Genes Important In The U.V. Survival Of Herpes Simplex Virus

CONSTRUCTION OF A HERPES SIMPLEX VIRUS TYPE 1 (HSV 1) INSERTION MUTANT CONTAINING THE BACTERIOPHAGE T4 DEN V GENE : GENES THAT ARE IMPORTANT FOR THE UV SURVIVAL OF HSV 1

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ABSTRACT

The den V gene from bacteriophage T4 codes for a small, pyrimidine dimer specific, endonuclease. Recent studies have shown that transfection of the gene into DNA excision repair deficient, Xeroderma Pigmentosum cells, can partially restore the excision repair ability of the cells and results in an increased resistance to UV light.

In this study the den V gene has been inserted into Herpes Simplex Virus type 1 (HSV 1) in order to determine if HSV 1 can be used as a suitable vector for studying DNA repair genes. A 1.9 kb cartridge containing the den V gene, the 3' LTR of Rous Sarcoma Virus as the promoter, and the SV40 polyadenylation signals was inserted at the thymidine kinase locus of the virus. Properly initiated transcription form the construct, HDV 1, was verified by primer extension analysis.

The Host cell reactivation of this virus and several other strains of HSV 1 were examined in normal and Xeroderma Pigmentosum cells. The results from these experiments suggest that both the viral DNA polymerase and thymidine kinase genes play important roles for the survival of UV irradiated HSV 1.

DEDICATIONS

This work is dedicated to my wife, Karen and my parents Kitty and Don for all their love and support which made this thesis possible.

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INTRODUCTION

Deoxyribonucleic acid (DNA) encodes the information required by a cell for proper function. In order for the cell to continue to function properly and maintain its heredity the DNA sequence must be stable. However, the DNA is constantly being altered, either spontaneously, through replication errors or through environmental agents such as chemicals or radiation.

The major source of error that occurs during normal metabolism of the DNA is mispairing of nucleotide bases during replication (Friedberg, 1985). There are many factors that affect replication fidelity including the presence of a proof reading function in the polymerase molecule and the presence or absence of certain DNA binding proteins (for a review refer to Loeb & Kunkel, 1981).

Spontaneous base mutations also occur at a high frequency in the cell. If a base undergoes a spontaneous tautomeric shift to an isoform the hydrogen bonding pattern for that base is altered and during replication can cause an incorrect base to be inserted in the growing strand (Watson, 1976). Spontaneous deamination may also occur which can change cytosine, adenine and guanine into uracil, hypoxanthine and xanthine respectively (Lindahl, 1979). The new products can then cause mispairing during replication. Loss of bases spontaneously also occurs in the cell which can lead to strand breakage at the newly created apurinic or apyrimidinic (AP) sites (Greer & Zamenhof, 1962).

Chemical agents from the environment can produce a

wide variety of alterations in the DNA. Alkylating agents react with either a nitrogen or oxygen molecule in the nucleotide base (Singer & Kusmieric, 1982). These alkylation events can then cause DNA-DNA cross links and protein-DNA cross links, both of which can inhibit transcription and replication (Smith & Hanawalt, 1969 ; Nietert *et al.*, 1974). Methylating agents, depurinization agents and base analogues also exist and serve to alter the DNA sequence by altering the nucleotide bases (Robbins *et al.*, 1974).

Ionizing radiation has the most potential to be immediately lethal because its most predominant effect on the DNA is single and double strand breaks (Ward, 1975). Minor effects of this type of radiation include base alterations of free bases and of those incorporated into the DNA (Hariharan & Cerutti, 1972).

The most studied type of damage and the one most used for studying DNA repair mechanisms is that produced by ultraviolet (UV) irradiation. The major type of damage is the formation of cyclobutane dimers of adjacent pyrimidines (Smith & Hanawalt, 1969). This type of damage can block transcription, replication and cause mutations (Friedberg, 1985). Minor damage caused by this type of irradiation include ; 6-4 pyrimidine-pyrimidone noncyclobutane type adducts, pyrimidine hydrolysis, production of thymine glycols, DNA cross links and single strand breaks (Kittler & Lober, 1977).

