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**Comparisons of the factors influencing intrinsic radiosensitivity
between two isogenic human ovarian carcinoma cell lines.**

submitted by

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In partial fulfilment of the requirements for
the degree of Master of Science in the Physiology graduate program

October 7, 2000
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ABSTRACT

The human ovarian carcinoma cell line, A2780s, and its variant, A2780cp, provide an isogenic tumour model for examining factors which determine intrinsic radiosensitivity. A2780s demonstrates sensitivity to ionization radiation while A2780cp is relatively radioresistant (surviving fraction following a 2 Gy dose is 0.083 and 0.43, respectively). The increased radioresistance of A2780cp can not be explained by greater cellular recovery, neither from sublethal nor potentially lethal damage. Moreover, A2780s demonstrates significantly greater ($P < 0.05$) sublethal and potentially lethal damage recovery than A2780cp. The induction of double strand breaks (dsb), as measured by asymmetric field inversion gel electrophoresis (AFIGE), is identical in these cell lines. Dsb rejoining efficiency is similar between A2780s and A2780cp. Initially, rejoining kinetic experiments reveal that A2780s is deficient in the slow phase of rejoining, while A2780cp is not (80% of dsb remain for A2780s vs 29% A2780cp) ($P < 0.01$). This deficiency however, is not reproduced in residual dsb experiments and further analysis reveals that it may be an artifact of the normalization process. When the level of residual dsb as a function of dose is examined, the slopes are similar for both cell lines and the only significant difference is seen for the highest dose used, 150 Gy (50% for A2780s and 33% for A2780cp) ($P < 0.05$). Finally, the cell-free fidelity assay demonstrates that for blunt-cut plasmid, no differences exist in the rejoining efficiency of nuclear extracts. The ligation infidelity of blunt-cut plasmid however, is significantly less ($P < 0.05$) in A2780cp extracts (0.021 for A2780s vs 0.0030 for A2780cp). Therefore, neither the induction of dsb, nor the efficiency of dsb repair can explain observed differences in radiosensitivity. The fidelity of rejoining however, is significantly greater in the resistant variant and may, in part, explain these differences.

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LIST OF ABBREVIATIONS

amp ^R	ampicillin resistance
AFIGE	asymmetric field inversion gel electrophoresis
ANOVA	analysis of variance
CHO	chinese hamster ovary
cisplatin	cis-dichlorodiamineplatinum(II)
cpm	counts per minute
D ₁	dose required, in Gy, to reduce survival to 1%
D ₁₀	dose required, in Gy, to reduce survival to 10%
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphates
dsb	double strand break
DTT	dithiothreitol
<i>E. coli</i>	<i>Escheri coli</i>
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid
FAR	fraction of activity released
Gy	gray
HDRI	high dose rate irradiation
IPTG	isopropyl-thio-β-D-galactopyranoside
LB	Luria-Bertani medium
LB-amp	selective Luria-Bertani medium containing 100 µg/ml ampicillin
LET	linear energy transfer
LIF	ligation infidelity
Mbp	mega basepairs
pBS	pBluescript phagemid
PFGE	pulsed field gel electrophoresis
PLD	potentially lethal damage
PMSF	phenylmethylsulphonyl fluoride
RE	restriction endonucleases
RR	recovery ratio
SEM	standard error of the mean
SF	surviving fraction
SF ₂	surviving fraction following a 2 Gy dose
SLD	sublethal damage
t _{1/2}	half-time (time required to repair 50% of damage)
TE	transformation efficiency
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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1. INTRODUCTION

1.1 DAMAGE DUE TO X-RAYS

1.1.1 Ionizing Radiation

Radiation can be considered as energy transmitted by waves (electromagnetic radiation) or a stream of particles (particulate radiation). Ionizing radiation is a type of radiation with sufficient energy, once absorbed, to eject one or more orbital electrons from an atom or molecule (termed ionization) (1) and is characterized by a localized release of a large amount of energy (33 electron volts per ionizing event). Ionizing radiation is energetic and penetrating, but despite its energetic nature, very little total energy is deposited within a cell or tissue by moderate doses of radiation. For example, a dose of 4 gray (Gy) would kill 80% to 90% of a population of mammalian cells (2) but if all its energy were converted to heat, it would raise the temperature of the cell or tissue by only 0.001°C.

There are two forms of ionizing radiation; electromagnetic, which includes x-rays and γ -rays, and particulate, which includes neutrons, electrons, protons, α -particles and heavy charged ions. X-rays and γ -rays are identical forms of electromagnetic radiation; x-rays are produced extranuclearly (via a linear accelerator, for example) and γ -rays are produced intranuclearly (radioactive isotopes).

To analyze the effect of radiation, a quantitative description of the energy deposited is necessary. The absorbed dose is defined as the energy imparted to matter by ionizing radiation per unit mass of irradiated material at the place of interest (1). The SI unit of absorbed dose is the gray (Gy), where 1 Gy = 1 Joule/kilogram. The old unit of absorbed dose is the rad (100 rads = 1 Gy).

1.1.2 Energy Deposition

Once radiation is absorbed, ionizations and excitations occur that are not randomly distributed, but tend to be localized along tracks of individual charged particles. The spatial distribution of the ionizing events produced by different particles varies enormously. X-rays are considered to be sparsely ionizing, in that the primary events are well separated in space. For any given particle, the density of ionization decreases as the energy increases.

Linear energy transfer (LET) is defined as the energy transferred per unit length of the track and is measured in kiloelectron volts per micron (KeV/ μm). Incident radiation consists of a wide spectrum of energetic particles, so LET can only be an average quantity. Nevertheless, LET is used as a simple, if naive, way of indicating the quality of different types of radiation. X-rays are considered to be low LET radiations (LET of about 0.2 KeV/ μm). For any given type of charged particle, the higher the energy, the lower the LET and the lower its relative biological effectiveness. Relative biological effectiveness is the ratio of the absorbed dose of a reference radiation to the absorbed dose of a test radiation necessary to produce the same level of biological effect. When relative biological effectiveness is plotted as a function of LET, relative biological effectiveness reaches a peak at a LET of about 100 KeV/ μm for a wide range of mammalian cells (3).

1.1.3 Direct and Indirect Action of Ionizing Radiation

Radiation damage at the level of an intracellular molecule occurs in several distinct phases. Following the radiation event, energy deposition onto a molecule occurs almost instantaneously (10^{-18}s). Energy is then transferred directly or indirectly to biological targets within 10^{-12}s and incurs damage (figure 1.1.3.1).

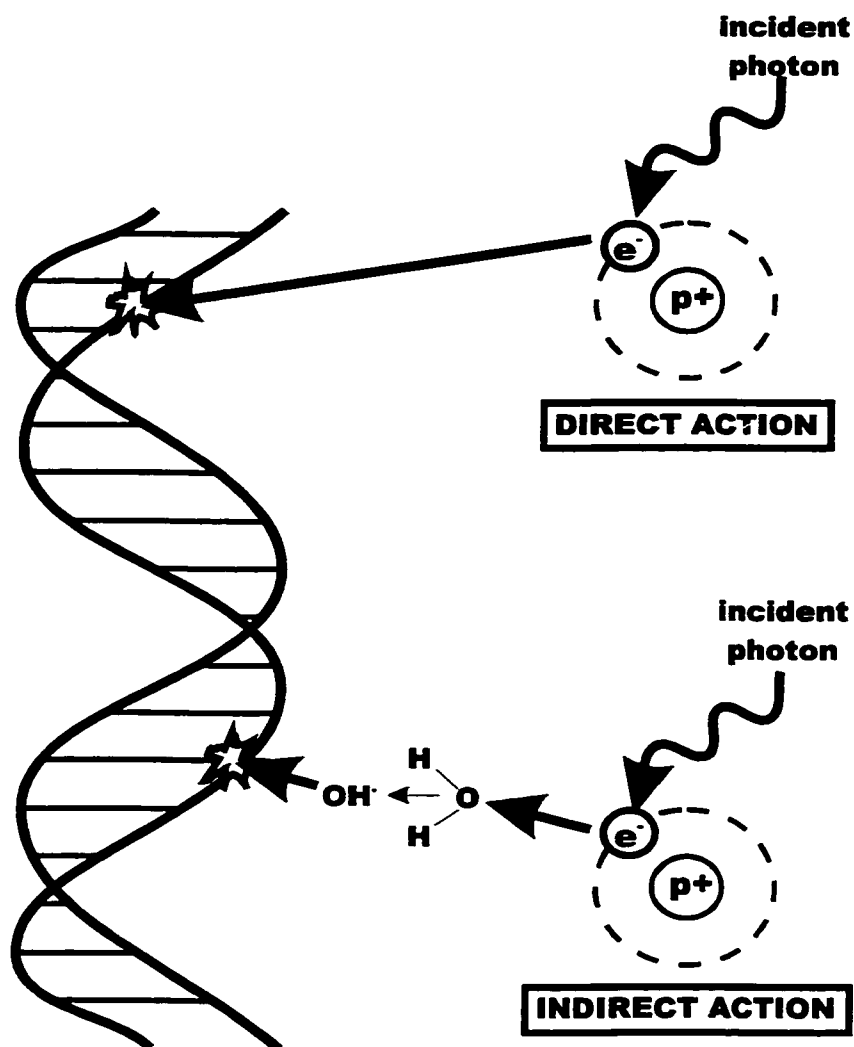
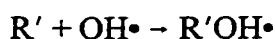
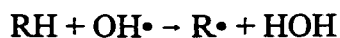


Figure 1.1.3.1 The direct and indirect action of ionizing radiation.
(modified from Hall EJ, *Radiobiology for the Radiobiologist*, 1995)

The direct effect of energy transfer is the dominant process for radiations with high LET, such as neutrons or α -particles. With the direct action, the radiation ionizes the atoms of the target itself, initiating a chain of events leading to biological damage. X-rays however, are considered to be low LET radiations, so the majority of damage occurs due to indirect action. The radiation interacts with other atoms and molecules producing free radicals which diffuse short distances to injure critical targets. Considering that cells are composed of 80% water,

the interaction of x-rays with water becomes important. X-rays can ionize water to produce three types of free radicals; hydrogen radicals ($\text{H}\cdot$), hydroxyl radicals ($\text{OH}\cdot$), and solvated electrons (e^-_{aq}) (4). These radicals can attack biological targets by H-abstraction or OH-addition:



Free radicals are highly reactive, unstable molecules, with a lifetime of only 10^{-5} s, and can only diffuse short distances to reach critical targets. The importance of free radicals in cell killing by x-rays has been demonstrated by the fact that free radical scavengers can reduce the effect of x-rays by a factor close to 3 (5). It has been estimated that two-thirds of the x-ray damage incurred by mammalian cells is due to hydroxyl radicals (6). Although the indirect process dominates for X-rays, radiation is still directly absorbed by critical targets in DNA. This leads to a more extended type of lesion; a multiply damage site (6).

1.1.4 Cellular Effects

Three possible consequences occur following exposure to radiation (figure 1.1.4.1), cells may: not receive any damage to a critical target and remain unaffected; accumulate lethal damage in critical sites and die in their next attempt at mitosis; or accumulate non-lethal damage. When non-lethal damage is accumulated a cell faces two fates: death (if it accumulates more damage which then becomes lethal) or survival (if it repairs the damage). Radiation damage to cells can be classified into three types; lethal, sublethal, and potentially lethal damage. The cellular consequences of ionizing radiation are displayed in a flow chart in figure 1.1.4.1

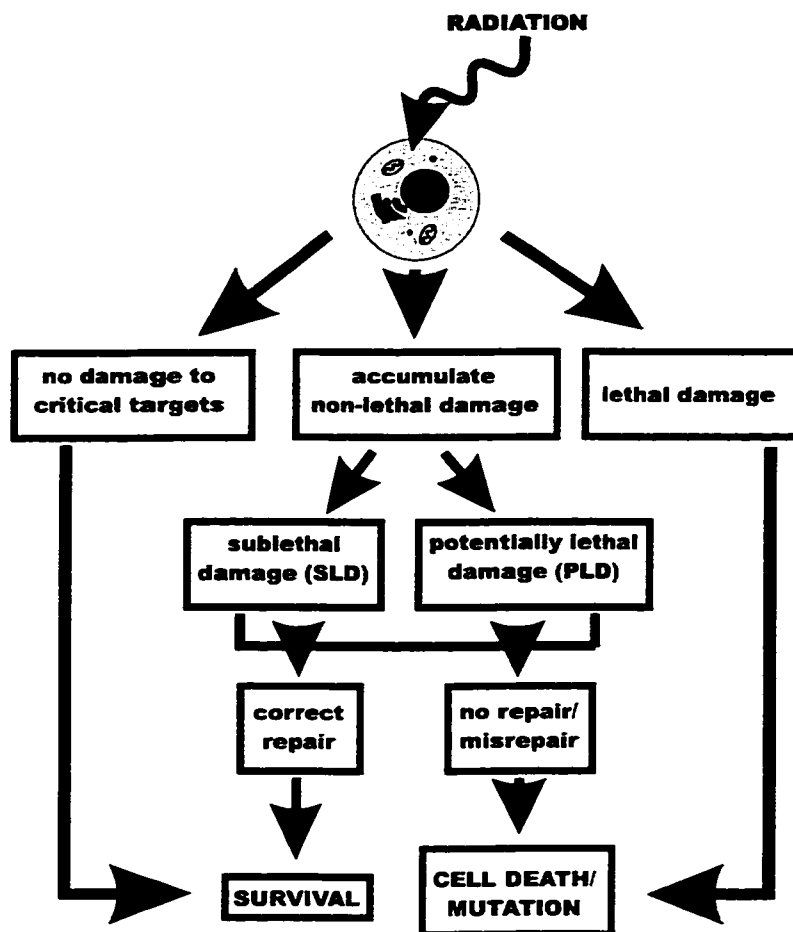


Figure 1.1.4.1 The cellular consequences of ionizing radiation absorption.

Lethal damage is irreparable and leads to cell death. Sublethal damage (SLD) is normally repaired if sufficient time is given for the cell to repair it before more damage is incurred. SLD recovery is observed as an increase in survival when a given dose is split into two equal fractions (7). By splitting the dose, cells are given the opportunity to repair the sublethal damage from the first fraction before accumulating additional damage during the second fraction. If the cell is unable to repair the first lesion before incurring another insult, the sublethal lesion can become lethal. It is well established that the SLD recovery is related

to the shoulder of the radiation survival curve (see section 1.2.1), and that the amount of SLD that can be accumulated and repaired is proportional to the size of the shoulder (8). Potentially lethal damage (PLD) is normally lethal, but under post irradiation conditions which prevent the cells from cycling (so that the cells do not attempt mitosis), PLD can be repaired (9). PLD recovery is seen as an increase in survival when the plating of plateau phase cultures is delayed, following a given dose or irradiation (9).

1.2 INHERENT RADIOSENSITIVITY

1.2.1 The Clonogenic Assay

The clonogenic assay is the most common technique used to deduce the intrinsic radiosensitivity of cells. It measures cell survival by colony formation and thereby assesses the ability of the cells to maintain their reproductive integrity following an experimental treatment. For the clonogenic assay, cell death is defined as a reproductive (not necessarily a functional) death. This is significant when considering that for a tumour to be eradicated it is only necessary that the cells be rendered unable to divide. A dose in the range 80 Gy to 120 Gy is required to destroy cell function in nonproliferating systems while a dose of less than 2 Gy will cause a loss of proliferative capacity. For cancer therapy, a reproductive cell death is generally the desirable endpoint.

The *in vitro* survival curve describes the loss of reproductive integrity as a function of dose, with survival being plotted on a logarithmic scale and dose being plotted on a linear scale (figure 1.2.1.1). For sparsely ionizing (low LET) radiations such as x-rays, the curve begins as a straight line and begins to bend at higher doses. This bending region extends over several Gy, and at very high doses the survival curve tends to straighten out again.

1.2.1.1 The Linear Quadratic Model

The linear-quadratic model is the model of choice to describe survival curves (10). The model assumes that there are two components of cell kill, one proportional to dose and one proportional to the square of the dose (equation 1.2.1.1):

$$SF = e^{-\alpha D - \beta D^2}$$

Equation 1.2.1.1

where SF is the surviving fraction of the cell after a dose (D) of radiation, and α and β are constants which depend on the cell type and irradiation conditions. It has been postulated that the α component is related to a DNA double strand break (dsb) produced by a single charged particle track and the β component to a dsb arising from a combination of two independent single strand breaks produced by unrelated particle tracks (figure 1.2.1.1)(11).

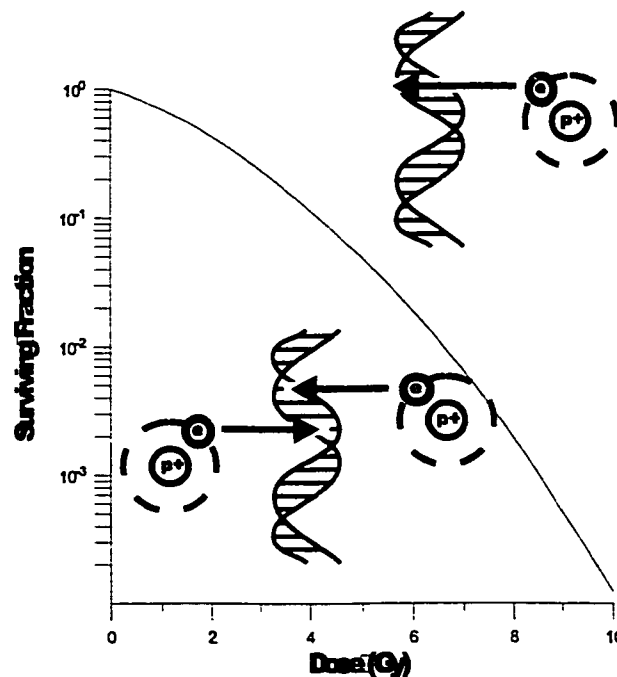


Figure 1.2.1.1 The linear quadratic model of cell survival following HDRI.

A characteristic of the linear-quadratic fit is that the resultant cell survival curve is continuously bending (there is no final straight region) but this does not coincide with what is observed experimentally. However, since the slope becomes linear again only after seven or more logs of cell death, the linear-quadratic relationship adequately represents the data for the first few decades of survival and up to doses used in single fractions in radiotherapy.

1.2.2 Intrinsic Radiosensitivity as a Prognostic Parameter for Radiotherapy

The ability to predict treatment response would be an invaluable tool in the planning of patient care. By individualizing therapy, major improvements could be made to local tumour control and patient survival rates, as well as reducing treatment morbidity. Recently, Mackay and Hendry have examined the effect of prescribing a patient's dose based on cellular radiosensitivity using the surviving fraction following a 2 Gy dose (SF_2) as a prognostic parameter. They have determined that if the patient population was stratified into 3 groups of intrinsic radiosensitivity (sensitive, normal, and resistant), gains of 20 % in tumour control probability could be achieved (12). Furthermore, if patients having a higher probability of recurrence could be identified, alternative and more radical therapies (which are usually costly, time consuming and risky) could be applied to those who most require them. Having a prognostic factor also improves the efficacy of clinical trials, as researchers would be able to select a smaller, more clinically defined group of tumours.

Several measurements have been considered as prognostic factors for the radiotherapy of cancer, such as tumour oxygenation (13), PLD recovery (8;14), DNA ploidy (15-17), proliferation (18), and *in vitro* radiosensitivity (19-21). Of particular interest are measurements of intrinsic radiosensitivity. The radiosensitivity which can be measured *in*

vitro represents an intrinsic quality of the cell and may be representative of the radiosensitivity *in vivo* and, therefore, the patient's response to radiotherapy. It was the work of Fertil and Malaise (19) which emphasized the importance of the intrinsic radiosensitivity of human tumours to radiotherapy. Through surveying the literature they found 36 published survival curves, 14 of which were obtained for the HeLa cell line. These survival curves were fit with the multi-target and linear quadratic models and it was found that the dispersion in radiosensitivity was greatest when judged on the basis of the initial slope of the curves. Fertil and Malaise took the actual survival at 2 Gy as a measure of the initial slope. Further analysis demonstrated a remarkably good correlation; the SF₂ was low for the most curable tumours and high for the refractory diseases. The results were confirmed when this study was repeated, incorporating new data and withdrawing the HeLa data. Using a five-point classification system of tumour responsiveness, SF₂ has been demonstrated to be statistically the best discriminator of radiosensitivity among cell lines. Despite differences in cell kinetics, hypoxia, etc, the intrinsic radiosensitivity of tumour cells is a significant determinant of radiotherapy outcome (22). Additional work on 110 tumour cell lines indicated that clinical radiosensitivity could only be correlated with parameters that describe the initial portion of the survival curve (23). Although SF₂ is the most common endpoint of intrinsic radiosensitivity, there are additional endpoints; α (the initial slope of the survival curve) (24;25), β (the final slope of the survival curve) (26), the dose required to reduce survival to 10% (D₁₀) (25;27), and the dose required to reduce survival to 1% (D₁) (28).

More recently, SF_2 has been used as a measure of the intrinsic radiosensitivity and has been examined as a prognostic parameter. Animal models, as reviewed by West and Hendry (29), have supported this finding: SF_2 as measured *in vitro*, could predict response to *in vivo* irradiation. For cervical carcinoma treated with radical radiotherapy, patients with a SF_2 above the median had a significantly lower 3 year survival level and greater frequency of local recurrence (30). Tumour radiosensitivity has also been correlated with patient morbidity. In a study involving 113 patients with a minimum of 3 years follow up it has been revealed that SF_2 is a significant prognostic factor for the probability of developing late complications (31). SF_2 has been demonstrated to be independent of disease stage, nodal status, tumour grade, patient age and tumour site (32). With the exception of a radiosensitive group of lymphomas, SF_2 has also been shown to be independent of tumour type (32). Within multi-variate analysis, it has been revealed that tumour SF_2 is an independent prognostic factor for treatment outcome (33). Another study has shown that with respect to local tumour control, α but not SF_2 is predictive of treatment outcome (34). However, for head and neck cancers it has been consistently shown that SF_2 is not predictive of local control (20;21;34-37). Some of these studies suggest that α correlates to a high risk of local failure (34;36) instead of SF_2 . All things considered, the clonogenic assay remains the best tool available for predicting a patient's response to radiotherapy.

Knowledge of the biochemistry and molecular basis of cellular sensitivity to ionizing radiation can provide invaluable information regarding carcinogenesis, cancer proneness, and patient responses to radiotherapy. By understanding the molecular mechanisms underlying variations in intrinsic radiosensitivity, novel means of controlling tumours and rapid

predictive assays of tumour radioresponsiveness can be developed. Therefore, the identification of the determinants of radiosensitivity of human tumour cell lines has become an important goal in radiobiology and is examined in this work.

1.3 DNA DAMAGE

It is generally accepted that DNA is the primary target for radiation-induced cell lethality (38). The importance of DNA in radiation-induced cell killing is demonstrated by experiments where the cell nucleus and the cytoplasm are selectively irradiated using either suitably constructed beams of ionizing (39;40), or radioactive compounds which are localized in DNA (41). The dose required in the nucleus to kill a cell is orders of magnitude less than that required in the cytoplasm. Additional evidence includes the observation that the incorporation of halogenated pyrimidines into DNA sensitizes cells to radiation exposure (42). Finally, radiation with an LET of 100 KeV/ μm is ideal in terms of producing a biological effect. For this LET, the average separation between ionizing events just about coincides with the diameter of the DNA double helix (3). Radiation with this density of ionization is most likely to cause a dsb by the passage of a single charged particle (5).

1.3.1 Types of DNA Damage and their Repair

Ionization radiation can induce a spectrum of DNA lesions (figure 1.3.1.1), including; sugar damage, base damage, cross-links, single strand breaks and dsb.

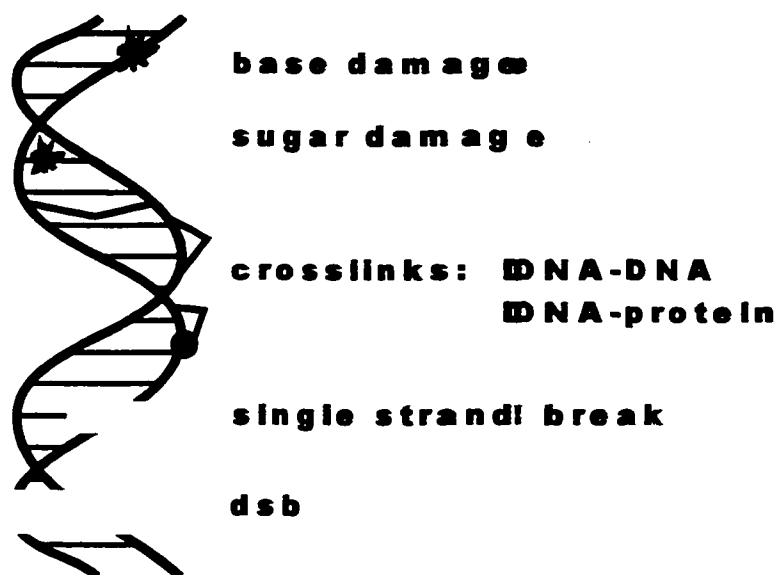


Figure 1.3.1.1 Possible lesions induced by ionizing radiation.

