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I am submitting herewith a dissertation written by Gloria Ann Greer entitled "The Use Rodent Mutant Cell Lines With Specific Repair Defects for the Study of Aberration Induction and for Identification and Characterization Human Repair Genes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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**The Use of Rodent Mutant Cell Lines With Specific Repair Defects
For The Study Of Aberration Induction and for Identification
And Characterization of Human Repair Genes**

A Dissertation

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Doctor of Philosophy

Degree

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ABSTRACT

In order to study the mechanism of induction of mutations and chromosome aberrations by ionizing radiations, it is particularly useful to have available radiation-sensitive mutants. While several X-ray sensitive rodent cell lines are available, they have been selected rather non-specifically. I determined that selection for resistance to the DNA replication/repair inhibitor cytosine arabinoside (ara-C) would enable me to produce a set of X-ray sensitive mutant cell lines that would be defective in the resynthesis step in excision or recombination repair. Such mutant cells could also be used for the isolation and characterization of human DNA repair genes. In particular, it was predicted that the repair gene defective in individuals with ataxia telangiectasia (AT) might be amenable to study with ara-C resistant (X-ray sensitive) mutants, since additional studies in our laboratory had shown that AT cells were resistant to ara-C. In the long term, it is hoped that determining the specific defect in AT might lead to an understanding of the possible role of defective repair in tumor induction and/or progression.

The general approach used to isolate ara-C resistant CHO cell mutants was to treat cells with EMS and select in increasing concentrations of ara-C. Although several mutants have been isolated, one in particular, AraC^R213, has been subject to the most extensive studies. It was selected largely because it shows the greatest sensitivity to X rays. Survival curves show that AraC^R213 cells are hypersensitive to the killing effect of X rays with an LD₅₀ of 2.5 Gray as compared to the wild-type cells

that had an LD₁₀ of 6 Gray. An analysis of the cell cycle showed that the S-phase is about 2 hours longer in the mutant than in the parental cell line. Further, the mutant showed an increased frequency of X-ray induced chromosomal aberrations in the G₁ and G₂ stages of the cell cycle compared to wild-type frequencies. There was no increase in sister chromatid exchange levels. All of these observations in AraC^R213 are very similar to those made with AT cells in our and other laboratories. Even more importantly, complementation analysis of AraC^R213 x AT hybrid cells indicated that the gene responsible for X-ray sensitivity of AT was also mutated in the mutant cell. Thus, AraC^R213 appears to have a phenotype and probably genotype that is very similar if not exactly the same as those of AT. This makes it quite different from other X-ray sensitive cells that have been isolated in other laboratories.

Procedures are now underway to identify the "repair" gene that appears to be defective in both AT and AraC^R213 cells. The hamster mutant has been transfected with DNA from wild-type human cells by electroporation. Ten primary transformants were isolated that showed wild-type repair after irradiation and one of these was used to generate a secondary transformant. A human-specific oligonucleotide primer was used to PCR amplify *Alu* sequences in the hamster transformants. Southern blot analysis of the PCR product using the human *Alu* fragment from BLUR8 plasmid verified the presence of human *Alu* DNA.

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PROLOGUE

It is rapidly becoming clear that specific genetic alterations in cells are responsible for the development of tumors and the production of many birth defects. These changes for cancer induction or development are chromosome alterations (translocations and deletions), specific mutations in oncogenes that can alter expression or activity of the product, and changes of expression in normal cellular genes through the activity of transcription factors. There are human genetic disorders that have mutations that alter cellular processes, specifically, processes in repair and/or replication, that can increase the probability of chromosome alterations and gene mutations, and thus present the probability of increasing the risk of tumor development. One such genetic disorder is ataxia telangiectasia (AT). AT individuals are radiation sensitive and have an increased risk of developing cancer. AT is estimated to affect one in 40,000 individuals (Sedgwick and Boder, 1972). The lifetime risk of an AT patient of developing cancer is estimated to be 10 to 20% (Gatti and Good, 1971; Spector *et al.*, 1982). Of 57 autopsy reports of AT sufferers, 21% of the deaths were due to malignancy alone, 47% to pulmonary disease alone, and 27% to a combination of both (Boder, 1985). The families of AT patients also show an increased risk of developing cancer. With an estimated heterozygote frequency of 1.4%, it has been calculated that as many as 5% of all cancer deaths before the age of 45 occur in AT heterozygotes (Swift *et al.*, 1976, 1987). Specific cell types in AT

individuals are radiation sensitive for the induction of chromosome aberrations, cell killing and mutations (Taylor *et al.*, 1975; Cox *et al.*, 1986; Cox and Masson, 1980). To determine the possible relationship between the increased radiation sensitivity for these end-points and neoplastic transformation, it is necessary to determine the specific defect leading to radiosensitivity and its importance in the induction of various genetic end-points. Such studies will necessarily involve an understanding of the mechanism of induction of chromosome aberrations and mutation by radiation, and also the development of a mammalian cell model system for attempting to isolate and characterize the DNA repair gene that is probably defective in AT cells. The production of such a model system needs to be made in a directed way, i.e. to select mutants for a specific cellular function, and to represent a genotypic alteration which would have the same effect as the deficiency seen in AT cells. The purpose of the studies described in this thesis is to select such a mammalian cell, AT-equivalent mutant, with an emphasis on how this cell line can aid in an understanding of aberration induction and in isolation and characterization of the human DNA repair gene that is altered in AT cells.

Ionizing radiation is a ubiquitous environmental hazard. Different radiation types deposit their energy in the medium through which they pass at different rates. The rate is characterized as the linear energy transfer (LET) of the radiation. The damage to DNA in mammalian cells is generally considered to arise from two sources; the direct effect (damage originates from energy deposited in the DNA molecule) and an indirect effect (damage is induced in the DNA by reactive species produced by ionizations in other molecules, particularly water). With low-LET radiation, the direct

effect results in single-strand breaks and double-strand breaks in the DNA, and the indirect effects are largely base and sugar damages. The indirect effects are dominant for low-LET radiations, whereas with high-LET double-strand breaks are greatly increased (for a review, see Ward, 1988).

The majority of single strand breaks are rapidly repaired at the rate of 50% within 2 minutes (Van der Schans *et al.*, 1983). Directly induced double-strand breaks are the result of one or two ionization tracks (either two single-strand breaks less than five bases apart or a single track through both strands of the DNA). DNA double-strand breaks can also be induced indirectly by the action of a single-strand endonuclease at the site of a single-strand gap (Natarajan and Obe, 1978).

Base damages are the preponderant lesion produced by ionizing radiation (for a review, see Cerutti, 1976; Wallace, 1983). In Figure I a schematic diagram of the various DNA damages induced by X rays is depicted. The loss of a base (purine or pyrimidine) will result in an alkali labile lesion. At the apyrimidinic site the sugar ring is opened and the pyrimidine base is missing. This type of damage is probably produced by hydroxyl radical attack on the C-2' of the deoxyribose moiety (Isildar *et al.*, 1981). The other abasic site shown is the apurinic site, which has an intact sugar, is produced by hydroxyl radical attack on the base itself (Isildar *et al.*, 1981). Two radiolysis products of thymine are also shown (thymine glycol and urea). The information on other DNA bases modified by radiation is scarce, but examples of cytosine and guanine damage are discussed in Ward (1975).

The first reported evidence that X rays induced structural chromosomal aberrations was made by Muller (1927) based on genetic changes in *Drosophila* after

exposure to X rays. Shortly afterwards, Painter and Muller (1929) confirmed these findings using cytogenetic methods. By the late 1930s and early 1940s cytogenetics had become accepted as an experimental science and during this time Karl Sax described the induction of chromosome aberrations in *Tradescantia* microspores by X rays (Sax, 1938).

In the next few years, Sax and his colleagues determined the chromosome aberration dose-response curves for X rays and neutrons. From these studies the "breakage first" hypothesis was developed for the origin of X-ray-induced chromosomal aberrations (Sax, 1941). The hypothesis was defined in terms of one-hit or two-hit kinetics, now more appropriately described as one-track or two-track. The initial event was presumed to be the breakage of chromosomes with subsequent rejoining of the broken ends leading to the different classes of chromosome aberrations observed. Other, particularly significant concepts arising from this early work were: firstly, that the lesions involved in the production of chromosome aberrations were capable of being "repaired" and, secondly, that the types of aberrations and their frequencies were dependent upon the cell cycle stage irradiated. Chromosome-type aberrations were induced prior to DNA synthesis and chromatid-type during or after replication.

Several years later, Revell (1955) proposed his exchange hypothesis for the origin of chromosomal aberrations, based upon his careful studies of dose-response curves for X-ray-induced chromatid-type aberrations. His model proposed that all aberrations (not just interchanges) were the consequence of an inter- or intrachromosomal exchange, with two "lesions" in the DNA being necessary to initiate

the exchange, and if the rejoining of the resulting "breaks" was not completed, a deletion would result.

Although both of these hypotheses are still under debate, it would seem to be reasonable to conclude that some proportion of aberrations, including chromatid and isochromatid deletions and chromosome-type terminal deletions, are formed by an exchange process, and a proportion are formed by a breakage-first mechanism. It was not until 1964 that the process by which DNA could be repaired was described (Setlow and Carrier, 1964; Pettijohn and Hanawalt, 1964). It is now generally accepted that damage to cellular DNA provokes the repair machinery of the cell. The primary lesions are either directly repaired or converted to secondary lesions which in turn may be repaired to the original status or may lead to chromosomal aberrations and /or gene mutations, by misrepair. In addition, if the lesions are left unrepaired at the time of replication, misreplication can result (Evans, 1977). That is to say, that a chromosomal aberration or a gene mutation is the result of a mistake in cellular repair mechanisms or a consequence of damage remaining unrepaired.

This significant realization gave specific direction for future studies of the mechanism of aberration production. A model for the repair of double-strand breaks involving recombination was proposed by which a double-strand gap could be repaired by two rounds of single-stranded repair synthesis (Resnick, 1976; Szostak *et al.*, 1983). There is recent evidence that the most likely way by which specific exchange aberrations could be induced is by a recombination-like process (Hastie and Allshire, 1989; Vernick *et al.*, 1988; Wahls *et al.*, 1990). Suffice it to say, damages can be repaired to apparently normal products, however misrepair or misreplication

can occur with a very low probability, resulting in chromosome aberrations and mutations. Misrepair can occur at more than one step but, in general, for double-strand breaks it occurs during ligation, either directly or indirectly by a recombination process, and with base damages during the resynthesis step of excision repair. Because evidence in the literature supported the suggestion that repair of base damage was defective in AT whereas, double-strand break repair was normal (Lehmann, 1982; Cox *et al.*, 1986) the emphasis in these studies was on the resynthesis step of excision repair for the selection of mutants.

The cell lines most commonly used for selection of rodent cell lines to be used as models for human syndromes are Chinese hamster for several reasons. Large areas of the hamster genome appear to be functionally haploid, which greatly increases the chances of isolating a mutant with a recessive mutation (Hickson and Harris, 1988). Hamster cells grow well in culture and plating efficiencies are high. Transformation by DNA of mutant hamster cells is more efficient due to the high uptake of exogenous DNA compared with human cell lines. Most importantly, fusions of hamster and human cells suggests a similarity of DNA repair processes in the two species.

The repair inhibitor used most extensively in this laboratory is 1- β -D-furanosylcytosine arabinoside (ara-C). Ara-C is a base analog of cytosine (Figure II) which inhibits DNA polymerases α (Furth and Cohen, 1968; Momparler, 1969; Graham and Whitmore, 1970) and β (Dunn and Regan 1979; Yoshida *et al.*, 1977; Ohno *et al.*, 1988). There is no available information regarding the effects of this agent on DNA polymerase δ . Experimental results obtained using this inhibitor have shown a good

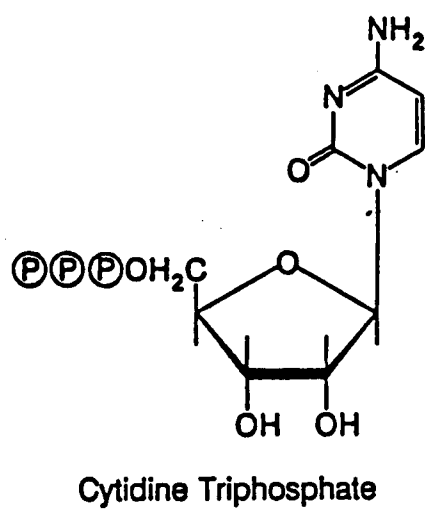
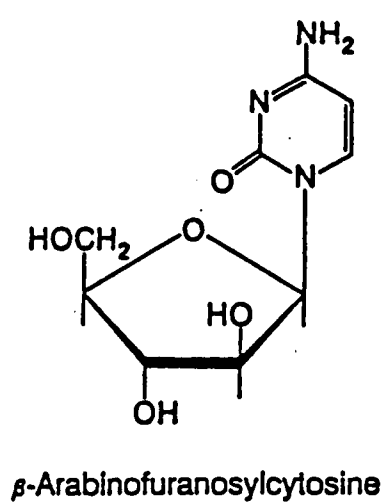


Figure II. Structure of ara-C, an analog of cytidine.

correlation between the effect of ara-C (as a repair inhibitor) in normal cells and the intrinsic defect in AT cells. Therefore, we reasoned that a mutant (CHO) isolated as resistant to the inhibitor might prove to be X-ray sensitive and would therefore have characteristics of AT cells. In addition, these mutants could be used for the identification and characterization of human DNA repair genes. The following chapters present a logical series of studies that address these several hypotheses. More specific details of DNA repair processes and their role in the production of chromosome aberrations and background information pertinent to the studies can be found in the Introduction and Discussion sections of each chapter.

CHAPTER 1

MUTANT ISOLATION AND CHARACTERIZATION

Summary

Twelve repair defective, Chinese hamster ovary cell mutants were selected as being resistant to ara-C, an inhibitor of DNA replication and repair synthesis. These mutants were also, as predicted, hypersensitive to X rays. They showed a range of sensitivities to X-ray-induced cell killing (LD_{10} of 2.4 Gy to LD_{10} of 4.5 Gy compared to wild-type cells with an LD_{10} of 6 Gy). The general approach taken for isolation of the resistant cell lines was to mutagenize CHO cells with EMS and then select in $5 \times 10^{-5}M$ ara-C. Ara2.1, one of the original cell lines, was mutagenized a second time and incubated in $10^{-3}M$ ara-C to select the mutant AraC^R213, which proved to be the most X-ray sensitive. When the mutant cell lines Ara1, Ara2.1, and AraC^R213 were treated for 3 hours post X-irradiation with ara-C, no effect on survival was observed, while the wild-type CHO cells showed a decrease in survival due to the inhibition of repair. The repair pathway normally inhibitable by ara-C is no longer functional in the mutant cells.

The Ara1 mutant cell line appears to be phenotypically similar, in part, to Bloom Syndrome cells exhibiting a high frequency of background sister chromatid exchanges (24.9 per cell compared to the wild-type with 13.7 per cell). This cell line also showed a sensitivity to BrdU which consequently resulted in a delay of the cell cycle and an increased frequency of tetraploid cells.

Ara2.1 and AraC^R213 cell lines share features in common with the human syndrome AT, with respect to X-ray sensitivity, the frequency of sister chromatid exchanges, and an extended S-phase of the cell cycle.

Introduction

Mechanisms of the repair of X-ray-induced DNA damage may be resolved through detailed studies of cellular mutant phenotypes that exhibit increased sensitivity to radiation. One such mutant is found in human cells from patients suffering from AT. Studies of fibroblast cells derived from several AT patients have identified a hypersensitivity to the effects of ionizing radiation due to putative defects in repair (Taylor *et al.*, 1975). This aspect of the disease in conjunction with the high incidence of associated neoplasia (Morrell *et al.*, 1986) makes it a model system that can be used to study the mechanisms of repair and, further, the possible role of misrepair in the induction of cancer.

Attempts have been made to isolate rodent X-ray sensitive mutants with the same genetic defect as AT (Jeggo and Kemp, 1983; Stamato, *et al.*, 1983; Schultz, *et al.*, 1981; Giaccia, *et al.*, 1985; Robson, *et al.*, 1985; Zdzienicka and Simons, 1987;

Jones, *et al.*, 1987; Fuller and Painter, 1988), but to date none have been particularly successful.

We took a different approach based on earlier experiments performed in our laboratory. In the past few years a considerable amount of knowledge of the mechanisms of DNA repair has been obtained by the use of repair inhibitors. Cytosine arabinoside (ara-C), an inhibitor of repair resynthesis and DNA replication (Fram and Kufe, 1985), has been used extensively in our laboratory to investigate the mechanisms of induction of chromosome aberrations by radiation and chemicals (Preston, 1980, 1981, 1982a, 1982b, 1983). In addition, previous experiments had shown that although AT cells had the observed increase in X-ray sensitivity to cell killing with X rays alone, when ara-C was used as a repair inhibitor in conjunction with X rays, there was no additional increase in cell killing. In contrast, a large increase in cell killing was observed with the normal cells with post-irradiation incubation in ara-C compared to X rays alone (Greer *et al.*, 1991). Also, AT cells were shown to be resistant to the effect of ara-C on the frequency of X-ray-induced chromosome aberrations in G₂ cells. These observations allowed us to propose a procedure for the isolation of a repair deficient CHO mutant (*i.e.* X-ray sensitive) that could be used as a model for the study of AT. The hypothesis was that if a CHO cell was selected as ara-C resistant and was also X-ray sensitive, it could potentially be deficient in the same repair function as AT cells.

In this chapter a description of the method used to isolate 12 ara-C resistant, X-ray sensitive mutants will be presented. Three of these mutants have been further characterized for sister chromatid exchange levels and the length of cell cycle phases.

The role these mutants might play in the study of human repair syndromes will be discussed.

Materials and Methods

Cell Culture Conditions

Chinese hamster ovary cells (CHO-K1) and ara-C resistant mutant cell lines were maintained in Ham's F12 (GIBCO) supplemented with 5% heat-inactivated (56°C, 55 minutes) fetal bovine serum (FBS, Hazelton), 50 units/ml penicillin (Squibb), and 50ug/ml streptomycin (Lilly). The mutants were grown intermittently in ara-C at the concentration at which they were selected. Cells were maintained as monolayers at 37°C in 5% CO₂ at 95-100% humidity.

Mutant Isolation

The initial mutant cell lines, Ara1 and Ara2, were isolated from a mutagenized population of CHO cells. CHO cells (5×10^5 cells/100 mm plate) were plated 24 hours prior to treatment with 125µg/ml methanesulfonic acid ethyl ester (EMS, Sigma) for 3 hours in serum free Ham's F12 medium. The cells were rinsed 3 times with Hank's balanced salt solution (BSS). The mutagenized cells were subcultured every two days for 1 week to allow expression of the mutation. A concentration of 5×10^{-5} M ara-C (Calbiochem) was used for selection of resistant mutants. Colonies were isolated approximately 10 days later.

A second group of ara-C resistant mutants was selected from the X3/5^{hprt} cell line, (a CHO hprt deletion mutant cell line with wild-type repair kinetics). The cells were mutagenized with 250-400 $\mu\text{g/ml}$ of EMS and selected at concentrations of ara-C varying from $5 \times 10^{-5}\text{M}$ to 10^{-4}M in an attempt to isolate a set of mutants with a broader range of X-ray sensitivities. Five ara-C resistant cell lines were isolated.

A third set of mutants was generated by mutagenizing Ara2.1, a single cell clone from the population of Ara2 cells, a second time with 200 $\mu\text{g/ml}$ of EMS. The cells were grown for 1 week as before and then mutants were selected at a higher concentration of ara-C (10^{-3}M). Five colonies were isolated.

