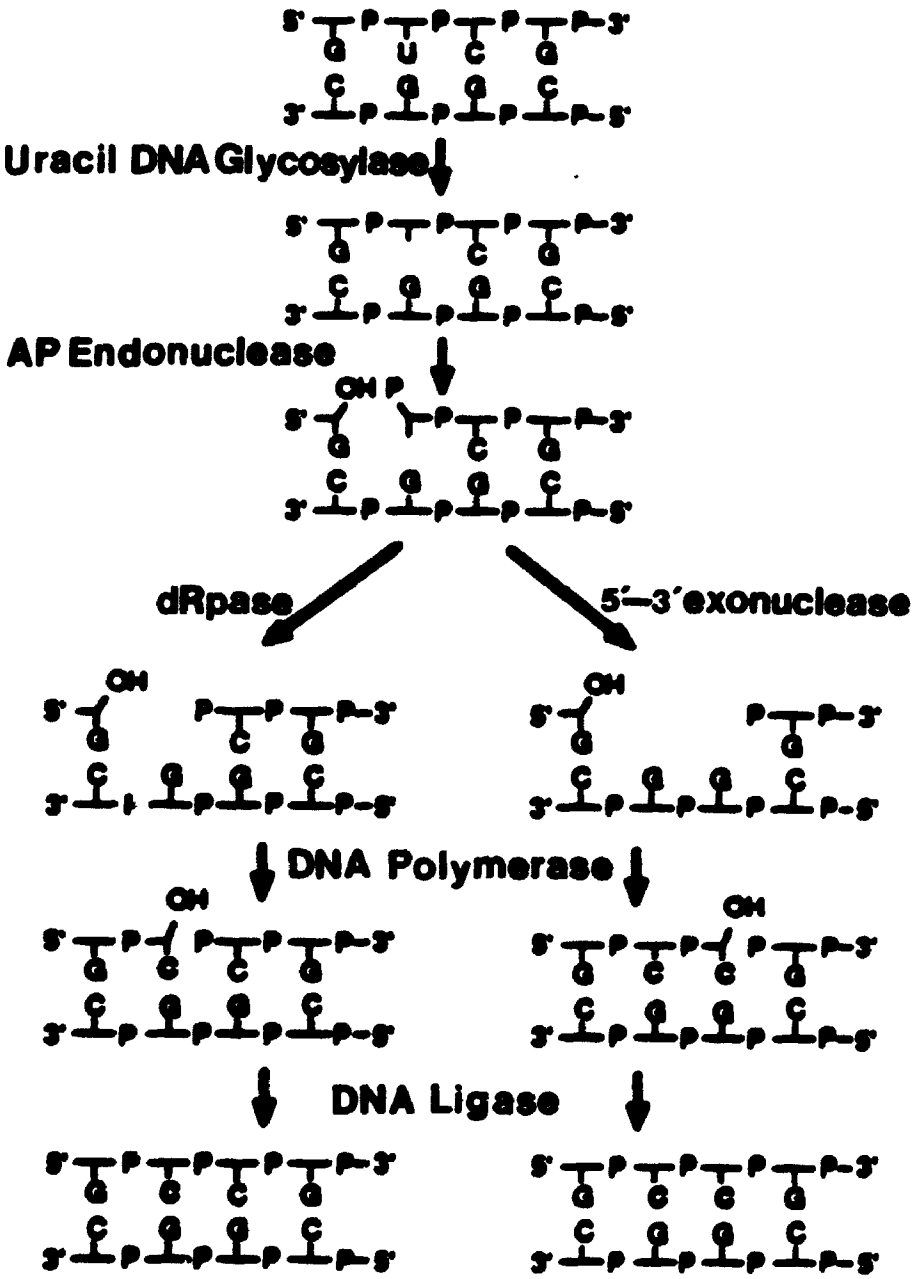
Mammalian and *E. coli* cell free extracts have been used to study the patch size in base excision repair on a synthetic double stranded DNA containing a dUMP residue. The molecular mechanism of base excision repair is schematically depicted in Figure 1.2. The patch size was determined by restriction analysis and incorporation of radioactivity in the vicinity of the uracil lesion. By using this approach, the patch size in the base excision repair turned out to be a single nucleotide for both *E. coli* and mammalian cell free extracts. The nucleotide insertion appeared to be carried out by DNA polymerase β, which was based on studies with inhibitors of DNA polymerases. Before inserting the correct base at the lesion site, the modified base must first be removed by DNA glycosylases, followed by hydrolysis of the phosphodiester bond at either 5′ or 3′ to the AP site. These AP sites are further processed by enzymatic trimming to remove various terminal groups that block DNA synthesis and ligation. The oxidized derivative of deoxyribose and 3′ phosphate groups at DNA termini are removed by deoxyribophosphodiesterase (dRPase) and exonuclease, then DNA polymerase β carries out repair synthesis and repair is completed by DNA ligation. Thus, the pathway for base excision repair is quite different than that for nucleotide excision repair (Franklin and Lindahl, 1988; Dianov et al., 1992; Boorstein et al., 1989).

1.10.4 PCNA as a Nucleotide Repair Protein

The proliferating cell nuclear antigen (PCNA) is required for DNA replication, and human PCNA has a stimulatory effect on DNA polymerase δ and polymerase ε. An interaction between PCNA and DNA polymerase δ has

FIGURE 1.2 THE BASE EXCISION REPAIR PATHWAY FOR REPAIR OF A DEAMINATED CYTOSINE (URACIL) RESIDUE IN DNA

AP sites in DNA are generated after cleavage of base-sugar bond of the dUMP residue either by DNA glycosylases or by non-enzymatic hydrolysis of base-sugar bond. AP endonuclease incises the phosphodiester bond. The repair is further facilitiated by action dRPase or a 5′-3′ exonuclease. dRPase replaces a single nucleotide, while 5′-3′ exonuclease removes at least two nucleotides. DNA polymerase fill in the gap and DNA ligase seals the remaining nick. (This model was adapted from Dianov et al., 1992.)


Uracil DNA Glycosylase
AP Endonuclease
dRpase
5′—3′exonuclease
DNA Polymerase
DNA Ligase

been reported (Jaskulski et al., 1988; Zuber et al., 1989, Wang, 1991). *In vivo* studies have demonstrated that PCNA can be detected in human nuclei following UV-irradiation. It has also been shown that PCNA can be seen at DNA replication sites during DNA replication (Celis and Madson, 1986; Toschi and Bravo, 1988; Celis et al., 1987; Wilcock and Lane, 1991). On the basis of these observations, it was assumed that PCNA might be involved in DNA repair synthesis.

The resolution and reconstitution of human cell free extracts has provided the evidence that PCNA is required for synthesis of short repair patches generated by excision of damaged nucleotides (Shivji et al., 1992; Nichols and Sancar, 1992). These studies indicate that a considerable overlap exists between the repair and replication processes. PCNA is not required for the initiation step of excision nuclease since excision of thymine dimers takes place even in the absence of PCNA and dNTPs. This suggests that PCNA is working at some post incision stage such as polymerization. DNA polymerases δ and ϵ are most likely involved in interacting with PCNA since antibodies against DNA polymerase α in cell extracts do not affect the repair synthesis (Shivji et al., 1992; Nichols and Sancar, 1992).

1.10.5 Role of Poly (ADP-ribosylation) in DNA Repair

Poly (ADP-ribose) polymerase (PARP) catalyzes the transfer of ADP residues from NAD^+ onto various nuclear proteins. This polymerase itself has a site for autopoly (ADP-ribosylation) on its C terminus (Kameshita et al., 1984; Grandwohl et al., 1990; Desmarais et al., 1991). PARP is involved in various processes such as differentiation (Thibodeau et al., 1989), transcription (Ohtsuki

et al., 1984), recombination (Cleaver and Morgan, 1991), DNA replication (Cesarone et al., 1990), and DNA repair (Boulikas, 1988). The *in vitro* repair synthesis assay was again exploited to characterize the role of poly (ADP-ribose) synthesis in DNA repair single strand breaks. The assay system consisted of a human cell extract prepared from HeLa or lymphoblastoid cells supplemented with Mg^{2+} dNTP's, ATP generating system, and gamma irradiated open circular plasmid DNA. The repair of the strand break was monitored by the rate of generation of closed circular DNA from open circular nicked DNA on agarose gel (Satoh and Lindhal, 1992). An *in vitro* repair synthesis assay provided the following information: 1) repair was dramatically enhanced by the addition of NAD^+; 2) the addition of the PARP inhibitor aminobenzamide suppressed the repair; 3) the cell extract depleted of PARP efficiently catalyzed the repair of the single strand break both in the presence and absence of NAD^+; and 4) the addition of purified PARP to the depleted assay condition required NAD^+ for repair of the single break.

On the basis of these characteristics of PARP in the *in vitro* assay, the following model was developed to account for the role of poly (ADP-ribose) in the repair of single strand breaks. PARP binds to the single strand break site and auto-poly (APD-ribosylation) of this enzyme causes its release from the break site, thereby making the lesion available to repair enzymes such as exonuclease, phosphatase for trimming the oxidized termini and DNA polymerase and ligase for patching and sealing (Satoh and Lindhal, 1992). This model is in agreement with the shuttle mechanism proposed in 1982 by . .. K. Ebisuzaki of this department (Zahradka and Ebisuzaki, 1982).

1.10.6 Complementation Between Interspecies Cell Extracts

The use of cell free systems has made it possible to assess the interspecies complementation for nucleotide excision repair. By using this cell free system it has been demonstrated that when cell extracts from the repair deficient CHO 43-3B (*ERCC-1*) cell line and the human lymphoblastoid XP-B cell line were mixed repair synthesis was found to be substantially higher than the level achieved by either extract alone. However, when extract from *ERCC3* mutant CHO-27-1 was mixed with the extract from the human lymphoblastoid XP-B cell that is defective in *ERCC3*, no complementation of excision repair was found, but this actually led to the dilution of residual repair synthesis found in non-mixed individual extracts.

Similarly, repair synthesis could be achieved by mixing extracts from XP-A cells and extracts prepared from either repair deficient CHO 43-3B or CHO 27-1 cells. These cell lines are defective in *ERCC-1* and *FRCC3*, respectively. The ability of cell extracts prepared from one species to complement the repair synthesis in cell extract of another species showed not only the potential of the system but also constituted a new way of purifying and characterizing the active protein(s); eg, using this system as an assay, the *XPA* and *XPG/ERCC5* gene products were isolated (Biggerstaff and Wood, 1992; Robins et al., 1991; O'Donovan and Wood, 1993).

1.11 Excision Repair in Extracts from Chinese Hamster Ovary Cell Lines

Extracts prepared from the repair proficient CHO-9 line could carry out repair synthesis on UV-irradiated plasmid DNA and this was as great as was observed with human extracts in terms of the total femtomoles of nucleotides

incorporated per reaction. CHO extracts prepared from repair deficient cell lines were unable to catalyze repair synthesis on UV-irradiated plasmid DNA. (Biggerstaff and Wood, 1992; Wood, 1988).

1.12.0 Excision Repair in *Xenopus Laevis* Oocyte Extract

A cell free extract from *Xenopus laevis* oocyte was found to repair efficiently a 3-hydroxyl-2-hydroxy methyl tetrahydrofuran residue on covalently closed circular DNA. This was regarded as a model for the repair of AP sites (Matsumoto and Bogenhagen, 1989. 1991; Takeshita et al., 1987). Surprisingly, AAF lesions were not repaired in the *Xenopus* extracts although they were readily repaired by *Xenopus laevis* oocytes. Although extract systems supported transcription very efficiently, it was suggested that some factor(s) in the cell free extracts were limiting or became inactivated. (Orfanoudakis et al., 1990; Chen et al., 1991).

1.12.1 DNA Repair in *Xenopus Laevis* Oocytes

A DNA repair system has been characterized by microinjecting ultraviolet light irradiated plasmid DNA along with radiolabelled nucleotides into *Xenopus laevis* oocytes. The oocytes supported the repair synthesis on supercoiled and nicked DNA 50-fold more efficiently than on linear DNA (Legerski et al., 1987). The remarkable propensity of the oocytes for the repair of pyrimidine dimers was demonstrated by injecting UV-irradiated ^{32}P-labelled plasmid DNA into oocytes and analyzing the extracted plasmid with UV-endonuclease treatment and alkaline agarose gel electrophoresis. (Saxena et al., 1990; Hays et al., 1990).

The repair of UV-damaged plasmid DNA in *Xenopus laevis* oocytes was

found to be processive where repair occurred by an all or none mode (Gruskin and Lloyd, 1988a,b; Saxena et al., 1990). The patch size of excision repair on plasmid DNA measured *in vivo* was 27-29 nucleotides and a fragment of the same size has been detected and further studied. When this 27-29 nucleotides long fragment was treated with T4 poly 3'-5' exonuculease, a fragment of 23-25 nucleotide long was generated, confirming that *Xenopus* oocytes removed cyclobutane pyrimidine dimers by a mechanism similar to the one found in human cell extracts (Svoboda et al., 1993; Huang et al., 1992).

1.13.0 Excision Repair in Extracts from *Schizosaccharomyces pombe* and *Saccaromyces cerevisiae*

An *in vitro* cell free extract system has demonstrated that the repair of pyrimidine dimers and single stranded breaks in plasmid DNA can be achieved. The ideal conditions for the repair of single stranded breaks and cyclobutane dimers are different which suggested that there are different repair mechanisms for UV and ionizing radiation induced damages (Sidik et al., 1992). The *S. cerevisiae* cell free system was unable to support nucleotide excision repair but was efficient in promoting base excision repair. The patch size for the base excision repair was reported to be about four fold smaller than that previously observed for nucleotide excision repair (Wang et al., 1992, 1993; Huang et al., 1992).

1.14.0 Xeroderma Pigmentosum

This syndrome was first described more than a century ago as an inherited form of skin disorder (Kaposi, 1882). The patients inflicted with this autosomal, recessively inherited disorder show abnormal pigmentation and a high occurrence of skin cancer, eye lesions and in some cases, neurological

abnormalities (Cleaver, 1983; Kraemer et al., 1987; Cleaver and Kraemer, 1989). An assay of DNA repair was developed which involved the incorporation of high specific activity [^{3}H] thymidine into DNA at all stages of cell cycle following UV-irradiation. This assay of DNA repair was called unscheduled DNA synthesis (UDS) because of the unusual incorporation of [^{3}H] thymidine into DNA during the non-S-phases of the cell cycle. Normally this incorporation occurs only in S-phase cells. The unscheduled DNA synthesis (USD) could be quantified from the number of silver grains generated by autoradiography of the cells. By using this assay, a DNA repair defect was first demonstrated in fibroblasts from a patient with xeroderma pigmentosum by showing that there was no detectable UDS following UV-irradiation (Cleaver, 1968).

The genetic heterogeneity of XP cells was demonstrated by fusing the cells from different patients and comparing the level of UDS in heterodikaryons and unfused monokaryons. It was found that cells from different XP patients could complement one another to restore the level of UDS to normal. Systematic cell fusion analysis has led to the identification of at least seven complementation groups designated XP-A through XP-G plus a clinically recognized form called XP variant that did not show a DNA repair defect. The different XP complementation groups showed that a number of proteins are defective in XP cells (Cleaver, 1983; Bootsma et al., 1970; DeWeerd-Kastelein et al., 1972; Giannelli et al., 1982). This genetic disorder is manifested at a frequency of one to four per million and its carrier frequency is estimated to be about 0.2 - 0.4% (Paterson et al., 1984). The availability of these naturally occurring XP mutants as well as synthetically generated mutants has provided

a unique way of dissecting the normal repair process by understanding the defective excision repair in mammalian cells.

1.14.1 Xeroderma Pigmentosum Complementation Group A

XP complementation group A is both the most common and most debilitating form of the repair disorder, and represents about one quarter of the patients. XPA cells are defective at the initial incision step of the excision repair and repair could not be restored by microinjection of *E. coli* uvrABC proteins (Fornace et al., 1976; Zelle and Lohman, 1979, Erixon and Ahnstrom, 1979; Zwetsloot et al., 1986; deJonge et al., 1983; 1985). By means of DNA-mediated gene transfer, a mouse gene was cloned that would correct XPA cells and isolation of the mouse *XPA* complementing gene made it possible to isolate the human *XPAC* correcting gene. This was a most difficult feat not only because spontaneous revertants were generated, but also, human cells were much more recalcitrant to transfection by exogenous DNA than rodent cells (Tanaka et al., 1989; Tanaka et al., 1990; Mayne et al., 1988a). The human *XPAC* gene is located on chromosome 9q34.1 and the protein encoded by this gene is rich in glutamic acid, lysine, and alanine. The characterization of its cDNA showed a single reading frame encoding a protein of 273 amino acids (Tanaka et al., 1990).

The cloning of the human *XPA* gene has made it possible to map the location of the molecular defect in different XPA afflicted patients. The defect usually results from a nonsense mutation in the fifth exon of *XPA* genes (Satokata et al., 1990; 1992a,b). XPAC protein has been partially purified and turned out to have a molecular mass of 40 kDa. XPAC protein could not be

detected in extracts prepared from XPA cells by means of an antibody against XPAC proteins indicating that the mutated gene either produced unstable proteins or RNA (Robins et al., 1991; Satokata et al., 1992a,b).

1.14.2 Xeroderma Pigmentosum Complementation Group B

There are only a few cases of XPB that have been reported. XPB is a special case in the sense that it is not only a severe form of the disorder, but also a hybrid of XP and Cockayne's syndrome, and XP patients display phenotypic characteristics of both syndromes (Venema et al., 1990b). XPB was corrected by the human gene *ERCC3* which was originally found to correct the DNA repair rodent mutant belonging to complementation group 3. Thus the XPB gene and *ERCC3* were identical. The predicted 782 amino acid sequence of ERCC3 protein displayed several domains including the helicase motif. The XPB defect generates a 4 bp (GCAC) insertion in its mRNA that disrupts the open reading frame in the part corresponding to the C terminus of the protein. The correction of XPB by *ERCC3* was only possible by the technique of microinjection since no immortalized cell line was available for a transfection experiment (Weeda et al., 1990a,b,1993).

The genomic structure of *ERCC3* is spread over 45 kbp and contains at least 14 exons. The promoter region is 259 bp upstream of the initiation site and is without CAAT and TATA boxes. The transcription level of *ERCC3* is low and there is no inducible response after UV-irradiation. The mRNA of *ERCC3* is very stable, and does not fluctuate with the cell cycle stages, which shows that even limiting concentrations are vital for the repair process (Weeda et al., 1991). The human BTF2/TF11H is a multisubunit transcription factor possessing

several enzymatic activities such as ATPase, a kinase activity on a 62 KDa polypeptide that phosphorylates the largest subunit of RNA polymerase II and a helicase activity on a 89 kDa polypeptide. The sequence of the 89 kDa polypeptide turned out to be the same as that predicted for ERCC3 and appears to fulfill a DNA unwinding function required for the late step in the complex process of transcription initiation (Schaeffer et al., 1993; Bootsma and Hoeijmakers, 1993).

There is a homolog of *ERCC3* in *S. cerevisiae* identified as *SSL2 (RAD25)* which is 54% identical with *ERCC3*. The *SSL2* gene encodes a 95 kDa protein with helicase activity. There are an extra 58 amino acids at the N-terminus and it is not known how they influence the function. It is proposed that SSL2 protein helps to overcome the obstacle of the stem-loop secondary structure in translation of mRNA. An alternative speculation is that both ERCC3 and SSL2 are involved in translational control of a family of genes, some of which may control the expression of repair proteins (Gulyas and Donahue, 1992; Weeda et al., 1990b). A number of mutants in the *haywire* gene of *D. melanogaster* mimic the phenotypic characteristics of xeroderma pigmentosum and Cockayne's syndrome. The *haywire* gene is a homolog of *ERCC3*. It is suggested that this gene may encode a helicase activity involved in DNA nucleotide excision repair (Mounkes et al., 1992).

1.14.3 Xeroderma Pigmentosum Complementation Group C

XP group C patients are less prone to the neurological abnormalities (Robbin et al., 1974) and XPC cells, unlike other groups such as XPA and XPD, are capable of restoring the UV-induced inhibition of RNA synthesis (Mayne and

Lehmann, 1982). These cells display a higher UV-resistance and about 15-20% overall repair capacity is present (Kantor, 1988). When excision repair at the gene level was monitored in these cells, it was observed that repair was very efficient in the transcriptionally active *ADA* and *DHFR* genes, while repair was very inefficient in a transcriptionally inactive locus. When the strand specificity of repair was examined in these genes it turned out that there was about 70% repair of dimers within 8 hours in transcribed strands, while 30% of the dimers were removed in non-transcribed strands (Venema et al., 1990b, 1991a; Venema, 1991b). This showed that the repair activity is targeted towards the repair of transcribed strands of active genes (Carreau and Hunting, 1992). The cloning of the *XPC* gene, unlike other XP groups, has been accomplished by the use of an Epstein-Barr virus based shuttle vector system that can efficiently transform and be maintained extrachromosomally in human cells (Yates et al., 1985). The *XPC* gene expressed a hydrophilic protein of 823 amino acids and was specific for correcting *XPC* mutant cell lines. There was no correction of repair deficient cell lines of XPA-, XPF-, and CHO-derived UV135 when *XPC* gene was expressed in these mutant cell lines (Legerski and Peterson, 1992).

1.14.4 Xeroderma Pigmentosum Complementation Group D

XPD in many respects is similar to XPB, and shows a combined occurrence of XP, Cockayne's syndrome and trichothiodystrophy. The inclusion of XPH within XPD has increased the clinical heterogeneity that was associated with trichothiodystrophy (Vermeulen et al., 1991; Stefanini et al., 1986; Johnson et al., 1989). UV-induced UDS in XPD cells was found to be greater than in XPA, XPC, and XPG cells, therefore it was proposed that the XPD mutation

results in the accumulation of an intermediate in the excision repair pathway. (Paterson 1982; Paterson et al., 1987). Moreover, it was found that little or no gene specific DNA repair occurred in XPD cells (Evan et al., 1993).

XPD cells have been reported to be corrected by the *ERCC2* gene (Weber et al., 1990; 1991). When human chromosome 19 was transferred into XPD cells UV-resistance was conferred and there was a correction of defective nucleotide excision repair (Fleijter et al., 1992; Mohrenweiser et al., 1989). The *ERCC2* gene is considered to be essential since its yeast homolog, when defective, was found to be lethal to the haploid cell. The identification of mutations in the *ERCC2* gene will provide information on the role and function of the gene involved in XPD cells.

1.14.5 Xeroderma Pigmentosum Complementation Group E

There are certain proteins that bind specifically to damaged DNA and are called damage recognition proteins, DRPs. These proteins are presumed to be involved in DNA repair (Chu and Chang 1988; Patterson and Chu, 1989; Hirschfeld et al., 1990). The DRP activity is reported to be absent in some XP group E cells of Caucasian origin but present in XPE cell lines derived from Japanese patients. It is proposed that this difference may arise from defects in binding and functional domains of the protein (Kataoka and Fujiwara, 1991; Keeney et al., 1992). Exact enzymatic and functional aspects of DRP remain to be established; however, this protein binds selectively to (6-4) photoproducts rather than cyclobutane pyrimidine dimers (Treiber et al., 1992). This XPE protein has been purified to homogeneity, has a Mr of 125 kDa and it is also present in high quantity in drug resistant cells, which suggests it has a role in

DNA repair. It is also suggested that DRPs are human homologs of photolyase (Patterson and Chu, 1989; Chu and Chang, 1988, 1990; Hwang and Chu, 1993, Treiber et al., 1992).

1.14.6 Xeroderma Pigmentosum Complementation Group F

It has been shown that transfer of human chromosome 15 into XPF cells by microcell-mediated chromosome transfer restored about 20% of the wild type resistance to killing by UV-irradiation. This transfer also partially restored repair synthesis of DNA measured as unscheduled DNA synthesis. This complementation of cells by chromosome 15 was specific for complementation Group F since this transfer of chromosome 15 did not correct UV-sensitivity exhibited by XPA, XPC or XPD cells. Another characteristic found in XPF cells was that less repair occurred in an active gene than the overall genome (Saxon et al., 1989; Evan et al., 1993). Different explanations have been proposed for the partial complementation of XPF cells by human chromosome 15; one of them is that two copies of wild type *XPF* genes are required for the complete complementation (Barnes, 1992; Weeda et al., 1990b). Another explanation is that more than one gene is defective and complete complementation would require some other chromosome in addition to chromosome 15 (Lambert and Lambert, 1985).

1.14.7 Xeroderma Pigmentosum Complementation Group G

The serendipitous cloning of a frog cDNA and subsequently a human homolog of the yeast excision repair gene, *RAD2*, have been described. The *Xenopus* cDNA encodes a predicted acidic protein of 1196 amino acids. The human cDNA encodes an acidic protein of about 1186 amino acids and showed

50.6% homology to the *Xenopus* gene and 24.3% homology to *S. cerevisiae RAD2*. When a full length cDNA was cloned into an Epstein-Barr virus-based vector and expressed in lymphoblastoid cell lines, it restored the normal sensitivity to UV-light and unscheduled DNA synthesis of lymphoblastoid cells from XP group G. There are at least 10 copies of XPG mRNA present in XPG cell lines which suggests that UV-sensitivity is due to a subtle change of a normally active XPG allele (Scherly et al., 1993; Bootsma and Hoeijmaker, 1993).

The use of *in vitro* cell free systems has also provided the evidence that XPG protein is defective in cell extracts of XPG cells. This defect was corrected by mixing a XPG cell extracts with extract from different repair defective cell lines. Extract prepared from CHO group 5 was unable to complement the XPG extract. This suggested that ERCC5 protein and XPG protein are one and the same. XPG protein has been purified 1000 fold from HeLa cells and turned out to have a relative molecular mass of 260 kDa. The *XPG* gene has been mapped to human chromosome 13q32-33 by *in situ* hybridization and the defective gene in the CHO cell line, UV135 (Group 5), has been localized to chromosome 13q14-34 by somatic cell hybridization (O'Donovan and Wood, 1993).

1.14.8 Xeroderma Pigmentosum Revertant (XP129)

The XP129 revertant cell line was generated when SV40 immortalized XPA cells (XP12RO) were mutagenized and these revertant cells showed wild type characteristics with respect to UV-resistance (Cleaver et al., 1987; Vuksanovic and Cleaver, 1987). The characterization of revertants has demonstrated that there was a change at the location of the premature stop codon of the original strain XP12RO. The stop codon (nucleotide 619) in the

revertant has an arginine codon in the normal *XPA* gene. This substitution might be restricted to one allele of the *XPA* gene while the other had no change of codon. The level of revertant protein was much reduced and this reduction could be from poor stability of protein or mRNA (Jones et al., 1992). Further investigation showed that the XP 129 revertant displayed the characteristics of CHO cells. They could efficiently repair 6-4 photoproducts as well as cyclobutane pyrimidine dimers in transcriptionally active genes but were deficient in overall repair of cyclobutane pyrimidine dimers in the genome (Mitchell and Nairn, 1989; Lommel and Hanawalt, 1993). The CHO-like behaviour exhibited in the revertant could be attributed to one or a combination of the following reasons: 1) a reduced level of revertant XPA protein is responsible for well-balanced economical exploitation of available protein by repairing only essential genes; 2) the substitution of the amino acid glycine is responsible for the selective targeting of protein for strand specific repair as observed in CHO cells; and 3) amino acid substitution affects the stability or conformation of XPA protein.

1.15.0 Cockayne's Syndrome

Cells from patients with Cockayne's syndrome have a normal overall nucleotide excision repair and are clinically characterized by retarded growth, neurological degeneration, retinal disorders, but no elevated risk of skin cancer. There is also no recovery of RNA synthesis after UV-irradiation as there is in repair proficient cells (Ahmed and Setlow 1978; Lehmann, 1987; Nance and Berry, 1992; Mayne and Lehmann, 1982).

The preferential repair of the actively transcribed gene is defective in

both CSA and CSB groups but inactive domains of chromatin are normally repaired. These CS mutants are just opposite to XP group C cells in their DNA repair characteristics (Venema et al., 1990a,b; Venema, 1991; Hanawalt, 1991; Mayne et al., 1982, 1988b). CS cells functionally resemble the *mfd* mutant of *E. coli* where coupling of transcription and repair is also defective (Selby and Sancar, 1993). A human gene has been cloned in CHO mutant UV61 and is designated as *ERCC6*, which restored to normal the sensitivity of UV61 cells to UV-light. The expression of this gene encoded a protein of 1493 amino acids containing domains diagnostic of nuclear localization and RNA helicases and this protein also corrected CS group B cells (Troelstra et al., 1990; 1992b). A number of mutations have been detected in CS patients that cause an A-T transversion or a splicing mutation resulting in non-functional truncated proteins. The *ERCC6* gene has been localized to human chromosome 10q11-q21 (Troelstra et al., 1992a; 1993; Troelstra, 1993).

1.16 Fanconi's Anaemia

There are a number of diseases other than XP that show an abnormal response to DNA-damaging agents; one of them is Fanconi's anaemia, which is characterized by congenital physical abnormalities, aplastic anemia, chromosomal instability and a deficiency in processing DNA lesions (Hanawalt and Sarasin, 1986; Schroeder, 1982, 1964; Fanconi, 1967). An increased sensitivity to DNA cross linking agents such as mitomycin C, diepoxybutane and photoactivated psoralens is the principal character of this disorder. On the basis of this specific hypersensitivity of FA, it was suggested the FA cells are deficient in DNA crosslink recognition and processing (Auerback and Wolman,

1976; Ishida and Buchwald, 1982; Fujiwara, 1982).

It has also been suggested that the lack of detoxification of activated oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals in cells could be responsible for the chromosomal instability in FA cells because SOD and catalase are present in low amounts in these cells and the enzymes exert stabilizing effects on chromosomes (Nordenson, 1977; Joenji and Gille, 1989). Another hypothesis was that a defective protein(s) directly involved in repairing the crosslinks is responsible for this disorder. It remains to be established that a molecular relationship exists between defective repair of crosslinks and oxygen byproducts in all four complementation groups.

However, a cDNA that corrects complementation group C has been cloned by using an Epstein-Barr virus (EBV) based cDNA expression shuttle vector which facilitated the transfection and gene cloning. The gene that corrects the defect is designated as *FACC*. A mutation at base 1913 in FA changed the codon 553 from leucine to proline. *FACC* gene is ubiquitously expressed in a variety of tissues such as the brain, muscle, liver, and fibroblasts tissue. The predicted FACC protein is 557 amino acids long with many hydrophobic amino acids, it possesses no transmembrane domains and no sequence homology is found with other proteins (Strathdee et al., 1992).

