Mechanisms of DNA Double-Strand Break Repair in Mammalian Cells.

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To my family.

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Abbreviations

AT	Ataxia Telangiectasia
A,T,C,G	Adenine, Thymine, Cytosine, Guanine
bp, kb	base pair, kilobase pair
β-gal	β-galactosidase
BSA	Bovine Serum Albumin
^{o}C	degrees centigrade
CIP	Calf Intestinal Phosphatase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-Acetic acid
EMS	Ethyl methane sulphonate.
FCS	Foetal calf serum
G_{418}	Geneticin
g, µg, ng	gram, microgram, nanogram
kd	kilodalton
$MgCl_2$	Magnesium Chloride
M, mM	Molar, millimolar
MMC	Mitomycin C
O.D.	Optical density
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
NaAc	Sodium Acetate
NaCl	Sodium Chloride
SDS	Sodium Dodecyl Sulphate.
TCA	Trichloroacetic acid.
UV	Ultra Violet
XP	Xeroderma Pigmentosum

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Abstract

The DNA double strand break (DSB) is the lesion believed to be responsible for the cytotoxic effects of ionising radiation. A number of cell lines hypersensitive to ionising radiations have been shown to have a defect in DSB repair. However, the *irs* series of mutants derived from V79 hamster cells are extremely radiosensitive but show no defect in DSB repair detectable by neutral elution. Previous studies using transfection of plasmid containing a DSB induced by restriction endonucleases into these cell lines suggested that one of the *irs* mutants was deficient in the fidelity of rejoin of such a DSB.

The present study describes an assay for the capacity of nuclear extracts prepared from radiosensitive and wild type cells to rejoin an endonuclease induced DSB *in vitro*. Endonuclease treatment was used to linearise plasmid DNA producing DSB substrates with either 3' or 5' cohesive termini which were incubated with nuclear extracts from wild type or radiosensitive cell lines. The efficiency and fidelity of DSB rejoining was asessed by bacterial transformation and Southern blot hybridisation of reaction products.

Nuclear extracts prepared from V79 and *irs-1*, a radiosensitive mutant derived from V79, efficiently catalyse the faithful religation of an endonuclease induced DSB as measured by increase in bacterial transformation of plasmid DNA. The level of DSB rejoin is dependent on the amount of nuclear extract added. In comparison, nuclear extracts prepared from another radiosensitive mutant of V79, *irs-2*, are unable to rejoin such a DSB to give molecules capable of transforming bacteria. The addition of V79 nuclear extract to *irs-2* is shown to compensate for the *irs-2* defect in production of a transforming molecule. Incubation of linear plasmid with nuclear extracts prepared from V79 or *irs-1* gives rise to a molecule migrating faster than the linear form on electrophoresis and resistant to λ-exonuclease treatment. This form is presumed to be closed circular plasmid DNA. It is not detected after reaction of linear plasmid with nuclear extract prepared from *irs-2* cells. However *irs-2* is not deficient in all pathways of DSB ligation, since nuclear extracts from this line catalyse the rejoin of linear substrate to high molecular weight concatemers as efficiently as extracts prepared from wild type or *irs-1* cells. These linear concatemer forms are the products of a ligation reaction proceding with equivalent efficiency and fidelity in nuclear extracts from all cell types. Consistent with the capacity of nuclear extracts from *irs-2* cells to catalyse concatemer formation is the finding that the protein concentration response, inducibility and fractionation characteristics of DNA ligases I and II are comparable in extracts from *irs-2* and V79 wild type cells.

Experiments designed to address the role of concatemers as substrates for the production of a circular molecule by recombination failed to show a difference between *irs-2* and V79. The addition of specific purified protein activities to *irs-2* extracts in attempts to restore wild type activity are also described. No compensation of the *irs-2* defect was observed on addition of T_4 ligase, gyrase or topoisomerase I activities.

CHAPTER ONE: INTRODUCTION

Introduction

1.1 AIM OF THIS STUDY

It is widely believed that the major lesion responsible for the cytotoxic effects of ionising radiations is the DNA double strand break (DSB). A number of cell lines hypersensitive to such radiation have been shown to have a defect in DSB repair (Frankenberg et al. 1984; Kemp 1984; Resnick and Martin 1976). However mammalian cell lines have been described which while sensitive to ionising radiations show no apparent defect in the repair of DSB. Cell lines derived from patients suffering from the cancer prone syndrome Ataxia Telangiectasia (AT) (Taylor et al. 1975) and the *irs* series of mutants derived from V79 hamster cells (Jones et al. 1987) are hypersensitive to γ-irradiation but show no DSB repair defect as measured by neutral elution. Thacker (1989 b, 1986) described a defect in the fidelity of DSB repair in an AT line and in one of the *irs* mutants *(irs-1)*. In these studies repair of DNA DSB was analysed by transfecting DNA containing an endonuclease induced DSB into mutant and wild type parental cells and comparing the fidelity of DSB rejoin.

The aim of the work described in this thesis was to extend the analysis of DSB rejoin in these mutants to a cell free system to enable a biochemical description of the defect leading to the radiosensitivities of these lines. It was hoped to elucidate further the nature of some of the biochemical reactions involved in the repair of ionising radiation induced damage.

1.2 IONISING RADIATION

1.2.1 Definition and general effects.

Ionising radiations can be defined as those causing ionisation of atoms by the ejection of electrons. Other types of non ionising radiations for example most wavelengths of UV light may excite electrons to a higher energy level but have insufficient energy to cause ejection of electrons. The most obvious long term effects of ionising radiations upon cells are killing, induction of mutation and conversion to a precancerous state (Gillies 1987). A number of forms of cellular damage are induced by ionising radiation which may in turn lead to one or other of the effects mentioned above. Ionising radiations can induce in cells reproductive death, interphase death, division delay, raised incidence of sister chromatid exchange and other chromosome aberrations as well as malignant transformation and mutation (Cole et al. 1980). Ionising radiation can also induce genetic changes in the germ-line leading to alteration in the phenotype of the organism. For instance, if germ cells are irradiated prior to fertilization, such radiations can induce mutations and structural changes in chromosomes leading to lethality, malformations and genetic disease in subsequent generations (Searle 1987).

1.2.2 Lesions induced.

A large body of evidence has been accumulated to suggest that the cellular and biological consequences of exposure to ionising radiations are due to damage induced in DNA. Studies by many investigators using modifiers of radiation response implicate chromatin as the primary target for ionising radiation damage. Such modifying parameters include oxygen concentration, chemical enhancing and protecting agents cell temperature before during and after irradiation, cell cycle stage during irradiation, repair incubation, radiation dose, dose rate, dose fractionation, distribution and linear energy transfer (Tolmach 1990; Ward 1990; Cramp et al. 1984; Elkind 1985; Cole et al. 1980; Painter 1980; Ward 1986; Powell and McMillan 1990). The effects of ionising radiation upon cells are consistent with the notion that damage to DNA as opposed to other chromatin associated molecules is the relevant immediate target for induced damage by ionising radiation but of course additional

putative targets exist in the shape of proteins associated with DNA in chromatin (Ekind 1985; Gillies 1987).

Four main types of lesion are induced in DNA by ionising radiation. These are double strand breaks (DSB), single strand breaks (SSB), various kinds of base damage and DNA protein crosslinks (Natarajan et al. 1986; Cole et al. 1980; Ward 1986; Painter 1980; Cerutti 1975; Cramp 1984; Chiu et al. 1990; Oleinick 1990).

1.2.3 The DNA DSB as the main cytotoxic lesion caused by ionising radiation.

It has been shown that of the different lesions induced by ionising radiation the induction of DSB best correlates with the cytotoxicity of increasing doses of ionising radiation (Cole et al.1980; Painter 1980; Natarajan et al. 1986). This is further supported by observations that treatments affecting removal of DSB also have an impact on response to radiation (Bryant 1988; Evans et al. 1984; Natarajan et al.1986). High linear energy transfer (LET) radiation is more efficient in inducing both DSB and chromosomal aberrations than the same dose at low LET again suggesting a correlation between the induction of DNA DSB and the production of a specific biological endpoint (Cole et al. 1980). It was proposed that DSB were involved in the generation of the chromosomal aberrations seen in response to X-irradiation. This hypothesis was substantiated by the work of Natarajan et al.(1986) in which X-irradiated cells were permeabilised and treated with an endonuclease from *Neurospora crassa*. This treatment has the effect of converting single strand breaks into DSB. Following such treatment a corresponding increase in chromosomal aberrations was observed. This strongly implies an involvement for DSB in the generation of chromosome aberrations and as a toxic lesion. In a study using various radiomodifying chemicals and conditions to vary the ratios of the various DNA lesions caused by ionising radiation Radford (1985) showed a linear relationship between lethal lesions and DSB induced. This correlation is not necessarily a simple one and it is probably more accurate to consider cellular radiosensitivity as the result of competition between processes of damage fixation and the repair of such damage.

A number of yeast (Ho 1975), and mammalian cell (Jeggo et al. 1983; Kemp et al. 1984; Costa and Bryant 1990) mutants hypersensitive to ionising radiations have been shown

to be deficient in the repair of DSB. In the case of mammalian cell mutants higher than usual frequencies of chromosomal aberrations are also seen (Bryant 1988). In radiosensitive yeast mutants deficient in DSB repair approximately one DSB per cell corresponds to a lethal event suggesting once again that the unrepaired DSB is a potentially lethal lesion (Frankenberg et al. 1984). Frankenberg-Schwager and Frankenberg (1990) describe two modes by which DSB might confer cell lethality. An unrepaired DSB might be lethal on its own or two such breaks may interact to form the lethal lesion. These authors also show that the operationally described cellular phenomena of sublethal and potential lethal damage repair and the effects on survival curves of radiation dose rate can be explained in terms of the repair of DSB. Bryant (1988) documents studies whereby the induction of DSB by treatment of cells with restriction endonucleases produces some of the cellular effects of ionising radiation (eg mutations, chromosomal aberrations and cell death) and Frankenberger et al. (1984) have shown that a yeast mutant unable to repair DSB has the type of radiation survival curve characteristics expected if cell lethality were caused by misrepair of DSB.

Ward (1990) reviewed the evidence that the number of DSB induced by radiation is linearly related to dose and that the yields of DNA damage per dose is constant. In the absence of differences in chromatin structure altering the amounts of DNA damage induced, variations in radiosensitivity are thought to arise from differences in the speed or accuracy with which repair of DNA DSB proceeds.

The evidence suggests then that the unrepaired or misrepaired DSB is the main lesion responsible for the cytotoxic effects of ionising radiation.

1.2.4 Explanation of cellular survival curves by hypotheses of DNA damage and repair.

Typically a plot of cell survival against dose of radiation has a shoulder at low doses where there is no increase in cell death in response to an increased dose of radiation. Models to account for this shoulder phenomenon postulate a repair process which is eventually saturated as the dose of radiation increases (Alper 1984). Goodhead (1989) suggested that models to describe a shouldered survival curve should be based on the idea that cellular processes can handle low levels of radiation damage but fail to operate either accurately or

efficiently with increasing amounts of damage. If radiation is given in fractions allowing time between each irradiation it is found that a repair competant cell has a higher survival for a given dose of radiation than if the dose had not been fractionated.

Inherent in a repair saturation model (& also implied by split dose experiments where eventually a dose is reached which, even if fractionated will still have a lethal effect) is the hypothesis that repair must occur within a given time or damage will become irreversible or "fixed". Ward (1986) suggests that cell survival can be reduced by decreasing the rate of damage repair, by causing usually repairable damage to be unrepairable or by increasing the rate of damage fixation.

A situation analagous to damage being irreparable is when a cell has little or no capacity to repair such damage. Repair deficiency can indeed be indicated by a reduced or absent shoulder in survival curves and the failiure of radiation dose fractionation to bring about a decrease in the cell kill caused by that dose (Haynes et al. 1984). The differences in the shapes of survival curves generated in response to UV irradiation of haploid RAD wild type and rad1-1 UV sensitive yeast cells have been shown to agree quantitatively with measurements of the relative efficiencies of pyrimidine dimer excision between the two lines (Game 1974)

In summary the evidence suggests that the biological effects of ionising radiation arise due to DNA damage. Of the types of damage induced by such radiations the DSB is most consistently implicated as the main cause of cytotxicity. Models postulated to explain the shape of cell survival curves are consistent with the hypothesis that the outcome of an irradiation depends on the result of competition between processes of damage fixation and damage repair. Experiments with a variety of mutants hypersensitive to ionising radiation further support these ideas in that inefficient or inaccurate repair of DSB induced by ionising radiation correlates well with increased chromosomal aberration.

1.3 INTRODUCTION TO DNA REPAIR

In order to survive and enable genetic continuity in the face of environmental damage cells must maintain the integrity of their DNA. It was originally believed that the resistance of DNA to the numerous physical and chemical agents for which it is a target was due to the double helical structure of the molecule. All information is carried in duplicate but the primary structure of DNA is all that is required for its transfer. It is now realised that efficient mechanisms exist for the repair of DNA damage (Sancar and Sancar 1988; Hanawalt et al.1979). There are a variety of constitutive and inducible DNA repair pathways. On being presented with a lesion there are a number of possibilities open to the cell; the lesion may be ignored, reversed, removed or bypassed (Laval and Laval 1980). Different cellular responses to a lesion may thus lead to total reinstatement of the initial message, mutation or loss of genetic information and cell death.

Damage may be defined as any modification of DNA altering its coding properties or normal function. To be repairable damage must be recognised by a protein that can initiate a sequence of biochemical reactions leading to its elimination and the regeneration of an intact duplex DNA molecule (Hanawalt et al.1979). Many agents can damage DNA for example UV and ionising radiations, electrophilic reagents alkylating or arylating the DNA directly or after activation. A number of classification schemes have been proposed for DNA damage and the enzymes involved in its repair. Classification of adducts according to the agent causing the damage was realised to be misleading when it became clear that different agents could induce the same adduct and in general each agent produces more than one type of lesion. Three classes of lesion were postulated by Grossman, 1. monoadducts in which a single base is modified, 2. diadducts in which two bases are involved in the final product (for example the pyrimidine dimers produced on reaction with UV, or cross links induced by mitomycin or psoralens and light). The third class of adduct is that of apurinic or apyrimidinic sites where a base is lacking. Cerutti emphasized the effect of a lesion on the conformational structure of the DNA helix. Again lesions were classified into three groups. Monofunctional adducts causing negligible helical distortion and no impairment in base pairing for example 7-alkylguanine; monofunctional lesions causing minor helix distortion and slight base pairing alteration (eg base elimination leading to apurinic or apyrimidinic sites); lesions causing major

helical distortion and impairing base pairing properties by the formation of bulky substituents or difunctional lesions. Such adducts include the thymidine dimers induced by UV light and the adducts produced by the reaction of DNA with the antitumour agent cisplatin. A further method of classification of agents was proposed by Regan and Setlow according to the form of repair observed in human cells by the damage an agent caused. Some type of agents for example ionising radiation were thought to induce a "short "form of repair in which the damage is repaired by the removal and resynthesis of three to four nucleotides during a period of about an hour. The damage caused by others was seen as requiring a "long" type of repair in which about 100 nucleotides are excised over a longer period of 18-20 hours. This type of repair was seen after treatment with UV light (Laval and Lava 1980; Hanawalt et al. 1979;Linn et al.1982; Sancar and Sancar 1988).

The correlation of a specific repair pathway or enzyme with an in vivo response to a particular damaging agent is also complicated by the damage induced by a given agent (itself not necessarily confined to one type of adduct or lesion) being dealt with in several different ways. For example UV light gives rise to many types of lesion including pyrimidine dimers, thymine glycols, baseless sites, protein:DNA cross links, DNA:DNA crosslinks. The pyrimidine dimer might be repaired by photoreactivation, error free excision repair or recombinational repair. An error prone pathway might be induced to repair the dimer or it might be copied by an error prone DNA replicative system. Another alternative is that the lesion remain unrepaired.

The best characterised systems of DNA repair involve pathways of excision repair. Two types of excision repair are recognised. Nucleotide excision repair was first described following studies on the removal of cyclobutane pyrimidine dimers from DNA after UV irradiation (Setlow and Carner 1964; Boyce and Howard-Flanders 1964) and subsequently found to function in the repair of a wide range of helix distorting lesions. Base excision repair is relatively specific for simple forms of base damage for example that produced by monofunctional alkylating agents (Lindahl 1979). This type of repair involving the action of two classes of repair enzymes -DNA glycosylases and apyrimidinic/apurinic (AP) endonucleases.

The classical nucleotide excision repair model derived from study of *E.coli* involves firstly endonuclease incision at the site of damage and removal of an oligonucleotide containing the adduct to be repaired by an exonuclease activity. Polymerase resynthesis of the excised residues and finally the sealing of the single stranded nick in the phosphodiester backbone by a ligase activity complete the process. In *M.Luteus* and Bacteriophage T_4 it appears that the incision activity is a small monomeric protein (Riazuddin and Grossman 1977a; 1977b; Friedberg 1975), in other organisms it appears this activity comprises a complex of a number of proteins. In *E.coli* excision repair is initiated by an "exinuclease" complex of the uvrA, -B, and -C products. The uvrA protein binds to the damaged region followed by complexing of uvrB and -C products (Sancar and Rupp 1983; Sancar and Sancar 1988; Seeberg et al. 1976; Seeberg 1978). It has been recognised for some time that as well as the removal of the addduct for example pyrimidine dimers as small oligonucleotides a number of other mono and dinucleotides are released. The amount of extra material thus removed has been estimated by different authors using different assays as varying from 12 to 30 nucleotides. The most recent determination of Sibghat-Ullah et al. (1990) using a defined substrate and conditions to minimise enlargement of the gap through nick translation (thought to lead in some instances to artificially high patch size estimates) giving a value of 12 nucleotides. The involvement of a number of activities in the initial stage of the pathway of nucleotide excision repair in *E.coli* (& in eucaryotic systems) is suggested by Lindahl (1979) to allow greater flexibility of the system. Adducts other than pyrimidine dimers can be recognised and so repaired but the "recognition" of structural distortion of DNA present as a result of normal processes of DNA metabolism is precluded. After incision at the site of damage an activity must prevent the single strand gap produced in the phosphodiester backbone from being a substrate for a DNA ligase. The 5' phosphate might be removed or a protein bind the ends of the scission preventing their ligation. It is suggested that the enzymes responsible for the exonuclease step of the reaction are distinct from the incision activity (Grossman et al. 1978) with the exonuclease activity of DNA polymerase I possibly fulfilling this role in *E.coli* cells.

Base excision repair provides a model for the repair of some forms of DNA damage whereby the initial event is the release of an altered base by a DNA glycosylase (Lindahl 1976). A number of DNA glycosylases have been identified, each cleaving the base-sugar bond of an altered nucleotide residue releasing the damaged base and leaving an baseless apurinic or apyrimidinic (AP) site (Lindahl et al. 1979; Duncan 1981; Lindahl 1982; Friedberg 1985). Olsen et al. (1989) report the identification and cloning of a human uracil DNA glycosylase by virtue of homology to previously characterised activities from other organisms. It is found that the human and bacterial enzymes are most closely related being 73.3% homologous. The human enzyme also shares considerable homology with those from a number of DNA viruses and also yeast. There are two major classes of enzymes known to cleave DNA at AP sites, the development of assays using a synthetic DNA substrate should facilitate the further characterisation of these activities.

In a sense the simplest mechanism of DNA repair is that of direct repair in which bases are neither removed or replaced. The covalent modification of DNA is simply reversed. Three examples of this type of repair are known: direct removal of methyl groups from DNA by methyltransferases, yeast spore specific repair and photoreactivation mediated by DNA photolyase.

The most extensively characterised of these is photoreactivation in which light energy is used by photolyase enzymes to break the cyclobutane ring joining the pyrimidines of pyrimidine dimers. Photolyases have only been extensively characterised from *E.coli* and two yeast species but enzymatic photoreactivation has been identified in a number of organisms. Using an in vitro system a factor binding to damaged DNA was found to be lacking in cells from one complementation group of the human excision repair deficient syndrome Xeroderma Pigmentosum (XP) (Chu and Chang 1988). It is thus implied that this protein plays a role in excision repair in normal cells. Searches for a similar factor in the yeast *S.cerevisiae* revealed an activity with many characteristics of the originally described XPE binding protein (Patterson and Chu 1989). This binding activity appears to correspond to photolyase implying that XPE cells are deficient in the human homologue of yeast photolyase and that this activity may be important in excision repair.

Alkylation of DNA (for example at the O^6 position of guanine producing O^6-methyl-guanine) creates substrates for repair processes effecting the transfer of an alkyl group to a cysteine residue in the repair protein (Olsson et al. 1980). Thus the removal of the methyl group from O^6-methyl-guanine (O^6MeG) formed when DNA is treated with N-methyl-N-nitrosourea is catalysed by O^6-methyl-guanine transferase. The final reaction product is methanol and no free O^6-methyl-guanine is detected so it is assumed that guanine is regenerated. One in vitro assay for this activity involves the use of substrate DNA prepared by reaction with radiolabelled MNU. The removal of O^6MeG can be followed by the use of high performance liquid chromatography before and after reaction with cell extracts (Cooper et al. 1982). An alternative assay described by Margison et al. (1985) measures the transfer of radiolabelled methyl groups from substrate DNA to the methyltransferase enzyme. This assay is not specific for O^6MeG transferase, measuring the activity of all methyltransferases however it is rapid and sensitive. Using this assay Margison et al. screened extracts of bacteria harbouring an *E.coli* genomic DNA library in a plasmid vector and so cloned the O^6MeT (ada) gene of *E.coli.* Such activities have also been identified in yeast and human cells. The gene coding for the *S.cerevisiae* enzyme has been cloned. The yeast O^6MeT appears to bear more functional relation to the mammalian enzymes than the bacterial (Sassanfour and Samson 1990). This is encouraging in that it implys that yeast system may provide a model for some repair events in mammalian cells. Hayakawa et al. (1990) cloned the human gene by means of complementation by gene transfer of a cDNA library from O^6MeGT positive (mer+) cells of deficient (mer-) human tumour cells. The relevant DNA was amplified using the polymerase chain reaction and seqeunced to reveal extensive homology with the previously cloned bacterial enzymes.

In bacteria though not in yeast O^6MeT is inducible (Sassanfour and Samson 1990). In yeast however the enzyme is only present in significant quantity in exponentially growing cultures. Pretreatment of *E.coli* with low doses of N-methyl-N'-nitroso-guanidine induces increased survival and resistance to mutation in cells subsequently challenged with high concentration of mutagen (Samson and Cairns 1977). This is correlated with an increased

rate of disappearance of O^6-methylguanine from adapted cells, while O^6-methyladenine is eliminated at a similar rate in adapted and nonadapted cells.

A further class of enzymes involved in reversion of lesions are purine insertases. A DNA-binding activity in human fibroblasts (Deutsch and Linn 1979a; 1979b) specific for partially depurinated DNA has been described. This enzyme inserts purines into apurinic sites in a reaction that is apparently energy independent. Such activities have been identified in *E.coli* (Livneh et al.1979) and Drosophila (Linn et al. 1982).

Incorrect bases inserted during replication which are not corrected by a proofreading system inherent in the replication process may be substrates for a mismatch repair system (reviewed by Modrich 1987).

Repair of single and double strand breaks has also been demonstrated. Breaks more complex than single strand scissions and so possibly not subject to direct ligation may be substrates for the excision repair pathway (Sancar and Sancar 1988). In some cases a double strand break results from two single strand lesions close together on opposite strands. If both breaks are substrates for ligases repair would be postulated to require only that the two ends be kept together until ligase action is complete. If both breaks involve base or sugar damage only faulty templates would be availiable for excision repair. In such instances a recombinational mechanism has been implicated (Rupp et al. 1971).

Recombinational repair is also important in replicative repair. On encountering some lesions during DNA replication the polymerase stops and reinitiates about 1000bp beyond the adduct generating a single stranded gap containing the lesion (Rupp et al. 1971). This discontinuity is filled in by the recA protein in bacteria by transfer of the complementary strand from the sister duplex into the gap. This transfer procedes by the same mechanism as the recombination events generally mediated by the recA protein (Howard-Flanders et al. 1984). Thus it is assumed that RecA, polymerase I a "nicking in trans" activity, ligase and Holliday resolvase are required (Ruppert et al. 1971; Cox and Lehman 1987; Livneh and Lehman 1982; Howard-Flanders et al. 1984; Ross and Howard-Flanders 1977). Recombinational repair may contribute to cellular survival in two ways, by bypassing adducts posing a block to replication and by making the region containing the adduct double stranded, the modified

base or bases then being a substrate for excision repair. This manner of repair is also used to deal with interstrand cross links (Linn et al. 1982; Laval and Laval 1980; Sancar and Sancar 1988).

The study of excision repair of UV damage and indeed of other repair pathways in *E.coli* has provided a basis for much of the knowledge which is now being extrapolated to other systems. Studies on bacteria and on yeast systems are facilitated by the existence of a vast array of repair mutants. The identification of gene products involved in repair in mammalian cells by these methods is limited by the number and variety of mutants availiable, as such mutations must be consistent with the survival of the organism. Studies in higher organisms must involve cells in culture. During adaptation to growth in culture selection pressures may lead to an established line with different properties (possibly including repair activities) from those in vivo.

The use of information from bacterial systems in the interpretation of results with mammalian cells is further complicated by the structural organisation of DNA into chromatin. Comparison between bacteria and mammalian cells involves the analysis of what are in effect two different substrates both for damage induction and its repair. In *E.coli* DNA is essentially naked while in mammalian cells damage and repair processes are acting on a highly structured DNA protein complex. This has been suggested to impose constraints on repair activities in terms of accessibility and allowing repair only under certain conditions (Morse and Simpson; Mattern 1984; Smerdon et al. 1990; Ljungman 1989; Collins and Squires 1984).

The consideration of DNA repair mechanisms is further confused in eukaryotic cells by differences in repair efficacy between a) regions of the genome or particular types of DNA sequence (Hanawalt 1987; Venema et al. 1990; Thomas et al. 1988; Wolfe et al 1989; Bohr et al. 1987; Govan et al. 1990); b) strands of a particular coding region of the DNA duplex (Mellon and Hanawalt 1989 ; Smerdon and Thoma 1990; Scicchitano and Hanawalt 1989) ; c) coding or non coding sequences (Bohr and Wassermann 1988); d) transcriptionally active as oposed to inactive sequences (Terleth et al. 1989). Repair capacity or regional differences between repair capacities may vary in a cell cycle dependent fashion (Terleth et al. 1990) or be evident only upon induction (Mellon and Hanawalt 1989). On a grosser level repair

capacity may vary from organ to organ and also with development (Mitchell and Hartman 1990) as well as between species.

Some repair activities may be present under normal circumstances at a low constitutive level only to be induced to a much higher level in response to DNA damage. The paradigm for such responses is the SOS system of *E.coli* (Walker et al. 1984) in which a number of genes are activated by the action of the RecA protein speeding the autocatalytic cleavage and concommittant inactivation of the lexA repressor (Slilaty et al. 1987). This activates genes responsible for excision repair, mutagenic damage bypass systems, genes involved in cell division and a number of genes whose functions are unknown. Divers repair pathways are stimulated and cell division is inhibited allowing time for repair. Also in bacteria the activation of a set of genes by a positive regulator protects against cell kill and mutagenesis by alkylating agents and a third type of inducible activity is seen in response to oxidative stress (Sancar and Sancar 1988).

A number of mechanisms thus exist for the repair of damage sustained by DNA. Studies of reactions in bacteria have provided a wealth of information which has in many instances facilitated the elucidation of repair mechanisms in eucaryotic systems. In extrapolating from results obtained in bacterial systems the very different nature of the structural organisation of the prokaryotic and eukaryotic genomes must be taken into account.

1.4 STUDY OF DNA REPAIR

- Use of mutants hypersensitive to DNA damaging agents.

Much of the current knowledge of DNA repair mechanisms has been derived from analysis of *E.coli* mutants. Over 60 show some alteration in aspects of DNA repair. The use of this range of cells has enabled the cloning of most of the relevant genes, the purification of their products and reconstitution of their activities in vitro with defined substrates (Lindahl et al.1982; Van Houten 1990). There is a strong interest in attempting to apply the same approach to mammalian systems by isolation and characterisation of mutants of mammalian cells with a defect in a pathway of DNA repair. It is expected that, while not all mutants hypersensitive to DNA damaging agents will be altered in pathways of repair (as opposed to those of drug uptake or metabolism) a number will be. An expanding range of such lines is providing much fundamental information. For example cross sensitivities to various agents allow inferences about the particular type of repair process defective in a given mutant. The detailed genetic and biochemical analysis of such mutants should provide the framework for the identification of most catalytic, structural and regulatory components involved in mammalian DNA repair. The situation in mammalian cells is likely to be more difficult than that in *E.coli* to unravel not least because of the much greater genetic complexity of mammalian as opposed to bacterial cells. The organisation of the mammalian genome into chromatin and this chromatin into nucleosomal structures (Morse and Simpson 1988) further complicates the situation. It might be supposed that the total number of repair mutants in mammalian cells will be considerably larger than that of all bacterial mutants. A larger proportion of mammalian mutants than bacterial would be expected to be regulatory mutants with complex phenotypes perhaps not as amenable to analysis.

The general approach taken in the isolation of mutants in repair processes has been to isolate sublines hypersensitive to a particular DNA damaging agent. It is then assumed that the mutation involved will be at one stage of a pathway for the repair of the lesion(s) caused by the agent used.

The majority of known mammalian DNA repair mutants (excluding the human syndromes thought to be caused by a defect in DNA repair) originate from the two Chinese hamster lines V79 and CHO, though there are some murine mutants. These lines have a high plating efficiency and grow well in culture. Furthermore they contain many regions of hemizygosity. The chances of isolating recessive mutations are thus much increased (Hickson and Harris 1988; Collins and Johnson 1987).

A population of mammalian cells is mutagenised and clonal isolates screened for sensitivity to a particular DNA damaging agent. In many cases a replica plating technique is used allowing clones killed by a dose of selective agent on a test plate to be isolated from an untreated master plate (Thomson et al.1988). In some cases a "suicide" approach is taken to enrich for the small population with a repair mutation in the originally mutagenised pool. After treatment with a DNA damaging agent the cells are incubated with a DNA precursor analogue which will be incorporated into the DNA of normal cells in the course of repair synthesis and prove lethal. If the cells are defective in DNA repair they will survive unless they are in S phase (Collins and Johnson 1987). This approach relies on a DNA damaging treatment inducing a DNA resynthesis response. The damage may be sufficient to remove from the population any highly sensitive mutants, the final population will thus contain a disproportionately high proportion of mutants with only moderately severe defects. An alternative suicide enrichment involves the infection of mutagenised cells with virus previously inactivated with a DNA damaging agent. Repair proficient cells repair the cytotoxic virus to wild type and so are killed while repair deficient subpopulations survive.

Identification by virtue of reduced growth or cell division following a low level of DNA damage is the technique most used to select clones representing repair defects from a mutagenised population. Individual colonies most affected by such treatment can be picked after microscopic examination (Thomson et al. 1988). Alternatively the colonies can be identified which do not increase in size during a given time interval after such a treatment.

Replica plating techniques where the pattern of clones on one plate is duplicated precisely on another allows isolation without exposure to a genotoxic agent. In this type of protocol clones which have not survived or show altered characteristics on the treated plate

following exposure to the relevant agent are identified and isolated from the untreated master plate. Treated and untreated replica clones may also be compared using asays for relevant types of repair for example unscheduled DNA synthesis (UDS) following exposure to UV. Following their identification repair mutants must be characterised in terms of the degree of sensitivity to genotoxic agents. As well as clonogenic assays for the effects of cytotoxic drug sensitivity specific assays have been used to address repair capacity. One is that mentioned above of UDS (or repair synthesis) as a parameter of the capacity of a line for excision repair. Various techniques including sucrose gradient sedimentation, nucleoid sedimentation, alkaline unwinding and filter elution have been used to study the removal of strand breaks which are produced on treatment with many DNA toxic agents. Further by preventing the later stages of excision repair by the use of DNA synthesis inhibitors mutants can be identified in the earlier stages for example incision (Collins and Johnson 1987).

After isolation of mutants on the basis of sensitivity to a particular drug the pattern of cross sensitivities to other DNA damaging agents allows the deduction of information as to the types of DNA lesion repaired by the same pathway. Interrelationships in terms of shared pathways of repair of the lesions caused by a number of a different agents may be revealed and unrecognised similarities in the mode of action of a set of genotoxic agents highlighted. For instance, of four mutants isolated for sensitivity to mitomycin C (MMC) only one is also UV sensitive, two (including the UV sensitive MMC-2) are cis-platinum sensitive (Hickson and Harris 1988; Collins and Johnson 1987). This suggests common processes in the pathways of repair of MMC, UV and cisplatin. This is perhaps not surprising as the lesions caused by both types of agent are bulky and helix distorting so might well be substrates for excision repair. The adducts caused by UV and cisplatin are however also revealed to be repaired by pathways with at least one step peculiar to each type of adduct.

In general it is assumed that hypersensitive mutants arise from single gene defects based on the frequency with which mutants are isolated. A direct test of this is provided on complementation by transfection of a specific gene, where if all cross sensitivities are corrected simultaneously they were due to one defect. It is only in a few cases that genes are availiable for this type of transfection complementation.

Complementation analysis may also be done by fusing cells and testing the cross sensitivities of the resulting hybrids. This allows classification of newly isolated mutants into complementation groups. The number of complementation groups identifiable for sensitivity to a given agent can provide information as to the number of activities involved in the repair of the lesion it causes. There are at least six complementation groups for sensitivity to ionising radiation (Jones et al. 1988) and five for UV sensitivity in hamster cells (Thompson et al. 1981). The conclusion is that a vast array of genes are involved in the repair of UV lesions. Microinjection of cell extract of one cell into another has also been used to restore repair activity (Hoeijmakers 1987). Cell free extracts can also be assayed for their capacity to restore repair activity in vitro when mixed if an assay for the activity exist (Wood et al. 1988). Similar studies have been performed by the addition of *E.coli* repair proteins to a cell free extract (Hansson et al. 1990).

The main purpose of isolation of mutants hypersensitive to genotoxic agents has been the eventual cloning of human DNA repair genes (Bootsma et al. 1988). To this end genomic DNA or a cDNA expression library can be transfected into certain hamster or mouse cells with high efficiency. Complementation of the sensitivity for which the mutant was selected and for concomittant correction of one or more of its cross sensitivities can be tested. In this manner the human repair genes ERCC-1 (Westerveld et al. 1984) -2 (Weber et al. 1988) and -6 (Troelstra et al. 1990) correcting the defects in UV sensitive rodent cells from complementation groups 1,2 and 6 respectively were cloned. An in vitro mixing approach can be used to purify repair proteins from cell extracts by virtue of their ability to complement mutant function in vitro (for example the isolation of the XPA gene described later).

Permanent cell lines have been derived from primary cultures of naturally occuring human DNA repair mutants. Immortalised lines have been derived from patients with syndromes such as the UV repair deficient XP the ionising radiation sensitive syndrome Ataxia Telangiectasia (AT) (Debenham et al. 1987), Blooms syndrome and Fanconi's anaemia (Plooy et al. 1985; Collins and Johnson 1987). The existance of such lines allows processes of DNA repair in human cells to be studied. The mixing of cell free extracts from complementation groups A and C of XP has been shown to restore the level of excision repair

to that of normal cells (Wood et al. 1988). The repair deficiency of XP cell extracts can also be restored by the addition of purified uvrABC proteins from *E.coli* (Hansson et al 1990). The mouse gene complementing the UV sensitivity of XP group A cells has been cloned (Tanaka et al. 1989) and its human equivalent isolated (Tanaka et al. 1990).

In summary the study of bacterial DNA repair mutants has provided a large part of the current knowledge of the pathways of DNA repair. In attempting to extrapolate to processes occuring in mammalian cells increased complexity arises due to the much greater size and more complex organisation of the genome. In at least the case of the complementation of the XP repair defect by uvrABC proteins referred to above however, it is clear that there is functional conservation of repair activities from bacteria to mammalian systems. The use of hamster mutants hypersensitive to DNA toxic agents is facilitating the analysis of repair in mammalian cells. It has already proved possible to clone a number of repair genes using approaches of complementation by DNA transfection of such phenotypes. Mixing of cell extracts from repair deficient cells has also allowed the identification of proteins able to restore excision repair. It is clear from the number of complementation groups of the rodent and human mutants availiable that DNA repair in mammalian cells is a complex set of processes involving many genes. As yet only one overlap has been found between UV sensitive rodent cells and any XP complementation group. It is also notable that the majority of mutants sensitive to UV are defective in the initial incision stage of excision repair (rare XP variants have a defect in a poorly characterised process of post replication repair). This implies that there is some bias in the generation of mutants and that by no means all the genes in even the relatively well characterised pathway of excision repair are represented.

A eucaryotic system (thus with a genome complexity and organisation more closely resembling that of human cells than bacterial) with an extensively characterised genetic system is that provided by the yeast *Saccharomyces cerevisiae*. Yeast cells are easily manipulable in culture, can be transfected by exogenous DNA (Hinnen et al. 1978) and a variety of vectors exist to allow gene cloning (Beggs 1981; Stinchcomb et al. 1979; Struhl et al. 1979). There are also indications that the organism may provide an adequate model for at least some processes of mammalian DNA repair.

1.5 S.CEREVISIAE AS MODEL FOR STUDY OF DNA REPAIR

1.5.1 Introduction.

Following the isolation of a large number of yeast mutants sensitive to UV and ionising radiations it was agreed that such mutants should be designated *rad*, with identifying locus and allele numbers. Locus numbers 1-49 refer to strains primarily UV sensitive and those greater than 50 to strains primarily sensitive to ionising radiations (Friedberg 1988). Mutants have been selected on the basis of sensitivity to monofunctinal alkylating agents like methyl-methane-sulphonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (Nisson and Lawrence 1986), to cross linking agents like psoralens and nitrogen mustards (Henriques and Moustacchi 1980a; 1980b) as well as for sensitivity to both UV and ionising radiations (Prakash 1977a; 1977b; Prakash and Prakash 1979). Mutants have also been selected for altered levels of spontaneous and induced mutation or mitotic recombination (Rodarte-Ramon and Mortimer 1972). Studies on the relative sensitivity of single and double *RAD* mutants facilitated the organisation of yeast DNA repair genes into different epistasis groups. Each epistasis group represents mutations in a distinct pathway of repair. If the sensitivity of a double mutant to a particular agent is no greater than that of a single mutant in either gene the two genes are considered epistatic. Such genes are likely both to be involved in the same pathway of repair of the damage caused by that agent. Approximately 30 mutant loci have been analysed leading to the classification of cells sensitive to radiation into three epistasis groups exemplified by the RAD3, RAD52 and RAD6 genes (Friedberg et al. 1983; Haynes and Kunz 1981). These three groups are thought to represent largely nonoverlapping functions. Loci in the RAD3 group are involved in nucleotide excision repair, those in the RAD6 group in mutagenesis and those in the RAD52 group are believed to reflect the existance of recombinational responses. Studies on the sensitivities of some of the RAD3 mutants to alkylating agents suggest that the repair of some types of monofunctional alkylation damage involves subsets of genes involved in repair of UV damage (Cooper and Waters 1987). Different mutants in the RAD3 group are sensitive to different alkylating agents and furthermore sensitivity to many of these agents is not confined to the RAD3 group mutants.

1.5.2 RAD3 epistasis group - excision repair genes

All loci in the RAD3 group confer some aspect of the normal response to UV light (Haynes and Kunz 1981). This is not an exclusive correlation in that as described above a number of RAD3 loci are also involved in the response to alkylating agents. There are also indications that genes in this epistasis group can also be involved in repair of X-ray damage. Double mutants defective in genes in RAD6 and RAD52 groups are less radiosensitive than triple mutants also defective in RAD3 genes. The implication is that there exists a type of radiation damage repairable by all three types of RAD pathway. Such lesions will not be lethal unless genes in all three pathways are mutated.

Direct analysis of strand breaks brought about by incision at pyrimidine dimers after UV treatment has been used to reveal an incision defect in some members of the RAD3 group (specifically a number of rad1, rad2, rad3 and rad4 mutations). The loss of pyrimidine dimer specific endonuclease sites also identified incision defective RAD3 mutants. It has been shown that cells carrying mutations in RAD1, RAD2, RAD3, RAD4, and RAD10 genes do not carry out detectable incision of their DNA during postirradiation incubation while those mutant in the RAD7, RAD14, RAD16, RAD23 and MMS19 genes have a significant capacity for incising DNA at sites of pyrimidine dimers. Indeed mutants deleted in the entire rad7 and rad23 genes are not wholly deficient in excision repair of pyrimidine dimers. Friedberg (1988) suggests that these members of the RAD3 epistasis group may not primarily be involved in the incision of DNA at sites of pyrimidine dimers having only a secondary role in such a process. In extrapolation from studies with bacteria it is possible that RAD1, RAD2, RAD3, RAD4, and RAD10 encode essential activities and the others play a regulatory role. It is also possible that the complexity of the excision repair system in yeast arises to cope with the inherent problems encountered due to the structural organisation of the eucaryote genome. Accessibility of DNA to repair activities might be presumed to pose the major stumbling block in terms of efficient repair. This may account for observations that excision repair is more efficient in actively transcribed sequences. It also suggests that the multiplicity of genes required for or involved in excision repair might reflect a process performed, not by single

proteins but by multiprotein complexes the three dimensional structure of which provides the biochemical basis for the specificity of the requisite interactions.

The development of cell free systems to study excision repair in yeast has not proved as succesful as in bacterial systems. An in vitro system where extracts from repair proficient cells reproducibly catalyse removal of pyrimidine dimers has yet to be described. Such a system which should accurately reflect the ability of the various rad strains to complement each other would be invaluable in purification of repair activities.

A number of genes in the RAD3 epistasis group have now been cloned including RAD1 (Higgins et al. 1983a; White and Sedgwick 1985), RAD2 (Higgins et al.1984; Naumovski and Friedberg 1984), RAD3 (Higgins et al. 1983b; Naumovski and Friedberg 1982), RAD4 (Fleer et al.1987) and RAD10 (Wiess and Fieidberg 1985). The amino terminal end of the deduced rad3 protein contains a region closely resembling a nucleotide domain present in a number of other proteins, such as ATPases. There is striking similarity between this region of Rad3 and regions in the amino acid sequence of the *E.coli* excision repair proteins UvrA (Hussain et al. 1986), UvrD and UvrB (Arikan et al. 1986) and the recombinational repair proteins RecA (Sancar et al. 1980), RecC and RecD (Sancar and Sancar 1988). UvrA, UvrD and RecA have also been shown to be ATPases and there is a suggestion that ATPase activity is stimulated by interaction with UvrB revealing a cryptic ATPase activity of this protein. RAD3 and UvrD share homology with a yeast gene required for recombination repair of mitochondrial DNA. The RAD3 protein has been purified and shows ATPase activity in vitro. It is possible that the function of RAD3 involves interaction with another (distinct) ATPase activity of *S.cerevisiae* which also has associated helicase and DNA synthesis stimulatory functions in vitro (Sugino et al. 1986). This activity (ATPaseIII) could possibly have a role in excision repair and might concievably be the product of an as yet uncloned RAD3 epistasis group member. Random mutagenesis of RAD3 shows that single missense mutations scattered about the gene are capable of inactivating the excision repair function of this gene (Naumovski and Friedberg 1986). Site specific mutagenesis shows the putative purine nucleotide binding domain to be essential for excision repair activity. Disruption of the chromosomal gene is lethal in haploids and recessively lethal in

diploids (Naumovski and Friedberg 1983; Higgins et al. 1983b). This is not so for any other of the four RAD genes required for excision repair. The nature of the RAD3 essential function is unknown. Some mutants completely deficient in excision repair are still viable implying that repair functions are distinct from those essential for viability. It is possible that both functions are products of the same catalytic domain but that the excision repair function is more sensitive to missense mutations or to a reduction in total protein content while that of viability represents the complete abscence of protein in the correct coformation.

The RAD10 gene is not essential for viability (Wiess and Friedberg 1985) and there is no evidence for its induction by treatment with DNA damaging agents (Friedberg 1988). It contains a reasonable match for an helix-turn-helix DNA binding domain (van Duin et al. 1986) and has been shown to be involved (with at least RAD1) in a mitotic recombination pathway distinct from that of the RAD52 group (Schiestl and Prakash 1990). The translated amino acid sequence of RAD10 bears strong homology to a human gene ERCC1 cloned by virtue of its ability to complement the excision repair defect of hamster mutants in complementation group 2. Indeed it is reported (Lambert et al. 1988) that the RAD10 gene partially complements the defect in a number of independent UV sensitive CHO lines in this complementation group though not those in other groups for UV sensitivity. This indicates a degree of functional conservation between repair genes in yeast and mammals. That complementation was incomplete is not surprising given that the sequences of the yeast RAD10 and human ERCC1 genes are not identical. Furthermore it might be anticipated that a certain degree of species specificity would characterise the various pathways of DNA repair. This result underlines the use of yeast as a model system for analysis of repair pathways.

Other genes in the RAD3 epistasis group which have been cloned include the thymidylate kinase gene CDC8 (Hartwell 1971) originally isolated as being required for cell cycle progression. Mutants in the CDC9 DNA ligase gene show enhanced UV sensitivity (Hartwell et al. 1973; Johnston and Nasmyth 1978).

As well as an involvement in excision repair, genes in the RAD3 epistasis group are implicated in other repair pathways. Thus double mutants in RAD3 and RAD6 pathways are more sensitive to psoralens than either type of single mutant. Many rad mutants isolated on

the basis of UV sensitivity have also been shown to be sensitive to bifunctional agents such as nitrogen and sulphur mustards. This type of repair also appears to involve genes in the RAD50 group. Genes in the RAD3 group also appear to have an involvement in the repair of the damage caused by alkylating agents as do those in the RAD6 (Friedberg 1988;1985; Friedberg et al. 1987).

1.5.3 Photreactivation in S.cerevisiae.

There are indications that two distinct photolyase activities are present in yeast cells. Two genes PHR1 and PHR2 have been identified in mutants deficient in photoreactivation in vivo. The two genes are believed to be linked but distinct. The PHR1 gene appears to code for one of the photolyase activities mentioned above. Oddly mutants in PHR1 are totally deficient in photoreactivation. Thus it is speculated that PHR2 has a regulatory function. The predicted product of PHR1 is a 53,000 protein with marked homology to the *E.coli* enzyme. This size is also consistent with the biochemical data on the activities mentioned above (Iwatsuki et al. 1980; MacQuillan et al. 1981; Resnick 1969; Resnick and Setlow 1972) .

1.5.4 RAD52 epistasis group - repair of ionising radiation induced damage

Mutants primarily sensitive to ionising radiation are designated with locus numbers from RAD50 upwards. Among the RAD52 epistasis group rad51, rad52, and rad54 are extremely sensitive to ionising radiations (Haynes and Kunz 1981; Henriques and Moustacchi 1980; Schild et al. 1983). Mutants at these loci show almost completely deficient mitotic recombination. They show a greater than normal chromosome instability, both spontaneous and radiation induced. These mutants also show a sharp reduction of the X-ray resistance normally associated with mating type heterozygosity.

The rad50, rad53, rad55, rad56 and rad57 are also classified in the RAD52 epistasis group although they are typically less sensitive than those mutants mentioned above and show less extreme deviations from the normal phenotypic responses to ionising radiation (Game 1983).

The observation that RAD52 group mutants are deficient in meiosis (which requires a number of recombination events) has led to the suggestion that this group represents genes involved in a recombination mechanism. The inference is then that ionising radiation induced damage is repaired by a recombination mechanism (Friedberg 1988).

The RAD50 gene has been cloned (Kupiec and Simchen 1984) and from the amino acid sequence is predicted to encode a protein which carries a domain with significant homology to characterised purine nucleotide binding domains. It does not appear to be induced either at the RNA or the protein level by DNA damage by UV or MMS. Deletion of this gene does not prevent viability so the gene is non essential.

The RAD52 gene (also non essential) was isolated by the phenotypic complementation of appropriate mutants (Schild et al. 1983; Adzuma et al. 1984). There are suggestions that it is damage inducible. RAD54 has been found to be induced 3- to 12- fold in a cell cycle independent fashion by treatment of cells with X-rays, UV and MMS and also by double strand breaks introduced into the genome by expression of the endonuclease EcoRI. There is no evidence as to whether RAD54 and RAD52 (which is not cell cycle regulated) are induced by the same mechanism.

1.5.5 RAD6 epistasis group.

Loci in this epistasis group affect the sensitivity of mutants to both ionising and UV radiation (Haynes and Kuntz 1981; Game 1983). The RAD6 gene has been cloned (Kupiec and Simchen 1984) and while nonessential its absence causes cells to grow more slowly and contributes to cell lethality. The RAD6 protein has been shown to bear significant homology to human ubiquitin conjugating proteins and demonstrated to have ubiquitin conjugating activity in vitro (Jentsch et al. 1987). It is possible that the ubiquitination of histones (a major substrate for the type of ubiquitin conjugating enzyme of which RAD6 is an example) is an important prerequisite for the remodelling of chromatin required for some processes of DNA metabolism including repair. Expression of RAD6 has been reported to be regulated in a cell cycle specific fashion (Kupiec and Simchen 1986) and there are also suggestions of inducibility by DNA damage.

1.5.6 Mismatch repair in yeast.

Mimatched nucleotides can be generated as a result of replicative infidelity during DNA synthesis, during recombination or as a result of spontaneous base damage such as the deamination of cytosine to uracil. In yeast evidence for mismatch repair is provided by tetrad analysis where classical models suggest that such correction events will lead to deviations from the usual Mendelian segregation patterns (Hotchkiss 1974; Muster-Nassal and Kolodner 1986; Meselson and Radding 1975). Mismatch repair has been demonstrated using an exogenous DNA heteroduplex as a probe both in vivo and in vitro. For in vivo experiments yeast cells were transformed with plasmids containing 8 or 12 bp insertion mismatches or A.C or C.T single mismatches. Such substrates are seen to be repaired in wild type cells and this repair to be altered in the PMS (postmeiotic segregation) mutant *pms1-2*. Cell free studies involved synthetic oligonucleotide DNA substrates constructed with 4- or 7- bp insertion/deletion mismatches or with each of the eight possible single base mismatches. Extracts of mitotic cells were found to catalyse the correction of mismatches. It was found that the system exhibited a strong preference for repair of A.C and G.T mismatches with these being repaired with great efficiency while the other six single base mismatches were corrected with a much lower efficiency. Mismatch correction was accompanied by detectable repair synthesis, patches of up to 20 nucleotides located at or near the sites of the repaired mismatches (Muster-Nassal and Kolodner 1986).

1.5.7 *S.cerevisiae* as a system for analysis of DNA repair -summary and conclusions.

In summary the availability of a large number of mutants sensitive to DNA toxic agents has provided a genetic framework for the analysis of repair in *S.cerevisiae*. The current set of mutants is unlikely to include all the loci involved in DNA repair and it is clear that the situation is highly complex with pathways being interrelated at various points and the apparent existence of a large number of regulatory genes. Extrapolation from bacterial systems to mammalian must be cautious given the more complex genomic organisation in yeast cells.

A number of yeast repair genes have been cloned. The proteins encoded by many of these show no clear homologies with those previously identified although in some cases functions consistent with a role in repair of DNA damage can be assigned to them. The use of *S.cerevisiae* in the investigation of mechanisms of DNA repair is likely for a variety of reasons to be a valuable tool in the analysis of repair in mammalian cells. The relative facility of genetic experiments in the organism enables detailed analysis of mutants defective in repair. The successful establishment of an *in* vitro system (ie that for the analysis of mismatch repair described above) provides a model for the creation of similar systems for the assay of other processes. There is also evidence of functional conservation between repair proteins of yeast and mammalian cells since the RAD10 gene is able to partially complement the UV sensitivity of excision repair defective CHO cells from complementation group 2 (Lambert et al. 1988). The defect in XP group E cells identified originally as a damage binding factor missing in these cells has been identified as the homologue of a yeast photolyase (Patterson and Chu 1989) again suggesting functional overlap between the two systems.

Table 1.1 adapted from Hoeijmakers and Bootsma summarises the information availiable on yeast genes in the RAD3 epistasis group. It includes mention of the recently described yeast homologue of the human repair gene ERCC3. ERCC3 (Weeda et al. 1990) corrects the defect in hamster UV sensitive cells in complementation group 3.

Table 1.1

Properties of nucleotide excision repair genes of *S.cerevisiae.*

Gene	Remarks
RAD1	acidic carboxy terminus, involved in recombination.
RAD2	transcription UV inducible.
RAD3	homologous to human ERCC2, nucleotide and DNA binding, 5'-3' DNA helicase, acidic carboxy terminus, essential.
RAD4	DNA binding? acidic carboxy terminus
RAD7	acidic stretches, membrane association? partial excision defect
RAD10	homologous to human ERCC1, DNA binding? involved in recombination
$ERCC3^{Sc}$	homologous to human ERCC3, nucleotide and DNA binding, DNA helicase? acidic stretches, essential.

A "?" denotes a function or property postulated on the basis of amino acid sequence homology to functional domains in other proteins; direct proof at the protein level is lacking.

1.6 DNA REPAIR IN HUMAN CELLS

The strategy most commonly used for isolation of human repair genes is based on attempting to correct some or all aspects of a mutant phenotype by transfection of human genomic DNA from repair proficient cells (Hoeijmakers and Bootsma 1990; Bootsma et al. 1988;). The human excision repair gene ERCC1 corrects the excision repair defect in CHO mutants of complementation group 2 (Westerveld et al. 1984). This gene resides on chromosome 19q13.2-13.3. It is about 15kb in length with 10 exons (van Duin et al. 1987). The ERCC2 gene corrects the defect in the incision step of excision repair of group 1 hamster UV sensitive mutants (Weber et al.1988). ERCC6 corrects the cyclobutane dimer repair

defect in the moderately UV sensitive complementation group 6 hamster mutants (Troelstra et al. 1990).

Microinjection of the previously cloned ERCC3 gene into nuclei of the rare XP complementation group B was found to restore the repair synthesis ability of these cells (Weeda et al. 1990a). ERCC3 corrects the defect in CHO UV sensitive mutants in complementation group 3 (Weeda et al. 1990b). The predicted protein product of ERCC3 has putative nucleotide and chromatin binding sequences. It carries a consensus helix-turn-helix DNA binding motif and seven consecutive motifs conserved between two superfamilies of DNA and RNA helicases. There is thus a strong possibility that ERCC3 is a DNA helicase.

Another approach to the isolation of human DNA repair genes is by virtue of homology to repair genes from other species. Thus a sequence from a mouse gene complementing the defect in human UV sensitive XP cells of group A (XPAC Tanaka et al. 1989) was used as a probe to identify the corresponding human gene (Tanaka et al. 1990). The human XPAC gene encodes a protein of 273 amino acids and contains a motif suggestive of a DNA binding "zinc finger".

There is good evidence that the XPAC gene isolated by Tanaka et al.(1990) is the site of the primary defect in complementation group A XP patients. The mRNA is greatly reduced in cell lines from a number of severely affected Japanese patients due to a G to C transversion at a splice acceptor site. Some group A patients with milder symptoms have normal mRNA levels but carry a missense mutation in the cDNA. Moreover in situ hybridisation locates the gene to human chromosome 9q34.1 an assignment in agreement with chromosomal transfer experiments (Wood and Lindahl 1990). The mutation in the XP group B gene (ERCC3 described above) that has been identified is also a transversion at a G-C pair within a splice acceptor site (Weeda et al. 1990).

A human uracil-DNA glycosylase has been cloned by purification of the protein and using the amino acid sequence to generate oligonucleotides. These were used as probes to isolate the relevant gene from human placental DNA in a cDNA library (Olsen et al. 1989). This study compared the sequence of the human gene with those from yeast, *E.coli* and a number of DNA viruses. The bacterial gene was found to be most closely related to the

human (73.3% if conservative amino acid changes are included) though all the enzymes showed striking homology. Regions of homology were seen to be confined to several discrete boxes. It is thus clear that this class of repair enzymes are highly conserved between different species. Presumably the regions of homology represent domains of the protein conserved because of their importance in the three dimensional structure or the catalytic functions of the enzyme.

Other systems developed for the analysis of repair in human cells have involved the transfection of a plasmid previously damaged in a defined manner into repair proficient and deficient cells. If the damaged plasmid carries a marker gene coding for an assayable activity (for example the chloramphenicol acetyl transferase or "CAT" gene) increased expression of this gene implies the repair of the damaged plasmid. This can then be compared in repair proficient and deficient cells. Thus Chu and Berg (1987) found much lower levels of CAT activity in XP as compared to excision repair proficient cell lines following transfection of a CAT carrying plasmid previously inactivated by treatment with the cytotoxic drug cisplatin.

Cell free systems have been used to demonstrate complementation between the various XP groups in vitro. An assay for repair synthesis on a damaged plasmid incubated with cell extract showed XP cells to be deficient compared to wild type. This defect was found in representative extracts from all XP complementation groups tested. Mixing of extracts from two different complementation groups resulted in full restoration of repair synthesis capacity (Wood et al. 1988).

A slightly different *in vitro* approach is to add proteins involved in repair which have been purified from other organisms to extracts from human repair deficient cell lines. Hansson et al. (1990) complemented the repair synthesis defect in XP extracts by addition of the *E.coli* incision complex of Uvr ABC proteins. This allows confirmation of the repair defect in these XP cell lines as being in the incision (UvrABC catalysed) stage of excision repair. Arrand et al.(1987) in an analagous *in vivo* approach restored excision repair proficiency (in terms of UV response) to XP cell lines of group D by expression of a transfected *denV* gene encoding the pyrimidine dimer specific endonuclease of bacteriophage T_4.

Making the assumption that repair of a DNA adduct requires firstly that it be recognised assays are being developed to detect specific recognition proteins in normal repair proficient cells. Chu and Chang (1988) report the identification of a protein binding UV damaged DNA. This protein is deficient in XP cells belonging to complementation group E. Patterson and Chu (1989) found an equivalent factor in extracts from yeast and present evidence that this XPE factor is a photolyase.

A variety of complementary approaches have thus been taken to analyse DNA repair in human cells. A number of excision repair genes have been isolated and in some cases the mutations responsible for the repair defect in human cell lines with deficient excision repair characterised. Complementation by DNA transfection of mammalian UV sensitive mutations has proven an extremely succesful method for the isolation of human repair genes. Sequence homology analysis of cloned genes leads to interesting speculations as to structure function relationships and shows a large degree of conservation between the repair activities of mammalian cells and those in evolutionarily distant organisms. Table 1.2 (also adapted from Hoeijmakers and Bootsma) summarises the properties of cloned human genes involved in excision repair.