Survival curves

Four hours prior to X-ray exposure, cells were trypsinized, counted (Coulter counter) and plated (three 60mm plates per treatment). The number of cells (CHO or mutants) plated per dose was estimated such that 100 cells per plate would form colonies after X-irradiation. For those survival curves in which ara-C was used in conjunction with X-rays, $5 \times 10^{-5}\text{M}$ ara-C was added to the plates 30 minutes prior to irradiation. Three hours post-irradiation all plates were rinsed 3 times with Hank's BSS and 10^{-4}M deoxycytidine (dC, Sigma) added to reverse the inhibitory action of ara-C.

After 7 days incubation, colonies containing more than 50 cells were scored as the progeny of surviving cells. Relative cell survival was calculated as the ratio of number of colonies to the number of cells plated, corrected for the survival (plating efficiency) in control, non-irradiated cultures.

X-irradiations

X-irradiations were with a 320-kVp Gemini Industrial X-Ray Unit operated at 250 kV and 12 mA with 1mm aluminum filtration at a dose rate of 1 Gy per minute.

Cell Cycle Evaluation

Actively dividing, asynchronous cells (plated 24 hours prior to each experiment) were incubated with ^3H -thymidine (1.9 Ci/mmol, 1 $\mu\text{Ci/ml}$, New England Nuclear) for 30 minutes. Cells were fixed (Korthor, 1986) at 2 hour intervals for 20 hours and dropped onto clean microscope slides. Slides were prepared for autoradiography by dipping them in emulsion (NTB2) and keeping them in the dark for 5 days. Slides were developed and the percentage of labeled metaphases recorded at each time-point.

Sister Chromatid Exchanges

CHO cells (5×10^4 cells/100mm plate), Ara1, Ara2, and AraC^R213 cells (all at 10^5 cells/100mm plate) were incubated in 10 μM BrdU for about 2 cell cycles: CHO for 30 hours; Ara1 and Ara2.1 for 34; and AraC^R213 for 30 hours. Metaphase cells were fixed, dropped onto slides, and two days later stained by a modified FPG (fluorescence plus giemsa) technique (Perry and Wolff, 1974) as follows. Slides were stained with 1 $\mu\text{g/ml}$ of Hoechst 33258 stain for 30 minutes, rinsed in distilled water and then exposed to black light in McIlvaines buffer for 30 minutes at 56°C. Differential staining was done with 3% Giemsa.

Results

Survival Curves

Ara1 and Ara2 were the first two ara-C ($5 \times 10^{-5} \text{M}$) resistant mutants isolated from an EMS mutagenized population of CHO cells. The mutant cell line Ara2 will be hereafter referred to as Ara2.1 (a single cell clone picked from the Ara2 population). As shown by the survival curves, Ara1 and Ara2.1 were both found to be X-ray hypersensitive: Ara1 had an LD_{10} of 4 Gy and Ara2.1 had an LD_{10} of 3.5 Gy compared to an LD_{10} of parental CHO cells of approximately 6 Gy (Figure 1.1).

Another set of mutants was isolated from the parental X 3/5^{hgprt} cell line (wild-type X-ray sensitivity), since a mutant with a greater sensitivity would be more useful in later experiments. This time the mutants were selected at higher concentrations of ara-C, but the mutants isolated in 10^{-4}M ara-C (no surviving cells at higher concentrations) were no more sensitive to X rays than the ones isolated at $5 \times 10^{-5} \text{M}$. The range of sensitivities of the mutants, AraX4, AraX5, AraX7, AraX8, and AraX9 are shown in Figure 1.1. The most sensitive mutant isolated was AraX8 with an LD_{10} of 3.2 Gy.

It was then decided that a step-wise selection approach might be more successful in isolating a mutant with a greater X-ray sensitivity. Therefore, one of the original ara-C resistant mutants, Ara2.1, was mutagenized a second time and resistant colonies were selected with a 20-fold higher concentration of ara-C (10^{-3}M). Of the five surviving colonies, AraC^R211, AraC^R212, AraC^R213, AraC^R214, and AraC^R215, one (AraC^R213) was found to be more sensitive to cell killing by X rays than Ara2.1. This

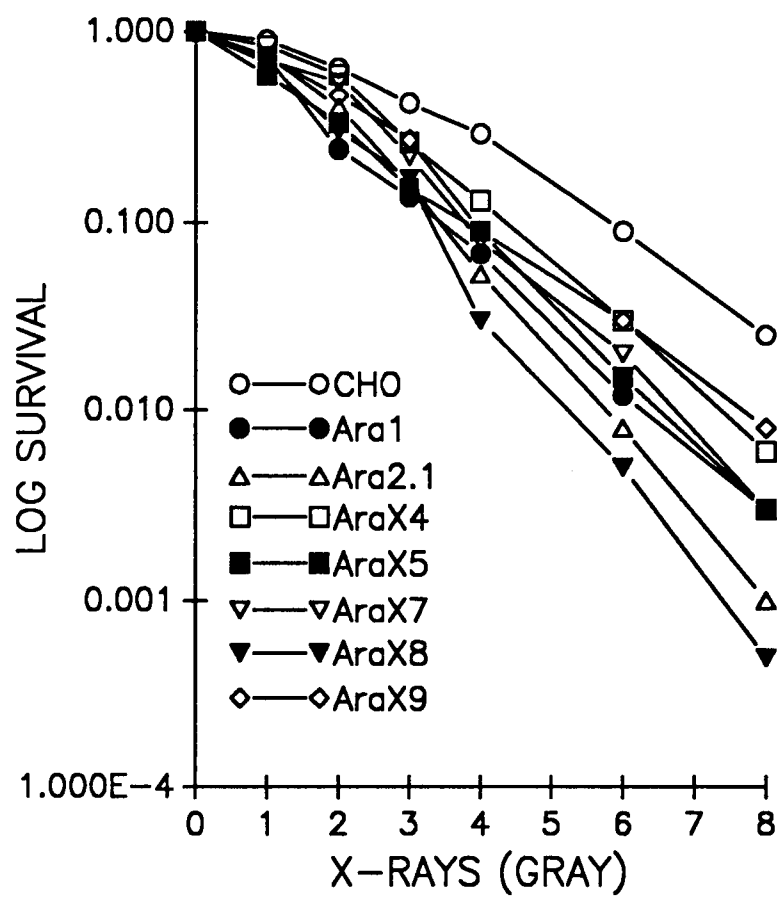


Figure 1.1. Survival curves showing the range of X-ray sensitivities of the mutants isolated from the parental CHO cell line, (Ara1 and Ara2.1) and from the X3/5^{hprt} cell line (AraX4, AraX5, AraX7, AraX8, and AraX9).

mutant shows a greater than 2-fold increase in X-ray sensitivity (LD_{10} of 2.5 Gy) compared to wild-type cells (Figure 1.2). In addition a set of survival curves are shown in Figure 1.3 generated using ara-C as a repair inhibitor of X-ray-induced damage. When the mutant cell lines tested (Ara1, Ara2.1 and AraC^R213) were incubated in 5×10^{-5} M ara-C post-irradiation for 3 hours followed by the addition of dC (10^{-4} M) to reverse ara-C inhibitory effects, there was no change in the number of surviving cells compared to those X-irradiated alone (Figure 1.3). In contrast, when the wild-type CHO cells were incubated in ara-C post-irradiation, the LD_{10} was 2.5 Gy (the same LD_{10} as AraC^R213 with or without ara-C). The repair function normally inhibitable by ara-C in wild-type cells appears to be no longer functional in the ara-C resistant mutant.

Sister Chromatid Exchanges

The level of background SCEs in Ara1, Ara2.1 and AraC^R213 is presented below. Two cell cycles in the presence of BrdU are necessary for differential staining of sister chromatids. The chromatid with two substituted strands will stain lightly and the chromatid with one substituted strand and one strand containing only thymidine will stain darkly.

Ara1 showed a significant increase in SCEs with 24.9 per cell as compared to 13.7 per cell in the CHO wild-type cells. The frequency of SCEs in Ara2.1 and AraC^R213 cell lines was comparable to that of wild-type CHO cells.

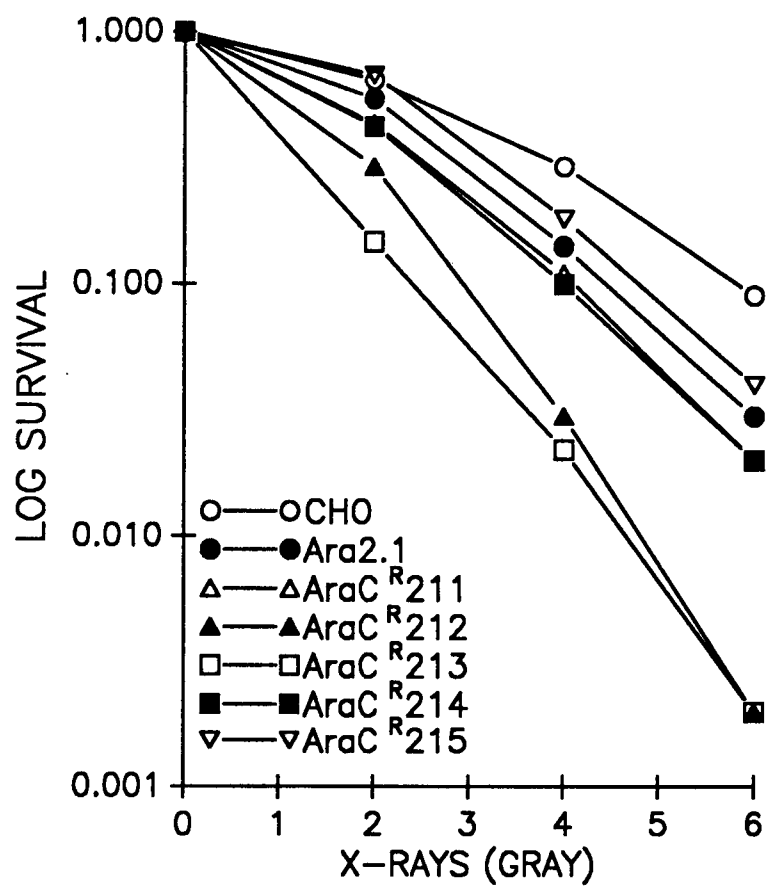


Figure 1.2. Survival curves of mutant cell lines isolated from mutagenized Ara2.1 cells: AraC^R213 was the most sensitive cell line isolated.

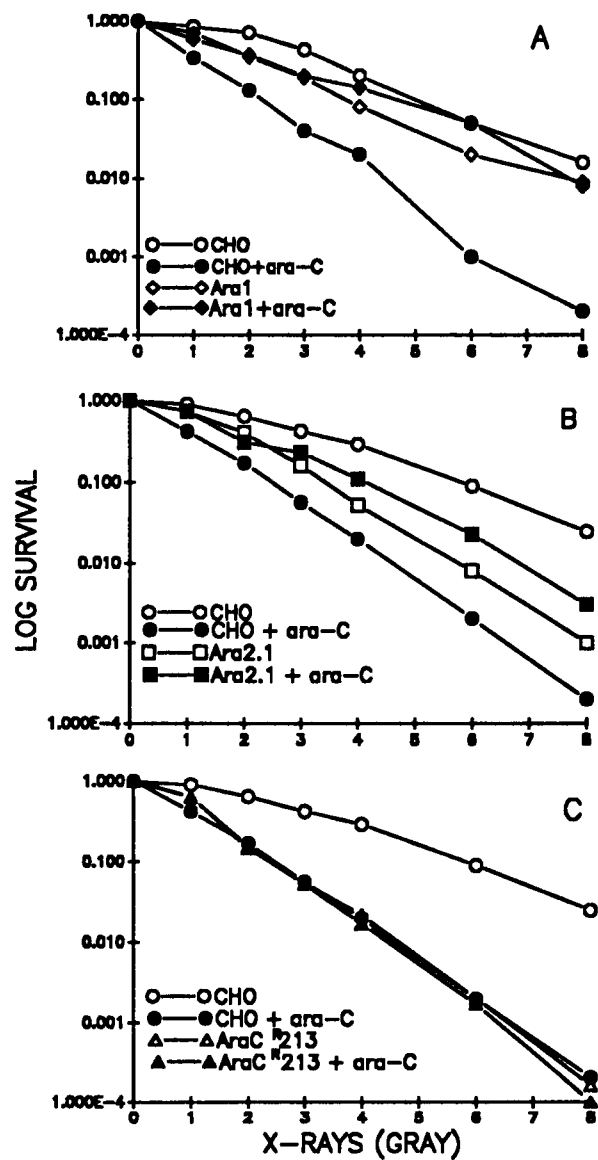


Figure 1.3. Survival curves for X-irradiated cell lines with and without the presence of ara-C.

Background Sister Chromatid Exchanges

Cell Type	Time Grown In	SCE/Cell
	10 μ M BrdU	
CHO	30 hours	13.7 $\pm$ 5.2
Ara1	34 hours	24.9 $\pm$ 6.9
Ara2.1	34 hours	14.2 $\pm$ 3.8
AraC ^R 213	30 hours	12.4 $\pm$ 4.1

At the selected sampling time, there were very few second divisions in the mutant cell line Ara1. Since this raised the possibility that the mutant may be BrdU sensitive, an analysis was performed of first and second divisions. The first division metaphases are uniformly stained dark because each chromatid has one BrdU substituted strand and one original thymidine-containing strand. As described above, the second divisions will appear as one dark chromatid and one light chromatid. After a third replication in the presence of BrdU, theoretically, 50% should be differentially stained, in fact less than half usually are. After 30 hours in BrdU, the CHO cells were 9% first divisions, 79% seconds, and 12% thirds. The mutant cell line, Ara2.1, after 34 hours incubation revealed 1% first divisions, 54% seconds, and 45% thirds. But Ara1 had 46% first divisions and 54% seconds with no third divisions. This would represent a cell cycle time of approximately 23 hours, which is not consistent with the estimated doubling time of 17 hours. It is also notable that 40% of the Ara1 cells examined were tetraploid after incubation in BrdU, and only 9% tetraploids were counted in metaphase preparations of mutant cells in the absence of BrdU. The frequency in the parental CHO cells was less than 2%. The frequency of

chromosomal aberrations was also much higher in this mutant after incubation in BrdU than in normal CHO cells.

Cell Cycle Evaluation

The length of each phase of the cell cycle was calculated by counting labeled metaphases for 20 hours after a 30 minute incubation in ^3H -thymidine. The number of labeled metaphases increased as the cells that were in S-phase at the time of incubation in ^3H -thymidine progress through the cell cycle into mitosis. The length of G_2 was estimated from time 0 hour to the amount of time required for 50% of the metaphases to be labeled. As the percentage of labeled metaphases continued to increase, S-phase was estimated as the width of the % labeling curve at the 50% levels. The time from 50% labeling on the descending portion of the curve until the labeled cells started a second round through the cell cycle was taken as the length of G_1 and mitosis combined.

Examination of the curves for percent labeled mitoses showed the phases of the cell cycle to be:

CHO	Ara1	Ara2.1
2.5 hour G_2	2.5 hour G_2	2 hour G_2
7 hour S-phase	9 hour S-phase	9 hour S-phase
5 hour G_1 /mitosis	3 hour G_1 /mitosis	4 hour G_1 /mitosis

The mutants, Ara1 and Ara2.1, exhibit an extended S-phase as compared to the wild-type parental CHO (Figure 1.4). The time required for progression through the cell

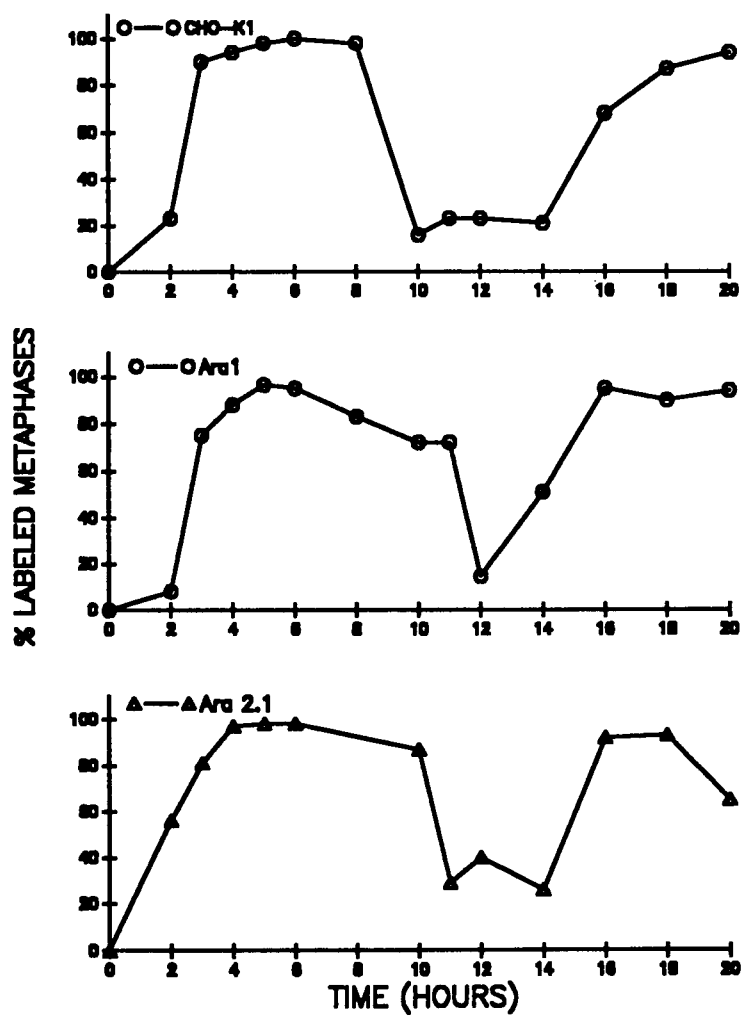


Figure 1.4. Percent of labeled metaphases seen in the parental CHO and the two ara-C resistant mutants, Ara1 and Ara2.1.

cycle was approximately the same in the mutant cells and the parental CHO cells (15 hours). The estimated 23 hour cell cycle time of mutant Ara1 seen in the SCE experiment was caused by a delay due to BrdU sensitivity. Ara2.1 showed a slightly faster progression into S-phase, but the duration of S-phase was about 2.5 hours longer than that for wild-type cells.

Discussion

The results presented above for the ara-C resistant mutants indicate that they are very useful cell lines for studying the mechanisms of induction of chromosome aberrations by radiation, for characterizing the basis for radiation sensitivity, and for the isolation of human DNA repair genes.