1.17 Trichothiodystrophy

Trichothiodystrophy (TTD) was first reported in 1968 as an autosomal recessive disorder characterized by heterogenous symptoms such as sulfur-deficient, brittle hair, physical and mental retardation of varying severity, and photosensitivity in some patients (Pollitt et al., 1968; Price et al., 1980, King et

al., 1984; Lehmann et al., 1988). It has also been shown that TTD is associated with an excision repair defect (Yong et al., 1984; Van Neste et al., 1985; Stefanini et al., 1986). Cell fusion studies have demonstrated that there is a lack of complementation between XP group D and cells from four photosensitive patients with TTD and it has been suggested that TTD and XPD are defective in the same gene (Stefanini et al., 1986; Stefanini et al., 1987). The *ERCC2* gene has been reported to correct the XPD cell. The characterization of mutations in the two disorders would help to unravel the relationship between them (Weber et al., 1988; 1990; 1991; Fleijter et al., 1992).

1.18.0 CHO Mutants

The artificially generated UV-sensitive CHO cells have been used to dissect the excision repair process and have increased our understanding of this process. These mutants have been very useful because they take up and integrate high molecular weight DNA efficiently while XP cells are poor in this regard (Hoeijmaker et al., 1987). This characteristic has facilitated cloning of several human repair genes into rodent cells which showed a striking overlap between human and CHO repair processes (Weeda et al., 1993).

There are three categories of CHO cells that are biochemically and genetically characterized according to sensitivity to different damaging agents such as 1) ultraviolet light, 2) ionizing radiation and 3) cross linking agents. There are at least 12 well established complementation groups identified in UV-sensitive CHO cell lines. It is possible that even this large body of CHO mutants might still not represent all the components of the excision repair process, however, they have proved to be extremely valuable in investigating

the nucleotide excision repair pathways (Hickson and Harris, 1988; Busch et al., 1989; Robson et al., 1985; Collins, 1993; Hoeijmaker, 1993b; Weeda et al., 1993).

1.18.1 CHO Complementation Group I

The representative UV-sensitive cell lines of this group are UV20, UV4, CHO43-3B. These cell lines are also sensitive to mitomycin C, cis-platinum, diepoxybutane (Thompson et al., 1980; Hoy et al., 1985a,b; Wood and Burki, 1982; Hickson and Harris, 1988; Collins, 1993). The CHO43-3B mutant has proved extremely valuable in cloning a human excision repair gene, *ERCCI*, which corrected the excision repair defect of this UV-sensitive mutant. The cDNA cloning and characterization of the gene also has been reported (Westerveld et al., 1984; van Duin et al., 1986). *ERCCI* encoded a protein of 297 amino acids with strong homology with yeast RAD 10 protein. The *ERCCI* gene is located on human chromosome 19 and a region containing the classical promoters is not present on *ERCCI*. The mouse homolog of human *ERCCI* gene has also been cloned (van Duin et al., 1986, 1988; Thompson, 1989a). The introduction of cloned *ERCCI* gene into XP cells does not correct the UV-sensitivity of any of the XP comlementation groups, which shows that the known *XP* mutants do not represent all aspects of the molecular defects of nucleotide excision repair (van Duin et al., 1989). It has also been shown that ERCCI protein is part of a high molecular weight repair enzyme complex. The XP group F complementing factor, ERCC4 and ERCC11, are associated with this complex (Biggerstaff et al., 1993; van Vuuren, et al., 1993).

1.18.2 CHO Complemention Group 2

The represention UV-sensitive mutant cell lines of this group are UV-5,

UVLI, VHI and they also are sensitive to crosslinking agents (Thompson et al., 1990a,b; Mitchell et al., 1986; Zdzienicka et al., 1989a; Collins, 1993). A human gene, *ERCC2*, has been isolated and characterized by utilizing the UV-5 cell line. This gene was found to be located on human chromosome 19 (Weber et al., 1988, 1990; Thompson et al., 1989a).

ERCC2 protein is 760 amino acid long with a molecular weight of 87 kDa. This protein has striking sequence homology with RAD3 yeast protein. A region of 14 basic amino acids present on ERCC2 protein is considered as a putative nuclear localization signal. The sequence promoter region on *ERCC2* possesses classic consensus promoter elements such as GC, CAAT (reverse orientation) and TATA box. *RAD3* gene has been demonstrated to code for an ATP dependent helicase. Since both *RAD3* and *ERCC2* are strikingly homologous, *ERCC2* gene product was considered as a putative DNA helicase. ERCC2 also corrects XP-D cells (Fleiter et a., 1992; Sung et al., 1988; Hirosh et al., 1989; Weber et al., 1990; Weber et al. 1991).

1.18.3 CHO Complementation Group 3

Representative cell lines of this group are UV24, CHO-27-1 and these mutants display characteristics of cells from XP patients who are sensitive to alkylating agents as well as UV-irradiation and they are defective in the incision step of nucleotide excision repair (Busch et al., 1980; 1989; Wood and Burki, 1982; Robson et al., 1985; Thompson et al., 1982; Collins 1993). The *ERCC3* gene specifically corrected the defect of the UV-sensitive rodent mutant 27-1 and ERCC3 protein is also a part of transcription factor TFIIH (Weeda et al., 1990a,b; Schaeffer et al., 1993).

1.18.4 CHO Complementation Group 4

The representative members are UV41 and UV47, which are incision defective and were derived from the wild type CHO AA8-4 cell line. These cell lines are sensitive to UV light as well as to cross linking agents such as mitomycin C and diepoxybutane (Busch et al., 1980; Thompson et al., 1981; Collins, 1993). The *ERCC4* gene has not been cloned probably because of the absence of highly repetitive DNA sequences which are used to locate it. UV 41 cells also have been reported to be less efficient in transformation .Complementation of the UV41 CHO mutant mediated by human DNA transfer has been reported and transfectants displayed the wild type level of resistance. It has been concluded from repair proficiency of transfectants that the complementation was the result of human DNA uptake into the defective UV41 mutant (Rubin et al., 1983; Westerveld et al., 1984; Dulhanty et al., 1988).

1.18.5 CHO Complementation Group 5

This group is represented by UV135 and Q31 which are deficient in incision activity. These cells are also sensitive to mitomycin C and 4NQO (Busch et al., 1980; Sato and Hieda 1979a; Thompson et al., 1982; Collins, 1993). The *ERCC5* gene that corrected CHO UV135 mutant has been sucessfully isolated on overlapping cosmids that reconstituted a functional 32 Kbp gene by homologous intercosmid recombination in UV135 transformants and cosmid transformed UV 135 were shown to be able to repair the cytotoxic damage to levels about 70% of normal (Mudgett and MacInnes, 1990; MacInnes and Mudgett, 1990).

1.18.6 CHO Complementation Group 6

The representative members of this group are UV61 and UV86. Besides a moderate sensitivity to UV light, they are also sensitive to cross linking agents (Collins, 1993; Yang et al., 1991; Regan et al., 1990). The sensitivity of UV61 to UV irradiation was less than that of UV5 cells and the kinetics for repairing cyclobutane pyrimidine dimers were found to be in between the wild type and CHO 5 cell type with repair confined to the transcribed strand (Lommel and Hanawalt, 1991). A human gene, *ERCC6*, that corrects UV61 has been cloned. Transfection of the cDNA into the deficient cell corrected its sensitivity to UV light including the recovery of RNA synthesis. This gene also corrected the Cockayne's syndrome cell group B. The genetic organization and description of the gene has been reported also (Troelstra et al., 1990, 1992b, 1993).

1.18.7 Salient Characteristics of Other CHO Complementation Groups

V-B11 is a representative member of CHO complementation group 7. This mutant is derived from V79 and showed slight sensitivity to EMS and MMS. The defect in this mutant was characterized as a reduced level of incision and dimer removal from the active *HPRT* gene. The gene correcting this mutation (*ERCC7*) has not yet been cloned (Zdzienicka et al., 1988b, 1991a). The mutant US31 from a mouse lymphoma cell is a representative member of the 8th complementation group. The human correcting gene (*ERCC8*) has not been cloned and the defect in this gene was suggested to be at the level of incision and removal of UV lesions (Thompson et al., 1988b; Collins, 1993).

The representative members of the 9th and 10th complementation groups are CHO7PV and CHO4PV. These mutants are less UV sensitive but show

moderate sensitivity to DNA cross linking agents. Genes correcting these two mutants have not been cloned (Stefanini et al., 1989, 1991; Botta 1991). The mutants UVS1 and UVHO have been reported to constitute complementation groups 11 and 12. This mutant could complement all other complementation groups including CHO7PV and CHP4PV. This mutant was reported to display low UV sensitivity but was moderately sensitive to DNA cross linking agents. This mutant possessed a low ability to perform the incision step of repair of UV induced damage. The members of the last three goups provide new opportunities to characterize new repair genes (Riboni et al., 1992; Troelstra, 1993).

1.19.0 Inhibition of Preferential Repair

The transcriptionally active genes are repaired more efficiently than the inactive genes (Smith and Mellon, 1990; Hoeijmakers, 1993ab). The following approaches were adopted to dissect the correlation between strand specific repair and transcription.

1.19.1 Repair in Presence of Transcription Inhibitors

α-Amanitin inhibits the activity of RNA polymerase II and prevents chain elongation by binding to its 140 KDa subunit . α-Amanitin dramatically inhibits the repair of cyclobutane pyrimidine dimers from the transcribed strand in *DHFR* gene in CHO cells (Christians and Hanawalt, 1992). Repair is 3 times faster on the transcribed strand than on the non-transcribed strand in the metallothionein gene induced by dexamethansone. This strand specific repair is absent in the regulatory region of the gene and when the gene is uninduced. This accelerated repair on the transcribed strand is abolished by α-Amanitin

indicating that transcription directs the repair to the transcribed strand. XP group C cells remove cyclobutane pyrimidine dimers only in the transcribed strand. α-Amanitin completely inhibited repair in XPC cells which shows that repair of cyclobutane pyrimidine dimers are dependent on transcription (Venema et al., 1990b, 1991a; Venema, 1991b; Carreau and Hunting, 1992).

1.20.0 Preferential Repair in Transcriptionally Active and Inactive Loci of *Saccharomyces cerevisiae*

There are two loci *MATα* and *HMLα* in yeast which differ only in their transcriptional activity and provide the best example of comparison under normal physiological conditions, without the need for induction, in the same cells. The *MATα* locus is transcriptionally active while *HMLα* is inactive. In the *MATα* locus about 20% of the UV dimers are repaired in 2 to 3 hours while 6 to 7 hours are required for the same extent of repair in the *HMLα* locus. This locus becomes active in the *Sir-3* mutant and as a result, repair of both loci is to the same extent at all times after UV-irradiation (Terleth et al., 1989).

1.20.1 Strand Specific Repair in *Saccharomyces cerevisiae*

Transcription is inhibited at elevated temperature in a yeast strain with a mutated *rpbI-1* gene that encodes the largest subunit of RNA polymerase II. This simple system was used to investigate the repair not only on chromosomal DNA but also on a plasmid-containing copy of the same gene. There was fast efficient repair on the transcribed strand of *RPB2* gene both in wild and mutant strains at permissive temperature whereas the rate of strand specific repair was abolished to the level of non-transcribed strand at non-permissive temperature. These observations demonstrated the correlation between strand specific repair and transcription on the same gene, not only in chromosomal DNA but also on

plasmid containing copy of the same gene in *S. cerevisiae*. Moreover, the strand specific repair rate observed on a single copy plasmid was similiar to the repair rate observed for a multicopy plasmid (Sweder and Hanawalt, 1992; Smerdon and Thoma 1990).

1.21 Strand Specific Repair in *Drosophila melanogaster*

The *Gart* and the *Notch* genes are transcriptionally active, whereas the *white* gene is inactive in *D. melanogaster*. Preferential and strand specific repair was examined in these three genes after UV-irradiation of immortalized cell lines from repair proficient *D. melanogaster*. The cyclobutane dimers were almost completely repaired within 24 hours from all of these genes with the same rate and kinetics as observed for the genome overall (deCock et al., 1991). Moreover, UV-induced dimers in transcribed strands were repaired at the same rate and to the same extent as observed for non-transcribed strands in all of these three genes showing the lack of strand selectivity in *D. melanogaster* (deCock et al., 1992a). The *ß3 tubulin* gene is not transcribed under normal culture conditions and its transcription is induced upon addition of steroid hormone to the culture medium. When repair was monitored in the induced and uninduced gene, it was found that there was no difference in repair rate and repair was comparable to that of *Gart*, *Notch* and *white* genes. There was no strand bias in both the induced and uninduced *ß3 tubulin* gene. (deCock et al., 1992b). These observations demonstate that no strand specific repair is operative in the permanent cell line from *D. melanogaster*.

1.22.0 Effect of Cellular Differentiation on Repair of DNA Damage

Proliferating mouse cells were found to be more repair proficient

compared to non-dividing differentiated cells.(Murray and Meyn, 1987; Wheeler and Wierowski, 1983). The alkaline elution technique was used to demonstrate that repair of x-ray-induced strand breaks is more efficient in proliferating stem cells than in terminally differentiated 3T3-T cell. Similarly, differentiation results in a reduction of the cell's proficiency to carry out the excision repair of UV-induced damage (Tofilon and Meyne, 1988). Repair in these examples was monitored in total genomic DNA which yielded no information regarding the transcriptionally active genes.

1.22.1 The Repair Heterogeneity Across the Genome in Differentiated Cells

There is a lack of the characteristically distinctive strand specificity in the repair of transcriptionally active genes such as the muscle isoform of pyruvate kinase in L8 rat myoblasts (Ho and Hanawalt, 1991). In differentiating 3T3-T stem cells, UV-induced pyrimidine dimers were repaired at a faster rate and to a greater extent in actively transcribed *ADA* and *DHFR* gene than the non-transcribed *LPL* and 70-38 sequence. On the other hand actively transcribed *β-actin* gene in 3T3-T stem cells was repaired like that of the inactive *LPL* and70-38 sequences rather than *ADA* and *DHFR* which showed that actively transcribed genes are not always repaired preferentially and transcription is not the sole determinant in preferential gene repair. However, in the differentiated TD 3T3-T cells the repair rate of all these genes was similar to that of *LPL*, 70-38 sequences and *β-actin* in stem cells. Thus, there was the loss of repair heterogeneity with differentiation, which was not due to a change in basal repair activity but rather to disappearance of the fast repair of the active domains. The underlying mechanism behind the loss of domain specificity

expecially in housekeeping genes remains to be elucidated at the molecular level in differentiated cells (Bill et al., 1991).

There are several factors that can be responsible for controlling preferential repair. The chromatin becomes more condensed during differentiation. It has been hypothesized that the condensed chromatin reduces the accessibility of repair enzymes (Wheeler and Wierowski, 1983; Szabo et al., 1987). An alternative explanation is that there are common components in both repair and replication processes (Shivji et al., 1992; Nichols and Sancar, 1992). The synthesis of replication machinery is suppressed in non-dividing muscles tissue which also affects the repair process (Stockdale, 1971; Hahn et al., 1971). Neurons are non-proliferating specialized cells and neuronal degeneration in DNA repair disorders such as xeroderma pigmentosum may be exclusively due to fixation of unrepaired DNA damage (Robbins, 1983).

CHAPTER 2

Rationale

When this project was initiated, it had been established by others that UV-light damage in mammalian cells (cyclobutane pyrimidine dimers) were repaired much more rapidly in several transcriptionally active genes than in inactive genes, and this more rapid rate of repair extended to the DNA strand that was being transcribed. A similar phenomenon was seen to occur in *E. coli* with the demonstration that the induced *lacZ* gene was repaired much faster than the uninduced gene and this rapid repair was confined to the transcribed strand of the active *lacZ* gene (Smith and Mellon, 1990). The mechanism by which this transcription coupled repair was accomplished was completely unknown, nor was it known how the repair path for the slower reaction differed from the repair path for the faster reaction.

The use of plasmid DNA and the technique of transfection appeared to offer an interesting and useful model for further studies of DNA repair in mammalian cells. It was of inherent interest to learn if the "rules" that governed repair of chromosomal DNA also applied to plasmid DNA because, although the plasmid DNA that entered the nucleus was fashioned into a nucleosome type of structure, it was still possible that it was subject to different regulatory control than the chromosomal DNA. It seemed experimentally advantageous that damage could be delivered to the target DNA in the absence of damage to

the host cell. The biological activity of the plasmid, once it has been transformed into the cell, could be followed by a simple, very sensitive enzymatic assay. The plasmid could be divided functionally into distinct domains characterized by their ability to be transcribed and these domains could be analyzed separately by employing restriction endonucleases to physically separate the domains. The ability to cut the plasmid into readily mappable fragments made it possible to prepare sequence and strand specific probes which could be directed at the individual domains of the plasmid. This, of course, allowed DNA repair to be measured in an individual domain or even in the separate strands of the domain.

Since multiple copies of the plasmid made their way into the nucleus following transfection, it was thought that this would make the analysis of the extent of repair easier than it had been in the case of amplified chromosomal genes. Finally, the genetic background of the host could be varied by choosing a repair mutant such as exemplified by xeroderma pigmentosum cells. In summary then, it was hoped to establish the essential experimental conditions for studying the repair of damaged plasmids following their transfection into mammalian cells with the long term goal of elucidating various features of the DNA repair mechanism. In particular, it was desired to learn whether a transcribed domain of the plasmid was repaired more rapidly than a non-transcribed domain of the plasmid and if so, was there also a bias in the repair rate of the two strands.

Since it transpired that this experimental goal could not be achieved, it

was decided to investigate similar problems in *E. coli* cells. In this case, the plasmids were resident in the cells and UV-irradiation was delivered to the cells as well as the plasmids. This situation, however, obviated the problems of inefficient transformation of naked plasmids and the delay that would occur between transformation and extraction of plasmids. The latter experimental situation might make it difficult to measure accurately the expected rapid repair kinetics. As with the mammalian cell system, the primary goal was to learn whether transcribed regions of the plasmid were repaired more rapidly than non-transcribed regions, and whether repair occurred preferentially in the transcribed strand.

CHAPTER 3

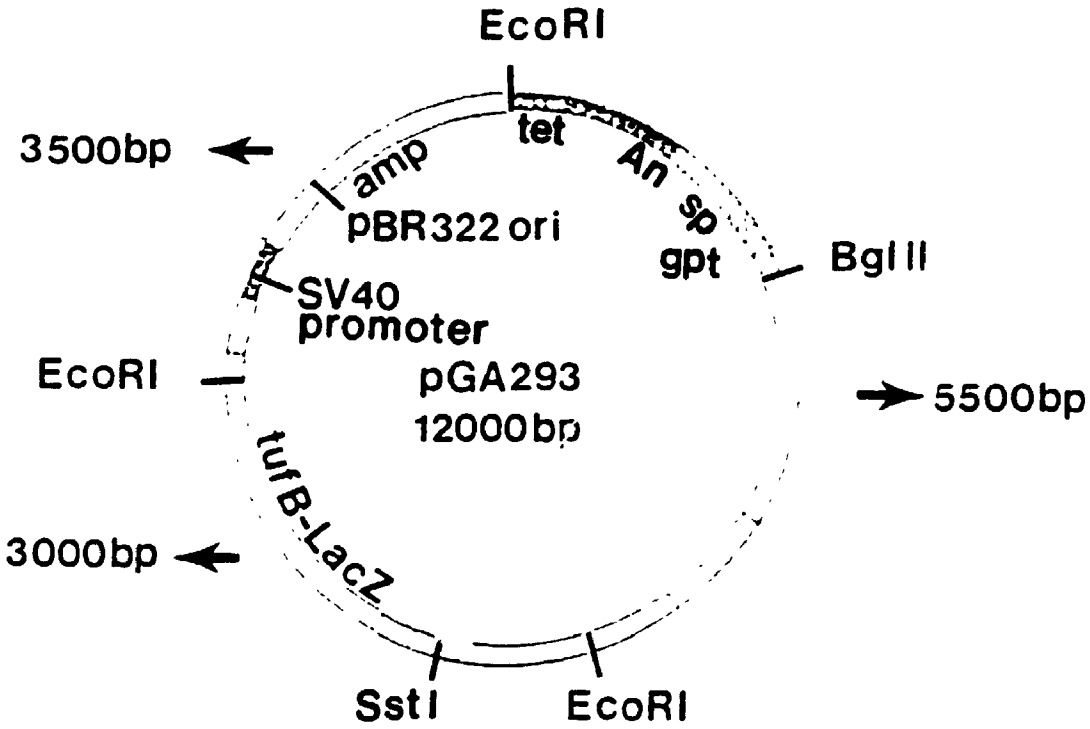
3.0 Materials and Methods

3.1 Description of pGA293

Plasmid pGA293 was a gift from Dr. L. Siminovitch, Mount Sinai Hospital, Toronto [An et al. 1982]. It was grown in *E. coli* strain JM109 and isolated by a lysozyme method (Sambrook et al., 1989). When examined by neutral gel electrophoresis, about 90% of the plasmid migrated as a single band. A diagram of the plasmid with some of its restriction endonuclease cutting sites is shown in Figure 3.1. *Eco*RI digestion of this plasmid generates three fragments, 3, 3.5 and 5.5 kbp. The 3 kbp fragment contained mostly a hybrid DNA sequence in which part of the *E. coli tufB* gene is fused to the *E coli lacZ* gene. The fused gene still yields an active ß-galactosidase, under the control of the SV40 early promoter. The 3.5 kbp fragment contains a 2.3 kbp DNA sequence derived from pBR322 in which are located the *ColEI* replication origin, *bla* gene encoding ß-lactamase and the SV40 early promoter (Mulligan and Berg, 1981; Lodge et al., 1989).

The 5.5 kbp *Eco*RI fragment of pGA293 contains a small portion of the tetracycline resistance gene (*tet*) and beyond this region is the intervening sequence of the SV40 early transcript (sp), polyadenylation signal (An) and part of the xanthine/guanine phosphoribosyl transferase (*gpt*) DNA sequence. Further digestion of this fragment with *Bgl*II generates fragments of 2.4 kbp and 3.1 kbp (An et al., 1982, Mulligan and Berg, 1981).

Figure 3.1: THE PLASMID pGA293 AND ITS RESTRICTION ENDONUCLEASE CUTTING SITES. *Eco*RI digestion generates three fragments of 3, 3.5 and 5.5 Kbp which are shown by arrows around the plasmid diagram. The 3.0 Kbp domain contains the *lacZ* sequence. The 3.5 Kbp contains the *ColE1* replication origin, *ß-lactamase* (*amp*) gene, and the SV40 early promoter. *Bg1*II digestion of the 5.5 Kbp fragment further generated two subfragments of 2.4 Kbp and 3.1 Kbp. The 2.4 Kbp fragment contains a tetracycline resistance gene (*tet*), an intervening sequence of the SV40 early transcript (sp), a polyadenylation signal (An) and part of the xanthine/guanine phosphoribosyl transferase (*gpt*) DNA sequence. The sizes of fragments of pGA293 generated by *Eco*RI, *Eco*RI-*Bg1*II and *Eco*RI-*Sst*I were determined from marker fragments generated by digestion of lambda DNA with *Eco*RI, *Eco*RI and *Hind*III.


EcoRI
3500bp
tet
amp
An
sb
gpt
pBR322 ori
BglII
SV40
promoter
EcoRI
pGA293
12000bp
5500bp
tufB-LacZ
3000bp
SstI
EcoRI

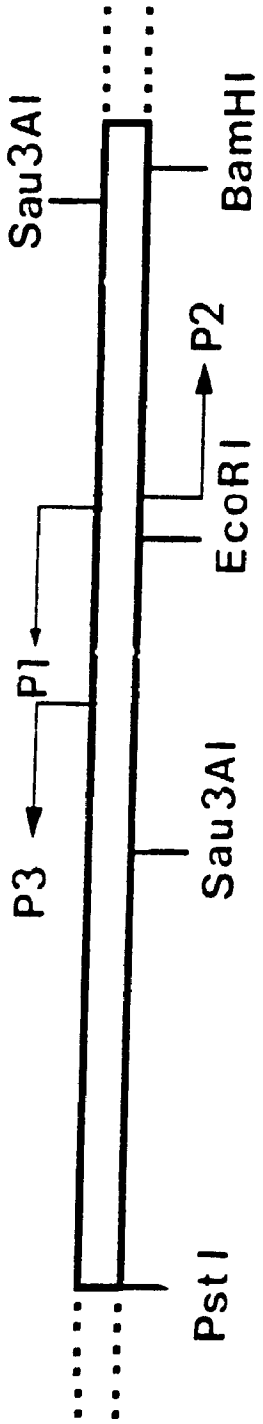
3.2 Bacterial promoters in pGA293

In the construction of pGA293, elements of pBR322 were retained that included a 2.3 kbp DNA sequence around the *Eco*RI site that separated the 3.5 kbp domain from the 5.5 kbp domain (An et al. 1982, Mulligan and Berg, 1981). In this region are three known bacterial promoters, P1, P2 and P3. The position and orientation of these bacterial promoters is shown in Figure 3.2. P3 is the natural promoter for the *ß-lactamase* gene (*amp*) and was reported to be constitutively transcribed on the 3.5 kbp domain of pGA293. P1 was artificially generated during plasmid construction when two different DNA sequences were ligated at the *Eco*RI site and, this promoter was reported to be functionally operative *in vivo* and also controlled transcription of the *amp* gene (Russell and Bennett, 1981; Brosius et al., 1982). The third promoter P2, the natural promoter for the *tet* gene, is located on the 5.5 kbp domain very close to P1 but on the opposite strand, and with opposite direction. (Brosius, et al 1982). It is presumed, but not established, that the truncated *tet* sequence, and perhaps beyond, is transcribed in pGA293 under the control of promoter P2. The bacterial promoter that preceded the *tuf-lacZ* gene was deleted during the construction of pGA293 and apparently there is no other classical bacterial promoter present for this hybrid gene. Therefore the *ß-galactosidase* gene of pGA293 should not be transcribed in *E. coli*, but weak expression attributable to this gene was noted (An et al. 1982; this thesis).

3.3 Description of pZH10

A diagram of pZH10 with some of its restriction endonuclease cutting

Figure 3.2: THE ORGANIZATION OF THE BACTERIAL PROMOTERS IN pGA293. The promoters P1, P2 and P3 are located near the *Eco*RI site at the junction of the 3.5 kbp and 5.5 kbp domains and these promoters control the transcription of the *amp* gene (P1 and P3) and the partial *tet* gene (P2) in pGA293.


Sau3AI
BamHI
P2
EcoRI
P1
P3
Sau3AI
Pst I

sites is shown in Figure 3.3. This is a pGEM-3Z (Promega, Madison WI, USA) based plasmid containing a 1 Kbp fragment of the *lacZ* gene, inserted between the T7 and SP6 RNA polymerase that originally flanked the multiple cloning region of the vector (Mellon and Hanawalt, 1989; Ganesan and Spivak, 1988). It was received as a gift from Dr. Mellon, grown thereafter in *E. coli* strain JM109 cells and isolated by an alkaline lysis method (Sambrook et al., 1989). The size of purified pZH10 was confirmed by restriction endonuclease cutting and mapping the generated fragments with respect to molecular markers that consisted of lambda DNA restricted with *Hind*III and *Eco*RI plus *Hind*III.

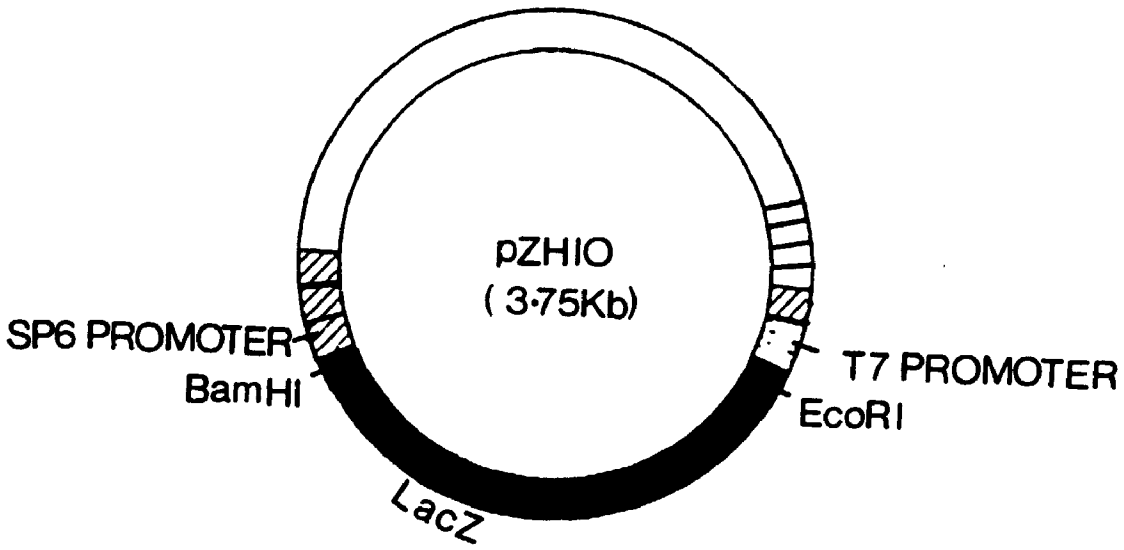
3.4 UV Irradiation of Plasmids

For initial calibration studies, plasmid was diluted in water to a concentration of 30 ng/µl and 50 µl drops in an open petri dish were irradiated at an incident fluence of 2 J/m^2 from a 15 watt germicidal lamp. The output of the lamp was calibrated with a UVX radiometer equipped with a UVX-25 probe from UVP, San Gabriel CA. For quantification of the yield of pyrimidine cyclobutane dimers in the plasmid, the UV fluence range was between 5-30 J/m^2. For transfection studies, the plasmid was diluted to a concentration of 0.1 µg/µl in TE solution and a higher dose of UV light was required to achieve the desired yield of about 1 dimer per 3 kbp of DNA.

3.5 *E. coli* Transformants

E. coli recombination deficient strain JM109 (*recA1, supE44, endA1, hsd17, gyrA96, relA1, thi, delta*(*Lac-proAB*)) and recombination proficient strain MM294 (*supE44, hsdR, endA1, pro, thi*) were kindly supplied by Dr. G.A. Mackie

Figure 3.3: THE STRUCTURE OF PLASMID pZH10. The plasmid pZH10 was derived by cloning the 1 kbp *Eco*RI-*Sst*I *lacZ* fragment into the multiple cloning site of the 2.7 kbp plasmid vector, pGEM-3Z, which also contains the *ß-lactamase* gene, the SP6 promoter, and the T7 promoter.



A

of this department. They were transformed with pGA293 by a procedure developed by Hanahan (1983). The purified pGA293 plasmid from these *E. coli* strains were analyzed by restriction endonuclease cutting and sizes of generated fragments were determined with respect to lambda DNA restricted with *Hind*III and *Hind*III plus *Eco*RI. *Eco*RI digestion of pGA293 from both *E. coli* strains generated the fragments of 3, 3.5 and 5.5 Kbp. The sizes and positions of some of these fragments are shown in Figure 3.1.