As was mentioned in section 1.1.3, two-thirds of the cellular damage produced by ionizing radiation is due to hydroxyl radicals (6). These radicals produce singly damaged sites (6) which can result in single strand breaks, base damage and sugar damage (43). Dsb, however, are more complex lesions and result from single energy deposition events with sufficient energy to produce multiple lesions in the DNA (6;44;45). Table 1.3.1.1 provides an example of the types of damage induced in mammalian cells by ionizing radiation and gives the estimates of the number of induced lesions.

Damage Induced in a Mammalian Cell Nucleus from 1 Gy of low-LET Radiation	
Damage	Amount
Initial physical damage	
Ionizations in cell nucleus	~ 100 000
Ionizations directly in DNA	~ 2 000
Excitations directly in DNA	~ 2 000
Biochemical Damage	
DNA single strand breaks	1 000
8-Hydroxyadenine (base damage)	700
Thymine damage	250
DNA-protein cross links	150
DNA dsb	40
Cellular Events	
Lethal events	~0.2 - 0.8
Chromosome aberrations	~ 1
Hprt mutations	~ 10 ⁻⁵

Table 1.3.1.1 An example of types of damage induced by 1 Gy of low-LET radiation (adapted from Goodhead, D.T. *Int.J.Radiat.Biol.* 1994; 65:7-17 and Ward, JF. *Prog.Nucleic.Acid.Res.Mol.Biol.* 1988; 35:95-125)

DNA is subject to damage by numerous agents, and cells must be able to repair DNA damage to survive. DNA repair can be classified into two broad categories; error-free repair and error-prone repair. All hydroxyl radical damage is rectified by base excision repair (43), an error-free process (46). Base excision repair is a well understood process; the damaged part of the DNA is excised, the gap is filled in by repair polymerase and the nick in the DNA strand is sealed by a DNA ligase (47) (figure 1.3.1.2).

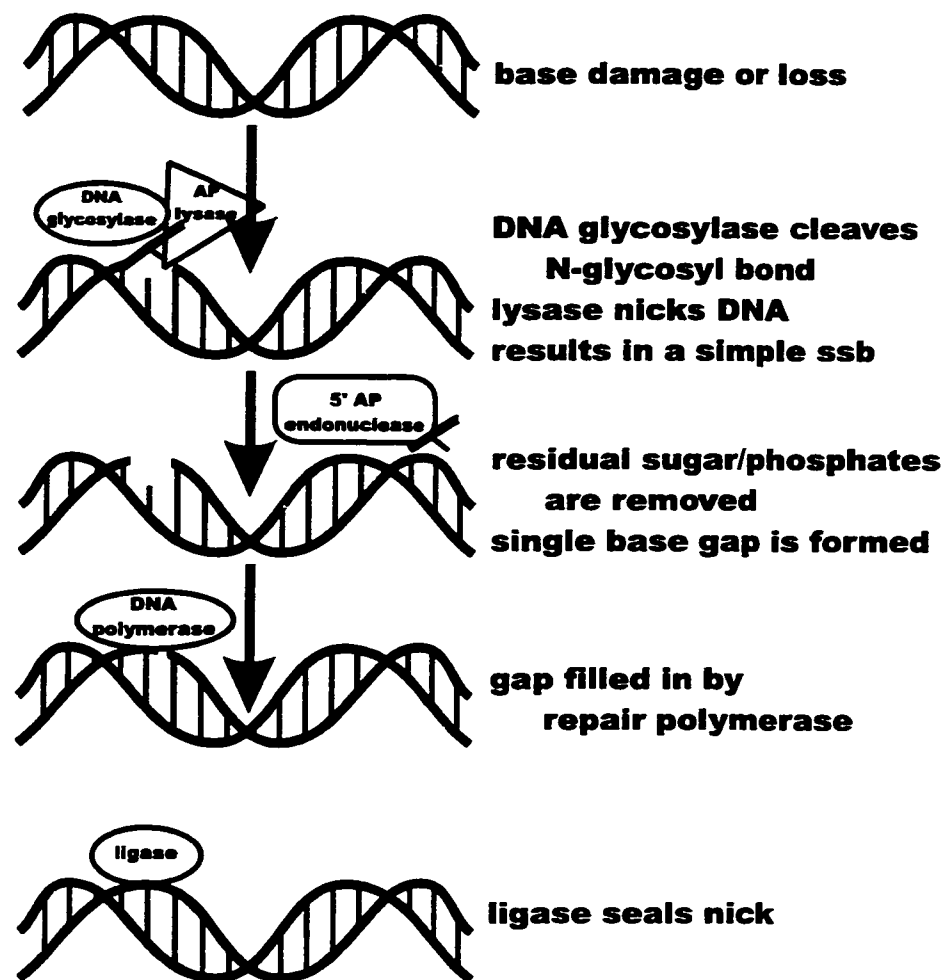


Figure 1.3.1.2 The repair of hydroxyl damage by base excision repair
(modified from Wallace SS, *Rad Res* 1998, 150: S60-S79)

By contrast, dsb repair occurs primarily through two recombination processes. Homologous recombination is an important process in lower eukaryotes whereas the majority of dsb induced in mammalian cells are repaired through non-homologous end joining. Non-homologous end joining is a rather complex process, and the genes involved have only recently been cloned. A model for non-homologous end joining in mammalian cells is presented in figure 1.3.1.3 (48).

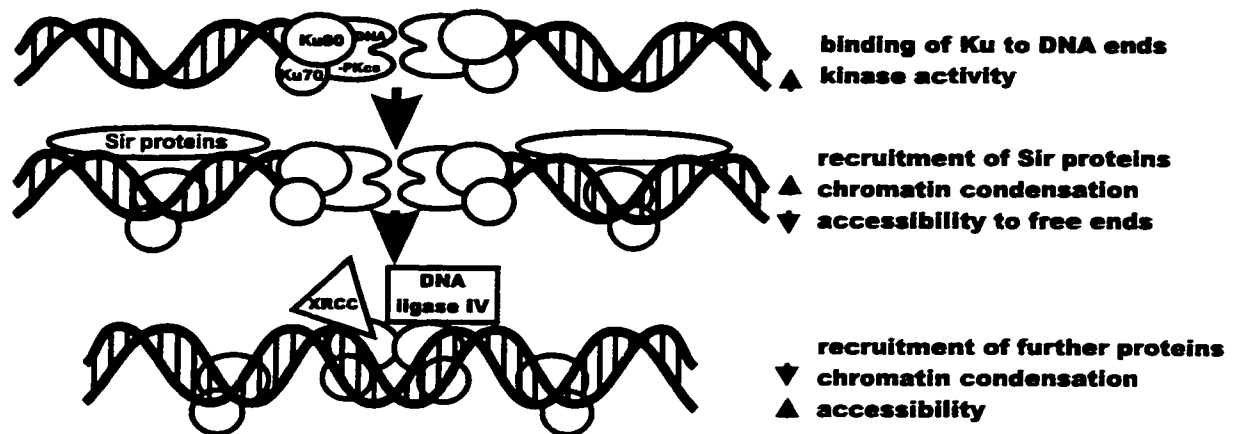


Figure 1.3.1.3 The repair of dsb by non-homologous end joining
(adapted from Jeggo PA, *Rad Res* 1998, 150: S80-S91)

Ku-autoantigen end-binding protein binds to double-stranded DNA ends to recruit and activate DNA-protein kinase (DNA-PK). This recruits Sir proteins which causes chromatin condensation and decreased accessibility of the DNA ends. Additional proteins are recruited, resulting in the release of the Sir proteins and chromatin decondensation. DNA ligase IV can now gain access to the site and rejoin the break.

1.4 DOUBLE STRAND BREAKS

1.4.1 Dsbs are the Lethal Lesion

Ionizing radiation produces a broad spectrum of DNA lesions and no single lesion has been identified which leads irrevocably to cell death. To the contrary, ultraviolet irradiation and cytotoxic drugs have well defined lethal lesions. Dsbs however, are thought to be primarily responsible for cell death (49;50). Intuitively, it would seem that dsb damage would be the most severe as damage to both strands of the DNA molecule will deprive the cellular repair system of the template required for efficient and accurate restoration of the molecule. Dsb repair involves an error-prone recombination event, whereas other types of

DNA damage are subject to error-free repair (4). Furthermore, as displayed in table 1.3.1.1, only 40 dsb are produced per lethal event, compared with 1 000 single strand breaks, 700 damaged base sites and 150 DNA-protein cross links. That fewer dsb are associated with cell lethality stresses the significance of a dsb in the cellular effects of ionizing radiation. A direct association has been made between cell killing and DNA dsb in bacteriophage systems (51). In yeast, a direct correlation between unrejoined DNA dsb and cell killing has been demonstrated in DNA dsb repair deficient mutants (52;53). For mammalian systems, there is no direct evidence that dsbs are the lethal lesion. Some of the evidence is based on a process of elimination, as no other lesions have been correlated with cell killing (54;55). Furthermore, agents which produce dsbs specifically, such as a restriction endonuclease (RE), cause chromosome aberrations and cell killing in a manner similar to that of ionizing radiation (56). For a group of human fibroblasts demonstrating a wide range of intrinsic radiosensitivities, a correlation was demonstrated between the sensitivity of the cell lines to x-rays and to a RE which produces blunt ends (57). Convincing evidence also comes from genetic mutants of mammalian cells (58). Mutant rodent cell lines such as xrs (59), scid (60) and XR1 (61) which are deficient in the repair of dsb also exhibit severe intrinsic radiosensitivity. In addition, the xrs and XR1 mutants are cross-sensitive to the radiomimetic drug bleomycin (59;61). Although no causal relationship has been established between DNA dsbs and cellular radiosensitivity, the indirect evidence is compelling and indicates that DNA dsb are the primary lesion responsible for damage due to ionizing radiation. Controversy remains however, as to which aspect of DNA dsb induction and repair can explain observed differences in intrinsic radiosensitivity.

1.4.2 Asymmetric Field Inversion Gel Electrophoresis (AFIGE)

Gel electrophoresis is a technique which separates charged biological molecules, such as protein, DNA, and ribonucleic acid, according to their molecular weight. In conventional gel electrophoresis a constant electric field is applied across the gel and the biological molecules (if they are negatively charged) migrate towards the anode. The separation of DNA molecules depends largely on the sieving properties of the gel matrix, although other factors such as the composition and concentration of the gel and buffer, the temperature and the magnitude of the electric field can affect mobility. Because of the sieving properties of the gel, DNA is separated according to size. Smaller molecules migrate more easily through the gel than do larger ones and therefore move with a greater velocity. Conventional gel electrophoresis uses a static electric field and can separate DNA molecules as large as 30 kilo basepairs. Molecules larger than 30 kilo basepairs will migrate at approximately the same rate. The DNA will elongate and align with the field, and therefore the radius becomes independent of molecule size and the molecules can no longer be separated according to size.

It was observed that elongated DNA molecules relaxed back to their unperturbed state once an applied electric field was removed and that the rate of relaxation was dependent on the length of the DNA. Pulsed field gel electrophoresis (PFGE) was developed from these observations (62) to separate larger DNA molecules, such as yeast chromosomes of several hundred kilo basepairs. Periodically changing the orientation of the electric field forces the DNA molecules to realign under the physical constraint of the gel matrix, and larger molecules require more time to reorient than smaller ones. Because the larger DNA molecules spend more time realigning, they have less time to migrate and therefore travel

a shorter distance in the gel. PFGE can separate DNA molecules of up to several megabasepairs (Mbp). Field inversion gel electrophoresis is an adaptation of PFGE, with the electric current periodically reversing (180° change) directions. Net forward migration is obtained by using longer pulse times and/or a higher field in the forward direction. AFIGE was developed by Stamato and Denko (63) and is a further modification of the field inversion gel electrophoresis technique. The electric fields are 180° to each other, but the strength of the voltages are different in each direction. The forward current is weaker than the reverse current, and the DNA is forced to migrate in the low field direction by using relatively long pulse times in that direction. This modification allows separation of DNA of up to 9 Mbp. AFIGE is used for the measurement of DNA dsb. The smallest human chromosome is 50 Mbp long and will not migrate in the gel matrix using the AFIGE technique. However, fragments of DNA caused by DNA dsb will migrate through the gel under an electric field and can be quantified. AFIGE specifically detects DNA breakage due to dsb (63-65). Migration of DNA into the gel is seen for purified DNA exposed to the RE *Xho*I (an enzyme which specifically induces dsbs) and migration increases in direct proportion to exposure time (63). In contrast, treatment with supralethal doses of hydrogen peroxide, a single-strand breaking agent, results in no migration of DNA (63;65). Other assays exist for measuring DNA dsbs such as the modified neutral velocity sedimentation procedure, neutral filter elution assay and clamped homogenous electric field gel electrophoresis (66). AFIGE is economical and sensitive down to a few Gy (63-65). Therefore, AFIGE will be used to quantify dsb induction, dsb rejoining kinetics and residual dsb.

1.4.3 Dsb Induction

It has been suggested that cells can differ in the amount of DNA damage which is detected immediately after irradiation, in the absence of DNA repair. Radford was the first to report a correlation between dsb induction and cell killing (54). Since these findings were published, numerous reports demonstrate significant differences in dsb induction between tumour cultures with differing intrinsic radiosensitivities (67-72). However, only a few of these reports observed a correlation between dsb induction and the intrinsic radiosensitivity of these cell lines (67;72). To the contrary, much of the literature suggests that dsb induction does not correlate with the intrinsic radiosensitivity of human tumour cell cultures (26;70;73-79). Even when working with genetic mutants of rodent (80-83) and murine (84) cell lines, dsb induction could not explain the extreme differences in intrinsic radiosensitivity.

That differences in dsb induction can account for differences in inherent radiosensitivity still remains a possibility. A given dose of x-rays would not necessarily produce the same amount of damage in different tumours or tumour cultures. Differences in the chemical fixation of damage (such as hypoxic versus oxic conditions), the presence of free radical scavengers (such as sulphydryls) and differences in DNA conformation and chromatin structure (85) are all factors which can influence damage induction. Therefore, dsb induction will be examined to determine whether it can explain any observed differences in radiosensitivity between the radiosensitive ovarian carcinoma cell line, A2780s, and its more radioresistant variant, A2780cp.

1.4.4 Dsb Rejoining

The repair of dsb is a rather complex process. When dsb rejoining is evaluated in the laboratory, three aspects must be considered; the kinetics, the quantity, and the fidelity of rejoining. Rejoining kinetics and residual dsb will be examined using the AFIGE technique.

1.4.4.1 Rejoining Kinetics

The level of DNA damage due to dsb decreases as cells are allowed to incubate at 37°C following irradiation, with the repair curve measured for mammalian cells being generally described as biphasic (86). Rejoining kinetics are often described in terms of half-times ($t_{1/2}$); which represent the time required to repair 50% of the damage. The biphasic dsb rejoining curve has an initial fast component with a $t_{1/2}$ of 20-40 min followed by a slow phase with a $t_{1/2}$ of several hours (87). If repair processes are considered to be in competition with fixation processes, then faster rejoining kinetics could lead to radioresistance. The kinetics of dsb rejoining have not been as extensively examined in the literature as dsb induction or residual dsb. In studies involving rodent cell lines, an increased $t_{1/2}$ was seen for some (80;81;88-90), but not all (83), of the radiosensitive mutants. When examining groups of human tumour cell lines with differing inherent radiosensitivities, correlations between the rejoining kinetics and SF_2 have been reported (67;79). Other reports, however, indicate no differences in rejoining kinetics (75-77). For human tumour cell lines and their radiosensitive clones, differences in repair kinetics have been observed (73;79) by some groups, while other groups suggest no differences (71;91). As there is no consensus as to the role of dsb rejoining kinetics in explaining intrinsic radiosensitivity, it will be examined in A2780s and A2780cp.

1.4.4.2 Residual Dsb

Cell killing has been suggested to result from non- and misrepaired dsb leading to chromosomal damage such as deletions and dicentrics (92). Therefore, the number of residual dsb should directly reflect the amount of lethal damage and, thus, should be an adequate parameter of cellular radiosensitivity. Much research has been devoted to examining the possible correlation between residual dsb and intrinsic radiosensitivity. When five mammalian cell lines were compared, a significant correlation was found between intrinsic radiosensitivity and the amount of non-repaired dsb (84). Convincing evidence also comes from the study of five repair competent and four repair-deficient mutant mammalian cell lines which exhibit an extreme range of radiosensitivities. It was found that while induction was the same for all lines, there was a good correlation between residual dsb and SF_2 (93). Other reports suggest that there is a correlation between non-repaired dsb and intrinsic radiosensitivity of human tumour cells (26;74;94) and genetic mutants (80). However, there are also many studies which indicate that the level of residual dsb do not explain differences in the cellular radiosensitivity of human tumour cells (67;75-77;79). When comparing human tumour cell lines and their variants, no differences in the level of non-rejoined dsb have been shown (71;91). If the consequence of an unrejoined dsb is a chromosomal aberration, one would expect levels of residual dsb to correlate with radiosensitivity. The literature, however, presents conflicting evidence and therefore the levels of residual DNA damage due to dsbs will be examined in A2780s and its radioresistant clone, A2780cp.

1.4.5 Rejoining Fidelity

Dsb rejoining is an important, but not the sole, determinant of intrinsic radiosensitivity. As outlined in section 1.4.4, dsb rejoining in many cases does not correlate with *in vitro* radiosensitivity. Rejoining fidelity focuses on the accuracy of DNA dsb repair as distinct from techniques (such as AFIGE) that monitor changes in the amount of DNA damage. Conventional dsb assays are able to discriminate between the rejoining and non-rejoining of dsb, but are unable to distinguish accurate rejoining from misrejoining.

Previous investigators have reported that repair fidelity correlates to intrinsic radiosensitivity (95;96). It has also been shown that ataxia-telangiectasia transformed fibroblasts, which are highly radiosensitive, have errors in the repair of linear plasmid (97-99). Furthermore, when transfected with linear plasmid, radiosensitive human tumour cell lines exhibit low repair fidelity compared to resistant cells. When the transfected plasmid was analyzed by Southern analysis, it was demonstrated that the types of misrepair varied between cell lines, and the type of digested plasmid used (100). Finally, work with human tumour cell lines and their clones demonstrate that ligation fidelity reflects differences in inherent radiosensitivity (91;101;102). This area of dsb repair however, has not been thoroughly examined.

1.4.5.1 The cell-free fidelity assay

The cell-free fidelity assay was developed by North (97) and adapted by Britten (96). Briefly, linearized plasmids are incubated with nuclear protein extracts from the cell lines of interest and transformed into *Escheri coli* (*E. coli*). This assay uses a RE-induced dsb in a plasmid as a model for a dsb induced by ionizing radiation. REs were first used in the

1980s to mimic radiation damage (103). REs work by recognizing a unique base sequence and cleaving double-stranded DNA at that specific site. Since a RE recognizes a specific base sequence, the site of cleavage can be determined in advance. The plasmid used in this assay, pBluescript, contains two markers; an ampicillin-resistance gene and the LacZ gene (figure 1.4.5.1.1).

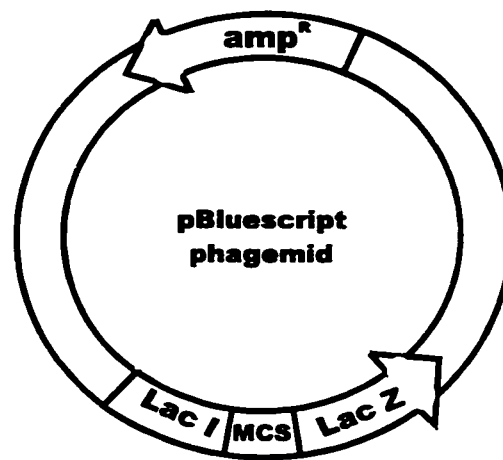


Figure 1.4.5.1.1 A schematic of the pBluescript phagemid.

The protein product of the LacZ gene is β -galactosidase which catalyzes a blue colour reaction. The plasmid is linearized by one of two REs; *EcoRI* produces a linear plasmid with staggered (cohesive) ends while *EcoRV* produces a linear plasmid with blunt ends (figure 1.4.5.1.2).



Figure 1.4.5.1.2 Cutting action of the restriction endonucleases, *EcoRI* and *EcoRV*.

Therefore, a plasmid cut with *EcoRI* is representative of a dsb resulting from two single strand breaks and a plasmid digested with *EcoRV* is similar to a dsb induced from a single event. The cleavage sites for both REs are in the multiple cloning region of the LacZ gene, so that a plasmid linearized by these REs contains an interrupted LacZ gene. The linearized plasmid is incubated with nuclear extracts from the cells of interest and then transformed into *E. coli*. The bacteria are then plated onto media which selects for ampicillin-resistance. Any colonies that grow in the selection media have been successfully transformed. The integrity of the second, LacZ, gene can be assessed through blue/white selection since blue colonies have a functional LacZ gene while colonies which have a nonfunctional LacZ gene remain white.

Accurate ligation of the plasmid results in restoration of the LacZ gene and a blue colony while misrejoining results in a defective LacZ gene and a white colony. Nonrejoining will result in no colonies since linear plasmids are not successfully transformed (76) and the bacterial cells will not contain the resistance gene necessary to grow on ampicillin plates. Thus, both rejoining capacity and rejoining fidelity can be evaluated with this assay. Rejoining capacity can be assessed by comparing the transformation efficiencies of plasmids incubated with different protein extracts. For example, plasmids incubated with nuclear extracts from a cell line with a greater rejoining capacity are expected to generate a higher transformation efficiency. The fidelity of rejoining can be examined by comparing the white to blue ratio of colonies. In theory, plasmids incubated with nuclear extracts from a cell-line with a superior rejoining fidelity will yield a lesser white to blue ratio compared to plasmids incubated with extracts from a error-prone cell line.

1.5 A2780s and A2780cp

The clinical radiosensitivity of a tumour is believed to be due, at least in part, to the inherent radiosensitivity of its component cells. If radiosensitivity is an intrinsic quality of a cell, then it is most likely determined by the cell's genetic makeup. It would be prudent, therefore, to study cell lines of differing radiosensitivities which are genetically similar. Human tumour cell lines and their radiosensitive or radioresistant variants provide an excellent isogenic model for studying the importance of dsb induction and repair in determining inherent radiosensitivity.

The human ovarian carcinoma cell line, A2780s, and its variant, A2780cp, provide an excellent tumour model for examining the role that dsb induction and repair play in determining *in vitro* radiosensitivity. A2780s was derived from an untreated ovarian carcinoma patient and found to be sensitive to the chemotherapeutic agent, cis-dichlorodiamineplatinum(II) (cisplatin). Through stepwise incubation with cisplatin, a resistant variant was derived which was 100-fold less sensitive to the drug (104). It was later demonstrated that A2780cp was also resistant to x-irradiation when compared to the parent line (105).

Many reports in the literature examine the correlation between dsbs and intrinsic radiosensitivity, but only a handful of these, use isogenic rodent (82;83) and human tumour cells (71;73;91;102) as models. The chinese hamster ovary (CHO) cell line and its radiosensitive mutant, xrs-5, have been extensively examined. Xrs-5 is known to be deficient in dsb rejoining (59). The parental CHO AA8 and the radiosensitive clones NM2 and EM9 are an example of another CHO system which has been investigated. No clear

relationship could be demonstrated between dsb and *in vitro* radiosensitivity in this system (83). Of the reports which used isogenic human tumour cell lines, differences in intrinsic radiosensitivities were associated with differences in induction (71), rejoining (73), and repair fidelity (71;102). It is obvious that further work needs to be done in such isogenic systems before the role of dsb in inherent radiosensitivities becomes clear.

1.6 Hypothesis and Specific Aims

The hypothesis of this thesis is that differences in the intrinsic radiosensitivity of the human ovarian carcinoma cell line A2780s and its variant, A2780cp, are due to differences in the induction and/or repair of DNA dsb.

