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INDUCTION OF A DNA RECOVERY RESPONSE IN BENZO[a]PYRENE DAMAGED MAMMALIAN CELLS

by

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ABSTRACT

The induction of a DNA recovery response in CV-1P African green monkey kidney cells treated with the environmental carcinogen Benzo[a]pyrene 7,8 diol, 9,10 epoxide (BPde) was studied. The induction of this response was assayed by an increase in survival of UV irradiated SV40 virus grown in BPde pretreated cells over control treated cells. Concentrations of BPde which correspond to the shoul- region of the clonogenic survival curve (0.16 μM and lower) did not enhance viral survival. An enhancement of survival was seen when cells were pretreated with concentrations of BPde corresponding to the linear or exponential portion of the survival curve (0.33 μM and greater). A time course of enhanced viral survival at 0.66 μM BPde showed this response to be fully induced by 24 hours and to enhance viral survival after 96 hours.

CHAPTER 1

INTRODUCTION

One of the earliest observations of chemical carcinogenesis was recorded in 1775 when Sir Percival Pott noted a high incidence of skin cancers in chimney sweeps, attributing it to coal and soot exposure (1). The identification of and human exposure to many carcinogenic chemicals in the environment has led to the estimate that 80 - 90% of all cancers are environmentally caused (2).

The role of chemicals in the origins of human cancer was furthered by the proposal of the somatic mutation theory. First proposed in 1914, Boveri thought cancer could be caused by wrongly arranged chromosomes, thus implicating DNA abnormalities in developing the malignant state. The Millers expanded the theory, postulating that: 1) changes in information coded by the base sequence (mutations) of non-germ line (somatic) cells initiate the transformation process, 2) most chemical carcinogens require metabolic activation to an ultimate carcinogen, and 3) the ultimate carcinogenic form of a chemical carcinogen is an electrophilic compound which reacts covalently with nucleophilic cellular macromolecules, such as DNA, RNA and proteins (3). They concluded that the covalent binding of carcinogens to informational macromolecules leads to mutations in DNA, thus initiating carcinogenesis.

Other evidence also implicates DNA as the target in the transformation process by chemical (and physical) insult. Work by Ames (4)

shows that after metabolic activation, carcinogens are mutagens in the Salmonella test system. Polycyclic hydrocarbon metabolite binding to DNA, rather than RNA or protein, is related to the carcinogenicity of an agent (5).

One such polycyclic hydrocarbon, benzo[a]pyrene (BP), is a ubiquitous contaminant in our environment. This compound arises as the result of incomplete combustion of organic materials. The practice of discharging combustion products from fossil fuels into the environment for many decades has led to the widespread distribution of BP in our air, soil, water, food and vegetation. In the United States alone, it is estimated that 1300 tons of BP are introduced into the environment annually (6). Automobile exhaust emitted during one mile of driving contains 2.5 - 3.5 µg of BP (7). BP is also found in cigarette smoke, roasted coffee, smoked meat and fish and fruits and vegetables, making human exposure to this compound inevitable (8). Investigation of the effects of BP on biological systems is of great interest, therefore warranting its use in this study.

Like most chemical carcinogens, BP is unreactive and must be metabolized to a chemically reactive form, or ultimate carcinogen. The metabolism of BP to an ultimate carcinogen is diagrammed in figure 1. In a detoxification process the cellular microsomal fraction, containing mixed function oxidases and cytochrome p450, metabolizes the parent compound. The enzyme aryl hydrocarbon hydroxylase converts BP to an epoxide. This compound can undergo a number of further reactions including spontaneous isomerization to a phenol, conjugation to