The Importance Of DNA Repair Mechanisms

The above alterations could potentially change the coding sequence of the DNA or impair its ability to be transcribed and replicated which can lead to cell death. Sequence maintenance is not only important for cell survival but also for whole organism survival. This is witnessed by the fact that alterations in the DNA sequence can lead to the development of certain diseases, cause premature aging of cells and most importantly, lead to the transformation of a normal cell into a malignant one (Zasukhina, 1987).

The cellular systems which provide DNA homeostasis are the DNA repair mechanisms. Support for the idea that DNA repair mechanisms are necessary for cell or organism survival comes from many human diseases including, the human disease Xeroderma Pigmentosum (XP). Skin fibroblasts taken from XP patients have a decreased ability to repair UV damage to their DNA and these patients also have an increased rate of sunlight induced skin cancers (Friedberg, 1985).

Bacterial DNA Repair Mechanisms

In the past, studies on DNA repair and its associated mechanisms were performed mainly on the prokaryotic system and the enzymology of the bacterial system is well understood (Little & Mount, 1982). The study has been aided by the isolation and characterization of a large number of mutants deficient in DNA repair enzymes (Hanawalt *et al.*, 1979).

There are five known mechanisms that allow a bacterial cell to either repair DNA lesions or bypass them. These include; enzymatic photoreversal (Sutherland, 1978), post replication repair (Lehmann & Karran, 1981), branch migration - bypass replication (Higgins *et al.*, 1976) and excision repair (Hanawalt and Setlow,1975).

The repair mechanism that is by far best understood is excision repair. Excision repair in bacteria is mediated by a large number of enzymes (Friedberg, 1985). There are two modes of excision repair that exist in prokaryotes, they are termed base excision repair (Friedberg *et al.* 1978) and nucleotide excision repair (Hanawalt, 1978).

The enzymatic event that characterizes base excision repair is the hydrolysis of the N-glycosylic bond linking the damaged or altered base to the DNA backbone (Duncan, 1981). The enzymes that catalyze this event are DNA glycosylases which are highly specific for a certain type of damage. Removal of the altered base by a DNA glycosylase generates an apurinic apyrimidinic (AP) site in the DNA. Experiments have shown that the site can be restored by an insertase enzyme which replaces the correct base at the altered site (Deutsch & Linn, 1979). However, most of the AP sites become targets for AP endonucleases which hydrolyze the phosphodiester backbone, thereby creating a nick, in the DNA (Lindahl, 1979). The post incision events for base excision are the same as for nucleotide excision and will be discussed below.

Nucleotide excision repair is utilized for repairing damage that causes severe helical distortions such as pyrimidine dimers, cross links and other various chemical adducts (Grossman, 1981). The initial incision step is performed by a complex of proteins; UVR A (Sancar *et al.*, 1981a), UVR B (Sancar *et al.*, 1981b) and UVR C (Yoakum *et al.*, 1981). The following model has been presented by Seeberg and Steinum 1982 for the mode of action of this complex. DNA damage induces the protease activity of the Rec A protein. This in turn degrades the Lex A protein which is bound to the UVR genes promoters and has them shut off. The stimulation of the UVR genes then allows the initial incision step in the following manner. UVR A protein binds to the DNA near the damaged site. UVR B then binds to A and the two translocate to the direct site of the damage and upon the binding of UVR C the backbone is incised. In the past, it was thought that the incision was made immediately 5' to the damaged site and the actual excision performed by a 5' to 3' acting exonuclease (Hanawalt *et al.*, 1979). However, more recent *in vitro* studies have shown that two incisions occur. The first incision occurs always at the eighth phosphodiester bond 5' to the damage and the second occurs at the forth or fifth 3' to the damage (Rupp *et al.*, 1982). This, therefore, releases an oligomer of approximately 12 bases. It is still not known which of these mechanisms act in the cell and it is possible that both may act at the same time, but, it is now felt that the second mechanism is the one most probably employed by the

cell (Husain et al., 1985).

The enzymes that perform the post incision events are the same for both modes of repair. It appears that the first activity is a 5' to 3' exonuclease activity. This could be performed by a number of known enzymes, of, which some are associated with polymerase activities (Grossman, 1981). This, therefore, leaves a gap in the DNA and resynthesis of the excised patch must take place.

