# CONSTRUCTION OF AN HSV-1 RECOMBINANT EXPRESSING THE BACTERIOPHAGE T4 DEN V GENE

# CONSTRUCTION OF A HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) EXPRESSION VECTOR CONTAINING THE BACTERIOPHAGE T4 DEN V GENE: EFFECT OF THIS GENE ON UV-SURVIVAL OF HSV-1 IN NORMAL AND XERODERMA PIGMENTOSUM FIBROBLASTS

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# ABSTRACT

In order to examine the potential of HSV-1 as a vector to study the expression of DNA repair genes in mammalian cells, a recombinant virus containing the den V gene from bacteriophage T4 has been constructed. This gene encodes a pyrimidine dimer-specific endonuclease that has the capacity to initiate excision repair of DNA. Transfection studies indicate that excision repair deficient xeroderma pigmentosum (XP) group A cells are able to carry out excision repair initiated by the den V gene product. This gene along with the 3' LTR of Rous Sarcoma Virus and the SV40 polyadenylation signals were inserted into the non-essential glycoprotein I gene of HSV-1. Immunoprecipitation studies confirmed the production of the den V protein in virus infected cells. The uv survival of this HSV-1:den V recombinant virus was examined in various primary cell types.

The cells examined in this study were primary fibroblasts from a normal individual, a Trichothiodystrophy patient and five XP patients as well as a mouse L cell line. The ability of the virally encoded den V gene to restore the excision repair deficiency in these cells was measured by monitoring the uv survival of HSV-1:den V as compared to wildtype HSV-1. Increased survival of HSV-1:den V was detected in Trichothiodystrophy cells, and in cells from XP complementation groups A, C and D, but not in XP cells from complementation groups E and F or in mouse L cells. These results demonstrate that HSV can be effectively used to study the expression of a cloned DNA repair gene in a variety of cell types. HSV has a substantial

capacity of gene insertion and a wide host range including cells of human and rodent origin.

# DEDICATIONS

This thesis is dedicated to my parents, Lawson and Mary for their endless love and support throughout my education.

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# INTRODUCTION

Living cells have evolved by investing a considerable portion of their genetic material in DNA repair processes. Replication and repair are essential processes that maintain the continuity and structural integrity of the genetic material. Within cells there exists an intricate network of biochemical repair systems to protect the DNA from the accumulation of DNA damage and permanant mutations. A living cell is constantly subjected to alterations in the DNA either spontaneously or through environmental agents. Spontaneous base mutations such as a tautomeric shift during replication can cause an incorrect base to be inserted into the growing strand (Watson, 1976). Alternatively, chemical agents or ultraviolet (uv) radiation can produce a wide variety of lesions, including alkylation, crosslinking and cyclobutane pyrimidine dimers in cellular DNA. These lesions, if unrepaired, may have lethal, mutagenic, or carcinogenic consequences.

The maintenance of a DNA sequence is not only important for the individual cell's survival, but also for the organism as a whole. Alterations in this sequence can lead to the development of diseases such as Xeroderma pigmentosum (XP), Ataxia telengectasia (AT) and Fanconia's Anemia (FA) among others. Cells from individuals with such cancer prone genetic diseases often have defects in important pathways that may normally be involved in cancer prevention. By examining the precise nature of these defects, one may gain some insight into cancer etiology and prevention.

## BACTERIAL DNA REPAIR PROCESSES

Studies of DNA repair processes were initially performed primarily using the prokaryotic system. The isolation and characterization of a large number of repair deficient mutants has led to a sound understanding of the enzymology of the bacterial system (Hanawalt *et al.*, 1979). Within the prokaryotic system, five mechanisms of DNA repair have been identified. These include photoreactivation (Sutherland, 1978) excision repair (Hanawalt and Setlow, 1975), post replication repair (Lehmann and Karran, 1981) and SOS repair (Little and Mount, 1982).

## PHOTOREACTIVATION

A simple and direct method of DNA repair in prokaryotes is known as enzymatic photoreactivation (PR) of pyrimidine dimers. Rupert *et al.* , (1958) identified an enzymatic system in bacteria and yeast capable of thymine dimer monomerization. The enzyme DNA photolyase specifically recognizes and binds to pyrimidine dimers. The resulting enzyme-dimer complex absorbs light in the 300-600 nm range and uses the energy to monomerize the pyrimidine dimers (Sutherland, 1978). This enzymatic system is considered of interest since similar photolyase activity exists in a large number of higher plants and animals (Rupert, 1975; Friedberg, 1985).

## EXCISION REPAIR

There are two fundamental excision repair processes in *E. coli.*, base excision repair (Friedberg *et al.*, 1979) and nucleotide excision repair (Hanawalt *et al.*, 1978). Both processes are mediated by a large number of enzymes, which can remove a variety of pyrimidine dimers, and DNA-DNA

or DNA-protein crosslinks (Hanawalt, *et al.*, 1979). Base excision repair occurs when base modifications are recognized by specific glycosylases that hydrolyse the N-glycosylic bond linking the lesion to the DNA backbone (Duncan, 1981). After cleavage by the 5' apurinic-apyrimidinic (AP) endonuclease, the resulting 5' deoxyribose-phosphate group can then be excised by either a 3' AP endonuclease or a 5' - 3' endonuclease (Lindahl, 1976, 1982). The gap that is left in the strand is then filled in by DNA polymerase and sealed by DNA ligase.

UV irradiation, however, predominantly results in the formation of bulky base lesions, hence an additional repair process is needed. Nucleotide excision repair functions to repair damage that causes severe helical distortions such as pyrimidine dimers, cross links and ethyl methane sulphonate damage. In *E. coli* this is accomplished by the UvrABC endonuclease complex (Howard-Flanders *et al.*, 1966). The 114,000 dalton uvrA gene product binds single stranded irradiated DNA with higher affinity than unirradiated DNA, and exhibits ATPase activity (Seeberg and Steinum, 1982). The remaining two genes, uvrB and uvrC, each code for a protein of 70,000 daltons (Sancar *et al.*, 1981a, Sancar *et al.*, 1981b). These three genes are under the control of the RecA-LexA regulatory system (Little and Mount, 1982). DNA damage stimulates the protease activity of RecA, which then degrades the LexA proteins that are bound to the promoters of the uvr genes. It is the stimulation of these genes that allows for the initial incision step. The uvrA protein first non-specifically binds to DNA and then binds with the uvrB protein to form a more stable complex. In an ATP dependent process, this dimer translocates along the DNA to a damaged site. The uvrC protein then joins the complex, making it catalytically active and able to excise an

oligonucleotide from the damaged region. Excision is accomplished by two nicks made in the deoxyribose-phosphate backbone. The first incision occurs at the eighth phosphodiester bond 5' to the damage site and the second occurs at the fourth or fifth phosphodiester bond 3' to the damage (Rupp *et al.*, 1982). This process liberates an oligomer of 12 bps. Just as in base excision repair, this gap is then filled in by DNA polymerase and sealed by DNA ligase.

POST REPLICATION REPAIR AND SOS REPAIR

Post replication repair occurs when a lesion is not corrected prior to the time of DNA replication. This damage can act as a block to DNA synthesis at that site. The arrested polymerase will either re-initiate downstream from the damage producing a gap in the daughter strand or trans-lesion synthesis can take place through an error prone polymerase that is able to overcome the dimer block (Hanawalt *et al.*, 1979). It is these gaps which are the targets for post replication repair (Lehmann and Karran, 1981). It has been suggested that the gaps are repaired by the process of sister chromatid exchange, in which non-reciprocal recombination with the intact sister DNA acts as a template for the resynthesis of the deleted strand (Rupp *et al.*, 1971). The gap left in the parental molecule by this strand exchange can then be repaired by conventional mechanisms.

An alternative to the post replication repair process is trans-lesion synthesis. Since the information as to the correct base to insert opposite the lesion is unclear, an error prone process results. The SOS repair system of *E. coli* exemplifies such a process. The current model of the SOS system indicates the involvement of the RecA-LexA repressor system. Activated by DNA damaging agents the RecA protein cleaves the LexA repressor which

now allows for the expression of the SOS genes. As the DNA damage is repaired, the signal level drops and Rec A loses its protease function. The repressor molecules accumulate and the level of gene expression is lowered (Little and Mount, 1982).

## MAMMALIAN DNA REPAIR MECHANISMS

The complex secondary structure of eukaryotic DNA as well as its association with DNA binding proteins and the nuclear matrix clearly suggest the requirement for a more elaborate DNA repair mechanism than that seen in prokaryotes. While knowledge of the precise mechanism involved in eukaryotes is limited, both base and nucleotide excision repair are known to exist in mammalian cells. Moreover, they are thought to occur by relatively the same basic steps as in the bacterial system (Lehmann & Karran, 1981). Mammalian DNA glycosylases can act to repair a wide range of base damage (Lindahl, 1979; Cathcart & Goldthwait, 1981; Duncan, 1981). As in bacterial cells, these enzymes can either replace the damaged base directly (Lindahl, 1979) or the AP site can be targeted by an AP endonuclease (Linn *et al.*, 1978).

Similar to the bacterial system, nucleotide excision repair in mammalian cells requires the recognition and incision of damaged DNA, excision of the lesion, repair synthesis and ligation of the newly synthesized DNA. A great deal of work has focused on the incision step of excision repair. Fischer *et al.* (1985) have shown that cells from XP complementation groups (A through G) are deficient in the initial incision step. This suggests that seven genes are possibly involved in a complex similar to the bacterial uvrABC complex. The large number of genes necessary may be required for preincision events such as the processing of the chromatin to allow access to

the DNA (Karentz & Cleaver, 1986a). Alternatively, it is possible that some of these genes may code for molecules that regulate the other genes in the repair pathway (Karentz & Cleaver, 1986b).

A possible model for the incision step of excision repair in mammalian cells was proposed by Paterson *et al.* (1987). They suggest that the first step is the hydrolysis of the internal phosphodiester bond of the pyrimidine dimers. This step is thought to create a severe helix distortion that is recognized by the putative repair complex, which can then hydrolyze the phosphate backbone. The damaged lesion must be next excised and resynthesis must be performed. In bacteria, these two functions are achieved with the single polypeptide, Pol I. Such a dual functioning molecule has yet to be isolated in mammalian cells. However, a eukaryotic alpha polymerase molecule does exist as part of a 640 kd multipolypeptide complex, the alpha polymerase holoenzyme (Ottiger *et al.*, 1987). This holoenzyme has been shown to have a polymerase function, a 3' - 5' exonuclease function and a weak 5' - 3' exonuclease function (Skarnes *et al.*, 1986). Whether or not this holoenzyme represents the mammalian homologue of the bacterial Pol I has yet to be determined. In either case many mammalian exonucleases have been isolated and shown to be able to excise dimers from DNA (Grossman, 1981). As well any one of the four eukaryotic polymerases ($\alpha, \beta, \delta, \gamma$) that have been isolated (Weisback, 1977) can perform the repair synthesis required. The final step in the excision repair pathway is the ligation of the newly made strand which is accomplished by the polynucleotide ligase molecule (Lindahl and Edelman, 1968).

Just as in bacterial cells, both long patch and short patch repair occur in mammalian cells. Short patch repair acts on damage caused by X-rays or

chemicals that cause damage similar to that produced by ionizing radiation. This repair process has patch sizes between 3 and 4 nucleotides and repair is usually accomplished within a couple of hours (Regan & Setlow, 1974). Long patch repair operates on damage caused by uv light and chemicals which cause uv light type damage. The size of the repair patch ranges from 15 to 100 nucleotides and requires up to 24 hrs to complete (Smith, 1978).

## BACTERIOPHAGE T4 ENDONUCLEASE V GENE (DEN V)

Bacteriophage T4 is a double stranded DNA virus able to code for a DNA excision repair enzyme. Initial studies on T4 indicated a higher resistance to uv light as compared to the other T-phages (Luria, 1947). It was initially believed that T4 lacked a gene present in the other T-phages which made them more sensitive to killing by uv light (Streisinger, 1956), however subsequent studies showed that T4 actually carried a gene possessing a thymine dimer specific endonuclease activity (Friedberg & King, 1971). This gene is now termed the T4 pyrimidine dimer DNA glycosylase (den V). It is capable of reparing only thymine dimers (Friedberg, 1972) and is specific for dimers on one strand only (Simon *et al.*, 1975). Further characterization of this gene revealed a catalytic site for a 3' apurinic-apyrimidinic (AP) endonuclease activity (Valerie *et al.*, 1984). While this activity has only been detected in vitro, it is also postulated to occur in vivo (McMillian *et al.*, 1981).

Friedberg (1985) has proposed a model for the action of this 16 kd DNA glycosylase. Briefly, this molecule first locates and cleaves the 5' glycosyl bond of the dimer, thus leaving the pyrimidine base attached to its 3' partner through a cyclobutane ring (Lindahl, 1982). The 3' AP endonuclease activity cleaves the backbone between the dimerized bases to monomerize it. The

damage is then excised and the gap filled in by DNA polymerase. Hence, the den V gene product appears to bind to both irradiated and unirradiated DNA and scans it for the presence of thymine dimers (Ganesan *et al.*, 1986).

The den V gene has been isolated, cloned and sequenced (Valerie *et al.*, 1984). Numerous studies have been conducted to determine if this gene can complement excision repair deficient bacterial cells. Initial studies by Shimizu and Sekiguchi (1979) involved the introduction of the den V protein into permeabilized *E. coli* cells. They showed that uv survival of uvrA, B and C mutants were increased relative to wild type cells, thus indicating that the den V gene product can complement the defect in the mutant cells. DNA mediated transfection experiments were greately facilitated by the availability of this gene in both prokaryotic and eukaryotic expression vectors (Lloyd & Hanawalt, 1981; Valerie *et al.*, 1985; Recinos *et al.*, 1986). Moreover, the contamination problems associated with the use of partially purified extracts in the earlier protein mediated studies could now be eliminated. Recinos *et al.* (1986) introduced one of these expression plasmids into uvrA, B or C mutant *E. coli*cells and found an increase in the uv survival of the cell.

Since the den V gene product was able to incise damaged DNA in prokaryotes, it was speculated that the same might be true in eukaryotes. If the den V gene could complement the repair defect in mammalian cells, then a homologous but defective mammalian enzyme may be responsible for the mutant cell phenotype. Initial studies were conducted using XP cells since they were known to be deficient in the incision step of excision repair (Friedberg, 1985). Sendai virus fusion studies and microinjection experiments (Tanaka et al., 1975 and de Jonge et al., 1983) introduced purified den V protein into XP cells (groups A through G) and showed that DNA

repair as measured by unscheduled DNA synthesis (UDS) could be restored to near normal levels.

Transfection studies involving den V DNA were initially conducted using Chinese Hamster Ovary (CHO) cell mutants because of the ease with which transformants could be isolated and grown (Valerie *et al.*, 1985). These mutant cell lines are hypersensitive to killing and to mutagenesis by uv light. Five complementation groups have been identified thus far. Each of the mutants exhibited less than 10% of the incision events present in normal CHO cells. When the den V gene was transfected and stably integrated into the genome of one such mutant (UV-5), an increase in the uv survival of these cells was seen. Survival in this study was assayed for by colony forming ability and unscheduled DNA synthesis. Although the den V gene could complement the defect in CHO cells, it was only able to restore uv cell survival to an intermediate level.

More recently, similar DNA mediated transfection experiments were conducted using XP cells. Using SV40 transformed XP group A cells, Valerie *et al.* (1987) transformed with the den V gene. After stablely integrating into the genome, this gene was able to partially restore the defect in the XP group A cells. Cell survival was assayed for by repair synthesis and by colony forming ability after uv irradiation. The presence of the den V protein in these cells was confirmed using monoclonal antibodies and immunofluorescence. Similar results were obtained when Arrand *et al.* (1987) examined XP-D/Hela hybrid cells. These hybrids were able to retain the repair characteristics of the parental XP cells. The transfected den V gene was able to restore 15.4-22.5% of the repair defect in these hybrid cells as assayed for by colony forming ability.

Since XP cell extracts have the ability to incise thymine dimers from naked DNA, it is thought that an inability to process the chromatin in order to access the DNA may reflect the defect in XP cells. It is speculated that the small size of the the den V protein provides it with easier access to the DNA (Valerie *et al.* , 1987). In spite of this accessibility, the den V gene is still unable to complement 100% of the defect in XP cells. Since a mammalian pyrimidine dimer specific glycosylase has yet to be isolated, it is unlikely that the den V gene is complementing the normal cells repair pathway but may instead be initiating a novel or salvage pathway (Arrand *et al.*, 1987). This novel pathway may not cooperate efficiently with the host enzymes, thus generating harmful long lived nicks or gaps in the DNA ( R. Johnson *et al.*, 1987). Moreover, pyrimidine dimers are only one type of damage induced by uv irradiation. A 6,4 pyrimidine-pyrimidone molecule appears to be a second class of potentially mutagenic uv photoproduct. This product makes up 20-35% of the total uv damage and has been shown to be removed from human genomic DNA more rapidly than the pyrimidine dimers (Mitchell, 1986), which suggests that this 6,4 pyrimidine-pyrimidone photoproduct may represent the major cytotoxic lesion induced by uv (Mitchell, 1988). Since den V can only repair one of these two types of lesions, complementation of XP cells is likely to be only partial.

## XERODERMA PIGMENTOSUM

Xeroderma Pigmentosum (XP) was first described by Moritz Kaposi (1882) who noted the inherited nature of the syndrome and the skin tumors on the sunlight-exposed portions of the body. When subjected to uv irradiation, XP cells were seen to have reduced rates of UDS as measured by

tritiated thymidine incorporation (Cleaver, 1968). This rare, autosomal, recessive human disease is clinically characterized by the early onset of severe photosensitivity of exposed skin to sunlight, a very high incidence of skin cancers and frequent neurological abnormalities (Setlow, 1978). Cells from XP patients are hypersensitive to uv light in terms of cell killing, mutagenesis and in vitro transformation (Paterson *et al.*, 1984; McCormick *et al.*, 1986; Lambert & Lambert, 1987). All XP complementation groups are impaired in the initial endonuclease-mediated incision step of nucleotide excision repair (de Jonge *et al.*, 1985; Rubin, 1988; Hansson *et al.*, 1990). It is not clear however, whether the problem resides at the level of the incising endonuclease (Wood *et al.*, 1988; Hansson *et al.*, 1990) or involves one or more factors functioning in unfolding the chromatin structure to facilitate the accessibility of the damaged sites in DNA to large repair complexes (Lambert and Lambert, 1987; Bohr *et al.*, 1988).

Studies using plasmid vectors have provided a great deal of insight into the DNA repair defect in XP cells. Such experiments involve damaging the plasmid in vitro with uv radiation or another DNA damaging agent prior to introducing it into human cells. The cellular machinery then acts upon the damaged DNA, performing repair, replication or mutation. After 2 days of transient expression the plasmid is harvested and the alteration assayed. This assay measures the ability of the host cell to correct the damage in the plasmid DNA and is known as a "Host Cell Reactivation" assay (Protic-Saliljic and Kraemer, 1986).

Genetic complementation analysis by cell fusion experiments has led to the identification of seven mutually complementing XP groups, designated A through G and one variant (Friedberg, 1985; Robbins, 1989; Johnson, 1989;

Bootsma *et al.*, 1989). The XP variant was first thought to have impaired post replication repair (Fischer *et al.*, 1985), but recent studies suggest that it also has a defect in excision repair (Konodo *et al.*, 1987; Wood *et al.*, 1988). By using antibodies against thymidine dimers, Roth *et al.* (1987) was able to show a lower rate of loss of antigenicity to antibodies with time in the XP variant cells as compared to normal cells.

XP complementation group A (XP-A) has one of the largest repair defeciencies of all the complementation groups (Kraemer *et al.*, 1987; Lambert and Lambert, 1987). The repair defect in XP-A cells is known to occur at the level of the initial endonuclease mediated, incision step (Cleaver, 1974; Setlow, 1978) but the molecular mechanisms involved in this defect is still unclear. After extensive genomic DNA transfection experiments, Tanaka *et al.* (1989) has cloned the mouse gene able to correct SV-40 transformed XP-A fibroblasts. This cloned gene is able to confer uv resistance to group A XP cell lines but not to XP cell lines of groups C, D, F or G, which suggests that this gene may be the mouse homologue of the group A XP human gene. This same research group more recently described the isolation and sequence of the human XP-A cDNA (Tanaka *et al.*, 1990). This XP-A correcting gene encodes a hydrophilic protein of relative molecular mass 31,000 daltons which contains many charged residues. The presence of a distinct zinc-finger motif in this protein reflects its ability to interact directly with the DNA, suggesting a possible role in an enzyme complex involved in incision.

The presence of seven XP complementation groups indicates that there are at least seven genes involved in the repair of bulky DNA lesions. Since chromatin rearrangements/unfolding and nucleosome structure are believed to play a role in this process (Liebermann, 1981; Sidik and Smerdon, 1987), it is

possible that some of the XP complementation groups are not defective in the incising activity per se, but simply cannot gain access to the DNA properly. Support for this idea is seen when protein extracts from cells with limited repair abilities (XP-A, C and G) are able to remove dimers from naked DNA almost as well as protein extracts from normal cells (Mortlemans *et al.*, 1976). Thus, it is thought that the repair process in mammalian cells may involve either a multi-step pathway or a holoenzyme complex (Friedberg, 1985).

In addition to the XP group cells, there exists a second class of excision repair deficient mutants derived from Chinese Hamster Ovary Cells (CHO). Recently, a number of human repair genes have been isolated which can complement the excision defect in these CHO cells. These genes are designated as Excision Repair Cross Complementing (ERCC). To date six ERCC genes have been identified and all but the ERCC-4 gene has been cloned. Previous attempts to locate any overlap between these XP and CHO mutants proved to be unsuccessful. Weeda *et al.* (1990) was however, able to demonstrate that the human ERCC-3 gene specifically corrected the defect in XP group B cells. Moreover, since XP is known to be associated with a predisposition to skin cancer, ERCC-3 was thought to be a tumour preventing gene. More recently, the ERCC-2 gene, which is known to complement the excision repair defect in the CHO cell line mutant UV-5, was shown to correct the deficiency in XP-D cells (Weber, *et al.*, 1991). The large degree of homology that exists between these genes and the yeast helicases suggests a putative DNA helicase function for ERCC-2 and ERCC-3.

TRICHOTHIODYSTROPHY (TTD)

Trichothiodystrophy is a rare, genetic disease characterized by brittle hair, mental and physical retardation and peculiar faces (Nuzzo and Stefanini, 1989). The presence of photosensitivity has been reported in about 20% of the patients identified. Three categories of response to uv have been identified for TTD cells (Broughton *et al.*, 1990). The first category of cells exhibit a completely normal uv response. Category two cells however, are deficient in excision repair and have properties indistinguishable from those of XP group D cells. The third category is characterized by normal uv cell survival and normal rates of removal of cyclobutane pyrimidine dimers. The removal of 6-4 photoproducts however, is reduced in these cells which correlates with a reduced repair synthesis rate (Broughton *et al.*, 1990) and a reduced incision rate (Lehmann *et al.*, 1988). The genetic analysis of the DNA repair defect in TTD cells was performed via complementation studies. Cells from different TTD strains were fused with XP fibroblasts of complementation groups A, C and D. The results of these studies indicated that in all of the TTD repair deficient strains the genetic defect is the same as that present in XP-D cells (Stefanini *et. al.*, 1986).

It is unlikely that the presence of this mixed TTD/XP-D phenotype in patients is due to a sporadic event since it would require the co-inheritance of two very rare diseases. The simplest explanation for this association is the occurence of two independent, homozygous mutations. Other possibilities could include the presence of two heterozygous, defective alleles within the same locus in a trans configuration (Lambert and Lambert, 1985). More recently however, Lehmann and Norris (1989) proposed a model in which the excision deficient TTD phenotype was generated first by a deletion in a

"TTD gene" that extended into the XP-D repair gene and then by an independent mutation in a locus governing immunosurveillance. They speculated that the various observed repair phenotypes in TTD could possibly be due to different length deletions. A recent study involving a search for consanguinity within and among the familites of TTD/XP-D patients revealed that this genetic defect may be due to remote multiple inbreeding (Nuzzo et al., 1990). This implies that if two mutations are responsible for the TTD/XP-D phenotype, they have to have been transmitted together throughout nine generations to the heterozygous carrier and then to the affected offspring. Consequently, this would require that the two mutation occur either at closely linked loci or within a single gene.

## EFFECT OF TRANSFORMATION ON THE DNA REPAIR PROCESS

Experiments involving the use of primary cultures must overcome two major obstacles, the limited life span of primary cells and the low transfection efficiency of primary cultures. These two factors makes the isolation of transformed primary human cell lines very difficult, because by the time a cell colony is isolated and propagated the cells are already starting to senesce. It is not surprizing therefore that many studies requiring the establishment of stable transformed lines have involved the use of immortalized cell lines. Many excision repair studies have utilized SV40 transformed cells. (Gantt *et al.*, 1984).

Although transformed cells are easier to work with they may not accurately reflect the cellular mechanisms of the parent cell. When a cell is transformed, the expression of their proteins may be altered. In a study conducted by Bravo et al. (1982) the expression of 58 out of 1300 polypeptides

had changed by at least 40% in lung fibroblasts that were transformed with SV40. The DNA repair ability of transformed cells compared to their parent line has been extensively examined. The sensitivity to an alkylating agent N-Methyl N' nitro-N-nitroguanidine (MNNG) was studied by Day *et al.* (1980). Upon examination of tumor lines and SV40 transformed lines, they concluded that these cells were unable to reactivate MMNG treated Adenovirus type 5 to the same levels as that seen in the parent cells. Chemically untreated virus however, were replicated to the same levels in both primary and transformed cell types. This suggests that the tumor lines and the SV40 transformed lines possess a defect in their ability to repair MNNG damage. Moreover, Heddle and Arlett (1980) likewise treated the SV40 transformed normal and XP cell lines with a DNA damaging chemical, ethyl methane sulphonate (EMS), and compared DNA repair ability to the untransformed lines. The transformed cells showed an increased sensitivity to EMS as assayed for by increased sister chromatid exchange and decreased colony survival.

This inability of the transformed cells to correct chemically damaged DNA was also apparent in studies examining uv damaged DNA. Using SV40 transformed lung fibroblasts, Squires *et al.* (1982) showed a 50% decrease in the rate of the initial incision step of excision repair when examining uv irradiated cells. Previous work conducted in our lab (Rainbow, 1989c) employed HCR of viral structural antigen ($V_{ag}$) expression to examine the DNA repair capacity of SV40 transformed human cells. A deficiency in the ability to repair uv damaged DNA was seen in the SV40 transformed cells. This indicates that SV40 transformed humans cells may not reflect the actual DNA repair abilities of the untransformed parent. As a result, the use of

SV40 transformed repair deficient cell lines as recipients in DNA transfection experiments may preclude the restoration of "normal" levels of repair.

## USE OF VIRUSES TO STUDY DNA REPAIR

Mammalian viruses have been extensively used to study DNA repair, recombination and mutagenesis in various mammalian cells (for reviews refer to Rainbow, 1981; Defais *et al.*, 1983). Most of these types of studies have utilized nuclear replicating, double stranded DNA viruses such as SV40, herpesvirus or adenovirus to examine host cell reactivation, cellular capacity and enhanced reactivation.