Table 1.2

Human genes involved in Excision repair.

Gene	Comments
XPAC	chromosome 9, DNA binding
ERCC1	chromosome 19q13.2, DNA binding? homologous to yeast RAD10 homology to E.coli UvrA and UvrC
ERCC2	chromosome 19q13.2,homologous to RAD3 of yeast, *nucleotide* and DNA? binding, DNA helicase? essential
ERCC3/XPBC	chromosome 2q21,yeast homolog is $ERCC3^{Sc}$ nucleotide and DNA binding, helicase? essential? acidic stretch
ERCC5	chromosome 13q,
ERCC6	chromosome 10q11

"?" denotes a function postulated on the basis of amino acid sequence homology to known functional domains in other proteins; direct proof at protein level is lacking.

1.7 HETEROGENEITY IN DNA REPAIR

The discussion above has largely assumed that DNA repair is a homogeneous process with all sequences of the genome repaired with an equivalent efficiency and the capacity of a cell to repair being invariant. In fact this is an oversimplification. In eucaryotes the extremely tight packaging of DNA in the nucleus and the organisation of chromatin might be expected to render large portions of the genome inaccessible. The proportion of DNA inaccessible to repair enzymes would not however be expected to remain constant. There are indications that processes of transcription and replication require extensive remodelling of

chromatin structure. Further it is possible that regions of the genome never subject to transcription and remaining in a fully condensed state except at replication might have different repair characteristics from regions of chromatin containing active genes. Transcribed regions might be repaired differently from neighbouring untranscibed sequences. A corollory to these considerations is that damage induction and the lethality of damage once induced may not be uniform throughout the genome. So what is the evidence for these postulates?

Bohr et al. (1987) in a review addressing heterogeneity in DNA damage and repair presents a large body of evidence for the action of carcinogens being sequence specific. Bohr et al. (1987) also present evidence for the preferential binding of a number of carcinogens to chromatin in the more open configuration (euchromatin). Ljungman (1989) suggests that treatment with some DNA damaging agents for example UV light actually alters chromatin structure in such a way as to render DNA more susceptible to subsequent attack by damaging agents.

A number of methods have been developed enabling the quantification of adducts and so the measurement of DNA damage and repair in defined sequences. One involves the restriction digestion of genomic DNA containing the adducts of interest followed by treatment with an enzyme for example T_4 endonuclease V (Bohr et al. 1985) or the Uvr ABC excision nuclease of *E.coli* (Thomas et al. 1988). Incision by one of these enzymes at a substrate adduct can be quantified by subjecting the digested fragments to Southern analysis and hybridisation with sequences complementary to that under investigation. Reduction in hybridisation intensity gives a measure of the number of adducts in this DNA sequence. Govan et al. (1990) describe the use of an assay based on the polymerase chain reaction (PCR) for measuring DNA damage and repair in small genomic segments. This approach relies on the inability of DNA polymerases to synthesise across a damaged nucleotide. It was reasoned that if quantitative PCR conditions could be established the fraction of gene segments bearing one or more damaged nucleotides would be inversely proportional to the amount of PCR product. DNA repair at the gene level should then be measurable as a restoration in the level of amplified product. These authors were able to detect UV and 4-NQO induced damage and follow its repair in three transcriptionally active genes.

There is much evidence to suggest that repair in mammalian cells proceeds with different overall efficiency depending on the sequence in question (Bohr et al. 1987; Bohr and Wasserman 1988). It was demonstrated that repair of the DHFR gene in hamster (CHO) cells was much more efficient than the repair over the genome as a whole. In repair proficient human cells the whole genome is repaired more efficiently than in rodent cells. Essential genes are repaired faster than noncoding sequences. Preferential DNA repair has been demonstrated in matrix associated regions. In some instances different genes are repaired with differing efficiency in the same cell. For example in a study comparing the rates of repair in mouse cells of a number of proto-oncogenes the *c-abl* gene was found to be repaired much faster than *c-mos*. This correlates with the relative transcriptional activity of the two genes in these cells. The correlation with transcriptional activity and efficiency of repair was further supported by the observation that repair in CHO cells of the metallothionein gene is markedly more efficient when the gene is transcriptionally active. This implies a connection between the machinery of DNA repair and that of transcription. In bacteria it is reported that upon induction of transcription of the lactose operon repair of the transcribed strand is much faster than that of the nontranscribed strand. Furthermore, prior to induction no such difference in the efficiency of repair of the two strands is evidenced (Mellon and Hanawalt 1989). However this coupling of repair to transcription seems only to apply to some adducts. In contrast to the preferential repair of pyrimidine dimers from the transcribed strand of active as opposed to inactive genes seen in CHO cells Scicchitano and Hanawalt (1989) observe in the same cells equivalent removal of N-methylpurines in both strands of the DHFR gene and a downstream noncoding region. Repair in the DHFR domain does seem however to be more efficient than that of the genome overall. Studies of the repair of the DHFR gene in CHO cells have shown the existence of a repair domain. The preferential repair of this gene is confined to a 60-80 kb region centred round the 5' end of the gene. The initial frequency of pyrimidine dimers is similar in all fragments in and around the DHFR gene. The level of repair differs markedly being maximal at the 5' end of the gene. The repair domain has the same size as the loops or domains into which chromatin is organised and it is possible that DNA repair processes are regulated or organised within such loops or domains.

Differential repair of actively transcribed genes has also been demonstrated in yeast (Terleth et al.1989) and this difference observed in defined cell cycle stages (G1 and S but not in G2) (Terleth et al. 1990).

Transfection into repair deficient CHO cells of the human repair gene ERCC-1 was shown by Bohr et al.(1988) to restore the normal pattern of preferential repair of the active DHFR gene.

Cells from patients with Cockaynes syndriome (CS) are hypersensitive to UV irradiation but appear to remove pyrimidine dimers from the genome with normal efficiency. The genetic defect in CS seems to lie in the pathway of repair of transcriptionally active genes such that (Venema et al. 1990a) in a number of CS lines from different complementation groups there is no preferential repair of transcribed over nontranscribed sequences.

Other results in human repair deficient lines from XP group C also indicate the possible existance of independent repair pathways. These lines are deficient in overall repair but efficiently repair active genes. At least some are repaired with an efficiency equivalent to that seen in normal repair proficient lines. The authors (Venema et al.1990b) suggest a pathway of repair of active chromatin different from that of inactive.

In summary, there is extensive evidence for DNA repair being a non homogeneous process. Techniques are being developed enabling the analysis of damage and repair in very specific regions of the genome. It has been shown that repair efficiency correlates with chromatin openness and also with transcriptional activity. Some human repair deficient syndromes appear to have a defect in repair of actively transcribed regions. There are implications that the processes of repair of "active" and "inactive" chromatin are different. These indications of the complexity of DNA repair imply that care must be taken in interpretation of results from studies on model systems or upon situations which might differ significantly from that pertaining *in vivo*. It has been demonstrated in yeast that DNA repair in a minichromosome and indeed a small plasmid accurately reflects that in the genome overall (Smerdon and Thoma 1990; Smerdon et al. 1990). As previously described the pattern of repair heterogeneity in yeast appears analagous to that in mammalian cells thus these

systems should be valid models for many of the processes involved in DNA damage and repair.

1.8 ANALYSIS OF REACTIONS INVOLVED IN DNA MEATABOLISM and REPAIR

In this section I shall describe some of the methods used to elucidate the nature of reactions required in the metabolism of undamaged DNA and also those specifically designed to analyse repair of DNA damage. There are two reasons for considering systems for the analysis of DNA metabolism with those addressing DNA repair. One is that the reactions in both cases are likely to exhibit considerable overlap with regards to proteins involved as well as in particular pathways. Further, repair activities form part of the "normal" metabolism of DNA being required as a vital part of the replication process. A second reason for not separating the systems is that the types of approach originally applied to the analysis of DNA replication and metabolism are now being applied to the study of repair pathways.

1.8.1 In vivo systems for the analysis of reactions of DNA

A number of systems have been developed for the analysis of recombination reactions. Many groups have used an approach based on the transfection of defined DNA substrates into cells and monitoring their fate. Wilson et al. (1982) constructed substrates for the analysis of nonhomologous recombination by inserting pBR322 sequences into the genome of SV40 virus and demonstrated efficient recombination after transfection into CV-1 cells.This system was further exploited to yield further information about the sequence requirements and the mechanisms of homologous (Rubnitz and Subramani 1984) and nonhomologous (Roth et al. 1985) recombination.

Shapira et al. (1983) demonstrated recombination in a system also used by Small and Scangos (1983) whereby thymidine kinase negative (TK^-) mouse L cells were transfected with plasmids carrying an inactivated TK gene. Cotransfection of inactivated plasmids with non overlapping deletions led to a significant proportion of recipient LTK^- cells becaming TK^+ as detected by selection with hypoxanthine/aminopterin/thymidine (HAT).

Rubnitz and Subramani (1985) transfected substrate plasmids into monkey COS cells. Recombination was assayed by isolating DNA and using it to transform recombination deficient bacteria. In this fashion it was demonstrated that a DSB in one substrate had a stimulatory effect upon the recombination process.

The TK selection system for recombination in mouse L cells provided evidence that repair of DSB or double strand gaps is a major mechanism for homologous recombination between exogenous DNAs (Brenner et al. 1986). These authors conclude that DSB and double strand gaps are recombinogenic because they serve as intermediates in homologous recombination by double strand gap repair.

Partially homlogous substrates were used by Abastado et al. (1987) to asess the contribution of a single DNA end on the process of recombination as it occurs in *E.coli* and also in Xenopus oocytes. A single DNA end was found to stimulate recombination if located in the region of homology. Kucherlapati et al. (1988) also demonstrated that generating a double strand gap in the region of homology greatly increases the recombination frequency. Brouilette and Chartrand (1987) used an assay based upon recombination between a mammalian and a bacterial vector transfected into mammalian cells to demonstrate the recombinogenic nature of a DSB. de Saint Vincent and Wahl (1983) used chinese hamster cells with a mutant CAD gene requiring exogenous uridine for survival to assay recombination between transfected substrates containing overlapping fragments of the CAD gene.

Folger et al.(1985) and Wong and Cappecchi (1986) used microinjection to introduce DNA into mammalian cells and also demonstrated efficient recombination. These groups too found the introduction of a DSB into one or both substrates to have a marked stimulatory effect.

A number of groups have used transfection mediated introduction of exogenous DNA into cells to probe the capacity of cells to repair a given type of damage introduced in vitro. Popoff et al. (1987) modified pBR322 in vitro with cis-platinum and studied the transformation of UV repair mutant *E.coli* by such substrates. These studies showed the uvrB gene to be essential for the repair of cis-platinum induced DNA damage. A functional recA gene was of minor importance. This set of experiments also emphasised the difference in the pathways of

repair of DNA treated with cis-platinum and the trans isomer trans-platinum. *In vitro* incision by the UVRABC excinuclease complex was found to proceed more readily if DNA modified with cisplatin as opposed to transplatin was the substrate.

White and Sedgwick (1987) report a much reduced frequency of transformation of UV sensitive mutant strains of *S.cerevisiae* by plasmid damaged *in vitro* by UV irradiation. This study allowed the assignment of a role in post incision UV repair to the products of the RAD1, 4, 7 and 14 gene products.

Knox et al (1987), Chu and Berg (1987), Vos and Hanawalt (1989) introduced DNA damage into plasmid substrates *in vitro* prior to transfection into repair competant and deficient mammalian cells. The results of these studies were confused by the finding that one effect of DNA damage is to stimulate integration of that DNA into the host cell genome. There were suggestions however that if a plasmid damaged with a DNA toxic agent was transfected into a cell line sensitive to that agent its transfection frequency was reduced implying a lack of repair.

Some evidence for an inducible DNA repair system analagous to that observed in bacteria was obtained (Protie et al. 1988) using a reactivation system in which treatment of monkey cells with UV light or mitomycin C 24-48 hours prior to transfection with a UV damaged plasmid greatly stimulated the subsequent reactivation of the CAT gene. This enhancement of excision repair capacity was not seen in cells from the repair deficient syndrome XP.

The transfection approach has demonstrated that mammalian cells posses the relevant machinery to perform homologous and nonhomologous recombination. Manipulation of substrate DNA prior to transformation has shown double strand breaks to be recombinogenic though there is not a consensus as to the mechanism by which this effect operates.

Transfecting a plasmid damaged in vitro into cells and assaying for reactivation has met with limited succes in mammalian systems compared with bacterial and yeast systems. It is clear that DNA maintained exogenously is treated in a very different manner from chromosomal thus reactivation of episomal plasmids may provide only limited information as

to the capacity of a cell to deal with DNA damage to its genome. Furthermore the reactivation of some substrates after treatment with a particular agent has not been found to mirror the cellular response to that agent. Indeed the very treatment of plasmid with a damaging agent may increase its capacity to stably integrate into the host cell genome rendering the interpretation of the results of such studies more complex.

1.8.2 In vitro systems

A number of groups have used extracts from mammalian cells (Darby and Blattner 1984, Kucherlapati et al. 1985, Mortelmans et al. 1976), mammalian nuclei (Lopez and Coppey 1987, 1989, Holmes et al. 1990, Wierbauer and Jiricny 1989,1990, Rauth et al. 1986, North et al. 1990, Sibghat-Ullah 1989), Drosophila nuclei (Holmes et al. 1990) Xenopus egg extracts (Thode et al. 1990, Pfeiffer and Vielmetter 1988) extracts from yeast (Symington et al. 1983), Tetrahymena (Robinson et al. 1989) or purified proteins (Evans and Linn 1984, West 1990) to study various mechanisms of DNA joining, recombination or repair.

Soluble cell free systems were developed capable of mediating transcription of purified genes from class I, II, or III promoters. These have been improved upon by separating cytoplasmic or nuclear protein fractions thus obtaining an enrichment for proteins from each compartment (Dignam et al. 1983). Stillman and Gluzman (1985) showed that nuclear (& to a lesser extent cytoplasmic) extracts of human cells could catalyse SV40 DNA replication and supercoiling. Reaction products were analysed by incorporation of radioactive nucleotides followed by TCA preipitation and CsCl equilibration gradient centrifugation of samples labelled with BUdR and [^{32}P]dAMP to asess the extent of DNA synthesis or gel electrophoresis to look at supercoiling.

Recombination reactions have been characterised *in vitro* in two sorts of system (Kucherlapati and Moore 1988). In one type appropriately designed substrates are incubated with cell free extracts and the reaction products used to transform recombination deficient (RecA-) bacteria (Symington et al.1984; Kucherlapati et al. 1984,1985; Rauth et al. 1986; Darby and Blattner 1984; Lopez and Coppey 1987). The bacteria are thus a tool for isolation of recombination end products. Knowing the nature of the substrate and of the end products

deduction of the processes invoved in generation of recombinant molecules is possible. The second approach involves using cell free extracts or fractions of such extracts to catalyse the formation of intermediates detected by specific assays. The availiability of appropriate substrates and assays to detect specific intermediates should enable the purification of proteins or complexes capable of catalysing a particular reaction. These approaches are obviously complementary in that the eventual aim is to use purified proteins to reconstitute a system capable of performing all the necessary reactions in the correct sequence to transform substrate into recombination products analogous to those formed in vivo.

The existence of an *in vitro* assay for an activity allows the monitoring of the purification of that activity from crude extracts. Thus an electrophoretic assay and the use of electron microscopy for detection and analysis of recombination intermediates was used by Hsieh et al.(1986) to partially purify a recombinase by fractionation of crude extract from human B lymphoblasts and by Eisen and Camerini-Otero (1988) to isolate a similar activity from nuclear extracts of Drosophila melanogaster embryos.

Similarly an activity catalysing deletion-ligation reactions (which appear similar if not identical to a set of developmentally controlled reactions thought to be involved in differentiation *in vivo*) was identified in and partially fractionated from cell free extracts of Tetrahymena by Robinson et al. (1989).

Pfieffer and Vielmetter (1988) and Thode et al. (1990) studied DNA ligation reactions using plasmids cut with restriction endonucleases to produce sets of linear molecules with defined termini. These substrates could then be used to ask very specific questions as to the nature of ligation and circularisation activities present in extracts. Substrates were incubated with extracts from Xenopus and reaction products isolated, cloned and the region of joining sequenced. Circle formation was also visualised by gel electrophoresis followed by Southern blotting. In this manner a novel type of ligation reaction was identified and the existence of a previously undescribed activity holding broken ends of DNA together prior to their ligation postulated to explain it. Attempts can now be made to purify and characterise this alignment protein.

A number of groups have used *in vitro* systems to look at excision repair. Evans and Linn (1984) studied the capacity of a previously defined (Mosbaugh and Linn, 1983) enzyme system capable of repairing AP sites and pyrimidine dimers to repair UV treated DNA packaged as SV40 minichromosomes. They could compare the efficiency of each constituent step and of the reaction as a whole to that of repair of naked DNA. It was found that the overall proficiency of repair in chromatin was reduced and that only a subset of damage was repaired. These authors are currently using this system to investigate the nature of the activities producing this differential effect.

Another application of cell free systems is in the study of repair deficient mutants. If a defect in an extract from a mutant can be identified in such an assay, complementation using wild type extract, purified proteins or fractions of wild type extract provides a means of identifying a biochemical basis for a phenotypic effect. If the genetic localisation of the mutation is known then the assignment of an activity to that gene may be made possible by this kind of approach.

Mortelmans et al.(1976) showed crude cell extracts prepared by sonication from wild type and from cells of XP complementation groups A, C and D to be able to excise thymine dimers from purified UV irradiated *E.coli* DNA, but that XP group A extracts could not excise dimers from DNA of UV irradiated cells that still had associated chromatin proteins present. This result suggests a defect in XP group A in the handling of damage in DNA packaged as chromatin but not in the repair of UV damage in DNA per se. This type of result was confirmed by Kano and Fujiwara (1983) who also found that similarly prepared cell free extracts from XP-C and XP-G cells were deficient in excision of pyrimidine dimers from native but not partially deproteinised chromatin.

Using whole cell extracts Hansson and Wood (1989) and Sibghat-Ullah et al.(1989) have looked at excision repair *in vitro* using an assay for repair synthesis of DNA measuring the incorporation of radioactive nucleotides into a DNA substrate damaged *in vitro*. Results show that extracts from cells in XP group A are deficient in excision repair of a substrate damaged by UV, psoralen and cis- or trans-platin.

These are in contrast to the results described above (Mortelmans et al. 1976, Kano and Fujiwara 1983) where no deficiency in repair of uncomplexed DNA is found in extracts from XP cell lines. Results using permeabilised cell systems to study UV induced incision and repair synthesis (Ciarrochi and Linn 1978, Dresler and Lieberman 1983, Kaufman and Briley, 1987) and also the partial purification of repair activities by microinjection into XP cells of wild type extract or fractions from such an extract (Yamaizumi et al. 1986,) lend support to the defect in at least XP-A being in the type of activity measured by the repair synthesis assay. This discrepancy could be explained by the different methods used to prepare cell extacts. Another explanation could be that the assays used measure different activities, both involving the protein(s) of the XP gene(s).

In 1988 Wood et al. showed, using the repair synthesis assay that extracts from XP lines of complementation groups A, B, C, D, H and V, all fail to act on circular DNA containing thymine dimers or psoralen adducts but are proficient in repair synthesis of UV irradiated DNA containing incisions generated by *M.luteus* pyrimidine dimer DNA glycosylase. This suggests that the sole defect lies in the incision step of excision repair. Mixing of extracts of group A and C leads to reconstitution to wild type of DNA excision repair activity showing that these lines are deficient in different activities (although they are both involved in incision). In 1990 Hansson et al. demonstrated that the defects in extracts of XP A, C, D and G could be complemented in terms of the repair synthesis assay by the addition of the *E. coli* Uvr ABC incision complex. These studies confirm the nature of the defect in these lines as being one of incision and thus imply (all XP groups tested can complement the excision repair defect of the others) that this step in the excision repair of bulky adducts is complex requiring many activities.

Wiebauer and Jiricny (1989) studied mismatch repair in vitro by placing a single G.T mismatch in a 90-mer synthetic substrate rendering it resistant to SalI restriction enzyme cleavage. This was visualised as the disappearance of a fast migrating band on denaturing polyacrylamide gel electrophoresis. After incubation with HeLa nuclear extract in the prescence of ATP Mg^{2+} and the four dNTPs a proportion of isolated product became susceptible to SalI digestion indicating repair of the mismatch. The same authors then used

this test in combination with biochemical assays for and antisera against previously identified enzymes to begin to characterise the mismatch repair pathway biochemically and break it down into discrete stages (Wiebauer and Jiricny 1990). Holmes et al. (1990) incubated circular heteroduplex substrates containing a single mismatch which inactivated two restriction sites, with HeLa or Drosophila nuclear extracts. Depending on which strand was corrected the restoration of one or the other restriction site implies mismatch repair. These types of assay will lead to the isolation of factors involved in mismatch recognition, activities determining which strand to repair and those affecting the type of mismatch preferentially repaired is being attempted.

1.8.3 Advantages of an in vitro approach.

It is clear from the foregoing discussion that much information has been gained by the analysis of the reactions occuring after introduction into a cell of defined substrates. There are a number of pitfalls in such a system however especially when it is desired to make comparisons between cell lines. The processes of introducing substrate (mainly by transfection) into cells may not result in all cell lines in comparable proportions of cells in a population transfected, or in equivalent DNA uptake by those cells. After purification of substrate subsequent to incubation it may also be hard to decide how great a part general cellular metabolic processes (as opposed to the reaction it is desired to study) have played in giving rise to the observed outcome. In the analysis of repair of a damaged substrate complications can arise if the damage itself affects the uptake or integration of DNA. A number of groups have adopted an approach studying the reactions of substrate molecules with cell free extracts. Such approaches have the advantage over transfection systems of being free of the complications arising from interactions of cellular metabolism with the process under investigation. Cell free systems also provide a means for purification of proteins involved in specific reactions either by assaying protein fractions for a defined activity in a model system (Eisen and Camerini-Otero 1988) or by restoration of the relevant activity (Wood et al. 1988, Hansson et al 1990). Inhibitors of enzymes postulated to be involved in a specific process can be added to extracts and the effect on the assay in question asessed

(Holmes et al. 1990). Similarly the effect of adding to the assay antibodies against a defined protein may be investigated, if inhibition of activity results that protein is assumed to play a part in the process in question (Wiebauer and Jiricny 1990). Further the cofactor, metal ion, salt, pH and nucleotide triphosphate requirements as well as time and temperature optima of the process can be elucidated by varying assay conditions and noting the effect on the efficiency of the reaction.

1.9 ANALYSIS OF REPAIR OF IONISING RADIATION INDUCED DNA DAMAGE

1.9.1 The link between radiosensitivity and DSB misrepair.

Work on the nature of the lesions induced in DNA by ionising radiation has implicated the DSB as a major lesion responsible for the cytotoxic effects of this type of radiation. A number of reports (eg Radford 1986; McMillan et al. 1990) suggest a correlation between repair and induction of DSB and radiosensitivity of tumour cell lines with radiosensitive cell lines suffering more DSB damage. In yeast there is also evidence that radiosensitive mutants are defective in processes involving DSB rejoin (Frankenberg et al. 1984). The work of Bryant (reviewed 1988) in mammalian cell lines using restriction endonucleases to study relationships between DNA DSB and some of the end points associated with the cytological effects of ionising radiation further supports the correlation between deficiency in DSB repair and sensitivity to ionising radiations. Six X-ray sensitive CHO mutants showing no deviation from the normal in terms of repair of SSB showed a decrease in the ability to rejoin ionising radiation induced DSB as measured by neutral elution techniques (Kemp et al. 1984). These authors point out the resemblance in this respect between these mutants and the *rad52* mutant yeast strains which also show a defect in DSB rejoining (Resnick and Martin 1976).

There are, in contrast to these findings reports of a number of cell lines showing a sensitivity to ionising radiation but no defect in DSB repair. Mutants of yeast (Kroga and Schroeder 1987), a number of radiation sensitive hamster cells for example the *irs* mutants (Jones et al. 1987) and V-C4, V-E5 and V-G8 (Zdzienicka et al. 1989) and cell lines derived from individuals with the cancer prone syndrome Ataxia Telangiectasia (AT) show no abnormality in DSB induction or repair in neutral elution studies (Jones et al. 1989; Thacker 1990).

1.9.2 Radiosensitive cell lines with no defect in DSB repair in neutral elution assays.

1.9.2a Ataxia Telangiectasia.

Ataxia Telangiectasia is an autosomal recessive defect the clinical features of which include cerebellar ataxia and occular telangiectasia (Miller 1982). Patients also show immunological abnormalities (Waldmann 1982), neurological defects (Sedgwick et al. 1982) and cancer susceptibility (Harnden and Bridges 1982; Spector et al. 1982). Patients show a heightened sensitivity to radiotherapy and ionising radiations induce a higher than normal number of chromosomal aberrations in leucocyte cultures from AT patients. This clinical radiosensitivity is also manifest at the cellular level (Taylor et al. 1975). Cell lines derived from AT fibroblasts exhibit cytogenetic abnormalities and high levels of chromosomal instability (Taylor 1982).

The radiosensitivity of AT cells has most commonly been interpreted as a defect in DNA repair. Clonogenic survival experiments with normal and AT fibroblasts using various post irradiation culture conditions, dose rate and quality of radiation suggest AT lines to be deficient in the repair of radiation induced damage (Cox 1982). AT cells have also been found to be hypermutable by ionising radiation (Cole pers. comm.) and show evidence of altered repair of ionising radiation induced potential lethal damage (Arlett and Priestley 1984). AT cells show a failiure to inhibit DNA synthesis after irradiation (Houldsworth and Lavin 1980; Painter and Young 1980). Post irradiation recovery defects in DNA synthesis inhibition are also found in other radiosensitive cell lines for example the *xrs* lines (Thacker and Stretch 1985) derived from CHO hamster cells. Notably this defect is also seen in the RAD 52 mutant of *S.cerevisiae*. This strain is recombination deficient and defective in the repair of DNA DSB (Rao et al.1980). The *xrs* strains too show defective DSB repair (Kemp et al. 1984) as measured by neutral filter elution. Thus a correlation is implied for these mutants between cellular recovery from radiation induced damage and repair of DSB.

In neither AT lines nor the V79 derived mutants of Zdzienicka et al. (1989) and one of the *irs* mutants (Jones et al. 1987) mentioned above has it been possible to demonstrate a defect in the repair of DSB by the standard techniques (Thacker 1990; Jones et al.1989).

Other evidence however, is consistent with a strand break processing defect in these cell lines. AT cell lines show cross sensitivities to agents other than radiation causing DNA strand breaks eg mitomycin C and bleomycin (McKinnon 1987; Jones et al.1987; Thacker 1990). The enhanced frequency of chromosomal damage induced in AT cells as compared to normal by ionising radiation or the anti tumour drugs mentioned above has been suggested to correlate with a greater number of DSB remaining unrepaired in AT as opposed to normal cell lines. The levels of increase over normal in the DSB remaining would probably be undetectable by standard biochemical methods (Thacker 1990).

1.9.2b The *irs* mutants of V79.

The *irs* series of radiosensitive mutants were derived by Jones et al.(1987) to facilitate the study of the cellular responses to ionising radiation and the pathways involved in repair of the damage. The V79 line was chosen as a parent line for derivation of such a set of mutants as it contains different characteristic chromosome alterations to the CHO lines used in many other studies. Further the V79 line would be expected to have monosomies of different genes from those present in CHO cells so the screening of this line should yield different mutants from those already isolated and characterised. V79-4 Chinese hamster cells were mutagenised by treatment with the powerful point mutagen ethylnitrosourea (ENU). Populations of mutagenised cells were screened for radiation sensitivity by isolating single colonies, splitting each colony between two replica 96 well plates. One plate was then irradiated. Irradiated and unirradiated minicultures were subsequently compared for the amount of growth that had occurred and also for the presence of dead and mitotic cells. Putative X-ray sensitive clones were retested under a variety of conditions to provide an accurate description of their radiosensitivities.

In this fashion three clearly and one slightly radiosensitive mutant sublines were isolated at a frequency in agreement with that obtained for mutation induction in EMS mutagenised CHO cells (much greater than that expected for detection of recessive mutation in disomic genes). A high reversion frequency is reported for the *xrs* mutants following treatment with 5-azacytidine. This implies that some DNA repair loci in hamster cells may be

functionally monosomic due to DNA methylation. The *irs* mutants were not found to be readily revertable by 5-azacytidine and did not show a high rate of spontaneous reversion. *irs-1*, *-2*, and *-3* were found to be recessive to wild type and also to complement each other and the xrs mutations (Jones et al. 1988) thus suggesting the existance of at least six complementation groups for ionising radiation sensitivity in Chinese hamster cells.

Table 1.3 is taken from Jones et al.(1987). It details the radiation sensitivities of the irs mutants as compared to the parental V79 cells and also shows the cross sensitivities of these cell lines to a range of cytotoxic drugs.

Table 1.3

D_{37} values of V79 and *irs* mutants for different genotoxic agents

Cell line	Cloning effic.	X-rays (Gy)	UV (J/m2)	EMS (mM)	MMC (x106M)
V79	1.00	4.20	8.84	35.3	1.81
irs-1	0.25-0.45	1.34	3.09	3.7	0.03
irs-2	0.75-1.00	1.41	7.40	12.8	1.37
irs-3	0.10-0.20	2.06	7.24	14.1	0.25

All data from Jones et al. (1987).

Table 1.3 shows the X-ray sensitivities of the mutants to vary in sensitivity in the order *irs-1*; *irs-2*; *irs-3* with *irs-1* the most sensitive. Survival curve data presented by Jones et

al.(1987) show both *irs-3* and *irs-2* to exhibit, like V79 a curvilinear dose response curve with that of *irs-2* being substantially shifted towards greater radiosensitivity than that of *irs-3* and V79. In contrast the dose response of *irs-1* is linear over the range tested.

This linear response of *irs-1* as opposed to a curvilinear of *irs-2*, *irs-3* and V79 is also seen upon treatment with UV, MMC and EMS to which this mutant is noticably more sensitive than either wild type or the *irs-2* and *-3* lines. *irs-2* shows a small but reproducible sensitivity as compared to wild type in respect to UV, EMS and MMC. *irs-3* is more markedly sensitive to MMC and slightly less so to EMS than *irs-2*. The UV sensitivities of *irs-2* and *-3* are similar.

There was no evidence for instability of the mutant phenotype during drug sensitivity testing and specific tests for reversion both spontaneous and 5-azacytidine induced revealed no detectable reversion.

The *irs* mutants are distinct from other previously described radiosensitive mutants in terms of cross sensitivities and show radiosensitivies at the less severe end of the spectrum exhibited by other rodent mutants. In comparison with human radiation sensitive mutations (AT showing ionising radiation sensitivity but being relatively UV resistant and XP showing UV sensitivity but not usually an increase in sensitivity to ionising radiation) *irs-1* is classified by its broad range of sensitivities as being comparable to some Cockaynes syndrome lines while *irs-2* and *-3* appear more AT like.

Jones et al. (1988) used cell fusion analyis to show the *irs* mutants to complement the radiosensitivity of each other and the previously isolated Xray sensitive mutants *xrs-1*, EM7 and XR-1. The *irs* phenotypes are recessive to wild type in cell fusion analysis. They suggest that there must thus be at least 6 complementation groups for ionising radiation sensitivity in Chinese Hamster cells.

In extension of these studies Jones et al. 1990 showed that in hybrids formed between *irs-1* and human lymphocytes the mitomycin C sensitivity of *irs-1* was corrected. These cell hybrids also showed concomittant correction of γ ray, UV and EMS sensitivities. The implication of this is that the *irs-1* phenotype is due to a single gene mutaion. These authors also showed the mitomycin C sensitivities of four CHO mutants to complement that of *irs-1*. Using alkaline and neutral elution techniques to measure the production and repair of

single and double strand breaks respectively *irs-1* and *irs-2* showed kinetics indistinguishable from those of V79. Looking at the rate of DNA synthesis following irradiation *irs-1* showed a dose dependent inhibition similar to that seen in V79 while *irs-2* exhibited DNA synthesis significantly resistant to irradiation.

In terms of the failiure to detect abnormalities in the induction or repair of DSB or SSB in reponse to γ irradiation by the techniques of neutral and alkaline elution both *irs-1* and *-2* are similar to AT cell lines and distinct from other radiation sensitive Chinese hamster mutants for example XR-1, the *xrs* series and EM9 which have a deficiency in strand break repair.

1.9.3 Application of a transfection assay for repair of DSB by radiosensitive cells.

Thacker and co workers (Thacker 1988; Thacker and Debenham 1988; Debenham et al. 1988; Cox et al. 1984; Thacker 1986; Debenham et al. 1987) used an endonuclease scission to model an ionising radiation induced DSB and developed an assay for DSB rejoin in wild type and radiosensitive cells based on DNA transfection. A plasmid carrying two selectable genes is cut with a restriction endonuclease, introducing a DSB into one of these genes. This substrate is transfected into either radiosensitive or wild type cells. Selection is imposed for the undamaged marker. The percentage of these colonies also positive for the damaged marker gives an indication of the fidelity with which the original cut was ligated. Table 1.4 summarises some of the relevant data from such experiments.

Table 1.4

Percentage of colonies selected for an undamaged marker subsequently found to be positive for a marker initially inactivated.

Cell type	Cut site		
	uncut	cut in gpt	distant site
1. HAMSTER			
V79 (wild type)	59%	11%	55%
irs-1 (sensitive)	50%	2.7%	54%
irs-2 (sensitive)	56%	10%	ND
2. HUMAN			
MRC-5 (wild type)	83%	55%	87%
AT5BI (sensitive)	74%	9%	80%

Data selected from Thacker 1988; Experiments involved the transfer into cells of plasmid pMH16 carrying the gpt and neo selectable markers. "cut in gpt" refers to the transfected plasmid having been cut with kpnI in the coding region of the gpt gene, figures in this column represent the percentage of colonies selected first for the neo marker and then for expression of the gpt gene. "distant site" refers to an endonuclease induced DSB having been introduced at a site distant from the coding regions of both the neo and gpt genes. Mean values are given with errors where shown representing one standard deviation from the mean. Numbers in parenthesis are numbers of independent experiments.

Table 1.4 shows that the wild type V79 cells and the two radiosensitive *irs* lines tested are equally efficient in the uptake of uncut plasmid DNA. An endonuclease induced DSB in a site distant from either of the two selectable markers gives rise to an equivalent percentage of

colonies positive for both markers in all the hamster lines. This implies an equivalent capacity for rejoin of a DSB but does not require conservation of information at the break point. The data for rejoin of a DSB introduced into the gpt gene does give an indication of rejoin fidelity as presumably information must be intact at the break point for effective gene expression. One of the radiosensitive mutants *irs-2* is seen to rejoin an endonuclease induced DSB with a fidelity equivalent to that of the wild type V79 cell line while *irs-1* cells exhibit a five fold drop in the fidelity of such repair.

In comparison with the data from similar experiments transfecting plasmid into human cell lines it is clear that the human lines both radiosensitive and wild type show less nonspecific damage of exogenous DNA as indicated by the greater number of colonies positive for both markers carried on uncut plasmid. This is also seen in the case where the transfecting plasmid contains a DSB at a site distant from both selectable markers. As compared to wild type (MRC-5) the radiosensitive mutant line derived from an AT patient AT5BI shows a drop in rejoin fidelity of the same order of magnitude as that shown by *irs-1* in comparison with V79.

By this assay then *irs-1* cells show an AT like phenotype. *irs-1* and AT also share other characteristics for example elevated γ ray induced chromosomal aberrations and possibly chromosomal instability (Jones et al. 1988). In other respects *irs-1* differs from AT significantly. *irs-1*. unlike AT lines is somewhat UV sensitive and extremely so to mitomycin C. Another respect in which *irs-1* does not resemble all AT lines so far tested is in its radiosensitive DNA replication. Thus *irs-1* and wild type but not AT lines show post irradiation inhibition of DNA synthesis.

Jones et al. consider it likely given the broad range of agents to which *irs-1* is sensitive that the defect causing this phenotype is not simply one of DSB repair but involving a gene product important in the repair of various lesions.

irs-2 shares the property of radioresistant DNA synthesis with AT cells and like AT lines *irs-2* cells are highly sensitive to ionising radiations but not to UV. However *irs-2* does not show the chromosomal instability reported for AT and by the assay described above differs in being capable of repairing DSB with fidelity.

1.9.4 Summary and conclusions.

In a number of cases the radiosensitivity of a cell line can be correlated with a defect in the handling of DSB as measured by conventional techniques of neutral elution. This correlation is not absolute however in that a number of cell lines for example AT lines and the *irs* mutants of V79 show no abnormality in DSB repair by this kind of assay.

The transfection system of Thacker and co workers described above extends the correlation of radiosensitivity with misrepair of DSB in that an AT line and one of the *irs* mutants (*irs-1*) are found to be deficient as compared to wild type in the fidelity of repair of an endonuclease induced DSB. The assay of Thacker however reveals no DSB repair defect in the highly radiation sensitive mutant *irs-2*.

The nature of the processes involved in the DSB repair event assayed by this system cannot be further dissected using this sort of assay. It is impossible in this system to differentiate between repair by simple ligation (postulated to require few activities) or by a more complex recombination mechanism. The evidence quoted above from studies on mechanisms of recombination using a DNA transfection based approach would certainly imply that such events would occur with a high frequency under the conditions of the assay just described.

1.10 AIMS OF PRESENT STUDY

The reactions involved in the repair of lesions induced in DNA by ionising radiation are less clearly understood than reactions in the excision repair pathway. In the case of excision repair the availiability of a variety of bacterial mutants deficient in repair of UV light induced DNA lesions provided the basis for models of repair pathways. Using a variety of approaches reactions making up the pathway of nucleotide excision repair were gradually elucidated and there is now a fairly thorough understanding of the prokaryotic modes of excision repair. Many of the steps in the recognition, incision, removal and resynthesis stages of excision repair can be performed in vitro with purified bacterial proteins.

A large degree of functional homology between many repair activities has meant that conclusions drawn from the analysis of bacterial repair systems can be extrapolated to

eucaryotic pathways of excision repair. The yeast *S.cerevisiae* has proven a highly useful system for the study of DNA repair mechanisms combining the advantages of a wide variety of repair mutants with eucaryotic DNA structural organisation. Increasing attention is being turned on the derivation of mammalian mutants in DNA repair processes and the subsequent elucidation of normal pathways of repair by the analysis of these mutants. A number of mammalian excision repair genes have now been cloned and the bases for defects in some of the complementation groups of the human excision repair deficient syndrome xeroderma pigmentosum established.

The same sorts of approach are beginning to yield results with respect to repair of ionising radiation induced damage. A number of bacterial, yeast and mammalian mutants hypersensitive to the effects of ionising radiation have been identified and their phenotypic and biochemical characterisation undertaken. Many of these mutant lines were found to have a defect in the processing of the DNA DSB which has come to be regarded as the major lesion responsible for the cytotoxic effects of ionising radiation. Neutral elution assays have been used to reveal defects in the removal of DSB induced by treating cells with ionising radiations. Some ionising radiation sensitive mutants were however found not to have such a DSB repair defect detectable by such assays. These include cell lines from the human ionising radiation sensitive, cancer prone syndrome Ataxia Telangiectasia and the *irs* mutants of V79 hamster cells. A transfection assay described by Thacker's group revealed a defect in fidelity of repair of an endonuclease induced DSB in AT cells and the *irs-1* radiosensitive mutant of V79 as compared to wild type cells.

In the case of the analysis of excision repair the setting up of assays for the in vitro repair of damage to defined DNA substrates has been invaluable in allowing characterisation of the reactions involved.

A main aim of the work described in the following chapters was to analyse the repair of ionising radiation induced damage in hypersensitive cells showing no DSB repair abnormalities by neutral elution, in particular AT and *irs* lines. In order to elucidate the biochemical defect of the AT and *irs* cell lines it was hoped to establish a cell free system to assess the capacity of extracts from ionising radiation hypersensitive cell lines to repair a

DSB introduced into a defined substrate. The work of Bryant (reviewed 1988) implies that in many ways an endonuclease generated DSB can be considered a reasonable representation of an ionising radiation induced cytotoxic lesion. This type of DSB was considered an appropriate substrate for use in such an assay.

The establishment of an assay to measure both the efficiency and fidelity of rejoining in cell free extracts of mammalian cells was a prime objective. We hoped to use such an assay to dissect the reactions involved in the repair of ionising radiation induced lesions and determine the biochemical nature of this type of repair. We then hoped to be able to define the biochemical alterations responsible for the radiosensitivities of the *irs* and AT mutant cell lines.

Specific objectives were as follows;

* To check that the phenotypes of the cells under study have been maintained regarding cellular sensitivities and to extend this data to include previously undescribed cytotoxic agent dose response curves.

* To establish a cell free assay to analyse the capacity of extracts of mammalian cells to repair an endonuclease induced DSB.

* To use such an assay to analyse extracts of radiosensitive mutant cell lines in particular the *irs-1*, *-2* and *-3* mutants derived from V79.

* To characterise in detail any defect so observed.

* To analyse previously identified enzyme activities that could be involved in DSB repair in the *irs* mutants, for example ligase activities.

CHAPTER TWO: MATERIALS & METHODS

Materials and methods

2.1 MATERIALS

Chemicals: BDH; Sigma; Biorad; Aldrich; BRL; Koch-light; BS & S;

Enzymes & Buffers: Boehringer Mannheim; BRL; (One-Phor-All Plus from Pharmacia)

Cytotoxic drugs: Sigma;

Antibiotics: Sigma; Vestric (Hospital dept.);

Nucleotides & Oligonucleotides: Pharmacia (except $\alpha P_3 A$ synthesised in house)

Radiochemicals: Amersham International;

Tissue culture: Media & supplements from Gibco except RPMI from NBL; Flasks & Culture bottles from Becton Dickinson;

Bacterial culture: Luria Broth base from Gibco; Bacto-Agar from Difco; Petri dishes from Becton Dickinson;

Transfer membranes: Du Pont;

Nick Columns: Pharmacia;

Spinex Tubes: Costar;

Filters: Nalgene; Anderman & Co.;

Biogel A1.5 fine: Biorad;

Dialysis tubing & Colloidon bags: Sartorius;

Lambda HindIII DNA: BRL;

Film: Kodak; Polaroid U.K.;

Bacterial strains: *E.coli* JM83 (Yanisch-Perron et al.1985); K12 derived, thi, SupE, D(lac-proAB), [F-, traD36, proAB, lacIqZDM15].

2.2 BACTERIOLOGICAL METHODS

2.2.1 Bacterial culture

Bacteria were maintained on Luria agar plates at 4°C & stored for long periods in L-broth / 50% v/v glycerol at -20°C.

Media:

L-broth:

1.0% w/v tryptone

0.5% w/v yeast extract

1.0% w/v sodium chloride

Ampicillin when included at 100ug/ml.

L-agar:

L-broth / 1.5% agar. Autoclaved & allowed to cool prior to pouring plates. Ampicillin at 100ug/ml & the chromogenic substrate X-gal at 250ug/ml being added after sterilisation as required.

2.2.2 Plasmids

pIC20H (Marsh et al. 1984)

2.2.3 Transformation of competent bacteria

100ul aliquots of competent bacteria were added to 10 - 50ng of transforming DNA & left on ice for 30 min. After 5 min. heat shock at 37°C 1ml L-broth was added & the samples vortexed before incubation for 1 to 1.5 hr at 37°C. Appropriate dilutions were plated on selection medium using glass beads to distribute transformed bacteria. Plates were incubated at 37°C.

2.2.4 Preparation of competent bacteria

Bacteria were streaked onto L-agar & incubated o/n at 37°C. A single colony was innoculated into 25ml L-broth & incubated with shaking o/n at 37°C. 250µl of this culture were used to innoculate into 25 ml L-broth which was incubated at 37°C with shaking for 2-3hrs until growth was exponential as indicated by an optical density at 600nm ($O.D_{600}$) of 2.5-5 units. The culture was placed on ice for 10 min. & cells pelleted at 3000 rpm for 10 min. at 4°. Pellet was resuspended in 12.5ml ice cold 100mM CaCl2 10mM Tris. pH 7.5 & incubated at 4°C for 1 hr. Bacteria were pelleted as before & resuspended in 2.5ml CaCl2- Tris & kept o/n at 4° before using. Typically this procedure yielded bacteria with transformation efficiency of $5x10^5$ to $1x10^6$ colonies/ug uncut pIC20h.

2.2.5 Plasmid mini preps (Holmes & Quigley 1981).

STET Buffer:

8% sucrose

5% Triton X-100

50mM EDTA

50mM Tris pH 8.0

A 25ml o/n culture of bacteria carrying the desired plasmid was spun down & the pellet resuspended in 700µl STET. 60µl 10mg/ml lysozyme was added the samples vortexed & then boiled for 1-2min. Debris was pelleted & removed & an equal volume of isopropanol added to the supernatant. After 10 min at room temp. DNA was spun down in a microcentrifuge, the pellet washed with 70% & then 100% ethanol, air dried & then resuspended in 20-50ul TE. Plasmid preparations were checked by restriction endonuclease digestion & agarose gel electrophoresis.

2.2.6 Rapid large scale plasmid preparation (Clewell & Helsinki, 1970)

5ml of a 10ml o/n culture was used to innoculate 500ml of L-broth containing appropriate antibiotics & this culture grown to stationary phase o/n. Bacteria were pelleted & resuspended in

10ml 50mM Tris pH 8.0 25% sucrose. 5ml of 10mg/ml lysosyme were added & samples incubated on ice for 3 min. 5ml 0.2M EDTA was added, samples mixed & incubated on ice for 20-25 min. 600ul 10% NP-40 in Tris-sucrose was added & the tubes inverted until the sample became very viscous indicating complete lysis of bacteria. The lysate was cleared by spinning at 15K for 30 min. The supernate was first extracted with an equal volume of phenol (mixed for 10 min & spun 3K 20 min.) and then with an equal volume chloroform (24:1 chloroform:isoamyl alcohol). RNA was digested with Ribonuclease A at 20ug/ml for 30 min at 37^{o}C, the RNase inactivated & removed by phenol & chloroform extraction. DNA was precipitated using 1/2 volume 7.5M sodium acetate & 1 volume isopropanol at room temp. for 30 min to 1 hr & pelleted by spinning at 3K for 15-20 min. The pellet was washed in 100% & then 70% ethanol, dried & resuspended in 1ml TE.

2.2.7 DNA preparation for plasmid sequencing

Bacteria harbouring the plasmid of interest were pelleted from a 15ml o/n culture by spinning at 10K for 15 min., resuspended in 1ml 0.4% lysosyme, 50mM glucose, 25mM Tris pH8.0, 10mM EDTA pH8.0 & incubated at room temp. for 5 min. 1.5ml of a 6M potassium acetate were added, the sample mixed gently & incubated on ice for 5 min. before spinning 10K for 15 min at 4^{o}C. The supernatant was extracted with one volume phenol & one volume chloroform:isoamyl alcohol (24:1) and nucleic acid precipitated by the addition of 2 volumes 100% ethanol, incubation at room temperature for 5-30 min followed by centrifugation at 10K for 15min at 4^{o}C. The pellet was washed in 70% ethanol & freeze dried before resuspension in 100ul of10mM Tris pH 8.0, 1mM EDTA. RNase A was added to a final concentration of 10ug/ml & incubated at 37^{o}C for 30 min. 60ul of 20% PEG, 2.5M sodium chloride was added & the sample incubated on ice for 1hr before being spun for 5 min in microfuge. The pellet was washed in 70% ethanol, dried & resuspended in 20ul TE. The preparation was checked by agarose gel electrophoresis & stored at -20^{o}C.

2.3 DNA MANIPULATION

2.3.1 General techniques

Disposable gloves were worn. All buffers & solutions, glass & plasticware sterilised by autoclaving or filtration as appropriate. Most techniques from Maniatis et al 1982 (first edition) or Sambrook et al 1989 (second edition) "Molecular Cloning a Laboratory Manual". All chloroform was equilibrated 24 volumes chloroform to one of isoamyl alcohol.

2.3.2 General Buffers

TE:

10mM Tris-HCl pH7.5

1mM EDTA

TEN:

40mM Tris-HCl pH7.5

1mM EDTA

150mM NaCl

2.3.3 Phenol extraction

DNA in solution was mixed with an equal volume phenol (AR grade, equilibrated with TE) the organic & aqueous phases separated by centrifugation & the aqueous phase reextracted with an equal volume chloroform:isoamyl alcohol (24:1).

2.3.4 Ether extraction

An equal volume of ether was added to DNA in solution, mixed well & the phases separated by centrifugation. The top organic phase was discarded & residual ether allowed to

evaporate from the lower aqueous phase. Ether extractions were performed to remove traces of solvents remaining after previous extractions which might inhibit subsequent reactions.

2.3.5 Precipitation

2.3.5.1 Ethanol precipitation

DNA was precipitated from solution by the addition of appropriate amounts of a solution of monovalent cations to give a final concentration of 2.0M-2.5M ammonium, 0.8M lithium, 0.2M or 0.3M sodium. This depended on wether the anion was chloride or acetate. 1/10 volume 3M sodium acetate pH 5.2 (pH adjusted with acetic acid) being the most usual. 2.5 volumes 100% ethanol were added followed by incubation at -20^{o}C or -70^{o}C for at least 30 min. before pelleting DNA by microcentrifugation at 13K for at least 15 min. at room temperature or in the cold room. The pellet was washed twice with 70% ethanol & dried under vacuum. DNA was resuspended in either TE or sterile distilled water & stored at -20^{o}C.

2.3.5.2 Isopropanol precipitation

Having adjusted the cation concentration of the DNA solution as described above, one volume of isopropanol was added prior to incubation at -20^{o} followed by centrifugation & 70% ethanol washes as above. Use of isopropanol reduces sample volume but the solvent is less volatile than ethanol so harder to remove & solutes such as sucrose or sodium chloride are more likely to coprecipitate with DNA. Thus ethanol precipitation was used unless it was important to keep sample volume to a minimum. Isopropanol precipitation (1/10 vol. 3M sodium acetate, 0.6 vol isopropanol) is also the method of choice when it is desired to selectively precipitate longer DNA molecules from short oligonucleotides.

2.3.6 Restriction endonuclease digestion of DNA

Digests carried out (in conditions specified by manufacturers) using buffers provided, or for double digests using enzymes with different optimal salt conditions in "one phor all plus" from pharmacia. Inactivation of enzymes was performed as suggested in Maniatis et al.(1982) using EDTA, heat, phenol extraction or a combination of methods as appropriate.

2.3.7 Exonuclease Treatment

Exonuclease III Buffer:

50mM Tris-HCl pH8.0

5mM MgCl2

10mM BME

λ-Exonuclease Buffer:

67mM glycine-KOH

2.5mM $MgCl_2$

50ug/ml BSA

Bal 31 buffer as supplied with enzyme.

Exonuclease treatment was performed under conditions of time & temperature as determined for individual reactions or as specified in manufacturers instructions.

2.3.8 Phosphatase treatment

10X Low Salt Buffer:

60mM Tris-HCL pH7.4

60mM NaCl

60mM$MgCl_2$

60mM BME

Conditions of time & enzyme concentration for phosphatasing were determined for specific reactions. In all cases incubation was at 37^{o}C & in Low Salt Buffer. Calf intestinal phophatase was inactivated by heating to 80^{o}C for 10 min, on the addition of TCA (for ligase

assays; Chapter 4) or by the addition of proteinase K. Bacterial alkaline phosphatase is more active than the calf intestinal enzyme but far more resistant to inactivation by heat or detergent so was used only when total inactivation was not essential (establishing conditions for ligase assays; Chapter 4)

2.3.9 Ligation of DNA

T_4 DNA ligase was used according to manufacturers instructions, reactions being performed in the buffer supplied & carried out under conditions suggested for a particular reaction by Maniatis et al (1982).

When used as control in ligation assays or ligase assay reactions (Chapter 4) conditions were as required by the particular experiment.

2.3.10 Kinase labelling of DNA

10X Low Salt Buffer

60mM Tris

60mM NaCl

60mM $MgCl_2$

6mM BME

1-50pmoles of 5' termini were incubated in 1XLSB with 50pmoles [γ-32P]ATP (sp. act. 3000Ci/mmole; 10uCi/ul), 10-20 units of T_4 polynucleotide kinase the reaction being made up to 50ul with sterile distilled water. Labelling was carried out at 37^{o}C for 30-45min. The enzyme was inactivated by the addition of 1ul 0.5M EDTA & heating to 70o for 10 min. DNA was ethanol precipitated using glycogen as acarrier & the pellet washed twice with 70% ethanol prior to resuspension in TE or sterile water.

Purification of labelled DNA from unincorporated ^{32}P-ATP was by 8% polyacrylamide gel electrophoresis & elution versus TE or using a sephadex G-50 packed nick column eluting with TEN or TEN/0.1% SDS.

2.3.11 Fill In Reactions

10X Klenow buffer:

0.5M Tris-Cl pH7.6

0.1M $MgCl_2$

10 units of Klenow enzyme were used to fill in 1-50pmol of ends in a solution containing 1X Klenow buffer, 2mM dNTP made up to a total volume of 25ul with water. Incubationwas at 22°C for 15-30 min. The reaction was heated to 70°C for 5 min. to inactivate enzyme. As the Klenow fragment works efficiently in most buffers used for enzyme reactions the buffer used in each case was determined by previous and subsequent manipulations.

2.3.12 Sequencing of plasmid DNA

(All buffers from Sequenase kit).

Double stranded template was denatured, primer added & coprecipitated with template. The pellet was resuspended in sequenase buffer, warmed to 65°C for 2 min & allowed to cool to room temperature. To each annealed mix was added DTT, labelling mix, diluted sequenase buffer & [35-S] dATP. Labelling reactions were incubated at room temp for 5 min. then added to the appropriate termination mix. Incubation was continued at 37°C for 5 min. & stopped with stop solution. Samples were denatured by heating & then loaded on a denaturing polyacrylamide gel as described below.

2.4 ELECTROPHORESIS OF DNA

2.4.1 Agarose

2.4.1.1 Buffers

10x TBE:

900mM Tris-base

889mM Boric acid

25mM EDTA

50x TAE:

2.0M Tris-base

1.0M Acetic acid

0.2M EDTA

Loading buffer:

20% Ficoll

0.25% Bromophenol blue

0.25% Xylene cyanol FF

10mM Tris-HCL pH 7.5

1.0mM EDTA

2.4.1.2 TBE gels

Horizontal slab gels containing 0.6-1.0% agarose (depending on the size of fragments to be separated) were used to check plasmid preparations, restriction digestions & to quantify amounts of DNA. The appropriate amount of agarose was disssolved in TBE using a microwave, the volume made up with distilled water & ethidium bromide added to a final concentration of

0.5ug/ml. Gels were run in 1xTBE also containing 0.5ug/ml ethidium bromide until the dye front had migrated the desired distance & then photographed under ultra violet light.

In some instances where it was important to minimise nicking of DNA (preparation of concatemers Chapter 4) TBE gels were run without ethidium in gel or buffer, individual marker samples being run with ethidium to enable location of desired fragment under UV illumination. TBE gels were electrophoresed submerged in buffer at 50 to 125V.

2.4.1.3 TAE gels

When a gel was to be used for Southern transfer agarose was dissolved in TAE without ethidium & the gel run o/n with circulation of buffer (also without ethidium).
TAE gels were run at 15 to 35V.

2.4.1.4 Molecular wieght markers

HindIII restricted lambda DNA either "cold" (TBE analytical gels), or ^{32}-P end labelled (Southerns) was run in parallel with samples to provide fragments of 23.6, 9.46, 6.72, 4.34, 2.26, 1.98 & 0.58 kb.

2.4.2 Acrylamide.

2.4.2.1 Non Denaturing

6X Gel loading buffer:

0.25% bromophenol Blue

0.25% xylene cyanol

30% glycerol in water

Non denaturing gels were used for gel retardation assays & purification of oligonucleotides. Vertical systems were run at the appropriate temperature with circulating buffer. A 29:1 acrylamide-N,N'-methylenebisacrylamide stock solution was diluted to the appropriate concentration (from 5-20% depending on the separation required) in water containing TBE to a

final concentration of 0.2-1X. The solution was then degassed using a nalgene 5u filter assembly & polymerising agents ammonium persulphate (10% stock 700ul/100ml acrylamide solution) & TEMED (35ul/100ml acrylamide solution). After pouring the gel was overlayed with isobutanol to exclude oxygen which would inhibit polymerisation. Gels were preelectrophoresed & run at 150V in TBE of the same concentration as that of the acrylamide solution.

Wells were cleaned before preelectrophoresis & again before loading samples to remove unpolymerised acrylamide. Samples were loaded in buffer described above, run to obtain the desired resolution the gel then dried down prior to autoradiography or in the case of preparative gels exposed & the purified oligonucleotide excised.

2.4.2.2 Denaturing gels

Sequencing gel-loading buffer:

98% deionized formamide

10mM EDTA pH8.0

0.025% xylene cyanol FF

0.025% bromophenol blue

(loading buffer described for non-denaturing gels was also sometimes used)

Denaturing gels were used to resolve sequencing reactions & to check preparation of oligonucleotide substrates for DNA ligase assays (Chapter 4).

Urea was added to an acrylamide:bis solution to give a final concentration of 8M, gels were 8-20% acrylamide & 1X TBE. The acrylamide solution was degassed before the addition of 700ul/100ml 10% ammmonium persulphate & 35ul/100ml TEMED. Preelectrophoresis brought the gel up to the required temperature (usually 55^{o}C), again wells were cleaned before preelectrophoresis & also before loading samples. Samples were denatured in loading buffer (described above) by heating to 95^{o}C for a few minutes then run to achieve the desired separation at 2000V. This type of vertical gel (BioRad system) was checked regularly & buffer replaced & current reduced (to prevent overheating) as necessary.

2.4.2.3 Molecular weight markers

[^{32}P γ]-ATP labelled 1kb ladder (Pharmacia).