3.6 UV Irradiation of *Escherichia coli*

Log phase cultures of E. coli strains MM294 and JM109 harbouring plasmid pGA293 were pelleted, diluted to a cell density of $2x10^8$ cells/ml in M9 medium (6 μg/ml Na_2HPO_4, 3 μg/ml K_2PO_4, 0.5 μg/μl NaCl, 0.01 μg/ml $CaCl_2$ $2H_20$) and cooled on ice. Ten ml aliquots in open 150 mm tissue culture dishes were then irradiated with UV-light at an incident dose rate of 7.8 $J/m^2/s$ for a total of 80 J/m^2. During irradiation, the culture dishes were constantly rotated. The cells were either immediately lysed after irradiation or were incubated in the dark at 37°C for 7.5, 15, 30, 45, and 60 minutes after pouring them into a 50 ml flask already containing 10 ml LB medium and 100 μg/ml ampicillin. Repair was terminated by chilling the pooled cells from two culture dishes on dry ice before lysing them by the alkaline method (Sambrook et al., 1989).

3.7 Extraction of Plasmid pGA293 from Irradiated *E. coli* Strains and Analysis of Repair

The chilled *E. coli* cells were resuspended in 400 μl ice cold resuspension buffer (25% sucrose, 50 mM Tris-HCl pH 8.0 and 40 mM EDTA). The cells were lysed immediately by adding 800 μl lysis buffer (1% SDS and 0.2M NaOH),

rocking gently for one minute and neutralizing with 400 µl of 3M sodium acetate pH4.8. The lysed cells were centrifuged for 10 minutes at 10,000 rpm and the supernatant passed through gauze to remove any unpelleted material and the DNA was precipitated by adding 3-5 ml of isopropanol to 400 µl TE of supernatant, then the DNA was extracted three times with phenol/chloroform and reprecipitated with ethanol. The precipitate was suspended in 40 µl TE and the concentration of recovered nucleic acid was then determined spectrophotometrically. These DNA samples were analyzed for the extent of repair by digesting them with *Eco*RI or *Eco*RI and *Bgl*II, followed by treatment with T4 endonuclease V, electrophoretic separation, transfer to nylon membrane and hybridization with ^{32}P labelled probes.

3.8 Mammalian Cell Culture

AA8 wild type Chinese hamster cells (repair proficient) and UV-41 mutant cells (repair deficient) were obtained from Dr. G.F. Whitmore, Ontario Cancer Institute, Toronto with the permission of Dr. L.H. Thompson, Lawrence Livermore National Laboratory, University of California (Thompson et al. 1988a,b). They were grown in 75cm^2 tissue culture flasks (Falcon) in alpha medium supplemented with 8% fetal bovine serum and 0.1 mg/ml of streptomycin sulfate and 100 units/ml penicillin. The cultures were routinely maintained as monolayers at 5-10% CO_2 in an incubator.

3.9 Transient transfection of CHO cells

The calcium phosphate technique described by Hoeijmakers et al. (1988) was employed. Each 100 mm tissue culture dish was seeded with 2-3x10^6 cells.

The following day, each dish received the plasmid DNA in amounts ranging from 0.2-10 μg in 500 μl of calcium phosphate suspension and was incubated for 6,10,15 or 16 hours. The cells were then exposed to a 10% solution of DMSO in medium for 30 minutes at 37°C. The DMSO medium was then replaced with fresh regular medium. Cells were then harvested and the plasmid isolated immediately or 6, 12, 18, 24 or 48 hours later. The end of the DMSO treatment was taken as time zero in each experiment.

3.10 Extraction of plasmid DNA from cell nuclei

For each time point, the cells were removed from 4-6, 100 mm tissue culture dishes by trypsinizing the cultures, then the cells were pooled and a part of the culture, usually 1/10 of the cells, was separately preserved for analysis of ß-galactosidase activity. The pooled cells were suspended in 5 ml of Hank's medium (185.5 μg/ml $CaCl_2$ $2H_20$, 400 μg/ml KCl, 60 μg/ml KH_2PO_4, 97.7 μg/ml $MgSO_4$, 8 mg/ml NaCl, 350 μg/ml $NaHCO_3$, 47.5 μg/ml $NaHPO_4$, 1 mg/ml glucose) and 150 μl of freshly prepared DNAaseI (2 mg/ml in 150 mM NaCl, and 50% glycerol) was added. The solution was then incubated for 25 min at 37°C. This DNAaseI treatment was designed to remove any plasmid DNA adsorbed on the cell surface. The use of DNAaseI to eliminate extracellular plasmids of transfected cells is a standard procedure to remove the DNA from cell surfaces (Graham and van der Eb, 1973; Sheibani et al., 1989; Mah et al., 1991).

The possibility that DNAaseI could have gotten into AA8 cells and nicked intracellular plasmid DNA seems a remote possibility. The animal cell

membrane is impermeable to many molecules such as nucleotides and regulatory proteins (Castellot Jr. et al., 1978). The animal cells must be permeabilized in order to introduce the exogenous enzymes and substrates by methods such as freezing and thawing, saponin-treatment and osmotic shock (Van Zeeland et al., 1981; Kaufman and Briley, 1987; Wilkins, 1973, Wilkins and Hart, 1974; Keeney and Linn, 1990). The exogenous enzymes, such as pancreatic DNAaseI, could not get into Chinese hamster cells and reduce the molecular weight as determined by sedimentation profiles of DNA from cells lysed directly on akaline sucrose gradients when a freezing and thawing step was omitted (Van Zeeland et al., 1981). The cells, after DNAaseI treatment, were spun down, washed with Hank's medium, and resuspended in 2 ml of Nuclei buffer (0.25 sucrose, 10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 0.5% Triton X-100). The suspension was homogenized by 20-30 strokes in a Dounce homogenizer with a tight fitting pestle. At this stage, the extent of cell lysis was checked with a phase contrast microscope which showed that lysis was essentially complete. The nuclei were centrifuged at 3000g for 10 minutes, resuspended in 1 ml of fresh Nuclei buffer without Triton X-100, and again centrifuged. The nuclear pellets were immediately frozen in liquid nitrogen and stored at -20°C until they were extracted by the Hirt or the proteinase K method (Hirt, 1967; Bohr et al., 1985).

In the Hirt method, the nuclei were lysed in 2 ml of 0.6% SDS, 10mM EDTA and 1M NaCl, incubated overnight at 4°C and then centrifuged at 1700g for 30 minutes. The plasmid DNA was recovered in the supernatant and

genomic DNA as a precipitate. When the precipitate was analyzed to determine the degree of plasmid DNA integration into genomic DNA and the extent of coprecipitation of plasmid with genomic DNA, this fraction was washed briefly in TE, suspended in 1 ml of TE and dialysed against TE (Hirt, 1967). Both plasmid and cellular DNA fractions were extracted three times with phenol/chloroform and finally precipitated with ethanol after adding 250 µg of carrier yeast RNA. The precipitates were washed with 70% ethanol and dissolved in 200 µl of TE.

Alternatively, the DNA was extracted from isolated nuclei by dispersing them in 1 ml of 10mM Tris-HCl, pH 7.5, 5mM EDTA, 0.5% SDS and 100 µg/ml proteinase K followed by incubation overnight at 37°C. Treatment with proteinase K at a concentration of 50-200 µg/ml at 37°C, improved the dissociation and degradation of proteins of the cell extract in the presence of SDS. Moreover, this treatment also resulted in the reduction of the amount of protein in the interphase between aqueous and phenol layers during phenol extraction. The resulting solution was extracted three times with phenol/chloroform and dissolved in 200 µl of TE (Bohr et al., 1985).

3.11 Analysis of isolated plasmid DNA

A 30-50 µl volume of isolated DNA from each time point was electrophoresed on 0.7% neutral agarose gel and transferred to a nylon support membrane by using a Pharmacia vacuum blotting apparatus. Transfer of DNA was carried out by the following sequence of steps: first the gel was treated with depurination solution for 5 min. (0.25M HCl), then with denaturation buffer

(1.5 NaCl, 0.5M NaOH) for 5 min. It was then treated with neutralizing buffer containing 1M Tris-HCl and 2M NaCl for 5min. Finally 20x SSC solution was used to carry out the transfer for 3-5 hours. The transferred DNA was fixed by irradiating the blot. The UV-irradiation treatment served to cross-link nucleic acid to the membrane. The primary amino groups which were present on support membrane were highly reactive with 254-nm light activated thymines in DNA to produce N-substituted thymines (Saito et al., 1981; Church and Gilbert, 1984). These blots were hybridized with a ^{32}P labelled probe. These blots not only showed the conformation of pGA293 and pZH10 in the cell nuclei but also allowed the concentration of the plasmid to be determined by scanning densitometry (LKB Ultrascan XL Laser Densitometer).

3.12 Scanning Densitometry

Autoradiographs were scanned with an LKB ultrascan XL Laser densitometer and only densities in the range of 0.25 - 1.4 optical density units, which fall within the linear response of the scanner, were used. In order to increase the accuracy of the scanning data, multiple, independent gels were prepared, autoradiographed without an intensifying screen which gave sharper bands, and scanned independently. This made it possible to calculate a mean and standard error for most of the data.

3.13 T4 Endonuclease V

T4 endonuclease V is found in T4-infected *E. coli* and generates an endonucleolytic cleavage specifically at sites of cyclobutane pyrimidine dimers. T4 endonuclease V has been purified to homogeneity, sequenced and cloned in

E. coli. The homogenously purified T4 endonuclease was a gift from Dr. R.S. Lloyd, University of Texas, Galveston, U.S.A. The purified enzyme was supplied at a concentration of 400 μg/ml in 25 mM Na_2HPO_4 (pH 6.8) 1 mM EDTA. The specific activity of purified T4 endonuclease V (units/mg) was 2.1×10^7 (Higgins and Lloyd, 1987; Price et al., 1991; Friedberg, 1985). The first enzyme preparation received from Dr. Lloyd, although highly purified, did possess a small amount of non-specific DNA strand cutting activity. This non-specific activity could be eliminated by including an excess of non-irradiated RNA in the reaction mixture.

3.14 Assay of Cyclobutane Pyrimidine Dimers (CPDs) in UV-irradiated pZH10

T4 endonuclease V reaction mixture contained 500 ng of irradiated or non-irradiated plasmid DNA, 20 mM Tris-HCl pH 7.5, 2mM EDTA, 100 mM KCl, 1 μl of BSA (50 mg/ml), 50 - 150 μg carrier yeast tRNA and 1 μl of T4 endonuclease in a volume of 30 μl. The reaction mixture was incubated at 37°C for 30 minutes. pZH10 DNA was extracted with phenol and purified by ethanol precipitation. The samples were electrophoresed on a neutral agarose gel and the gel was stained in 0.5 μg/ml ethidium bromide solution. A photographic negative of the stained gel was prepared and the proportion of supercoiled form I pZH10 to nicked form II pZH10 was quantified at each UV-dose by scanning densitometry. When quantifying the number of dimers at each time point, one must take into account the fact that supercoiled DNA is topologically restricted and binds less ethidium bromide than the relaxed form and therefore, the amount of supercoiled DNA was multiplied by the factor 1.42 to correct for this

effect (Lloyd et al., 1973).

3.15 Assay of Cyclobutane Pyrimidine Dimers (CPDs) in UV-irradiated pGA293

Since pGA293 was larger than pZH10, 12 Kbp compared to 3.75 Kbp, and it was desired subsequently to assess the removal of cyclobutane pyrimidine dimers from restriction fragments of pGA293, a modification of the assay was employed. In this method the plasmid, irradiated or control, was cut with *Eco*RI and extracted with phenol-chloroform, further purified by ethanol precipitation and resuspended in 65 µl T4 endonuclease V reaction buffer. The mixture was split into two 30 µl aliquots and one was treated with T4 endonuclease V and the other was sham treated. The samples were electrophoresed on 0.7% alkaline gel, transferred to a support membrane and hybridized with ^{32}p labelled DNA or RNA probes. Autoradiographs were made and scanned with a laser densitometer. The average number of cyclobutane pyrimidine dimers in each *Eco*RI generated fragment of pGA293 was calculated using the Poisson expression. The cyclobutane pyrimidine dimer frequency was determined by using both double stranded DNA probes and riboprobes.

3.16 Repair Analysis in Different Domains of the Plasmid pGA293

To measure DNA repair in different domains of the irradiated plasmid pGA293, the Bohr and Hanawalt assay was used. This assay can be used with any DNA sequence of interest for which a DNA probe or a riboprobe is available (Bohr et al., 1985; Bohr and Okumoto, 1988). The plasmid itself or cells harbouring it were irradiated and cells were lysed immediately or incubated for various time periods to allow DNA repair and then plasmid DNA was extracted.

An equal amount of DNA at each time point, determined by preliminary scanning densitometry of samples, was digested with 50 units of *Eco*RI in a volume of 100 µl for 4-6 hours at 37°C. The digested DNA was precipitated with ethanol after adding 100 µl of yeast carrier RNA (10µg/µl). After washing the precipitate with 70% ethanol, it was dissolved in 57 µl of water to which was added 6.5 µl of 10x T4 endonuclease V reaction buffer (200mM Tris-HCl pH 7.5, 20mM EDTA, 100mM KCl) and 2 µl of BSA (50 mg/ml).

Two 29 µl aliquots were placed in new Eppendorf tubes. One aliquot was treated with 1 µl of T4 endonuclease and the other was mock treated with 1 µl of enzyme storage buffer (25mM NaH_2PO_4 pH 6.8, 1mM EDTA and 100mM NaCl) (Sancar and Rupp, 1983). The aliquots were incubated in parallel for 30 min at 37°C. The reaction was terminated by adding 4 µl of 10x alkaline loading buffer (500mM NaOH, 10mM EDTA, 25% Ficoll and 2.5% Bromophenol purple) and 1 µl of 10% SDS. The samples were loaded in parallel onto a 0.7% alkaline gel, electrophoresed in 30 mM NaOH - 1mM EDTA, and then transferred to a nylon support membrane for hybridization.

3.17 Quantification of Cyclobutane Pyrimidine Dimer Frequency

The Poisson expression was used to calculate the frequency of CPDs per DNA molecule under the reasonable assumption that the distribution of CPDs is random within a given DNA molecule. The fraction of molecules containing no lesions was determined by comparing the intensities of the band corresponding to T4 endonuclease V treated and untreated samples on an autoradiograph (Bohr et al., 1985; Bohr and Okumoto, 1988). In practice, this

was done by scanning the bands, and cutting out and weighing the traces. P_o, the fraction of DNA fragments free of endonuclease sensitive sites (Zero class of the Poisson expression) is given by:

$$P_o = \frac{(+)\text{ T4 endonuclease V intensity}}{(-)\text{ T4 endonuclease V intensity}}$$

This zero class was used to calculate the dimer frequency per restriction fragment from the relationship:

$$\text{T4 endonuclease sensitive sites/fragment} = -\ln P_0$$

The percentage repair was calculated from the relationship (initial dimer frequency minus dimer frequency after repair divided by initital dimer frequency) multiplied by 100.

For example, the zero class and the logarithmic relationship between the zero class and the frequency of dimers in the 3 Kbp domain of pGA293 extracted from *E. coli* strain MM294 immediately after UV-radiation at 80 J/m^2 was determined on an autoradiograph hybridized with a ^{32}P labelled DNA probe. The densitometry of T4 endonuclease V treated and untreated bands yielded the values of 0.153 and 0.0435, respectively, and the fraction of the 3 Kbp molecules containing no lesions was calculated as follows:

$$P_o = \frac{0.0435}{0.153} = 0.284 \text{ (Zero Class)}$$

The frequency of dimers per 3 Kbp = -ln 0.284 = 1.26

3.18 Prehybridization and Hybridization Conditions

Prehybridization of membranes wrapped in nylon mesh was conducted in rolling tubes for 16-24 hours at 42°C in buffer containing 50% deionized

formamide, 5x SSC (0.75M NaCl, 75mM sodium citrate pH 7), 2-5% SDS, 5x Denhardt's solution (0.1% ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone), 200 μg/ml salmon sperm DNA and 100 μg/μl yeast tRNA. When riboprobes were used, hybridization was allowed to proceed for at least 20 hours at 42°C in 5-10 ml of the same buffer, after adding ^{32}P labelled RNA probe. When DNA probes were used, hybridization was allowed to proceed for at least 48 hours after adding the DNA probe previously heated at 100°C for 10 minutes.

After hybridization, membranes were washed 3 times in 2xSSC (0.30 M NaCl, 30 mM sodium citrate pH 7), 0.1% w/v SDS at room temperature for 15 minutes for each wash followed by 3 washes in 0.1x SSC, 0.1% w/v SDS at 48°C for 15 minutes for each wash. Each membrane was wrapped in a plastic sheet without drying the membrane and exposed to Kodak XAR-5 X-ray film usually with an intensifying screen at -75°C for different time periods ranging from a few minutes to several days. In most cases, the same blot was probed several times. Bound radioactive probe was removed by incubating the membrane in 400mM NaOH for 15-20 minutes at 42°C, followed by neutralizing the membranes in buffer containing 0.2M Tris-HCl pH 7.5, 0.5% SDS pH 7.5, 18 mM NaCl, 1mM Na_2HPO4 and 1mM EDTA.

3.19 Construction of ^{32}P labelled DNA probes

In order to detect repair in the 3 kbp, 3.5 kbp and 5.5 kbp domains of plasmid pGA293, specific DNA fragments corresponding to these domains were isolated from gels and purified using a Geneclean kit (Bio 101, LaJolla, CA) and

labelled by the random primer method (Feinberg and Vogelstein, 1983).

The probe for the *ß-galactosidase* gene, which is contained in the 3 kbp domain of pGA293, was a 1 kbp *Sst1-Eco*RI fragment isolated from pGA293. Similarly, for detecting the amount of repair in other domains, the 3.5 kbp and the 5.5 kbp *Eco*RI fragments and a 2.4 kbp *Eco*RI-*Bgl*II fragment were isolated from pGA293. The primer cocktail solution was prepared by first dissolving 50 units of random hexamer (Pharmacia) in 0.55 ml of 20mM tris-HCl pH 7.5 and 1mM EDTA solution which yielded a solution of 0.09 units of primer/µl. The primer cocktail solution was made by mixing 15 µl of 0.09 units of primer/µl, 25 µl 2M Hepes buffer pH 6.6 and 10 µl H_2O. Small aliquots of cocktail solution were made, kept frozen and then thawed just before use.

The DNA fragments were labelled by boiling 100-200 ng DNA in 56 µl H_2O for 10 minutes, quickly cooling on ice and adding 20 µl of primer cocktail solution, 10 µl of dATP, dGTP and dTTP solution each at a concentration of 200µm, 5 µl of ^{32}P alpha labelled dCTP (specific activity 3000Ci/mmol, 10 µCi/µl in aqueous solution, DuPont Canada Inc.), 1 µl of BSA (50 mg/ml) and 2-3 units of Klenow fragment (Boehringer Manheim). This cocktail was incubated overnight at room temperature. The reaction was terminated by adding 5 µl of 200mM EDTA and 5 µl yeast carrier tRNA (10 µg/ml). The cocktail solution was passed through a Sephadex G-50 Nick column (Pharmacia) in order to separate the unincorporated ^{32}P labelled nucleotides from labelled DNA. 500 µl fractions were collected and the 2nd fraction, which contained the labelled primer was further purified by ethanol precipitation and resuspended in 3mM EDTA.

Similarly, the 3.5kbp fragment or a sub fragment (*Pst*I-*Eco*RI) and a 5.5kbp fragment or its subfragment (*Eco*RI-*Bam*HI) were labelled and used for determining repair in the 3.5 kbp and 5.5kbp domains of pGA293, while whole pZH10 was labelled and used to monitor repair in this plasmid DNA. The specific activity of these probes was 3-5 x 10^8 cpm/μg as determined on a small sample of TCA precipitated probe. In most cases, 2-5x10^7 cpm of probe was used for each hybridization to detect the desired band on the blot. The DNA probe was heated at 100°C for 10 minutes before adding it to the blot.

3.20 Constuction and Description of Plasmids pMB1 and pIW1

3.20.1 pMB1

Plasmid pGA293 was digested with *Eco*RI and *Pst*I and the resulting DNA fragments were extracted with phenol/chloroform. The restriction fragments were separated by electrophoresis on 0.5% neutral low melting agarose gel. The 753 bp *Eco*RI-*Pst*I fragment containing most of the *amp* gene was excised from the gel. This excised fragment was purified by slicing the agarose fragment into very small pieces and suspending them in a NaI solution and incubating at 56°C. When the gel pieces were dissolved, glass milk was added at a ratio of 1 μg DNA per μl of glass milk. Finally, the DNA was extracted and purified according to the instructions of the supplier of the Geneclean kit (Bio 101, LaJolla CA). Plasmid pGEM3 was digested to completion with *Eco*RI and *Pst*I and the resulting linear products were subjected to phenol/chloroform extraction and the DNA was purified by ethanol precipitation.

The excised 753 bp fragment was directly ligated into *Eco*RI-*Pst*I cut pGEM3 DNA (Promega, Madison WI, USA) at 20°C in a ligation reaction mixture containing 66 mM Tris-HCl pH 7.5, 6.6 mM $MgCl_2$, 10 mM DTT and 66 μM ATP. The incubation was carried out overnight. After diluting the ligation product, 50 ng of it was used to transform competent JM109 strain *E. coli* cells and ampicillin resistant recombinant colonies were selected. The plasmid was isolated from some of the colonies by an alkaline lysis miniprep method and examined by electrophoresis. Some of the plasmids that contained an insert were grown into large cultures and tested by restriction fragment analysis in order to obtain pMBI which contained the desired insert (Birnboim and Doly, 1979).

3.20.2 pIW1

A 375 bp fragment was generated by digesting the plasmid pGA293 with *Eco*RI and *Bam*HI. The position and size of bands on agarose gels was determined from marker fragments which were generated by restricting lambda DNA either with *Hind*III or *Eco*RI and *Hind*III. These DNA markers were included in all gels to map the sizes and positions of restricted pGA293 used either in subcloning or in constructing the ^{32}P labelled double stranded DNA. The 375 bp fragment was cloned into the *Eco*RI and *Bam*HI sites of pGEM3 plasmid. Cultures of JM109 were transformed with the ligation products. The purified plasmid yielded a single monomeric supercoiled form on ethidium bromide-stained agarose gels. The size of this plasmid was confirmed by digesting with restriction endonucleases. The sizes and position of restricted

fragments of this plasmid were mapped with respect to molecular markers that consisted of *Eco*RI-*Bam*HI digested pGEM3 and lambda DNA restricted with *Hind*III, *Eco*RI and *Hind*III.

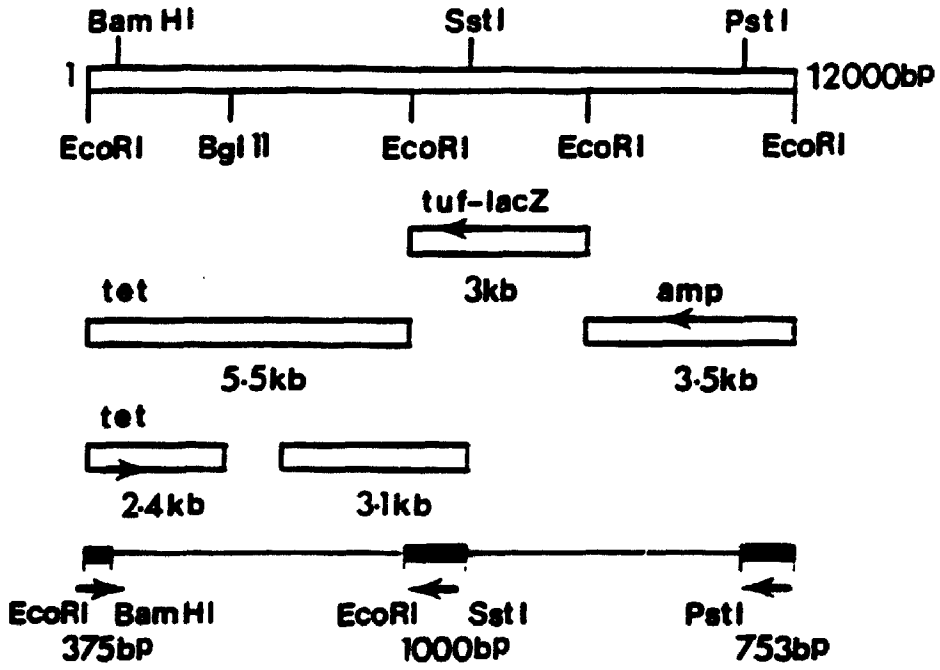
3.21 Construction of ^{32}P Labelled Riboprobes for Strand Specific Repair Analysis of Different Domains of pGA283.

Plasmids pMB1, pZH10 and pIW1 were used to generate riboprobes that detected transcribed and non-transcribed strands of the *amp*, *lacZ* and *tet* genes, respectively (Figures 3.4A and 3.4B). The appropriate plasmid was first linearized either with *Eco*RI when using SP6 RNA polymerase or *Hind*III when using T7 RNA polymerase. Linearized plasmid pMBI DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in water at a concentration of 2 μg/30 μl, then frozen in Eppendorf tubes and thawed just before generating ^{32}P labelled riboprobes. *In vitro* RNA synthesis from linear DNA templates was carried out in a reaction volume of 100 μl containing 40mM Tris-HCl pH 7.8, 6mM $MgCl_2$, 2mM spermidine, 10mM dithiothreitol, 75 units of RNA guard (RNAase inhibitor from human placenta), 500 μM GTP, ATP, UTP and 7.5 μM CTP, 6 μl ^{32}P labelled CTP (specific activity 3000Ci/mmol, 10 μCi/μl in aqueous solution, Amersham) and 100-150 units of SP6 or T7 RNA polymerase. Typically, RNA polymerase was added last and RNA synthesis was carried out for one hour at 37°C (Melton et al., 1984; Milligan and Uhlenbeck, 1989; Milligan et al., 1987).

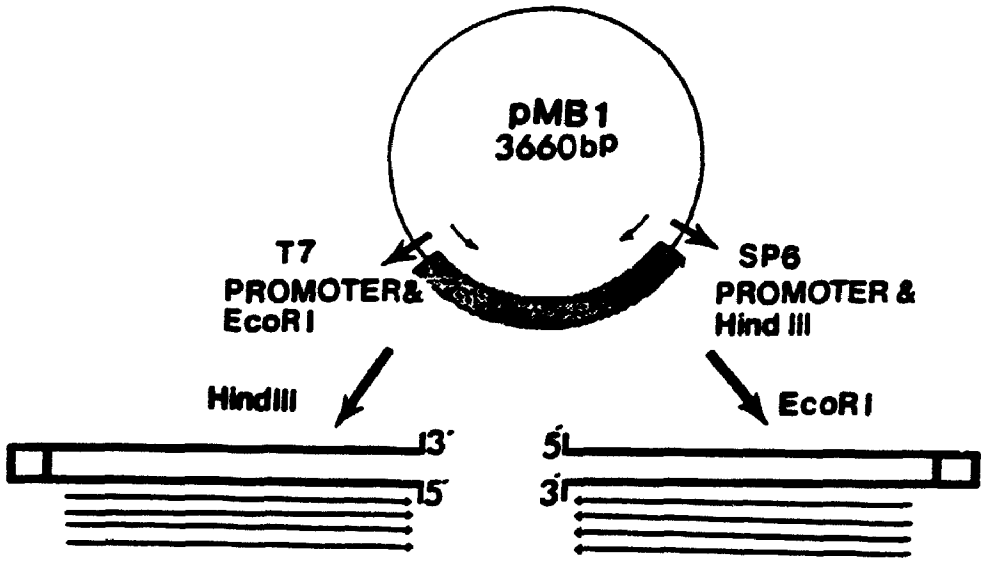
The components of the transcription reaction were prepared at room temperature from sterile stock solutions stored at -20°C. The 5x transcription buffer contained 200mM tris-HCl pH 7.5, 30mM $MgCl_2$ and 10mM spermidine.

Figure 3.4A: RELEVANT RESTRICTION MAP OF pGA293: The position of the *amp*, *lacZ* and *tet* genes on the 3 kbp, 3.5 kbp and 5.5 kbp fragments of plasmid pGA293 DNA generated by *Eco*RI are shown. The transcribed strand and the direction, of the *amp*, *lacZ* and *tet* genes, are indicated by arrows on the different fragments. The 5.5 kbp fragment was further restricted with *Bgl*II to generate two fragments of 2.4 kbp and 3.1 kbp but only the 2.4 kbp fragment was subjected to repair studies. When this 5.5 Kbp fragment was digested with *Bam*HI that generated two fragments of 5.1 Kbp and 0.37 Kbp, *Pst*I digestion of 3.5 Kbp fragment further generated two subfragments of 2.7 Kbp and 0.75 Kbp. The 3.0 Kbp fragment was also further digested with *Sst*I that generated two fragments of 2 Kbp and 1 Kbp. The sizes of the fragments of pGA293 generated by *Eco*RI, *Eco*RI-*Bgl*II, *Eco*I-*Bam*HI, *Eco*RI-*Pst*I and *Eco*RI-*Sst*I were determined from marker fragments generated by digestion of lambda DNA with *Eco*RI and *Eco*RI plus *Hind*III. The location of pGA293 DNA sequences used to construct pMB1, pZH10 and pIW1 are indicated by black bands on the bottom line.

Figure 3.4B: THE STRAND SPECIFIC RIBOPROBES: The synthesis of the strand specific riboprobe for the *amp* gene (*β-lactamase*) is illustrated. The 753 bp *Pst*I-*Eco*RI fragment, shown as a stippled arc, was derived from pGA293 and inserted into the multiple cloning site of vector pGEM-3 to yield a new 3660 bp plasmid, pMB1. The insert in pMB1 is flanked by two different phage RNA polymerase promoters, SP6 and T7, oriented in opposite directions. The direction of transcription of the *amp* gene in pGA293 is from the *Eco*RI site to the *Hind*III site. Therefore when pMBI was linearized with *Hind*III and transcribed with T7 RNA polymerase, it generated RNA transcripts that detected the transcribed strand of the *amp* gene. On the other hand, when pMBI was linearized with *Eco*RI and transcription was driven by SP6 RNA polymerase it generated a RNA probe that detects the non-transcribed strand of the *amp* gene. Similarly, strand specific riboprobes were made for the *lacZ* and *tet* genes.



A



^{32}P RNA PROBE
DETECTS TRANSCRIBED STRAND OF THE AMPICILLIN GENE.

^{32}P RNA PROBE
DETECTS NONTRANSCRIBED STRAND OF THE AMPICILLIN GENE.

B

Dithiothreitol and NTP stocks were prepared in DEPC water. The mixing of the ingredients of the transcription reaction buffer was not done on ice because spermidine caused the precipitation of DNA at the low temperature. After the ^{32}P labelled RNA synthesis, the template DNA was not removed from the reaction mixture by DNAase-treatment because the addition of DNAase in the range of 0.5-2 μg to the reaction mixture caused the degradation of labelled RNA molecules, probably due to RNAase contamination in commercially available DNAases.

