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THE ROLE OF PROTEIN SYNTHESIS IN THE REPAIR
OF SUBLETHAL X-RAY DAMAGE IN A MUTANT CHINESE
HAMSTER OVARY CELL LINE

M.J. Yezzi
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ABSTRACT

A temperature-sensitive mutant for protein synthesis, CHO-TSH1, has been compared to the wild-type cell, CHO-SC1, in single- and split-radiation-dose schemes. The cells were normally grown at 35°C, but for purposes of inducing the temperature-sensitive phenotype, a temperature of 40°C was used. When the exponentially growing TS mutant and the wild-type cells were treated at 40°C for up to 2 hrs prior to graded doses of X rays, the survival curves were identical and were the same as those obtained without heat treatment. If the cultures were incubated at 40°C for 2 hrs before a first dose and maintained at 40°C during a 2 hr dose fractionation interval, repair of radiation damage was reduced in the mutant compared to the wild type. These observations implied that a pool of proteins was involved in the repair of sublethal X-ray damage. However, if repair was measured by the alkaline-unwinding technique under the same time and temperature schemes, no difference in the kinetics of DNA strand rejoining was observed. Misrepair processes may permit restoration of DNA strand integrity but not allow functional repair.

The effect of diminished repair under conditions of inhibition of protein synthesis was found to be cell-cycle dependent in survival studies with synchronized mutant cell populations. Repair was found to

be almost completely eliminated if the temperature sequence described above was applied in the middle of the DNA synthetic phase (S-phase-10 hours post-mitosis). Treatment of cell populations in the middle of G₁-phase (2 hours post-mitosis) yielded repair inhibition comparable to that observed with the asynchronous cells.

Distinct perturbations in the cell-cycle progression were noted following heat alone or heat with radiation. Using ³H-TdR pulse labelling and autoradiography, a delay in the progression of synchronized G₁-phase and S-phase cells was demonstrated after inhibition of protein synthesis. In addition, treated S-phase cells showed a transient increase in the percent labelled cells after the cells were returned to their normal growth temperature of 35°C. This observation was suggestive of an unusual pattern of DNA synthesis during the recovery period.

Split-dose experiments were done using pre-incubation with cycloheximide to chemically inhibit protein synthesis. WT cells and TS cells were treated with cycloheximide at 35°C for 2 hrs before a first dose and during a 2 hr dose fractionation interval. Under these conditions both cell lines showed a reduction in the repair of sublethal damage comparable in magnitude to that observed in the TS cells when they were treated with 40°C. Both the chemical and thermal inhibition of protein synthesis substantiate its necessity for the repair of sublethal damage.

Conrad S. Tobias

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CHAPTER I

INTRODUCTION

PERSPECTIVE

Soon after X rays began to be used clinically, it was recognized that their biological effectiveness was usually reduced if lower-dose rates were used or if the total dose was delivered in fractions rather than as a single exposure. Radiobiological investigations of dose fractionation helped to elucidate the sparing effect that was observed. Elkind and Sutton (1959) were the first to examine this phenomenon in mammalian cells. Their work led to an interest in understanding the biochemical nature of the sparing effect that had been observed on a cellular level. Many investigators have undertaken studies to examine the biochemistry involved with any available tools. Over the past decade mutant cell lines with specific biochemical defects have become available. The objective of this thesis is to reexamine the role of protein synthesis in the sparing effect observed during a dose fractionation scheme with a mutant cell line.

GENERAL REVIEW

A. Radiation survival

Biological effects of a perturbing agent are usually quantitated as a function of the dose of the agent. The term dose used in a radiobiological sense is defined as the absorbed dose, that is the

energy absorbed per unit mass. The System International (SI) unit of dose is one joule per kilogram and this is called one Gray (abbreviated Gy). An older unit of dose, the rad, is defined as an energy absorption of one hundred erg per gram. One Gray, therefore, is equal to one hundred rad.

The biological effects due to a dose of radiation can be many and varied. They can include decreased enzyme activity, reduced infectivity of virus or phage, inhibition of DNA synthesis, chromosome aberrations, mutations and cell killing to mention a few. The term cell killing can apply to two different aspects of cultivated mammalian cells. One is a loss of metabolic activity with subsequent loss of cellular function leading to "interphase death", and the other is loss of the cell's ability to produce viable progeny capable of continued reproduction. The expression "reproductive death" has been used to describe the loss of capacity for sustained proliferation.

A radiobiological cell survival curve is a dose-effect relationship between reproductive death and radiation dose. A way to measure reproductive death is to measure the fraction of cells that survive a given exposure. A survivor is defined as a cell that can produce a colony of at least a minimum number of daughter cells, usually 50 or 60. Dose-effect curves of this type for bacteria and fungi have been known for quite some time. However the use of mammalian cells in tissue culture to measure cell survival versus radiation dose is relatively recent (Puck and Marcus, 1956). The usual convention in representing a cell survival curve is to plot surviving fraction on a logarithmic scale and dose on a linear scale. Mammalian cells exposed to low linear energy transfer (LET) radiation commonly have an initial low dose region

within which a smaller proportion of cells is killed per unit dose than after higher doses. Survival curves representing this type of cell killing have the appearance of a "shoulder", followed by a straight or nearly straight portion of the curve.

Dose-effect curves that seem to show a threshold effect have been explained in several ways. One explanation was to consider the idea that damage must be accumulated before the lethal effect is expressed. The damage that is presumed to accumulate without killing the cell is called "sublethal damage". Consistent with this interpretation is the idea that there may be targets in the cell requiring more than one hit for inactivation or that there are multiple targets each requiring one or more hits for inactivation. The concept of target theory has been in use in radiation biology for a number of years (Lea, 1956; Elkind and Whitmore, 1967). Target theory has shown itself to be simple in description and allows comparison between survival curves because they can be characterized by a small number of parameters. The form of the theory that is in general use today is referred to as the multitarget-single-hit (MTSH) model. The straight or nearly straight portion of the survival curve can be extrapolated to the logarithm of surviving fraction axis. The intersection point is designated N , the extrapolation number, which corresponds to the number of targets. The slope of the straight portion of the curve is used to identify another parameter, defined as $D_0 = -1/\text{slope}$. D_0 can be thought of as a measure of the sensitivity of the targets. The form of the MTSH model predicts that as the dose approaches zero, the survival curve will approach a line with zero slope. Few cell survival curves have that property.

Most of them display a survival curve that approaches a line with negative slope as the dose approaches zero.

There are other proposals, however, based on the idea of targets and hits that have subsequently been offered as explanations for the observed survival curves. One of these proposals is referred to as the linear-quadratic (LQ) model. It too has the appeal of a simple mathematical form with a small number of parameters. This equation relates the logarithm of surviving fraction to a polynomial in dose. Specifically there are two terms; one linear in dose and the other quadratic. Several attempts to provide a biological explanation for the linear-quadratic equation have been put forth (Kellerer and Rossi, 1972; Chadwick and Leenhouts, 1973). The LQ model is better suited to describe the shape of most survival curves at low doses because mathematically the low-dose approximation of the equation gives a line of negative slope.

An alternative group of models to explain a threshold or shouldered survival curve is based on the idea that cells have a mechanism for repair that becomes less effective as the dose increases, until it ceases to function. This description was suggested by Powers (1962) and modified by Green and Burki (1972) in their repair-saturation model. More recently another idea referred to as the repair-misrepair model (Tobias et al., 1980) has been proposed to explain the shape of cell survival curves. These models tend to be more complicated biologically and mathematically requiring three parameters to describe the phenomena which result in a survival curve of specific form.

B. Repair

1. Cellular

Elkind recovery

For many years it has been known that a variety of biological effects of radiation diminish as the dose rate is decreased or as the interval between two fractionated doses is increased (Lea, 1956). Using the dose fractionation method with mammalian cells in vitro, Elkind and Sutton (1959) demonstrated that these cells can recover from radiation-induced damage. Their experiment showed that more cells survive an exposure to a given total dose of radiation if the dose is divided into two fractions spaced at variable time intervals. To explain this phenomenon one can go back to the idea of damage accumulation presented by the MTS model of the survival curve. A given dose of radiation can hit all targets in some cells and those cells will be killed. Likewise radiation can spare the other cells by damaging just a few of the targets. That is, the surviving cells may be sublethally damaged. If enough time is allowed to pass before another radiation dose is given, some of the sublethal damage is repaired and therefore more cells will survive the second exposure. This recovery process is often referred to, in the literature, as repair of or recovery from sublethal damage. It is also called Elkind recovery. Since there are other models and theories which attempt to account for the shape of the survival curve, it is less confusing and more accurate to define this repair process without reference to a particular model of survival. Elkind recovery is then operationally defined as the increased cell survival observed when a total dose of radiation is divided into two fractions separated by varying time intervals.

The curve of survival versus time between the dose fractions, that Elkind and Sutton observed, and others have subsequently seen for a variety of mammalian cells *in vitro* and *in vivo*, has two distinct components. The first feature is an initial rise in survival which reaches a maximum in 2 to 6 hours, and a subsequent dip in survival reaching a minimum value from 4 to 11 hours. The nature of the phenomenon behind these observations is illustrated in the work of Sinclair and Morton (1964), and Elkind et al. (1965). Elkind and co-workers observed that if the temperature of incubation for the interval between the doses was kept at 37°C they saw the rise and subsequent dip in survival. However if the incubation temperature was kept at 24°C the initial rise in survival was observed but the subsequent dip was eliminated. These observations suggest that the initial rise is due to repair of radiation damage, from the first dose. This repair goes on regardless of the incubation temperature used. The dip in survival can be accounted for by considering the partial synchrony in a heterogeneous population of cells that results due to the first dose of radiation. Those cells in the more radiosensitive phases of the cell cycle are damaged lethally by the first dose leaving the more radioresistant cells less damaged. As time goes on these radioresistant cells progress to more radiosensitive phases of the cell cycle and sustain more lethal damage with the second dose. This change in radiosensitivity can be great enough to counteract the effects of the repair that have occurred after the first radiation exposure. If cell progression is inhibited by holding the cells at 24°C, the response of the movement of cells through the cycle is avoided and no subsequent dip in survival is observed. Repair goes on in all stages of the cell cycle

as demonstrated by Sinclair and Morton (1964) and the asynchronous response is a composite effect of repair and progression effects for cells at different stages of the cell cycle at the time of first irradiation.

Another type of experiment that illustrates Elkind recovery is produced by giving a first dose then waiting for a time interval before giving a second series of doses so that a new survival curve is produced using the survivors of the first dose as the starting population. What is commonly observed is that the presence of the shoulder on this survival curve is dependent on the time interval between the doses. The straight portion of this new survival curve is often parallel to the single dose curve. If an appropriately long interval is introduced for repair to be complete, the original shoulder on the single dose survival curve is reproduced (Elkind et al., 1967b).

Soon after the existence of the repair phenomenon was demonstrated by the experiments of Elkind and Sutton, questions as to the nature of this process arose. As was mentioned earlier, room temperature incubation of the cells during the dose fractionation interval had no effect on Elkind recovery (Elkind et al., 1965). Several investigations have attempted to study the dependence of the recovery process on the biochemistry of the cell. Actinomycin D, an RNA synthesis inhibitor, did show an effect on Elkind recovery. Elkind et al. (1964) showed that the presence of actinomycin D during the interval between the dose fractions suppressed the recovery phenomenon in Chinese hamster V79 cells. This observation suggested that RNA synthesis is necessary for Elkind recovery. Similar experiments using FUDR, an

inhibitor of DNA synthesis, showed no effect on split dose recovery in HeLa S-3 cells (Kim et al, 1964).

Several investigators examined the effect of protein synthesis inhibition, using chemical inhibitors, on Elkind recovery. Berry (1966) used puromycin and cycloheximide on HeLa S-3 cells to study their effect on split dose recovery. In those experiments where he added the drug immediately before the first dose, he saw no reduction in recovery. He also performed experiments in which he treated cells for up to 20 hours, with the inhibitors, before giving the first dose of radiation. He continued to treat the cells with the inhibitor, until the second dose. Again he saw no reduction in recovery. Kim et al. (1966), used puromycin during the time interval between the two doses and observed no effect on Elkind recovery in synchronized HeLa S-3 cells. Elkind et al. (1967a) performed a series of experiments on Chinese hamster V79 cells using excess thymidine (TdR), to inhibit DNA synthesis, or actinomycin D or puromycin to observe the effect of these macromolecular inhibitors on split-dose recovery. They noticed no effect on split-dose recovery using the DNA synthesis inhibitor or with puromycin even when the inhibitor treatments were started an hour or two before the first dose.

In confirmation of their earlier experiments with actinomycin D, they did notice a reduction in split-dose recovery with this inhibitor. Extensive testing of the effect of puromycin and actinomycin D on DNA, RNA and protein synthesis showed that puromycin was as effective in inhibiting RNA synthesis as actinomycin D. This raises some questions as to whether RNA synthesis is really involved in Elkind recovery as was suggested by the earlier work with actinomycin D (Elkind et al., 1964). Since actinomycin D inhibition of RNA synthesis seems to involve binding

to the DNA, a likely explanation of any effect on Elkind recovery may be through this mechanism rather than any effect on RNA synthesis. It should be noted that the level of protein synthesis inhibition achieved in these previously mentioned experiments was in the range of 60% - 70% of the level of protein synthesis in untreated cells as measured by the incorporation of radioactive amino acids.

The effect on Elkind recovery of the inhibitor of oxidative phosphorylation, 2,4-dinitrophenol (DNP), has also been studied. In the same series of experiments as those reported with puromycin and cycloheximide, Berry (1966) used DNP in the same fashion. He treated the cells for hours before the first dose and continued the drug between the dose fractions or treated with DNP only during the split-dose interval. In either case he observed no inhibition of Elkind recovery. Dalrymple et al. (1969) performed experiments on mouse L cells treated with DNP in the time interval between the dose fractions. They also observed no inhibition of Elkind recovery. In fact, they report enhanced amounts of Elkind recovery in cells treated with DNP compared to control cells left in medium alone. The observations reported here suggest that Elkind recovery is not well understood as a biochemical process in the cell. Inhibition of macromolecular synthesis seems to have no clear effect on the cell's ability to carry out this type of repair process.

Recovery from potentially lethal damage

Another type of recovery phenomenon which resembles that of Elkind and Sutton has been named recovery from potentially lethal damage (Phillips et al., 1966). Operationally, this recovery is defined in a

similar way as in the split-dose recovery discussed before. The difference being that instead of giving a second radiation dose at various time intervals after a first radiation dose, one subjects the cells to a treatment involving a modification of the normal cellular environment to one that is not conducive to cellular progression through the division cycle, in place of the second radiation dose. These changes in the cellular environment can include raised or lowered temperatures, placing cells in a balanced salt solution, or the addition of various chemicals that inhibit DNA synthesis, RNA synthesis or protein synthesis. Generally the agent used in the treatment is not toxic to the cells when given in the absence of radiation (Altman et al., 1970). The idea here is that radiation causes damage to cells that if unrepaired will lead to cell death and that appropriate postirradiation treatment can lead to either an increase or decrease in cell survival. That is, the treatment alters the ability of the cells to repair potentially lethal damage (PLD).

There is general agreement that PLD is repaired and the fraction of cells surviving a given X-ray dose is increased if post-irradiation conditions are suboptimal for growth. This can be achieved by placing the cells in a balanced saline solution or holding them in stationary phase in culture (Hahn and Little, 1972). In contrast to these conditions, lowered temperature has produced contradictory results. In some instances a lower temperature (29°C) has promoted repair of PLD and increased cell survival as in the case of saline, while in other cases it has decreased cell survival and interfered with the repair of PLD. Cell survival was decreased following postirradiation incubation with DNA synthesis inhibitors, while incubation with cycloheximide, a protein

synthesis inhibitor enhanced PLD repair and enhanced survival (Hall, 1978).

Experimentally it does not seem possible for Elkind recovery to influence the observed repair of PLD since only one dose is involved in the latter case. However the converse is not true since repair of PLD is probably going on in the time interval between the dose fractions used to measure Elkind recovery. The operational definition of both types of repair are distinct, but whether common lesions or repair enzymes are involved is not clear. There have been some comparisons between the general properties of each repair process in specific cell types. Repair of PLD in exponentially growing cells placed in saline and/or at reduced temperature after radiation occurs at the same rate as Elkind recovery (Winans et al., 1972). Conversely repair of PLD in stationary phase cultures held in saline or conditioned medium has a much longer repair time than is usually reported for Elkind recovery (Hahn and Little, 1972). Differences exist between the two repair processes in terms of their respective sensitivity to drugs that interfere with macromolecular synthesis. Some of these have been mentioned already. Koch et al. (1977) reported on the repair of PLD and Elkind recovery in stationary phase cultures and exponentially growing cultures of Chinese hamster ovary cells under conditions of extreme hypoxia. They conclude that the oxygen and/or metabolic energy requirement for each repair process is different. Utsumi and Elkind (1979) report on the effect of anisotonic saline solution on the repair of PLD and Elkind recovery in exponentially growing Chinese hamster cells. They found that repair of PLD is reduced by anisotonicity, but Elkind recovery is not. The time required to complete repair of each

process was found to be different. The repair of PLD was shown to be complete in one hour after irradiation, while Elkind recovery was complete in 4-6 hours. The accumulation of evidence suggests that there is at least one component of the repair of PLD and Elkind recovery which is not common to both phenomena.

2. Molecular

DNA as the target

The mechanism whereby cells lose their reproductive integrity as a result of X irradiation is not fully understood. As discussed earlier, theoretical models attempt to link cell survival with critical targets inside the cell. However these models tell us nothing about the nature of these targets. What is established, is that the sensitive sites are located in the nucleus as opposed to the cytoplasm. A rather clever experiment conducted by Von Borstel and Rogers (1957, 1958) illustrates this idea. The eggs of the wasp, Habrobracon juglandis, have a nucleus which occupies an eccentric position in the cytoplasm. They were able to differentially irradiate the nucleus and cytoplasm with α particles. They reported a 10^6 -fold difference in the number of particles to reduce the hatchability to 37%, depending on whether the nucleus or the cytoplasm was irradiated. An experiment conducted by Ord and Danielli (1956) on amoebae is also striking. Since the large size of these organisms allow easy manipulation, they could remove the nucleus from the cell and separately irradiate the nucleus or the cytoplasm. They then combined the irradiated and unirradiated parts to measure survival. Their observations support the idea that the nucleus is the important site for cell survival.