Host cell reactivation as its name implies relies in part on the host cell mechanisms to repair damaged viruses. It is assumed here that the viral DNA is at least partially processed by the same enzyme systems that function on the host cellular DNA (Rainbow, 1981). In this way the ability of a cell to repair uv or chemically damaged viral DNA becomes a measure of the cell's DNA repair capacity (Day, 1978; Rainbow, 1981). Various endpoints can be used to measure survival. These include V antigen formation of adenovirus (Rainbow, 1978), intranuclear inclusion body formation of adenovirus (Rainbow & Mak, 1972), plaque formation of herpesvirus (Ryan & Rainbow, 1986) and viral DNA synthesis. Previous work in our laboratory has demonstrated the use of an adenovirus vector system to study DNA repair genes (Colicos, 1988). A recombinant Ad 5 den V virus was constructed and examined for percent host cell reactivation (%HCR) of $V_{ag}$ production for uv irradiated virus. The %HCR was significantly greater for Ad 5 den V as compared to the control virus in xeroderma pigmentosum cells.

Cellular repair capacity can be measured by pretreating cells with DNA damaging agents before infecting with untreated virus. Since the cell must repair the damage in its genome before it can support viral growth, the yield of virus depends on the cells ability to perform this repair (Coohill *et al.*, 1977). Consequently, it is not surprizing that as the the time between treatment of the cells and infection is increased the decrease in the capacity of cells to support virus infection becomes less. It is thought that this time period enabled the cells to repair the lesions in its cellular DNA (Coppey *et al.*, 1979). Capacity studies using HSV in normal and XP cells have demonstrated that there is a greater decrease in capacity in XP cells than in normal cells due to the presence of repair defects in XP cells. Unlike normal cells, the restoration of capacity was not possible in treated XP cells (Lytle *et al.*, 1976; Coppey *et al.*, 1979). Since many human fibroblasts have poor clonogenic abilities, the use of such a virus system in a capacity study would provide information on cellular repair abilities that would not otherwise be attainable (Coohill, 1981).

The enhanced survival of virus combines the first two techniques into one assay. Chemically treated or irradiated virus is used to infect cells which have also been pretreated with some form of DNA damaging agent (Defais *et al.*, 1983). This pretreatment of cells has been shown to increase the survival of UV irradiated nuclear replicating viruses in a variety of host cells including normal, XP (Lytle *et al.*, 1976), CS (Coppey & Menezes, 1981) and Monkey kidney cells (Bockstahler *et. al.*, 1976).

The exact mechanism of uv enhanced reactivation is not known, but does not appear to depend on excision repair since excision repair deficient XP cells are capable of uv enhanced reactivation (Lytle *et al.*, 1976). Since drugs

that block DNA synthesis such hydroxyurea and cycloheximide, have been shown to enhance SV40 reactivation, it is hypothesized that enhanced reactivation represents a repair pathway activated by the blockage of DNA synthesis (Sarasin & Hanawalt, 1978). Although the mechanism of enhanced reactivation is not clear it may be related to other putative mammalian SOS responses such as provirus induction (Zamansky *et al.*, 1980) and enhanced mutagenesis (Lytle, *et al.*, (1980; Sarasin & Benoit, 1980).

Previous studies have demonstrated that enhanced reactivation of HSV may be accompanied by enhanced mutagenesis (Das Gupta & Summers, 1978). The mutation rate of viruses grown in irradiated or chemically treated host cells can be used as a measure of inducible error prone repair in some mammalian systems. Su *et al.* (1981) using uv irradiated parvovirus demonstrated the simultaneous enhancement of reactivation and mutagenesis in human cells treated with nitronapthofuran. Moreover, studies in which CV-1 monkey kidney cells were pretreated with chemical carcinogens or uv-light produced very large increases in the mutation frequency of the surviving SV40 viruses (Sarasin & Benoit, 1980).

Of the three different assays previously discussed for studying DNA repair mechanisms, host cell reactivation (HCR) is the most widely used . Numerous studies have shown that the yield of virus in an infection initiated with uv irradiated HSV was much lower in XP fibroblasts than in normal fibroblasts (Rabson *et al.*, 1969; Takebe *et al.*, 1978; Ryan & Rainbow, 1986 and Bueschleb, 1987). Reduced host cell reactivation for XP cells as compared to normal cells was also demonstrated for HSV treated with N-acetoxy-2-actylaminoflourene (Selsky & Greer, 1978), but not for HSV treated with X-rays (Lytle *et al.*, 1972).

HCR by excision repair has also been shown to be primarily an error free process. Lytle *et al.* (1982) demonstrated this by using HSV in a thymidine kinase mutation assay. They infected XP group A cells and normal cells with uv irradiated HSV and looked for forward mutations in the thymidine kinase gene (tk). A comparison of the mutation frequency between excision repair proficient normal cells and excision repair deficient XP-A cells were indistinguishable when normalized to the same number of lethal hits to the virus. Hence the excision repair of normal cells which provides host cell reactivation by removing lethal uv damage also removes mutagenic lesions from the virus with the same efficiency.

## HERPESVIRUSES

### STRUCTURE

Herpesviruses have linear, double stranded DNA, an icosodeltahedral capsid and an envelop derived from the hosts nuclear membrane (Roizman and Batterson, 1985). The alpha virus, HSV-1 has a short replication cycle, a broad host range and is able to produce persistent infections. The HSV-1 genome contains 150 kilobase pairs and consists of two covalently linked components, designated as long (L) and short (S). The long sequence comprises approximately 82% of the viral DNA and the short sequence makes up the remaining 18%. Each component consists of largely unique sequences flanked by inverted repeats (Varmuza and Smiley, 1985). By inverting the long and short regions with respect to each other, four genomic arrangements are possible. The capsid and envelop contain somewhere between 15 and 35 structural proteins (Cassai *et al.*, 1975). Six of these are major capsid proteins

(Gibson and Roizman, 1972) and seven others are glycoproteins (Ligas and Johnson, 1987).

REPLICATION

HSV-1 infects cells by binding to cell surface receptors (Ligas and Johnson, 1987), fusing with the plasma membrane (Fuller and Spear, 1985) and by entry of the capsid into the cytoplasm of the cell. This capsid migrates to the nucleus and deposits its DNA there via the nuclear pore (Batterson *et al.*, 1983). The viral genome then acts to shut off DNA synthesis (Auerelian and Roizman, 1965), reduce protein synthesis (Roizman *et al.*, 1965) and alter RNA synthesis (Roizman and Furlong, 1974) such that its own viral transcripts are transcribed by RNA Pol III (Stringer *et al.*, 1977).

There are three classes of HSV-1 genes which are sequentially expressed during infection. They are the Immediate Early (IE), Early (E) and Late (L) genes (Roizman and Batterson, 1985). The IE genes are transcribed in the absence of de novo protein synthesis and function in stimulating transcription of E and L genes (Preston, 1979). After E gene synthesis the genome of the virus is replicated. There are two origins of replication, one in each of the long and short regions. Rolling circle replication occurs such that large concatamers of full length genomes are generated (Ben-Porat and Takezewski, 1977). Following synthesis of the late genes, virus assembly occurs. A full length genome is cleaved off the concatamer (Roizman and Batterson, 1985) and packaged into a capsid. Budding of the capsid occurs at a region of the nuclear membrane where HSV-1 glycosylated proteins have been inserted (figure 1).

HSV-1 AS A VECTOR

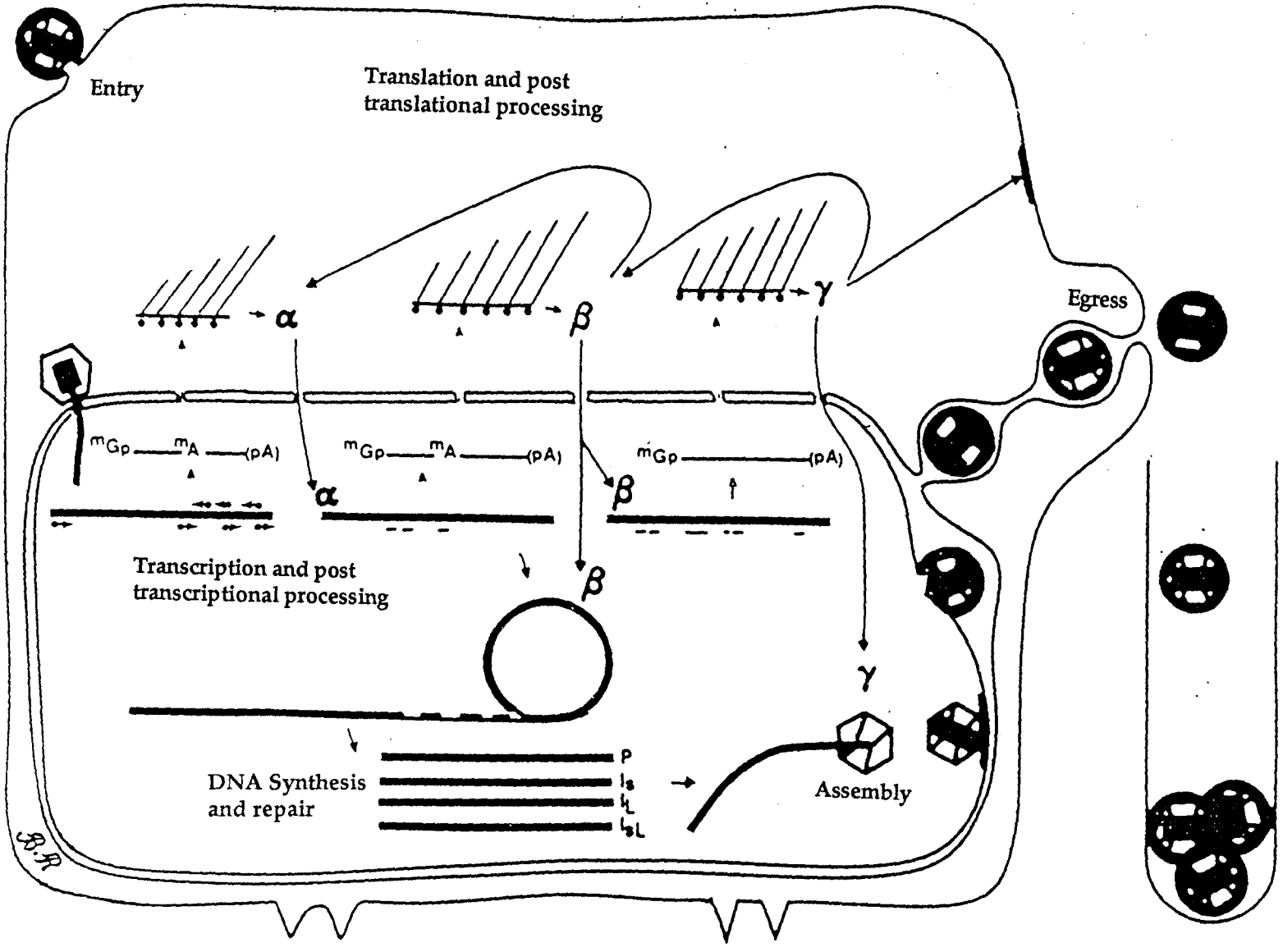
It was initally thought that HSV-1 would distinguish foreign promoters from its own (Tackney *et al.*, 1984) and therefore would not be an appropriate expression vector. Smiley et al., (1987) however, was able to construct a non defective recombinant virus carrying the rabbit beta globin gene and promoter inserted into the thymidine kinase locus of HSV-1. This foreign promoter expressed properly inititated RNA transcripts to high levels during productive infection of Vero cells. More recently, Salloukh (1991) inserted 7904 bps from the Human Papilloma Virus 16 into the gI locus of HSV-1 to generate a recombinant known as R1 (personal communication). It is therefore possible for an unmodified cellular gene not normally induced by HSV infection to be abundantly transcribed when inserted into the HSV-1 genome.

Previous studies in our laboratory have attempted to construct an HSV-1 mutant containing the den V gene inserted into the thymidine kinase (tk) locus (Intine, 1988). Although properly initiated RNA for den V transcription was produced in the infected cells, the functional den V protein was still unable to complement the excision repair defect in XP cells. Since a previously constructed Ad 5 recombinant expressing den V was found to increase the survival of the virus (Colicos, 1988), this lack of an effect with the HSV-1 recombinant was believed to be due to the concomitent loss of tk activity in the HSV virus. A number of other investigators have also indicated an involvement of the tk gene in excision repair (McKenna *et al.*, 1985; Lopez & Coppey, 1987; Rainbow 1989a, b).

By utilizing tk mutants, Intine & Rainbow (1990) demonstrated that the viral tk gene was involved either in excision repair or a process dependent on

## Figure 1: Herpes Simplex Virus Replicative Cycle

A summary of the major features of the HSV replicative cycle as modified from Virology ed. Fields et al., 1985. Chapter 25, Roizman & Batterson.


Entry
Translation and post
translational processing
α
β
γ
Egress
mGp
mA
(pA)
Transcription and post
transcriptional processing
DNA Synthesis
and repair
P
Assembly

excision repair. Inspite of the unsuccessful first attempt to construct an effective HSV-1 recombinant virus, HSV-1 is still be believed to be a suitable expression vector for DNA repair genes. Moreover its versatility is seen in its broad host range, rapid life cycle and large capacity for gene insertion.

In order to examine the potential of HSV as a vector to study the expression of DNA repair genes in mammalian cells, a recombinant HSV-1 expressing the T4 den V gene was constructed containing a functional tk gene. The uv survival of this recombinant was examined in several cell types. By combining the use of HSV-1 as a mammalian expression vector with the uv survival of HSV-1 as a measure of the DNA repair capacity of the the infected cell, this would allow an examination of the ability of a DNA repair gene to complement a repair deficient cell.

# MATERIALS AND METHODS

## 1.0 CELLS

**1.1 Human Diploid Fibroblasts** (purchased from the NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, 401 Haddon Ave., Camden, NJ, 08103).

Normal GM00037D

Xeroderma Pigmentosum GM5509A (XP12BE complementation group A)

Xeroderma Pigmentosum GM00677 (XP2BE complementation group C)

Xeroderma Pigmentosum GM03615 (XP1BR complementation group D)

Xeroderma Pigmentosum GM02415B (XP2RO complementation group E)

Xeroderma Pigmentosum GM04313B (XP2YO complementation group F)

**1.2 Other Human Cell Lines:**

XP-4: These cells are resistant to the drug G418 and were obtained by cotransfecting GM4429B cells with the pSV2-neo plasmid (Southern and Berg, 1982) and the pR plasmid (Marcucci et. al., 1986)

293 cells: These cells were obtained by transfection of human

embryonic kidney cells with sheared human adenovirus type 5 DNA (Graham *et al.*, 1977).

Xeroderma Pigmentosum GM4429B: These cells were made by transforming GM5509 cells with SV40.

Trichothiodystrophy cells (TTD8PV): These human diploid fibroblasts were obtained from patients exhibiting photosensitivity and a deficiency in excision repair that is indistinguishable from those of XP-D patients (Miria Stefanini, personal communication).

**1.3 Non Human Cell Lines:**

Vero cells: These cells were initiated from the kidney of normal adult African green monkeys and were obtained from Dr. J. Smiley, Dept of Pathology, McMaster University.

VD 60 cells: These cells are capable of expressing high levels of glycoprotein D (gD) after infection with HSV and in so doing can complement viruses with a mutation in the gD gene. They were generated by transfection of Vero cells with pSV2HISgD, which contains the BAM H1 'J' fragment of HSV-1 inserted into the BAM H1 site of pSV2HIS (Hartman & Mulligan, 1988).

LPM2A cells: This mouse cell line is resistant to the drug G418 and was obtained by transfection of mouse LTA($tk^-$) cells with the pSV2-neo plasmid (Southern and Berg, 1982).

## 1.4 Growth and Culturing of Cells:

### 1.41 Media

**Alpha-MEM medium** (Gibco/BRL, cat.# 410-2000, Gibco Canada Inc., 2270 Industrial Street Burlington, Ont., L7P 1A1) was purchased in powdered form, made in double glass distilled water and filtered sterilized using a 0.22 micron filter. This media was used for all cell lines and strains.

### 1.42 Media Supplements

**Antibiotic-Antimycotic Solution** (penicillin 10,000 u/ml, fungizone 25 mcg/ml and streptomycin 10,000 mcg/ml, BRL cat. # 600-52401) was added to the medium at a 1:100 dilution.

**Newborn Calf Serum** (NCS, BRL Cat # 200-6010AJ) was added to the media to make up 15% of the total volume for fibroblasts and 10% of the total volume for the other cell lines.

### 1.43 Other Solutions

**Trypsin** (BRL Cat # 610-5400AG) was used in subculturing all cell lines and strains.

**Phosphate Buffered Saline** (PBS) without calcium and magnesium ( 0.8 g NaCl, 2 g KCL, 1.15 g $Na_2HPO_4$ and 0.2 g $KH_2PO_4$ dissolved in 1 L double distilled water and autoclave sterilized) was used to wash the cell monolayers.

**1.5 Procedure to Passage Cells:**

Cells were grown in 150 $cm^2$ plastic Nunc dishes (Nunclon Intermed, Denmark) in a humidified incubator at 37°C with 5% $CO_2$. When confluent the media was aspirated off and 1 ml of trypsin was added to the dish which was then returned to the incubator for 1-2 mins. When the cells were rounded up the dish was vigorously tapped to dislodge the cells. 10 ml of media was then added to each dish and cells were pipetted up and down to break up any clumps. The suspension was then divided and added to new dishes for further growth and passaging.

## 2.0 VIRUSES

**2.1 Viral Strains:**

**HSV-1:kos** is a wildtype HSV-1 strain and was obtained from Dr. J. Smiley, Dept. of Pathology, McMaster University, Hamilton, Ont.

**HSV-1:FGDβ** is a recombinant virus in which the gD gene is replaced by the E.coli β-galactosidase gene (Ligas & Johnson, 1988). It was propagated and assayed on VD 60 cells.

**HSV-1:den V** is a recombinant virus which was generated by insertion of the bacteriophage T4 gene (den v) into the gI gene of HSV-1:kos. (Constructed in this study)

**2.2 Preparation of Virus Stocks:**

Confluent 150 $cm^2$ dishes of Vero cells were infected with a 0.2 ml suspension of virus in 2.0 ml of straight media at a multiplicity of infection of approximately 0.05 pfu/cell. The dishes were then incubated at 37°C for 2 hrs

with manual rocking every 10-15 mins. 10 ml of alpha mem containing 1% NCS was added to each dish and placed in the incubator for about 48 hrs. until all the cells had lifted off or rounded up. At this time the cells were scraped off, pooled together and pelleted in 50 ml Falcon tubes at 1000 rpm and 4°C. The pellets were resuspended in 2 ml of cold alpha MEM containing 1% NCS and sonicated on ice for five 30 sec intervals separated by 30 secs using a probe sonicator (Biosonik III, Model BPIII40T, Bronwill Scientific). 1 ml suspensions were aliquoted into freezing vials for storage at -70°C.

**2.3 Titre of Virus:**

Viruses were titred on either Vero cells or human fibroblasts. Confluent 150 $cm^2$ dishes of cells were collected in the same manner as was used for passaging. These cells were resuspended in a total volume of 24 ml and then seeded into two 24-well Linbro plates (Nunc, cat # 1-43982) by adding 0.5 ml of the cell suspension into each well. The plates were then incubated at 37°C to reach confluency either 24 or 48 hrs later. Ten fold serial dilutions of virus were added to the wells and the plates were incubated for 90 mins. Each well was then overlayed with media containing 1% NCS and 0.1 % Human Immune Serum Globulin (Connaught Laboratories Ltd.) After 3 days, the plates were fixed and stained using 0.2 ml of crystal violet stain (2 gm crystal violet dissolved in 20 ml methanol, 144 ml PBS and 36 ml formaldehyde) for 20 min. and then washed with cold water. The plaques were counted to determine the titre.

# 3.0 RECOMBINANT DNA TECHNIQUES

## 3.1 Plasmids:

**PSS17BG** consists of a copy of the Bam H1 'J' fragment derived from HSV- 1:kos inserted into PUC 19. It contains a unique Bgl II cloning site positioned within its gI gene (Ligas and Johnson, 1988).

**PTKE59,** a generous gift of Robert Intine (McMaster University, Dept of Biology, Master's Thesis, 1988) contains a den V expression cartridge inserted into the HSV-1:kos tk gene of pTKSB.

**PMT21** was constructed by inserting the 1.9 kb Bam H1 fragment from pTKE59 into the Bgl II site of pSS17BG (Ligas and Johnson, 1988).

## 3.2 Restriction Enzyme Digests:

All restriction enzymes were purchased from Boehringer Mannheim Canada, and used with the recommended reaction buffers supplied by the company. A typical digest consisted of 10 units of enzyme in a final volume of 30 µl. The digests were carried out in microfuge tubes at 37°C for 2 hrs.

## 3.3 Ligation Reactions:

Cohesive end ligations were performed using T4 DNA Ligase in a final volume of 20 µl. The reaction mix consisted of 0.1 µg of vector DNA, 50 molar excess of foreign DNA, 1X ligase buffer, 5 mM ATP, 5 units of ligase and dd$H_2O$ to the desired final volume. The reactions were incubated in microfuge tubes at 16°C for 1-4 hrs.

### 3.4 Transformation of Bacteria (Electroporation):

Competent DH-5 Alpha cells (Gibco, cat. # 8265SA) were thawed at room temperature and placed on ice. The Bio Rad Gene Pulser (cat. # 165-2075) was set to 25 μF, 2.25 kV and 200 ohms. Approximately 1.0 - 2.0 μl of the ligation reaction was added to 50 μl of the thawed cells. This mixture was transferred to a prechilled cuvette and placed in a chilled safety chamber within the Gene Pulser unit. A charge was sent through this mixture for a few seconds. 1 ml of SOC media (2% Bactotryptone, 0.5% Bactoyeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was immediately added to the cuvette and then transferred to an innoculum tube which was incubated at 37°C with agitation for 1 hr. This bacterial mixture was then plated on ampicillin 100 mm plates and incubated at 37°C overnight. The plates were prepared by adding 15 g Bacto-Agar (Difco, cat. # 0140-01, Difco Labs Detroit Mich., USA), 10 g LB broth base to 1 L of $ddH_2O$ which was then autoclave sterilized. When this had cooled, 25 μg/ml ampicillin was added to the plating media, which was then poured into petri dishes (Fisher, cat.# 8-757-12, Fisher Scientific P.O. Box 9200 Terminal Ottawa, Ont., K1G 4A9) and allowed at least 2 hrs to solidify.

## 4.0 Screening of Transformants:

### 4.1 Small Scale Plasmid Preparations (Birnboim Minipreps)

Bacterial colonies that were produced from the transfections were picked with a wooden stick and used to innoculate 2 ml of LB containing 25 μg/ml ampicillin. These minicultures were grown overnight at 37°C with shaking. 1.5 ml of this culture was then transferred to an eppendorf tube and

centrifuged for 15 secs. The supernatant was aspirated off and the pellet resuspended in 100 ul of ice cold lysozyme solution (25 mM Tris-Cl pH 8.0, 50 mM glucose, 10 mM $Na_2EDTA$ and 10 mg/ml Lysozyme). After sitting on ice for 10 mins, 200 ul of Alkaline SDS (0.2 N NaOH, 1 % SDS) was added and left for 15 mins on ice. This solution was neutralized by adding 150 ul of 3 M NaOAc pH 5.2 with a further incubation of 30 min on ice. The bacterial debris was removed by a 5 min centrifugation. The supernatant was then transferred to a new tube and 1 ml of cold 95 % ethanol was added and immediately vortexed and centrifuged for 15 mins. The pellet was then redissolved in 100 µl Tris-Acetate solution (0.1M NaAc and 0.05 M Tris-HCl pH 8.0) and reprecipitated with 200 µl isopropanol. This pellet was dried and dissolved in 50 µl TE buffer and placed at 4°C for storage.

## 4.1 Agarose Gel Electrophoresis

The extracted DNA was digested with the appropriate enzyme as described previously and the fragments run out on an agarose gel. All DNA electrophoresis was performed in Horizontal gel boxes. The gel buffer for all gels was TPE (0.08 M Tris-phosphate, 0.002 M EDTA) and all gels were made with 300 ml 0.8 % agarose (Ultrapure, Cat # 5510UB). Included in the gel was 0.5 µg/ml ethidium bromide in order to visualize the bands. All electrophoresis was performed overnight (16-24 hrs) at 25 volts. The marker used was lambda DNA (BRL, Cat # 5250SA) cut with either Bst E or with Hind III.

4.2 Photographing Gels

All ethidium bromide stained gels were photographed with a Polaroid 545 land camera using Polaroid 57 film (Hall Photographic Inc., Hamilton, Ont., Canada). The gels were illuminated with U.V. light using a transilluminator. (Fotodyne, New Berlin, Wisconsin 53151, USA)

4.3 Large Scale Plasmid Preparations

The method used was essentially the one described by Maniatis et. al., 1982. Briefly, 1 L of overnight culture was spun down at 5000 rpm for 10 min in the Cryofuge 8000. The cell pellet was resuspended in 40 ml of lysozyme solution(25 mM Tris-Cl pH 8.0, 50 mM glucose, 10 mM $Na_2EDTA$ and 10 mg/ml Lysozyme) and left at room temperature for 20 mins. Next, 80 ml of Alkaline-SDS(0.2 N NaOH, 1 % SDS) was added and the mixture was incubated on ice for 5 mins. 40 ml of 3 M NaAcetate was then added and the mixture was incubated on ice for 30 min. 10 ml of $ddH_2O$ was added to the mixture before it was centrifuged for 10 min at 2,000 rpm. The supernatant was filtered through cheesecloth into 250 ml Nalgene centrifuge bottles and spun at 5,000 rpm for 10 mins. The supernatant was decanted into a centrifuge bottle containing 0.6 volumes of isopropanol and left at room temperature for 30 mins before centrifuging at 3,000 rpm for 10 mins. The resulting pellet was redissolved in 15 ml of Tris-Acetate and transferred to a centrifuge tube containing 30 ml of 95 % ethanol. After incubating for 30 mins at -20°C, the mixture was spun down for 10 mins at 3500 rpm. The pellet was then redissolved in 5 ml 0.1X SSC and 2 ml of a 50 mM Tris/10 mM EDTA solution before the addition of 8.4 g CsCl and 0.5 ml of Ethidium Bromide (10 mg/ml). This mixture was transferred to a Beckman Quick Seal

centrifuge tube and spun in an Ultracentrifuge at 55,000 rpm and 15°C overnight. The plasmid band was isolated from the tube and extracted with an equal volume of CsCl saturated n-propanol to remove the ethidium bromide. The DNA was precipitated down using 2 volumes of 95 % ethanol and redissolved in TE buffer.