Following the completion of incubation, the reaction was stopped by adding 100 μl of 8M ammonium acetate pH 7.5, 10 μl (10 μg) yeast RNA, 90 μl DEPC treated water and 100 μl of 0.5M tricine solution. The reaction mixture was extracted with phenol/chloroform and precipitated by addition of 2.5 volumes of ethanol. The pellet was resuspended in 100 μl of 0.5M tricine solution and precipitated twice. At this stage, an insignificant number of counts were detected in the supernatant. Finally, the pellet was resuspended in 500 μl tricine solution and 5 μl of it was counted to determine the degree of ^{32}P incorporation in synthesized RNA molecules. In most cases, 3.5 - 5 x 10^7 cpm of riboprobe was used for each hybridization to detect the individual strands of desired band on the blot.

Summary

When pMBI was linearized with *Hind*III and transcription was driven by T7 RNA polymerase, it generated ^{32}P labelled RNA species that detected the transcribed strand of the *β-lactamase* gene in the 3.5 kbp domain of pGA293.

FIGURE 4.6 REPAIR OF THE TRANSCRIBED AND NON-TRANSCRIBED STRANDS OF THE 3.5 Kbp DOMAIN IN pGA293

Scanning data were generated by densitometry of autoradiographs such as those shown in Figure 4.5. The dimer frequency was calculated from the relative DNA signal intensities from samples treated or not treated with T4 endonuclease V for each point. The percentage repair was then determined from the dimer frequency at each time and these were averaged. The standard error of the mean is indicated at each time point (See appendices I and II). Dashed line; averaged data for repair of transcribed strand (T) of 3.5 Kbp domain of pGA293. Solid line; averaged data for repair of non-transcribed (NT) strand of 3.5 Kbp domain of pGA293.

thus the position and sizes each band on autoradiographs were confirmed.

Moreover, the position of *Eco*RI and *Eco*RI-*Bgl*II generated fragments on autoradiographs were confirmed by superimposing the different autoradiographs generated by using riboprobes that detected the transcribed and non transcribed strands of different domains. The perfect alignment of the transcribed and non-transcribed strands of each domain confirmed that ^{32}P labelled riboprobes generated by linearizing the substrate molecules detected what they should be detecting. When the same blot used for detecting the domains of pGA293 was again hybridized with ^{32}P labelled double stranded DNA probes, they detected the same positions that the riboprobes were detecting. These independent autoradiographs aligned perfectly with respect to each other and confirmed that the riboprobes detected what they should be detecting.

3.22 ß-Galactosidase Assay

Cell samples at each time point were obtained by trypinization. They were then pelleted, washed with phosphate buffered saline and resuspended in water. The cells were disrupted by freeze-thawing and the cell debris was sedimented by centrifugation. A 20 µl sample of supernatant was incubated with 80 µl of substrate solution at 37°C for 30 minutes, after which the reaction was quenched and fluoresence developed by adding 2 ml of 0.5M sodium carbonate. The fluoresence standard was prepared by adding 2 ml of 0.5M sodium carbonate to 100 µl of 1 µM 4-methylumbelliferone (4MU) in ß-galactosidase buffer. For fluorescence measurements, the wave lengths for

excitation and emission were 365 nm and 448 nm, respectively. The substrate solution was prepared by dissolving 34 mg of 4MU-ß-galactosidase in 1 ml of DMSO. This stock solution was stored at -20°C. The stock solution is diluted 1:100 in ß-galactosidase buffer (50mM KH_2PO_4, 4mM $MgCl_2$, 0.15% triton X-100 adjusted to pH 7.8 with KOH). 4MU standard: A 10mM solution of 4MU is prepared in ethanol and then diluted to 1 µM in ß-gal buffer (J. Jongkind, Erasmus University, The Netherlands: personal communication).

CHAPTER 4

4.0 Studies on the repair of pGA293 in *E. coli*

4.1 Quantification of cyclobutane pyrimidine dimers in irradiated DNA

An example of the technique is provided in Figure 4.1 which shows that the extent of conversion of the supercoiled form (S) to the relaxed form (R) by the T4 endonuclease V increases as the dose of UV-irradiation is increased. These results were quantified by the densitometry of the supercoiled form and the relaxed form. The Poisson expression was then used to generate the number of cyclobutane pyrimidine dimers (CPDs) per plasmid. A linear relationship was found between the dose of UV-irradiation and the number of cyclobutane pyrimidine dimers per plasmid (Figure 4.2).

4.2 Relationship Between pGA293 and Chromosomal Encoded Expression of β-galactosidase in *Escherichia coli*

β-galactosidase should not be expressed from pGA293 in *E. coli* because its bacterial type promoter has been deleted. However, it was reported that a *lacZ*⁻ strain of *E. coli* carrying pGA293 yielded pale blue colonies when grown on x-gal plates indicating that the *β-galactosidase* gene in pGA293 was transcribed weakly (An et al., 1982). Measurements of the levels of β-galactosidase in hosts with and without pGA293 were, therefore, necessary in order to evaluate the transcriptional activity in the 3 Kbp domain of pGA293 which in turn might influence the rate of repair in this region.

In order to examine β-galactosidase expression by pGA293 and by the chromosomal encoded gene in *E. coli*, the levels of β-galactosidase were

FIGURE 4.1 AGAROSE GEL PATTERN SHOWING THE SENSITIVITY OF PLASMID pZH10 TO T4 ENDONUCLEASE V FOLLOWING INCREASING DOSES OF UV-IRRADIATION

Following UV-irradiation, the plasmid samples were treated with T4 endonuclease V and electrophoresed in an agarose gel to separate the supercoiled form (S) from the relaxed form (R). The gels were stained with ethidium bromide and viewed under UV-light.

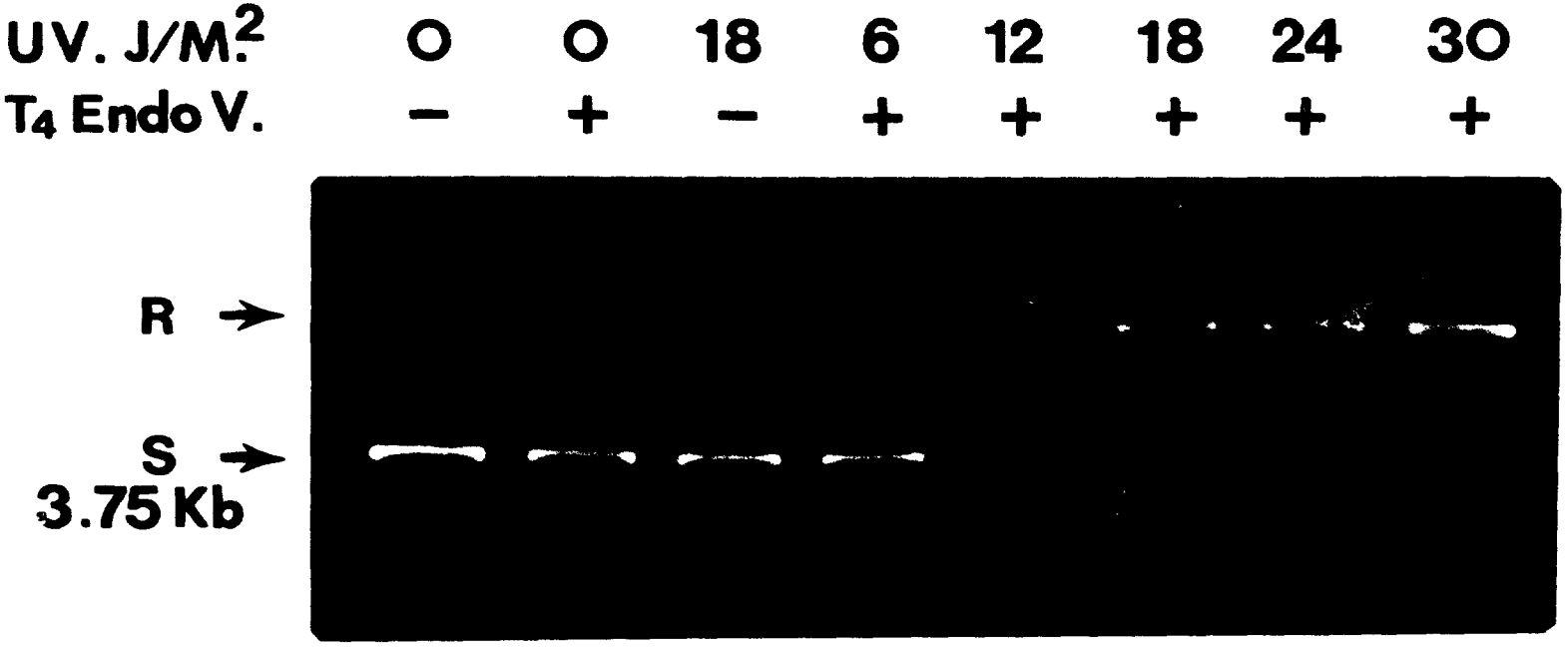
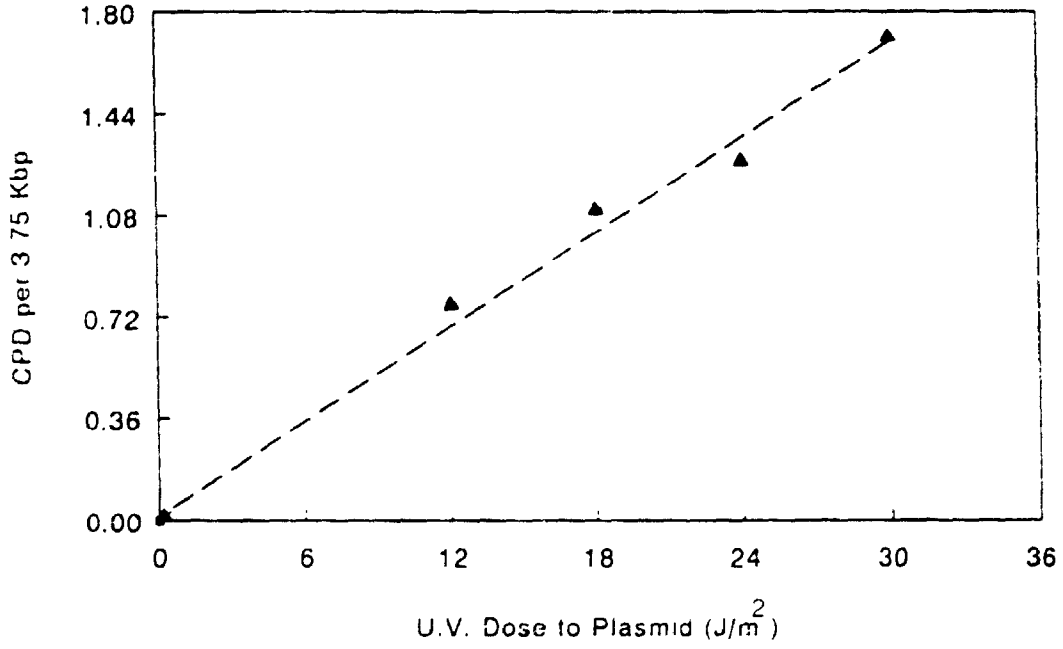

UV. J/M.2
0 0 18 6 12 18 24 30
T4 Endo V.
– + – + + + + +
R
S
3.75 Kb

FIGURE 4.2 QUANTIFICATION OF T4 ENDONUCLEASE V SENSITIVE SITES IN pZH10 IRRADIATED *IN VITRO* IN THE RANGE OF 0-30 J/m²

The photographic negative of the ethidium bromide agarose gel shown in Figure 4.1 was scanned with a laser densitometer and these data were used to determine the number of cyclobutane pyrimidine dimers (CPDs) in pZH10 at each UV dose.


1.80
1.44
1.08
0.72
0.36
0.00
CPD per 3 75 Kbp
0
6
12
18
24
30
36
U.V. Dose to Plasmid (J/m2)

compared in *E. coli* strain MM294 with and without pGA293 plasmid. The activity of β-galactosidase was measured in cell extracts prepared by freeze-thawing equal numbers of the two kinds of *E. coli* cells. The activities of β-galactosidase in cell extracts were assessed fluorometrically using 4-methyl umbeliferone-β-galactoside as a substrate. The β-galactosidase activity in *E. coli* cells without pGA293 was 93 pmol 4 MU/4.8 x 10^7 cells/30 mins. On the other hand, the enzyme activity of cells with pGA293 was determined to be 380 pmol 4 MU/4.8 x 10^7 cells/30 mins. These enzyme activities showed the basal level of β-galactosidase was about 4 times higher in the host cells harbouring pGA293 than in those lacking the plasmid.

4.3 Ultraviolet Irradiation Induced Inhibition of DNA Synthesis

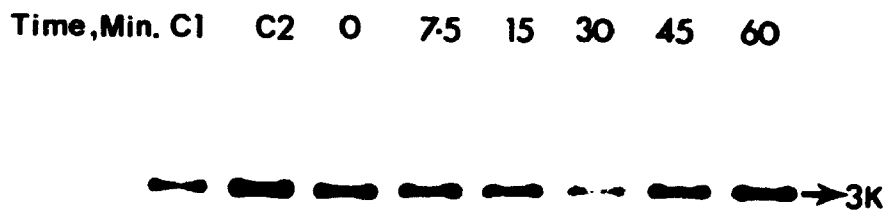
The extent of repair of cyclobutane pyrimidine dimers from pGA293 will be overestimated if replication of pGA293 in *E. coli* strains MM294 and JM109 is going on after UV damage. Therefore, in order to determine if replication of pGA293 occurred over the one hour period following UV-irradiation, plasmid was extracted from equal volumes of the *E. coli* cultures at different times after UV-irradiation, digested with *Eco*RI and fractionated on a neutral agarose gel along with a known amount of *Eco*RI-digested pGA293. After Southern blotting and probing with different probes corresponding to each of the fragments, autoradiographs were prepared, such as the one shown in Figure 4.3A. The amount of plasmid remains constant for at least one hour after UV-irradiation at 80 J/m^2. In order to confirm that inhibition of pGA293 replication in *E. coli* strains MM294 and JM109 is due to UV-irradiation, plasmid pGA293 was

FIGURE 4.3A AMOUNT OF pGA293 EXTRACTED FROM *E. coli* STRAIN MM294 AT DIFFERENT TIMES AFTER UV-IRRADIATION AT 80 J/m²

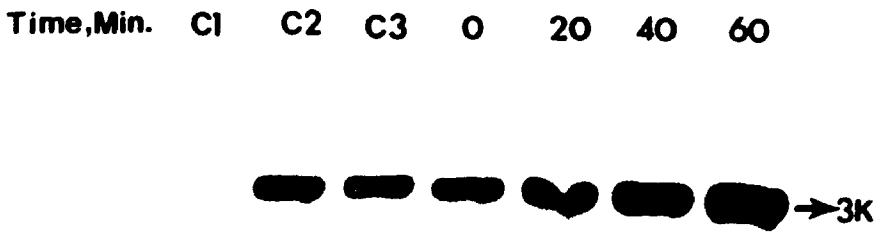
Cultures of *E. coli* strain MM294 were irradiated at ice temperature and then incubated at 37°C. At each time point equal volumes of cells were taken for extraction of plasmid and subsequent treatment with *Eco*RI in order to remove the catenated forms of DNA before electrophoresis on neutral agarose gel. A ^{32}P-labelled double stranded DNA probe was used that detected both strands of the 3Kbp domain of pGA293. Lane C1 contains a known amount (6 ng) of *Eco*RI-cut pGA293. C2 contains pGA293 DNA extracted from non-irradiated MM294 cells that were kept on ice for at least one hour before DNA extraction. The decrease in hybridization signals at 30 min reflects simply an electrophoresis artifact.

FIGURE 4.3B AMOUNT OF pGA293 EXTRACTED FROM UNIRRADIATED *E. coli* STRAIN JM109 AND INCUBATED FOR DIFFERENT TIMES

Cultures of *E. coli* which had been kept on ice were incubated at 37°C. Equal volumes of cells were taken for plasmid extraction at 0, 20, 40, and 60 minutes and digested with *Eco*RI before electrophoresis on neutral agarose gel. A ^{32}P-labelled riboprobe that detected the transcribed strand of 3 Kbp domain of pGA293 was used. Lane C1 contains a known amount (2 ng) of *Eco*RI-cut pGA293. Lanes C2 and C3 contain the plasmid pGA293 extracted from non-irradiated *E. coli* strain JM109.



A



B

isolated under the same conditions from unirradiated *E. coli* cultures after increasing incubation times and processed in the same way as the irradiated samples. A typical autoradiograph is shown in Figure 4.3B. Analysis of scanning data obtained from multiple autoradiographs showed that the signal intensity of pGA293 DNA isolated from unirradiated samples continued to rise with increasing incubation time whereas a constant DNA signal intensity was observed in all post UV-irradiated samples for a one hour period (Figure 4.4). These results demonstrated that pGA293 replication remains inhibited for at least one hour after the various doses of radiation used.

4.4 The copy number of pGA293 in the transformed *Escherichia coli* cells.

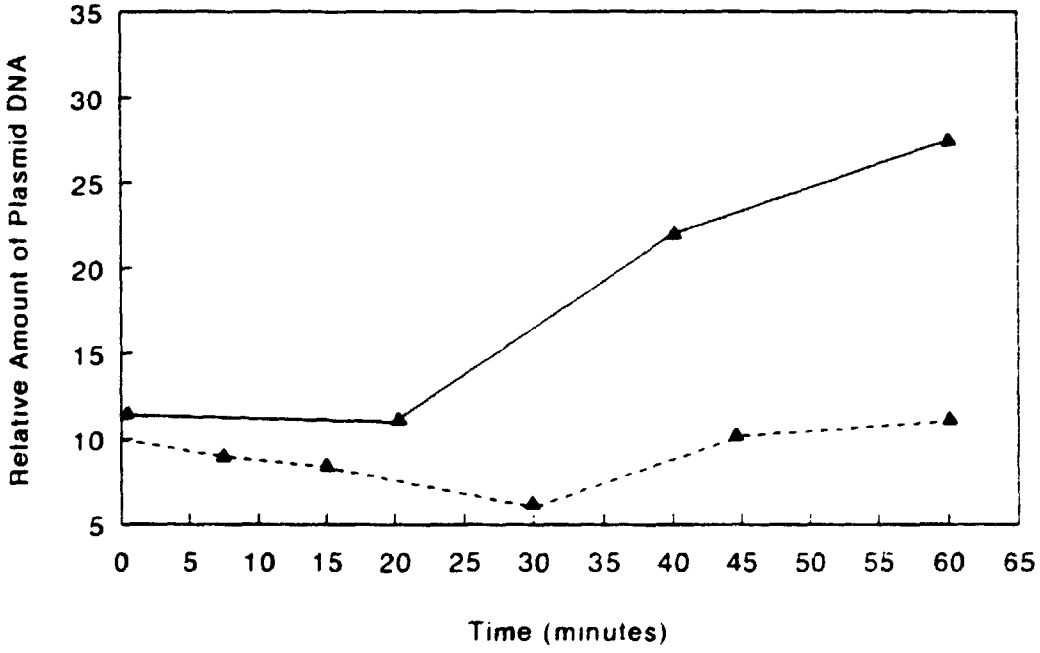
The experimental results described in 4.3 were used to determine the copy number of the plasmid. The density of the autoradiographic band obtained from a known amount of *E. coli* cells was compared to the density of a known amount of pGA293 run on the same gel. For both strains, JM109 and MM294, the average value was 20 copies per cell. This copy number in these *E. coli* strains is comparable with multicopy number plasmid ColE1 which is normally maintained at 10-20 copies in its host cells (Gelfand et al., 1978; Muesing et al., 1981; Novick, 1987; Brendel and Perelson, 1993).

4.5 Repair of transcribed and non-transcribed strands of the 3.5 Kbp domain of pGA293

The 3.5 Kbp domain of pGA293 contains the *β-lactamase* gene along with its natural promoter P3. This domain constitutes a transcription unit in which repair in individual strands can be studied. It has been demonstrated that the

FIGURE 4.4 AMOUNT OF pGA293 EXTRACTED FROM IRRADIATED AND NON-IRRADIATED SAMPLES OF *E. coli*

The autoradiographs shown in Figure 4.3A,B were scanned on a laser densitometer and from these the amount of DNA per sample was calculated at each time point. Solid line; the amount of pGA293 extracted from non-irradiated *E. coli* strain JM109. Dashed line; the amount of pGA293 extracted from irradiated *E. coli* strain MM294.

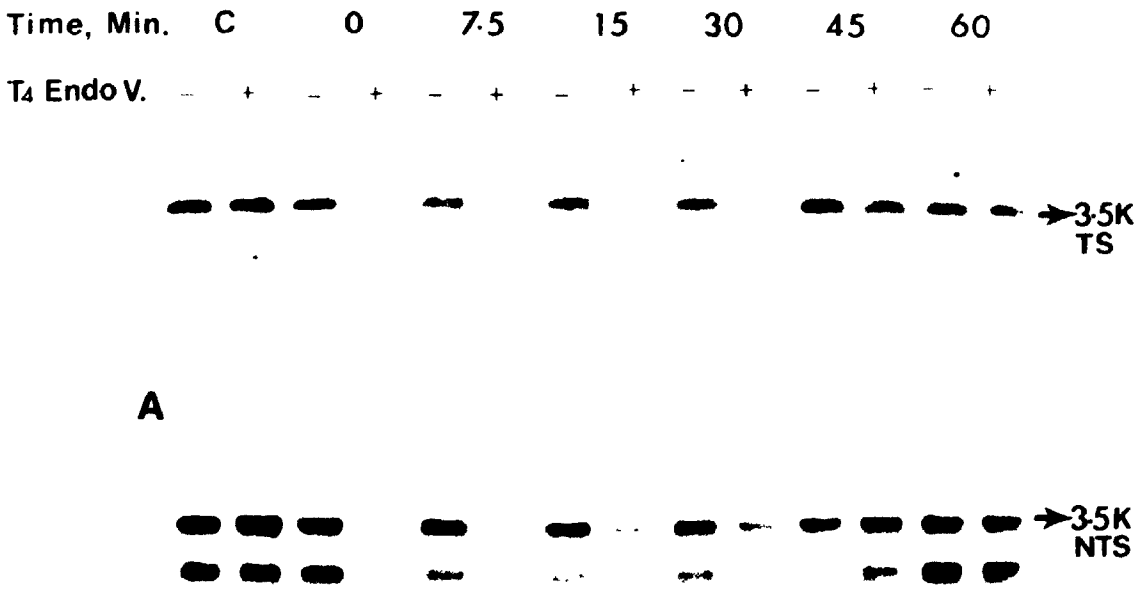

Relative Amount of Plasmid DNA
35
30
25
20
15
10
5
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (minutes)

transcribed strand of the transcriptionally active *lacZ* gene of *E. coli* is more rapidly repaired than the non-transcribed strand (Mellon and Hanawalt, 1989). In order to determine whether the strand selectivity phenomenon is operative on the *ß-lactamase* gene of pGA293, repair was monitored in the individual strands using the strand- specific probes described earlier. *E. coli* strain MM294 harbouring pGA293 was UV-irradiated and the plasmid was extracted immediately to determine the initial frequency of dimers. Other samples after UV-irradiation were incubated in darkness for 7.5, 15, 30, 45 and 60 minutes before extracting the plasmid. The incubation was conducted in the dark was done to avoid repair mediated by photoreactivation. An aliquot of the plasmid from each sample was incubated with *Eco*RI and then two equal portions of this were taken, one for treatment with T4 endonuclease V and the other not. First the riboprobe that detected the transcribed strand was used. A representative autoradiograph is shown in Figure 4.5A. Inspection of the relative DNA signal intensities from the various samples treated or not treated with T4 endonuclease V at each point on the autoradiograph showed that as the incubation time increased fewer and fewer cyclobutane pyrimidine dimers were left and, therefore, repair was taking place. Repair was complete within 45 minutes since a full length restriction fragment was recovered after T4 endonuclease V treatment.

Four independent blots were prepared and autoradiographs were made for each blot. The extent of repair at each time was calculated and the individual values obtained are recorded in Appendix I. From the four blots an

FIGURE 4.5 AUTORADIOGRAPH TO DEMONSTRATE THE REPAIR OF THE 3.5 Kbp *Eco*RI DOMAIN IN pGA293

E. coli strain MM294 cells harbouring pGA293 were irradiated at ice temperature with 80 J/m^2 of UV-light and incubated for various times at 37°C. At each time point, the extracted plasmid samples were digested with *Eco*RI and then split into two equal aliquots. One was digested with T4 endonuclease V (+) and the other was sham treated (-). Lane C contained plasmid DNA isolated from non-irradiated MM294 and analyzed in the same way as the other samples. Control and UV-irradiated samples were subjected to electrophoresis through 0.7% denaturing agarose gel and examined by Southern blot analysis using riboprobes. A) Repair analysis of the transcribed strand of the 3.5 Kbp domain of pGA293. B) Repair analysis of the non-transcribed strand of the 3.5 Kbp domain of pGA293 on the same blot.


Time, Min. C 0 7.5 15 30 45 60
T4 Endo V. – + – + – + – + – + – + – +
3.5K TS
A
3.5K NTS
B

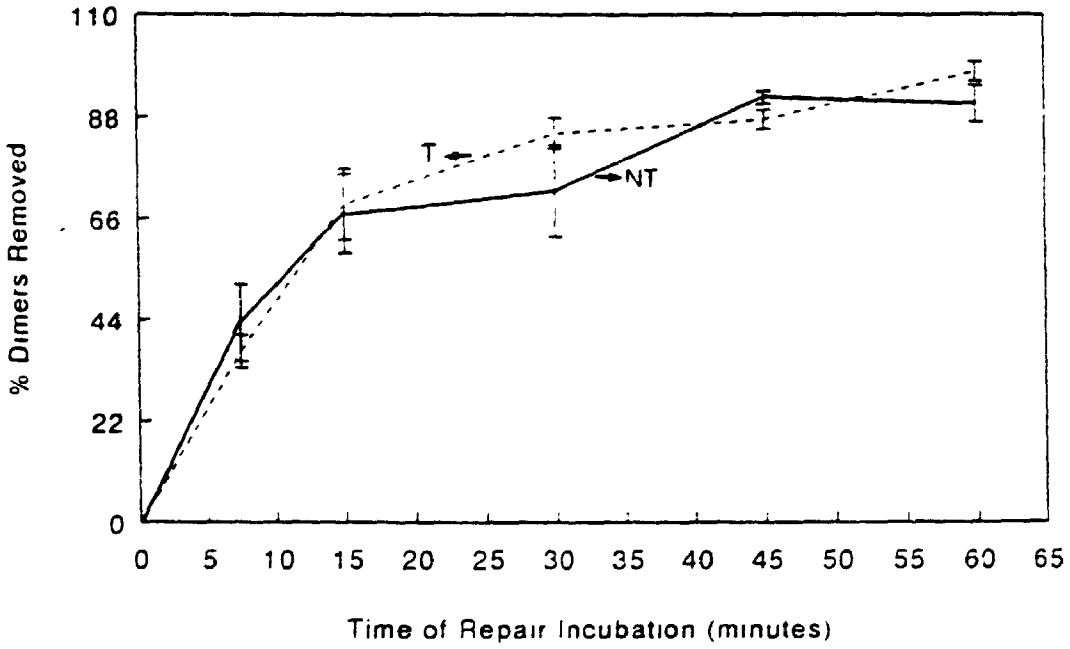
average initial dimer frequency of 1.46 dimers/3.5 Kbp was obtained. The values for the extent of repair at each time were averaged and the average values with their standard errors of the mean are shown graphically in Figure 4.6 The same blots were deprobed and then hybridized with a riboprobe that detected the non-transcribed strand. A representative autoradiograph for the non-transcribed strand of the 3.5 Kbp fragment is shown in Figure 4.5B. The four previous independent blots plus an additional one were probed for the non-transcribed strand and the individual densitometric data are recorded in Appendix 2. The initial level of damage in the non-transcribed strand was 1.43 cyclobutane pyrimidine dimers per 3.5 Kbp which was obtained by averaging the values from all five blots. The values for the extent of repair at each time-point were averaged and these values with their standard errors of the mean are also shown in Figure 4.6. The initial level of damage in the non-transcribed strand is identical to the level found in the transcribed strand which showed that neither strand was preferentially shielded from UV damage in the *E. coli* cells and that the number of adjacent pyrimidine dimers was similar in both strands. When the results for the repair of the transcribed and non-transcribed strands are compared (Figure 4.6) no dramatic difference is apparent. Even during the initial 30 minutes after irradiation where one might expect to see a difference, it is clearly demonstrated that there is no strand bias in the repair of the *β-lactamase* gene located in the 3.5 Kbp domain of pGA293.

4.6 Repair of transcribed and non-transcribed strands of the 3.0 Kbp domain of pGA293.

The same methodology that was used to assess repair in the 3.5 Kbp

FIGURE 4.6 REPAIR OF THE TRANSCRIBED AND NON-TRANSCRIBED STRANDS OF THE 3.5 Kbp DOMAIN IN pGA293

Scanning data were generated by densitometry of autoradiographs such as those shown in Figure 4.5. The dimer frequency was calculated from the relative DNA signal intensities from samples treated or not treated with T4 endonuclease V for each point. The percentage repair was then determined from the dimer frequency at each time and these were averaged. The standard error of the mean is indicated at each time point (See appendices I and II). Dashed line; averaged data for repair of transcribed strand (T) of 3.5 Kbp domain of pGA293. Solid line; averaged data for repair of non-transcribed (NT) strand of 3.5 Kbp domain of pGA293.


110
88
66
44
22
0
% Dimers Removed
T
NT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time of Repair Incubation (minutes)

domain was applied to the 3.0 Kbp domain of pGA293. That is, the individual strands of the 3.0 Kbp domain were examined with riboprobes that would detect the transcribed strand and the non-transcribed strand of the *β-galactosidase* gene. Three blots were made for the transcribed strand and two blots were made for the non-transcribed strand. Autoradiographs for each were prepared. Representative examples are shown in Figure 4.7A and 4.7B. It is obvious from a visual inspection of these autoradiographs that repair is proceeding in both strands. Each of the autoradiographs was analyzed densitometrically and from these data the number of cyclobutane pyrimidine dimers at each time was calculated. For the transcribed strand, the initial dimer frequency was 1.19/3.0 Kbp; for the non-transcribed strand, the initial frequency was 0.74/3.0 Kbp. From the dimer frequency at each time, the percentage repair was calculated. The individual data for the transcribed and non-transcribed strands are recorded in Appendix III and Appendix IV, respectively.