These observations were extended to include mammalian cells by Munro (1970). He irradiated Chinese hamster cells, attached to a glass surface, with α particles from a polonium tipped microneedle. The α particles from polonium have a definite well-defined range and it is possible, by appropriate positioning of the needle, to irradiate only the cytoplasm or mainly the nucleus of the cells. He reported that penetration of the nucleus by a few α particles could be lethal while large numbers of particles delivered to the cytoplasm, corresponding to a dose in excess of 250 Gy, had no effect on cell proliferation.

As to the important targets inside the nucleus, chromosomes would seem to be likely targets for lethality. A number of experiments over the years have added strong support for this idea. One such experiment is the idea of suicide. Radioactive atoms can be added to biologically important molecules. If the molecule is specific enough, the cell will use it only in certain macromolecules. Burki and Okada (1968) used this idea to incorporate radioactive atoms in cellular DNA, RNA or protein. They examined the differential survival of these cells, with radiation damage being accumulated at specific sites. They observed that radiation damage to the DNA was most crucial to cell survival.

Another series of experiments involved the use of structural analogs of the nucleoside, thymidine, which is only incorporated into DNA. In particular, halogenated pyrimidines, such as BrUdR, were given to a wide range of organisms, from viruses to mammalian cells to partially substitute the thymidine (TdR) present in the DNA. It was observed that those organisms with the substituted nucleoside showed an increased sensitivity to ultraviolet light and ionizing radiation (Szybalski, 1967).

Over the years much information on the radiation sensitivity of different organisms has accumulated. Sparrow et al. (1967) examined this collection of data and found that if the logarithm of the D_0 dose was plotted versus the logarithm of the chromosome volume for eight classes of organisms, a series of parallel lines resulted. The eight classes show no relation to classic taxonomy. This data presentation shows that the same amount of energy must be expended per individual chromosome to kill each of the eight classes of organisms, regardless of chromosome number, chromosome size and DNA content per chromosome. All these experiments point toward DNA and the chromosomes to be very important sites of radiation damage leading to cell lethality.

The evidence presented for DNA being the critical target leading to cell death is compelling but not conclusive. In fact there are cells that do not divide in an organism, such as nerve and muscle, for which enough radiation will lead to a cessation of metabolic activity, i.e., death. The radiation damage to these types of cells results in altered permeability of the cell membrane and intracellular membranes, leading to a shift of molecules within subcellular components of the cell, and changes in ion balance. These changes could be responsible for death in the case of nondividing cells. However, in reproductive death there is no evidence of direct involvement by membrane damage. In fact, cultured mammalian cells receiving a moderately lethal dose of radiation will divide at least once before they die. So if membranes are damaged they do not seem to prevent the cell from carrying on the necessary metabolism to pass through their life cycle (Altman et al., 1970).

Over the years evidence has accumulated for the role of membranes in the organization of the DNA. In bacteria, the membrane serves as a site

of attachment and replication for DNA (Tremblay et al., 1969). In eukaryotic cells close association of condensed chromatin with the nuclear membrane can be seen in electron micrographs. The use of radioactively-labelled DNA precursors indicates that the sites of replication are at or near the nuclear membrane (Sparvoli et al., 1976). All this evidence suggests that although DNA is important as far as cell survival is concerned, it may be only part of the sensitive site to radiation damage in prokaryotic and eukaryotic cells.

DNA damage and repair

Damage to DNA can be defined as any modification that alters its coding properties or its normal function in replication or transcription. These modifications may result in an altered DNA sequence or distortion of the DNA structure. Three broad categories of modification have come to be recognized. The first is base damage which includes missing bases, incorrect bases and altered bases. Damage of this type threatens the integrity of genetic information and can also distort the helical structure as is the case with pyrimidine dimers and bulky adducts. The second class of damage is cross-links. These are covalent bonds formed between the complementary strands of DNA or between protein and DNA. These generally cause distortion of the helical structure. Interstrand cross-links are particularly serious since neither strand can function directly as a template for error-free resynthesis. The third category of damage is strand breaks. These include single- and double-strand scissions in the helix of DNA. These breaks can be between a phosphate and the ribose unit or may involve partial destruction of the ribose unit (Hanawalt et al., 1979).

Breakage of the DNA molecule was noted early on as one of the effects of ionizing radiation on DNA irradiated extracellularly. However, since DNA is sensitive to shear forces, the reproducibility and sensitivity of this method is dependent on the method used to isolate DNA from the cells. McGrath and Williams (1966) introduced an innovation whereby DNA could be irradiated intracellularly, cells were then lysed in an alkaline solution on top of a sucrose gradient, and the resulting single-stranded DNA could be centrifuged into the sucrose to observe the size distribution of irradiated DNA. They applied their method to bacteria while Lett et al. (1967) reported on the use of mammalian cells. It was observed that as the dose of radiation increased, the size of the DNA pieces became smaller. Since the alkali solution totally denatures the DNA, their method gives information about the total number of breaks introduced into the DNA. It was also observed that if some time was allowed to pass before the cells were lysed that the size of DNA pieces started to return to that observed in unirradiated samples. This indicated that these breaks were being repaired. A variation of this method involves lysing the cell and freeing the DNA at the top of a sucrose gradient at a neutral pH. In this way the strands of DNA are not denatured and smaller pieces of DNA can only be produced from breaks that sever both strands of the helix. Double-strand breaks can be measured with this technique. From these experiments it became apparent that sites existed on damaged DNA that would break in the presence of alkali. These contribute to the yield of strand breaks and are called alkali-labile lesions. It is believed that these lesions may be sites of base damage (Elkind and Redpath, 1977).

The results of the velocity sedimentation technique suggest that there are two components of the repair of strand breaks. One component is very fast requiring about four minutes and can take place in buffer instead of growth medium. The other component takes about 60 minutes and requires growth medium. The suggestion is that single-strand break repair is characterized by the fast component, and double-strand break repair is characterized by the slow component. Some of the characteristics of single-strand break repair in mammalian cells are: it occurs under both aerobic and hypoxic conditions; it occurs in all ages of the synchronized population; it is not inhibited by hydroxyurea, actinomycin D, or puromycin, and it is almost completely absent at 5°C (Altman et al., 1970). Enzymatic repair of double-strand breaks has been reported to occur in bacteria (Burrell et al., 1971) and yeast (Resnick and Martin, 1976). Repair of double-strand breaks in mammalian cells has been reported but not conclusively documented (Hutchinson, 1978).

The connection between repair of strand breaks and repair of PLD and Elkind recovery is not clear. It would seem that based on the repair rates of strand breaks that single-strand breaks are repaired too rapidly to be related to either of the cellular repair processes. Double-strand break repair may however be related to repair of PLD and Elkind recovery. The idea that when both strands of the DNA molecule are broken a template for error-free resynthesis is not available suggests that survival of a cell may be more closely linked to the correct repair of a double-strand break. It is not known what role the rejoining of double-strand lesions play in the capacity of a cell to perform Elkind recovery or repair of PLD.

If an exponentially growing population of cells is given a brief exposure to ^3H -TdR, only cells synthesizing DNA at that time will take up the labelled molecule. In an autoradiograph this would appear as blackened silver grains in the photographic emulsion lying above the cells that incorporated the isotope while other cells not synthesizing DNA would have no grains above them. This indicates that only a certain fraction of the cells are synthesizing DNA at any one time (S phase) and the other cells have already completed their synthesis (G_2 and M phases) or have not yet started (G_1 phase). It was observed by Rasmussen and Painter (1966) that if HeLa cells were irradiated with X rays before giving the pulse of ^3H -TdR, that all cells took up the label. This DNA synthesis in non-S stage cells became known as unscheduled DNA synthesis (UDS). Similar observations have been reported in other cell lines such as mouse L cells and human lymphocytes (Altman et al., 1970). The idea behind these observations was that radiation-induced damage in the DNA was being repaired by the insertion of new nucleotides. UDS is sometimes referred to as repair replication or repair DNA synthesis.

Another method used to demonstrate the insertion of nucleotides into the DNA of irradiated cells is the technique of density-gradient centrifugation. The use of this technique to study repair replication was developed by Painter and Cleaver (1967). HeLa cells were incubated for 16 hrs with BrUdR, then irradiated with X rays and incubated with ^3H -TdR for an additional 2-3 hrs. The presence of the Br in the DNA changes the density of this DNA. When the DNA is subjected to neutral CsCl gradient centrifugation, two layers of DNA appear: a denser one with Br and a less dense one without Br. In nonirradiated cells the ^3H activity coincides with the light density DNA only. However, in

irradiated cells, ^3H activity shows up in light and heavy density DNA. An alkaline CsCl gradient centrifugation, will separate the two strands of the DNA molecule. In nonirradiated cells the heavy density DNA and ^3H activity do not overlap. However, in irradiated cells the heavy DNA also had ^3H activity. This experiment clearly demonstrates that insertion of nucleotides occurs in the middle of DNA molecules which have already been duplicated. Painter and Cleaver (1967) also examined the effect of hydroxyurea (HU), a DNA synthesis inhibitor, on repair replication. They found that it was not inhibited by this drug.

Gautschi et al. (1973) examined the ability of HeLa S-3 cells and Chinese Hamster Ovary cells to perform repair replication, on UV and X-ray damage, in the presence of cycloheximide. They observed that the ability of the cells to perform repair synthesis did not depend on the ability to synthesize protein. The type of damage being repaired here is probably a combination of base damage and strand breaks. Similar to the situation with strand-break rejoining, no correlation can be made between repair synthesis and the repair of PLD or Elkind recovery.

Chromosomal damage and repair

Radiation damage to mammalian cells can be measured by another method not available for prokaryotes. Variations in the number, and physical appearance of the chromosomes at the first mitosis following radiation exposure can be scored. Usually the scoring is done during metaphase or anaphase. Since chromosome variations are observable only in mitosis, two limitations occur: nondividing cells cannot be studied and, until recently, the effects of irradiation in different stages of the cell cycle could not be studied until the cell reached mitosis. The

cell fusion technique of Johnson and Rao (1970) allows premature chromosome condensation (PCC) to occur. The PCC technique permits X-ray-induced chromosomal damage in interphase cells to be examined (Hittleman and Rao, 1974; Waldren and Johnson, 1974). The study of radiation damage in mammalian cells is further complicated by the large number of chromosomes present. Many plant cells contain fewer and larger chromosomes, consequently they are used extensively as a test system (Hall, 1978).

There are two classes of chromosomal changes that are observed at metaphase. Chromosome aberrations result if a cell is irradiated early in interphase, before the DNA and chromosome material has duplicated. If there is a break in the chromatin strand it will be copied when the S phase is complete. This leads to a chromosome at mitosis that has breaks at identical sites on the two chromatid areas of the chromosome. Chromatid aberrations result if a cell is irradiated late in interphase after the DNA and chromosome material has duplicated. In this case radiation damage will be visible as a break in only one chromatid area of the chromosome (Hall, 1978). For a more complete description of these two classes into further groups, the reader is referred to Elkind and Whitmore (1967). The dose-effect relationship for aberrations is usually represented as a polynomial in dose. The different powers of dose included in the equation depend on the type of aberration being examined, the LET of the radiation, a limited number of exchange sites, cell age and restitution or rejoining processes (Elkind and Whitmore, 1967).

When cells are irradiated with X rays, breaks are produced in the chromatin. These broken ends appear to be "sticky" and once formed can

rejoin to give the original configuration (called restitution), can fail to rejoin and give rise to a deletion of chromatin, or can reassort and rejoin with other sticky ends (called reunion) to give rise to the aberrations seen at mitosis (Hall, 1978). An understanding of the process of reunion and restitution would seem to be very important for understanding aberrations and cell survival.

In order for two breaks to participate in an aberration they must coexist in time and space. Wolff et al. (1958), calculated that the maximum distance over which rejoining could occur is less than 0.3μ . Split-dose experiments of Dewey and Humphrey (1964) showed that the yield of exchanges decreased as the time between the doses increased. In fact the yield drops in about ten minutes to a value that remains constant for a time interval of up to 60 minutes between doses. The yield did not drop to the value expected if full restitution had occurred between the doses. This is suggestive that some breaks may be incapable of restitution. In the same report the authors tested the ability of cells in a particular cell stage to undergo restitution. The results indicate that cells irradiated in G_1 can reconstitute breaks and this occurs in about 10 minutes to a level that indicated full restitution between the dose fractions. However in S phase cells there was no restitution of breaks. This observation does not support a possible relationship between Elkind recovery and restitution of chromosome breaks, since S phase cells show the greatest degree of Elkind recovery and the least amount of restitution.

Evans (1966) reported on fractionated dose experiments with Vicia faba root tips and human leukocytes. The frequency of two-hit aberrations, dicentric and rings, decreased to a minimum around 2 hours

between dose-fractions then increased to a maximum around 6 hours. In fact, the yield of aberrations at a 6-hour split-dose interval was comparable to that at zero time between the fractions. This pattern bears a striking resemblance to an Elkind recovery curve for cell survival. Evans also tested the effect of actinomycin D on the yield of two-hit aberrations in a split-dose scheme and found no effect. This is in contrast to the results reported on Elkind recovery with actinomycin D where it was shown to inhibit repair (Elkind et al., 1964).

The biochemistry of the rejoining process was studied by Wolff and Luippold (1955), using Vicia faba seeds. They performed split-dose experiments, in which the environmental conditions between the dose fractions were changed to observe what effect these modifications would have on restitution. Treatment at 0°C prevented restitution. Treatment with carbon monoxide (CO) in the dark or KCN prevented restitution. These last observations suggest that generation of adenosine triphosphate (ATP) is involved in the repair process. However, attempts to perform these experiments in the absence of oxygen, using a vacuum or N₂ gas, failed to prevent restitution. But experiments with Na₂S₂O₄, a reducing agent, and vacuum did inhibit restitution. This suggests that the amount of oxygen needed is very small and with the vacuum or N₂ gas alone, there was enough oxygen present to allow restitution to occur. Other experiments using DNP, an inhibitor of oxidative phosphorylation, showed inhibition of restitution.

Wolff (1959) also reported on the use of chloramphenicol and aureomycin, two protein synthesis inhibitors, on Vicia faba seeds. He found that restitution was prevented when these chemicals were present between the dose fractions. In an attempt to extend the plant cell

studies, Wolff (1972) used unstimulated (G_0) and stimulated (G_1) human lymphocytes. He examines the yield of dicentrics in a split dose experiment and finds that X-ray-induced chromosome breaks restitute in about 4-5 hours. However, if cycloheximide is present between the dose fractions, restitution is prevented, and the yield of dicentrics remains at the level observed if the total dose is given at one time.

The experiments performed seem to indicate no simple relationship between Elkind recovery and restitution of chromosome breaks, as far as trying to relate cellular repair with molecular repair. That chromosome aberrations are somehow involved in cell lethality in some cases is clear, but it is not plausible that every lethal event should be associated with a detectable change in chromosome morphology.

C. Proteins

1. Translation

Protein synthesis in eukaryotic cells is a multi-step process. Much of the information about the process of translation was first obtained from the study of bacteria. There are differences between the two types of organisms and our understanding of the exact nature of protein synthesis in eukaryotic cells is still not complete (Lehninger, 1975; Alberts et al., 1983).

One of the necessary precursors for the process of protein synthesis is activated amino acids. Activation is the process by which each of the 20 different amino acids are each esterified to a particular transfer-RNA molecule specific for that amino acid. The joining of an amino acid and a transfer-RNA molecule requires a highly specific enzyme called a synthetase which is capable of recognizing each amino acid and

the particular transfer-RNA with the correct anti-codon coding for that amino acid. This biochemical reaction requires ATP (Lehninger, 1975).

The first step in protein synthesis is the formation of the initiation complex. In bacteria the first amino acid in a growing polypeptide chain was found to be a derivative of methionine. Although this amino acid is often later removed it is a necessary starting point for protein synthesis. In eukaryotic cells a methionine residue is also found at the beginning end of the polypeptide chain. There appears to be a specific transfer-RNA molecule which accepts the methionine and has the anti-codon for the start codon (AUG). Also needed are the ribosomal units which in eukaryotic cells are designated by their sedimentation values, 40S and 60S. These subunits, made of ribosomal-RNA and protein, combine to form a ribosome with a sedimentation value of 80S. The complete formation of the initiation complex also requires small proteins called initiation factors, guanosine triphosphate (GTP) as an energy source and, of course, messenger-RNA to be translated (Lehninger, 1975).

The next step is the process which involves the elongation of the polypeptide chain. Small proteins called elongation factors bind to an aminoacyl-transfer-RNA that has the anti-codons for the next codon to be read. GTP is again required to bind this complex to the ribosome. A peptide bond is formed between the growing polypeptide chain and the amino acid attached to the transfer-RNA molecule. Translocation, requiring GTP, occurs so that the next codon is in position to be read and is prepared for the binding of another transfer-RNA molecule. This process of elongation continues until the end of the "message" is signaled by one of three special termination codons (Lehninger, 1975).

The final step in making a polypeptide chain involves the release of the protein from the ribosome. After the special termination codon is in place release factors bind which cause the last transfer-RNA molecule to break the bond holding the protein to it. After this occurs the unit consisting of the messenger-RNA, ribosome and transfer-RNA dissociate. The parts can be recycled to be used to synthesize another protein (Lehninger, 1975).

Some of the information about the molecular mechanisms responsible for the translation process have been elucidated through the use of particular chemical compounds which selectively inactivate or inhibit different parts of the protein synthetic machinery. These substances have also proved invaluable for providing tools with which to examine various biological effects of inhibiting protein synthesis. Cycloheximide binds to the 60S ribosomal unit and blocks peptide bond formation during the elongation step. Puromycin resembles an aminoacyl transfer RNA and can bind to the ribosome during elongation. A bond is formed between the peptide chain and the puromycin molecule. This prevents any more amino acids from being added to the already started polypeptide chain and causes release from the ribosome of an incomplete protein chain (Lehninger, 1975; Alberts et al., 1983).