## 5.0 PRODUCTION OF RECOMBINANT VIRUS

### 5.1 Isolation of Infectious Viral DNA:

Ten 150 $cm^2$ tissue culture dishes of just confluent vero cells were infected with HSV-1: FgDβ virus at an moi of 5 and incubated at 37°C for 20-24 hrs. The cells were then scraped and spun down. The cell pellet was resuspended in 5 ml of Viral Lysis Buffer (0.01 M Tris-Cl pH 7.8, 0.01 M EDTA) and placed on ice for 10 mins. An additional 5 ml of viral lysis buffer containing 0.2% Triton X-100 was added to this mixture before transferring it into a Dounce Tissue Homogenizer (Wheaton Scientific, cat. # 357544). The cell membranes were sheared using 5 strokes of the pestle. The debris was removed by centrifuging for 5 mins at 5,000 rpm. The supernatant was transferred to an Oakridge centrifuge tube and spun for 1 hr at 25,000 rpm and 4°C using a Ti50 rotor (Beckman). The resulting pellet was resuspended in 2 ml Viral DNA Extraction Buffer (0.15 M NaCl, 0.01 M Tris-Cl pH 7.5, 0.01 M EDTA). Next, 100 ul of 10% SDS was added to the suspension and left on ice for 5 mins. The mixture was then gently extracted with phenol/chloroform three times ensuring that each transfer was done with a large bore pipet to limit shearing. The resultant supernatant was dialysed against 1X TE for 2-3

days at 4°C with several changes of buffer. The DNA was stored at 4°C until ready to use.

**5.2 Transfection Procedure:**

Twenty-four hours prior to the transfection, vero cells were set up in 100 mm dishes to be 50% confluent. 10 μg of plasmid DNA, 100 μl of infectious HSV-1:FgDβ DNA, 1.0 ml of 2X Hepes buffer and $ddH_2O$ to make up a final volume of 1.9 ml was added to a microfuge tube. After gently mixing, 100 ul of 2.5 M $CaCl_2$ was added dropwise with mixing after each drop and the tube was left at room temperature for 10-15 mins. 1.0 ml of this mixture was added to the 100 mm dish containing 10 ml alpha-mem 7% NCS and 50% confluent vero cells. The dish was incubated for 4-5 hrs at 37°C and then glycerol shocked with alpha-mem 1% NCS 15% glycerol for 60 secs. The glycerol solution was removed and the cells washed with complete media. After two days, the media was replaced with 10 ml of alpha-mem 1% NCS. After incubation at 37°C for 3-5 days, well-isolated plaques were picked with a wooden stick. The wooden sticks were then touched to the media of a well in a 24 well dish that contained confluent vero cells. These plates were incubated until all cells had lifted or rounded. They were then collected and ministocks of virus were made from each well. Each virus stock was then diluted an used to infect 6-well dishes of vero cells. After incubation at 37°C for 2 days, well-isolated plaques were picked and more ministocks were made of each plaque pick.

## 5.3 Screening of Recombinant Viruses:

### 5.31 Viral DNA Extraction

60 mm dishes of vero cells were infected with 200 µl of the plaque picks and incubated overnight at 37°C. The infected cells were scraped from the dishes and spun down at 2,000 rpm for 10 mins. The viral DNA was isolated using the Hirt Viral DNA Extraction procedure. Briefly, the cell pellet was resuspended in 500 ul of TE (10 mM Tris-Cl pH 7.6, 10 mM EDTA), then lysed with 23 µl of 20% SDS for 5 mins. Cellular DNA was removed by adding 132 µl of 5M NaCl and incubating for 2 hrs on ice. After centrifuging for 10 mins, the supernatant was removed to a fresh tube to be phenol/chloroform extracted. The viral DNA was precipitated down with 95% ethanol, washed with 70% ethanol and dried. The pellet was then resuspended in 50 ul of TE buffer and placed at 4°C for storage. Each pellet was digested with the appropriate enzyme and the fragments separated on an agarose gel. The DNA was then transferred to Gene Screen Plus (Dupont, cat. # NEF-976) for Southern blotting.

### 5.32 Southern Blotting

After electrophoresis the gel was denatured for 30 mins in a solution of 4 M NaOH and 5 M NaCl, then neutralized for 30 mins in 5 M NaCl and 2 M Tris pH 7.5. The DNA in the gel was then transferred to Gene Screen Plus (Dupont, cat. # NEF-976) in the following manner. A sheet of Whatman filter paper soaked with 10X SSC was draped over a plexiglass support contained within a tray of 10X SSC. The gel was placed on top of this

Whatman paper. The Gene Screen Plus membrane presoaked in 10X SSC was then placed on the gel followed by 6 sheets of Whatman paper soaked in the same 10X SSC solution. Ten sheets of Quick Draw Blotting Paper (Bio Rad, cat. #P8171) was then placed on top followed by a weight. This was allowed to sit at room temperature for 4 hrs. Following this incubation the membrane was immersed in excess 0.4 N NaOH for 30-60 sec to completely denature the immobilized DNA. The membrane was then immersed in an excess of 0.2 N Tris-HCl pH 7.5 and 2X SSC. This membrane was allowed to dry before probing.

5.33 Nick Translation Labelling of DNA

The radioactive labelling of DNA was performed using the BRL standard nick translation kit (BRL, cat. # 8160SB) and under the recommended reaction conditions. The label used was $^{32}P$ labelled dCTP purchased from Amersham (cat. # PB10205). The reaction involved the use of 50 μCi of isotope to label 1 μg of plasmid DNA. This was incubated at 15°C for 60 mins. The reaction mix was passed through a spin column of Sephadex G-50 beads (Pharmacia, cat. # 170043 01) and the eluent was stored on ice until needed.

5.34 Probing the Blot

The membranes were prehybridized for at least 2 hrs in plastic heat sealed bags at 68°C in 20 ml of the following solution, 6X SSC, 0.5% SDS, 5X Denhardt's solution and 100 μg/ml denatured, sonicated salmon sperm DNA (Sigma, cat. #D9156). The radioactive probe was then boiled for 10 mins and

added to the plastic bag containing the membrane and hybridization fluid (6X SSC, 0.01 M EDTA, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured, sonicated salmon sperm DNA). This was then incubated at 68°C overnight. After this time period the membrane was removed from the bag and washed three times. The first wash was 2X SSC and 0.5% SDS at room temperature for 5 mins. The second wash was 2X SSC and 0.1% SDS at room temperature for 15 mins. The third was was 0.1X SSC and 0.5% SDS for 30 mins at 37°C and 30 more mins at 68°C. The blot was then dried at room temperature on Whatman paper, wrapped in Saran Wrap and exposed to XAR-5 film (Kodak, Xomat AR Film, Eastman Kodak Company, Rochester N.Y., 14650). The length of exposure varied with the amount of activity on the blot.

## 6.0 IMMUNOPRECIPITATION

### 6.1 Antibodies:

**Rabbit Anti-T4 Endonuclease V Polyclonal antibody** (cat. # 550-ABD) was acquired from Applied Genetics Inc. Antibodies were raised in New Zealand white rabbits against the purified native and active T4 endonuclease v(cat. # 350-DEN). The polyclonal antibodies were prepared by ammonium sulfate precipitation and chromatography on DEAE-Blue. The protein was freeze dried and was stored refrigerated.

### 6.2 Labelling and Immunoprecipitation Procedure:

Vero cells grown to confluency in 6-well tissue culture dishes were infected at an moi of 10 pfu per cell. At various times post infection (2-8 hrs.),

the overlay media was aspirated off and the infected cells were washed three times with 2 ml 199-Met media (containing 1 % NCS and 1% Hepes pH 7.4 but lacking methionine). Then 0.5 ml 199-Met containing 50 μCi $^{35}$S-Met (Amershan, cat. # SJ1515) and 1% NCS was added to the monolayers and the infection was allowed to proceed for varying lengths of time (4-8 hrs.). The proteins were then extracted as follows. The media was aspirated off the cell sheets and 2 ml of ice cold NP40/DOC (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.5% Sodium deoxycholate and 1% NP40) plus inhibitors (1 mg/ml BSA and 1 mM PMSF) was added to each well. The solution was pipetted up and down to ensure that all the cells had been lysed and washed off the bottom. The resultant lysates were transferred to Oakridge tubes and centrifuged at 25,000 rpm and 4°C for 1 hr. In an eppendorf tube, 500 ul of this lysate was combined with 10 ul of anti-sera and kept on ice for 90 mins. At the end of this incubation 100 ul of Protein A/Sepharose beads (0.5 g beads swelled in 20 ml NP40/DOC for 2 hrs. at 4°C) were added and the mixture was mixed end-over-end for 90 mins at 4°C. The tubes were then spun for 1 min and the supernatant removed. The beads were washed by adding 1 ml NP40/DOC buffer 3 times. After the last wash the beads were resuspended in 100 ul 2X Protein gel loading buffer (100 mM Tris pH 6.8, 20 % glycerol, 4% SDS, 0.25% Bromophenol blue and 2% Beta Mercaptoethanol). The tubes were boiled for 10 min, chilled on ice for 5 mins and finally centrifuged for for 2 mins. The supernatant was collected to be run on a gel.

**6.3 SDS Polyacrylamide Gel Electrophoresis:**

All protein samples were run on a 15 % SDS polyacrylaminde gels (15 ml of acrylamide/DATD 30:1.2, 7.5 ml of 1.5 M Tris-Cl pH 8.8, 9.7 ml dd$H_2O$,

degassed for 10 min. then add 150 µl 20% SDS, 200 µl 10% ammonium persulfate and 10 µl temed) with a 5% stacking gel (3.34 ml acrylamind/datd, 5.0 ml 0.5 M Tris-Cl pH 6.8, 11.46 ml dd$H_2O$, degassed for 5 min. then add 100 ul 20% SDS, 200 ul 10% ammonium persulfate and 20 µl temed). The vertical gels were run overnight at 40 volts in Lameli buffer (0.25 M Tris, 1.92 M Glycine and 1% SDS for a 10X stock). The protein markers used was the $^{14}C$-labelled Rainbow markers purchased from Amersham (cat. # RPN.756). 5 µl of marker was mixed with sample buffer before loading in the well.

### 6.4 Fixing and Drying:

After electrophoresis the gel was dimethyl sulfoxide (DMSO) - polyphenoloxide (PPO) treated to enhance the radioactive signal. First the gel was soaked in DMSO for 30 mins., then transferred into fresh DMSO for another 30 mins. The solution was then changed to one containing 22% PPO in DMSO for 1 hr. The gel was then first rinsed in distilled water and then soaked in distilled water for 15 mins. After this time the gel was dried for 2 hrs. at 60°C and then exposed to fast film.

## 7.0 SURVIVAL ASSAYS

### 7.1 Preparation of Cells:

As described earlier cells were grown to confluence in 150 $cm^2$ dishes before being collected and plated into 24-well dishes. One dish of cells were resuspended in 24 ml of media and seeded into two linbro plates by adding 0.5 ml of the cell suspension into each well. The plates were then incubated at 37°C to reach confluency either 24 or 48 hrs. later.

**7.2 Irradiation of Virus:**

UV irradiation of virus was performed using a germicidal lamp (General Electric Germicidal Lamp G8T5) emitting a wavelength of predominantly 254 nm. The method employed was essentially the same as that described previously (Ryan and Rainbow, 1986). Stock virus was diluted 10 to 100 fold with cold alpha-MEM without serum, and an aliquot of virus suspension no greater than 1.6 ml was irradiated in a 35 mm diameter petri dish (Falcon Plastics) with the dish cover removed, kept on ice, with constant swirling during the irradiation. Under these conditions the incident dose rate was about 2 $J/m^2/sec$ as determined using a J-225 shortwave UV meter (Ultraviolet Products, San Gabriel, CA.)

**7.3 Plaque Assay Procedure:**

The method employed was similar to that described previously (Ryan and Rainbow, 1986). Cells were seeded into 24-well plastic Linbro tissue cultrue plates (Flow Laboratories Inc., Hamden, CT) with 0.5 ml of growth medium per well and incubated at 37°C in a $CO_2$ incubator. When the monolayers were confluent, the growth medium was aspirated off the cells. Each well was then inoculated with 0.2 ml of an appropriate dilution of the virus in alpha-MEM without serum to produce between 1 and 50 plaques per well. After a 90 min. adsorption period, the cells were overlayed with 0.5 ml of alpha-MEM containing 1% NCS and 0.1% Human Immune Serum Globulin. Plaques were scored after 3 days. The growth medium was aspirated off the cells, and the monolayers were simultaneously fixed and stained with crystal violet solution. Plaques were scored in duplicate at the

three serial dilutions and the data points fitted to a straight line through the origin using least-squares analysis. Taking into account the dilution factor, the slope of the line was used to determine the plaque titer.

## 8.0 MATHEMATICAL ANALYSIS OF THE DATA

### 8.1 Surviving Fractions

In each experiment, monolayers of the appropriate cells were infected with three two-fold serial dilutions of virus in duplicate plaques were stained and counted three days post infection. Plaque counts were plotted as a function of virus concentration and the data points fitted to a straight line through the origin using least squares analysis. Taking into account the dilution factor, the slope of the line was used to determine the plaque titre. The surviving fraction at each dose point was calculated by dividing the titre of the uv irradiated virus by the titre of the unirradiated control virus. The data from two or more experiments for the uv survival of a given viral strain, after infection of a given cell type, was pooled. The log of the surviving fraction values for the same uv doses in the different experiments were averaged to produce a single logarithmic mean value and a standard deviation of the mean. These logarithmic mean values were fitted to two straight lines by linear regression analysis. The turning point of the survival curve was determined by using the points which yielded the highest correlation coefficients in this analysis.

## 8.2 $D_0$ Values

$D_0$ values for the second component of the uv survival curves were calculated and used as a measure of virus survival in a specific cell type. The $D_0$ value was calculated by pooling the individual survival points for all experiments involving one virus on one cell type. Using linear regression analysis a line was fitted through these pooled values and a slope value obtained. $D_0$ values were calculated using this slope value and the error expressed in terms of a 90% confidence interval. A comparison of the $D_0$ values between normal and repair deficient cells provides an indication of the DNA repair capacity of the infected cell. The percent host cell reactivation (%HCR) is the $D_0$ for the uv survival curve of the virus in the cells of interest expressed as a percentage of the $D_0$ value obtained in normal repair proficient cells.

# RESULTS

## 1.1 CONSTRUCTION OF AN HSV-1 RECOMBINANT VIRUS ENCODING THE DEN V GENE OF BACTERIOPHAGE T4

### 1.1 The Den V Expression Cartridge

The den V eukaryotic expression cartridge was constructed by Valerie *et al.* (1987). It consists of 55 bps from pBR322 followed by the 3' LTR of RSV. The transcription start site is at position 545 with the tata box located at position 510. The coding sequence for the den V gene begins at position 591 and ends with the TAA opal stop located at position 1060. The SV40 small t antigen intron and the large T antigen polyadenylation splice site follows this translation termination. (figure 2)

### 1.2 Cloning Strategy

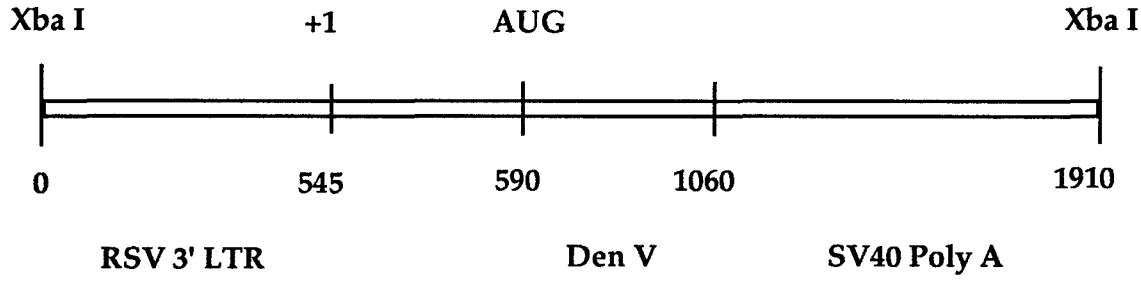
The strategy used in cloning the den V gene into HSV-1 is outlined in figures 3 and 4. The den V gene was first inserted into the HSV-1 gI gene that was contained within a plasmid. This plasmid was then transfected into cells along with infectious HSV-1 FgDβ DNA. It was through site specific recombination that the den v cartridge became integrated into the viral genome.

### 1.3 Plasmid Construction

The den V expression cartridge was first isolated from the plasmid

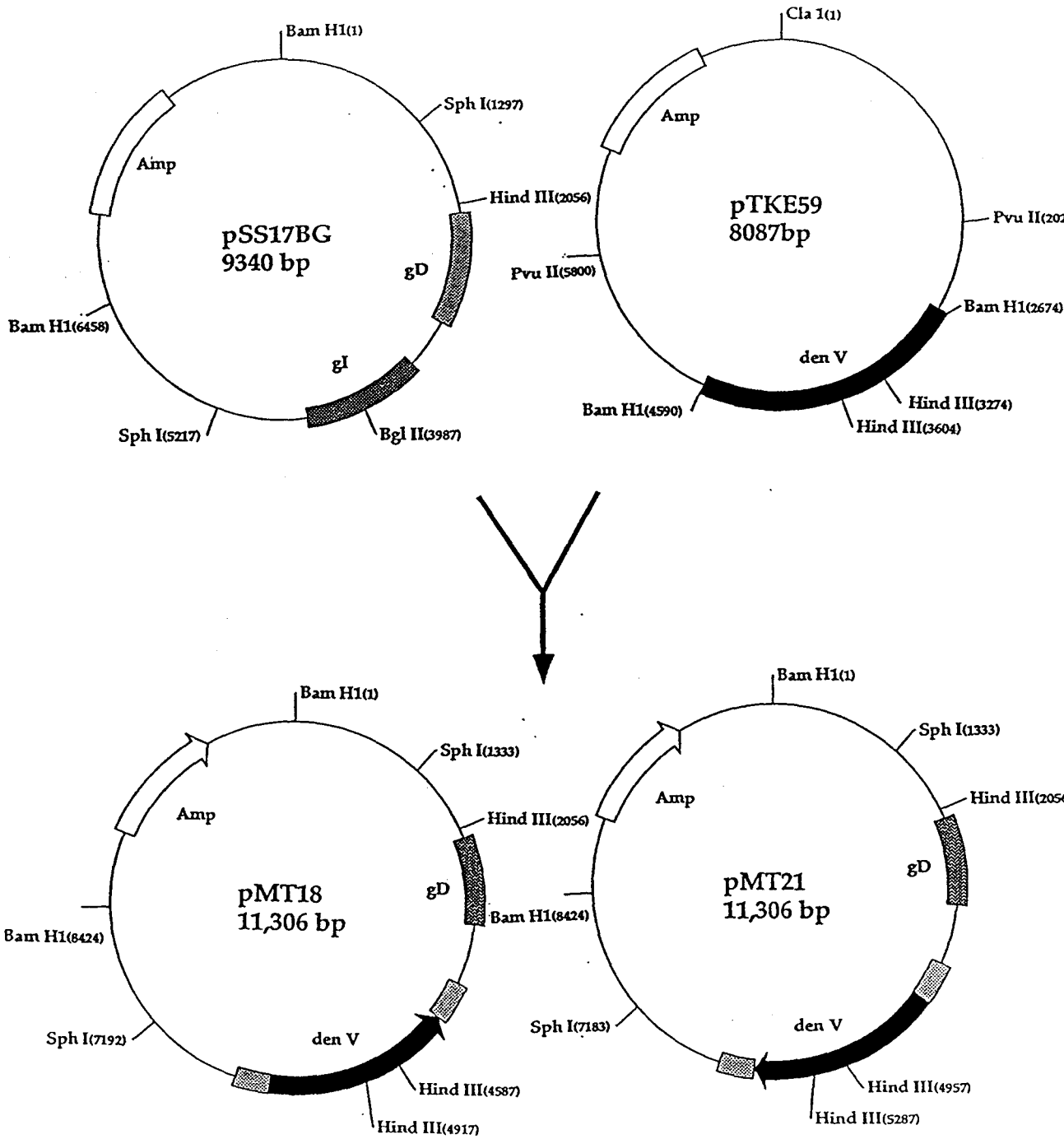
## Figure 2: The Den V Expression Cartridge

This expression cartridge was constructed by Valerie et al. (1987). It consists of den V coding sequences that run from 590 to 1060 and is flanked by a 3' LTR of Rous Sarcoma Virus and SV40 polyadenylation splice site.


Xba I
+1
AUG
Xba I
0
545
590
1060
1910
RSV 3' LTR
Den V
SV40 Poly A

## Figure 3: Cloning Strategy

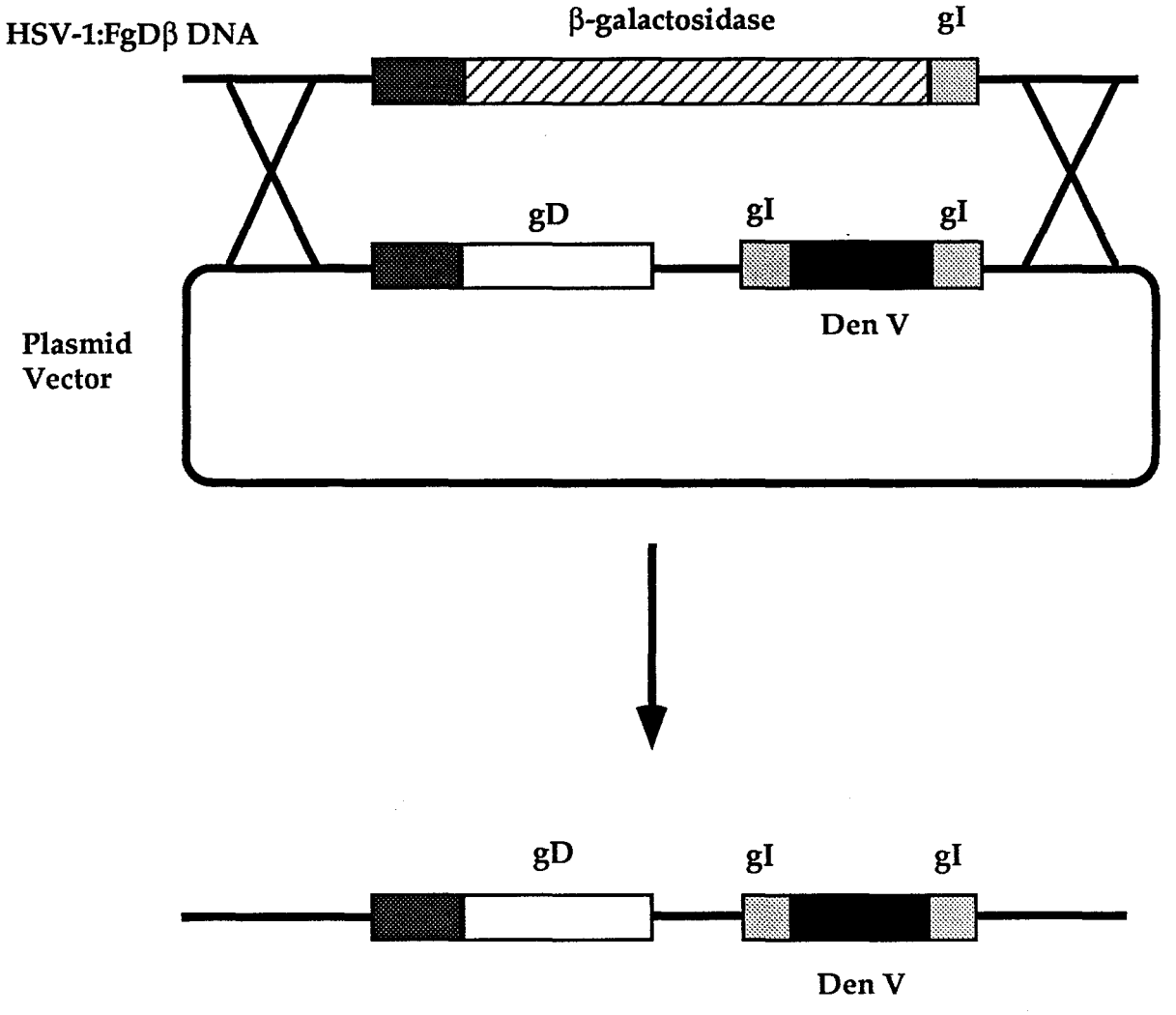
Plasmid pTKE59 was cut with Bam H1 to release the den V expression cartridge. This 1.9 kb fragment was gel isolated and ligated to the Bgl II site of pSS17BG. Two possible orientations of insertion of the expression cartridge resulted in plasmid pMT18 and pMT21.


Bam H1(1)
Sph I(1297)
Amp
Hind III(2056)
pSS17BG
9340 bp
gD
Bam H1(6458)
gI
Sph I(5217)
Bgl II(3987)
Cla 1(1)
Amp
pTKE59
8087bp
Pvu II(2024)
Pvu II(5800)
Bam H1(2674)
den V
Bam H1(4590)
Hind III(3274)
Hind III(3604)
Bam H1(1)
Sph I(1333)
Amp
Hind III(2056)
pMT18
11,306 bp
gD
Bam H1(8424)
Sph I(7192)
den V
Hind III(4587)
Hind III(4917)
Bam H1(1)
Sph I(1333)
Amp
Hind III(2056)
pMT21
11,306 bp
gD
Bam H1(8424)
Sph I(7183)
den V
Hind III(4957)
Hind III(5287)

## Figure 4: Homologous Recombination

The plasmid vector containing the den V cassette was cotransfected into Vero cells with HSV-1:FgDβ DNA using the calcium chloride method (Graham & Van der Eb, 1973). Homologous recombination between the DNA and the plasmid produced the recombinant HSV-1:den V virus.

# HOMOLOGOUS RECOMBINATION




HSV-1: Den V Virus

pTKE59 by digesting with Bam H1. This fragment was gel isolated by electroelution and ligated to the Bgl II site of the plasmid pSS17BG. Figure 5 (upper panel) shows the results of this cloning step. PSS17BG digested with Bam H1 produces two fragments of approximately 6,600 bp and 2,800 bp (lane 4). Lanes 1 and 2 shows the two plasmids containing the den V insert. The presence of the den V gene should increase the size of the larger fragment by 1.9 kb. As expected, a Bam H1 digest of the new plasmid produced two fragments of approximately 8,500 bp and 2,800 bp.

The orientation of the cartridge within the plasmid was determined by a Hind III digest (lower panel). Fragment sizes of approximately 6,400 bp, 2,600 bp and 2,000 (lane 2) indicate that the den V cartridge was inserted in the opposite orientation with respect to the existing pSS17BG genes. This plasmid was named pMT18. A plasmid containing the cartridge in the other orientation was also isolated and named pMT21. A Hind III digest of this plasmid produced fragment sizes of approximately 6,000 bp, 3,000 bp and 2,000 bp (lane 1).

### 1.4 Transfection and Screening of Recombinants

The plasmid pMT21 was cotransfected with HSV-1:FgDβ DNA into vero cells using the calcium chloride method of Graham & Van Der Eb, 1973. In vivo homologous recombination between the DNA resulted in the formation of HSV-1:den V. Plaques were isolated from the transfection plates and plaque purified on vero cells. Ministocks of the plaque picks were made in 24 well dishes and screened for the presence of the den V insertion (figure 6).