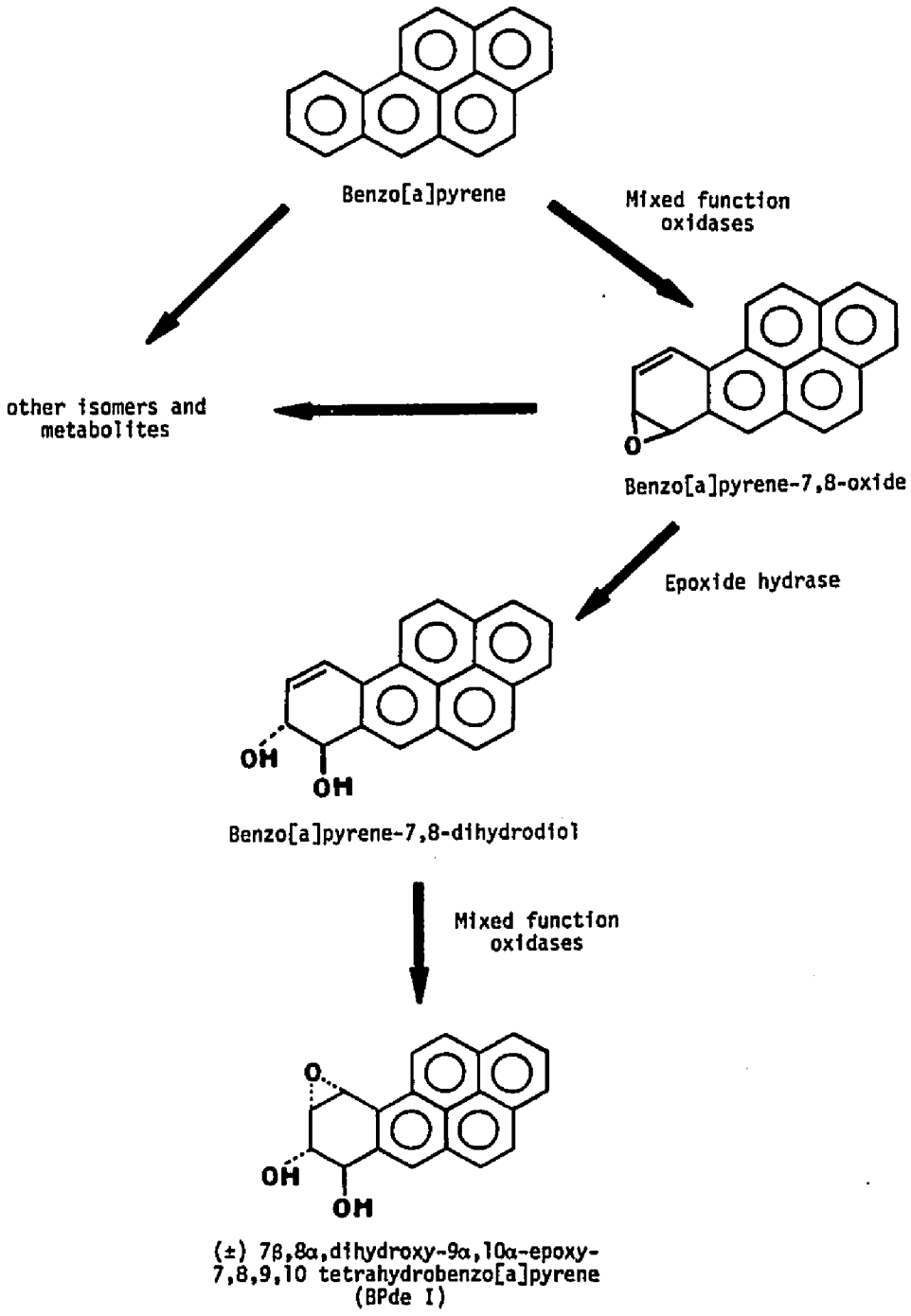

Figure 1. Metabolism of Benzo[a]pyrene to an Ultimate Carcinogen

glutathione, macromolecule binding or conversion to diols by epoxide hydrase (9, 10). Further metabolism leads to a variety of compounds such as quinones, dihydrodiol phenols, diol epoxides, dihydroxy derivatives and quinone phenols (11). The metabolite which is thought to be the ultimate carcinogenic form of BP is the BP 7,8 diol, 9,10 epoxide (BPde). Evidence supporting this conclusion includes the observation that *in vitro* binding of BPde to nucleic acids gives the same adduct profile as that of DNA from cultured mammalian cells exposed to BP *in vitro* and *in vivo* (12). More specifically, of the isomeric forms possible, the most active is thought to be (±) 7β,8α, dihydroxy-9α,10α-epoxy-7,8, 9,10 tetrahydrobenzo[a]pyrene or BPde I (13). Further evidence incriminating BPde I as the ultimate carcinogen is the observation that BPde I is a strong mutagen in both bacterial and mammalian cells (14, 15).

Inherent in a discussion of DNA damage leading to mutation is the question of DNA repair. Many repair pathways have been observed (16) in both mammalian and bacterial systems for a variety of damaging agents. Of interest to this study is an inducible, possibly error-prone recovery pathway in mammalian cells.

The first observation of an induced recovery response was noted in bacteria by Weigle (17). It was observed that UV irradiated phage showed greater survival when grown in *E. coli* that had been irradiated prior to λ infection. Along with the increase in phage survival, there was a high level of phage mutagenesis. These phenomena are known as Weigle reactivation and Weigle mutagenesis.

In addition to increased survival and mutagenesis observed by Weigle, other diverse responses are seen in E. coli upon irradiation or exposure to other agents. These include induction of prophage, turn-off of respiration and inhibition of cell division, or filamentation (18). Besides UV irradiation, these functions are induced by other DNA damaging agents, such as X-rays, alkylating agents and cross-linking agents, or when DNA replication is inhibited by such treatments as nalidixic acid or thymine deprivation (19). Thus there appears to be a number of coordinately controlled responses to treatments which are deleterious to the cell.

This diverse set of responses, often called the SOS response (20), is under the control of a complex genetic regulation system. This system is controlled through the lex A gene and rec A gene. The Lex A protein is a repressor which represses genes involved in the SOS response. Derepression of this system is through Rec A protein, a protease which cleaves the Lex A repressor. The Rec A protease is believed to be activated by an 'inducing signal' produced in response to an inhibition of DNA replication, though specifics of this signal are not known (21).

The observation of this induced global response in bacteria to DNA damage has led to an investigation of such a response in mammalian cells. Indeed, there does seem to be a similar response in mammalian cells exposed to agents which interfere with DNA replication.

Perhaps analogous to prophage induction in bacteria as part of the SOS response, is induction of covalently integrated oncogenic virus

from mammalian cells by light. Both RNA and DNA oncogenic viruses have been induced from transformed cells by light, ionizing radiation, certain chemicals or light plus chemicals (22).

Early work by Bockstahler and Lytle demonstrate induced reactivation of virus in mammalian cells. These workers used cultured African green monkey kidney cells (CV-1) and herpes simplex virus type 1 (HSV) to assay for increases in survival of irradiated virus grown in cells infected immediately after treatment. Enhanced reactivation of irradiated virus was found when cells were treated with UV light (23) as well as X-irradiation (24). These studies show not only an enhanced survival dependence on the amount of damage recieved by the cells, but also upon damage recieved by the virus. A UV dose resulting in less than 2% viral survival was needed to induce reactivation. Depending on the UV dose given to the virus prior to infection, survival was maximally enhanced 2 - 8 fold in treated cells. It was also found that other UV-like damaging agents such as aflatoxin B_1 and N-acetoxy-acetylaminofluorene metabolites induce reactivation in the CV-1 HSV system (25). If infection of the virus was delayed up to 5 days post-cell treatment, an even greater enhancement of virus survival was noted (26).

The inducibility of enhanced reactivation implies that protein synthesis is required for this response. Das Gupta and Summers (27) have looked at protein synthesis requirement for UV-induced reactivation using HSV and African green monkey kidney cells. This phenomenon

was blocked by cycloheximide, an inhibitor of protein synthesis, thus leading to the conclusion that new protein synthesis is required.

A variety of cell and virus systems have been used to demonstrate enhanced reactivation in eukaryotic organisms. This phenomenon has been observed in human cells, both normal fibroblasts and repair deficient Xeroderma pigmentosum cells, when infection is delayed after cell treatment by UV light (28). Besides studies using HSV, simian adenovirus 7 (SA7) and simian virus 40 (SV40) show enhanced reactivation in African green monkey kidney cells. Studies using the cytoplasmic replicating viruses vaccinia and poliovirus show no increases in survival of irradiated particles, indicating that the processes involved in this phenomenon are probably localized in the cell nucleus (29). Because virus are dependent on host cells to reproduce, it may be reasonable to conclude enhanced reactivation is a property of the host cell rather than the virus. The fact that HSV and SA7 code for their own polymerases and have other unknown functions still leaves the possibility that enhanced reactivation is a property of the virus. The use of SV40 circumvents this possibility.

The system utilizing SV40 to assay for enhanced reactivation is advantageous. This small mammalian virus has a genome consisting of 3.6×10^6 base pairs and has been completely sequenced. It is found to code for only 5 proteins, none of which are polymerases or repair enzymes, thus making it totally dependent on the host cell for replication and repair functions. SV40 produces a lytic infection in

African green monkey kidney cells and these cells are used for plaque assaying (30).

Further studies using SV40 have better defined the enhanced recovery phenomenon. Sarasin and Hanawalt (31) have found a number of chemical carcinogens which enhanced the survival of UV-irradiated SV40 grown in treated CV-1P cells. Agents tested which have UV-like effects include aflatoxin B_1 metabolites and N-acetoxyacetylaminofluorene (AAAF) and X-ray-like compounds such as methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS). Pretreatment of the CV-1P cells with cycloheximide or hydroxyurea, which inhibit DNA synthesis without producing lesions, enhanced UV-irradiated SV40 survival. Time course experiments with UV-irradiation or aflatoxin B_1 treatment of the cells showed maximal enhancement when infection was delayed two to three days.