Diphtheria toxin is an enzyme that catalyzes a reaction that inactivates elongation factor two. The inactive elongation factor inhibits translocation of the ribosome so no completed peptide chain is produced. Abrin and ricin inactivate the 60S subunit of the ribosome and block elongation. There are chemical inhibitors of RNA synthesis which have an indirect effect on protein synthesis. One of these is actinomycin D which binds to DNA and blocks movement of the RNA

polymerase molecule, thereby stopping transcription. A messenger RNA synthesis inhibitor is α -amanitin which binds to RNA polymerase II (Lehninger, 1975; Alberts et al., 1983).

2. Chromosomal protein synthesis

In eukaryotic cells this process of translation occurs in the cytoplasm where there are free and bound ribosomes. Most proteins are synthesized continuously throughout the cell cycle. Some proteins have varying patterns of protein synthesis. There are those that show a rise in synthesis at a particular part of the cell cycle, and then synthesis is reduced to a low level after a period of time. There are others that show a rather abrupt initiation of synthesis and then remain on for the remainder of the cell cycle (Mitchison, 1971). Histones are classes of proteins synthesized mainly during the S phase. The regulation of histone translation is at the level of the lifetime of the messenger RNA, which is controlled by the level of transcription and the amount of degradation. (Hereford et al., 1981). DNA synthesis and histone synthesis seem to be coupled since the messenger RNA for histones is very unstable whenever DNA synthesis is stopped. (Gallwitz, 1975). However, there is also a close coupling between overall protein synthesis and overall DNA synthesis. Overall DNA synthesis is reduced, at the level of replication fork movement and at the level of initiation frequency, in a coordinated fashion, so that it is nearly equal to the reduction in overall protein synthesis, when different protein synthetic inhibitors are used (Stimac et al., 1977).

Histones are a highly conserved, lysine- and arginine-rich, group of five classes of proteins called H1, H2A, H2B, H3 and H4. They are found

complexed with DNA in a ratio of approximately 1:1 by weight. The classes of histones form complexes that interact with DNA to create structures called nucleosomes. The nucleosomes are stacked and arranged in different ways to form the fiber-like structures in the nucleus called chromatin (Bradbury et al., 1981).

There are other proteins associated with DNA, collectively called non-histone chromosomal proteins. The non-histone proteins include the enzymes required for DNA replication and transcription, enzymes needed for histone modifications and "scaffold" proteins involved in organizing and shaping the metaphase chromosomes. The synthesis of non-histone proteins is not directly coupled to the replication of DNA but instead seems to vary throughout the cell cycle (Stein and Borun, 1972). A subgroup of the non-histone proteins have been identified by their great mobility on a polyacrylamide gel. This subgroup of small, highly charged proteins is called high mobility group (HMG) proteins. HMG proteins have been found in all tissues and higher organisms so far examined. The variations, in amino acid sequences, between different organisms for homologous HMG proteins is greater than that observed for homologous histones. There is some evidence to suggest that two of the HMG proteins function as DNA-unwinding proteins. Another protein that may function to regulate the transcriptional activity of chromatin is ubiquitin. It is a highly conserved protein found in prokaryotes and eukaryotes. Ubiquitin can be found covalently linked to 1 in 10 of the histone H2A molecules where it may play a role in higher order chromatin structures (Bradbury et al., 1981).

D. Repair deficient mutants

Mutant cell lines provide very powerful tools for the further understanding of the repair and metabolism of DNA. Bacteria and lower eukaryotic cells provide a foundation with which to understand some of the processes going on inside the mammalian cell. As more mammalian cell mutants are discovered and the properties of those we already have come to light our understanding of the radiation effects on these cells will advance.

1. Prokaryotes (Bacteria)

Most of our information about cellular responses to DNA damage has been obtained from studies of cyclobutane dipyrimidines (pyrimidine dimers) in UV-irradiated Escherichia coli. The presence of an identifiable lesion produced by UV radiation greatly facilitates the processes of understanding how these lesions are removed. This is in contrast to X-radiation which causes multiple lesions which are varied in chemical nature. The well-defined genetic system of E. coli has facilitated the understanding of the many enzymatic steps involved in the response to UV-induced damage.

The simplest mechanism for repairing pyrimidine dimers is direct reversal by photoreactivation. This depends on a single enzyme that binds at the site of the dimer, and in a process catalyzed by light of a particular frequency, monomerizes the dimers without causing a break in the DNA helix (Ganesan et al., 1979).

The other repair mechanisms are more complex than photoreactivation. The most extensively studied is excision repair. This is a coordinated series of biochemical reactions that breaks a phosphodiester bond near

the dimer, removes the damaged area by exonuclease activity, replaces the removed nucleotides by using the complementary strand as template and ligates the repaired region to the rest of the strand. The incision step has become characterized by at least three genes *uvrA*, *uvrB*, *uvrC*. The discovery of mutant bacteria that are sensitive to the effects of UV-radiation have elucidated the role of these three genes. There are three DNA polymerases that operate in *E. coli*. Polymerases I and III have a 5'-exonuclease activity associated with it and are therefore good candidates for the process of coupled excision of damage and resynthesis (Ganesan et al., 1979).

Mutants deficient in polymerase I are UV sensitive although not as sensitive as *uvrA*, *uvrB* or *uvrC* mutants. Mutants deficient in polymerase III have only been obtained as conditional lethals and it has not been possible to assess their UV sensitivity under conditions in which the enzyme is not functional. Excision repair can act on defective or incorrect bases also. There may be specific endonucleases that remove the altered base leaving a site which is apurinic or apyrimidinic (AP). AP endonucleases which recognize and incise DNA at AP sites have been found. Presumably, the subsequent steps of the excision process would be followed after the incision is made. There is also evidence that AP sites can be filled directly by a base insertion enzyme, insertase (Ganesan et al., 1979).

The further use of *E. coli* mutants has brought to light another repair pathway. This repair mechanism was discovered in excision-deficient mutants. This process was first termed post-replication repair. It centered on the idea that if a dimer existed at the time the replication fork was moving past, there would

not be any way for the dimer to be read, so that a gap across from the dimer would result. The filling in of these gaps is the process of post-replication repair. Evidence has accumulated that a recombinational process is involved where the already duplicated complementary strand is used to fill in the gap. Mutants of E. coli in the rec genes have provided information on this recombinational repair. It is interesting to note that a recA, uvrA double mutant is so sensitive that a single pyrimidine dimer is lethal (Howard-Flanders and Boyce, 1966). This observation suggests that no other pathways play a major role in the repair of UV damage (Ganesan et al., 1979).

The repair processes outlined here are effective against other agents such as some chemical damage caused by nitrous acid, 4-nitroquinoline-1-oxide (4NQO), mitomycin C and psoralen plus UV light. There may be a collection of damage-specific endonucleases that can start the excision process by recognizing damage and breaking a phosphodiester bond. It is reasonable to conclude that some ionizing radiation damage is repaired in a similar fashion. (Ganesan et al., 1979).

2. Eukaryotes

Non-mammalian organisms

The existence of DNA repair processes has also been reported in several lower eukaryotic cells. The yeast, Saccharomyces cerevisiae and the fruit fly, Drosophila melanogaster have been widely studied in terms of their ability to repair radiation damage. Since the genetics of these systems have been extensively researched, they provide some biochemical understanding of the repair process on a level comparable to

that afforded by E. coli. About 55 loci exist in yeast and 20 in *Drosophila* that seem to affect sensitivity to radiations and chemical mutagens.

Yeast

The loci in yeast seem to fall into three groups named for the prominent loci in each. The RAD3 group is involved in the excision of UV-induced pyrimidine dimers. This group bears a resemblance to the *uvr* mutants of E. coli. The RAD6 group controls UV-induced mutagenesis. Error-prone repair is also controlled by this group. This is a repair that is usually categorized as a different pathway than excision repair which is considered to be error-free. The RAD52 group controls the repair of X-ray-induced double-strand breaks. They do not convey marked sensitivity to U.V. unless in combination with a RAD3 mutant. The RAD52 group is particularly interesting because it seems to control double-strand break repair, which is absent in E. coli. This repair process also requires that a haploid G_2 sister, or diploid homologue DNA molecule be present. The RAD52 group is also involved in recombination events in meiosis and mitosis. It is believed that these three groups cover all the repair pathways present in yeast since a haploid triple mutant, containing one allele from each group, can be killed with a UV dose for which one lethal hit produces only one or two dimers (Haynes et al., 1978).

The presence of a third repair system in yeast as opposed to two in E. coli increases the complexity of the repair processes. Considering the large number of loci in each group it is suggestive that a large number of gene products are required for the control and coordination of repair in yeast. The three groups may be indicative of three complex

and functionally coordinated macromolecular structures which are required to control excision repair, replication and recombination (Haynes et al., 1978).

Fruit flies

In Drosophila five genes have been identified by analysis of mutagen sensitive strains to influence DNA repair. Some of these mutagen sensitive strains also show some deficiency in meiotic functions. Photoreactivation occurs in this organism. The mei-9 mutants are deficient in excision repair of dimers and also show sensitivity to X rays, methyl methanesulfonate (MMS), nitrogen mustard, 4NQO and benz-(a)-pyrene. Meiotic recombination is also reduced in females homozygous for the mei-9 locus. These mutants are deficient in repair replication after UV and X rays (Boyd, 1978).

The mei-41 and mus-101 mutants are deficient in meiotic recombination and post-replication repair following U.V. Both these mutants also show sensitivity to X rays. Two other mutants mus-104 and mus-302 are also deficient in post-replication repair after UV exposure. All these mutants repair single-strand breaks (Boyd, 1978).

Mammalian organisms

The ideas developed from repair studies of bacteria, yeast, and fruit flies are helpful in developing concepts of repair that may operate in mammalian cells. However, a limitation encountered in studying repair of DNA damage in mammalian cells is the narrow spectrum of mutants available. Nearly all have been obtained from humans with genetic disorders which represent a small set of mutations that do not

prevent growth and development of the organism. Interpretation of results is also complicated by the organization of DNA into chromatin, a highly complex structure of DNA and protein.

Human

The hereditary disease Xeroderma pigmentosum (XP) has become the most extensively studied example of defective DNA repair in humans (Cleaver, 1968). This is an autosomal-recessive disease characterized by hypersensitivity of the skin to sunlight. Excision repair may be visualized autoradiographically by incorporation of ^3H -TdR into UV irradiated cells in G_1 phase or G_2 phase cells. This phenomenon is sometimes referred to as unscheduled DNA synthesis (UDS). XP cell strains are found to be deficient in UDS. The classification of XP strains into multiple complementation groups is based on cell fusion experiments between different cell strains of XP. If normal levels of UDS result then the two cell types are assumed to represent mutations in different genes. The genes involved may be regulatory as well as structural in nature. There may also be several different genes belonging to a particular complementation group, each involved with a particular step in the excision process (Bootsma, 1978).

All complementation groups show defective removal of dimers. Evidence has accumulated that these complementation groups reflect an inability to incise the DNA near the site of damage. Experiments with extracts from some XP strains mixed with UV-irradiated E. coli DNA show that excision repair can occur normally (Cook et al., 1975; Mortelmans et al., 1976). The results suggest that because of the complicated nature of the chromatin in eukaryotic cells, additional steps may be

required to facilitate the interaction of repair enzymes with the DNA. Group A cells were found to be defective in DNA-protein cross-link repair (Fornace and Kohn, 1976) but could repair interstrand cross-links caused by mitomycin C (Fujiwara et al., 1977). The A group is also defective in removing adducts formed by 4NQO (Ikenaga et al., 1977) and N-acetoxy-2-acetylaminofluorene (AAAF) (Amacher and Lieberman, 1977). Extracts of the D group showed altered apurinic endonuclease activity (Kuhnlein et al., 1976). All these results seem to confirm that DNA repair in mammalian cells is a relatively complicated process compared to bacteria (Bootsma, 1978).

There are other groups of XP cells that show no defect in UDS. These are referred to as XP variants (Lehmann et al., 1975). The XP variants show a slow rate of postreplication repair. This repair presents itself as a bypass mechanism allowing DNA replication to pass DNA lesions present in the parental DNA strand. Gaps occurring in the daughter strands are slowly sealed in XP variants as compared to normal cells. The molecular mechanism of this gap filling is unknown. One can speculate that some type of recombinational process similar to bacteria is involved, but evidence is contradictory (Bootsma, 1978).

Another rare hereditary disorder called Ataxia telangiectasia (AT) shows sensitivity to X rays in an analogous way as XP to UV irradiation. AT strains are found to be proficient in single-strand and double-strand break repair, but half of those studied seem to be deficient in repair replication of γ -ray damage. A striking feature of AT cells is that DNA synthesis is much more resistant to ionizing radiation than in normal diploid cells. DNA damage that inhibits replicon initiation and chain elongation in normal human cells fails to do so in AT cells (Painter and

Young, 1980). This effect is seen in those strains classified as proficient or deficient in repair replication (Painter, 1981). AT strains do not show sensitivity to U.V. (Arlett, 1977). They are able to carry out normal excision repair and postreplication repair after U.V. Another feature of these cells is the presence of chromosome instability in the presence or absence of an external agent. They have increased levels of numerous types of chromosome aberrations but not sister chromatid exchanges (Patterson, 1978).

Bloom's syndrome is another rare autosomal recessive disorder characterized cytogenetically by an increased frequency of chromosomal aberrations and sister chromatid exchanges (Chaganti et al., 1974). Yet, these cells appear to be normal with regard to excision and postreplication repair of U.V. damage. They also possess normal DNA polymerase activities. Single-strand breaks due to X rays are repaired normally. However, there is some evidence that DNA replication may be defective in cells of this type (German, 1978).

Fanconi's anemia, an autosomal recessive disorder, shows high sensitivity to cytotoxic and chromosome breaking action of bifunctional alkylating agents. Some researchers report that the cells appear to have a reduced ability to repair DNA interstrand cross-links. The cells are capable of repairing X-ray- or γ -ray-induced DNA strand breaks. The molecular mechanisms involved in cross-link repair are not well understood (Sasaki, 1978).

The study of human mutant cells in culture is proving to be valuable but there are limitations. Some repair genes may not be represented in the human genetic diseases since the gene products may be essential for development of the embryo. These human strains are not transformed,

which makes them difficult to manipulate genetically. Since the human cells are diploid it is more difficult to obtain mutants since they must be homozygous which tends to be a rarer condition. The established animal cell lines may provide a better system in which to produce mutants.

Chinese hamster

The Chinese hamster cells (strains CHO and V79) are particularly useful since they are highly versatile in handling and growth properties, they have an attractive karyotype for cytogenetic studies, and they have a proven history of yielding a wide variety of mutant phenotypes. UV sensitive CHO cells have been isolated and divided into five complementation groups. All seem to be defective in excision repair. In fact they seem to lack the ability to cause incision just as the XP cells are unable to do. These UV sensitive CHO cells are also sensitive to agents that form bulky covalent DNA adducts. Two of the complementation groups also show sensitivity to mitomycin C, a cross-linking agent. This is a similar property to that of Fanconi's anemia cells (Thompson, 1984).

A CHO mutant designated EM9 was found to be X-ray sensitive as well as sensitive to the alkylating agents MMS and ethyl methanesulfonate (EMS). It is defective in rejoining strand breaks and has a high rate of spontaneous sister chromatid exchanges (SCE). The biochemical defect in this cell line is not known. The high rate of SCE's resembles that of Bloom's syndrome (BS) cells; however, BS cells do not show a defect in DNA strand break repair (Thompson, 1984).

Another UV-sensitive CHO cell called UV-1 has been isolated (Stamato and Waldron, 1977). Excision repair after UV irradiation appears to be normal but postreplication repair after UV exposure is defective. This cell line is similar to an XP variant cell type in its characteristics (Thompson, 1984).

Another ionizing radiation sensitive CHO mutant has been isolated (Stamato et al., 1983). It is referred to as XR-1. This cell type is also sensitive to bleomycin, an agent known to produce double strand breaks. The response of XR-1 to EMS, which produces abundant single-strand breaks, is normal. This suggests that the defect in this cell may involve double-strand break repair. The cell cycle response to X rays in this mutant shows a pronounced G_1 sensitivity (Thompson, 1984).

Mouse

Other cell types besides Chinese hamster cells have been used to select for mutants. A wide variety of mutants in the mouse lymphoma line L5178Y have been found. With respect to UV sensitivity, four complementation groups have been isolated. These cells show sensitivity to 4NQO and mitomycin C, but not to X rays (Sato and Hieda, 1979; Shiomi et al., 1982). In fact these mutants show a defect in the incision step of the excision repair to UV damage (Sato and Setlow, 1981).

E. Statement of the problem

Mammalian cell mutants and particularly temperature-sensitive ones provide a very specific system to selectively alter cellular metabolism in a controlled manner. The results of experiments which use chemicals

to affect the biochemistry of a cell are not always easy to interpret. There may be problems associated with knowing whether or not all of the chemical has been removed after treatment. Lingering traces of these chemicals could alter the response one is interested in studying. Also chemicals can have wider reaching consequences than may be first thought based on a particular mode of action. For example, the effect of puromycin on RNA synthesis and the effect of actinomycin D on Elkind recovery (Elkind et al. 1967a). Ideally we would like to have tools available to probe the biochemistry of the cell which are effective only against a particular enzymatic pathway, or can alter the function of a specific component of the cell's metabolic apparatus. Specific genetic defects which have been characterized in mammalian genomes, therefore enable us to ask fundamental questions to elucidate mechanisms of action.

Thompson et al. (1973) reported on the isolation and characterization of a mutant of the Chinese hamster ovary cell line which is temperature-sensitive for protein synthesis. They discovered that the particular defect is in the cells ability to activate the amino acid leucine when the temperature is raised. This means that the transfer-RNA molecule with the leucine anti-codon does not have a leucine molecule attached to it. Specifically, they discovered that the leucyl-transfer-RNA synthetase is the site where the temperature effect on protein synthesis is acting. This temperature-sensitive cell type was designated CHO-TSH1.

"The low leakiness and low reversion rate of this mutant, combined with the specificity of the defect in its protein synthesizing machinery, make it an appealing tool for investigating regulatory

mechanisms in animal cells," (Thompson et al., 1973). It was my thought that now one could reexamine the role of protein synthesis in Elkind recovery and avoid some of the problems caused by chemical inhibitors. This temperature-sensitive mutant cell line, together with its wild-type parent cell line (CHO-SC1), could provide a self-contained system to manipulate the internal protein biochemistry of the cell by using temperature shifts as an external switch. The question therefore addressed in this dissertation is, "What is the role of protein synthesis in the repair of sublethal X-ray damage in this temperature-sensitive cell line?"