## Figure 5: Restriction Digest Analysis of Plasmids

Large scale plasmid DNA was digested with Bam H1 or Hind III for 2 hrs. at 37°C before being electrophoresed in a 0.8% agarose gel overnight. This ethidium bromide stained gel was then photographed.

**Upper Panel: Identifying the Presence of the Den V Cartridge**

Marker lane: Lambda DNA cut with Hind III to give fragment sizes of 23,130 bp, 9,416 bp, 6,682 bp, 4,361 bp, 2,322 bp and 2,027 bp. Fragment sizes 564 bp and 125 bp are not indicated.

pMT21 lane: A Bam H1 digest gives fragments of 8,500bp and 2,800 bp.

pMT18 lane: A Bam H1 digest gives fragments of 8,500bp and 2,800 bp.

pSS17BG lane: A Bam H1 digest gives fragments of 6,600 bp and 2,800 bp.

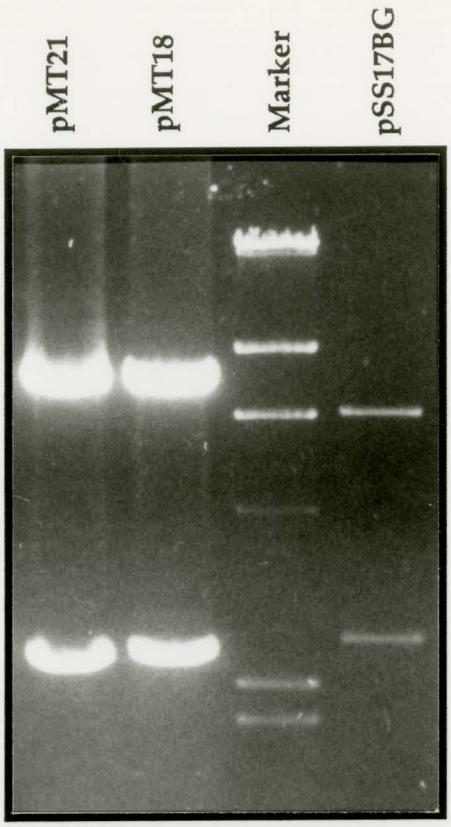
**Lower Panel: Identifying the Orientation of the Den V Cartridge**

Marker lane: Lambda DNA cut with Hind III to give fragment sizes of 23,130 bp, 9,416 bp, 6,682 bp, 4,361 bp, 2,322 bp and 2,027 bp. Fragment sizes 564 bp and 125 bp are not indicated.

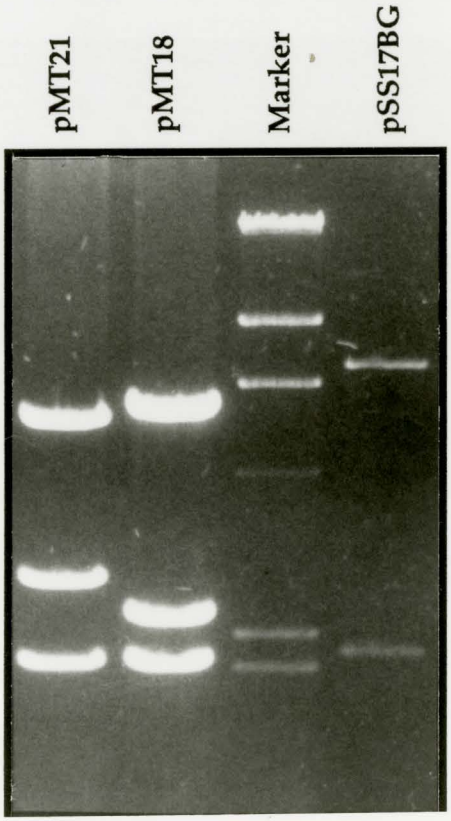
pMT21 lane: A Hind III digest gives fragment sizes of 6,000 bp, 3,000 bp and 2,000 bp.

pMT18 lane: A Hind III digest gives fragment sizes of 6,400 bp, 2,600 bp and 2,000 bp.

pSS17BG lane: A Hind III digest gives fragment sizes of 7,200 bp and 2,100 bp.



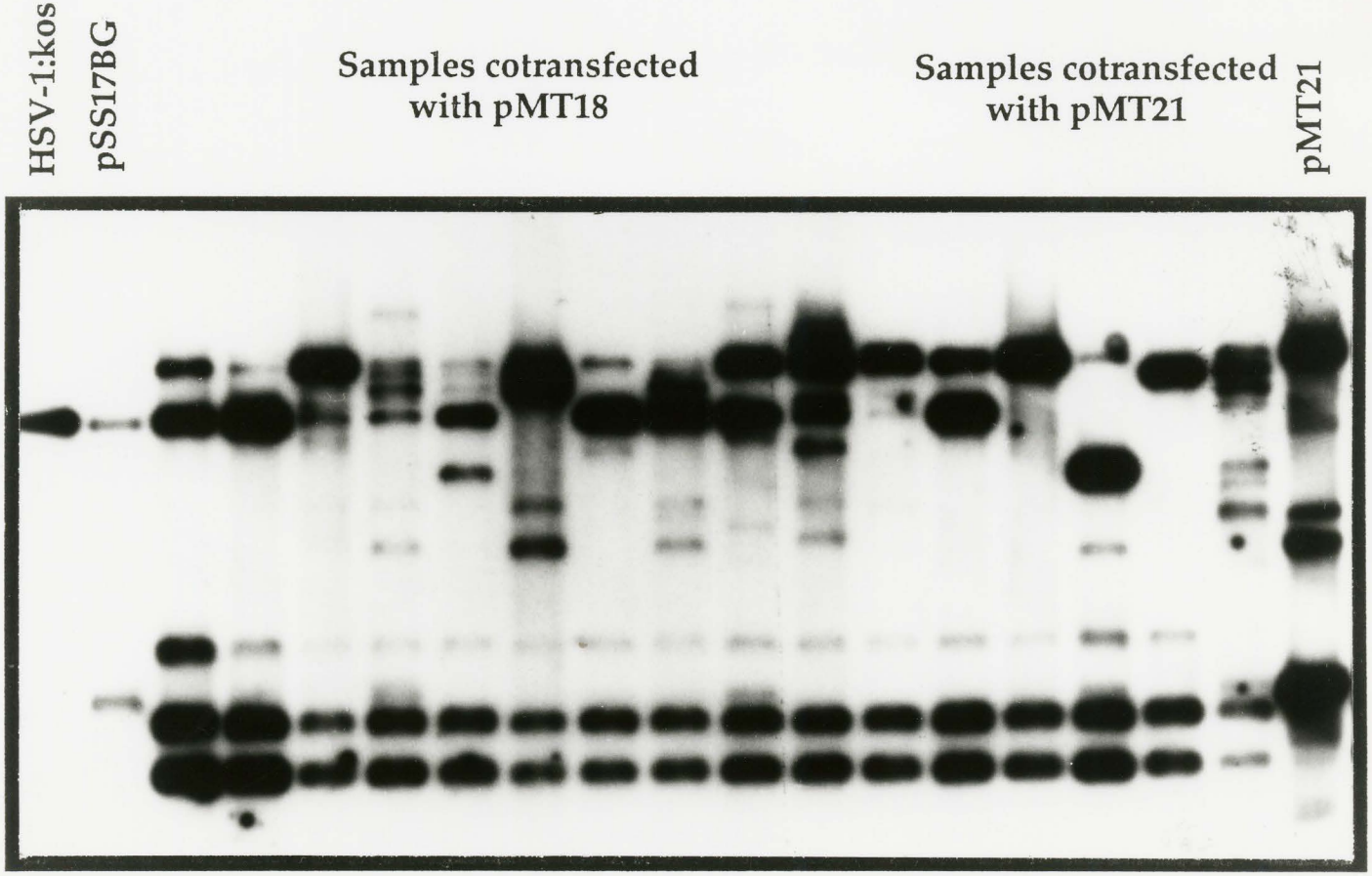

Bam H1
Digestion




Hind III
Digestion

## Figure 6: Initial Screening of Transfection Stocks for Recombinant Viruses.

Screening was performed by extracting viral DNA, digesting it with Bam H1 and electrophoresing the products on a 0.8% agarose gel. The gel was blotted onto Gene Screen Plus and probed with nick translated pSS17BG. This figure represents the autoradiogram of the southern blot. Since each sample contained a mixture of viruses, a number of them were further plaque purified to isolate a sample containing only the recombinant virus.




PSS17BG PROBE

Screening was performed by extracting viral DNA from each infected culture, digesting it with Bam H1 and eletrophoresing the products on a 0.8% agarose gel. The gel was blotted onto Gene Screen Plus and probed with nick translated pSS17BG or the 1.9 kb den V expression cartridge (figure 7). Since the pSS17BG plasmid was derived from the Bam H1 'J' fragment of HSV-1, it hybridizes to only one band in the wildtype HSV-1:kos digest (lane 1). Since pSS17BG consists of two Bam H1 fragments, the probe hybridizes to itself showing two bands (lane 2). Similarly, pMT21 contains two Bam H1 fragments, one of these is longer due to the 1.9 kb den V insert, and therefore migrates more slowly as compared to wildtype (lane 4). Upon incorporation of the den V gene into the HSV-1 genome, the Bam H1 'J' fragment of the recombinant becomes larger and co-migrates with the pMT21 fragment and not the wildtype fragment.

To ensure that this slower migrating band actually does contain sequences specific to the den v cartridge, an identical blot was probed with the den v expression cartridge (figure 7, right panel). As expected, one band is present in the pMT21 and HSV-1: den V lanes and none is present in the other lanes.

**1.5 Expression of the Den V Protein by HSV-1:den V**

Monolayers of vero cells were left uninfected, or were infected with HSV-1:kos or recombinant HSV-1:den V and then subsequently labelled with $^{35}$S-Methionine for 2-6 hrs. after infection. At 6 hrs. after infection, the cell extracts were mixed with rabbit anti-endonuclease V antibody. The immunoprecipitated proteins were eluted and electrophoresed on SDS-PAGE gels and subjected to autoradiography. The autoradiogram of the

## Figure 7: Verification of the Presence of the Den V cartridge in the Recombinant Virus.

Viral DNA was isolated from recombinant virus infected Vero cells, digested with Bam H1 and electrophoresed in a 0.8% agarose gel. This gel was southern blotted and probed with either nick translated pSS17BG (right panel) or the 1.9 kb den V expression cartridge (left panel).

**Left Panel:** Autoradiogram of Southern Blot probed with the pSS17BG plasmid

**Right Panel:** Autoradiogram of Southern Blot probed with the Den V cartridge.

pSS17BG probe
Den V probe
HSV-1:kos
pSS17BG
HSV-1:den V
pMT21
HSV-1:FgDβ
HSV-1:kos
pSS17BG
HSV-1:den V
pMT21
HSV-1:FgDβ

## Figure 8: Immunoprecipitation of Den V protein from HSV-1:den V infected cells

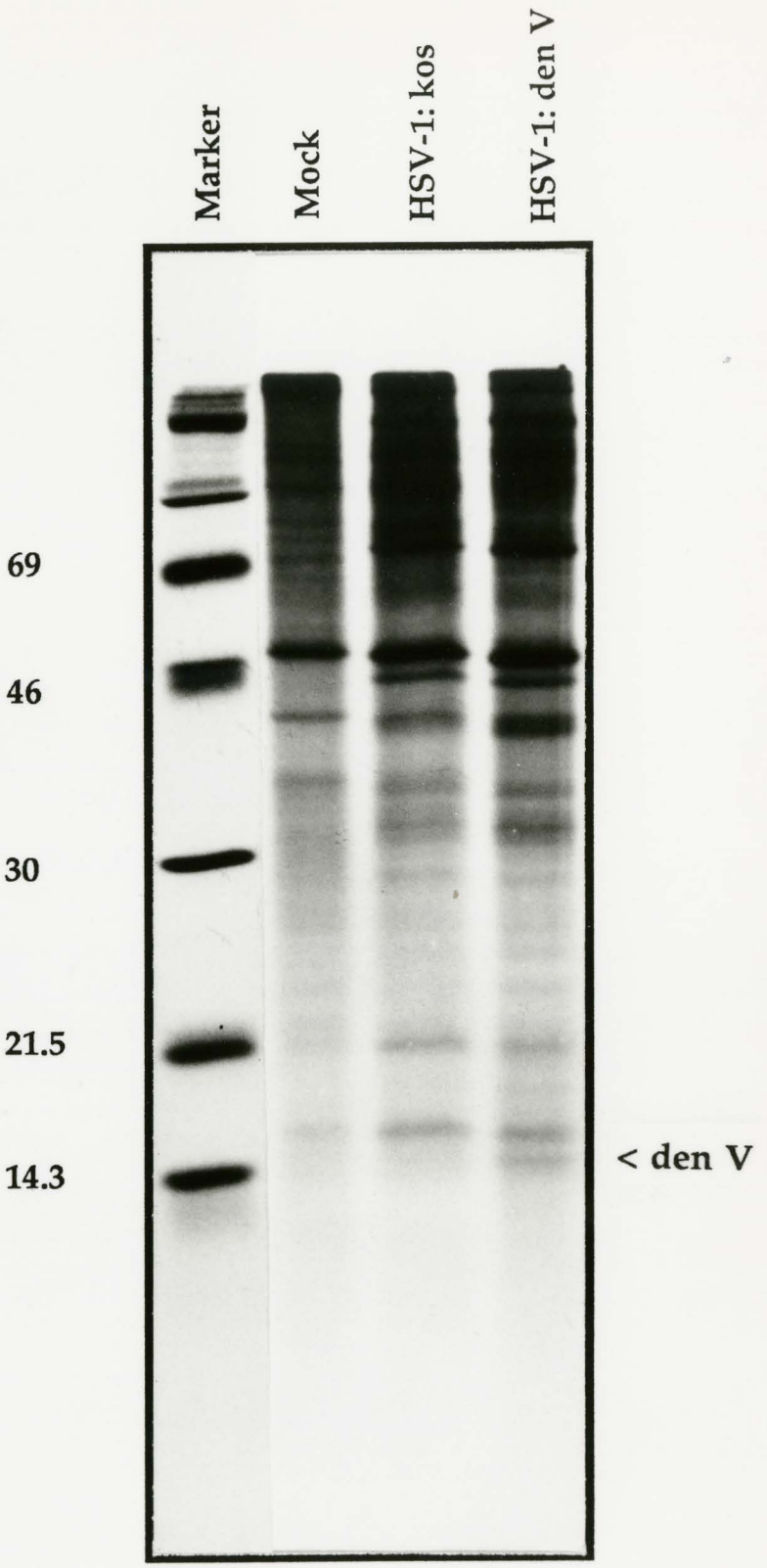
Monolayers of Vero cells were infected with the appropriate virus and subsequently labelled with $^{35}$S-Methionine for 6 hrs. Cell extracts were mixed with rabbit anti endonuclease V antibody. The immunoprecipitated proteins were eluted and electrophoresed on SDS PAGE gels and subjected to autoradiography.

**Lane 1:** Rainbow Marker purchased from Amersham with sizes of 69,000 dal, 46,000 dal, 30,000 dal, 21,500 dal and 14,300 dal.

**Lane 2:** Mock infected cells

**Lane 3:** Wildtype (HSV-1:kos) infected cells

**Lane 4:** Recombinant (HSV-1:den V) infected cells


Marker
Mock
HSV-1: kos
HSV-1: den V
69
46
30
21.5
14.3
< den V

immunoprecipitated proteins is illustrated in figure 8. A band of 16 kd (the size of the den V protein) is seen in HSV-1:den V infected cell extracts (lane 3), but is absent in the mock infected cell extracts (lane 1) and the wildtype infected cell extracts (lane 2).

## 2.0 EFFECT OF THE VIRAL ENCODED DEN V GENE ON UV SURVIVAL OF THE VIRUS IN MAMMALIAN CELLS

UV survival of the plaque forming ability of virus was used to determine if the virally encoded den V gene could increase the survival of uv irradiated recombinant virus in various excision repair deficient cell types. In this way one could determine the suitability of HSV-1 as a vector for studying the expression of DNA repair genes. The uv survival of the recombinant virus as compared to wildtype was examined in repair proficient and repair deficient cells. These experiments were performed by seeding cells into 24-well linbro dishes 24 or 48 hrs. prior to virus infection. Virus suspensions were irradiated with three doses, and then subjected to three two fold dilutions before being plated out in duplicate. An unirradiated control sample was also plated out in duplicate after three two fold dilutions. Plaques were scored after 3 days at the three serial dilutions and the data points fitted to a straight line through the origin using least squares analysis. Surviving fractions of the virus at each dose point were determined by dividing the titre of the irradiated virus by the titre of the unirradiated control.

A plot of the log of the surviving fraction against the dose to the virus produces a straight line for a 1-component system or two straight lines for viruses exhibiting a 2-component survival curve. By using the slope of the straight line the $D_0$ value can be determined. $D_0$ is the dose required to

reduce the level of survival of any point along the straight line to 0.37 of that level. HCR could then be determined by dividing the $D_0$ value for the virus in the cell of interest by the $D_0$ value for the virus in normal cells.

As mentioned earlier, the T4 encoded den V gene produces a greater T-phage survival after uv irradiation in excision deficient bacteria because of its ability to complement the repair deficiency. Previous studies including microneedle injection and DNA mediated transfection of den V into XP group A cells have demonstrated a partial restoration of DNA repair capacity as measured by cell survival post uv irradiation. XP cells have been shown to have a reduced ability to reactivate uv damaged HSV-1 (Bueschleb, 1987; Lytle, 1971) and HSV-2 (Ryan & Rainbow, 1986). This quantitative measure of the host cells ability to reactivate damaged virus provides an indication of the cell's DNA repair capacity. The survival of uv irradiated HSV-1: den V as compared to the control wildtype virus was determined in a number of human fibroblast cell lines in order to investigate the ability of this gene to enhance the cellular DNA repair capacity.

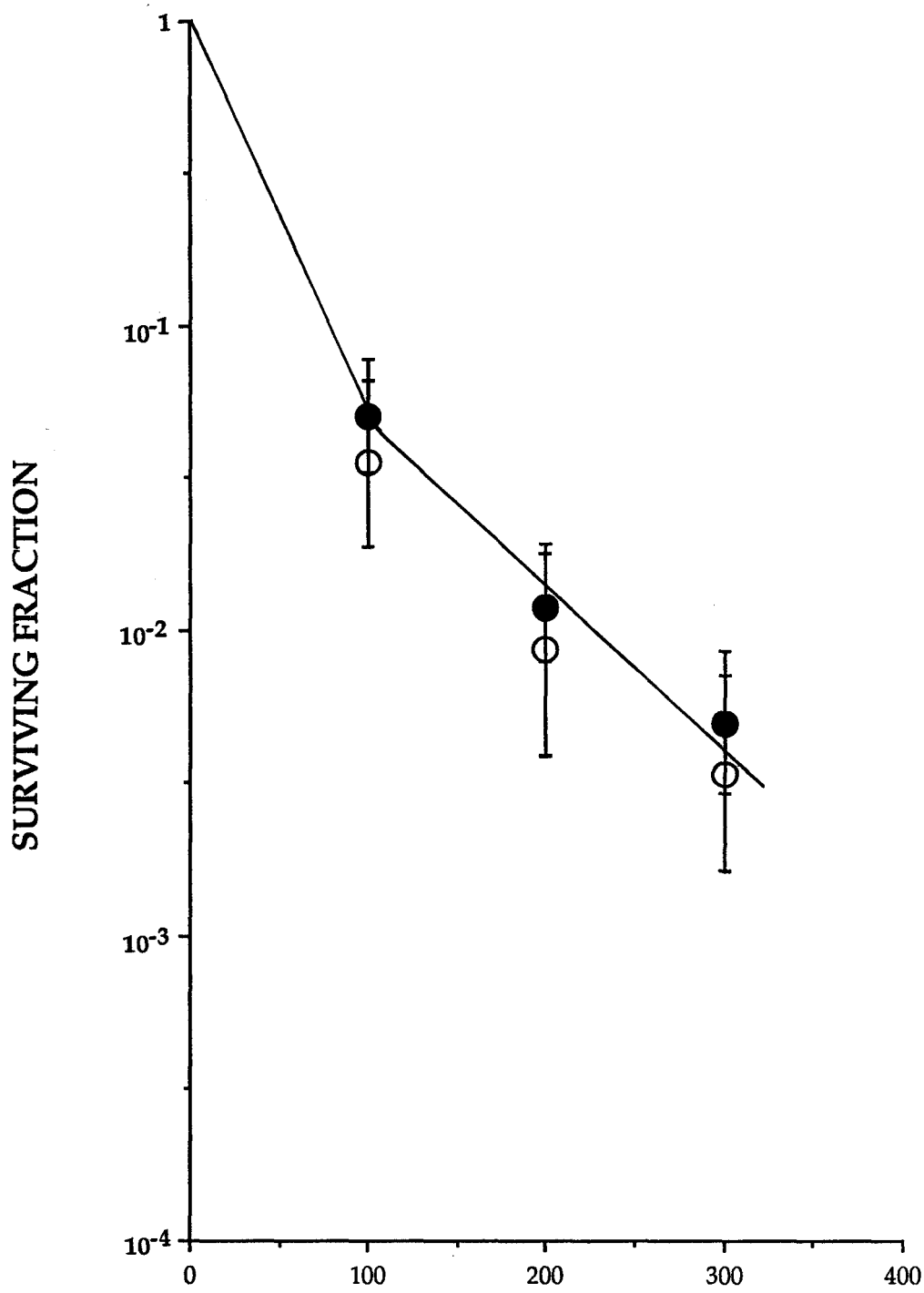
**2.1 UV virus survival in 293 and XP-4 cells**

The plaque survival of uv-irradiated HSV-1:kos and HSV:den V in 293 and XP-4 cells are shown in figures 9 and 10. The survival curves display a two-component nature, as has been reported previously (Ryan and Rainbow, 1986; Lytle *et al.*, 1972, 1982; Hall *et al.*, 1980). As previously described, each graph depicts the survival curves for the logarithmic mean value at each dose point for HSV-1:kos and HSV-1:den V in 293 cells (normal) and XP-4 cells. The $D_0$ values for HSV-1:kos and HSV-1:den V in 293 cells are $85.34 \pm 28.41$ $J/m^2$ and $84.54 \pm 27.58$ $J/m^2$ respectively. These values reflect the second

**Figure 9: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V viruses in 293 cells.**

This figure illustrates the pooled results of three individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

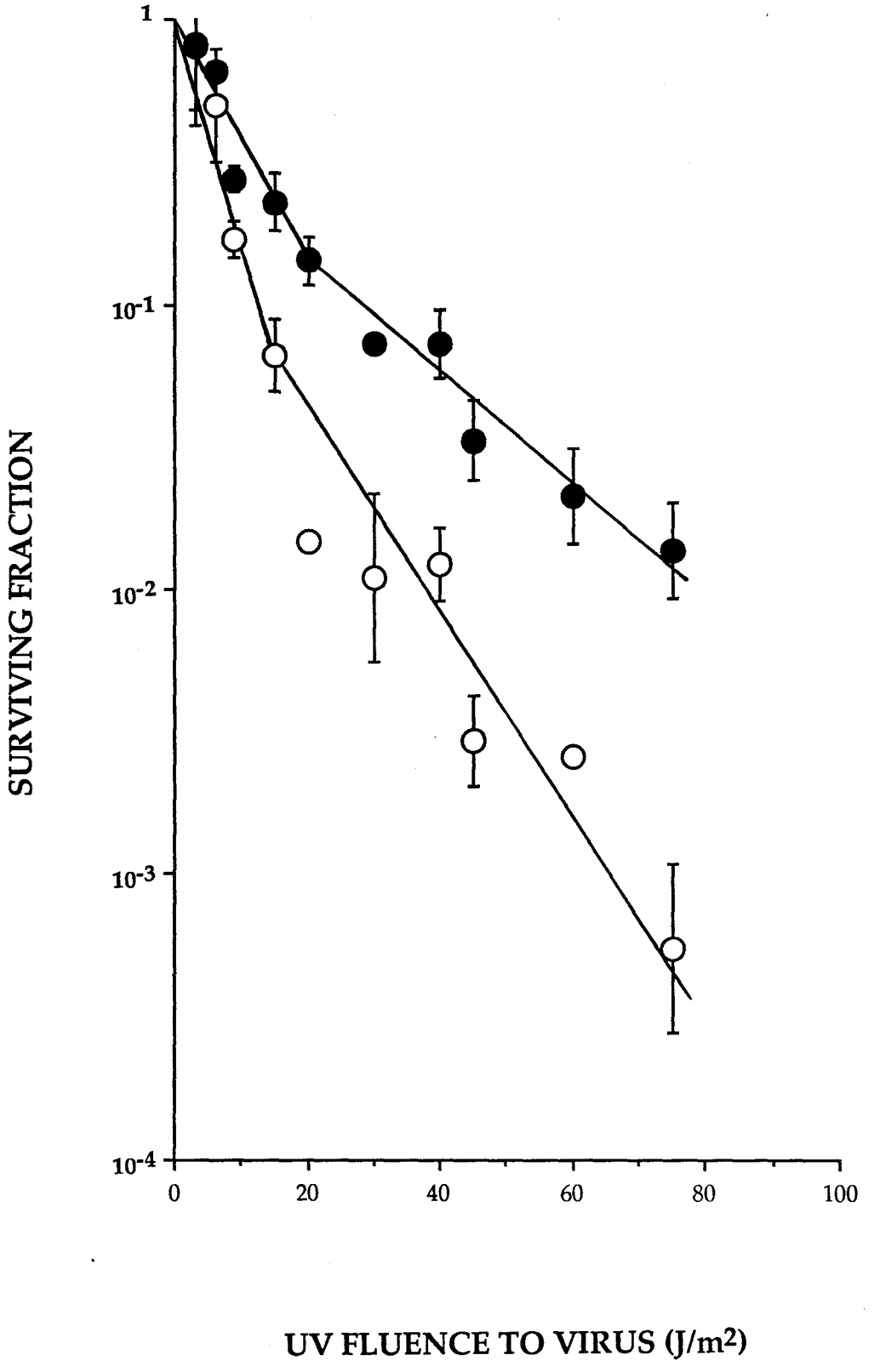
# 293 CELLS


SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
400
UV FLUENCE TO VIRUS ($J/m^2$)

## Figure 10: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V viruses in XP-4 cells

This figure illustrates the pooled results of five individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# XP-4 CELLS


SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
20
40
60
80
100
UV FLUENCE TO VIRUS (J/m$^2$)

component of the survival curve and are reported with a 90% confidence interval as are all the $D_0$ values quoted in this study. These results indicate that the uv survival of both types of viruses is the same in 293 cells.