There are three DNA polymerases known to exist in bacteria that could resynthesize the excised DNA. Pol I is thought to be the prime candidate for this function because it is the only one that is able to bind nicks in the DNA (Hanawalt *et* al., 1979). This enzyme would also be ideal, because it has an exonuclease activity associated with it and could, therefore, perform two necessary functions.

However, Pol 1 is not the only polymerase that can resynthesize the patches. It has been shown that mutants deficient in pol 1 can carry out excision repair and in fact synthesize more DNA than pol 1^{+} strains (Cooper and Hanawalt, 1972a). In reality, more DNA is synthesized, but, less repair occurs. This is because the patch size in the mutant strains can be up to several hundred nucleotides, where in the wild type the patch size is somewhere between 20 and 30 nucleotides (Cooper and Hanawalt, 1972b).

The final step of excision repair is the ligation of the newly synthesized DNA. The enzyme that performs this step is a DNA ligase (Gottesman *et al.*, 1973).

Mammalian Repair Systems

Four mechanisms of DNA repair have been postulated to exist in mammalian cells; these include enzymatic photoreactivation, post replication repair, an sos type of repair and excision repair (Hanawalt, 1979).

Very little is known about the molecular mechanisms of excision repair in eukaryotes due to the lack of mutants available for study. However, studies have been stimulated by the availability of excision repair deficient cell lines from patients suffering from the autosomal recessive disease xeroderma pigmentosum (XP), of, which there are 9 complementation groups (Thielmann *et al.*, 1986)

As in the prokaryotic system, both base and nucleotide excision repair exist in mammalian cells and are thought to occur by relatively the same basic steps as in the bacteria (Lehmann & Karran, 1981).

There are several DNA glycosylases present in mammalian cells which act to repair a wide variety of base damage (Duncan, 1981 ; Lindahl, 1979 ; Cathcart & Goldthwait, 1981). Similar to the bacterial system enzymes are present that can either replace the base directly (Lindahl, 1979) or the AP site becomes a target for the action of one of the many isolated AP endonucleases (Linn et al., 1978).

As in bacterial cells, nucleotide excision repair in mammalian cells requires recognition of the damaged DNA followed by an incision event, excision of the damage, repair

synthesis and finally ligation of the newly synthesized DNA. The enzymology of all of these steps is very poorly understood.

Most of current research in this area is being focused on the incision step. Strains from all of the complementation groups of XP cells have been studied and it is apparent that all nine groups are deficient in the initial incision step (Fischer *et al.*, 1985). This suggests that there may be as many as nine loci involved in a mammalian complex like the UVR ABC complex in bacteria. Explanations for the large number of loci needed include the idea that not only is an incising activity necessary for incision but preincision events (ie., processing of the chromatin to allow access to the DNA) are also needed (Karentz & Cleaver, 1986). Another explanation may be that some of the loci code for genes that have a regulatory function in controlling the other loci involved in the pathway (Karentz & Cleaver, 1986b).

Until recently, it has always been thought that the initial incision in the damaged DNA was either to the 3' or 5' side of the lesion. But, the most recent model for the incision step in mammalian cells has been proposed by Paterson *et al.*, (1987), based on experiments performed in XP group D (XP D) cells. From the data, it is suggested that hydrolysis of the internal phosphodiester bond of the dimerized pyrimidines constitutes the first incision in the DNA. This leaves the strand held together by just the

dimerized bases. It is postulated that this initial step is performed in order to create a severe helix distortion which the putative repair complex can recognize and then hydrolyse the backbone either 5' or 3' to the damaged bases.

Once the backbone has been incised the excision of the damage and resynthesis must be performed. As seen earlier in the bacterial system, pol I combines both exonuclease and polymerase activities and may perform both steps on its own. In mammalian cells, no single polypeptide has been isolated that can perform both of these functions. However, polymerase alpha is involved in a 640 kD multipolypeptide complex which is termed the alpha polymerase holoenzyme (Ottiger *et al.*, 1987). This holoenzyme has been shown to have a polymerase function, 3' - 5' exonuclease function and a weak 5' - 3' exonuclease function amongst others (Skarnes *et al.*, 1986). If mammalian cells do not use an exonuclease that is associated with a polymerase to excise the damage then it is possible that they use one of the many exonucleases that have been isolated and shown to be able to excise dimers from DNA (Grossman *et al.*, 1981).