CHAPTER II

MATERIALS AND METHODS

CELL CULTURE

A. Cell lines

For all the studies reported, two clones of a proline-requiring Chinese hamster ovary cell line (CHO) were used. A clone of the original cell line started by Puck et al. (1958) was isolated by L. Thompson of the Lawrence Livermore Laboratory. He designated this cell line CHO-SC1. It was referred to as the wild-type cell. Upon treatment of this cell line with MNNG and selection under conditions that would allow the temperature-sensitive mutants to grow (Thompson and Baker, 1973), Thompson isolated a clone he referred to as CHO-TSH1. Under subsequent biochemical testing it was discovered that this cell was temperature-sensitive for protein synthesis (Thompson et al., 1973). The optimal growth temperature for the mutant clone was less than 37°C. Molnar et al. (1975) discovered that the rate of protein synthesis was also affected by the concentration of leucine in the medium. At the non-permissive temperatures above 37°C, when greater than 52 µg/ml leucine was present, the decrease in protein synthesis was less pronounced compared to conditions where less than 52 µg/ml leucine was present in the growth medium. Phenotypic expression of the mutation was dependent therefore on leucine concentration in coordination with temperature.

The temperature-sensitive defect in protein synthesis in the CHO-TSH1 cell was located in its leucyl-transfer-RNA synthetase (Thompson et al., 1973). This is the enzyme that catalyzes the transfer of leucine to the t-RNA molecule specific for leucine. Apparently without a sufficient supply of "charged" t-RNA molecules at the non-permissive temperature, protein synthesis is inhibited. In subsequent research with the CHO-TSH1 mutant cell line it was discovered that the pool of uncharged t-RNA molecules, produced at the non-permissive temperature, affected the initiation of new protein synthesis, not the elongation of synthesis already initiated (Stanners, 1977). It is interesting to note that in stringent bacteria the generation of uncharged transfer-RNA molecules results in a number of diverse changes probably mediated by the unusual nucleotides, guanosine tetraphosphate and guanosine pentaphosphate. These changes include a rapid decrease in RNA synthesis, selective changes in the transcription of various genes, an increase in protein degradation and changes in transport properties of membranes (Cashel et al., 1974). The synthesis of these nucleotides requires ribosomes, uncharged transfer-RNA and a protein called stringent factor. Stanners (1977) investigated whether a similar stringent response occurred in the CHO-TSH1 cell type under the non-permissive temperature conditions. He reported that there was no production of these unusual nucleotides and that these CHO cells do not show the same type of molecular responses as stringent E. coli.

B. Growth conditions

Both cell types, SC1 and TSH1 were grown in Minimal Essential Medium (MEM) with Earle's salts (Gibco) for maintenance of stock cultures and

for colony growth for survival experiments. The MEM was supplemented with 0.25 mg/ml L-glutamine (Gibco), 43 units/ml potassium penicillin G (M.A. Bioproducts), 43 µg/ml streptomycin sulfate (M.A. Bioproducts), 1.94 mg/ml NaHCO₃ (Gibco), 10% v/v fetal calf serum (Gibco) and 0.31 mM MEM non-essential amino acid solution (Gibco). For experiments with synchronized cells the McCoy's 5a medium (Gibco) used was supplemented with 0.25 mg/ml glutamine, 43.3 units/ml potassium penicillin G, 43.3 µg/ml streptomycin sulfate, 10% v/v fetal calf serum and 17.3 mM of Hepes Buffer (Gibco). Both the MEM and McCoy's 5a medium used contained leucine at a concentration of 52 µg/ml. All cells grown in MEM with Earle's salts and the bicarbonate buffer system were maintained in an incubator at 35°C in an atmosphere of 95% air plus 5% CO₂. With McCoy's 5a medium and the Hepes Buffer System, the cultures were grown at 35°C but the vessels were sealed off to the air and CO₂ atmosphere. Under the growth conditions described, the population doubling times of the SC1 and TSH1 cell lines with either MEM or McCoy's 5a medium were 14-16 hours.

C. Mycoplasma testing

Approximately every two weeks the cells were tested for contamination by mycoplasma, using a modification of the technique described by Chen (1977) with the fluorescent dye Hoechst 33258 (American Hoechst Corp.). Briefly, about 5×10^5 cells were seeded on to a sterile glass microscope slide resting in a 100 mm tissue culture dish with 20 ml of MEM present. About 24 hours later the cells were fixed for 5 min with 20 ml of fresh Carnoy's fixative which was added directly to the culture dish. The solution was removed and

approximately 20 ml of fresh Carnoy's fixative was added to the dish for 5 min. After this was removed, the slide was air dried. Immediately before each test a solution of H33258 was made up by diluting a stock solution of H33258 1:100 in distilled water to give a final concentration of 0.5 $\mu\text{g/ml}$. The slide was stained for 30 sec and rinsed with distilled water. A mounting solution of citric acid disodium phosphate buffer (see APPENDIX A) was added and a cover slip applied. The edges of the cover slip were sealed with euparal (Roboz Surgical Instruments Co.). After 12-24 hours the slide was observed with a Zeiss UV-fluorescent microscope system using 53/44 barrier filters with BG-3 exciter filter to screen for the presence of fluorescing organisms. A total magnification of 312.5X was used. During the course of this work all mycoplasma tests of both the wild-type and mutant cell lines were negative.

EXPERIMENTAL TECHNIQUES

A. Survival curves

1. Survival assay

For both cell lines, colony formation was the survival assay used to test reproductive integrity. In all experiments, both control and treated cultures were trypsinized and plated for survival in an identical fashion as rapidly as possible after the treatment. This was usually accomplished by 30 min. to at most 1 hour after treatment. This involved removing the medium from the 25 cm^2 flask (Co-Star) adding 2 ml of a 0.05% trypsin (1:250) and 0.2% EDTA solution (Gibco) to rinse the cell surface. This solution was removed and 2 ml of the trypsin

solution was added again. The flask was placed in a 37°C incubator for 3-5 minutes to remove the attached cells. The solution was repeatedly pipetted to form a single cell suspension and 1-2 ml was placed into a tube with 4 ml of MEM. After further pipetting a cell count was made on a 1:10 dilution of the cell suspension in isotone (Coulter Electronics, Inc.) using a Coulter Counter (Coulter Electronics, Inc.) model ZB1 (machine settings were 1/amplification = 16; 1/aperture current = 1/2). Two to four counts per vial were done. Dilutions of the cell suspensions into MEM for survival plating were made based on the number of cells counted and the anticipated survival level. A one ml volume of cells was plated into each 25 cm² flask (Falcon) which was pre-filled with 5 ml of warmed (35°C) MEM. Six to eight flasks were inoculated for each control or treated sample.

Colony formation was allowed to occur at 35°C in a humidified incubator with a 5% CO₂ and 95% air atmosphere. The cells were left for 8 days to allow adequate time for growth of survivors into macroscopic colonies. The medium was then removed and a staining solution of 0.1% crystal violet (Manufacturing Chemists) in a 25% Ethanol water solution was added to stain and fix the cells to the bottom of the culture vessel. The cultures were stained for 15-30 minutes; then the flasks were carefully rinsed with cold water twice, and they were allowed to air dry before the colonies were counted.

Macroscopic colonies containing 50 cells or more were counted as survivors. These cells tend to be loosely attached to the plastic flasks during colony formation. In some cases (control and low-dose samples) the colonies that formed had tails of extended growth, presumably from the same clone. Under microscopic examination these

tails seemed to be formed from cells that drifted off from the parent colony and settled down to form a sweeping tail 3-4 colony diameters long. Colonies that looked like they were derived from these tails were not counted. In the moderate- to high-dose cultures, however, colonies with tails were rarely seen. About 100-200 colonies were counted per culture vessel. Plating efficiencies were in the range of 80%-100% and surviving fraction was calculated by dividing the average number of colonies per vessel, at a particular dose, by the number of cells plated per vessel and dividing that number by the plating efficiency. The range of surviving fraction covered in these experiments was from 0.001 to 1.0.

2. Survival curve analysis

A computer-calculated least-squares dose fit was made through the survival data points using the linear-quadratic model where survival is represented by the expression $S/S_0 = Ze^{-\alpha D - \beta D^2}$. S/S_0 is the surviving fraction at dose D , Z is the best fit plating efficiency. The Z parameter allows a best fit of the data without forcing the survival curve through the 100% survival, zero-dose point. The dose and raw surviving fraction were entered into the computer. The raw surviving fraction was found by dividing the average number of colonies per vessel by the number of cells plated per vessel. The plating efficiency was entered as the raw surviving fraction corresponding to zero dose. The best fit of all the data was made and the linear- and quadratic-inactivation coefficients α and β were calculated with standard errors and a best-fit plating efficiency (Z) with standard errors. The

parameter Z is only used in plotting the associated data points. The software used in the curve fitting was written by N. Albright.

B. Cellular repair measurements

Cellular repair was operationally defined as the increased survival observed, when cells were exposed to two fractions of radiation separated by several hours, compared to that when the total exposure was given at one time. This is a conventional measurement of split-dose recovery of sublethal damage as defined by Elkind and Sutton (1959). About 48 hours in advance of the start of these experiments $\sim 2 \times 10^5$ cells in a 1 ml aliquot were placed into 25 cm² flasks (Co-Star) prefilled with 5 ml of MEM. At the start of these experiments all flasks were filled with warmed ($35^\circ\text{C} \pm 0.1^\circ\text{C}$) and pH-adjusted (7.3 ± 0.1) MEM. All the flasks were then placed into the circulating water bath at 35°C . At the appropriate time, flasks were removed from this bath and placed in the circulating 40°C water bath if a temperature treatment before irradiation was called for. In the case of the cycloheximide treatment, the flasks were placed back into the 35°C water bath after the medium with cycloheximide was added. The time in these experiments was measured from the point when the flask entered a water bath. The flasks were removed from a water bath for irradiation. There was some heat loss during the time of irradiation but a typical irradiation took no more than 5 minutes. After irradiation the flasks were returned to the appropriate water bath. The time was again measured from the point the flask was placed back into a bath. After further incubation at the prescribed temperature the flasks were removed from the water bath for a second radiation dose. After the second dose

the flasks were again placed in the appropriate water bath. In the case of the cycloheximide treatment, the medium with cycloheximide was removed and replaced with drug-free medium. After all treatments had been completed the flasks were returned to the circulating 35°C water bath to await plating for survival, usually within the hour. Samples that were to receive only one dose of radiation were handled in a similar way.

Two types of experiments were used to quantitatively measure cellular repair of sublethal damage. One experiment involves irradiating cells with a total dose of radiation split into two fractions separated by various time intervals. In this experiment the surviving fraction was measured as a function of the time between the dose fractions. The other type of experiment involves administering a fixed first dose of radiation, waiting for a specific time interval, then giving a second dose which was variable in amount.

C. Synchronization techniques

Cell synchrony was achieved using the method of selection by mitotic shake-off (Terasima and Tolmach, 1961). The CHO cell lines used in this work were very well suited to this technique because they become very rounded and loosely attached in late mitosis and early G₁-phase. By agitating the medium over the cells these loosely attached ones were removed from an exponentially growing monolayer and collected for experiments requiring a cell population with a narrow window of cell age. During the course of the work two techniques were used to select mitotic cells. These techniques were performed in a temperature controlled warm room (Forma Scientific, Inc.) set at 35°C.

1. Reciprocal method

In early experiments a reciprocating shaker table (Eberbach) was used with a stroke length of 2 inches and a frequency of 120 cpm. Cells were set up at a density of 2×10^6 cells in a 75 cm² flask (Falcon) with McCoy's 5a medium approximately 48 hrs before the experiment. It was found that 20 minutes between shakes gave a good yield of synchronized cells (2%) as confirmed by cell volume distribution analysis with a Coulter Channelanalyzer (Coulter Electronics, Inc.). The volume distributions indicated the cells that were selected by shake off were mainly G₁-phase (70%) and M-phase cells (30%). The use of a reciprocating shaker table, however, did not produce enough cells due to a limitation in the number of flasks that could be shaken simultaneously.

2. Rotary method

To obtain a large enough population (1×10^7 cells) to complete the necessary experiments a machine designed for handling large batches of cells was used. This device called a Cell Cycle Analyzer System (Talandic Research Corp.), has 4 large, plastic roller bottles (Corning) with a total growing surface of 3200 cm² which can be programmed to automatically collect and distribute the loosely attached cells. Depending on the time of the experiment, enough cells were inoculated into each roller bottle to yield approximately 1.4×10^8 cells per roller bottle at the time the experiment starts. The bottles were slowly rotated at ~ 0.5 rpm on a Cell Production Roller Apparatus (Bellco Glass, Inc.) to allow the cells to become attached for growth and feeding. Prior to the start of the experiment the bottles were

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cycle. This was done by removing the medium from the flasks at a specific time when the cultures represented the age of interest. Pre-warmed McCoy's 5a medium containing ^3H -TdR (0.05 $\mu\text{Ci/ml}$) was added for 15 minutes at 35°C. After the pulse, the radioactive medium was removed and the flask was rinsed twice with 5 ml of phosphate buffered saline (PBS-Gibco) and the cells were fixed for 5 min in 5 ml of Carnoy's fix (1 part glacial acetic acid: 3 parts Ethanol). The fix was removed and 5 ml of 70% Ethanol-water solution was added for storage of the cells until the photographic emulsion was added. The flasks were kept in a refrigerator at this point. Several days to a week later, the top of the plastic flask was removed and the cell surface was allowed to dry out. In a dark room, Kodak NTB-2 photographic emulsion diluted 1:1 with distilled water was added to the surface to cover the cells with a thin film. The flasks were allowed to set in the dark for 1 week to allow sufficient disintegrations to occur to expose the grains of the film. The flasks were then developed and fixed using Kodak products. The flasks were stained with Hematoxylin (see APPENDIX A) to provide contrast between the cell nucleus and the cytoplasm. The flasks were examined under a Zeiss microscope with a magnification of 630X using oil. Cell nuclei with 3 or more grains are considered labelled. At least 500 cells are counted per flask. Cell cycle parameters were measured by plotting percent labelled cells vs time after synchronization. The generation time was found to be ~ 14 hrs, with $G_1 \sim 5.5$ hrs, $S \sim 7.5$ hrs, and $G_2 + M \sim 1$ hr from cells obtained by both synchronization methods.

D. Protein synthesis measurements

The rate of protein synthesis was observed by measuring the amount of ^3H -Leucine incorporated into the cold trichloroacetic acid (TCA) insoluble fraction of exponentially growing wild-type and mutant cells. About 48 hrs prior to the start of the experiment $\sim 2 \times 10^5$ cells in a 1 ml aliquot were placed into 25 cm² flasks (Co-Star) prefilled with 5 ml of MEM. The flasks were filled with warmed ($35^\circ\text{C} \pm 0.1^\circ\text{C}$) and pH-adjusted (7.3 ± 0.1) MEM. At the appropriate time during or after treatment, a solution of ^3H -Leucine (60 Ci/mM, New England Nuclear) was added to each flask to yield a final concentration of radioactivity of 3 $\mu\text{Ci/ml}$ and remained in contact with the cells for 5 minutes. After the pulse the radioactive medium was removed and the cell surface was rinsed with cold trypsin. 2 ml of Trypsin (Gibco) was added to remove the cells from the surface by incubating at 37°C for 3 minutes. 4 ml of MEM was added to neutralize the trypsin and the suspension was repeatedly pipetted to form a single cell suspension. An aliquot (0.5 ml) of the cell suspension was added to 9.5 ml of isotone (Coulter Electronics, Inc.) to find the concentration of cells present. 5 ml of the suspension was added to a syringe with a 0.22μ Millipore filter attached. The solution was forced through the filter, where the cells become lodged. First, the filter was rinsed twice with 10 ml each time of ice-cold PBS. Second, the cells were washed twice with 10 ml each time of an ice-cold 5% TCA solution. Finally the cells were washed twice with 10 ml each time of ice cold 95% ethanol. The filters were air dried and placed in scintillation vials with 10 ml of scintillation fluid (Instagel, Packard Instrument Co., Inc.) A background sample was also prepared by adding non-labelled cells to a filter and following the

same procedure as for labelled cells. The vials were counted twice in a Beckman Liquid Scintillation Counter for 10 minutes each time, and the disintegrations per minute were found from internal standards and quenching corrections. The activity of a sample was the average activity found from the two counts. The activity in the background sample was used to correct the activity found in the labelled samples. The activity per cell was determined by dividing the activity of each sample by the number of cells placed on the filter. The percent rate of incorporation was the background corrected activity per cell of the treated sample divided by the background corrected activity per cell of the sample kept at 35°C and not treated.

E. DNA strand-break measurements

Velocity sedimentation of DNA has been a major technique for studying radiation-induced strand breaks and their repair (McGrath and Williams, 1966). Several investigators reported, however, that DNA isolated in this way had anomalous sedimentation behavior (Elkind and Kamper, 1970; McBurney et al., 1972). Ahnstrom and Erixon (1973) modified the technique to remove these sedimentation anomalies. After lysing the cells and centrifuging the DNA into a pellet, they sonicated the DNA to reduce the molecular weight. The DNA was then placed on a hydroxylapatite column where elution with different concentrations of phosphate buffers yielded single-stranded and double-stranded DNA fragments. The relative proportion of these types of fragments could be correlated with dose. These modifications made the procedure more reproducible and sensitive to doses in the range where mammalian cells can still survive and divide. Ahnstrom and Edvardsson (1974) further

modified the technique by removing the centrifugation step. Rydberg (1975) improved the sensitivity of the assay, performed extensive studies, and developed models for the strand separation process. S. Okada brought this method, often referred to as the alkaline-unwinding technique, to the laboratory and helped me adapt it for my purposes.