The $D_0$ values for HSV-1:kos and HSV-1:den V in XP-4 cells are 13.57 $\pm$ 3.39 J/m$^2$ and 22.86 $\pm$ 4.80 J/m$^2$ respectively. Figure 10 compares the survival of HSV-1:kos and HSV-1:den V in repair deficient XP-4 cells. It is apparent that both components of the curve show increased survival with the presence of the den V gene in the viral genome. This suggests that the den V protein can partially complement the DNA repair deficiency in these cells. An examination of the %HCR values from Table 2 indicate that there is no significant difference between the values of 15.9 $\pm$ 9.3% and 27.0 $\pm$ 14.3%. Initial HCR studies were conducted with 293 and XP-4 cells because of the speed at which they doubled, their plating efficiency and the ease with which their plaques could be scored. The majority of studies however, involved the use of primary fibroblasts.

## 2.2 UV virus survival in Normal Fibroblasts

HSV-1:kos and HSV-1:den V were assayed for their uv survival in normal repair proficient fibroblast. The logarithmic mean values at each dose point were used to plot survival for the two viruses. Figure 11 shows the pooled results of four separate experiments. The $D_0$ values of the two viruses HSV-1:kos and HSV-1:den V are 43.43 $\pm$ 6.24 J/m$^2$ and 47.82 $\pm$ 5.65 J/m$^2$ respectively. These results show that there is no difference between the HSV-1:kos and HSV-1:den V virus survivals in normal cells as measured by plaque forming ability. This indicates that there is no intrinsic enhanced survival of the HSV-1:den V virus in normal fibroblasts.

**Figure 11: Survival of plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V viruses in GM37D (Normal) cells.**

This figure illustrates the pooled results of four individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM37D (NORMAL)

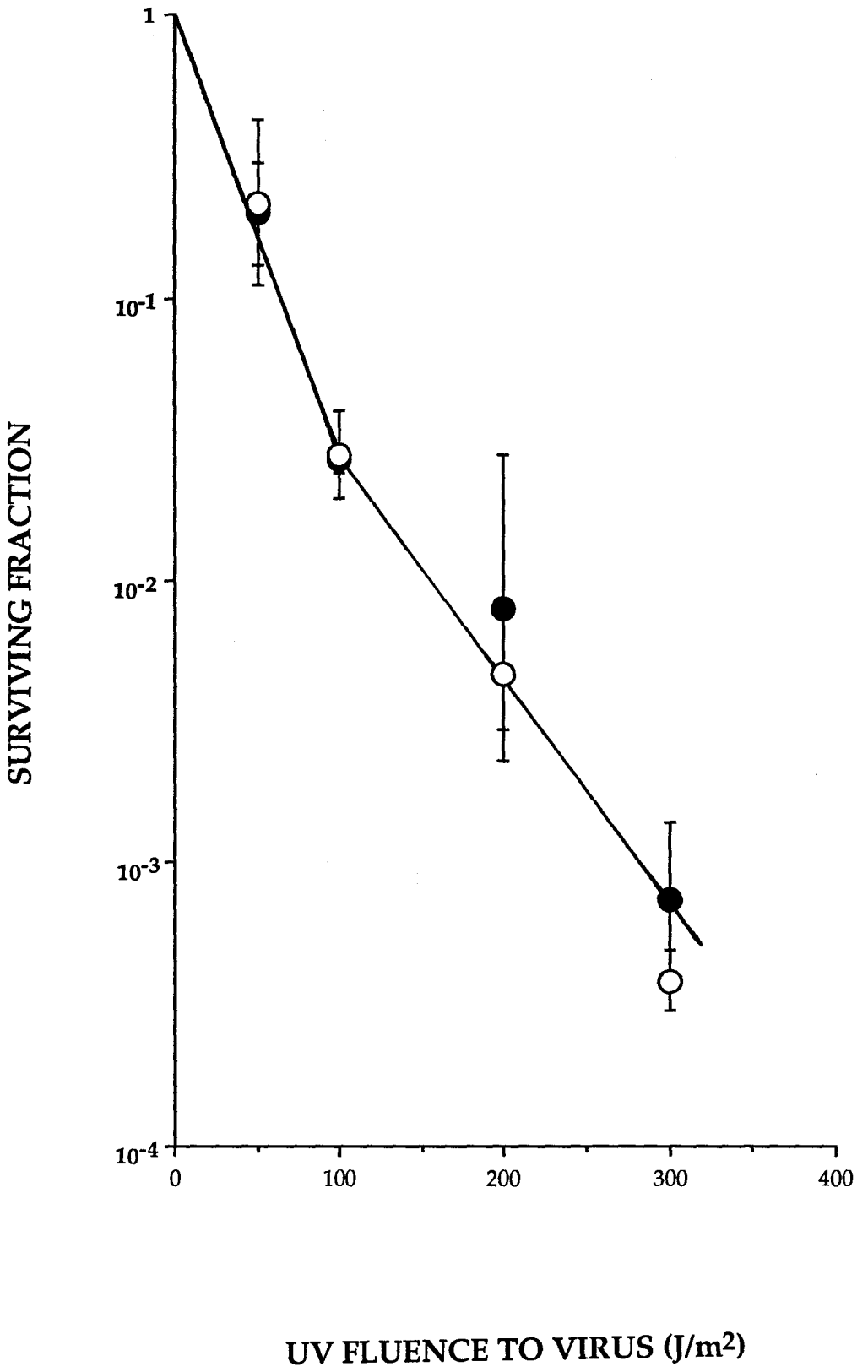


**Figure 12: Survival of UV irradiated HSV-1:kos in GM37D (Normal) cells as compared to GM5509A (XP12BE group A) and GM4313B (XP2YO group F) cells.**

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis.

△ GM37D (Normal)
□ GM4313B (XP2YO group F)
● GM5509A (XP12BE group A)

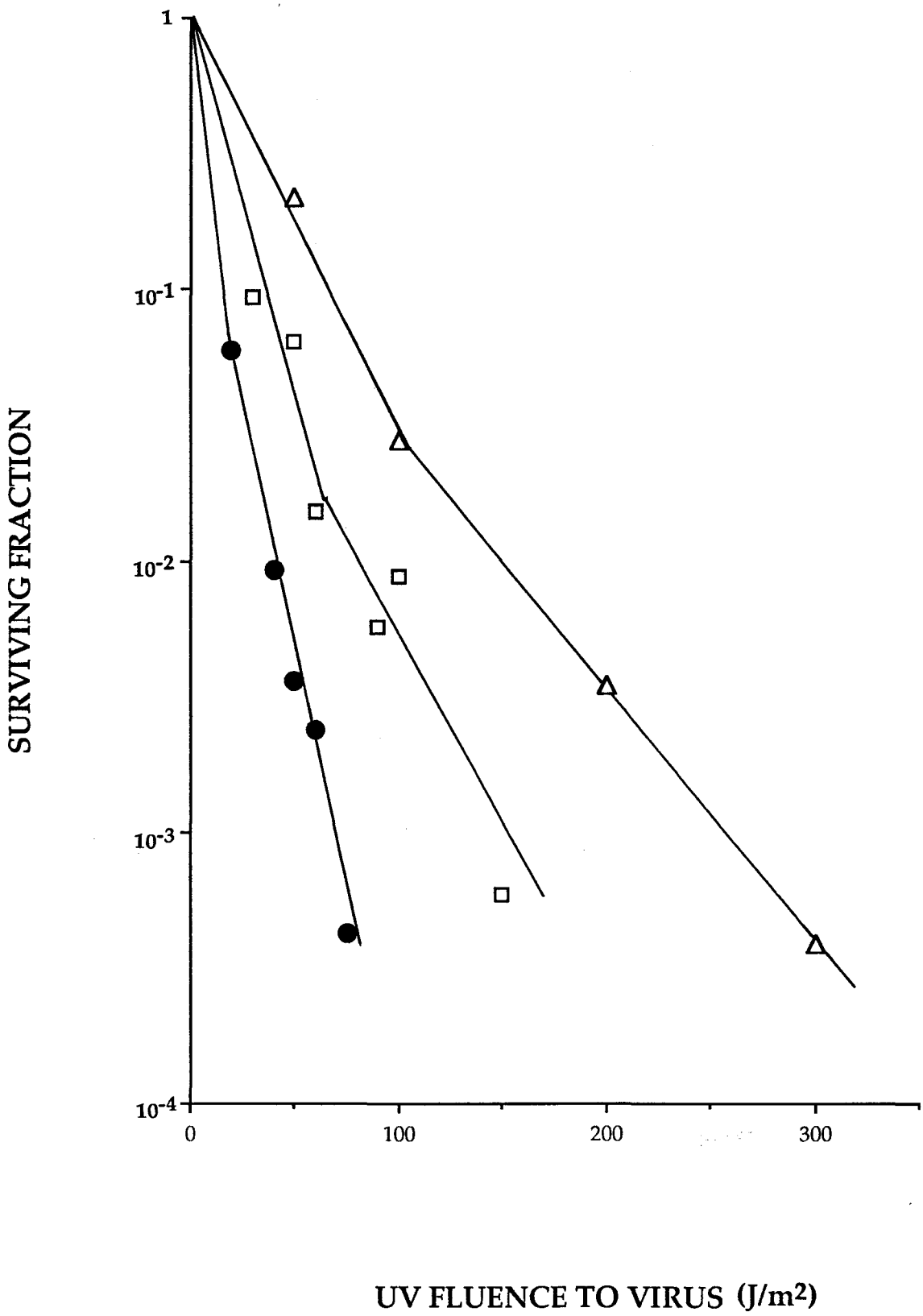
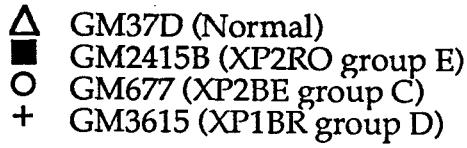
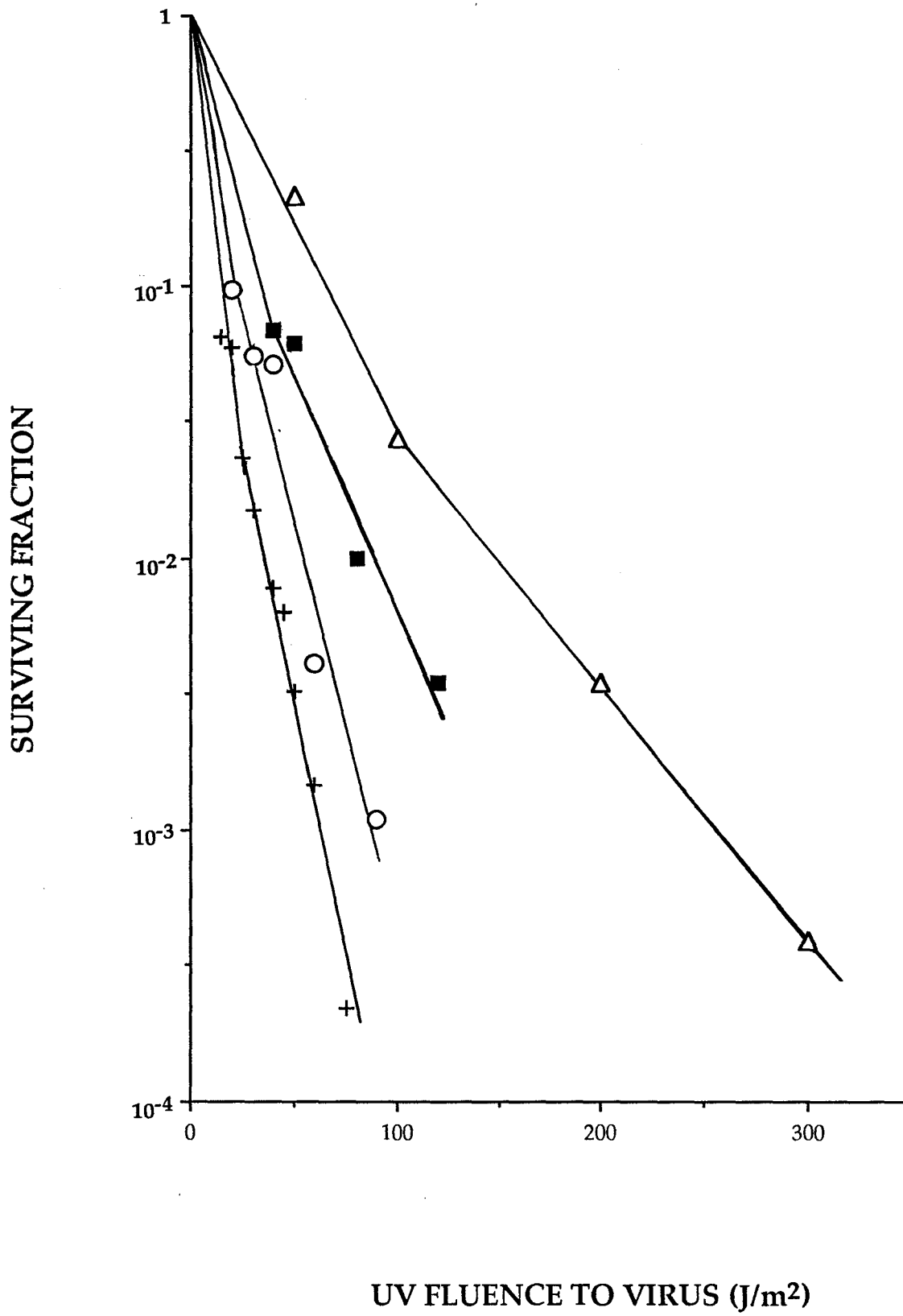

SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
UV FLUENCE TO VIRUS ($J/m^2$)

**Figure 13: Survival of UV irradiated HSV-1:kos in GM37D (Normal) cells as compared to GM677 (XP2BE group C), GM3615 (XP1BR group D) and GM2415B (XP2RO group E) cells.**

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis.




SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
UV FLUENCE TO VIRUS (J/m2)

## 2.3 UV virus survival in Xeroderma Pigmentosum Fibroblasts

The construction of the viral vector carrying the den V gene enables one to examine the effect of the den V gene product in a wide variety of cell types. UV survival of HSV-1:den V was examined in cells from five xeroderma pigmentosum complementation groups. The results are summarized in Tables 1a and 2 and figures 14 to 18. In group A cells there was a two-fold increase in uv viral survival of the recombinant as compared to wildtype (figure 14). The $D_0$ values for HSV-1:kos and HSV-1:den V in XP group A cells are 11.74 $\pm$ 1.90 J/m$^2$ and 21.71 $\pm$ 1.50 J/m$^2$ respectively. The %HCR values of the two viruses are 27.0 $\pm$ 8.2% and 45.4 $\pm$ 8.5%. From the survival curves it can be seen that the second component shows increased survival in the presence of the den V gene product.

Similarly, a significant difference in uv virus survival was seen in XP group D cells (figure 16). The enhanced HSV-1:den V survival in group D cells is reflected by the $D_0$ and %HCR values. As indicated in Tables 1a and 2, the $D_0$ values for HSV-1:kos and HSV-1:den V are 11.37 $\pm$ 1.50 J/m$^2$ and 19.28 $\pm$ 2.5 J/m$^2$ and the %HCR values are 26.2 $\pm$ 7.2% and 40.3 $\pm$ 9.98% respectively. Hence, it appears that the HSV-1:den V virus survives significantly better in both XP group A and group D cells than in its wildtype counterpart. From the survival curves in figures 14 and 16, it can be seen that there is a 25.2% restoration of the second component in XP-A cells and a 19.1% restoration of the second component in XP-D cells.

One of the remaining three XP complementation groups examined showed a small increase in uv virus survival. As illustrated in figure 15, the presence of the den V gene in the viral genome was able to enhance the uv survival of the virus by a small amount in XP group C cells. The calculated

**Figure 14: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in GM5509A (XP12BE group A) cells**

This figure illustrates the pooled results of five individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM 5509A (GROUP A)

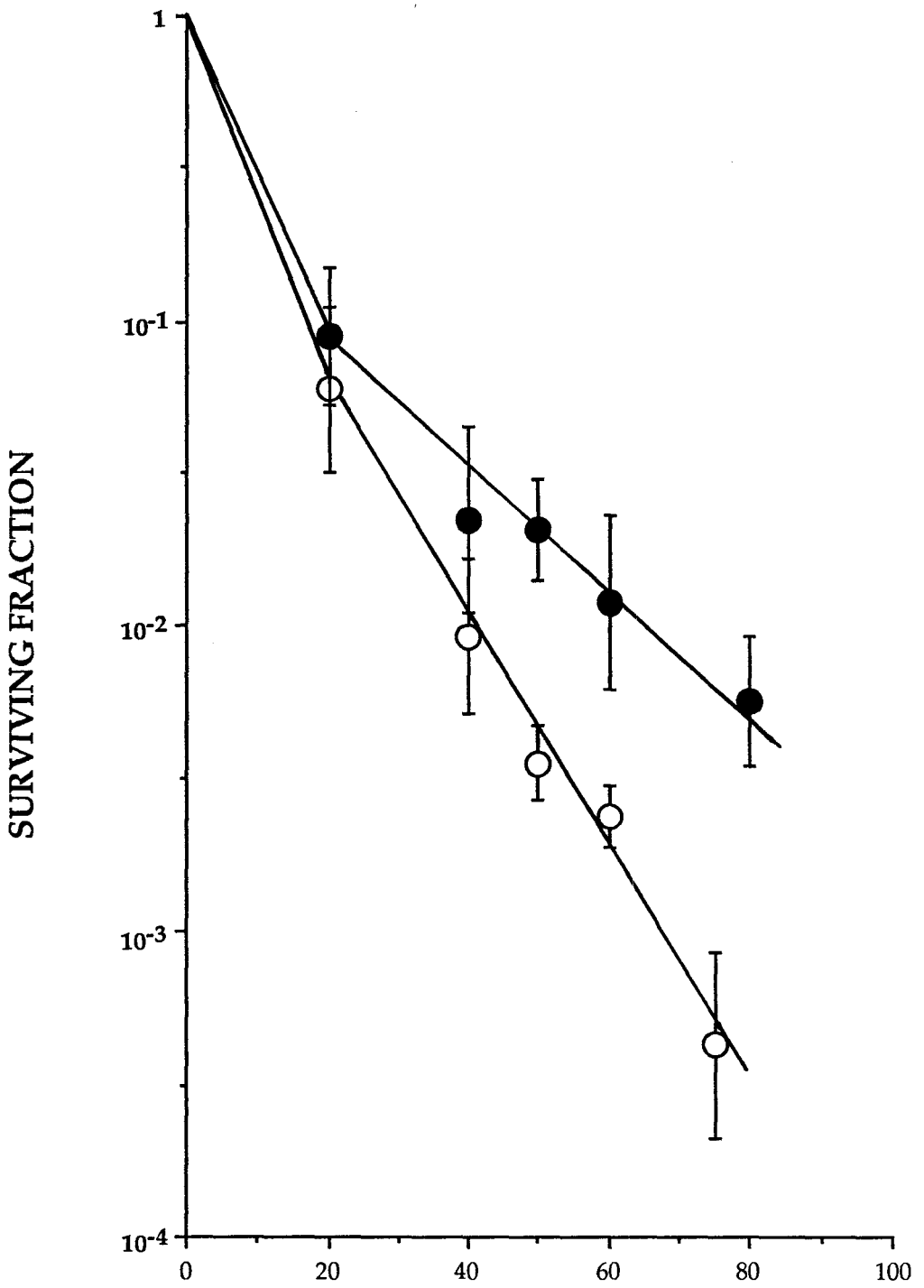


**Figure 15: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in GM677 (XP2BE group C) cells**

This figure illustrates the pooled results of five individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM677 (GROUP C)

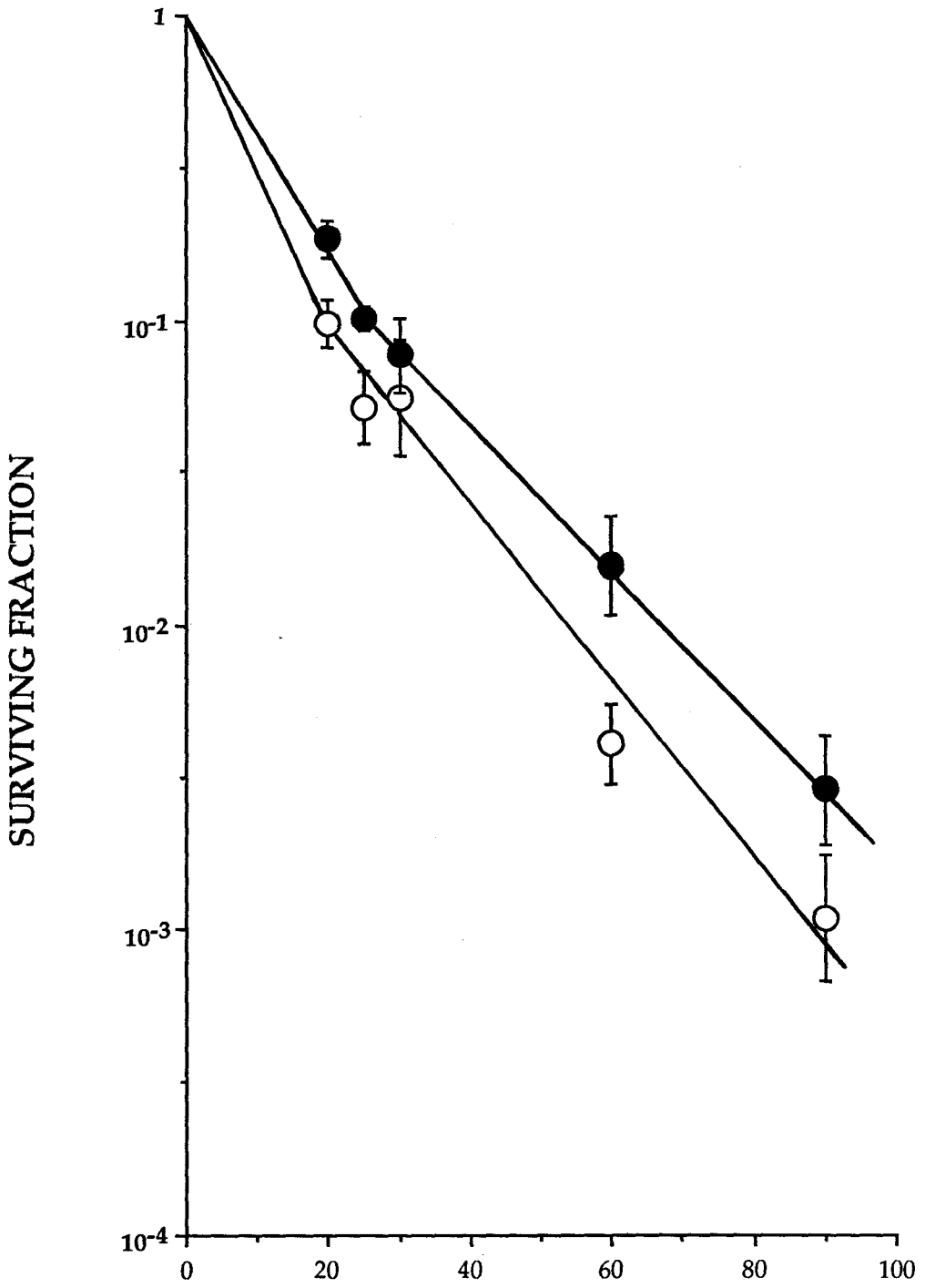


**Figure 16: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in GM3615 (XP1BR group D) cells**

This figure illustrates the pooled results of six individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM3615 (GROUP D)

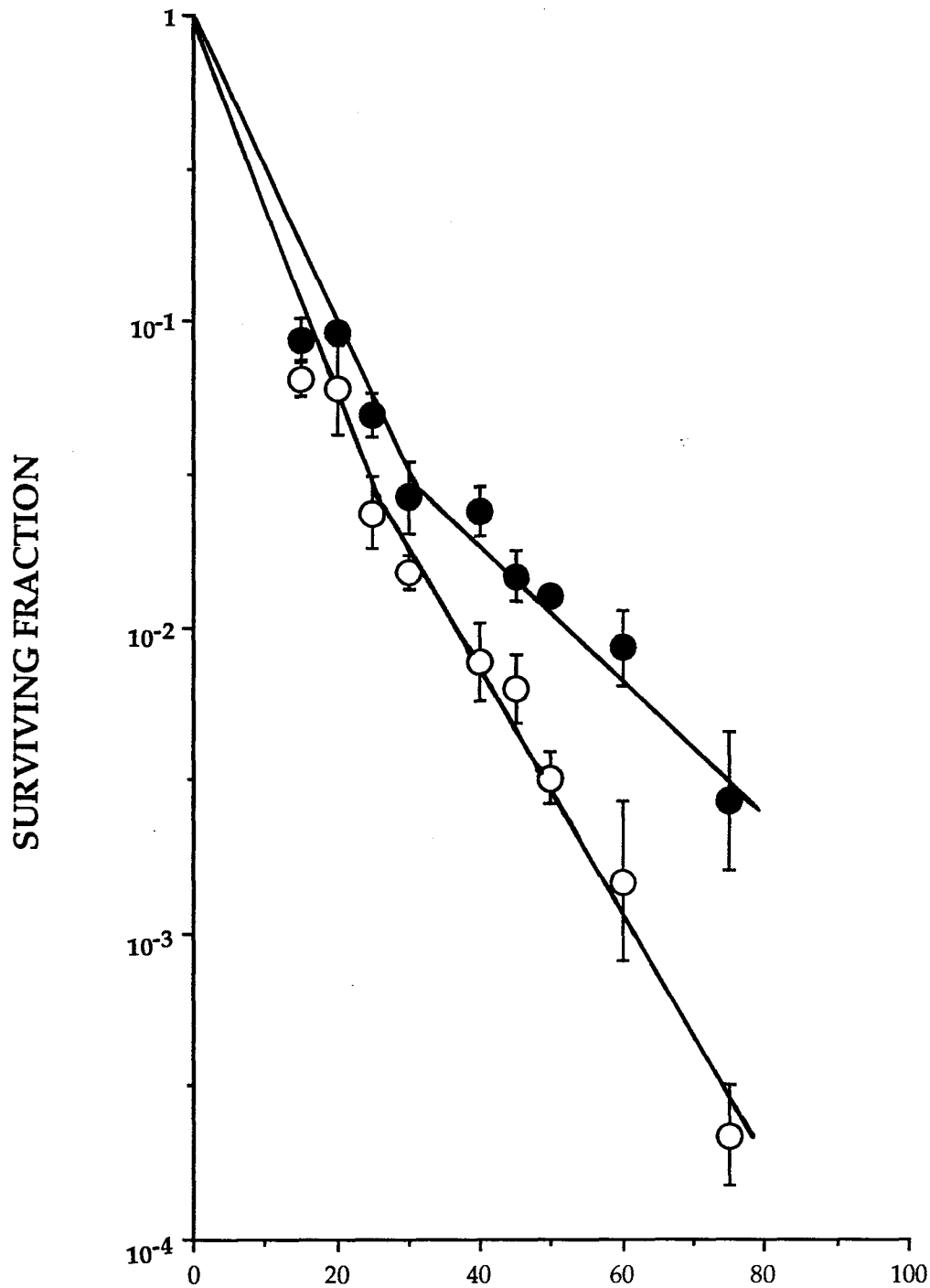


**Figure 17: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in GM2415B (XP2RO group E) cells**

This figure illustrates the pooled results of four individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM2415B (GROUP E)