The first point of interest is the method of selection of ara-C resistant, X-ray sensitive mutants. The majority of X-ray sensitive mutant cell lines have been established by replica plating and repeated exposure to radiation. Isolating an X-ray sensitive mutant by this type of screening is very laborious. Jones *et al.*, (1987) screened 5000 surviving clones for X-ray sensitivity using the microwell technique and successfully isolated 4 sensitive mutants: *irs1*, *irs2*, *irs3*, and *irs4*. The replica-plating technique was used to isolate the *xrs1* to *xrs7* cell lines (Jeggo and Kemp 1983), the XR-1 line (Stamato *et al.*, 1983), the *irs1SF* line (Fuller and Painter, 1988), the V-G8 line (Zdzienicka *et al.*, 1987) and, the most recently isolated X-ray sensitive mutant, XR-V15B (Zdzienicka *et al.*, 1989). Mutants defective in repair of X-ray damage have been isolated on the basis of sensitivity to chemicals. The EM9 cell line was isolated

on the basis of hypersensitivity to the killing effect of ethyl methanesulfonate (EMS). Thompson *et al.*, (1982) and Robson *et al.*, (1985) used bleomycin hypersensitivity to isolate the BLM-2 X-ray sensitive strain. Again, replica-plating was required for screening. This is a time-consuming method, that results in the selection of a rather non-specific mutant; the phenotype is simply radiation sensitivity. From previous work in our laboratory (Hiss and Preston, 1977; Preston, 1980; Heartlein and Preston, 1985a; Heartlein and Preston, 1985b), we had established that ara-C inhibits the resynthesis step in the excision repair of X-ray-induced base damages. Although the precise mechanism responsible for this inhibitory effect is not yet known, a considerable amount of data has been directed toward understanding the mechanism of its therapeutic and cytotoxic effects. After ara-C is transported across the cell membrane, it must be phosphorylated to 5'-monophosphate by deoxycytidine kinase (Plagemann *et al.*, 1978). Ara-CMP is then sequentially phosphorylated to ara-CTP (the cytotoxic form) by pyrimidine nucleoside monophosphate kinase and nucleoside diphosphokinase. Ara-CTP is a potent inhibitor of mammalian DNA polymerases α (Graham and Whitmore, 1970) and β (Yoshida *et al.*, 1977). Initial studies emphasized that the competitive inhibition of polymerase α by ara-CTP at the dCTP binding site could inhibit replication (Furth and Cohen, 1968; Momparler, 1969). Recent studies have demonstrated that ara-C is a weak competitive inhibitor of this enzyme and that the observed cytotoxic effects of ara-C are better explained by the consequences of incorporation of ara-C into the DNA (Kufe *et al.*, 1980). In experiments using the pH-step alkaline elution technique, Ross *et al.*, (1990) isolated and quantitated nDNA replication intermediates (Okazaki fragments, short length nDNA from replicon origins,

longer lengths but subgenomic, and full length nDNA) to look at the effects of ara-C on replication in the HL-60 human promyeloblast cell line. In agreement with previously published data (Ohno *et al.*, 1988) they found that incorporation of ara-C into the DNA caused marked inhibition of chain elongation rather than absolute chain termination. Additional information was provided by Ohno *et al.*, (1988) from studies in which M13 phage DNA templates of defined sequences (single and contiguous dGMP residues that would code for the incorporation of ara-CMP) were used to study the effects of ara-CTP on purified DNA polymerase β . The results demonstrated that DNA strand elongation was progressively slowed at sequences of two, three and four contiguous sites of cytosine incorporation, i.e. the chain terminating effects of ara-C are sequence specific. They also confirmed experiments of Hiss and Preston (1977) that the inhibitory effects of ara-C were reversed by competition with deoxycytidine-triphosphate for incorporation into the DNA. It has been shown that ara-C is incorporated into DNA undergoing repair resynthesis (Kufe *et al.*, 1984). Thus, an ara-C residue incorporated into the repair patch could act, as it does in the inhibition of normal DNA replication, as a relative chain terminator. Kufe *et al.*, (1984) further suggested that another mechanism of ara-C inhibition is by incorporation of ara-CMP into the DNA, after which the polymerase will be forced to stutter at this site eventually leading to sequestration of repair enzymes making them unavailable to perform functions at other damaged sites.

It seemed quite plausible, therefore, that if cells were selected for ara-C resistance they would be mutant for one of the polymerases and would be X-ray sensitive as a consequence of a defective excision repair process, either in fidelity or

rate. This was borne out by the fact that of the 12 ara-C resistant mutants that have been partially characterized all were X-ray sensitive, albeit to different extents. The major advantages of this approach are that a positive selection system is used, and hopefully a set of mutants with a specific defect can be obtained. We have yet to establish this latter point.

The spectrum of X-ray sensitivities ranges from an LD₁₀ of 2.4 to an LD₁₀ of 4.74 (parental LD₁₀ is 6 Gy). The most sensitive mutant isolated, AraC^R213, shows a 20-fold increase in X-ray sensitivity as compared to wild-type cells at a dose of 8 Gy. X-ray sensitive mutants described in the literature show sensitivities that range from the extreme with an LD₁₀ of 1 Gy in the XR-V15B cell line (Zdzienicka *et al.*, 1989), an LD₁₀ of 1.5 Gy of the XR-1 cell line (Stamato *et al.*, 1983), an LD₁₀ of 1.5 in Jeggo's (1982) *xrs6* line, and an LD₁₀ of 2.25 Gy in the *irs1SF* cell line (Fuller and Painter, 1988), to the less extreme of cell lines *irs1*, *irs2*, *irs3*, and *irs4* with LD₁₀s of 3 to 4 Gy and the V-G8 line with an LD₁₀ of 3.25 Gy (Zdzienicka *et al.*, 1987). The degree of sensitivity is not the major point to be considered. We propose the important issue to be the rather non-specific approach taken to isolate the mutants. This point is demonstrated by the fact that *irs1*, *irs2*, *irs3*, *xrs1*, EM7, and XR-1 have all been shown to belong to different complementation groups (Jones *et al.*, 1988).

The data on cell killing by X rays in the presence or absence of ara-C for the CHO parent cells and the mutant cell line, AraC^R213 (the mutant most similar to AT at this point), can be interpreted in terms of the X-ray sensitivity and ara-C resistance of the mutant. AraC^R213 is considerably more sensitive to cell killing by X-irradiation than the parental CHO cell line. However, when CHO cells were incubated with ara-C

during and after irradiation, they were as sensitive as AraC^R213 showing that inhibition of DNA repair by ara-C "converts" the wild-type cells into the equivalent of the AraC^R213 cells. In contrast, incubating AraC^R213 cells with ara-C during and after X-ray exposure does not cause any change in the sensitivity to cell killing. This suggests that the repair process normally inhibited by ara-C is indeed insensitive to the inhibitor in the mutant cell line indicating a mutation in a step in excision repair, most likely resynthesis.

It is important to determine the specific defect in the ara-C resistant mutants and how this relates to the increased X-ray sensitivity of the cells. The first experiment to address this question was SCE analysis of the mutant cell lines to examine the fidelity of replication. The frequency of background SCEs in Ara1 showed a significant increase when compared to wild-type cells. From what is known about the mechanism of induction of SCEs, it would appear that Ara1 cells are less proficient (in terms of rate and/or fidelity) at replicating a template containing the halogenated base BrdU. In this regard, Heartlein *et al.*, (1983) and O'Neill *et al.*, (1983) showed that the frequency of SCEs is proportional to substitution of thymidine by BrdU or CldU during replication, and that this observed increase required BrdU or CldU to be present during the first of two DNA replications. Replication of the substituted template was the determining factor, rather than mistakes caused by the incorporation of an altered base. In addition the presence of CldU resulted in 5-7 times as many SCEs as with BrdU, indicating that in wild-type cells the particular substituted base can affect the frequency of errors leading to SCE.

Also, Ara1 showed a considerable delay in the cell cycle when grown in BrdU. According to Painter (1980), hindrances to the polymerase complex caused by an altered base can result in stalling at the replication fork. Since the DNA is single stranded at this point, a recombination event may occur resulting in an SCE.

The higher frequency of SCEs in Ara1 makes it comparable to Bloom Syndrome. Lymphocytes from individuals with Bloom syndrome contain 8-10 times as many SCE as normal cells when grown for two rounds of replication in BrdU (Shiriashi *et al.*, 1983). It was further shown that the increase in SCE was due exclusively to replication errors at the second replication in BrdU. Complementation analysis of Bloom X Ara1 hybrid will show if indeed Ara1 shares a common mutation with Bloom Syndrome. The only other reported CHO mutant cell line to show an increase in SCEs (12-fold above wild-type) is the EM9 line isolated by Thompson and *et al.*, (1982).

It will be important to test the induction of SCEs by X-rays in the ara-C resistant cell lines since X rays are known to be poor inducers of SCEs in wild-type cells. A logical assumption would be that, since the mutants are deemed to be deficient in base damage repair, an increased frequency would be seen. In fact, cytogenetic studies of the X-ray sensitive cell line *xrs6* showed an increase in SCEs induced by X rays (Darroudi and Natarajan, 1987), and the authors suggested that the possible defect in the *xrs6* cells was in DNA base damage repair.

Both Ara1 and Ara2.1 showed extended S-phases. Cell cycle studies of AraC^R213 were not deemed necessary since this was not a critical issue and the time points used in the SCE experiment gave essentially the same proportion of second

and third divisions (data not given) as the mutant Ara2.1 (the cell line from which AraC^R213 was isolated). An extended S-phase has been reported to exist in AT cells by Painter (1985). He explains this observation as a result of abnormal replicon initiation processes with fewer initiations per unit time compared to normal cells. Another explanation could be that the polymerases that are normally affected by ara-C are deficient or slower in polymerization functions in both AT and in the hamster mutants.

The AraC^R213 cell line is the most attractive one for further experiments involving "repair" gene isolation because of the sensitivity to X-irradiation and, because, thus far, it appears to be the most similar to AT cells. Additional support for this hypothesis will be demonstrated in the following chapter by examining chromosome aberration induction by X rays.

CHAPTER 2

X-RAY SENSITIVITY AS ASSESSED BY ABERRATION INDUCTION IN G₁ AND G₂ STAGES OF THE CELL CYCLE

Summary

Two of the ara-C resistant, X-ray sensitive mutants, Ara2.1 and AraC^R213 have been further characterized with regard to cytological responses to X-irradiation and the influence of ara-C, when used as an inhibitor of DNA repair, on the yield of chromosomal aberrations induced by X rays. X-irradiation of synchronized mutant cells in G₁ induced both chromosome- and chromatid-type aberrations. The frequency of chromosome-type aberrations was 2-fold greater than for wild-type, while a 3-fold higher frequency of chromatid-type aberrations was seen. The mutant, AraC^R213, showed no increase in aberration frequency over X rays alone when treated with X rays plus ara-C while a 3-fold increase in aberrations above that seen with X rays alone was seen in the wild-type CHO cells.

The AraC^R213 cells were further evaluated for G₂-phase specific sensitivity to X-ray-induced chromosome aberrations. There was a 2.5-fold increase over wild-type aberration frequencies with no change in aberration frequency with a post-irradiation

incubation with ara-C. In contrast, the wild-type cells showed a 6- to 8-fold increase when treated with X rays in conjunction with ara-C compared to the frequency with X rays alone. Therefore, we propose that the mutation in AraC^R213 which confers ara-C resistance affects a process that is involved in repair in both G₁ and G₂ phases of the cell cycle.

The untreated control groups from the experiments above were used to evaluate the frequency of spontaneous chromosomal aberrations in the mutant cell lines. The incidence in the mutant was approximately 4-fold greater than for the wild-type.

Introduction

The mode of action of ara-C inhibition, ie., the polymerization step of replication repair, would suggest that it would not inhibit the repair of clean double-strand breaks (Hiss and Preston, 1977), but it would most likely play a major role in the inhibition of base damage repair. Essentially, the process for the repair of base damage is the removal of the damaged base leaving an apurinic or apyrimidinic (AP) site. An AP-specific endonuclease nicks the DNA adjacent to the deoxyribose that does not have an attached base, and an exonuclease excises the AP-site and a few adjacent bases resulting in a single-strand gap (Lindahl, 1972). If repair replication and subsequent ligation is inhibited by ara-C, single-strand gaps would accumulate.

In order for an aberration to be formed two or more lesions must interact which requires that they have to be repairing at the same time and within the rejoining

distance. Thus, since ara-C accumulates gaps by inhibiting repair, the probability of two or more interacting to form an aberration will be greatly increased on reversing the inhibition by addition of deoxycytidine compared to that in the absence of ara-C (Preston, 1980, 1981, 1983). Essentially the requirement for coincident repair is eliminated. If base damage is left unrepaired at the time of replication, it can be converted into chromatid-type aberrations as a consequence of errors of replication on a damaged template. An increase in chromatid-type aberrations was observed in X-irradiated AraC^R213 cells, as described below.

Subsequent information that is needed to equate AraC^R213 to AT is an analysis of cytogenetic features. Cytogenetic findings on cells from individuals with AT have demonstrated an increased spontaneous chromosomal aberration frequency (Hecht *et al.*, 1966; Pfeiffer, 1970; Oxford *et al.*, 1975; Al Saadi *et al.*, 1980; Aurias *et al.*, 1980; Taylor *et al.*, 1981; Hecht and Kaiser-McCaw, 1982). Others have reported cell-cycle-specific aberration induction by X rays at an increased frequency in AT cells (Natarajan and Meyers, 1979; Littlefield *et al.*, 1981; Taylor, 1982; Bender *et al.*, 1985; Nagasawa *et al.*, 1985). Further, if AT cells are X-irradiated in G₀, there is an enhancement in aberration frequency which is largely due to an increase in chromatid-type aberrations (Taylor, 1978). Paterson *et al.*, (1976) have proposed that AT cells are deficient in base damage repair.

Cytogenetic studies using AT cells in our laboratory have provided additional experimental evidence that AT cells may be deficient in base damage repair. It is also possible that the ara-C inhibitable repair process is non-functional or inefficient in AT. A study with AT cells to evaluate the effect of ara-C on aberration production

after exposure to X rays (Greer *et al.*, 1991) gave some very informative results. AT5BI fibroblasts were treated with X rays in the G₂ phase of the cell cycle, and the cells were incubated with ara-C after exposure. Reflecting the X-ray sensitivity of the cell line, the aberrations produced by X rays alone were 2-3-fold higher in AT5BI cells compared to normal cells. However, the frequency of aberrations in AT cells remained the same when ara-C was used in combination with X rays, whereas there was an enhancement in normal fibroblasts irradiated and incubated with ara-C. These initial results give a phenotypic correlation between the repair defect in the ara-C resistant mutant and the deficiency in AT.

This chapter presents a study of aberration induction by X rays alone and by X rays in conjunction with ara-C in the ara-C resistant, X-ray sensitive cell lines, Ara2.1 and AraC^R213 and the parental CHO cells.

Materials and Methods

Cell Culture Conditions

CHO-K1 cells and the mutant cell lines, Ara2.1 and AraC^R213, were maintained in Ham's F12 (GIBCO) supplemented with 5% heat-inactivated (56°C, 55 minutes) FBS (Hazelton), 50 units/ml penicillin (Squibb), and 50ug/ml streptomycin (Lilly). Ara2.1 cells were grown intermittently in 5x10⁻⁵M ara-C and Ara-C^R213 cells were grown intermittently in 10⁻³M ara-C. Cells were maintained as monolayers at 37°C in 5% CO₂ at 95-100% humidity.

X-Irradiations

X-Irradiations were with a 320-kVp Gemini Industrial X-Ray Unit operated at 250 kV and 12 mA with 1mm aluminum filtration at a dose rate of 1 Gy per minute.

X-ray-induced Chromosome Aberrations in G₁ Cells

The preliminary experiments were carried out with synchronized cells (Ara2.1 and CHO) obtained by mitotic shake-off. One hour after plating, to allow the mitotic cells to enter the G₁ phase of the cell cycle, the cells were X-irradiated with .5, 1, or 1.5 Gy. Ara-C ($5 \times 10^{-5} \text{M}$) was added to the appropriate plates 30 minutes prior to irradiation. Three hours post-irradiation, the plates were rinsed to remove the ara-C and dC (10^{-4}M) was added to reverse inhibition. Colchicine ($1 \times 10^{-7} \text{M}$) was added when the cells began to round-up as they entered mitosis (18 hours for the CHO cells and 20 hours for the Ara2.1 cells). The fixation, slide preparation, and staining procedures followed standard methods (Korthor, 1986).

The experiment was repeated using AraC^R213 cells with a change in the procedure. To enable a distinction to be made between late G₁ and early S-phase cells at the time of irradiation, the cells were pulsed immediately after X-ray exposure with ³H-thymidine for 15 minutes to label all S-phase cells. X-irradiations and incubation times followed the same format as above. Cells were fixed and dropped onto slides. A drop of 1% acetic-orcein was placed on each slide and covered with a temporary coverslip for aberration analysis. The coordinates of analyzed metaphases were recorded and the slides were developed by autoradiography. Any

labeled cells were excluded from the data. A total of 100 unlabeled metaphases per group were analyzed for aberrations.

X-ray-induced Chromosome Aberrations In G₂ Cells

All cell lines were plated 24 hours prior to the experiment at appropriate densities to ensure actively dividing cultures. Each cell line was set up in two different treatment groups; one group was treated with X rays alone while the second group was treated with X rays in conjunction with ara-C. Ara-C ($5 \times 10^{-5} \text{M}$) was added to the appropriate cultures 30 minutes prior to irradiation and remained in the medium until cells were harvested. Cells were irradiated at doses of .2 .5, or .75 Gy. The controls included one culture that was untreated and one culture treated with ara-C alone. Colchicine ($1 \times 10^{-7} \text{M}$) was added to all plates immediately after irradiation. Again, the fixation, slide preparation, and staining procedures followed standard methods. Two populations of cells were scored: those entering mitosis during the first hour after irradiation and the cumulative population of the two hours after irradiation. A minimum of 100 metaphases was analyzed per treatment group.

Results

X-ray-induced Chromosome Aberrations in G₁ Cells

The results of the preliminary experiment using CHO and Ara2.1 cells are presented to emphasize three points: first, the high frequency of spontaneous aberrations seen in the mutant cells; second, the higher frequency of X-ray-induced

chromosome-type aberrations in Ara2.1 cells; and third, the presence of chromatid-type aberrations in the G₁ irradiated Ara2.1 cells. Ara2.1 cells had a 2-fold increase in total aberrations compared to wild-type cells in the groups treated with X rays alone (Figure 2.1 and Table 2.1). Not only was the frequency of chromosome-type aberrations increased in Ara2.1 irradiated in G₁, but there was a higher frequency of chromatid-type aberrations. These results implicated a sensitivity to X rays in Ara2.1 as assessed by increased aberration formation. A higher frequency of spontaneous aberrations was seen in the mutant cell line with 0.26 total aberrations per cell in the controls while the CHO cells had only 0.08 total aberrations per cell (over a 3-fold increase). The data on the CHO cell line treated with ara-C plus X rays from this experiment were not used due to the high standard errors. Only 22 metaphase cells were found in the CHO group treated with 1 Gy of X rays plus ara-C.

Since the preliminary data showed an increase in chromatid-type aberrations, a second experiment was deemed necessary in which one could rule out the possibility that this increase was due to S-phase cells in the cultures at the time of irradiation. Therefore, the cells were incubated in ³H-thymidine immediately after irradiation to label any replicating cells. The results presented in Figure 2.2 and Table 2.2 were compiled from scoring unlabeled G₁ cells. Chromosome-type aberration frequencies in AraC^R213 cells showed about a 2.5-fold increase at all doses. For example, at a dose of 1.5 Gy there were 0.56 exchanges (dicentric and rings) and 0.65 deletions per cell in the mutant compared to 0.28 exchanges and 0.20 deletions in the wild-type cells (Table 2.2). When CHO cells were incubated with ara-C during and after X-irradiation, there was a 3-fold increase in exchanges

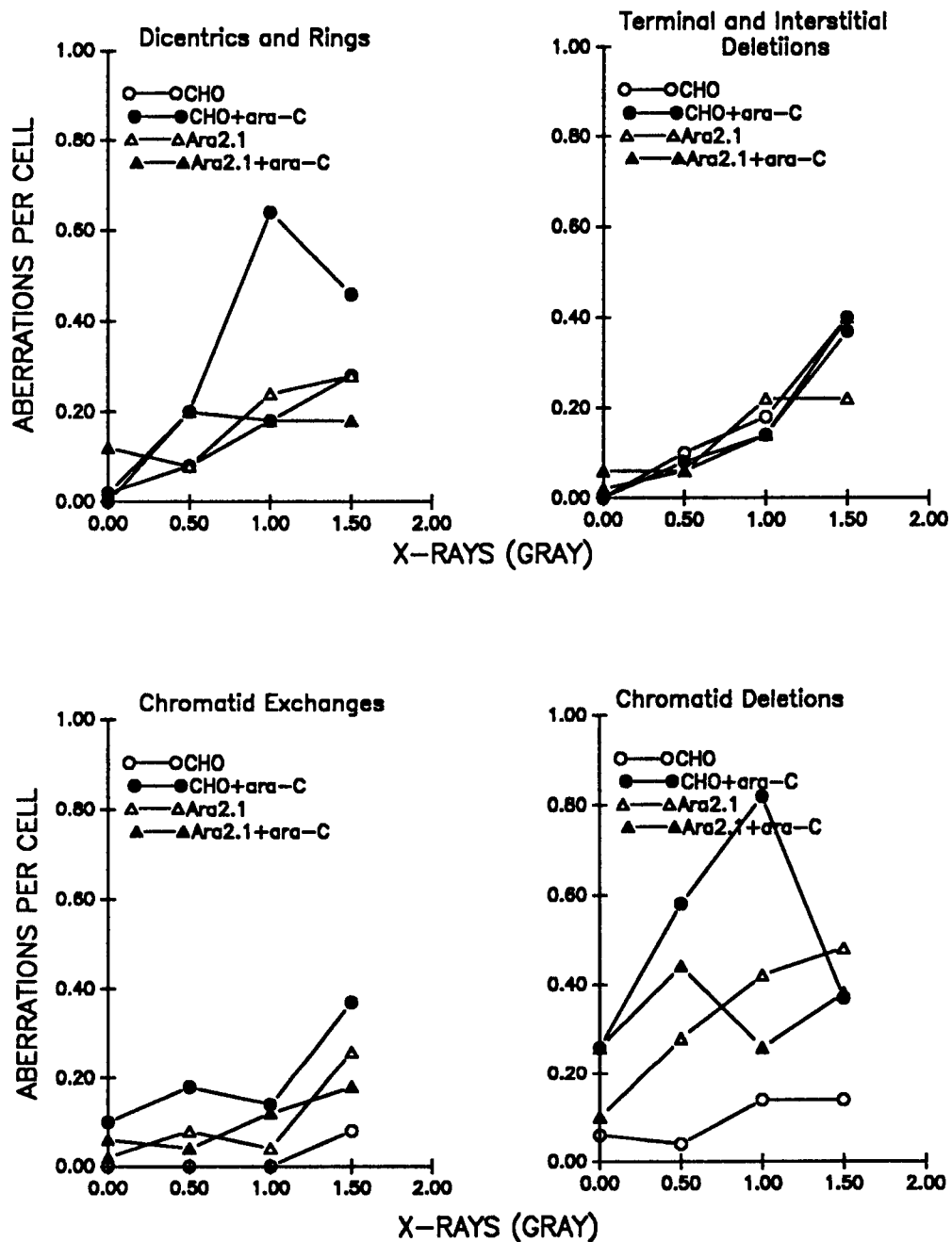


Figure 2.1. Chromosome aberration frequencies of G₁ X-irradiated Ara2.1 cells.