The time courses for repair of the transcribed and non-transcribed strands are compared in Figure 4.8. The time course of repair of the transcribed strand is hyperbolic while that for the non-transcribed strand is linear. Thus, the initial repair of the transcribed strand is greater than that of the non-transcribed strand. However, completion of repair is reached at about the same time in both strands. Repair of the 3 Kbp domain was also monitored using a ^{32}P-labelled double stranded DNA probe. An autoradiograph of this trial is shown in Figure 4.7C. The repair kinetics were very similar to those obtained with the

FIGURE 4.7 AUTORADIOGRAPH TO DEMONSTRATE THE REPAIR OF THE 3.0 Kbp *Eco*RI DOMAIN IN pGA293

E. coli strain MM294 cells harbouring pGA293 were irradiated at ice temperature with 80 J/m^2 of UV-light and incubated for various times at 37°C. At each time point, the extracted plasmid samples were digested with *Eco*RI and then split into two equal aliquots, one was digested with T4 endonuclease V (+) and the other was sham treated (-). Lane C contains plasmid DNA isolated from non-irradiated MM294 and analyzed in the same way as the other samples. Control and UV-irradiated samples were subjected to electrophoresis through 0.7% denaturing agarose gel and examined by Southern blot analysis using different probes. A) Repair analysis using a ^{32}P-labelled riboprobe that detected the transcribed strand of the 3.0 Kbp domain of pGA293. B) Repair analysis using a ^{32}P-labelled riboprobe that detected the non-transcribed strand of the 3.0 Kbp domain of pGA293. C) Repair analysis using a ^{32}P-labelled double stranded DNA probe that detected both strands of the 3.0 Kbp domain of pGA293.

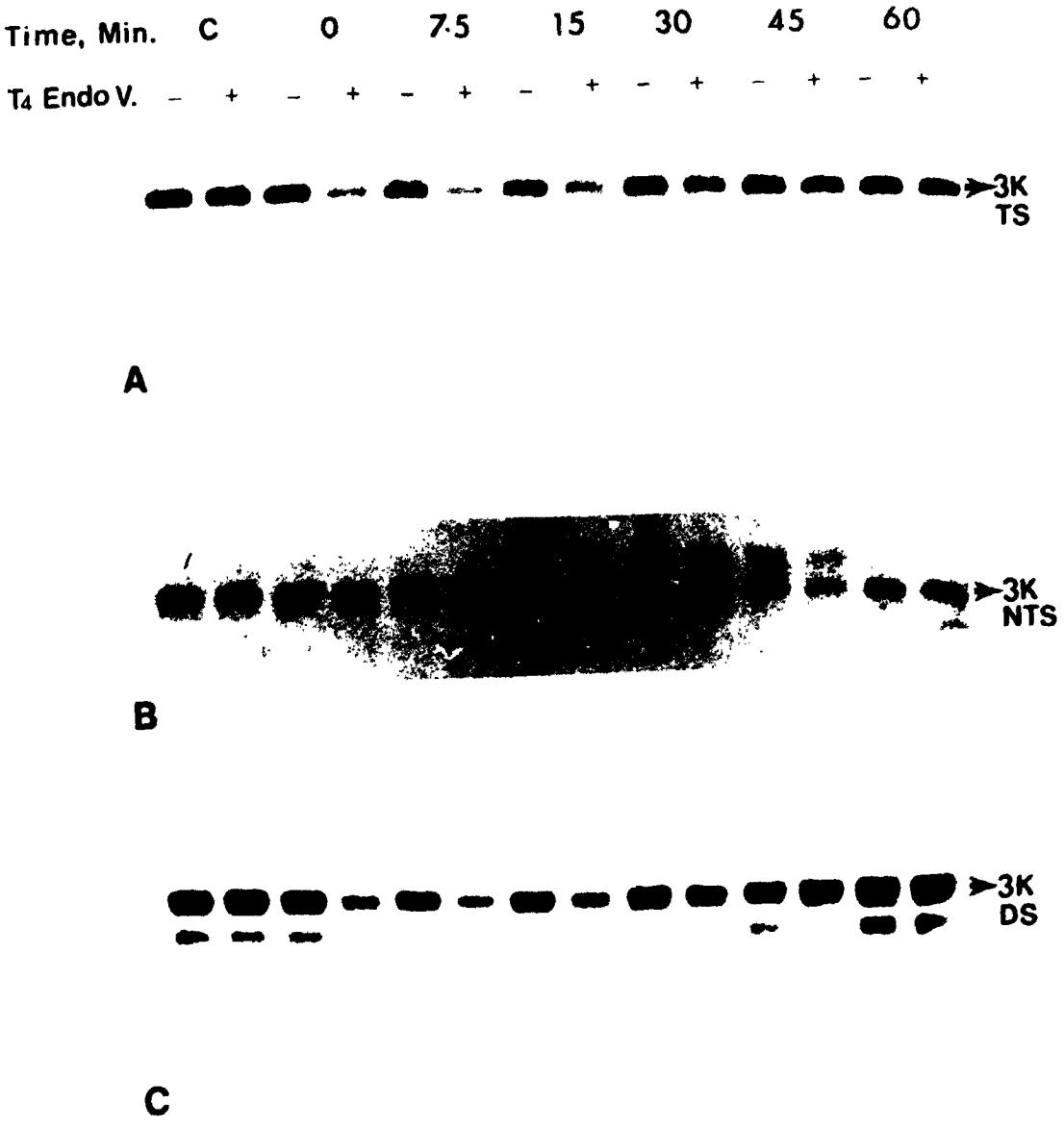
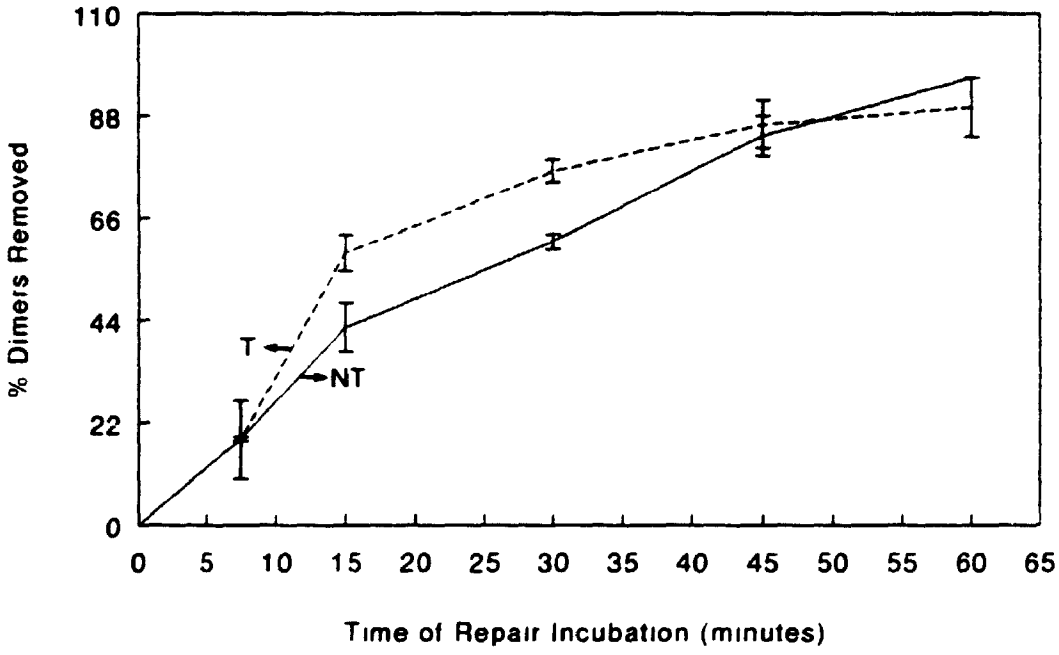

Time, Min. C 0 7.5 15 30 45 60
T4 Endo V. – + – + – + – + – + – + – +
3K TS
A
3K NTS
B
3K DS
C

FIGURE 4.8 REPAIR OF THE TRANSCRIBED AND NON-TRANSCRIBED STRANDS OF THE 3 Kbp DOMAIN IN pGA293

Scanning data were generated by densitometry of autoradiographs such as the one shown in Figure 4.7. The dimer frequency was calculated from the relative DNA signal intensities from samples treated or not treated with T4 endonuclease V for each point. The percentage repair was then determined from the dimer frequency at each time and these were averaged. The standard error of the mean is indicated at each time point (See Appendices III and IV). Dashed line; averaged data for repair of transcribed strand (T) of 3 Kbp domain of pGA293. Solid line; averaged data for repair of non transcribed strand (NT) of 3 Kbp domain of pGA293.


110
88
66
44
22
0
% Dimers Removed
T
NT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time of Repair Incubation (minutes)

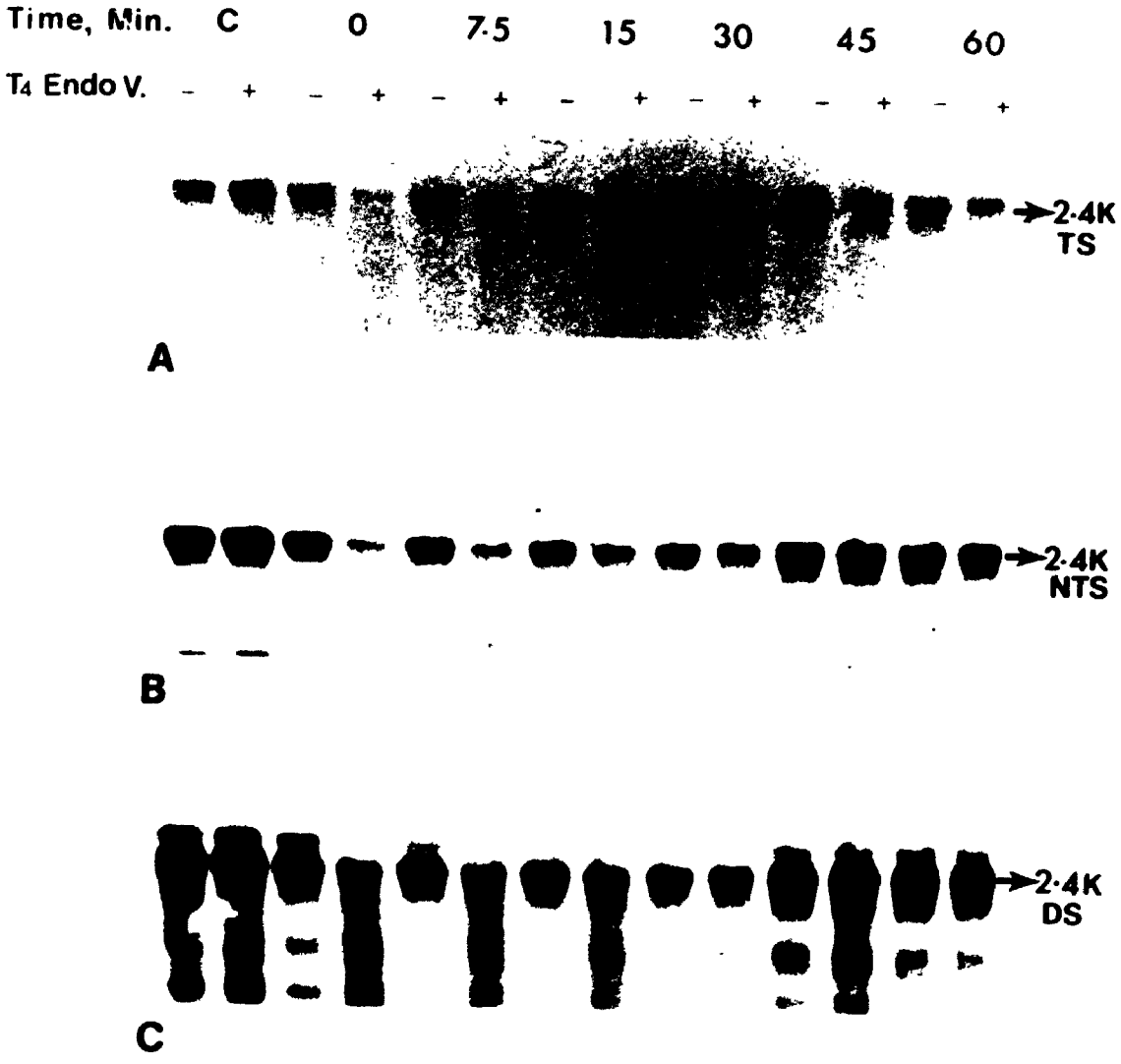
strand specific riboprobes. For the sake of clarity, the results were not shown.

4.7 Repair of Transcribed and Non-transcribed Strands of the 2.4 Kbp Domain of pGA293

During the course of this study, a technical difficulty was experienced in working with the whole 5.5 Kbp domain of pGA293. Hybridization with its specific probe was inefficient and 10-15 times as much DNA as usual was required to monitor the repair in the whole 5.5 Kbp domain. It appeared that the fragment was susceptible to some form of strand cleavage. Therefore, the 5.5 Kbp domain was further restricted with *Bgl* II which generated 2.4 Kbp and 3.1 Kbp fragments. Repair studies were confined to the 2.4 Kbp fragment which was easy to manipulate and could be easily probed following electrophoresis in an alkaline agarose gel. This fragment contains a truncated form of the *tet* gene, an intervening sequence of the SV40 early transcript and the polyadenylation signal of SV40 early transcripts. It is presumed but not established that the *tet* gene is transcribed in pGA293 in MM294 because its original promoter is intact (Peden, 1983; Sutcliffe, 1978). Repair was monitored in this domain by first using a riboprobe generated from *Bam*HI linearized plasmid pIWI and transcribed by T7 RNA polymerase. This probe detected the strand of the 2.4 Kbp domain that was designated the transcribed strand of the *tet* gene. Only a single blot was examined to characterize the repair in this domain and its autoradiograph is shown in Figure 4.9A. When the autoradiograph was scanned on the laser densitometer the initial dimer frequency was found to be 0.97 dimer per 2.4 Kbp. When the same blot was reprobed with a ^{32}P labelled riboprobe that detected the strand designated the

FIGURE 4.9 AUTORADIOGRAPH TO DEMONSTRATE THE REPAIR OF THE 2.4 Kbp *Eco*RI -*Bgl* II DOMAIN IN pGA293

E. coli strain MM294 cells harbouring pGA293 were irradiated at ice temperature with 80 J/m^2 of UV-light and incubated for various times at 37°C. At each time point, the extracted plasmid samples were digested with *Eco*RI plus *Bgl*II and then split into two equal aliquots, one was digested with T4 endonuclease V (+) and the other was sham treated (-). Lane C contains plasmid DNA isolated from non-irradiated MM294 and analyzed in the same way as the other samples. Control and UV-irradiated samples were subjected to electrophoresis through 0.7% denaturing agarose gels and examined by Southern blot analysis using different probes. A) Repair analysis using a ^{32}P-labelled riboprobe that detected the transcribed strand of the 2.4 Kbp domain of pGA293. B) Repair analysis using a ^{32}P-labelled riboprobe that detected the non-transcribed strand of the 2.4 Kbp domain of pGA293. C) Repair analysis using a ^{32}P-labelled double stranded DNA probe that detected both strands of the 2.4 Kbp domain of pGA293.


Time, Min. C 0 7.5 15 30 45 60
T4 Endo V. - + - + - + - + - + - + - +
2.4K TS
A
2.4K NTS
B
2.4K DS
C

non-transcribed strand of the *tet* gene of pGA293, an initial dimer frequency of 1.34 dimers/fragment was found. The autoradiograph of the non-transcribed strand of the *tet* gene is shown in Figure 4.9B. When the repair kinetics for the transcribed and the non-transcribed strands of the 2.4 Kbp domain were compared as shown in Figure 4.10, they appeared to be very similar. It was concluded that there is no strand bias in the repair of the 2.4 Kbp fragment. In order to confirm the repair rate pattern obtained with riboprobes for this domain, a double stranded DNA probe was used on the same blot. The autoradiograph obtained with this double stranded probe is shown in Figure 4.9C. From scanning data, the initial dimer frequency was 0.96 dimer/fragment. The repair kinetics using the double stranded DNA probe were very similar to those obtained with the strand specific riboprobes. For the sake of clarity, the results were not shown.

4.8 An Examination of Excision Repair of Cyclobutane Pyrimidine Dimers in Different Domains of pGA293 in *E. coli* strain JM109.

JM109 is a *recA*$^-$ strain of *E. coli* that contains a small number of ABC excinuclease complexes relative to a *recA*$^+$ strain such as MM294 that has been induced via the SOS signalling system. It was of interest to examine repair in JM109 where the ABC excinuclease activity was much lower. JM109 cells carrying pGA293 were UV-irradiated at 80 J/m^2 at ice temperature and then incubated at 37^{0}C as was done with MM294. Repair in the 3.5 Kbp domain of pGA293 was monitored at 0, 20, 40, and 60 minutes after UV-irradiation using the a ^{32}P-labelled riboprobe that detected the transcribed strand. A representative autoradiograph is shown in Figure 4.11. Autoradiographs of

FIGURE 4.10 REPAIR OF THE TRANSCRIBED AND NON-TRANSCRIBED STRANDS OF THE 2.4 Kbp DOMAIN IN pGA293

Scanning data were generated by densitometry of the autoradiographs shown in Figure 4.9. The dimer frequency was calculated from the relative DNA signal intensities from samples treated or not treated with T4 endonuclease V for each point and in this case only one determination was included at each point. The percentage repair was then determined from the dimer frequency at each time. Dashed line; repair of the transcribed strand of pGA293. Solid line; repair of the non-transcribed strand of pGA293.

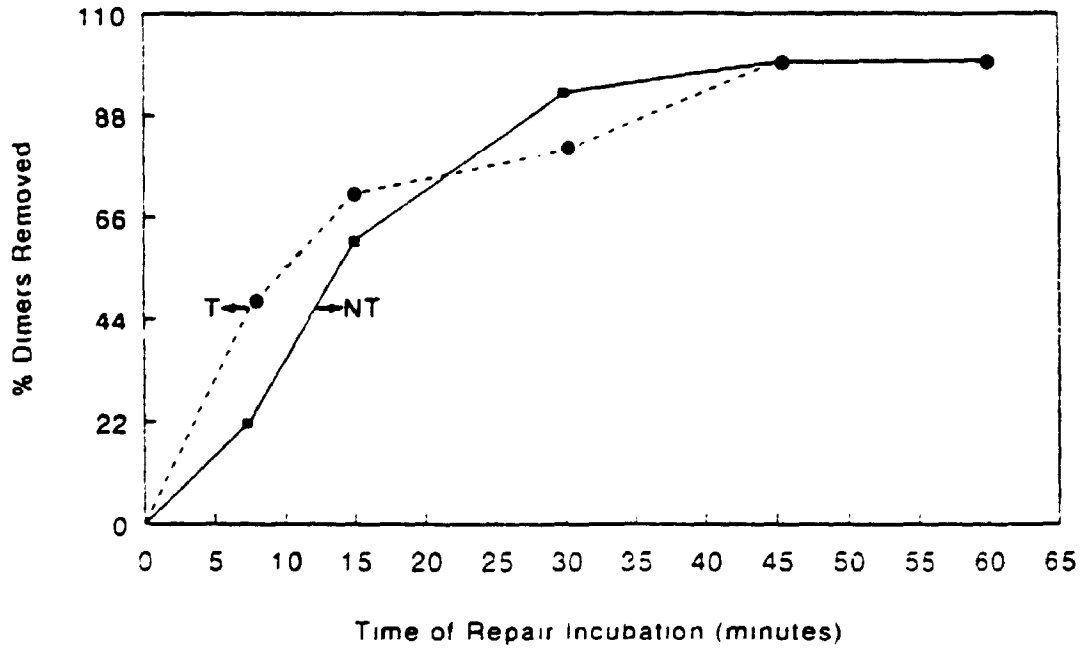
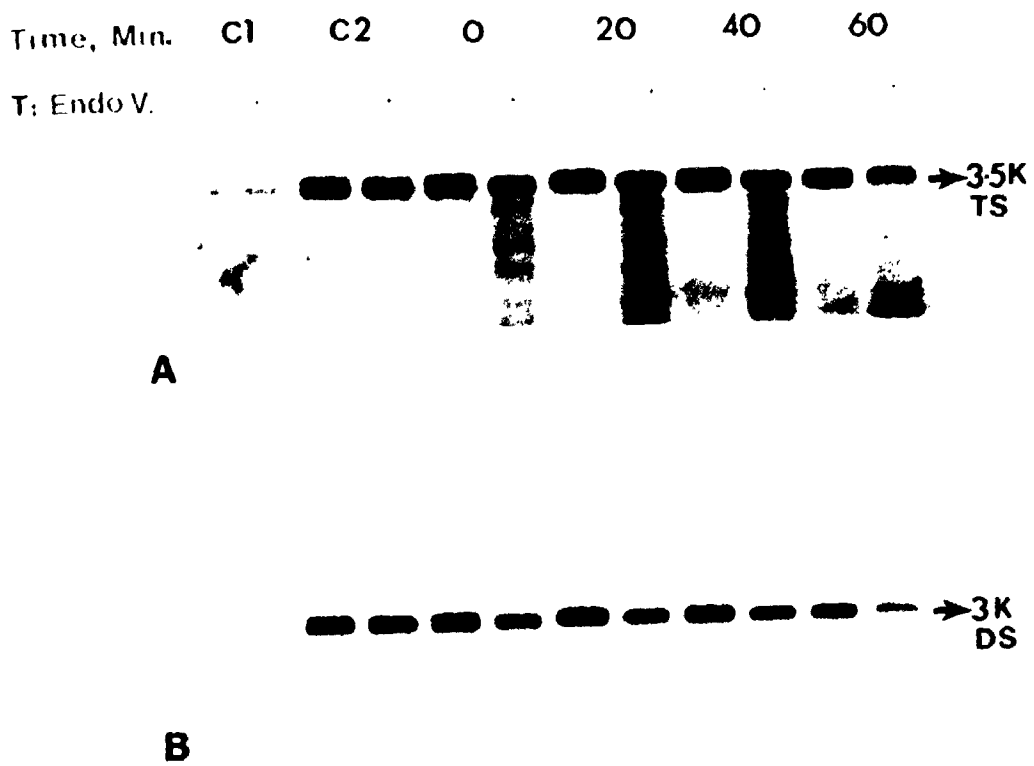

110
88
66
44
22
0
% Dimers Removed
T
NT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time of Repair Incubation (minutes)

FIGURE 4.11 AUTORADIOGRAPH TO DEMONSTRATE THE LACK OF REPAIR OF THE 3 Kbp AND 3.5 Kbp DOMAINS OF pGA293 IN *E. coli* JM109

E. coli strain JM109 cells harbouring pGA293 were irradiated with 80 J/m^2 of UV-light and incubated for various times. At each time point, the extracted plasmid samples were digested with *Eco*RI and then split into two equal aliquots, one was digested with T4 endonuclease V (+) and the other was sham treated (-). Lane C1 contains unirradiated plasmid DNA. Lane C2 contains plasmid DNA isolated from non-irradiated JM109 and analyzed in the same way as the other samples. Control and UV-irradiated samples were subjected to electrophoresis through 0.7% denaturing agarose gel and examined by Southern blot analysis using different probes. A) Repair analysis by using a ^{32}P-labelled riboprobe that detected the transcribed strand of the 3.5 Kbp domain of pGA293. B) Repair analysis by using a ^{32}P-labelled double stranded DNA probe that detected both strands of the 3 Kbp domain of pGA293.

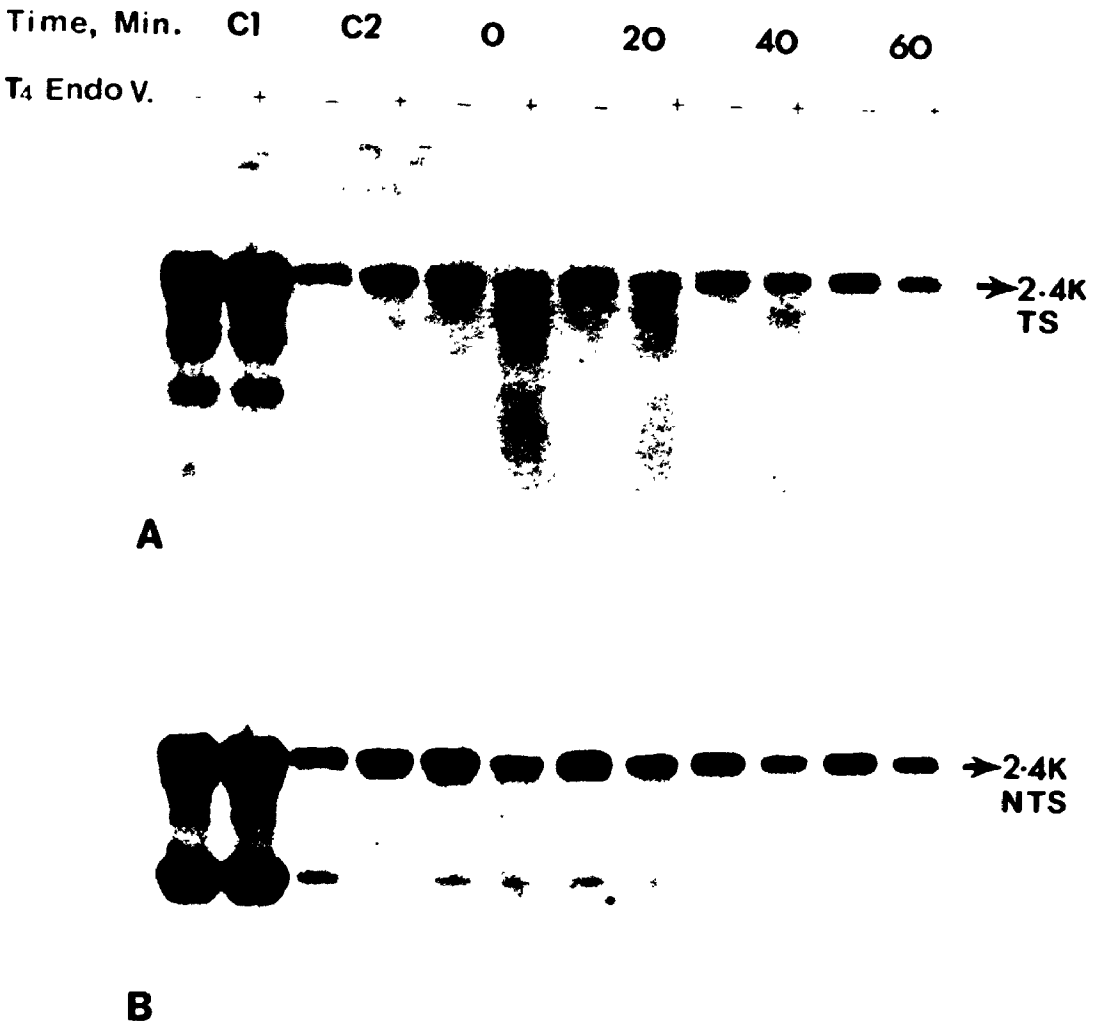

Time, Min.
C1
C2
0
20
40
60
T4 Endo V.
3.5K
TS
A
3K
DS
B

Southern blots were scanned and they showed that no detectable repair was observed up to one hour after irradiation.

When excision repair was examined in the 3 Kbp domain of pGA293 by using a double stranded DNA probe, it was found that there was a lack of repair in this domain of pGA293 also. A representative autoradiograph showing no repair up to 60 minutes is given in Figure 4.12. Repair in the 2.4 Kbp domain of pGA293 was examined using two different ^{32}P-labelled riboprobes that detected the transcribed and non-transcribed strands. Autoradiographs of the blots are shown in Figure 4.12A,B. The scanning analysis showed that there was no detectable repair at 45 minutes in either strand of this domain.

FIGURE 4.12 AUTORADIOGRAPH TO DEMONSTRATE THE LACK OF REPAIR OF THE 2.4 Kbp DOMAIN OF pGA293 IN *E. coli* JM109

E. coli strain JM109 cells harbouring pGA293 were irradiated with 80 J/m^2 of UV-light and incubated for various times. At each time point, the extracted plasmid samples were digested with *Eco*RI plus *Bgl* II and then split into two equal aliquots, one was digested with T4 endonuclease V (+) and the other was sham treated (-). Lane C1 contains unirradiated plasmid DNA. Lane C2 contains plasmid DNA isolated from non-irradiated JM109 and analyzed in the same way as the other samples. Control and UV-irradiated samples were subjected to electrophoresis through 0.7% denaturing agarose gel and examined by Southern blot analysis using different probes. A) Repair analysis by using a ^{32}P-labelled riboprobe that detected the transcribed strand of the 2.4 Kbp domain of pGA293. B) Repair analysis by using a ^{32}P-labelled riboprobe that detected the non-transcribed strand of 2.4 Kbp domain of pGA293.


Time, Min.
C1
C2
0
20
40
60
T4 Endo V.
2.4K TS
A
2.4K NTS
B

4.9 DISCUSSION

There is an increasing body of evidence suggesting that excision repair of cyclobutane pyrimidine dimers occurs more rapidly in transcriptionally active genes of mammalian cells, yeast cells, and *E. coli* than in inactive genes of these organisms. This more rapid rate of repair is confined to the transcribed strand of the active genes (Smith and Mellon, 1990; Smerdon and Thoma, 1990; Sweder and Hanawalt, 1992). There is no information regarding intragenomic DNA repair heterogeneity and strand specificity of plasmid DNA in *E. coli*. The objective of my work was to investigate whether intragenomic heterogeneity and strand specificity of repair is exhibited in plasmid DNA in *E. coli*. This system was considered to be potentially useful for investigating preferential repair because not only the plasmid, which is to undergo repair, can be manipulated, but in addition, the genetic background of the host can be varied. Therefore, repair of UV-induced cyclobutane pyrimidine dimers was monitored in three different domains of the plasmid pGA293 resident in *E. coli* strains MM294 and JM109. *Eco*RI digestion of pGA293 generates three domains of different sizes, which made it possible to monitor the repair in each domain using, in most cases, the same blot. This approach avoided the experimental variability resulting from unequal loading of separate gels and thereby provided reliable comparisons of repair rates.

In order to assess repair in the individual strands of each domain of pGA293 strand, specific riboprobes were used. The DNA sequence which served as a template for riboprobe construction was cloned into a plasmid

vector between SP6 and T7 bacteriophage RNA polymerase promoters. Prior to transcription, the construct was first linearized by digestion with an appropriate restriction endonuclease to control the size of the transcript formed. Care was taken to use an excess of restriction enzyme and to incubate the reaction mixture for a sufficiently long time, so that no uncut DNA would remain. The complete digestion of the plasmid DNA with restriction endonucleases was checked on an agarose gel by running these samples along with the uncut plasmid and DNA markers that were generated by restricting lambda DNA with *Eco*RI and *Hind* III. This was necessary because uncut DNA would serve as a template for a DNA sequence extending into the vector. Moreover, a small quantity of uncut plasmid may result in the incorporation of a large proportion of nucleotides into a higher molecular weight product. RNA polymerases are very active on linear templates but more so on supercoiled templates. It was also found that T7 RNA polymerase was less active than SP6 in synthesizing the riboprobes. Therefore, in order to get approximately equal counts in both SP6 and T7 polymerase generated RNA transcripts, the incubation time for the T7 polymerase reaction was increased (Melton, et al., 1984; Milligan et al., 1987; Cunningham et al., 1991).