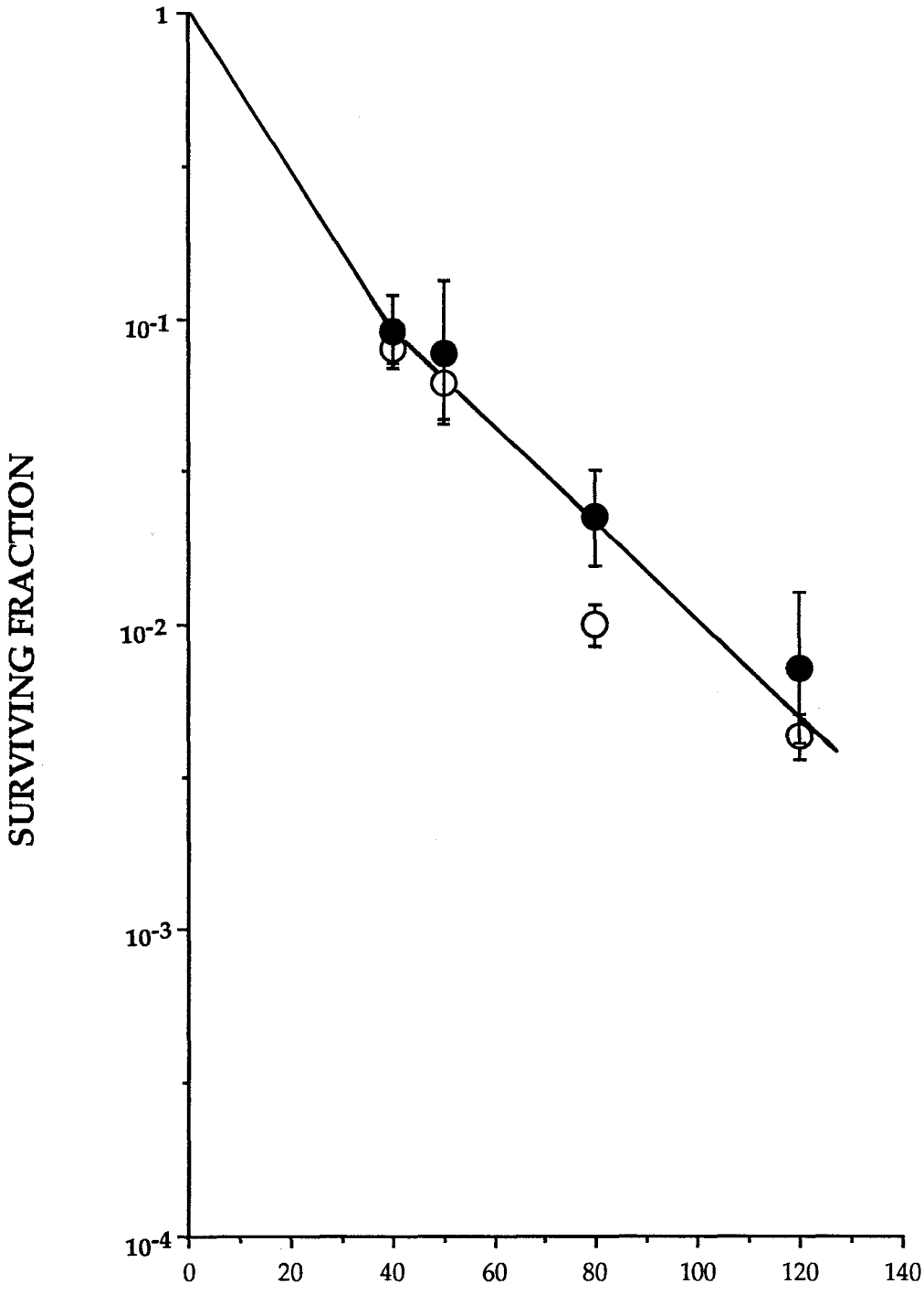
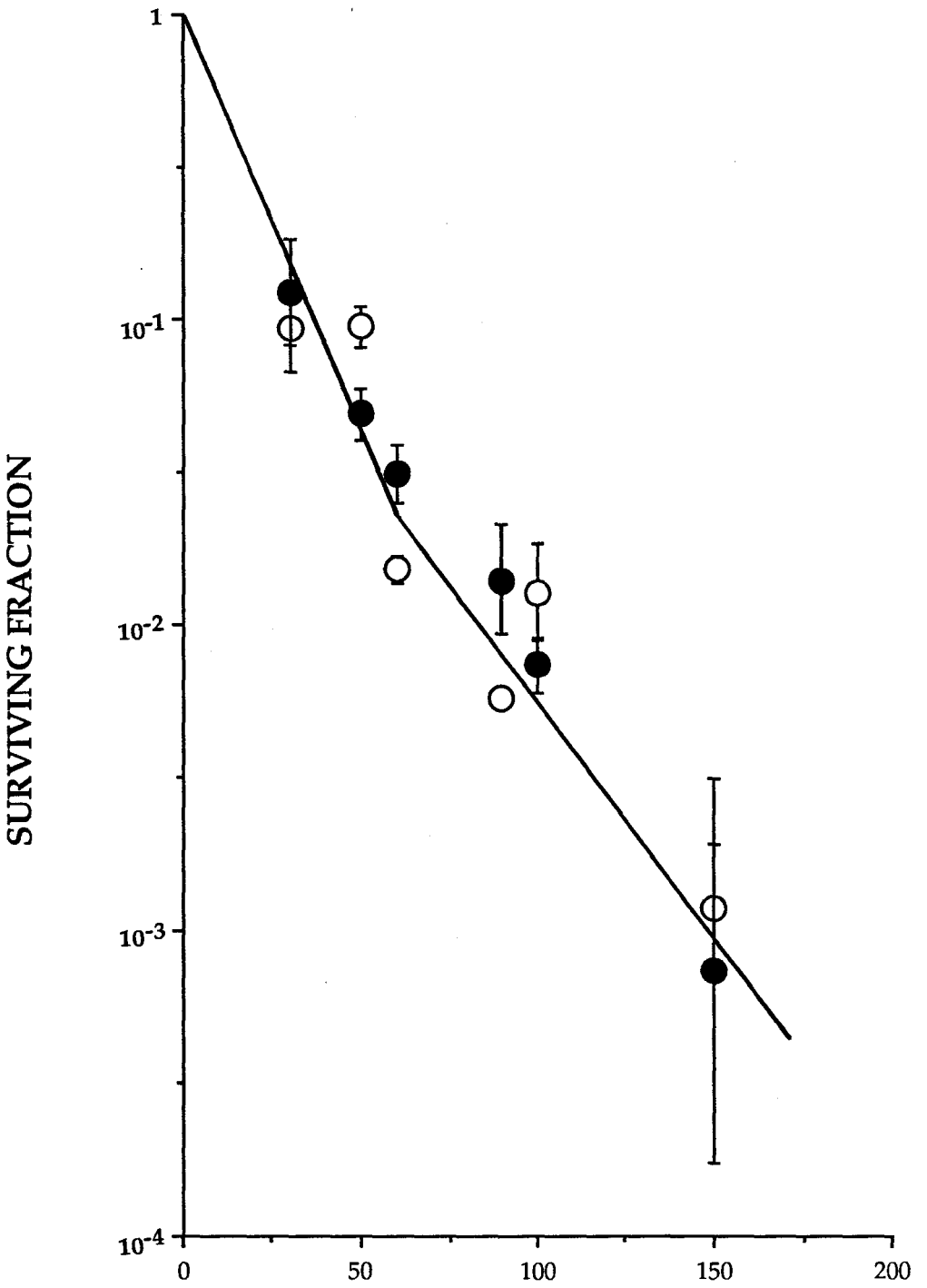


**Figure 18: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in GM4313B (XP2YO group F) cells**

This figure illustrates the pooled results of three individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols to HSV-1:den V.

# GM4313B (GROUP F)



$D_0$ values for HSV-1:kos and HSV-1:den V were determined to be 14.35 $\pm$ 2.87 $J/m^2$ and 17.56 $\pm$ 3.23 $J/m^2$ and are not statistically different from each other (90% confidence). Figures 17 and 18 illustrates the uv-viral survival curves for XP groups E and F cells. A significant difference in survival of uv-irradiated virus was not dectected in these cell types following infection with HSV-1(den V). The calculated $D_0$ values for the two viruses in these two cell lines are summarized in Table 1a.

**2.4 UV virus survival of HSV-1:FUS-7 kan and HSV-1:kos in XP Fibroblasts**

It was speculated that the insertion into the gI gene during the cloning procedure may have in some manner contributed to the increased survival observed in the XP fibroblasts. To investigate this possiblity, the uv survival of a $gI^-$ mutant virus, HSV-1:FUS-7 kan (Johnson *et al.*, 1988) containing the kanamycin gene instead of the den V gene inserted into the gI locus of HSV-1, was assayed in two XP fibroblasts.

From figures 21 and 22, it can be seen that no significant difference in uv survival exists between this recombinant and the wildtype counterpart. The calculated $D_0$ values for both cell lines are summarized in Table 1b. These results indicate that any increases in survival observed in the various cells are specifically due to the inserted den V gene and not to the absence of the gI gene.

**2.5 UV virus survival in Trichothiodystrophy (TTD) cells**

One of the three existing categories of TTD cells have been shown to be photosensitive and deficient in excision repair (Broughton, *et al.*, 1990). These primary fibroblasts exhibit characteristics indistinguishable from XP

## Figure 19: Survival of UV irradiated HSV-1:kos and HSV-1:den V in GM37D (Normal) cells and GM5509A (XP12BE group A) cells

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to the HSV-1:kos virus and the closed symbols refer to HSV-1:den V virus. The triangles represent the results of four separate experiments involving GM37D (Normal) cells and the circles represent the results of five experiments involving GM5509A (XP12BE group A) cells.

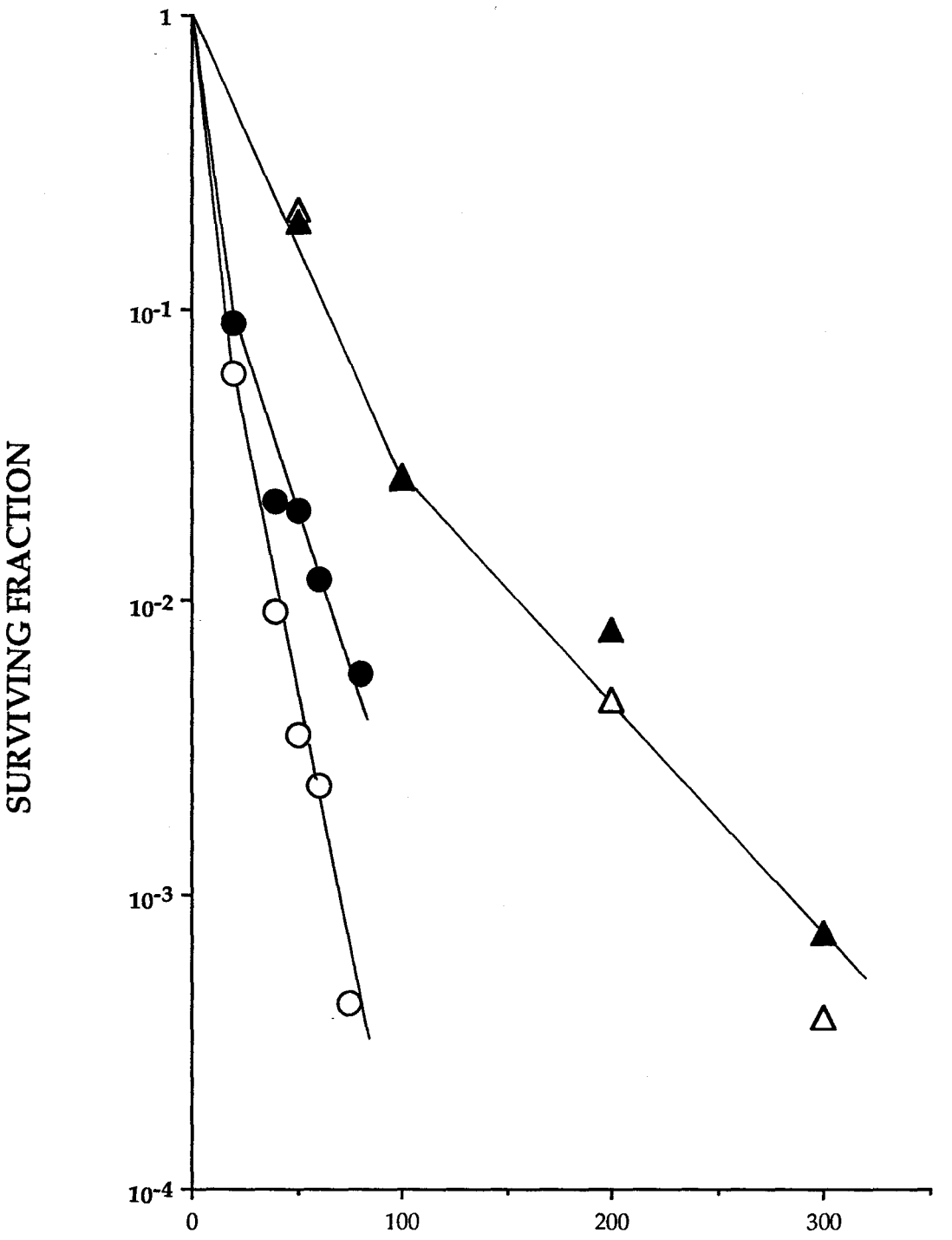
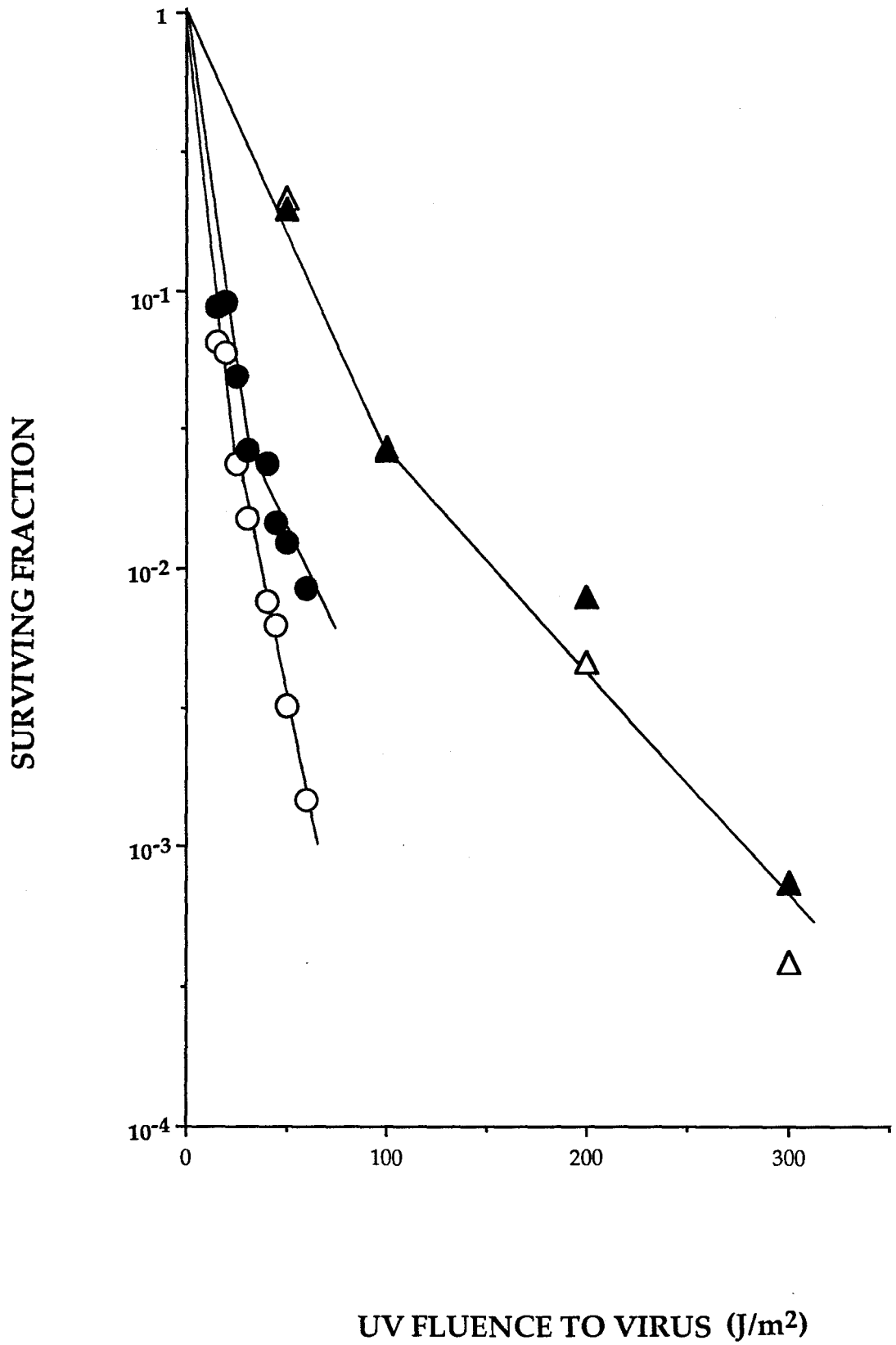

SURVIVING FRACTION
1
10-1
10-2
10-3
10-4
0
100
200
300
UV FLUENCE TO VIRUS (J/m2)

**Figure 20: Survival of UV irradiated HSV-1:kos and HSV-1:den V in GM37D (Normal) cells and GM3615 (XP1BR group D) cells**

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to the HSV-1:kos virus and the closed symbols refer to HSV-1:den V virus. The triangles represent the results of four separate experiments involving GM37D (Normal) cells and the circles represent the results of six experiments involving GM5509A (XP12BE group D) cells.


SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
UV FLUENCE TO VIRUS ($J/m^2$)

## Figure 21: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:FUS-7 kan in GM5509A (XP12BE group A) cells

This figure illustrates the pooled results of two individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols refer to HSV-1:FUS-7 kan.

# GM5509A (GROUP A)

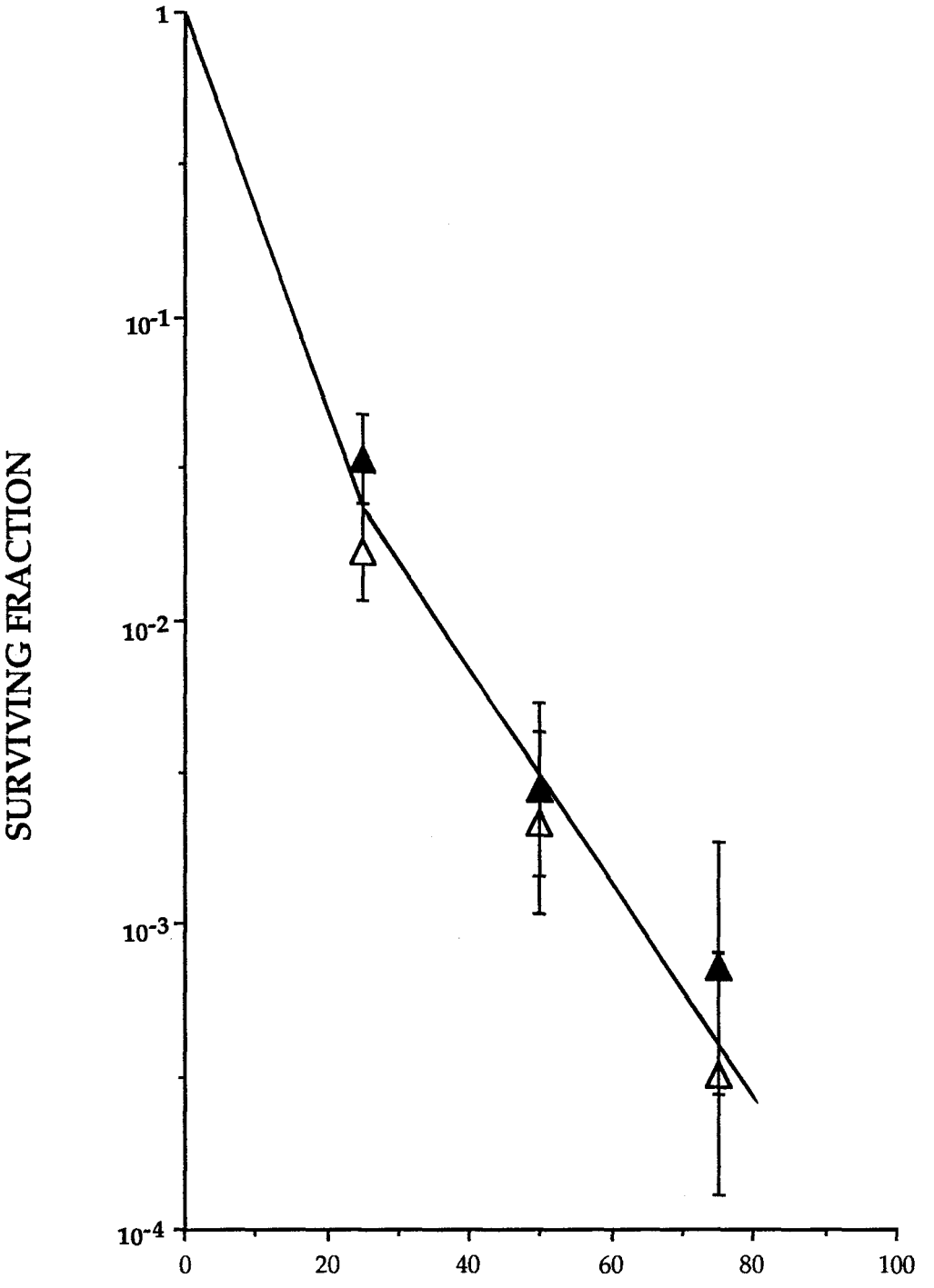


**Figure 22: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:FUS-7 kan in GM3615 (XP1BR group D) cells**

This figure illustrates the pooled results of two individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols refer to HSV-1:FUS-7 kan.

# GM3615 (GROUP D)

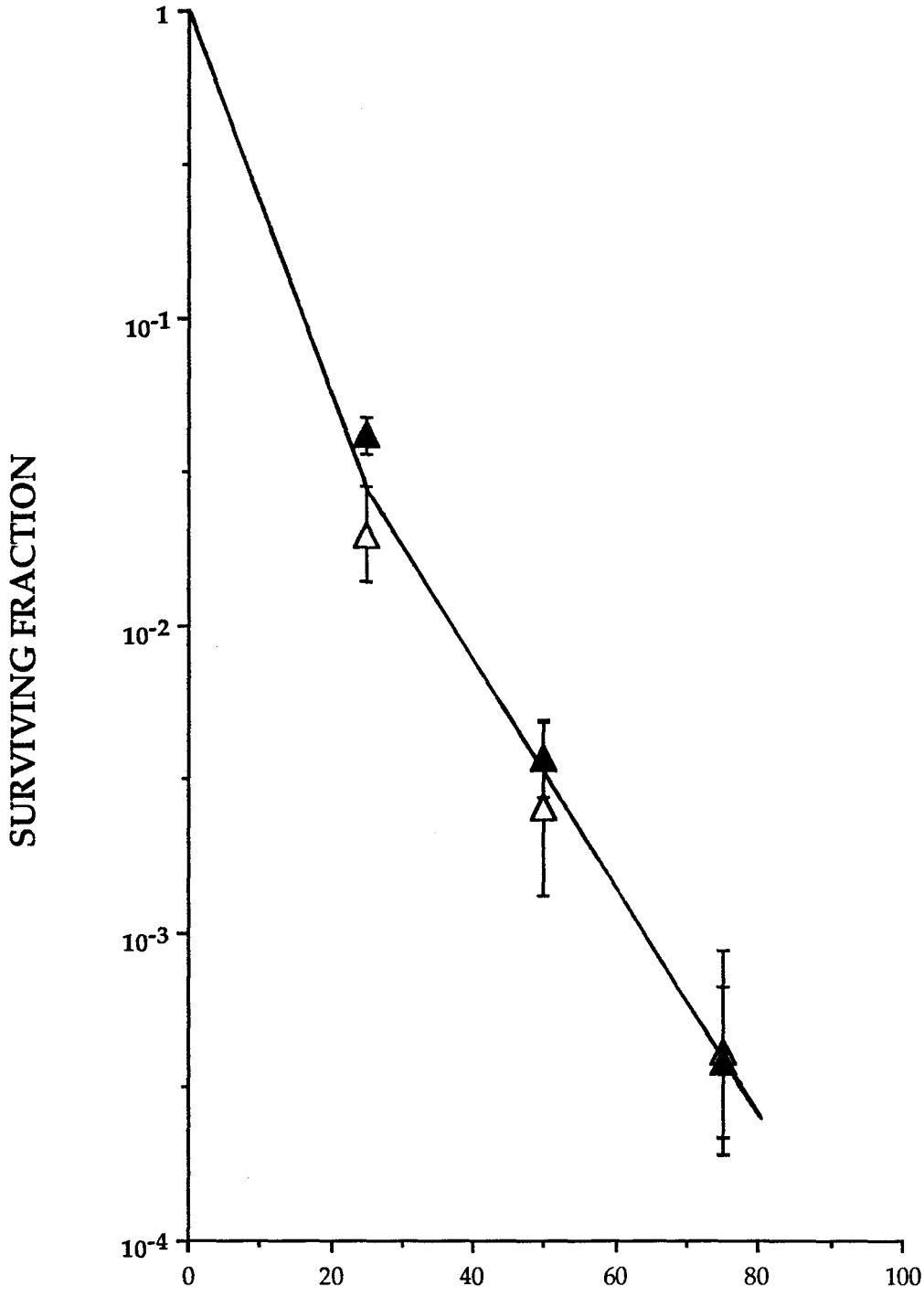


**TABLE 1a:** **$D_0$ values for the second component of uv irradiated virus.**

| | VIRUSES | | |
|---|---|---|---|
| | **HSV-1: kos** $D_0$ ($J/m^2$) | **HSV-1: den V** $D_0$($J/m^2$) | **# Expts** |
| CELLS | | | |
| 293 Cells | 85.34 ± 28.41 | 84.54 ± 27.58 | 3 |
| XP-4 Cells | 13.57 ± 3.39 | 22.80 ± 4.80 | 5 |
| GM37D(Normals) | 43.43 ± 6.24 | 47.82± 5.65 | 4 |
| GM5509A (XP12BE Group A) | 11.74 ± 1.90 | 21.71 ± 1.50 | 5 |
| GM677 (XP2BE Group C) | 14.35 ± 2.87 | 17.56 ± 3.23 | 5 |
| GM3615 (XP1BR Group D) | 11.37± 1.50 | 19.28 ± 2.50 | 6 |
| GM2415B (XP2RO Group E) | 27.14 ± 5.09 | 31.02 ± 4.43 | 4 |
| GM4313B (XP2YO Group F) | 27.56 ± 4.59 | 23.35 ± 2.60 | 3 |
| Trichothiodystrophy (TTD8PV) | 10.86 ± 1.90 | 11.74 ± 1.59 | 2 |
| LPM2A | 53.98 ± 33.25 | 55.49 ± 29.59 | 2 |