2.4.2.4 Discontinuous SDS-Polyacrylamide gels

Tris-glycine elerctrophoresis buffer:

25mM Tris

250mM glycine (electrophoresis grade, pH 8.3)

0.1% SDS

Gel-loading buffer:

50mM Tris.Cl pH 6.8

100mM DTT

2% SDS (electrophoresis grade)

0.1% bromophenol blue

Used for the analysis of proteins in nuclear & whole cell extracts (Chapter 4). A resolving gel was first prepared, with the desired acrylamide concentration (commonly 10%) in a buffer containing Tris pH8.8 at 0.375M & 1% SDS. Polyacrylamide was added (from a 1% stock) to a final concentration of 0.1%. After the addition of ammonium persulphate & TEMED the gel was poured & overlayed with either 0.1% SDS (for gels below 8% acrylamide) or isobutanol (gels of 10% acrylamide or above). When polymerisation was complete the overlay was poured off & the gel drained. A stacking gel was then added of 5% acrylamide in a buffer of 0.25M Tris pH 6.8, 0.1% SDS, 0.1% polyacrylamide (again polymerisation agents being ammonium persulphate & TEMED). These gels were not preelectrophoresed in order not to destroy the dicontinuity of the buffer systems. Samples denatured in SDS gel-loading buffer (described above) by heating to 100^{o}C for 3 min were loaded & the gel run at 50-80V until the dye front had moved into the resolving gel & then at 100-150V until the bromophenol blue reached the bottom of the gel. The gel was then stained (in 5 gel volumes of 0.25% Coomassie brilliant blue R-250, 50% methanol,

10% acetic acid for at least 4hr at room temp with shaking), destained (in sucessive changes of 5% methanol, 7.5% acetic acid at room temp with shaking) & photographed.

2.4.2.5 Molecular wieght markers (Sigma)

Carbonic Anhydrase M.W. 29000

Ovalbumin M.W. 45000

Bovine Serum Albumin M.W. 66000

Phosphorylase b M.W. 97000

B-Galactosidase M.W. 116000

Myosin M.W. 205000

2.4.3 Recovery & purification of DNA from agarose gels

2.4.3.1 Electroelution

Used for preparation of "recombination substrates" (Chapter 4). A slice of gel containing the fragment of interest of the smallest size possible was cut out & placed in (preboiled) dialysis tubing which was then filled with either TBE or TAE depending on the type of gel & clipped (excluding air bubbles) just above the slice. The bag was placed in buffer & current (15V o/n) passed through it, the polarity of the current was then reversed for one minute & again for 30 seconds. The buffer surrounding the slice was recovered, DNA ethanol precipitated & then resuspended in TE or water.

2.4.3.2 Spinex tubes

The slice containing the fragment of interest was placed in the upper chamber of a spinex tube & microfuged for 15 to 30min. DNA thus eluted can then be precipitated & used as required.

2.4.3.3 Low melting point agarose gels

Electrophoresis having been carried out at 4oC the gel slice containing the band of interest was excised & incubated (in 5 volumes 20mM Tris.Cl pH 8.0, 1mM EDTA pH 8.0) for 5 min at 65^{o}C. The sample was cooled to room temp. & extracted once with an equal volume phenol, once with phenol: chloroform & once with chloroform & then precipitated at room temp. with 0.2 volumes 10M ammonium acetate & 2 volumes ethanol.

2.4.4 Recovery of DNA from acrylamide gels

Used in preparation of oligonucleotide probe for gel retardation assays. A slice containing the purified end labelled oligonucleotide identified by autoradiography was incubated at 37^{o}C o/n in TE & the buffer containg the eluted oligonucleotide removed.

2.4.5 Southern Blotting of agarose gels

Denaturation Buffer

1.5M NaCl

0.5M NaOH

Neutralisation Buffer

3.0M NaCl

0.5M Tris.HCl pH7.0

20X Gene Screen

0.5M Na2HPO4

0.5M NaH2PO4 pH 6.5

20X SSC

3M NaCl

0.3M Na Citrate

Prehybridisation mix:

50% formamide

6X SSC

1X Gene Screen

5X Denhardts (50X Denhardts: 5g Ficoll 5g Polyvinylpyrrolidone 5g BSA)

0.5% SDS

100 ug/ml denatured, fragmented salmon sperm DNA

After electrophoresis the gel was incubated at room temperature in two changes of denaturation buffer for 20 min. each, washed in water, incubated in two changes of neutralisation buffer for 30 min then in three changes of 1X gene screen for 20 min. Transfer was by capillary action to Gene Screen nitrocellulose using 1X gene screen buffer as the transfer buffer. Blots were baked for 2hours at 80^{o}C, wetted in gene screen/0.1% Triton & prehybridised at 42^{o}C for at least 4 hours. Nick translated probe was then added & hybridisation was at 42^{o}C O/N. Filters were washed three times at room temperature for 5 min in 2X SSC 0.1% SDS & then for 35 min to 1 hr at 65^{o}C in 0.1X SSC 0.1% SDS before being sealed in a polythene bag & exposed to film at -70^{o}C.

2.4.6 Nick Translation of DNA

Kit from Amersham:

Solution 1: 100uM each dATP, dGTP & dTTP in Tris/HCl pH7.8, MgCl2 & BME

Solution 2: Each 5ul aliquot contains 5 units

DNA polymerase 1 & 100pg DNase 1 in Tris/HCl pH7.5, MgCl2, glycerol & BSA

Solutions 1 & 2 were aliquoted & stored at -20^{o}C. All radiolabelled probes for hybridisation to Southern blots were produced by incubation for 2-3 hr at 15^{o}C of 150ng uncut plasmid with 10ul of solution 1, 100uCi [α32-P]dCTP & 5ul solution 2 made up to 50ul with water. Unincorporated nucleotides were removed by chromatography through a Sephadex G-50 packed "Nick Column". At least 5x10^{7} cpm were added to each hybridisation.

2.4.7 Gel Retardation Assays

Used to analyse functional protein content in extracts from nuclei.

Oligonucleotides kinase end labelled & purified as described above, 20-100 cps used in each binding reaction.

Binding was carried out under conditions of time, temperature & salt determined as optimal for each particular oligonucleotide used. Non specific competitors used were pUC18 & poly dI:dC. Binding reactions were stopped by the addition of loading buffer & run on non denaturing polyacrylamide TBE gels (usually 5% acrylamide, 0.2-0.5 XTBE). Gels were run such that free oligonucleotide migrated to the bottom of the gel but was not run off the end, dried & exposed at -70^{o}C.

2.5 TISSUE CULTURE

2.5.1 General Methods

All cell lines were mycoplasma free. Long term storage was in liquid nitrogen in growth medium containing 10% DMSO. Cells were maintained subconfluent in the appropriate medium at 37o in an atmosphere of 5% CO2.

2.5.2 Cells

V79 hamster fibroblasts (Chu et al. 1969); *irs* mutants derived from V79 by Jones et al. (1987). MRC-5 human fibroblasts immortalised with SV40 (Huschtascha & Holliday 1983). AT5BI fibroblasts from an Ataxia Telangiectasia patient immortalised with SV40 (Day et al. 1980). Other

Ataxia Telangiectasia cell lines were kindly provided by Dr Colin Arlett. Early passage human foetal fibroblast (HFF) cells kindly provided by Dr Ken Parkinson.

2.5.3 Media

V79 & *irs* cells grown in SLM supplemented with 2mM glutamine, 10% foetal calf serum, 20-50ug/ml penicillin. MRC5 & AT lines grown in RPMI 1640 supplemented as above. HFF cells grown in M199 supplemented as above.

2.5.4 Drug sensitivity assays

2.5.4.1 MTT

Cells were plated at 500 per well in triplicate 96 well plates (leaving the end rows blank) & incubated to allow attatchment & growth for 2-3 days. Cytotoxic drug was then added at eight concentrations (one each row of wells with a row of control wells at each end) & removed after the desired incubation time (2hrs or 24hrs). Cells were incubated in fresh medium for about one more doubling time then fed with medium containing 10mM HEPES. MTT is then added at a concentration predetermined to give maximum absorbance while not being toxic to the cells, plates wrapped in tin foil & incubated for 4 hrs. Medium is then removed & the insoluble formazan crystals produced by reduction of MTT by metabolising cells then dissolved in DMSO, 0.1M glycine, 0.1M NaCl pH10.5 is added & plates read in an ELISA plate reader at an absorbance of 570nM.

2.5.4.2 Clonogenic

Cells were plated at a density of 500/ 25cm^2 flask & allowed to settle o/n. Medium was removed & fresh medium containing drug at the desired concentration added (triplicate or more flasks were treated for each concentration). Incubation for 2 hours at 37^{o}C followed, then drug was removed, fresh medium added & incubation continued for 10-14 days after which time colonies were counted & stained.

2.5.5 Ionising radiation sensitivity curves

Cells were plated at $1X10^5$ & $2X10^2$ & incubated o/n. Cells at the lower density were irradiated (3 flasks per dose point), incubated for 12 days then stained with giemsa diluted 1 in 10 with water & colonies counted. Cells at the higher density (2 flasks per dose point), were trypsinised after irradiation & each flask replated at $2X10^2$ per dish into 3 dishes. Dishes were incubated for 12 days then stained & counted as above.

Irradiation was at a dose rate of 130.3 cgray/min using a 1MeV cobalt source for various times to give doses of 0, 100, 200, 400, 600 & 1000 rads using a field size of 32 X 32cm.

2.5.6 Preparation of Nuclear Extracts

2.5.6.1 Buffers

TMS:

0.25M sucrose

5mM MgCl2

10mM Tris pH 7.5

E50:

50mM (NH4)2SO4

20mM HEPES pH 7.9

5mM MgCl2

0.1mM EDTA

0.1% Brij 35

20% glycerol

SB:

50mM NaCl

20mM HEPES pH 7.9

5mM MgCl2

0.1mM EDTA

20% glycerol

1mM DTT

2.5.6.2 Protease inhibitors

Leupeptin 0.1mg/ml, Chymostatin 0.1mg/ml, Benzamidine 50mM, Aprotinin 0.1mg/ml, Pepstatin 0.1mg/ml, PMSF 50mM (all at stock concentrations of 100X) added to all solutions.

2.5.6.3 Method

At least $1x10^8$ cells were harvested & washed with ice cold PBS osmotically swollen with a TMS wash & lysed in TMS-025% Triton. The pellet was washed three times in TMS & DNA content estimated by taking a reading ofthe $O.D._{260}$ of a small sample taken after sonication. The pellet was resuspended in TMS to a concentration of 7.5mg/ml DNA, NaCl added to a final concentration of 0.3M & proteins extracted by incubation on ice for 10 min. Debris was removed by a 15K spin & the supernatant clarified further by a 36K ultracentrifugation step. Proteins were pelleted by incubation on ice with ammonium sulphate at 0.35g/ml followed by a 10K spin. The pellet was dissolved in 0.6ml/0.5 O.D260 units & dialysed o/n vs SB. After a clarifying 36K spin the extracts were aliquoted & stored in liquid nitrogen.

2.5.7 Cytoplasmic Extracts

Prepared by taking the supernate from the Triton lysis step & precipitating proteins with 0.35g/ml ammonium sulphate. All subsequent steps as for nuclear extract.

2.5.8 Whole Cell Extracts

Extraction Buffer:

0.1M NaCl

50mM Tris-HCL pH 7.5

10mM β-2-mecaptoethanol

1mM EDTA

(plus protease inhibitors as above)

Cells (about $5X10^7$-$1X10^8$) were harvested & washed twice with PBS. The pellet was resuspended in approx 500ul extraction buffer & homogenised with 50-100 strokes in a 1ml hand held glass homogeniser (Jencons). The homogenate was left on ice for 1 hour, debris removed by microfuging for 10 min. & nucleic acids precipitated from the supernate by the addition of 0.1 vol. 1M NaCl, 0.1 vol. Polymin-P & incubation on ice for 30 min. After spinning down nucleic acids extracts were aliquoted & stored at -70^{o}C.

2.5.9 FPLC Fractionation of whole cell extracts

Column buffer:

50mM NaCl

50mM Tris-HCL pH7.5

10mM β-2-mercaptoethanol

1mM EDTA

A Superose-12 column was equilibrated with 60ml column buffer at a flow rate of 0.3ml/min., the pumps having been washed with the same buffer. 200ul of extract (typically at a protein concentration of 5-8mg/ml) were loaded & fractions of 0.5ml collected at a flow rate of 0.2-0.5ml/min. The column was then washed once with column buffer & once with 24% ethanol.

2.5.10 Estimation of protein content of extracts

In all cases performed using the BioRad kit method as manufacturers recommend & also by SDS discontinuous polyacrylamide gel electrophoresis.

2.6 METHOD FOR IN VITRO ASSAY OF DSB RELIGATION

50ng of restrction endonuclease linearised pIC20H was incubated at 14^{o}C for 90 minutes in a volume of 350µl. Nuclear extract volumes were made up to 100µl with SB and incubations were performed in a buffer containing 1mM ATP, 10mM $MgCl_2$ at a pH of 7.9. Final salt concentrations were 60mM NaCl; 6mMMgCl_2; 6% glycerol and 1mM ATP.

Reactions were set up on ice, nuclear extracts being thawed on ice, added to reaction mixture last and returned to -70^{o}C as soon as possible. After incubation proteins were phenol/chloroform extracted from samples and DNA ethanol/ Sodium acetate precipitated. DNA was dried by lyophilisation and resuspended in TE or sterile distilled water. These reaction products were stored at -20^{o}C prior to being used for bacterial transformation or Southern analysis.

2.7 METHOD USED FOR ASSAY OF MAMMALIAN DNA LIGASE I AND II ACTIVITIES

(Arrand et al. 1986)

Ligase assay substrates were prepared by annealing ^{32}-P end labelled oligo$(dT)_{25-30}$ with poly(dA) or poly(rA). 5µg labelled oligo(dT) was incubated with 5µg poly(dA) or 5µg poly(rA) at 95^{o}C for 2-5 minutes in a volume of 100µl and then allowed to cool slowly to room temperature.

Incubations were performed in triplicate incubating 0.5-1µl of such a substrate with whole cell extract in a total volume of 50µl with a final Mg^{2+} concentration of 5nM and ATP concentration of 1mM. Incubation was at 16^{o}C for one hour following which samples were incubated at 80^{o}C for 30 minutes to 1 hour to inactivate ligase enzymes and denature substrates. Samples were treated with calf intestinal phosphatase (2 units for 2 hours at 37^{o}C) and TCA precipitated onto nitrocellulose filters. TCA precipitation was as follows: 500µl 5% TCA and 0.5µg salmon sperm DNA were added to each sample, samples were incubated for 5 minutes on ice and loaded onto folters using a vacuum suction filtration flask. Samples were

washed once with 10% TCA, twice with 5% and then once with 70% ethanol. Filters were air dried and then counted in EcoScint scintillation fluid using a Beckman scintillation counter.

CHAPTER THREE: CHEMOSENSITIVITIES & RADIORESPONSE OF CELL LINES

Chemosensitivities and radioresponse of cell lines

3.1 INTRODUCTION

3.1.1 Cell lines

Double strand breaks (DSB) have been suggested in a number of different studies to be a lesion responsible for many of the cytotoxic effects of ionising radiation (Natarajan et al. 1986; Bryant 1988; Elkind 1985; Painter 1980; Kemp et al. 1984; McMillan et al. 1990; Frankenberg et al. 1984; Gillies 1987; Frankenberg-Schwager & Frankenberg 1990; Radford 1985; Ward 1990 & reviewed in Chapter One). Cellular radiosensitivity has been found to correlate with an incapacity to deal with DNA DSB as measured by neutral elution in six Chinese hamster ovary mutants (Kemp et al.1984). However a number of ionising radiation sensitive cell lines have no detectable defect in DSB processing as measured by conventional assay techniques. Thus two Neurospora crassa mutants (Koga & Schroeder 1987), human cell lines derived from Ataxia Telangiectasia (AT) patients & some Chinese hamster mutant lines (the *irs* series of Jones et al. 1987 & V-C4, V-E5 & V-G8 of Zdzienicka et al. 1989) are extremely radiosensitive but show no abnormality in their neutral elution profiles after X irradiation (Jones et al. 1990; Thacker 1989b).

The *irs* radiosensitive mutants derived from V79 (Jones et al. 1987) provide an ideal system for the study of DSB repair. Three *irs* mutants were originally described, the present study deals for the most part with *irs-1* and *irs-2* and unless otherwise specified "irs mutants" refers to *irs-1* and *irs-2*. These lines are relatively well characterised but no specific defect has been identified as associated with their radiosensitivities (Jones et al. 1987;1990). The V79 parental line has been shown to be karyotypically stable retaining a predominant 23 chromosome karyotype since its isolation (Thacker 1981). The phenotypes of the radiosensitive *irs* mutants are suggested (Jones et al. 1990) to result in each case from a single genetic change. Thus it is reasonable to assume that each *irs* line differs from V79 & the other *irs* lines only at loci important for the radiosensitive phenotype. Given the correlation between radiosensitivity & DNA DSB repair abnormalities these loci are postulated to be involved in pathways of DSB repair. The present study was initiated with the aim of establishing an assay using cell extracts to facilitate the elucidation of mechanisms involved in the repair of double strand breaks. Identification of a defect in the *irs* mutants compared to V79 wild type cells in an appropriate assay should enable the identification of proteins involved in repair of DSB & so by inference in radiosensitivity.

A system for the analysis of radiosensitivity in human cells is provided by cell lines from patients suffering from Ataxia Telangiectasia. The transfection experiments of Thacker & co workers described in the introduction used the radiosensitive AT5BI (Day et al. 1980) cell line in comparison to the normal MRC-5 line (Huschtascha & Holliday 1983). This system provides a means toward the identification of human genes & proteins involved in repair of ionising radiation induced damage.

To ensure that the cell lines to be used in the present study had maintained their phenotypes during growth in culture drug and radiation sensitivity assays were performed. The *irs* mutants are described by Jones et al. (1987) as being sensitive to mitomycin C (MMC). The MMC sensitivities of the *irs* lines and the V79 parental line were thus checked. To ensure that our AT5BI line retained its radiosensitivity the radiation response of this line was compared to that of the normal MRC-5 line. We also checked the radiation responses of V79 and one of the *irs* mutants *irs-2* since much of the work subsequently described concerned detailed analysis of this mutant. Finally we extended the characterisation of the cross sensitivities of *irs-1* and *-2* by studying the sensitivity in comparison to V79 of these lines to the cytotoxic commonly used anticancer drug cisplatin.

3.1.2 MTT assay

Drug sensitivities were assessed using a short term viability assay the MTT assay described in Materials and Methods. Cells are plated at a low density and exposed to a cytotoxic agent for a defined length of time. Drug is then removed and the cells allowed to recover and pass through two to three doubling times. Surviving cell number is then determined by the ability of live but not dead cells to reduce the tetrazolium dye MTT. The D_{37} or D_{50} value of a drug (being the drug concentration required to reduce the absorbance to 37% or 50% of that of control untreated cells respectively) can then be calculated from a graph of absorbance (as a measure of viability) against drug concentration. The optimum MTT concentration to ensure a linear relationship between cell number and MTT formazan production was first determined. It was found that a concentration of 2.5mg/ml MTT diluted in PBS gave maximum absorbance without significant toxicity.

Cells were treated with drug for two hours at 37^{o} in serum free medium keeping conditions identical to those described in Jones et al.(1987).

3.2 MMC SENSITIVITIES OF *IRS* MUTANTS

Figure 3.1 shows the graphs obtained by combining the results from at least nine independent MTT assays asessing the response of V79 and the *irs* mutants to MMC. Each assay involves eight independent determinations for each dose point so the points in the graphs of figures 3.1-3.3 represent 72 independent observations. It can be seen from Figure 3.1 that the sensitivities observed by Jones et al. are maintained, *irs-1* being significantly sensitive to MMC and the *irs-2* line less so. Table 3.1 shows the D_{37} and D_{50} values for MMC for V79 and the radiosensitive mutants.

Table 3.1

Comparison of D_{50} and D_{37} values for cell kill by mitomycin C as measured in V79 and the *irs* mutants by the MTT assay.

Cell line	D_{50} x 10^{-5} μg/ml	fold sens.	D_{37} x 10^{-5} μg/ml	fold sens.
V79	1.5	1	2.5	1
irs-1	0.028	54	0.038	62.5
irs-2	0.75	2	2.0	1.25

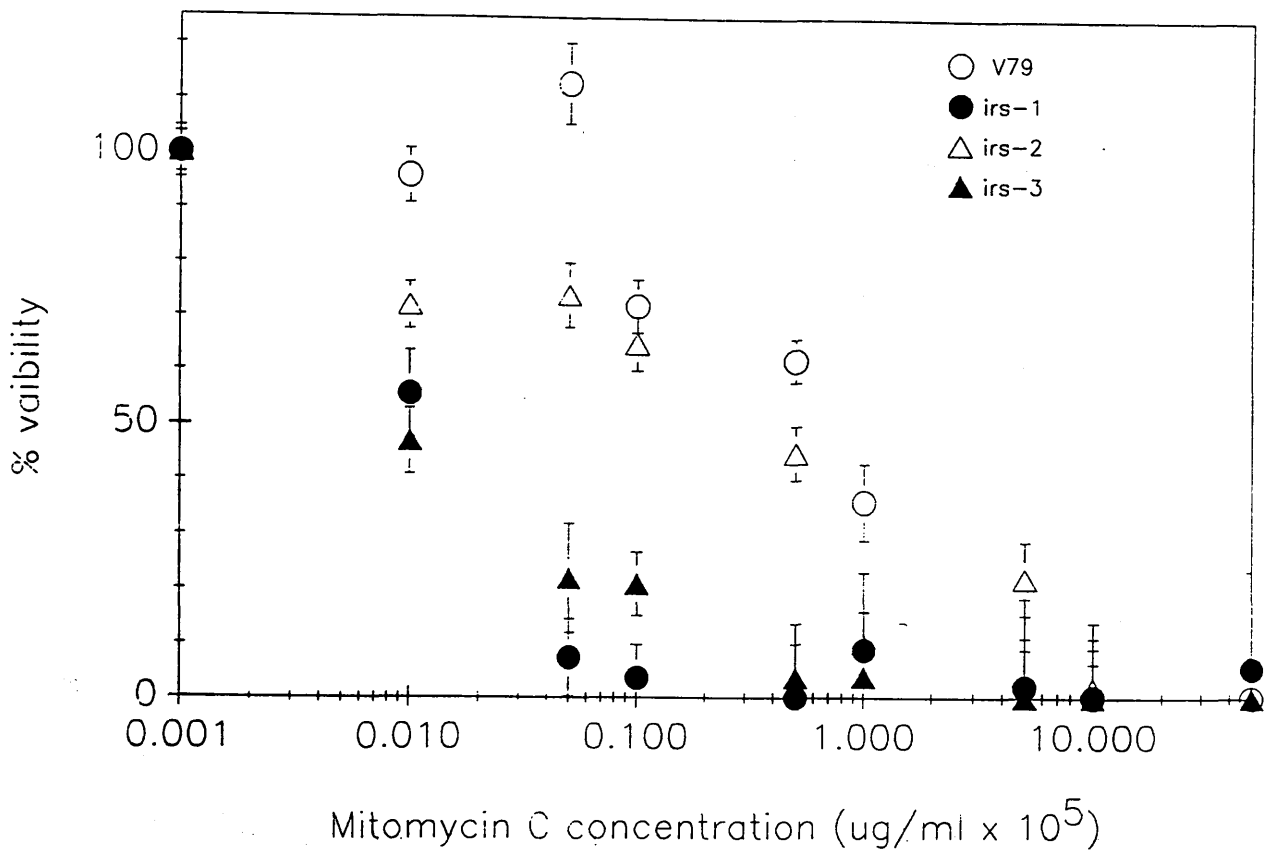
Fold sensitivities calculated by dividing the relevant D_{50} or D_{37} value for V79 with that for the mutant in question. All other values calculated from curves shown in Figure 3.1.

Table 1.3 (Introduction) shows the MMC sensitivities of V79 and the *irs* mutants (Jones et al.1987) using a clonogenic assay. It is clear comparing Table 3.1 with Table 1.3 that the MTT and clonogenic assays yield extremely similar results. By the clonogenic assay of Jones et al. (1987) *irs-1* is 60 fold more sensitive than V79 to MMC and *irs-2* 1.32 times more so. By the MTT assay *irs-1* is 54 fold more sensitive if D_{50} values are compared and 62 fold more sensitive

comparing D_{37} values. *irs-2* is again slightly less sensitive to MMC using the MTT assay if D_{50} values are compared (2 fold compared with 1.32) while if D_{37} values are compared the value is very close to that obtained by Jones et al. (1987) using the clonogenic assay.

Figure 3.1

Mitomycin C sensitivity of V79 and *irs* mutants



Graph of percentage viability as a function of MMC concentration for V79 and the *irs* mutants. MTT assays performed as described in Materials and Methods and in text. MMC was applied at each concentration specified for 2hrs in serum free medium. Each point represents at least 24 independent observations. Mean values are shown with error bars represnting standard errors of the mean.

It is concluded that the MTT assay can reasonably be used as an alternative to the more time consuming clonogenic assay for the study of drug sensitivities. More importantly the cell lines have each been shown to retain the phenotypes described by Jones et al. (1987) and thus not to have undergone genetic change or cross contamination. Large frozen stocks of these cells were used in all subsequent experiments, to minimise periods of growth in culture.

3.3 CISPLATIN SENSITIVITY OF *IRS* MUTANTS

The MTT assay was then used to generate data for the response of V79 and the *irs* lines to cisplatin. It was important to extend the range of drugs for which the response of these mutants was characterised for a number of reasons. The first is that characterisation of the spectrum of responses of a mutant gives information as to the nature of the defect involved - if a mutant isolated by virtue of hypersensitivity to one agent is cross sensitive to another the defect in that mutant must lie in a pathway of metabolism or damage repair common to both. Another reason for extending the knowledge of drug cross sensitivities of the mutants used in the present study was to facilitate drug selection systems that could be used for gene transfer experiments to complement the hypersensitivity. Microcell-mediated gene transfer into the irs mutants using genetically marked human chromosomes was explored as a method of gene transfer but was eventually not pursued.

The broad range of sensitivities of *irs-1* to agents with differing modes of action suggest that this line is not deficient in an activity involved in a pathway of repair of a specific DNA adduct but rather in an enzyme important in the repair of a broad spectrum of lesion types. This line is sensitive both to damage by UV light causing bulky adducts to DNA and to γ radiation induced DNA damage not believed to be repaired by excision repair. The implication is that the pathways for repair of these different types of lesion may share a number of activities.

For complementation analysis by cell fusion or gene transfer it is desirable to have as wide as possible a range of agents to which the sensisivity of the mutant in question is known. This renders such experiments easier, particularly for radiation sensitive mutants as screening large populations for recovery of normal radiation response is cumbersome. It is thus preferable to be able to apply primary selection for a cytotoxic drug so reducing the population that must be

tested for complementation of radiation response. Furthermore cells in which it is believed the primary defect has been rectified can then be tested for the co-complementation of other sensitivities. In this manner a picture can be built up of the relationships between pathways of repair of different adducts and as relevant activities are identified these can be fitted into such a scheme.

It was decided to look at the response of the *irs* and V79 lines to the antitumour cytotoxic agent cisplatin. Cisplatin causes inter and intra strand DNA crosslinks. There is evidence to suggest that these DNA lesions are responsible for the cytotoxic effect of the drug. Some cisplatin lesions are repaired by the pathway of excision repair. There are a number of reports of the identification of proteins recognising cisplatin-DNA adducts, presumed to be the first step in the removal of such an adduct (Chu and Chang 1990, Mclaughlin pers comm.).

MTT assays were performed and D_{37} values calculated for the response of the *irs* mutants to cisplatin. Table 3.2 shows the values obtained for the sensitivity of V79 and the *irs* mutants to cisplatin.

Table 3.2

D_{37} values for the sensitivity of V79 and the *irs* radiosensitive mutants derived from it measured using the MTT assay.

Cell line	**D_{37} in response to cisplatin (x10-6M)**	
		fold sens.
V79	16.3	1
irs-1	0.7	23
irs-2	10.25	1.6

D_{37} values calculated from curves of at least 12 independent determinations over nine dose points. Fold sensitivities obtained by dividing the V79 value by that for the mutant line in question.

Table 3.2 shows *irs-1* to be extremely cisplatin sensitive and *irs-2* slightly so. These results are consistent with the defect in *irs-1* being in a protein required for the repair of a wide variety of types of DNA lesion. Cisplatin as mentioned above is thought to cause DNA adducts some of which are repaired by the pathway of excision repair. Since excision repair pathways can repair damage induced by cisplatin and UV the cross sensitivity of *irs-1* to UV and cisplatin is not surprising. The slight sensitivity to both UV and cisplatin of *irs-2* suggests that the excision repair pathway and that of radiation damage repair which is altered in *irs-2* have some functional overlap.

3.4 RADIATION RESPONSE

3.4.1 Introduction

Radiation response curves were obtained as described in the materials and methods section. Cells were irradiated in medium (using a 1MeV Cobalt 60 source) at a dose rate of 130.3cGy/min. Prior to irradiation cells had been seeded at densities of either $1x10^5$ or $2x10^2$ and incubated overnight. After irradiation those seeded at $2x10^2$ were incubated for about twelve days before staining and counting of colonies. Cells seeded at $1x10^5$ were divided among three flasks prior to this incubation.

3.4.2 Radiation response of human cell lines

Figure 3.2 shows the dose response curve obtained by irradiating the SV40 immortalised human fibroblast human lines MRC-5 (normal fibroblast), AT5BI (classical AT) and a primary fibroblast line derived from a skin biopsy of a patient suspected of having AT (ATG). This patient had a severe response to radiotherapy. Since a characteristic of AT is extreme cellular radiosensitivity and adverse reaction to radiotherapy, it was important to assess the radiosensitivity of these cells prior to subsequent radiotherapy of the patient. It is clear that the AT line is significantly more sensitive than the MRC-5 line and gives a radioresponse curve of a very different nature showing the lack of a "shoulder" characteristic of the radioresponse of AT lines. The ATG line gives a response curve superimposable upon that of MRC-5.

Thus the radiosensitivity of our "AT5BI" line was confirmed and it was shown that the the

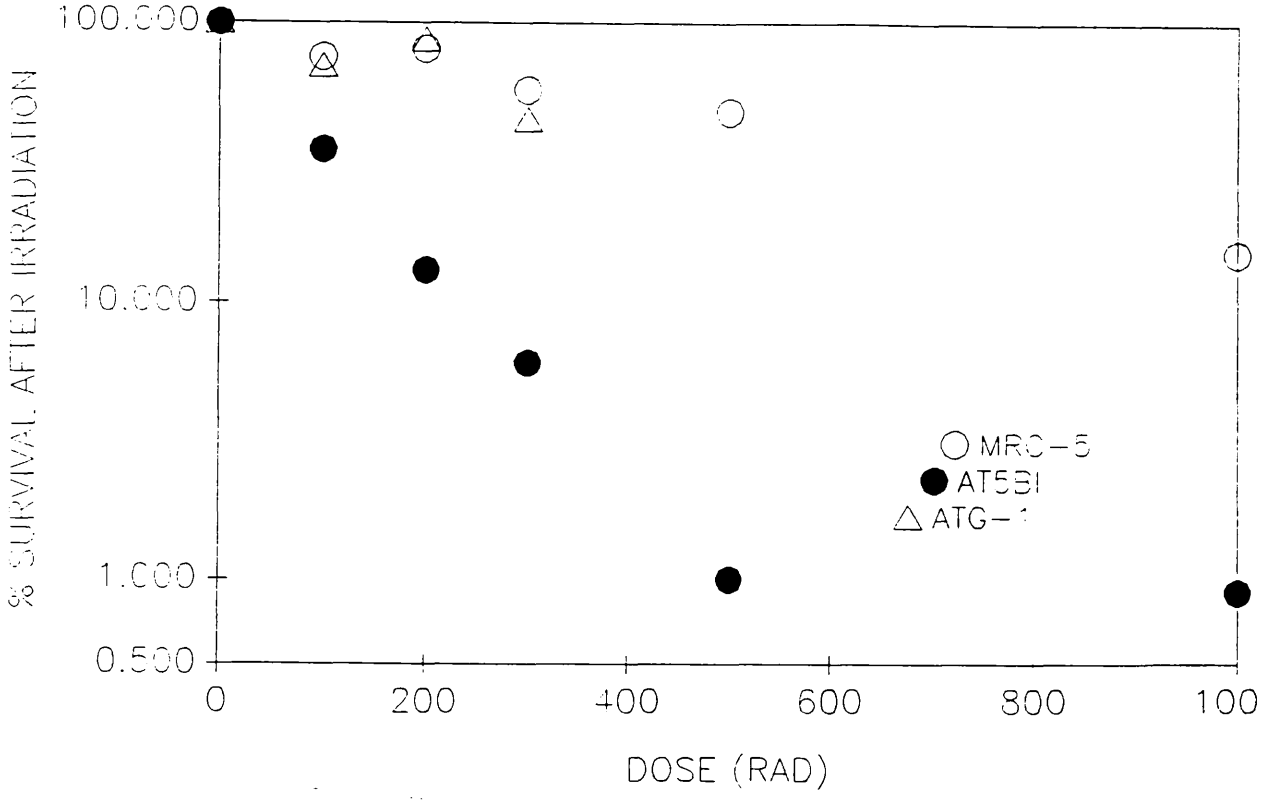
ATG cells had normal radiosensitivity.

3.4.3 Radiation response of hamster cell lines

Figures 3.5 and 3.6 show the radiation response curves of the hamster V79 and *irs-2* lines respectively. *irs-2* gives a different response curve to that of V79, appearing to lack a shoulder at low doses. It is clear that the *irs-2* cells used in the present study are significantly more radiosensitive than the wild type.

Figure 3.2

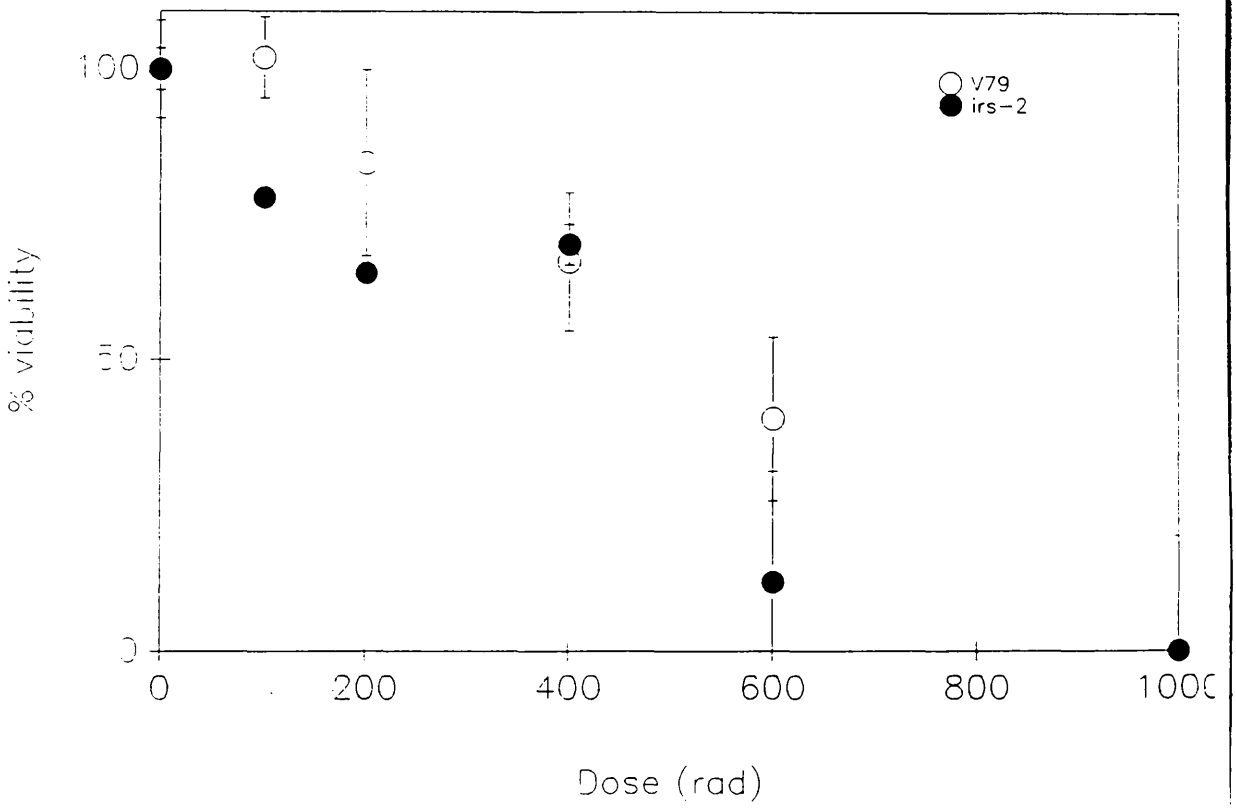
Radiosensitivities of MRC-5, AT5BI and ATG cell lines



Assays performed as described in text and materials and methods. Irradiation was with a 1MeV Cobalt ^{60} source for time calculated to give the doses indicated. Each point represents the mean of at least three independent observations.

Figure 3.3

Radiation response of V79 and *irs-2* cell lines



Cells were plated and incubated for 24hrs. Assays performed by irradiating cells with a 1MeV Cobalt ^{60} source for times calculated to give the doses indicated. Cells were then refed and split to allow determination of survival values by counting of colonies after incubation for 10 to 15 days.

3.5 CONCLUSION

The results presented in this chapter show that the cell lines used in the present study have the expected MMC and γ radiation sensitivity profiles. Thus no inadvertant cross contamination or confusion of the cell lines has occurred. Further no phenotypic change in sensitivities during growth has ocurred. The same large stocks of frozen cells were drawn on for all subsequent studies ensuring a stable phenotype.

It has been demonstrated that results obtained using the simple and rapid MTT assay for drug sensitivity are comparable to those gained using the more laborious clonogenic type of assay. The availiable data on the cross sensitivity of the *irs* mutants to agents other agents than ionising radiation has been extended to include response to cisplatin. *irs-1* has been found markedly (20 fold) more sensitive than V79.

CHAPTER FOUR: IN VITRO DSB RELIGATION BY HAMSTER NUCLEAR EXTRACTS

In vitro DSB religation by hamster nuclear extracts

4.1 IN VITRO ASSAY FOR DSB REPAIR BY MAMMALIAN CELL EXTRACTS.

4.1.1 INTRODUCTION.

4.1.1.1 The DSB as the basis for the cytotoxicity of ionising radiation.

Ionising radiation causes a variety of forms of cellular damage including reproductive death, interphase death, division delay, chromosome aberrations, mutation and transformation (Cole et al 1980). It is now accepted that the target of most relevance to the cytotoxic effects of ionising radiation is DNA (Cole et al. 1980, Frankenberg et al. 1984, Painter 1980, Elkind 1985, Cramp et al. 1984, Haynes et al. 1984). Further the outcome in terms of survival of an irradiated cell population will depend on the nature of the interaction between induction of DNA damage and DNA damage repair in that cell type (Haynes et al. 1984, Ward 1986, Alper 1984). Ionising radiation may induce many types of DNA damage; for example single and double strand breaks, base damage, DNA-DNA and DNA-protein cross links (Cerutti 1975). Experiments attempting to correlate cell responses to increasing doses of ionising radiation and the various forms of damage incurred have suggested the double strand break (DSB) as the lesion most important in terms of cytotoxicity (Cole et al 1987 and 1980, Painter 1980, Natarajan et al 1986). The result of irradiating a cell population depends on the induction and repair of DNA double strand breaks under specific conditions of irradiation. There is now a substantial body of evidence supporting this view, for instance it has been shown that the capacity to remove DSB correlates well with radiosensitivity. Treatments affecting removal of DSB also have an impact on response to radiation (Bryant 1988; Evans et al. 1984; Natarajan 1986). Bryant (1988) documents studies whereby the enzymic induction of DSB by treatment of cells with restriction endonucleases produces some of the same cellular effects as ionising radiation (eg mutations, chromosomal aberrations and cell death). Frankenberger et al (1984) show that a yeast mutant unable to repair DSB has the radiation survival curve characteristics predicted if cell lethality were caused by misrepair of DSB. Work involving radiosenstive mutants of yeast (Ho 1975) and mammalian cells (Jeggo et al 1983, Kemp et al 1984, Costa and Bryant 1990) shows a number of cases where deficiency in repair of double strand breaks correlates with radiation hypersensitivity.

Repair assays in these instances involve following the disappearance of DSB by neutral elution or nucleoid sedimentation.

4.1.1.2 Measurement of induction and removal of DSB

DNA filter elution methods for the assessment of DNA damage and repair are based on the selective retention on a filter of longer DNA strands when an eluting solution is pumped through a cell lysate. To eliminate the measurement of DNA-protein cross links the cell lysate can be treated with proteinase K, the type of adduct measured is then determined by the pH of the eluent, alkaline elution giving an indication of single strand breaks while DSB can be quantified using neutral pH to preserve the DNA duplex.

Although neutral elution is a convenient method for measuring DSB in cellular DNA it remains controversial. The dose response relationship, the contribution of other adducts (eg SSB and DNA protein cross links) and the discrepancies with results obtained using neutral sedimentation give rise to concern. Evidence is cited by supporters of each technique to show that the other is more susceptible to these distorting factors (Peak 1990). Comparisons of DSB induction and removal between cell lines yield similar results using either method. Thus it is acceptable to consider data from both types of study when looking for relationships between induction, repair of DSB and sensitivity to ionising radiation. These "classical" techniques of adduct quantification are however limited in the extent to which they can provide information of damage induction and repair heterogeneity between cells in a population as well as between different regions of the genome.

More recently a trend has emerged favouring measurement of DNA damage (& repair) using methods allowing analysis at the level of the single cell. Such assays, eg Halo assay of Roti-Roti and Wright (1987) and Comet assay of Olive et al (1990), involve embedding a small number of cells individually after a damage inducing treatment, lysing them, separation of the DNA by electrophoresis. However these assays cannot address the measurement of a particular type of damage so it is impossible to correlate induction and repair of a specific lesion with cellular response. At present the experimental

variations intrinsic in the preparation of samples for these assays also complicates the interpretation of results. However as conditions are optimised for the detection of particular types of damage and technical inconsistencies are removed this type of assay will doubtless prove very powerful.

Application of pulsed-field gel electrophoresis (PFGE) to the measurement of DSB is being explored. PFGE resolves high molecular weight DNA fragments in agarose gels by means of alternately pulsed, perpendicularly oriented, electrical fields at least one of which is non uniform. These methods are based on embedding cells in a gel matrix (preventing shearing during cell lysis) and quantifying the fraction of DNA migrating from the wells under PFGE. The data suggest that after calibration and careful establishing of conditions DSB resulting from doses as low as 3-4 Gy can be detected in yeast (Contopoulou et al. 1987) and now in mammalian cells (Blocher and Kunhi 1990, Blocher 1990, Ager 1990). Such a technique allows relatively unambiguous quantification of DSB.

4.1.1.3 Radiosensitive cell lines with no detected defect in DSB repair or induction.

The correlation between induction or repair of a particular radiation induced lesion (DSB) with radiosensitivity is confused by the existence of a number of mammalian cell lines with extremely radiosensitive phenotypes and abnormal radioresponse curves but no detectable alteration in induction or handling of DSB as measured by neutral elution or sedimentation techniques.

Cells derived from patients with the cancer prone syndrome Ataxia Telangiectasia (AT) show enhanced radiosensitivity (Taylor et al 1975) and abnormal handling of radiation induced damage. Such lines exhibit deficient recovery from potentially lethal damage (Cox 1982, Lehman 1982, Arlett and Preistley 1984) and a lack of inhibition of DNA synthesis following irradiation (Painter and Young 1980, Houldsworth and Lavin 1980). AT lines do not however appear altered in the induction or repair of DSB in response to ionising radiation as measured by neutral elution (Paterson et al 1984).

Radiosensitive mutants of the yeast *Neurospora crassa* (Koga and Schroeder 1987) and two series of mammalian cell mutants have been derived in vitro which

resemble AT cell lines in that they have no apparent defect in repair of radiation induced double strand breaks (Jones et al 1990, Zdzienicka et al. 1989).

A series of mammalian radiation sensitive lines, *irs-1*, *irs-2* and *irs-3* were derived from V79-4 Chinese hamster cells by ENU mutagenesis followed by replica plating and selection for X-ray sensitivity (Jones et al. 1987). The radiosensitivity of each was shown to be recessive to wild type and to complement the ionising radiation sensitivity of other rodent mutants *xrs-1* (or *xrs-7*) (described in Jeggo and Kemp 1983), EM7 and XR-1 (described in Stamato et al. 1983) (Jones et al. 1990). The *irs* mutants show differing cross sensitivities to mitomycin C (MMC), ultra violet light (UV) and ethyl methane sulphonate (EMS) (Jones et al. 1987).

The molecular bases for the radiosensitivities of *irs-1*, *irs-2* and *irs-3* are not yet clear. In comparison with wild type V79 cells no gross alteration in the number of double (or single) strand breaks induced by radiation is evident. Similarly no alteration is seen in the removal of these lesions (Jones et al. 1990). The authors consider it probable that the assay used (neutral elution at pH9.6) would identify any existing gross defect of this type as it detects defective DSB repair in three genetically distinct radiosensitive lines (Kemp et al. 1984). The same authors determined the rate of DNA synthesis following γ-irradiation in V79, *irs-1* and *irs-2* by measuring the amount of [^{3}H]thymidine incorporated in a 60 minute interval after irradiation of cells. V79 and *irs-1* showed a rapid dose dependent inhibition, the rate of synthesis dropping to about 60% of the control level at 25 Gy. DNA synthesis in *irs-2* was in contrast markedly resistant to inhibition with a rate of synthesis 90% of that of controls at 25 Gy. The authors postulate that *irs-2* fails to respond appropriately to radiation damage which in normal cells inhibits replicon initiation. Cytogenetic evidence suggests that a very high level of chromosomal aberrations are induced in *irs-1* on exposure to ionising radiation (Jones et al. 1990) implying the presence of unrepaired or misrepaired strand breaks (Natarajan et al 1986).

4.1.1.4 DNA repair assays

Biochemical analysis of DNA metabolism enzymes which play a part in the repair of cytotoxic lesions would facilitate the identification of the defect leading to a particular mutant phenotype as well as enable characterisation of processes involved in repair of DNA damage. Transfection and in some cases microinjection (Folger et al. 1985) systems have been employed to study mechanisms of recombination between exogenously introduced plasmids in normal cells (Lin et al 1990, Miller and Temin 1983, Lin et al 1985, Roth and Wilson 1986, Abastado et al. 1987, Vos and Hanawalt 1989, Wilson et al. 1982,Roth and Wilson 1988, Kucherlapati and Moore 1988, Suberamini and Seaton 1988, Shapira et al. 1983, Wong and Capecchi 1986,Roth et al. 1985, Rubnitz and Subramini 1984, 1985, Brenner et al. 1986, Subramini and Berg 1983, Brouillette &, Chartrand 1987,Kucheralpati et al. 1985, de Saint Vincent and Wahl 1983, Rauth et al. 1986). A number of authors describe the application of such an approach to the analysis of repair defects in cells hypersensitive to particular cytotoxic agents (Knox et al. 1987, Runger and Kraemer 1989 Protie et al. 1988, Chu and Berg 1987, Moore et al. 1986). A system involving transfection into cells of a plasmid cut with a restriction enzyme in a selectable marker gene or genes has been developed to assay for the repair of DSB. (Thacker 1986, Cox et al. 1984, Debenham et al. 1988, Thacker 1989b, Thomson 1988, Hamilton and Thacker 1987) Cut plasmid is introduced into cells and selection for an uncut marker applied thus eliminating non transfected cells. Selection is then applied for the cut marker and the number of transfectants positive for this gene scored as a measure of cellular ligation or recombination capacity. DNA isolated from such colonies can then be sequenced to yield information as to the precise nature of the rejoin process. This type of experiment suggest that when compared to normal human fibroblasts AT cells repair double strand breaks with reduced fidelity and further that one of the *irs* mutants (*irs-1*), in comparison with the isogenic wild type V79 parental cell line, also shows such a lack in fidelity of DSB rejoin (see general introduction for more detailed discussion).

There are disadvantages inherent in such an approach where unknowns must

arise from the largely undefined interactions of input DNA with cellular metabolism. The processes of uptake of exogenous DNA by a recipient cell in terms of copy number and of events occurring inside the cell leading to its (more or less) stable maintenance are not fully understood. Transfection approaches do not readily lend themselves to the dissection of particular repair pathways to discrete enzymatic steps as they involve the action of an undefined set of enzymes upon a substrate which may be altered during the transfection procedure. Matters are further complicated by wide variations in the capacity of cell lines to be transfected and variations in amount of DNA taken up rendering comparison between diferent cell types problematic. Some of these difficulties can be alleviated to a degree by the use of two gene vectors (Thacker 1989b, Debenham et al.1987) where the selection for transformants is applied prior to that for repair of damage.

As described in the Introduction a number of groups have developed assays to enable the analysis of the capacity of extracts from mammalian cells (Darby and Blattner 1984; Kucherlapati et al. 1985; Mortelmans et al. 1976), mammalian nuclei (Lopez and Coppey 1987; 1989; Holmes et al. 1990; Wierbauer and Jiricny 1989;1990; Rauth et al. 1986; North et al. 1990; Sibghat-Ullah 1989), Drosophila nuclei (Holmes et al. 1990), extracts from Xenopus eggs (Thode et al. 1990; Pfeiffer and Vielmetter 1988) extracts from yeast (Symington et al. 1983), tetrahymena (Robinson et al. 1989) or purified proteins (Evans and Linn 1984; West 1990) to perform various recombination, ligation or repair reactions upon defined substrates *in vitro*.

The repair of DSB in mammalian cells has not been extensively studied *in vitro*. The strong links between recombination and DSB repair in a number of yeast systems and in phage λ (Szostak et al. 1983, Thaler and Stahl 1988, Sun et al. 1989) inspired Moore et al. (1985) to test the radiosensitive Chinese hamster mutant cell line *xrs-5* (Jeggo and Kemp 1983) in both the in vivo (transfection) and in vitro recombination assays developed by Kucherlapati and coworkers. *xrs-5* is defective in the repair of DSB as measured by neutral elution (Kemp et al. 1984) and was observed to have a reduction in integration of transfected DNA (Moore et al 1988, Hamilton and Thacker, 1987). Taking

a transfection approach Moore et al. (1986) found a fourfold reduction in homologous recombination - a reduction of the same order of magnitude as that observed for the DSB repair defects. The implication was that the primary defect in *xrs-5* is thus in recombination. However, assay of recombination *in vitro* revealed no significant difference in the capacities of nuclear extracts of *xrs-5* and the parental CHO line to catalyse recombination. Several reasons to explain this discrepancy between results from *in vivo* and *in vitro* assay are offered (Kucherlapati and Moore 1988), it could be that the defect in *xrs-5*, is not rate limiting in extracts. Alternatively another activity may replace it *in vitro* or its function be performed by the bacteria used to recover and assay recombinant molecules.

4.1.2 AIMS IN DEVELOPING IN VITRO ASSAY

We wished to devise an assay for the repair of double strand breaks by nuclear extracts from wild type and radiosensitive cells. Restriction endonuclease lesions were used in the transfection assays of Thacker and colleagues to model DSB induced by ionising radiation. These enzymes recognise specific sequences in DNA and generate DSB at sites defined with respect to these recognition sequences. Depending on the particular enzyme the DSB generated may have "blunt" ends or "cohesive" ends with varying degrees of overlap and either 5' or 3' termini. Thus it is possible to generate DSB of various configurations equivalent to those induced by radiation in the DNA of cells. All DSB induced by restriction digestion however have ends with 3' hydroxyl and 5' phosphate groups which theoretically could be repaired by a simple ligation reaction (though there is no evidence to suggest that this is in fact the case). Ionising radiation would on the contrary be expected to cause DSB without such clean ends which might require more complex processing (Bryant 1988). However as described in the Introduction work reviewed by Bryant (1988) suggests that restriction endonuclease induced DSB can lead to a similar spectrum of cellular responses as those induced by ionising radiation. It was thus concluded that the use of a DSB induced by restriction digestion as a substrate for potential repair enzymes was not too great a conceptual compromise.

The results of experiments described above using a transfection assay to measure DSB repair in AT and the *irs* cells (reviewed in Thacker 1988) imply that the radiosensitivities of these lines might result from a reduction in fidelity as opposed to efficiency of DSB repair. It is thus important that a cell free assay can measure the fidelity of DSB repair as well as efficiency.

One method of detecting religation is bacterial transformation. Plasmid DNA carrying a gene conferring resistance to a bacteriocidal drug for example ampicillin is linearised by restriction endonuclease cleavage. Following incubation with repair activities plasmid is isolated and used to transform competent bacteria. The number of ampicillin resistant bacterial colonies then gives an indication of the efficiency of religation. If the

DSB is introduced into a gene such as the lacZ gene thereby inactivating it. Fidelity of religation can be assessed by scoring the proportion of ampicillin resistant colonies positive for the lac Z gene.

The approach I have used to assay DSB repair was to introduce a restriction endonuclease DSB into the lac Z gene of plasmid pIC20H which also carries the gene for ampicillin resistance. Linear substrate was then incubated with extract and reaction products used to transform competent bacteria. Reaction products were also visualised directly by agarose gel electrophoresis followed by Southern blotting, using uncut pIC20H as a radiolabelled probe.

Incubation with T_4 ligase provided a positive control for the capacity of substrate to be ligated. Incubation with extract inactivated by boiling or by proteinase treatment gave a negative control, as did incubation of substrate without the addition of extract.

4.1.3 RESULTS

4.1.3.1 ESTABLISHING CONDITIONS FOR THE IN VITRO ASSAY OF DSB REPAIR

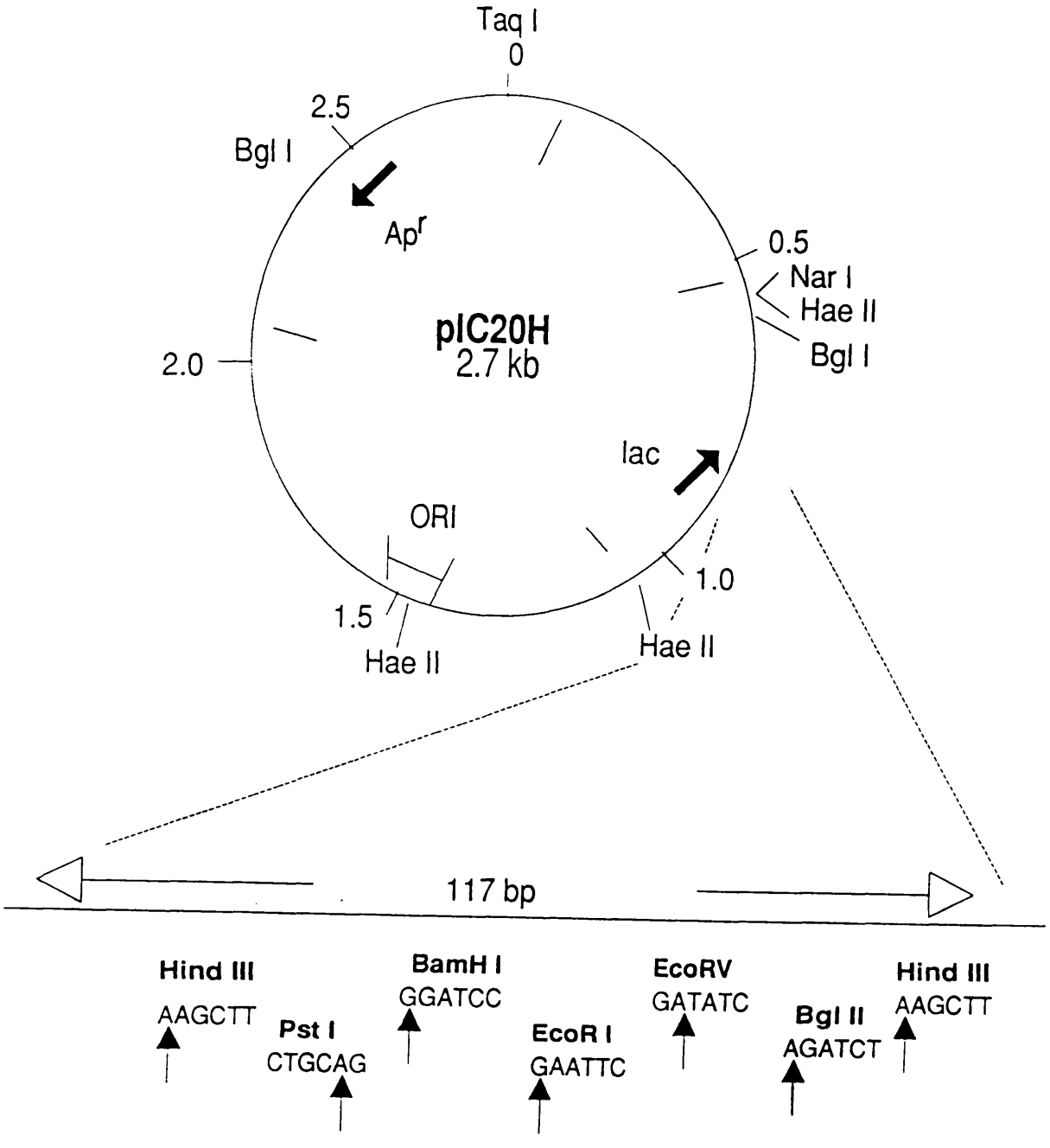
4.1.3.1.1 Substrate.

Figure 4.1 is a simplified diagram of pIC20H showing the main restriction sites present in the body of the plasmid and those in the polylinker within the *Lac Z* gene. The presence of the ampicillin resistance gene distant from the cut site enables selection for efficient recircularisation by transformation of bacteria with DNA after reaction with nuclear extracts. The plasmid pIC20H as indicated contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase (*lac Z*) gene. Inserted in this coding region is a polycloning site containing recognition sequences for a number of restriction enzymes. This sequence does not disrupt the reading frame, resulting in the harmless insertion of a small number of amino acids into the amino terminal fragment of β-galactosidase. In a host cell such as JM83 coding for the carboxy terminus of β-galactosidase (Yanisch-Perron et al. 1985) complementation can occur with association of the two portions of the β-galactosidase protein, each non functional alone, to give an activity rendering the bacteria lac^+ and capable of hydrolysing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside). The use of this strain allows selection by α complementation. If the plasmid portion of the *lac Z* gene (or of course the bacterial) is non functional then no complementation can occur and a white colony will result after plating on X-gal. A DSB repaired without fidelity might be expected to disrupt the β-galactosidase coding capacity of the *lac-Z* gene precluding α complementation giving a white as opposed to a blue colony on X-gal. Therefore the fidelity of rejoin of a DSB introduced into the *lac Z* gene should then be indicated by the number of ampicillin resistant colonies that are blue.