Repair synthesis is performed by one of the four polymerases that have been isolated from mammalian cells (Weissbach, 1977). Of the four, only polymerase gamma has not been implicated to have a role in DNA repair. Studies performed in order to elucidate which polymerase molecule is most important have been done using selective inhibitors such as aphidicolin (Ikegami *et al.*, 1978). This drug has been

shown to inhibit polymerase alpha (Ikegami *et al.*, 1978) and more recently polymerase delta (Mitchell *et al.*, 1986). Delta polymerase has been shown to have both a polymerase function and a highly accurate 3' - 5' exonuclease function (Wahl et al., 1986).

When aphidicolin is added to normal fibroblast cells, XP variant fibroblast cells (normal excision repair levels), Cockayne syndrome fibroblasts, Bloom syndrome fibroblasts or XP D fibroblasts, U.V sensitivity of the cells increases to levels that approach the sensitivity of XP A cells (lowest levels of excision repair). However, when it is added to XP A cells the cells are not further sensitized which indicates that the excision repair pathway is dependant on an aphidicolin sensitive polymerase (Tyrell & Amaudruz, 1987).

XP C cells have U.V. sensitivity levels between that of normal cells and XP A. It was therefore expected that these cells would be sensitized by the aphidicolin like the other cells that have intermediate levels of excision repair. But when the experiments were performed the XP C cells were found not to be sensitive to aphidicolin. This implies that there may be more than one excision repair pathway functioning at the same time in the cell and that XP C cells may lack the aphidicolin sensitive pathway but be proficient in another pathway which performs a minor amount of repair. It has been suggested that the difference in the pathways is due to the difference in the polymerase enzyme used. Through the use of aphidicolin it has also been shown that both an

aphidicolin sensitive polymerase and an aphidicolin insensitive pathway may act together on the same lesion sites to repair the damage with the aphidicolin sensitive polymerase performing between 85 and 90 % of the repair in normal cells (Tyrrell *et al.*, 1985). Therefore, it has been suggested that possibly the repair synthesis performed in XP C cells may be carried out by the aphidicolin insensitive pathway which has been postulated to include polymerase beta.

Using aphidicolin to inhibit the polymerase molecules leaves the possibility that repair synthesis is done by either polymerase alpha or polymerase delta. Most recently, it was noted that polymerase delta is more sensitive to ddTTP than polymerase alpha (Wahl et al., 1986). When cells are pretreated with ddTTP, it has been shown that they become highly sensitive to UV irradiation, which suggests that polymerase is most likely responsible for repair synthesis (Dresler and Kimbro, 1987).

In the above study it was shown that not only is repair synthesis decreased, but, actual incision of the backbone also decreases. Therefore, it appears that the aphidicolin sensitive polymerase involved in repair appears to be involved in the repair pathway to a greater extent than just resynthesizing the excised patch. These results can be best explained if the polymerase is involved with the incision enzymes in a reversible complex.

Excision repair synthesis has been shown to be error free. In 1980 Yang *et al.* showed that although normal

fibroblasts survived much better than XP group A fibroblasts (either no or very little excision repair ability) both cell types had the same mutation frequency when the cells were exposed to (±)-7B,8 -dihydroxy-9 ,10 -epoxy-7,8,8,9,10-tetrahydrobenzo[a]pyrene and normalized to the same cell survival (Yang *et al.*, 1980). This type of result has also been reported for Host Cell Reactivation Studies of UV irradiated HSV 1 (Lytle *et al.*, 1982). In this study, both normal and XP group A fibroblasts were infected with UV irradiated HSV 1. Mutations in the thymidine kinase (TK) gene were then assayed for by titering the progeny with a drug against TK + virus. It was found that the mutation rates were the same for both types of cells, when they were normalized to the same number of lethal hits. This again indicates that the excision repair pathway is error free.