**TABLE 1b:** **$D_0$ values for the second component of uv irradiated virus.**

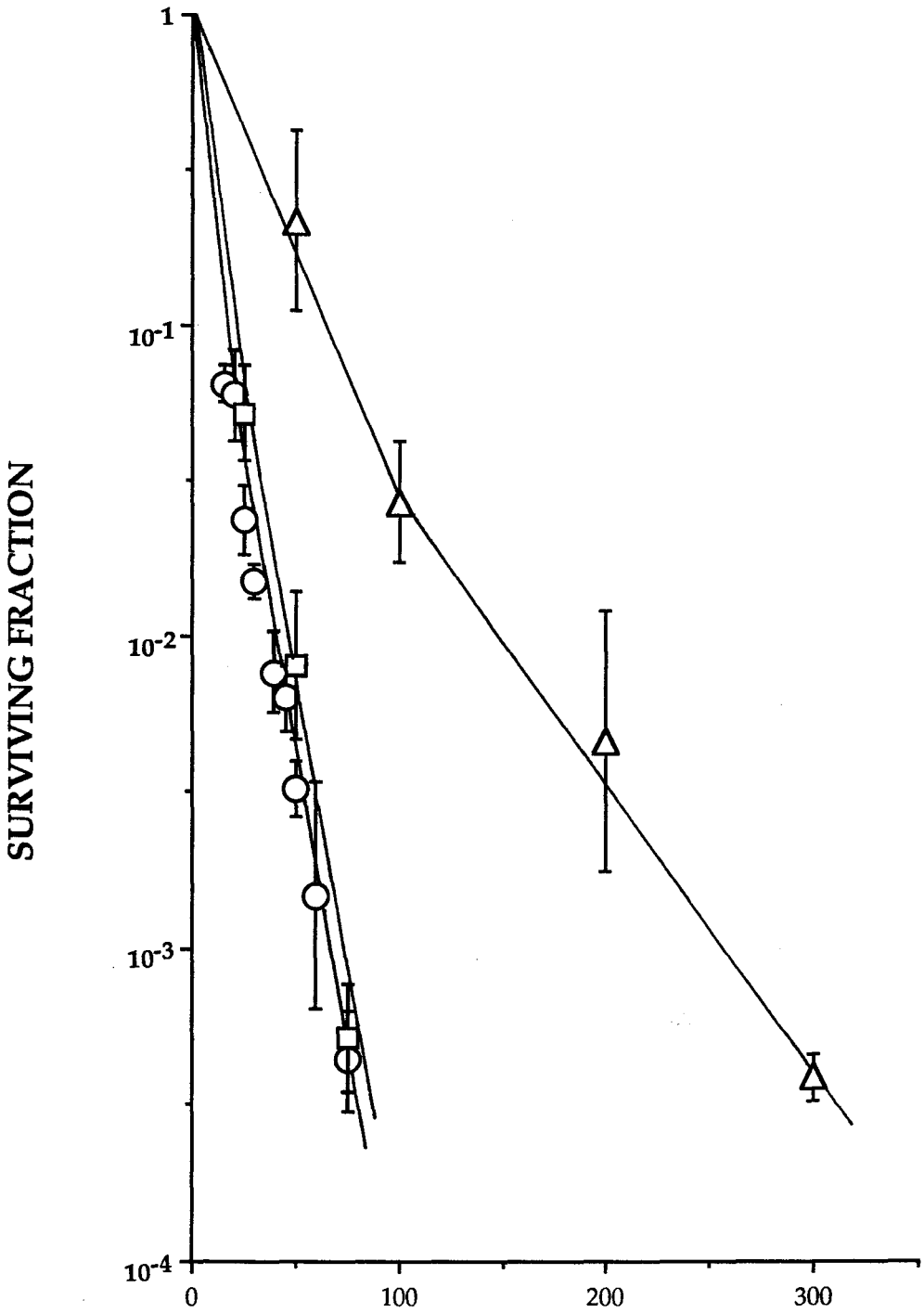
| | VIRUSES | | |
|---|---|---|---|
| CELLS | HSV-1: kos $D_0$ ($J/m^2$) | HSV-1: FUS-7 kan $D_0$($J/m^2$) | # Expts |
| GM5509A (XP12BE Group A) | 12.77 ± 1.88 | 13.16 ± 2.33 | 2 |
| GM3615 (XP1BR Group D) | 12.77 ± 2.63 | 10.59 ± 1.29 | 2 |

**TABLE 2:** **%Host Cell Reactivation for the second component of uv-irradiated virus.**

| | VIRUSES | | |
|---|---|---|---|
| CELLS | HSV-1: kos % HCR | HSV-1: den V %HCR | %Restoration |
| XP-4 Cells | 15.9 ± 9.3 | 27.0 ± 14.7 | 13.2 |
| GM5509A (XP12BE Group A) | 27.0 ± 8.2 | 45.4 ± 8.5 | 25.2 |
| GM677 (XP2BE Group C) | 33.0 ± 11.3 | 36.7 ± 8.7 | 5.5 |
| GM3615 (XP1BR Group D) | 26.2 ± 7.2 | 40.3 ± 9.98 | 19.1 |
| GM2415B (XP2RO Group E) | 62.5 ± 20.7 | 64.9 ±17.0 | 6.4 |
| GM4313 (XP2YO Group F) | 63.5 ± 19.7 | 48.8 ±11.2 | -40.3 |

**Figure 23: Survival of UV irradiated HSV-1:kos in GM37D (Normal) cells, GM3615 (XP1BR group D) cells and Trichothiodystrophy (TTD8PV) cells**

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The triangles represent the results of four separate experiments involving GM37D (Normal) cells, the circles represent the results of six experiments involving GM5509A (XP12BE group D) cells, and the squares represent the results of two experiments involving TTD8PV cells.


SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
UV FLUENCE TO VIRUS ($J/m^2$)

## Figure 24: Survival of Plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in Trichothiodystrophy (TTD8PV) cells

This figure illustrates the pooled results of two individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols refer to HSV-1:den V.

# TRICHOTHIODYSTROPHY (TTD)

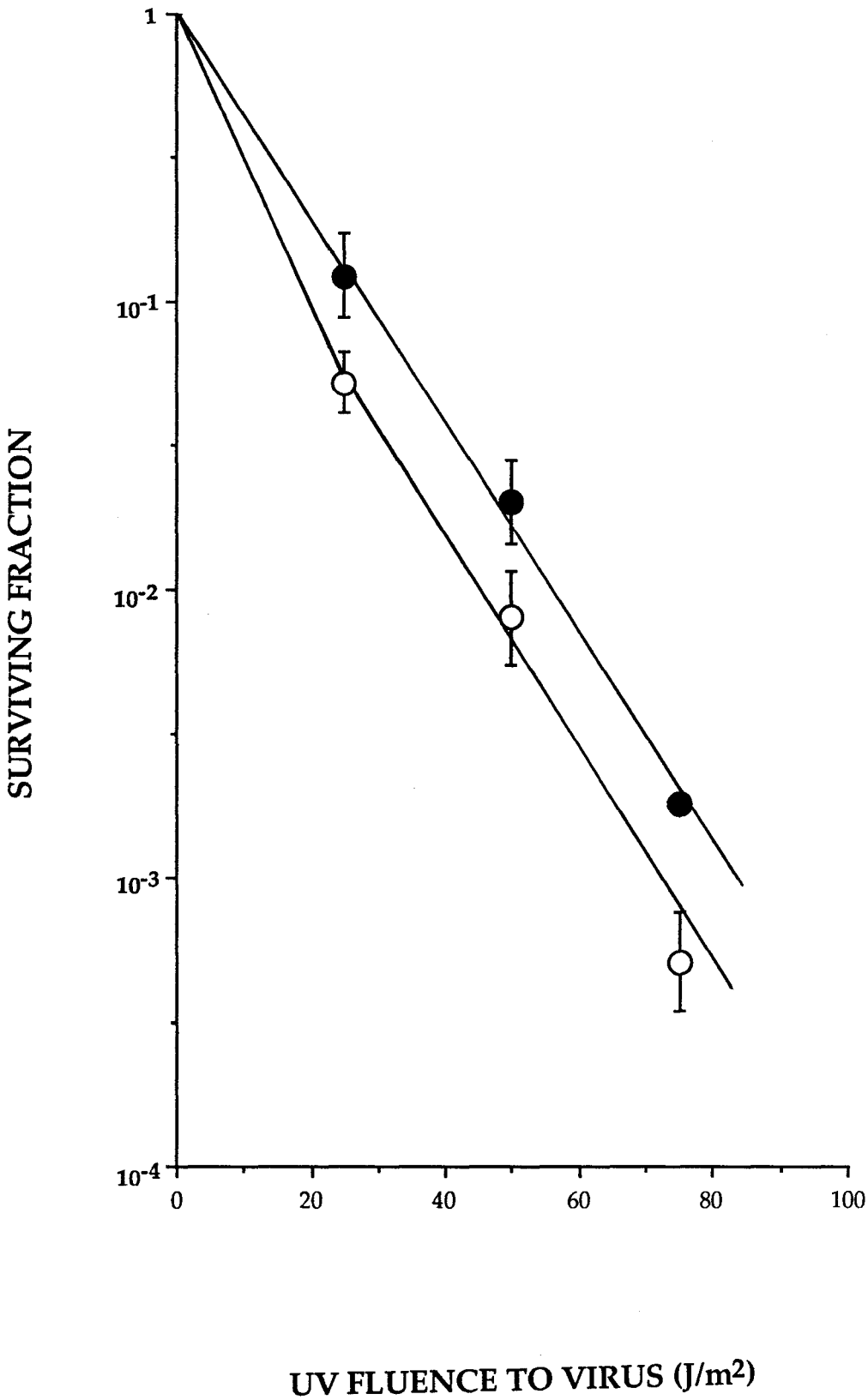
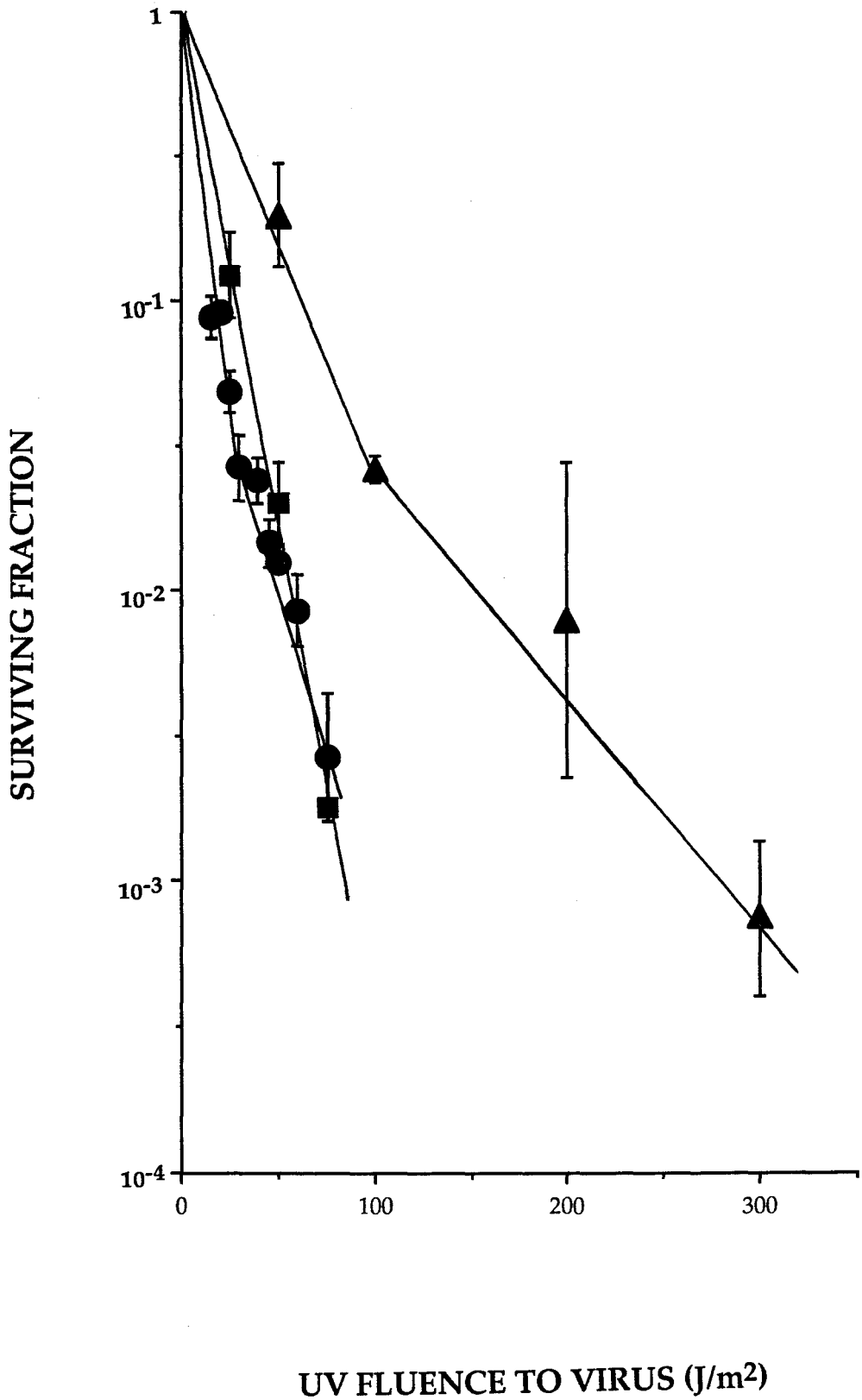


**Figure 25: Survival of UV irradiated HSV-1:den V in GM37D (Normal) cells, GM3615 (XP1BR group D) cells and Trichothiodystrophy (TTD8PV) cells**

Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The triangles represent the results of four separate experiments involving GM37D (Normal) cells, the circles represent the results of six experiments involving GM5509A (XP12BE group D) cells, and the squares represent the results of two experiments involving TTD8PV cells.


SURVIVING FRACTION
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
100
200
300
UV FLUENCE TO VIRUS (J/m2)

group D cells which include a reduced ability to support uv-irradiated virus (figure 23). The ability of the den V gene to complement this excision defect was determined using the HSV-1:den V recombinant virus. Figure 24 shows the pooled results of two separate experiments. The presence of the den V gene product was able to increase viral survival by a small amount. The Do values for the second component however, were not significantly different and are indicated in Table 1a as 10.86 $\pm$ 1.90 J/m$^2$ and 11.74 $\pm$ 1.59 J/m$^2$. This suggests that the effect of the den V protein may be related to the first component of the survival curve.

**2.6 UV virus survival in LPM2A cells**

Although rodent cells exhibit significantly less excision repair than human cells, they are able to survive equally well when exposed to similar levels of uv radiation. Different types of rodent cells have varying degrees of excision repair ability. Previous studies have shown that HCR of uv irradiated HSV in mouse cells is reduced as compared to unirradiated virus (Lytle, 1971; Glazier *et al.*, 1982). By using the recombinant HSV-1:den V virus, we proceeded to examine the ability of the den V gene product to alter virus survival in the mouse cell line LPM2A. Figure 26 shows the pooled results of two separate experiments. The den V gene product was unable to increase virus survival in these cells. The $D_0$ values for the two viruses are 58.38 $\pm$ 33.25 J/m$^2$ and 51.50 $\pm$ 29.59 J/m$^2$ and are not statistically significant.

3.0 CELLULAR CAPACITY

A number of studies involving HSV have shown that there is a greater decrease in capacity in XP cells than in normal cells due to the presence of

**Figure 26: Survival of plaque forming ability of UV irradiated HSV-1:kos and HSV-1:den V in LPM2A cells.**

This figure illustrates the pooled results of two individual experiments. Each point represents the logarithmic mean of the pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The survival points were fitted to two straight lines using linear regression analysis. The open symbols refer to HSV-1:kos and the closed symbols refer to HSV-1:den V.

# LPM2A CELLS

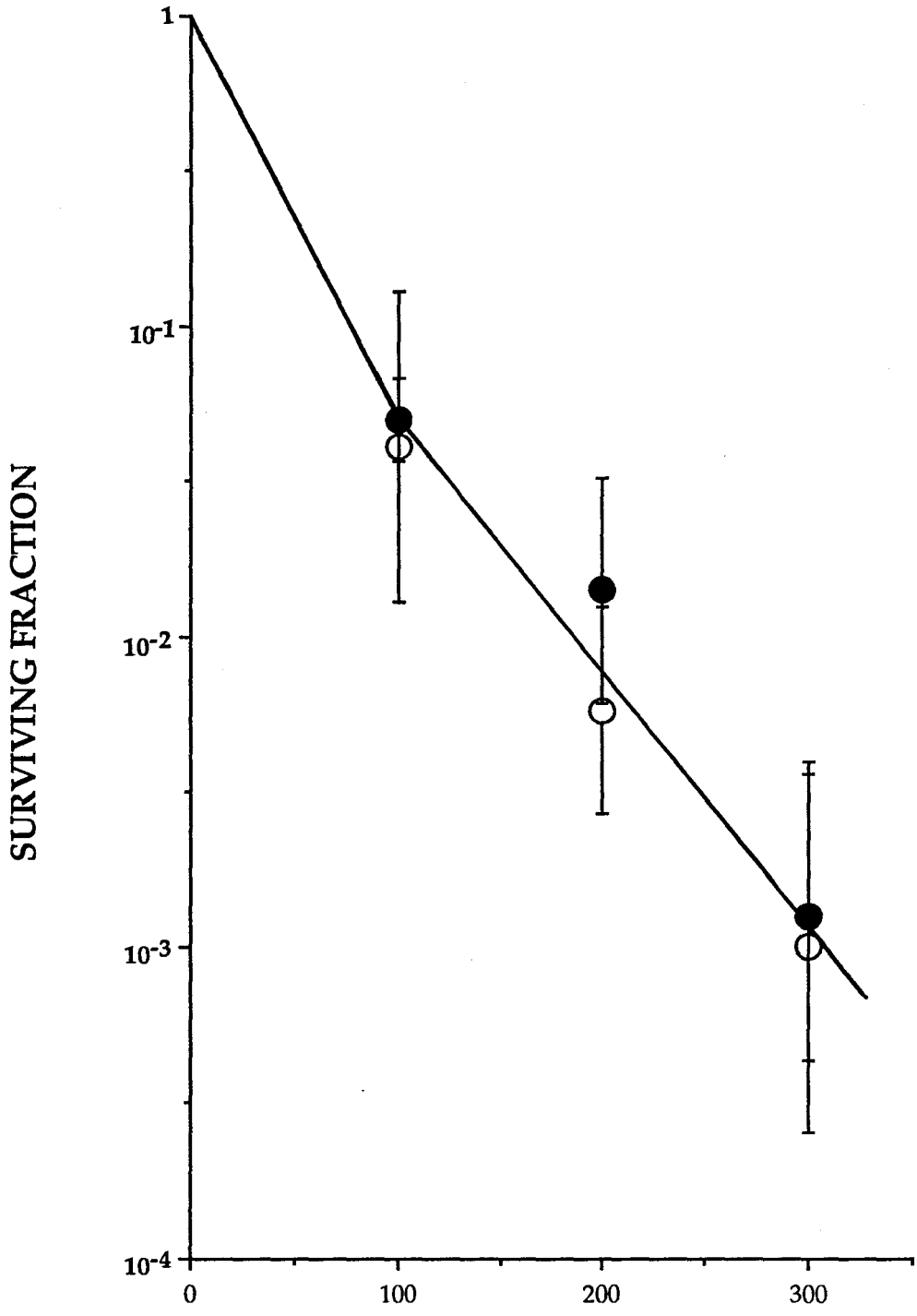
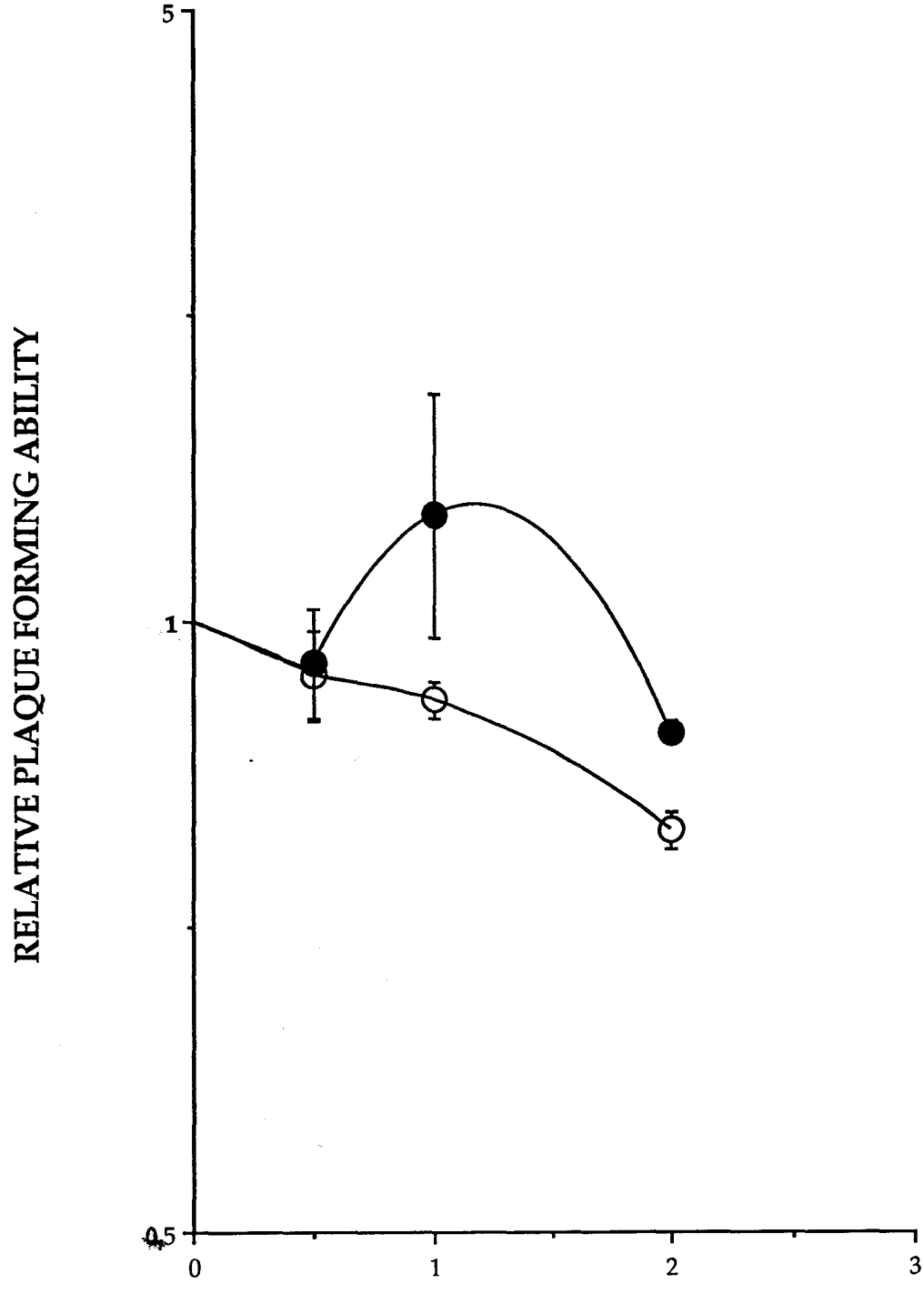

1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
SURVIVING FRACTION
0
100
200
300
UV FLUENCE TO VIRUS (J/m²)

**Figure 27: Capacity of UV irradiated GM5509A (XP12BE group A) cells to support HSV-1:kos and HSV-1:den V plaque formation**

Monolayers of cells were irradiated immediately prior to infection with unirradiated virus. The relative plaque forming ability was calculated as a function of uv fluence to the cells. Each point represents the logarithmic mean of pooled data at each uv fluence, and the standard deviation of the mean is shown by the bar or is contained within the symbol. The open symbols refer to HSV-1:kos and the closed symbols refer to HSV-1:den V.


RELATIVE PLAQUE FORMING ABILITY
5
1
0.5
0
1
2
3
UV FLUENCE TO CELLS (J/m2)

repair defects in the XP cells. In the present study, the cellular repair capacity of XP12BE(group A) cells was measured by pre-irradiating the cells prior to infection with virus. Figure 27 shows the pooled results of two separate experiments in which XP12BE(group A) cells were infected immediately after irradiation. The survival of the recombinant virus in these cells was not significantly different from wildtype virus.

Experiments in which cells were infected with virus prior to irradiation were likewise conducted. It was thought that this would enable the virally encoded den V gene product to be synthesized and made available for use in the cell before uv irradiation. This approach however, did not significantly alter the survival of the recombinant virus in these cells.

## 4.0 PLAQUE SIZE DIFFERENCES BETWEEN VIRUSES

It was observed in this study that the plaque sizes of $gI^-$ viruses were smaller when infecting human fibroblasts but not when infecting vero, XP-4 or 293 cells. Twenty randomly selected $gI^-$ and $gI^+$ plaques were measured under a microscope at 2.5X magnification. The average size of a $gI^+$ plaque was $1.85 \pm 0.33$ mm, where as the average size of the $gI^-$ plaque was $0.48 \pm 0.06$ mm in XP12BE (group A) cells.

# DISCUSSION

The main objective of this study was to demonstrate the suitability of HSV-1 as a vector for the expression of DNA repair genes in primary fibroblasts. The majority of DNA repair studies have utilized transformed cells in DNA mediated transfection experiments (Gantt *et al.*, 1984; Thompson *et al.*, 1987; Green *et al.*, 1987). Since the DNA repair profiles of these transformed cells have been altered, they may no longer reflect the actual DNA repair abilities of their untransformed parents. The technique used in this study overcomes this problem by using a vector to deliver a repair gene into primary fibroblasts. The effect of this novel gene can then be examined by assaying for survival of the virus containing DNA damaged by radiation or chemical agents in the cells of interest.

## CONSTRUCTION OF THE HSV-1:DEN V VIRUS

The system used to construct the HSV-1:den V recombinant virus in this study has been successfully applied by other investigators to express foreign genes in various cell types. Gene inserts have ranged in size from 1.9 kb (in this study) to as much as 7.9 kb (Hasham Salloukh, 1991). The HSV-1:den V recombinant virus was constructed as outlined in materials and methods and figures 3 and 4. A den V expression cartridge containing the den V gene flanked by the long terminal repeat promoter of Rous Sarcoma Virus and the Simian Virus polyadenylation splice signals (figure 2) was inserted into the Bgl II site of the plasmid pSS17BG (figure 3). A Hind III

digest identified the two possible orientations by which the cassette could have been inserted as pMT18 and pMT21 (figure 5). Through homologous recombination with HSV-1:FgDβ DNA, the recombinant virus HSV-1:den V was generated (figure 4). The presence of the den V sequences within the viral genome was confirmed by southern blot analysis (figure 7).