In applying the blotting/hybridization technique, an unexpected phenomenon of double band formation was experienced. When detecting the so-called non-transcribed strands of the 3.5 Kbp and 3 Kbp domains and when a double stranded DNA probe was used to detect the 3 Kbp domain, a reasonable explanation for the phenomenon could not be provided since it was

confined to the use of riboprobes for the non-transcribed strands and not for the transcribed strands of the 3.0 Kbp and 3.5 Kbp domains. Running the gels at a lower voltage did not change the pattern. When transcription templates for riboprobes were generated by linearizing the plasmid DNA with enzymes that yield blunt or protruding ends, this doublet band phenomenon could not be overcome. Whatever might be the reason for this phenomenon, the position of the correct band was confirmed on each autoradiograph by superimposing the same blots generated by using sense and antisense probes on the same blot.

The *β-lactamase* gene (*amp*) was presumed to be transcribed since *E. coli* was grown at each stage in the presence of 100 μg/ml ampicillin. The *β-lactamase* gene on pGA293 is expressed constitutively even in the absence of ampicillin. It was an unexpected finding that repair in the transcribed and the non-transcribed strands of the *β-lactamase* gene which lies in the 3.5 Kbp domain of pGA293 occurred at the same rate and to the same extent, and was complete within 45 minutes. A similar result was found for the repair of the *tet* gene in the 2.4 Kbp domain of pGA293. The repair kinetics observed in the transcribed strand of the 3 Kbp domain are almost identical to those found in the individual strands of the 3.5 Kbp and the 2.4 Kbp domains and the time course of this repair can be described as hyperbolic. However, repair in the non-transcribed strand of the 3 Kbp domain occurs in a linear fashion. Thus, the repair rate is slower initially for the non-transcribed 3 Kbp strand but nevertheless is completed by about the same time as the other strands.

The lack of strand selectivity of repair in the 3.5 Kbp and 2.4 Kbp domains

contrasts with the selectivity demonstrated in the *lacZ* gene of *E. coli* and the *uvrC* gene in an *in vitro* system (Mellon and Hanawalt, 1989; Selby and Sancar, 1991). Mellon and Hanawalt demonstrated that the removal of cyclobutane pyrimidine dimers from the transcribed strand of the *E. coli lacZ* gene occurred rapidly and completely after the transcription of the gene was released from *lacI* control. By contrast, removal of cyclobutane pyrimidine dimers from the non-transcribed gene occurred more slowly. These results are reproduced in Figure 4.13. In light of subsequent work, it can be concluded that rapid repair of the transcribed strand is the result of the participation of the transcription repair coupling factor (TRCF) (Selby and Sancar, 1993).

Since the time course for the removal of cyclobutane pyrimidine dimers from the transcribed and non-transcribed strands of the 3.5 Kbp and 2.4 Kbp domains as well as the transcribed strand of the 3.0 Kbp domain are very similar, these data were combined and were plotted in Figure 4.13 so that they could be compared with the results obtained by Mellon and Hanawalt. The time course for the removal of the cyclobutane pyrimidine dimers from the non-transcribed strand of the 3.0 Kbp is also shown in Figure 4.13.

How are the repair kinetics obtained for the different domains of pGA293 to be interpreted? One possibility is that transcription coupled repair is not operative or works poorly on the plasmid DNA and that both DNA strands in each of the 3 domains are repaired at essentially the same rate but with individual differences. The average rate of this repair is somewhat greater than that seen for the repair of the non-transcribed strand of genomic *lacZ*. This

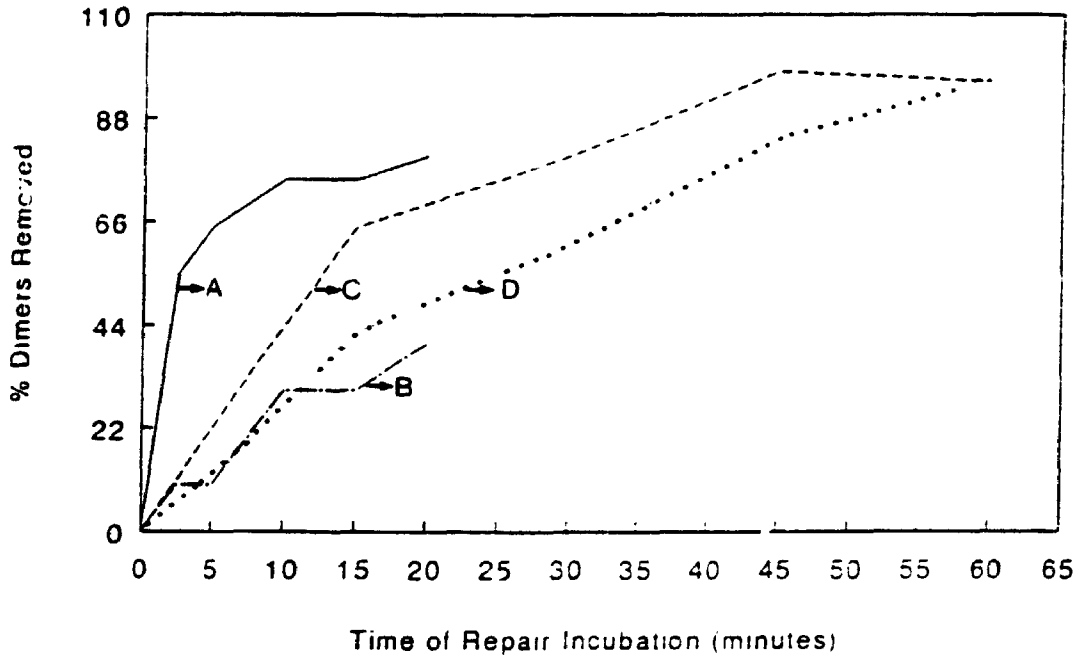
FIGURE 4.13 COMPARISON OF REPAIR OF *lacZ* GENE OF *E. coli* AND DOMAINS IN pGA293

Solid line (A); repair of the transcribed strand of the induced *lacZ* gene of *E. coli* (redrawn from Mellon and Hanawalt, 1989).

Dashed and dotted line (B); repair of the non-transcribed strand of the *lacZ* gene of *E. coli* (redrawn from Mellon and Hanawalt, 1989).

Dashed line (C); averaged data for repair of transcribed and non-transcribed strands of 3.5 and 2.4 Kbp domains and the transcribed strand of 3 Kbp domain of pGA293.

Dotted line (D); averaged data for repair of the non-transcribed strand of the 3 Kbp domain of pGA293.


110
88
66
44
22
0
% Dimers Removed
A
C
D
B
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time of Repair Incubation (minutes)

could be interpreted as being due to different rates for genomic DNA and plasmid DNA based on different arrangements of the DNAs in their chromatin forms.

Another possibility is that the transcribed and non-transcribed strands of the 3.5 Kbp and 2.4 Kbp domains as well as the transcribed strand of the 3.0 Kbp domain are undergoing transcription coupled repair. However, the time course of repair of these strands is slower than that of the genomic *lacZ* transcribed strand because there are 10 times as many copies of them and they contain about 2 times the number of dimers per Kbp. As part of this possibility, it appears that the non-transcribed strand of the 3.0 Kbp domain does not show transcription coupled repair and in fact, its time course is very similar to that of the non-transcribed strand of the genomic *lacZ* gene. These two possibilities could be resolved by examining the repair of a plasmid in an *mfd* mutant of *E. coli* where the transcription coupling factor is missing (Selby and Sancar, 1993). It would also be informative to examine the repair of a transcriptionally active gene such as *lacZ* that was present in both the bacterial genome and the plasmid. The genes could be distinguished on the basis of different sized restriction fragments (Sweder and Hanawalt, 1992).

Why are the non-transcribed strands of the 3.5 Kbp and 2.4 Kbp domains repaired as rapidly as the transcribed strands? One explanation is that these strands are in fact being transcribed. A promoter has been described which lies on the non-transcribed strand of the *β-lactamase* gene and is located between the gene and the Co1E1 origin. This promoter, called RNA I, directs

the synthesis of a RNA transcript of 108 nucleotides that is involved in the control of replication and this might direct transcription repair coupling (Chan et al., 1985; Cesareni et al., 1991; Sutcliffe, 1978; Polisky, 1988; Kues and Stahl, 1989). It seems likely that the non-transcribed strand of the 2.4 Kbp domain is being transcribed for the reason that the *gpt* gene, which is in close proximity to the *tet* gene, contains a transcriptional promoter and a protein encoding sequence that would express XGPRT in the transformed *E. coli* host (Mulligan and Berg, 1980).

The *tufB-β-gal* gene located in the 3.0 Kbp domain had its bacterial promoter removed during construction of pGA293; nevertheless it shows some expression. It was reported that a *lacZ*⁻ strain of *E. coli* carrying pGA293 produced light blue colonies on x-gal plates (An et al., 1982) and in this study it was demonstrated that the β-galactosidase activity in MM294 cells carrying pGA293 was 4 times that in MM294 without the plasmid. This represents a very weak transcriptional activity when compared to the 400 fold increase reported to occur following induction (Miller, 1972). Nevertheless, it may be sufficient to allow transcription-coupled repair to occur. The basis for this "promoterless" expression is not known; however, a promoter has been described that controls the synthesis of a primer RNA, called RNA II, which primes the synthesis of DNA. RNA polymerase transcribes this primer at a location 555 base pairs upstream of the origin of replication. Transcription of this RNA II continues until a termination signal is reached which depends on the downstream sequence of the specific plasmid. The RNA II is then

processed by RNase II to generate a 550 nucleotide sequence that is used as a primer by DNA polymerase I to initiate leading strand synthesis. The read through transcription originating at the RNA II promoter could be the basis for the more rapid repair that is observed (Sutcliffe, 1978; Backman et al., 1978; Itoh and Tomizawa, 1980; Tomizawa and Mosukata, 1987; Polisky, 1988; Chan et al., 1985; Cesareni et al., 1991).

In order to examine how much difference exists in the repair kinetics of Rec A^+-dependent and -independent repair pathways, the repair of pGA293 was monitored in *E. coli* strain JM109 that carries a mutation in the Rec A protein. There was no detectable repair up to 60 minutes after UV-irradiation. This showed that the Rec A^+ phenotype was necessary for the rapid repair of pGA293. It appears that low concentrations of several enzymes in the absence of the SOS process limits the repair of pGA293 in JM109.

This plasmid repair system has potential use for investigating the effect of promoters with different efficiencies. Also, with an appropriate promoter one can have rigid control of inducible expression. There are a number of proteins involved in the organization of bacterial chromatin such as HU, IHF and H-NS and their role in repair could be studied with this system. HU, the most abundant of the histone-like proteins in *E. coli*, interacts with DNA and changes its topology (Pettijohn, 1988). IHF is a multifunctional protein with similarities to HU. It is involved in several processes including transcriptional and translational control. This protein can bend DNA by binding to specialized sequences (Friedman, 1988; Nash, 1990). H-NS plays a more active role in the

regulation of chromatin structure, DNA topology, gene expression, and stablizing the *E. coli* genome. It binds linear and supercoiled DNA and increases the compactness of DNA in reporter plasmids. Mutations in H-NS result usually in increased transcription of *proU, bglY, ompC,* and the *pilus* adhesin gene. It is also known that some mutations in H-NS increases the mean linking number of reporter plasmid DNA (Dorman et al., 1990; Rimsky and Spasskey, 1990; Higgins et al., 1988; Lejeune and Danchin, 1990; Goransson et al., 1990). Finally, it could also be used to determine whether repair coupling at the transcription level is operative when different sigma factors are replaced under a stress condition, such as heat shock (Neidhardt et al., 1985).

CHAPTER 5

STUDIES ON THE REPAIR OF pGA293 IN CHO CELLS

5.1 Expression of β-Galactosidase After Transfecting UV-Irradiated pGA293 into AA8 and UV41 Cells

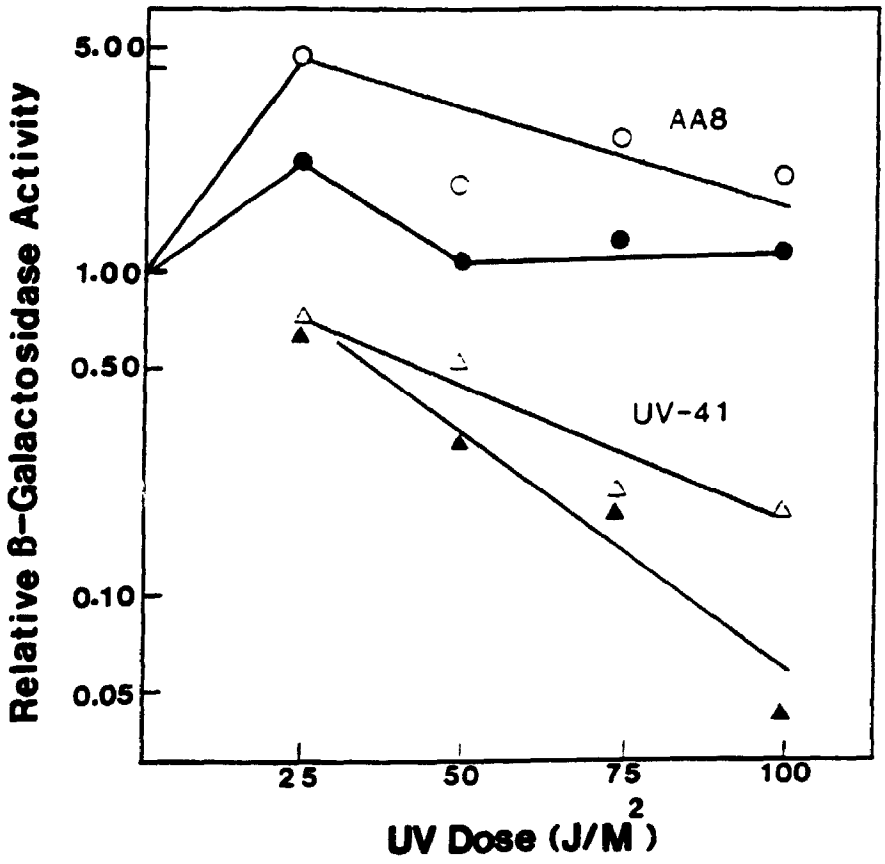
The validity and feasibility of a plasmid-based system was examined in pursuit of developing a simplified system to study nucleotide excision repair, which would be reminiscent of repair occurring in endogenous chromosomal DNA. The repair of *in vitro* UV-irradiated recombinant plasmid pGA293 was examined after transfecting it into repair proficient AA8 and repair deficient UV41 cell lines. In this host reactivation assay, the transient expression of *β-galactosidase*, under the control of the SV40 promoter, was monitored. UV-induced cyclobutane pyrimidine dimers inhibit the transcription of the reporter gene and a resumption of its expression would reflect the repair capacity of the recipient cells (Sauerbier and Hercules, 1978). In the experiment shown in Figure 5.1, the expression of *β-galactosidase* was greatly inhibited when UV41 cells were the recipients of the irradiated plasmid but only slightly when AA8 cells served as the host. This result indicates that AA8 cells can repair UV-damaged plasmid following its transfection into the cells.

5.2 Effect of Transfection Protocol on the Expression of β-galactosidase Activity

In the calcium phosphate coprecipitate method, the cells are incubated with the coprecipitate for a period of time to allow adsorption of the coprecipitate onto the cells. A subsequent brief exposure of the cells to 10% DMSO is designed to enhance the uptake of the coprecipitate. The effect of

FIGURE 5.1 EXPRESSION OF β-GALACTOSIDASE FROM UV-IRRADIATED pGA293 AFTER TRANSFECTION INTO AA8 OR UV41 CELLS

AA8 or UV41 cells were incubated with irradiated plasmid for 16 hours and then shocked with 10% DMSO. The β-galactosidase activites were determined fluorometrically at 18 hours (closed symbols) or 24 hours (open symbols) after DMSO shock using 4-methyl umbelliferone β-galactoside as a substrate. The transfected CHO cells were harvested at 18 and 24 hours and assayed for β-galactosidase activities because the time for maximal enzyme expression was not known.


Relative β-Galactosidase Activity
5.00
1.00
0.50
0.10
0.05
AA8
UV-41
25
50
75
100
UV Dose (J/M²)

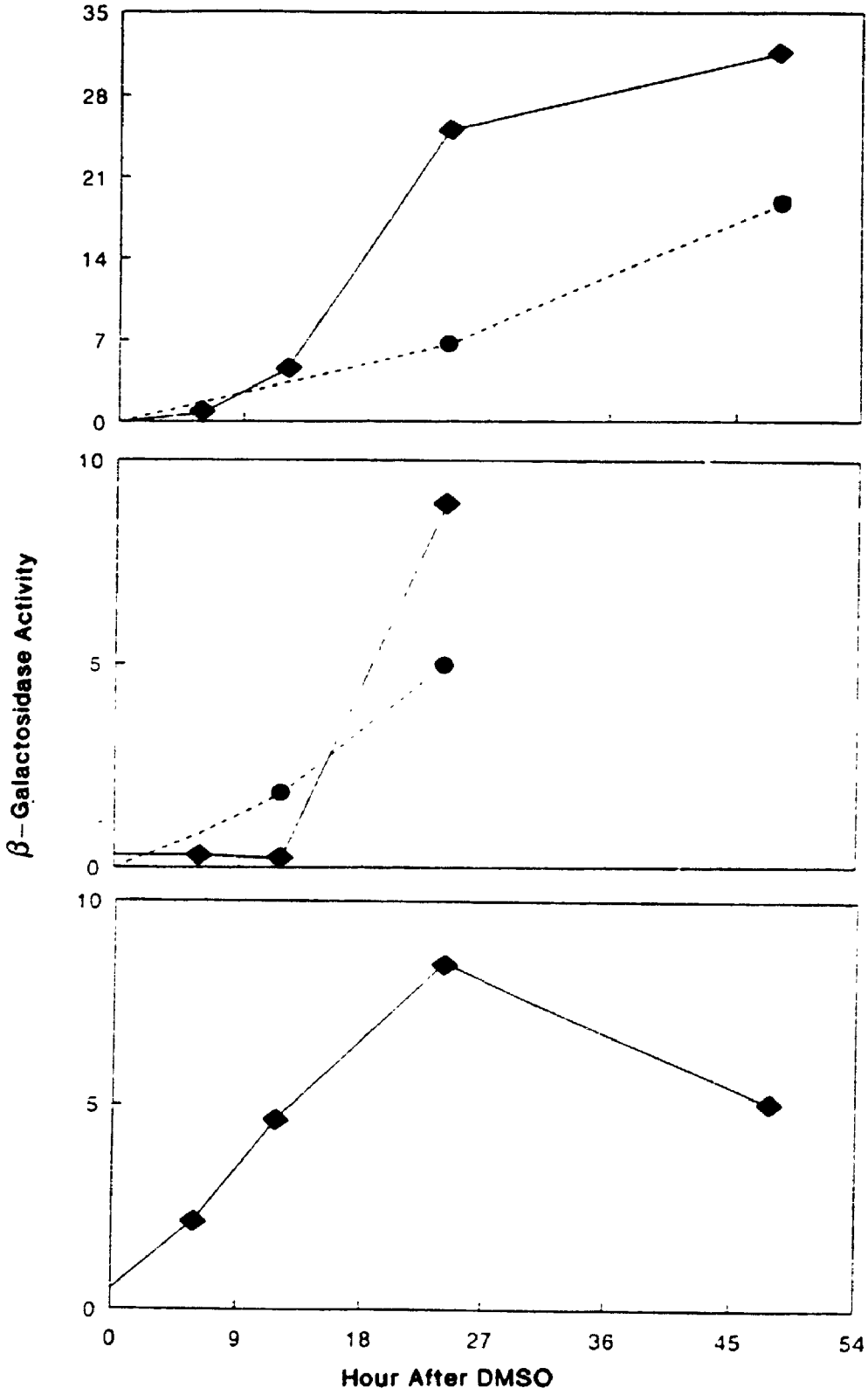
varying the incubation time prior to the DMSO shock on β-galactosidase activity was investigated. Figure 5.2 shows the results of three trials in which the incubation times prior to the DMSO shock were 6, 10, and 15 hours. First, it should be noted that the irradiated plasmid is not only expressed, but is expressed to a greater extent than unirradiated plasmid. This shows that the UV-damaged plasmid has not only been repaired but it is a preferred substrate for transcription. As will be discussed later, this phenomenon may be related to the assembly of minichromosomes prior to transcription. The various expression patterns obtained are thought to reflect the efficiency of entry of the plasmid into the cells and then the fate of the plasmid, whether it is enzymatically degraded or converted into a transcriptionally active structure.

5.3 Validation of the Method for Extracting Plasmid DNA from Nuclei

Before proceeding with the analysis of the repair of transfected pGA293, it was necessary to know if a detectable amount of plasmid had integrated into the genome of AA8 cells. If the extent of integration proved to be negligible, it would not be necessary to use the more elaborate Hirt procedure to analyse the plasmid DNA. Rather, unfractionated nuclei could be analysed. When the high molecular weight fraction obtained by the Hirt method was further purified, restricted and subjected to Southern blotting, there was no apparent signal indicative of pGA293 integration even after long autoradiographic exposure. This showed, as expected, that integration frequency was extremely low and was not an analytical concern. In addition, the identical pattern of relaxed, linearized, and supercoiled pGA293 was observed when DNA samples were

FIGURE 5.2 EXPRESSION OF β-GALACTOSIDASE ACTIVITY IN AA8 CELLS FOLLOWING TRANSFECTION WITH pGA293 OR IRRADIATED pGA293 USING DIFFERENT PROTOCOLS

The amount of plasmid transfected was 5 μg per 2 x 10^6 cells per 100 mm dish and DMSO treatment was given 6, 10, or 15 hours (panels A, B and C respectively) after exposing the cells to the plasmid-calcium phosphate coprecipitate. The β-galactosidase activities in AA8 cells following transfection with pGA293 (dots) or UV-irradiated pGA293 (squares) are expressed as nanomoles of 4-methyl umbelliferone β-galactoside hydrolyzed in 30 minutes by one 100 mm tissue culture dish of cells.


35
28
21
14
7
0
10
5
0
10
5
0
0
9
18
27
36
45
54
β-Galactosidase Activity
Hour After DMSO

extracted from nuclei by the Hirt method or when the SDS-proteinase K method was applied to nuclei. Any plasmid remaining adsorbed to the cell membrane was removed by DNAase-I treatment and plasmid in the cytoplasm was eliminated by rupturing the cells and isolating the nuclei very carefully. Following these preliminary results, plasmid DNA was extracted only by the SDS-proteinase K method. Recovery of pGA293 was found to be somewhat higher by this method because some of the pGA293 was lost in separating the plasmid DNA from higher weight molecular genomic DNA in the Hirt method.

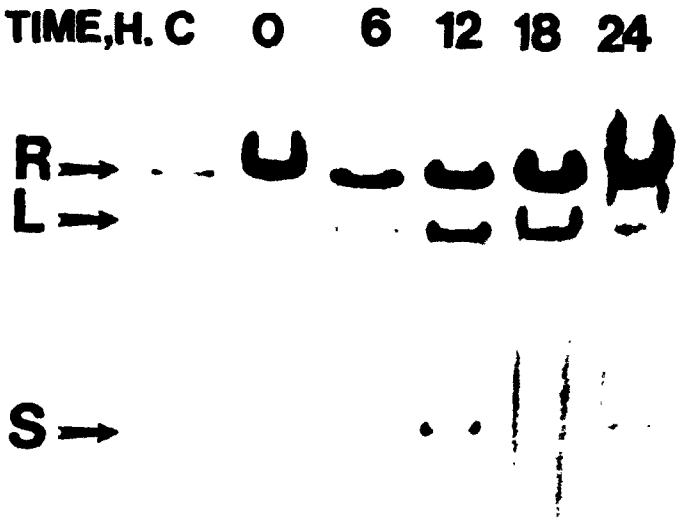
5.4 Early Experiments to Assess Repair of UV-irradiated pGA293 Transfected into AA8 Cells

In an early experiment to examine excision repair at the gene level, 100 mm dishes containing 2 x 10^6 cells were incubated for 16 hours with 10 μg of pGA293 that had been irradiated with 50 J/m^2 of UV-light. The cells were exposed to 10% DMSO for 30 minutes and then were incubated in regular medium for 6, 12, 18, and 24 hours. The cells were harvested at these times and plasmid extracted from the nuclei. Equal volumes from each sample were electrophoresed in a neutral agarose gel. The gels were blotted to a membrane, probed with a ^{32}P labelled preparation of pGA293 and autoradiographs were made. One of these is shown in Figure 5.3. It can be seen that the amount of plasmid in the nucleus increased with time following DMSO shock.

Portions of the same plasmid samples were cut with *EcoRI*, then analyzed by the Bohr-Hanawalt method. This involved dividing each *EcoRI* digest into two equal parts and treating one with T4 endonuclease V, while the other was sham treated with T4 endonuclease V buffer. These samples were

FIGURE 5.3 AUTORADIOGRAPHS TO SHOW THE AMOUNT AND CONFORMATION OF pGA293 EXTRACTED FROM AA8 CELLS AT DIFFERENT TIMES AFTER TRANSFECTION

AA8 cells were treated with the pGA293 calcium phosphate coprecipitate and incubated for 16 hours before exposing the cells to 10% DMSO for 30 minutes. The end of the DMSO treatment was taken as zero time. The DMSO containing medium was replaced with regular medium and incubation was continued for 6, 12, 18, and 24 hours. At each time, plasmid was extracted, purified, and electrophoresed in 0.7% neutral gel, transferred to a membrane and hybridized by ^{32}P labelled pGA293. C refers to control pGA293 that was not transfected but included along with other samples in order to determine the amount and position of pGA293 extracted from AA8 cells at different times after transfection. Supercoiled (S), relaxed (R) and linear (L) forms of pGA293 are shown by arrow marks.

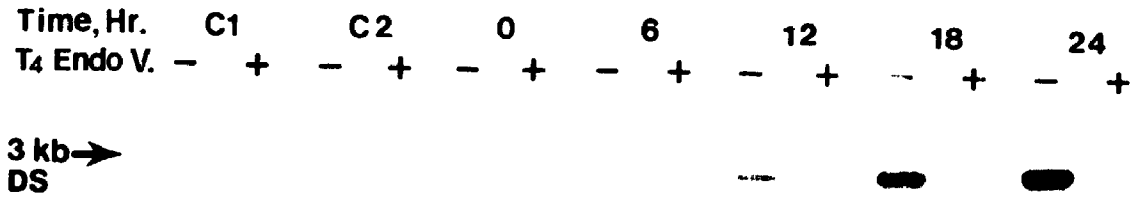

TIME,H. C 0 6 12 18 24
R→
L→
S→

then electrophoresed as pairs on a denaturing agarose gel to separate the different fragments of pGA293. The DNA was transferred to a support membrane and hybridized with a ^{32}P labelled probe that detected the *lacZ* containing 3 Kbp domain of pGA293. An autoradiograph of this gel is shown in Figure 5.4A. By densitometry the average number of cyclobutane pyrimidine dimers per 3 Kbp was determined from the ratio of the band intensities of T4 endonuclease V treated to untreated samples using the Poisson expression, and these are recorded in Table 5.1. The results obtained with the double stranded probe indicated that there were 1.03 dimers in the 3 Kbp domain of pGA293 before transfection and these were reduced to 0.59 dimers at 0 hour. This corresponded to 43% repair. There was an even more dramatic decline in the number of dimers by 6 hours when only 0.13 dimers were left corresponding to 88% repair. However, the number of dimers unexpectedly increased after this, reaching values of 0.39, 0.50, and 0.69 dimers at 12, 18, and 24 hours. We interpretated this pattern as the result of plasmids entering the cells and were being repaired during the 16 hours of incubation. Subsequently, when DMSO treatment was given, a large amount of unrepaired plasmid entered the cell and diluted the already repaired plasmid.

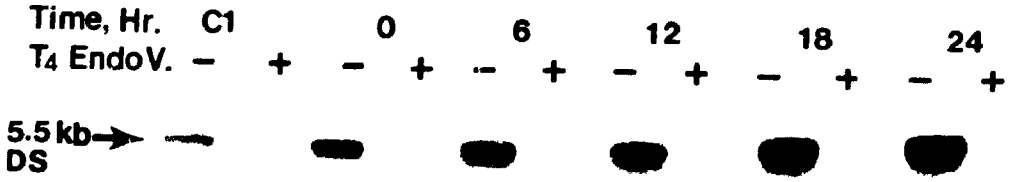
In order to determine whether there was any strand bias in the repair detected in the 3 Kbp fragment, ^{32}P labelled riboprobes specific for the transcribed strand and non-transcribed strand were hybridized to blots, similar to that described above. An autoradiograph showing the strand specific repair is shown in Figure 5.5A,B. The number of dimers formed in the individual

FIGURE 5.4 AUTORADIOGRAPHS FROM AN EARLY EXPERIMENT IN WHICH REPAIR APPEARS TO HAVE BEEN DEMONSTRATED IN THE 3 Kbp DOMAIN BUT NOT THE 5.5 Kbp DOMAIN OF pGA293

The AA8 cells were incubated with 10 μg of irradiated plasmid per dish containing 2 x 10^6 cells for 16 hours, then shocked with 10% DMSO, and further incubated in regular medium. Plasmid was extracted from the cell samples by the Hirt method and digested with *Eco*RI. The digests were split into two equal aliquots and one was treated with T4 endonuclease (+) and the other was sham treated (-). A) Repair analysis using a ^{32}P labelled double stranded DNA probe that detected both strands of the 3 Kbp domain of pGA293. C1 and C2 refer respectively to irradiated and non-irradiated pGA293 samples that were not transfected but analyzed in the same way as samples treated after transfection. B) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 5.5 Kbp domain of pGA293. C1 refers to irradiated pGA293 samples that were not transfected but analyzed in the same way as samples treated after transfection.