The last step in the excision pathway is ligation of the newly synthesized DNA to the rest of the molecule. In the mammalian system it appears that the molecule polynucleotide ligase (Lindahl *et al.*, 1969), is the enzyme responsible for performing this final step.

Mammalian cells like their bacterial counterparts perform long patch and short patch repair. However, there exist very few similarities between the two systems. Short patch repair in the mammalian system operates on damage caused by ionizing radiation such as X rays or chemicals that produce X ray like damage. The size of the repair patch is between 3 and 4 nucleotides and repair is complete within a

couple of hours (Regan & Setlow, 1974). Long patch repair acts on damage caused by U.V. light and chemicals which cause UV light damage. This repair process has patch sizes from 15 to 100 nucleotides and can take up to 24 hrs. to complete (Smith, 1978).

Xeroderma Pigmentosum

The major clinical manifestations of the disease, XP, are ; increased photosensitivity, a high incidence of skin cancer and neurological abnormalities (Setlow, 1978). Through the use of cell fusion experiments 9 complementation groups (A-I, plus one variant) of XP cells have been classified (Sidik and Smerdon, 1987).

The exact defect in XP cells is not known but a large number of phenotypic alterations have been reported which show that XP cells are deficient in excision repair. Cells taken from XP patients, in general, have reduced post UV colony forming ability (Andrews *et al.*, 1978), reduced unscheduled DNA synthesis (repair synthesis) (Friedberg *et al.*, 1979) and also a decreased ability to reactivate UV damaged viruses including ; Herpes Simplex Virus type 1 (HSV 1) (Bueschleb, 1987 ; Lytle 1971), HSV 2 (Ryan & Rainbow, 1986), Adenovirus (Rainbow, 1980 ; Day, 1974) and SV40 (Abrahams & Van der Eb, 1976). The severity of these defects differ for each of the different complementation groups (Thielmann & Edler, 1986). HCR studies of adenovirus type 2

have enabled a ranking of the complementation groups in terms of their repair abilities. Groups A and D have between 3-7 % normal repair, group B, 11 %, group C, 11-35 %, group F, 22 %, group E, 47 % and the variant between 57 % and 100 % of normal levels (Rainbow, 1981). The reason the variant is so high is thought to be due to the fact that the defect in these cells is in post replication repair and not excision repair (Lehmann *et al.*, 1977).

The step in which the XP cells appear to be deficient is in either the preincision or the actual incision step (Friedberg *et al.*, 1979). Initial studies were done by looking at the single strand molecular weight of DNA from XP versus normal cells shortly after UV irradiation (Fornace *et al.*, 1976). In these studies, the XP DNA versus normal DNA was of higher molecular weight indicating a lack of incision ability. Other studies looked for endonuclease sensitive sites in the DNA from cells which were given sufficient time to repair following UV irradiation. In these cases (XP A C & G) pyrimidine dimer specific endonucleases from either bacteriophage T^4 or M. *luteus* are used to nick extracted DNA. It has been shown that the single strand molecular weight of the DNA from XP cells is much lower, therefore, more dimers were present (ie. less repair) (Zelle & Lohman, 1979).

Knowing that the defect is in the incision step and there are 9 complementation groups leads to the question ; why are there so many loci involved in the incision step ? Besides the obvious incision process, in order for excision

repair to take place it has been shown that chromatin rearrangements (unfolding) are necessary for the pathway to proceed (Lieberman *et al.*, 1981). Also, it has been shown that nucleosome structure may play a role in the repair pathway (Sidick & Smerdon, 1987). Therefore, it is possible that some of the XP complementation groups are not defective in the incising activity, but, are unable to gain access to the DNA properly. Further support of this idea is the fact that cells from groups A, C and G have limited repair abilities but protein extracts from these cells can remove dimers from naked DNA almost as well as protein extracts from normal cells (Mortlemans *et al.*, 1976). Recent evidence suggests that in normal cells the amount of repair and the speed with which it is performed is greater in essential DNA versus bulk DNA, therefore, there may be targeting functions as well (Mellon *et al.*, 1986). This is further supported by the fact that group C cells, which survive 11 - 30 % as well as normal cells, actually have 100 % repair abilities clustered on 25 % of the genome and none on the other 75 %. This data however could be an artifact due to the processivity of the repair complex (Ganesan *et al.*, 1986).