Table 2.1. Preliminary evaluation of chromosome aberration frequencies induced by X rays in G₁ cells, with or without ara-C (3 hours) post-treatment.

Cell Type	Treatment	Chromosome-type Aberrations		Chromatid-type Aberrations		*Total Aberrations per Cell
		Deletions	Exchanges	Deletions	Exchanges	
CHO	Control	0.0	0.02 ± .02	0.06 ± .05	0.0	0.08 ± .06
	0.5 Gy	0.10 ± .06	0.08 ± .06	0.04 ± .04	0.0	0.22 ± .09
	1.0 Gy	0.18 ± .08	0.18 ± .08	0.14 ± .07	0.0	0.50 ± .14
	1.5 Gy	0.40 ± .13	0.28 ± .10	0.14 ± .07	0.08 ± .06	0.90 ± .19
Ara2.1	Control	0.02 ± .03	0.12 ± .07	0.10 ± .06	0.02 ± .03	0.26 ± .10
	0.5 Gy	0.06 ± .05	0.08 ± .06	0.28 ± .10	0.08 ± .06	0.50 ± .14
	1.0 Gy	0.22 ± .09	0.24 ± .10	0.42 ± .13	0.04 ± .04	0.92 ± .19
	1.5 Gy	0.22 ± .09	0.28 ± .10	0.48 ± .14	0.26 ± .10	1.24 ± .22
CHO	ara-C	0.0	0.0	0.26 ± .10	0.10 ± .06	0.36 ± .12
	0.5 Gy + ara-C	0.08 ± .06	0.20 ± .09	0.58 ± .15	0.18 ± .08	1.04 ± .20
	1.0 Gy + ara-C	0.14 ± .17	0.64 ± .36	0.82 ± .41	0.14 ± .17	1.74 ± .60
	1.5 Gy + ara-C	0.37 ± .15	0.46 ± .17	0.37 ± .15	0.37 ± .15	1.57 ± .25
Ara2.1	ara-C	0.06 ± .05	0.02 ± .03	0.26 ± .10	0.06 ± .05	0.40 ± .13
	0.5 Gy + ara-C	0.06 ± .05	0.20 ± .04	0.44 ± .13	0.04 ± .04	0.74 ± .17
	1.0 Gy + ara-C	0.14 ± .07	0.18 ± .08	0.26 ± .10	0.12 ± .07	0.70 ± .17
	1.5 Gy + ara-C	0.40 ± .13	0.18 ± .08	0.38 ± .12	0.18 ± .08	1.14 ± .21

*50 or less metaphase cells scored per group

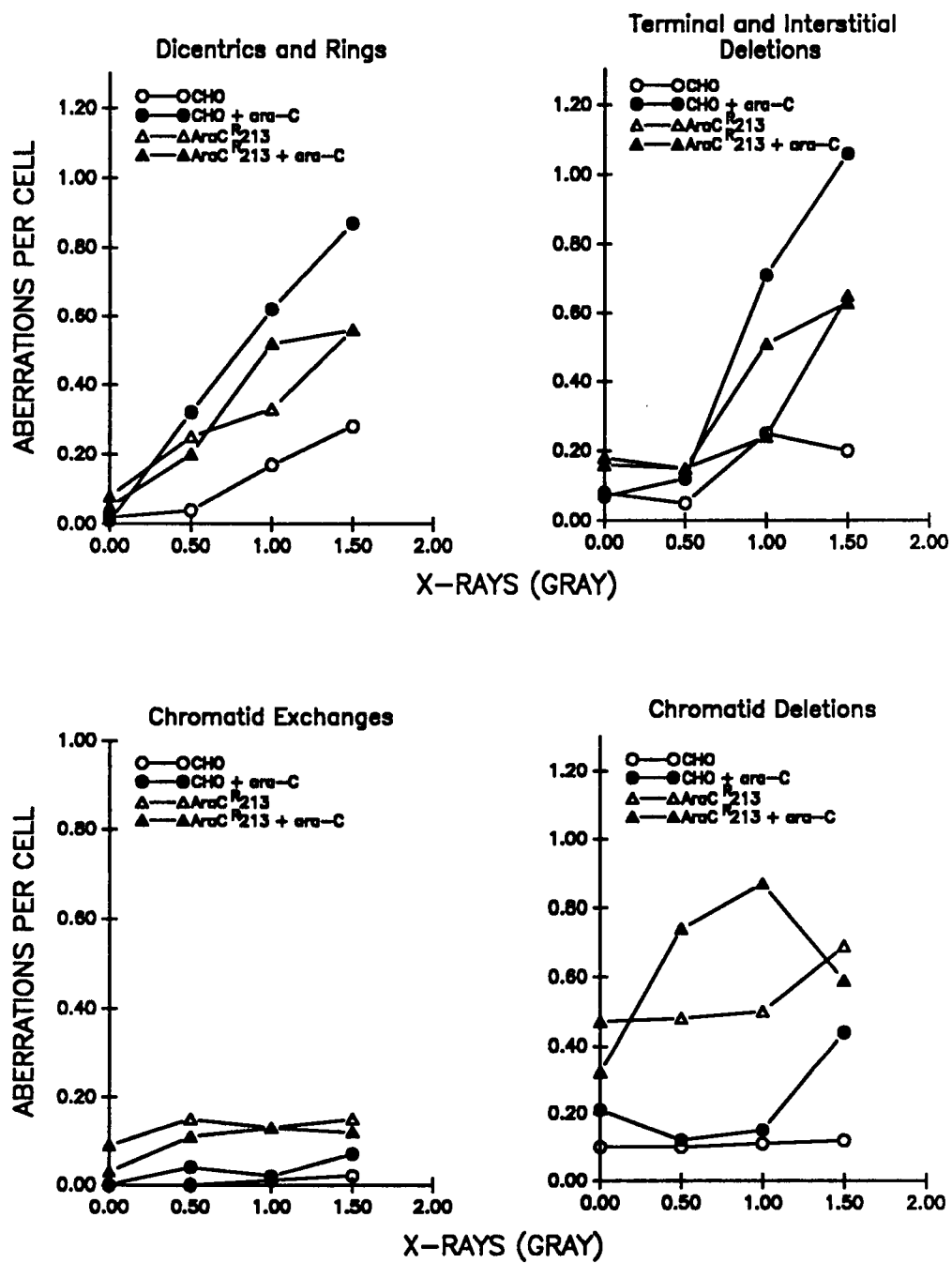


Figure 2.2. Chromosome aberration frequencies in G_1 , X-irradiated AraC^{R213} cells.

Table 2.2. Chromosome aberration frequencies* induced by X rays in G₁ cells, with or without ara-C (3 hours) post-treatment.

Cell Type	Treatment	Chromosome-type Aberrations		Chromatid-type Aberrations		Total Aberrations per Cell
		Deletions	Exchanges	Deletions	Exchanges	
CHO	Control	0.08 ± .03	0.02 ± .01	0.10 ± .03	0.0	0.20 ± .04
	0.5 Gy	0.05 ± .02	0.04 ± .02	0.10 ± .03	0.0	0.19 ± .04
	1.0 Gy	0.25 ± .05	0.17 ± .04	0.11 ± .03	0.01 ± .01	0.54 ± .07
	1.5 Gy	0.20 ± .04	0.28 ± .05	0.12 ± .03	0.02 ± .01	0.62 ± .08
AraC ^R 213	Control	0.18 ± .04	0.08 ± .03	0.47 ± .07	0.09 ± .03	0.82 ± .09
	0.5 Gy	0.15 ± .04	0.25 ± .05	0.48 ± .07	0.15 ± .04	1.03 ± .10
	1.0 Gy	0.24 ± .05	0.33 ± .06	0.50 ± .07	0.13 ± .04	1.20 ± .10
	1.5 Gy	0.65 ± .08	0.56 ± .07	0.69 ± .08	0.15 ± .04	2.05 ± .14
CHO	ara-C	0.07 ± .03	0.01 ± .01	0.21 ± .05	0.0	0.29 ± .05
	0.5 Gy + ara-C	0.12 ± .03	0.32 ± .06	0.12 ± .03	0.04 ± .02	0.60 ± .08
	1.0 Gy + ara-C	0.71 ± .08	0.62 ± .08	0.15 ± .04	0.02 ± .01	1.50 ± .12
	1.5 Gy + ara-C	1.06 ± .10	0.87 ± .09	0.44 ± .07	0.07 ± .03	2.44 ± .16
AraC ^R 213	ara-C	0.16 ± .04	0.05 ± .02	0.32 ± .06	0.03 ± .02	0.56 ± .07
	0.5 Gy + ara-C	0.15 ± .04	0.20 ± .04	0.74 ± .09	0.11 ± .03	1.20 ± .10
	1.0 Gy + ara-C	0.51 ± .07	0.52 ± .07	0.87 ± .09	0.13 ± .04	2.03 ± .14
	1.5 Gy + ara-C	0.63 ± .08	0.56 ± .07	0.59 ± .08	0.12 ± .04	1.90 ± .14

*100 unlabeled metaphase cells scored per group

(0.87/cell) and a 5-fold increase in deletions (1.06/cell) compared to the frequencies with X rays alone. In contrast, with the mutant, AraC^R213, there was no increase in chromosome-type aberrations when the cells were incubated in ara-C (0.56 exchanges and 0.63 deletions per cell), compared to the frequencies with X rays alone. These results essentially parallel those reported in Chapter 1 for cell survival.

Due to the high frequency of spontaneous chromatid-type aberrations in the untreated AraC^R213 cells, an evaluation of their relative frequencies for the different treatment groups was somewhat complicated. Suffice it to say that the frequency of X-ray-induced chromatid-type aberrations were higher in the mutant than in the parental cells. Table 2.2 shows that there is an 11-fold increase in frequency of chromatid-type deletions (0.02 deletions in CHO cells and 0.22 deletions in AraC^R213 cells) in the mutant G₁ cells after an X-ray treatment of 1.5 Gy. This point is considered further in the discussion.

X-ray-induced Chromosome Aberrations in G₂ Cells

The frequencies of aberrations induced in G₂ cells by X rays are shown in Table 2.3. It can be seen that the mutant, AraC^R213, had consistently higher frequencies of X-ray-induced chromosomal aberrations than wild-type CHO cells. For example, there was a 2.5-fold increase at a X-ray dose of 0.75 Gy when cells were fixed at 1 or 2 hours after irradiation. When cells were incubated with ara-C, and fixed 1 hour after irradiation (Figure 2.3A), the aberration frequency in the mutant was the same as with X rays alone, whereas, there was an increase in deletions in the parental CHO cells incubated with ara-C. When cells were fixed 2 hours after X-irradiation

Table 2.3. Chromosome aberration frequencies* induced by X rays in G₂ cells, with or without ara-C (1 or 2 hours) post-treatment.

Cell Type	Treatment	1 Hour Fixation Time		2 Hour Fixation Time	
		Deletions	Exchanges	Deletions	Exchanges
CHO	Control	0.03 ± .02	0	0.04 ± .02	0
	.20 Gy	0.46 ± .07	0	0.32 ± .06	0
	.50 Gy	0.74 ± .09	0.03 ± .02	0.45 ± .07	0.11 ± .03
	.75 Gy	0.91 ± .10	0.12 ± .03	0.68 ± .08	0.10 ± .03
AraC ^R 213	Control	0.11 ± .03	0.01 ± .01	0.05 ± .02	0
	.20 Gy	0.69 ± .08	0	0.46 ± .07	0.07 ± .03
	.50 Gy	1.72 ± .13	0	1.09 ± .10	0.08 ± .03
	.75 Gy	2.43 ± .16	0	1.66 ± .13	0.15 ± .04
CHO	ara-C	0.21 ± .05	0	0.37 ± .06	0
	.20 Gy + ara-C	0.78 ± .09	0	1.72 ± .13	0
	.50 Gy + ara-C	1.17 ± .10	0	3.21 ± .18	0
	.75 Gy + ara-C	3.14 ± .18	0	5.19 ± .23	0
AraC ^R 213	ara-C	0.15 ± .04	0	0.06 ± .02	0.02 ± .01
	.20 Gy + ara-C	0.70 ± .08	0	0.39 ± .06	0
	.50 Gy + ara-C	1.36 ± .12	0	0.87 ± .09	0.06 ± .02
	.75 Gy + ara-C	2.68 ± .16	0.03 ± .02	2.01 ± .14	0.18 ± .04

*100 metaphase cells scored per group

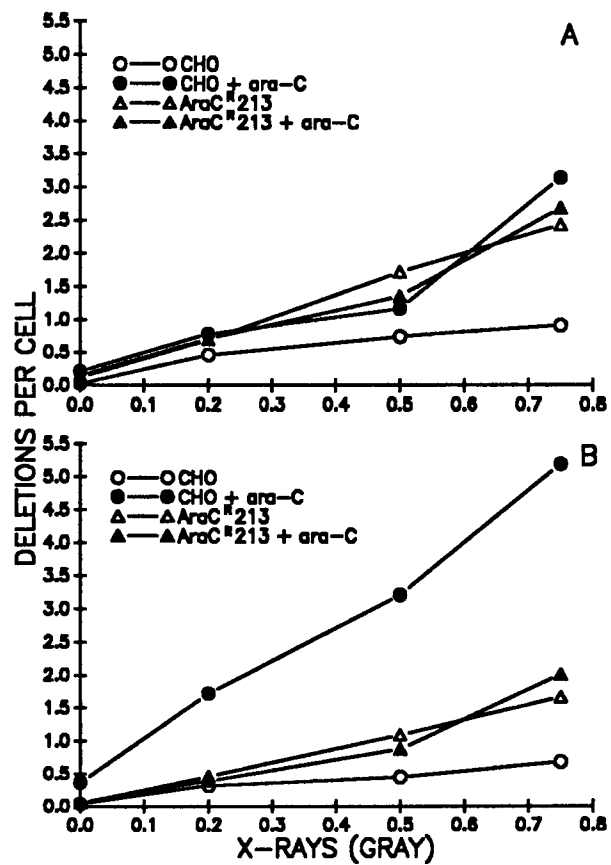


Figure 2.3. X-ray-induced chromatin deletions in G_2 treated cells: (A) 1 hour after treatment, (B) 2 hours after treatment.

(Figure 2.3B), there was no significant change in deletion frequency with AraC^R213 cells, whereas the frequency of deletions increased 6- to 8-fold in the CHO cells. The effects of post-irradiation incubation with ara-C were essentially the same for the 1 hour fixation: no change with AraC^R213 cells and a large increase with CHO cells.

The exchange frequencies presented in Figure 2.4 (A without ara-C; B with ara-C) show the effect of ara-C on X-irradiated wild-type CHO and AraC^R213 cells. It is interesting to note that although X rays alone induced exchanges in both cell types, only AraC^R213 cells contained exchanges in the presence of ara-C and for the 2 hour fixation only. The frequency was similar to that with X rays alone.

Discussion

The AraC^R213 mutant cell line has been studied in detail with regard to cytological responses to X-irradiation and the influence of ara-C when used as an inhibitor of DNA repair on the yield of chromosomal aberrations induced by X rays. Cytogenetic analysis of the mutant showed that there was a marked increase in the levels of X-ray-induced chromosomal aberrations in both G₁ and G₂ phases of the cell cycle. X-irradiation of the synchronized mutant cells in G₁ significantly induced both chromosome- and chromatid-type aberrations. Characteristics of AT cells, equivalent to those presented here for AraC^R213 mutant cells, have been documented. Chromosome breakage is a basic cytogenetic feature of AT (Hecht *et al.*, 1966; Harnden, 1974). The breaks are non-random in location and give rise to breakage products such as dicentric chromosomes and other types of rearrangements, i.e.

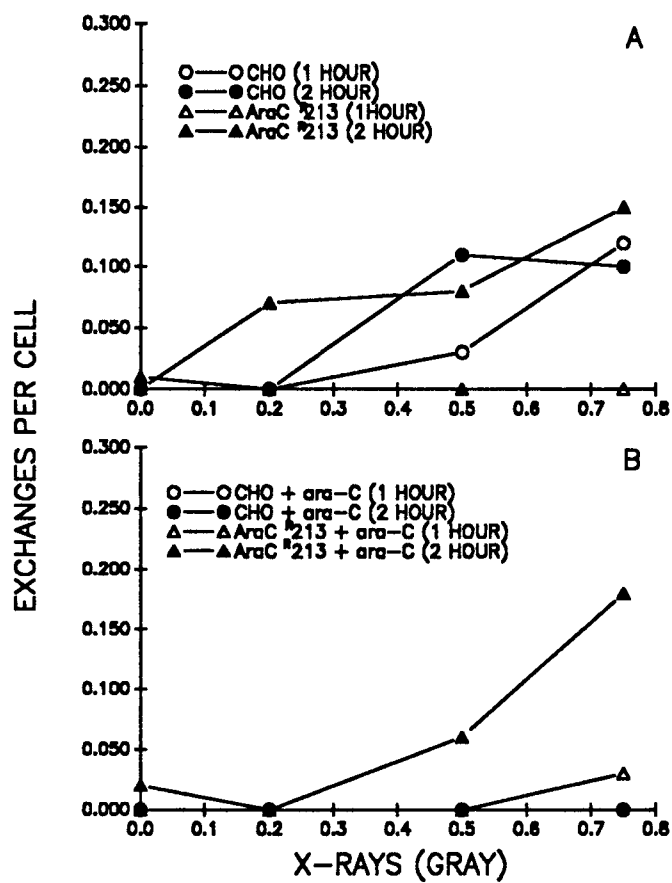


Figure 2.4. X-ray-induced chromatid exchanges in G_2 treated cells: (A) without ara-C, (B) with ara-C.

acentrics, rod deletions and duplications, rings, inversions, and translocations. Chromatid breakage products such as polyradials are also observed in AT cells. These aberrations occur in AT patients *in vivo* and are occasionally observed in direct bone marrow preparations. The cell clones with marker rearrangements also provide ample confirmation that breaks in chromosomes do occur *in vivo* in patients with AT, not merely *in vitro* in laboratory cultured cells (Hecht and Hecht, 1985). When AT fibroblasts were irradiated in G₁, a significant increase in chromatid gaps, breaks and interchanges was observed in addition to an increased frequency of chromosome-type aberrations (Nagasawa *et al.*, 1985). In addition, studies done in this laboratory showed that there was no increase in chromosome aberrations in AT cells X-irradiated in G₂ when incubated with ara-C in contrast to a large increase in normal human cells with the same treatment (Greer *et al.*, 1991).