The specific aims are to:

- ▶ determine the intrinsic radiosensitivity of A2780s, and its variant, A2780cp, using the clonogenic assay.
- ▶ establish the capacity for the two cell lines to repair SLD and PLD, using the clonogenic assay.
- ▶ examine the induction of dsb through the AFIGE technique.
- ▶ analyze both the rate of dsb rejoining as well as the amount of residual dsb left unrejoined by using the AFIGE assay.
- ▶ assess the accuracy of dsb repair by means of the cell-free fidelity assay

2. MATERIALS AND METHODS

2.1 IRRADIATION

All experiments were conducted using high dose rate irradiation (HDRI). X-rays were delivered by an industrial X-ray unit (Pantak) at a dose rate of 142cGy/min. All HDRI was carried out on ice (4°C) in order to minimize the amount of repair occurring during irradiation. For all clonogenic and dsb rejoining experiments, cells were irradiated in 25 cm² flasks (T-25s) as confluent monolayers. For the DNA dsb induction experiments, cells were suspended in low melting point agarose (see 2.4) and irradiated as agarose plugs.

2.2 TISSUE CULTURE METHODS

All cell manipulations were carried out under sterile conditions in a laminar flow hood. Cells were grown in the absence of antibiotics in a mixture of DMEM/F12 (Gibco BRL) media containing 0.1mM non-essential amino acids (Gibco BRL), 20 mM HEPES (Boehringer Mannheim) and 10 mM sodium bicarbonate (Sigma) and supplemented with 15% fetal bovine serum (Wisent). Cells were retained in an incubator set at 37°C in 97.5% air, 2.5% CO₂ and 100% humidity. Cells were grown and maintained in 80 cm² flasks (T-80s) and experiments were set up and conducted in T-25s. Cells were subcultured by rinsing with isotonic citrate saline (134 mM potassium chloride (KCl) (BDH), 17.6 mM citric acid (Sigma)) and then adding 1.0 ml of trypsin (0.2% weight/volume (w/v) in citrate saline) (Gibco BRL) in a T-80 or 0.5 ml in a T-25. Flasks were incubated at 37°C for 5 min and then cells were resuspended in 11 ml of complete medium, with a 0.1 ml aliquot being reserved in order to count the cells using a haemocytometer (BriteLite). For experiments, 10⁵ cells were seeded per T-25 in 4 ml media, fed on day 5 and reached confluence on day

8. All experiments were done on plateau phase cells (day 8) in order to minimize cell cycle effects as well as mimic the tumour environment *in vivo*.

2.3 CLONOGENIC ASSAY

The clonogenic assay measures cell survival by colony formation and thereby assesses the ability of the cells to maintain their reproductive integrity following an experimental treatment. Upon completion of the experimental treatment, the medium was removed from the flask, the cells were rinsed with citrate saline and 0.5 ml trypsin was added. After 5 min of incubation at 37°C, the cells were resuspended in 5 ml of complete medium and counted (section 2.1). Cells were diluted in complete medium to the desired concentration and plated onto dishes such that on each dish 50 surviving cells would grow to form colonies. For low concentration of cells, 60 mm² dishes were used containing 4 ml of complete medium. For concentrations of 4×10^4 cells/dish or greater, 100 mm² dishes were used with 10 ml complete medium.

Dishes were incubated at 37°C for 14 days to allow the individual cells to grow and form distinct colonies. On day 14 of incubation, the media was removed from the dish and cells were fixed with 100% ethanol. After a few minutes the ethanol was removed and colonies were stained with crystal violet (0.2% w/v in 70% ethanol). After 15 min of staining, the crystal violet was poured off and the dishes were rinsed and left to dry. A colony was counted if it contained 50 cells (when in doubt, a Fisher Stereomaster II 10X stereoscopic microscope was used to determine the number of cells) and the total number of colonies per plate was then counted by eye.

Three dishes of cells were plated for each experimental treatment as well as three

dishes for a control point. The control dishes were obtained from a sham experiment and used to calculate the plating efficiency (PE), as follows:

$$PE = \frac{\text{mean number of colonies per control plate}}{\text{number of cells seeded per plate}} \quad \text{Equation 2.3.1}$$

Plating efficiency was determined in order to assess the health of the cultures and ensure consistency between experiments. Using the mean of the three plates from the experimental point, and knowing the plating efficiency, the surviving fraction (SF) could be calculated according to the following formula:

$$SF = \frac{\text{mean number of colonies per plate}}{(\text{number of cells seeded per plate}) \times (PE)} \quad \text{Equation 2.3.2}$$

2.3.1 Modeling Cell Survival

2.3.1.1 HDRI Survival Experiments

HDRI survival curves were fit to the linear quadratic model by taking the natural logarithm of equation 1.2.1.1 and plotting the $\ln(SF)$ versus the dose. This was then fit to a second order polynomial using nonlinear regression in the GraphPad Prism (GraphPad Software Inc) plotting program. The data was then normalized to the zeroth order coefficient which had the effect of setting the zero dose point to a surviving fraction of 1. The first and second order polynomial coefficients were taken to be α and β , respectively.

2.3.1.2 SLD and PLD Survival Experiments

Both SLD and PLD were examined using the clonogenic assay. SLD recovery is observed as an increase in cellular survival when a dose of HDRI is split into two equal fractions which are separated by an incubation at 37°C (7). For SLD experiments, the first

fraction of HDRI (3 Gy or 2 Gy) was given on ice, the cells were transferred to a 37°C waterbath for the appropriate incubation interval, and then placed on ice again to receive a second fraction of HDRI (3 Gy or 2 Gy). At the end of the second fraction, the cells were immediately trypsinized, plated onto dishes and left to form colonies in an incubator for 14 days. PLD recovery is observed as an increase in cellular survival when, following a dose of HDRI, cells are prevented from cycling (9). This can be achieved by delaying the plating of confluent cultures. For PLD experiments, a 6 Gy dose of HDRI was delivered on ice, following which the confluent monolayers were incubated in a waterbath set to 37°C for 0 to 8 h. After completion of the post-irradiation incubation, cells were immediately trypsinized, plated and left to incubate for 14 days. For both SLD and PLD experiments, the SF (equation 2.3.2) was calculated and the recovery ratio (RR) was determined, as follows:

$$RR = \frac{SF_{t=t}}{SF_{t=0}} \quad \text{Equation 2.3.2.1}$$

By calculating RR, data was normalized to the survival when no repair time ($t=0$) was given. Normalization facilitated comparisons between cell lines and was necessary since a given dose was not necessarily equally toxic to each cell line.

2.4 AFIGE

The AFIGE technique used was developed by Stamato and Denko (63). Cells were grown to confluence in complete media containing 0.02 $\mu\text{Ci/mL}$ of [^{14}C]thymidine (Dupont NEN Products). Cultures were constantly exposed to [^{14}C]thymidine during incubation in order to radiolabel genomic DNA. Just prior to irradiation, excess media and [^{14}C]thymidine was removed and replaced with depleted media which was free from [^{14}C]. Cells were either

irradiated and then suspended in agarose or vice versa, depending on the experiment. Dsb induction experiments allow no repair time, so cells were first suspended in agarose and then irradiated and lysed. For dsb rejoining experiments, the cells were irradiated as monolayers and allowed to repair in flasks (so that the cells could repair normally) prior to being suspended in agarose and lysed.

2.4.1 Plug Preparation

Cells were washed twice with ice-cold (4°C) Dulbecco's phosphate-buffered saline (Gibco BRL) and then were trypsinized by adding 0.025% trypsin. Following an incubation at 37°C for 5 min, the cells were resuspended in complete media. A 100 µl aliquot was diluted 10-fold and cells were counted using a haemocytometer. The remaining cells were centrifuged for 5 minutes at 2,500 XG at 4°C. The supernatant was removed and the cell pellet was resuspended to achieve a final concentration of 2.5×10^6 cells/ml in 1.0% w/v low melting point agarose (Gibco BRL) in phosphate buffered saline at 37°C. Working quickly, the cell suspension was drawn up into 3-mm plastic tubing (cut into 5 cm lengths) with a 1-cc syringe and allowed to solidify on ice. Once solidified, air pressure from the same syringe was used to extrude the agarose and the agarose was then cut into 5 mm lengths. The 5 mm long plugs were then ready for irradiation (dsb induction experiments) or lysing (dsb rejoining experiments).

2.4.2 Plug Lysis

Agarose plugs were lysed in 0.5 mL of lysis buffer (0.5 mM ethylenediamine-tetraacetic acid (EDTA) (Sigma), 1.0% w/v N-lauryl sarcosine (Sigma)) containing 1.0 µg/ul proteinase K (Gibco BRL) by heating at 50°C for 24 hours in a dry bath incubator (Fisher

Scientific). Following incubation, plugs were dialyzed twice against 0.5 ml of TE buffer (10 mM Tris base (Gibco BRL), 1 mM EDTA) for 1 hour each. Ribonucleic acid was removed by digesting with 0.5 ml of TE buffer containing 0.1 ug/l of RNAase A (Sigma) for 2 hours at 37°C. The plugs were now ready for gel electrophoresis and, if needed, could be stored in TE buffer at 4°C for several weeks prior to being run in a gel.

2.4.3 Gel Preparation

A 0.8% w/v agarose gel was prepared by adding 1.6 g of electrophoresis grade agarose (Gibco BRL) to 200 ml of 0.5X TBE (0.9 M Tris base, 0.9 M boric acid (BDH), 25 mM EDTA) and heating in a microwave oven until dissolved. The agarose was then allowed to cool to 50°C and then poured into a leveled HE97X 15 x 20 cm casting tray (Hoefer). Bubbles were removed with a pipette and the well combs put in place. The gel was allowed to solidify at room temperature, the combs were removed and the gel was stored at 4° C in 0.5X TBE buffer until needed. One plug was placed per well with DNA size standards flanking each row to act as controls. *Schizosaccharomyces pombe* (*S. pombe*) and *Saccaromyces cerevisiae* (*S. cerevisiae*) were used as standards, having markers between 3 and 6 Mbp and 200 and 2000 kilo basepairs, respectively. They represent a good approximation of the resolution limits of the AFIGE system. Each well was covered with low melting point agarose.

2.4.4 Running the Gel

The gel was placed in a HE99X MAX horizontal agarose submarine unit (Hoefer) and covered with 1.5 L of 0.5X TBE buffer. The gel was run with a forward cycle of 1.25 V/cm for 900 s and a reverse cycle of 5 V/cm for 900 s. To ensure that DNA migrated into

the lane, the ratio of forward cycles to reverse cycles was 12.9 to 1. A Fisher Biotech FB570 power supply was used with a Pulsewave 760 electrophoretic field switcher and a Pulsewave 760 programming block (BioRad). A diode box, constructed by a previous graduate student, was added to the system to control the pulse heights in each direction independently. The gel ran for a total of 40 h, during which the buffer was circulated through a RTE-100 water cooling bath (Neslab) set at 12°C.

Upon completion of the gel run, the gel was removed from the buffer and stained with 1 L of 0.5X TBE containing 100 µl of 10 mg/ml ethidium bromide (Sigma) and agitated for 1 h on a rotating plate. The gel was destained by removing the TBE + ethidium bromide buffer and agitating gently in warm water for 15 min. The DNA was viewed by fluorescing the ethidium bromide on a 312 nm variable intensity transilluminator (Fisher Biotech) and then photographed.

2.4.5 Analysis

To quantify the amount of DNA migrated out of the plugs, it was necessary to determine the amount of DNA in the lane versus the total amount of DNA in the lane and plug (well). In order to accomplish that the gel was cut so that each lane and well pair were isolated from other lane and well pairs, and then the lane and well were separated from each other. The gel pieces were placed in labeled glass scintillation vials and 50 ml of 10 M HCl was added. Each vial was placed on a hot plate until the gel pieces melted. The HCl prevented repolymerization of the gel pieces upon cooling. Once the pieces were melted, 10 ml of Ecolite scintillation fluid (ICN Radiochemicals) was added to each vial and mixed by vigorous inversion. The ^{14}C content of each vial (which was representative of the DNA

content) was counted for 4 minutes in a Tri Carb 1900 TR scintillation analyzer (Packard). The counts per minute (cpm) for each sample was determined and the fraction of activity released (FAR) from each well was determined as follows:

$$FAR = \frac{cpm \text{ in lane}}{(cpm \text{ in lane} + cpm \text{ in well})} \quad \text{Equation 2.4.5.1}$$

2.4.6 Dsb Induction

The AFIGE technique was used to measure the induction of DNA damage due to dsb. Agarose plugs were prepared from confluent cell monolayers and placed on ice. The plugs were given increasing doses of HDRI (0 to 60 Gy) on ice and then immediately lysed. The FAR (equation 2.4.5.1) was calculated and plotted versus the dose. Data was normalized to the intercept of the FAR axis so that a zero dose yielded a FAR of zero. The data was fit with nonlinear regression using the GraphPad Prism plotting program.

2.4.7 Rejoining Kinetics

Rejoining kinetics were also examined using the AFIGE method. Confluent cell monolayers were irradiated with a 30 Gy dose of HDRI on ice. Following irradiation, the sealed flasks were immediately immersed in a 37°C waterbath for various incubation intervals (0 to 8 h). Following incubation, plugs were prepared and lysed. The agarose gel was run and analyzed, and the FAR was determined. Data was normalized by calculating the FAR remaining, as follows:

$$FAR \text{ remaining} = \frac{FAR_{t=x}}{FAR_{t=0}} \quad \text{Equation 2.4.7.1}$$

where $FAR_{t=0}$ represents the FAR when no post-irradiation incubation is given and $FAR_{t=x}$

represents the FAR following an incubation interval of length t (in h). FAR remaining was then plotted versus the incubation time, in h, using the Prism software.

2.4.8 Residual Damage

Residual damage is the DNA dsb remaining when repair is complete. Confluent cell monolayers were given a 0 to 150 Gy dose of HDRI on ice. Immediately following irradiation the sealed flasks were immersed in a 37°C waterbath and left to incubate for 6 h. Upon completion of the incubation the cells were promptly prepared into plugs which were lysed, ran in a gel and analyzed. The FAR was determined and plotted versus dose. Data was normalized to the intercept of the FAR axis such that a zero dose yielded a FAR of zero. The data was fit by linear regression using the GraphPad Prism software.

2.5 CELL-FREE FIDELITY ASSAY

The cell-free fidelity assay was first developed by North (97) and the method used for these experiments was adapted from that of Britten (96). It is a technique by which the fidelity of the ligation step of dsb rejoining may be assessed. The overall assay is described briefly here. Details of individual steps are given in subsequent sections.

A single, specific dsb is introduced into the phagemid, pBS, by a RE (section 2.5.2). Nuclear proteins are extracted from the cell lines of interest (section 2.5.3). The linearized plasmid is incubated with the nuclear extracts (section 2.5.4) to allow for potential ligation. Following incubation, pBS is recovered from the ligation mix and transformed into *E. coli* (section 2.5.6). The transformed *E. coli* are streaked onto selective bacterial plates and incubated overnight at 37°C. Rejoining efficiency can then be estimated by the transformation efficiency, and the rejoining infidelity is indicated by the white to blue ratio

of the resultant colonies (section 2.5.7). Luria-Bertani medium (LB) containing 10 g/l peptone from casein (BDH), 5 g/l yeast extract (BDH) and 10 g/l NaCl (Sigma) was used for all bacterial work. Selective medium (LB-amp) was prepared by adding 100 µg of ampicillin (NEC) per ml of LB. LB agar plates were prepared by adding 20 g of agar-agar (BDH) per litre of LB, heating until dissolved, pouring into 100 mm² dishes, and leaving to cool until solidified. Selective LB-amp agar plates were prepared in a similar manner, by adding 100 µg/ml of ampicillin to LB agar which had been cooled to 50°C and then pouring the plates.

2.5.1 Preparation of pBS

A single colony was picked from a freshly streaked selective plate and used to inoculate a starter culture of 5 ml of LB-amp. The starter culture was incubated for 8 h at 37°C with shaking at 250 rpm. Following the incubation, the starter culture was diluted 1000-fold into LB-amp and grown at 37°C for 16 h with shaking at 250 rpm. The Bluescript phagemid was purified using the Qiagen MaxiPrep kit (Qiagen) which is based on a modified alkaline lysis procedure (106). The bacterial cells were harvested by centrifugation at 6,000 XG for 15 min at 4°C, and all traces of supernatant were removed. The pellet was resuspended in 10 ml of resuspension buffer (50 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA and 100 µg/ml RNAase A). 10 ml of lysis buffer (200 mM NaOH, 1% w/v sodium dodecyl sulphate) was added, the tubes were mixed by inversion and left to incubate at room temperature for 5 min. Following lysis, 10 ml of ice-cold neutralization buffer (3.0 M potassium acetate, pH 5.5) was added, the tubes were mixed by inversion and then incubated on ice for 20 min. After neutralization was complete, the suspension was mixed by inversion again and immediately centrifuged at 20,000 XG for 30 min at 4°C. The supernatant was

removed promptly following centrifugation and ran through a filter paper (Whatman) pre-wetted with neutralization buffer. The filtered supernatant was applied to a Qiagen-tip (an anion-exchange resin) which had been prepared with 10 ml of equilibration buffer (0.75 M NaCl, 20 mM MOPS, pH 7.0, 15% v/v isopropanol, 0.15% Triton X-100). The supernatant was allowed to pass through the tip by gravity flow. The tip was then washed twice with 30 ml of wash buffer (1.25 M NaCl, 50 mM MOPS, pH 7.0, 15% v/v isopropanol). The phagemid DNA was eluted into a fresh collection tube by adding 15 ml of elution buffer (1.6 M NaCl, 50 mM MOPS, pH 7.0, 15% v/v isopropanol) to the Qiagen tip. pBS was precipitated from the eluate by adding 10.5 ml isopropanol and centrifuging at 15,000 XG for 30 min at 4°C. The supernatant was decanted and the pBS pellet was washed with 5 ml of 70% v/v ethanol and re-centrifuged at 15,000 XG for 15 min at 4°C. The wash was decanted and the pellet was left to air-dry. Once dry, the pellet was resuspended in TE buffer, pH 8.0. The yield of pBS was determined by spectrophotometry, as follows:

$$\text{concentration of pBS } (\mu\text{g/ml}) = (OD_{260\text{nm}}) \times (50 \mu\text{g/ml}) \times (\text{dilution factor}) \quad \text{Equation 2.5.1.1}$$

where $OD_{260\text{nm}}$ is the optical density for a wavelength of 260 nm. To assess the quality of purification, the phagemid to protein concentration was calculated as follows:

$$\text{pBS:protein} = OD_{260\text{nm}} / OD_{280\text{nm}} \quad \text{Equation 2.5.1.2}$$

A ratio of 1.7 to 2.0 indicated adequate purification.

2.5.2 RE digestion of pBS

The Bluescript SK +/- phagemid (Stratagene) is a 2958 basepair phagemid derived from pUC19 which contains an amp^R gene and a LacZ gene (figure 1.4.5.1.1). The amp^R gene is used for antibiotic selection of successfully transformed bacteria. The LacZ gene provides α -complementation for blue/white colour selection of transformed bacterial colonies. pBS contains a single *EcoRI* and a single *EcoRV* restriction site located in the multiple cloning site of the LacZ gene. The multiple cloning site does not interfere with normal translation of the LacZ gene. However, when the multiple cloning site is cut, through RE digestion, the LacZ gene is interrupted.

Prior to digestion of the plasmid, a diagnostic was done in order to ensure that the REs linearized the plasmid as expected. 1 μg of pDNA was digested with 10 μl of 10 units/ μl *EcoRI* or *EcoRV* (Gibco BRL) in a total volume of 30 μl of React buffer (Gibco BRL). The digestion cocktail was incubated at 37°C for 1.5 h and then ran on gel. A 50 ml gel made of 1% (w/v) electrophoresis grade agarose in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA) containing 2 ml ethidium bromide was cast in a small casting tray (Hoefer). The gel was placed in a small gel box, covered with TAE and run at 80V for 1 h. DNA bands were visualized by ultraviolet illumination and photographed.

Once the diagnostic showed proper linearization of the phagemid, 10 μg of pBluescript was digested with 3 μl of RE in a total volume of 60 μl of React buffer and incubated at 37°C for 24 h. Ostensibly, 1 unit of RE incubated at 37°C for 1 h is sufficient to digest 1 μg of plasmid DNA. A 72-fold over digest (30 units/ μg x 24 h) was used to ensure complete digestion. To inactivate any remaining RE, the digestion cocktail was

incubated at 65°C for 20 min. Following digestion the plasmid needed to be recovered and purified before being transformed. Once digestion was complete, the volume of the cocktail was increased to 200 ml with TE buffer (pH 8.0). The organic layer of phenol:chloroform (1:1) was added in a 1:1 (v:v) ratio to the digestion cocktail. The mixture was vortexed for 30 s and microcentrifuged at 12,000 XG for 5 min. The aqueous layer containing the DNA was transferred to a new centrifuge tube. The phagemid DNA was extracted by adding CH₂Cl-IAA (chloroform:isoamylalcohol) in a 1:1 v:v ratio to the aqueous layer. The mixture was vortexed and microcentrifuged at maximum speed for 5 min. The aqueous layer was transferred to a new tube, the volume determined and 10% v/v sodium acetate was added. The phagemid DNA was precipitated by adding 2.5 X 100% ethanol and vortexing for 30 s. The DNA was allowed to precipitate on ice for 15 min and was then microcentrifuged at 12,000 XG for 15 min. The excess ethanol was removed and the pellet was dried in a DNA spin vac. The pellet was resuspended in TE buffer (pH 8.0) to achieve a final concentration of 1 µg/µl. 1 µg of the linearized plasmid was run on a 1% agarose gel (see above) in TAE buffer at 80 mV for 1 h. DNA bands were visualized by ultraviolet illumination and photographed.

2.5.3 Nuclear Protein Extraction

The fast and simple method of extracting nuclear proteins was adapted from the method developed by Olnes and Kurl (12). Confluent cultures were washed three times with ice-cold phosphate buffered saline and the cells were scraped into the third wash. The wash was then centrifuged at 200 XG for 10 min at 4°C. The supernatant was aspirated and the cell pellet was resuspended in 0.5 ml of phosphate buffered saline and transferred to a

microcentrifuge tube. The 0.5 ml suspension was microcentrifuged at 4°C for 5 min at 200XG, the supernatant aspirated and the pellet resuspended in 400 µl of HKEE buffer (pH 7.9) containing 10 mM HEPES, 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM ethyleneglycol-bis-(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA) (Sigma), 1 mM dithiothreitol (DTT) (Sigma), 0.5 mM phenyl methylsulfonyl fluoride (PMSF) (Sigma) and 1% (v/v) aprotinin (ICN). The cell pellet was resuspended in the buffer by repeatedly pipeting with a 200-1000 µl pipette tip. The cell solution was then cooled on ice for 15 min. To lyse the cells, 1.0% v/v Nonidet P-40 (BDH) was added and the cells were mixed by inversion several times. The nuclei were microcentrifuged at 200 XG for 5 min at 4°C. The supernatant was aspirated, and the nuclei were lysed by resuspending in 100 µl of ice-cold HNEE buffer containing 20 mM HEPES (pH 7.9), 0.4 mM sodium chloride (Sigma), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% (v/v) aprotinin, 10% v/v glycerol (BDH) and shaking for 60 min on ice. The lysate was microcentrifuged at 12,000 XG for 20 min at 4°C. The supernatant was aliquoted into a new microcentrifuge tube, 0.025 mg/ml of leupeptin (Boehringer Mannheim) was added and stored at -80°C until needed.

2.5.3.1 Assessing the Quantity and Quality of the Nuclear Protein Extracts

To determine the quantity of protein, the BioRad protein assay (107) was used. Dye reagent was prepared by diluting 1 part dye reagent concentrate (Coomassie Brilliant Blue G-250, Bio-Rad) in 4 parts distilled deionized water and filtering through a Whatman #1 filter. Protein standards were composed of bovine serum albumin (Sigma) diluted in HNEE buffer, with concentrations ranging from 0 to 1.0 mg/ml. Nuclear protein extracts were diluted 4-fold in HNEE buffer. The diluted protein was then diluted 50-fold in the prepared dye

reagent and incubated at room temperature for 30 min. A standard curve was established by measuring absorbance of the standards at 595 nm. The protein concentration of the nuclear extracts was determined by measuring absorbance at 595 nm and interpolating from the standard curve.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis used to assess the protein quality and to ensure no degradation had occurred. The polyacrylamide gel was discontinuous, with an upper stacking gel and a lower separating gel. The separating gel was made by combining 6.66 ml of monomer solution (30% w/v acrylamide (Sigma) and 2.7% w/v N,N'-methylene bis-acrylamide (Sigma)), 5 ml 1.5 M Tris-hydrochloride (pH 8.8), 200 μ l of 10% w/v sodium dodecyl sulphate, 100 μ l of 10% w/v ammonium persulfate (Sigma), 10 μ l temed (Sigma), and 8 ml of ddH₂O. The separating gel solution was quickly poured into a 1.5 mm thick gel casting apparatus (Hoefer Scientific), a H₂O saturated n-butanol layer (10% ddH₂O and 90% n-butanol) was layered over top and the gel was left until solidified (at least 30 min). Once the separating gel was set, the butanol layer was poured off and the gel was rinsed 3 times with distilled water. The stacking gel was prepared by combining 1.34 ml of monomer solution, 2.5 ml of 0.5 M Tris HCl, pH 6.8, 100 μ l of 10% w/v sodium dodecyl sulphate, 50 μ l of 10% w/v ammonium persulfate and 4 μ l of temed just prior to pouring. The stacking gel was poured over the separating gel, a well comb was inserted and bubbles were removed. The gel was then left until set. Once the gel was set, it was placed in a TE Series Transphor electrophoresis unit (Hoefer Scientific). The combs were removed, tank buffer (0.025 M Tris, 0.192 M glycine, 0.1% w/v sodium dodecyl sulphate, pH 8.3) was added, and the gel was allowed to equilibrate for 15 min. 10 μ g of nuclear extracts were

mixed with 10% v/v loading buffer (0.125 M Tris HCl, 4% w/s sodium dodecyl sulphate, 20% v/v glycerol, 10% v/v mercaptoethanol, 0.1 mg bromophenol blue, pH 6.8). Samples were boiled for 90 s and then left to chill on ice. The gel was loaded with the boiled samples and let run at a constant current of 60 mA for 1.5 h. The gel was removed from the running apparatus and stained with a solution of 0.025% w/v Coomassie Brilliant Blue R-250, 40% v/v methanol and 7% v/v acetic acid (Sigma) for 2 h with agitation. The staining solution was then decanted, and excess was removed by covering the gel with destain solution (7% v/v acetic acid, 5% v/v methanol) for at least 15 min with agitation. Quality nuclear proteins were detected as numerous distinct bands.