The polylinker of pIC20H (fig. 4.1) contains a number of restriction sites enabling the generation of different types of DSB substrate ie DSB with either 3' hydroxyl or 5' phosphate protruding ends or blunt ends. As each cut has a precise recognition site it is possible to create DSB with different end sequences. This might be important in determining the kinetics of a

Figure 4.1

pIC20H



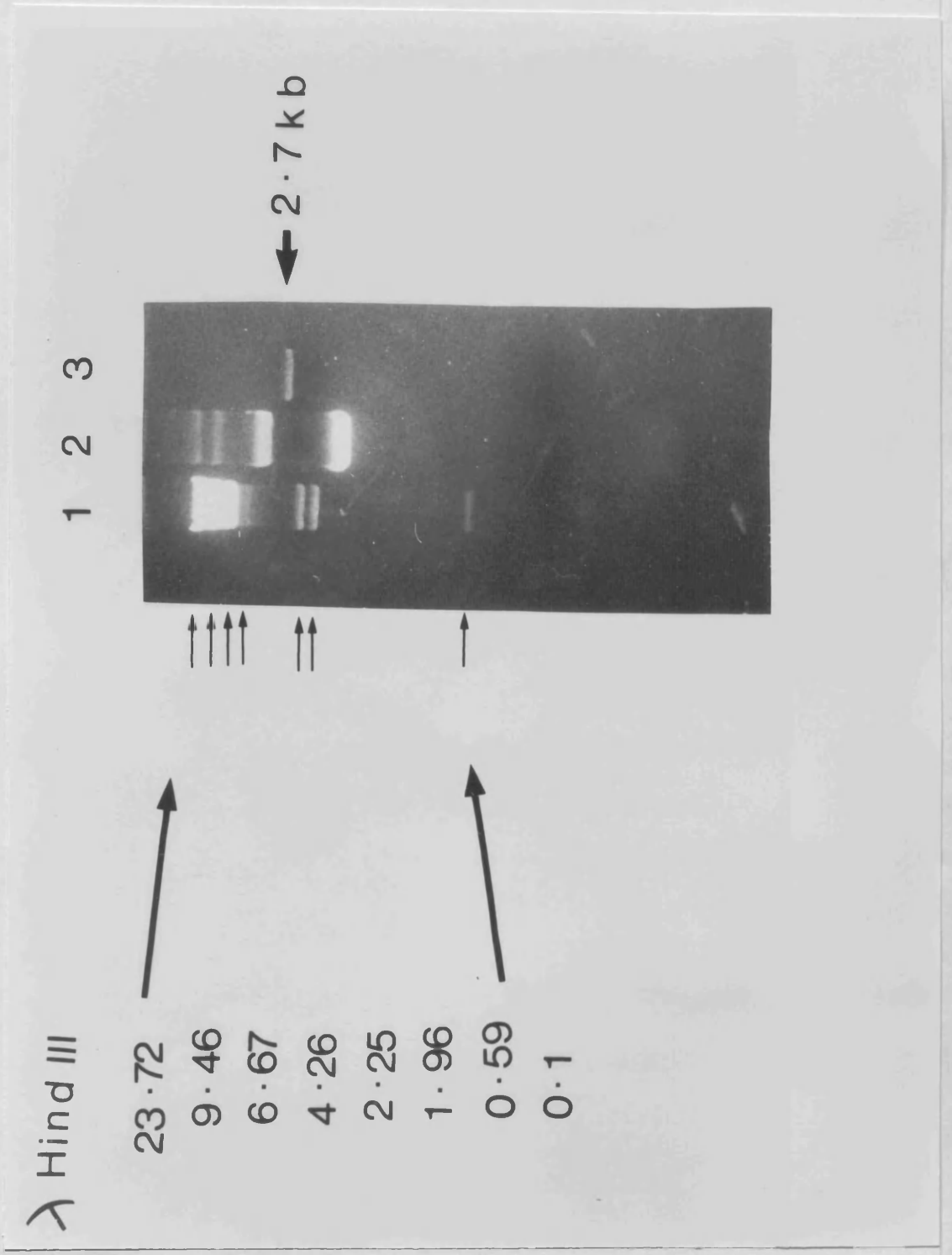
Simplified from Marsh et al. (1984). Bold arrows indicate the direction of transcription of the gene for ampicillin resistance (ap^r) and the lacZ gene (lac). ORI is the origin of replication. Selected restriction sites in the body of the plasmid are shown. Some of the restriction sites in the polylinker inserted into the lacZ gene are shown with their recognition sequences. Arrows indicate the cleavage points for each enzyme within these sequences.

rejoin reaction. To maximise the chances of detecting ligation *in vitro* we thought that a cohesive, as opposed to blunt ended DSB would be an appropriate initial choice for a substrate as such ends are more readily ligated by characterised ligation activities. PstI digested pIC20H - a DSB with a four nucleotide (TGCA) 3' protruding terminus was the substrate used to develop the repair assay. Figure 4.2 shows the Pst I digestion of pIC20H. PstI cuts the plasmid once, in the polylinker (Fig. 4.1) producing a linearised 2.7kb molecule, seen in FIgure 4.2 as a single band. To confirm that the endonuclease reaction had gone to completion a further restriction digest of an aliquot of cut plasmid was performed. Cleavage of uncut pIC20H with BglI which cuts twice in the plasmid backbone should give two bands one of approximately 1kb the other of 1.7kb. Cleavage of linearised plasmid should again yield the 1kb fragment, the 1.7kb band should however be replaced by two fragments. Figure 4.3 shows such a BglI double digest of pIC20H originally linearised with PstI, Hind III (giving rise to 5' protruding ends) and EcoRV (producing a blunt ended DSB). All these enzymes cut once in the polylinker of pIC20H. A BglI digestion of uncut plasmid is run for comparison. It is clear that the samples in lanes 3 (PstI cut pIC20H), 4 (HindIII cut pIC20H), 8 (HindIII cut pIC20H) and 9 (EcoRV cut pIC20H) were efficiently linearised as they give the expected three bands while samples in lanes 5 (EcoRV cut pIC20H) and 7 (PstI cut pIC20H) were only partially digested.

Conditions of DNA concentration to favour recircularisation were chosen by consultation of the equation of Dugaizyk et al.(1975). A factor *j* is involved in the theory of cyclization of DNA, where *j* is the effective concentration of one end in the neighbourhood or volume of the other end of the same molecule. Above a DNA concentration given by [DNA]= 51.1/(MW)1/2 the factor *i* (total concentration /ml of self complementary duplex DNA ends) will be greater than *j* and linear n-mer formation will be favoured. Below this concentration (ie $i<j$) more circularisation should occur. It was important that recircularisation be favoured over end to end ligation (which would of course also involve "repair" of a DSB) in order for the bacterial transformation system to allow asessment of both efficiency and fidelity of rejoin.

Figure 4.2

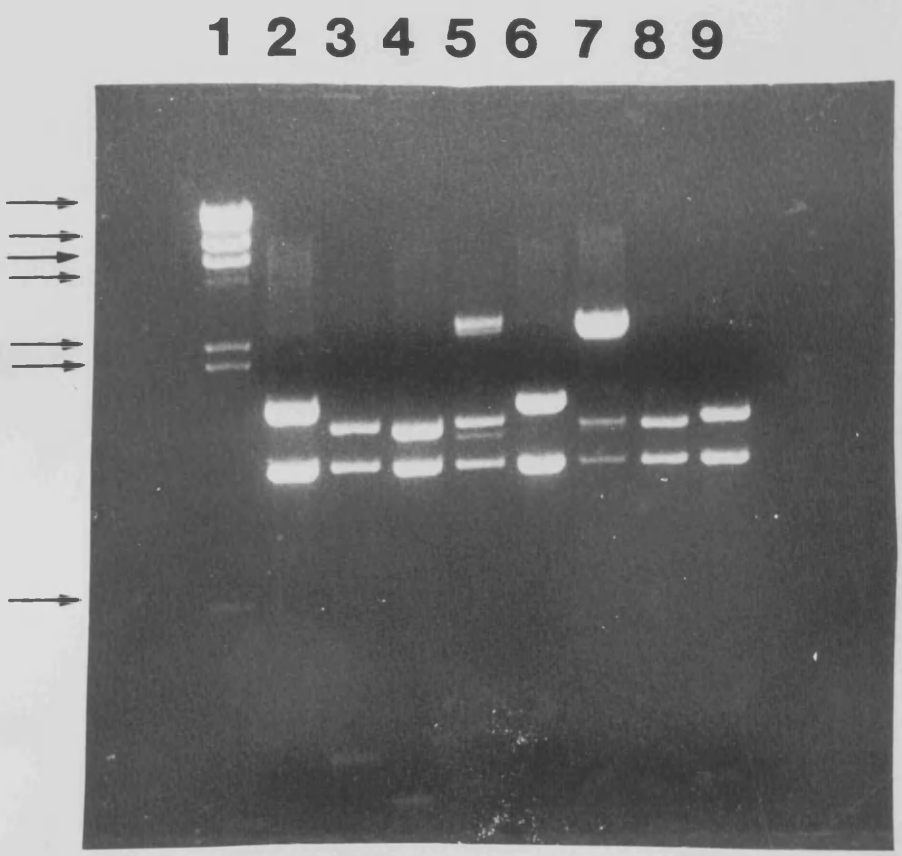
PstI digestion of pIC20H



10µg pIC20H was digested overnight at 37°C adding enzyme in two aliquots of 10 units in the buffer supplied by the manufacturer. Lane 1: size markers; Lane 2: uncut pIC20H; Lane 3: PstI cut pIC20H.

Figure 4.3

BglI double digest to check linearisation of pIC20H



Restriction reactions performed overnight adding enzyme in two aliquots under conditions specified by manufacturer. Lane 1: λ HindIII size markers; Lanes 2 to 9 all digested with BglI. Lane 2 and 6: uncut pIC20H; Lanes 3 and 7: PstI cut pIC20H; Lanes 4 and 8: HindIII cut pIC20H; Lanes 5 and 9: EcoRV cut pIC20H.

Before attempting to use this substrate to analyse repair capacities of extracts it was necessary to show that the endonuclease induced DSB had ends that could be ligated and that bacteria could be effectively transformed by this DNA. Linearised plasmid was thus incubated with T_4 ligase under the conditions to be used in the assay. Aliquots of this reaction were then used to transform competent JM83 Rec A- *E.coli* (Yanisch - Perron et al. 1985). Transformed cells were plated on L-agar containing ampicillin and X-Gal. Total colony number provides a measure of the efficiency of ligation and the number of blue colonies indicates the fidelity of this ligation. For comparison aliquots of cut plasmid substrate and of uncut pIC20H were used in parallel transformations. The transformation frequency of uncut plasmid provides a measure of the efficiency with which bacteria are transformed by a closed circular population and can be used to standardise experiments. Table 4.1 shows the results of such control transformations. I have shown the results using an EcoRI (giving rise to a four base AATT 5' overhang) cut plasmid as well as the original PstI cut substrate since while the PstI induced DSB was used as a substrate in the developement of the assay a number of experiments were later performed investigating the religation of an EcoRI induced DSB.

Table 4.1

Transformation of bacteria to ampicillin resistance by PstI cut, EcoRI cut and uncut pIC20H compared to T_4 ligated cut plasmid.

Treatment	**Colony number per ngDNA transformed**
Pst I cut pIC20H	7.5+/-2 (14)
EcoRI cut pIC20H	5.3+/-2 (6)
T_4 ligated pIC20H	236+/-51 (20)
Uncut pIC20H	1737+/-478 (14)

Standard errors of the mean are shown with the number of independent incubations in parenthesis.

The above Table (4.1) shows that the DSB induced by restriction enzyme cleavage can be ligated by at least one well characterised ligase activity. Furthermore this ligation can be visualised by transformation of competent *E.coli* JM83 to ampicillin resistance. In these experiments the increase in ampicillin resistant colonies with ligase is 37 times higher than that shown on transformation of cut plasmid without incubation with ligase. There is no transformation to ampicillin resistant β Gal negative white colonies, all colonies are blue, thus T_4 ligation proceeds with fidelity, regenerating a functional β Gal gene.

Table 4.1 suggests that some "background" rejoin of cut plasmid occurs giving rise to a low frequency of blue ampicillin resistant colonies following transformation with cut plasmid not previously T_4 ligated. We took this religation to be a property of the bacterial host cells. Another explanation would be that the substrate preparation was not fully linearised, and that low levels of uncut plasmid were not detected using the restriction enzyme digests shown in figure 4.3. This was tested by incubation of DNA isolated after repair incubation with λ-exonuclease. λ exonuclease has a requirement for DSB for its nuclease activity (Little 1967), so digests linear but not circular DNA even if single strand DNA breaks are present. If background transformation was due to a bacterial ligase activity, treatment with λ-exonuclease before transformation should abrogate the capacity of DNA isolated after incubation of cut plasmid alone to give ampicillin resistant colonies but have no effect on the transformation efficiency of uncut or T_4 ligated plasmid. There would be no reduction in background transfromation however if this was due to uncut molecules present in the linearised substrate. Table 4.2 shows the effect of λ exonuclease treatment of DNA isolated after various "control" treatments.

Table 4.2

The effect of treating DNAs isolated after control reactions with λ exonuclease.

Treatment before λ exo	Colony number per ng DNA transformed
PstI cut pIC20H	0.4+/-0.2 (6)
EcoRI cut pIC20H	0.3+/-0.2 (6)
T4 ligated pIC20H	218+/-83 (11)
Uncut pIC20H	1704+/- 753 (4)

In each case the standard error of the mean of the number of ampicillin resistant colonies per ng DNA used to transform is shown with numbers of independent incubations in parenthesis.

The colony number of PstI and EcoRI cut pIC20H shown in Table 4.2 compared to Table 4.1 demonstrates that the products of incubation of cut plasmid alone are sensitive to λ exonuclease treatment. This implies the background recircularisation observed is a property of the bacterial host. In contrast T_4 ligated plasmid and uncut plasmid are λ exonuclease resistant as might be expected since these incubations give rise to circular forms.

Other exonucleases were investigated as possible tools for the analysis of reaction products. Bal 31 exonuclease acts processively at both ends of linear DNA removing oligo or mononucleotides from 5' and 3' ends at an equal rate, utilising both blunt and protruding terminii of a double stranded DNA as substrate (Sambrook et al 1989). Table 4.3 shows an experiment to look at the effect of increasing concentrations of Bal 31 upon products of incubations treated with Bal 31 prior to being used to transform bacteria.

Table 4.3

Effect of incubating products of control reactions with various concentrations of Bal 31 exonuclease prior to transformation.

Treatment before Bal 31	**Colony number per ng DNA transformed** Bal 31 concentration (µl)		
	1	1/10	1/100
PstI cut pIC20H	0.02	0.14	0.1
T_4 ligated pIC20H	3	5.5	15
Uncut pIC20H	4	100	190

Each value represents the reult of incubation of the product of the reactions detailed in the first column with Bal 31 under the appropriate salt, time and temperature conditions (materials and methods for details) and at an enzyme concentration as indicated.

Table 4.3 makes it clear that only the highest concentration of Bal 31 is as effective as λ exonuclease in reducing the background transformation frequency shown by cut plasmid incubated alone. At this concentration however there is significant effect upon uncut and T_4 ligated plasmid. This is presumably due to nicks being a substrate for the single strand specific nuclease also associated with this enzyme. Thus in contrast to λ exonuclease Bal 31 might be expected to digest nicked circular molecules. At lower concentrations the effect on uncut and T_4 ligated plasmid is less extreme but unligated cut substrate is not digested to the same efficiency. It was decided to use λ exonuclease in future experiments of this type.

To summarise, a large well defined stock of substrate was prepared by restriction digestion of pIC20H. This linear plasmid was shown to be capable of being ligated to a form capable of efficiently transforming bacteria to ampicillin resistance. Treatment of linear plasmid with λ-exonuclease prior to transformation markedly reduces the background transformation suggesting this background to be due to religation in the

bacterial host. The residual transformation observed shows that less than 0.01% of the plasmid molecules remain uncut by the restriction enzyme. Such a substrate was used in all experiments.

4.1.3.1.2 Choice of bacterial host for detection of ligation.

For the bacterial transformation detection system to function effectively it is important to know that any ligation detected is a property of incubation with extract as opposed to the result of a bacterial process. It is necessary to use a host strain itself deficient in repair of an endonuclease induced DSB and therefore unable to rejoin pIC20H to a form capable of giving rise to an ampicillin resistant colony. To enable asessment of fidelity of ligation the bacteria must carry sequences coding for the C-terminal portion of the β-galactosidase protein.

The host cell chosen for these studies was *E.coli* JM83 (Yanisch-Perron et al.1985). This strain is inactivated in the Rec A pathway of recombination. It is not as recombination deficicient as some *E.coli* strains as it still has residual recombination activity conferred by the Rec B and C functions. A major advantage of JM83 over such strains is that it carries the C-terminal portion of the lacZ protein on its chromosome as opposed to on a plasmid. This renders the capacity to complement the lacZ peptide of pIC20H stable. We considered that, given the success of JM83 as a host for detection of ligation in early experiments it was not worth engineering a host cell with the advantage of a stable lac Z complementing genotype and inactivated in more repair pathways. In all subsequent experiments JM83 was the host cell used to detect ligation by nuclear extracts.

4.1.3.1.3 Preparation of nuclear extracts.

As the activity(ies) involved in religation of a DSB must at least at some point be DNA associated and so located in the nucleus it was decided to use nuclear extracts as the source of "repair" activity to be assayed. By the use of nuclear as opposed to whole cell extracts it was hoped to enrich for proteins important in repair of such a lesion. The

method used to prepare nuclear extracts was as in Dignam et al.(1983). In essence exponentially growing cells (at least 10^8) are harvested and lysed by treatment with Triton X-100. Nuclei are pelleted and proteins extracted with 0.3M sodium chloride. An ammonium sulphate concentration step is included, proteins are pelleted, resuspended according to nuclear equivalents (measured by DNA content in a sonicated sample of extract) and dialysed against a storage buffer containing 20% glycerol.

In this manner nuclear extracts were prepared from the hamster cell line V79 and the radiosensitive mutants derived from it *irs-1, irs-2* and *irs-3.*

To compare the properties of extracts of different cell lines it is critical to show that each extract is of equivalent protein concentration and contains functional proteins. This was particularly important as the repair assay was being designed to facilitate exposure of a defect in a radiosensitive line as compared to wild type. Such a lack of activity must be demonstrated not to be due to an overall absence of functioning proteins.

Protein concentration -Biorad.

Protein concentrations of all extracts were determined using the Bio-Rad kit assay . This assay is based on the fact that the absorbance maximum for a solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm on binding to protein. The selection of an appropriate dye volume to sample concentration ratio allows accurate quantitation of protein concentration. Compared to other chemical methods of protein determination the Bio-Rad assay suffers little from interference due to reaction of dye with chemicals in the assay sample (Bio-Rad Instruction Manual) although it does give variation in estimation of concentration of different proteins. It provided a reasonable method of estimating protein concentration in nuclear extracts. The method of extract preparation being uniform artefacts brought about by chemical interference or variations in spectra of protein content should be minimal. In general extract concentration assessed by this method was about 5mg/ml. Extracts were diluted in storage buffer as necessary to obtain equal amounts of protein nuclear equivalents in equal volumes. Thus results may be expressed in terms of volume in µl of extract added to a reaction.

Gross protein composition of extracts by SDS-PAGE

Total protein composition of extracts was further compared by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4.4, protocols as described in Materials and methods). Soluble proteins were fractionated in a discontinuous SDS-buffer system using a 4% to 10% exponential gradient acrylamide concentration. This analysis depends on the dissociation of all proteins into polypeptide subunits. Upon heating at 100^{o}C with excess SDS and thiol reagent (to cleave disulphide bonds) most polypeptides bind SDS in a constant weight ratio. The SDS-polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels according to polypeptide size (Hames 1981).

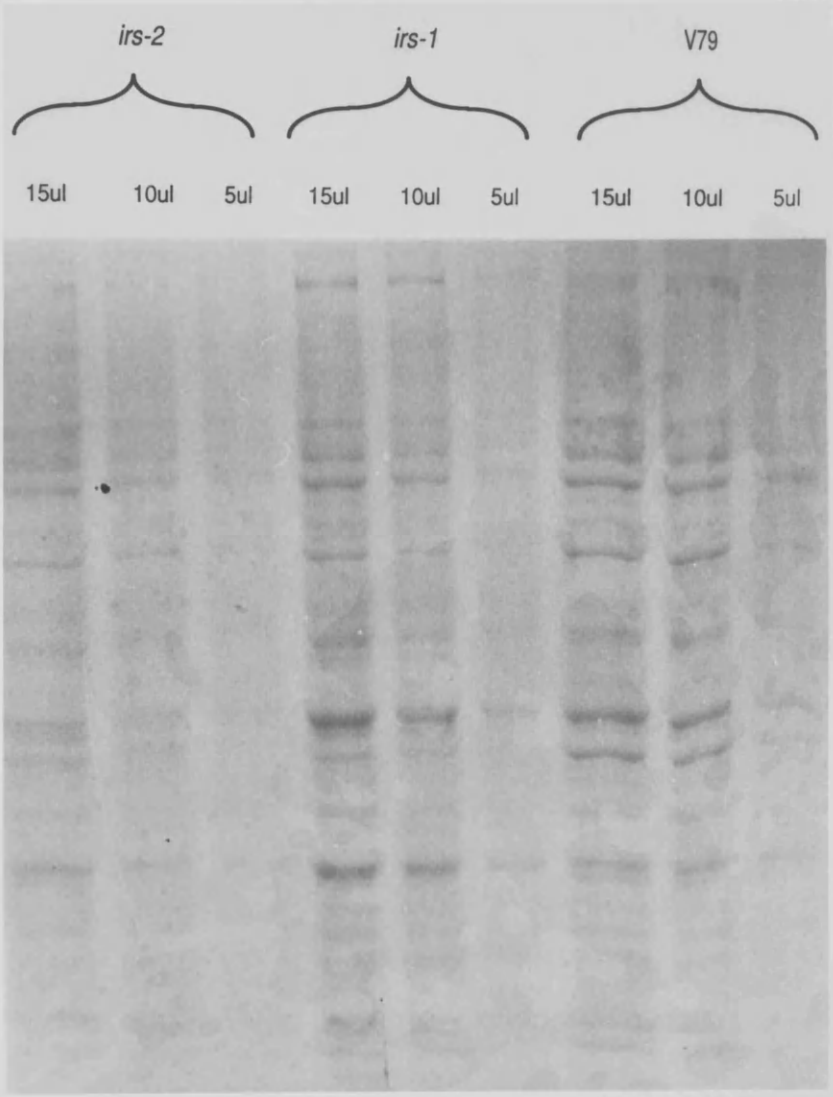
No alterations were observed in the banding patterns or relative band intensities of nuclear proteins (after Coomassie staining of gels run as descibed) of either *irs-1* or *irs-2* in comparison to the wild type V79. This type of analysis gives a qualitative indication of large differences in protein composition. It cannot reveal subtle alterations in a particular protein. The denaturing of proteins prior to loading also means that altered conformation will not be revealed. It does suggest that in comparison to V79 wild type the radiosensitive mutants *irs1* and *irs2* have not suffered a mutation leading to complete loss of a major peptide (perhaps not surprisingly given the similar growth properties and relatively subtle phenotypic effects as compared to other rodent radiosensitive mutants).

Gel Retardation analysis of nuclear extract

As a means of checking that proteins remained intact and functional after the manipulations involved in extract preparation band shift or "gel retardation" assays were performed. This type of assay (see Materials and Methods for details) involves detection of protein binding to a radioactively end labelled oligonucleotide by virtue of the associated reduction in the speed with which such an oligonucleotide migrates through a polyacrylamide gel.

Figure 4.4

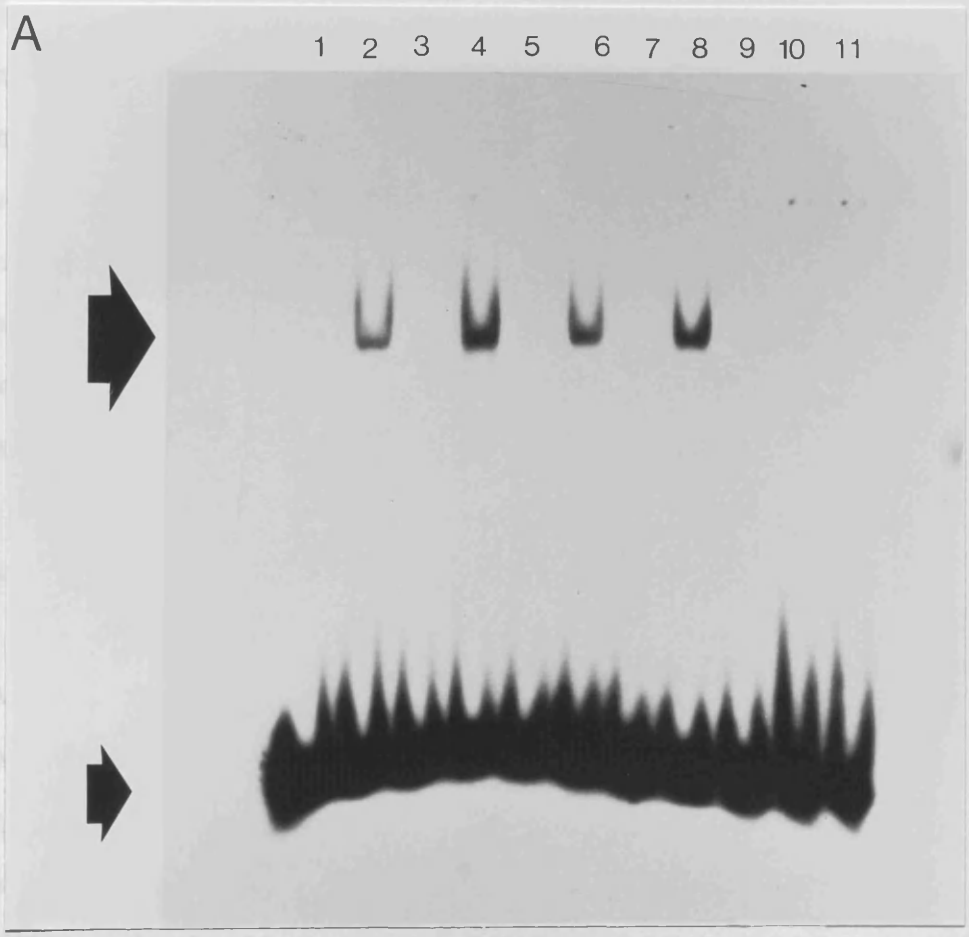
Denaturing SDS-PAGE analysis of nuclear extracts from V79. *irs-1* and *irs-2* cells



Nuclear extract proteins were separated following denaturing at 100^{o}C in SDS gel loading buffer. The stacking gel was 4% acrylamide 0.1% SDS pH 5.8 and the resolving gel 10% acrylamide 0.1% SDS pH8.8. The gel was Coomassie stained prior to destaining and photography as described in section 2.4.2.4.

Figure 4.5

Band shift assay of αP3A binding activity in nuclear extracts from V79 *irs-1*, *irs-2*, *irs-3* and whole cell extract from V79 cells.



B

GATCCAAACCAGCCAATGAGAACTGCGCCA

A: Band shift assay performed as described in materials and methods. Binding reactions were performed with 100-200 cps [^{32}-P]-γ ATP end labelled αP3A incubated for 1 hour at 4^{o}C in a volume of 20μl. 4μl of a 3mg/ml solution of a nonspecific competitor DNA poly d(I).d(C) representing a vast excess was present in all reactions. Volumes were made up with SB as described in materials and methods but containing 150mM NaCl. Lanes 3; 5; 7; 9 and 11 also included excess of unlabelled αP_3A as a specific inhibitor of binding to labelled oligonucleotide. 5μl of nuclear extract was included in binding reactions as follows: Lane 1: no extract; Lanes 2 and 3: V79; Lanes 4 and 5: *irs-1*; Lanes 6 and 7: *irs-2*; Lanes 8 and 9: *irs-3*. Reactions in lanes 10 and 11 contained 5μl V79 cytoplasmic extract.

The large arrow marks the position of migration of the specific αP_3A binding complex and the small the migration of unbound labelled oligonucleotide.

B: Nucleotide sequence of oligonucleotide aP_3A with recognition site for CCAAT box binding protein (CBP) underlined.

Thus if an oligonucleotide is incubated with a solution containing a sequence specific DNA binding protein for which it carries the consensus recognition site, the protein will bind its cognate sequence. This will be seen as a retardation of the migration of oligonucleotide as nucleoprotein complex as compared to the migration of free oligonucleotide ie a "band shift". Conditions of salt concentration, time and temperature may be established such that binding of the protein in question to its recognition sequence is favoured over non specific binding. That this binding is indeed specific can be indicated by running competition reactions in parallel in which excess unlabelled oligonucleotide is used to compete for binding activity precluding binding of protein to labelled oligonucleotide so preventing appearance of a retarded band on autoradiography following electrophoresis.

All extracts described in this study were assayed for the binding activity of the ubiquitously expressed CCAAT box binding protein (CBP) described by Cohen et al. 1986. This protein binds an oligonucleotide αP_3A (Plumb et al. 1989) containing its CCAAT consensus recognition sequence. Figure 4.5 shows one such assay from which it is clear that all extracts tested (in this instance V79 wild type and *irs1 2* and *3*) have an activity binding αP_3A to form a single tight retarded band. A cytoplasmic extract from V79 shows no αP_3A binding protein. In all extracts binding is shown to be specific by competition experiments, adding excess unlabelled αP_3A to the reaction and observing apparent disappearance of the shifted band. The appearance of a single specific band in Figure 4.5 suggests that the hamster extracts from V79 and the *irs* mutants contain all activities necessary for the formation of a complete αP_3A binding complex. Any nuclear extracts found not to have specific αP_3A binding activity were discarded (including a large series inactivated due to failure of the -70^{o}C freezer).

A panel of nuclear extracts from the wild type V79 and the radiosensitive *irs* mutants *irs-1*, *irs-2* and *irs-3* and a number of human extracts were prepared. Protein concentration was determined as described and extract volume adjusted to give equal numbers of nuclear equivalents of protein in equal volumes of each extract. All extracts used in subsequent studies contained functional CAATT binding proteins. There is no

obvious reason why DNA binding proteins should be more resistant to deleterious effects of the preparation protocol than any other class so the presence of an $\alpha P_3 A$ binding activity was taken as being a reasonable indicator of extract integrity.

4.1.3.1.4 Conditions for repair incubations.

To compare capacity of extracts of different origin to rejoin the cohesive compatible termini produced by PstI or EcoRI digestion of pIC20H the conditions of incubation favouring such a ligation must be determined. Under such circumstances DSB ends can anneal. In a solution containing only ligase activities this is a passive process depending solely on kinetic considerations. If the temperature of the solution is above a critical value (which depends on the GC content of the cohesive ends in question) the extent of random motion of the DNA and so its ends will favour dissociation. Lower temperatures will result in less random motion of ends and annealing will result. A ligase activity can then seal the phosphodiester backbone leading to regeneration of a duplex DNA molecule.

The situation when such ends are incubated with protein extracts is more complex. The outcome will now depend on the result of competition between activities favouring ligation and those favouring degradation of ends. Ends must come together in an intact form, with the rest of the molecule in a conformation such that annealing is possible. Annealing must occur and the resulting "pre ligation" complex be stable enough to resist degradation prior to sealing by a ligase. Any activity promoting these processes would render ligation of cohesive ends more probable, while activities slowing or inhibiting one or more of the stages outlined above might tip the balance in favour of degradation.

A number of experiments (Pfieffer and Vielmetter 1988; Thode et al. 1990)) looked at the processes of ligation of a number of different DSB ends catalysed by extracts from Xenopus eggs. The suggestion was made that an alignment protein is required to hold DNA terminii together prior to ligation. This protein would protect DNA ends from nuclease activities and hold them in the correct conformation for ligation. Thacker et al.(1988) postulate an end binding protein required to protect DNA DSB ends prior to their eventual ligation to explain their data (reviewed in Chapter one) on the rejoin

of DSB *in vivo*.

Even the measurement of a simple final ligation event in the pathway of DSB repair may thus be more complex than immediately obvious, conditions being required to reveal a ligation masked as little as possible by degredation.

On consideration of the manipulations to be performed in purification of reaction products it was decided to incubate reactions in a total volume of 350µl. DNA concentration of substrate must (Dugaiczyk et al.1975) satisfy the condition $i<j$ in order that recircularisation of linear substrate be favoured over end to end ligation. The equation of Dugaiczyk et al. (1975) states that $i=j$ when [DNA] = 51.1 / $(MW)^{1/2}$ where [DNA] is the concentration of DNA in g/l and MW is the molecular weight of the DNA involved. pIC20H is approximately 2.7kb, so taking the average MW of a base to be 300 the MW of pIC20H is $2.7x10^3$ x 300 for each strand ie approx $1.6x10^6$. The concentration of pIC20H above which formation of linear multimers is favoured is thus 40mg/l which is equivalent to 14µg in 350µl. This is much higher than the 50ng in 350µl (equivalent to 0.14mg/l) I used as a substrate concentration (5µl of a 10ng/µl stock) in the repair assay. The use of 50ng of pIC20H should be well within the limit of concentration favouring recircularisation of linear substrate. Detection of circularisation of the plasmid by bacterial transformation should thus be favoured.

The best incubation conditions to reveal the activity of the repair protein(s) were uncertain as the activities in question were undefined. As a starting point I considered the conditions for optimum activity of characterised ligases. T_4 DNA ligase which ligates adjacent 3' hydroxyl and 5' phosphate groups (Wiess et al. 1968) works efficiently in a wide range of buffers and is not inhibited by the prescence of dNTPs. It has a requirement for magnesium and ATP (Sambrook et al. 1989). The slightly different *E.coli* DNA ligase which seals nicks and cohesive ends only (Panasenko et al. 1977; 1978) also requires magnesium and ATP. The major mammalian DNA ligase, ligase I, apparently acts by the same mechanism and with the same requirements as the T_4 ligase. The other characterised ligase activity of mammalian cells DNA ligase II, exhibits a different substrate specificity but requires similar conditions for optimal activity (Soderhall and

Lindahl 1975). Vaccinia virus and yeast DNA ligases also exhibit the requirement for divalent cation and ATP (Kerr and Smith 1989). Thus it seems that the spectrum of conditions for efficient ligase activity is fairly broad, the essentials being an energy source (ATP or AMP) and a divalent cation (Mg2+). There are however ligation activities which deviate from these requirements. For example the ligation activity of Topoisomerase I is ATP independent and may even be inhibited at high concentrations of ATP. Similarly Mg^{2+} at a moderate concentration stimulates this activity but is not essential and at high concentration is inhibitory (Zijlstra et al. 1990). Mammalian DNA ligase II has a slightly higher pH optimum (7.9) than ligase I (7.6) but functions efficiently under the same conditions. The buffer recommended for use with T_4 ligase (Maniatis et al. 1982) contains ATP at a final concentration of 1mM, Magnesium at 10mM and DTT at 1mM, the pH is buffered by 50mM Tris HCl pH 7.6.

Polyethylene glycol (PEG) is commonly used to stimulate T_4 ligation, particularly of blunt ends. The effect appears to be one of promoting non enzymic cohesion of ends by macromolecular crowding (Zimmerman and Harrison 1985), high concentrations of PEG 8000 causing more than 2000 fold increase in the annealing of complementary cohesive bacteriophage λ DNA ends. The rate of enzymic ligation of blunt or cohesive ends was also increased by orders of magnitude on addition of high concentrations of PEG. Pheiffer and Zimmerman (1983) suggest conditions of PEG concentration that increase intramolecular circularisation, this is accompanied however by a much stronger stimulatory effect on the formation of linear multimers by intermolecular ligation. In terms of the theory of Dugaiczyk et al.(1975) PEG alters (increases) the effective DNA concentration such that even at *j:i* ratios favouring recircularisation the major product will be linear multimers. PEG was omitted from the ligation repair incubations as although it has a general stimulatory effect upon ligation, this effect is markedly greater upon intermolecular ligation than intramolecular rejoin. Although the addition of PEG to a repair reaction might stimulate the postulated activity it would not increase the probability of detecting such an activity using the bacterial transformation system. Bovine serum albumin, also recommended for T_4 ligase activity was omitted for the same reason.

Repair reactions were set up in a volume of 350µl in a buffer containing 1mM ATP and 10mM Magnesium at a pH of 7.6. Extracts were added in a volume of 100µl buffered in 50mM NaCl, 5mM $MgCl_2$, 20% glycerol at pH7.9. The final concentrations of salt in repair incubations were thus 60mM NaCl; 6mM $MgCl_2$; 6% glycerol and 1mM ATP.

Conditions of time and temperature were selected by considering the conditions used to assay characterised ligase activities, the conditions for T_4 ligation (Maniatis 1982) and the necessity of forcing the ligation / degradation equilibrium in the direction of ligation of ends. The challenge is to obtain the best balance in terms of stimulation of enzymic ligating activities ie long incubation times at physiological temperatures without adversly affecting the annealing of ends required prior to enzymic ligation. Assays for mammalian DNA ligases I and II (Arrand et al. 1979) are performed at 14^{o}C, many T_4 reactions are also performed at this temperature (Maniatis 1982) so this was chosen as the incubation temperature for repair reactions.

With respect to length of incubation time Table 4.4 below shows the results of incubation at 14^{o} of PstI cut or uncut plasmid for either 24 hours or 90 minutes expressed as the number of ampicillin resistant colonies per ng DNA after transformation of competent JM83 with the reaction products.

Table 4.4

Effect of incubation time on "repair" reactions at 14°

Treatment	Colony number per ng DNA transformed Time of incubation	
	90 mins	24 hours
uncut	1737	N.D.
uncut + 50µl V79	580	9
PstI cut	7.5	6
PstI cut + 50µl V79	77	0.7

All incubations were performed under buffer conditions and at substrate concentration described in the text. N.D. is "not determined".

Incubation of cut plasmid with V79 extract for 24 hours gives rise to products capable of transforming JM83 with an efficiency lower than that of cut plasmid incubated alone. This implies that the longer time of incubation is allowing the "ligase/degradase" equilibrium to be shifted in favour of degradation as opposed to ligation of cohesive ends. In contrast incubation with V79 nuclear extract under the conditions stated for 90 minutes can be seen in Table 4.4 to lead to a ten fold increase in transformation of JM83 to ampicillin resistance over that of background. The indication was that an incubation time of 90 minutes would enable detection of "repair" of a restriction endonuclease induced DSB.

Incubation of uncut plasmid with V79 extract leads to a drop in transformation frequency. This might suggest that this extract contains nucleases degrading uncut plasmid. The reduction in transformation seen after treatment of uncut plasmid with V79 extract is much greater on incubation for 24 hours than 90 minutes confirming the latter

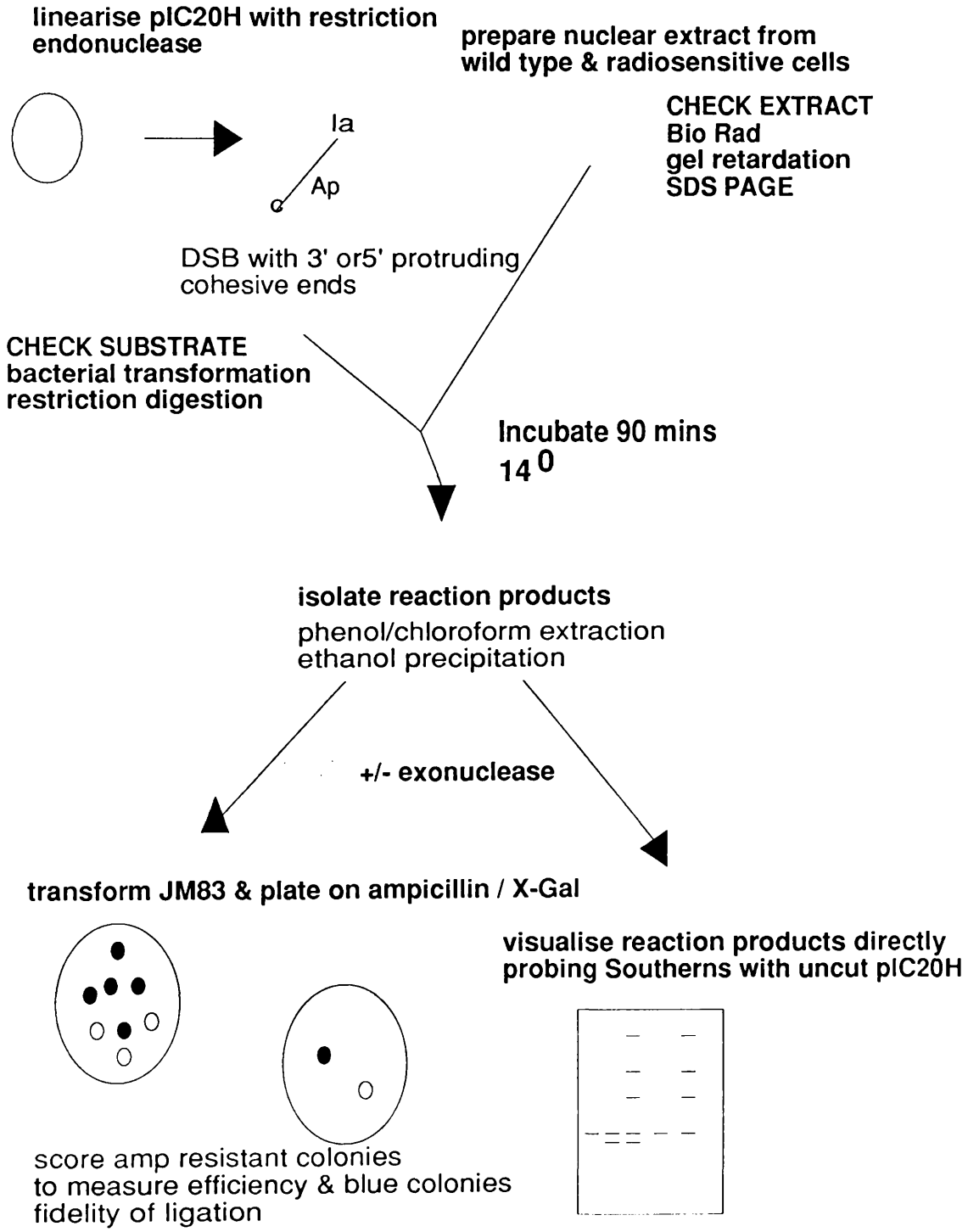
as a preferable time for repair incubation.

Figure 4.6 summarises the method and conditions established for detection of *in vitro* DSB rejoin. In summary, plasmid pIC20H was linearised in the *lac Z* gene and incubated in a solution containing ATP and magnesium at a DNA concentration to favour recircularisation. Incubations were performed for 90 minutes at 14^{o}C, with nuclear extracts from wild type or radiosensitive cells or T_4 ligase providing repair activity. Reactions without extract and with extract inactivated by boiling or proteinase treatment were included for comparison. DNA products of such reactions were phenol /chloroform extracted and ethanol precipitated before being used to transform competent JM83 bacteria. Transformation of an aliquot of uncut pIC20H was included in each experiment to show that competent cells could be transformed with comparable efficiency. Ligation efficiency was then asessed by the number of ampicillin resistant colonies resulting and ligation fidelity by the number of blue colonies when such transformed cells were plated on Xgal.

To visualise reaction products directly aliquots of DNA isolated after a repair reactions were subjected to Southern analysis , blots being probed with radiolabelled uncut pIC20H

Figure 4.6

Schematic representation of protocol established for the detection of DSB ligation in vitro



4.1.3.2 INCUBATION OF pIC20H WITH NUCLEAR EXTRACTS FROM V79 AND THE *IRS* MUTANTS

Table 4.5 summarises the data obtained by using the products isolated from various repair reactions with nuclear extracts from V79, *irs-1* and *irs-2* cells performed under the conditions just described to transform competent JM83 *E.coli.*

4.1.3.2 INCUBATION OF pIC20H WITH NUCLEAR EXTRACTS FROM V79 AND THE *IRS* MUTANTS

Table 4.5 summarises the data obtained by using the products isolated from various repair reactions with nuclear extracts from V79, *irs-1* and *irs-2* cells performed under the conditions just described to transform competent JM83 *E.coli*.

Table 4.5

Rejoin of an endonuclease induced DSB by nuclear extracts assayed by transformation of JM83 *E.coli*

Treatment[b]	Colony number per ng DNA transformed[a]	
	-λexonuclease	+λexonuclease
uncut	1737+/-478(14)	1704+/-753(4)
uncut + V79	580+/-220(6)	ND(c)
uncut + *irs-1*	690+/-250(6)	ND
uncut + *irs-2*	640+/-280(6)	ND
PstI cut	7.5+/-2.4(14)	0.4+/-0.2(6)
PstI cut + T_4 ligase	214+/-48(14)	208+/-88(5)
PstI cut + In extract	5.6+/-1.0(30)	0.8+/-0.2(16)
PstI cut + V79	77+/-20(9)	55+/-5.5(3)
PstI cut + *irs-1*	119+/-34(5)	43+/-4.4(3)
PstI cut + *irs-2*	12+/- 8(6)	3.2+/-2.4(3)
EcoRI cut	5.3+/-2.1(6)	0.3+/-0.2(6)
EcoRI cut + T_4 ligase	257+/-53(6)	227+/-77(6)
EcoRI cut + In extract	4.1(2)	0.4(2)
EcoRI cut + V79	65(2)	59(2)
EcoRI cut + *irs-1*	42(2)	49(2)
EcoRI cut + *irs-2*	8.5(2)	2.0(2)

(a) Each value represents the number of ampicillin resistant colonies per ng DNA used to transform JM83 with standard error indicated. For each treatment results using a number of different nuclear extract and substrate preparations are represented. The number of independent incubations is given by the figures in parenthesis.(b) Where extract was included in reactions 50μl was added. "In" refers to inactivated extract. (c) "ND" is not determined.

Effect upon uncut plasmid

Considering first the effect of nuclear extract upon uncut ie mainly closed circular DNA it is clear that V79 wild type and both *irs-1* and *irs-2* extracts have a significant effect in reducing transformation efficiency even at an incubation time of 90 minutes. This may be due to the presence of nucleases in the extracts or could be a passive effect. The lack of transformation inhibition by inactivated extracts suggests that the process is an active one (Brown unpublished). The effects of all extracts are equivalent, suggesting that neither *irs-1* nor *irs-2* compared to V79 have an excess of a nuclease activity with closed circular DNA as its substrate.

It is uncertain exactly what type of activity causes the reduction of transformation with uncut plasmid. The loss of transformation efficiency could be due to the loss of linear molecules present in the "uncut" plasmid preparation which would otherwise be recircularised by a bacterial activity. If the uncut plasmid preparation contained significant quantities of linear molecules one would expect a sharp decrease in transformation frequency on pretreatment with a nuclease that uses linear but not circular molecules as a substrate for example λ-exonuclease. Treatment of uncut plasmid with λ-exonuclease prior to transformation does not have a significant effect implying that the uncut population consists mainly of circles ie λ-exonuclease resistant forms.

If the reduction of uncut plasmid transformation after incubation with extract is due to nuclease activity it must be an endonuclease using either a closed supercoiled or a relaxed circular molecule as substrate. If the nuclease were causing loss of information, a proportion of the products of this reaction would lose β-galactosidase complementation activity. This would be seen as an increase in the ratio of white (β-gal negative) colonies after transformation of bacteria, however no increase is observed in the ratio of white colonies after plating on X Gal.

It is clear then, that all extracts have an activity reducing the transformation efficiency of uncut plasmid and that extract prepared from radiosensitive cells has an equivalent inhibitory effect to that from wild type.

Effect on cut plasmid

Treatment of PstI cut (DSB with 3' cohesive ends) substrate plasmid with 50µl V79 nuclear extract leads to a highly reproducible approximately one hundred fold increase over background λ-exonuclease resistant transformation (Table 4.5). Background is taken as being that shown after incubation of cut plasmid alone or of cut plasmid with nuclear extract inactivated by proteinase K treatment or by boiling for three to five minutes.

Comparing the net ligation activity shown by V79 with the activity of T_4 ligase there is a substantial drop of over 50% in the total ligation possible seen as ampicillin resistant colonies after transformation. This I suggest is due to the presence in the nuclear extract but not in the pure T_4 enzyme of nuclease, phosphatase and other activities. These activities may be acting either before ligation to reduce the effective concentration of substrate, or after to render ligated molecules replication or transcription incompetent. Alternatively the ligase activities present in V79 are intrinsically less efficient than that of T_4 or the concentration of V79 nuclear extract used in these incubations might not be sufficient for optimum activity. This latter possibility is unlikely since as Figure 4.7 (see later) makes clear the level of religation seen after incubation with 50µl V79 extract is maximal for this extract. Increasing the amount of extract added to the incubation to 100µl does not increase the number of ampicillin resistant colonies formed after transformation of reaction products. It is probable that due to the presence of conflicting activities the ligation seen in an extract will never be as efficient as the activity of a pure preparation of T_4 ligase.

Incubation with nuclear extract prepared from *irs-1* a radiosensitive mutant of V79, is seen in Table 4.5 to give rise to an increase equivalent to that observed for V79 extract in transformation of bacteria to ampicillin resistance. As with V79 the amount of rejoin exhibited by extract is less than that of the T_4 positive control, the considerations discussed above presumably also apply here, this drop being due to the requirement for ligase activity to compete with degradative (eg nuclease) or activities preventing ligation (eg phosphatases).

After incubation with V79 or *irs-1* extracts all colonies produced after transformation of competent bacteria and plating on X-gal are blue. This indicates that extract mediated ligation of the DSB introduced into the *lac Z* gene has occurred with fidelity, restoring the coding capacity of the gene and so allowing α-complementation.

The results given in Table 4.5 show nuclear extracts prepared from the radiosensitive *irs-2* cell line to be deficient in the religation of DSB substrate. Thus the addition of 50μg of *irs-2* nuclear extract produces little increase in ampicillin resistant colonies over background when reaction products are isolated and used to transform bacteria. This is in contrast to the situation with extracts prepared from V79 wild type or from the radiosensitive *irs-1* mutant. A Student's T test using the "minitab" computer program was applied to results from incubation with V79 and *irs-2* nuclear extracts. The probability of the difference between the number of ampicillin resistant colonies seen after incubation with 50μl V79 nuclear extract and that seen after incubation with inactivated extract having arisen by chance was found to be P=0.012. The difference between the means of the two sets of data is thus highly significant. The difference in the number of ampicillin resistant colonies resulting from incubation with inactivated *irs-2* extract and 50μl of active extract was not significant, P=0.54. (any low residual ligation activity of *irs-2* proceeds with the same fidelity as that of V79 and *irs-1*, since the ampicillin resistant colonies produced after incubation with *irs2* nuclear extract are blue.)

Table 4.5 also shows the effect of incubating an EcoRI induced DSB with nuclear extracts. EcoRI cleavage of pIC20H produces a cohesive ended DSB with four bp AATT 5'protruding ends. The levels of transformation following incubation of cut plasmid alone or with inactivated extract are very similar to those obtained using the PstI induced DSB. Further, the effect of T_4 ligase upon this plasmid seen as ligation to a λ-exonuclease resistant form is also equivalent.

Treating an EcoRI induced DSB with 50μl V79 wild type extract produces an increase over background religation equivalent to that given on treatment of a PstI induced DSB. As with a PstI induced DSB this religation occurs with a high degree of fidelity -no white colonies being observed after bacterial transformation by reaction

products. Again nuclear extract prepared from one of the radiosensitive mutants *irs-1* catalyses this rejoin with the same efficiency and fidelity as wild type. Extract prepared from another radiosensitive mutant *irs-2* shows on the contrary a vastly reduced capacity to religate an EcoRI induced DSB to give λ-exonuclease resistant transformation above background levels.

A slight difference is observed between the ligation by extract of the PstI cut and the EcoRI cut substrates. Incubation of nuclear extract with a PstI induced DSB substrate gives slightly more colonies than does incubation with an EcoRI cut substrate after bacterial transformation without treatment with λ-exonuclease (table 4.6). This could be a reflection of the different stability of the pre ligation complexes formed by the two sorts of molecule due to their differing end sequences. PstI ended (TGCA) DSB would thus be expected to form pre ligation complexes more readily and to produce annealed but not ligated cirles of greater stability than the EcoRI cut DSB with the less GC rich (AATT) ends. Incubation at 37^{o} for λ-exonuclease treatment would be postulated to overcome this slight but possibly significant difference in bond energy rendering both types of complex equally likely to dissociate and so be subject to exonuclease attack.

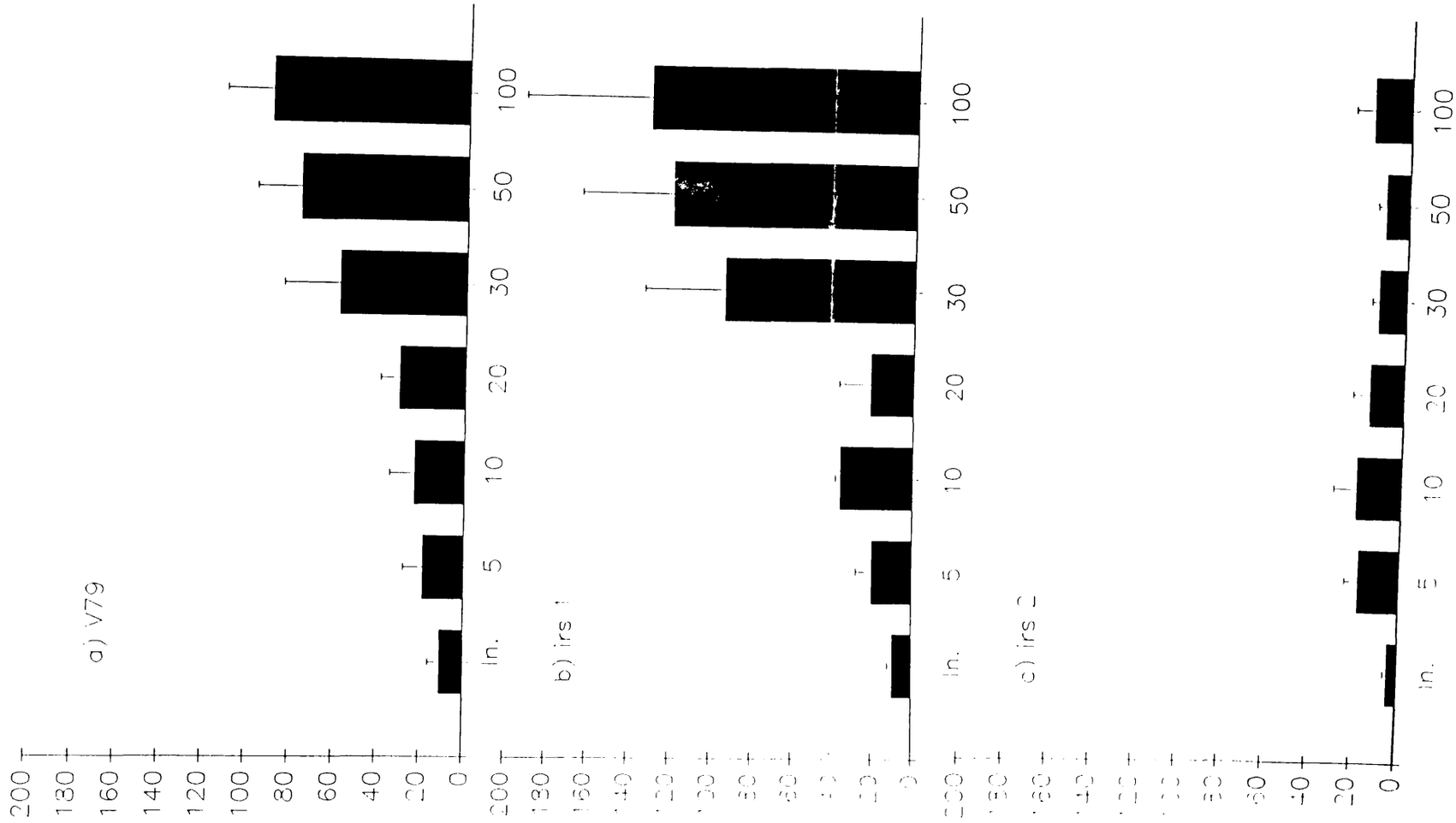
Figure 4.7 presents as a histogram the collected data on the dose response characteristics of the PstI induced DSB repair activity of V79, *irs-1* and *irs-2*. The comparison in each case is with the increase in capacity of reaction products from incubation of inactivated (In) extract to transform bacteria to ampicillin resistance. V79 wild type and *irs-1* show a clear dose response with increasing amounts of extract producing increasing numbers of ampicillin resistant *lac Z* positive colonies. In none of the extracts does the proportion of white colonies increase with dose of extract, ie all ligation procedes with fidelity. *Irs-2* nuclear extracts show no ligation dose response. Ligation catalysed by *irs-2* extracts never significantly rises above background even at the highest concentrations. Figure 4.8 shows a similar histogram for the dose response of the ligation activity of *irs-3* nuclear extracts. This data is more preliminary representing the results of few experiments and using only one extract preparation. It suggests that *irs-3* may have a religation capacity and extract concentration response intermediate between that of the

ligation deficient *irs-2* and wild type V79 extracts.

The results of experiments using V79, *irs-1* and *irs-2* nuclear extracts having been more conclusive comparison of these extracts and their further investigation was seen as a higher priority than refining the *irs-3* results. Thus in further investigation into DSB repair by extracts from wild type and radiosensitve cells I took V79 and *irs-2* cell lines as the system for study. In as many cases as possible results were extended by examination of the behaviour of *irs-1*.