1. Cell preparation

About 48 hours before the start of the experiment about 2×10^5 cells in a 1 ml aliquot were inoculated into 25 cm² flasks (Co-Star) prefilled with 5 ml of MEM. At 24 hours before the experiment a solution of ¹⁴C-TdR (52-58 Ci/mM, New England Nuclear) was added to the growing cells to give a final concentration of radioactivity of 0.01 μ Ci/ml so as to have at least unifilar labelling of the cellular DNA. At the time of the experiment the radioactive medium was removed and the flasks were completely filled with warmed ($35^\circ\text{C} \pm 0.1^\circ\text{C}$) and pH-adjusted (7.3 ± 0.1) nonradioactive MEM. At the time of irradiation the medium was removed and replaced with 5 ml of PBS, and the flasks were either irradiated at room temperature or chilled during irradiation in an ice bath. If the rejoining of breaks was being measured, the PBS was removed after irradiation and was replaced with warmed and pH-adjusted MEM for the required time of post-irradiation incubation.

2. Unwinding procedure

At the appropriate time the medium was removed, and the cell surface was rinsed with 5 ml of cold PBS. After the rinse was discarded, the cells were treated with 3 ml of an 0.03 M NaOH solution for 1 hour. An hour of alkali treatment was necessary for complete lysis. The

published experiments with the alkaline-unwinding technique used cells in suspension and treated them with the alkaline solution for 30 minutes. Cells attached to a plastic substrate may limit exposure to the alkali solution, hence the requirement for longer incubation times to complete the lysis procedure.

The 0.03M NaOH alkali solution was made fresh for each experiment from a stock solution of 0.9 M NaCl and 0.01 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. The pH of this solution was ~ 12.0 . Before the experiment it was necessary to know how much of the acid neutralizing solution must be added to the alkali solution to yield a pH of 7.0 ± 0.1 . This was accomplished by accurate volume measurements throughout this part of the procedure. A 0.036 M HCl solution was used for neutralizing the alkali solution. At the end of the hour the acid solution was forcefully injected with a Pipetman into the cell solution in the flask. This insured a rapid neutralization. The contents were poured into a 15 ml centrifuge tube and sonicated for 15-20 sec at a power output of 45 watts using a Heat System - Ultrasonics Inc. sonicator.

After sonication, a 2% sodium dodecyl sulfate (SDS-K & K Laboratories, Inc.) solution was added to give a final concentration of 0.1% SDS in the centrifuge tube. The solution was mixed using a Vortex Genii. The samples were placed in an ice bucket until the experiment was completed. After this, the samples were stored in a freezer for several days to a week before completing the rest of the handling.

3. Hydroxylapatite chromatography

Column preparation

A column of hydroxylapatite (HA) was prepared to separate the

single- and double-stranded DNA that resulted from the earlier treatment of the samples with alkali and sonication. Potassium phosphate buffers of different concentrations were used in the column preparation. A stock solution of 0.5 M K_2HPO_4 and 0.5 M KH_2PO_4 was mixed to produce a 0.5 M potassium phosphate buffer (KPB) at a pH of 6.9 ± 0.1 . The other concentrations of KPB were produced by diluting this stock and adjusting the pH to 6.9 ± 0.1 . Econo columns from Bio Rad Laboratories were used. They are polypropylene with a porous glass filter disc at the bottom.

The hydroxylapatite was also from Bio Rad Laboratories and is their DNA grade. A suspension of the hydroxylapatite was made with a 0.01 M KPB solution. Enough solution was made to put 2 ml of the suspension into each column and have about 0.4 gm of hydroxylapatite present. The columns were placed in a test tube rack, anchored in each hole with rubber stoppers. The whole rack was placed in a circulating water bath at 60°C with the water level adjusted almost to the top of the column. A multiport peristaltic pump (Manostat) was connected to each column. After the solution of hydroxylapatite was added to the column the solution was drawn very slowly by the pump to insure a gentle, even settling of the hydroxylapatite into the bottom of the column. It was important not to let the hydroxylapatite become too dry at any time in this procedure. To ensure proper settling of the HA, 1-2 ml aliquots of the 0.01 M KPB were added during pumping. Once the HA had settled at the bottom and no holes appeared in the column, the DNA was added.

DNA elution

It was discovered that proper elution of the DNA depended on not overloading the column with too much DNA. The best results occurred when

there was less than 1×10^5 cells/g of HA loaded onto the column. After thawing the previously frozen samples, the cells were diluted 1:5 in distilled water. One ml of this diluted solution was added to each column. The solution was eluted through the column at a rate of 0.3-0.4 ml/min. The DNA bound to the column was rinsed once with 3 ml of 0.01 M KPB. The single-stranded DNA was eluted from the column by adding 3 ml of 0.125 M KPB, three times to the column. The double-stranded DNA was eluted by adding 1.5 ml of 0.25 M KPB, three times to the column.

To regenerate the column after all fractions were collected, 1.5 ml of 0.5 M KPB was run through the column twice. The column was rinsed three times with 3 ml of 0.01 M KPB. After this the column was ready for another DNA sample. The column can be reused two-three times. The volume of the double-stranded elution was increased with distilled water to equal that of the corresponding single-stranded elution which usually was corrected to a total volume of 10 ml. This makes the salt concentration the same in these elution volumes so that the solubility of an aliquot of each in the scintillation fluid was the same.

Scintillation counting

The activity in each of the elutions was measured by adding 2 ml of each sample to 13 ml of scintillation fluid (Instagel, Packard Instrument Co., Inc.). To test that all the radioactivity in the sample appeared in the single- and double-stranded fractions after elution, a control vial was prepared by adding a 0.5 ml aliquot of the sample that was added to the column, 0.5 ml of 0.25 M KPB, and 1 ml of 0.125 M KPB to adjust the salt concentration to that of the single- and double-stranded elution samples. Thirteen ml of scintillation fluid was

also added to this vial. A background vial was also prepared by mixing 0.5 ml of distilled water, 0.5 ml of 0.25 M KPB, and 1 ml of 0.125 M KPB to 13 ml of scintillation fluid. After thorough mixing, the samples were placed in a Beckman Liquid Scintillation Counter. The samples were counted twice, each time for 10 minutes. Disintegrations per minute (DPM) were calculated using internal standards and quenching corrections. The activity of a sample was taken as the average of the two counts. The counts of the control sample were compared to that of the single- and double-stranded samples to determine a percent recovery by calculating

$$\frac{2.5 (DPM_{\text{single-stranded}} + DPM_{\text{double-stranded}})}{DPM_{\text{control}}} \times 100$$

Recovery was typically in the 95%-105% range. The activity of each sample was corrected by the background activity.

F. Temperature Control

The temperature treatments of cells were performed in water baths (B. Braun) held at $35^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $40^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$. The water in the baths was circulated using a Thermomix 1460 unit (B. Braun) and the flasks were totally immersed in the water. A temperature versus time profile for taking a flask from the 35°C water bath to a 40°C water bath and visa versa was measured using a Colt-Parmer Digital Thermometer. Measurements indicated that within 5 minutes of transferring the flask the temperature inside the flask was within 1°C of the water-bath

temperature. It took 10 minutes for the temperature inside the flask to be within 0.1°C of the water bath.

G. Irradiation technique

Irradiations were performed with a Philips therapy X-ray machine operated at 225 kVp with a current of 15-17 mA. The X rays produced by this machine were filtered with 0.35 mm Cu and had a half value layer of 1 mm of Cu. The X-ray tube to cell-layer distance in the survival experiments was 38 cm while the tube to cell-layer distance for the DNA rejoining experiments was 17 cm. These differences were necessary to change the dose rate. In the survival studies the dose rate was ~ 1.7 Gy/min and for the DNA rejoining studies the rate was ~ 7.4 Gy/min. Dosimetry for each experiment was done with a Victoreen R meter. The probes for this device were periodically calibrated at the National Bureau of Standards. Corrections for air temperature and pressure were made. The Roentgen to Gray conversion used was 9.45×10^{-3} . All the irradiations were performed on exponentially growing cellular monolayers. The X-ray beam was normally incident to the cell surface.

H. Cycloheximide treatment

Cycloheximide (Sigma Chemical Co.) was found to dissolve readily in complete MEM. After dissolving the drug, the medium was filtered through a 0.22μ 500 ml capacity filter (Nalgene) to insure sterility. At the start of the experiments the MEM in the prefilled flasks was removed and replaced with warmed ($35^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$) and pH-adjusted (7.3 ± 0.1) complete MEM with cycloheximide. After the appropriate treatment time this medium was removed. The cell surface was rinsed once with

warmed and pH-adjusted MEM without cycloheximide. The flasks were then completely refilled with this medium and placed in the 35°C water bath to await further handling.

CHAPTER III

RESULTS

CHARACTERISTICS OF THE CELL LINES

A. Cell growth measurements

Cell growth measurements were made with the Chinese hamster ovary wild-type (WT) and temperature-sensitive (TS) mutant cell lines in MEM and McCoy's 5a media, using the culture methods described in the Materials and Methods (CHAPTER II, CELL CULTURE, Section B). In exponential growth, at the permissive temperature of 35°C, the population doubling time for both cell lines was 14-16 hours (see Figure 1). The cell density used for all experiments using asynchronous cells was $\sim 8 \times 10^3$ cells/cm². Cells inoculated at this density were in exponential growth phase within 48 hours.

B. Protein synthesis

To examine the temperature dependence of protein synthesis in the mutant and the wild type cells, the effect of a treatment at 40°C on the percent rate of incorporation of ³H-Leucine was measured. The percent rate of incorporation was measured by comparing the uptake of ³H-Leucine in cells treated at 40°C with those kept at 35°C. The time at the particular temperature was measured at the point the flask entered the appropriate water bath. Experiments were also performed to examine the recovery of protein synthesis, as measured by ³H-Leucine incorporation, after various times at 40°C. The results of all of these experiments

XBL 852-6980

Figure 1. Growth characteristics of the WT cells and TS cells after they were set up at different cell densities in different media at the permissive temperature of 35°C.

can be seen in Figure 2. There was a rapid reduction in the percent rate of incorporation in the mutant cells after the flask of cells was placed in a 40°C water bath. A decrease in the percent incorporation to 50% occurred within 10 minutes, and the level dropped to 10% of the initial level of incorporation in 30 minutes. After that time there appeared to be a further decline in the percent rate incorporation so that in 4 hours, it had reached a level of 3% and seemed to remain at this level at least up to 6 hours (data not shown). The residual protein synthesis that seemed to remain may have been mitochondrial in nature. The mitochondria has its own protein synthetic machinery, and the mutated cytoplasmic synthetase enzyme would not affect its ability to synthesize protein (Wallace et al., 1975). The wild-type cells showed an increase in the percent rate incorporation during the same time period. This seemed to indicate that 40°C speeds up protein synthesis in the wild-type cells.

The reversible nature of this mutation in the mutant cells after a 2-hour or 4-hour treatment at 40°C can be observed in Figure 2. After 2 hrs or 4 hrs of 40°C, the rate of recovery of protein synthesis as measured by the percent rate of incorporation was very similar and seemed to be a mirror image of the reduction in the percent rate of incorporation after 1 hour of 40°C. That is, there was at least a 10-fold change in the percent rate of incorporation. However, in the mutant the return of the percent rate of incorporation to control levels was prolonged. It appeared to take as long as 4 hours before the percent rate of incorporation seemed to return to control levels after a 2-hour treatment at 40°C. Figure 2 indicates that after a 4-hour treatment at 40°C, the return to control levels will take even longer.

TEMPERATURE DEPENDENCE OF PROTEIN SYNTHESIS

Figure 2. Percent rate of ^3H -Leu incorporation into the TCA insoluble fraction of the WT cells and TS cells during and after a temperature treatment at 40°C. The percent rate of incorporation was calculated relative to the uptake of labelled Leucine into each cell type at 35°C. Error bars representing the standard deviation of the mean are shown when larger than the symbol size.

XBL 852-6973

An inhibition of protein synthesis with this mutation may start to affect the machinery of protein synthesis, and repair or replacement of lesions may account for the long recovery time. The effect of the 40°C treatment may be more severe than just a delay period after which cellular metabolism can start up again as if nothing had happened.

C. X-ray response

To examine the phenomenon of sublethal damage repair in the mutant- and wild-type cells, it was necessary to know the inherent radiation sensitivity of each cell type. Preliminary experiments indicated that there was no inherent difference in the radiation response of the two cell lines to single doses of X rays. This observation was substantiated throughout the asynchronous cellular part of this work, since every graded-dose response experiment involving single doses and split doses had a control curve of one or both cell types, representing the single dose response at 35°C. A composite curve for each cell type from four different experiments is shown in Figure 3. The composite α and β parameters for the best-fit linear quadratic (LQ) curves to these data are shown in Table 1. Within the standard error indicated, the α and β parameters for the WT and TS cells are not significantly different.

D. Reproductive integrity

The survival studies on the temperature-sensitive mutant were started by looking at the effect of different times of treatment at 40°C on reproductive integrity. This was done by measuring the percent survival of cells treated at 40°C for up to 12 hours. The results of

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Figure 3. Survival curves for asynchronous populations of each cell type after exposure to single doses of 225 kVp X rays. Data for each cell type was taken from four different experiments.

TABLE 1. Composite α and β parameters for the best-fit LQ curves to the X-ray response of WT cells and TS cells (Figure 3).

	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$
WT	3.32 ± 0.29	4.39 ± 0.33
TS	3.12 ± 0.33	4.70 ± 0.36

this investigation are shown in Panel A of Figure 4. There does not appear to be a significant drop in reproductive integrity until the time at 40°C had exceeded 6 hours. A steady decrease in survival was observed to occur, beginning after 6 hours of 40°C and continuing for up to 12 hours of 40°C. Based on this result, in order to minimize irreversible effects of prolonged 40°C, all experimental protocols described herein which required 40°C treatments did not exceed 6 hours in duration.

REPAIR OF SUBLETHAL X-RAY DAMAGE

To demonstrate the phenomenon of the repair of sublethal damage, I first irradiated the TS cells with a total dose of 7.0 Gy that was split into two equal fractions separated in time from 0 to 12 hours. The cells were kept at 35°C during this time. The results are shown in Panel C of Figure 4. The classical survival versus time between dose fraction pattern was evident. Repair of sublethal damage seemed to reach a maximum in 2-4 hours before cell progression effects started to alter the radiation sensitivity and mask the repair.

I was also interested in what would happen to the TS cells if during the interval between doses the cells were kept at the nonpermissive temperature of 40°C. The results of that experiment can also be seen in Panel C of Figure 4. Again the repair of sublethal damage occurred and seemed to reach the same extent of repair as observed in the cells kept at the permissive temperature. However, there were differences noted for longer incubation times between the dose fractions. In particular the second rise in surviving fraction starting at about 8 hours was

Figure 4. Panel A: Survival of unirradiated TS cells exposed to 35°C or 40°C for varying amounts of time. Panel B: Survival of TS cells after receiving a single X-ray dose of 3.5 Gy followed by varying amounts of time at 35°C or 40°C. Panel C: Survival of TS cells exposed to a first X-ray dose of 3.5 Gy followed by varying amounts of time at 35°C or 40°C before receiving a second X-ray dose of 3.5 Gy. All TS cells used were asynchronous.

EFFECTS OF 35°C OR 40°C ON THE RADIATION RESPONSE OF CHO-TSH1 (TS) CELLS

Figure 4.

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missing. The surviving fraction continued to fall. This could be explained by noting that inhibition of protein synthesis was probably interfering with cell progression. The cells may be blocked in G_2 phase and this relatively radiosensitive phase overwhelms the increase in survival due to repair. In conjunction with the split dose studies, I was also interested in what effect a 40°C treatment after the first dose of 3.5 Gy would have on the single-dose survival. Panel B of Figure 4 shows the results of that experiment. There appeared to be enhanced cell killing if the cells were kept at 40°C for longer than 4 hours after the 3.5 Gy exposure. The split-dose survival shown in Panel C of Figure 4 was corrected by this decrease in single-dose survival.

Preliminary experiments done on split-dose samples that were pretreated at 40°C before the first dose, in addition to having 40°C in the dose fractionation interval, showed that the split-dose recovery pattern could be changed. To examine this observation more carefully, I performed a series of experiments with the TS and WT cells that involved a single dose of 8.0 Gy separated into two fractions of 4.5 Gy and 3.5 Gy spaced in time from 0 to 4 hours. The temperature for the time between doses was 35°C for the control. For all the other treatments the cells were kept at 40°C between the dose fractions. In addition, groups of cells were held at 40°C for various times in half hour increments from 0 to 3 hours before the first dose to observe the effect on the rise in survival in the 0-4 hours after the first dose. The results are shown in Figure 5.

Cells of both types only treated with 40°C between the dose fractions showed results similar to the control patterns. For pre-first dose exposures to 40°C ranging from 0 to 1 hours results similar to the

Figure 5. Survival of asynchronous WT cells and TS cells that were exposed to a first X-ray dose of 4.5 Gy followed by varying amounts of time at 35°C (top panel) or 40°C (other panels) before receiving a second X-ray dose of 3.5 Gy. The time in hours shown within the panels represents the pretreatment interval. That is, the amount of time the cells were kept at 40°C before the first dose was given.

SPLIT-DOSE X-RAY RESPONSE OF CHO-SC1 ($\blacktriangle$) AND CHO-TSH1 ($\bullet$) CELLS

Figure 5.

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control patterns were seen. However for pretreatment times of 1.5 hours or longer there was a reduction in the amount of recovery seen in the TS cells compared to the WT cells. This indicated that the repair of sublethal damage was dependent on the pre-irradiation conditions of the TS cells.

In an attempt to quantitate this phenomenon, I defined a recovery ratio. This ratio was found by dividing the surviving fraction at 2 hours between the dose fraction by the surviving fraction of the total dose given at one time for each cell type in each panel of Figure 5. If the recovery ratio for each cell type at 35°C was used to normalize the ratios found from the other panels, a relative recovery ratio could be plotted. The relative recovery ratio plotted against the time of pretreatment at 40°C is shown in Figure 6. This showed rather dramatically the effect on split dose recovery in the TS cells compared to the WT cells when the pretreatment interval was extended beyond 1.5 hours.