The frequency of chromatid-type aberrations in control cultures was considerably higher in AraC^R213 cells than in CHO cells (0.56 per cell vs. 0.1 per cell). This is perhaps not surprising since ³H-thymidine was used to detect S-phase cells, and the AraC^R213 cells are radiation sensitive. The beta rays from ³H-thymidine have been reported to increase chromosome breakage in AT cells presumably via a mechanism similar to that for other types of ionizing radiation (Natarajan and Meyers, 1979; Zampetti-Bosseler and Scott, 1981). This finding and the increased frequency of chromatid-type aberrations in G₁ X-irradiated cells support the theory that DNA damage incurred during G₁ in the mutant cells remains unrepaired at the time of DNA replication. This further supports the conclusion that AraC^R213 is radiation sensitive because of a defect in repair and that it has a similar phenotype to AT.

If we can define the specific defect in AraC^R213 cells, we can endeavor to relate this defect to the increased X-ray sensitivity of the cells. In order to address this question, we have called upon previous experiments in our laboratory that show that X-ray-induced chromosome aberrations and cell killing are a consequence of misrepair of induced double-strand breaks (dsb) and base modifications (reviewed by Preston, 1991). The probability of misrepair of base damages (ara-C inhibitable damage) leading to chromosome aberrations increases with increasing dose, whereas, the probability of misrepair of directly induced dsb being converted into aberrations is constant with dose (Heartlein and Preston, 1985b; Preston, 1991). This leads to the suggestion that the linear component of the quadratic dose response for X-ray-induced aberrations is a reflection of misrepair of dsb, and that the dose-squared component is a reflection of misrepair of base damages. It is further proposed that ara-C specifically inhibits the excision repair of base damages. This hypothesis is, in part, in contrast to the often-held one that only dsb misrepair is involved in aberration and mutation induction and cell killing. In support of this latter view, some of the X-ray sensitive mutants that have been described are defective in ssb or dsb repair ((Thompson *et al.*, 1982; Schwartz *et al.*, 1987; Fuller and Painter, 1988; Kemp *et al.*, 1984; Giaccia *et al.*, 1985; Wlodek and Hittleman, 1987; Robson *et al.*, 1989). This defective strand break repair would be expected to increase radiosensitivity, and would be predicted, based on our general hypothesis, to influence the linear component of the aberration dose response curve. However, it is important to add that this would not negate the idea that another set of mutants, such as the ara-C resistant ones reported here, are defective in a second repair

process, namely, that of base damages. In support of this, it is interesting to note that the X-ray sensitive mutants, *xrs* 5 and *xrs* 6, (isolated by Jeggo, 1982) show no synergistic effect of ara-C with X rays (Darroudi and Natarajan, 1987). It is suggested that this is due to the fact that these mutant cells also are defective in the repair pathway normally inhibited by ara-C, i.e. base damage repair.

Additional support for the hypothesis that defective repair of base damages can result in increased X-ray sensitivity comes from studies with AT cells. These X-ray sensitive cells are not apparently deficient in the repair of ssb and dsb (Lehmann, 1982). It has additionally been reported that the removal of gamma endonuclease sensitive sites (ESS) is similar in normal and AT fibroblasts (Fornace *et al.*, 1986). This, however, does not negate the hypothesis that the repair of base damages is defective, since we would propose, based upon our studies with AT and ara-C resistant mutants, that incision and excision of base damages is normal, but the resynthesis step is altered. Based upon our chromosome aberration data with wild-type and araC-resistant Chinese hamster cells, we further suggest that the resynthesis step is slowed in the mutant cells as compared to the normal cells, either in initiation or progression. In the presence of ara-C, the frequency of X-ray-induced chromatid deletions in CHO cells increases in G_2 exposed cells with increasing time of incubation with ara-C. No chromatid exchanges were observed. The deletions are suggested to be due to the inhibition of excision repair by ara-C. This is supported by the fact that no rejoining leading to exchanges takes place. In contrast, chromatid exchanges were observed in the ara-C resistant mutant cells when X-irradiated and incubated with ara-C for 2 hours but not with a 1 hour incubation. Thus, some

rejoining leading to exchanges can take place in the mutant, although not in less than 1 hour. The frequency of deletions in the mutant cells was considerably less than that in the wild-type CHO cells with a 2 hour incubation in ara-C, whereas it was about the same with a 1 hour post-irradiation incubation in ara-C. This suggests that in the mutant cells some excision repair can be completed between 1 and 2 hours after irradiation. However, it takes longer to complete resynthesis in the mutant cells than it does in wild-type cells in the absence of ara-C. It remains to be determined if a similar alteration in excision repair synthesis is present in AT cells. However, our results do at least indicate that AraC^R213 cells are phenotypically similar to AT cells, and that there is a high probability of their sharing the same deficiency in repair.

CHAPTER 3

HYPERSENSITIVITY OF AraC^R213 TO ABERRATION INDUCTION BY THE RESTRICTION ENZYME Rsa I

Summary

Characterization of AraC^R213 has enabled parallels to be drawn between it and the human DNA repair-defective syndrome, AT. In addition to a hypersensitivity to X-irradiation, AT cells have been shown to have an increased sensitivity to restriction enzyme-induced double-strand breaks, although they do not appear to have a reduced capacity to repair X-ray-induced double-strand breaks. An analysis of aberration induction of endonuclease-induced damage in the mutant could provide further means of comparison of the repair defects in AraC^R213 and AT.

The repair of a portion of restriction enzyme-induced double-strand breaks is inhibitable in wild-type cells by ara-C, an inhibitor of DNA repair synthesis. When aphidicolin, an inhibitor of α and δ polymerases, is used to inhibit repair of restriction enzyme-induced double-strand breaks, the results are essentially the same as with ara-C. Since it is known that the pathway of repair which is normally inhibitable by ara-C in wild-type cells is no longer functional in the X-ray sensitive, ara-C resistant mutant, AraC^R213, it would be particularly informative to evaluate the mutant's fidelity of repair of double-strand breaks induced by restriction enzymes.

Synchronized populations of AraC^R213 and CHO cells were treated in the G₁ phase of the cell cycle with the restriction endonuclease Rsa I, a blunt-end cutter. An analysis of chromosome-type aberrations showed a 3-fold increase in exchanges (dicentrics and rings) and a 2-fold increase in deletions in AraC^R213 as compared to wild-type CHO cells. An increase in chromatid-type aberrations was also seen in the mutant, but not in the wild-type cells.

In light of these results, the explanation offered is that the polymerases (inhibitable by ara-C and aphidicolin) which are involved in resynthesis during excision repair of X-ray-induced damage also play a role in blunt-end double-strand break repair (probably through recombinational repair) and that this function is deficient in AraC^R213 and in AT.

Introduction

The observation that AT cells are specifically sensitive to ionizing radiation both in terms of induction of chromosome aberrations (Taylor, 1982) and loss of reproductive capacity (Taylor *et al.*, 1975; Cox *et al.*, 1978) has been the motivation for the isolation and characterization of X-ray sensitive mutants. Studies with such mutants have attempted to relate radiosensitivity to specific defects in DNA repair and/or replication. The aim of studying various aspects of DNA repair in the X-ray sensitive, ara-C resistant CHO mutant, AraC^R213, is to identify a particular DNA repair defect which can ultimately account for the AT phenotype.

Ionizing radiation produces a variety of damages to DNA including single- and double-strand breaks, alkali labile lesions, base damages, sugar damages and both DNA-DNA and DNA-protein crosslinks (for an extensive review see Ward, 1988). This broad spectrum of DNA damages complicates elucidation of mechanisms of how specific DNA lesions can be converted into chromosome aberrations. It is, therefore, desirable to utilize agents that produce a specific type of DNA lesion. Type II restriction endonucleases produce either blunt- or cohesive-end double-strand breaks at specific sequences in the DNA. The restriction enzyme used in the present experiment, *Rsa* I, is a blunt-end cutter with an estimated cutting frequency (based on nearest neighbor analysis) of once every 416 base-pairs in the middle of the recognition site GT|AC. This enzyme was chosen because of the availability of data generated in this laboratory on the effectiveness of aberration induction and dose-response curves. A number of investigators have shown that restriction enzyme-induced double-strand breaks can induce chromosome aberrations (Bryant, 1984; Natarajan and Obe, 1984; Obe and Natarajan, 1985; Obe and Winkel, 1985; Obe *et al.*, 1985, 1986; Stoilov *et al.*, 1986; Winegar and Preston, 1988;) regardless of whether the enzyme forms cohesive-ends or blunt-ends.

If, in combination with the induction of specific double-strand breaks, repair inhibitors are used, the role of a specific step in the repair process can be analyzed. Two such inhibitors are aphidicolin, an inhibitor of α and δ polymerases, and ara-C, an inhibitor of α and β polymerases (it has not been shown if polymerase δ is inhibitable by ara-C). Each has been shown to enhance aberration induction of both X-ray-induced damage and restriction enzyme-induced chromosome damage

(Preston, 1980; Natarajan and Obe 1984; Obe and Natarajan, 1985; Chung *et al.*, 1991). The step in repair that is inhibitable by ara-C is intrinsically deficient in the X-ray sensitive mutant, AraC^R213, as indicated by the fact that ara-C has no effect on the frequency of X-ray-induced aberrations, in contrast to the significant increase in wild-type CHO cells. A similar response to ara-C was seen when AT5BI fibroblasts were exposed to X rays and then incubated in ara-C post-irradiation (Greer *et al.*, 1991). Much like AraC^R213 cells, no enhancement in aberration induction was seen in the presence of ara-C compared to the frequency of aberrations-induced by X rays alone, whereas there was a significant increase in X-irradiated normal human fibroblasts incubated with ara-C.

Since it appears that the step in repair that is inhibitable by ara-C also plays a role in the repair of at least a portion of restriction enzyme-induced double-strand break damage, and since AraC^R213 is deficient in this function, one would predict an increased sensitivity of AraC^R213 to aberration induction by some, if not all, restriction endonucleases.

The study described here will attempt to correlate (1) the repair function(s) that is inhibitable by ara-C which is non-functional in the mutant cell, (2) the mechanism of repair of radiation and restriction enzyme damage, and (3) the repair deficiency in AT cells.

It is not the aim of these studies to analyze the utility of using restriction enzymes as a surrogate for studying the mechanisms of aberration induction by ionizing radiations nor to debate the effectiveness of blunt-end versus cohesive-end double-strand breaks for the production of chromosomal aberrations, but to attempt

to elucidate the way in which a specific double-strand break, in this case a blunt-ended one, can be converted into chromosome aberrations. In addition, they will serve to provide further comparisons between AraC^R213 and AT cells.

Materials and Methods

Cell Culture Conditions

CHO-K1 cells and AraC^R213 cells were maintained in Ham's F12 (GIBCO) supplemented with 5% heat-inactivated (56°C, 55 minutes) FBS (fetal bovine serum) (Hazelton), 50 units/ml penicillin (Squibb), and 50ug/ml streptomycin (Lilly). The mutant was grown intermittently in 10⁻³M araC. Cells were maintained as monolayers at 37°C in 5% CO₂ at 95-100% humidity.

Restriction Endonuclease Treatment

Cells were synchronized by the mitotic shake-off method. It was estimated that 20 confluent flasks of 6x10⁶ cells/flask were needed if 5% were in mitosis at the time of shake-off. Mitotic cells were plated for one hour to allow progression into G₁ and then removed from the plate by trypsinization and counted with a Coulter counter. Cells were electroporated with 300 volts at a capacitance of 250μF (Gene Pulser, BRL) in cold phosphate-buffered sucrose solution at a concentration of 2x10⁶ cells/.7ml with 5 units of Rsa I. The controls were electroporated with the restriction enzyme storage buffer alone. Effective time constants were 29 to 31 milliseconds. Cells were replated in growth medium. Colchicine (1x10⁻⁷M) was added to the

cultures when rounded-up mitotic cells were visible under the microscope and the cells were harvested two hours later. CHO cells were fixed 24 hours after treatment and AraC^R213 cells were fixed at 24, 26, and 28 hours.

Slide Preparation

The fixation method followed the protocol of Korthor (1986). Cells were centrifuged, resuspended in .075 M KCl and incubated at 37° for 12 minutes. After centrifugation, the cells were resuspended in 4 ml of methanol and held at room temperature for 5 minutes. The cells were centrifuged for 30 seconds at medium speed in a clinical centrifuge and then resuspended in fixative of 3 parts methanol to 1 part glacial acetic acid. After 5 minutes the cells were centrifuged for 1 minute at medium speed. Cells were dropped onto wetted microscope slides. The slides were stained with Giemsa and 100 metaphases were scored per treatment group.

Results

The results given in Table 3.1 show a striking increase in aberration production in AraC^R213 over wild-type CHO cells when treated with 5 units of Rsa I. Three different sampling times were evaluated in the mutant to cover any variations in the cell cycle as a result of increased recovery time after electroporation. Cells analyzed at 24 hour, 26 hour, and 28 hour harvest times show the same increase in aberrations, indicating that they are similarly sensitive throughout G₁. The harvest time of 24 hours for the CHO cells was determined by the amount of time required for

Table 3.1. Aberration induction by Rsa I (5 units)

Cell Type	Treatment	Fixation Time	Aberrations per cell				Total Aberrations per Cell
			Chromosome-type		Chromatid-type		
			Deletions	Exchanges	Deletions	Exchanges	
CHO	Storage buffer	24 hours	0	0.01 ± .01	0.01 ± .01	0	0.02 ± .01
	Rsa I	24 hours	0.28 ± .05	0.31 ± .06	0.04 ± .02	0.03 ± .02	0.66 ± .08
AraC ^R 213	Storage buffer	24 hours	0.05 ± .02	0.13 ± .04	0.15 ± .04	0.03 ± .02	0.36 ± .06
	Rsa I	24 hours	0.62 ± .08	1.23 ± .11	0.30 ± .05	0.10 ± .03	2.25 ± .15
	Rsa I	26 hours	0.25 ± .05	1.03 ± .10	0.32 ± .06	0.37 ± .06	1.95 ± .14
	Rsa I	28 hours	0.44 ± .07	1.15 ± .11	0.36 ± .06	0.21 ± .05	2.16 ± .15

the synchronized population to reach mitosis i.e., when the cells began to round up. Other experiments done in this laboratory show that at 24 hours the treated cells contain predominantly chromosome-type aberrations which is indicative of G₁ treated cells.

AraC^R213 cells treated with Rsa I show a 3-fold increase in chromosome-type exchanges and a 2-fold increase in deletions as compared to wild-type cells. This high number of exchanges (24 hour fixation group), included 1 pentacentric, 2 quadracentrics, 7 tricentrics, 86 dicentrics, and 15 centric rings. The CHO cells contained only dicentrics. The mutant showed an increased frequency of chromatid-type aberrations. This phenomena is seen consistently in the mutant as would be expected if DNA damage is still unrepaired at the time of replication resulting in a high probability for the production of chromatid-type aberrations.

Discussion

AraC^R213, the repair deficient mutant, is a very informative tool for studies of mechanisms of DNA repair processes. Not only is the mutant hypersensitive to X-ray damage as are others such as *xrs5*, *xrs6* (Jeggo, 1982), *1rs1*, *irs2*, *irs3* (Jones, *et al.*, 1987) *irs1SF* (Fuller and Painter, 1988) and V-C4, V-E5, V-G8 (Zdzienicka and Simons, 1987) but it was selected as being ara-C resistant. This aspect of AraC^R213 provides a specific focus on the synthesis step of excision repair, even though the exact nature of the mutation is not yet clear.

The mechanism by which ara-C, a base analog of cytidine, inhibits repair synthesis and replication is discussed in detail in chapter 1. Briefly, the inhibition by ara-C during repair occurs at the resynthesis step of excision repair of DNA damage. The degree of inhibition is correlated with the extent of incorporation of ara-CMP into the DNA. Ara-C behaves as a poor primer end for chain elongation and breaks accumulate in the DNA in a concentration-dependent, time-dependent manner. Sequestration of DNA polymerases could result from stuttering by the polymerase after encountering ara-C moieties incorporated into the repair patch. Distortion of the helical structure due to ara-C incorporation could result in interference with hydrogen bonding (Fram and Kufe, 1985). The inhibitory action of ara-C can be reversed by excess deoxycytidine (Hiss and Preston, 1977).

It can be reasonably stated that the mutant is hypersensitive to X rays due to a deficiency in repair synthesis. This deficiency would be reflected in the repair of any lesion that requires a polymerization step. Preston (1980) demonstrated the enhancement of aberration yield after X-irradiation by ara-C in experiments with G_0 phase human lymphocytes. The cells were exposed to X rays and then incubated for 1, 2, or 3 hours with ara-C. The data showed that post-irradiation incubation in ara-C increased the aberration yield as compared to X rays alone and that the frequency of aberrations increased as time of incubation in ara-C increased over a 3 hour period. The conclusion was that the process inhibited by ara-C, the resynthesis step of excision repair of base damages, was involved in the production of chromosome aberrations-induced by X rays.

As would be expected, ara-C had very little effect on the repair of X-ray damage in the repair-deficient cell line, AraC^R213. This was demonstrated when G₁ and G₂ AraC^R213 cells were exposed to X rays and then incubated in ara-C post-irradiation (Chapter 2). The total number of aberrations with X rays alone was 2-fold higher than the total aberrations seen in wild-type cells but this frequency did not change with incubation in ara-C in contrast to wild-type cells, i.e. ara-C had no effect on aberration induction in the mutant.

Is ara-C an effective inhibitor of restriction enzyme-induced double-strand breaks, i.e. is a polymerization step involved in their repair? Inhibition of the repair of restriction enzyme-induced double-strand breaks by ara-C was demonstrated in experiments by Winegar and Preston, (unpublished) and Chung *et al.*, (1991). Restriction enzymes Rsa I, a blunt-end producing enzyme and Sau3A I, a 4-base overlap cohesive-end producing enzyme, were introduced into CHO cells by the method of osmolytic shock of pinocytotic vesicles. When ara-C was added prior to and after restriction enzyme treatment, an increase in exchanges and deletions was seen with Rsa I at a 2.8-fold and 2.4-fold increase respectively. With Sau3A I treated cells a 2-fold increase in exchanges and a 4.5-fold increase in deletions was observed. In contrast to the X-ray enhancement by ara-C where an accumulation of aberrations was seen over a 3-hour period, no additional enhancement of aberration induction was seen when ara-C exposure was longer than 1 hour. The reason presented to account for this difference was that the restriction enzyme performs the "incision step" that is normally rate-limiting for X-ray damage. Further evidence that ara-C potentiates restriction endonuclease-induced aberration production was demonstrated for G₁ CHO

cells when treated with Pvu II in the presence of ara-C; aberration yields were doubled (Natarajan and Obe, 1984; Obe and Natarajan, 1985). Thus, a portion of the breaks produced by restriction enzymes is clearly not directly ligated or "rejoined" as demonstrated by the fact that their repair is inhibitable by ara-C.

In summary, some portion of the repair of DNA damage induced by X rays or by restriction enzymes is inhibitable by ara-C, an inhibitor of DNA synthesis. Therefore, a mechanism that requires the action of a polymerase is involved in the repair of both types of damage. The CHO mutant, AraC^R213, and AT5BI cells are deficient in this mode of repair and when ara-C is used as an inhibitor of repair in these cell types very little effect is seen. The implications are that recombinational repair is involved, in part, in the repair of restriction enzyme-induced double-strand breaks.