Figure 4.7

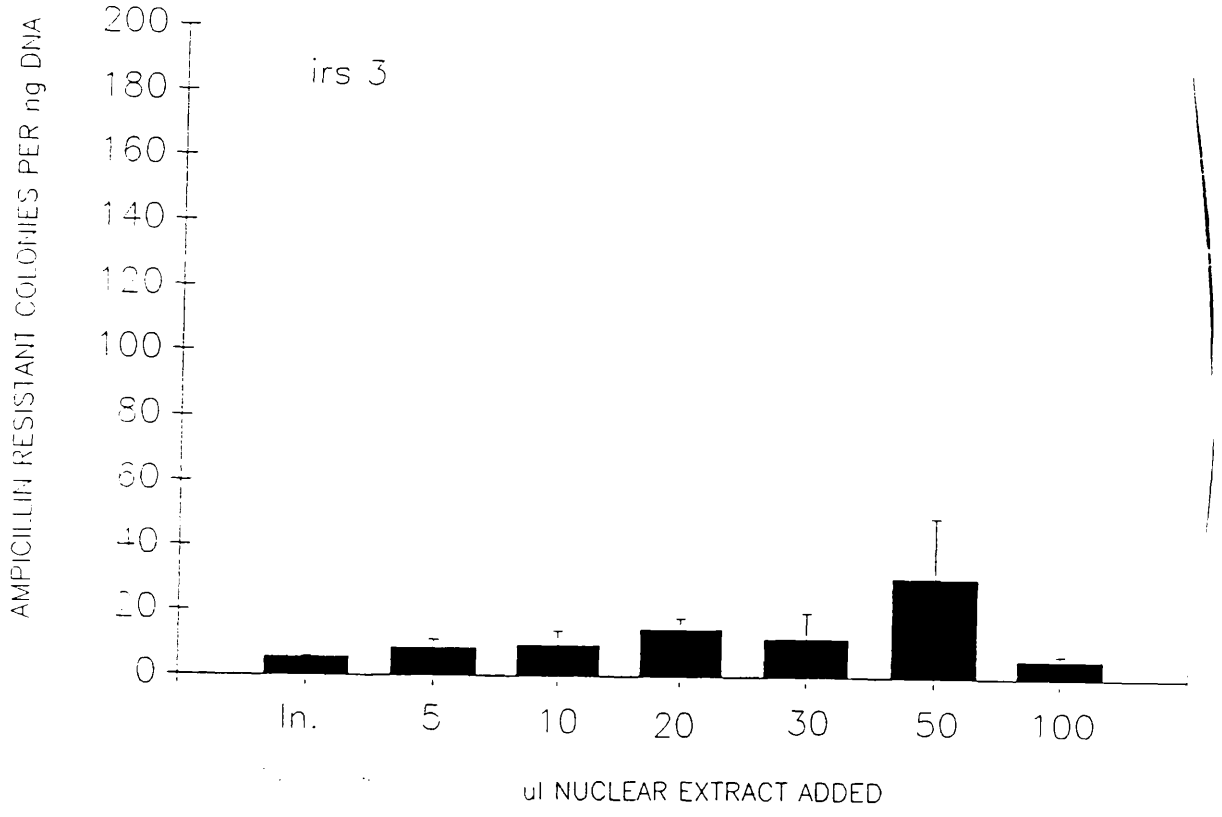
Dose response of ligation of a PstI induced DSB by nuclear extracts from V79, *irs-1* and *irs-2* cells.



Incubations performed as described in text. "In" refers to incubation with extract inactivated by boiling for 3-5 minutes. Each block represents the mean of at least nine independent determinations using at least three different preparations of nuclear extract. Errors are standard errors of the means.

Figure 4.8

Dose response of ligation of a PstI induced DSB by nuclear extract from *irs*-3 cells.



As for Figure 5.7 incubations performed under conditions described in text. "In" indicating incubation with inactivated extract. Blocks represent at least two independent observations. In some cases results from a single preparation of nuclear extract are given. Errors are standard errors of the mean.

To summarise:

* The *in vitro* assay for rejoin of an endonuclease induced DSB has allowed the detection by bacterial transformation of an activity recircularising linearised plasmid in nuclear extracts from V79 cells.

* This activity produces 15-20 times more ampicillin resistant colonies after transformation of bacteria by reaction products than control incubations with inactivated extract.

* Religation catalysed by V79 nuclear extract occurs with high fidelity, the number of white colonies seen after bacterial transformation and plating on X-gal being less than 0.1% of the total.

* Nuclear extracts prepared from one of the radiosensitive mutants *irs-1*, derived from V79 show the same capacity as those from V79 to rejoin an endonuclease induced DSB to a λ-exonuclease resistant form. Extracts from *irs-1* catalyse rejoin with similar efficiency and fidelity as V79.

* Nuclear extracts from *irs-2* cells show a much reduced capacity to catalyse ligation of linearised plasmid.

* DSB induced by PstI (3'protruding ends) and EcoRI (5' ends) are treated in an equivalent fashion by all extracts.

* Nuclear extracts from V79 and the radiosensitive mutants contain an activity reducing transformation efficiency of uncut plasmid. This activity appears similar in all extracts.

A number of questions are posed by the data so far presented. The first is the relative drop in maximal ligation activity seen in wild type V79 nuclear extract in comparison to that of the T_4 ligase. At the highest concentrations tested the activity of V79 is substantially lower than that of T_4. This I believe can be explained as suggested above by the presence in extracts (as opposed to pure ligase preparations) of activities inhibiting ligation making religation but one of a number of possible substrate fates.

Another anomaly is the complete absence of white colonies on transformation of

repair reaction products. If ligation in an extract is considered to be the result of a competition between ligation and degradation of substrate it would be expected that some molecules at least would lose information prior to ligation and so give rise to a white colony on transformation of bacteria.

Lastly an explanation is obviously required for the observed defect in ligation exhibited by *irs-2* as compared to V79 and *irs-1*.

4.1.3.3 MODEL DESCRIBING POSSIBLE ACTION OF NUCLEAR EXTRACTS UPON DSB SUBSTRATE

To clarify some of the experiments to be described I shall put forward a model describing the possible fates of a cohesive ended DSB substrate treated with a nuclear extract. I will discuss results so far presented in this context and amend the proposed scenario as discussion of further data requires.

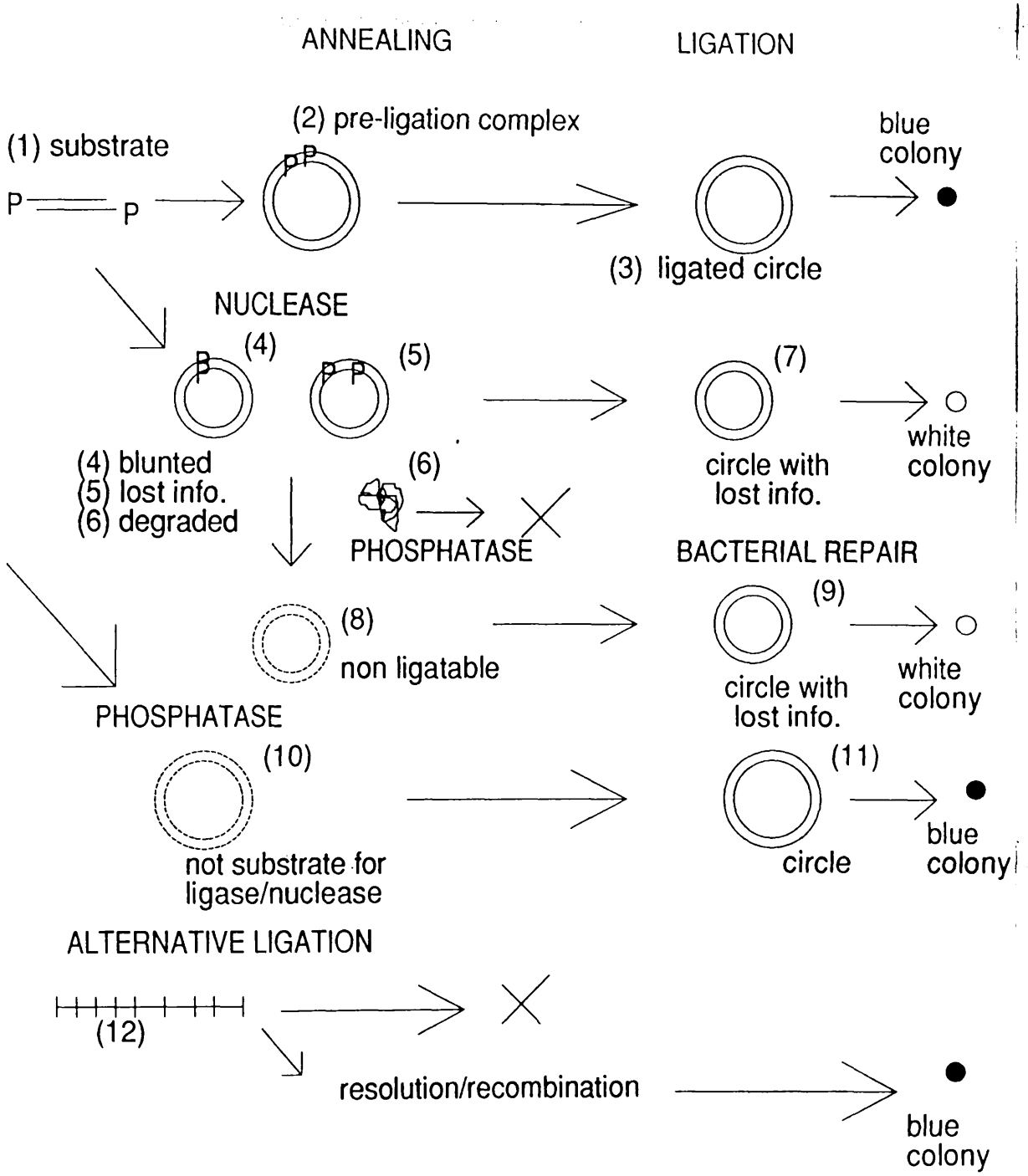
The main observation is the production of a blue colony after transformation of bacteria with the products of an incubation of a linear molecule having cohesive ends with nuclear extract (top line in Figure 4.9).

The simplest route for such a result would be ligation to form a circular molecule capable of transforming bacteria (ie from 1 direct to 3 and thence to a blue colony in Figure 4.9). This is postulated to be the situation on incubation of substrate with T_4 ligase. The process can be dissected into discrete stages, ie ends must come together in a more or less stable conformation to form what I have termed a "pre ligation" complex (2). This may then be sealed by ligase action to form a molecule (3) capable of transforming bacteria to lac Z positive ampicillin resistance giving a blue colony.

A number of activities present in an extract act to complicate this simple representation (see Figure 4.9). Firstly the action of nucleases on substrate might lead to a number of different pre ligation complexes. One (4) is the result of removal of protruding ends to produce a blunt ended pre ligation form. Although this molecule has the requisite end structure for ligation (3' hydroxyl and 5' phosphate groups) it will be less readily ligated by any activity analagous to previously characterised ligases. The presence of a blunt end would also be expected to make annealing less likely and further to give rise to a less stable annealed complex. Another product of the action of nucleases on substrate is a molecule with loss of information but with protruding ends (5). This molecule would be expected to anneal more readily and form a more stable complex than its blunt ended counterpart. Nuclease action could also result in the effective destruction of substrate giving molecule (6).

Figure 4.9

Possible fates of substrate molecules upon incubation with nuclear extract.



Input linear substrate is represented by molecule (1). Suggested fates of this molecule may be followed accross the Figure. Detailed description appears in text.

Of these products of nuclease digestion (6) would be lost, resulting in reduction of total colony number. (4) and (5) could be ligated to form circular molecules, (4) the blunt ended pre ligation complex with less efficiency than (5) the cohesive ended. Both these ligations would produce a circular molecule with information lost at the original DSB site (7), which, on transformation would give rise to a white colony.

The action of phosphatases on either of the pre ligation complexes formed from the products of nuclease action on substrate would render them unligatable by extract ligases giving molecule (8). A proportion of these would be stable to extraction and transformation (fewer of those derived from the blunt ended preligation complex than from that with cohesive ends) After transformation these might be repaired by bacterial kinase and ligase activities to produce a replication competent circular molecule again with loss of information (9). These would give rise to white colonies.

Phosphatases acting directly on substrate would lead to the formation an annealed preligation complex, molecule (10) with ends susceptible neither to nuclease action nor capable of being ligated. A proportion of this population being stable to isolation and transformation could be repaired in bacteria giving molecule (11). No loss of information would occur as phosphatase action was prior to nuclease attack, so a blue colony would result.

Another activity or set of activities competing for linear substrate might be alternative ligation reactions giving rise to more complex plasmid forms. The most likely of these is the production of linear multimers of substrate joined end to end in an intermolecular ligation (12). This type of molecule would not be expected to be capable of transforming bacteria. Such forms would only give rise to a blue colony after undergoing conversion to a circular molecule.

This scheme is guilty of a number of oversimplifications -it is of course the balance in any given extract between opposing activities for example nucleases and fill in polymerases, phosphatases and kinases that determines the outcome for any one of the molecules depicted in Figure 4.9. At any point proteins may be acting to stabilise or destabilise a particular molecule thus shifting the equilibrium between formation of that

molecule and its degredation. Furthermore it does not take into account either the role of DNA topology or torsional state or the enzymes such as gyrases, topoisomerases and helicases involved in maintaining and altering these parameters. Lastly the possibility exists that substrate DNA may become complexed in chromatin. If this were the case differences between extracts in terms of structural proteins might be important and again proteins altering and maintaining such structures could be pivotal.

To summarise, a model has been proposed in which the results obtained on incubating cut plasmid substrate with nuclear extract are seen as the result of the action of ligation activities, nucleases and phosphatases. The effects of these activities are complicated by the existance of alternative ligation pathways for ligation of substrate to a molecule unable to transform bacteria. Other considerations include the effects of topoisomerases and helicases as well as end binding proteins. The possible impact of chromatin structure must also be considered. It must be emphasised that the final outcome of any repair reaction will depend on the balance of activities acting on particular subpopulations of input substrate.

4.1.3.4 INCUBATION WITH A MIXTURE OF V79 and *IRS-2* EXTRACTS

Having identified a defect in nuclear extracts prepared from *irs-2* cells it was important to determine if this was a due to a loss of function. The result described might arise from lack of or deficiency in an activity required for recircularisation of linear substrate, or to an increase in an activity opposing this process. If the *irs-2* alteration were the loss of an activity the addition of V79 nuclear extract as a source of a complete set of ligation enzymes should restore the capacity of *irs-2* extract to seal a retriction endonuclease induced DSB.

Table 4.6 compares the effect on substrate (linearised with PstI or EcoRI) of addition of either V79 or *irs-2* nuclear extract alone with that of incubation with a mixture consisting of equal parts of *irs-2* and V79 extracts. The addition of a mixture of equal concentration V79 wild type and *irs-2* to a substrate with either a PstI or an EcoRI induced DSB gives an ampicillin resistant colony number above that produced by incubation of cut plasmid alone or with inactivated extract. This religation is shown (for the PstI cut substrate) to convert substrate to a λ exonuclease resistant form. It is equivalent to approximately half the rejoin capacity of V79 extract alone. It appears that the defect observed in *irs-2* can be corrected by the addition of V79 extract. Wild type extract is not inhibited in its recircularisation activity by the presence of *irs-2* proteins. These results indicating a recessive alteration are consistent with those from cell fusions (Jones et al. 1988, 1989) suggesting a recessive phenotype for the radiation sensitivity of *irs-2*.

Table 4.6

Comparison of the rejoin activity above that of inactivated extract of a 50:50 mixture of V79 and *irs-2* extracts with that of either extract alone.

Treatment	Colony number per ng DNA per µl extract	
	-λ-exonuclease	+λ-exonuclease
PstI cut + V79	1.4+/-0.2(24)	1.2+/-0.3(13)
PstI cut + *irs-1*	1.7+/-0.4(12)	1.1+/-0.3(8)
PstI cut + *irs-2*	0.2+/-0.05(25)	0.08+/-0.05(14)
PstI cut + V79/*irs-2*	0.6+/-0.3(12)	0.4+/-0.1(4)
EcoRI cut + V79	2.1(4)	1.6(2)
EcoRI cut + *irs-1*	1.7(3)	1.4(2)
EcoRI cut + *irs-2*	0.2(4)	0.04(2)
EcoRI cut + V79/*irs-2*	0.5(3)	ND

Data is shown as colony number above background levels per ng DNA per µl extract. Numbers in parenthesis indicate independent experiments, errors where shown are standard errors of the mean. For each set of results data from experiments involving a number of independent nuclear extract preparations are included. All colonies are blue, ie β-gal positive as well as ampicillin resistant.

4.1.3.5 VISUALISATION OF REACTION PRODUCTS BY SOUTHERN ANALYSIS

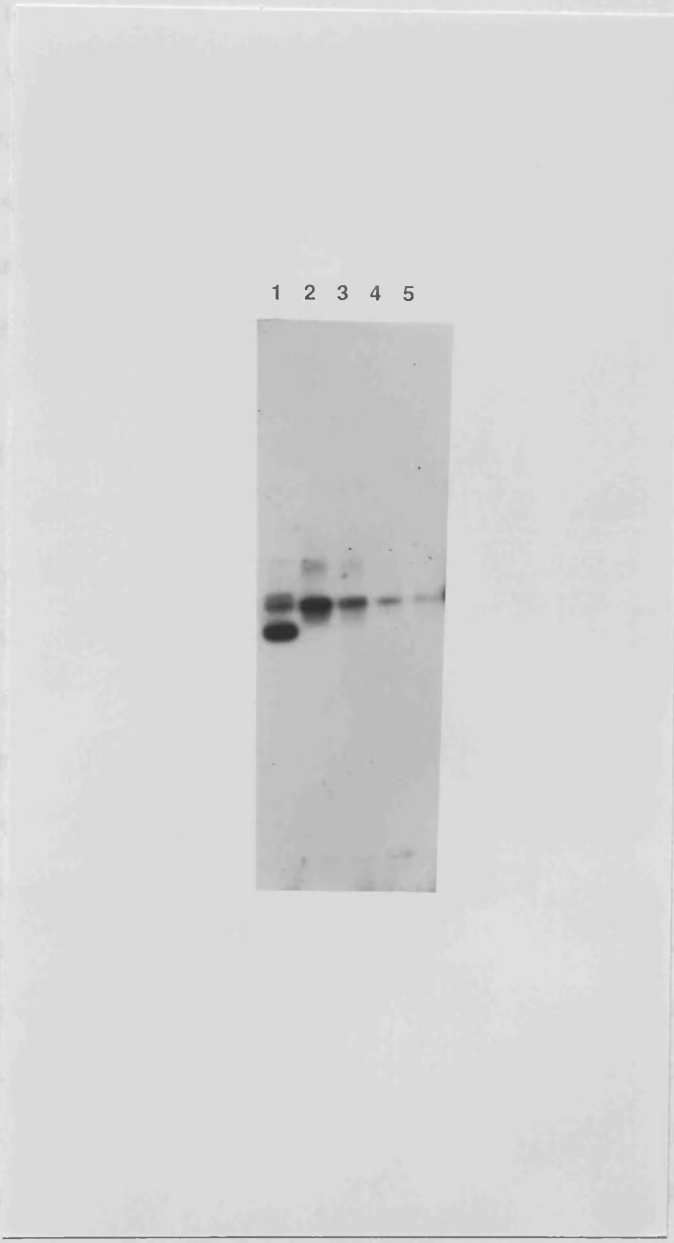
Reaction products from repair incubations were directly visualised by Southern blot hybridisation. DNA was isolated and precipitated and the various forms separated by electrophoresis through 0.8-1.0% agarose/ TAE gels (run without ethidium bromide unless otherwise stated). DNA was transferred to nitrocellulose and visualised by hybridisation to radiolabelled nick translated uncut pIC20H.

Reaction of nuclear extracts with uncut plasmid.

Figure 4.10 is a Southern blot showing the products resulting from incubation of pIC20H alone and with 20µl of nuclear extracts prepared from V79, *irs-1*, *-2* and *-3* cell lines. The resolution of this gel allows the visualisation of four forms of untreated uncut plasmid. The fastest migrating being closed circular molecules, those above nicked circles or linear forms. Migrating above these are multimeric forms. Under the conditions used nicked circles and linear molecules cannot be distinguished. Treatment with extract removes the closed circular fast migrating form. This could be a visualisation of the activity reducing transformation frequency of uncut plasmid implied by the results shown in Table 4.5. This is consistent with the assumption that circular molecules are required for efficient transformation of bacteria. The unequal loading of samples on the Southern in Figure 4.10 means it is not possible to say whether the effect on uncut plasmid is equivalent in all extracts.

Figure 4.10

The effect on uncut pIC20H of nuclear extract from V79, *irs-1*, *irs-2* and *irs-3* cells.



Lane 1: uncut pIC20H; Lanes 2-5: products of incubation of uncut pIC20H under standard conditions with nuclear extracts from; lane 2: V79; lane 3: *irs-1*; lane 4: *irs-2*; lane 5: *irs-3*.

Reaction of nuclear extracts with cut plasmid.

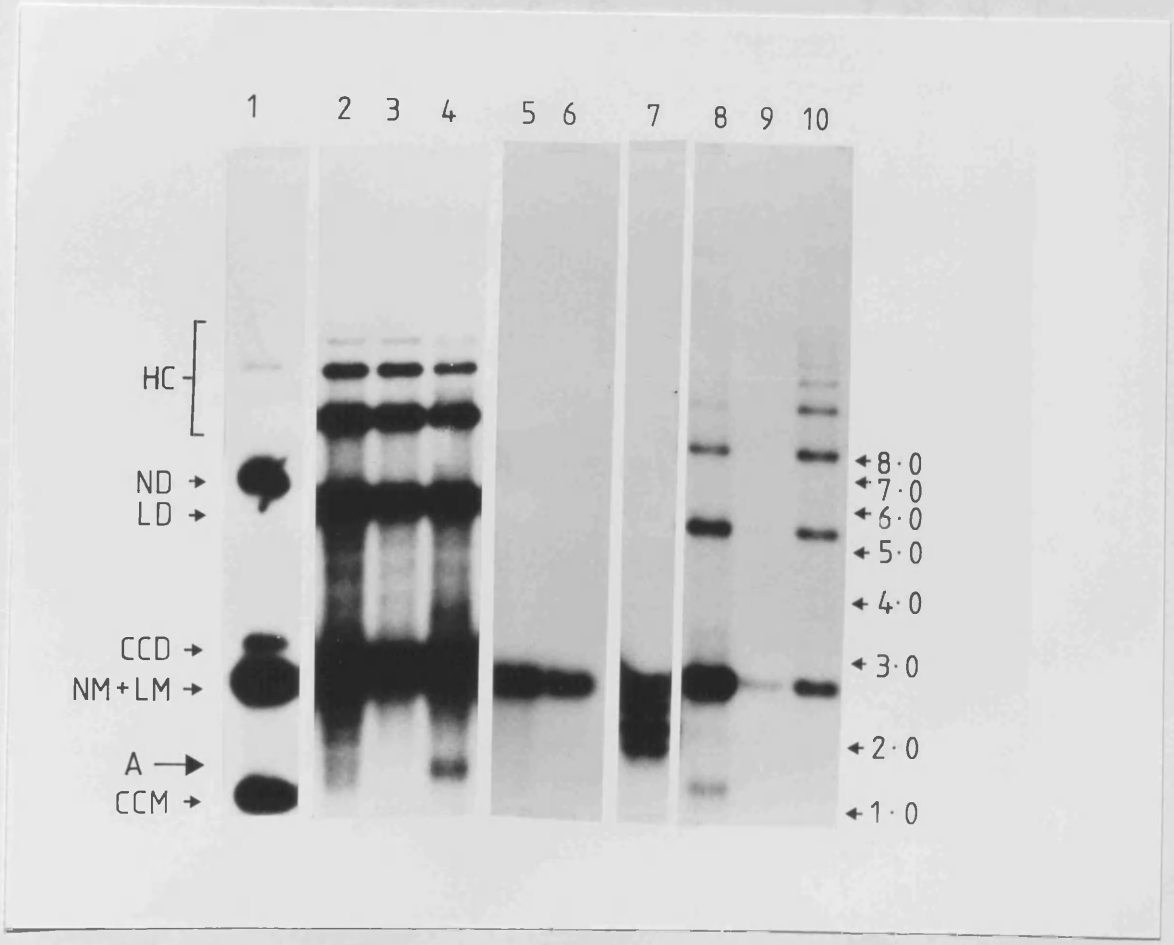
Figure 4.11 is a Southern showing the products of reaction of PstI cut pIC20H with nuclear extract from V79 wild type, *irs-1* and *irs-2* cell lines. This Figure exemplifies two consistent findings. The first is the production by all three extracts of high molecular weight forms. The second, the presence among the reaction products produced by incubation with V79 and *irs-1* nuclear extracts of a fast migrating closed circular form. This circular form is not seen after incubation with *irs-2* extracts.

Figure 4.11 also shows the effect of T_4 ligase and extracts from V79 and *irs-2* on an EcoRI cut substrate (5' four base overhang as opposed to PstI with a 3' four base protrusion). The findings from experiments using this substrate are equivalent to those involving a PstI induced DSB. T_4 ligase catalyses the production of a number of circular forms migrating ahead of the cut plasmid. Under these conditions of electrophoresis a relaxed circular molecule and a linear molecule migrate to the same point while a closed circular supercoiled molecule migrates ahead of these forms. V79 and *irs-2* extracts both give rise to high molecular weight concatemers. Incubation with V79 but not with *irs-2* extract produces among the reaction products a fast migrating form. This band (lane 8) is more discrete than that in for example lane 2. This variability probably arises from the presence of different amounts of catenating and decatenating activities in individual extract preparations.

Comparing Table 4.5 with figure 4.11 the visualisation among the products of a repair reaction of a fast migrating circular band (A) correlates well with the ability of such products to transform bacteria to ampicillin resistance. Thus such a band appears consistently on Southern analysis of the reaction products of incubations with V79 and *irs-1* but is not detected after incubation with *irs-2*. Incubation of cut plasmid with a mixture of V79 and *irs-2* extracts would be expected to give rise to such a fast migrating form. Figure 4.12 shows that this is indeed the case, products of reaction with T_4 ligase, V79 extract alone and a 50:50 mixture of V79 and *irs-2* nuclear extract containing 50µl V79 including a closed circular fast migrating band. This form is not detectable after reaction with *irs2* alone or with mixture containing only 25µl of V79.

Figure 4.11

Southern visualisation of products of reaction of PstI and EcoRI cut pIC20H with T_4 ligase and nuclear extracts from V79, *irs-1* and *irs-2* cells.



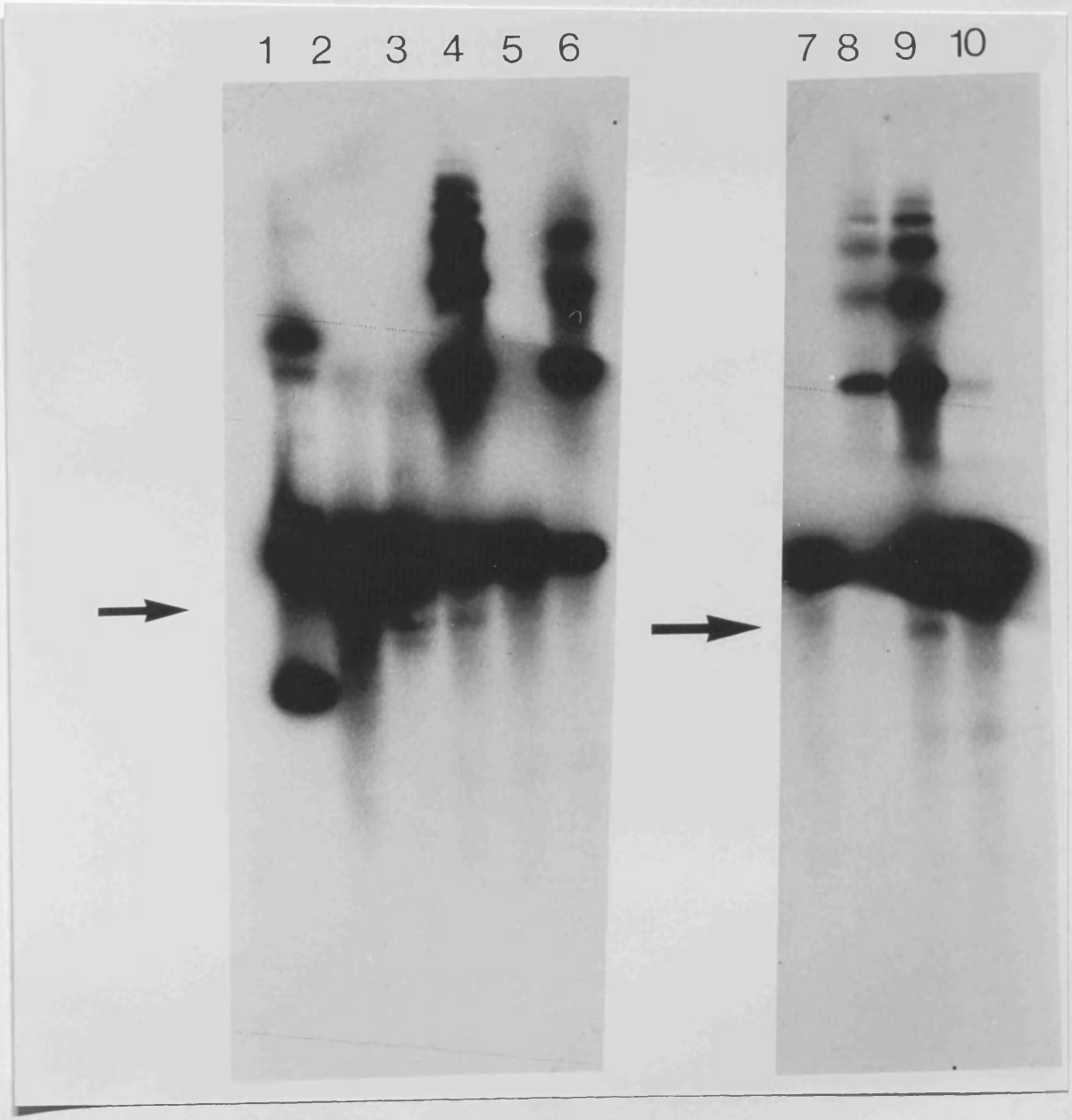
Gel run and Southern blot analysis performed as described for Figure 4.10 and in materials and methods.

Lane1, uncut plasmid pIC20H. Lanes2-7, PstI cut pIC20H and lanes 8-10 EcoRI cut pIC20H incubated with: lane2, V79 extract; lane3, *irs-2* extract, lane4, *irs-1* extract; lane5, no extract; lane6, inactivated V79 extract; lane 7, T_4 ligase; lane8, V79 extract; lane 10, *irs-2* extract. Numbers on the right of the figure indicatesizes in kb. Interpretation of bands: CCM, closed circular monomer; NM, nicked monomer; LM, linear monomer; LD, linear dimer; CCD, closed circular dimer; ND, nicked dimer, HC, high molecular weight concatamers.

"A" indicates the position of migration of the form presumed to be closed circular monomeric present among the reaction products of incubation of linear pIC20H with V79 or *irs-1* extracts but not after incubation with *irs-1* extract.

Figure 4.12

Southern visualisation of products of reaction of PstI cut pIC20H with T_4 ligase, V79 nuclear extract and a mixture of V79 and *irs-2* nuclear extracts.



Conditions of electrophoreisis and Southern blotting as described for Figure 4.10.

Lane 1: uncut pIC20H; lane 2: PstI cut pIC20H incubated alone; lanes 3-10: visualisation of products of incubation of PstI cut pIC20H with; lane 3: T_4 ligase; lane 4: 50µl V79 nuclear extract; lane 5: inactivated V79 nuclear extract; lane 6: 50µl *irs-2* nuclear extract; lane 7: inactivated *irs-2* extract; lane 8: 50µl of a 50:50 mixture of V79 and irs-2 nuclear extracts; lane 9: 100µl of a 50:50 mixture of V79 and *irs-2* nuclear extracts; lane 10: inactivated 50:50 mixture of V79 and *irs-2* extracts.

Arrows indicate position of migration of form presumed to be closed circular.

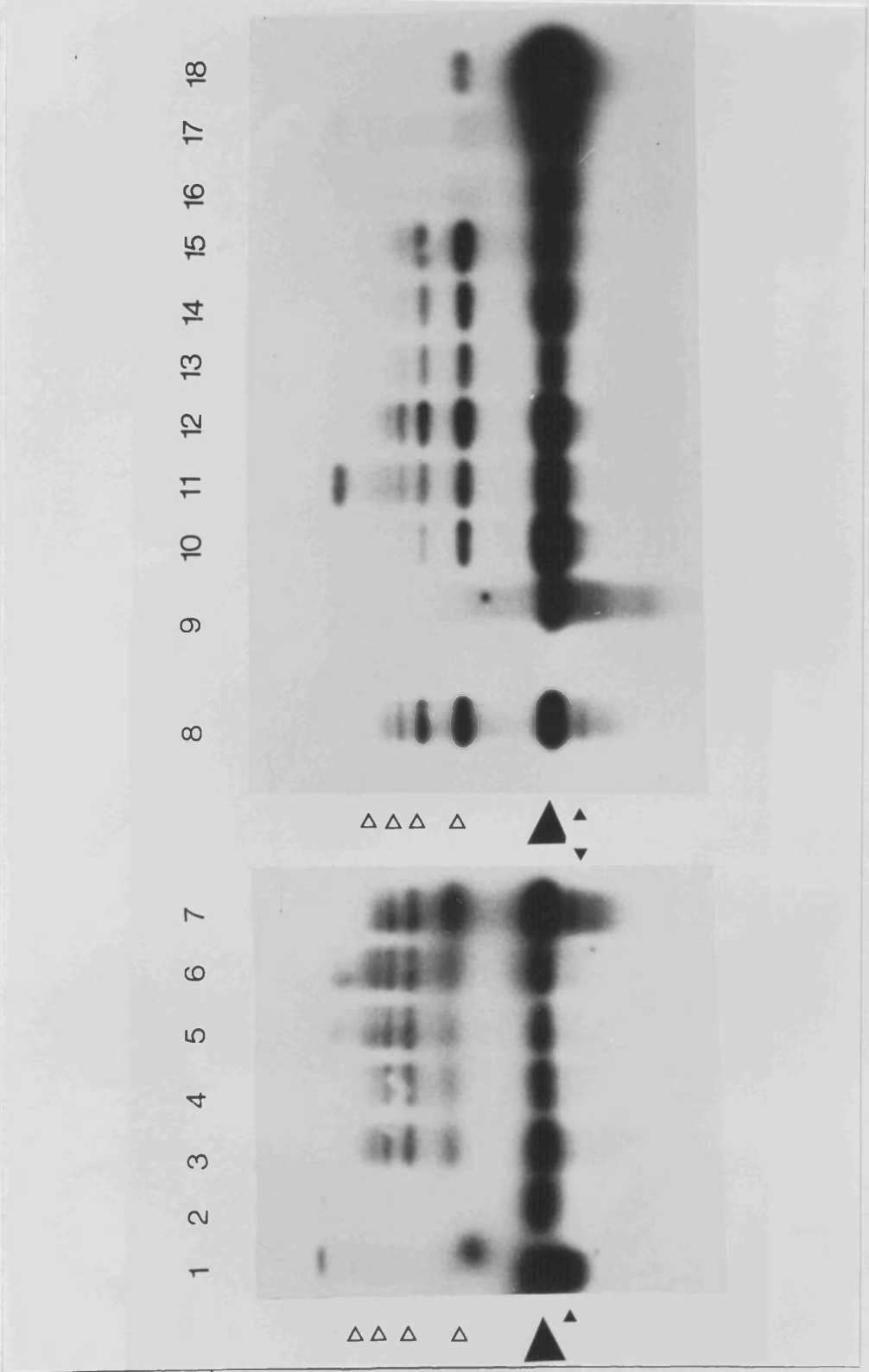
All active extracts catalyse the formation of high molecular weight concatemers. That detectable circle formation requires relatively high concentrations of V79 extract is also suggested in other experiments where the concentration of V79 required to produce this form is 50µl.

Figure 4.13 is a Southern visualisation of the products of the reactions of cut plasmid with increasing concentrations of V79 and *irs2* nuclear extracts. At even the lowest concentrations tested (5µl) both extracts show efficient concatamerisation. This activity is not dose responsive under these conditions appearing to have reached a maximum at the lowest level of extract. In contrast the recircularisation to produce a fast migrating molecule is only exhibited at higher concentrations of V79 extract ie those above 50µl. This separation of the two effects of V79 nuclear extract implies the involvement of different mechanisms in the generation of the two types of product. Circle formation and catalysis of the formation of a transforming molecule (but not concatemerisation) exhibit dose responsiveness over the concentrations of extract tested. Thus the results seen in Figure 4.14 increase the validity of the correlation between circle formation and bacterial transformation by products of reaction of plasmid substrate with nuclear extract.

The capacity of *irs-2* extracts to catalyse the formation of concatemers with an efficiency comparable to that of the equivalent activity seen in V79 and *irs-1* suggests that *irs-2* is capable of at least this type of ligation reaction. If the products isolated after incubation of a PstI cut substrate with nuclear extract from V79 cells or any of the *irs* mutants are recleaved with PstI the high molecular weight forms are abolished, Southern analysis showing only forms migrating at monomer size. Therefore the concatamers produced by treatment with all extracts are products of faithful ligation reactions. The capacity of *irs-2* extracts to perform faithful ligation might seem anomalous on consideration of the data presented in Table 4.5 where V79 and *irs-1* extracts are able to rejoin an endonuclease induced DSB as assayed by bacterial transformation while *irs-2* extract is not.

Figure 4.13

Southern visualisation of products of reaction of PstI cut pIC20H with T_4 ligase and increasing concentration of nuclear extracts from V79 and *irs-2* cells.



(Figure legend on following page)

Electrophoresis and Southern blotting as described for Figure 4.10.

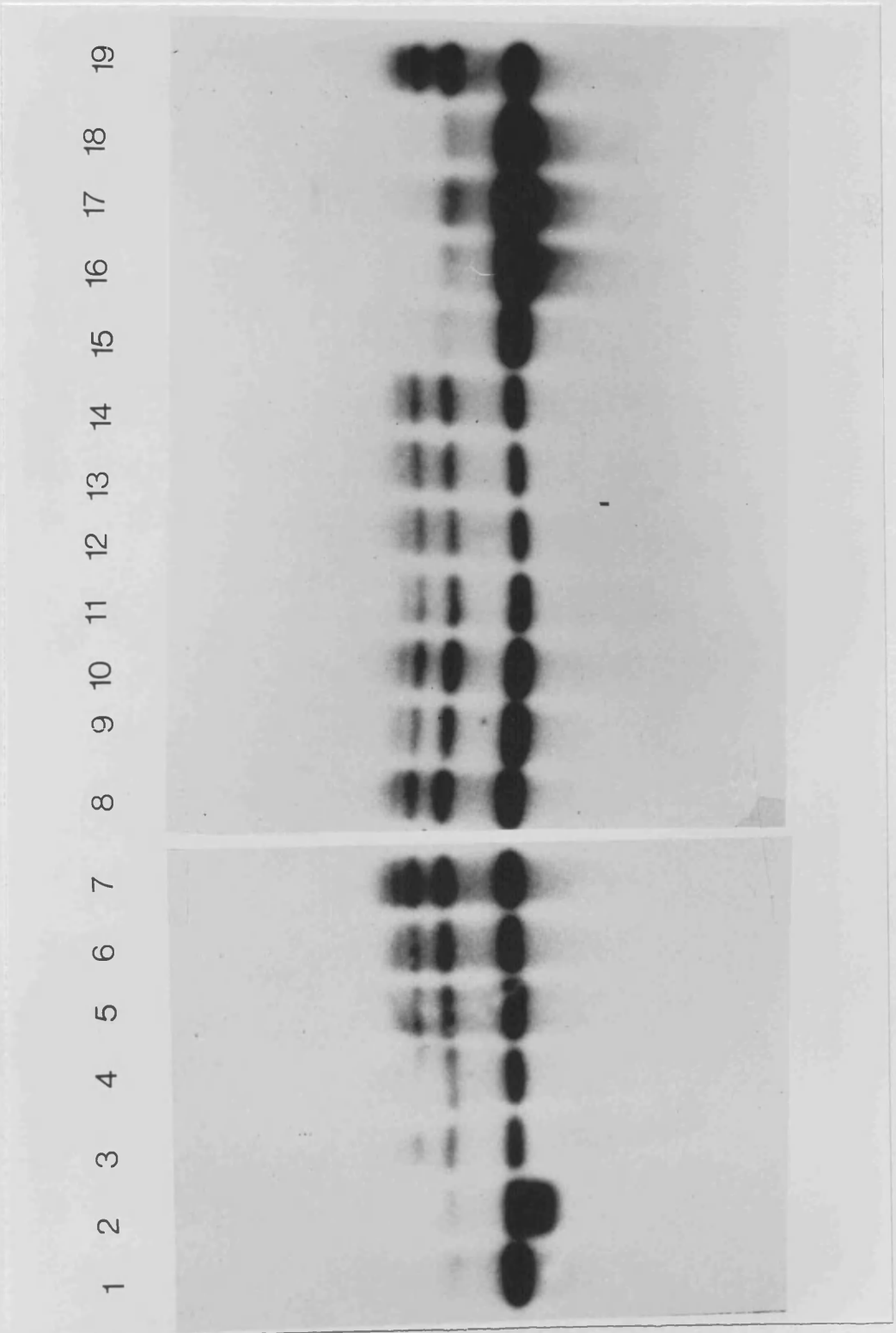
All lanes show products of incubation of PstI cut pIC20H under standard conditions. Lane 1: incubation with T_4 ligase; lanes 2 and 9: no extract; lanes 3-8 incubation with V79 nuclear extract, lane 3: 5µl; lane 4: 10µl; lane 5: 15µl; lane 6: 25µl; lane 7: 50µl; lane 8: 100µl. Lanes 10-15: incubation with *irs-2* nuclear extract, lane 10: 5µl; lane 11: 10µl; lane 12: 15µl; lane 13: 25µl; lane 14: 50µl; lane 15: 100µl. Lanes 16-18 inactivated extract. lane 16: boiled V79 extract; lane 17: boiled *irs-2* extract; lane 18: proteinase K treated V79 extract.

Open arrows indicate position of migration of high molecular weight concatamers. Large filled arrow shows position of migration of linear plasmid monomer and small filled arrows show position of preumed closed circular molecules.

Figure 4.14 is a Southern showing the reaction products isolated following incubation of cut plasmid with increasing concentrations of nuclear extracts from V79, *irs-1*, *2* and *-3*, cells. The gel was run under conditions leading to a lower resolution than seen in Figure 4.11 thus there is no visualisation of a fast migrating closed circular form on the addition of V79 or *irs1*. Similarly the forms produced by incubation with T_4 ligase can not be resolved from linear molecules or each other. All extracts, ie those produced from V79 wild type and *irs-1*, *irs-2* and *irs3* radiosensitive mutants as well as a mixture of V79 and *irs-2* extracts, can efficiently catalyse the formation of high molecular weight forms. Reaction with inactivated extract does not give rise to these forms indicating that concatemerisation is an active process. Over the range of concentrations tested here there is no detectable increase in concatemerisation with increasing extract concentration. In contrast the capacity of V79 and *irs-1* nuclear extracts to catalyse rejoin of cut plasmid substrate to a molecule capable of giving rise to bacterial transformation is strongly concentration dependant over this dose range (Figure 4.7). One possibility consistent with this is that concatamers cannot efficiently transform bacteria, transformation to ampicillin resistance requiring the circular form seen in the reaction products of V79 and irs-1 extracts.

Figure 4.14

Southern visualisation of products of reaction of PstI cut pIC20H with T_4 ligase and nuclear extracts from V79, *irs-1*, *irs-2*, *irs-3* and a mixture of extracts from V79 and *irs-2* cells.



(Figure legend on following page)

Conditions of electrophoresis and Southern blotting as described for Figure 4.10, in text and in materials and methods.

All lanes show products of reaction of PstI digested pIC20H. Lane 1: cut plasmid alone; lane 2: incubation with T_4 ligase; lanes 3-5: incubation with 5µl, 10µl and 50µl V79 nuclear extract; lanes 6-8: incubation with 5µl, 10µl and 50µl *irs-1* nuclear extract; lanes 9-11: incubation with 5µl, 10µl and 50µl *irs-2* nuclear extract; lanes 12-14: incubation with 5µl, 10µl and 50µl *irs-3* nuclear extract; lanes 15-18: incubation with inactivated extract, lane 15: V79; lane 16: *irs-1*; lane 17: *irs-2* and lane 18: *irs-3*. lane 19: incubation with 50µl of a 50:50 mixture of V79:*irs-2* extract.

Summary

* All nuclear extracts are shown to have an effect upon closed circular uncut pIC20H removing plasmid forms migrating at the distance expected of closed circles.

* The reaction products of incubation of cut substrate with all hamster extracts include high molecular weight plasmid concatamers. This effect is seen at the lowest concentration of extract tested and is not dose responsive over the range of concentrations used. In terms of this activity nuclear extracts from V79, *irs-1* and *irs-2* are equivalent.

* V79 and *irs-1* extracts but not *irs-2* catalyse the formation of fast migrating circular forms. This does seem to be a dose responsive effect and correlates with the increase in capacity of reaction products to transform bacteria to ampicillin resistance.

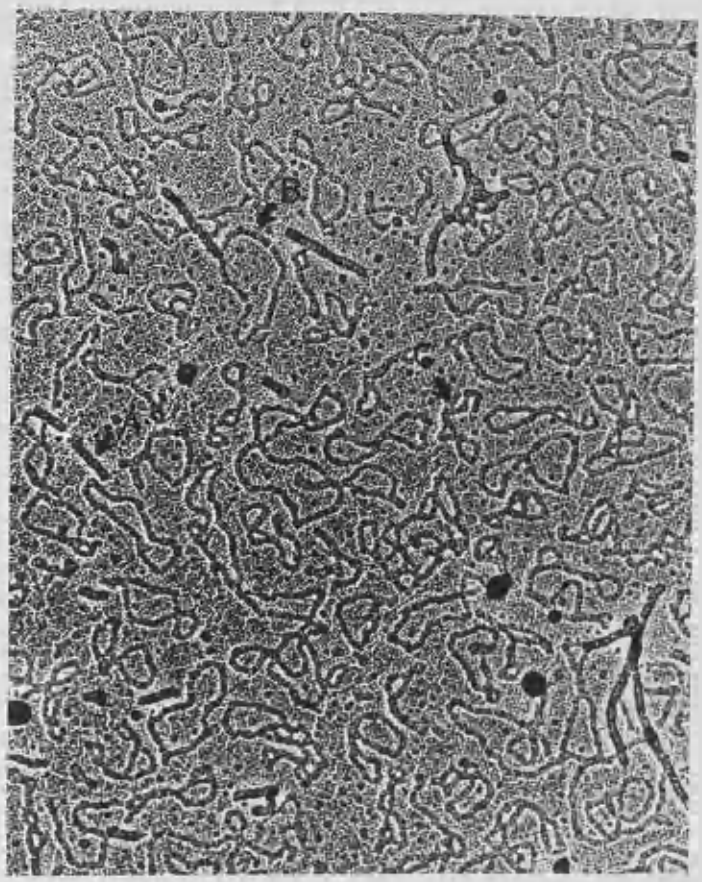
4.1.3.6 VISUALISATION OF REACTION PRODUCTS BY ELECTRON MICROSCOPY

Visualisation by Southern analysis does not allow discrimination between high molecular weight linear multimers and equivalent intertwined circular forms. In collaboration with Dr Coggins (Beatson Institute) we analysed the products of reaction of V79 extract with PstI cut substrate under the transmission electron microscope (EM).

DNA in solution exists as a three dimensional random coil, for visualisation by electron microscopy this must be converted to a two dimensional array of unaggregated molecules. To achieve this basic protein is mixed with a solution of nucleic acid and used to form a monolayer at a water-air interface. The resulting complex can then be adsorbed to a supporting film mounted on an EM grid. To avoid subjecting the sample to the shearing forces methods involving diffusion have been introduced as means of producing a nucleic acid-protein monolayer for EM examination (Coggins 1987). Figures 4.15, 4.16 and 4.17 are electron micrographs resulting from the transmission EM examination of the results of incubations of uncut pIC20H (Fig 4.15), cut plasmid with V79 nuclear extract (Fig 4.16) and cut plasmid with inactivated nuclear extract (Fig 4.17). A preparation of uncut plasmid (Fig 4.15) can be seen to consist of a number of different circular forms, representing different topoisomers of closed and open circular DNA molecules. The products of reaction of cut plasmid with V79 nuclear extract (Fig 4.16) comprise mainly of linear molecules of varying lengths each an integral multiple of that of monomer. A relatively small (only one is observed in the field shown in Figure 4.17) population of monomer sized circles is also seen. No catenated high molecular weight circular forms can be discerned. Figure 4.17 shows as expected monomer sized linear molecules, indicating the lack of effect of inactivated extract upon uncut DNA. The implication is that concatamers are produced in great excess over circles and that these concatamers are linear multimers as opposed to circular forms.

Figure 4.15

Electron microscope visualisation of incubation of uncut pIC20H without nuclear extract.

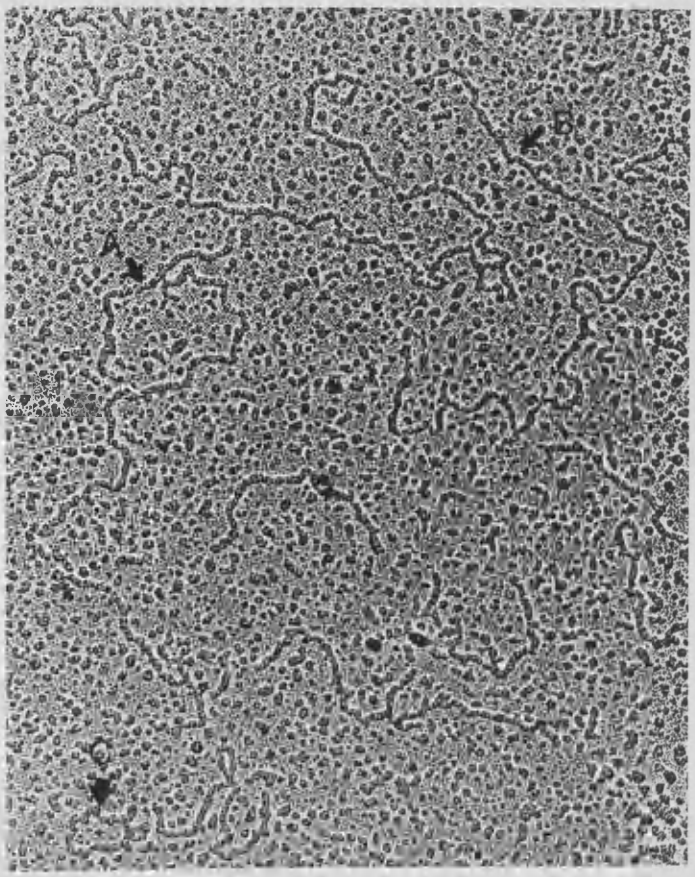


Electronmicrograph of uncut pIC20H isolated after incubation under standard conditions without nuclear extract. Transmission electron microscopy performed by Dr Coggins using spreading technique as described in text and in Coggins (1987).

Arrows labelled "A", "B" and "C" indicate different topological forms of the uncut pIC20H. A and C: closed circular molecules with different superhelical densities; B: open circular.

Figure 4.16

Electron microscope visualisation of products of incubation of PstI cut pIC20H with V79 nuclear extract

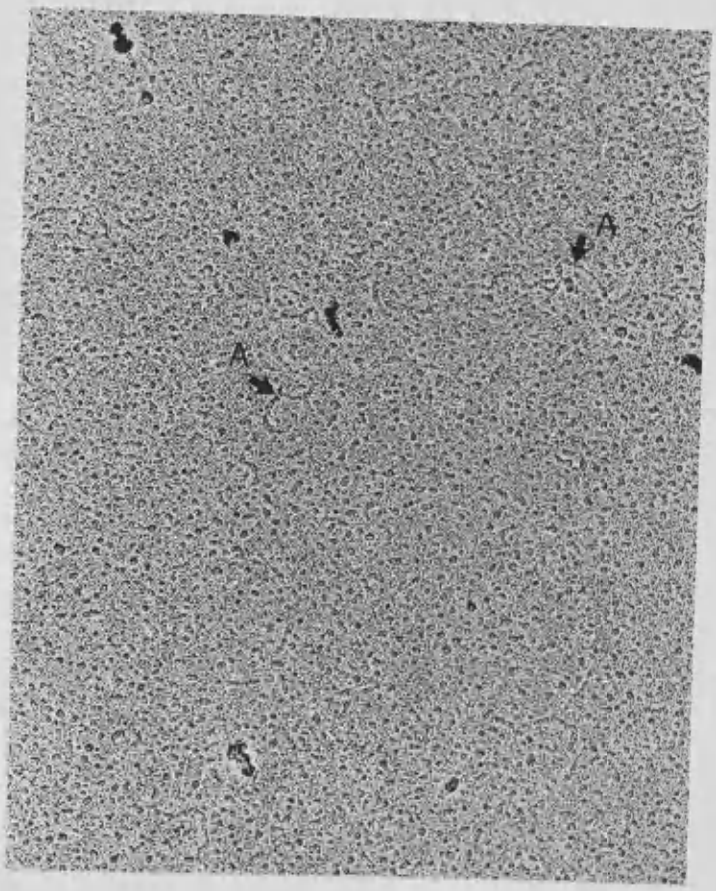


Electron microscopy as described for Figure 4.15.

This electron micrograph shows molecules isolated after reaction of PstI linearised pIC20H with V79 nuclear etract under standard conditions. A: linear monomer; B:linear multimer; C: open circular form.

Figure 4.17

Electron microscope visualisation of products of incubation of PstI cut pIC20H with inactivated V79 extract.



Electron micriscopy as for Figure 4.15.

This Figure shows products of reaction of PstI cut pIC20H with V79 nuclear extract inactivated by boiling. A: indicates representative linear monomeric forms.

4.1.3.7 BACTERIAL TRANSFORMATION BY LINEAR CONCATAMERS and CIRCULAR MONOMERS OF pIC20H

In terms of the model outlined in Figure 4.9 concatamer formation could be a competing activity reducing the amount of substrate availiable for recircularisation to a transforming molecule. This would require that concatamers be incapable of transforming bacteria to ampicillin resistance. Table 4.7 shows data from an experiment performed to test this in which gel purified concatamers were used to transform competent JM83 bacteria in parallel with cut plasmid, cut plasmid after T_4 ligation and uncut plasmid. The concatamers used in this experiment were dimers, trimers and quadrimers of cut pIC20H produced by overnight incubation with T_4 DNA ligase and then purified away from monomeric circular forms.

Table 4.7

Comparison of the efficiencies of transformation of competent JM83 to ampicillin resistance by pIC20H as circular, concatemeric, linear or uncut forms.

Treatment	Colony number per ng DNA transformed
uncut	274
EcoRI cut	4
EcoRI cut + T_4	231
concatamers	7*

Each value represents the average of two independent determinations. The EcoRI cut + T_4 value is taken as that of a population in which ligation to circle forms has gone to completion.

* In this experiment all ampicillin resistant colonies were blue ie X-gal positive except for an average of one per ng of concatamer DNA transformed (white colony frequency for cut control below level of detection ie less than 1 per ng transformed).

Table 4.7 shows concatamers to be incapable of efficiently transforming bacteria. This adds further weight to the suggestion that monomeric circular molecules as visualised among the reaction products of V79 and *irs-1* extracts but not among those of *irs-2* are required for efficient transformation of bacteria.

4.1.3.8 EFFECT OF λ-EXONUCLEASE TREATMENT UPON REACTION PRODUCTS

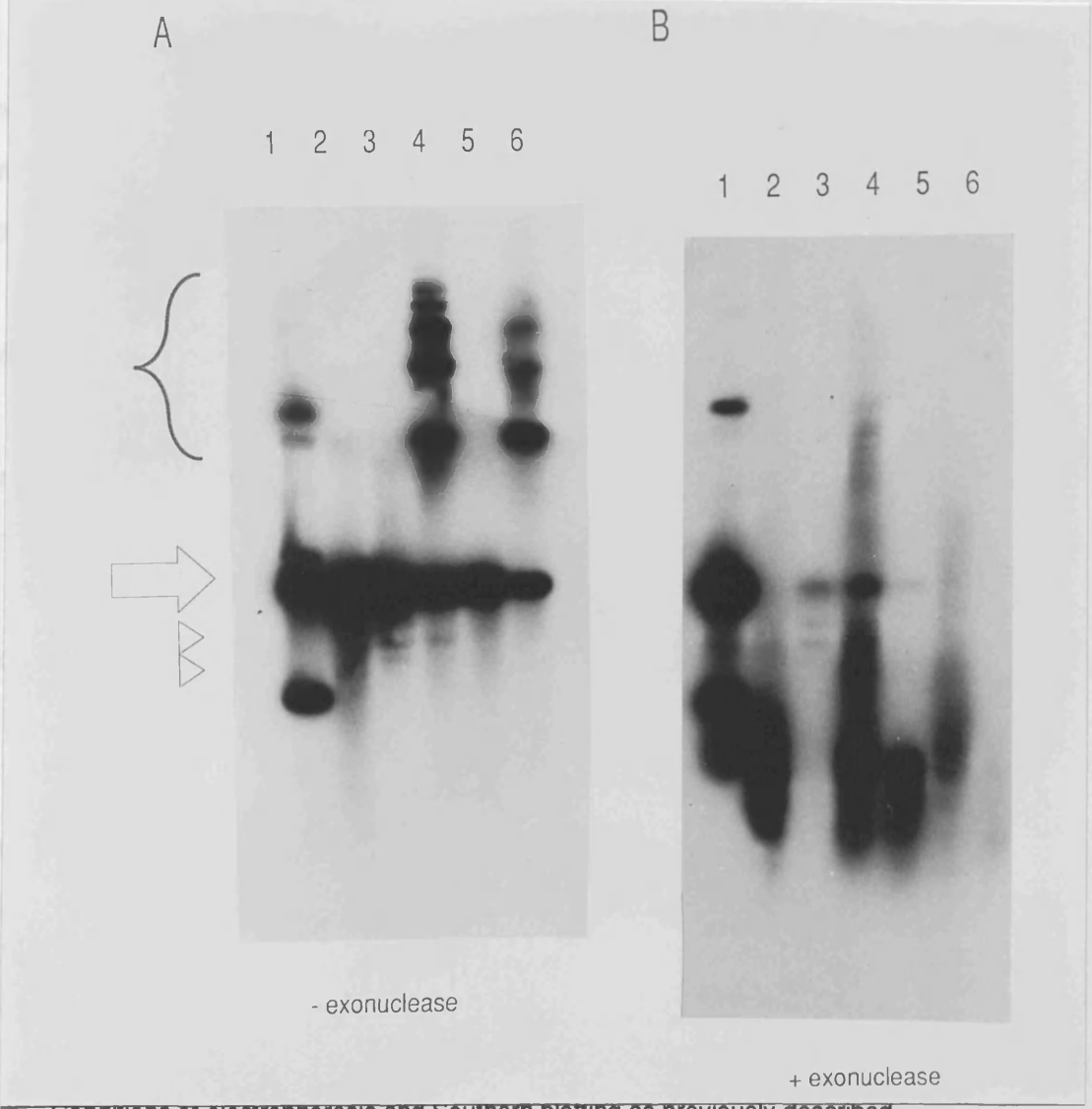
The data shows that in comparison to V79 and *irs-1*, *irs-2* extracts cannot efficiently catalyse the ligation of an endonuclease cut plasmid to produce a molecule capable of transforming JM83 bacteria to ampicillin resistance. There is evidence from Southern analysis that this deficiency correlates with an inability to recircularise this substrate to give a closed circular form migrating ahead of the linear.