Excision repair deficiencies are not the only defects in XP cells. Extracts from some complementation groups of XP cells have also been shown to have a reduced amount of photoreactivating enzyme (Wagner *et al.*, 1975) and a reduction in post replication repair (Lehmann, 1978).

The Bacteriophage T_4 Endonuclease V Gene (den V)

Bacteriophage T_4 is one of few viruses known to code for an enzyme that is used specifically for DNA repair (Frieberg, 1985). The first studies conducted on the phage showed that the T_4 virus was approximately twice as resistant to UV irradiation than T_2 or T_6 (Luria, 1947). This finding prompted many studies to be conducted on the T_4 virus. At first, it was thought that T_4 was lacking a gene that the T_2 and T_6 had which rendered them more sensitive (Streisenger, 1956). But, through coinfection experiments and genetic crosses it was shown that T_4 actually carries a gene that conferred UV resistance and this gene was termed the v gene (Harm, 1961). The next studies showed that the gene is required for the removal of thymine dimers in the T_4 DNA (Setlow & Carrier, 1968). This requirement was further defined to show that the gene product was necessary for incision of the DNA and that it acted solely on UV irradiated DNA (Sekiguchi *et al.*, 1970). The gene was then termed DNA endonuclease V (den V) (Wood & Revel, 1976).

The nomenclature for this gene is a bit misleading because den V more recently has been shown to be a 16 kDa pyrimidine dimer specific DNA glycosylase *in vivo* (Radany & Friedberg, 1982). DNA sequencing and studies using amber mutations have revealed a catalytic site for a 3' AP endonuclease activity (Valerie *et al.*, 1984). Unfortunately, this activity has been detected only *in vitro* but is

postulated to work *in vivo* as well (McMillian *et al.*, 1981).

A model for the action of the den V gene has been proposed (Friedberg, 1985). In the first step, the glycosylase activity catalyses the hydrolysis of the 5' glycosyl bond of the dimer. This leaves the pyrimidine attached to the 3' partner of the dimer through the cyclobutane ring (Lindahl, 1982). Cleavage of the base leaves an AP site in the DNA at which the 3' AP endonuclease activity can act to cleave the backbone leaving a nick in the DNA. The subsequent repair steps are performed by cellular enzymes.

The direction of more recent research performed on the den V gene has been to determine if it can complement cells deficient in the incision step of excision repair. Initial studies in this line were performed using purified or partially purified preparations of the den V protein. In 1979, Shimizu and Sekiguchi showed that upon introduction of the den V protein into permeabilized *E. coli* cells an increase in UV survival of the cells was observed (Shimizu & Sekeguchi, 1979). The increase was observed in UVR A, B, and C mutants but not in wild type cells indicating that the den V gene can complement the defect in the mutant cells. More recently, the den V gene has been cloned by several groups and is available in both prokaryotic and eukaryotic expression vectors (Lloyd & Hanawalt, 1981 ; Valerie *et al.*, 1985 ; Recinos *et al.*, 1986). Introduction of one of these expression plasmids into UVR A, B or C mutant *E. coli* cells

results in an increase in the UV survival of the cell (Recinos *et al.*, 1986). Thus, the DNA mediated rescue of the phenotype shows the den V genes abilities to complement an incision defect and rules out any arguments of contamination problems like in the earlier protein mediated rescue experiments.

The next logical progression was to determine if the den V gene can complement an incision defect in mammalian cells. Initial studies were done using Sendai virus fusion techniques (Tanaka *et al.*, 1975) or microinjection experiments (de Jonge *et al.*, 1983). In these experiments, purified protein preparations of den V protein were introduced into XP cells (groups A-G) and it was found that the den V gene could restore unscheduled DNA synthesis to near normal levels.