The possibility that this induced recovery is error-prone in mammalian systems has been investigated by several laboratories. Attempts to answer the question of mutagenicity have employed specific gene loci to look at mutations in UV reactivated virus. A number of studies have used HSV to study mutagenic reactivation induced by UV light. Das Gupta and Summers (27) investigated mutation frequencies by measuring the forward mutation of TK^+ (thymidine kinase) to TK^- of HSV grown in African green monkey kidney cells (Vero). Their results showed an approximate 2.5 fold increase in mutation frequency when virus were grown on irradiated cells. Interestingly, the same increase in mutation frequency was observed for both

irradiated and unirradiated HSV grown on irradiated cultures. Their protocol utilizing a high multiplicity of infection (MOI of one used) has been criticized in that multiplicity reactivation (MR) mutagenesis might have been observed rather than UV induced 'SOS' mutagenesis (32). Lytle has done similar experiments with HSV and scoring TK^- mutants using different MOIs to distinguish between induced reactivation and MR mutagenesis. Results showed that mutation frequency was higher if there was a treater MOI. He concluded that when enhanced reactivation and MR were present toghether, they were mutagenic, but could make no conclusion about either separately based on his data (33).

The use of temperature sensitive (ts) SV40 mutants has allowed mutation frequencies to be determined by reversion to wild type rather than forward mutation assays. Sarasin and Benoit (34) used two ts mutants of SV40 and screened mutants by the ability to grow at the non-permissive temperature. No mutations were detected in undamaged SV40 grown in UV irradiated cells. An increase in mutation frequency was observed as the UV fluence to the virus increased, when grown in irradiated cultures. They concluded that induced reactivation is error-prone or mutagenic. In similar studies using ts SV40 mutants, Cornelis et. al., (35) found a 16 - 17 fold increase in mutagenesis of UV irradiated virus grown in both irradiated and unirradiated cells.

Thus there appears to be some similarities between prokaryotes and eukaryotes in terms of an induced 'SOS' response. Mammalian cells show an enhanced reactivation similar to Weigle reactivation to several DNA damaging agents, as well as induction of some lysogenic viruses.

The mutagenicity of this response in mammalian systems is still questionable.

The studies presented in this thesis were undertaken to investigate possible BPde enhanced reactivation in mammalian cells. The virus chosen was SV40 for reasons given above and cells used were CV-1P, a line of African green monkey kidney cells which support a lytic infection of SV40 necessary for a plaque assay to monitor virus survival. While pretreatment of the cells was with BPde, the virus was damaged with UV irradiation prior to infection.

The reactivation experimental protocol used was similar to that of Sarasin (36). Many of the previous studies discussed above have used the same cells (treated or untreated) to repair virus and assay for survival. The protocol used in my studies allows the virus approximately one replication cycle to recover in the treated or untreated cells. The virus is then collected and survival is measured in undamaged cells by plaque assay. This allows a distinction between viral recovery and survival measurement processes.

The protocol used is necessary for mutation frequency determination. The SV40 virus used in this study was a ts mutant, tsA58, to investigate mutagenesis by BPde. This virus cannot grow at a nonpermissive temperature and mutation by reversion to wild type can be determined by plaque assay at this elevated temperature.

A statistical analysis of the data was done in this study. The use of computer facilities allowed for data analysis by analysis of variance and analysis of covariance.

CHAPTER 2

MATERIALS AND METHODS

Cells

Cells used in this study were CV-1P, a line of African green monkey kidney cells, obtained from Jennifer D. Hall, Department of Cellular and Developmental Biology, University of Arizona. The cells were grown in Dulbecco's modified Eagle medium (DME), supplemented with 10% fetal calf serum (FCS, Biocell, Carson, CA), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Grand Island, NY). Subculturing was done every three to four days and stocks maintained in T-75 flasks (Falcon, Oxnard, CA) in a humidified, 10% CO_2, 37° incubator. Cells were periodically tested for Mycoplasma contamination and were found to be negative (37).

Virus

A temperature sensitive mutant of SV40, tsA58, was obtained from Edward L. Kuff, National Cancer Institute. This mutant has a temperature sensitive mutation in the A region of the genome, which involves the large T antigen, and is unable to initiate DNA synthesis at a nonpermissive temperature, 40.3° in our laboratory. A lytic infection of the CV-1P cells resulted when they were grown at 33°, the permissive temperature. The virus was plaque purified in CV-1P cells by growing stocks from progeny of a single virus at 33°. Stocks were

grown by infecting near-confluent monolayers of CV-1P cells in 100 mm dishes at a multiplicity of infection (MOI) of 10^{-3}. The monolayers were maintained in DME containing 5% FCS. When lysis of the cells occured (about 21 days), the remaining cells were scraped off the plates with a sterile rubber policeman and the virus and cell suspention put into sterile tubes. The stocks were freeze-thawed three times to disrupt cellular membranes and release virus particles. The SV40 stocks were stored at -20°. Viral stocks were titered in 6-well plates (Costar, Cambridge, MA) to determine the plaque forming units/ml (PFU/ml) by the plaque assay described below.

Benzo[a]pyrene

Racemic 7β,8α,-dihydroxy-9α,10α-epoxy-7,8,9,10 tetrahydrobenzo-[a]pyrene (BPde) was obtained from J. Keith of the National Cancer Institute, Chemical Repository (ITT Research Institute, Chicago, IL) through D.G. Longfellow of the Chemistry Operational Unit, Carcinogenesis Branch of the National Cancer Institute. BPde was dissolved in anhydrous dimethyl sulfoxide (DMSO, Pierce Chemical Co., Rockford, IL) to a concentration of 1 mg/ml and stored at -90°. For each experiment this stock was diluted to the appropriate concentration in DMSO (0.1% DMSO in the medium). Work involving BPde was done under subdued lighting to prevent photolysis.

Survival Assay

Exponentially growing cultures of CV-1P cells were treated with varying concentrations of BPde or DMSO for 1 hour. The cells

were trypsinized and resuspended in fresh DME medium. From 100 to 1000 cells were plated into 60 mm dishes and allowed to incubate 14 days at 37° for colony formation. The colonies were fixed with 3% glutaraldehyde in distilled water, stained with 0.2% methylene blue, counted and percent survival calculated. The average plating efficiency for the DMSO control was 66%.

UV Irradiation of SV40

The virus was irradiated under a Sylvania germicidal light (30 watts, 253.7 nm) at a fluence rate of 5 $J/m^2/s$ as determined by a Blak-Ray UV meter (UV products, San Gabriel, CA). The viral suspension was placed in a sterile culture dish and swirled continuously during UV irradiation to ensure a uniform exposure. For the reactivation studies or unless otherwise noted, the virus received a UV dose of 1360 J/m^2. Appropriate dilutions of the virus were made for infection after irradiation in serum-free DME.