The defect in *irs-2* nuclear extracts could be one of recircularisation. Southern analysis carried out as described does not resolve open circular forms from the linear. Treatment of reaction products with λ-exonuclease removes linear molecules. If after such treatment reaction products are visualised migrating to the same distance as linear plasmid they can be assumed to be open circular.

Figure 4.18 shows the Southern visualisation of reaction products of incubation with uncut plasmid and of cut plasmid alone and with T_4 ligase, V79 or *irs-2* extracts and inactivated extract. Panel A shows an aliquot run directly after isolation and panel B aliquots of the same reaction products treated with λ-exonuclease prior to Southern analysis. Uncut plasmid is seen to be unaffected by exonuclease treatment. The linear forms from reactions of cut plasmid alone or with inactivated extract are reduced to a fast migrating smear, as are the high molecular weight concatamers produced by incubation with either active V79 or active *irs-2* extract (providing further evidence that these are linear forms). Incubation with V79 nuclear extract or T_4 ligase is seen to give rise to a number of closed circular forms migrating ahead of linear plasmid, these are λ-exonuclease resistant. Furthermore a proportion of the molecules resulting from these incubations migrating with linear substrate are unaffected by λ-exonuclease treatment. These are presumed to be nicked circular forms. In contrast the reaction products of incubation of substrate with *irs2* include no λ-exonuclease resistant forms all being reduced to a smear. Nuclear extract prepared from V79 cells is thus shown to catalyse the formation of nicked circular forms migrating with linear pIC20H as well as the fast migrating closed circular molecules. Nuclear extract from *irs-2* cells shows no detectable ability to catalyse the formation of either type of circular form.

Figure 4.18

Southern visualisation of the effect of λ-exonuclease treatment of products of reaction of PstI cut pIC20H with T_4 ligase and nuclear extracts from V79 and *irs-2* cells.



Conditions of electrophoresis and Southern blotting as previously described (Figure 4.10 and materials and methods). All incubations performed under standard conditions.

Panel A: Products of incubation of lane 1: uncut pIC20H alone; lanes 2-6 PstI cut pIC20H, lane 1: alone; lane 2: with T_4 ligase; lane 3: with 50µl V79 nuclear extract; lane 4: with inactivated nuclear extract; lane 6: with 50µl *irs-2* nuclear extract.

Panel B: A 25ng aliquot of each of the products shown in panel A was treated under manufacturers recommended conditions with λ-exonuclease. 5ng aliquots of the products of these reactions were then subjected to Southern analysis as previously described. Lanes 1-6 as in panel A.

The bracket shows the positions of migration of high molecular weight concatamers, the large arrow that of linear plasmid and nicked circular forms and the arrow heads indicate positions of migration of closed circular forms.

4.1.4 SUMMARY AND CONCLUSIONS

* Conditions have been established allowing the ligation of an endonuclease induced DSB to be detected by bacterial transformation.

* Nuclear extracts from V79 wild type and one radiosensitive mutant derived from it *irs-1* catalyse rejoin of an endonuclease induced DSB producing about an hundred fold increase in the number of ampicillin resistant colonies over that seen transforming bacteria with cut plasmid incubated alone.

* Another radiosensitive mutant of V79, *irs-2* shows a much reduced capacity to catalyse this reaction.

* All ligation occurs with high fidelity shown by the production of a blue colony when plating on X-gal. This implies faithful religation of the DSB originally introduced into the lacZ gene.

* Mixtures of irs-2 and V79 nuclear extracts produce an increase over background in the number of ampicillin resistant colonies equivalent to that expected from the amount of V79 present in the mixture.

* Southern analysis of reaction products from incubation with extracts from wild type and *irs-1*, *irs-2* and *irs-3* cells shows all extracts to catalyse the formation of high molecular weight concatamers.

* This concatemerisation activity is maximal at all extract concentrations tested.

* The concatamers formed are shown by λ-exonuclease treatment and by electron microscopy to be linear molecules.

* Such high molecular weight linear molecules do not efficiently transform bacteria, transformation to ampicillin resistance appearing to require a circular form.

* Southern analysis detects the formation by V79 and *irs-1* extracts of fast migrating closed circular molecules.

* No closed or open circular forms can be detected after reaction of cut plasmid with *irs-2* extracts.

* The appearance of a fast migrating form amongst the reaction products of incubation with an extract seems to correlate with the ability of that extract to catalyse

ligation of cut plasmid to give a substantial increase over background numbers of ampicillin resistant colonies on bacterial transformation.

* Nuclear extracts prepared from V79 wild type cells, and from radiosensitive mutant lines *irs-1*, *irs-2* and *irs-3* all contain an activity or activities reducing transformation by uncut pIC20H.

4.2 BIOCHEMICAL NATURE OF *IRS-2* DEFECT.

4.2.1 INTRODUCTION.

4.2.1.1 Defect in Irs-2.

As already described the *irs-2* line consistently shows an inability to catalyse the religation of either a PstI induced or an EcoRI induced DSB to give molecules capable of transforming JM83 bacteria to ampicillin resistance. In contrast nuclear extracts prepared from *irs-1*, another radiosensitive mutant derived from V79 shows no such defect, reaction products transforming bacteria as efficiently as those isolated from incubations involving the same concentration of wild type extract.

This inability of *irs-2* to catalyse an increase over background transformation correlates with the lack of detection of circular molecules among reaction products of incubation with *irs-2* extract with endonuclease linearised pIC20H. Such circular molecules do appear on Southern blots of reaction products from incubations with wild type or *irs-1* extracts. Addition of wild type extract to *irs-2* allows religation of both types of DSB studied to produce molecules capable of transforming bacteria with an efficiency equivalent to that expected from the amount of V79 extract in the mixture. Southern analysis of the reaction products of incubation of linearised pIC20H substrate with a mixture of V79 and *irs-2* extracts shows that circular molecules are formed. Thus it appears that the difference observed between the wild type religation activity and that of *irs-2* is not due to the excess of an inhibitory factor but to loss of an activity present in V79. The recessive nature of the defect revealed by the assay is consistent with the cellular characteristics of the *irs* mutants which are recessive by cell fusion analysis to each other and to wild type in terms of radiation and drug sensitivities.

Irs-2 extract is capable of catalysing the formation of concatamers with an efficiency which, at the level of detection of Southern analysis, appears equivalent to that of V79. Furthermore these concatamers can be recut with the enzyme originally used to linearise the plasmid implying that this is a faithful ligation. Therefore the deficiency in *irs-2* is not a general ligation defect or even an incapacity to ligate PstI or EcoRI induced DSB with fidelity. Thus the *irs-2* defect is revealed here as an alteration in a specific set of

reactions required to convert a linear substrate to a molecule able to transform bacteria to ampicillin resistance.

To see if the specific ligation deficiency in *irs-2* revealed by the *in vitro* plasmid DSB assay could be ascribed to a defect in a characterised mammalian ligase activity we decided to assay these activities in V79 and *irs-2* cells.

4.2.2 DNA LIGASE ACTIVITIES OF V79 and *IRS-2*

4.2.2.1 Introduction

It has been suggested before that a DNA ligase activity would be required as the sole activity or the final step in the process of DSB rejoin. There is some evidence from studies on the *cdc-9* DNA ligase mutations of *S.cerevisiae* (Moore 1982a;1982b;1988) and the findings of Willis and Lindahl (1987), Chan et al. (1987) and Lasko et al. (1990a, 1990b) that ligase deficient mutants are defective in DSB repair and that this deficiency correlates with a sensitivity to ionising radiations. Obviously the situation is not as simple as this might imply since there are also reports of ionising radiation sensitive cells with defects in DSB repair but with no detectable ligase abnormality (Stamato and Hu 1987; Chan et al. 1984). Either the assays currently in use are unable to detect the ligase defects in these lines or, more likely, repair of DSB requires additional activities.

Nuclear extracts prepared from *irs-2* a radiosensitive mutant of V79 *irs-2* have been shown to be deficient in an activity ligating a DSB in the form of a plasmid linearised by restriction digestion to form a circular molecule capable of transforming bacteria and conferring ampicillin resistance. Although it was also shown that *irs-2* can efficiently perfom ligation reactions of a different type giving rise to concatemeric linear molecules there was no data availiable on the activity of DNA ligases I and II in this cell line. It was postulated that a defect in one of these enzymes might account in part or in total for the above observations. Thus it was decided to assay V79 wild type and the *irs-2* mutant line for activity of the characterised mammalian DNA ligases.

4.2.2.2 Conditions for assay of mammalian DNA ligases I and II.

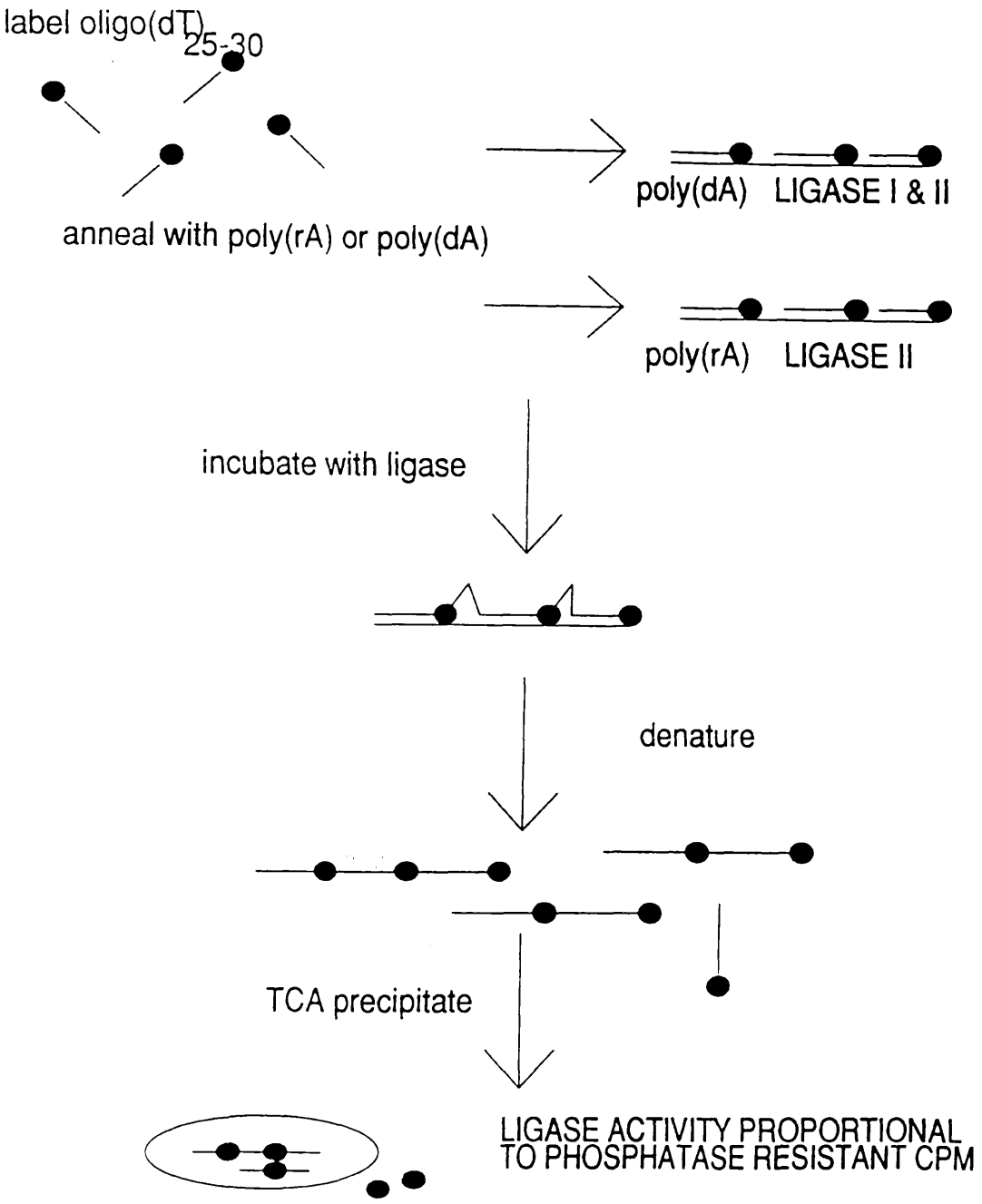
As there are suggestions that the two mammalian ligases have different roles (Lasko et al. 1990a,1990b) it was considered important to assay ligase activity in such a manner as to differentiate between the two enzymes. Such assays rely on the different substrate specificities of ligase I and II. Both enzymes can seal gaps in a substrate consisting of oligo(dT) annealed to poly(dA) but only ligase II can seal a hybrid substrate of oligo(dT) annealed to poly(rA) (Arrand et al. 1986). The assay chosen was essentially

that of Arrand et al. (1986). This assay (represented diagramatically in Figure 4.20) involves the preparation of two different substrates by annealing ^{32}-P labelled oligo(dT) to either poly(dA) or poly(rA). Reactions are then carried out with samples containing putative ligase activity in the presence of ATP and magnesium at 16^{o} for an hour. The reaction is terminated and substrates denatured by heating. Treatment with calf intestinal phosphatase (CIP) is used to remove any ^{32}-P labelled phosphate groups that have not been rendered phosphatase resistant by ligation. The samples are then TCA precipitated onto nitrocellulose and the radioactivity retained on each filter is a measure of the labelled phosphates protected from phosphatasing and thus a measure of the ligase activity present. The oligo(dT).poly(dA) substrate can be sealed either by ligase I or by ligase II. Use of this substrate gives an indication of total ligase activity. Ligase I is incapable of sealing nicks in the hybrid oligo(dT).poly(rA) substrate so incubation with this substrate provides a measure of ligase II activity.

Lasko et al.(1990b) used immunocytochemistry and indirect immunofluorescence to determine the subcellular location of DNA ligase I and found the enzyme present in the nucleus but not the nucleolus. Soderhall and Lindahl had previously (1975) suggested from the results of fractionation studies a nuclear location for ligase I but found the enzyme to leach into the cytoplasmic fraction following cell lysis. DNA ligase II has been reported as being more tightly retained in nuclei than ligase I (Lasko et al. 1990a). It was decided to use whole cell extracts to assay V79 and *irs-2* for ligase I and II activity. Use of this type of extract has advantages over the use of a nuclear extract in that fewer cells are required and extract preparation is considerably simpler. Published assays for ligase activities use whole cell extracts to determine these activities in cells. Of course it must be remembered that the in vitro plasmid DSB assay which established the existence of a ligation defect in the *irs-2* cell line used nuclear extracts. Thus use of a whole cell extract might preclude the detection of an alteration in a ligase activity relevant in nuclear extracts.

Figure 4.19

Protocol for the assay of DNA ligase activities in extracts from mammalian cells.



Simplified representation of assay procedure described in Arrand et al. (1986) and in text.

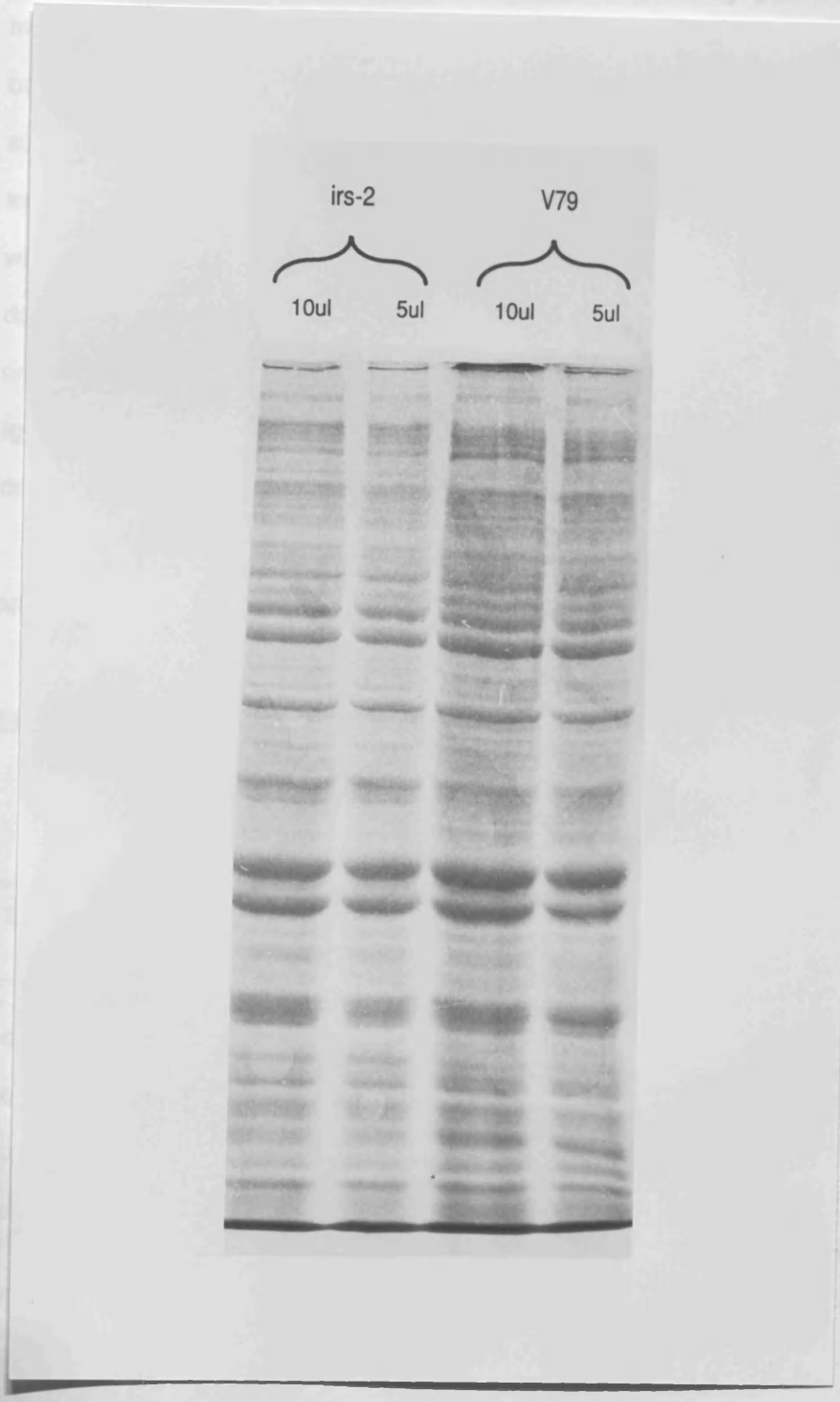
On balance however, particularly since it is reported (Soderhall and Lindahl 1975) that some ligase I activity leaches from the nucleus during cell lysis it was felt that whole cell extracts provided the best system for making comparisons between ligase activities in the two cell lines. Whole cell extracts were prepared as described by Arrand et al.(1986) and in materials and methods. Approximately 10^8 cells were pelletted and the pellet washed twice with PBS to which had been added protease inhibitors (pepstatin, leupeptin, peptidase A, benzamidine and PMSF all at 50mg/ml). The pellet was then resuspended in 500-1000µl extraction buffer (0.1M NaCl; 50mM Tris pH7.5; 10mM β-2-mercaptoethanol; 1mM EDTA) and homogenised with about 50 strokes using a 1ml capacity hand held glass homogeniser (Jencons). The homogenate was incubated on ice for an hour and debris pelletted by microfuging at 13K for 10 minutes. To the supernatant was added 1/10 volume 1M NaCl and 1/10 volume of polymin-P (Sigma). After a half hour incubation on ice nucleic acids were precipitated by a further 10 minute microfuge step. The supernate was aliquoted and stored prior to use at -70^o. Each aliquot was only thawed once.

The protein content of each extract was estimated using the Bio Rad assay. Typically crude extract concentration was between 5 and 10 mg/ml protein. Extracts were shown to be equivalent in quality by SDS polyacrylamide gel electrophoretic analysis. Figure 4.21 shows the SDS PAGE separation of proteins from V79 and *irs-2* whole cell extracts. The two cell lines show identical banding patterns.

To prepare substrates with which to assay extracts from V79 and *irs-2* lines oligo(dT)25-30 (Pharmacia) was labelled by the exchange reaction of polynucleotide kinase (10mg oligo 100 units PNK) with [γ^{32}P]ATP (100mCi) as in materials and methods by incubation at 37^o for 45 minutes in a total volume of 50µl. The reaction was stopped by the addition of EDTA and heating, the labelled oligo ethanol precipitated in the presence of glycogen and washed with 70% ethanol. The oligo was purified over a "nick column" and the peak activity fraction split into two. One half was annealed to 5µg poly(dA) in a total volume of 100µl and the other to 5µg poly(rA). In both cases annealing was performed by heating to 95^o and then slow cooling to room temperature. These oligonucleotide substrates were then stored at -20^o.

Figure 4.20

SDS-PAGE separation of proteins in whole cell extracts prepared from V79 and *irs-2* cells.



Denaturing SDS polyacrylamide gel electrophoresis performed as described for Figure 4.4. Whole cell extract type and quantity loaded as stated above lanes.

Prior to attempting to assay ligase activity using these substrates it is of prime importance to show that they can be ligated by the sorts of enzyme it was hoped to detect. The ligase activity of mammalian ligase I resembles that of T_4 ligase in terms of mode of action and the reaction intermediates involved in the ligation process. The bacteriophage enzyme is capable however, unlike ligase I, of sealing nicks in a hybrid substrate (Arrand et al. 1986). An aliquot of each set of substrate molecules was incubated with T_4 ligase. The products of these T_4 ligations were then analysed in two ways. One was essentially to perform the steps of a ligase assay, heating samples to denature the two strands, treating with calf intestinal phosphatase (CIP) to remove unligated radiolabelled phosphate groups and then TCA precipitation in the fashion of the ligase assay. The other was to separate products of ligation with T_4 DNA ligase by denaturing polyacrylamide gel electrophoresis.

Table 4.8 shows values obtained from the TCA precipitation of a number of preparations of substrates before and after T_4 ligation.

Table 4.8

TCA precipitation of kinased oligonucleotide ligase assay substrates.

Substrate	**Acid precipitable CPM**
oligo(dT).poly(dA)	726+/-167(5)
oligo(dT).poly(dA) + T_4	34282+/-7758(5)
oligo(dT).poly(rA)	1610+/-458(5)
oligo(dT).poly(rA) + T_4	30227+/-7437(7)

Mean values are shown in each case with the number of substrate preparations represented shown in parenthesis. Errors are standard errors of the mean values. Each individual value making up the means shown is the result of a single precipitation.

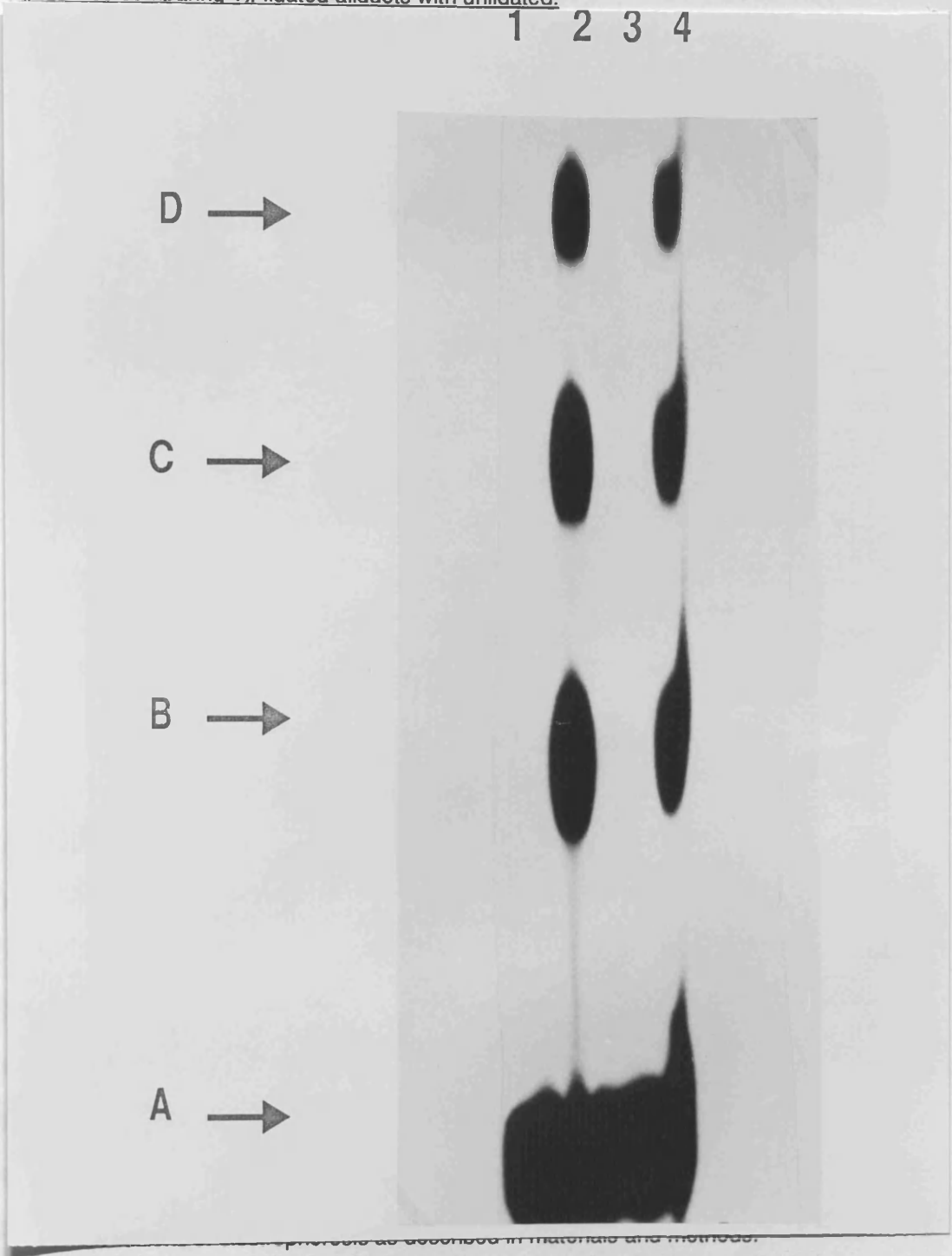
Table 4.8 shows that reaction with T_4 ligase renders an oligo(dT).poly(rA) substrate about 50 times more resistant to dephosphorylation by alkaline phosphatase than unligated kinased substrate. The oligo(dT).poly(dA) substrate is 20 times more resistant to dephosphorylation. This difference is not surprising in view of the description of the activities of T_4 ligase (Maniatis et al. 1982) whereby the most characterised and efficient activity is that of sealing cohesive breaks or single stranded nicks in duplex DNA molecules. It is to be expected that nicks in a hybrid substrate would be annealed less efficiently than those in a DNA/DNA oligonucleotide.

Figure 4.21 shows a representative denaturing polyacrylamide gel separation of the products of T_4 ligation reactions performed with one preparation of oligonucleotide ligase assay substrates. The Figure compares an aliquot of T_4 ligated (lanes 2 and 4) oligo(dT).poly(dA) or oligo(dT).poly(rA) with aliquots of unligated substrates (lanes 1 and 3). T_4 ligation would be expected to seal the single stranded nicks in such molecules. On denaturing prior to sample loading and during electrophoresis the labelled oligo (dT) monomers and higher forms produced by ligation would be separated from the unlabelled polynucleotide backbone. Unligated samples should, after electrophoresis show only monomer (25-30bp) length bands while ligation of either substrate should lead to the visualisation of slower migrating bands of lengths integral multiples of 25-30. Figure 4.21 shows that both the oligo(dT).poly(dA) (lanes 1 and 2) and the oligo(dT).poly(rA) (lanes 3 and 4) substrates can be ligated by T_4 at 15^{o} to give discrete populations of dimers, trimers, quadrimers (& higher forms which have been cut off the gel shown in the Figure).

It is clear from Figure 4.21 that while the loading in each case is approximately equal T_4 ligation leaves a population of substrate molecules (about 30%) unligated. The amount of T_4 ligase added to each analytical reaction would be expected to be in excess thus even if a proportion of molecules contained unlabelled oligo(dT) strands this should not prevent the labelled molecules from being ligated. The particular gel shown in Figure 4.24 is in fact an extreme case probably due to the high specific activity of this particular substrate preparation. In other instances the ligation by T_4 converts almost all the labelled substrate to higher forms.

Figure 4.21

Denaturing polyacrylamide gel separation of a preparation of ligase assay substrates comparing T_4 ligated aliquots with unligated.



Lanes 1 and 2: oligo(dT).poly(dA) substrate, lane 2: T_4 ligated. Lanes 3 and 4: oligo(dT).poly(rA) substrate, lane 4: T_4 ligated. "A" indicates the position of migration of monomer 25-30 mer oligonucleotide, "B" dimers, "C" trimers and "D" quadrimers formed by T_4 ligation of the monomer.

Under conditions of the precipitation assay unligated substrate is dephosphorylated with calf intestinal phophatase (CIP) prior to TCA precipitation. This treatment removes the radiolabel from these molecules. They will not then contribute to the TCA precipitable counts measuring ligase activity.

Table 4.8 makes it clear that the conditions used do give rise to a background level of acid precipitable counts after phophatase treatment of unligated substrate. This could be due to incomplete denaturation of double stranded substrate allowing unligated labelled phosphates to be protected from the action of the phosphatase by virtue of secondary structure of the molecule rendering unligated phosphate groups inaccessible. Alternatively the phosphatasing reaction might not be proceding with maximum efficiency. Experiments were thus performed to assess the effects of incubation time and phosphatase enzyme concentration to attempt to establish conditions under which the maximum difference between the values for acid precipitable counts of ligated and unligated substrate could be obtained.

Table 4.9 shows the results of experiments where the temperature of incubation was constant at 37^{o} (that recommended for calf intestinal phosphatase (BCL)). The volume of incubation in each case was 50µl, that to be used in the ligase assay of extracts. Substrate was a 50:50 mixture of oligo(dT).poly(dA) and oligo(dT).poly(rA). The use of these substrates was hoped to enable optimisation of conditions of phosphatasing for both substrates.

The results shown in the Table are calculated in two stages:

Stage 1: For both T_4 ligated and unligated substrates under each set of reaction conditions the efficiency of dephosphorylation is calculated. The number of TCA precipitable counts per minute (TCA ppt. cpm) of a sample previously CIP dephosphorylated is subtracted from the TCA ppt. cpm of an identical sample not CIP treated.

Stage 2: A value for the relative efficiency of dephosphorylation of unligated as compared to unligated is obtained. For each set of conditions the efficiency of CIP dephosphorylation of the ligated substrate is subtracted from the efficiency of

dephosphorylation of the unligated substrate.

The higher the final value obtained the greater the efficiency of dephosphorylation of unligated as opposed to ligated substrate and thus the better that set of dephosphorylation conditions for the detection of ligase activity.

Table 4.9

Effect of time of incubation and CIP concentration upon efficiency of CIPing of oligonucleotide ligase assay substrates.

Incubation time at 37°	Units of CIP added	relative CIP efficiency
30 minutes	0.5	60421
	1	68947
	2	71938
	4	65025
1 hour	0.5	65301
	1	54575
	2	57240
	4	56671
2 hours	0.5	80059
	1	71495
	2	69546
	4	70749
o/n	0.5	63408
	1	64319
	2	75803
	4	61971

Each value determined as described above from results of single incubations. o/n = over night.

Table 4.9 shows that the greatest difference between counts lost by ligated and unligated substrate occurs with a two hour incubation (over night incubation at 37° perhaps giving rise to significant nonenzymic hydrolysis of both types of substrate). It is

probable that as between the calf intestinal phosphatase concentrations for the two hour incubation time (& all the other time points) the differences in values shown in Table 4.9 are not significant given the intrinsic variability in TCA precipitation procedures and counts added originally to each reaction.

A further complication is the fact that the substrates were not denatured prior to calf intestinal phosphatasing as they would be under the ligase assay protocol. This would be expected to affect all conditions equally and so not to bias the results in favour of any particular time/calf intestinal phosphatase concentration combination. It is likely then that in all cases if the substrates had been denatured the unligated counts would have been lower as the presence of the complementary strand might well protect phosphates at unsealed nicks from the action of calf intestinal phosphatase. This probably suggests that the differences evidenced in Table 4.9 between the ligated and unligated substrates are artificially low. It is not known however how much the presence of extract proteins might affect the efficient action of CIP.

In an attempt to minimise the effect of substrate concentration variability between samples and also to counteract the variability inherent in the procedure of TCA precipitation all ligase determinations were performed in triplicate. It was hoped that denaturation of substrate after ligation reaction at 80^{o} for 30-45 minutes would be sufficient to render the subsequent phosphatasing reaction effective.

To recapitulate, the ligase assay protocol involves incubation of oligonucleotide substrate comprised of end labelled oligo$(dT)_{25\text{-}30}$ annealed to either poly(dA) or poly(rA) with a source of ligase. The oligo(dT).poly(dA) substrate detects DNA ligases I and II while only ligase II activity is revealed by incubation with the oligo(dT).poly(rA) substrate. Reactions of substrate with whole cell extract or FPLC fractionated whole cell extract were incubated in triplicate. Incubation was at 16o for one hour. Ligase was inactivated and substrate denatured by heating the reaction to $80^{o}C$ for 30-45 minutes. Two units of CIP were then added to all except "no CIP" control reactions and phosphatasing reactions performed at $37^{o}C$ for two hours. Reactions were then either stored at $-20^{o}C$ prior to precipitation or TCA precipitated immediately. Filters were air

dried and counted. Ligase activity was then expressed as the percentage of total CPM added to the assay (acid precipitable CPM in a sample incubated without extract and without calf intestinal phosphatasing ie the "no CIP no ligase" control value) rendered phosphatase resistant by the ligation reaction.

4.2.2.3 Dose response of DNA ligase activities in whole cell extract from V79 and *irs-2.*

Table 4.10 shows the results of in each case at least eight independent determinations of total ligase activity measured using the oligo(dT).poly(dA) substrate as described above in at least three different preparations of whole cell extracts of V79 and *irs-2.* For each dose point the amount of extract to add to give equivalent protein concentrations in the extracts from the two cell lines was calculated and then made up to 40µl with Extraction Buffer.

Table 4.10

Protein concentration response of ligase I and II activity in whole cell extracts of V79 and *irs-2.*

Protein added (µg/ml)	**% phosphatase resistant CPM** V79	*irs-2*
50	16.8+/-2.8 (9)	15.0+/-2.2 (12)
100	14.9+/-2.0 (8)	12.4+/-1.6 (12)
500	25.2+/-2.5 (9)	21.7+/-2.3 (12)
1000	38.9+/-2.5 (8)	31.0+/-2.5 (11)

Values represent the means of results from a number of ligase assays performed in triplicate as described above incubation being with the oligo(dT).poly(dA) oligonucleotide substrate detecting the activity of both mammalian ligases. Standard errors are shown and numbers of independent determinations given in parenthesis after each value.

Table 4.11 presents results assaying the protein concentration response of ligase II activity in whole cell extracts from these lines. Again assays were performed as described above but measuring the sealing of nicks in the ligase II specific hybrid substrate oligo(dT).poly(rA) as opposed to the (dT).(dA) ligase I and II substrate.

Table 4.11

Protein concentration response of ligase II activty in whole cell extracts from V79 and *irs-2*.

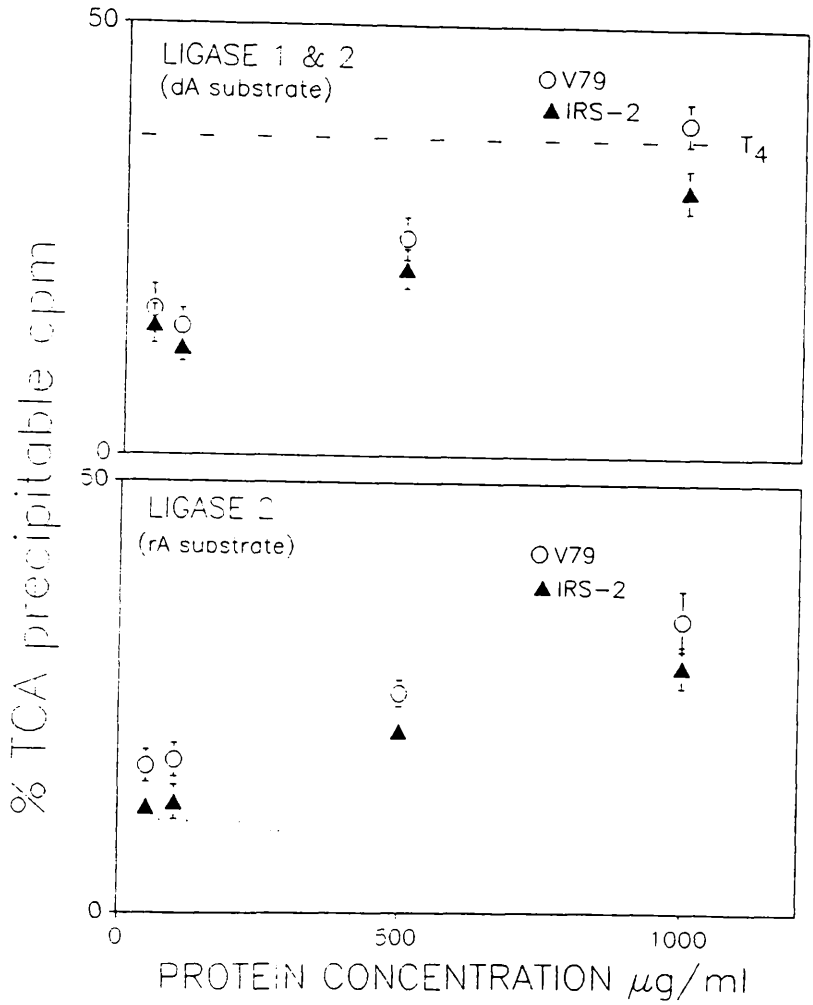
Protein added (µg/ml)	**% phosphatase resistant CPM** V79	*irs-2*
50	16.9+/-1.9 (8)	12.2+/-1.5 (6)
100	17.6+/-2.0 (12)	12.8+/-2.0 (8)
500	25.4+/-1.5 (12)	21.0+/-1.4 (8)
1000	34.1+/-3.6 (12)	28.6+/-2.4 (8)

Values represent the means of the results of a number of determinations of ligase II activity using the oligo(dT).poly(rA) substrate. As for Table 4.10 at least three different extract preparations from each extract were tested at each protein concentration. Standard errors are shown and figures in parenthesis represent in each case the number of independent incubations.

Figure 4.23 is a graphical representation of the data shown in tables 4.12 and 4.13. It is clear from tables 4.12 and 4.13 and from Figure 4.26 that there is no significant difference between whole cell extracts of V79 and *irs-2* in terms of the concentration response of either of the activities measured by the oligonucleotide substrates. Thus the dose responses of ligase I and II activity and of ligase II activity are virtually identical in both cell lines. The slight apparent differences observed between the cell lines at some dose points are not statistically significant.

Figure 4.22

Graphs showing concentration response of total ligase and ligase II activity in whole cell extracts from V79 and *irs-2* cells.



Ligase assays for mammalian ligases I and II and specifically for ligase II performed as described in text following the method of Arrand et al.(1986).

Each point represents the mean of at least nine independent observations. Errors are standard errors of the mean.

The graphs shown in Figure 4.22 do not pass through the origin. This is presumably a reflection of background TCA precipitation due incomplete removal of phosphates from non ligated substrate molecules. As discussed above this could be due either to incomplete denaturation of substrate precluding effective CIPing or to the CIP conditions themselves being too mild. It is also possible that the extracts contain factors acting to protect labelled substrate from phosphatasing. In this instance the assay is being used to compare directly the ligase activities of two cell lines rather than to measure absolute units of ligase activity. Thus it was felt that it was not necessary to attempt to correct for this background.

4.2.2.4 Assay of ligase activities in fractions of whole cell extracts from V79 and *Irs-2.*

The concentration response data presented above strongly suggests that there is no difference in the activity of either of the two characterised ligase enzymes in *irs-2* whole cell extracts as opposed to the wild type V79. To confirm this the FPLC separation characteristics of the two enzymes in both cell lines were studied. To this end a number of whole cell extracts of both V79 and *irs-2* were subjected to gel filtration fast protein liquid chromatography (FPLC) and fractions assayed for ligase activity in the manner described above.

Gel filtration determines the size and molecular weight of proteins by a fractionation based on the differential diffusion of various molecules into gel pores of a particular size. Proteins of high molecular weight are excluded from the gel pores and so pass through the fluid volume of a column of gel particles faster than those with lower molecular weights. Proteins thus elute from such columns of porous gel particles in order of decreasing molecular weight (Sigma Technical Bulletin GF-3 1987).

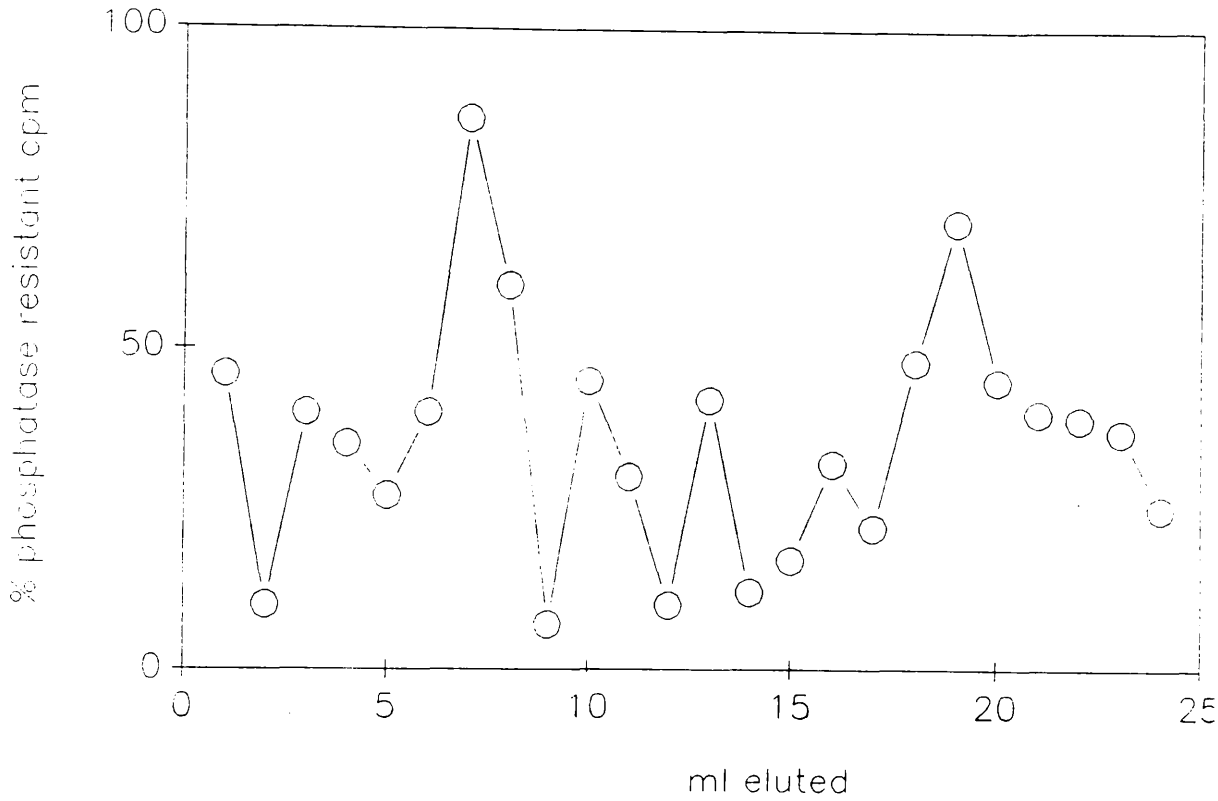
Whole cell extracts were fractionated by FPLC gel filtration as described in Materials and Methods over a "Superose-12" (Pharmacia) column packed with a cross linked agarose based gel filtration medium (Pharmacia 1985).

Figure 4.23 shows the profile of DNA ligase activity assayed using the

oligo(dT).poly(dA) substrate from a typical fractionation. This figure shows two distinct peaks of ligase activity. The first eluting at about 7ml corresponding to ligase I enzyme and the second corresponding to the smaller ligase II enzyme. Table 4.12 indicates the fractions in which the peaks of activity of DNA ligases I and II eluted following the fractionation of V79 and *irs-2* extracts as described above. Determination of ligase activity employed the oligo(dT).poly(dA) oligonucleotide substrate (the point at which ligase II elutes was confirmed in extracts from each cell line by reaction with the oligo(dT).poly(rA) substrate) exactly as described for the concentration response experiments above. The results shown are from assay of fractions derived from at least two or more independently prepared whole cell extracts. In each case the peaks described were the only ones observed.

Figure 4.23

Elution profile of ligase activities in whole cell extracts of V79 cells fractionated on a Superose 12 column.



200µl (approximately 1mg protein) of crude V79 whole cell extract was loaded onto a superose 12 column. Fractionation was performed at a rate of 0.2-0.4ml/minute as described in materials and methods. Samples were collected on ice and stored at -70^{o}C prior to being assayed for total ligase activity. Ligase assays were as described in text and in materials and methods with the results calculated and expressed as described in the text.

Table 4.12

Peaks of ligase activity eluted from a superose column by fractionation of whole cell extracts of V79 and *irs-2*.

	Ligase I Fraction	**Ligase II** Fraction
V79	17.5(2) **8.75ml**	34.7(3) **17.3ml**
irs-2	18.8(5) **9.4ml**	31.7(3) **15.8ml**

The value in ordinary typeface in each case represents the actual fraction number at which the relevant peak of activity was eluted with the number of independent determinations in parenthesis. Values in bold face are the actual volumes of column buffer (see Materials and Methods) required to elute the relevant activity.

Table 4.12 suggests that the elution profiles of the two ligase activities are very similar in both cell lines. It was found that as between experiments background "no ligase" phosphatase resistant CPM varied widely. Therefore it was not possible to standardise results to render them strictly comparable in terms of units of enzyme activity. Further within each experiment it was not feasible to perform triplicate TCA precipitations so no errors can be assigned to the values obtained for each fraction. Thus it was considered that it was preferable to obtain data from a number of different extract fractionations using different substrates and then to compare peaks of enzyme activity. The elution patterns of both enzymes was similar in both cell lines (Table 4.12) and it had already been shown that the assayed activity of both ligase I and ligase II in *irs-2* whole cell extracts was equivalent to that in those from V79. Obviously if any indication of an alteration in the activity or elution profile of either of the ligases had been observed in *irs-2* as opposed to V79 then it would be vital to obtain complete activity profiles with each point statistically significant.

Comparison of the fractions at which the two enzymes elute with those reported

by Willis and Lindahl (1987) shows the normal human ligase I enzyme to elute off an identical column at about 12.5ml and ligase II at about 15.5ml. These values are in good agreement with the data above showing the hamster ligase I to elute from a superose 12 column at about 9ml and ligase II at 16 ml. It is not clear if the difference in the elution of ligase I represents a true interspecies variation in this enzyme or is due to differences inherent in the experimental procedures used.

The results shown in tables 4.12, 4.13 and 4.14 and in Figure 4.23 give no indication of any functional or structural alteration of either ligase enzyme in the *irs-2* cell line as opposed to V79. Before ruling out a ligase defect as the basis for the in vitro ligation deficiency exhibited by *irs-2* however the possibility of an alteration in the regulation of the enzymes was considered. Levels of a number of enzymes with a putative role in DNA repair reactions in bacteria (Hanawalt et al. 1979; Sancar and Sancar 1988), yeast (Cole et al 1987; Madura and Prakash 1990; Madura et al. 1990; Jones et al. 1990) and mammals including human cells (Ben-Ishai et al 1990; Hirschfeld et al. 1990; Glazer et al. 1989; Gupta and Sirover 1980) have been shown to increase upon treatment with DNA damaging agents. This upregulation has mainly been seen to occur at the transcriptional level. It would be difficult to postulate a defect in induction of a DNA ligase as the reason for the ligation deficiency (& the cellular radiosensitivity) of *irs-2* as the nuclear extracts used to reveal the ligation defect were not produced from cells previously treated with an inducing agent. However it is feasible that a defect in a ligase which in one system manifests itself as an anomaly in induction might in another be seen as an incapacity to catalyse a particular form of ligation, ie the same basic alteration could be postulated as having a number of phenotypic effects.

4.2.2.5 Induction of DNA ligases in *irs-2* and V79

Ligase II has been reported (Lasko et al. 1990a and refs therein) to be induced on treatment of cells with the alkylating agent dimethyl sulphate and by poly-ADP-ribosylation (considered a response to DSB). Ligase II is also induced in mouse L cells and in V79 nuclear extracts after treatment of cells with methylnitrosourea. Chan et

al.(1984) report similar induction of ligase II (2.0-2.6 fold) in CHO cells treated with methyl methanesulphonate (MMS). They find no difference in the response to MMS in terms of ligase activity between wild type CHO and the mutant EM9. EM9 is hypersensitive to ethyl methanesulphonate and MMS, has an increased frequency of sister chromatid exchange (SCE) and is defective in rejoin of DNA strand breaks after treatment with EMS, MMS or Xrays. Thus a defect in the induction of a DNA ligase has not yet been correlated either with a sensitivity to radiation or a deficiency in the rejoin of DNA strand breaks. However given the probable role of DNA ligases in the repair of DSB and so in radiation damage repair it was decided to extend the study of ligase activity in V79 and *irs-2* to cover induction.

The choice of inducing agent was somewhat arbritary. Ideally ionising radiation would have been used but practically this was not possible. If a difference in the ligase induction response to DNA damage was to be observed in *irs-2* as opposed to V79 it might be most readily seen in response to an agent to which *irs-2* is hypersensitive. *irs-2* is reported (Jones et al.1987) to be mildly (about 2.5 fold see Introduction and Chapter 3) sensitive to EMS so this agent was selected for use in attempting analysis of induction of ligases. Chan et al.(1984) demonstrated induction of ligase II by treating CHO AA8 and the EM9 mutant cells with MMS for an hour immediately prior to making extract for ligase assays. No expression time was allowed between drug treatment and extract preparation so it is probable that this is an effect acting at a post transcriptional level.

For EMS induction experiments V79 and *irs-2* cells were treated with 10ml of a 10mg/ml EMS solution for one hour. The EMS was then removed, cells harvested and whole cell extracts prepared. Ligase assays were then performed exactly as described above. In each case parallel non EMS treated extracts were also prepared. It was thought important to attempt induction with a concentration of EMS high enough to allow reasonable certainty of detecting an induction effect if it existed. It is not necessary to consider cytotoxicity as extracts are made immediately after exposure to the drug. Concentrations can thus be much higher and less precisely defined than if time were to be allowed for damage expression. Treatment of V79 wild type cells with 2.5mg/ml EMS

for 2 hours leads to 20% cell survival. It was thought likely that even a short term treatment with 10mg/ml EMS should, if this type of treatment induces ligase enzymes at all, be effective in this respect in V79 and so presumably in the more sensitive *irs-2* line.

Table 4.13 shows the protein concentration response of total ligase activity measured in whole cell extracts from EMS treated V79 and *irs-2* cells compared with extracts from non treated cells treated using a oligo(dT).poly(dA) substrate. Ligase activity is expressed as before as the percentage of total counts added to the assay converted to phophatase resistance by "ligation" incubations at 16^{o} for an hour.

Table 4.13

Ligase I and II activity in whole cell extracts from EMS treated V79 and *irs-2* cells compared with that in extracts from non treated cells.

Protein added	**% phosphatase resistant CPM**	
(μg/ml)	V79	V79-EMS
50	16.8+/-2.8(9)	66.1+/-24.0(8)
100	14.9+/-2.0(8)	57.3+/-21.5(6)
500	25.2+/-2.5(9)	61.6+/-18.6(7)
1000	38.9+/-2.5(8)	101.0+/-19.1(7)
	irs-2	*irs-2*-EMS
50	15.0+/-2.2(12)	66.7+/-31.5(6)
100	12.4+/-1.6(12)	61.9+/-19.5(9)
500	21.7+/-2.3(12)	72.3+/-16.2(9)
1000	31.0+/-2.5(11)	94.1+/-25(8)

Assays were performed as described using the oligo(dT).poly(dA) substrate. All TCA precipitations were done in triplicate. Values are means and standard errors are shown with the number of independent incubations for each dose point given in parenthesis. Data for the uninduced extract has been shown before in Table 4.10 and is shown again here only for comparison. Each value represents incubation with at least two independent whole cell extract preparations in the case of induced extract and at least three in the case of uninduced. "V79-EMS" and "*irs-2*-EMS" refers to whole cell extract from cells treated with EMS prior to harvesting while "V79" and "*irs-2*" refers to extract from untreated cells.

Table 4.14 gives the data comparing ligase II activity in whole cell extracts from V79 and *irs-2* cells treated prior to harvesting with EMS to that in whole cell extracts prepared from untreated cells. Assays were performed as described using the oligo(dT).poly(rA) hybrid substrate. Once more the data on ligase activity in extracts not treated with EMS has been presented before (Table 4.11) and is included again for ease of comparison.

Table 4.14

Comparison of ligase II activities in whole cell extracts prepared from V79 cells treated with EMS to that in extracts from untreated cells.

Protein added	**% phosphatase resistant CPM**	
(µg/ml)	V79	V79-EMS
50	16.9+/-1.9(8)	45.0+/-7.1(5)
100	17.6+/-2.0(12)	54.3+/-10.0(4)
500	25.4+/-1.5(12)	52.7+/-9.2(8)
1000	34.1+/-3.6(12)	88.2+/-12.8(6)
	irs-2	*irs-2*-EMS
50	12.2+/-1.5(6)	37.0+/-7.8(5)
100	12.8+/-2.0(8)	67+/-10.4(4)
500	21.0+/-1.4(8)	40.8+/-4.3(6)
1000	28.6+/-2.4(8)	47.3+/-9.2(6)

Figures represent mean values for ligase II activity in whole cell extracts, at every dose point including in each case determinations of activity in at least two (EMS induced extracts) or three (uninduced extracts) different extract preparations. Standard errors are shown and the numbers in parenthesis represent the numbers of independent incubations. "V79" and "*irs-2*" represents whole cell extract prepared from cells not treated with EMS prior to extraction and "V79-EMS" and "*irs-2*-EMS" refers to extract prepared from cells pretreated with EMS.

As tables 4.13 and 4.14 show induction of total ligase activity and also specifically of ligase II was stimulated approximately three fold in V79 cells by treatment with EMS. The mechanism for this is unclear. Presumably the treating of cell cultures immediately prior to extract preparation (leaving no time for expression of the DNA damage caused by EMS in terms of mutation, chromosome abnormalities and cytotxicity) and the short duration of the treatment itself (one hour) renders it unlikely that altered gene transcription is involved. For the purposes of this study however the precise mechanism by which ligase enzymes are induced in response to treatment by a DNA damaging agent is not important. The interest in these experiments lies in the comparison of the effect of EMS treatment on the wild type V79 cell line with that upon the radiosensitive mutant *irs-2*. It is clear from the results shown above that there is no significant difference between the inducibility of ligase activity measured by incubation with either the oligo(dT).poly(dA) or the oligo(dT).poly(rA) substrate between the two cell lines. Thus as compared to V79 there is no alteration in the *irs-2* cell line with respect to induction of either total ligase activity or of ligase II measured separately. The results shown here indicating a roughly three fold induction of ligase II are consistent with observations reported in Lasko et al. (1990) where 2.5 fold induction of this enzyme is said to have been seen on treating V79 with methylnitrosourea. However no data is given on induction of ligase I which is not generally considered inducible by DNA damage (Lasko et al. 1990a; Tomkinson et al.1990;) levels of this enzyme being thought to be regulated more in a cell cycle specific fashion (Soderhall and Lindahl 1975; Mezzina and Nocenti 1978; Hoffman and Collins 1976) increasing upon stimulation to divide. The experiments described here however do not specifically measure ligase I activity, the induction seen in reactions with the oligo(dT).poly(dA) substrate can not thus be ascribed to ligase I as opposed to ligase II. The question of induction of ligase I could be addressed by assaying fractions of EMS induced whole cell extracts for ligase activity and comparing activity levels of peak fractions for each of the two enzymes between induced and uninduced extracts. As the question under investigation was one of the basis of the observed defect in *irs-2* and it appeared that ligase enzymes were identical in the two cell lines in terms of activity,

physical character and regulation in response to DNA damage it was decided not to pursue this further. There does remain the possibility that the use of whole cell extracts to assay ligase activity is masking an effect important when the activity in nuclei is considered. For example a defect in nuclear localisation of a ligase enzyme could conceivably lead to nuclear extracts of *irs-2* containing less of such an activity than those prepared from V79 cells. This could be tested by assaying nuclear extracts for ligase activity. However since no indication of any alteration in either activity had been suggested by the results of any of the experiments described above this was not considered high priority.

4.2.2.6 Summary and conclusions.

To summarise, the recircularisation defect shown by nuclear extracts of *irs-2* using the in vitro assay does not appear to have its basis in a defect in either of the characterised mammalian DNA ligases. This is consistent with the finding that *irs-2* extracts can catalyse ligation to yield linear concatamers with an efficiency equivalent to that seen in V79. The production of linear concatamers presumably occur via pathways also requiring DNA ligase activities.