Time, Hr.
C1
C2
0
6
12
18
24
T4 Endo V.
3 kb
DS

A


Time, Hr.
C1
0
6
12
18
24
T4 Endo V.
5.5 kb
DS

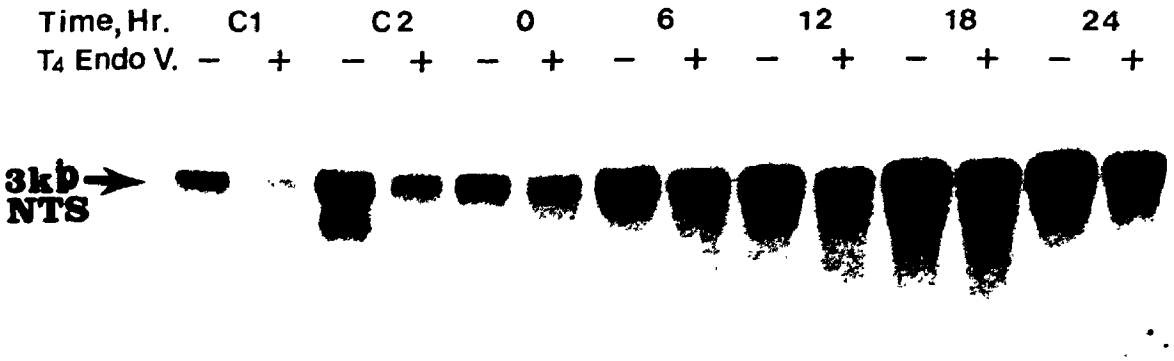
B

FIGURE 5.5 AUTORADIOGRAPHS FROM AN EARLY EXPERIMENT IN WHICH REPAIR APPEARS TO HAVE BEEN DEMONSTRATED IN THE TRANSCRIBED AND NON-TRANSCRIBED STRANDS OF THE 3 Kbp DOMAIN IN pGA293

This is a further analysis of the experiment described in Figure 5.4 but independent blots were used in each case. A) Repair analysis using a ^{32}P labelled riboprobe that detected the transcribed strand [TS] of the 3 Kbp domain of pGA293. C1 and C2 refer to non-irradiated and irradiated pGA293 samples that were not transfected but analyzed in the same way as samples extracted after transfection. B) Repair analysis using a ^{32}P labelled riboprobe that detected the non-transcribed strand [NTS] of pGA293. C1 and C2 refer to irradiated pGA293 samples that were not transfected but analyzed in the same was as samples extracted after transfection.


Time, Hr. C1 C2 0 6 12 18 24
T4 Endo V. – + – + – + – + – + – + – +
3kb
TS

A


Time, Hr. C1 C2 0 6 12 18 24
T4 Endo V. – + – + – + – + – + – + – +
3kb
NTS

B

strands of the 3 Kbp fragment using this technique are recorded in Table 5.1. The number of dimers found in the transcribed strand decreases up to 6 hours but rises thereafter. The number of dimers found in the non-transcribed strand shows a gradual decline over the 24 hours following the DMSO shock. Clearly, the results obtained with the double stranded DNA probe and the strand specific riboprobe are aberrant. Thus, the two results obtained with the strand specific probes do not accord well with the result obtained with the DNA probe. And, it is not reasonable for the number of dimers to increase during the time when they should be removed. Nevertheless, these results were considered sufficiently encouraging to continue with this type of analysis and it was believed that the aberrant results would be obviated by modifying the transfection protocol.

The removal of cyclobutane pyrimidine dimers from the 5.5 Kbp fragment was also examined using a double stranded DNA probe. An autoradiograph of this analysis is shown in Figure 5.4B. No evidence for the removal of dimers is seen. It was thought that this result could be due to a lack of transcription of this domain or because there were twice the number of dimers per fragment due to its larger size.

5.5 Modification to the Transfection Protocol: Varying the Duration of the Incubation Period Prior to DMSO Shock

The inconsistent repair pattern reported in section 5.4 might be due to an excessive amount of pGA293 entering the cell nuclei over the 16 hour incubation period prior to the DMSO shock. In an attempt to overcome this problem, the cells were incubated with irradiated pGA293 for 10 or 6 hours

TABLE 5.1 CYCLOBUTANE PYRIMIDINE DIMERS IN THE 3 Kbp DOMAIN OF pGA293 AFTER TRANSFECTION INTO AA8 CELLS.

Hours after DMSO	Number of dimers found in: Both Strands	Transcribed Strand	Non-transcribed Strand
Before Transfection	1.03	1.16	0.86
0	0.59	0.69	0.15
6	0.13	0.45	0.57
12	0.39	1.15	0.57
18	0.50	0.76	0.46
24	0.69	0.98	0.38

before giving the DMSO shock and then DNA was extracted from the cells 0, 6, 12, and 24 hours later. After cutting with *Eco*RI, the DNA samples were processed as described in the previous section in order to assess the extent of repair occurring in the three domains of pGA293. Figure 5.6 shows some autoradiographic results from the 10 hour trial. In panel A a double stranded DNA probe was used to detect the 3 Kbp domain. After deprobing, a riboprobe specific for the transcribed strand of the 3 Kbp domain was used (panel B). In panel C, a double stranded DNA probe specific for the 5.5 Kbp domain was employed. Visual inspection of T4 endonuclease V treated and untreated samples at various time periods provided no evidence of repair at any time and this was confirmed by densitometry.

The results obtained for the 6 hour trial were essentially the same and some autoradiographs from this trial are shown in Figure 5.7. In panel A, the 3 Kbp domain was detected with a double stranded DNA probe. In panel B, the transcribed strand of the 3 Kbp domain was detected with a riboprobe. In panel C the 3 Kbp domain was detected with a double stranded DNA probe. At the 6 hour point in these autoradiographs, the dimer frequency was found by densitometry to be about 54% of the initial frequency, but because there was no indication of dimers removed at the other times, this finding was regarded as an artifact.

5.6 Modification to the Transfection Protocol: Varying the Amount of Plasmid

Since a consistent repair pattern was not obtained with an input of 10 μg plasmid per dish and times of 6, 10, or 16 hours before the DMSO shock, one

FIGURE 5.6 ATTEMPT TO DEMONSTRATE REPAIR OF pGA293: INCUBATION OF PLASMID WITH CELLS FOR 10 HOURS BEFORE DMSO SHOCK

Irradiated plasmid and AA8 cells (10 μg pGA293 per 2 x 10^6 cells) incubated for 10 hours, then shocked with 10% DMSO for 30 minutes. The plasmid DNA was extracted by the Hirt method at various times after the DMSO shock and equal amounts were digested; the digests were split into two equal aliquots; one was treated with T4 endonuclease V (+) and the other was sham treated (-). C1 and C2 refer respectively to non-irradiated and irradiated samples that were not transfected but analyzed in the same way as samples extracted after transfection. A) Repair analysis using a ^{32}P labelled double stranded DNA probe that detected both strands of the 3 Kbp domains of pGA293. B) Repair analysis using a ^{32}P labelled riboprobe that detected the transcribed strand of 3 Kbp domain of pGA293. C) Repair analysis using a ^{32}P labelled double stranded DNA probe that detected both strands of the 5.5 Kbp domain of pGA293.

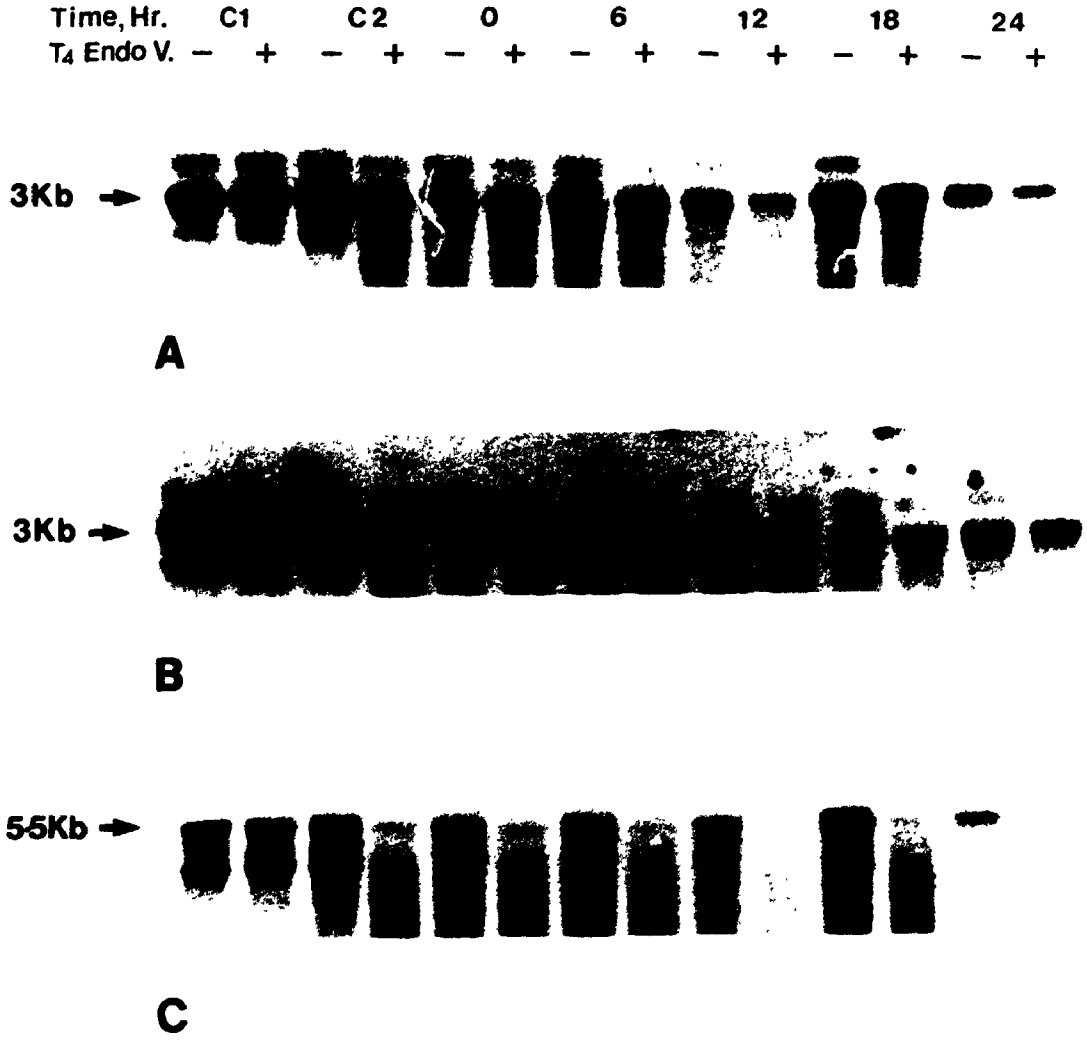
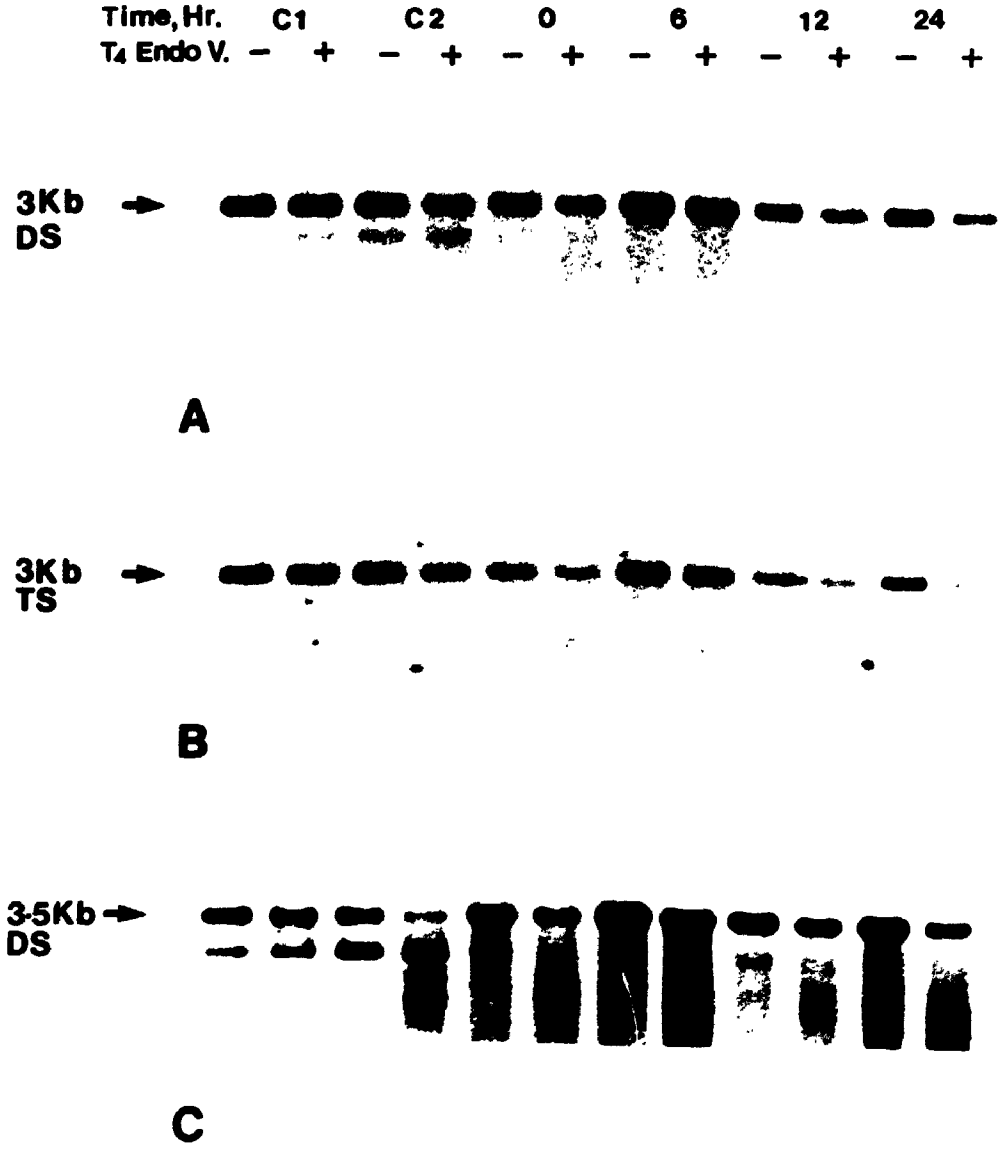

Time, Hr. C1 C2 0 6 12 18 24
T4 Endo V. − + − + − + − + − + − + − +
3Kb
A
3Kb
B
5.5Kb
C

FIGURE 5.7 ATTEMPT TO DEMONSTRATE REPAIR OF pGA293: INCUBATION OF PLASMID WITH CELLS FOR 6 HOURS BEFORE DMSO SHOCK

The experimental conditions were the same as described in Figure 5.6 except that the incubation period prior to DMSO treatment was 6 hours. C1 and C2 refer to non-irradiated and irradiated samples that were not transfected but analyzed in the same way as samples extracted after transfection. A) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3 Kbp domain of pGA293. B) Repair analysis using a ^{32}P labelled riboprobe that detected the transcribed strand [TS] of the 3 Kbp domain of pGA293. C) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3.5 Kbp domain of pGA293.


Time, Hr. C1 C2 0 6 12 24
T4 Endo V. − + − + − + − + − + − +
3Kb
DS
A
3Kb
TS
B
3.5Kb
DS
C

possible explanation for the aberrant repair pattern or total lack of repair might be the limitation of the cellular machinery for repair. The result could be that a small amount of repaired plasmid would go undetected in the presence of a large amount of unrepaired plasmid.

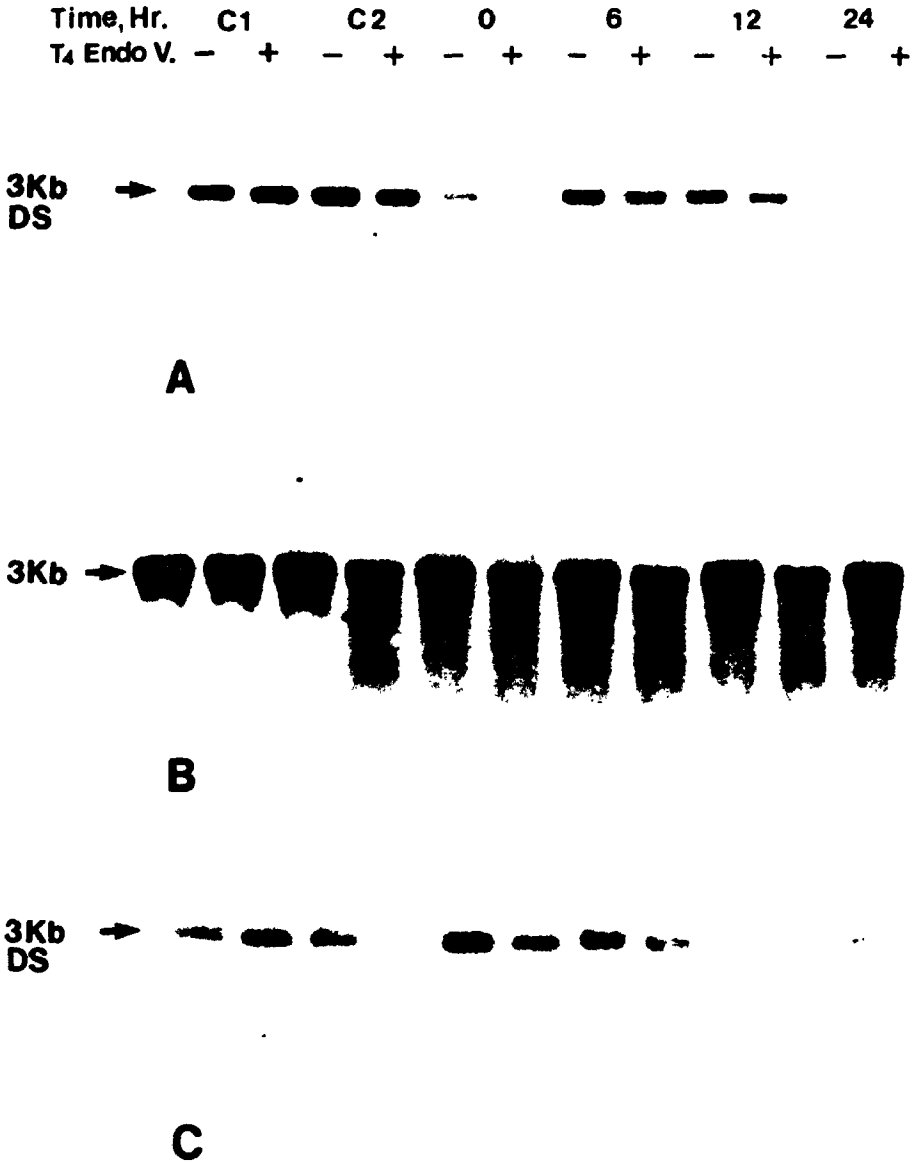
To test this possibility, the amount of plasmid transfected was reduced and the incubation time before the DMSO shock was fixed at 6 hours. The amounts of DNA chosen for this trial were 8, 5, and 2 μg per dish because this was the concentration range used by many investigations for transient transfection purposes and with lower amounts of plasmid, gene expression was not detectable. DNA was extracted from the cells, 0, 6, 12, and 24 hours after DMSO treatment and after cutting with *Eco*RI. The DNA samples were processed as described in section 5.4 in order to assess the extent of repair occurring in the domains of pGA293. Some representative autoradiographs are shown in Figure 5.8 as follows. In panel A, the amount of plasmid used was 8 μg and the 3 Kbp domain was detected with a double stranded DNA probe. In panel B, the amount of plasmid used was 5 μg and the transcribed strand of the 3 Kbp domain was detected. In panel C, 2 μg of plasmid was used to detect the 3 Kbp domain. No repair was evident from visual inspection of these autoradiographs and densitometry confirmed this observation. Repair was also looked for in the other domains using appropriate probes, but it was not detected.

5.7 Repair Analysis of Different Domains of pGA293 Transfected into AA8 Cells Without DMSO Shock

The DMSO shock in the transfection protocol has been used to facilitate

FIGURE 5.8 ATTEMPT TO DEMONSTRATE REPAIR OF pGA293: VARYING THE AMOUNT OF PLASMID TRANSFECTED

For the results shown in panels A and C, pGA293 was irradiated at a concentration of 100 ng/ul at an UV dose of 50 J/m^2 and 8 μg [A] and 2 μg [C] of the plasmid were incubated with 2 x 10^6 AA8 cells/dish for 6 hours before DMSO shock. For the result shown in panel B, pGA293 was irradiated at a concentration of 30 ng/ul at an UV dose of 75 J/m^2 and 5 μg [B] of plasmid was incubated with 2 x 10^6 AA8 cells/dish for 6 hours before the DMSO shock. In each experiment, the plasmid DNA was extracted at 0, 6, 12, and 24 hours after DMSO shock, digested with *Eco*RI, and analyzed for repair. C1 refers to the non-irradiated and C2 refers to the irradiated plasmid, both of which were not transfected but analyzed in the same manner as samples extracted after transfection. A) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3 Kbp domain of pGA293. B) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3 Kbp domain of pGA293. C) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3 Kbp domain of pGA293.


Time, Hr. C1 C2 0 6 12 24
T4 Endo V. – + – + – + – + – + – +
3Kb
DS
A
3Kb
B
3Kb
DS
C

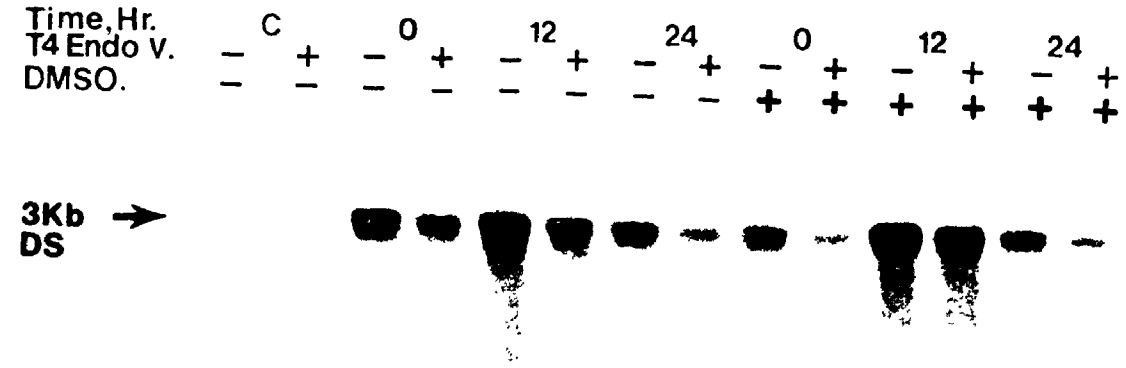
the uptake of the plasmid-calcium phosphate coprecipitate into the cells. It was thought that the DMSO shock might be causing a rapid influx of unrepaired pGA293 which in turn could be responsible for the inconsistent repair pattern as recorded in Table 5.1 or lack of repair shown in Figure 5.9. To examine the effect of DMSO shock on the influx kinetics of pGA293 into nuclei of AA8 cells and on the plasmid repair, pGA293 irradiated at 50 J/m^2 was transfected into AA8 cells in two sets of plates. One was subjected to DMSO shock while the other was not after the initial 6 hours incubation period. The cells were harvested at 0, 12, and 24 hours and pGA293 was extracted by the Hirt method from the isolated nuclei. Equal volumes of extracted DNA from both DMSO treated and non-treated samples at each time were digested with *Eco*RI in order to generate the three domains of pGA293.

These DNA samples were further split into two equal aliquots, one was treated with T4 endonuclease V to generate the nick at the site of dimers, while the other part of the sample was sham treated. The DNA samples were electrophoresed in pairs on a denaturing agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P labelled riboprobe that detected the transcribed strand of the *lacZ* gene of pGA293. An autoradiograph is shown in Figure 5.9A. The DNA hybridization signals are very similar in both DMSO treated and non-treated samples.

When the same blot was stripped and reprobed with a ^{32}P labelled double stranded DNA probe that detected both strands of the *lacZ* gene of pGA293, the DNA signal intensities were similar in both DMSO-treated and non-

FIGURE 5.9 ATTEMPT TO DEMONSTRATE REPAIR OF pGA293: THE DMSO TREATMENT

pGA293 was irradiated at a concentration of 30 ng/ul at a UV dose of 50 J/m^2. AA8 cells were incubated with 10 μg of irradiated plasmid per 2 x 10^6 cells in 100 mm dish for 6 hours and one set of dishes was shocked with 10% DMSO (+) and the other was not (-). The plasmid DNA was extracted by the Hirt method just after DMSO shock at 12 and 24 hours later. Equal amounts of pGA293 at each time were digested with *Eco*RI and subjected to repair analysis. C refers to irradiated pGA293 that was not transfected but analyzed in the same way as samples extracted after transfection. A) Repair analysis using a ^{32}P labelled double stranded [DS] DNA probe that detected both strands of the 3 Kbp domains of pGA293. B) Repair analysis using a ^{32}P labelled riboprobe that detected the transcribed strand [TS] of the 3 Kbp domain of pGA293. C) Repair analysis using a ^{32}P labelled riboprobe that detected the non-transcribed strand [NTS] of the 3 Kbp domain of pGA293.



A

B

C

treated AA8 cells. An autoradiograph is shown in Figure 5.9B. The same blot was once more deprobed and probed with a double stranded DNA probe that detected the 3.5 Kbp domain of pGA293. An autoradiograph is shown in Figure 5.9C. It can be seen that there is no significant difference between the DMSO-treated and non-treated samples.

This experiment shows that application of a DMSO shock is not causing a flood of pGA293 molecules into the nucleus and cannot be the reason for the aberrant repair pattern described in section 5.4. It also failed to demonstrate repair in either the 3 Kbp or the 3.5 Kbp domains of pGA293.

5.8 Copy Number of pGA293 in Nuclei of Transfected Cells

In order to ascertain how many plasmid molecules were being presented for repair in the nucleus of a transfected cell, the plasmid copy number per nucleus was measured under several conditions of transfection. Total DNA was extracted from the nuclei of transfected cells by the SDS-proteinase method to minimize the loss of plasmid DNA which occurs with the Hirt method because some plasmid, particularly in the case of a large plasmid like pGA293, accompanies the high molecular weight fraction.

Contamination contributed by plasmid adsorbed to the cell surface was eliminated by first treating the cell with DNAaseI, and cytoplasmic pGA293 was removed by isolation of the cell nuclei. The nuclei were further washed twice to remove all traces of contamination. The amount of DNA in the plasmid samples was determined by a Southern blotting procedure in which known amounts of pGA293 were electrophoresed along with the plasmid samples.

From cell counts made at the time of cell harvest, it was then possible to calculate the number of plasmid molecules per nucleus. The results from three trials are represented in table 5.2 where the total plasmids were expressed per 10^7 cells rather than 2 x 10^6 because the number of AA8 cells was increased at different times after transfection. In trial I with an input of 5 μg of irradiated pGA293 and a 6 hour incubation period before the DMSO shock, there was a large influx of plasmid into the nucleus in the following 6 hours. Between 12 and 48 hours, there was a dramatic drop in the plasmid count.

In trial II, the cells were incubated with 5 μg of irradiated pGA293 for 15 hours and then were treated with 10% DMSO. With this treatment, the number of plasmids reaching the nucleus was not nearly as great but again, the number fell off between 12 and 48 hours. The pattern of appearance of plasmids in the nucleus is presumably the result of the rate of passage of plasmid through the cell membrane, the rate of degradation both before and after crossing the membrane, and then degradation inside the nucleus.

From the point of view of this study, the conclusion to be drawn is that when 5 μg of plasmid are used for the transfection, a large number of plasmids enter the nucleus. It was thought that the large number of plasmids created difficulties for the detection of the small number of repair events. The results of these trials prompted us to look for repair of pGA293 when the amount of irradiated plasmid used in the transfection was greatly reduced. To this end, 0.2 and 0.5 μg amount of UV-irradiated pGA293 were incubated with the cells for 15 hours and then a DMSO shock was given, although the effectiveness of

TABLE 5.2 PLASMIDS PER NUCLEUS WITH DIFFERENT TRANSFECTION CONDITIONS

	Hours After DMSO	Total Plasmids x 10^{-7}	Total Cells x 10^{-7}	Plasmid per Nucleus
I.	5 μg pGA293 incubated with ~ 2 x 10^6 cells for 15 h before DMSO shock			
	0	234	4.08	57
	6	320	5.04	63
	12	343	5.04	68
	24	203	7.36	28
	48	140	9.75	14
II.	5 μg pGA293 incubated with ~ 2 x 10^6 cells for 6 h before DMSO shock			
	0	507	2.06	246
	6	2636	2.43	1085
	12	4602	3.16	1456
	48	47	11.34	4
III.	0.2 μg (A) or 0.5 μg (B) pGA293 incubated with ~ 2 x 10^6 cells for 15 h before DMSO shock			
	(A) 12	82	4.12	20
	(B) 12	148	4.12	36

The plasmids per nucleus were measured by a Southern blotting-hybridization method.

FIGURE 5.10 ATTEMPT TO DEMONSTRATE REPAIR OF pGA293: USING SMALL AMOUNTS OF PLASMID

pGA293 was irradiated at concentration of 30 ng/ul at an UV-dose of 100 J/m^2. AA8 cells were incubated with 0.2 and 0.5 μg of the plasmid for 15 hours after the DMSO shock and digested with *Eco*RI. The DNA samples were then analyzed for repair. C1 is non-irradiated pGA293. C2 and C3 contain 5 ng and 10 ng, respectively of irradiated DNA. These three controls were not transfected but analyzed in the same way as samples after transfection. They were used to determine the copy number of pGA293 in the nuclei of AA8 cells. Panels C and E show the repair analysis for the 0.2 μg and 0.5 μg amount of plasmid, respectively, using a ^{32}P labelled double stranded [DS] DNA probe that detected the 3 Kbp domain of pGA293. The smear is the result of having to apply a large amount of nuclear DNA to the gel.


Time, Hr.
T4 Endo V.
UV 100J/m2
C1
C2
12
C3
12
3K
DS
A
B
C
D
E

DMSO with this protocol was uncertain. Twelve hours later, the cells were harvested for repair analysis and for a determination of the copy number.