Reactivation Studies

Cells were seeded into 100 mm dishes at a density of 1×10^6 cells per dish and allowed to grow for 2 days. The log phase cells were treated with the appropriate concentration of BPde or DMSO (control) for 1 hour, then the medium was replaced with fresh medium containing 5% FCS and incubated for various times at 37° before viral infection. Prior to viral infection a cell count of a control plate was made to determine the viral dilution needed to give the desired MOI. Both the irradiated and unirradiated virus were diluted in the

same manner to give an MOI of 5 X 10^{-2} PFU/cell (unirradiated virus). For the infection, the cells were washed once with serum-free DME and 700 µl of viral suspention put on each monolayer. The virus was allowed to adsorb for one hour with frequent agitation to prevent the cells from drying out. After the adsorption period, the viral suspension was removed and DME containing 5% FCS was put on the cells. The infected cells were incubated at 33° for 72 hours, which is approximately the time for a replication cycle of the virus. After 72 hours, the cells and virus were scraped into the medium with a sterile rubber policeman and put into sterile tubes. These were freeze-thawed three times before titering was done.

Titer Determination - Plaque Assay

Cells were seeded onto 60 mm dishes and allowed to grow to near-confluence before infection. The virus was diluted appropriately in serum-free DME, with at least two dilutions consisting of five plates each used for determining the titer of each treatment. The monolayers were infected with 250 µl viral suspention and allowed to adsorb one hour with frequent swirling of the cultures. The remaining suspension was removed and 5 mls of DME containing 5% FCS and 0.45% agarose (MCB, Cincinnati, OH) was put onto the infected cells. After eight days of incubation at 33°, another 5 mls of DME agarose was added to feed the cells. Nineteen days after infection the cells were stained with 7 mls of DME agarose containing 0.01% neutral red stain. Plaques were counted one day after the stain was added. PFU/ml

was determined by dividing the number of plaques counted by the suspension volume (250 µl for 60 mm dishes) and then dividing by the dilution factor.

Mutagenesis Assay

Virus used in this determination were irradiated and grown in treated or control treated cells as described above. The titer was determined by the plaque assay at 33°. For mutation determination (reversion to wild type), 100 mm dishes of near-confluent cells were infected with 700 µl of the virus. The cells were overlayed with DME containing 5% FCS, 1.25% agarose and incubated at the non-permissive temperature, 40.3°. A feeder layer of DME agarose was added after 5 days and cells were stained after 12 days of infection. Possible plaques were picked off the plates and put on monolayers of CV-1P cells in 60 mm dishes. These were grown at 40.3° and observed for cell lysis to determine mutant stability.

Calculations and Statistical Analysis

The PFU determination was done as explained in Titer Determination. The PFU value was calculated per ml, though there were 9 mls total suspention for each sample in the reactivation studies. Surviving fractions were calculated in the following manner:

$$\text{Surviving fraction (SF)} = \frac{\text{PFU/ml of +UV SV40}}{\text{PFU/ml. of -UV SV40}}$$

Reactivation factors were calculated from the surviving fraction in the following manner:

$$\text{Reactivation factor (RF)} = \frac{\text{SF of virus in BPde treated cells}}{\text{SF of virus in DMSO treated cells}}$$

A statistical analysis of the data was done by Judy Parsells and Dr. D'Alice Sim of the Division of Radiation Oncology and Cancer Center, resp. at the University Hospital. Computer analysis of the data included an analysis of variance (ANOVA) for determining PFU/ml and an analysis of covariance (ANCOVA) for surviving fractions (38). Programs were from the University of California at Los Angeles (39) and run on a Cyber 175 computer.

CHAPTER 3

RESULTS

BPde Effects on CV-1P Survival

A clonogenic assay was used to determine the survival of CV-1P cells in the presence of BPde. Logarithmically growing CV-1P cells were treated with various concentrations of BPde dissolved in DMSO for one hour. DMSO treatment was used as a control to determine plating efficiency and did not show any toxic effects in CV-1P cells. The survival curve in figure 2 shows a shoulder region at the lower doses of BPde, 0.08 μM and 0.16 μM. A linear, exponential decrease in survival is seen as the concentration of BPde increases. The highest concentration used in this study, 1.11 μM, resulted in a 0.2% survival.

Enhanced Reactivation at Low Concentrations of BPde

The ability of low concentrations of BPde to enhance viral survival was studied using concentrations corresponding to the shoulder region of the survival curve for CV-1P cells. These included one hour treatments of logarithmically growing cells with 0.01 μM, 0.08 μM or 0.16 μM BPde. The cells were infected 12, 24 or 36 hours after BPde treatment with irradiated (1360 J/m^2) or unirradiated SV40 virus. A low MOI (5×10^{-2} PFU/cells) was used to prevent multiplicity reactivation. After the virus were allowed to grow 72 hours, the cells and media were collected with a total volume of 9 mls. The surviving

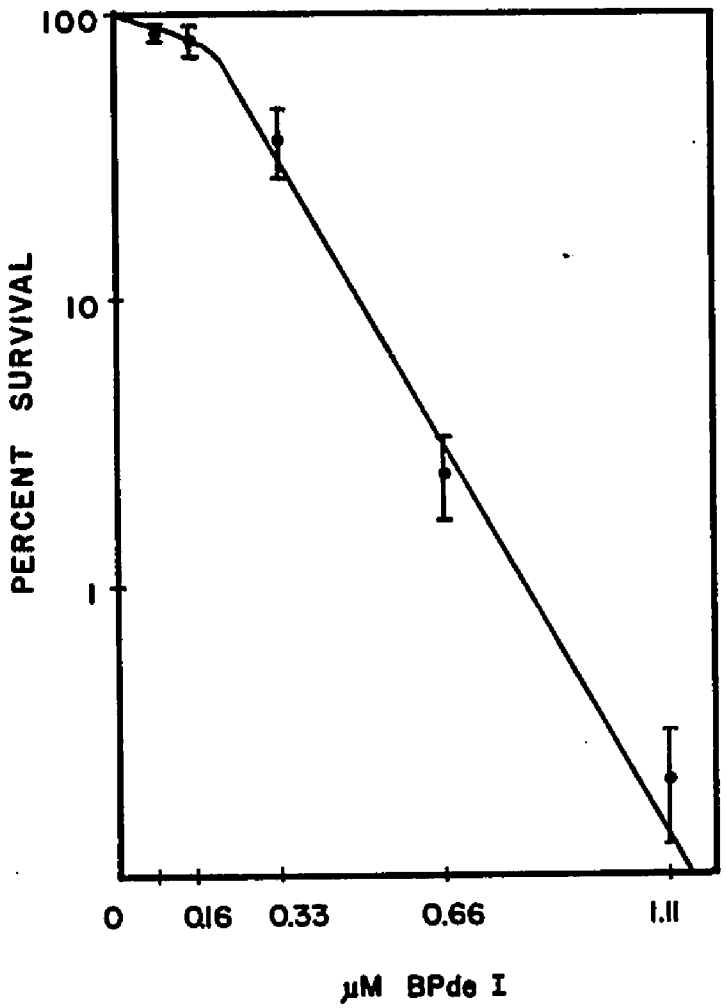

Figure 2. Survival of CV-1P cells exposed to BPde.