4.2.3 MODIFICATION OF IN VITRO ASSAY FOR ANALYSIS OF RECOMBINATION.

4.2.3.1 Introduction.

It has been shown that the defect in *irs-2* is not one in a characterised mammalian ligase detectable by the ligase assay used. There is an obvious requirement for ligation of a 3' hydroxyl to a 5' phosphate group in the repair of a DNA DSB. A tentative link between deficiency in repair of DSB, ionising radiation sensitivity and ligase deficiency is exemplified by Blooms syndrome cell lines (Lasko et al.1990a). There are precedents, however, for the observation of a cell line defective in DSB repair and sensitive to ionising radiation without a detectable ligase abnormality (Chan et al.1984; Stamato and Hu 1987). It is reasonable to postulate then that DSB repair requires activities in addition to ligase enzymes. Indeed, the evidence suggests that *irs-2*, shown to be deficient in recircularisation of a linearised plasmid is fully capable of the ligation of a DSB. To explain the deficiency in DSB repair exhibited by this cell line it is necessary to consider more complex processes than simple ligation events. It is clear that while *irs-2* is deficient at some stage in the pathway of conversion of linear substrate to circular molecules capable of transforming bacteria there is no observed alteration in activities which would be postulated to be involved in a "direct recircularisation" process. Thus there is strong evidence that ligation (so presumably the formation of a preligation complex) can proceed efficiently in *irs-2* extracts and circumstantial evidence against an excess of nuclease or phosphatase activity. Further it is clear that nuclear extracts prepared from *irs-2* are as efficient at forming high molecular weight concatamers as extracts prepared from the wild type V79 cells.

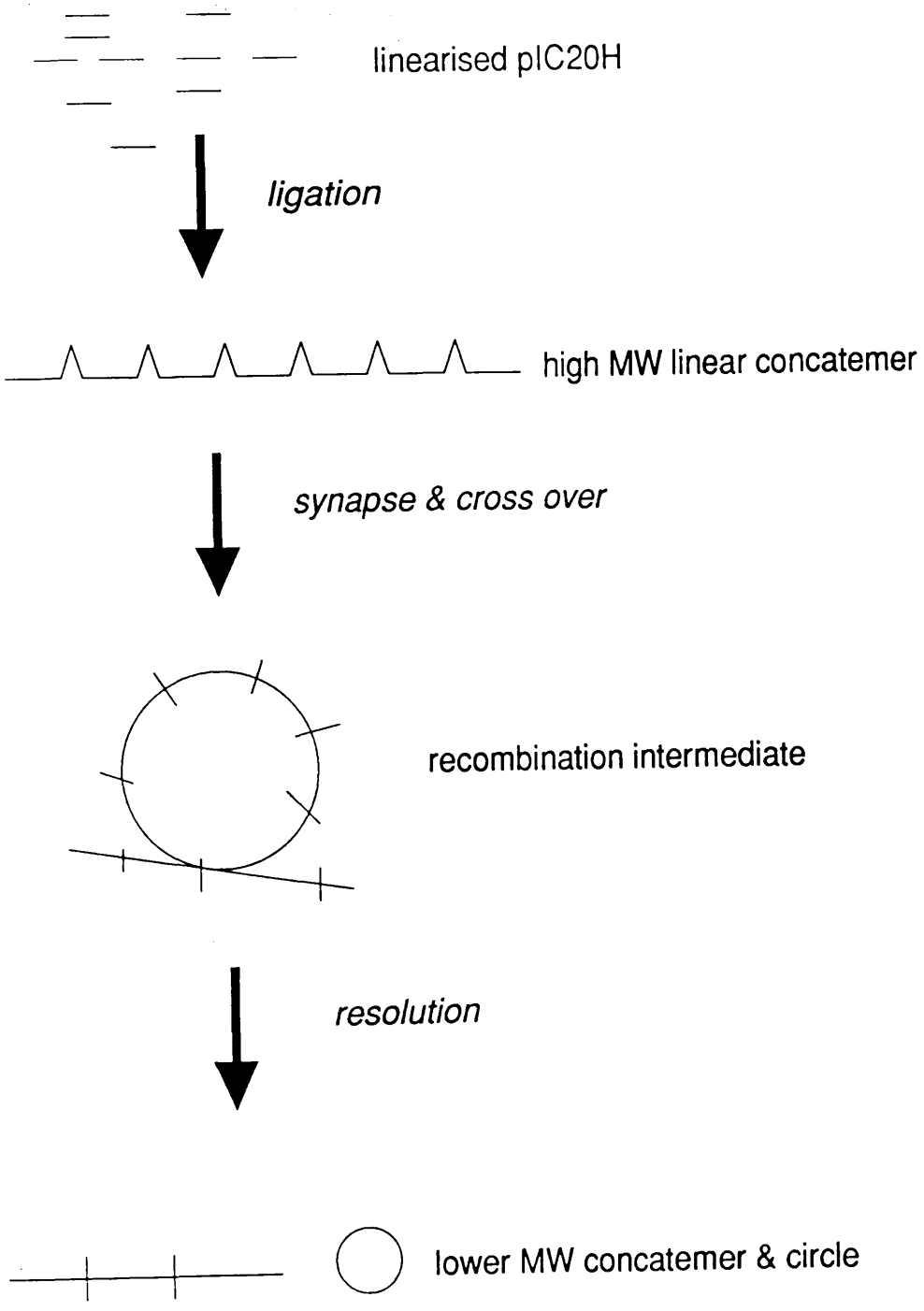
The formation of concatamers is implied in Figure 4.9 to be an alternative "dead end" fate for linear substrate, competing with and precluding recircularisation. This might in fact not be the case and concatemerisation of linear substrate far from abrogating its formation into circular molecules may be instead a prerequisite for recircularisation. It might then be postulated that the concatamers produced by *irs-2* nuclear extracts can not be processed in the manner of those formed in incubations with V79. This might be a defect in the concatamers themselves preventing their conversion to circular forms or the

defect might reside in an activity required to effect such a conversion. Figure 4.24 is a diagram giving one model for the production of a circular form by recombination of concatamers. Other scenarios involving more complex mechanisms could also be postulated but the sequence shown in the diagram is probably the most straightforward.

DSB repair has been linked in a number of systems with recombination (Moore et al.1985; Cao et al. 1990; Ho 1975; Jeggo and Kemp 1983; Kemp et al.1984; Costa and Bryant 1988). It was thus decided to attempt to modify the in vitro plasmid DSB assay to investigate the role of concatamers in the generation of circular molecules and so characterise further DSB repair in wild type cells and the defect observed in *irs-2*. This type of analysis could take two forms. One could either address the possibility of concatamer formation by *irs-2* extracts being somehow different from that by V79 extracts. Alternatively it could be assumed that the problem in *irs-2* lies in an activity or reaction required to process concatamers. There is no evidence to suggest that the concatamers formed by *irs-2* nuclear extracts are different from those produced on incubation of linear substrate with V79 and some evidence to suggest that they are identical for example in terms of concentration response and fidelity of joining. It should be noted that no information is available as to the directionality of ligation to form concatamers in the two types of extract and it is conceivable that the orientation in which linear molecules are linked might have an impact upon their subsequent processing.

Figure 4.24

One mechanism by which circular molecules could be produced by a pathway involving recombination of concatamers.



The diagram above represents one mechanism by which circular molecules might be formed via linear concatamers. Monomeric linear pIC20H is ligated to a high molecular weight linear form. This molecule forms an intermediate in an intramolecular recombination event, the intermediate being resolved to produce a linear multiple of fewer substrate monomers and a circular form.

4.2.3.2 Preparation of substrate and assay protocol.

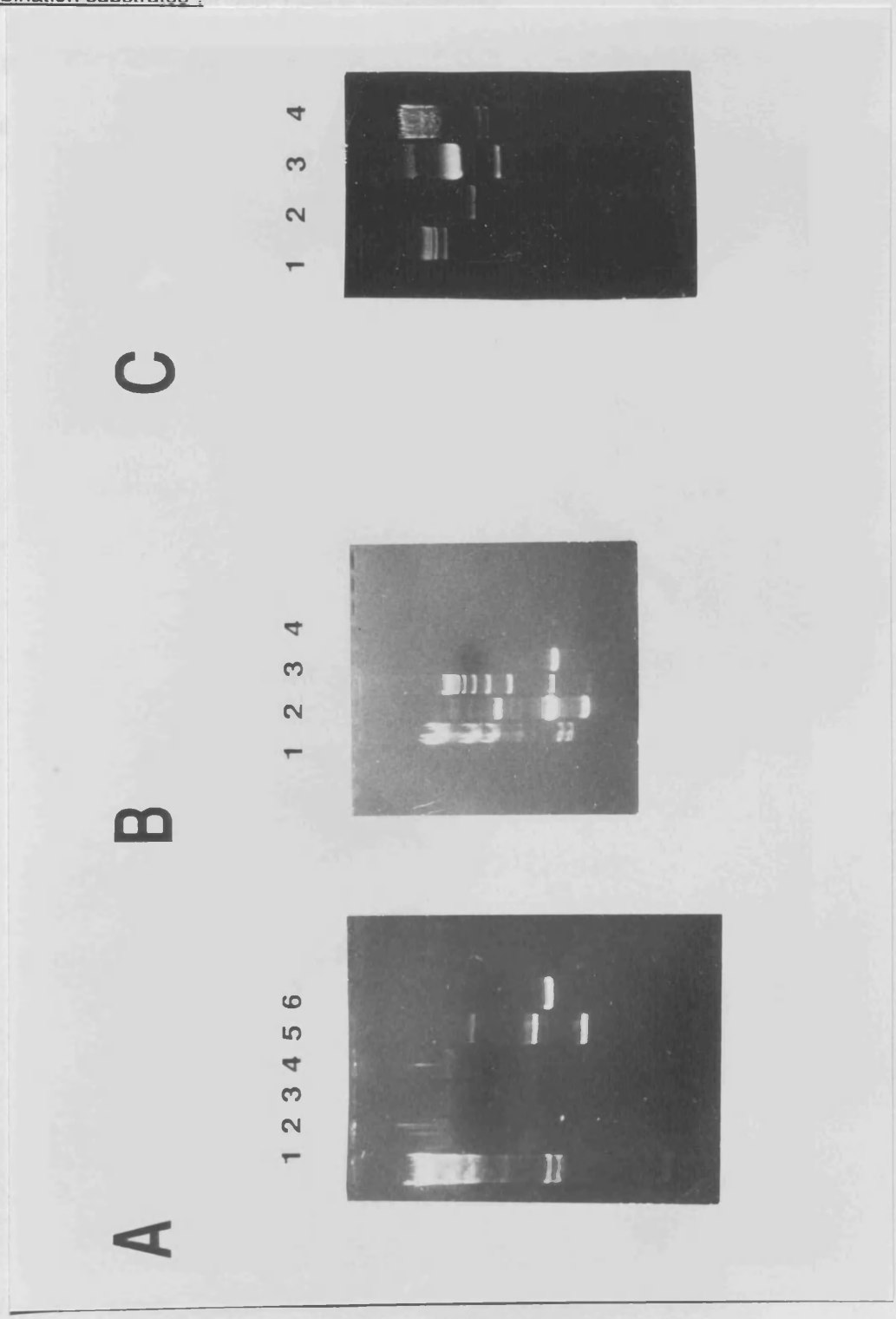
Before considering reaction mechanisms postulating concatamer resolution it is necessary to demonstrate that concatamers actually can give rise to circular forms under the relevant conditions. To attempt to see if this conversion could occur a population of dimers and higher forms (analogous to the concatamers produced under in vitro plasmid DSB assay conditions on reaction of linear plasmid substrate with nuclear extract (recombination substrates)) were prepared and incubated with nuclear extracts from *irs-2* and V79 cells under the conditions of the in vitro assay. The products of these reactions were then subjected to Southern analysis as described for the products of repair reactions.

Concatamer recombination substrates were prepared by the incubation of linear pIC20H with T_4 ligase under conditions under which it was expected that the reaction would proceed to completion (incubation with buffer supplied by and at an enzyme concentration suggested by manufacturers overnight at 14^{o}). The products of this reaction were analysed by 1% agarose gel electrophoresis with ethidium bromide running an aliquot alongside size markers, linear plasmid and uncut plasmid controls. Panel A in Figure 4.25 is a photograph of such a gel showing typical products of such a ligation reaction. It is clear that ligation has proceeded to an extent producing either a single high molecular weight species or a collection of molecules too large to be resolved by the conditions under which the gel was run. This ligated population was then restriction enzyme digested under conditions to render cutting incomplete, the aim being to produce the type of mixed population of multimeric forms seen after incubation with nuclear extract. Panel B of Figure 4.25 shows the 1% agarose gel analysis of such a "partialling" reaction. It is clear that while there are indeed a number of high molecular weight multimer forms produced by such a reaction the original ligation products are cleaved to produce amongst the products molecules of monomer length. It was necessary to include in the protocol for recombination substrate preparation a purification step to separate higher forms from monomers. Recombination substrates were thus routinely separated on a 0.4% agarose gel run without ethidium bromide to avoid the introduction of nicks. To

identify substrates an aliquot was run with ethidium alongside the bulk of the sample.

Figure 4.25

Preparation of dimers, trimers and higher forms of pIC20H for use as "recombination substrates".



(Figure legend on following page).

Electrophoresis performed under conditions described in the text and in materials and methods.

Panel A: Lane 1: size markers -λ DNA digested with HindIII. Sizes of fragment (kb); 23.72, 9.46, 6.6, 4.26, 2.25, 1.96, 0.59, 0.1. Lanes 2, 3 and 4: pIC20H T4 ligated to completion. Lane 5: uncut pIC20H. Lane 6: linear pIC20H.

Panel B: Lane 1: size markers as above. Lane 2: uncut pIC20H. Lane 3: T4 ligated pIC20H after a partialling reaction. lane 4: linear pIC20H.

Panel C: Lane 1: isolated recombination substrates. Lane 2; linear pIC20H. Lane 3: uncut pIC20H. Lane 4: size markers as for panel A.

The gel was then UV transilluminated and it was possible to identify the position of migration of dimers, trimers and higher multimeric forms. Also included for orientation were aliquots of uncut plasmid and cut plasmid monomer. A gel slice corresponding to the expected position of the migration of the desired multimeric recombination substrates was excised and substrates electroeluted. This procedure was found to give much higher yields than purification by the "gene clean" method or by overnight dialysis and so was adopted as the method of choice in the preparation of recombination substrates. Panel C of Figure 4.25 shows an aliquot of one such preparation of recombination substrates run on a 1% agarose gel in the presence of ethidium bromide alongside aliquots of cut and uncut plasmid. Such preparations can be seen to contain no monomer sized molecule consisting of a variety of high molecular weight forms.

Incubations were performed in the same volume (350µl) under the same salt conditions and making up the volume of nuclear extract added to each reaction to 100µl exactly as described for the in vitro plasmid DSB rejoin assay. The major unknown was the concentration of concatamer recombination substrate to add. It was possible that the difference between V79 and *irs-2* lay in the speed of concatamer formation ie the defect in *irs-2* would be essentially one of concentration of a necessary intermediate. This would obviously not be revealed in the modified "recombination" in vitro DSB rejoin assay described. However the primary purpose of these first experiments was to establish whether concatamers were a substrate for recombination or resolution to circular forms. Study of Southerns visualising the products of reactions under the standard in vitro assay conditions suggests that by the end of the ninety minute incubation period approximately half of the input linear substrate remains unconverted. It was decided to use a recombination substrate concentration of 20ng (linear plasmid being added in the standard assay at a concentration of 50ng in the 350µl reaction volume).

The protocol of the in vitro DSB rejoin assay modified to look at recombination involves the incubation of 20ng recombination substrate in a reaction mixture otherwise exactly as described for the original repair assay. Incubation with and without the addition of nuclear extract is then performed as usual at 14^{0} for ninety minutes. Reaction products

are then isolated and visualised by Southern analysis probing as before with nick translated uncut pIC20H.

4.2.3.3 Results and Discussion.

Figure 4.26 shows an example of such a Southern. Each of these experiments included reactions performed under the standard in vitro assay fashion with cut plasmid substrate to act as a control for nuclear extract activity. Thus it is clear that both the V79 and the *irs-2* nuclear extracts are eficiently catalysing the formation of concatamers. Incubation with V79 but not *irs-2* extract also gives rise to a fast migrating circular form. Both the nuclear extracts used in the reactions visualised in this Figure are functional in the standard repair assay and behave as expected under those conditions. It is also clear that the separation of high molecular weight concatamers from monomer forms during the preparation of the recombination substrates was highly effective with no monomer form being detectable in the lanes of recombination substrate without nuclear extract. It is also clear however that incubation of recombination substrates with nuclear extracts from neither V79 nor *irs-2* produced detectable circular molecules.

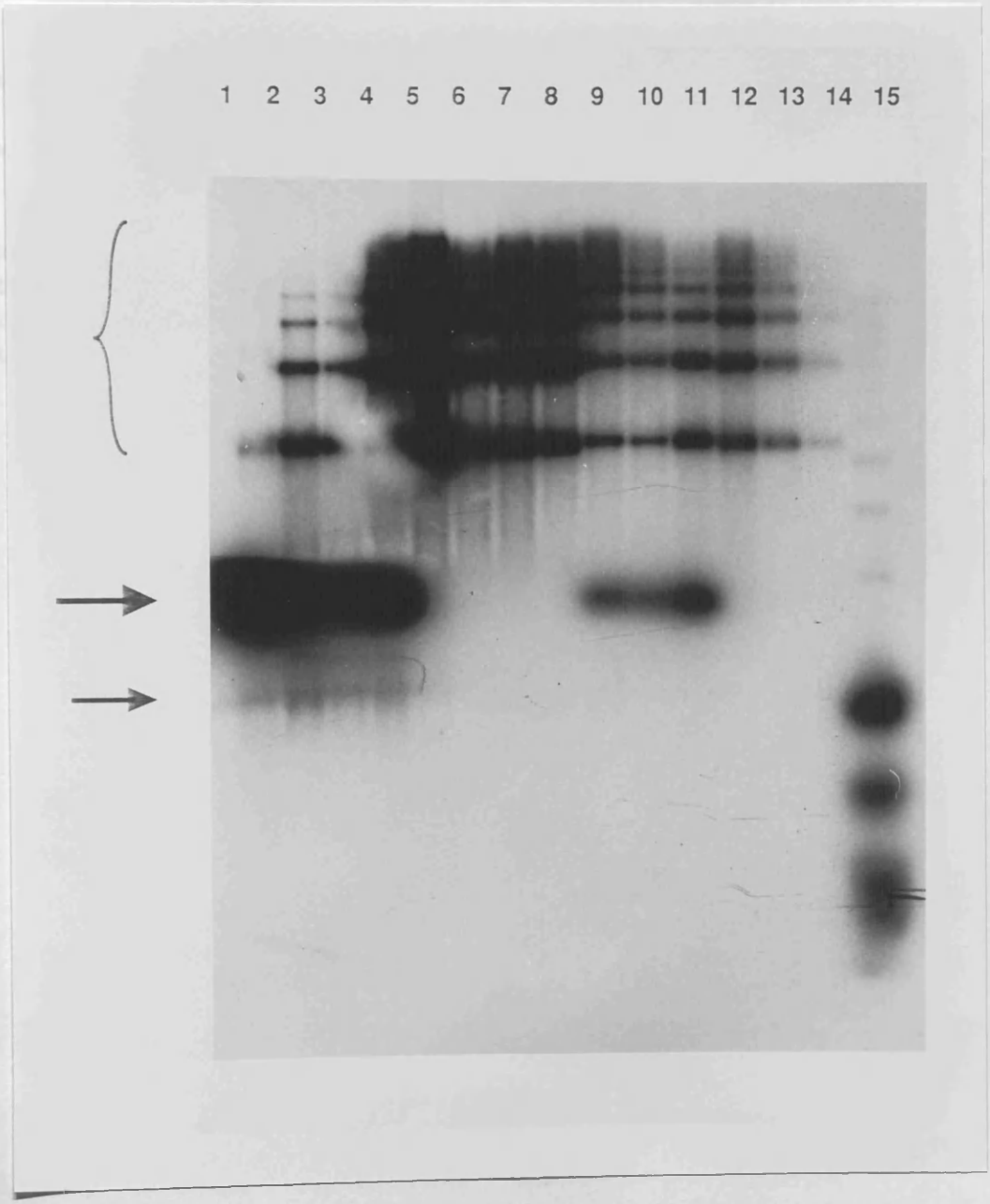
There are a number of possible explanations for the failiure to detect extract catalysed recombination of concatamer substrates. The most obvious is of course that concatamers are not an intermediate in the conversion of linear molecules to circles in the in vitro assay.

Before making this conclusion however it is worth considering other possibilities. It could be that the concentration of extract used in the reactions in Figure 4.26 are not sufficient to catalyse concatamer resolution. Alternatively formation of recombination intermediates and resolution of multimers might require the presence of monomeric circle (or linear) forms to act as a template for the recombination complex.

Figure 4.27 shows the visualisation of in each case reactions of V79 extract (the same extract as that used for the reactions shown in Figure 4.26 and thus known to be competent in terms of concatamer formation and recircularisation of linear plasmid) with

Figure 4.26

Southern visualisation of the products of reactions of concatamer recombination substrates and linearised pIC20H ligation assay substrate with nuclear extracts from V79 and *irs-2* cells.



(Figure legend on following page)

Conditions of electrophoresis and Southern blotting as previously described.

Reactions performed under standard conditions for in vitro assay reactions and with the modifications described in the text for recombination assay reactions.

Substrates were, in lanes 1-4 and 9-11 EcoRI linearised pIC20H and in lanes 5-8 and 12-14 20ng of concatamer recombination substrate.

Lane 1: EcoRI cut pIC20H incubated alone; lane 2: with 5µl; lane 3: with 10µl and lane 4: with 25µl V79 nuclear extract.

Lane 5: recombination substrate incubated alone; lane 6: with 5µl; lane 7: with 10µl and lane 8: with 25µl V79 nuclear extract.

Lane 9: EcoRI linearised pIC20H incubated with 5µl; lane 10: with 10µl and lane 11: with 25µl *irs-2* nuclear extract.

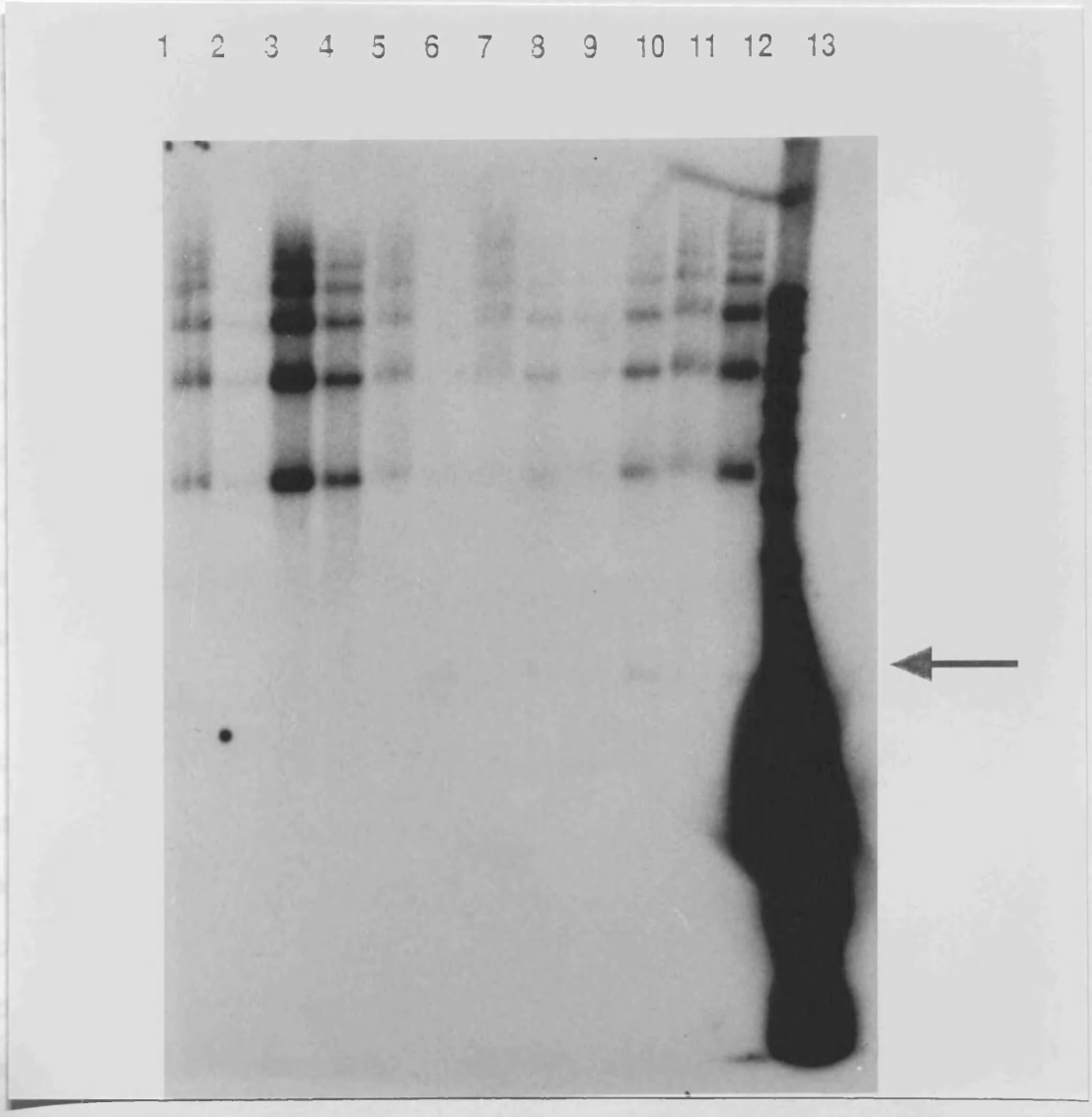
Lane 12: recombination substrate incubated with 5µl; lane 13: with 10µl and lane 14: with 25µl *irs-2* nuclear extract.

Lane 15: size markers -λ 1kb ladder BRL

Bracket indicates the positions of migration of high molecular weight concatamers, both those formed on incubation of linearised pIC20H with nuclear extracts and the recombination substrate concatamers. The larger arrow shows the position of migration of linear pIC20H and the smaller arrow the presumed closed circular form produced on incubation of linear pIC20H with V79 nuclear extract

Figure 4.27

Southern visualisation of products of reaction of recombination substrates with concentrations of V79 nuclear extract from 25ml to 100ml and showing the effect of addition of a small amount of linear or uncut pIC20H to the reaction.



Reactions, electrophoresis and Southern blotting performed as previously described.

Lanes 1-12 include 20ng concatamer recombination substrate. Lane 1: incubated alone; lane 2: with 25µl; lane 3: with 50µl and lane 4: with 100µl V79 nuclear extract. Lanes 5 and 6 include 1ng linear pIC20H; lane 6 also includes 50µl V79 extract. Lanes 7 and 8 include 0.5ng linear pIC20H; lane 8 also includes 50µl V79 extract. Lanes 9 and 10 include 1ng uncut pIC20H; lane 10 also including 50µl V79 extract. Lanes 11 and 12 include 0.5ng uncut pIC20H; lane 12 also including 50µl V79 nuclear extract.

Lane 13: λ 1kb ladder size markers.

Arrow indicates the position of migration of presumed circular forms.

recombination substrates. The reactions visualised here addresed the questions mentioned above. Recombination substrates were incubated with increasing concentrations of V79 extract. Reactions were also performed including small quantities of uncut or cut pIC20H. If resolution of recombination substrates were to occur under such conditions it would be vital to be able to differentiate input monomers from monomers formed by resolution of concatamers. Thus parallel incubations were performed including each type of monomer addition at each concentration tested without the addition of nuclear extract.

It can be seen that increasing the concentration of V79 extract as high as 100µl does not produce any evidence of resolution of concatamers. This is well above that required for recircularisation in all extracts tested. Furthermore this particular extract appears especially active -Figure 4.28 shows that the formation of a circular molecule from linear monomer input is catalysed even at a concentration as low as 5µl. It would thus be expected that, were V79 capable of concatamer resolution these concentrations should be sufficient.

The reactions involving the addition of a very low (0.5 and 1ng) concentration of uncut or cut monomer plasmid are shown in Figure 4.27. It is clear that the Southern gel was very unequally loaded so one must be careful not to overinterpret the data it presents. There is a suggestion of a molecule migrating well in front of the recombination substrates appearing only in the lanes in which extract as well as monomer pIC20H is added. It seems that either cut or circular plasmid monomer can stimulate the production of a fast migrating form and at both concentrations tested. Although the markers are grossly overloaded rough comparison with this lane and with Figure 4.26 suggests that the position of migration of the presumptive resolution product is not inconsistent with it being a monomeric form of pIC20H.

Assessing the impact of concatamer concentration might be important if it were found that both extracts are indeed equivalent in their processing of the recombination substrates using the current protocol. This set of experiments have demonstrated that the in vitro assay can be adapted to attempt to dissect reactions involved in pathways of DNA

repair. Thus as well as pursuing the line of investigation described the system might be exploited in slightly different ways. It is not possible to be certain that the effect of extract upon the artificial substrates generated as described is an accurate reflection of the treatment of concatamers produced in situ. Another approach would be to isolate concatamers formed by incubation of linear substrate with nuclear extracts prepared from V79 (and *irs-2*). These could be used as substrates for the action both extracts. This type of protocol might enable further study of the "concatamer defect " suggested as perhaps applying to *irs-2*.

4.2.4 COMPENSATION OF *IRS-2* DEFECT BY ADDITION OF PURIFIED PROTEINS.

4.2.4.1 Introduction.

An approach that has yielded much information in the study of the repair deficient syndrome Xeroderma Pigmentosum (XP) is that of adding purified protein to *in vitro* repair assays (Wood et al.1988; Hansson et al.1990). Wood et al.(1988) found extracts from XP cells to be deficient in repair synthesis of covalently closed circular DNA containing pyrimidine dimers. If UV irradiated DNA was incised by treatment with *M.luteus* pyrimidine dimer-DNA glycosylase prior to repair incubations extracts from XP cell lines showed fully competent repair synthesis. Furthermore these authors show that mixing of extracts from XP complementation groups A and C leads to full reconstitution of repair activity. Hansson et al.(1990) showed extracts from XP cell lines of four complementation groups to be incapable of repair synthesis on a DNA template treated with cis- or trans- platin or UV irradiated. Pretreatment of damaged DNA with purified *E.coli* UvrABC proteins enabled full repair synthesis by XP extracts. These studies give unequivocal support to the defect in the XP complementation groups studied being in the incision stage of excision repair. Using an in vitro repair synthesis assay in which XP cell lines are shown to be deficient Coverly et al.(1991) showed a requirement for the single stranded DNA binding protein (SSB also called RF-A and RP-A) in human excision repair.

As another approach towards characterisation of the biochemical defect in *irs-2* nuclear extracts it was decided to begin experiments to assess the capacity of purified proteins which might play a role in the repair of DSB to restore to *irs-2* extracts the capacity to form fast migrating circular molecules from a linear substrate.

Thus reactions were performed as in the standard in vitro assay of plasmid DSB rejoin using 50ng of cut pIC20H as substrate for the repair activities of *irs-2* nuclear extract alone or with the addition of purified proteins postulated to have a role in the repair of DSB.

Problems might arise due to repair incubation conditions not being optimal for the activities of the "complementing" proteins. Furthermore activities in extracts might

abrogate the repair activities of added proteins. It was thus important as far as possible to include controls for activity of added proteins as well as the usual extract activity controls.

As an end point in the study of possible correction of the *irs-2* defect it was chosen to examine circle formation visualising reaction products by Southern analyis.

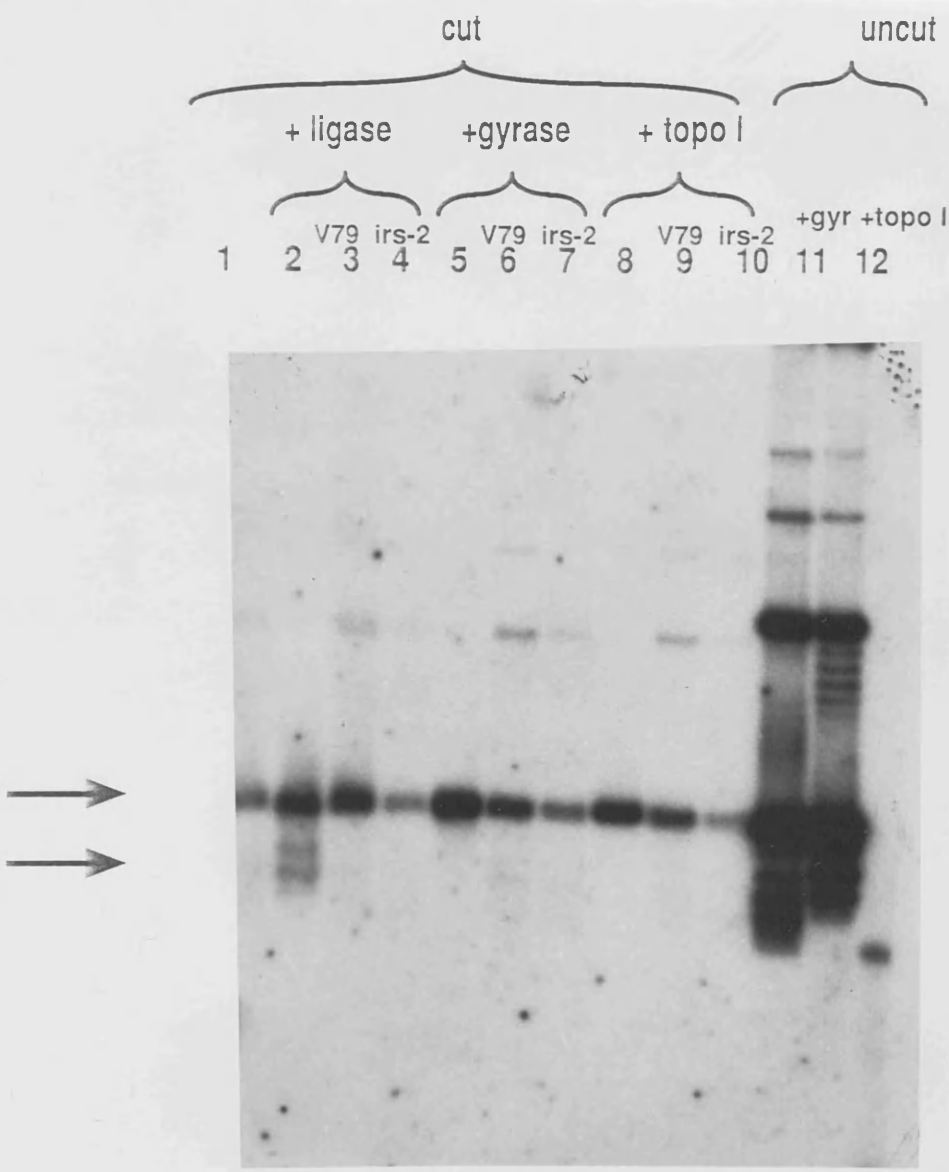
4.2.4.2 Results and discussion.

Figure 4.28 shows the addition of purified ligase, gyrase and topoisomerase I proteins to reactions including cut plasmid alone or with nuclear extract from V79 or *irs-2* cells.

The result of incubation of cut plasmid with T_4 ligase without nuclear extract shows that the T_4 used in these experiments is active in recircularising cut plasmid substrate. The addition of either V79 or *irs-2* extract to this reaction appears to inhibit this activity. The addition of gyrase alone has no effect upon cut plasmid substrate. On addition of V79 extract as well as gyrase there appears to be some recircularisation and the presence of gyrase does not inhibit the concatamerisation activity of V79. Unfortunately the extracts used for these reactions proved somewhat weaker than usual, as evidenced by the relative inefficiency of concatamer formation. Thus the presence of a fast migrating band (always less intense than those of concatamers) is only just detectable in the V79 and gyrase lane. The lane corresponding to incubation of cut substrate with *irs-2* also appears underloaded in comparison with the V79 plus gyrase incubation. There does however appear to be a faint band migrating below the cut plasmid substrate. This may of course be artifactual but it is still not possible to rule out the supposition that a gyrase activity can correct the deficiency in circle formation of *irs-2*. Incubation with topoisomerase I either alone or in the presence of V79 or *irs-2* nuclear extracts has little effect on cut plasmid. There is a very slight suggestion of a fast migrating band in the V79 lane but this is not strong enough to be interprable.

Figure 4.28

Southern visualisation of products of reactions of EcoRI linearised pIC20H with "complementing" proteins and nuclear extracts from V79 and *irs-2* cells.



Conditions of reactions, electrophoresis and Southern blotting as described previously.

Lanes 1-10: linear pIC20H; lane 1: incubated alone; lanes 2-4: incubated with T_4 ligase; lanes 5-7: with *E.coli* gyrase and lanes 8-10: with topoisomerase I.

Lanes 3, 6 and 9 also include V79 nuclear extract. Lanes 4, 7 and 10 *irs-2* extract.

Lanes 11 and 12 show results of incubation of uncut pIC20H with in lane 11: *E.coli* gyrase and lane 12: topoisomerase I.

Arrrows indicate the positions of migration of linear and circular forms of pIC20H.

That both the gyrase and topoisomerase I enzymes are active under the assay conditions is shown by the dramatic effect of both enzymes on uncut DNA. Thus Figure 4.28 presents no certain conclusions except that the enzymes added to extracts are functional under the conditions of the assay. A high priority would be to attempt to follow up this type of appraoch. It might well be however that even if reactions were performed with extracts of the highest quality results would still be equivocal due to the difficulty of interpreting a negative result. Thus it is possible that it is not the fact of the V79 extract being weak which is preventing detection of its recircularisation activity but some effect of the addition of gyrase or topoisomerase. In future experiments this could be addressed by the inclusion of exhaustive controls for extract activity as well as for enzyme activity.

The conclusions that can be drawn from these initial experiments are that the action of T_4 ligase is inhibited by the addition of (even rather weak) nuclear extract from V79 or *irs-2*. This implies that the recircularisation activity of T_4 proceeds by a different pathway from that of the wild type hamster extract.

No hard conclusions can be drawn from the data on gyrase or topoisomerase additions except that these enzymes are functional under the conditions of the in vitro assay. Their role in the activity seen in V79 and defective in *irs-2* is speculative and requires further study.

CHAPTER FIVE: LIGATION OF DSB BY NUCLEAR EXTRACTS FROM HUMAN CELLS

LIGATION OF AN ENDONUCLEASE INDUCED DSB BY NUCLEAR EXTRACTS FROM HUMAN CELLS

5.1 Introduction

The results of Thacker (1989a) and Jones et al.(1990) show cell lines derived from patients with the cancer-prone syndrome Ataxia Telangiectasia (AT) to have characteristics overlapping with those of particular *irs* mutants of V79. Both an AT cell line and *irs-1* show a lack of repair fidelity in the transfection assay for DSB ligation and the pattern of drug cross sensitivities of AT is similar to that of *irs-1*. *Irs-2* shares with AT lines the phenomenon of radioresistant DNA synthesis. We were interested in using the *in vitro* plasmid DSB assay for DSB repair to study AT cells. Nuclear extracts were prepared from AT and normal human MRC-5 cell lines. The MRC-5 line is not isogenic with the AT5BI line used but it is normal with respect to radiosensitivity. The two lines are those used in the transfection assay studies of Thacker and coworkers. These lines were felt to provide an adequate system for extending the plasmid DSB in vitro assay to look at human DSB repair. Nuclear extracts from human cells were prepared just as described for hamster lines in Chapter Four.

5.2 Results

5.2.1 Gel shift assays of human nuclear extracts for αP_3A binding activity.

Experiments assaying extracts of human cell lines for αP_3A binding activity gave rise to a pattern of results different from that seen for hamster extracts. Specific binding to αP_3A was shown to produce band shifts of differing mobility depending on the particular nuclear extract supplying the protein. Figure 5.1 shows one such band shift in which the extracts derived from "normal" human cell lines MRC-5 and HeLa exhibit an αP_3A binding activity producing a band shift equivalent to that seen after incubation with the hamster extracts, incubation of αP_3A with an extract derived from an AT line however gives rise to a faster migrating form. Competition experiments show both forms to be specific. It was thought possible that this represented an alteration in the CAAT box binding activity in this cell line. We pursued this, considering that this could represent an alteration common to AT cell lines or even be a manifestation of the AT defect. It was of interest to see if addition of MRC-5 extract (giving rise to the high form αP_3A band shift) to AT5 binding reactions would alter the pattern of binding of the AT5 nuclear extract. Figure 5.1 also shows band shift assay of the αP_3A binding activities of extracts from mixtures of

Figure 5.1

Band shift assay of αP_3A binding activity of MRC-5, HeLa and AT5 nuclear extracts.

Binding reactions and electrophoresis performed exactly as for hamster nuclear extracts, described in Chapter 4.

Free oligonucleotide has run off the end of this gel. All lanes contained radiolabelled αP_3A; lanes 3; 5; 7; 9 and 11 contained unlabelled competing oligonucleotide. Binding reactions included; Lanes 2 and 3: MRC-5 extract; lanes 4 and 5: a mixture of equal parts MRC-5 and AT5 extracts; lanes 6 and 7: AT5 extract; lanes 8 and 9: a mixture of equal parts AT5 and HeLa extracts; lanes 10 and 11: HeLa extract.

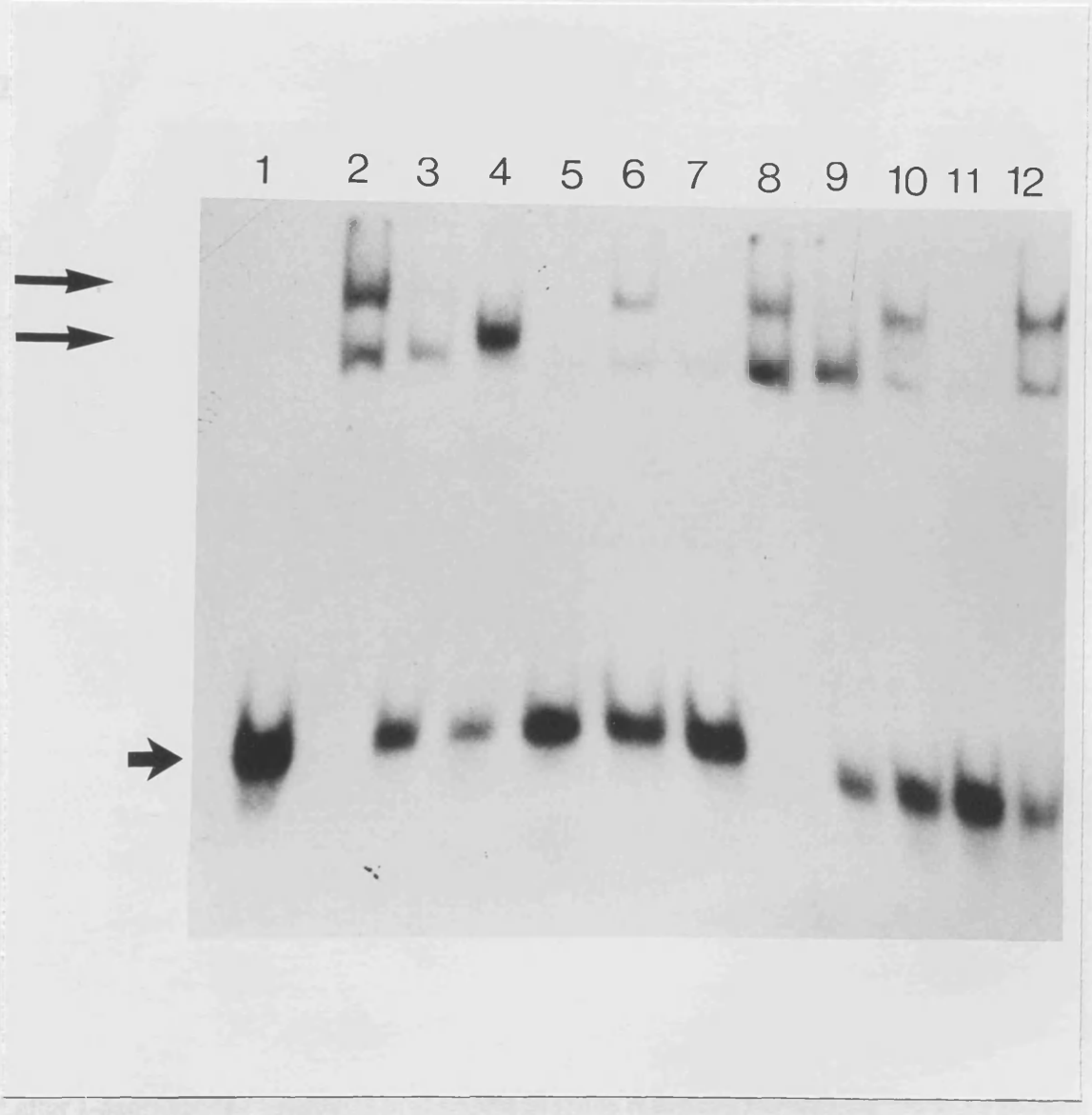
Arrows mark the positions of the bound αP_3A oligonucleotide/protein complexes.

extracts containing AT and HeLa or MRC-5. In this assay unbound αP_3A has been run off the end of the gel. Incubation of αP_3A with mixtures of the two extracts gives rise to both the faster and the slower migrating bands. This implies that neither of the two forms of binding activity is recessive to the other and is consistent with the fast migrating binding activity being a property of AT lines. We next assayed the αP_3A binding activity of nuclear extracts prepared from other AT cell lines and lines derived from parents of AT patients -presumptive heterozygotes for the AT gene(s) (Provided by Colin Arlett of the MRC radiobiology unit at Sussex University). Figure 5.2 shows a band shift assay of the αP_3A binding of nuclear extracts from AT1 and AT4 lines compared with that of two AT parental lines, MRC-5 and AT5. The MRC-5 extract is here shown to contain activities giving rise to both the high and the low band shifts, with the lower (fast migrating form) being more difficult to compete out with unlabelled αP_3A. The AT5 extract again gives rise only to the lower fast migrating activity. Nuclear extracts from both AT4 and AT1 and also the AT parental lines show both binding activities. All binding is specific. Thus the possession of a faster migrating form of αP_3A binding activity was shown not to correlate exclusively with an AT phenotype and the slower migrating activity did not correlate exclusively with a phenotype of normal radiation response. This was confirmed in subsequent assays where different preparations of nuclear extract from the same cell line showed different αP_3A binding patterns.

It is probable that a band shift assay is not revealing a single activity binding to a specific sequence but a complex interaction involving a number of proteins, one binding the relevant recognition sequence on DNA (which would in itself give a band shift) and then further proteins binding this complex via interaction with the first. One explanation of the results described above is that the slowest migrating (ie most retarded band) is the result of "complete" complex formation and that faster migrating (smaller) bands are due to the formation of incomplete complexes. Such a result implies that not all the proteins required to associate to form the slowest migrating complex (seen as the top band on autoradiography) are present in the extract in a functional form or that the proteins required for the formation of the higher molecular weight complexes are present at a lower concentration or in a form precluding binding.

Figure 5.2

Band shift assay of αP_3A binding activity of nuclear extracts from AT and AT parental lines compared to MRC-5.



Conditions of binding and electrophoresis as described in Chapter four.

All lanes include labelled αP_3A; Lanes 3, 5, 7, 9 and 11 include excess unlabelled competing oligonucleotide. Extract added to binding reactions as follows; Lanes 2 and 3: MRC-5; lanes 4 and 5: AT5; lanes 6 and 7: AT1; lanes 8 and 9: AT parental 108; lanes 10 and 11: AT4; lane 12: AT parental 103.

Small arrow marks the position of migration of unbound oligonucleotide, Large arrows mark the positions of migration of the high form and low form αP_3A binding complexes.

5.2.2 Assay by bacterial transformation

Table 5.1 shows data using the products of incubation with MRC-5 extract performed under the standard in vitro plasmid DSB assay conditions to transform bacteria. These results are the averages of in some cases only two incubations so are by no means conclusive.

Table 5.1

The effect upon uncut and PstI cut substrate of incubation with MRC-5 human fibroblast nuclear extract

Treatment	Colony number per ng DNA transformed
uncut	75
uncut + 10µl MRC-5	19
uncut + 50µl MRC-5	14
uncut + In MRC-5	51
PstI cut	0.3*
PstI cut + T_4 ligase	1
PstI cut + 10µl MRC-5	0.07*
PstI cut + 50µl MRC-5	0.7*
PstI cut + In MRC-5	0.3*

*** after λ-exonuclease treatment of products of these reactions transformation to ampicillin resistance was below the level of detection.**
Since numbers refer to very few incubations errors are not shown. "In" refers to inactivated MRC-5 extract.

Table 5.1 makes it clear that the human MRC-5 extract has a significant effect upon uncut pIC20H, reducing the transformation frequency after isolation of reaction products by a factor of four. This is assumed to reflect an activity degrading DNA. Comparison with with the data from the hamster system (Table 4.5) suggests that this represents a higher concentration of

nuclease activity in this extract than that found in the hamster extracts . This effect appears dose dependent but given the low transformation rates and the few incubations for each value this may be artefactual. The process is an active one -incubation with inactivated extract giving almost equivalent colony numbers as plasmid incubated alone.

Interpretation of the data for the rejoin by MRC-5 of the PstI induced DSB is complicated by the low transformation efficiency overall and also the low T_4 ligase value. Incubation with active MRC-5 leads to no significant rejoin of an endonuclease induced DSB to a λ-exonuclease resistant form (compare the increase over background value for the addition of 50μl V79 in Table 4.5 with that in Table 5.1 for addition of 50μl MRC-5). Furthermore, a high proportion of the colonies formed after incubation of substrate with MRC-5 nuclear extract were white as opposed to blue. Thus ligation of a PstI DSB appears to proceed with low efficiency and also low fidelity (compare again V79 in Table 4.4 where no white colonies are seen ie all ligation visualised occurs with high fidelity). One possibility is that the MRC-5 extract contains a high concentration of nuclease activities causing degradation or loss of information before cut plasmid substrate can be ligated.

Figure 5.3 shows restriction analysis of plasmids rescued from white colonies produced on transformation of competent JM83 with DNA isolated after reaction of cut plasmid with human extracts and in one rare case T_4 ligase. All but one of these (lanes B5, 6 and 7) are found to have lost the PstI site at which the plasmid was originally linearised, the exception has gained a novel PstI site giving an anomolous two band pattern on electrophoresis. On cutting these plasmids with BglI it was clear that while two plasmids exhibited the normal pattern (A7, B10) one of the six analysed had lost information in the smaller BglI band ie that spanning the lacZ polylinker where the original cut was introduced (A10). Two others appeared to show deviant BglI patterns (B4 and B7) where the smaller fragment spanning the polylinker remains intact but there is a considerable increase in size of the larger spanning the body of the plasmid. These two plasmids ran anomolously as uncut molecules however so these alterations may be an artefact. These results support the idea that the action of nucleases producing loss of information might result in white colonies. Explanation of gain of sequence information would require some form of recombination occuring on incubation of cut plasmid with extract. This could be either an

homologous event between substrate molecules as linear monomers or as multimers. Alternatively it could be a non homologous recombination event between substrate molecules and genomic DNA. The extracts used in this study are not believed to contain large amounts of genomic nucleic acids. In general nuclear extracts prepared by the method used have ratios of optical densities at 260nm to those at 280nm (O.D.260/280 ratios) of about 1.0. Assuming the absorption by protein (280nm) to be about twenty times less efficient than that of nucleic acids (260nm) this suggests an excess of protein over nucleic acids, furthermore it is believed that most of the nucleic acid remaining in the extract preparation is RNA not DNA (Dr M. Plumb personal communication). Although this indicates an actual physical excess of protein over DNA competition for extract activities by genomic DNA can not be discounted entirely.

Figure 5.3

Restriction analysis of plasmid rescued from white colonies produced after transformation with products of reaction with nuclear extracts from human cells and T_4 ligase

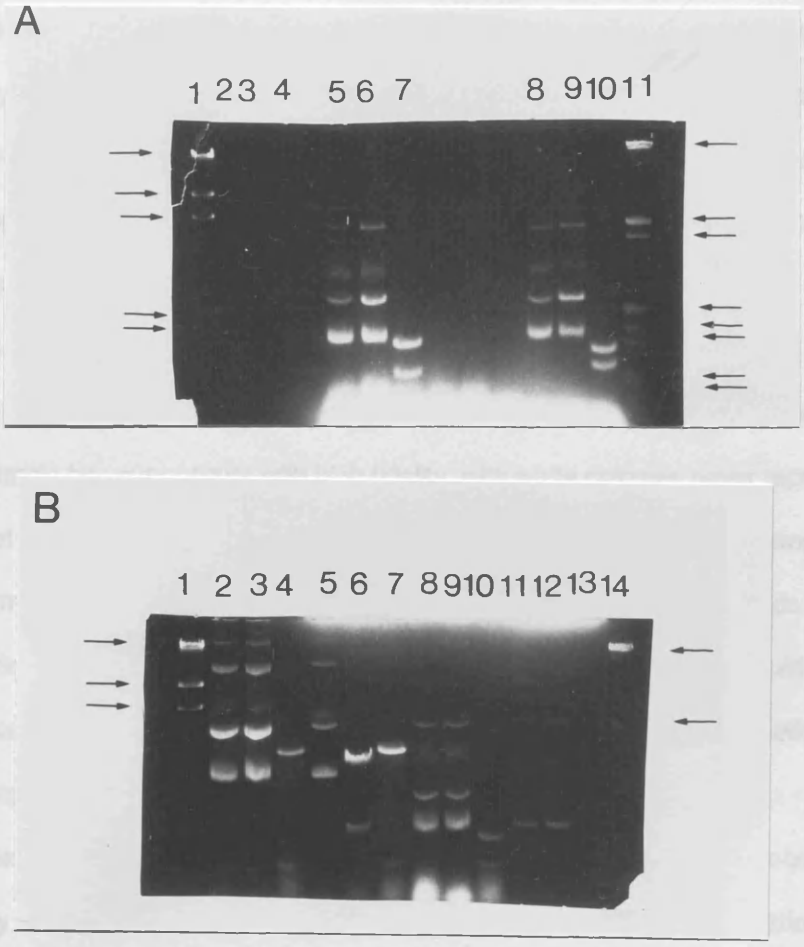


Figure 5.3 shows 1% TBE/agarose gels run with ethidium bromide. Approximately 1µg of each DNA was loaded in each lane. After electrophoresis DNA was visualised by UV transillumunation and gels were photographed.

Panel A: Lane 1: λ/HindIII size markers. Sizes of fragments: 23.72kb; 9.46kb; 6.67kb; 4.26kb; 2.25kb; 1.96kb; 0.59kb; 0.1kb. Lane11: I EcoRI/HindIII size markers. Sizes of fragments: 21.8kb; 5.24kb; 5.05kb; 4.21kb; 3.38kb; 1.96kb; 1.91kb; 1.62kb; 1.32kb; 0.93kb; 0.88kb; 0.59kb; 0.01kb. Lanes 2, 5 and 8: uncut plasmid. Lanes 3, 6 and 9: PstI cut plasmid. Lanes 4, 7 and 10: BglI cut plasmid. Lanes 2, 3 and 4: pIC20H. Lanes 5, 6 and 7: plasmid isolated from white colony after transformation of bacteria with products of reaction of cut substrate with T_4 DNA ligase. Lanes 8, 9 and 10: plasmid isolated from white colony after transformation of bacteria with reaction products of incubation of cut plasmid alone.

Panel B: Lane 1 as lane 1 and lane 14 as lane 11 in panel A. Lanes 2, 5, 8 and 11 uncut plasmid. Lanes 3, 6, 9 and 12 Pst I digested plasmid. Lanes 4, 7, 10 and 13 BglI digested plasmid. Plasmids isolated from white colonies after transformation of bacteria with reaction products of incubations of cut substrate with; Lanes 2, 3 and 4 and also 5, 6 and 7: nuclear extract from kidney cells. Lanes 8, 9 and 10 and also 11, 12 and 13: nuclear extract from MRC-5 cells. The reaction products giving rise to colonies harbouring these plasmids had all been treated with λ-exonuclease prior to transforming bacteria.

Dr Gill Ross (Depts. Medical Oncology and Radiation Oncology CRC Beatson labs. Glasgow) is currently using the in vitro plasmid DSB assay system described to address questions about the religation capacity of nuclear extracts from human tumour cell lines of varying radiosensitivities. These extracts do not give evidence of concatemerisation. Furthermore even at very low extract concentrations there is efficient recircularisation of substrate. In these human extracts there is little "alternative ligation" activity competing with recircularisation and the situation approximates more closely to that we expected when establishing the assay in that recircularisation of input linear molecules is the main reaction observed.

It is expected that an extract containing nucleases would catalyse rejoin of substrate to give at least some proportion of products with abrogated β-gal transcription. All ligation in the hamster extracts however occurs with high fidelity, with white colonies never representing more than 0.1% of the total and concatemers retaining a PstI site. An activity protecting substrate from loss of information could be acting. This could be a specific protein binding ends and preventing their degradation prior to ligation. Alternatively the "protection" of substrate could arise as a consequence of the concatemerisation activity or as a result of the relative kinetics with which nuclease degradation, ligation and phosphatasing reactions proceed.

In extracts from the human lines which Dr Ross is studying ligation proceeds with slightly lower fidelity with 2% of colonies being white. This value increases with incubation time. Preliminary results suggest that one of the extracts -from a Glioma cell line with radiosensitivity equivalent to that of the wild type V79 hamster cells gives as dose of extract is increased many more white colonies, with at the higher concentrations tested up to 50% of colonies being white. Neither another Glioma line with equivalent radiosensitivity nor two lines more sensitive (analagous to the *irs* mutants of V79) show this dose dependent increase in the proportion of white colonies. Perhaps an explanation for these anomalies lies in part with the lack of concatemerisation exhibited by the human extracts.