2.5.4 Phagemid Ligation

2 µg of phagemid DNA was combined with 0 to 1.0 µg of nuclear protein extract in a 50 µl buffer solution containing 20 mM Tris-hydrochloride (Gibco BRL), 10 mM magnesium chloride (BDH), 1 mM adenosine triphosphate (Sigma), 1 mM DTT, a 50 µM mix of d-nucleotide triphosphates (dNTPs) (Gibco BRL), and 5% (w/v) polyethylene glycol 8000 (Sigma). The phagemid DNA and nuclear proteins were incubated in the buffer for 24 h at 16°C in a RTE-100 water cooling bath (Neslab). The next day, the phagemid DNA was recovered in an Ultrafree*MC-Probind centrifugal filter system (Millipore), diluted 5X in TE buffer (pH 7.6) and stored at -20°C until needed.

2.5.5 Preparation of Competent *E. coli*

DH5α, a strain of *E. coli*, was rendered competent using the standard calcium chloride method. 5 ml of LB was inoculated with a single colony, obtained from a previously streaked LB agar plate, and left to shake at 300 rpm overnight at 37°C. The next

day the starter culture was diluted into 500 ml of LB and left to shake at 300 rpm at 37°C. Optical density readings at a wavelength of 600 nm (OD_{600}) were taken every hour. Once the culture achieved an OD_{600} of 0.3-0.4 the culture was cooled by immersion in an ice-water bath for 5 min. The culture was then transferred to pre-chilled polypropylene tubes and incubated on ice for 10 min. The cells were harvested by centrifugation at 2500 XG for 10 min. The medium was decanted and allowed to drain thoroughly by inverting the tubes for 1 min. The cell pellet was resuspended in 100 ml of 0.1 M calcium chloride. The cells were harvested once again by centrifugation at 2500 XG for 10 min. The supernatant was decanted and the tubes were inverted for 1 min. The pellet was resuspended in 50 ml of a solution containing 0.1 M calcium chloride, 5 mM Tris, and 10% glycerol (BDH), pH 7.6. The cells were harvested again by centrifugation at 2500 XG for 10 min. The supernatant was decanted and tubes were inverted for 1 min. The cell pellet was resuspended in 4 ml of 0.1 M calcium chloride, 5 mM Tris, and 10% glycerol, pH 7.6 and left on ice. Working on dry ice, 200 μ l aliquots of the cell suspension was quickly transferred to pre-chilled microtubes. The cells were then stored at -80°C until needed. Once thawed, cells were discarded because if refrozen, they were no longer competent.

2.5.6 Heat Shock Transformation

Cells which had been stored at -80°C were allowed to thaw on ice. 200 μ l of competent DH5 α was aliquoted into a prechilled polypropylene tube (Falcon). 16 ng of pBS was added, the tubes were swirled and left to incubate on ice for 30 min. At the end of the incubation period, the tubes were immersed in a 42°C waterbath for 90 sec and then incubated on ice for 2 min. 800 μ l of LB was added and the cells were left to recover by

shaking at 1,000 XG at 37°C for 45 min. Once recovery was complete, the transformation mix was aliquoted onto LB-amp agar plates. 40 µl of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, 20 mg/ml) and 10 µl of isopropyl-thio-β-D-galactopyranoside (IPTG, 25 mg/ml) was added to each aliquot of transformed cells and the mixture was streaked using a sterile glass spreader. The plates were left at room temperature just until the liquid had been absorbed and then inverted and left to incubate for 17 h at 37°C.

2.5.7 Analysis of Ligation

Both ligation efficiency and fidelity were determined from the fidelity assay. Transformed DH5α were grown on selective LB-amp agar plates. Any (white or blue) colonies which grow on the selective plates have been successfully transformed. Since transformed bacteria contain ligated phagemids, the TE is representative of the ligation efficiency of the nuclear extracts with which the linear phagemid was incubated. The plating efficiency of the DH5α cells was assessed by streaking DH5α transformed with circular pBS onto LB-amp agar plates, and calculated as follows:

$$PE = \frac{\text{colonies per control plate}}{\text{volume streaked}} \quad \text{Equation 2.5.7.1}$$

The TE was then calculated according to the following formula:

$$TE = \frac{\text{colonies per plate}}{(\text{volume streaked}) \times (PE)} \quad \text{Equation 2.5.7.2}$$

Any colony which grew on LB-amp agar plates was successfully transformed, but only a blue colony received a phagemid with a functional LacZ. Ligation infidelity (LIF) was assessed as follows:

$$LIF = \frac{\text{number of white colonies}}{\text{total number of colonies}} \quad \text{Equation 2.5.7.3}$$

2.6 STATISTICAL ANALYSES

To statistically examine the differences between means, P values were calculated by a variety of tests. All statistical analyses were done using the GraphPad Prism software. To examine differences between the cell lines for one specific treatment (i.e. 2 h of incubation, 10 Gy dose) an unpaired student's t test was used. To compare numerous treatments for one cell line, one-way ANOVA (analysis of variance) was conducted. To compare numerous experimental treatments simultaneously between the cell lines (i.e. comparing the cell lines over the entire incubation or dose range), it was necessary to use two-way ANOVA. Finally, to compare whether the mean response (i.e. FAR, LIF) differed significantly from zero, a one-column t test was employed. In order to determine how well a regression line fit the data, R^2 values were calculated which gave an estimation of the goodness of fit.

3. RESULTS

3.1 CLONOGENIC SURVIVAL

3.1.1 HDRI Survival

The *in vitro* survival curve following HDRI is used to assess the intrinsic radiosensitivity of cells. For HDRI survival, confluent monolayers are given a dose of irradiation on ice, and then immediately trypsinized, counted, plated onto dishes and left to incubate for 14 days. On day 14, colonies are fixed, stained, and counted. HDRI survival is assessed by plotting the surviving fraction (SF, equation 2.3.2), as determined by the clonogenic assay (section 2.1), versus dose. The surviving fraction is plotted on a logarithmic scale and the dose is plotted on a linear scale (figure 3.1.1.1).

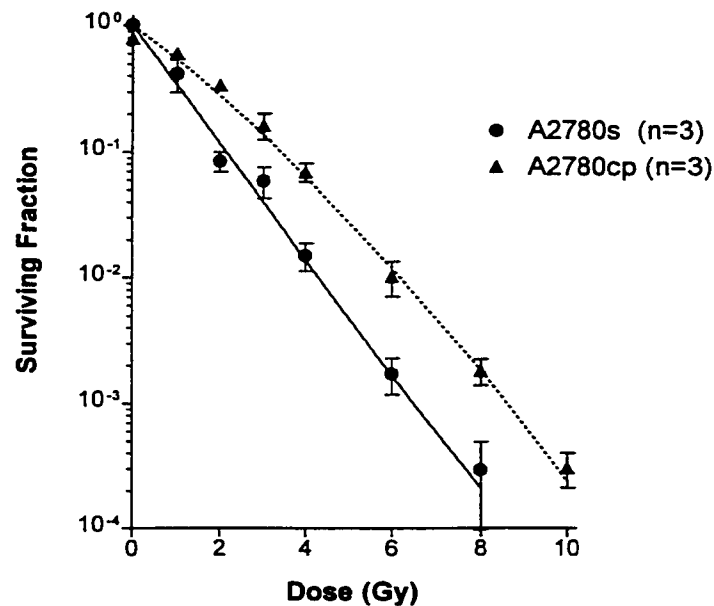


Figure 3.1.1.1 Cell survival immediately following HDRI. Confluent cell monolayers were irradiated on ice and immediately plated to assess clonogenic survival. Data points represent the mean SF of n independent experiments. Data has been fitted using the linear quadratic model (equation 1.2.1.1). Error bars represent the SEM between experiments.

SF data are fit with the linear quadratic model (equation 1.2.1.1) and the α and β parameters are determined. As can be seen from figure 3.1.1.1, the linear quadratic model fits the data very well (R^2 is 0.96 for A2780s and 0.98 for A2780cp). When comparing the HDRI survival curves of A2780s and A2780cp, conspicuous differences are seen. The parent curve is practically linear while the variant curve has a distinct shoulder region. Differences in survival are seen at doses as low as 2 Gy, and these differences are magnified at higher doses. From figure 3.1.1.1 qualitative differences in the radiosensitivity of A2780s and A2780cp are clear. These observations are quantified in table 3.1.1.1.

Endpoint	A2780s (n=3)	A2780cp (n=3)	P value
α	0.99 ± 0.0822	0.59 ± 0.0057	0.016 [†]
β	-0.014 ± 0.014	0.019 ± 0.0072	0.53
SF ₂ (data)	0.083 ± 0.015	0.43 ± 0.043	0.0016 [†]
SF ₂ (fits)	0.13 ± 0.018	0.28 ± 0.029	0.012 [†]
D ₁₀	2.23 ± 0.13	3.4 ± 0.227	0.0089 [†]
D ₁	4.36 ± 0.29	6.2 ± 0.30	0.011 [†]

Table 3.1.1.1 Comparisons of endpoints of intrinsic radiosensitivity between A2780s and A2780cp. α and β are obtained from the linear quadratic fits of the data. SF₂ (data) is the normalized SF at 2 Gy as determined by the clonogenic assay. SF₂ (fits) is the SF at 2 Gy calculated from the fitted curves of individual experiments. D₁ and D₁₀ are estimated from the fitted curves. [†] the difference between the means is significant (P<0.05)

Numerous endpoints of intrinsic radiosensitivity are displayed in table 3.1.1.1. As mentioned in section 1.2.1.1, α represents the linear component of cell kill while β represents the quadratic component. SF₂ is an isodose (equal doses are given) endpoint and is a measure of survival following a dose of 2 Gy. In contrast, D₁ and D₁₀ are isosurvival

endpoints and indicate the doses, in Gy, required to reduce survival to 1% and 10%, respectively. To establish any significant differences P values were computed via a two-tailed, unpaired student's t test. Significant differences exist in the value of α between the two cell lines (0.99 vs 0.59, $P < 0.05$), while β is not significantly different (-0.0142 vs 0.019, $P > 0.05$). SF_2 has been calculated two ways and both yield significant differences between the cell lines. Significant results (0.13 vs. 0.28, $P < 0.05$) are obtained when the SF_2 values calculated from the individual linear quadratic fits are considered. Very significant differences (0.083 vs 0.43, $P < 0.01$) are seen, however, when comparing the normalized SF_2 values obtained from the clonogenic assay. The differences in the isosurvival endpoints, D_1 and D_{10} , are also striking ($P < 0.05$). To reduce survival to 10%, a dose of approximately 2 Gy is required for A2780s while 3 Gy is required for A2780cp. Likewise a dose of 4 Gy in A2780s results in 1% survival, compared to 6 Gy for A2780cp. Taken together, this data indicates that A2780cp is qualitatively and quantitatively radioresistant compared to its parent, A2780s. Furthermore, parameters that describe the initial portion of the survival curve are more powerful in identifying differences in radiosensitivity than those that describe the final portion. Significant differences are found in the value of α but not β ($P < 0.05$ vs. $P > 0.05$, respectively), and D_{10} yields more significant differences than D ($P < 0.01$ vs. $P < 0.05$, respectively).

3.1.2 SLD Survival

SLD is normally repaired if sufficient time is given before more damage is incurred. Elkind and Sutton (7) were the first to describe split dose experiments. They observed that if a dose was split into two equal fractions with an intervening repair time, survival was

greater than if the dose was given in one fraction. For this work, SLD recovery has been examined using isodose and isosurvival irradiation. In both cases, the two fractions are separated by repair intervals during which confluent cell monolayers are incubated at 37°C for 0 to 8 h. Cells are plated immediately following the second fraction. The results are presented in two ways: SF and recovery ratio (RR). SF is an absolute measurement of survival, while RR is a relative measurement of the increase in survival seen when a repair interval is given between doses compared to when no repair time is given (equation 2.3.2.1).

For the isodose experiments, cells received a 6 Gy dose of HDRI split into two fractions of 3 Gy. Figure 3.1.2.1 illustrates the increase in survival observed with increasing repair intervals between fractions.

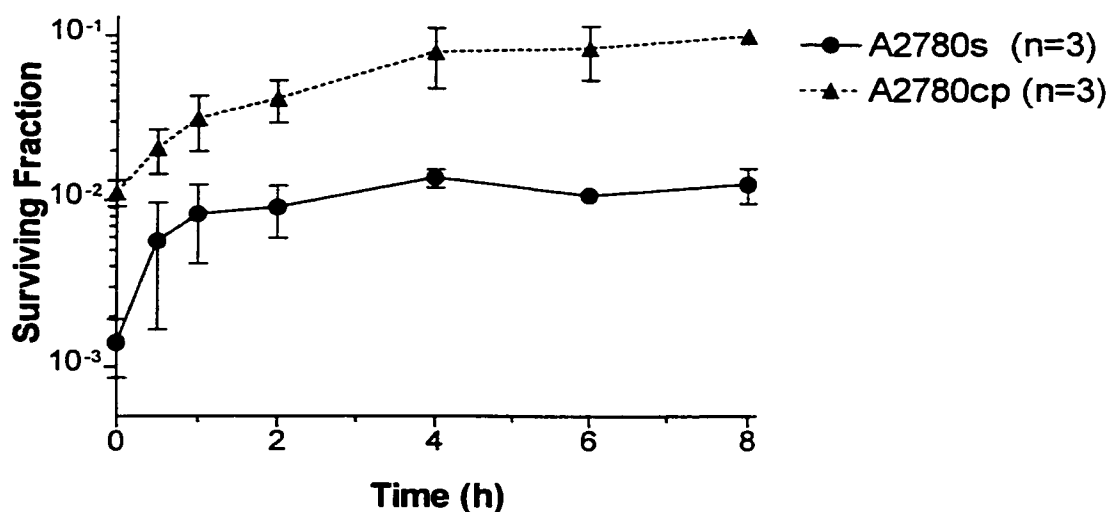


Figure 3.1.2.1 Survival of cells following an isodose split dose. Two fractions of 3 Gy were separated by incubation at 37°C, with the time ranging from 0 to 8 hours. Points represent the mean SF of n independent experiments. Error bars represent the SEM between experiments.

It is evident from figure 3.1.2.1 that there is an initial log difference in survival when no repair time is given. Initial split dose survival is examined in table 3.1.2.1. To ensure that the radiosensitivity of the cell lines is not changing with time, the split dose SF when no repair time is given (split dose $SF_{t=0}$) is compared to the SF following a 6 Gy dose (SF_6) on the HDRI curve (figure 3.1.1.1)

	split dose $SF_{t=0}$ (n=3)	SF_6 (n=3)	P value
A2780s	0.0014 ± 0.00053	0.0017 ± 0.00055	0.70
A2780cp	0.011 ± 0.0020	0.012 ± 0.0052	0.91
P value	0.0090 [†]		

Table 3.1.2.1 A comparison of isodose SLD survival when no repair time is given between fractions (split dose $SF_{t=0}$) and the HDRI survival following a 6 Gy dose (SF_6). The mean $SF \pm SEM$ of n independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant ($P < 0.01$).

There is an initial log difference in survival which is significant ($P < 0.01$). This is expected as a 6 Gy dose is not equitoxic to both cell lines (see figure 3.1.1.1). Indeed, it is evident from table 3.1.2.1 that the split dose survival when no repair time is given is not significantly different ($P > 0.05$) than the SF_6 on the original survival curve dose for either cell line. For A2780s the surviving fraction increases rapidly following 1 h of repair, then increases slightly as the repair interval is increased to 4 h and remains relatively constant for longer incubation times. However, the error for shorter repair intervals is very large, so the initial survival is difficult to describe. In contrast that seen for A2780s, the survival of A2780cp steadily increases up to 4 hours of repair time and then remains steady.

Because of the initial log difference in survival, it is prudent to compare SLD recovery between these two cell lines by examining their relative recovery, via RR. Figure 3.1.2.2 demonstrates the relative increase in survival observed as the repair interval between fractions lengthens. Comparisons of survival are displayed in table 3.1.2.2.

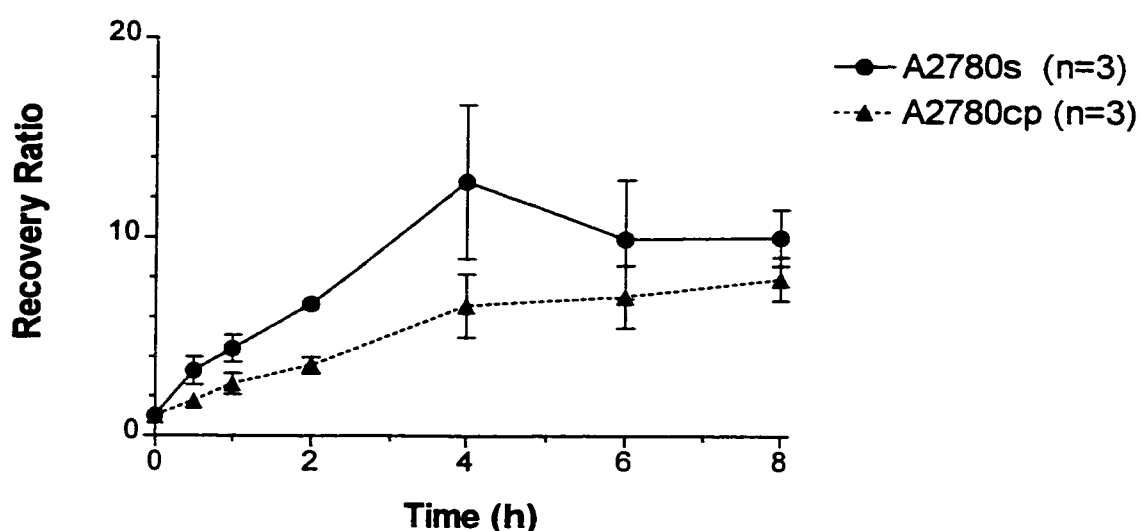


Figure 3.1.2.2 Recovery of cells following an isodose split dose. Fractions were separated by an incubation at 37°C ranging from 0 to 8 hours. Recovery is measured as the increase in survival for a given repair time compared to the survival with no repair time. Points represent the mean RR of n independent experiments. Error bars represent the SEM between experiments.

Length of repair interval (h)	A2780s (n=3)	A2780cp (n=3)	P value
2.0	6.7 ± 0.27	3.6 ± 0.42	0.0034 [†]
8.0	10 ± 1.4	7.9 ± 1.1	0.37

Table 3.1.2.2 A comparison of isodose SLD recovery between A2780s and A2780cp. The mean RR ± SEM of n independent experiments is displayed. P-values are calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant (P<0.01).

From table 3.1.2.2 it can be seen that when 2 h of repair is allowed between fractions, A2780s is far superior ($P<0.01$) at SLD recovery compared to its resistant variant. This significant difference however, disappears as the repair interval is lengthened ($P>0.05$). Two-way ANOVA analysis is used to compare the SLD recovery of the cell lines over the range of repair intervals studied. No significant interaction exists between the length of the repair interval and cell line ($P=0.53$). Over the entire dose range, A2780s is capable of significantly greater SLD recovery ($P<0.0001$) than A2780cp

SLD recovery was also examined for isosurvival doses. From figure 3.1.1.1, doses were selected which were approximately equitoxic to both cell lines (4 Gy for A2780s and 6 Gy for A2780cp) and were then split into equal fractions (2 Gy + 2 Gy for A2780s and 3 Gy + 3 Gy for A2780cp). Figure 3.1.2.3 shows how survival increases with increasing repair intervals.

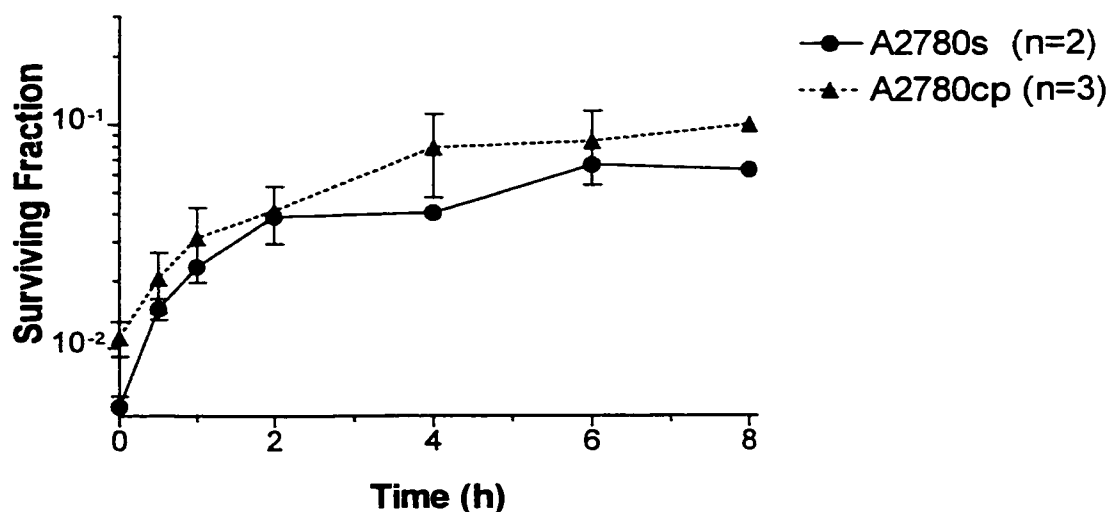


Figure 3.1.2.3

Survival of cells following an equitoxic split dose. Fractions are separated by an incubation at 37°C ranging from 0 to 8 h. Points represent the mean SF of n independent experiments. Error bars represent the SEM between experiments.

Table 3.1.2.3 compares the initial survival following an equitoxic split dose. To check for consistency between experiments, the split dose survival when no repair time is given (split dose $SF_{t=0}$) is compared with the survival following a 6 Gy dose (SF_6) for A2780cp and to survival following a 4 Gy dose (SF_4) for A2780s on the original survival curve (figure 3.1.1.1).

	split dose $SF_{t=0}$	SF_4 or SF_6 (n=3)	P value
A2780s	0.0055 ± 0.00063 (n=2)	0.015 ± 0.0036	0.15
A2780cp	0.011 ± 0.0020 (n=3)	0.012 ± 0.0052	0.91
P value	0.12		

Table 3.1.2.3 A comparison of equitoxic SLD survival when no repair time is given between fractions (split dose $SF_{t=0}$) and the HDRI survival following a 4 Gy dose (SF_4 , for A2780s) or a 6 Gy dose (SF_6 , for A2780cp). The mean $SF \pm$ SEM of n independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's t test.

It should be noted that the initial survival (no repair interval) of the two cell lines is not significantly different ($P > 0.05$). Thus, these doses are indeed equitoxic. Also, the initial split dose survival is not significantly different ($P > 0.05$) for either cell line compared to their corresponding HDRI survival (SF_4 for A2780s and SF_6 for A2780cp). The SLD recovery is similar in both cell lines. SLD survival increases steadily as the incubation between fractions is increased to 2 h, but increases in survival are less swift for longer intervals.

Recovery ratio was also used in the isosurvival experiments to compare SLD recovery between these two cell lines, as shown in figure 3.1.2.4 and the analysis of specific time points is displayed in table 3.1.2.4.