The clonogenic survival of CV-1P cells exposed to various concentrations of BPde for one hour is shown. The error bars represent standard error of the mean, calculated from 3 independent determinations.

fractions of irradiated virus for each of the cell treatments is shown in figure 3. There is a slightly higher survival fraction for virus grown in treated cells. In table I the corresponding reactivation factors are shown. While the reactivation factors are slightly greater than one, when survival fractions (used to calculate reactivation factors) are subjected to statistical analysis by analysis of covariance, the values are not found to be significant at $p<0.05$, thus indicating little or no reactivation occuring at these low BPde concentrations for the times studied.

Enhanced Reactivation at Extended Times for Low BPde Concentrations

The possibility of a delayed induced reactivation occuring for a low BPde concentration was studied. Using a one hour cell treatment of 0.16 μM BPde, a time course was done extending to four days. The surviving fraction of irradiated virus is shown in figure 4 for control and BPde treated cells. There is a slight increase in survival for both treated and untreated cells, however, there is little or no surviving fraction increase for treated cells over virus grown in control treated cells during the time course. Statistical analysis by analysis of covariance shows no significant increase in survival at $p<0.05$.

Enhanced Reactivation of SV40 by Higher BPde Concentrations

The ability of higher concentrations of BPde (those corresponding to a linear decrease in cell survival) to induce reactivation of irradiated virus was studied. Cells were exposed to BPde

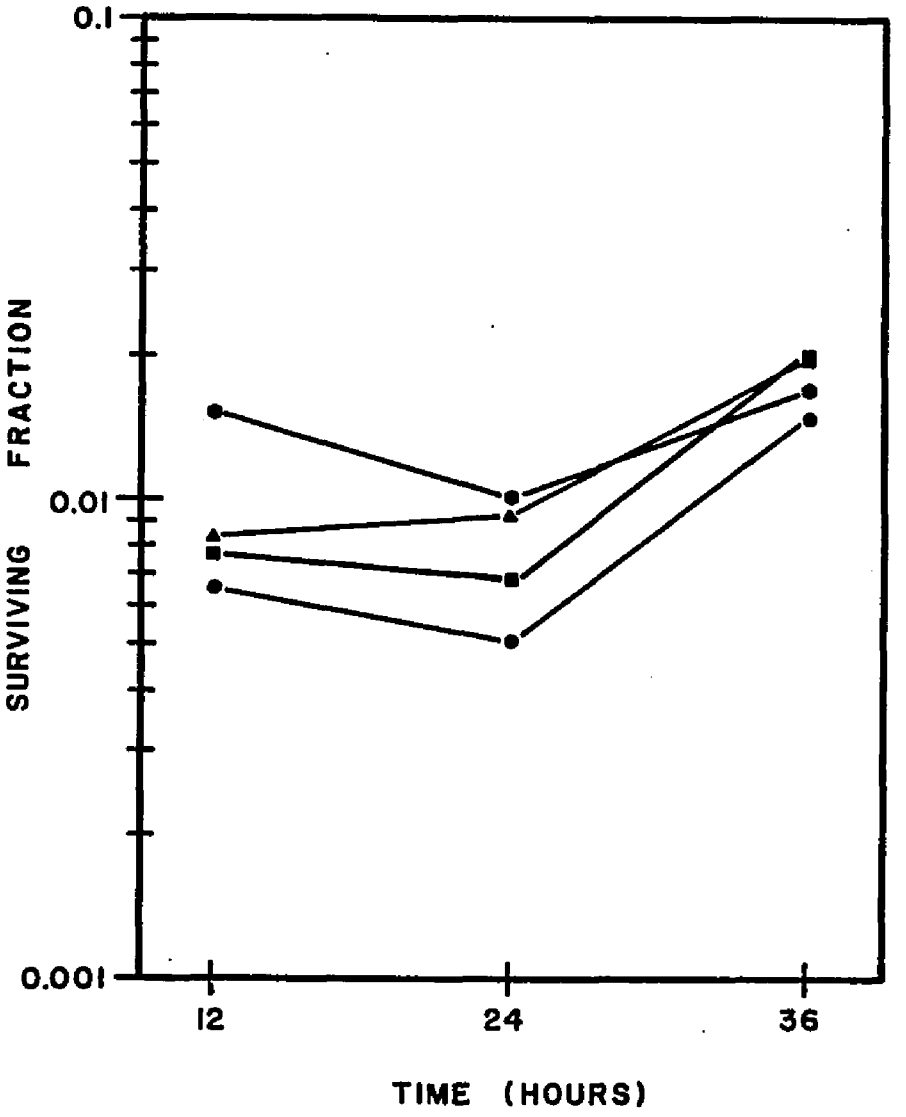

Figure 3. Surviving fractions of SV40 grown in CV-1P cells treated with low BPde concentrations.

CV-1P cells were control treated with DMSO or treated with BPde for one hour. At the times indicated the cells were infected with irradiated (1360 J/m^2) or unirradiated virus. ● , DMSO treated; ■ , 0.01 μM,BPde; ▲ , 0.08 μM BPde; ⬢ , 0.16 μM BPde.

Table I. Reactivation factors of SV40 grown in BPde treated CV-1P cells.

Reactivation factors calculated from surviving fractions in Fig. 2 for different doses of BPde and differing times after BPde treatment.

Concentration of BPde	Time of infection after BPde treatment		
	12 hours	24 hours	36 hours
0.01 μM	1.18	1.36	1.34
0.08 μM	1.28	1.85	1.30
0.16 μM	2.30	2.00	1.13