I decided at this point to examine the repair of sublethal damage using the other type of experiment described in the Materials and Methods (CHAPTER II, EXPERIMENTAL TECHNIQUES, Section B), that is, a first dose followed by a fixed time interval, then graded second doses. Since the earlier experiments showed a reduction of the repair of sublethal damage with a pre-first dose exposure to 40°C, I included in this experiment 2-hr exposures to 40°C before the first dose. The experimental scheme involved irradiating the WT and TS cells with 4.5 Gy, waiting for 2 hours, then giving a second dose that varied from 0 Gy to 5.5 Gy. The temperature the cells were held at before the first dose and between the dose fraction, was either 35°C or 40°C. A whole

DEPENDENCE OF SPLIT-DOSE X-RAY
RESPONSE ON 40°C PRETREATMENT TIME

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Figure 6. Relative recovery ratios, for all the 40°C panels in figure 5, plotted as a function of the amount of time at 40°C before the first dose. Recovery ratios from each 40°C panel for each cell type were normalized by the recovery ratio for each cell type at 35°C.

survival curve for each cell type was also completed so the split-dose repair could be illustrated more clearly. The results of this experiment are shown in Figure 7. The extent of repair of sublethal damage was illustrated by the partial reappearance of the shoulder on the split dose survival curve when the temperature was continuously held at 35°C. There was a reduction in the repair of sublethal damage in the TS cells when the temperature before the first dose and between the dose fractions was kept at 40°C. There was no reduction seen in the WT cells under these same conditions indicating that this was not just an effect of a 40°C treatment. No reduction in repair was seen with any other sequence of temperature and radiation. The α and β parameters for the best-fit LQ curves to these data are shown in Table 2. The α parameter of the TS cells seems to be most affected in the case where repair of sublethal damage was inhibited.

To examine this phenomenon more closely I decided to try a split-dose experiment in which the interval of time between the dose fractions was subdivided into time intervals at different temperatures. This was a similar experiment to the one in which progressively longer exposures to 40°C before the first dose were given. A reduction in the repair of sublethal damage occurred when 2 hours of 40°C preceded the first dose and continued in the 2 hour interval between doses (Figure 7). No reduction in repair was observed when 2 hours of 40°C preceded the first dose and the temperature was shifted to 35°C for the 2-hour interval between doses (Figure 7).

I wanted to examine what would happen to repair in the TS cells between these two results if the 35°C between the dose fractions was replaced in half-hour increments with 40°C after the first dose. The

X-RAY SPLIT-DOSE RESPONSE

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Figure 7. Survival of asynchronous WT cells (left side) and TS cells (right side) that were exposed to 2 hour intervals of various temperatures, before the first dose and between the dose fractions, in a split-dose irradiation scheme. The second dose was varied from 0 Gy to 5.5 Gy. The response of each cell type to single X-ray doses is shown by the dotted curves.

Table 2. α and β parameters for the best-fit LQ curves to single- and split-dose response of WT cells and TS cells (Figure 7).

	WT		TS	
	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$
Single dose	3.36 $\pm$ 0.34	4.32 $\pm$ 0.37	4.05 $\pm$ 0.4	3.99 $\pm$ 0.43
2 hrs of 35°C + 4.5 Gy + 2 hrs of 35°C + graded doses	3.70 $\pm$ 0.46	6.09 $\pm$ 0.81	4.36 $\pm$ 1.24	3.96 $\pm$ 2.15
2 hrs of 35°C + 4.5 Gy + 2 hrs of 40°C + graded doses	4.11 $\pm$ 1.24	3.49 $\pm$ 2.15	4.36 $\pm$ 0.85	5.36 $\pm$ 1.49
2 hrs of 40°C + 4.5 Gy + 2 hrs of 35°C + graded doses	4.73 $\pm$ 0.94	4.10 $\pm$ 1.64	5.16 $\pm$ 0.74	2.81 $\pm$ 1.29
2 hrs of 40°C + 4.5 Gy + 2 hrs of 40°C + graded doses	3.09 $\pm$ 1.15	7.25 $\pm$ 2.01	7.37 $\pm$ 0.78	2.35 $\pm$ 1.36

results are shown in Figure 8. The α and β parameters for the best-fit LQ curves to these data are shown in Table 3. The results indicated a progressive reduction in repair as the interval of 40°C extending from the pre-first dose interval into the interval between the doses became longer. This was a similar response to that observed in Figure 5 with the TS cells when the amount of pretreatment was varied from 0 hrs to 2 hrs.

The effect noted so far was not very large. I tried further variations of time and temperature to see if I could enhance the effect. One variation was to continue treating the TS cells at 40°C after the second dose for an additional 2 hours. In this split-dose experiment, a 2-hour interval at 40°C preceded the first dose of 4.5 Gy. The cells were held at 35°C or 40°C during the 2-hour dose-fractionation interval before second doses ranging from 0 Gy to 5.5 Gy were given. The results of this experiment are shown in Figure 9. The α and β parameters for the best-fit LQ curves to these data are shown in Table 4. Here again, the time and temperature scheme that seemed to inhibit repair of sublethal damage had the greatest effect on the α parameter. A further reduction in the repair of sublethal damage occurred as the 40°C treatment was extended after the second dose in the scheme where 40°C was present in the pre-first dose interval and in the intradose interval. In fact, it appeared to almost eliminate the repair of sublethal damage.

EFFECT OF 40°C TREATMENT TIME ON SPLIT-DOSE X-RAY RESPONSE

Figure 8. Split-dose survival of asynchronous TS cells when a 2 hour interval of 40°C precedes the first dose of 4.5 Gy and the 2 hour interval between the dose fractions was subdivided into intervals at different temperatures. The second dose was varied from 0 Gy to 5.5 Gy. The response to single X-ray doses is shown by the dashed curve.

TABLE 3. α and β parameters for the best-fit LQ curves to single- and split-dose response of TS cells (Figure 8).

	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$
Single dose	2.69 ± 0.3	4.81 ± 0.32
2 hrs of 40°C + 4.5 Gy + 2 hrs of 35°C + graded doses	1.95 ± 0.33	6.87 ± 0.64
2 hrs of 40°C + 4.5 Gy + 0.5 hr of 40°C + 1.5 hrs of 35°C + graded doses	3.63 ± 0.46	4.68 ± 0.88
2 hrs of 40°C + 4.5 Gy + 1.0 hr of 40°C + 1.0 hr of 35°C + graded doses	2.76 ± 1.15	5.97 ± 2.22
2 hrs of 40°C + 4.5 Gy + 1.5 hrs of 40°C + 0.5 hr of 35°C + graded doses	3.62 ± 0.5	5.75 ± 0.96
2 hrs of 40°C + 4.5 Gy + 2 hrs of 40°C + graded doses	2.96 ± 0.8	7.27 ± 1.54

Figure 9. Split-dose survival of asynchronous TS cells to a 2 hour interval of 40°C before the first dose of 4.5 Gy. The temperature during the 2 hour intervals, between the dose fractions and after the second dose, was changed to examine all possible combination of 35°C and 40°C. The second dose was varied from 0 Gy to 5.5 Gy. The response to single X-ray doses is shown by the dashed curve.

EFFECT OF THE DURATION OF PROTEIN SYNTHESIS INHIBITION ON SPLIT-DOSE X-RAY RESPONSE

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Figure 9.

TABLE 4. α and β parameters for the best-fit LQ curves to single- and split-dose response of TS cells (Figure 9).

	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$
Single dose	2.62 ± 0.36	4.72 ± 0.41
2 hrs of 40°C + 4.5 Gy + 2 hrs of 35°C + graded doses + 2 hrs of 35°C	3.38 ± 0.67	3.51 ± 1.29
2 hrs of 40°C + 4.5 Gy + 2 hrs of 35°C + graded doses + 2 hrs of 40°C	2.69 ± 0.24	5.75 ± 0.47
2 hrs of 40°C + 4.5 Gy + 2 hrs of 40°C + graded doses + 2 hrs of 35°C	3.74 ± 0.79	5.49 ± 1.51
2 hrs of 40°C + 4.5 Gy + 2 hrs of 40°C + graded doses + 2 hrs of 40°C	6.68 ± 0.49	3.25 ± 0.93

RESPONSE TO SINGLE DOSES AND EXPOSURE TO 40°C

In all these experiments it was important to observe any effect on the single-dose survival of cells treated at 40°C before and after the radiation exposure. To be able to say something about split dose repair it was important to correct for any effect the 40°C had on a single exposure. A series of experiments on the TS cells in which various times of 40°C preceded and/or followed single doses of radiation was performed. Figure 10 shows the results of this set of experiments. The α and β parameters for the best-fit LQ curves to these data are shown in Table 5.

The time intervals of 40°C were chosen to be the same as those encountered in the previously reported split dose experiments. The results of these temperature treatments on the single-dose X-ray survival were not dramatic. The data indicated some sensitization to the X rays if 40°C were given before and after the radiation exposure. This was more evident at high doses. The treatment scheme that provided the longest exposure to 40°C, 2 hours before and 4 hours after, seemed to show the greatest amount of sensitization.

This general effect was sometimes observed in the split-dose experiments in which a first dose was given and incubation occurred for the appropriate time period but a second radiation dose was not given. The survival of this point was sometimes below that of the single-dose control curve. Also, all data points of the set receiving 0 Gy to 5.5 Gy as a second dose seemed to be consistently lower. In those graphs the curve, for the multiple second exposures, was moved upward to account for this general decrease in survival in order to demonstrate

EFFECTS OF TEMPERATURE ON THE SINGLE DOSE X-RAY RESPONSE OF CHO-TSH1 (TS) CELLS

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Figure 10. Effect of different time intervals of 40°C before and/or after single X-ray doses on asynchronous TS cells. Broken line curves are reproductions of curves with data points shown in other panels.

TABLE 5. α and β parameters for the best-fit LQ curves to single-dose response with 40°C of TS cells (Figure 10).

	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$
35°C	3.00 ± 0.28	4.16 ± 0.32
2 hrs of 40°C + X rays	3.18 ± 0.47	4.55 ± 0.51
X rays + 2 hrs of 40°C	3.22 ± 0.22	4.36 ± 0.25
2 hrs of 40°C + X rays + 2 hrs of 40°C	1.99 ± 0.36	5.65 ± 0.41
2 hrs of 40°C + X rays + 4 hrs of 40°C	2.44 ± 0.19	5.88 ± 0.22

the effect of the overall time and temperature treatment on split-dose recovery.

MOLECULAR EXPERIMENTS

A. DNA strand breaks

It has been well established that DNA is a prime target for radiation damage. To further extend this thesis I was interested in observing what effect the ability to synthesize protein would have on DNA breaks and the rejoining of DNA breaks. Since the cell-survival studies showed that the repair of sublethal damage in the mutant cells was being affected by a 40°C treatment while repair in the wild type cells was not, I extended my studies using the DNA assay to include only the mutant cells. The alkaline-unwinding technique of Rydberg (1975) seemed to be the most appropriate assay to evaluate molecular damage by radiation. The assay allows for the use of doses in the range of those I have used in the survival experiments. In this way it may be possible to see a direct effect on DNA breaks and break rejoining in the mutant cell under the nonpermissive conditions for protein synthesis.

Figure 11 shows a dose-response curve for the fraction of double-stranded DNA versus dose for the mutant cells. The cells in this experiment were not irradiated on ice. The data falls along a straight line in this semi-log plot with a slope equal to $-[1.53 \times 10^{-3} \text{ cGy}^{-1} \pm 0.14 \times 10^{-3} \text{ cGy}^{-1}]$ as determined by the best-fit curve to the data. This linear relationship was consistent with the description of the radiation effect on breaking up the DNA as described by Rydberg (1975). Holding the TS cells at 40°C without radiation for various times up to

Figure 11. Double-stranded fraction versus X-ray dose for asynchronous TS cells measured by the alkaline-unwinding technique. Data from 5 experiments is shown.

10 hours introduced no more breaks than when the cells were kept at 35°C without radiation for various times up to 10 hours.

B. Rejoining kinetics: single dose

For the first experiments a dose of 10 Gy was chosen since it gave a large amount of breakage and was in the dose range used in the survival experiments. It was also apparent that to study the time course of the rejoining of breaks one needs to start out with a significant number of breaks to be able to follow the changes in the fraction of double-stranded DNA versus time. I wanted to examine the effect of 2 hrs of 40°C before a single dose of 10 Gy followed by various times of 40°C up to 6 hours. I wanted to use the same pretreatment time and temperature scheme which caused an alteration in the cell-survival studies. This would be compared to the response of cells given only 35°C before and after the 10 Gy dose. The results of this experiment are shown in Figure 12. The percent of double-stranded DNA in the irradiated samples was normalized to the percent double-stranded DNA in the unirradiated control samples to determine the double-stranded fraction. The 10 Gy experiments were performed such that the irradiations took place at room temperature. There appeared to be no difference in the rate of repair for short times (less than 30 minutes) or in the final extent of repair. There was also no difference in the initial amount of damage observed in the two temperature schemes.

Since no difference in the rejoining kinetics for 10 Gy was observed I thought that a higher dose might be necessary to elucidate some damage that is less apparent at a low dose. The single dose kinetics experiments were repeated but the dose was increased to 100 Gy. Figure

Figure 12. The main figure shows the double-stranded fraction for asynchronous TS cells after receiving a single X-ray dose of 10 Gy or 100 Gy followed by varying amounts of time up to 6 hours at 35°C or 40°C. The insert shows the results after a 100 Gy dose that was followed for up to 16 hours at 35°C or 40°C. Cells that were incubated at 40°C after irradiation were also incubated for 2 hours at 40°C before irradiation. Error bars representing the standard deviation of the mean are shown when larger than the symbol size.

DNA REJOINING KINETICS: SINGLE DOSES

Figure 12.

12 shows the results of that experiment. The samples for the 100 Gy group were cooled to ice temperature before irradiation and kept on ice during irradiation because of the lengthy time needed to complete the radiation exposure. The major difference between the 10 Gy curve and the 100 Gy curve was the fact that the return to control levels was much slower at the higher dose. In the 10 Gy case the damage was completely repaired after 4 hours, whereas in the 100 Gy experiment only about 70 percent of the damage had been repaired in that time. However, there again was no detectable difference between the samples in which protein synthesis had been inhibited and those in which it had not been inhibited.

At times less than 30 minutes there were large fluctuations in the double-stranded fraction. This was due to the large amount of radiation damage present in the cells. At the particular salt and temperature conditions of the alkali treatment, this large dose saturated the cellular DNA with damage in a non-resolvable manner. To get the zero-time and early kinetics would require little or no salt for a 20°C alkali treatment. However, this alkali treatment would show saturation for small amounts of damage and the longer time kinetics could not be followed. This illustrates a fundamental problem in using the hydroxylapatite technique.

For times longer than 6 hours after a dose of 100 Gy the double-stranded fraction was followed. The insert in Figure 12 shows this data. The 40°C treatment was found to cause a reduction in the fraction of double-stranded DNA at very long times after irradiation. This could be explained by considering that just as plating efficiency started to drop after long times at 40°C, in a heavily-irradiated cell,

which was prevented from synthesizing protein, DNA may start to degrade. I did notice that the activity in those samples that were incubated for 10 hours or more at 40°C after the 100 Gy dose was less than similar samples held at 35°C. Since all samples had the same conditions for incorporation of ^{14}C -TdR it seemed reasonable to conclude that the loss of label was due to degradation of the DNA.

C. Rejoining kinetics: split dose

I extended the single dose studies to look at the effects on the rejoining of DNA if another 10-Gy dose was given 2 hours after the first 10-Gy dose. After the second dose both sets of cells were at 35°C. That is, the cells were treated for 2 hours before the first dose with 40°C or 35°C, and the respective temperatures were continued in the 2-hour interval between the two 10-Gy exposures. This scheme of time, temperature, and radiation exactly duplicates the survival experiments in which a reduction in split-dose recovery was observed. In this experiment the cells at the time of irradiation were at room temperature. As can be seen in Figure 13, there appeared to be no difference in the early rate of rejoining or the final extent of rejoining in the mutant cells whether protein synthesis was inhibited or not in a split-dose scheme. Since DNA does rejoin at room temperature, even though the time of irradiation was about 1.5 minutes, I repeated the split-dose studies by cooling the cells to ice temperature before irradiation and held them on ice during the irradiation. After irradiation growth medium at the appropriate temperature was added back. There was a slight drop in the fraction of double-stranded DNA to 0.15-0.2 after each dose at the zero time point, indicating that there

DNA REJOINING KINETICS: SPLIT DOSES

Figure 13. Double-stranded fraction for asynchronous TS cells as a function of the time at 35°C or 40°C after receiving a first dose of 10 Gy (open symbols) and after receiving a second dose of 10 Gy (closed symbols) two hours after the first dose. The incubation temperature of the cells after the second dose is 35°C in all cases. Those cells maintained at 40°C between the two 10 Gy doses also received 2 hours of 40°C before the first 10 Gy dose. Error bars representing the standard deviation of the mean are shown when larger than the symbol size.

was rapid repair going on at room temperature. There was no shift in any of the other time points during the incubation period between doses or after the second dose.

SYNCHRONIZED CELLS

The work with asynchronous cells had given a clear indication that repair of sublethal damage could be affected by inhibiting protein synthesis. The effects noted though were sometimes small, and work with asynchronous cells has the added complication that effects observed represent that of a heterogeneous population. It is well known that the radiation sensitivity of mammalian cells varies dramatically through the cell cycle (Terasima and Tolmach, 1961; Sinclair and Morton, 1965). This may account for some of the experiment-to-experiment variation that was sometimes observed in the asynchronous survival experiments. If cell-cycle perturbations were introduced because of slightly different temperatures or set-up conditions from experiment to experiment, then the results of an asynchronous experiment may show variation because the subpopulations were slightly different each time. To investigate this possibility, it appeared to be of interest to study more homogeneous cell populations.

Mitotic synchronization allowed a way to obtain a more well-defined population of cells. Work with synchronized cells has the added advantage of allowing the experimenter to know a little better some of the biochemistry going on in the cell at particular stages in the cell-cycle phase. In this way a correlation between the radiation response and cellular biochemistry may become apparent. I extended

these studies with synchronized cells to include only the TS cell type since only the mutant cells were affected by the 40°C treatment.

A. Cell cycle parameters

Pulse labelling of synchronized cells with ^3H -TdR can provide basic information as to the cell-cycle parameters of a particular cell line. Cell synchrony induced by the method of mitotic shake-off was used. The percentage of cells taking up the radioactive label, i.e. synthesizing DNA, was plotted against the time, after synchronization, when the pulse was given. The results for the mutant cells using the reciprocal-shaking method are shown in Figure 14. The generation time (T_c) in the TS cell line was about 14 hours. The length of S, the DNA synthetic period, was about 7.5 hours. The length of G_1 was about 5.5 hours. By calculation, this left the length of $G_2 + M$ to be about 1 hour in duration.