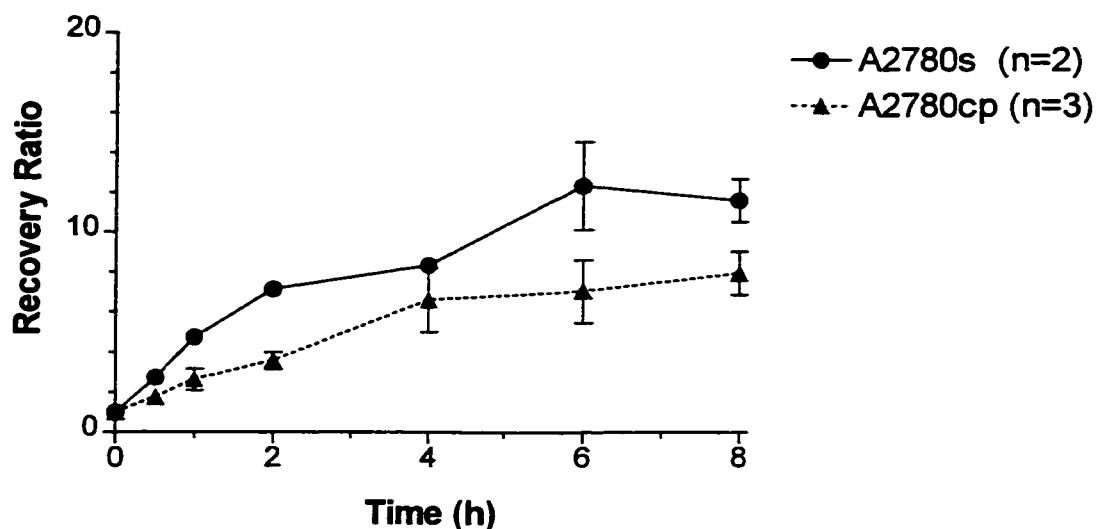


Figure 3.1.2.4 Recovery of cells following an equitoxic split dose. Fractions are separated by an incubation at 37°C, ranging from 0 to 8 h. Recovery is measured as the increase in survival for a given repair time compared to the survival when no repair time is given. Points represent the mean RR of *n* independent experiments. Error bars represent the SEM between experiments.

Length of repair interval (h)	A2780s (n=3)	A2780cp (n=3)	P value
2.0	7.1 ± 0.32	3.6 ± 0.42	0.0092 [†]
8.0	12 ± 1.1	7.9 ± 1.1	0.14

Table 3.1.2.4 Comparison of isosurvival SLD recovery between A2780s and A2780cp for different repair intervals. The mean RR ± SEM of *n* independent experiments is displayed. P values have been calculated using a two-tailed, unpaired, student's *t* test. [†] indicates that the difference between the means is significant (*P*<0.01).

The SLD recovery from equitoxic fractions is similar to what is observed for the isodose fractions. If 2 h of repair time is allowed between fractions, the sensitive parent has a greater recovery than its resistant variant, and this difference is significant (*P*<0.01). Once again, this difference wanes (*P*>0.05) with longer repair intervals. As before, no significant interaction (*P*=0.20) is seen between the length of the repair interval and cell line and the overall ability of A2780s to repair SLD is significantly greater (*P*=0.0011) than that of

A2780cp, as determined by two-way ANOVA analysis.

3.1.3 PLD Survival

Potentially lethal damage (PLD) is normally lethal, but under post irradiation conditions which prevent the cells from cycling (and thereby preventing the cells from attempting mitosis), PLD can be repaired (9). PLD recovery is observed as an increase in survival when the plating of plateau phase cultures is delayed, and this is demonstrated in figure 3.1.3.1.

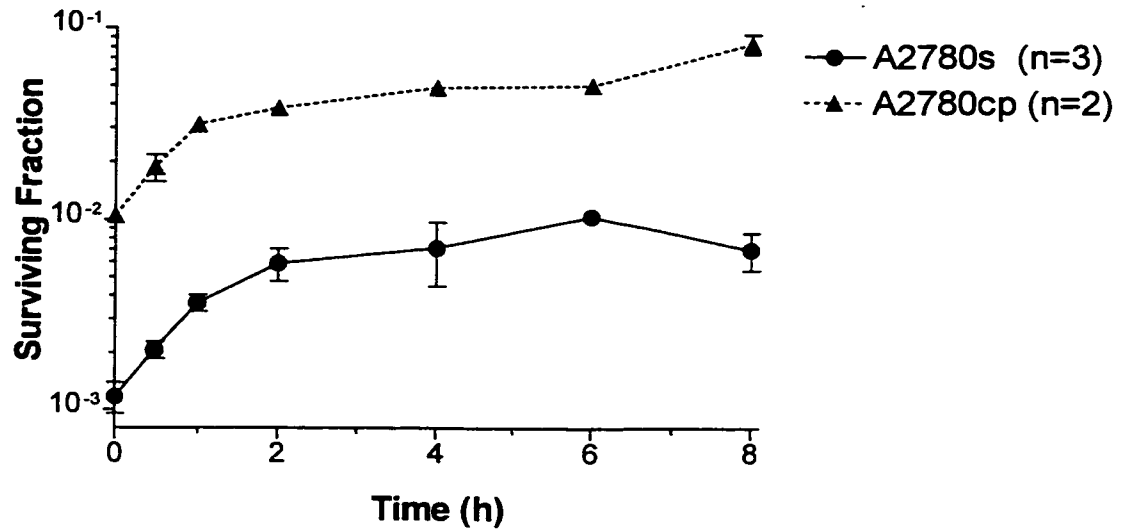


Figure 3.1.3.1 Survival of cells when plating of plateau-phase cultures is delayed. Following a 6 Gy dose of HDRI, cells were incubated at 37°C for 0 to 8 hours before being plated. Points represent the mean SF of n independent experiments. Error bars represent the SEM between experiments.

Table 3.1.3.1 compares the survival when plating is not delayed. To verify the consistency between experiments, table 3.1.3.1 also compares the PLD survival when plating is not delayed (delayed plating $SF_{\tau=0}$) to the survival following a 6 Gy dose from the HDRI survival curve (SF_6).

	delayed plating $SF_{t=0}$	SF_6 (n=3)	P value
A2780s	0.0012 ± 0.00022 (n=3)	0.0017 ± 0.00055	0.42
A2780cp	0.010 ± 0.000042 (n=2)	0.012 ± 0.0052	0.86
P value	$<0.0001^\dagger$		

Table 3.1.3.1 A comparison of PLD survival when plating is not delayed (delayed plating $SF_{t=0}$) and HDRI survival following a 6 Gy dose (SF_6). The mean $SF \pm SEM$ of n independent experiments is displayed. P values have been calculated using two-tailed, unpaired student's t test. † indicates the difference between the means is significant ($P < 0.005$).

Significant differences ($P < 0.005$) in survival are seen when plating is not delayed. This large difference in survival can be attributed to the differing radiosensitivities of the cell lines to a 6 Gy dose observed for HDRI survival (figure 3.1.1.1). Indeed, there is no significant difference ($P > 0.05$) between survival when plating is not delayed and survival following a 6 Gy dose on the original survival curve. Survival of both cell lines increases steadily as the delay time increases up to 2 h. For longer delay times the survival of both cell lines begins to plateau.

Recovery ratios were calculated from PLD experiments and are graphically displayed in figure 3.1.3.2. PLD recovery is examined following specific delay times in table 3.1.3.2.

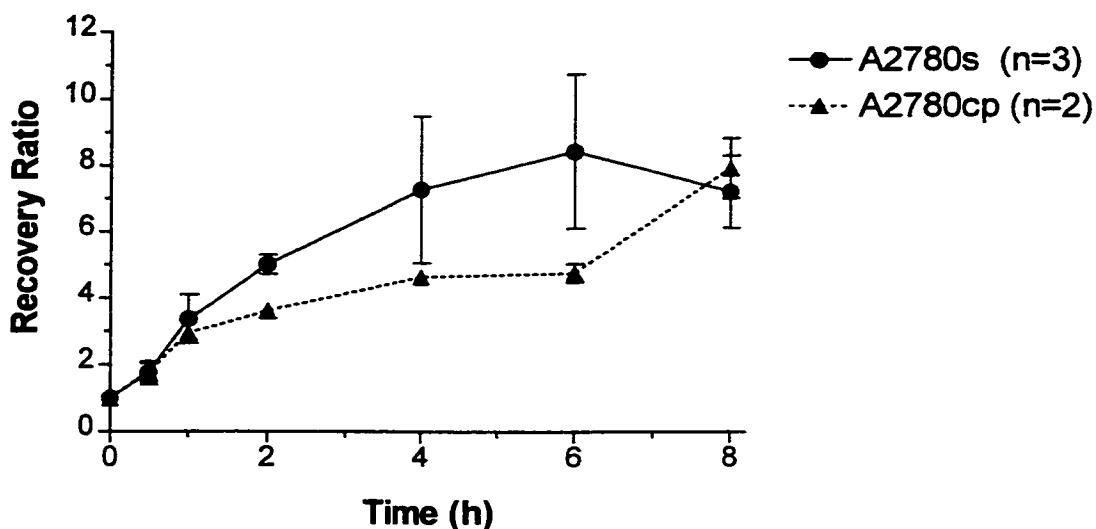


Figure 3.1.3.2 Recovery of cells when the plating of plateau-phase cultures is delayed. Following a 6 Gy dose of HDRI, cell monolayers are incubated at 37°C for 0 to 8 h. Recovery is measured as the increase in survival for a given repair time compared to the survival with no repair time. Points represent the mean RR of *n* independent experiments. Error bars represent the SEM between experiments.

Delay time (h)	A2780s (n=3)	A2780cp (n=2)	P value
2.0	5.0 ± 0.28	3.6 ± 0.16	0.036 [†]
8.0	6.4 ± 0.99	7.9 ± 0.91	0.38

Table 3.1.3.2 A comparison of PLD recovery between A2780s and A2780cp. The mean RR ± SEM of *n* independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's *t* test. [†] indicates that the difference between the means is significant (*P*<0.05).

PLD recovery is similar to that observed for SLD experiments. If plating is delayed by 2 h, A2780s is significantly (*P*<0.05) superior to A2780cp at PLD recovery following a 6 Gy dose. However, as plating is delayed further, any differences become insignificant (*P*>0.05). As before, two-way ANOVA analysis is employed to elucidate differences in PLD recovery over the entire range of delay times. Naturally, no significant interaction (*P*=0.23) is observed between the cell lines and the delay interval. Furthermore, the PLD recovery

A2780s is significantly greater overall ($P=0.040$).

3.1.4 Comparisons of SLD and PLD

Some controversy exists to whether the repair of SLD and PLD are similar processes involving the same basic lesion or distinct mechanisms of cellular recovery. Thus, the recovery ratios of the isodose SLD (RR_{SLD}) and PLD (RR_{PLD}) experiments are compared in table 3.1.4.1 for the A2780s cell line and in table 3.1.4.2 for the A2780cp cell line.

Repair time (h)	RR_{SLD} (n=3)	RR_{PLD} (n=3)	P value
2.0	6.7 ± 0.27	5.0 ± 0.287	0.014 [†]
8.0	10 ± 1.4	7.2 ± 1.1	0.257

Table 3.1.4.1 A comparison of SLD and PLD recovery for A2780s. The mean $RR \pm SEM$ of n independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant ($P<0.05$).

Repair time (h)	RR_{SLD} (n=3)	RR_{PLD} (n=2)	P value
2.0	3.6 ± 0.42	3.6 ± 0.16	0.95
8.0	7.9 ± 1.1	7.9 ± 0.91	1.0

Table 3.1.4.2 A comparison of SLD and PLD recovery for A2780cp. The mean $RR \pm SEM$ of n independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's t test.

From table 3.1.4.1, A2780s exhibits significantly greater ($P<0.05$) SLD recovery than PLD recovery when 2 h of repair time are given. However, if an 8 h delay is allowed, similar recovery from SLD and PLD is achieved. Table 3.1.4.2 clearly shows that for A2780cp, identical SLD and PLD recovery is attained for both 2 h and 8 h of repair. Because of the interaction between cell line and RR, two-way ANOVA analysis can not be used to determine if any differences exist between SLD and PLD recovery.

3.2 AFIGE

The AFIGE assay quantifies DNA dsbs as the fraction of activity released (FAR). That is, the amount of radio-labeled DNA which migrates out of the well into the lane following a specified treatment (section 1.4.2). AFIGE is used to assess both the induction and rejoining of DNA dsbs.

3.2.1 Dsb Induction

The induction of dsbs by x-rays can be measured by irradiating the cells on ice and processing them immediately. Although no time is given for endogenous repair processes to act, other endogenous factors (free radical scavengers, DNA conformation, etc) can contribute to the fixation of x-ray induced damage. The induction of dsbs immediately following HDRI is presented in figure 3.2.1.1.

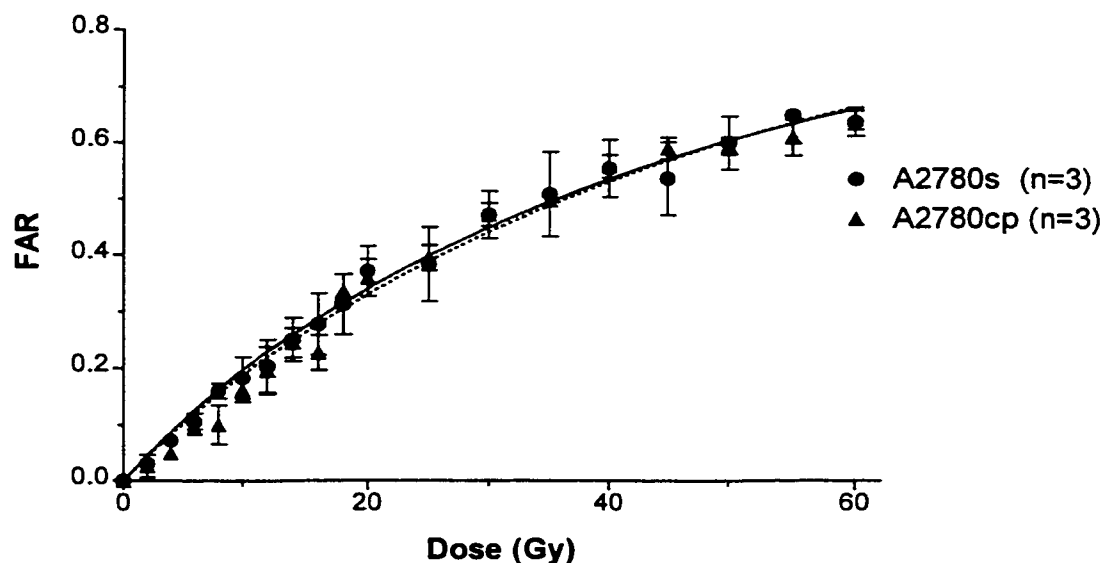


Figure 3.2.1.1

Dsb induction immediately following HDRI. Agarose plugs, containing DNA, were prepared from plateau phase cultures and irradiated on ice. Each point represents the mean FAR of n independent experiments. Error bars represent the SEM between experiments. Data points are normalized to the FAR following a 0 Gy dose. Curves were fit using nonlinear regression.

DNA damage due to dsb, as measured by FAR, increases with dose and approaches a plateau at doses above 60 Gy. The dsb induction curves for A2780s and A2780cp are best fit using nonlinear regression (R^2 is 0.89 and 0.96, respectively). The lines are curvilinear and essentially identical, with A2780s and A2780cp reaching a FAR of 0.66 and 0.64, respectively. There are no differences in the induction of dsb between these cell lines. The dsb induction curve extends over a wide range of doses, up to 60 Gy. The clonogenic assay, by comparison, only examines doses up to 10 Gy. If the clonogenic assay is to be considered the gold standard of intrinsic radiosensitivity, it would be logical to examine the AFIGE assay in a similar dose range. A highlight of the dsb induction curve (doses extending from 0 to 20 Gy) is presented in figure 3.2.1.2.

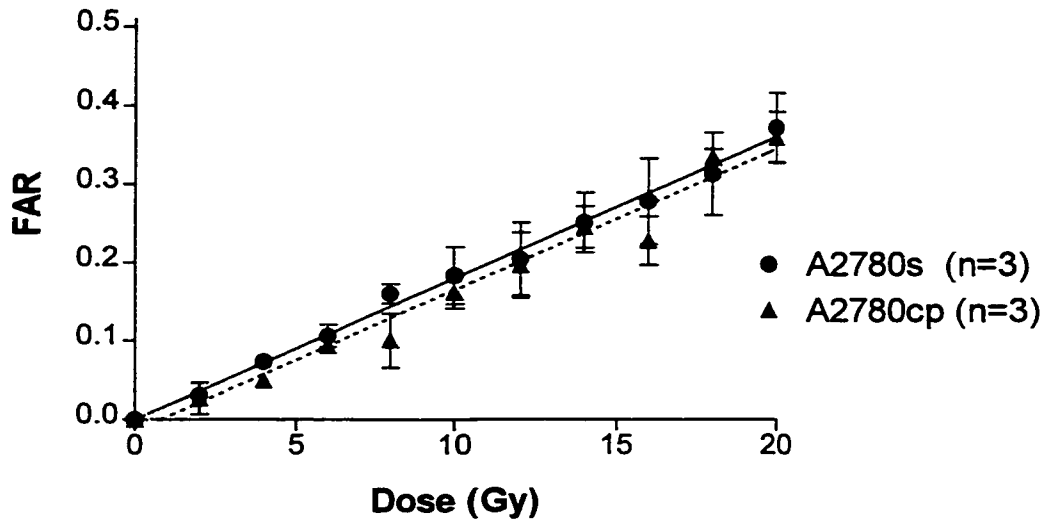


Figure 3.2.1.2 An amplification of the dsb induction curve (figure 3.2.1.1), displaying the lower dose region. Curves were fit using linear regression.

If only the dose range of 0 to 20 Gy is considered, the data are best fit using linear regression (R^2 is 0.81 and 0.89 for A2780s and A2780cp, respectively). The slopes of both A2780s and

A2780cp are identical. To examine the sensitivity of the AFIGE assay, two-way ANOVA analysis is employed to examine the 0 to 10 Gy dose range. No significant ($P=0.79$) interaction exists between cell line and dose. Although the curves are not significantly different, FAR increases significantly ($P<0.0001$) with dose. Thus, AFIGE is sensitive to doses in the clonogenic dose range. One-way ANOVA with the Newman-Keuls post test is used to further examine AFIGE's sensitivity in the 0 to 10 Gy dose range. When the data from the A2780s cell line is examined, one-way ANOVA reveals a significant difference between doses ($P=0.007$). The Newman-Keuls post test suggests that significant differences ($P<0.05$) can be observed when the dose differs by 6 Gy. Similar results are found when the A2780cp data is examined; significant differences in FAR are detected in the 0 to 10 Gy dose range ($P=0.003$, one-way ANOVA) with a sensitivity of 6 Gy ($P<0.05$, Newman-Keuls post test). Although no significant differences were found between cell lines, it is clear that the sensitivity of the AFIGE is indeed powerful as it is capable of detecting differences in FAR when the delivered dose differs by 6 Gy.

3.2.2 Rejoining Kinetics

The dsb rejoining kinetics were examined for monolayer cultures irradiated with 30 Gy. Following irradiation on ice, the monolayers were allowed to incubate at 37°C for 0 to 8 h prior to plug preparation and AFIGE analysis. Figure 3.2.2.1 illustrates the decrease in FAR observed when cells are incubated at 37°C prior to AFIGE preparation and analysis. Table 3.2.2.1 displays the analysis of FAR when no repair delay is given (rejoining kinetics $FAR_{t=0}$) compared to the FAR induction following a 30 Gy dose of HDRI (induced FAR_{30G}).

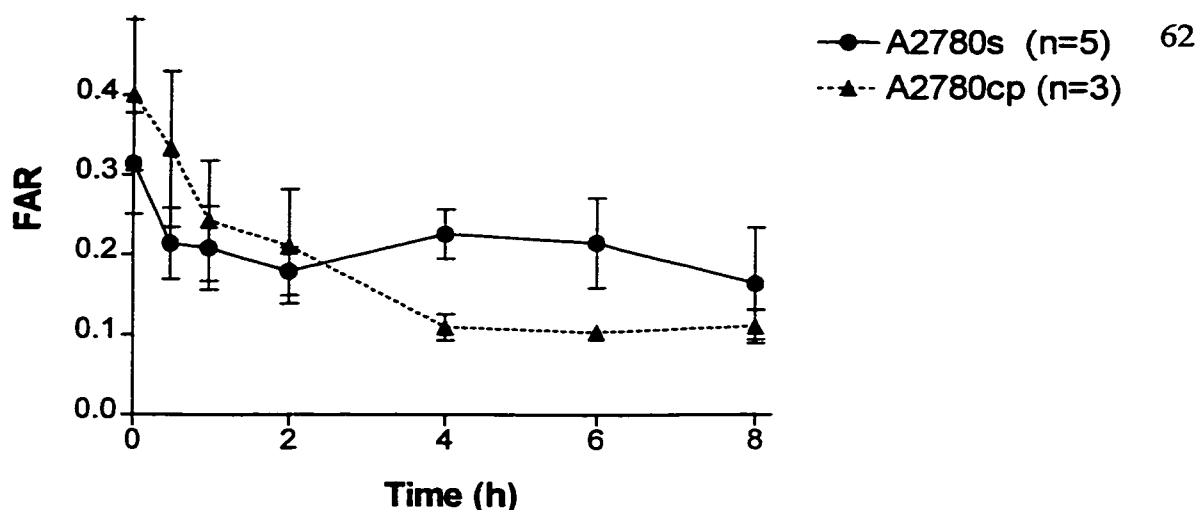


Figure 3.2.2.1 Rejoining kinetics following a 30 Gy dose of HDRI. Cell monolayers were incubated at 37°C following irradiation. Plugs were prepared at the end of the incubation period and then analyzed. Points represent the mean FAR of n independent experiments. Error bars represent the SEM between experiments.

	rejoining kinetics FAR _{t=0} (n=5)	induced FAR _{30Gy} (n=3)	P value
A2780s	0.28 ± 0.061	0.57 ± 0.069	0.041 [†]
A2780cp	0.40 ± 0.095	0.56 ± 0.011	0.17
P value	0.46		

Table 3.2.2.1 A comparison of rejoining kinetics when AFIGE preparation is not delayed (rejoining kinetics FAR_{t=0}) and dsb induction (induced FAR_{30Gy}). The mean FAR ± SEM of n independent experiments is displayed. P values have been calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant (P<0.05).

It is expected that when no repair is given the FAR from the rejoining kinetic experiments would be the same as the FAR following a 30 Gy dose from the induction curve. This is true for A2780cp (P>0.05), but is not the case for A2780s (P<0.05); the FAR when no repair time is given is only half of the FAR value obtained from the induction experiments. To compare cell lines, the FAR remaining (equation 2.4.7.1) is depicted in figure 3.2.2.2, and analyses are listed in table 3.2.2.2..

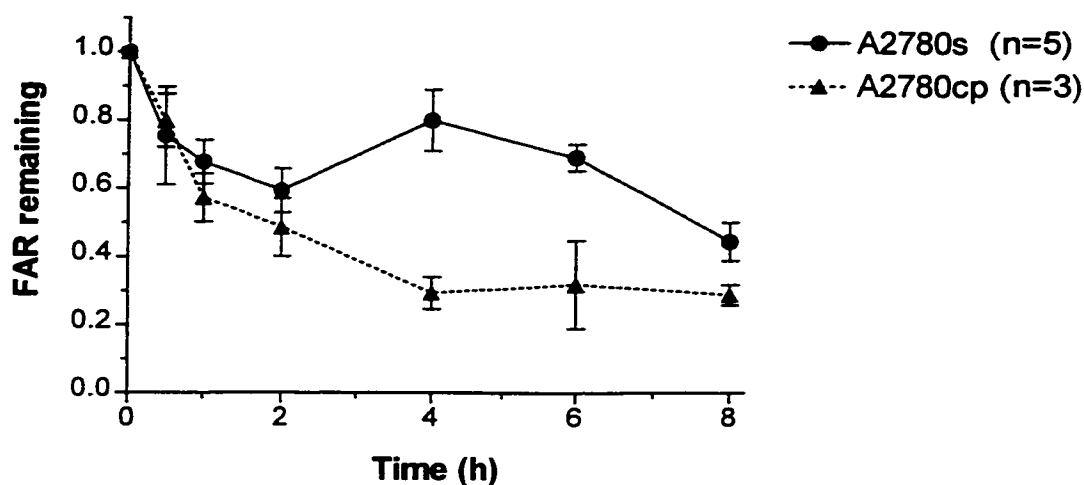


Figure 3.2.2.2 FAR remaining when cell monolayers are incubated at 37°C following a 30 Gy dose of HDRI. The data has been normalized; FAR remaining is calculated by dividing the FAR at time t by the FAR at time 0. Points represent the mean FAR remaining of n independent experiments. Error bars represent the SEM between experiments.