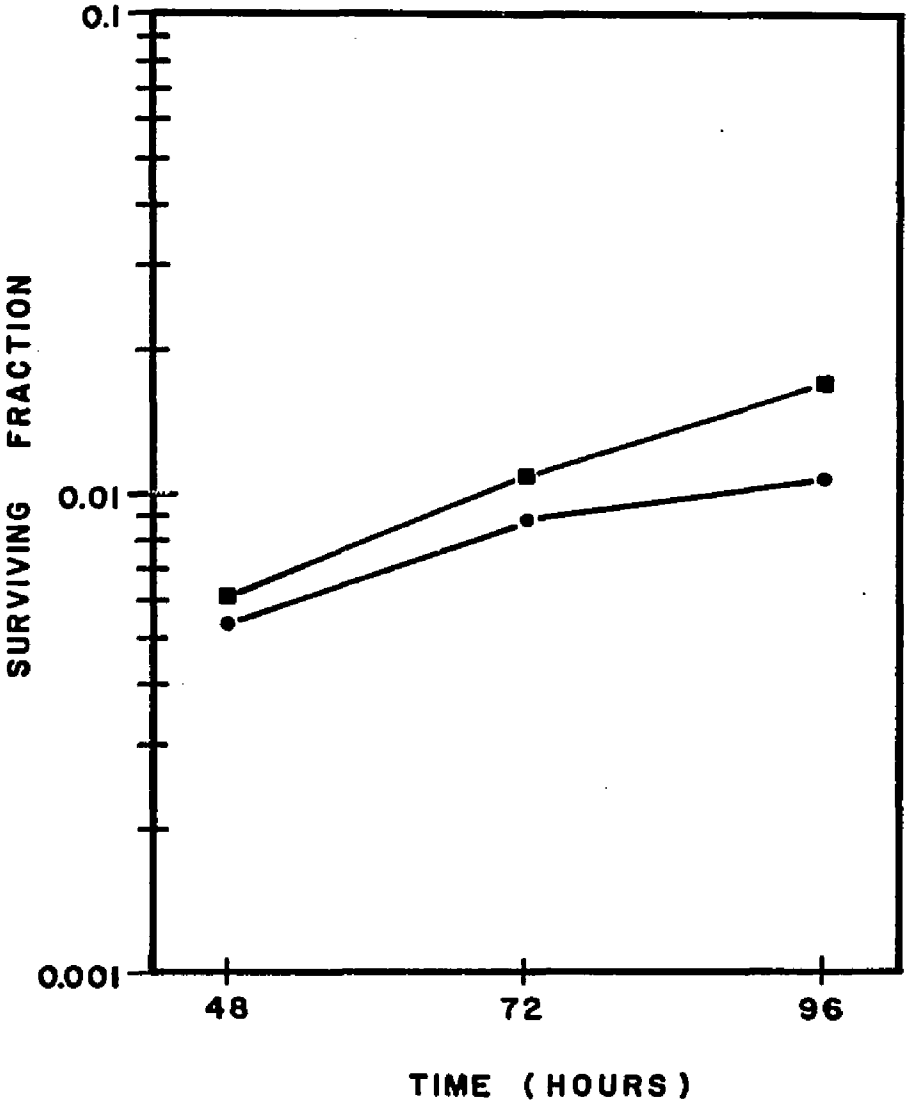

Figure 4. Surviving fractions of SV40 grown in CV-1P cells at extended times after 0.16 μM BPde treatment.

CV-1P cells were infected at extended times after a one hour DMSO control or 0.16 μM BPde treatment. Irradiated virus recieved 1360 J/m^2. ● , DMSO treated; ■ ,0.16 μM BPde.

concentrations of 0.16 μM, 0.33 μM, 0.66 μM, and 1.11 μM for one hour and infected with irradiated (1360 J/m^2) or unirradiated virus 24 hours later. The surviving fraction of the irradiated virus for the various BPde concentrations is shown in figure 5. Again, little or no enhanced survival is seen at the low dose, 0.16 μM. The higher doses resulted in a greater than two-fold enhancement of viral survival at 24 hours. Statistical analysis by analysis of covariance showed this to by significant at $p<0.05$.

Time Dependence of Enhanced Viral Survival After BPde Treatment

The time course of the induced recovery response was studied. Using a BPde concentration of 0.66 μM, which gave a high enhanced survival of irradiated virus, a time course extending to 96 hours was done. The increased survival of virus growing in treated cells is apparent and is shown in figure 6. This enhancement, which is about an order of magnitude higher, is sustained out to 96 hours. Thus, this enhanced reactivation response is incuced within 24 hours post-BPde treatment with very little, if any, reduction after 4 days. A paired t-test show the lines in figure 6 to be significantly different at $p<0.01$.

Mutagenesis Screen for Induced Reactivation

A mutagenesis screening assay for induced reactivation was developed. Cells were treated with a high concentration of BPde, 0.66 μM, to induce enhanced survival of irradiated virus. The cells were allowed to incubate 96 hours before infection with virus

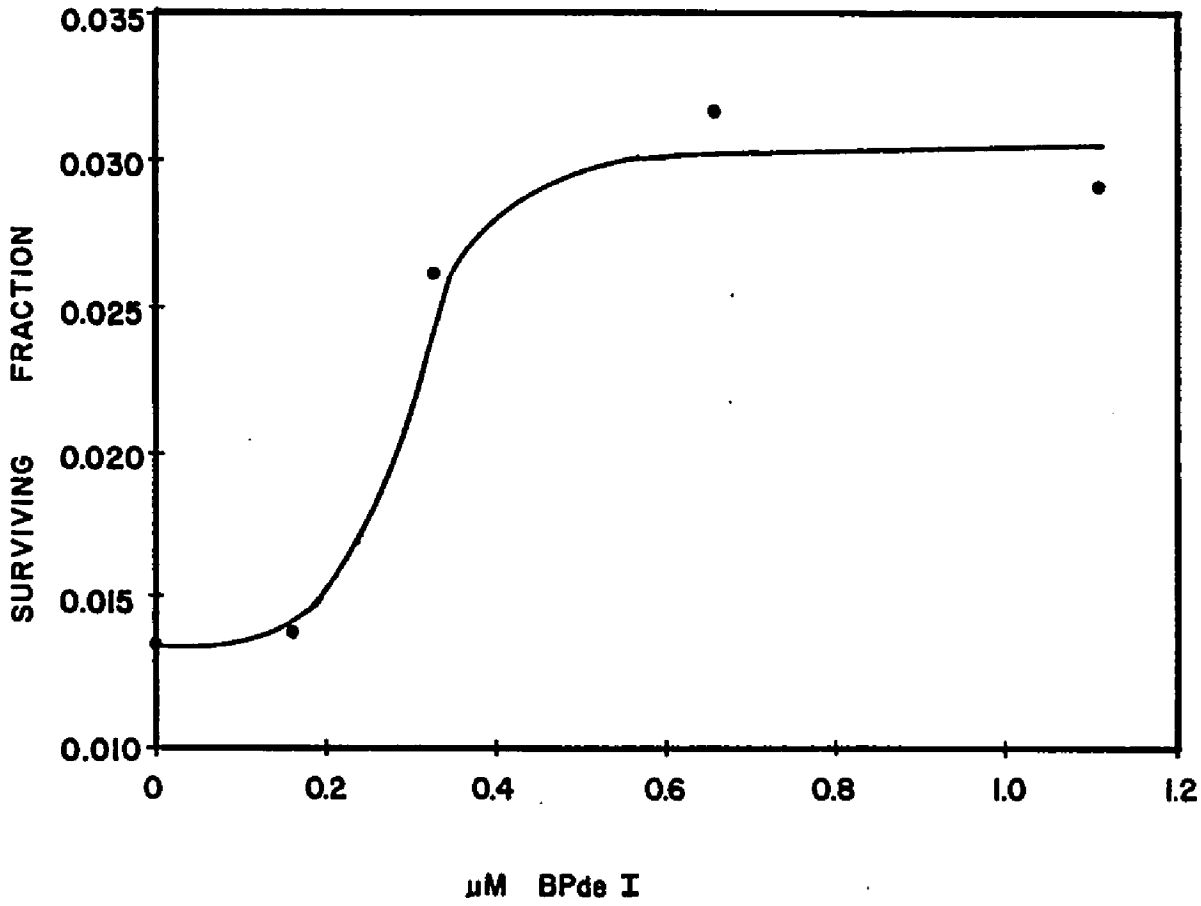

Figure 5. Surviving fraction of SV40 grown in CV-1P cells treated with differing concentrations of BPde.