There is a body of literature that supports an active role of recombinational repair of double-strand breaks in mammalian cells (Ayares, *et.al.*, 1986; Brenner *et.al.*, 1986; Lin *et.al.*, 1990; Jessberger and Berg, 1991). A model for double-strand break repair via a recombinational event has been presented by Szostak *et al.*, (1983) which suggests that a double-strand gap is initiated at the site of a double-strand break by the action of exonuclease and then the gap is repaired by using a strand from an uncut recombination partner as a template for DNA synthesis.

The factors that determine whether a double-strand break will be repaired by simple ligation or by a recombinational event are not understood at present. It has been shown that transcriptionally active genes are preferentially repaired in both human and rodent cells (Blackwell, *et.al.*, 1986; Thomas and Rothstein, 1989). The

constraints that chromatin structure might impose on recombination could determine the pathway chosen for repair (Painter, 1982). Lopez *et.al.*, (1989), found that the end structure at the break site can be a determining factor as to which method of repair the cell will utilize. Dephosphorylation of the extremities of the break prevents the ligation pathway but does not affect the recombination pathway: blunt or 5' protruding ends can be repaired by recombination, whereas 3' protruding ends cannot. This could be due to the polarity of the exonuclease and recombinase activities associated with the repair process. It has also been reported that the probability of a homologous recombinational event increases when the break occurs in areas of repetitive sequences such as *Alu* sequences (Winegar and Preston, 1988). Short stretches of perfect homology rather than long stretches of partial homology were found to govern the efficiency of recombination. Wahles *et.al.*, (1990) found that when a break was introduced into hypervariable minisatellite sequences, recombination was stimulated over and above that caused by double-strand breaks in other sites in the constructed plasmid. Efficient recombination in mammalian cells can be maintained with a homologous stretch of DNA as short as 134-232 bp (Waldman and Liskay, 1988).

A scheme commonly employed to study recombinational repair involves monitoring the reconstruction via recombination between two defective sequences of a gene encoding a selectable marker such as the thymidine kinase gene (*tk*) studied for example in mouse L^{tk} cells (Brenner *et al.*, 1985 and 1986; Song, *et.al.*, 1985). In order to study the influence of double-strand breaks on recombinational events, a cut was introduced very near one of the homologous recombining sequences with a

restriction enzyme and a 10-fold enhancement in extrachromosomal recombination was observed. With placement of a break in both of the homologous recombining sequences a synergistic effect was seen with a 100-fold increase in recombination.

AT cells are efficient at rejoining double-strand breaks (Lehmann, 1982) but they rejoin DNA double-strand breaks with a higher frequency of misrepair than normal cell lines (Cox et al., 1986). An approach similar to that described above was used to evaluate the fidelity of extra-chromosomal recombinational repair in AT cells. A unique restriction enzyme-induced double-strand break was introduced in the coding sequence of a selectable gene (gpt). A second dominant and selectable gene (G418^r) in the same vector was used as a control for DNA transfer and gene expression. If the double-strand break introduced into the non-selected gene was not rejoined or was rejoined incorrectly within the recipient cell, the gene is nonfunctional. The frequency of misrepair was 7- to 10-fold higher in the AT cells. One explanation offered was that in AT cells a protein factor/complex present in normal cells that concomitantly protects the termini from exonuclease activity and promotes their correct repair is altered or deficient so that sequence information is more frequently lost at the termini of a DNA double-strand break and, consequently this leads to a degree of misrepair. While this is a logical assumption, alternative explanations can be given. In light of the experiments done with restriction enzymes in combination with ara-C, it would be reasonable to assume that infidelity of polymerization during recombinational repair could lead to similar results. This propensity for misrepair was also observed in AT cells by North and Thacker (1989).

A similar conclusion can be drawn concerning the fidelity of "rejoining" of double-strand breaks in AraC^R213. Although the experiment presented here was not specifically focused on the study of recombination, information can be gleaned from the data available. When AraC^R213 cells were treated with a restriction enzyme, a 3-fold increase in exchanges (chromosome-type) was observed as compared to wild-type cells. These exchanges included dicentrics, tracentrics, quadracentrics, a pentacentric, and rings. A dicentric is indicative of incorrect rejoining of coincidentally repairing double-strand breaks in two different chromosomes in close proximity to each other. The probability of five chromosomes undergoing repair at the same time close enough in space for a pentacentric (five centromeres) to be formed is very low, but the probability is directly proportional to the time that the breaks remain open. If the polymerase involved in repair is error prone and incorrect bases are incorporated as a result, an increased exonuclease activity would be expected which could stall the polymerase complex at the site of the damage and consequently the breaks would be left open and accessible for exchange. Similarly, a defective polymerase could be inefficient in initiating or completing resynthesis during excision repair resulting in double-strand breaks being available for longer periods of time and able to misrejoin when repair is completed. A 2-fold increase in deletions (incomplete exchanges) was also observed. Polymerase complexes sequestered at sites of damage would not be available to continue the repair that requires DNA synthesis, and an incomplete exchange could occur. Although it is apparent that deletions are a result of non-joined ends, it cannot be determined if the ends have the "potential" to be ligated, i.e. have they been processed for ligation. An additional indication of

damage left unrepaired as the cell enters the S-phase after treatment in G₁ is the high number of chromatid-type aberrations seen in the mutant. Thus, it is suggested that the mutant, AraC^R213, is efficient in joining double-strand breaks but the incidence of "misjoining" is elevated above that observed in wild-type CHO cells.

In conclusion, the intent of this discussion is to provide additional support for the hypothesis that the mutant, AraC^R213, shares an analogous mutation with AT which is observed phenotypically as a repair deficiency. Not only are both cell lines hypersensitive to X rays, but they share the characteristic of poor fidelity in repair of restriction enzyme-induced double-strand breaks. It is feasible that the common factor that links ara-C resistance, X-ray hypersensitivity and infidelity of rejoining double-strand breaks is a mutated polymerase.

CHAPTER 4

COMPLEMENTATION ANALYSIS OF AraC^R213 X AT FUSION PRODUCTS

Summary

To investigate the possibility that the X-ray sensitivities seen in AT and AraC^R213 might be due to a mutation in analogous genes, cell fusion experiments were performed using cell lines X3/5^{hprt-} (CHO^{wt}), AraC^R213^{hprt-}, GM606 (normal human lymphoblastoid) and GM717 (AT lymphoblastoid). Putative hybrids were cultured as fibroblasts in HAT selective medium. Hybrids formed between AraC^R213^{hprt-} X GM606 and hybrids formed between X3/5^{hprt-} X GM717 both showed X-ray sensitivities that are indistinguishable from wild-type. In contrast, the hybrid formed between AraC^R213^{hprt-} and AT showed a radiosensitivity that corresponded to that for the original AraC^R213 mutant ie., the two cell lines lie in one complementation class. The results suggest that the gene defective in AraC^R213 is most likely homologous to the gene responsible for the phenotype of AT and that this gene is genetically recessive.

Introduction

A weight of experimental evidence presented in the previous chapters has demonstrated a considerable similarity between AraC^R213 and AT. It is appropriate, at this point, to examine the genetic similarity between these two cell lines by complementation analysis. Genetic analysis of AT cultured fibroblast cells has suggested the existence of at least five (A-E) different complementation groups (Jaspers *et al.*, 1985; Jaspers *et al.*, 1988). Two cell lines are said to fall into different complementation groups if, upon cell fusion, they give some endpoint which is regarded as normal, and they are assigned to the same complementation group if they do not. It could be either inter- or intragenic complementation or both that is being followed. The AT cell line used for fusion experiments presented here is a lymphoblastoid cell line, GM717. Lymphoblastoid cells have been used to study complementation of the radiosensitivity of AT, and four (1-4) different complementation groups were identified in seven unrelated AT patients (Chen *et al.*, 1984), but the relationship of these groups to the fibroblast groups has not been examined. GM717 belongs to group 3.

Recently, the gene for AT has been localized to chromosome 11q22-23 by linkage analysis of a 61-member Amish pedigree belonging to complementation group A (Gatti *et al.*, 1988). Preliminary analyses of a large group C pedigree appear also to support linkage to 11q23 (Shiloh *et al.*, 1989). Groups A and C comprise approximately 80%-85% of tested AT families (Jaspers *et al.*, 1988). Others have

reported the restoration of radiation resistance to group D AT fibroblasts by introducing a normal chromosome 11 (Komatsu *et al.*, 1990). Therefore, which AT cell line is used for complementation analysis is not an important issue at this point.

With these concepts defined, it is a logical experimental approach to consider complementation analysis of AraC^R213 and AT using radiosensitivity as a marker for complementation of the mutations. Features of the AraC^R213 cell line have been shown to be consistent with those seen in AT cells, i.e. response to ionizing radiation and enhanced frequency of chromosomal aberrations (spontaneous and X-ray induced). The data presented in this chapter establish a genetic similarity between AraC^R213 and AT.

Materials and Methods

Cell Culture Conditions

Chinese hamster ovary cells and AraC^R213 mutant cells were maintained in Ham's F12 (GIBCO) supplemented with 5% heat-inactivated (56°C, 55 minutes) FBS (fetal bovine serum) (Hazelton), 50 units/ml penicillin (Squibb), and 50ug/ml streptomycin (Lilly). The mutant was grown intermittently in 10⁻³M ara-C. Cells were maintained as monolayers at 37°C in 5% CO₂ at 95-100% humidity.

The normal human lymphoblastoid cells (GM606) and ataxia telangiectasia lymphoblastoid cells (GM717) were maintained in RPMI 1640 medium (GIBCO) supplemented with 15% FBS and antibiotic conditions as given above.

X-Irradiations

X-Irradiations were with a 320-kVp Gemini Industrial X-Ray Unit operated at 250 kV and 12 mA with 1mm aluminum filtration at a dose rate of 1 Gy per minute.

Fusions

The AraC^R213^{hgp^r-} cell line was selected as a spontaneous mutant by incubating AraC^R213 cells in hypoxanthine free Ham's F12 medium (GIBCO) with 10⁻⁵M thioguanine (TG)(Sigma) supplemented with 5% FBS. The wild-type CHO cell line X3\5 (hgp^rt deletion mutant) and the AraC^R213^{hgp^r-} cell line were fused with normal human lymphoblastoid cells, GM606, and with ataxia telangiectasia (AT) lymphoblastoid cells, GM717. X3\5 and AraC^R213^{hgp^r-} cells were plated 16-20 hours before fusions at 1x10⁷ cells per 100mm plate. The plates were washed with serum free F12 and all medium aspirated completely. The two human lymphoblastoid cell lines, GM606 and GM717, were centrifuged and resuspended in Hanks BSS (GIBCO) at a concentration of 5x10⁷ cells in 10 mls with 250 µl of a 1-mg stock of phytohemagglutinin (PHA) per ml (Wellcome Diagnostics). Five milliliters of lymphoblastoid cells (2.2 x 10⁷ cells) were added to each plate of fibroblasts. Plates were incubated for 15 min at 37°C. The lymphoblastoid cells, now sticky due to treatment with PHA, became adherent to the attached fibroblast cells and the excess solution was aspirated. Two milliliters of 50% polyethylene glycol 1450 (PEG) was added to the layered cells for 1 minute at room temperature. The plates were rinsed gently 3 times with F12 and incubated for 30 min at 37° C. The medium was aspirated and 10 mls of F12 with 5% FBS added to the plates overnight. The putative hybrid cells were trypsinized and plated for selection at 5x10⁵ cells/plate in F12

containing 10^{-7} M hypoxanthine, 10^{-5} M aminopterin, 4×10^{-5} M thymidine, (HAT medium) and 5% FBS. After 7 to 10 days, surviving colonies in each group were pooled and three days later were assessed for X-ray sensitivity by survival studies and chromosome analysis.

Results

Complementation Analysis

From the survival curves for hybrid cells, the mutation in AraC^R213, which is phenotypically expressed as resistance to ara-C and X-ray hypersensitivity, appears to be the same as that responsible for the increased sensitivity to X rays seen in AT cells (Figure 4.1). The X-ray hypersensitivity was maintained in the fusion product, AraC^R213 X GM717, since the LD₁₀ of 2.75 Gy is essentially the same as that for AraC^R213 (LD₁₀ of 2.5 Gy). In contrast, the hybrid CHO X GM717 has the same sensitivity as the CHO parent, and the AraC^R213 X GM717 hybrid has the same sensitivity as the normal GM606 cells. Thus, AraC^R213 and AT mutations are non-complementary, whereas, the mutant phenotype can be complemented by the wild-type hamster or human cells. The data in Figure 4.1 were duplicated in at least three separate but identical experiments, each done on different days.

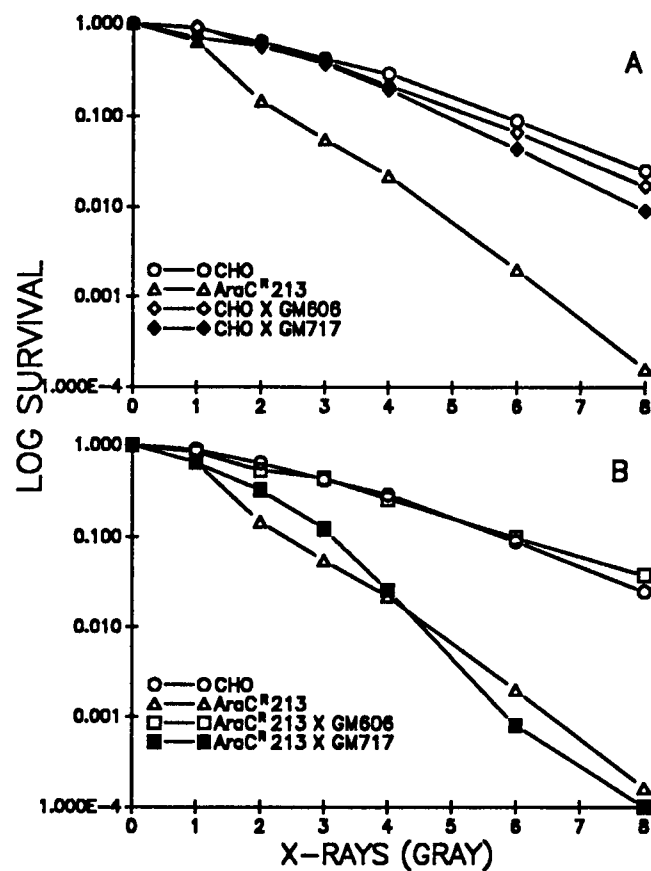


Figure 4.1. Survival curves for X-irradiated human/hamster hybrid cell lines: (A) CHO fused to normal human (GM606) or AT (GM717) cells, (B) AraC^R213 mutant fused to GM606 or GM717 cells.

Discussion

It is possible to correct mutations in Chinese hamster cells with human genes, and vice versa, suggesting a similarity of DNA repair processes in the two species. This has been demonstrated by the data presented here. Hybrids formed between AraC^R213 and normal human lymphoblastoid cells were isolated in which the X-ray sensitivity of AraC^R213 was corrected by complementing human chromosomal material. Likewise, the X-ray sensitivity of AT cells were complemented by wild-type hamster chromosomal material.

However, when the two "mutants" (AT and AraC^R213) were fused, there was no complementation of the X-ray sensitivity. It is therefore concluded that the genetic defect, the consequence of which is X-ray sensitivity, in AraC^R213 corresponds to that in AT. We are confident that this observation is not due to clonal expansion of a single hybrid cell that could have lost the chromosome of interest since the studies were carried out with heterogeneous populations approximately 9 days after the actual fusions.

It is also of note that a single mutation must underlie the observed radiosensitivity of AraC^R213 since there was no partial restitution of X-ray resistance in the AraC^R213 X GM717 hybrids.

These observations are extremely significant in the endeavor to isolate the "AT" gene. AraC^R213 is the only rodent mutant that has been shown by fusion experiments to be non-complementing with AT cells. Jones *et al.*, (1990) reported mutants *irs1*

and *irs2* to have similarities to AT cells, but the dissimilarities suggested that the primary defects are distinct. Their attempts to perform complementation analysis between *irs* mutants and AT were unsuccessful due to a failure to obtain satisfactory hybrids (Jones, 1987). An "ataxia-telangiectasia-like" Chinese hamster V79 cell mutant has been described previously (Zdzienicka *et al.*, 1989). This mutant does share some of the characteristics of AT, but no complementation analysis with AT cells was described. AraC^R213 appears to be the first rodent cell mutant isolated that does not complement the X-ray sensitivity of an AT cell line. Fusions with AraC^R213 cells and other AT cell lines and with other X-ray sensitive hamster mutants will provide additional information and allow for an assignment of complementation groups.

The primary criterion in evaluation and characterization of the repair deficient mutant was X-ray hypersensitivity as compared to the sensitivity seen in AT cells. Resistance to the inhibiting effects of ionizing radiation on the rate of DNA synthesis was not evaluated. Although radioresistant DNA synthesis is a pertinent characteristic of AT cells, variant forms of this disorder have been found with a rate of DNA synthesis comparable to normal cells after γ irradiation (Fiorilli, 1985). Further, the presence of radioresistant DNA synthesis (RDS) in cells of patients without AT strongly suggests that RDS is not causally related to the radiation sensitivity and DNA repair defect in AT patients (Young and Painter, 1989). This concept is further supported by the finding that AT cells transfected with normal human DNA have been recovered that are normal for radiation survival but still exhibit RDS (Lehmann *et al.*, 1986; Knapp and Painter, 1989).

In summary, the present data present a convincing picture that a single mutated gene causes the radiosensitive phenotype of AraC^R213 and the same gene is altered in AT. Indeed, AraC^R213 is a rodent model for AT which can facilitate the isolation of the "AT" gene.

CHAPTER 5

RESTORATION OF DEFICIENT REPAIR FUNCTIONS IN AraC^R213 CELLS BY SHEARED HUMAN DNA

Summary

The ara-C resistant, X-ray sensitive cell line, AraC^R213, was cotransfected by electroporation with sheared normal human DNA together with a selectable marker, the neo gene in pSV2neo. The colonies resistant to the antibiotic G418, and thus potentially radioresistant, were then exposed to 8 Gy of X rays once every 7 days for 4 weeks. Ten primary transformants were isolated that show wild-type sensitivity for killing by X rays, and one of these has been used to generate a secondary transformant. A human-specific oligonucleotide primer was used to amplify *Alu* sequences in the hamster transformants by PCR. Southern blot analysis of the PCR product using the human *Alu* fragment from BLUR8 plasmid verified the presence of human *Alu* DNA.

Introduction

The Chinese hamster X-ray sensitive (ara-C resistant) mutant, AraC^R213, can be used to isolate and characterize the DNA repair gene that is deficient in AT cells. The mutant has been shown to be deficient in the same repair gene as the AT cell line GM717 by a lack of complementation of X-ray sensitivity in the hybrid cell, AraC^R213 X AT (chapter 4). Repair deficient CHO cell lines have been used to isolate human UV-excision repair genes ERCC-1 (Westerveld *et al.*, 1984), ERCC-2 (Weber *et al.*, 1988) and ERCC-3 (Weeda *et al.*, 1990).

The general approach used to clone human repair genes is by DNA-mediated gene transfer using the calcium phosphate co-transfection method. Although the calcium phosphate method is known to be effective in establishing cell lines that carry integrated copies of the foreign DNA arranged in head-to-tail tandem arrays, the large amount of DNA taken into the cell by this method complicates identification of the gene of interest. Therefore, we chose the method of electroporation [application of brief, high-voltage electric pulses to the cells which leads to the formation of nanometer-sized pores in the plasma membrane (Neumann *et al.*, 1982)] to introduce foreign DNAs into the cells. Electroporation, in contrast to calcium phosphate-mediated transfection, gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA (Boggs *et al.*, 1986).