Delay time (h)	A2780s (n=4)	A2780cp (n=3)	P value
0.5	0.75 ± 0.14	0.80 ± 0.078	0.82
1.0	0.66 ± 0.052	0.57 ± 0.070	0.34
4.0	0.80 ± 0.090	0.29 ± 0.047	0.0067 [†]
6.0	0.69 ± 0.039	0.32 ± 0.13	0.025 [†]
8.0	0.44 ± 0.056	0.29 ± 0.030	0.071

Table 3.2.2.2 A comparison of FAR remaining between A2780s and A2780cp when AFIGE preparation and analysis is delayed. The mean normalized FAR remaining ± SEM of n independent experiments. P values have been calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant (P<0.05).

The fast component of repair is similar in both cell lines. No significant differences (P>0.05) exist between the parent and its variant when either 0.5 h or 1 h of repair time are given. Differences are seen however, in the slow phase of repair. While the parent rejoins

very little additional dsbs following the initial stage (0.5 h), the resistant variant continues to rejoin damage up to 4 h of repair time. A2780cp is capable of significantly greater ($P < 0.05$) dsb rejoining when cells are incubated for 4 h or 6 h following irradiation and prior to AFIGE preparation. Although differences still exist, their significance disappears ($P > 0.05$) when the repair time is increased past 6 h. Two-way ANOVA has been used to compare the two rejoining kinetics curves. Surprisingly, a significant ($P = 0.010$) interaction exists between the length of the repair interval and cell line, making further differences between cell lines difficult to substantiate.

3.2.3 Residual Damage

Residual damage has often been cited as an explanation for differences in radiosensitivity. From figure 3.2.2.1 it is clear that rejoining is essentially complete following 4 h of repair time. Thus, for residual damage experiments, cell monolayers were given various doses of HDRI and allowed to incubate at 37°C for 6 hrs prior to being prepared for AFIGE analysis. The results of these experiments are displayed in figure 3.2.3.1. Table 3.2.3.1 compares residual dsb between A2780s and A2780cp.

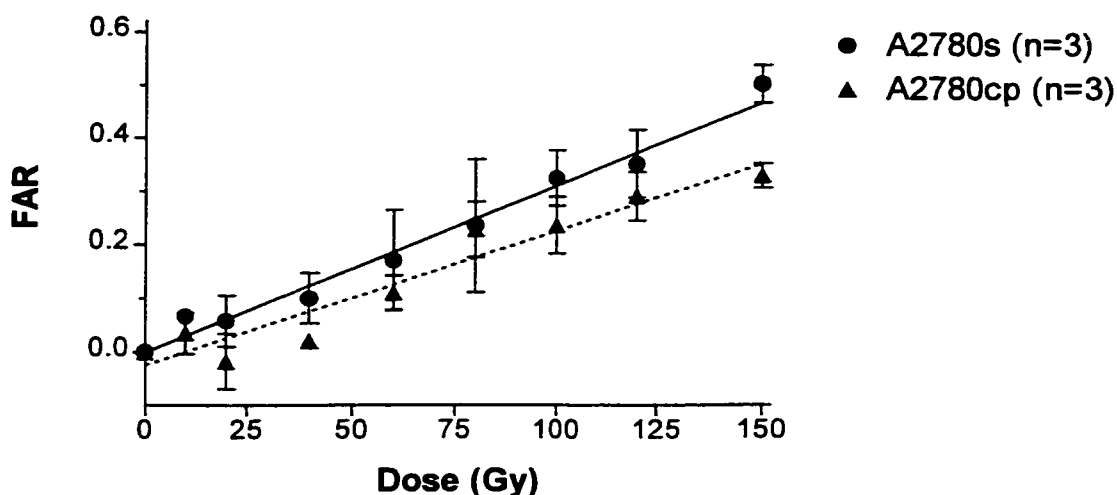


Figure 3.2.3.1 Residual FAR when 6 h of incubation is given following a dose of HDRI. Cell monolayers were irradiated on ice and then incubated at 37°C for 6 h. Plugs for AFIGE analysis were prepared at the end of the incubation period. Points represent the mean normalized FAR for n independent experiments. Error bars represent the SEM between experiments. FAR has been normalized to the intercept of the FAR axis, so that for dose of 0 Gy FAR is zero. Curves were fit using linear regression.

	A2780s (n=3)	A2780cp (n=3)	P value
slope	0.0031 ± 0.00041	0.0022 ± 0.00030	0.21
R^2	0.71	0.78	--
residual FAR _{150Gy}	0.50 ± 0.035	0.33 ± 0.023	0.023 [†]

Table 3.2.3.1 A comparison of parameters describing the residual damage curve between A2780s and A2780cp. Slope is determined by linear regression and the mean slope $\pm$ SEM of n independent experiments is listed. R^2 describes the goodness of fit. Residual FAR_{150Gy} is the FAR remaining when 6 h of repair time are given after a dose of 150 Gy. The mean FAR remaining $\pm$ SEM of n independent experiments is given. P values are calculated using a two-tailed, unpaired student's t test. [†] indicates that the difference between the means is significant ($P < 0.05$).

From figure 3.2.3.1 and table 3.2.3.1 it is evident that there is linear relationship between dose and residual FAR, as the straight lines fit the data quite well ($R^2=0.71$ for A2780s and $R^2=0.78$ for A2780cp). Linear regression analysis indicates that the residual damage lines are quite shallow, and yet they do differ significantly from zero ($P<0.0001$ for both). No significant differences ($P>0.05$) exist however, between the slopes of A2780s and A2780cp.

From figure 3.2.3.1, the only significant difference in residual FAR occurs following highest dose used, 150 Gy ($P=0.023$). If the residual FAR was analyzed at higher doses, perhaps the difference between the cell lines would be more distinct, but its relevance to intrinsic radiosensitivity must be considered.

The residual damage experiments reveal no differences in the level of residual dsbs between these cell lines, even though rejoining kinetic experiments demonstrated a difference in the slow phase of dsb repair. The two experiments are normalized differently, so it is beneficial to compare the unnormalized data. Table 3.2.3.2 compares the unnormalized FAR following a 30 Gy dose and 6 h of repair from the rejoining kinetic experiments (rejoining kinetics $FAR_{t=6}$) and the unnormalized FAR following a 40 Gy dose and 6 h of repair from the residual damage experiments (residual FAR_{40Gy}). Residual dsbs following a 30 Gy dose were not measured in these experiments, so the residual FAR following a 40 Gy dose must be used for comparison purposes.

	rejoining kinetics FAR _{t=6} (n=5)	residual FAR _{40Gy} (n=3)	P value
A2780s	0.24 ± 0.052	0.19 ± 0.034	0.43
A2780cp	0.14 ± 0.037	0.13 ± 0.011	0.82
P value	0.21	0.16	--

Table 3.2.3.2 A comparison of FAR between the rejoining kinetic and residual damage experiments. The mean FAR ± SEM of n independent experiments is listed. Data has not been normalized. P values are calculated using a two-tailed, unpaired student's t test.

If the unnormalized data is examined, no significant ($P > 0.05$) differences are observed between the FAR values obtained from rejoining kinetic and residual dsb experiments. Furthermore, there are no significant ($P > 0.05$) differences between the FAR values of A2780s and A2780cp in the FAR following a 6 h of repair in the rejoining kinetic experiments. Thus, it appears that differences in rejoining kinetics may have arisen from the normalization process. The residual damage curve can also be compared with the induction curve as shown in figure 3.2.3.2. A comparison of induction and residual DNA damage following a 60 Gy dose is presented in table 3.2.3.3.

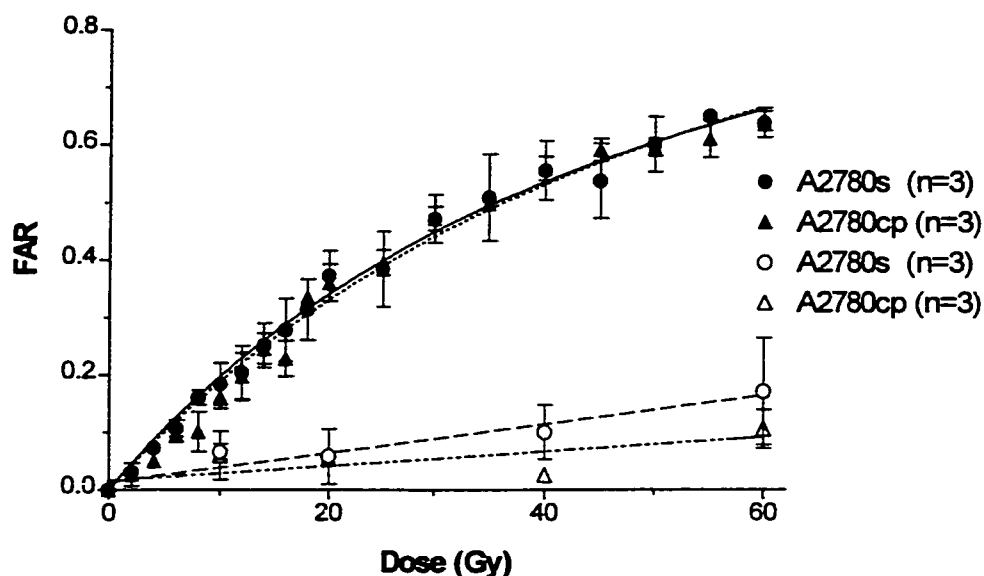


Figure 3.2.3.2

A comparison of the residual damage and induction curves. Solid symbols represent the mean induced FAR (FAR measured immediately after HDRI). Open symbols represent mean FAR remaining (FAR detected when cell monolayers are allowed to incubate for 6 h at 37°C following HDRI). The induction curve is fit with nonlinear regression. The residual damage curve is fit with linear regression. Error bars represent the SEM between n independent experiments.

	A2780s	A2780cp	P value
FAR induction	0.64 ± 0.026 (n=3)	0.66 ± 0.021 (n=3)	0.54
FAR remaining	0.17 ± 0.093 (n=3)	0.10 ± 0.033 (n=3)	0.54
% FAR rejoined	0.73	0.84	--

Table 3.2.3.3 A comparison of FAR induction and residual FAR following a 60 Gy dose between A2780s and A2780cp. FAR has been normalized as described previously. The normalized FAR ± SEM between n independent experiments is listed. % FAR rejoined is calculated as (FAR induction - FAR remaining)/(FAR induction). P values are calculated using a two-tailed, unpaired student's t test.

As mentioned previously, the residual FAR curves are quite shallow and figure 3.2.3.2 demonstrates the striking ability of mammalian cells to repair the damage induced by HDRI.

If 6 h of repair time at 37°C is given following a 60 Gy dose, A2780s and A2780cp are capable of rejoining approximately 73% and 84% of the dsbs incurred by a 60 Gy dose,

respectively. Because of the proficiency of dsb rejoining in these cell lines, any real differences could be difficult to discriminate.

3.3 CELL-FREE FIDELITY ASSAY

The fidelity assay, in addition to detecting rejoining itself, attempts to distinguish between accurate rejoining and misrejoining. pBluescript (pBS) is a phagemid with two selectable markers; a amp^R gene and a LacZ gene. Rejoining is detected as colonies capable of growing in antibiotic selection media (a circular plasmid containing an intact amp^R gene). Accurate rejoining is indicated by blue colonies (an intact LacZ gene) and misrejoining by white colonies (interrupted LacZ gene). Transformation efficiency (TE, equation 2.5.7.2) is calculated to examine rejoining efficiency and ligation infidelity (LIF, equation 2.5.7.3) is computed to analyze the accuracy of rejoining. Once nuclear protein was extracted from the cell lines, it was necessary to determine the optimum protein concentration for the fidelity incubations. Two types of linearization were used. Staggered-cut plasmid is obtained by digesting pBS with *EcoRI* while blunt-cut plasmid is obtained by digesting pBS with *EcoRV*. A range of nuclear extract concentrations (0.1 μg to 1.0 μg) were incubated with either of the pBS digests in order to determine their effect on TE. Purified T4 DNA ligase was used as a positive control for ligation. The ability of various nuclear extracts concentrations and T4 DNA ligase to ligate pBS and therefore increase TE are compared in figure 3.3.1.

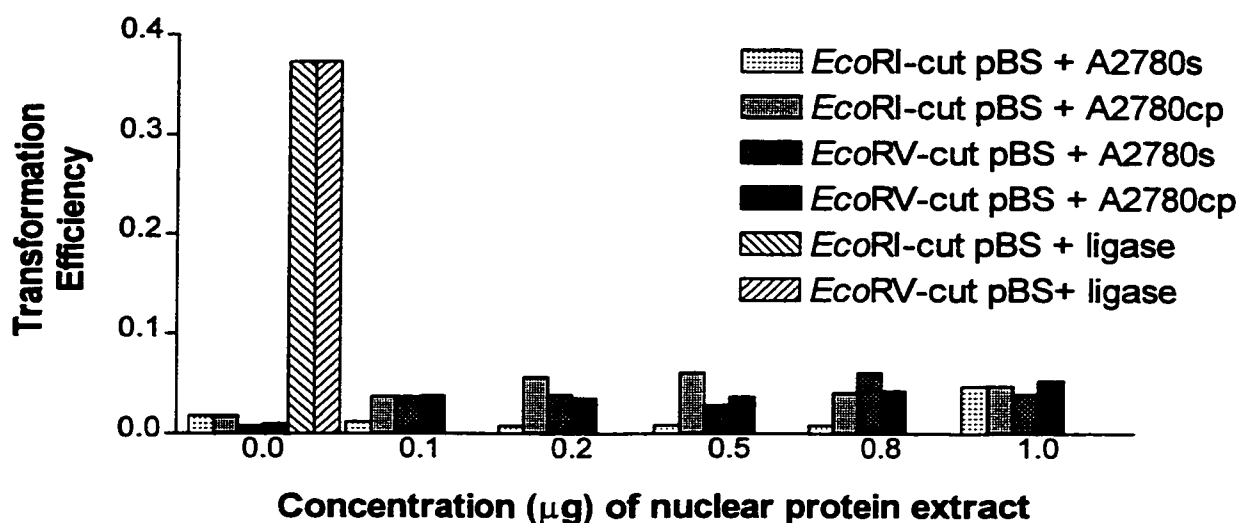


Figure 3.3.1 Titration of nuclear extract concentrations - a single experiment. A range of concentrations were incubated with 2 μg of digested pBS and 1 nM dNTPs in 50 ml of ligase buffer. Following a 20 h incubation at 16°C, pBS was recovered and transformed into DHG5α. The transformed DH5α was streaked onto LB-amp agar plates along with X-Gal and IPTG and incubated overnight at 37°C. The next day blue and white colonies were counted.

Note from figure 3.3.1 that *EcoRI*-cut pBS and *EcoRV*-cut pBS incubated in the absence of nuclear proteins are capable of transforming into DH5α and giving rise to colonies (TE > 0). This is surprising, particularly since gel electrophoresis shows no evidence of circular (undigested) plasmid prior to transformation (data not shown). Also, it is thought that linear plasmid is not efficiently transformed (76). These unexpected findings are in accordance with those seen in other labs (Britten, verbal communication). It is also evident from figure 3.3.1 that T4 DNA ligase is far superior than any of the nuclear extract concentrations at ligating pBS. This is not surprising, considering that commercially purified ligase would be more concentrated than that found in nuclear extracts. Figure 3.3.2 contains the same information presented in figure 3.3.1, minus the ligase control data, in

order to facilitate comparisons between nuclear protein concentrations. Since there is some background transformation, data has been normalized so that the TE is zero for linear plasmids incubated in the absence of nuclear protein.

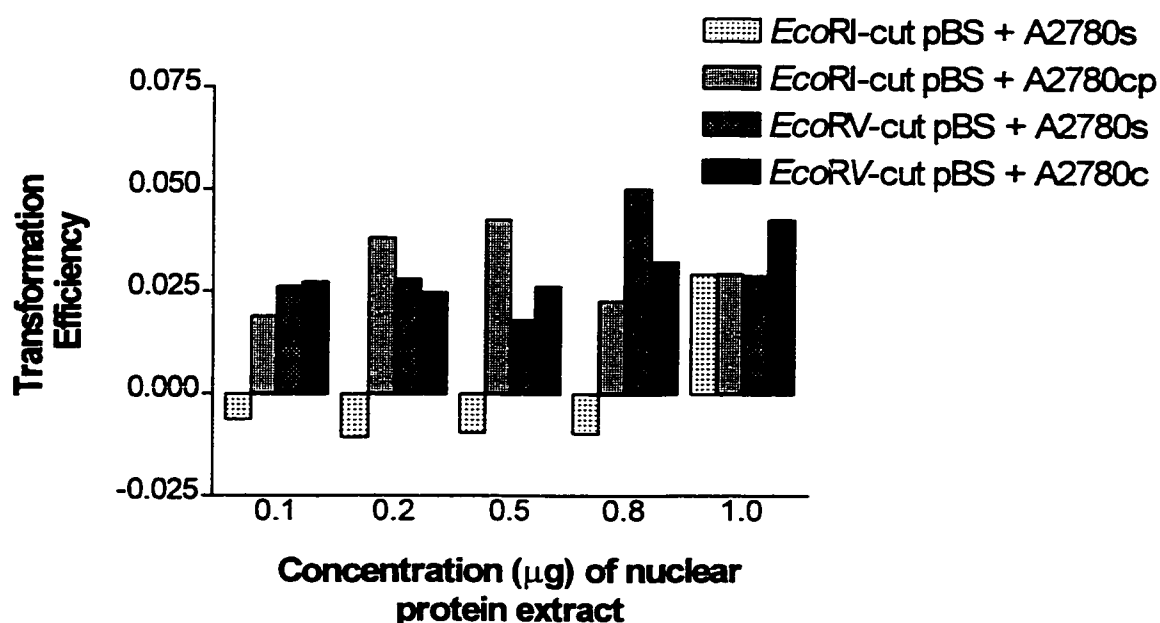


Figure 3.3.2 A portion of figure 3.3.1, displaying only the nuclear extract data. By removing the ligase data, comparisons between extract concentrations are more easily made. Data has been normalized so that TE=0 when pBS is incubated in the absence of nuclear proteins.

Negative TE values indicate that the TE was less for treated pBS than for pBS incubated alone. It is surprising that nuclear extracts could reduce TE below background levels, but this has been observed in other laboratories. There is no clear relationship between nuclear extract concentration and TE. For *EcoRI*-cut pBS, nuclear extracts from A2780s have little or a negative effect on TE. 0.1 μg to 0.8 μg of A2780s extracts actually decreases TE below background levels. This suggests that these extracts digest pBS, perhaps due to excessive nuclease activity. An even greater concentration of protein (1.0 μg)

however, had a positive effect on TE, albeit small. *EcoRI*-cut pBS incubated with A2780cp nuclear protein exhibits a more typical dose-response; TE increases as the protein concentration increases from 0.1 μg to 0.5 μg and then decreases as the extract concentration increases to 1.0 μg . For *EcoRV*-cut pBS incubated with nuclear extracts from either A2780s or A2780cp, TE exceeds background levels, but varies with no apparent relationship with extract concentration. Because there is no clear relationship between extract concentration and TE, experiments were carried out for several protein concentrations and analyzed separately. TE was first analyzed for nuclear protein concentrations of 0.8 μg and results are presented in figure 3.3.3 and listed in table 3.3.1.

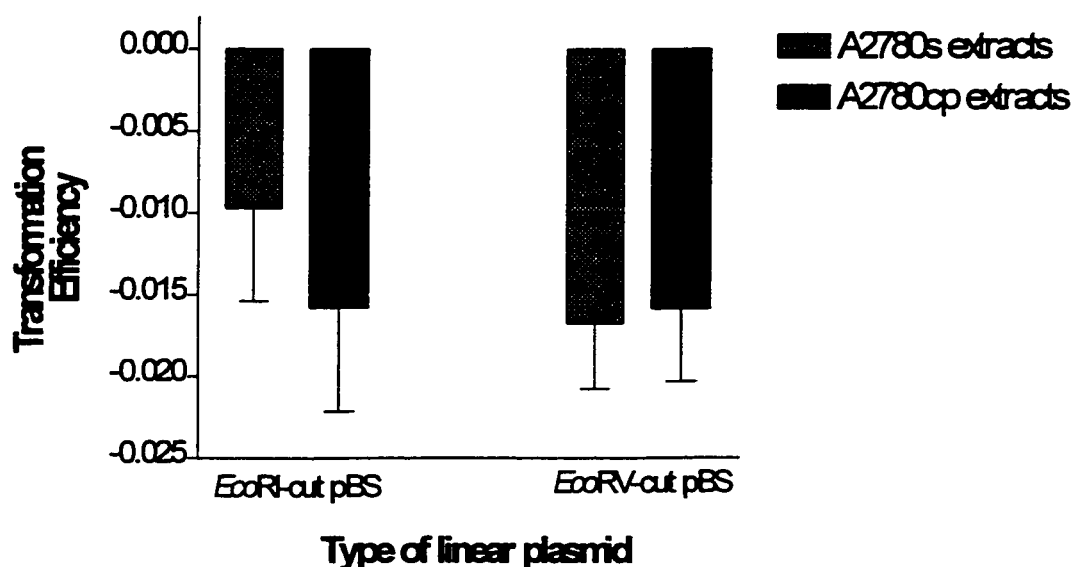


Figure 3.3.3 A comparison of TE between *EcoRI*-cut and *EcoRV*-cut pBS incubated with 0.8 μg of nuclear extracts. pBS and extracts were incubated with 1 nM dNTPs in 50 μl of ligase buffer for 20 h at 16°C. Recovered pBS was transformed into DH5 α , and cells were plated onto LB-amp agar plates along with X-Gal and IPTG. The total number of colonies were counted. Bars represent the mean TE of n independent experiments. Data has been normalized so that TE=0 when pBS is incubated without nuclear proteins. Error bars represent the SEM between experiments.

	A2780s	A2780cp	P value (s vs cp)
<i>EcoRI</i> -cut pBS (n=2)	-0.0097 ± 0.0056	-0.016 ± 0.0064	0.55
<i>EcoRV</i> -cut pBS (n=2)	-0.016 ± 0.0040	-0.016 ± 0.0045	0.90
P value (<i>EcoRI</i> vs <i>EcoRV</i>)	0.42	0.99	

Table 3.3.1 A comparison of the TE of *EcoRI*-cut pBS and *EcoRV*-cut pBS incubated with 0.8 µg of nuclear proteins from A2780s and A2780cp. P values have been calculated using a two-tailed, unpaired student's t test.

In contrast to the titration experiment, this concentration (0.8 µg) has a negative effect on TE regardless of the type of digested plasmid or extract used. No significant differences ($P > 0.05$) exist in TE of plasmid incubated with A2780s or A2780cp. Likewise, no significant differences ($P > 0.05$) exist between the TE of *EcoRI*-cut pBS and *EcoRV*-cut pBS. From these results it is clear a lower concentration of extract should be employed. These experiments do not agree with the single titration experiment, and reflect the great variability seen between experiments. Given the negative effect these extracts have on TE, LIF has not been calculated.

Figure 3.3.4 illustrates the TE of *EcoRI*-cut pBS and *EcoRV*-cut pBS incubated with 0.5 µg of nuclear extracts from A2780s and A2780cp. *EcoRV*-cut pBS incubated with nuclear protein from A2780cp is the only treatment which leads to an increase in TE. The remaining treatments all have a negative effect on TE and are associated with large error bars. Table 3.3.2 lists the results from these experiments.

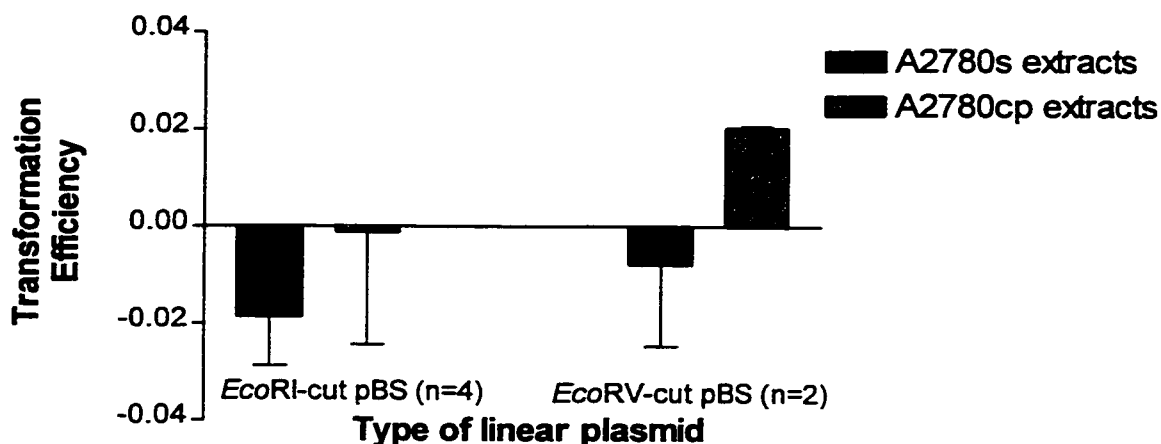


Figure 3.3.4 A comparison of TE between *EcoRI*-cut and *EcoRV*-cut pBS incubated with 0.5 µg of nuclear extracts. pBS and extracts were incubated with 1 nM dNTPs in 50 µl of ligase buffer for 20 h at 16°C. Recovered pBS was transformed into DH5α, and cells were plated onto LB-amp agar plates along with X-Gal and IPTG. The total number of colonies were counted. Bars represent the mean TE of n independent experiments. Data has been normalized so that TE=0 when pBS is incubated without nuclear proteins. Error bars represent the SEM between experiments.