Surviving fraction was calculated from PFU/ml values. Viral infection was 24 hours after a one hour BPde treatment at the concentrations shown.

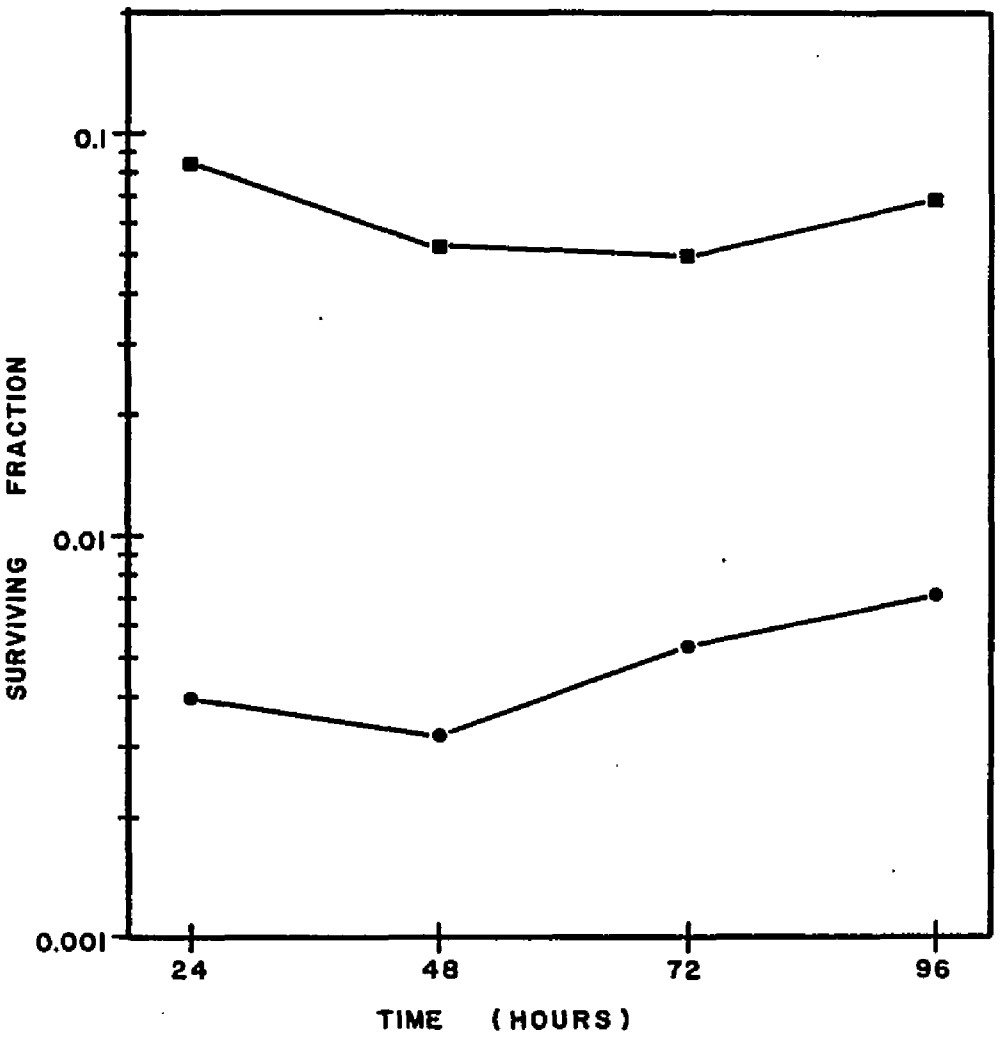

Figure 6. Surviving fractions of SV40 grown in CV-1P cells treated with 0.66 μM BPde.

Viral infection of CV-1P cells was at various times after 0.66 μM BPde treatment and survival calculated from PFU/ml values. ■, 0.66 μM BPde; ●, DMSO control treated.

(irradiated virus received 1750 J/m^2). Titering at 33° was done to determine the surviving fractions. Surviving fractions for DMSO treated was 1.4×10^{-2} and BPde treated cells was 2.9×10^{-2}. Virus assayed for mutagenesis were put on CV-1P monolayers and titered at 40.3°. At this temperature the monolayer could be maintained for at least two weeks and the tsA58 mutant SV40 was unable to form plaques. Results seen in table II show that two plaques were observed from virus grown in BPde treated cells. These were confirmed by the ability to lyse out a monolayer (replicate) at 40.3°. It is not possible to conclude from this experiment whether BPde treatment increases mutation frequency (observed frequency is 3×10^{-6}). An insufficient number of virus from the DMSO treated cells were obtained which would give a similar mutation frequency (3.4×10^5 PFUs would have needed to be screened to obtain one mutant).

Table II. Total virus and mutants observed after induced reactivation.

Cells were treated for one hour with 0.66 μM BPde and infected 96 hours later. Irradiated virus recieved 1750 J/m^2. The total PFUs collected were determined by multiplying PFU/dish from the plaque assay by the number of dishes used for the study.

Cell treatment, virus treatment	PFU/dish	# dishes	Total # PFU collected	# mutants observed
DMSO -UV	6.05×10^5	-	-	-
BPde -UV	1.58×10^6	-	-	-
DMSO +UV	8.52×10^3	24	2.04×10^5	0
BPde +UV	4.57×10^4	15	6.86×10^5	2

CHAPTER 4

DISCUSSION

The evidence presented in this study shows that BPde is able to induce reactivation of irradiated SV40 virus grown in CV-1P monkey cells. This observation is in agreement with other studies (23, 24, 31) showing an enhanced DNA recovery response in a number of mammalian cells exposed to DNA damaging agents. Besides DNA damaging agents, hydroxyurea and cycloheximide induce this response, suggesting that this process is not induced by specific types of damage, but rather an inhibition of DNA replication. This is analogous to the conclusions reached in bacterial systems that an inhibition of DNA replication, mediated by an unknown 'inducing signal' activates an SOS response (21).

A comparison of enhanced viral survival to cell survival after BPde treatment was made. There appears to be a BPde concentration threshold needed to induce this response. Low BPde concentrations, or those corresponding to the shoulder region of the cell survival curve, do not enhance reactivation. BPde concentrations corresponding to the exponential kill portion of the cell survival curve exhibit an induced recovery response. This analogy with the survival curve suggests that lethal damage (exponential portion of the survival curve) elicits an induced or 'SOS' type response as an attempt by the cell to survive this lethal damage.