5.2.3. Southern analysis of products of reaction with human nuclear extracts

Figure 5.4 is a Southern showing the products of an experiment in which MRC5 nuclear extract was incubated under standard conditions with uncut pIC20H. Native uncut plasmid can be seen to consist of a number of forms, comprising various topoisomers of closed circles, nicked circles and high molecular weight concatenated circular forms. The addition of active extract removes the fast migrating closed circular form and also the high molecular weight slower migrating forms. Boiled nuclear extract had no effect. In Table 5.1 the transformation efficiency of uncut plasmid is shown to be much reduced on incubation with MRC5 nuclear extract. These results presumably correlate with the removal of circular forms seen on Southern visualisation. The Southern analysis of these products adds weight to the hypothesis that the nuclear extract contains a high level of activity/ies nicking and degrading circular molecules. Figure 5.4 gives no information as to whether the molecules isolated after incubation of uncut plasmid with nuclear extract are nicked circular or linear since these migrate to the same distance under these gel conditions. Treatment of reaction products with λ exonuclease should resolve this question, as linear molecules but not nicked circles are substrates for its activity. Figure 5.5 shows Southern visualisation of the results of an experiment in which uncut pIC20H was incubated alone, with HeLa nuclear extract, or with inactivated HeLa extract. One aliquot was run immediately after incubation, another was treated with λ exonuclease prior to Southern analysis. The resolution and loading of the gel in Figure 5.5 do not allow the visualisation of as many isomers of the uncut plasmid as seen in Figure 5.4 but the presence of the products of incubation of uncut plasmid with inactivated extract and of plasmid alone allow comparisons to be made with the products observed after treatment with nuclear extract. It is clear from these results that the closed circular fast migrating form is abolished on extract treatment. This is not a linearisation as after exonuclease treatment there is little reduction in the signal corresponding to linear and closed circular plasmid forms. The reduction in closed circular forms is thus assumed to be due to their conversion to open circular. The loss of transformation frequency seen after treatment of uncut plasmid with nuclear extract presumably represents this population. The loss observed in transformation frequency after treatment with MRC-5 nuclear extract is possibly a function of the loss of a proportion of circular molecules as seen in the overall reduction in DNA content of the

lanes containing products of reaction of uncut plasmid with nuclear extract in Figure 5.4 and the left hand panel of Figure 5.5. The degradative effect of nuclear extracts upon uncut plasmid as seen using Southern analysis correlates well with the data following transformation of equivalent reaction products.

Figure 5.4

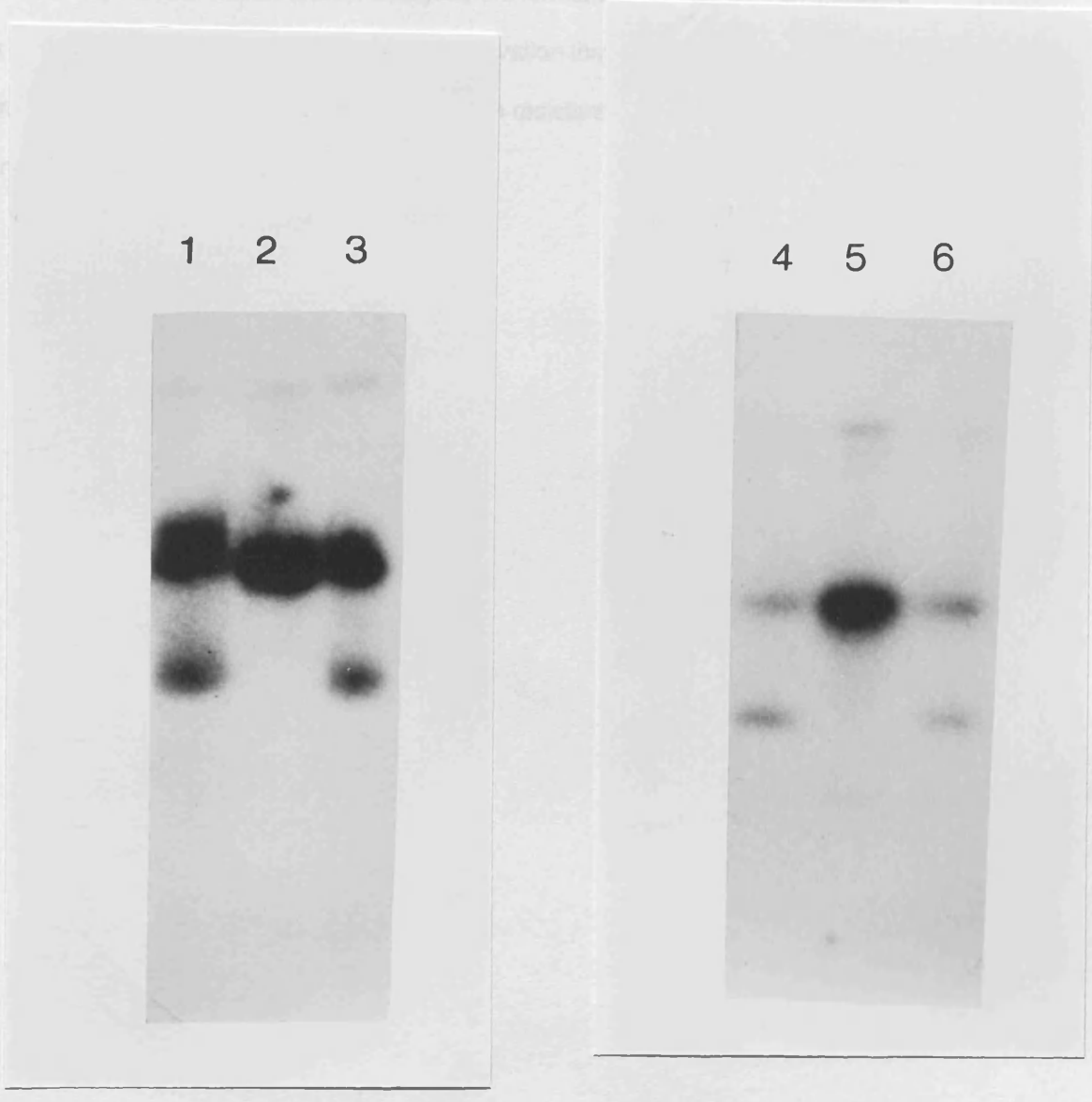
Southern visualisation of reaction of uncut pIC20H with nuclear extract from MRC-5 cells.

Electrophoresis and Southern blotting performed as described in Chapter four.

Visualisation of products of reaction under standard conditions, Lnae 1: uncut pIC20H alone. Lane 2: incubation of uncut pIC20H with inactive MRC-5 extract. Lane 3: incubation of uncut pIC20H with active MRC-5 extract.

Figure 5.5

Southern analysis of products of reaction of uncut pIC20H with HeLa nuclear extract before and after λ-exonuclease treatment.



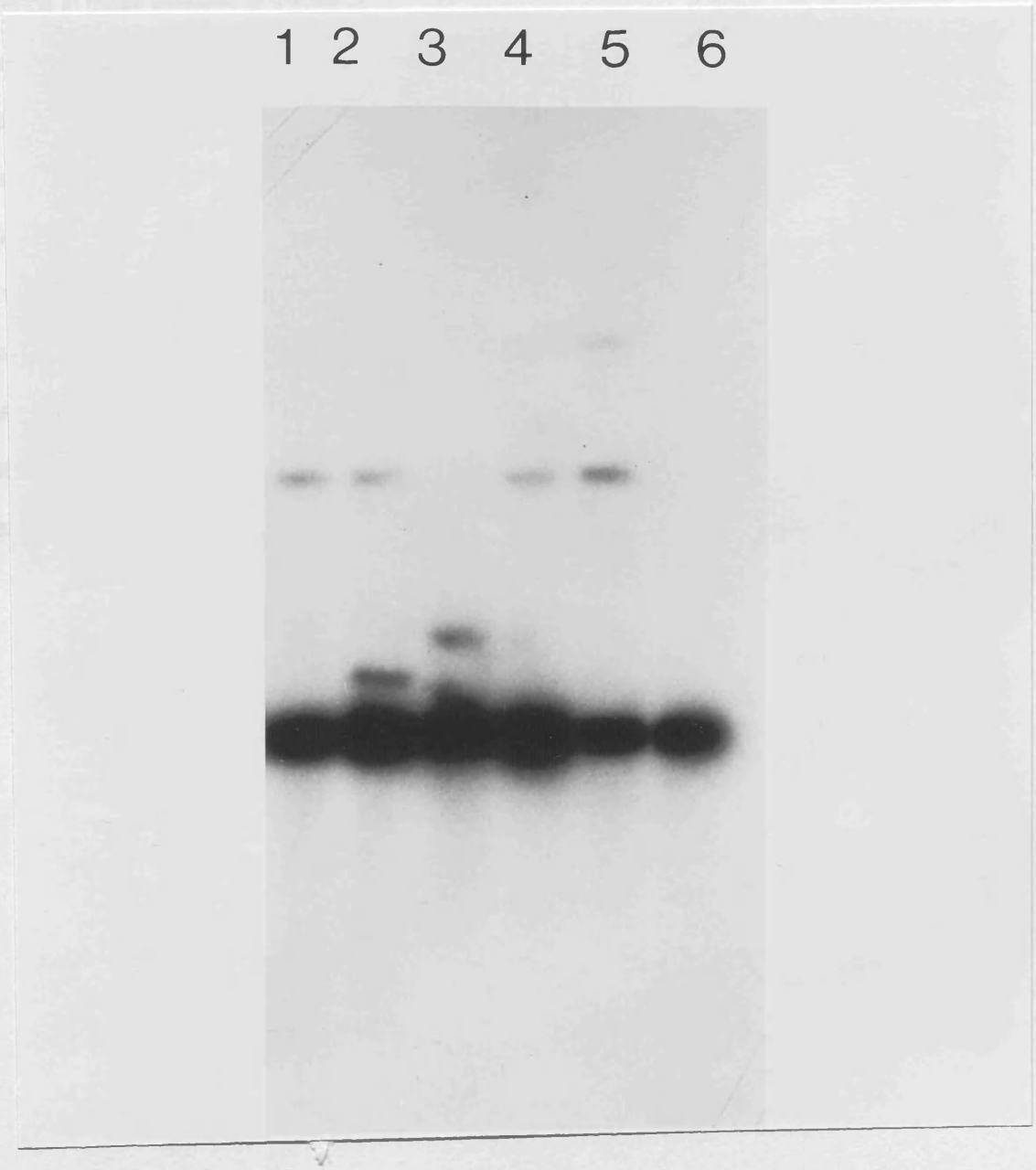
Conditions of electrophoresis and Southern blotting as previously described.

Lanes 1, 2 and 3: samples prior to λ-exonuclease treatment. Lanes 4, 5 and 6: aliquots of the same samples after λ-exonuclease treatment. Reaction products isolated after incubation under standard conditions of; Lanes 1 and 4: uncut pIC20H alone. Lanes 2 and 5: uncut pIC20H incubated with HeLa nuclear extract. Lanes 3 and 6: uncut pIC20H incubated with inactivated HeLa extract.

Figure 5.6 is a Southern showing the products of reaction of PstI linearised pIC20H with nuclear extracts from MRC-5 and AT5 lines. Both extracts show weak concatemerisation activity. Incubation with inactivated extract of either type does not give rise to these forms showing that this is an active process. Neither extract catalyses the formation of a fast migrating closed circular molecule. This correlates well with the observation that products of such reactions give rise to no significant increase in the level of ampicillin resistant bacterial colonies after transformation.

Figure 5.6

Southern analysis of reaction products from incubation of PstI linearised pIC20H with nuclear extracts form MRC-5 and AT5 cells.



Electrophoresis and Southern blotting as described previously.

This Figure shows reaction products of incubations under standard conditions of PstI cut plasmid with; lane 1: 30µl MRC-5; lane 2: 60µl MRC-5; lane 3: inactivated MRC-5; lane 4: 30µl AT5 extract; lane 5: 60µl AT5 extract; lane 6: inactivated AT5 extract.

5.3 Conclusions

It became clear that the MRC-5/AT system would not be as amenable to analysis in the in vitro plasmid DSB assay for DSB repair as we had hoped. We consider this to be due to the presence in these extracts of a balance of DNA degrading and DNA ligating activities favouring substrate degradation under the conditions of the assay. The effect of these extracts in reducing uncut plasmid transformation frequency was much more dramatic than that seen in the hamster extracts, this correlated with the removal from the reaction products (visualised by Southern blotting) of the closed circular form of the plasmid. Concatemerisation by these extracts was the only form of ligation visualised and this was in both cases a weaker activity than that seen in the hamster system. Transformation of bacteria with products of incubation of linearised pIC20H with human nuclear extracts gave rise to no significant increase over background in ampicillin resistant colonies. This correlates with lack of detection of a circular molecule on Southern visualisation of reaction products.

CHAPTER SIX: DISCUSSION

Discussion

6.1 LIGATION OF AN ENDONUCLEASE INDUCED DSB *IN VITRO* BY HAMSTER NUCLEAR EXTRACTS

The results presented show that rejoining of an endonuclease induced DSB in plasmid DNA by wild type V79 nuclear extract can be detected by means of bacterial transformation and selection for plasmid gene expression. The reaction products of such a repair reaction can also be visualised directly by Southern analysis. Religation of two types of cohesive DSB (with either 3'or 5'protruding ends) catalysed by V79 extract proceeds with comparable efficiency and in all cases with high fidelity. Although such DSB can only represent a small subset of the types induced by ionising radiation there is no indication that the processing of such a lesion differs from those induced by radiation damage. The assay can be considered relevant to the study of repair of ionising radiation induced damage.

The capacity of nuclear extracts prepared from ionising radiation sensitive mutants of V79 to recircularise cut plasmid substrate was assessed. One of these mutants *irs-1* showed the same efficiency and fidelity in religation of both types of DSB substrate as V79. A defect in religation efficiency in extracts prepared from *irs-2* was found. Incubation with *irs-2* extracts gave no significant increase in transformation of bacteria to ampicillin resistance. Southern analysis of the products of incubation of cut plasmid with extracts from wild type and radiosensitive cells showed all extracts to catalyse the conversion of substrate to high molecular weight concatemers with equal efficiency. Evidence from Electron Microscope (EM) studies and the use of λ-exonuclease suggests that these molecules are linear. I have shown that this type of molecule is incapable of transforming bacteria to ampicillin resistance. The presence of a circular molecule visualised on Southerns among the products of reaction with V79 and *irs-1* but not *irs-2* correlates quantitatively with increased transformation of bacteria to ampicillin resistance. Correlating better in terms of dose response with bacterial transformation is a circular molecule visualised on Southerns among the products of reaction with V79 and *irs-1* extracts but not *irs-2*. Such circles have been shown to transform bacteria with high efficiency. These data raise a number of questions, both in terms of the types of activity detected in the wild type V79 extract and with respect to the deficiency observed in *irs-2*.

I will discuss first the ligation activity detected in wild type V79 hamster cells and in one

radiosensitive mutant *irs-1*. Given that conditions of incubation were chosen to favour recircularisation over other forms of ligation, the formation of concatemers of input substrate at even the lowest concentrations of extract tested is unexpected. It appears that in the hamster extracts with which this study is concerned a pathway of concatemer ligation is favoured over recircularisation.

Low concentrations of extract, which only produce a slight increase in bacterial transformation efficiently induce concatemerisation. This implies that under these assay conditions the formation of concatemers is the primary reaction in V79 extracts, circle formation occuring with more complex kinetics. It is possible that a higher concentration of specific enzymes is necessary for recircularisation to compete successfully for substrate. Alternatively, the possibility exists that the transforming molecule is a product of a reaction involving concatemers and that the formation of concatamers is a prerequisite for circle formation. Thus a substantial population of input substrate could be converted to multimer form. As the concentration of extract is increased the multimers would be resolved to form circular molecules. Therefore the capacity of reaction products to transform bacteria would increase with increasing dose of extract. There appears to be no reduction in the amount of concatamers detected by Southern hybridisation at the highest extract concentrations, implying that the proportion converted to circles is small. However the possibility that the detection by Southern gel is saturated means that estimation of the proportion of concatemers converted is difficult.

If on the other hand concatemerisation is in fact a separate activity from that forming circles a mechanism might act to prevent the expected circle formation by imposing a topological constraint upon recircularisation. For example, the complexing of input substrate into nucleosomes or some other structure precluding recircularisation might favour the formation of linear concatemers. Alternatively the presence of a protein analogous to the "alignment protein" postulated to be present in Xenopus extracts by Pfieffer et al.(1988) might encourage the formation of concatemers. In either case there must be a mechanisn by which with increasing extract concentration concatemerisation is saturated and circle formation favoured.

It is a possibility that other activities in the extract contribute to circle formation as their absolute concentration is increased. For example, nucleases would be expected to have a more

significant effect on linear molecules than those circularised or even formed into a pre ligation complex.

6.2 THE *IN VITRO* PLASMID ASSAY FOR DSB REPAIR USED IN THIS STUDY IN THE CONTEXT OF OTHER APPROACHES.

Studies by Thacker et al. (reviewed Thacker 1989a) using an in vivo transfection system to assay for repair of an endonuclease induced DSB in V79 and the *irs* series of mutants (see Introduction) revealed a deficiency in *irs-1* in the fidelity of rejoin of such a DSB compared to wild type and also *irs-2*. Thus the in vivo transfection system and the *in vitro* plasmid DSB assay used in this study yield very different results. In our assay all extracts exhibit fidelity of rejoin and *irs-1* behaves as wild type V79 cells while *irs-2* (identical to wild type V79 cells in the assay of Thacker et al.) shows a marked defect in the efficiency of catalysis of one pathway of rejoin. In the *in vivo* transfection system described by Thacker et al. it is impossible to determine what proportion of molecules taken up by a cell are ligated. These assays also do not quantitatively address the question of efficiency of religation. The processes occuring between the application of a precipitate of DNA to the cell surface and its integration into the genome are poorly understood and while ligation events would be expected to be involved there is no certainty that a deficiency in ligation would be revealed by a drop in transfection frequency. Furthermore, the two assays may select for different processes of rejoining an endonuclease induced DSB. An obvious difference is in the much greater potential for recombination inherent in the assay system of Thacker, both between substrate molecules and during integration of the DNA. Further the amount of substrate present and the form in which the substrate exists over the period of incubation may well be very different in the two systems. Thus although direct comparison between these assays is complicated, both assays can be seen as giving valid indications of defects of DSB rejoining.

A similar discrepancy was found between the behaviour of the radiation sensitive mutant *xrs-5* (Jeggo and Kemp 1983) in assays for recombination. *xrs-5* is defective by neutral elution in the repair of DSB (Kemp 1984) and shows a deficiency in homologous recombination *in vivo*

similar in magnitude to its DSB repair deficiency (Moore et al 1985). Furthermore a comparable reduction in the nonhomologous integration of transfected DNA is observed (Moore et al. 1985, Hamilton and Thacker 1987) and it is suggested that the primary defect in this cell line is one in a process commmon to both pathways (Kucherlapati and Moore 1988). In vitro, however, nuclear extracts from *xrs-5* show comparable recombination activity to that of wild type (Moore et al. 1985).

A number of explanations are advanced for this, including the possibility that activities limiting in vivo are in excess in the in vitro situation or that activities unavailiable in vivo can be substituted for by different but related proteins (Kucherlapati and Moore 1988).

It is also possible that during extract preparation protein modifications (eg phosphorlyation or glycosylation state) are changed rendering the proportion in an active conformation in vitro different from that in vivo. The cellular environment is very different in a number of ways from the situation in vitro, factors such as compartmentalisation and subcellular localisation of proteins obviously not applying in vitro.

North et al.(1990) have recently described an *in vitro* assay for the ability of nuclear extracts from Ataxia Telangiectasia and MRC5 cell lines to catalyse the rejoin of DSB induced by a variety of restriction endonucleases. Using Southern blot analysis and bacterial transformation to monitor rejoin efficiency and fidelity these authors find that both extracts repair endonuclease induced DSB with equivalent efficiency. The efficiency of religation was found to depend on the type of DSB termini. DSB with 5' four base overhangs were repaired with greater efficiency than those with a 3' four base overhang, DSB with four base overhangs were repaired better than those with two base overhangs and all cohesive ended DSB were repaired more efficiently than blunt. Nuclear extracts from the radiosensitive AT cell line and from the normal MRC-5 line showed similar efficiency of religation of all types of DSB. At some, though not all, sites the AT line showed a drop in fidelity of religation.

The authors suggest that the differences in rejoin efficiency shown by both extracts between different types of DSB are due in part to differences in end cohesiveness. For example the low rejoin efficiency of HinclI (non-cohesive) or AccI (2 base overhang) cleaved ends by both MRC-5 and AT5 extracts is explicable by the lack of cohesiveness of these DNA ends. This

could also explain the difference in rejoin efficiency between an EcoRI and a SalI induced DSB, EcoRI termini (5'AATT, ie eight hydrogen bonds) being associated into a less stable pre ligation complex than SalI termini (5'TCGA, ie ten hydrogen bonds). Data for other sites suggests that other factors might be important, for example strand polarity of termini or possibly specific sequences at termini or the location in the plasmid of the break site.

The finding that nuclear extracts prepared from an AT line rejoin a DSB with comparable efficiency to that of extracts prepared from wild type MRC-5 is consistent with findings that DNA ligase activities are similar in both cell lines (Willis and Lindahl 1987). This observation extends the data from transfection of AT and MRC-5 lines (Thacker 1989b) described in Chapter One and discussed above in that it shows that the efficiency of rejoin, which cannot be asessed in the transfection assay, to be similar in both cell lines.

North et al. found nuclear extracts prepared from the AT line to give high mis-rejoin frequencies at some sites. Breaks rejoined with low efficiency by both extracts show low fidelity of rejoin by the AT extract. The implication is that where breaks are less efficiently rejoined some extracts will remove sequence from these ends prior to their rejoin. Two DSB sites having the same sequence of overhang but in reverse direction are rejoined with very different fidelity. Treatment of HindIII induced breaks (5'AGCT overhang) with AT extract gives a high efficiency of rejoin but a low fidelity while treatment of SstI (3' AGCT overhang) gives a lower efficiency of rejoin but at a much greater fidelity. North et al. suggest that the AT extract may contain specific means of ensuring that certain sequences are rejoined with fidelity. They suggest that the lack of fidelity in rejoin by AT extracts may be due to a lack of protection of ends allowing degradation processes access to DNA DSB ends prior to their ligation as proposed by Thacker (1989). Such protection could arise from hydrogen bonding of cohesive ends or more strongly from proteins binding such ends. A number of DNA binding proteins which might perform such a role have been identified in bacteriophage Mu and adenovirus infected cells. Studies of break rejoining in *E.coli* (Conley et al. 1986) as well as the studies of Pfeiffer and Vielmetter (1988) referred to previously have led to the suggestion that proteins in addition to ligation activities are required to maintain DNA termini in the correct conformation prior to rejoin.

In this AT cell line in comparison with MRC-5 a lack in such a protein might allow the

degradative activities present in the extract acces to broken DNA ends leading to loss of information and a drop in rejoin fidelity. North et al.(1990) mention that tests for nuclease activities show no difference between the two cell lines. This makes it improbable that the lack of rejoin fidelity exhibited by AT extracts is a consequence of excess nuclease activity.

In our assay the situation is somewhat different, with all hamster extracts showing equivalent fidelity of DSB ligation, differences being revealed in the efficiency with which the process occurs. It is possible that in the hamster extracts used in this study as opposed to in the human extracts used by North et al. proteins protecting ends of maintaining them in the appropriate conformation for ligation are not limiting. This is supported by the high efficiency of concatemer formation in all hamster extracts tested.

6.3 DEFECT IN *IRS-2* REVEALED BY IN VITRO ASSAY

In seeking explanations for the defect in *irs-2* revealed as an inability of nuclear extract to catalyse the rejoin of a plasmid linearised by endonuclease digestion the possible mechanisms of such repair must be considered.

6.3.1 Proteins with a possible involvement in repair of DNA DSB

For ease of description the types of activity postulated to have an involvement in the repair of DSB may be somewhat arbitrarily classified into three groups. The first, including activities such as ligases, nucleases and phosphatases are enzymes acting directly on substrate ends. The second are activities which might be required for the ligation of ends but are not ligation enzymes per se. In this category are proteins catalysing strand exchange, topoisomerases and helicases. The final set of proteins considered to play a part are structural components examples being the alignment protein postulated by Pfieffer, end binding or protecting proteins hypothesised by Thacker (1988) and strand binding activities. This third class are imagined not as having catalytic activity in themselves but as being necessary for the action of the enzymes of the other two classes. It is possible that histones and other proteins involved in DNA packaging might also be involved. There is some evidence for repair of adducts in naked

DNA being an inaccurate representation of events occurring during repair of adducts to DNA packaged as chromatin (Smerdon et al. 1990; Ljungman 1989). It is probable that questions of accessibility of the DNA adduct and the torsional conformation of the DNA molecule are the variable parameters. It should be emphasised that this classification of activities is simply for clarity of discussion and may have limited relevance to repair processes as they actually occur.

By the simplest model only a ligation event is required to recircularise linear substrate. A defect in a ligase activity could thus be postulated to account for the inability of *irs-2* to perform this reaction. Alternatively substrate could, in *irs-2* as opposed to V79 and *irs-1* extracts, be subject to attack by nucleases or phosphatases rendering it non-ligatable. Both these explanations are probably oversimplistic. Extracts prepared from *irs-2* clearly catalyse the production of concatemeric forms of the plasmid substrate and therefore have a capacity to ligate restriction endonuclease cut ends. To postulate a ligase deficiency it would have to be assumed that the ligation to form circles procedes via a reaction with an enzyme or complex different from that involved in the ligation to form concatemers.

An excess of nuclease activity in *irs-2* as opposed to either V79 or *irs-1* is unlikely as both the cellular data (Jones et al. 1988) and the results of the experiments involving mixtures of extracts argue that the defect in *irs-2* is recessive. It is hard to imagine a situation where nuclease excess would not be dominant. The same logic suggests that an increase in phosphatase activity in *irs-2* as opposed to either wild type or *irs-1* is not the explanation. It is possible however that the defect in *irs-2* results in an apparent excess of one of these activities. For example *irs-2* might be deficient in a protein required to protect substrate ends at a stage prior to their ligation. This would render substrate more susceptible to nucleases or phosphatases in *irs-2* extract without having to postulate the level of these enzymes being above that observed in wild type cells. It must then be hypothesised that under the conditions of the assay this protein is in sufficient excess in V79 cells such that on adding V79 extract to *irs-2* there is a concentration sufficient to compete with the "extra" nuclease or phosphatase activity added with the *irs-2* extract and so protect substrate allowing the observed ligation.

More complex models invoke a mechanism for the repair of the endonuclease induced DSB other than simple religation or one of a ligation requiring the activities of proteins in addition

to ligase enzymes. It is not necessarily the case that an event as intuitively simple as a ligation requires only one enzyme activity.

Characterisation of proteins involved in pathways of repair is most extensive in yeast and bacteria where the existence of a large number of mutants has facilitated such analysis (reviewed in Sancar and Sancar 1988 and in Hanawalt et al. 1979). The availiable evidence is consistent with there being substantial areas of overlap between enzymes involved in normal cellular DNA recombination and replication with repair activities.

Mammalian ligase I has been tentatively ascribed a function in DNA replication. The role of mammalian DNA ligase II is uncertain. There are suggestions of a repair function, for example Creissen and Shall (1982) use inhibitors of poly ADP ribosylation (which themselves sensitise to DNA damage) to abrogate the increase in DNA ligase II activity they see in response to DMS treatment of mouse leukaemic L1210 cells. These authors thus suggest that ligase II is important in the repair of DMS damage and that it is regulated by ADP ribosylation. A rise in cellular ADP ribosylation as briefly described above has been implicated as having a role in processes involved in the repair of many types of DNA damage (Lunec 1984). Therefore the correlation between its inhibition preventing induction of ligase II activity and increased cytotoxicity by DNA damaging agents implies a role for ligase II in repair. Similarly Li and Rossman (1989) found a 2.5 fold increase in ligase II activity in nuclear extracts of V79 cells after treatment with methylnitrosourea.

Only one ligase, the product of the *CDC9* gene has been thus far identified in *S.cerevisiae*. *cdc9* mutants have a temperature sensitive ligase activity and are defective in DNA synthesis and DNA repair at the restrictive temperature (Johnston and Nasmyth 1978).

Moore (1982a) treated ligase mutants of *S.cerevisiae* and wild type yeast cells with ionising radiation and measured the capacity of these cells to rejoin single strand scissions in prelabelled nuclear DNA. The single strand breaks produced by the cytotxic treatment were determined by velocity sedimentation through precalibrated alkaline sucrose gradients. The capacities of parental and mutant cells to rejoin these lesions was assessed in the absence of

medium (assuming that, under such conditions new ligase molecules should not be synthesised) after irradiation. DNA rejoining should then be proportional to the functional activity of ligase molecules present at the time of irradiation. Ligase-deficient yeast cells were defective in the rejoin of SSB and also more sensitive to the cytotoxic effects of ionising radiation. Furthermore two ligase mutants *cdc9-1* and *cdc9-9* were found in another study by the same author to be more sensitive than the parental *CDC9* strain to the radiomimetic drug bleomycin (Moore 1982b). At the nonpermissive temperature, ligase-deficient mutants repaired none of the breaks induced by bleomycin treatment while ligation-proficient strains removed 100% of such lesions. At a dose producing equivalent cell kill, although the wild type initially suffered more than five times as many strand breaks as the mutants, these were efficiently repaired. Ligase-deficient strains failed to catalyse significant removal of strand breaks even at the permissive temperature (Moore 1988).

At least two mammalian cell mutants *XR-1* (Stamato and Hu 1987) and *EM9* (Chan et al 1984) defective in DSB repair by neutral elution and sensitive to DNA damaging agents including ionising radiations have normal DNA ligase activities. This shows that in these cases a defect in repair of DSB does not correlate with a measurable ligase defect, ie ligase deficiency is not the basis of the increased sensitivity to DNA toxic agents of these lines. It does not however preclude the existence of such a correlation in another cell line or mean that ligases are not involved in the repair of DSB. If the misrepair of DSB is indeed the reason for the sensitivity of *XR-1* and *EM9* to DNA damaging agents (and the assays used give a true picture of ligase activities) these results indicate that repair of DSB lesions might, as previously implied be more complex than a single ligation event.

Currently the most promising candidate for a mammalian DNA ligase mutation is Blooms syndrome (Lasko et al. 1990a). Blooms is one of a number of rare, autosomal, monogenic chromosome-instability syndromes predisposing to cancer and leukaemia (Hecht 1988). Cell lines derived from patients show increased levels of sister chromatid exchange, elevated spontaneous mutation rates and a reduced rate of joining replication intermediates to high molecular wieght DNA. DNA polymerase α and β activities are normal (Lasko et al. 1990a). Lonn et al. (1990) labeled the replicating DNA of Blooms cells with tritiated thymidine and after

incubation for various times separated replication intermediates in agarose gels. Lymphocytes from Blooms syndrome patients showed a class of unusual replication intrermediates. Ligase I has been reported to be deficient in extracts from a number of Blooms cell lines (Willis and Lindahl 1987; Chan et al. 1987). Several lines were found to have reduced levels of an anomalously heat labile ligase I activity, in others the enzyme appeared as a dimer. There is thus a suggestion that the primary defect in Blooms syndrome is a mutation in ligase I. This is postulated to be a leaky mutation since ligase I is required for cell viability. Mezzina et al.(1989) however report levels of ligase I in Blooms syndrome cell lines to be higher than normal and claim the results of Willis and Lindahl to be due to the procedure used to make extract for the assay. The postulation being that ligase I precipitates with polymin-P during the clearing of debris. The original experiments of Willis and Lindahl have been repeated and the same conclusions reached (Lasko et al. 1990a). It was also shown that the pellet of debris postulated by Mezzina et al. to contain the "missing" ligase activity does not contain any protein crossreacting on immunoblots with ligase I antibodies. Further, using a method of ligase I fractionation without this precipitation step, ligase I activities from the Blooms lines were found to have a threefold reduced DNA joining activity per enzyme molecule.

Consistent with a DNA ligase defect in Blooms syndrome are the findings of Runger and Kraemer (1989) also showing an error prone DNA joining process and spontaneous hypermutability in these lines. Linearised plasmids with blunt or cohesive termini were transfected into Blooms syndrome and normal fibroblasts. The capacity of cells to catalyse religation of these substrates was measured by their ability to transform bacteria. The efficiency by which plasmids with both types of end were joined was from 1.3 to 3 times reduced in Blooms syndrome cells as opposed to normal. Mutation frequencies could be assessed by screening for the suppressor tRNA function carried by the substrate, for retention of restriction endonuclease sites or by sequencing. Spontaneous mutation frequency of the circular plasmid was found to be 2 to 21 fold higher in Blooms syndrome than in normal cells. In this context it should be noted that AT lines are hypermutable by X-irradiation and also show a lack of fidelity of DSB repair but have not been found to have altered ligase activities (Willis and Lindahl 1987).

Two classes of topoisomerase have been identified, type I changes the topological state of DNA by transiently breaking one strand of the double helix, passing the intact strand through the gap and resealing the DNA backbone, changing the DNA linking number by multiples of unity. Type II enzymes pass an entire DNA duplex through a double strand gap, altering the linking number by multiples of two (Smith 1990a; 1990b Liu 1989; Wang 1985). Both enzymes can effect supercoil relaxation. In addition, topoisomerase II has an unknotting and decatenating activity. The reactions of topoisomerase I are energy-\independent while the type II enzyme requires ATP hydrolysis to allow its release from DNA and so enable further strand passing reactions.

Work looking at the effect of topoisomerase inhibitors upon defined repair reactions (eg Mattern et al. 1982;) and results of studies on the response to DNA damage of yeast topoisomerase mutants (Boreham et al. 1990) suggest a connection between topoisomerase activity and DNA repair. It was reasoned that, since radiation inhibits replicative DNA synthesis, inhibitors of enzymes with a known role in the conformation and synthesis of DNA might have an influence on radiation lethality. Results show novobiocin, an inhibitor of topoisomerase II, to strongly inhibit the repair of radiation induced potentially lethal damage in V79 cells. The suggestion is made that this is due to the drugs potent effect on replicative DNA synthesis.

A number of mutant cell lines believed to be defective in DNA repair isolated by screening for sensitivity to the DNA damaging agents adriamycin (an intercalator) and bleomycin (a radiomimetic) are found to have cross sensitivities to topoisomerase II inhibitors (Robson et al. 1987; Davies et al. 1988). Some of these lines, eg *ADR-1* which appears to overproduce topoisomerase II, show altered topoisomerase activity (Robson et al. 1987). Others eg *ADR-4* and *ADR-5* also isolated by virtue of their sensitivity to intercalating agents and cross sensitive to topoisomerase II inhibitors (though not to ionising radiations), show no apparent change in either the level or activity of topoisomerase II. Studies with topoisomerase inhibitors must thus be interpreted carefully as it would appear that these compounds are not specific in their effects. Novobiocin, for example, inhibits nucleosomal histone-DNA complexing, the mitochondrial ATPase and DNA polymerase alpha as well as topoisomerase II (Downes and Johnson 1988). Pommier et al.(1986) report altered topoisomerase II activity in a line of Chinese hamster lung

cells resistant to topoisomerase inhibitors. The authors suggest that the resistance relative to the wild type of these cells is not however due to a drug-insensitive topoisomerase II or to a reduction in concentration of the enzyme but to the alteration of modulating factors related to topoisomerase I.

An AT line has been reported as having abnormally high levels of topoisomerase II at all stages in the cell cycle and this is associated with an increased sensitivity to the intercalator amsacrine (mAMSA) (Smith and Makinson 1989). Thus the cellular capacity to generate topoisomerase II-dependent DNA damage is a major determinant governing the sensitivity to at least one topoisomerase II inhibitor . A correlation of sensitivity to ionising radiation with that to inhibitors of topoisomerase I is provided by the work of Thacker and Ganesh (1990) in which the *irs-1* and *irs-2* mutants of V79 are found to be considerably more sensitive than wild type to camptothecin. The topoisomerase II inhibitors m-AMSA and VP 16 increased the frequency of chromosomal aberations in G2 cells and sister chromatid exchange in S phase of the ionising radiation sensitive CHO mutants *xrs-5* and *xrs-6* relative to the frequency induced in the parental CHO-K1 line (Darroudi and Natarajan 1989). The authors also report on the capacity of restriction endonuclease induced DSB to induce these effects and so correlate both the increase in chromosomal aberations and that of sister chromatid exchange with the incapacity of the *xrs* mutant lines to deal with DSB. The topoisomerase inhibitors in this instance presumably act by stabilising the "cleavable complex" as a protein (topoisomerase II) associated DSB which can then not be dealt with by the cell having a defect in DSB repair.

6.3.2 Role of mammalian ligases in *irs-2* defect.

We have assayed the activity of the two characterised mammalian DNA ligases in whole cell extracts of V79 and irs-2 cells. In terms of protein concentration response, FPLC separation characteristics and induction by the DNA damaging agent EMS the ligase activities of V79 and *irs-2* appear to be identical.

This data argues strongly against the basis for the deficiency in religation of an endonuclease induced DSB observed in vitro being in either DNA ligase I or II proteins. It is also circumstantial evidence to suggest that the repair of such a DSB requires more than ligase

activities. It can not be concluded however that a reaction more complex than simple ligation is occurring. The defect in *irs-2* may be one precluding ends being in the correct configuration for ligation. The assays for DNA ligase I and II activities were performed upon whole cell extracts while the in vitro assay for DSB religation uses nuclear extracts. It is possible that the defect in *irs-2* is one of nuclear localisation of a ligase activity. This would not be revealed by assay of whole cell extracts in which the division into nuclear and cytoplasmic compartments is not preserved. Alternatively repair of an endonuclease-induced DSB may indeed require additional proteins. *Irs-2* could be deficient in one of these.

6.3.3 Possibility that *Irs-2* is deficient in recombination of concatemers

Recombination has been implicated as important in the repair of DSB by studies of recombination deficient mutants of yeast, bacteria and mammalian cells which also have a defect in the handling of DSB (Moore et al. 1986; Ho 1975; Jeggo and Kemp 1983; Kemp et al. 1984; Costa and Bryant 1990). Studies of mechanisms of recombination have in some cases suggested that DSBs might be a prerequisite for recombination (Sun et al. 1989; Szostak et al. 1983). Furthermore a number of recombination mutants of yeast *S.cerevisiae* and *S.pombe* and Drosophila as well as mammalian cells are hypersensitive to DNA damaging agents suggesting that recombination and repair pathways have a number of common proteins (reviewed in Thomson 1988).

A further link between recombination and repair of DNA is provided by the evidence that the mutation giving rise to Severe Combined Immunodeficiency (SCID) in mice and humans involves loss of function of a transacting factor mediating lymphoid gene recombination . Specifically, the scid protein reportedly affects the final step of immunoglobulin gene VDJ rearrangement (Malynn et al. 1988). The VDJ recombinase in scid pre B cells (which may be the recently cloned recombination activating gene *RAG-1* (Schatz et al. 1989)) can correctly recognize heptamer-nonamer signal sequences and precisely incise these sequences. Malynn et al. suggest that the scid defect is due to the inability of affected lymphocytes to correctly rejoin the cleaved ends. Fibroblasts and myeloid cells from mice with the *scid* mutation show a marked sensitivity to ionising radiation (Fulop and Phillips 1990). The scid protein thus provides a further

example of a gene involved in DNA metabolism that also plays a role in repair.

Experiments with the concatamer recombination substrates were intended to provide information to clarify the mechanisms of repair occurring in nuclear extracts under the conditions of the in vitro assay. The hypothesis is that concatamerisation represents a stage in the pathway of conversion of an endonuclease linearised plasmid to form a circular molecule. It was hoped to test this by incubating the concatamers produced as described above with nuclear extracts from *irs-2* and V79 wild type cells. In order to make such experiments as relevant as possible to the context of the repair assay it is important to attempt to mirror the conditions in that assay. This is problematic since the only clearly defined points are that of initiating repair by addition of nuclear protein to linear substrate and that when reaction products are isolated and visualised.

Given the undefined nature of the reactions occuring during incubation of linearised plasmid with nuclear extracts the selection of conditions for investigation of the role of concatemers was somewhat arbitrary. In making as few modifications as possible to the basic assay we hoped that the reactions involved in the modified assay would be the same as those in th original form.

6.3.4 Role of topoisomerases and gyrases.

Work with bacterial mutants identifies at least three topoisomerase activities with distinct effects on DNA structure and similarly studies with yeast mutants indicate the existence of at least three distinct topoisomerase enzymes. The situation in bacteria and yeast is somewhat more complex than that so far elucidated in mammalian cells where to date two topoisomerases, one of each class have been described.

The reactions so far characterised of topoisomerases involve three steps: 1. cleavage of one or both DNA strands creating a transient gap; 2. passage of DNA strand(s) through this gap and 3. rejoin of the cleaved strand(s).

DNA topoisomerases controlling and modifying the topological states of DNA have been found to affect a number of cellular processes such as DNA replication. Topoisomerases are also implicated in transcription, recombination and chromosome segregation at cell division (Smith 1990; Wang 1985; Maxwell and Gellert 1986). Since considerable overlap appears to exist

between these processes and the mechanisms involved in repair it is worth considering the possibility that topoisomerases might play a role in the repair of radiation induced damage. A specific involvement in repair of strand breaks might be postulated as they have activities involving the introduction and resealing of DNA strand breaks. If a more complex reaction than ligation is involved in the nuclear extract-mediated repair of an endonuclease induced DSB DNA topology modifications might be required. There might be a role for the catenation and decatenation activities of topoisomerases in DSB repair.

In the preliminary experiments described in Chapter 4 the capacity of DNA topoisomerase I and the *E.coli* DNA gyrase (topoisomerase II functional equivalent) to enable *irs-2* to catalyse the formation of circular forms from linear plasmid was assayed. The effect of these pure proteins was compared with that of addition to *irs-2* nuclear extract of V79 extract.

It is clear from the effects of both topoisomerase I and the *E.coli* gyrase upon uncut pIC20H that both enzymes function efficiently under the conditions of the assay. The results obtained attempting to compensate the defect in *irs-2* nuclear extract were preliminary. A role for topoisomerases in the *irs-2* defect in repair of endonuclease induced DSB can not yet be excluded. One problem with this approach is the possibility of inhibition of the action of purified protein added to a repair reaction by nuclear extract. There is some evidence from this set of experiments that this does occur at least with respect to T_4 DNA ligase. While a positive result would be unequivocal a lack of compensation of the *irs-2* defect by addition of purified proteins would be harder to interpret, it being necessary to prove that this was not due to inhibition by nuclear extract. One way of doing this would be to compare the effect of purified topoisomerases on uncut plasmid (already shown to function under the conditions of the in vitro assay) with the addition of nuclear extract and without. If enzyme action upon uncut DNA was not inhibited by the addition of nuclear extract a lack of compensation of the defect in *irs-2* in terms of recircularisation of linear pIC20H could be taken as an indication that indeed altered topoisomerase activity is not the basis of that defect. If extracts were shown to prevent topoisomerase action the question of a role for these enzymes in the *irs-2* defect would have to be addressed in a different fashion.

6.4 COMPARISON OF *IRS-1* AND *IRS-2* PHENOTYPES WITH THAT OF ATAXIA TELANGIECTASIA.

If normal human cells are held after a dose of radiation under non-growth conditions, or are irradiated at very low dose rates, the surviving fraction is much greater than if cells are spread for colony formation immediately after a high dose of radiation. This effect is believed to be due to there being additional time for repair. In comparison, AT cells show little or no recovery under these conditions (Cox 1982). This type of post irradiation recovery defect is also seen in the radiosensitive *rad52* yeast mutants (Rao et al. 1980) and the *xrs* series of hamster radiosensitive mutants (Kemp et al. 1984). *rad 52* and the *xrs* mutants have a deficiency in the repair of DSB induced by ionising radiation as measured by neutral elution (Ho et al. 1975; Thacker and Stretch 1985). AT cell lines have no defect in DSB detectable by neutral elution but there is nonetheless evidence to suggest a strand break rejoin deficiency. Several AT cell lines have been found in a number of cases to have altered topoisomerase activities (Smith et al. 1989; Smith and Makinson 1989). The action of these enzymes, involving as it does strand breakage and rejoining, is consistent with an AT defect in strand break repair. AT cell lines are sensitive to DNA damaging agents causing strand breaks but not to those inducing bulky adducts (McKinnon 1987). Furthermore, results from in vivo and in vitro assay for DSB repair suggest a defect in the fidelity (though not the efficiency) of such rejoining reactions in AT as compared to normal human cells (Thacker and Debenham 1988; North et al. 1990).

Study of the induction and removal of DNA DSB in V79 and the *irs* mutants by neutral elution showed no difference between the normal V79 and either of the radiosensitive mutants *irs-1* or *irs-2* in the induction or handling of this type of damage. In this respect both *irs-1* and *irs-2* resemble AT lines.

Irs-1 shares with AT lines an elevation in induced chromosomal aberrations. In vivo analysis of DSB repair also suggest that *irs-1* shares with AT an incapacity to repair an endonuclease induced DSB. In our *in vitro* DSB rejoin assay no infidelity of such a rejoin is detected in *irs-1*. However, we have not tested AT lines under our assay conditions. Those of North et al. (which do detect an infidelity in in vitro DSB repair by an AT line) are substantially different. North et al. do not see a drop in overall efficiency of religation in AT lines in their in vitro

assay. This result is possibly comparable with our finding that *irs-1* repairs an endonuclease induced DSB with the same efficiency as wild type.

Irs-1, unlike AT lines is sensitive to UV irradiation and highly sensitive to MMC. Furthermore *irs-1* shows normal inhibition of DNA synthesis after irradiation while all AT lines tested show radioresistant DNA synthesis and reduced mitotic delay post γ-irradiation (Zampetti-Bossler and Scott 1981). This evidence suggests that the *irs-1* (assuming this phenotype to be caused by a single gene) and AT mutations are not in the same gene. However as yet it has been impossible to demonstrate complementation of the *irs-1* or AT defects in *irs-1*/AT hybrids.

Irs-1/human hybrids corrected for the MMC sensitivity of *irs-1* showed concommitant correction of sensitivity to g-rays, UV and EMS. Reversion to MMC sensitivity in these hybrids was accompanied by reversion to the other sensitivities. In all MMC corrected hybrids, an human chromosome 7 marker was detected in an otherwise random background of human chromosomes. The human gene correcting the MMC sensitivity of *irs-1* is probably on chromosome 7. It is possible that the *irs-1* phenotype is in fact due to mutation in two tightly linked genes (though this is unlikely given the mutation frequency). This would imply the existence of two putative repair genes on chromosome 7 and be an analogous situation to that of ERCC-1 and -2 both on human chromosome 19.

If the *irs-1* phenotype is caused by a single gene mutation this must be in a gene involved in a number of repair pathways -acting on diverse DNA lesions (Jones et al. 1990).

Unlike *irs-1*, *irs-2* shares with AT lines the property of radioresistant DNA synthesis. There are some similarities between *irs-2* and AT in the patterns of sensitivity to DNA damaging agents -both being highly sensitive to ionising but not UV radiations.

In the transfection assay of Thacker *irs-2* behaves as wild type while AT shows a substantial drop in DSB rejoin fidelity as compared to normal human cells. In contrast, in our in vitro assay *irs-2* shows a deficiency in DSB rejoin efficiency. The in vitro assay of North et al.(1990) referred to above also suggests a drop in fidelity of DSB religation by AT extracts as compared to normal but these authors see no drop in religation efficiency by AT extracts. Conclusions are hard to draw from this as we have not tested AT lines under our assay conditions and North et al. have not tested extracts from V79 and the *irs* mutants in their assay.

It does appear that while both *irs-1* and *irs-2* have some characteristics of AT lines neither represents a model of the total AT phenotype. There are suggestions that the radiosensitivity of AT lines and the phenomenon of radioresistant DNA synthesis can be separated in AT/ HeLa hybrids (Komatsu et al.1989). Mirzayans and Paterson (1991) find that the cytotocity to AT cells of a partially radiomimetic drug does not correlate with the impairment of DNA synthesis inhibition. These authors suggest that the AT phenotype is caused by a defect in a regulatory mechanism, postulated to control cell cycle division in particular and DNA synthesis in particular. They suggest that such a regulatory network might be responsible for the coordinate expression of multiple homeostatic processes involved in the normal response to ionising radiation. If such networks are also involved in regulating aspects of normal cell proliferation an explanation is provided for the extremely complex nature of the AT phenotype.

It is possible that the genes mutated in *irs-1* and *irs-2* are part of the network which the AT gene controls. Thus loss of either the gene mutated in *irs-1* or *irs-2* leads to some but not all of the aspects of the AT phenotype.

6.5 APPROACHES TAKEN AND POSSIBLE FURTHER APPLICATIONS.

6.5.1 *In vitro* plasmid DSB rejoin assay.

We have used the assay as described to identify a defect in *irs-2* nuclear extracts. By modification of the original assay we have shown that it can be extended to address questions raised by this original observation. The flexibility of this type of in vitro approach should allow further dissection of a number of aspects of repair of DNA DSB and radiosensitivity.

The mechanisms of DSB repair by nuclear extracts from V79, ie pertaining to cells with normal radioresponse, could be further investigated. Questions such as the speed with which concatemerisation of linear pIC20H occurs and further analysis of the relative kinetics of concatemer, open circle and closed circle formation being of high priority. The time course of concatemer formation has not been assessed. The speed at which each extract catalyses concatemer formation would be expected, if the process is crucial to eventual recircularisation, to have an impact on the extent of repair of original input linear plasmid. Obviously an extract forming concatemers faster during a set incubation time would have more time to catalyse their

resolution.

If concatemerisation was a prerequisite of circle formation, *irs-2* might have a defect in the pathway of concatemerisation itself, or in a pathway required to effect conversion to circular molecules. A more subtle defect, perhaps more difficult to detect, might involve speed of concatemer formation. Should the formation of concatemers in fact prove to be an integral part of the production of a transforming molecule it would be important to compare the time course of multimerisation in the two extracts.

These results could be compared with those obtained using nuclear extracts from a variety of different cell types to gain information as to the basic mechanisms involved in the religation of an endonuclease induced DSB and variations in these between different cell lines all with a normal response to ionising radiation.

Nuclear extracts from a variety of radiosensitive lines could also be tested to produce information covering a broad spectrum of repair capacities. This provides one method of looking for the connection between radiosensitivity and DSB repair.

6.5.2 Ligase assays.

The assays for mammalian DNA ligases described showed no difference in characterised activities between V79 and irs-2. These results are fairly conclusive, the only obvious extension of this type of study being the assay of DNA ligase I and II acvtivity in nuclear extracts. This would address the question of the defect in irs-2 being one of nuclear localisation of a ligase activity.

6.5.3 Modification of the in vitro assay for analysis of recombination of concatemers.

The results presented must be confirmed but it seems probable that the use of the recombination substrates produced as described may facilitate the breaking down of the process of religation of linear pIC20H into defined stages and so clarify understanding of the nature of this reaction. It could provide a tool to identify the biochemical alterations in cell lines with altered DSB handling or sensitivity to radiation. The same type of compensation by addition approaches as were tried using the original assay could then be applied to defects in the processing of the

recombination substrates.

To determine whether the concatemers produced by irs-2 nuclear extracts are different from those catalysed by V79, (in such a fashion perhaps as to render them impossible to resolve into circular transforming molecules) concatemers formed by the action of V79 and irs-2 nuclear extracts could be isolated and their processing compared.

This modification of the original assay should allow the investigation of the impact of one particular (still only possible) stage in a repair pathway to be investigated. In this it differs from other in vitro recombination assays which study the process as a whole (eg the assay of Kucherlapati et al. described previously and that of Jessberger and Berg (1991)).

6.5.4 Addition approach.

The appproach of attempting to compensate for an identified defect in a nuclear extract is a powerful one as described before. It is possible as mentioned above that it will prove difficult to interpret results using purified proteins as a complementing activity. As it has been shown that the *irs-2* defect can be complemented by the addition of V79 nuclear extract a way round this would be to fractionate V79 and assay fractions for the ability to give rise to circular forms on being added to *irs-2*.

If purified proteins are found to be functional in the presence of nuclear extract there are a number of activities it would be of interest to test. Helicases are enzymes catalysing the unwinding of the double helix by transient disruption of hydrogen bonds (Matson and Kaiser-Rogers 1990). There is strong evidence from the analysis of bacterial and yeast mutants that these enzymes are important in DNA synthesis and post incision stages of excision repair (Sung et al. 1988; Foury and Lehaye 1987). Sharing strong homology with the *RAD3* helicase of *S.cerevisiae* is *ERCC2*, a human gene cloned by its capacity to complement the excision repair defect in CHO complementation group 2 cell lines represented by UV5 (Weber et al. 1990). The human repair gene *ERCC-3*, cloned by its activity in correcting the UV sensitive phenotype of a complementation group three CHO mutant line, contains sequences suggesting nuclear localisation, DNA binding, and nucleotide binding activities. Furthermore *ERCC-3* shows significant homology with DNA helicases including *RAD3*. *ERCC-3* also complements the

excision repair defect in cell lines from the one patient with XP complementation group B (Weeda et al. 1990). Another gene sharing homology with *RAD3* and *UvrD* is the *PIF* gene involved in repair and recombination of mitochondrial DNA (Foury and Lahaye 1987). There is no evidence as to the involvement of this type of activity in repair of ionising radiation damage or DNA DSB, but, if it is assumed that DNA topology is important in these reactions, then helicase activities might have a role. Helicases are implicated as important in recombination, so, if circle formation by V79 extracts is found to occur by a concatemer resolution or recombination event, this activity of these enzymes might be important.

T_4 ligase should be able to catalyse the recircularisation of cut plasmid and indeed the fast migrating form found amongst the reaction products of incubation of V79 nuclear extract with cut pIC20H is also seen in reactions incubating cut substrate with T_4 ligase. Incubation with *irs-2* and T_4 would be expected to give rise to the fast migrating form. The finding that ligase enzymes appear unaltered in *irs-2* as compared to V79 suggests that in extracts as opposed to in reactions under the same conditions with T_4 DNA ligase an alternative type of ligation reation is occurring. The addition of nuclear extract might thus have an apparent inhibitory effect upon T4 ligase activity in recircularising linearised pIC20H resulting from competetion for substrate by such an alternative ligation reaction. This could be tested by using T_4 ligase in combination with nuclear extracts like those Dr Ross is using in her studies which do not appear to catalyse concatemer formation. This hypothesis would leave unexplained why, if concatemerisation were a step in the pathway of recircularisation, *irs-2* extracts efficient in concatemerisation cannot catalyse circle formation. It would have to be assumed that the concatemers produced by *irs-2* extracts were different from those generated by V79 or *irs-1* extracts in such a fashion as to mean they could not be resolved to circles. This could be tested by the study of the resolution of concatemers isolated after reaction with V79 nuclear extracts as compared to those isolated after reaction of cut substrate with *irs-2* extracts (mentioned above in the context of the modification of the in vitro assay to look at recombination).

6.6 OTHER POSSIBLE APPROACHES.

6.6.1 Analysis of rejoin by wild type cells using antibodies.

Another approach to dissecting the mechanism of recircularisation of linearised pIC20H by V79 extract would be experiments looking at the effect of antibodies against activities with a possible involvement in the process. The role of activities such as topoisomerases and also the single stranded binding protein might be studied in this fashion. If the addition of an antibody to V79 extract could be shown to abrogate circle formation the implication would be that the relevant activity is involved in the reaction. It would of course be vital to show that the action of the antibody was specific. A negative result, ie failiure of an antibody to inhibit recircularisation, would be more difficult to interpret. It would be necessary to demonstrate that the antibody was inhibiting the activity against which it was raised under in vitro assay conditions.

Antibodies could also be used to assess the levels in V79 and *irs-2* extracts of proteins with a possible involvement in repair using a Western blotting approach.

6.6.2 Isolation of genes responsible for radiosensitivities of *irs-1* and *irs-2*.

Gene transfer either by traditional cell fusion methods or using gene transfer techniques such as transfection or microcell mediated gene transfer is a method of gene isolation suited to the characterisation of the gene mutated in *irs-1*. These approaches would be facilitated as these cells are sensitive to a variety of cytotoxic agents. This allows a number of possible means of selection for complementation before screening for correction of radiosensitivity which is very laborious. The sensitivity of *irs-1* to a wide range of cytotoxic agents makes this line a better candidate for this type of approach than *irs-2* which is only markedly sensitive to ionising radiation.

Preliminary experiments we began, taking a microcell mediated gene transfer approach, involved attempts to reverse the hypersensitivity of *irs-1* to MMC. Microcells (three or four chromosomes enclosed in a membrane derived from nuclear or outer cell membranes) were to be produced from a population of early passage Human Foetal Fibroblast cells. These cells had been infected with a replication incompetent retrovirus carrying the gene for G418 resistance.

After fusion of *irs-1* cells with a population of microcells, fusants having taken up exogenous DNA can be selected for by virtue of G418 resistance and then screened for correction of the MMC sensitivity of *irs-1*. This approach proved dificult, problems arising with the production of microcells. It was decided that it would be more productive to concentrate on the results we were then beginning to obtain suggesting that *irs-2* was deficient in recircularisation.

Another approach arguably more applicable to the attempted isolation of the gene responsible for the *irs-1* phenotype than for the continued analysis of *irs-2* is to look at DNA binding proteins in nuclear extracts from wild type and radiosensitive cells.

The pattern of proteins binding to DNA treated with Cisplatin could be compared in V79 and *irs-1* nuclear extracts. This is based on the supposition that it is feasible that the protein lacking in *irs-1* leading to its increased sensitivity to cisplatin might form part of a complex binding a cisplatin adduct in vitro. A gel retardation system might reveal differences between V79 and *irs-1* nuclear extracts in this respect.

The most obvious approach to isolation of the gene responsible for the *irs-2* defect is to purify the relevant activity from V79 extracts using the in vitro assay to monitor its purification by compensation of the *irs-2* in vitro recircularisation defect. Having purified enough of such an activity for peptide sequencing a number of approaches exist to identify the relevant gene. The first is to screen an expression library with antibodies raised against partial sequences. The second is to synthesise sets of degenerate oligonucleotides and use these to probe a cDNA library and the third is to use amino acid sequences to generate sets of oligonucleotides to use as primers for amplification of the relevant gene using the polymerase chain reaction.

There are thus a number of approaches by which the manipulation of the cell free system for DSB rejoin described in the foregoing Chapters coupled with the use of the *irs* mutants should allow identification of genes and activities involved in DSB repair and the analysis of the role of such genes in conferring an ionising radiation hypersensitive phenotype.

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