As discussed earlier, three classes of HSV-1 genes are produced upon infection. They are the immediate early, early and late genes. Both immediate early and early classes reach peak synthesis prior to DNA replication, whereas the late genes do not reach maximum synthesis until after the genome has been replicated (Roizman & Batterson, 1985). Since the den V gene product is required for the incision step of excision repair, it must be expressed before DNA replication in order to help in the DNA repair of lesions which block DNA synthesis. As a result, this gene would have to be produced as either immediate early or early to be of value in increasing survival of the virus after uv. It is the sequences within the promoter of a gene that determine the class of the gene (Lang *et al.*, 1984).

In this study, the Rous Sarcoma Virus LTR was used to drive the den V gene. This promoter contains the the three elements believed to be necessary for a gene to be classed as early. These include a TATA box, a CAAT box and a G-C rich region for binding of the SP-1 transcription factor. An additional sequence thought to be necessary is the TAATGARAT enhancer element (Lang *et al.*, 1984). The RSV LTR contains two sequences which could be used as such an enhancer sequence. It was therefore, believed that the den V gene would likely be expressed as an immediately early or early gene if placed behind the RSV promoter. Previous work in this laboratory by Bob Intine (1988) has shown that the RSV promoter of a recombinant HSV-1:Paa$^r$5(den

V) virus can efficiently express the den V gene. Using RNA primer extension experiments, it was determined that in RNA extracted at 6 hrs. post infection, the amount of den V extension product was similar to the amount produced by an early protein, gD. This suggested that both these genes were in the same kinetic class and therefore, would be expressed at the same time.

Den V protein expression by the recombinant constructed in the present work was determined by immunoprecipitation studies using a rabbit anti-T4 endonuclease V polyclonal antibody. These antibodies were directed against the purified native and active T4 endonuclease V protein. Labelled protein extracts from wildtype, recombinant or mock infected vero cells were mixed with this antibody. The autoradiograph of the eluted, immunoprecipitated proteins can be seen in figure 8. A band of 16 kd (the size of the den V protein) is present in the recombinant infected cell extracts at 6 hrs. after infection, but is absent in the other lanes thus confirming the presence of the protein in the cell. These results further suggest that the den V gene is expressed as an early protein

## EFFECT OF THE VIRALLY ENCODED DEN V GENE ON THE UV-SURVIVAL OF HSV-1

In this study, the uv survival curves for HSV-1 plaque formation on mammalian cell monolayers exhibited a two component nature. Such two component curves have been reported previously by some (Hall *et al.*, 1980; Lytle *et al.*, 1982; Ryan & Rainbow, 1986; Intine & Rainbow, 1990) but not all (Takebe *et al.*, 1978; Selsky & Greer, 1979) investigators. The reason for the existence of the second component is not known. It has been shown, however, that the second component is not due to multiplicity reactivation

or a heterogenous virion population (Lytle, 1971). This second resistant component may reflect the presence of an additional repair pathway for uv damaged HSV that is activated at high uv doses. It is possible also that this component results from repair by a recombination mechanism in which DNA is exchanged between damaged genomes at high uv doses (Hall *et al.*, 1980). Furthermore, since the introduction of uv damaged DNA into unirradiated cells have been shown to activate a mutagenic pathway (Cornelis *et al.*, 1981; Dinsart *et al.*, 1984), it is possible that a repair pathway might also be activated by the presence of these large number of DNA lesions in the incoming HSV genome.

**UV survival of HSV-1:den V in Transformed cells**

The ability of the den V protein to complement the repair deficiency in a SV40 transformed repair deficient cell line was examined. XP-4 cells are immortalized cells derived originally form XP12BE(group A) fibroblasts. Figures 9 and 10 show the uv viral survival of HSV-1:kos and HSV-1:den V in XP-4 and 293 cells. The second component $D_0$ values obtained in this study for HSV-1:kos in 293 and XP-4 cells are 85.34 $\pm$ 28.41 J/m$^2$ and 13.57 $\pm$ 3.39 J/m$^2$ respectively, which are comparable to the previously reported values of 62.5 $\pm$ 7.81 J/m$^2$ and 7.97 $\pm$ 0.53 J/m$^2$ (Intine, 1988). The presence of the den V gene in the viral genome was shown to enhance its uv-survival in XP-4 but not 293 cells. This is indicated also by the second component Do value of 22.86 $\pm$ 4.80 J/m$^2$(90% confidence) for HSV-1:den V in XP-4 cells, which is significantly greater than the Do value for HSV-1:kos in these cells. These results suggests that the den V protein can partially complement the DNA repair deficiency in the XP-4 cells. The calculated %HCR value of 15.9 $\pm$ 9.3 %

for HSV-1:kos is comparable to the 12.8 $\pm$ 2.5% obtained in a previous study (Intine, 1988) for XP-4 cells. Since the cells used in this experiment have been altered by transformation, it is possible that the ability of the den V protein to enhance the DNA repair capacity of the infected cell was likewise altered. Previous work conducted in our laboratory (Rainbow, 1981c) have shown that SV40 transformed cells posses a deficiency in their ability to repair uv damaged adenovirus. As a result, it is possible that this process of transformation may preclude the ability of the den V gene to restore uv-survival of HSV-1 to the level found in excision repair proficient cells.

**UV survival of HSV-1:den V in Normal Fibroblasts**

Figure 11 shows that the uv survival of wildtype and recombinant virus was similar when infecting normal repair proficient fibroblasts. The second component $D_0$ values of these two viruses are 43.43 $\pm$ 6.24 J/m$^2$ and 47.82 $\pm$ 5.65 J/m$^2$ respectively, and are not statistically significant. All $D_0$ values quoted in this study refer to the second component of the viral survival curve, which is thought to be an appropriate measure of cellular DNA repair capacity for a specific cell type. The result of the present work show that no significant difference exists between HSV-1:kos and HSV-1:den V virus survivals as measured by plaque forming ability on excision proficient cells. More importantly, they indicate that there is no intrinsic enhanced survival of the den V containing recombinant virus in normal fibroblasts.

The second component $D_0$ value for wildtype HSV-1:kos in normal cells lies within the previously reported values of 47.6 $\pm$ 6.8 J/m$^2$ (Intine, 1988), 83 $\pm$16 J/m$^2$ (Bueschleb, 1987) and 30.6 $\pm$ 0.3 J/m$^2$ (Lytle *et al.*, 1982). The

large amount of variation in the $D_0$ values observed may be due to a combination of factors, some of which may include the viral strain used, the cell line used, the irradiation conditions and the time of plaque scoring. These $D_0$ values were also used to calculate the percent host cell reactivation (%HCR) of virus in repair deficient cell lines. A comparison of the uv survival of virus in normal and mutant cells provides a quantitative measure of the DNA repair capacity of the mutant cells. This value can then be compared to the values obtained in assays that measure other endpoints of repair such as, unscheduled DNA synthesis, DNA repair synthesis or colony forming ability.

**UV Survival of HSV-1:kos in Xeroderma Pigmentosum Fibroblasts**

As discussed earlier, xeroderma pigmentosum (XP) cells are excision repair mutant cells deficient in the initial endonuclease mediated incision step of excision repair. These cells are known to exhibit a reduced ability to reactivate uv damaged herpes virus. When the uv survival of wildtype virus was assayed in cells from five different XP complementation groups, a range of survival levels was seen (figures 12 and 13). The degree of reduction in virus survival in the XP cells relative to the normal cells provides an indication of the extent of the repair deficiency exhibited by each complementation group. From figures 12 and 13, it is apparent that cells from XP12BE(group A) and XP1BR(group D) are the most repair deficient, and XP2RO(group E) and XP2YO(group F) are the least repair deficient. This distribution of repair deficiencies are in reasonable agreement with the previously reported studies measuring cell survival (Friedberg *et al.*, 1979).

XP12BE(group A) cells were the first to be examined. The second component $D_0$ value obtained in this study for HSV-1:kos in these cells was 11.74 $\pm$ 1.90 J/m$^2$. This is comparable to the Do value of 8.7 $\pm$ 0.5 J/m$^2$ reported in a previous study examining the reactivation of uv irradiated HSV-1:kos in the same cell strain (Intine, 1988). The calculated %HCR value for uv-irradiated HSV-1 in this study was 27.0 $\pm$ 5.7%. Other previously reported values include 18.43 $\pm$ 3.57% for HSV-1:kos in XP12BE(group A) cells (Intine, 1988) and 28.0 $\pm$ 8.2 % for HSV-2 in XP25RO(group A) cells (Ryan & Rainbow, 1986).

Although XP group D strains exhibit relatively high levels of repair replication in response to uv, their colony forming and HCR abilities after uv are relatively poor (Friedberg *et al.*, 1979; Rainbow, 1981). An examination of HSV-1:kos survival in XP1BR(group D) cells futher supports this finding. The second component $D_0$ value and %HCR value obtained in this study were 11.37 $\pm$ 1.50 J/m$^2$ and 26.2 $\pm$ 7.2%, respectively. This level of HCR is comparable to a previously reported value of 33% for uv-irradiated HSV-2 in XP6BE(group D) cells (Ryan & Rainbow,1986). The measurement of uv-survival of HSV-1:kos in XP2BE(group C) cells generated a $D_0$ value of 14.35 $\pm$ 2.87 J/m$^2$ and a %HCR value of 33 $\pm$ 11.3%.

UV survival of HSV-1:kos was also examined cells from XP complementation groups E and F. The second component $D_0$ values for XP2RO(group E) and XP2YO(group F) cells are listed in Table 1a as 27.14 $\pm$ 5.09 J/m$^2$ and 27.56 $\pm$ 4.59 J/m$^2$ respectively. From these values %HCR was calculated to be 62.5 $\pm$ 20.7% and 63.5 $\pm$ 19.7% for XP2RO(group E) and XP2YO(group F) cells respectively.

Host Cell Reactivation studies for uv-irradiated Adenovirus type 2 have enabled a ranking of the xeroderma pigmentosum complementation groups in terms of their repair abilities (Rainbow & Howes, 1979). Groups A and D were found to be the most repair deficient, groups E and F appeared to be the most repair proficient and group C was intermediate to these. This distribution of the repair deficiencies of the complementation groups further support the results obtained in the present study.

**UV Survival of HSV-1:den V in Xeroderma Pigmentosum Fibroblasts**

By using the recombinant virus expressing the den V protein, we proceeded to examine the ability of this gene product to complement the different repair deficiencies exhibited by cells from the XP complementation groups. As mentioned earlier, XP12BE(group A) cells are one of the most repair deficient in terms of the the level of uv induced unscheduled DNA synthesis, colony forming ability and host cell reactivation ability. This repair deficiency was partially complemented by the presence of the virally encoded den V gene (figure 14). The second component $D_0$ value of 21.71 $\pm$ 1.5 J/m$^2$ is two-fold greater than that obtained for HSV-1:kos in these cells.

By comparing this value to HSV-1:den V survival in normal fibroblasts, a %HCR value of 45.4 $\pm$ 8.5% can be determined. This statistically significant increase in the %HCR value (90% confidence) suggests that the presence of the den V protein is able to enhance the DNA repair capacity of the infected cell. Previous investigators have been able to partially restore the repair deficiency in SV40 transformed XP12RO(M1) group A cells by the transfection of the den V gene (Valerie *et al.*, 1987). Intine (1988) calculated %restoration values from that study to be between 20-27.5%. In this study, the

percentage restoration of uv viral survival was determined to be 25.2%, which is comparable to the calculated values.

XP group D cells, like XP group A cells are among the most uv sensitive with virtually no dimer removal capability (Zelle and Lohman, 1979). The ability of the den V gene product to complement this deficiency was determined via HCR studies. The pooled results of six experiments comparing the uv survival of HSV-1:kos and HSV-1:den V in XP1BR(group D) cells is illustrated in figure 16. The presence of the den V gene in the viral genome was found to increase the uv survival of the virus in these cells. As in the case for XP12BE(group A) cells, this enhanced survival was reflected in a significantly increased second component $D_0$ value of 19.28 $\pm$ 2.5 J/m$^2$ (90% confidence) for HSV-1:den V as compared to 11.37 $\pm$ 1.5 J/m$^2$ for HSV-1:kos. The calculated %HCR values for HSV-1:kos and HSV-1:den V in XP1BR(group D) cells are 26.2 $\pm$ 7.2% and 40.3 $\pm$ 9.98%. From these values the percentage restoration of the DNA repair deficiency in XP-D cells was calculated. As mentioned earlier, Arrand *et al.* (1987) transfected the den V gene into XP-D/Hela hybrids and found a 15.4-22.5% restoration of the excision repair defect as assayed for by colony forming ability. The percentage restoration of uv viral survival of 19.1% obtained in this study is comparable to these values.

The results here suggest that the den V gene can effectively enhance virus survival in XP cells from complementation groups A and D. Previous studies suggest that the repair of lesions in XP cells is limited by the availability of damaged sites to repair complexes (Mirzayans *et al.*, 1991). Since the den V endonuclease is a relatively small molecule, it has the ability to access and repair lesions that would otherwise be unrepaired. This

restoration of excision repair activity by den V seems to have a more pronounced effect on the second component of the viral survival curve. The less noticeable effect on the first component may indicate that the deficiency which is reflected by this component is not due to the repair of pyrimidine dimers.

Since complementation by den V is only partial (figures 19 and 20), one may conclude that den V recognizes and initiates repair of uv induced pyrimidine dimers which block transcription, but may leave behind some lesions that are lethal to the cell. Furthermore, the 6,4 pyrimidine pyrimidone photoproducts is a potentially mutagenic lesion also induced by uv light, but unlike pyrimidine dimers, cannot be repaired by the den V protein. It is this form of damage that may be contributing to the remainder of the deficiency. It is also possible that the prokaryotic den V enzyme may not function as efficiently in the eukaryotic repair system. The mammalian repair enzymes may fail to or are slow to recognize and complete a repair site initiated by the den V protein. Finally, the small size of the den V protein enables it to gain access to and act on all dimers regardless of their potential lethality. Since the quantity of this enzyme is limiting, this lack of selectivity means that not all potentially lethal dimers will repaired.

XP group C cells possess characteristics distinct from the other XP complementation groups. Excision repair in XP-C cells occurs at about 20-30% of the normal levels. This limited repair occurs in an apparent random fashion throughout the genome in proliferating cells but is clustered in localized domains in non-dividing cells. Mullenders *et al.* (1986) noted that repaired sites in uv irradiated confluent XP-C cells are preferentially located near the attachment sites of DNA loops to the nuclear matrix. Since

transcriptionally active genes are located proximal to the nuclear matrix, the limited repair in XP-C cells is thought to be located primarily in these transcribed regions of the genome. The occurence of this preferential and efficient repair of actively transcribed genes would also explain the ability of XP-C cells to repair uv induced potentially lethal damage and to restore uv inhibited RNA synthesis, both of which are associated with repair of active genes (Mayne *et al.*, 1982). It seems likely therefore, that separate damage recognition systems may exist in human cells for active and inactive chromatin and that the deficiency in the XP-C cells reflects the lost ability to repair inactive chromatin.

The results presented in this study demonstrate that the den V gene was able to improve virus survival in XP2BE(group C) cells to a small but significant degree (figure 15). However, the calculated $D_0$ values of $14.35 \pm 2.87$ J/m$^2$ and $17.56 \pm 3.23$ J/m$^2$ for the second component of the survival curve were not significantly different from each other. It is possible that since this cell type contains a greater repair proficiency than group A or D cells, the detection of correction of the repair defect may not be possible. In fact, if the %restoration of viral survival by the den V gene in XP2BE(group C) cells were assumed to be the same as in XP12BE(group A) and XP1BR(group D) cells, the predicted %HCR value would be within the $36.7 \pm 11.1$% value obtained in this study. Consequently, the second component $D_0$ value obtained for HSV-1:den V in XP2BE (group C) cells is consistent with the values obtained for group A and D cells.

Furthermore, it is possible that the increased survival may be due to an activity in the first component. This effect of the den V gene on the first component of the viral survival curve may be due in part to a deficiency in

the ability to remove pyrimidine dimers from silent genomic domains in XP group C cells. That is, the limited increase in the first component may be attributed to the small sized den V protein accessing thymine dimers in nontranscribed domains that the host cell repair enzymes cannot.

XP cells from complementation groups E and F were also examined in this study. Previously these two cell types have not been investigated in any great detail using viral reactivation of HSV-1. In both these cell lines however, the repair deficiency is not as severe as those demonstrated in the other XP complementation groups. In fact, the levels of unscheduled DNA synthesis relative to normals for XP-E and XP-F cells are relatively high at 50-66.5% and 38.2-46.7% respectively (Yamaguchie *et al.*, 1990). The den V endonuclease functions by specifically recognizing and incising at pyrimidine dimers. XP-E and XP-F cells are known to contain significantly less endonuclease sensitive sites (pyrimidine dimers) after repair of uv damage than the other complementation groups (Zelle and Lohman, 1979). It is therefore possible that these repair deficient cells may still retain some inherent incising ability.

A comparison of uv survival of HSV-1:kos and HSV-1:den V in XP2RO(group E) cells and XP2YO(group F) cells is illustrated in figures 17 and 18. A significant difference in survival of uv-irradiated virus was not detected following infection of these cell types with HSV-1:den V. The second component $D_0$ values for XP2RO(group E) and XP2YO(group F) cells were determined to be $31.02 \pm 4.43$ J/m$^2$ and $23.35 \pm 2.6$ J/m$^2$ for HSV-1:den V and were not significantly different from wildtype. As discussed earlier for XP2BE(group C) cells, it is quite possible that the effect of the den V gene in these two cell strains may not be detectable. Both of these cell types possess a

relatively large degree of repair proficiency, which would make detection of any enhancement quite difficult. Results comparing the degree of removal of endonuclease sensitive sites from group E and F cells indicate that group F cells possess relatively less pyrimidine dimers following repair than group E cells. Consequently, restoration by the den V gene product is expected to be greater in group E cells than in group F cells, which is consistent with the results of this study.

**UV survival of HSV-1:kos and HSV-1:FUS-7 kan in XP Fibroblasts**

It was thought possible that the disruption of the gI gene in the Herpes virus could in some manner have contributed to the observed results in the XP fibroblasts. To rule out this possibility, a comparison of the uv-survival of a $gI^-$ mutant virus, HSV-1:FUS-7 kan, to the wildtype virus was conducted. Figures 21 and 22 illustrates the survival curves for these two viruses in cells from XP12BE(group A) and XP1BR(group D). The second component $D_o$ values for these viruses are $12.77 \pm 0.26$ J/m$^2$ and $13.16 \pm 2.33$ J/m$^2$ in XP12BE(group A) cells, and $12.77 \pm 0.41$ J/m$^2$ and $10.59 \pm 2.23$ J/m$^2$ in XP1BR(group D) cells (Table 1b). Since the level of survival of the gI mutant virus is the same as that of the wildtype virus in both XP group A and D cells, the observed enhancement of viral survival in the different cells must be specifically due to the inserted den V gene and not to the absent gI gene.

**UV survival of HSV-1:den V in Trichothiodystrophy cells**

The identification of cellular deficiencies in the ability to repair DNA damage in individuals with severe cancer prone genetic disorders suggests a possible association between defective DNA repair and cancer. Some TTD

patients however, contain a defect in repair of uv damaged DNA but are not cancer prone. Using cells from these individuals, one may be able to more clearly evaluate the connection between DNA repair capacity and cancer susceptibility.

Of the three existing categories of TTD cells, one category has been shown to be photosensitive and deficient in excision repair (Broughton et al., 1990). Complementation studies with XP fibroblasts have shown that in all of the TTD repair deficient strains the genetic defect is the same as that present in XP group D cells (Stefanini *et al.*, 1986). In this work, a primary fibroblast strain from this excision repair deficient group was shown to be indistinguishable from cells of XP1BR(group D) in their reduced ability to support uv-irradiated HSV-1:kos (figure 23). Since the den V gene product was shown in this and other studies to be able to partially complement the deficiency in XP-D cells, it was of interest to examine survival in TTD cells.

UV viral survival of HSV-1:kos and HSV-1:den V in TTD8PV cells is shown in figure 24. The presence of the den V endonuclease was found to significantly enhance uv survival of the virus to a similar level as found for XP1BR(group D) cells (figure 25). The second component $D_o$ values, however, were not significantly different between wildtype and recombinant viruses(Table 1a). These result support the current hypothesis that TTD and XP group D cells possess the same genetic defect.

The shape of the HSV-1:kos and HSV-1:den V survival curves in TTD8PV cells differs from that in XP1BR(group D) cells (figures 23 and 25). The clearly defined two-component nature of the curve exhibited in XP1BR(group D) cells was not seen in TTD8PV cells. This suggests that the effect of the den V protein on viral survival may be different in these two cell

types. In XP1BR(group D) cells, the DNA endonuclease was able to significantly alter the slope of the second component of the viral survival curve. The results in TTD8PV cells however, show no noticeable effect of this enzyme on the second component and a small effect on the first component of the survival curve.

**UV survival of HSV-1:den V in LPM2A cells**

Although rodent and human cells exhibit similar survival levels following uv irradiation, their repair efficiencies are quite different. UV irradiated human cells for example, are able to remove 76% of the T4 endonuclease sensitive sites, whereas rodent cells can only remove 25% of these sites (Van Zeeland *et al.*, 1981). Inspite of this lower overall repair efficiency, rodent cells are still able to achieve high survival because of their selective repair of essential genes (Bohr *et al.*, 1985, 1986, 1987; Smith, 1987; Hanawalt, 1991).

An alternate measurement indicative of excision repair is the ability of host cells to reactivate nuclear replicating viruses. Previous investigators have shown that HCR of uv irradiated HSV in mouse cells is reduced compared to that in human cells (Lytle, 1971; Glazier *et al.*, 1982). It was thought that the presence of a virally encoded DNA endonuclease might be able to enhance virus survival in these rodent cells possibly by increasing the level of pyrimidine dimer excision repair. As a result, the uv survival of HSV-1:kos and HSV-1:den V were examined in LPM2A cells. No significant difference in survival however, was seen for these two viruses (figure 26). This indicates that the lower level of excision repair present in rodent cells

was not the result of an insufficient amount of incision enzyme. Instead the results of this study lend support to the idea that the excision repair process in rodent cells may be concentrated on the correction of lesions located in vital areas of the genome.

CELLULAR REPAIR CAPACITY

The treatment of cells with DNA damaging agents prior to infection has been successfully applied to provide a measure of the cellular repair capacity (Lytle *et al.*, 1974; Coppey and Nocentini, 1976; Lytle & Hestler, 1976; Coppey *et al.*, 1979). The more common method of assaying for post uv colony forming ability was found to be inappropriate when dealing with cell strains that had inherently poor clonogenic ability. Moreover, this technique required that the cell be grown in isolation from its neighbours, which does not a accurately reflect the in vivo situation.

A reduction in the ability of human and monkey kidney cells to support herpes virus infection after treatment with uv light has been previously reported (Lytle *et al.*, 1976; Coppey & Nocentini, 1976; Ryan & Rainbow, 1986). A number of studies using HSV in normal and XP cells have shown a greater decrease in capacity in XP cells than in normal cells due to the presence of repair defects in the XP cells. In this study, the cellular repair capacity of XP12BE(group A) cells was measured by pre-irradiating the cells prior to infection with virus. Since the cell must repair this uv damage before it can support viral growth, the yield of virus reflects the cell's DNA repair capacity. In this and other studies the den V gene was shown to enhance virus survival by correcting pyrimidine lesions within its genome, therefore, it was thought that this viral protein might also enhance the cell's

ability to repair its uv damaged genome and in so doing improve viral survival. Figure 27 shows the pooled results of two separate experiments in which XP12BE(group A) cells were infected immediately after irradiation. The survival of the recombinant virus in these preirradiated XP cells was not significantly different from wildtype virus.

Experiments were also performed in which cells were infected with virus prior to irradiation. It was thought that this would enable the virally encoded den V gene product to be synthesized and made available for use in the cell before uv-irradiation. This approach however, likewise proved to be unsuccessful at improving the capacity of the cells for plaque formation of the recombinant virus. It is possible that the virally encoded den V gene product was not present in sufficient quantity to aid the cell in its DNA repair functions. The amount of den V protein produced by the virus may be only enough to repair the lesions occuring in the smaller sized viral genome and not in the larger cellular genome.

It is also possible that the effect of the den V gene product on cellular capacity may not be detectable using a plaque formation assay. Plaque scoring relies in part on a virus replicating within a cell to produce progeny, which is able to replicate and lyse neighbouring cells to generate a plaque. As discussed previously, irradiation of cells prior to viral infection reduces the cell's ability to support virus infection. If however, this cellular damage could be corrected by the den V protein before it becomes lethal, then the survival of the virus may be enhanced in these infected cells. The cells adjacent to these infected cells however, do not have the benefit of this protein before they are exposed to radiation, hence, their reduced capacity to support viral infection may be masking any den V effect that is present. A more appropriate assay

system to measure the effect of the den V gene may be to determine the quantity of progeny produced by the preinfected cells. With such a progeny assay (Lytle et al., 1982), one may be able to determine the ability of the den V gene product to enhance the cell's capacity to support virus.

## PLAQUE SIZE DIFFERENCES BETWEEN VIRUSES

The plaque size of the gI$^-$ viruses, HSV-1:den V and HSV-1:FUS-7kan, was substantially smaller than HSV-1:kos when infecting human fibroblasts, but not when infecting vero, XP-4 or 293 cells. The difference in plaque size between these two viruses occurred only when infecting fibroblasts and not when infecting vero cells. Initially it was thought that the overexpression of the den V gene might be toxic to either the cells or the virus, resulting in a smaller burst size. However, when a different recombinant virus, HSV-1:FUS7 kan, was used to infect fibroblasts, a similar difference in plaque size was produced.

Since the glycoprotein I gene (gI) was disrupted in these recombinant viruses, it is possible that the absence of this glycoprotein gene may be somehow responsible for this microplaque phenotype. Preliminary experiments from another laboratory involving a virus possessing a mutant gE gene has also generated microplaques (D. Johnson, personal communication). Both glycoproteins I and E have been shown in the past, to be genes dispensible for growth in tissue culture (Longnecker *et al.*, 1987). Moreover, these two glycoproteins are known to associate in a complex to produce an $F_c$ receptor of HSV-1 (Johnson *et al.*, 1988). Further studies need to be conducted to acquire a better understanding of the association between gI and gE to plaque size in human fibroblasts.

## CONCLUSIONS

In this study the uv survival of HSV-1:den V was shown to be significantly greater than wildtype HSV-1 when infecting cells from XP complementation groups A, C and D. The enhanced uv viral survival however, did not reach levels obtained in normal fibroblasts, indicating that restoration of the XP defect was only partial. This may be due in part to the fact that the den V gene product specifically repairs pyrimidine dimers while leaving behind other potentially lethal lesions.

The results of this work demonstrate that HSV can be effectively used as a vector for studying the expression of DNA repair genes. Moreover, the use of the 3' LTR promoter from RSV appears to be a suitable choice of eukaryotic promoter since abundant transcription was found to occur after infection by the virus. There are a number of advantages in the use of HSV for such studies, some of which include a large capacity for gene insertion, a rapid life cycle and a broad host range. This system also allows one to examine DNA repair fidelity by measuring forward mutations in the virally encoded thymidine kinase gene (Lytle *et al.*, 1974). Further studies employing such a system could provide information for a better understanding of DNA repair, mutagenesis and carcinogenesis.

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