	A2780s	A2780cp	P value (s vs cp)
<i>EcoRI</i> -cut pBS (n=4)	-0.019 ± 0.01020	-0.0012 ± 0.023	0.52
<i>EcoRV</i> -cut pBS (n=2)	-0.0078 ± 0.017	0.020 ± 0.00033	0.24
P value (I vs V)	0.59	0.57	

Table 3.3.2 A comparison of the TE of *EcoRI*-cut pBS and *EcoRV*-cut pBS incubated with 0.5 µg of nuclear proteins from A2780s and A2780cp. P values have been calculated using a two-tailed, unpaired student's t test.

It is clear from table 3.3.2 that much variability exists between separate extracts from the same cell line, and it is difficult to reproduce results. Because of the negative effect of the nuclear extracts on TE, it is meaningless to calculate the LIF for this data.

An even lower dose was chosen for the final set of fidelity experiments. 0.1 µg of nuclear proteins were incubated with 2 µg of *EcoRI*-cut pBS or *EcoRV*-cut pBS and the experiments were carried out as before. Experiments have been repeated several times to

account for inter-experimental variability. Furthermore, additional extractions have been conducted on both cell lines to obtain unique nuclear proteins (3 separate extracts for each cell line, in total). Thus, by repeating experiments and using nuclear proteins from unique extractions, it is hoped that real differences in plasmid rejoining can be elucidated. Figure 3.3.5 displays the effect of 0.1 μg nuclear extracts on the TE of linear pBS. The TE of *EcoRI*-cut pBS is very small, with large error bars. By comparison, there is a clear increase in the TE of *EcoRV*-cut pBS. To examine whether the mean TE actually differs significantly from zero, a one column t test is used and data are presented in table 3.3.3.

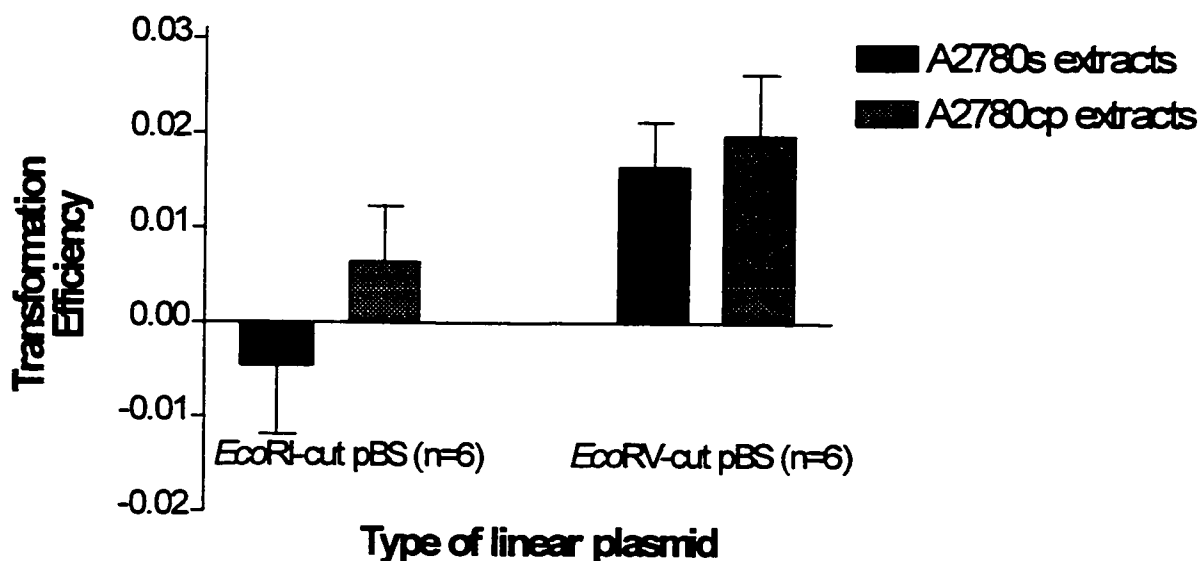


Figure 3.3.5 TE of *EcoRI*-cut and *EcoRV*-cut pBS incubated with 0.1 μg of nuclear protein extracts. pBS and nuclear protein were incubated with 1 nM dNTPs in 50 μl of ligase buffer for 20 h at 16°C. Recovered pBS was transformed into DH5 α , and cells were plated onto LB-amp agar plates along with X-Gal and IPTG. The total number of colonies were counted. Bars represent the mean TE of n independent experiments. 3 different extracts from each cell line were used, with some extracts used two to three separate experiments. Data has been normalized so that TE=0 when pBS is incubated without nuclear proteins. Error bars represent the SEM between experiments.

	<i>Eco</i> RI-cut pBS + A2780s (n=6)	<i>Eco</i> RI-cut pBS + A2780cp (n=6)	<i>Eco</i> RV-cut pBS + A2780s (n=6)	<i>Eco</i> RV-cut pBS + A2780cp (n=6)
TE	-0.0046 ± 0.0073	0.0064 ± 0.0059	0.016 ± 0.0048	0.020 ± 0.0065
P value	0.56	0.33	0.019 [†]	0.028 [†]

Table 3.3.3 A comparison of the mean TE of treated linear pBS to determine whether it differs significantly from zero. P values are calculated using a one column t test. [†]indicates that the mean TE differs significantly from zero (P<0.05).

It is clear from figure 3.3.5 and table 3.3.3 that the mean TE of *Eco*RI-cut pBS does not differ significantly (P>0.05) from zero when incubated with either extract. However, the mean TE of *Eco*RV-cut pBS incubated with either A2780s or A2780cp extracts is significantly (P<0.05) greater than zero. In other words, nuclear protein extracts do not efficiently rejoin staggered-ended linear plasmid (*Eco*RI-cut pBS), at least not above background levels. By contrast, blunt-ended linear plasmid (*Eco*RV-cut pBS) is rejoined by nuclear extracts with some efficiency. Given that only *Eco*RV-cut pBS demonstrated a significant increase in TE, *Eco*RI-cut pBS is not examined in further analyses of TE or LIF. The mean TE of *Eco*RV-cut pBS incubated with A2780s extracts approximately 0.016, and 0.020 for *Eco*RV-cut pBS incubated with A2780cp extracts. When analyzed with a two-tailed, unpaired student's t test, the difference between the means is found to be not significant (P=0.68). Thus, A2780s and A2780cp nuclear extracts are capable of rejoining *Eco*RV-cut pBS, and do so with similar efficiencies.

The LIF was compared only for *Eco*RV-cut pBS incubated with A2780s or A2780cp. It was not examined for *Eco*RI-cut pBS as no significant rejoining was observed (TE did not differ from zero). Figure 3.3.6 displays the LIF of *Eco*RV-cut pBS incubated with 0.1 µg of nuclear protein extracts and statistical comparisons are made in table 3.3.4.

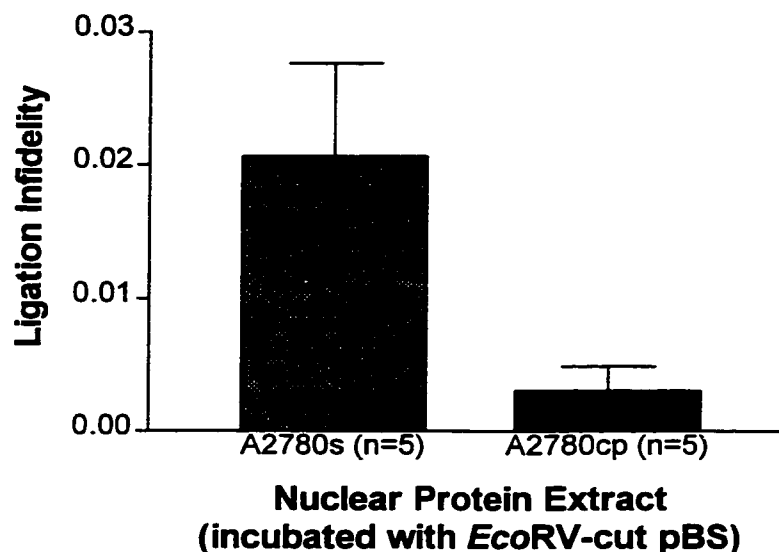


Figure 3.3.6 Ligation infidelity of *EcoRV*-cut pBS incubated with 0.1 μ g of nuclear protein extracts. LIF was calculated as the ratio of white colonies to total colonies, per plate. Bars represent the mean LIF of *n* independent experiments. Error bars represent the SEM between experiments.

	A2780s (n=5)	A2780cp (n=5)	P value (student's t-test)
LIF of <i>EcoRV</i> -cut pBS	0.021 $\pm$ 0.0070	0.0030 $\pm$ 0.0019	0.043 [†]
P-value (one-column t-test)	0.043 [†]	0.18	

Table 3.3.4 A comparison of the LIF of *EcoRV*-digested pBS incubated with 0.1 μ g of nuclear proteins from A2780s and A2780cp. The mean LIF $\pm$ SEM of *n* independent experiments is listed. P values are determined from a two-tailed, unpaired student's *t* test are used to compare the LIF between cell lines. To determine whether the mean LIF differs from zero, P-values are calculated using a one-column *t*-test. [†] indicates that the difference between the means is significant ($P < 0.05$).

Table 3.3.4 demonstrates that the LIF of *EcoRV*-cut pBS incubated with nuclear extracts from A2780cp is significantly ($P < 0.05$) less compared to the LIF of *EcoRV*-cut pBS incubated with A2780s. Although both extracts rejoin *EcoRV*-cut pBS with the same

efficiency, A2780cp extracts do so with greater accuracy than the parent. Furthermore, a one column t-test reveals that the mean LIF of the variant extracts does not differ significantly from zero ($P>0.05$) whereas the parent extracts exhibited a mean LIF which is significantly greater than zero ($P<0.05$). That is to say that the variant does not exhibit any significant misrepair, while its parent ligates 2% of *EcoRV*-cut pBS inaccurately.

4. DISCUSSION

4.1 INTRINSIC RADIOSENSITIVITY

The clonogenic assay is the gold standard for determining the intrinsic radiosensitivity of tumours. The aim of inherent radiosensitivity testing is to develop biological tests for dose prescriptions in radiotherapy that could lead to large improvements in tumour control probability (12). By understanding the factors influencing intrinsic radiosensitivity, novel means of controlling tumours and rapid predictive assays of tumour radioresponsiveness can be developed.

The preferred method of evaluating intrinsic radiosensitivity is by measuring the surviving fraction following a 2 Gy dose (SF_2). When considering the SF_2 obtained directly from the data, it is clear that A2780s is highly radiosensitive ($SF_2=0.083$), while A2780cp exhibits normal sensitivity ($SF_2=0.43$). These cellular radiosensitivities are consistent with those observed in other laboratories for ovarian carcinoma (74;108).

The linear quadratic model (equation 1.2.1.1) is the favoured method of fitting survival data (10), and has been used here to describe the survival curves. From the linear quadratic fit of the data, the radiosensitivity of A2780s is shown by the linear slope while relative radioresistance of A2780cp is manifested in the form of a shoulder region. The relative radioresistance of A2780cp is demonstrated by an increase in the width of the shoulder region, rather than a change in the terminal slope. The shoulder width is indicated by $1/\alpha$, while $1/\beta^2$ is representative of the terminal slope. Indeed, the greater shoulder of A2780cp seen in figure 3.1.1.1 is demonstrated by the significantly ($P<0.05$) decreased α value compared to its parent (0.99 vs 0.59 for A2780s and A2780cp, respectively). By

contrast there is no significant difference ($P>0.05$) in the value of β (-0.014 vs 0.019 for A2780s and A2780cp, respectively). A point should be made about the negative value of β seen here. β is determined mathematically, but a negative value is meaningless biologically. Some laboratories render β to zero if the linear quadratic model yields a negative value. In fact, the value obtained for the A2780s cell line approximates zero, so doing so would not appreciably affect the results.

The relative radioresistance of A2780cp is also demonstrated in differences in other radiosensitivity parameters. Both D_{10} and D_1 are significantly greater ($P<0.05$) for the variant; that is, a higher dose is required to kill 90% and 99% of A2780cp cells, respectively. As mentioned previously, SF_2 is significantly greater ($P<0.05$) for A2780cp. The SF_2 has been calculated in two ways; the mean SF_2 measured directly from individual experiments (SF_2 data), and the mean SF_2 calculated from the linear quadratic fits of individual experiments (SF_2 fits). It has been reported previously that the measured survival at 2 Gy is a better parameter than the calculated SF_2 from the linear quadratic model (109). The results from the A2780 cell lines are in accordance with this suggestion. Although both SF_2 (data) and SF_2 (fits) are significantly different, the SF_2 (data) has more statistical power than the SF_2 (fits) ($P<0.005$ vs $P<0.05$, respectively).

Parameters that describe proximal portions of the survival curve are better at distinguishing between the radiosensitivities of A2780s and its variant, A2780cp. Significant differences are found in the value of α but not β , and D_{10} ($P<0.01$) yields more significant differences than D_1 ($P<0.05$). A pitfall of the clonogenic assay is the impact of technical factors. Because the assay encompasses all aspects of cellular survival, the effects of media,

feeder cells, and technique can confound results. However, it has been observed that the distal part of the survival curve appears to be more dependent on technical factors, rather than the initial portion (19). Thus it is appropriate that parameters which describe the proximal portion of the survival curve are better indicators of intrinsic radiosensitivity.

Figure 4.1 depicts the approach taken to examine the factors influencing intrinsic radiosensitivity. These factors will be discussed in subsequent sections.

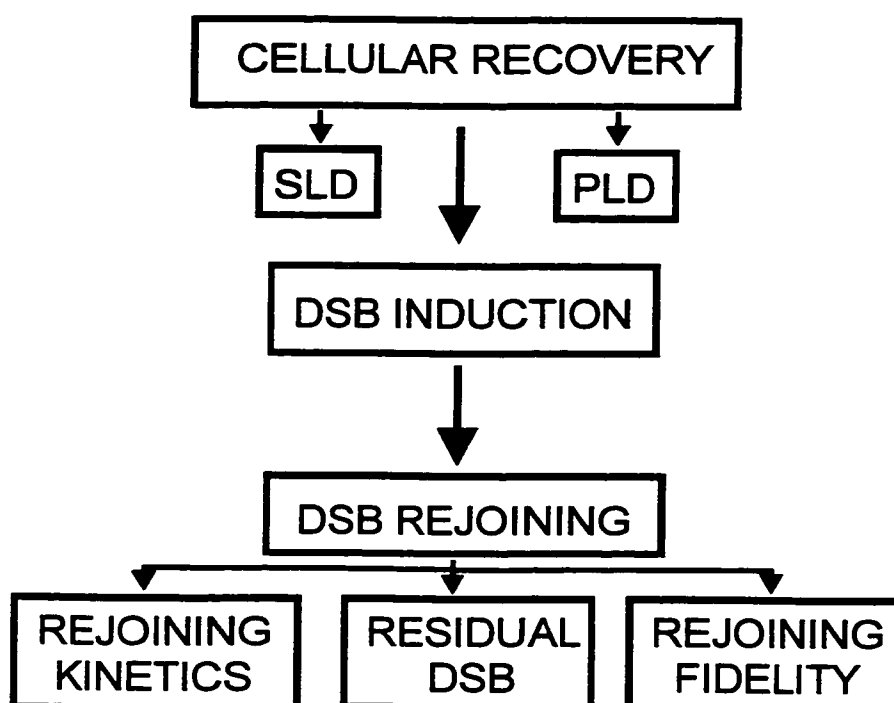


Figure 4.1 Possible determinants of intrinsic radiosensitivity.

4.2 CELLULAR RECOVERY

4.2.1 SLD Recovery

It is well established that the repair of SLD is related to the shoulder region of the radiation survival curve (8). If the linear quadratic equation is used as a model, a single strand break is an example of an SLD lesion. The single strand break becomes lethal if a

second single strand break occurs in temporally and spatially close enough to create a dsb. However, if sufficient time is allowed following the first insult, and the original single strand break can be repaired before the second single strand break is incurred, then the damage is non lethal.

The radiosensitive parent, A2780s, is capable of greater SLD recovery than its resistant variant, A2780cp, when a 6 Gy dose is split into two fractions. When 2 h of repair time is given between fractions, A2780s achieved a RR of 6.7; this is significantly greater than the RR of 3.6 attained by A2780cp ($P < 0.01$). It is surprising that the sensitive cell line exhibits greater SLD recovery. Because of the initial log difference in survival, this increased SLD recovery could be due to the fact that more damage is available (69) as it has been demonstrated that RR increases with increasing dose (110). To address this issue, SLD recovery is examined for equitoxic doses (4 Gy for A2780s and 6 Gy for A2780cp) split into equal fractions. As before, A2780s is capable of greater recovery following 2 h of repair time (RR of 7.2 vs 3.6 for A2780s and A2780cp, respectively) ($P < 0.01$). Furthermore, two-way ANOVA analysis reveals that A2780s has significantly greater SLD recovery overall for both isodose and equitoxic split dose experiments ($P < 0.001$).

These results are perplexing, as the shoulder region of the survival curve is thought to result from the accumulation of SLD. Given that A2780cp has a significantly greater shoulder ($1/\alpha$) than its parent, it would be expected to exhibit a greater recovery from SLD. These surprising results however, are in agreement with other reports which found that radiosensitive mammalian tumour cell lines exhibit greater SLD recovery than their radioresistant counterparts (69;110-113). However, one report studying a pair of isogenic

human malignant glioma cell lines with vast differences in radiosensitivity demonstrates that SLD recovery is minimal in the sensitive cell line (73). A recent study involving 17 head and neck tumour cell lines indicates that SLD recovery tends to be greater in radiosensitive cell lines, and that the β parameter of the linear quadratic model correlates well with the observed amount of SLD recovery (114). Nevertheless, no clear correlation between intrinsic radiosensitivity and SLD recovery has been established (115-118). Although the literature suggests that for human tumour cell lines, radiosensitive cells are more proficient at SLD recovery, this is not the case in rodent cell lines. Mutant rodent cell lines such as SCID (60), and *xrs* (59) which are known to be extremely radiosensitive and deficient in dsb repair are also deficient in SLD recovery (119;120). *Irs-20* is another radiation-sensitive mutant of CHO cells which exhibited no SLD recovery in plateau phase cultures (121). Although rodent studies suggest a possible link between dsb repair and SLD recovery, this has not been reproduced in human tumour cell lines. The data from the A2780 cell lines studied here, in agreement with the literature, suggests that radiosensitive tumour cell lines are capable of greater SLD recovery.

4.2.2 PLD Recovery

PLD is damage which would normally be lethal to the cell unless post-irradiation conditions were such that repair was facilitated (8). Delayed plating is one of many such conditions which allow for the repair of PLD. In 1982, Weichselbaum and colleagues reported that radiocurable tumours had lower PLD recovery than non-radiocurable tumours and suggested that PLD recovery capacity could predict clinical radioresponsiveness (122). In additional reports Weichselbaum continued to demonstrate a relationship between the

capacity of tumour cells to repair PLD *in vitro* and the local control rate *in vivo* (14;123-125). However, in a separate review of 22 human tumour cell lines taken from 19 published papers, no correlation was found between PLD recovery capacity and radioresponsiveness nor inherent radiosensitivity (126). An examination of published data on 81 human cell lines indicated that PLD recovery is a reasonable reflection of intrinsic radiosensitivity up to 2.2 Gy, but above 2.2 Gy the relationship is reversed where the greater the radioresistance, the lesser the PLD recovery (127).

Similar to what is observed for SLD recovery, A2780s is capable of greater PLD recovery compared to its resistant variant, A2780cp. Significant differences exist following 2 h of repair, (RR of 5.0 and 3.6 for A2780s and A2780cp, respectively) ($P < 0.05$). This significant difference disappears for longer repair intervals. However, when analysed over the entire range of delay times, two-way ANOVA indicates that significant differences exist between the PLD recovery curves of A2780s and A2780cp ($P < 0.05$). Most published reports of PLD examine the relationship between PLD recovery and tumour response to radiotherapy. Published reports comparing PLD recovery and the *in vitro* radiosensitivity of tumour cell lines are scarce. One such study examined the relationship between PLD recovery and cellular radiosensitivity in 22 human tumour cell lines (from previously published data) and did not observe any correlation (126). This confirmed the earlier findings of Marchese (128) and Hall (129). Any additional studies of PLD recovery and intrinsic radiosensitivity use non-human mammalian cell lines. The results presented here are in accordance with one published study which examined mouse L5178Y lymphoma cell lines. The extremely radiosensitive cell line was capable of greater PLD recovery than the

resistant line (130). Other reports however, indicate that radiosensitive mutants exhibit less PLD recovery. When a CHO cell line and its radiosensitive mutant, *irs-20* were compared, it was found that the mutant completely lacked the ability to repair PLD whereas the parent was competent at PLD recovery (121). *Xrs-5* is a radiosensitive CHO mutant known to be deficient in dsb repair and also demonstrates a complete lack of PLD recovery (131). Finally, another published report saw no differences in PLD recovery capacity when radiosensitive CHO cells EM9 and NM2 were compared to their parent, AA8 (132). It is clear from the data presented here and from published results that there is no clear relationship between PLD recovery capacity and inherent radiosensitivity.

4.2.3 Cellular Recovery - Comparing SLD and PLD

This work suggests that for A2780 ovarian carcinoma cells, increased intrinsic radiosensitivity does not result from decreased cellular recovery capacity, neither from SLD nor PLD. This leads to another question, namely, are SLD and PLD recovery unique processes or different manifestations of the same process operating on the same basic lesion? There remains much controversy regarding similarities and differences in the fundamental nature of SLD and PLD. Bedford and Cornforth examined chromosomal aberrations due to fragment loss and the rejoining of chromosome breaks along with SLD and PLD recovery (92;133). They concluded that recovery from SLD and PLD were different mechanisms for repairing the same lesion - a chromosome break. The lesion associated with SLD and PLD has yet to be discovered, although it is suggested to be the dsb (134;135). Most studies comparing SLD and PLD recovery in mammalian cell systems do not use human tumour cell lines. Reports examining CHO cells and their radiosensitive mutants are divided; most

studies indicate that SLD and PLD recovery are similar (119;131;132;136), while a couple of studies suggest that they are different processes (8;121). For the mouse lymphoma cell line, L5178Y, and its radiosensitive mutant, it was demonstrated that PLD and SLD recovery are different processes (130). Reports comparing SLD and PLD recovery in human tumour cell lines are scarce. One study examined SLD and PLD recovery together for ten human tumour cell lines of differing inherent radiosensitivities (137). SLD and PLD recovery were examined simultaneously using a new cellular recovery parameter, the recovery factor (RF). RF did not correlate to any intrinsic radiosensitivity measurements. However, the survival following a 4 Gy split dose correlated to the SF_2 determined from the linear quadratic fits of the data. It was suggested RF did not correlate with the cellular radiosensitivity because SLD and PLD recovery exerted different effects on overall recovery, depending on the cell line. This would imply that PLD and SLD recovery are different processes.

The results of the A2780 cell lines studied here indicate that SLD and PLD recovery are related processes. The RRs are comparable between the isodose split dose and PLD experiments (both experiments deliver a total dose of 6 Gy). For A2780s, significantly ($P<0.05$) greater SLD recovery (RR of 6.6) is achieved for 2 h of repair time compared to PLD recovery (RR of 5.0). However, as the repair time is increased to 8 h this significant difference disappears (RR of 10.0 and 7.2 for SLD and PLD recovery, respectively)($P>0.05$). SLD and PLD recovery are identical in the A2780cp cell line (RR of 3.6 for 2 h and 7.6 for 8 h, for both SLD and PLD experiments).