The Coulter Channelanalyzer gave a size distribution of a sample of the mitotically-selected population. The size distribution indicated that at least 70 percent of the cells were small in size (G_1 phase). Microscopic examination of the cell suspension revealed most of the cells present to be single- or double-small cells. Most of the mitotic cells present appeared to be in telophase as evidenced by the pinched cytoplasm and the formation of the cleavage furrow. Based on these observations I assumed that the percent labelled cells pattern would represent mainly the length of G_1 -phase from 0 hours to the point of half the maximum percentage of labelled cells. The convention is to take this distance as representing the length of G_1 -phase plus half of mitosis but that assumes that mitotic cells are shaken-off in metaphase

Figure 14. TS cells mitotically selected by reciprocal shaking of an exponentially growing monolayer and pulse labelled with ^3H -TdR. Cell cycle parameters are indicated at the level of half the maximum amount of labelling.

CELL CYCLE PARAMETERS OF CHO-TSH1 (TS) CELLS (35°C)

Figure 14.

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half way through mitosis. That clearly was not the situation encountered here. Cell-cycle parameters were also measured from the control experiments that used the Talandic cell synchronizer as the source of synchronized cells. The cell cycle parameters measured from these data were very similar to the data in Figure 14.

Independently of these measurements, I performed a pulse-labelled mitosis experiment on an asynchronous group of TS cells to find the length of $G_2 + M$. This was found to be 1.5 hours. This same experiment also gave information about the percentage of cells in S-phase and mitosis for an exponentially growing population at the time the pulse was given. I found that 55% of the cells were in S-phase, and 4% of the cells were in mitosis. Assuming a 14 hour generation time, this experiment gave an S-phase length of 7.7 hours and M phase length of 0.6 hours. All of these calculations were consistent with the data from the synchrony experiments.

B. Effects of 40°C on cell progression

Before conducting radiation studies on synchronized cells, it was necessary to examine the effect an exposure to 40°C would have on the progression of synchronized cells. This would make interpretation of the radiation studies clearer by accounting for variations in the cell cycle which would possibly profoundly alter radiation sensitivity. Since the asynchronous cellular work was performed with 2 hr or 4 hr exposures to 40°C I looked at subsequent cell progression following these treatments. I chose two populations of cells, one 2 hours after synchronization in the middle of G_1 -phase and the other 10 hours after synchronization in the middle of S-phase. I used the method of pulse

labelling with ^3H -TdR to follow the subsequent progression of each cell population after 2 hours or 4 hours of 40°C . Since the size of these experiments for just one population was very large I performed the progression experiment on the S-phase cells at a separate time from the G_1 -phase cells. Figure 15 shows the control curve, that is, cells kept at 35°C for the whole experiment, in a composite form for both the S-phase and G_1 -phase cell experiments. The area of overlap from 10 hours to 20 hours shows the reproducibility of the synchrony technique used for each experiment. The arrows in the figure indicate the two populations of cells used in the subsequent progression experiments.

One group of the G_1 -phase population was heated at 40°C for 2 hours while another group was heated for 4 hours. The cells were returned to 35°C after this heat treatment. The pulse labelling was started at the end of the respective heat treatments and continued for many hours after. The results are shown in Figure 16. The early part of the control curve (Figure 15) is shown for comparison. There was a rather pronounced alteration of the labelling pattern. If the time when the half maximum value of the percent labelled cells occurred was taken as the beginning of S then 2 hours of 40°C caused a delay of 5.5 hours, while 4 hours of 40°C treatment caused a 10-hour delay. This data gave striking evidence that there must be very crucial protein synthesis going on in G_1 -phase that allowed a cell to pass into its DNA synthetic phase.

A similar experiment was performed on the S-phase cells. The progression of these cells was followed after an exposure for 2 hours or 4 hours at 40°C . The results are shown in Figures 17 and 18. The control curve (Figure 15) is shown for comparison. Here again rather

Figure 15. Percent labelled TS cells after a pulse of ^3H -TdR was given to two population of cells used in subsequent progression experiments. This figure shows the effect on a sample of each population that is kept at 35°C . The arrows indicate the time after synchronization at which each population was chosen, G_1 (2 hours) and S (10 hours).

PROGRESSION OF UNTREATED CHO-TSH1(TS) CELLS (35°C)

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Figure 15.

Figure 16. Effect on the subsequent progression of a G_1 population of TS cells that were subjected to a 2 hour treatment at 40°C (closed symbols) or a 4 hour treatment at 40°C (open symbols). The G_1 population used in this experiment represents cells 2 hours after mitotic synchronization. The dashed curve is the progression of the untreated G_1 population (35°C) shown in figure 16.

XBL943-7638A

Figure 16.

Figure 17. Effect on the subsequent progression of an S population of TS cells that were treated for 2 hours at 40°C (closed symbols) or cells treated for 2 hours at 40°C followed immediately by an X-ray exposure (open symbols). The S population used in this experiment represents cells 10 hours after mitotic synchronization. The dashed curve is a reproduction of figure 16, the progression of an untreated (35°C, no radiation) group of cells.

PROGRESSION OF S PHASE CHO-TSH1(TS) CELLS

Figure 17.

XBL 843-7641D

Figure 18. Effect on the subsequent progression of an S population of TS cells that were treated for 4 hours at 40°C (closed symbols) or treated for 2 hours at 40°C followed immediately by an X-ray exposure and returned to 40°C for 2 hours more (open symbols). The S population used in this experiment represents cells 10 hours after mitotic synchronization. The dashed curve is a reproduction of figure 16, the progression of an untreated (35°C, no radiation) group of cells.

PROGRESSION OF S PHASE CHO-TSH1(TS) CELLS

Figure 18.

XBL 843-7641C

dramatic alterations in cell progression occurred due to the 40°C treatment. I observed that the percent labelled cells as shown by the first point after the respective heat treatment was decreased but not dramatically so compared to the control curve. Additional autoradiographic data taken after 1 hour and 3 hours of 40°C also showed a similar slight reduction below control levels. However, the number of grains per cell at these time points was very much reduced compared to that found in the control samples indicating that the rate of DNA synthesis was reduced. After the cells were released from 40°C the percent labelled cells increased and also the number of grains per cell increased. No mitotic figures or G₁-doublets were observed during the time of the peak in the percent labelled cells following the end of the 40°C treatment. Mitotic figures and G₁-doublets were observed at the time of the subsequent valley of percent labelled cells for each heat-treated group. If the time, when the half maximum value of the percent labelled cells for the leading edge of the second S-phase, was compared between the control and heat-treated groups, delays could be calculated. A treatment of 2 hours at 40°C gave a delay of 4.5 hours while 4 hours at 40°C resulted in an 8-hour delay.

C. Effects of X rays and 40°C on cell progression

The experiments discussed in the previous section showed that a 40°C treatment of S-phase cells altered the pattern of labelling subsequent to the treatment. It is well established that X rays can have dramatic effects on DNA synthesis (Altman et al., 1970). To have a clearer understanding in my experiments it became necessary to know about the combined effects of a 40°C treatment and X rays on the subsequent

labelling pattern. Experiments were performed in a similar fashion to those described in the previous section. These experiments were carried out on a different batch of S-phase cells. However, the control pattern of ^3H -TdR incorporation looked identical to that shown in Figure 15. The experiment was set up such that after the 10-hour population had received 2 hours of 40°C , an X-ray dose of 5.5 Gy was given to two groups of cells. One group had the temperature returned to 35°C after irradiation (Figure 17), and the other group was kept at 40°C for an additional 2 hours (Figure 18). Pulse labelling was performed on each group to follow the subsequent progression. Similar to the experiments done without X rays there appeared to be an extended period of DNA synthesis before the cells divided. X rays introduced about a 6-hour delay in the onset of mitosis as evidenced by the time of the valley in the percent labelled cells. In contrast to the labelling pattern seen with just the heat treatment alone, the peak of labelling immediately after the heat was removed, was broader, lower, and shifted to the right. It appeared that the extended period of DNA synthesis due to the heat treatment was still present, but the time course of this synthesis was altered by the X-ray exposure. Similar to the experiments with heat alone, mitotic figures and G_1 -doublets were only observed at the time of the subsequent valley of percent labelled cells for each treated group.

D. X-ray response

To begin to explore the phenomenon of the repair of sublethal damage in synchronized population of cells it was necessary to know something about the radiation sensitivity of each population to single doses of X rays. Survival curves for a G_1 population (2 hours after

synchronization) and an S population (10 hours after synchronization) are shown in Figure 19. The α and β parameters for the best fit LQ curves to these data are shown in Table 6. These two populations were heated at 40°C for 2 hours before single-graded doses were given. The reason for this pretreatment was to make these populations a better control for the subsequent split-dose studies. There was a broad shoulder on the S-phase survival curve, while the G_1 -phase survival curve had a less prominent shoulder.

E. Repair of sublethal damage

Split dose studies were undertaken on the synchronized mutant cell populations. A time and temperature scheme used earlier on the asynchronous cells was repeated here. Data from Figure 7 indicated that the same level of split-dose recovery occurred in cells that received only a 2-hour pretreatment of 40°C as cells that never were exposed to 40°C. Since experiments on synchronized cells can be complicated by variations of radiation sensitivity at different cell ages, the planning of split-dose studies to examine repair must have appropriate controls. Since the treatment scheme to produce the reduction in split-dose repair needed a pretreatment of 40°C before the first dose, as well as 40°C between the dose fractions, an appropriate control group for comparison was one that had also been exposed to 40°C before the first dose. In this way, variations, due to cell age differences between the two groups receiving the split radiation doses, would be minimized.

At the appropriate time after synchronization, each population of cells was held at 40°C for 2 hours. Doses of X rays were then given to produce a surviving fraction of about 0.1. In the case of the G_1

SINGLE DOSE X-RAY RESPONSE OF SYNCHRONIZED CHO-TSH1 (TS) CELLS

XBL 852-6970

Figure 19. Survival of two synchronized populations of TS cells to single X-ray doses. The G₁ population represents cells 2 hours after mitotic synchronization and the S population represents cells 10 hours after mitotic synchronization. Each population was treated for 2 hours at 40°C before irradiation. The data from two separate experiments is shown.

TABLE 6. α and β parameters for the best-fit LQ curves to single- and split-dose response of synchronized TS cells (Figures 19 and 20).

G_1 population

	α ($\times 10^{-3}$) cGy^{-1}	β ($\times 10^{-6}$) cGy^{-2}
Single dose	3.37 ± 0.3	5.72 ± 0.44
2 hrs of 40°C + 3.7 Gy + 2 hrs of 35°C + graded doses	5.18 ± 0.62	2.95 ± 1.48
2 hrs of 40°C + 3.7 Gy + 2 hrs of 40°C + graded doses	6.07 ± 0.38	3.8 ± 0.92

S population

	α ($\times 10^{-3}$) cGy^{-1}	β ($\times 10^{-6}$) cGy^{-2}
Single dose	0.75 ± 0.26	6.03 ± 0.29
2 hrs of 40°C + 5.5 Gy + 2 hrs of 35°C + graded doses	3.66 ± 0.44	4.11 ± 0.69
2 hrs of 40°C + 5.5 Gy + 2 hrs of 40°C + graded doses	7.44 ± 0.62	2.7 ± 1.05

population this was a dose of 3.7 Gy, while for the S population this was a dose of 5.5 Gy. After this first dose the population of cells was split into two groups, one being held at 40°C for an additional 2 hours and the other group being held at 35°C. After this 2-hour interval, graded doses of X rays were given. The results of this experiment are shown in Figure 20. The single-dose response (Figure 19) for each population was reproduced for comparison. The α and β parameters for the best-fit LQ curves to these data are shown in Table 6. Due to the size of each of these experiments, it was necessary to obtain the G_1 population and the S population from two different mitotic populations. To ensure that the cell progression for each batch of cells was comparable, pulse labelling was done at various intervals after synchronization. The percent labelled cells versus time for each batch of cells was inserted in the same figure. As can be seen the control pattern of labelling for the batch of cells used as the G_1 population was equivalent to that pattern for the batch of cells used as the S population.

As was seen with asynchronous cells a reduction in survival was observed in both cell populations when protein synthesis was inhibited before the first dose and in the interval between the dose fractions. In the G_1 -phase cells the effect seemed comparable in magnitude to that of the asynchronous cells, however in the S-phase cells repair of sublethal damage was almost eliminated. The S-phase cells showed a large change in the α parameter, while the G_1 -phase cells showed a small change in α . The change in α for the G_1 -phase cells was comparable in magnitude to that observed in the work with asynchronous cells. I did observe that irradiation followed by 2 hours of 40°C with no additional

Figure 20. Survival of two different synchronized populations of TS cells to split doses of radiation. The temperature before the first dose was kept at 40°C for 2 hours in all cases. The temperature between the dose fractions was either 35°C or 40°C. The single dose survival of each population shown in figure 20 is reproduced here as a dotted curve. Data from two separate experiments is shown. The insert shows the progression at 35°C of a sample of each population used in these experiments. Error bars representing the standard deviation of the mean are shown in the insert when larger than the symbol size.

SPLIT-DOSE X-RAY RESPONSE OF SYNCHRONIZED CHO-TSH1 (TS) CELLS

NSL 852 8200

Figure 20.

radiation gave data which fell below the dashed curves. This meant there was enhanced cell killing when 2 hours of 40°C was continued after the first dose. This enhanced cell killing was also observed with asynchronous cells. The effect on the split-dose survival can be better displayed by moving the split-dose data upward to correct for the additional cell-killing effect. This then allows all split-dose data for a given cell population to start at the same point on the single-dose survival curve.

CYCLOHEXIMIDE STUDIES

A. Effects on the cell lines

The previous methods of turning off protein synthesis in eukaryotic cells involved the use of chemical inhibition. Early radiation studies examined these chemical inhibitors to try to observe an alteration in split-dose recovery when protein synthesis was inhibited. Cycloheximide was one of these frequently used chemicals to inhibit protein synthesis. I wanted to explore what effect cycloheximide would have on protein synthesis in this CHO cell line in an attempt to gain additional evidence that the effects observed were related to the inhibition of protein synthesis caused by the non-permissive temperature.

Cytotoxicity of the cycloheximide was examined first. Since the survival of the mutant cells at 40°C was starting to decrease after about 6 hours, I decided that this would be the maximum length of time that I could treat the cells with cycloheximide. Different concentrations of cycloheximide were dissolved in complete growth medium and both cell types were treated for 6 hours at 35°C. The results are

shown in Figure 21. At a concentration of cycloheximide greater than 10 $\mu\text{g/ml}$ the survival slowly started to decrease. This concentration of cycloheximide was chosen for the subsequent studies.

I performed experiments to measure the percent rate of incorporation of ^3H -Leucine into the TS and WT cells after treating both with 10 $\mu\text{g/ml}$ of cycloheximide in complete growth medium at 35°C. I also examined the recovery of protein synthesis in each cell type after 2 hours or 4 hours of cycloheximide treatment at 35°C. The results are shown in Figure 22. There was a very rapid turnoff of protein synthesis in both cell types with the cycloheximide. The level of inhibition seemed comparable to that achieved in the mutant cell without cycloheximide when the temperature was shifted to 40°C for 2 hours. Also the recovery rate was very rapid, again a mirror image of the shutdown rate. The percent rate of incorporation for each cell type did not return to 100% even 2 hours after the cycloheximide treatment, just as was seen in the mutant cell without cycloheximide after treatment at 40°C for various times.

B. Split-dose effects

The observations based on the temperature treatment seemed to indicate that protein-synthesis inhibition before a first dose and between the dose fractions was necessary to demonstrate a reduction in the repair of sublethal damage. To test this hypothesis, I decided to use cycloheximide to inhibit protein synthesis during a split-dose experiment. If protein synthesis was involved, then a treatment of both the wild-type and temperature-sensitive cells with cycloheximide at 35°C should mimic the effect seen previously with only the TS cells at the nonpermissive temperature. A fractionation experiment was performed

XBL 852 6969

Figure 21. Survival of cells exposed to growth medium with cycloheximide for 6 hours. Data was normalized to the survival of cells exposed to growth medium without cycloheximide present.

EFFECTS OF CYCLOHEXIMIDE ON PROTEIN SYNTHESIS

XBL 852 6976

Figure 22. Percent rate of ^3H -Leu incorporation into the TCA insoluble fraction of the WT cells and TS cells during and after exposure of the cells to growth medium containing $10\text{ }\mu\text{g/ml}$ of cycloheximide. The percent rate of incorporation was calculated relative to the uptake of labelled Leucine into each cell type when the drug was absent from the growth medium.

with and without cycloheximide in which a single dose of 4.5 Gy was given to the cells, and 2 hours later a second graded dose from 0 Gy to 5.5 Gy was given. To simulate the non-permissive temperature I treated cells of both cell types for 2 hours before the 4.5 Gy dose and during the 2-hour fractionation interval with medium containing 10 $\mu\text{g/ml}$ of cycloheximide. A control group was also studied in which both cell types were not exposed to cycloheximide. The results of this experiment are shown in Figure 23. The α and β parameters for the best-fit LQ curves to these data are shown in Table 7. There was a reduction in the repair of sublethal damage in the TS and WT cells when they were exposed to cycloheximide under these conditions. The α parameter was changed in the WT cells by the cycloheximide treatment; however, the β parameter seemed to be altered in the TS cells by the cycloheximide treatment. The split-dose survival curves for both treated cell types look very similar to that seen in Figures 7,8, and 9 when temperature alone was used to inhibit protein synthesis in the mutant cells before the first dose and between the dose fractions.

Figure 23. Survival of asynchronous WT cells and TS cells to split doses of radiation in the presence or absence of growth medium containing 10 $\mu\text{g/ml}$ of cycloheximide. The first dose used in all the split dose experiments was 4.5 Gy. The second dose was varied from 0 Gy to 5.5 Gy. The two doses in all cases were separated by 2 hours. The treatment temperature in all cases was 35°C. The cells used in the split dose with cycloheximide experiments were treated for 2 hours before the first dose and during the 2-hour dose fractionation interval with growth medium containing cycloheximide. The cells used in the single dose and split dose without cycloheximide experiments were not exposed to the drug at all.

EFFECT OF CYCLOHEXIMIDE ON SPLIT-DOSE X-RAY RESPONSE

Figure 23.