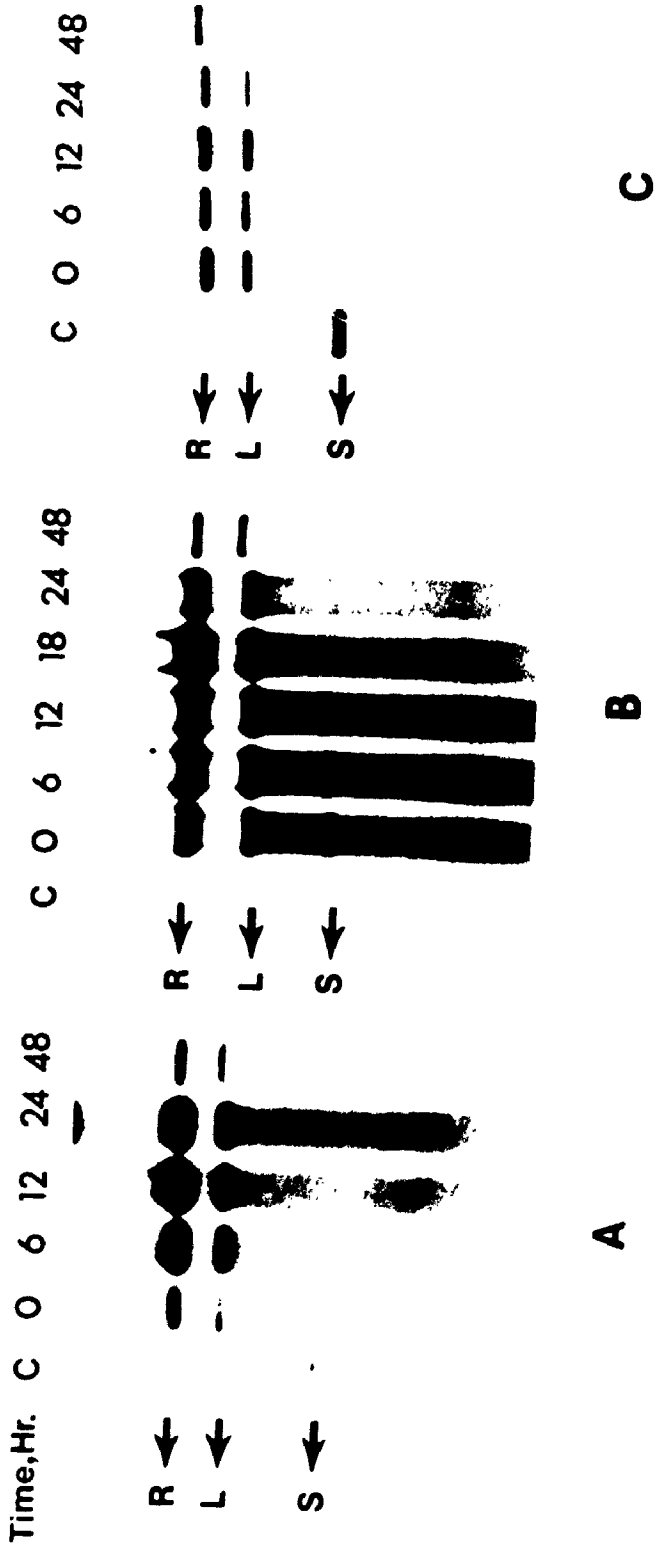
The copy number data are shown in trial III in table 5.2. Repair was assessed in the 3 Kbp domain using a double stranded DNA probe for detection and the autoradiographs are shown in Figure 5.10. Once again, no repair was evident in spite of having only 20-36 plasmids per nucleus.

5.9 The Fate of UV-Irradiated pGA293 Transfected into Repair Proficient AA8 Cells.

Cells were incubated with plasmid-calcium phosphate coprecipitate for 6, 10, and 15 hours, then were exposed to 10% DMSO for 30 minutes and then incubation was continued in regular medium. Cells were harvested at various times after this and plasmid was extracted from the nuclei by the Hirt method. Portions of extracted plasmid were separated by electrophoresis on an agarose gel, transferred to a membrane and hybridized to a ^{32}P labelled probe made from pGA293. An autoradiograph from one set of experiments is shown in Figure 5.11. Consider first the results obtained when the cells were incubated with plasmid for 6 hours before the DMSO shock. The extracted plasmid was found predominately in the relaxed and linear forms at all times, including zero time, which was extracted immediately after the DMSO shock. The supercoiled, relaxed and linear forms of pGA293 were distinguished by running standard samples of the pGA293 forms and detecting them autoradiographically or by ethidium bromide staining. There was no detectable signal of the supercoiled form even up to 48 hours presumably because of its small amount. The proportion of linear form of pGA293 was relatively low compared to the relaxed

Figure 5.11 AUTORADIOGRAPHS TO SHOW THE AMOUNT AND CONFORMATION OF pGA293 EXTRACTED FROM AA8 CELLS AT DIFFERENT TIMES AFTER TRANSFECTION

AA8 cells were treated with pGA293 (5-μg/2x10^6 cells) in a calcium phosphate coprecipitate and incubated for 6, 10, and 15 hours (Panels A, B, and C, respectively) before exposing the cells to 10% DMSO for 30 minutes. The end of the DMSO treatment was taken as zero time. The DMSO containing medium was replaced with regular medium and incubation was continued for 6, 12, 18, 24, and 48 hours. At each time, plasmid was extracted, purified, and electrophoresed in 0.7% neutral agarose gel, transferred to a membrane and hybridized by ^{32}P labelled pGA293. C in panels A, B and C refers to the control pGA293 that was not transfected but analyzed along with other samples in order to determine the amount and positions of pGA293 extracted from AA8 cells at different times after transfection. Supercoiled (S), relaxed (R), and linear (L) forms of pGA293 are shown with arrow marks.


Time,Hr.
C O 6 12 24 48
R
L
S
A
C O 6 12 18 24 48
B
C O 6 12 24 48
C

form. The amounts of both the linear and relaxed forms increased between 0 hour and 24 hours and then decreased markedly by 48 hours. However, when pGA293 was incubated with the cells for 10 hours before the DMSO shock, some of the supercoiled form was present in the first extracts. The amount of this form of pGA293 was increased at the 6 hour time point and had declined by 12 hours. Thereafter supercoiled pGA293 was not detectable. It is important to note that the amount of supercoiled DNA was far lower than the amount of linear and relaxed forms of pGA293. Both the linear as well as the relaxed forms of pGA293 started to disappear by 24 hours. There were very weak hybridization signals left by 48 hours, showing the degradation of pGA293 at this time. Moreover, the background level due to degradation was particularly strong between 0 and 12 hours.

The supercoiled form of pGA293 was also apparent in the Hirt extract when plasmid cells were incubated for 15 hours before the DMSO shock. The supercoiled form remained apparent up to 12 hours and thereafter disappeared. By 48 hours, very little of the relaxed and linear forms were present illustrating again the degradation of the plasmid over a 48 hours period. The pattern exhibited by the supercoiled form of pGA293 in my experiments is such that the amounts of the intracellular forms of supercoiled pGA293 varied with the conditions used to transfect the cells. The pattern is thought to reflect the plasmid relaxation, and minichromosome formation (reappearance of supercoiled form). It must be noted that pGA293 in my experiments was irradiated. The UV lesions themselves or the repair processes operative on irradiated pGA293

could cause the disappearance of supercoiled topology at a later time period.

In order to further elucidate this phenomenon of limited minichromosome formation reflected in the small amount of supercoiled form of pGA293 and to relate it to transcriptional activity of *lacZ* gene, β-galactosidase activity was assessed by staining cells as well as measuring enzyme activity in cell extracts. Plates containing approximately 1 x 10^6 cells were transfected with 5 μg of non-irradiated pGA293 and 26 hours later, the β-galactosidase activity was determined to be 165 picomole mol 4 mu/10^6 cell/minute. However, only about 1/1000 cells gave a positive stain using x-gal. The number of positively staining cells had not increased 48 hours after transfection. In a positive control reaction, a Chinese hamster cell line VBGC5, which carried a *β-galactosidase* gene in its genome, was examined and virtually all cells stained positively with x-gal. The specific activity of these cells was 2530 picomole mole 4 mu/10^6 cells/minute. These findings support the idea that only a small fraction of the input plasmid is converted into the functional minichromosome form.

5.10 The Fate of UV-irradiated pZH10 Transfected into Repair Proficient AA8 Cells

The 12Kbp plasmid pGA293 has been difficult to isolate in a supercoiled form from *E. coli* strain JM109. A part of this plasmid containing the *lacZ* gene is transcribed well in AA8 cells. In order to confirm that the topological changes observed for pGA293 were not due to the extraction process or to transcriptional activity, but a reflection of the intracellular processing of pGA293, the behaviour of a small supercoiled plasmid pZH10 was also examined. pZH10, which is presumably without a mammalian type of promoter,

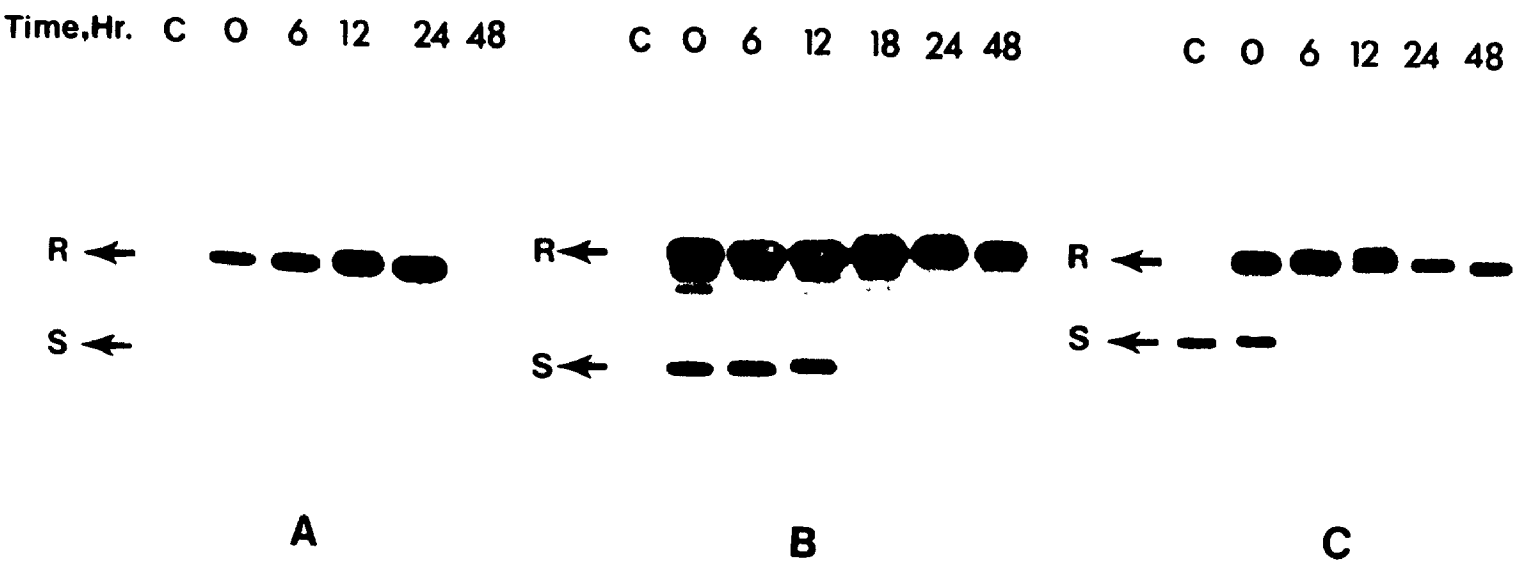
was transfected into AA8 cells. The amount of plasmid DNA and the experimental conditions were the same as adopted for pGA293. The pattern of appearance of the various forms of the plasmid extracted from the nuclei at various times after the transfection are shown in Figure 5.12.

When pZH10 was incubated with the AA8 cells for 6 hours before the DMSO shock, only the relaxed form of plasmid was seen in the nuclear extract prepared immediately after the DMSO shock or up to 48 hours later. The amount of plasmid found in the nucleus increased up to 24 hours but had largely disappeared by 48 hours. Unlike pGA293, no linear form of pGA293 is seen. When pZH10 was incubated with AA8 cells for 10 hours before the DMSO shock, two topological forms were extracted (Figure 5.12). The supercoiled form was present for the first 12 hours while the relaxed form of pZH10 was present in all samples. When pZH10 was incubated with AA8 cells for 15 hours before the DMSO shock, the pattern shown in Figure 5.3C was obtained. The supercoiled form of the plasmid is apparent only at 0 hour. The relaxed form of pZH10 is present at all times but has decreased in amount by 24 hours.

The patterns obtained with pZH10 are strikingly similar to those of pGA293 with respect to the appearance of supercoiled and relaxed forms of DNA. Only a small proportion of the extracted plasmid is seen in the supercoiled form and its appearance is transient. The relaxed form of the plasmid is present in all the samples and shows a rise in amount followed by a decline over the 48 hour post DMSO period. As in the experiments with

Figure 5.12 AUTORADIOGRAPHS TO SHOW THE AMOUNT AND CONFORMATION OF pZH10 EXTRACTED FROM AA8 CELLS AT DIFFERENT TIMES AFTER TRANSFECTION

AA8 cells were treated with a pZH10 (5μg/2x10^6 cells) as a calcium phosphate coprecipitate and incubated for 6, 10, or 15 hours (Panels A, B, and C respectively) before exposing the cells to a 10% solution of DMSO for 30 minutes. The end of the DMSO treatment was taken as zero time. The DMSO-containing medium was replaced with regular medium and incubation was continued for 6, 12, 18, 24, and 48 hours. At each time, plasmid was extracted, purified, and electrophoresed in 0.7% neutral agarose, transferred to a membrane and hybridized to ^{32}P labelled pZH10 as described earlier. C in panels A, B and C refers to the control pZH10 that was not transfected but analyzed along with other samples in order to determine the amount and positions of pZH10 extracted from AA8 cells at different times after transfection. Supercoiled (S), and relaxed (R) forms of pZH10 are shown with arrow marks.


Time,Hr. C O 6 12 24 48
R
S
A
C O 6 12 18 24 48
R
S
B
C O 6 12 24 48
R
S
C

pGA293, the exact pat ern depends on the experimental conditions of the transfection procedure and is considered to be the result of the efficiency of plasmid uptake followed by cellular processing. In the case of pZH10, no linear form of the plasmid is seen reflecting some difference in the processing of the two plasmids.

5.11 Discussion

Studies by other investigators have shown that cyclobutane pyrimidine dimers generated in DNA constituted blocks to transcription (Sauerbier and Herculese, 1978). When UV-irradiated pGA293 was transfected into repair proficient AA8 cells and repair deficient UV41 cells, expression of *β-galactosidase* was greatly inhibited in the UV 41 cells but only slightly in the AA8 cells. This host cell reactivation assay reflects the repair that has taken place and recovery of enzymatic activity was obtainable even after a UV-dose of 100 J/m^2 which shows that the repair machinery was not saturated even at this dose.

A strange phenomenon was observed for enzyme expression as a result of UV-irradiation. The expression of *β-galactosidase* was greater from irradiated pGA293 than from the unirradiated plasmid. It might be due to transcription-coupled repair of pGA293 in AA8 cells (Figure 5.1 and 5.2). The phenomenon was dose dependent and was absent in repair deficient UV 41 cells. Thus, it is clearly related to the repair process. Although the molecular mechanism behind this elevated expression remains to be determined, it seems to be analogous to studies where moderate doses of UV-irradiation to plasmids were

found to increase their ability to transform human cells (Spivak et al., 1988). This phenomenon was found to be dependent on the location of damage within the plasmid; cyclobutane pyrimidine dimers outside the transcription unit increased the transformation while dimers within the transcription unit reduced the transformation (Leadon et al., 1987). When a similar type of experiment was carried out using CHO cells, it was found that irradiation of the plasmid did not increase transformation (Nairn et al., 1988). Since CHO cells repair damage to the genome as a whole much more slowly than human cells, the association between repair and enhanced transformation is again apparent.

A complete understanding of the repair mechanism will require the reconstitution of a cell free system that can duplicate the basic repair reactions. An *in vitro* system in which repair is monitored by the incorporation of ^{32}P-labelled nucleotide into a damaged plasmid has been successfully exploited to dissect and purify the individual components of the repair machinery (Wood et al., 1988; Sibghat-ullah et al., 1989; Shivji et al., 1992; O'Donovan and Wood, 1993). However, with this assay, only a small portion of the plasmids are repaired thus showing that the repair process is very inefficient in this system; moreover, AAF adducts are not repaired in this system which further points out its deficiency. Perhaps in the cell extract the concentration of one or more of the repair factors is too low for efficient action on the non-chromosomal forms of the substrate or it is not well recognized by the repair machinery (Chen et al., 1991; Orfanoudakis et al., 1990).

Transfection studies have been adopted successfully to dissect the

mechanisms involved in replication (Cereghini and Yaniv, 1984; Yasui and Ryoji, 1989), transcription (Weintraub et al., 1986), and chromatin assembly (Ryoji and Worcel, 1984, 1985; Ryoji et al., 1989), and this encouraged us to examine repair at the gene level using this approach because with this system it is possible to vary the nature of the damage to the plasmid and the genetic background of the host could be manipulated. Moreover, one can examine the effect of transcription on repair either by changing the promoter or by using transcription and topoisomerase inhibitors. We wished to determine whether repair kinetics and overall repair efficiency could be improved by providing the damaged plasmid with closer physiological conditions. CHO cells were used because they were easily cultured and readily transfected and a number of well characterized mutants were available (Hoeijmaker et al., 1987, 1988; Hoeijmaker, 1993b).

In an early experiment in which UV-irradiated pGA293 was transfected into AA8 cells and the Bohr-Hanawalt technique for assessing repair in genes was applied to plasmid extracted from the cells, evidence was obtained that repair of the transcribed *β-galactosidase* gene in the plasmid had occurred. This finding is now regarded as an unexplained artifact, particularly as subsequent investigation has shown the amount of plasmid transfected, 10 μg per 2 x 10^6 cells, would overwhelm the repair system. There then followed a large number of experiments to try to demonstrate repair of the transcribed plasmid and these involved systematic variations of the transfection protocol and the amount of plasmid transfected.

None of the many trials was successful in demonstrating repair in any domain of the plasmid. A clue to the reason for the inability to demonstrate repair came in the observation that only a very small fraction of transfected cells were stained blue when exposed to the synthetic β-galactosidase substrate x-gal. However, an extract from the same cell sample contained what was considered to be an acceptable amount of β-galactosidase activity after correcting for endogenous ß-galactosidase activity. It was concluded that the enzyme assay on the cell extract, due to its sensitivity, was giving an inflated indication of the enzyme content of the cells. In turn, this led to the conclusion that many more plasmids had been repaired than in fact had been repaired.

A second clue came from a consideration of the topological forms of the plasmids and, their amounts, that were extracted from the nuclei of transfected cells. In brief, it was observed that the amount of the supercoiled form was much less than the amounts of relaxed or linear forms. Other investigators have concluded that soon after supercoiled plasmid is transfected into a cell, the plasmid is relaxed, presumably by the action of topoisomerase I, and then a small portion of it is assembled into a minichromosome. This event is signalled by the reappearance of the supercoiled form. It is presumed that it is the minichromosome form of the plasmid that is transcribed and in turn it is the transcribed sequence of the DNA that is efficiently repaired (Gargiulo and Worcel, 1983; Ryoji and Worcel, 1984, 1985).

Support for this concept can be found in the studies of other investigators. In particular, studies by Weintraub and his colleagues

(Weintraub et al., 1986) have shown the importance of the supercoiled form of the plasmid if it is to be transcribed. The pattern of the topological forms that they observed in plasmid extracted from nuclei is similar to ours. Other pertinent studies are the following: using a human cell free system and a plasmid containing an SV40 origin, minichromosome assembly was demonstrated. However, the amount of minichromosome formed was small compared to the total amount of plasmid (Stillman and Gluzman, 1985; Stillman, 1989; Smith and Stillman, 1989). When minichromosome formation from plasmid transfected into cos-1 or cv-1 monkey cells was examined, it was found that 13-15 hours after incubation only the relaxed form of the plasmid was seen but the supercoiled form had appeared by 24 hours (Myers and Tjian, 1980; Lusky and Botchan, 1980; Cereghini and Yaniv, 1984).

There are other reasons that can be given to explain the lack of success in demonstrating the repair of pGA293 at the molecular level. It has been demonstrated that DNA excision repair capacity is enhanced by exposing mammalian cells to moderate doses of UV-light irradiation before the damaged plasmid is transfected (Protic-Sabljic et al., 1988; Ikushina, 1987; Hirschfeld et al., 1990). This suggests that UV-irradiation of the cell enhances the repair machinery of the cell. This effect would be absent when damaged plasmid was transfected into an unirradiated cell. In this regard, the unirradiated cell behaves like a RecA$^-$ strain of *E. coli*. In yeast repair, genes are expressed at extremely low levels but a number of them are induced in response to DNA damage (Friedberg, 1988; Friedberg et al., 1991; Friedberg, 1991). Proliferating

cell nuclear antigen (PCNA) has recently been shown to be involved in excision repair and this factor accumulates in cell nuclei in all phases of the cell cycle following UV-irradiation (Shivji et al., 1992; Nichols and Sancar, 1992; Celis and Madsen, 1986; Toschi and Bravo, 1988). It is also possible that some uncharacterized regulatory controls determine the temporal or spatial level of repair enzymes. This spatial polarization phenomenon exists in the case of uracil DNA glycosylase which is in the cytoplasm in quiescent human cells whereas all glycosylase activity is found in the nucleus in proliferating cells. It is possible that a similar spatial polarization exists for components of the repair complex and their unavailability or lower concentrations in un-irradiated AA8 cells might yield a lower repair capacity (Cool and Sirover, 1989). Following UV-irradiation only 20-30% of the total genome of CHO cells is repaired in comparison to human cells where repair is almost complete within 24 hours. This suggests although, that overall repair capacity in CHO cells is low and that available repair enzymes are directed toward the most essential part of genome; the limited capacity of CHO might be saturated in uninduced cells to repair the flood of transfected pGA293. It is also possible that there might be some inhibitor in CHO cells that repressed the expression of repair enzymes under the non-UV-induced condition. Such a phenomenon has been reported for the photolyase gene; an inhibitor of this gene disappears after UV-irradiation. This repressor is bound to the promoter region and yields extremely low expression. If a similar inhibitor exists in CHO cells in the non-induced condition this might be responsible for the low repair of pGA93 in AA8 cells

(Sebastian and Sancar, 1991). The XPA revertant cell line XP129 displays the characteristics of CHO cells where repair of cyclobutane pyrimidine dimers is deficient in the overall genome but dimers are efficiently removed from the transcribed strand of active genes (Lommel and Hanawalt, 1993). The level of XPAC protein in the revertant is about 30 times less than that observed in normal cells (Jones et al., 1992). However, the reduced amount of this protein seems to be targeted to the repair of the essential transcribed domain of the genome. In the case of transfected pGA293, targeting to this DNA may have a low priority.

In summary, the transfected plasmid system I have used is not a good model for the study of nucleotide excision repair of genes at the molecular level. Although many of the transfected plasmid molecules find their way into the nucleus, only a few of them are processed into biologically significant forms namely, those that are assembled into transcriptionally active minichromosomes that presumably can be repaired efficiently. This rare event is probably hidden by the much larger amount of unrepaired plasmid.

CHAPTER 6

General considerations and future direction

From studies on the repair of pGA293 in *E. coli*, strain MM294, it was found that the transcribed and non-transcribed strands of the 3.5 Kbp domain which contains the *ß-lactamase* gene are repaired with the same kinetics. The 2.4 Kbp domain possesses the partial *tet* gene and both strands of this domain are also repaired with the same kinetics. Futhermore the repair rates for the two domains were very similar to each other. The repair rate for the transcribed strand of the 3.0 Kbp domain which contains the *ß-galactosidase* gene, was the same as found for the 3.5 Kbp and 2.4 Kbp domains. However, the repair rate for the non-transcribed strand of the 3.0 Kbp domain was slower than that observed for all other strands of pGA293.

When these repair kinetics were compared with those of the transcribed and non-transcribed strands of the *E. coli* chromosomal *lacZ* gene, it turned out that the repair rate of both strands of the 3.5 Kbp and 2.4 Kbp domains as well as the transcribed strand of the 3 Kbp domain was slower than that observed for the transcribed strand of the *E. coli lacZ* gene. However, the repair rate for the non-transcribed strand of the 3.0 Kbp domain was close to that of the non-transcribed strand of the *E. coli lacZ* gene.

The difference in the repair rates for the non-transcribed strand of the 3 Kbp domain and the strands of the other domains of pGA293 is likely due to transcription repair coupling operating on all strands of pGA293 except the non-transcribed strand of the 3 Kbp domain. It also seems possible from the

potential promoter sequences in the genes on the 3.5 Kbp and 2.4 Kbp domains that both strands of each sequence are transcribed. One possible reason for the slower repair of the pGA293 strands compared to that for the transcribed strand of the *E. coli* chromosomal *lacZ* gene could be due to the 10 fold greater copy number of pGA293 and the fact that they contained about twice as many cyclobutane pyrimidine dimers per unit of DNA.

An alternative explanation for the repair pattern observed in pGA293 in MM294 cells is that transcription repair coupling is not operating on the plasmid and that the slower rate of repair of the non-transcribed strand of the 3.0 Kbp domain is due to some other factor. This issue could be resolved by investigating the repair of pGA293 in the *mfd*⁻ strain of *E. coli* where coupling between transcription and repair is absent (Sancar and Selby, 1993).

If in this strain, each of the strands of pGA293 were repaired at the same rate as the non-transcribed strand of the 3.0 Kbp domain it would mean that transcription coupled repair had occured in the transcribed strands of pGA293 in the MM294 cells. If the repair kinetics of the non-transcribed strand of the 3.0 Kbp domain were slower than that of the other strands it would mean that non-coupled repair had occured in all strands of pGA293 in MM294 cells. It would also be informative to assess repair simultaneously in the chromosomal *lacZ* gene and the domains of pGA293 when the plasmid was resident in MM294. This would provide information about the effect of gene copy number on the repair kinetics.

This plasmid based system has the potential to address not only these

issues but could be used to examine the effect on the repair kinetics of promoter strength and of host factors such as HU, IHF and H-NS.

We were unable to demonstrate repair of irradiated pGA293 after transfection of the plasmid into AA8 cells. However, it is possible that by changing conditions such as exposing the AA8 cells to low doses of UV light prior to transfection, it could improve the repair capacity of AA8 cells (Hirschfeld et al., 1990) to the extent that it would be detected by the method used in this study. Another approach would be to use a resident plasmid as I did in MM294 cells. EBV-based shuttle vectors would appear to be appropriate because they are superior in transfection efficiency, expression and stability (James et al., 1989). These vectors can be maintained episomally in a variety of cell lines but cannot be maintained in rodent cell lines. There are about 10 copies of EBV-based plasmid per human cell, and monkey cells contain 5 copies (Yates et al., 1985; Margolskee et al., 1988).

It would be of interest to know whether the repair machinary is effectively targeted on the plasmid DNA when human cells harbouring these plasmids are irradiated. One can also ask whether the organization of the minichromosome affects the repair by using an agent like sodium butyrate. If repair in the shuttle vector turns out to be analogous to that in the yeast minichromosome, then one can examine the repair in proliferating cells and compare it with cell cycle arrested cells (Ho and Hanawalt, 1991).

It has been demonstrated that 8-methoxy psoralen cross-links and cross-linkable monoadducts in the *DHFR* gene of an EBV based episomal plasmid

maintained in a normal cell were repaired less well than in the chromosomal gene. This suggests that the repair machinery is preferentially targeted to the essential chromosomal genes (Dean, 1989). Is there a similar trend with respect to UV-damage? Even if the repair kinetics turned out to be slower on plasmid DNA it would be of interest to know if there is any strand selectivity.

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APPENDIX I REPAIR OF CYCLOBUTANE PYRIMIDINE DIMERS IN THE TRANSCRIBED STRAND OF THE 3.5 Kbp DOMAIN OF pGA293

	Trial I				Trial II			
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair
0M	0.25	1.30	100	0	0.2	1.61	100	0
7.5M	0.50	0.68	52	48	0.33	1.10	68	32
15M	0.53	0.63	49	52	0.46	0.77	30	70
30M	0.81	0.21	16	84	0.8	0.22	14	87
45M	0.79	0.23	17	83	0.86	0.15	10	90
60M	1	0	0	100	1	0	0	100

	Trial III				Trial IV				
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair	Average % Repair ± SEM
0M	0.23	1.45	100	0	0.23	1.46	100	0	0
7.5M	0.38	0.91	64	36	0.38	0.98	67	33	37.1 ± 3.6
15M	0.62	0.5	34	66	0.85	0.17	11.3	89	69.1 ± 7.7
30M	0.70	0.35	24	76	0.89	0.11	8	92	84.6 ± 3.3
45M	0.88	0.12	8	92	0.80	0.22	15	85	87.5 ± 2.1
60M	1	0	0	100	0.88	0.12	8.5	92	97.9 ± 2.1

APPENDIX II REPAIR OF CYCLOBUTANE PYRIMIDINE DIMERS IN THE NON-TRANSCRIBED STRAND OF THE 3.5 Kbp DOMAIN OF pGA293

	Trial I				Trial II			
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair
0M	0.29	1.21	100	0	0.22	1.5	100	0
7.5M	0.61	0.49	40	60	0.33	1.12	75	25
15M	0.82	0.20	17	83	0.46	0.79	53	48
30M	0.85	0.15	13	87	0.53	0.64	43	57
45M	0.92	0.09	7.4	93	0.85	0.16	11	90
60M	1.0	0	0	100	0.73	0.31	21	79

	Trial III				Trial IV			
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair
0M	0.18	1.69	100	0	0.27	1.30	100	0
7.5M	0.29	1.23	72	28	0.64	0.44	34	66
15M	0.54	0.62	36	64	0.88	0.13	10	90
30M	0.50	0.68	40	60	0.84	0.18	13	87
45M	0.9	0.11	6	94	0.86	0.16	12	88
60M	0.8	0.22	13	87	1.0	0	0	100

	Trial V				
Time	Zero Class	CPDs	% CPDs	% Repair	Average % Repair ± SEM
0M	0.24	1.43	100	0	0
7.5M	0.42	0.86	61	38	43.4 ± 8.4
15M	0.49	0.71	50	50	67.0 ± 8.6
30M	0.69	0.45	32	68	72.0 ± 10.0
45M	0.96	0.04	3	97	92.3 ± 1.4
60M	0.83	0.19	13	87	91.0 ± 1.0

APPENDIX III REPAIR OF CYCLOBUTANE PYRIMIDINE DIMERS IN THE TRANSCRIBED STRAND OF THE 3 Kbp DOMAIN OF pGA293

	Trial I				Trial II			
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair
0M	0.35	1.05	100	0	0.21	1.54	100	0
7.5M	0.50	0.68	65	35	0.25	1.36	88	12
15M	0.59	0.51	49	51	0.55	0.60	39	61
30M	0.74	0.29	29	72	0.72	0.33	21	79
45M	0.86	0.15	15	85	0.94	0.06	4	96
60M	1.0	0	0	100	0.86	0.14	9	91

	Trial III				
Time	Zero Class	CPDs	% CPDs	% Repair	Average % Repair ± SEM
0M	0.38	0.98	100	0	0
7.5M	0.41	0.90	92	82	18.4 ± 8.4
15M	0.7	0.35	36	64	58.6 ± 3.9
30M	0.75	0.29	29	71	73.9 ± 2.4
45M	0.80	0.22	22	78	88.8 ± 5.1
60M	0.80	0.22	22	78	89.7 ± 6.4

APPENDIX IV REPAIR OF CYCLOBUTANE PYRIMIDINE DIMERS IN THE NON-TRANSCRIBED STRAND OF THE 3 Kbp DOMAIN OF pGA293

	Trial I				Trial II				
Time	Zero Class	CPDs	% CPDs	% Repair	Zero Class	CPDs	% CPDs	% Repair	Average % Repair ± SEM
0M	0.47	0.75	100	0	0.48	0.72	100	0	0
7.5M	0.54	0.61	81	19	0.55	0.58	82	18	18.5 ± 0.4
15M	0.61	0.48	64	36	0.69	0.37	51	49	42.5 ± 5.3
30M	0.75	0.28	37	63	0.74	0.3	41	59	61.0 ± 1.6
45M	0.85	0.16	22	79	0.91	0.1	11	89	83.8 ± 4.3
60M	0.97	0.03	4	96	1	0	0	100	96.4 ± -