Since the repair gene of interest does not encode a selectable marker, the cells are simultaneously co-transfected with a mixture of two physically unlinked DNAs: sheared human DNA and a dominant selectable gene in an expression vector

(pSV2neo). Acquisition and expression of pSV2neo by the co-transfected cells is an indication that exogenous DNA has been taken into the cell. This vector is an SV40 hybrid plasmid vector (Southern and Berg, 1982) that contains the bacterial transposon Tn5 which encodes neomycin phosphotransferase whose expression confers resistance to aminoglycoside antibiotics (kanamycin, neomycin and G418) (Berg *et al.*, 1978). The selection for transformation of the mammalian cells relies on cell killing by G418. The structure of G418 resembles gentamicin, neomycin, and kanamycin but unlike these related compounds, G418 interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells (Davies and Jiminez, 1980). Once G418 resistant cells are obtained, a strategy for the isolation and efficient selection of repair-competent transformants is required. It was calculated from cell survival frequencies that an X ray dose of 8 Gy would kill most, if not all, of the cells that did not contain the human gene required to complement the deficient gene in the hamster mutant. After four rounds of X-ray exposure, any surviving cells should contain the gene of interest.

This approach enabled the isolation of 10 promising radioresistant clones one of which was used to generate a second-generation clone that displayed stable resistance to ionizing radiation. Further, partial characterization by Southern blot analysis showed the presence of human DNA in the primary and secondary transformants.

Materials and Methods

Cell Culture Conditions

AraC^R213 mutant cells were maintained in Ham's F12 (GIBCO) supplemented with 5% heat-inactivated (56°C, 55 minutes) FBS (fetal bovine serum) (Hazelton), 50 units/ml penicillin (Squibb), and 50 µg/ml streptomycin (Lilly) grown intermittently in 10⁻³M ara-C. Primary and secondary transformants were maintained in Ham's F12 (same antibiotics) with 5% FBS and 400 µg/ml (active concentration) G418. Cells were maintained as monolayers at 37°C in 5% CO₂ at 95-100% humidity. The human lymphoblastoid cells (GM606) lymphoblastoid cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 15% FBS and antibiotic conditions as given above.

Genomic DNA Isolation

To isolate large pieces of genomic DNA, the cells were first rinsed in ice-cold calcium free, magnesium free PBS. All reagents and samples were kept on ice at all times, and cell pellets were resuspended in 2 mls of Solution H (Jantzen *et al.*, 1986). To this a final concentration of .5% Triton X was achieved by adding 2 mls of a 1% solution of Triton X in Solution H and then mixing by inverting the tubes. This mixture was held on ice for 10 minutes and then pelleted in a refrigerated centrifuge at 2000-rpm for 10 minutes. At this point the pellet was half the original size and consisted of isolated nuclei. The previous step was repeated to wash the nuclear pellet. One

ml of T10/E10/N100 was added and nuclei were resuspended by pipetting gently. A 2X volume of preheated (65°) Hirt's solution (Hirt, 1967) was added to the nuclear suspension while mixing gently (mixture turns jelly-like). The salt concentration was adjusted to 100mM using 5M NaCl for optimal Proteinase K digestion conditions. Proteinase K was added to a final concentration of .1mg/ml and incubated at 50° overnight. RNase A (5mg/ml) was added to the suspension and incubation was continued for one hour. The salt concentration was adjusted to .5M. Phenol extractions were performed using an equal volume of phenol to each tube followed by chloroform extraction of the phenol. DNA was ethanol precipitated and resuspended in 500 μ l TE. A 1/2 volume of 7.5M NH_4OAc was added and then a second ethanol precipitation was performed. The expected DNA yield was 6pg/cell for a total of 2.7×10^8 pg.

DNA Transfection

High-molecular-weight human DNA (GM606 cells) isolated as described above was sheared to approximately 50 to 60 kb and used for cotransfection with a dominant marker, pSV2neo. AraC^R213 cells were trypsinized, counted with a Coulter counter and resuspended at a concentration of 3×10^6 cells/ml. Cells were rinsed twice in ice-cold electroporation buffer (phosphate-buffered sucrose solution) and .8 ml of the cell suspension was placed in an electroporation cuvette. Cotransfections were done with 5 μ g of human DNA and 1 μ g of EcoRI linearized plasmid by electroporation of the cells (Gene Pulser, BRL) with 300 volts at a capacitance of 250 μ F. Cells were plated at 5×10^5 cells per 100mm plate in Ham's F12 medium plus 5% FBS. After 24

hours G418 was added to a concentration of 400 μ g/ml (active concentration). Thereafter, the cells were maintained in the selective medium.

Isolation of Primary and Secondary Transformants

To select against the mutant cells that were not restored to wild-type repair of X-ray damage by exogenous DNA, the G418 resistant cells were irradiated once every 7 days with four treatments of 8 Gy of X rays. To estimate the dose necessary to kill all X-ray sensitive mutant cells but only a portion of cells with wild-type sensitivity, the following calculations were made. Theoretically, wild-type CHO cells grown for 7 days (12 divisions) would give 4096 (2^{12}) cells per colony. At a survival rate of .007 (taken from the survival curves) with 8 Gy of X rays, 28.7 cells with wild-type repair capacities would be expected to survive. The length of the cell cycle in the mutant cells, AraC^R213, is the same as the parental CHO cells and thus 4096 cells per colony would be expected after twelve divisions. However, the survival rate of the mutant at 8 Gy is only .0003 and only 1.2 cells would be expected to survive, some 20-fold difference from that calculated for the wild-type cells. Therefore, the procedure followed was to trypsinize the colonies and replate as single cells, irradiate with 8 Gy, culture for one week, trypsinize, replate, and irradiate through a total of four irradiations. The surviving colonies were isolated and cultured as individual clones for further testing.

The secondary transformants were generated by cotransfection of AraC^R213 cells with genomic DNA isolated from primary transformant PT 1.3 and the marker (10 μ g of PT 1.3 genomic DNA and .5 μ g pSV2neo). The G418 resistant colonies were

treated with X rays using the same procedure as given above. The surviving colonies were isolated as individual clones.

Survival Curves of Transformants

Four hours prior to X-ray exposure, cells were trypsinized and plated, (three 60mm plates per treatment). The number of cells (for cell lines PT 1.1, PT 1.2, PT 1.3, PT 2.3, PT 2.4, PT 4.2, PT 5.1, PT 5.2, PT 7.1.1, PT 7.1.3, PT 7.2.1, PT 7.2.2, PT 2B1, PT 2B3, PT 3B1, PT 3B3, ST 6.3 and ST 6.1) plated per dose was estimated such that if the cells were restored to wild-type, 100 cells per plate would form colonies after X-irradiation.

After 7 days incubation, colonies containing more than 50 cells were scored as the progeny of surviving cells. Relative cell survival was calculated as the ratio of number of colonies to number of cells plated, corrected for the survival in control, non-irradiated cultures.

X-Irradiations

X-Irradiations were with a 320-kVp Gemini Industrial X-Ray Unit operated at 250 kV and 12 mA with 1mm aluminum filtration at a dose rate of 1 Gy per minute.

PCRs and Primers

Two sets of conditions were used for polymerase chain reactions. The most favorable conditions with primers 125 and 126 were a total volume of 100 μ l containing 1 μ g of chromosomal DNA and primers at 1 μ M each in 10mM Tris-HCl pH 8.3 / 50mM

KCL / 4mM MgCl₂ / .001% gelatin / 200μM dATP / 200μM dCTP / 200μM dGTP / 200μM dTTP and 2.5 units of *Thermus aquaticus* polymerase (Perkin-Elmer Cetus) for 35 cycles with 94°C denaturation (1 min), 55°C annealing (45 sec), and 68°C extension (5 min) in an automated thermal cycler (Perkin-Elmer Cetus). The initial denaturation was 4 min at 94°C. The amplifications with primer TC-65 were performed in a 30μl reaction mixture of 6.7mM MgCl₂, 16.6mM (NH₄)₂SO₄, 5mM β-mercaptoethanol, 6.8μM EDTA, 67mM tris pH8.8, 10% dimethylsulfoxide with 1.5mM each deoxynucleotide, 66.7nM to 1.67μM primer and 4U Amplitaq. The reaction mixture was covered with 25μl paraffin oil and subjected to 30 cycles of 94°C/1min, 58°C/1min, 70°C/7min, preceded by 5 minutes at 94°C and followed by 13 minutes at 70°C, and then incubation at 4°C. The reaction products were then electrophoresed through 8% polyacrylamide gel for analysis (Rossiter *et al.*, 1991).

Primers for PCR were synthesized on an Applied Biosystems DNA synthesizer (model 391) PCR-Mate. After the deprotection groups were removed, the oligonucleotide was lyophilized, resuspended in 100-200μl of .5M NH₄OAc, vortexed briefly and centrifuged. The aqueous solution containing the oligonucleotide was removed leaving pelleted debris. Absolute EtOH was added to approximately .5ml total volume and the oligonucleotide was precipitated at -70°C for 10 minutes. The pellet was resuspended in 200 μl sterile water and amount determined by OD₂₈₀. The primer sequences were as follows: 125, ATGGTGATGCGTGCCTGGAAT; 126, AGATGGAGTCTCGCTCTGTC (Rubin *et al.*, 1980); TC-65, AAGTCGCGGCCGCTTGCA GTGAGCCGAGAT (Nelson *et al.*, 1989). The products of the amplification were analyzed by electrophoresis on 1.1% agarose gels.

Gel Electrophoresis and Southern Blots

Genomic DNAs (10 μ g) were digested overnight with *Eco*RI restriction endonuclease and electrophoresed for 20 hours on a .8% agarose gel. DNA was transferred to a nylon membrane (0.45 μ m pore size, Schleicher and Schull) using the Posiblot positive pressure transfer system (Stratagene) at 75mm Hg, for 30 minutes in 10X SSC. The membrane was dried completely (60°overnight) and prehybridized at 42°C overnight in 5X SSCP, 1X Denhardt's (Sigma), and 1% SDS 100 μ g/ml denatured salmon sperm DNA. Hybridization was performed in 5X SSCP, 1X Denhardt's solution, 0.1% SDS, 100 μ g/ml salmon sperm DNA, 1 Mm EDTA, 10% Dextran Sulfate, and 2-4x10⁶ cpm/ml of ³²P-labeled, (random prime, BRL) Nick column (Pharmacia) purified probe (preboiled for 10 minutes) at 65°C in a hybridization oven (HYBAID, Midwest Scientific) for about 20 hours. After hybridization, the blot was washed twice in 2X SSCP, 0.1%SDS: once for 20 minutes at room temperature and once at 65°C for 35 minutes. This was followed by a wash with 0.1X SSCP, and 0.1% SDS at 65°C for 35 minutes. The last wash was in 0.1X SSCP at room temperature. The washes were carried out in the hybridization bottles that came with the hybridization oven. The membrane was exposed to Kodak X-OMat film in a Kodak X-ray exposure cassette at -70°C.

The probe used for the Southern blot was a human *Alu* repeat isolated by a BamH1 digestion of the BLUR8 plasmid (Rubin *et al.*, 1980).

Results

Isolation of Repair-proficient Primary Transformants

To insure the isolation of several unique primary transformants, each group of co-transfected cells was individually maintained. As demonstrated in the schematic representation (Figure 5.1), after 4 treatments with X rays, G418-resistant AraC^R213 cells (cuvette 6) were all killed. But in several of the groups, (for example, cuvette 1), the number of surviving colonies continued to increase, i.e. survival frequencies appeared to be at wild-type levels. Survival curves of isolated colonies show 10 primary transformants (Figure 5.2) with restored repair. Some are comparable to wild-type but others showed an intermediate survival rate. For example, the clones numbered 1.3, 1.2, and 2.4 all show increased survival with an LD₁₀ of 5.75 Gy (parental wild-type LD₁₀ is 6 Gy) while clone 5.2 showed an LD₁₀ of 3.5 Gy.

Isolation of Secondary Transformants

Two secondary transformants (ST 6.1 and ST 6.3) were generated by cotransfection of AraC^R213 cells with DNA from PT 1.3 genomic DNA and pSV2neo. The isolated G418 resistant cells were then subjected to the same procedure as described above. Figure 5.3 shows the log survival of ST 6.3 with an LD₁₀ of 4.5 Gy that is similar to that of PT 1.3 (5.75 Gy) and quite different from that of AraC^R213 mutant cells (2.5 Gy). The survival curve of ST 6.1 is generally similar to that of ST 6.3.

COTRANSFECTION BY ELECTROPORATION

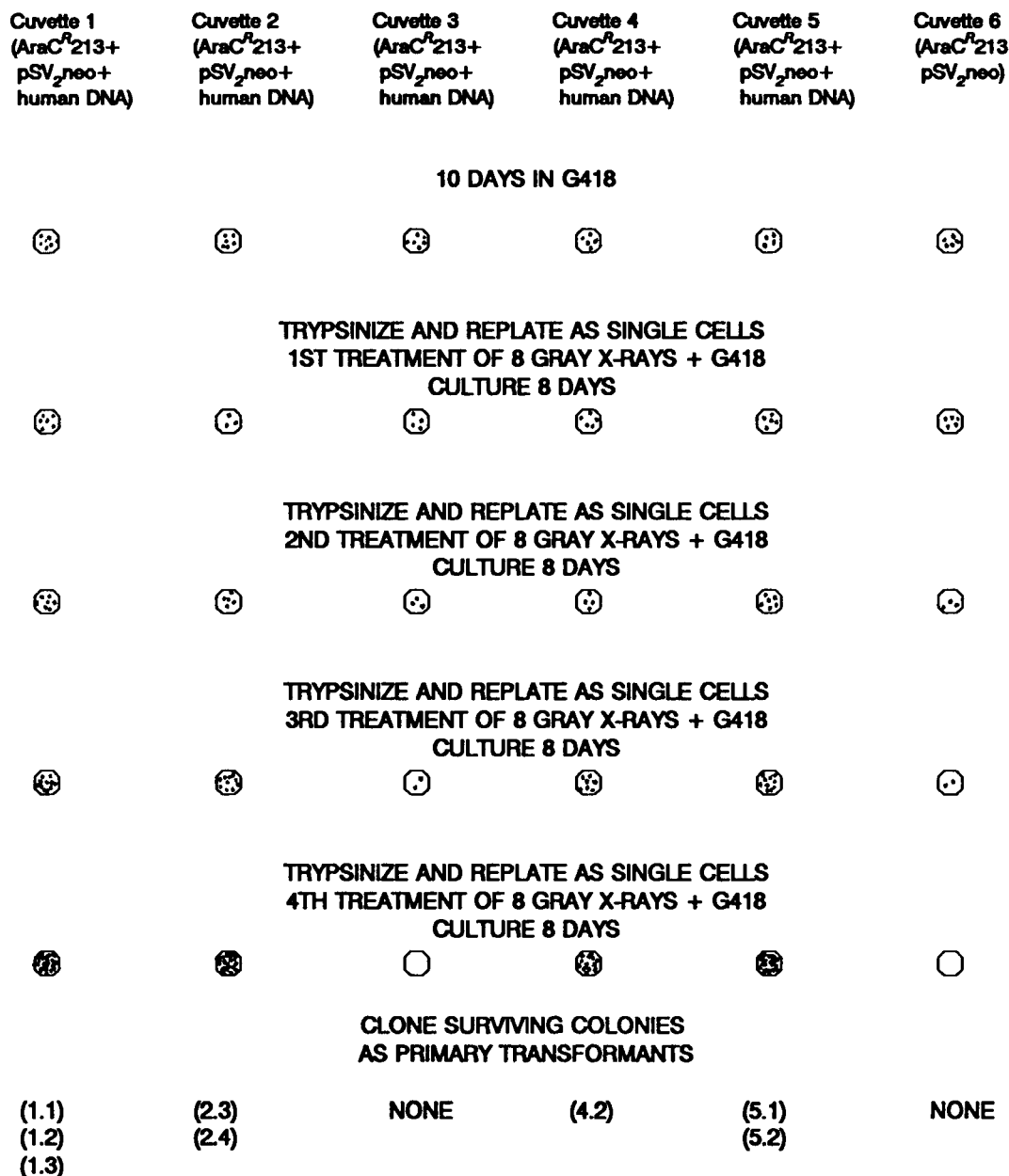


Figure 5.1. Schematic representation of procedure used to isolate primary and secondary transformants.

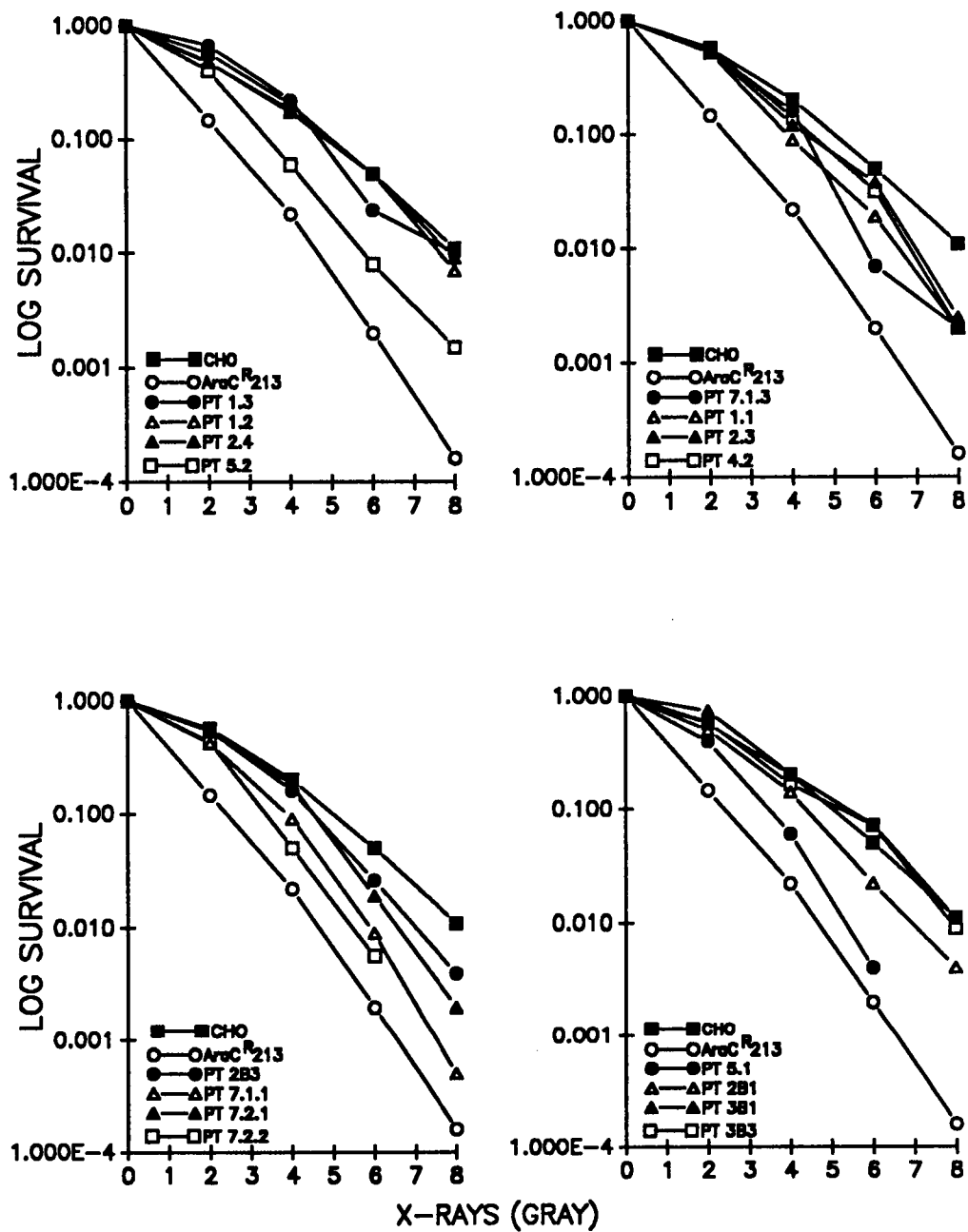


Figure 5.2. Survival curves of X-irradiated primary transformants (PT).