XBL 852 6979

Table 7. α and β parameters for the best-fit LQ curves to single- and split-dose response with or without cycloheximide of WT cells and TS cells (Figure 23).

	WT		TS	
	α ($\times 10^{-3}$) cGy $^{-1}$	β ($\times 10^{-6}$) cGy $^{-2}$	α ($\times 10^{-3}$) cGy $^{-2}$	β ($\times 10^{-6}$) cGy $^{-2}$
Single dose	1.88 ± 0.59	6.50 ± 0.73	2.99 ± 0.28	4.22 ± 0.32
Split dose without cycloheximide	2.25 ± 0.43	5.22 ± 0.83	3.38 ± 0.67	3.51 ± 1.29
Split dose with cycloheximide	3.72 ± 0.16	6.80 ± 0.38	3.16 ± 0.72	7.27 ± 1.38

CHAPTER IV

DISCUSSION

This dissertation undertook a reexamination of the question as to whether or not protein synthesis was a requirement for sublethal damage repair. The experimental approach was through the use of a mutant cell line that is temperature-sensitive for protein synthesis. The data indicated that protein synthesis was involved in the repair of sublethal damage or Elkind recovery. The relationship between the two only became evident when a very particular time sequence of inhibition was used. No effect on Elkind recovery was present when protein synthesis was inhibited only between the dose fractions. This observation suggested that the damage caused by the first dose of radiation did not appear to induce the synthesis of proteins which might have been involved in Elkind recovery. This same experiment also demonstrated that repair after the first dose can proceed normally when protein synthesis was inhibited. Experiments in which protein synthesis was inhibited only before the first dose indicated that the pretreatment introduced no additional damage compared to the non-inhibited cells. The fact that the cells needed to be inhibited before the first dose as well as between the dose fractions suggested that the proteins involved were constitutive and that a pool might exist. When this pool was depleted by the pretreatment interval a reduction in Elkind recovery resulted. It seemed that a 2.5 to 3 hour interval of protein synthesis inhibition before the first dose was sufficient to completely eliminate the repair of sublethal damage in an asynchronous cell population.

The recovery process seemed to occur rapidly, as evidenced by the data of Figure 8. In that experiment the intradose time interval was subdivided into periods of nonpermissive and permissive temperature for protein synthesis. As the time of inhibition of protein-synthesis was extended after the first dose, the data indicated that Elkind recovery was progressively reduced. The response to single doses and exposure to 40°C (Figure 10) added support for the idea that the recovery process and not the amount of damage introduced with each dose was responsible for the reduced cell survival observed. The results of Figure 9 add further evidence that the repair process going on immediately after the radiation exposure was affected. If a split-dose radiation scheme is envisaged to be a process of damage production, repair of that damage, introduction of more damage and the resultant repair of all accumulated damage then the effects observed in my experiments can be explained by considering that the repair process after each dose was dependent on ongoing protein synthesis.

The concept of a pool of chemical compounds involved in radiation survival was proposed by Powers (1962). He suggests that as the radiation exposure continues these substances are used up by the repair process. He proposes that Elkind recovery is a demonstration of the exhaustion of this pool by the first dose and replenishment during the interval between the dose fractions. A formalized mathematical treatment of these ideas is presented by Laurie et al. (1972). The ideas presented by these authors suggest that the shoulder on the survival curve and Elkind recovery are conceptually and experimentally distinguishable. The two phenomena are manifestations of two distinct

processes which are separated by the existence of a limited pool of intracellular constituents.

The idea of Elkind recovery being distinct from the shoulder on the survival curve is also suggested by Alper (1977). Alper thinks the radiobiological data that has accumulated over the years gives strong evidence that the shape of the survival curve can be attributed to a repair process operating at the outset of irradiation but becoming less and less effective as the dose increases. During a radiation-free interval, the surviving cells would recover their repair capacity, and that is how Elkind recovery would be defined. Alper believes that the view of the shoulder on a survival curve as representing the accumulation of damaged targets and Elkind recovery being the restoration of some of these damaged targets is misleading. From the concept of repair processes determining the survival curve shape comes the idea that the recovery phenomenon may depend on a pool of some factors inside the cell. In the simplest association the shoulder might be a demonstration of the concentration of these factors (Alper, 1979).

The idea of a pool of protein involved in the repair of sublethal damage has been suggested by Wienhard and Kiefer (1977). They examined the effect of cycloheximide on the repair of sublethal damage in yeast. Using exponentially growing cells and stationary-phase cells they conclude that there may be a pool of protein that functions in split-dose repair. The repair of sublethal damage can occur, but may not go to completion, if protein synthesis is blocked. Cells with depleted pools can be produced and these cells are unable to undergo split-dose repair unless protein synthesis is allowed to start.

The nature of the proteins that might be involved is open to speculation. Experiments of Sinclair and his colleagues contributed to the development of the idea of Q factor (Sinclair, 1972). This substance is postulated to vary in concentration throughout the cell cycle and account in part for the variation of radiation sensitivity observed during the cell cycle. Experiments with cysteamine and N-ethylmaleimide indicated that Q factor may be identified with a fraction of the intracellular sulfhydryl groups presumed to be involved in repair processes. These pools of sulfhydryl groups can be associated with proteins or non-protein compounds. The association of Q factor with one of these groups is not clear.

Since only a 2-hour treatment before the first dose was necessary to see a reduction in Elkind recovery in my experiments it seemed that whatever proteins were involved had a rapid depletion or turnover rate. Evidence of turnover times for proteins in mammalian cells is not abundant. Likely candidates for repair proteins or enzymes are endonucleases, glycosylases, DNA polymerases, ligases, or nucleotide phosphorylases. However, available evidence seems to show that their lifetimes are many hours in duration (Gautschi et al., 1973).

The DNA-rejoining experiments were an attempt to assay the activity of those proteins and enzymes involved in the physical rejoining of X-ray damaged DNA. I also wanted to assay other cellular responses to X rays to see whether the effects on split dose survival would also show up with other techniques. The results demonstrated that DNA was rejoined in the presence or absence of protein synthesis. This result was consistent with the work of Gautschi et al. (1973). The indication was that rejoining of DNA was not a process clearly linked to the

process of Elkind recovery. My experiments could only measure whether the DNA was rejoined, not whether the fidelity of the rejoined DNA was correct. It is very reasonable to assume that misrepair of DNA by insertion of incorrect bases could have profound effects on a cell's ability to survive and proliferate (Tobias et al., 1980). Also the alkaline-unwinding technique measured the total breaks which represented the sum of single-strand breaks, double-strand breaks and alkali-labile sites. If a very small subset of any of these lesions was crucial for cell survival it may not have shown up with this assay.

A technique for detecting damage caused by very low doses of ionizing radiation and also monitoring the repair of this damage has been developed (Cook and Brazell, 1976). Cells are lysed in the presence of non-ionic detergents and solutions of high-salt concentration, releasing structures called nucleoids. Nucleoids resemble nuclei in that they contain nearly all nuclear RNA and DNA but are depleted of nuclear proteins. The DNA is supercoiled and compact so that nucleoids sediment very rapidly in sucrose gradients. The sensitivity of this technique comes from the fact that a small number of strand breaks can alter the supercoiling of the DNA and change the sedimentation pattern of the nucleoids. Charles and Cleaver (1982) report on the comparison of nucleoid and alkaline-sucrose gradients to study the effects of DNA-repair inhibitors on human fibroblasts. They report very different observations with both techniques and suggest that the two techniques provide different views of the repair process. The nucleoid-sedimentation procedure may provide another way to look at the effects of protein synthesis inhibition on the repair of DNA damage that is different from the unwinding technique.

Another hypothesis to consider is that physical rejoining of DNA is only part of the repair process. For the damaged part of the chromosome to become fully functional again protein synthesis may be required to repair the chromatin structure of which the DNA is a part. Repairing chromatin may then involve the synthesis of new histones and non-histone chromosomal protein which both help to create the chromatin structure inside of mammalian cells. Support for this hypothesis comes from Wolff (1959; 1972). He showed that protein synthesis had an effect on the ability of chromosome breaks to reconstitute.

No clear correlations between the repair of DNA damage and chromosomal damage and the cellular repair processes of sublethal damage and potentially lethal damage has been made. In light of the present work described in this thesis, an appropriate follow-up study would be the reexamination of the role of protein synthesis in the repair of chromosomal radiation damage in mammalian cells. From this study a correlation between sublethal damage and potentially lethal damage and chromosome damage may be possible. The premature chromosome condensation (PCC) technique opens an area of research by permitting chromosomal damage and its repair to be examined in interphase cells.

The PCC technique allows a more rapid determination of chromosomal damage to be made instead of having to wait for the cell to proceed to mitosis before the scoring of damage can be done. CHO-TSH1 cells provide a very useful system in which to manipulate protein synthesis. Hittelman and Pollard (1982) try to relate γ -ray-induced DNA damage as measured by the elution technique (Kohn and Grimek-Ewig, 1973) to γ -ray induced chromosome damage as measured by the PCC technique in CHO cells. They use cycloheximide to observe what effect it will have on the repair

of the two types of damage. Their conclusion is that chromosome repair may involve not only the repair of damaged DNA but also protein synthesis. A very similar picture also emerges with regard to the radiomimetic drug, bleomycin. DNA damage and chromosomal damage, measured by the previously mentioned technique, are compared as to the effect of cycloheximide on their respective repair. The conclusion reached by the authors is that chromosomal repair might involve cycloheximide-sensitive pathways in addition to DNA repair (Sognier et al., 1979; Sognier and Hittelman, 1979). Cornforth and Bedford (1983) measure the rejoining rate of X-ray-induced chromosome breaks using the PCC technique in noncycling G_1 cells of a normal human fibroblastic cell line. They compare the rejoining rate with the rate of repair of PLD and find that the two processes have nearly identical kinetics. The PCC technique seems to be a very powerful tool for increasing our understanding of how radiation damage at the DNA or chromosomal level can result in cell lethality.

All of my early experiments were done with asynchronous cells. This provides an easily obtainable cell population but one that was heterogeneous in terms of the radiation sensitivity of the subpopulations that are present. The experiments with synchronized cells were chosen in an attempt to have more homogeneous populations. The results were most interesting when comparing the effect of protein-synthesis inhibition on the ability of S-phase and G_2 -phase cells to repair sublethal damage. S-phase cells which were the most resistant to single doses of X rays showed the greatest reduction of sublethal damage repair when protein synthesis was inhibited (Figure 20). Histones and probably some non-histone chromosomal proteins are

synthesized during S-phase. There is probably a great demand for these components of chromatin during the DNA synthesis period. It is possible to speculate that an interruption in protein synthesis would put severe stress on a cell in S-phase if it was also trying to repair radiation damage. These necessary protein components may be depleted very quickly under these conditions leading to more cell killing than was evident in G₁-phase cells. G₁-phase cells in comparison would have less demand for these protein components of chromatin.

The radiation sensitivity of cells in different stages of the cycle can be different. A concern here was that during the pretreatment or interdose intervals the cells had not progressed into a stage with markedly different radiation sensitivity. Variations in cell-cycle position of the different groups of cells used in the split-dose experiments seemed to be unlikely complications since the cell progression studies indicated that during the time of the experiment the two groups were not appreciably different in terms of cell age.

The split-dose experiments with cycloheximide were done to provide additional evidence that protein-synthesis inhibition was indeed responsible for the reduction in Elkind recovery. The results showed that a comparable level of reduction of Elkind recovery, to that observed in the TS cells using a temperature-induced inhibition of protein synthesis, could be achieved in both cell types using a chemically-induced inhibition of protein synthesis. The rate of incorporation of labelled leucine seemed to indicate that cycloheximide had a more rapid effect on protein synthesis than did the temperature-induced mutation. However, the rate of incorporation showed lower levels of inhibition with the elevated temperature than with

cycloheximide. I believe the differences in the kinetics of the two types of inhibition were a result of the way I chose to manipulate the temperature by placing medium-filled flasks into a water bath. The kinetics therefore reflect the process of temperature equilibration in addition to the temperature inhibition of synthesis. Stanners (1977) has reported that a quick temperature equilibration technique reduced the rate of protein synthesis below 5 percent within four minutes in the CHO-TSH1 cell line. The fact that a more severe depression of synthesis could be achieved with the mutation seems to indicate that cycloheximide may not be as strong an inhibitor as the temperature-induced mutation.

Although the mechanism of protein-synthesis inhibition by puromycin, cycloheximide and the temperature-sensitive mutation are different, one might expect to see similar effects on split-dose recovery. Puromycin and cycloheximide act to stop the elongation stage of protein synthesis, respectively, by bonding to the growing peptide chain and preventing peptide bond formation (Lehninger, 1975). The temperature-sensitive mutation, on the other hand, seems to block the initiation stage of protein synthesis by preventing the formation of the necessary activated amino acid (Stanners, 1977). The reason Elkind et al. (1967a) did not see any effect on split-dose recovery with puromycin could be that they did not pretreat the cells long enough before the first dose. My experiments showed that a pretreatment time of at least 1.5 hours was necessary before an effect became apparent. Also the level of protein-synthesis inhibition achieved by Elkind et al. may not have been severe enough to deplete the pool of crucial proteins which are required for repair. Kim et al. (1966) used puromycin but also did not pretreat the cells before the first dose. Berry (1966), using cycloheximide and

puromycin, did pretreat the cells for 18-20 hours before the first dose; however, the level of protein-synthesis inhibition was only down to 30 percent of control levels. It could be that a certain critical level of protein-synthesis inhibition is required before split-dose recovery is affected. One can speculate that the critical level of inhibition may be one that will affect the synthesis of essential proteins such as structural proteins involved in the organization of the chromosomes.

The results of a 40°C treatment or a 40°C treatment with X rays on the progression of S-phase cells were very intriguing. The progression of S-phase cells after a treatment of temperature alone seemed to indicate that the S period was extended in duration. What this means in terms of DNA synthesis is not clear. Several possibilities to account for the DNA-synthetic activity after treatment are that DNA synthesis was stopped during the temperature treatment and must be finished before the cells can proceed, that DNA was degraded during the treatment so that resynthesis must occur, or that reduplication of DNA already synthesized was triggered by the temperature treatment. Of course, any combination of these processes is also possible. X rays have a profound effect on DNA synthesis. At moderate doses the effect is one of inhibition of initiation of replicons. The results of my progression experiments in which X rays were given after or during the heat treatment also showed a reduction in the DNA synthetic activity that seemed to have been stimulated by heat alone.

The method of synchronization by mitotic shake-off provides a population of cells within a narrow window of cell age. The fact that in my experiments the percent labelled cells pattern for untreated (control) cells did not reach 100% during the middle of S-phase

indicated that there was a small subpopulation that may have already finished DNA synthesis at that time. The heat or heat with X rays treatment would be expected to have a different effect on the subsequent progression of that subpopulation. The lack of a very tightly synchronized cell population can make the interpretation of the percent labelled cells pattern more difficult.

The percent labelled cells versus time graphs gave information only about whether or not a cell was synthesizing DNA not how much was being synthesized. The autoradiographs for the heat and X-ray treated samples were counted to obtain information about the number of blackened grains per labeled cell. This is a measure of the rate of DNA synthetic activity. This data showed that during the conditions of heat plus X rays shown in Figure 17, ~ 1.5 times the normal amount of DNA was present in a cell entering G_2 and for Figure 18, ~ twice the normal amount of DNA was present in a cell entering G_2 . These observations suggest that the cells were not just synthesizing DNA to replace what was lost or not finished during the heat treatment. The results suggest that extra DNA may have been synthesized. However, one must be very cautious in using the grains per labelled cell data to measure DNA synthesis. Heat and X rays can alter the cell's ability to absorb biochemicals from the environment and can alter intracellular pools of these biochemicals. These changes could then mimic changes in the amount of DNA synthesized. A way to test the hypothesis of extra DNA being synthesized would be to take the treated cells and place them through a flow microfluorimetry system which would measure the DNA content of each cell. If a large amount of reduplication of DNA has occurred it should show up very clearly with this analysis system.

Another technique would involve looking at mitotic cells after the treatment to observe what type of chromosome configuration might be apparent. If whole chromosomes were reduplicated these would show up clearly in a mitotic preparation.

Aberrant replication or overreplication of DNA has been reported after transient inhibition of DNA synthesis in a number of organisms. There is a review by Schimke (1984) that covers these observations. One study by Woodcock and Cooper (1981) found that a pulse of cycloheximide resulted in double replication of chromosomal DNA segments in a human cell line. They also observed a similar effect with specific DNA synthesis inhibitors 1- β -D-arabinofuranosylcytosine (ara-C) and 9- β -D-arabinofuranosyladenine (ara-A). The DNA that is reduplicated is transitory in nature, usually being lost in subsequent mitoses. However, the amount of the genome reduplicated can be rather extensive (Mariani and Schimke, 1984).

In conclusion, the synthesis of proteins was essential for the complete repair of sublethal damage. Since the system used in these experiments was a general protein-synthesis inhibitor, it was difficult to gain knowledge as to which specific proteins might be involved. The work with synchronized cells revealed that the inhibition of sublethal damage repair appeared to be a cell-cycle specific effect in which S-phase cells were inhibited more in their repair capability than G₁-phase cells. This was suggestive that chromosomal proteins may be involved and that Elkind recovery involves not only the repair of damaged DNA but restitution of the chromatin structure in a mammalian cell.

APPENDIX A

I. Staining autoradiography plates with Hematoxylin

Dilute concentrated Hematoxylin stock solution 1:10 with distilled water. Filter stain twice through 0.45 μ Nalgene filters.

Hematoxylin stock solution:

Hematoxylin	0.5 g
$(\text{NH}_4)_2 \text{SO}_4 \cdot \text{Al}_2 (\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$	5.0 g
NaIO_3	0.1 g
glycerol	30.0 ml
glacial acetic acid	2.0 ml

Dissolve solids in 70 ml of distilled water. Add glycerol and acetic acid. This solution cannot be used immediately, but must be allowed to stand for at least 3 days.

II. Citric acid disodium phosphate buffer

2.22 ml of 0.1 M citric acid
 2.78 ml of 0.2 M Na_2HPO_4
 5.0 ml of distilled water

Make enough of this buffer to last a couple of months. It can be stored at 4°C for extended periods.

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