4.3 DSBs and INTRINSIC RADIOSENSITIVITY

4.3.1 Dsb Induction

A2780s and A2780cp exhibited identical dsb induction, as assessed by the AFIGE assay. Although many studies demonstrate significant differences in dsb induction for human tumour cell lines of differing inherent radiosensitivities (67-72;74), few have been able to demonstrate a correlation between intrinsic radiosensitivity and dsb induction (67;72). In fact, many reports fail to show any differences at all in dsb induction for human tumour cell lines of varying radiosensitivities (26;70;73-77;138). Thus, the bulk of the literature suggests that there is no relationship between inherent radiosensitivity and induction and this is supported by the data on the A2780 cell lines. This report demonstrates that the induction of dsb is identical in A2780s and its radioresistant mutant, A2780cp.

Dsb induction experiments do not allow for repair, although processes such as damage fixation and free radical scavenging can still occur. Also, factors such as chromatin structure and DNA conformation are capable of influencing dsb induction. When A2780cp was developed it was found to have intracellular glutathione levels that were elevated by three-fold compared to its parent (105). Additional experiments in this lab have confirmed these earlier findings, although for plateau phase cultures the difference was two-fold (139). Intracellular thiols, such as glutathione, are thought to be involved either in protection from radiation-induced damage or in the immediate reduction of free radicals formed by irradiation (140). In other words, increased levels of glutathione could render a given dose less toxic, in terms of free-radical damage. Butathione sulfoximine is a specific inhibitor of glutathione synthesis (141). Butathione sulfoximine was found to significantly increase

the radiosensitivity of A2780cp, although it did not completely return the sensitivity to the level of A2780s (105). Thus it would appear that increased glutathione levels could account for some, but not all, of the resistance acquired by A2780cp. Given the increased intracellular glutathione levels that contribute to the relative radioresistance of A2780cp, less dsb induction might be expected. A2780cp however, exhibited identical dsb induction compared with its parent. There are two possible explanations for the observed similarities in dsb induction. Firstly, cells were irradiated in agarose plugs for induction experiments and therefore it is possible that imbedding the cells interfered with the free-radical scavenging ability of glutathione. Other work with AFIGE however, has revealed that irradiating cells in agarose has no effect on dsb induction (63). A more likely explanation is that other free-radical scavengers, such as protein sulphydryls (142), compensate for the reduced glutathione levels in A2780s and obscure any effects of glutathione levels on dsb induction.

It is generally accepted, and has been demonstrated in assays which give absolute estimates, that dsbs are induced linearly with dose (143;144). The dsb induction curves of A2780s and its variant are clearly curvilinear over the entire dose range, whereas it is linear from 0 to 20 Gy. There are several explanations for the overall nonlinear relationship. Embedded cells are irradiated on ice and it is possible, particularly for high doses, that DNA degradation occurs during irradiation. However, experiments were designed so that control (unirradiated) and all treatment plugs received the same time on ice. The FAR values of the control plugs were subtracted from those of the experimental plugs, in order to factor out this effect. Another conceivable reason for the curvilinear fit is that high doses may lead to cross-links which can restrict fragment mobility, thereby reducing the FAR signal. The

alternative and most likely explanation, however, is that the curvilinear relationship reflects changes in the sensitivity of the assay in detecting dsbs, and not a decrease in the production of dsbs themselves.

The induction curve is linear from the 0 to 20 Gy dose range. Since dsbs are produced linearly with dose, the linear region reflects the most sensitive region of the assay, and delivers the most accurate value of FAR. The clonogenic assay, the best available method for assessing intrinsic radiosensitivity, encompasses the dose range extending from 0 to 10 Gy. The clonogenic dose range is within the sensitive region of the AFIGE assay, indicating the potential power of AFIGE as a predictor of inherent radiosensitivity. Furthermore, two-way ANOVA analysis reveals that within the 0 to 10 Gy dose range, FAR increases significantly ($P < 0.0001$) with dose. Further analysis with one way ANOVA and a Newmann-Keuls post test indicates that significant ($P < 0.05$) differences in FAR can be detected when the dose differs by at least 6 Gy. These results demonstrate the sensitivity of AFIGE, as it able to measure dsb induced by doses as low as 6 Gy.

4.2.2 Dsb Rejoining

Dsb rejoining is examined by two approaches in this report. The kinetics of rejoining are analysed by altering the time allowed for repair following a given dose. To study the effect of dose, residual dsbs are investigated by altering the dose while maintaining a constant time for repair.

4.3.2.1 Rejoining Kinetics

The rejoining kinetics curves of A2780s and A2780cp are biphasic, and this is representative of dsb rejoining curves. The typical dsb rejoining curve has an initial fast

component with a half time ($t_{1/2}$) of 20–40 min followed by a slow phase with a $t_{1/2}$ of several hours (87). The AFIGE assay improves sensitivity at the expense of resolution, so for experiments on the A2780 cell lines, dsbs cannot be quantified and half-times cannot be calculated. Nevertheless, it can be estimated from the rejoining curve that the fast phase is complete following 1 h of repair. The fast phase is similar ($P>0.05$) for both cell lines; following 0.5 h of incubation at 37°C A2780s rejoins about 25% of dsbs while A2780cp rejoins 20%. Dsb rejoining is also comparable following 1 h of repair; A2780s had rejoined 33% of dsbs while A2780cp has rejoined 43%. In other studies which use gel electrophoresis techniques to measure repair kinetics in human tumour cell lines, the dsb rejoining rates of the fast component varies widely; 15% to 85% of dsbs are rejoined within the first 30 min of repair time (67;77;91;138). The fast rejoining kinetics of the A2780 cell lines fall within the lower end of this range.

Although the fast component is similar for both A2780s and A2780cp, differences are observed in the slow phase of rejoining. Significant differences ($P<0.01$) are observed following 4 h of repair; only 20% of dsbs are rejoined for the A2780s cell line while 70% are rejoined for A2780cp. Previous observations in the literature report that 67% to 95% of dsbs were rejoined following 4 h of repair time, as measured by gel electrophoresis techniques (67;77;91). The aborted rejoining of dsbs in the parent is rather unusual whereas the slow phase rejoining of the variant is consistent with reported results. Further analyses however, reveal that these differences may be due, in part, to the normalization process. This will be discussed further in a subsequent section.

In a PFGE study of nine human tumour cell lines of various origins, it was found that the $t_{1/2}$ of the slow phase was significantly correlated to intrinsic radiosensitivity (67). This supports the findings presented here on the A2780 cell lines which indicate that significant differences may occur in the slow phase of rejoining. Other studies however, have found rejoining kinetics to be identical between human tumour cell lines and their clones, whether it was examined by PFGE (77), or neutral filter elution (71;91). The comet assay is another method of measuring dsbs, and it was found that the rejoining kinetics of 6 various human tumour cell lines are identical, despite their wide range of intrinsic radiosensitivities (75). Considering the conflicting data between the unnormalized FAR and FAR remaining, the increased dsb rejoining of A2780cp compared to its parent, A2780s (particularly in the slow phase) needs to be substantiated with additional experiments.

It is interesting to note that in the rejoining kinetics experiments, FAR never reaches background levels. This points to the possibility that non-specific DNA degradation is being measured alongside genuine DNA dsbs. It is conceivable that DNA degradation occurs during incubation or processing. DNA degradation by apoptosis, necrosis, and otherwise damaged cells will appear as smaller fragments while radiation-induced damage is manifested as larger fragments, in the Mbp range (88). AFIGE enhances sensitivity at the expense of resolution, and therefore can not distinguish between low and high molecular weight products. In a recent study of Ku80-deficient mouse fibroblasts, it was found that AFIGE was incapable of detecting complete dsb rejoining. However if the clamped homogenous electric field gel electrophoresis technique (which can distinguish between large and small fragments) was used, complete rejoining was observed when the low molecular

weight data was excluded (145). It seems then that the AFIGE technique may be limited by its inability to distinguish between degradatory and dsb damage. This problem could be further confounded by the use of plateau phase cultures. Plateau phase cultures are used in these experiments in order to mimic the crowded, hypoxic tumour environment, as well as reduce the effects of cell cycling and replication forks. However, plateau phase cultures do contain a proportion of dead and/or dying cells, which could lead to increased degradation and potentially disguise the contribution of *bona fide* dsbs to the FAR signal.

4.3.2.2 Residual Damage

The data from the rejoining kinetics experiments suggests that the variant may be capable of greater dsb rejoining when incubated for 4 to 6 hours following irradiation. To examine this in further detail, residual damage experiments were designed in which confluent monolayers were given 0 to 150 Gy doses of HDRI and then incubated for 6 h at 37°C. Despite the differences seen for rejoining kinetics, residual dsb levels were similar for A2780s and its variant A2780cp. No significant ($P>0.05$) differences are seen between the residual damage slopes of A2780s and A2780cp. Only following the highest dose point examined, 150 Gy, are differences in the residual level of dsbs significant ($P=0.02$). These results are surprising, as it is believed that the consequence of an unrejoined dsb is a chromosome aberration (92). Panels of human tumour cell lines have shown differences in the level of residual dsbs (67;74;76;138), and most have correlated these differences to parameters of intrinsic radiosensitivity such as SF_2 (74;138) or β (26). Despite this convincing data, when human tumour cell lines and their variants are examined, no differences in the level of residual damage are observed despite significant differences in

intrinsic radiosensitivity (71;91). These latter findings are consistent with the data on the A2780 cell lines.

It is generally accepted that dsb rejoining is dependent on dose (145), and the data on the A2780 cell lines indicates that residual FAR increases with increasing dose. The literature is divided on the nature of the relationship between residual dsbs and dose. Some studies using neutral filter elution demonstrate a curvilinear dose-effect relationship (84;93), although linear relationships have been demonstrated (83). Gel electrophoresis techniques are also divided as some demonstrate that, for doses from 0 to 50 Gy, the relationship is curvilinear (76;81;146), while others show that, for doses of up to 120 Gy, the residual damage curves are linear (79;147). From figure 3.2.3.1 it is clear that there is a linear relationship between residual dsbs and dose. These results are consistent with other gel studies which use higher doses. From the rejoining kinetic experiments, it is clear that rejoining is essentially complete following 6 h of repair (see figure 3.2.2.2), so any dsbs that remain are irreparable. Dsbs are induced linearly with dose (143;144), and since residual damage experiments permit 6 h of repair time, all dsbs detected are residual (irreparable), and therefore the FAR remaining would be expected to increase linearly with dose.

The slopes of the residual damage curves are very shallow (0.0031 for A2780s and 0.0022 for A2780cp), yet they do differ significantly from zero ($P < 0.0001$). When the residual curve is plotted on the same axes as the induction curve (figure 3.2.3.2), the powerful ability for these cells to rejoin dsbs *in vitro* is illustrated. As these cell lines rejoin such a large proportion of the induced dsbs, it is not surprising that significant differences in residual dsbs can not be discerned until doses above 150 Gy.

That no differences exist in the residual dsbs curves of A2780s and A2780cp opposes the rejoining kinetics data on the same cells. From the rejoining kinetics experiments, it was seen that A2780cp was capable of greater rejoining than its parent when 4 or 6 h of repair time were given. The residual damage experiments allow for 6 h of repair following each dose, so if any differences exist in the slow phase of repair should be reflected in the residual dsb experiments. This is not seen in the rejoining experiments despite the fact that the same experimental conditions were used. Data was normalized differently, and this could explain the seeming contradiction. In the kinetic experiments, the changes in FAR are measured relative to the FAR observed when no repair time is given. By contrast, the residual experiments measure the changes in FAR by subtracting the FAR obtained when no irradiation is given. When the unnormalized data is examined (table 3.2.3.2), there are no differences in either the FAR values of the rejoining kinetic and residual damage experiment, nor the absolute value of FAR between A2780s and A2780cp in the rejoining kinetic experiments following 6 h of repair. Perhaps it is the normalization process which creates significant differences in the kinetic experiments. Indeed, when no repair time is given the mean FAR of A2780s is half that of A2780cp in the kinetic experiments (see table 3.1.2.1). When the FAR is normalized ($\text{normFAR} = \text{FAR}_{t=t} / \text{FAR}_{t=0}$), the initial difference in FAR is superimposed on all following time points, and could create the significant differences observed. This analysis suggests that any observed differences in the kinetics of rejoining may be artifactual, and that no differences exist in the dsb rejoining as measured by AFIGE. Further experiments are needed to resolve this issue, to ensure that the initial ($t=0$) FAR of rejoining kinetic experiments is the same.

4.4 REJOINING FIDELITY

AFIGE analyses were incapable of detecting any differences in the induction or rejoining of dsbs caused by x-irradiation. Although AFIGE is a fast and simple method of measuring changes in the amount of dsbs, it does not examine the quality of induced dsbs nor the accuracy of their repair. The fidelity assay, in addition to assessing rejoining capacity, is capable of evaluating the precision of dsb rejoining. The fidelity assay uses a RE-induced dsb in the pBS phagemid as a model for a dsb induced in genomic DNA by ionizing radiation. Rejoining efficiency and fidelity are assessed by the ability of nuclear protein extracts to ligate an RE-induced dsb in pBS.

The rejoining capacity of nuclear proteins is reflected by the transformation efficiency (TE) of the linear pBS which they were incubated with. Experiments consistently reveal that linearized pBS incubated in the absence of nuclear proteins is capable of being transformed into DH5 α . This is not due to contaminating linear plasmid since prior to incubation, linearized pBS is visualized by gel electrophoresis to ensure complete digestion. All other reports using the cell free fidelity assay observe similar background TE (76;96;102;148). This background transformation could be due to linear plasmid transforming DH5 α , but it is generally accepted that this does not occur (76). Alternatively, this background TE could be a result of rejoining by the *E. coli* cells themselves (Britten, verbal communication).

Fidelity experiments also reveal the potential negative effect of nuclear extracts on the transforming ability of pBS. Oftentimes, linear pBS incubated with extracts actually has a decreased TE compared with linear pBS incubated in the absence of nuclear protein. This

would imply that the nuclear extracts are having a degradatory effect on the pBS. Although such results have not yet been published, this has been observed in other laboratories (148). Nuclear extracts contain endonucleases and exonucleases in addition to ligases, and therefore a potential balance between degradation and ligation exists. In fact, a recent study has shown that the DNA to protein ratio of the fidelity assay affects the type of rejoining products generated and possibly the activities involved (149).

The lowest protein concentration of 0.1 μg yields the most encouraging results for the cell-free fidelity assay. Even with this low concentration of nuclear proteins, the TE of *EcoRI*-cut pBS does not differ significantly from zero. Therefore, nuclear proteins from neither A2780s nor A2780cp rejoin *EcoRI*-cut pBS with an measurable efficiency. *EcoRI*-cut pBS contains a staggered cut with a overhang of 3 bases. The staggered ends of such a dsb are considered “sticky” and therefore easier to ligate than a blunt dsb such as that found in *EcoRV*-cut pBS. In fact, *EcoRI*-cut pBS incubated in the absence of nuclear extracts transforms DH5 α with twice the efficiency of *EcoRV*-cut pBS similarly incubated (data not shown). Given the higher background resulting from *EcoRI*-cut pBS, it would be more difficult to see any effects of the nuclear extracts. Indeed, it has been suggested that the efficiency of rejoining depends on the break site (i.e. cohesiveness of ends, polarity of termini, specific base sequences) (97). It has been shown that blunt-ended dsbs induced in chromatin are more likely to cause chromosome aberrations and cell death compared to staggered lesions (103), and this could explain, in part, the background TE of *EcoRI*-cut pBS. The reason for the greater TE of untreated *EcoRI*-cut pBS is not known and other groups have had success using this RE in the cell-free fidelity assay (96;97;102).

Encouraging results are obtained with *EcoRV*-cut pBS. Incubation with 0.1 µg of nuclear extracts from either A2780s or A2780cp resulted in a significant ($P<0.05$) increase in TE. For *EcoRV*-cut pBS incubated with A2780s a TE of 1.6% is achieved while when incubated with A2780cp extracts the TE is 2.0%, but these differences are not significant ($P>0.05$). Therefore, A2780s and A2780cp extracts are capable of ligating *EcoRV*-digested pBS with similar efficiencies. These results are consistent with those of the residual damage experiments which suggest that no differences exist in dsb rejoining. It is of interest that all dsb rejoining endpoints (kinetics, residual, and TE) suggest that A2780cp is superior, although not all demonstrate significance.

Rejoining fidelity is only assessed for *EcoRV*-cut pBS incubated with 0.1 µg of nuclear extracts. The mean LIF of *EcoRV*-cut pBS incubated with A2780cp extracts is 0.3% compared to 2% for *EcoRV*-cut pBS incubated with A2780s extracts, a significant ($P<0.05$) difference. Therefore, the variant extracts ligate *EcoRV*-cut pBS with greater accuracy than the parent, even though the efficiency of ligation is similar. In fact, the mean LIF of the variant is not significantly ($P>0.05$) different from zero whereas the mean LIF of the parent is different ($P<0.05$). These results are consistent with other published reports which use the cell-free assay (96;97;102) or transfection of tumour cells (95). The ligation step of the fidelity assay can not be separated from the process of integration of linear DNA. Therefore it is possible that rejoining fidelity could be due to differences in the number of integrated DNA (copy number). Even so, it has been observed that copy number is not related to rejoining fidelity, and that the copies of intact vs cut gene mirror the rejoining fidelity (95). Furthermore, although increasing the amount of plasmid transfected leads to

an increase in the amount of integrated plasmid, it had no effect on LIF (91). These experiments confirm that repair fidelity measures accuracy as independent of copy number or the number of repair events.

A criticism of the fidelity assay is that it is too artificial to be relevant. Ionizing radiation produces a non-uniform population of “dirty” dsbs, which may involve different repair pathways than those used by “clean” dsbs induced by REs. Research has shown however, that RE-induced dsbs have similar cytotoxic effects as dsbs induced by ionizing radiation (38;103;150;151). Previous work with this assay has shown that multiple proteins, in addition to the ligases, are required to rejoin RE-induced dsbs (152). The enzymatic activities required for dsb rejoining were compared between an *in vitro* assay based on plasmid DNA and an assay based on genomic DNA. Differences were found in the enzymatic activities, and it was suggested that the plasmid assay probably only detects dsb repair processes complementary to the pathway *in vivo* (153). Regardless, the fidelity assay is capable of detecting non-conservative rejoining pathways (102). When analysed, it was seen that misrepair events were mostly deletions (96;102) and most arose from illegitimate recombination (154). Work with RE-induced dsbs has led to the suggestion that they act as substrates for proteins involved in the abnormal conversion of dsbs into chromosome aberrations (155). Therefore, although the fidelity assay may not be an exact *in vitro* replication of dsb rejoining *in vivo*, it certainly involves complex pathways relevant to cellular survival. Reasons for the improved fidelity of the A2780cp is not known. A2780cp was initially selected for its resistance to cisplatin, and some cisplatin-DNA adducts can be repaired via non-homologous end joining (156). Perhaps the variant’s resistance to cisplatin

and radiation is due to a higher level of homologous recombination or a suppression of non-homologous end joining. This has been examined recently in the A2780 cell lines (102), but more work needs to be done with other tumour cell lines and their clones.

4.5 CONCLUSIONS AND FUTURE WORK

Data from the clonogenic assay, fit with the linear quadratic model, reveal clear differences in the radiosensitivities of the human ovarian carcinoma cell lines, A2780s and A2780cp. The parent, A2780s, is very sensitive to ionizing radiation with a SF_2 of 0.0829 while the variant, A2780cp, is relatively radioresistant with a SF_2 of 0.4277. Such differences in radiosensitivity are most obvious in the lower dose range of the survival curve. Differences in cellular recovery from either SLD or PLD do not explain the differences in intrinsic radiosensitivity between the cell lines. Moreover, both SLD and PLD recovery are clearly elevated in the sensitive parent line. Cellular recovery from SLD and PLD are strikingly similar, particularly for the A2780cp cell lines, suggesting that SLD and PLD are different methods of examining the same process operating on the same lesion. Dsb induction, as measured by AFIGE was identical in the parent and variant cells, despite the elevated glutathione levels previously observed in the variant. The most sensitive region of the induction curve is in the dose range of 0 to 20 Gy. The doses used in the clonogenic assay fall into this range, indicating the relevance of the AFIGE technique in measuring dsb in relation to intrinsic radiosensitivity. Dsb rejoining as measured by AFIGE is analysed in two ways; the kinetics of dsb rejoining and the relationship between residual breaks and dose. Rejoining kinetics reveals that rejoining is essentially complete following 4 h of repair. These experiments also suggest that differences exist in the slow phase of rejoining;

the parent does not appear to rejoin any additional dsb in the slow phase while the variant continues to rejoin dsb up to 4 h of repair time. Further analysis, however, reveals that this observed difference may be an artifact of the normalization process. Experiments which examine residual breaks as a function of dose indicate that no significant differences exist in the residual level of dsbs between A2780s and A2780cp, except at very high doses (150 Gy). The cell free fidelity assay is employed to examine the efficiency and accuracy of dsb ligation. No appreciable ligation of staggered-ended dsbs (induced by *EcoRI*) are observed by nuclear extracts of A2780s nor A2780cp. However, nuclear proteins from both A2780s and A2780 cp are capable of ligating a blunt-ended dsb induced by *EcoRV* with similar efficiency. Thus dsb rejoining, whether measured by AFIGE or the fidelity assay is similar for A2780s and its variant, A2780cp, and cannot explain the observed differences in inherent radiosensitivity between these cell lines. However, when the fidelity of the ligation of blunt-ended dsb is evaluated, it is observed that the resistant variant is capable of more accurate repair than its parent. Furthermore, A2780s exhibits a significant misrepair frequency of 2% whereas A2780cp does not exhibit any appreciable misrepair at all. The differences in the fidelity of dsb rejoining can explain, at least in part, the observed differences in cellular radiosensitivity. To summarize, figure 4.5 illustrates the conclusions drawn from this thesis work.

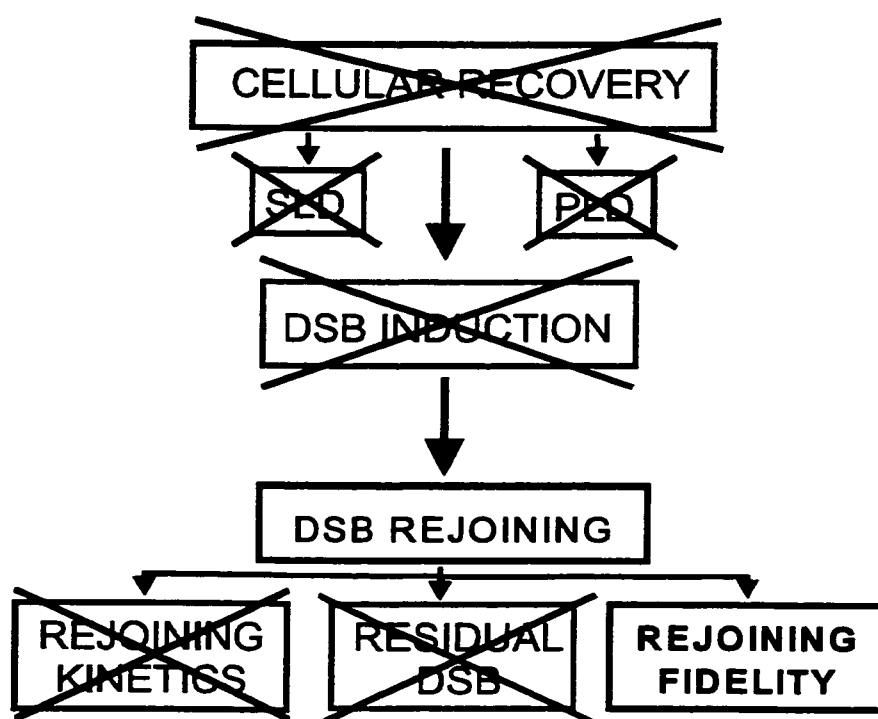


Figure 4.5 Illustration of the conclusions drawn regarding the factors influencing intrinsic radiosensitivity.

If the fidelity of ligation is indicative of intrinsic radiosensitivity, as this work and that of others suggests, then the cell-free fidelity assay holds much promise as a predictive assay of tumour radiosensitivity. The assay itself is relatively easy and fast, once all the contributing elements (nuclear protein concentration, incubation time, etc) are worked out. It would be even more powerful if a method for extracting nuclear proteins directly from a tumour biopsy could be developed. Developing such a technique would eradicate the need for the culturing tumour cells, a most time-consuming and ineffective process. Future work with these cell lines should involve examining the nature of misrepair in A2780s. Polymerase chain reaction (PCR) analysis of incorrectly ligated phagemid DNA from white colonies would reveal the type of event (i.e. deletion or insertion). By knowing the nature of the mis-rejoining event, it may be possible to ascertain the faulty mechanisms involved,

such as illegitimate recombination. Understanding the processes involved in misrepair would contribute to the current knowledge regarding the nature of intrinsic radiosensitivity.

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