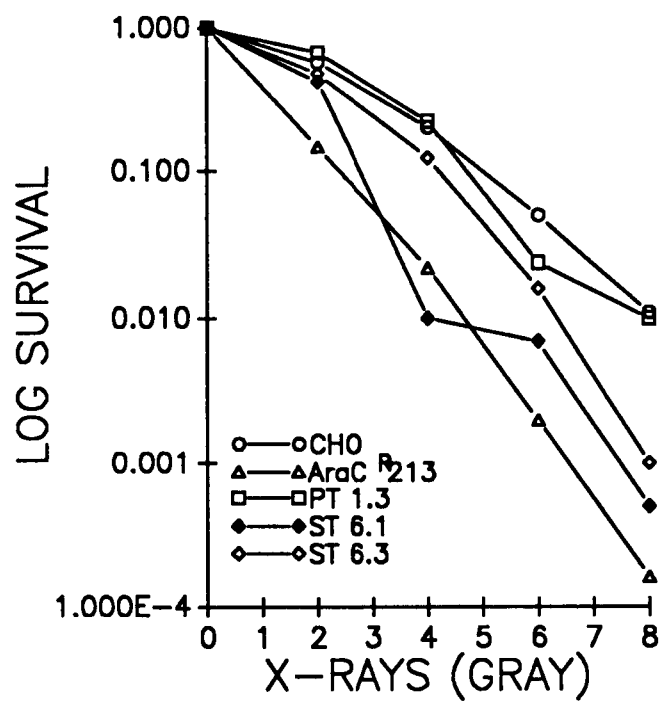


Figure 5.3. Survival curves of X-irradiated secondary transformants (ST).

Southern Blot Analysis

Southern blot analysis of genomic DNA digests of the primary transformants probed with whole human DNA or human-specific *Alu* failed to show any hybridization. These results could be accounted for by the electroporation technique used for cotransfection whereby only a small amount of DNA enters the cell and by the degree of specificity of the probes used. Also, the cells were maintained in culture for weeks through the selection procedures in which time nonessential foreign DNA could be lost.

We, therefore, turned to a different method to verify the presence of human sequences in the genome of primary and secondary transformants. The method is based on the preferential PCR amplification of human DNA in rodent/human hybrids using primers specific for the human *Alu* repeat element. Two different primers were used. The first primer sequence (T-65) was taken from a paper by Nelson *et al.*, (1989) which was assumed to be a specific human sequence, but false priming in the AraC^R213 cells failed to give definitive results. Lane 3 of Figure 5.4 shows PCR amplification of sequences in the hamster genome with this primer. Although, PT 1.3 does show a unique band of about 340bp, this band is not seen in the GM606 human cell. There is a common band between the human cells and PT 1.3 cells of approx. 220bp that is not seen in the mutant cell. Suffice it to say, that these data are inconclusive.

These results prompted us to synthesize different primers for PCR amplification using sequences from the *Alu* fragment of the BLUR8 plasmid. The agarose gel,

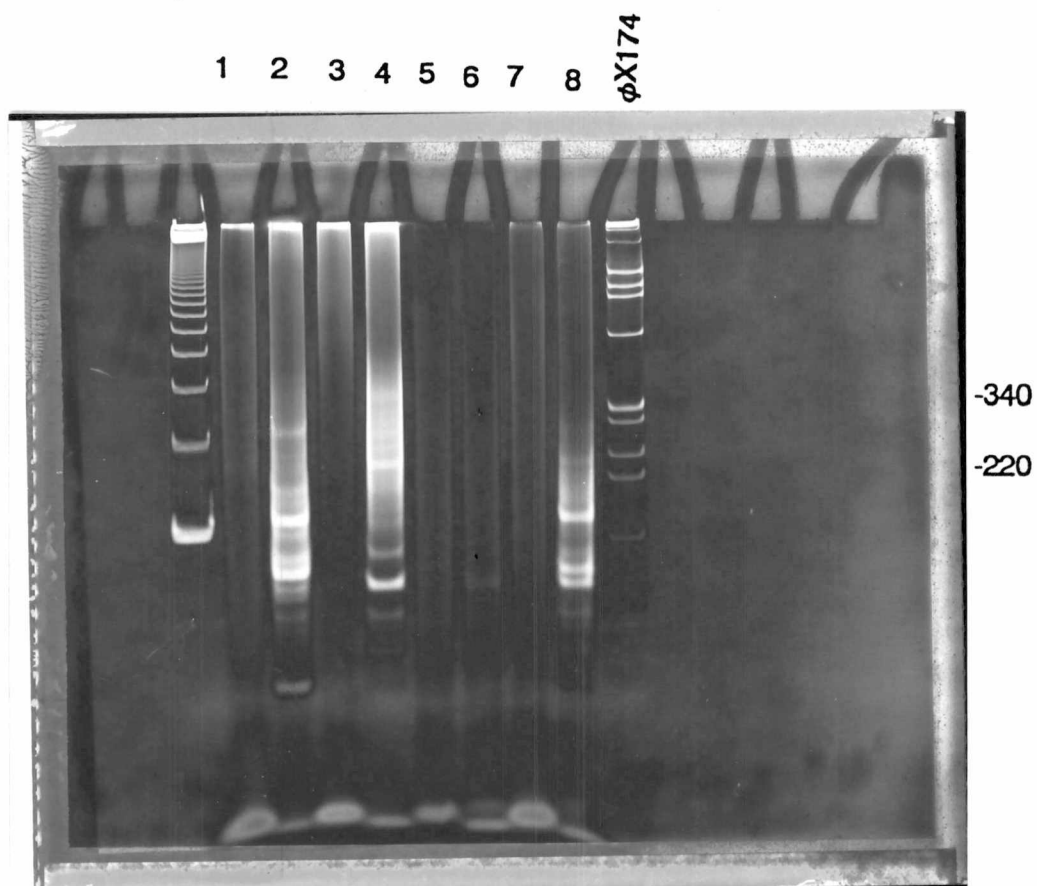


Figure 5.4. *Alu* primer TC-65 PCR products separated on an 8% acrylamide gel. (lane 2, AraC^R213; lane 4, PT 1.3; lane 6, ST 6.3; lane 8, GM606).

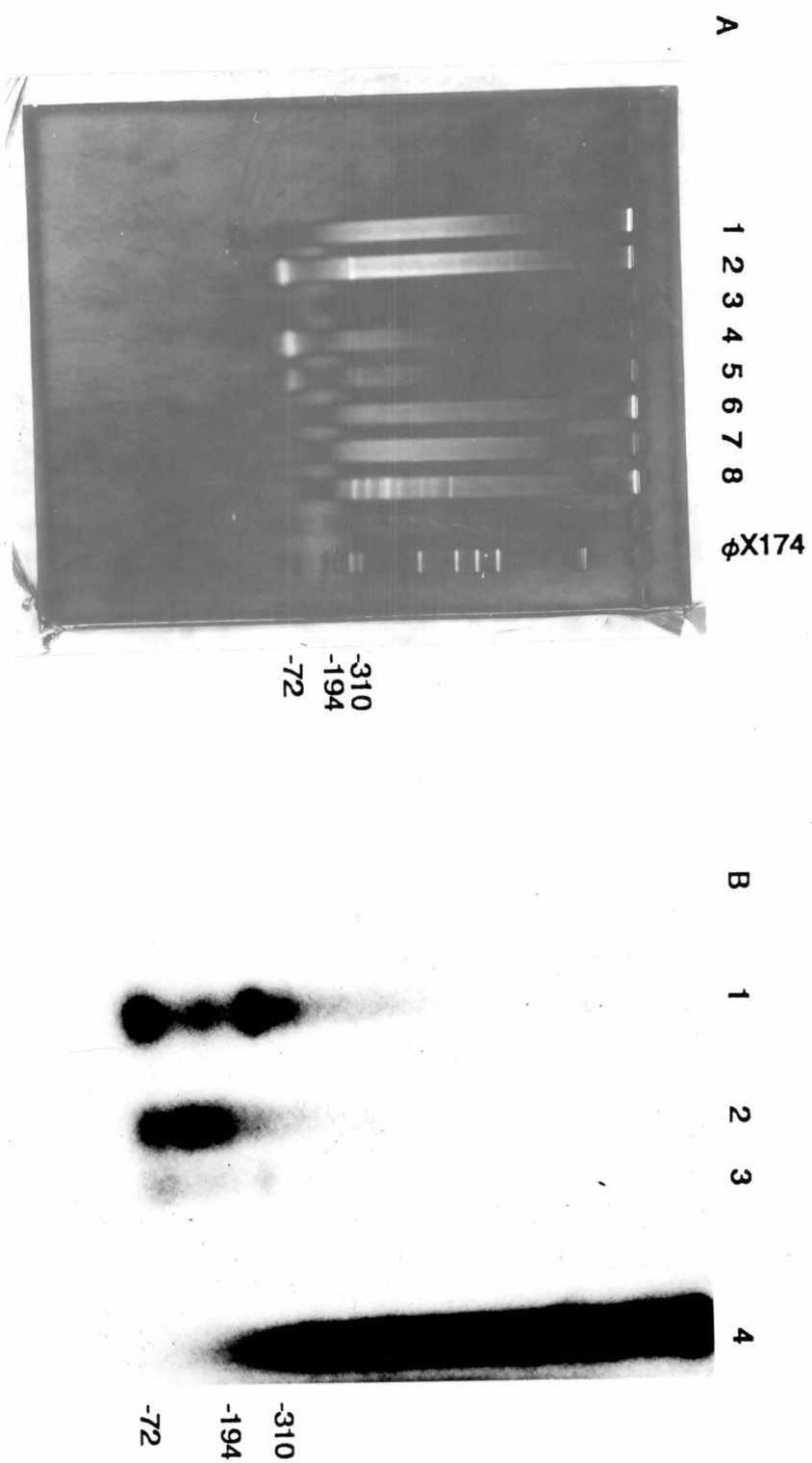


Figure 5.5. A) *Alu* primers 125 and 126 PCR products separated on 1.1% agarose gel before transfer for Southern blot transfer: lane 2, ST 6.3; lane 4, PT 1.3; lane 5, *AraC*^B213; lane 8, GM606. B) Southern blot analysis: lane 1, ST 6.3; lane 2, PT 1.3; lane 3, *AraC*^B213; lane 4, GM606.

Figure 5.5A, shows bands in the GM606 (lane 8), AraC^R213 (lane 5), PT 1.3 (lane 4), and in ST 6.3 (lane 2), but the Southern blot analysis of these PCR products using a human specific *Alu* probe (Figure 5.5B lane 2) shows a unique band in PT 1.3 cells of 180bp not seen on the agarose gel. The secondary transformant, ST 6.3 cells, shows a similar band although less intense as expected for a secondary transformant (Figure 5.5B lane 1).

Discussion

DNA-mediated gene transfer with the ara-C resistant, X-ray sensitive AraC^R213 mutant has permitted successful transfer of human DNA resulting in a repair-proficient phenotype in the hamster cell. It is particularly interesting to note the high interspecies conservation of encoded proteins that must exist between hamster and human. The human excision repair genes ERCC-1 (Westerveld *et al.*, 1984), ERCC-2 (Weber *et al.*, 1988), ERCC-3 (Weeda *et al.*, 1990), and ERCC-6 (Troelstra *et al.*, 1990) have all been cloned by using UV-sensitive, laboratory-induced CHO mutants. Further, the ERCC-1 gene product was found to be homologous to the *S. cerevisiae* excision repair protein *Rad10* (Van Duin *et al.*, 1986) and to parts of the *E. coli* UvrA and UvrC proteins (Van Duin *et al.*, 1988). ERCC-2 has been shown to be very similar to yeast *Rad3* (Weber *et al.*, 1990) and was shown to specify a 5' - to -3' DNA helicase (Sung *et al.*, 1987). One motivation for obtaining these excision repair genes was to isolate the correcting factor/s for the human hereditary disease Xeroderma pigmentosum (XP), but cell fusion experiments between the UV-sensitive hamster

mutants and human XP fibroblast cells showed no overlap between the two repair deficient groups (Stefanini *et al.*, 1985). ERCC-3 is the only gene reported to be involved in XP and it has been shown also to play a role in Cockayne's syndrome (Weeda *et al.*, 1990).

Kapp and Painter (1989) are in the process of isolating the "AT" gene by transfecting AT cells with a normal human DNA cosmid library and a selectable marker. The transformed cell line derived from AT5BI cells has acquired resistance to radiation and this resistance was transferred a second time by fusion of heavily irradiated primary transfectant cells to a second set of recipient AT5BIVA cells. The problem is, however, that the transformants contain at least four different fragments of DNA derived from the cosmid library. This will necessitate cloning out the desired sequence in the presence of three or more other neo-attached sequences that do not carry the desired information. There is no assurance that the transforming human DNA is still attached to the marker DNA.

Another approach for isolating human DNA repair genes was taken by Arrand *et al.*, (1989). A hamster DNA repair gene was isolated by cosmid rescue after transfection of a XP cell line. Complications with this approach are that most human cell lines have lower transfection frequencies (10 times lower than hamster cells and stably integrates 20-200 times less foreign DNA) and continued selective pressure is required for the cells to retain foreign integrated DNA (Hoeijmakers *et al.*, 1987). As was also the case with Kapp and Painter experiments, this has necessitated very large and complex studies. Also, analysis of the cloned hamster sequence for isolating the

human homolog would be hindered by any point mutations or major rearrangements of the gene.

The approach we have used appears to be the most efficient. Although, the small amount of DNA that is transferred into the cell by the electroporation method makes verification of human DNA in the hamster cells a more difficult task, it is still more desirable because there is less human DNA to interfere with the identification of the gene of interest. The initial Southern blot analysis of enzyme digested transformant DNAs showed no hybridization with a human-genomic DNA probe or with a human-specific-*Alu* probe. This problem was overcome by utilizing the PCR technique. It appeared plausible that the PCR method of amplifying DNA sequences could simplify the problem. Indeed, by Southern blot analysis of the PCR product after synthesis with a human-specific *Alu* primer, we were able to detect human-specific sequences in the primary and secondary transformants. Further applications of this technique will be investigated, but theoretically, if the extension product is long enough, it will include the sequence complementary to the gene of interest at the 3' end of the target sequence. If this target sequence has another human *Alu* repeat element integrated in the opposite orientation, the human DNA between would be amplified. The PCR product could be gel purified and then used as a probe to identify restriction fragments of genomic DNA containing the gene.

To examine whether the transfected DNA repair gene happened to be close to the cotransfected dominant marker, we tried linked secondary transfection with genomic DNA from PT 1.3 without the addition of extra pSV2neo. No G418 resistant colonies were obtained. This indicates that, in this particular clone, there is no

physical linkage between the repair gene and the dominant marker or that, because of low transfection frequencies, the number of cells used was insufficient to identify an actual transformant. There is the possibility that secondary transformants generated with the other nine primary transformants will have the dominant marker linked to the "repair" gene.

The stringent screening procedure of repeated X-irradiations makes us confident that human DNA isolated from these cells will be appropriate for characterizing the putative human DNA gene. We are confident that once secondary transformants are generated from the nine existing primary transformants, we will have a comprehensive representation of the gene we are attempting to isolate.

EPILOGUE

The proposed hypothesis that "CHO cells selected for ara-C resistance and shown to be X-ray sensitive could potentially be deficient in the same repair function as AT cells" has been demonstrated to be a correct. The ara-C resistant, X-ray sensitive mutant cell line, AraC^R213, was shown to be comparable to AT by several parameters: hypersensitivity to cell killing by X rays, normal background SCE, extended S-phase of the cell cycle, and hypersensitivity to X rays as assessed by increased frequencies of chromosome aberrations in both G₁ and G₂ phases of the cell cycle. The fact that the AT lymphoblastoid cell line (GM717) and AraC^R213 are in the same complementation group presents a convincing picture that AraC^R213 cells and AT have a defect in the same repair function. Isolation of the AT gene is imminent through the utilization of this rodent cell model. In fact, the ten primary transformants that have been isolated should provide a system for isolation of the entire human gene that is defective in AT.

The function deficient in AraC^R213 cells and AT cells is not clear, although, some speculations can be made. Resistance to ara-C has been acquired in human leukaemic cells during therapeutic treatment. One mechanism of resistance is correlated with an increase in intracellular concentrations of cytidine deaminase (Steuart and Burke, 1971). In man, ara-C is rapidly deaminated to produce arabinosyl uridine which has no therapeutic activity (Creasey *et al.*, 1966). Cells deficient in deoxycytidine deaminase have been selected at ara-C resistant (de Saint Vincent *et al.*, 1980). This class of variants have expanded dCTP and CTP pools and as a result

are thymidine-dependent. The inhibition of UDP reduction by CTP accounts for thymidine dependence imposed on the dCMP deaminase-deficient lines. Resulting pool imbalances could result in deficient repair processes (Weinberg *et al.*, 1981).

Characterization of a series of ara-C resistant mutants isolated from mouse teratocarcinoma cells showed that mutants isolated as highly resistant to ara-C ($2 \times 10^{-6} \text{M}$) were deficient in deoxycytidine kinase, their resistance being due to an inability to convert ara-C into its active form, ara-CTP (Aizawa *et al.*, 1983). These mutants were not cross resistant to aphidicolin. In contrast, mutants isolated as moderately resistant to ara-C ($1.5 \times 10^{-6} \text{M}$), and later shown to be equally resistant to aphidicolin, were shown to have altered dNTP pools (thymidine sensitivity), not an altered DNA polymerase- α as a mechanism of resistance (Aizawa *et al.*, 1983). Pool imbalance was thought to be a result of a mutated ribonucleotide reductase.

DNA polymerase α from leukemic cells of ALL patients that were resistant to ara-C was found to have altered K_m values for ara-C. These results indicate that DNA polymerase α from ALL blast cells has a decreased affinity to the arabinoside analog of dCTP which conferred resistance to ara-C in these cells (Tanaka and Yoshida, 1982).

It is possible that AraC^R213 cells and AT cells are resistant to inhibition by ara-C as a consequence of any of the mechanisms mentioned above and assays for the activity of these various enzymes would be an obvious direction for future experiments. We propose that AraC^R213 cells are more likely to be deficient in deoxycytidine kinase or have an altered polymerase than to be defective in cytidine deaminase activity or in ribonucleotide reductase activity. If resistance was acquired

through an increased deoxycytidine deaminase activity, the ara-C would simply be deaminated and one would not expect the cells to be X-ray sensitive as a consequence, therefore, this is probably not the case in AraC^R213 cells. If AraC^R213 cells are deficient in deoxycytidine deaminase, they would be thymidine-dependent since the concentrations of dCTP and dTTP are reciprocally related. We assumed that the increased sensitivity to ³H-thymidine seen in the aberration induction experiments (chapter 2) was a result of damage from beta rays, although, the sensitivity might be related to thymidine concentration. If the concentration was high before the addition of ³H-thymidine, the excess would only compound the negative effects of the mutation in the cell. Experiments with radioactively labeled ara-C (data not presented) showed that although ara-C enters the cell, it is not being incorporated into the DNA. If the deoxycytidine kinase is defective, the consequences would be 1) higher dTTP pools and 2) ara-C would not be utilized by the cell since the salvage pathway is required for utilization of ara-C via deoxycytidine kinase activity. Imbalance of dNTP pools has been examined in AT cells which showed a 20-fold increase in dTTP concentration (Kenne and Akerblom, 1990). Suffice it to say, these are only speculations, but our first effort will be to examine the cytidine kinase activity of these cells. If this proves to be normal, then the defect in the cells is most probably in a polymerase activity. The mammalian polymerases have been characterized, therefore, it would be possible to examine AraC^R213 cells or AT cells for alterations in protein structure. Of course, these experiments will be conducted in conjunction with our continued effort to isolate the human gene deficient in AT.

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VITA

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