DNA synthesis is affected by agents which damage DNA. Studies using the single stranded bacterial virus ϕX174 have shown that a UV induced dimer (40) or BPde adduct (41) block DNA synthesis. In vitro studies show UV dimers terminate DNA synthesis by E. coli polymerase I and eukaryotic polymerase α (42). These termination sites are at the base preceeding a dimer. In mammalian cells, UV irradiation decreases DNA synthesis. This is manifested by a decrease in the rate of DNA synthesis as well as a decrease in the size of nascent DNA (43, 44). The ability of UV irradiated cultures to synthesize control level molecular weight DNA is returned after a period of time (depending upon fluence of UV, 45). BPde has similar effects on replication. After treatment of monkey cells (Vero) with BPde there is a decrease in both the size and rate of synthesis of nascent DNA (46). This effect is maximal 2-3 hours post BPde treatment with recovery following this time. Clearly, replication must be facilitated to explain the increase in viral survival (increase in PFU recovered) from damaged cells as seen in the reactivation experiments presented in this thesis.

Two possibilities exist to explain the recovery of DNA synthesis in damaged cells. One is the repair of lesions inhibiting DNA replication. Studies using repair deficient Xeroderma pigmentosum (XP) cells suggest this does not occur (47). Irradiated XP cells were shown to recover the ability to synthesize control level molecular weight DNA by 24 hours after UV exposure. These cells are unable to remove dimers, thus indicating replication occurs on damaged

templates. Studies on the dimer content of UV irradiated Chinese hamster ovary (CHO) cells show little removal of dimers from DNA (48). Only 26 - 35% of the dimers could be removed in 24 hours of incubation while DNA replication was eventually able to proceed past these lesions.

The second possiblity, that a facilitated mode of replication is induced in damaged cells has been studied. CHO cells treated with a low dose of UV several hours before a larger dose of UV was found to increase the rate at which larger molecular weight DNA could be synthesized. If the cells were incubated with the protein synthesis inhibitor, cycloheximide, between irradiations, this enhanced recovery of the ability to synthesize larger DNA was not seen. It was concluded that the recovery in the ability to synthesize normal sized DNA (termed postreplication repair) requires de novo protein synthesis or is an inducible function (49, 50).

While UV induced dimers and BPde adducts are thought to inhibit elongation of nascent DNA, recent evidence has shown an inhibition of DNA initiation. Very low doses of UV (2.5 J/m^2) were shown to transiently inhibit DNA initiation in human fibroblasts (51). Studies in African green monkey kidney cells (Vero) also show an inhibition of initiation after treatment with a low (0.16 μM) BPde concentration (46). The BPde concentration of 0.16 μM did not significantly reduce survival of the Vero cells and did not inhibit elongation of DNA. This selective inhibition of DNA initiation was reversed by 4 hours after BPde treatment. Higher BPde concentrations, or those corresponding to the exponential portion of the cell survival curve,

characteristically inhibited elongation of nascent DNA. In comparing these effects on DNA initiation and elongation with the induction of enhanced viral survival, it appears that the inhibition of elongation may be related to the induction of the enhanced recovery response. Perhaps the signal for this response involves the blockage of DNA elongation.

The molecular mechanisms of the ability to replicate past DNA lesions is not understood. Villani et. al. (52) have suggested that an inhibition of the 3' → 5' proofreading activity of bacterial DNA polymerase I and III may be a result of SOS induction. Idling of the DNA polymerases (turnover of nucleoside triphosphates to free monophosphates) was observed on irradiated DNA templates. The proposed basis of idling is the incorporation of bases opposite dimers and the subsequent base removal by the 3' → 5' exonuclease activity. The inhibition of the editing function is suggested to account for replication past dimers as well as increased mutagenesis by an indiscriminate incorporation of bases opposite dimers.

The molecular mechanisms underlying facilitated replication on damaged templates in mammalian systems is not understood. The use of viral probes, such as SV40, may aid in elucidating these mechanisms. Sarasin and Hanawalt (53) have studied SV40 DNA synthesis in UV irradiated cells. This system has the advantage of a single origin of DNA replication and the ability to synchronize replication by the use of a tsA58 mutant SV40. Their observations also show UV irradiation blockage of DNA synthesis and an eventual bypass of

lesions with time. They concluded that there was an inducible mechanism to allow replication past dimers.

The chemical instability of BPde requires special consideration. BPde is subject to hydrolysis in aqueous solution, with a half-life of approximately ten minutes in media. It must be dissolved in anhydrous DMSO and is stored at -90° prior to treatments. The stability of BPde under these storage conditions is questionable and may account for some of the variability seen in the results and comparisons between experiments. Also, photolysis of BPde occurs readily and BPde must be manipulated only under subdued lighting. Comparison of results between experiments is difficult. For example, the magnitude of enhanced survival in fig. 5 and fig. 6 for a 24 hour infection after 0.66 μM BPde treatment shows a great deal of variation. In fig. 5, the survival of the virus is enhanced approximately two-fold, while in fig. 6 the survival enhancement is near twenty-fold. Possible inconsistencies in UV irradiation of the SV40 may also contribute to the variations observed. These variations make the use of statistical analysis very important in showing trends within experiments.

In the reactivation studies presented in this thesis, viral damage with BPde would be preferable to UV irradiation of the SV40. However, BPde reacts with other cellular macromolecules besides DNA. Unlike UV irradiation, which is primarily absorbed by DNA, BPde can bind to RNA and protein (54). In vitro BPde treatment of viral particles may lead to modification of coat proteins and may or may not lead to DNA adduct formation. The modification of viral coat proteins may

affect adsorption of the particles and alter infectivity. *In vitro* viral DNA modification and transfection into the cells would provide a BPde damaged template to study reactivation. This would be similar to the procedure used by Cornelis et. al. (35).

The ability of BPde to react with other cellular macromolecules besides DNA may pose an additional complicating factor in interpreting replication studies on BPde damaged templates. Studies using viral DNA as a probe for replication require very high levels of damage to introduce a number of lesions on the small viral genome (53). High BPde concentrations may interfere with cellular functions through modification of proteins or RNA, besides producing the desired effects of DNA adduct formation. Again, transfection of BPde modified viral DNA may overcome this problem in studying DNA replication on damaged templates.

Whether or not this induced recovery response is error-prone in mammalian cells is still questionable. It is possible that this recovery pathway is responsible for UV or BPde mutagenesis observed in mammalian cells. The system developed in this thesis should allow for investigation of the mutagenic potential of BPde during enhanced reactivation in mammalian cells.

In conclusion, the data presented in this work shows that BPde is able to induce enhanced survival of irradiated virus grown in mammalian cells. This response is associated with concentrations of BPde which result in an exponential decrease in survival of the CV-1P cells. The inhibition of nascent DNA elongation by these concentrations of

BPde may provide an inducing signal to facilitate DNA replication on damaged templates. The mutagenicity of this induced response is under continued investigation in the laboratory.

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