The first mammalian cells that the den V DNA were transfected into was Chinese Hamster Ovary cells (CHO) because of the ease with which transformants can be isolated and grown (Valerie *et al.*, 1985). Rodent cells in general have reduced abilities to repair UV induced DNA damage and have little nucleotide excision directed against pyrimidine dimers (Yagi, 1982). There is, however, an XP like CHO cell line, UV5, which, although, is not identical to the XP defect, also cannot perform the initial incision step (Hoy *et al.*, 1985). Therefore, this system is like the human XP versus normal system. Upon stable integration of den V DNA into the genome it was found that the den V gene could

increase survival of UV5 cells but had no effect on normal cells. This was assayed for by colony forming ability, unscheduled DNA Synthesis and detection of dimer incision. From these three assays it was determined that the den V gene could complement approximately 50 % of the UV5 defect.

These types of experiments most recently have been conducted using XP cells. The first report of this nature came from the lab of Valerie *et al.* in 1987. In this study SV40 transformed XP group A cells were transformed with the den V gene. Partial stable complementation of the UV survival defect was produced upon stable integration of the DNA into the genome (Valerie *et al.*, 1987a). Survival was assayed by colony forming ability after UV irradiation and the ability to perform repair synthesis. These assays revealed the fact that the den V gene could complement 20-27.5 % of the defect in the XP group A cells. These authors also showed the presence of the den V protein in the cells through immunofluorescence using monoclonal antibodies raised to the den V gene. Studies involving XP D / Hela hybrid cells, which retain the repair characteristics of the XP cell, have also been shown to be complemented by stable integration of the den V gene into the genome (Arrand *et al.*, 1987). In this case, 14- 27.5 % of the defect was complemented by the den V gene as assayed by colony forming ability and recovery of replicative DNA synthesis.

One of the possible defects in XP cells is the inability to process the chromatin in order to access the DNA

properly. From the above studies, it appears that the den V gene is able to access the DNA without chromatin alterations. It is possible that the den V protein is so small it does not need these alterations in order to access the DNA (Valerie *et al.*, 1987a). There have been several reasons suggested why the den V gene cannot complement 100 % of the defect in XP cells. The first of these is the fact that there has not been a pyrimidine dimer specific glycosylase isolated from mammalian cells (La Belle & Linn, 1982). Therefore, it is likely that the den V gene is not directly complementing the normal cells pathway but is indirectly complementing it by initiating a novel pathway (Arrand *et al.*, 1987). Thus by initiating a novel pathway the den V gene may not work in concert with other host enzymes very well. This would lead to long lived nicks or gaps in the DNA which can be very harmful and has been reported (Johnson *et al.*, 1987). Another reason for the lack of full complementation could be due to the fact that den V is pyrimidine dimer specific and this type of damage is not the only one induced by UV irradiation. Recent evidence in fact shows that this damage may not be the major cytotoxic lesion induced by UV irradiation. Cleaver (1988) constructed an XP A revertant cell line which cannot repair cyclobutane dimers but has near normal levels of UV survival. It was also shown that this line upon reversion was able to repair the 6-4 pyrimidine - pyrimidone type of damage which indicates that this form of damage may be more cytotoxic than previous studies indicated. However, it is seen that the den

V gene has proven to be a useful tool in studying excision repair.

DNA Repair Alterations In Simian Virus 40 Transformed Cells

Research in human systems has been greatly limited by the fact that human cells have a limited life-span of approximately 40 doublings and also the fact that transfection efficiency of primary cultures is low compared to that of immortilized cells (Gantt *et al.*, 1984). This makes the isolation of transformed primary human cell lines very difficult because by the time a colony is isolated and had time to grow, the cells will have reached the end of their life-span and will have started to senesce. For this reason, most studies that are done on mammalian cells where it is necessary to obtain stable transformed lines have been done using cells that have been transformed (immortilized) with Simian Virus 40 (SV40) and the same is true for excision repair studies (Gantt *et al.*, 1984).

However, there exists a problem in using transformed cells in order to study cellular mechanisms. This problem lies in the fact that upon transformation of a cell, expression of proteins can be altered. In fact, it has been shown that upon transformation of normal lung fibroblasts with SV40, 58 out 1300 polypeptides have altered expression by at least 40 % (Bravo *et al.*, 1982).

The ability of a transformed cell to repair its DNA,

compared to the parent line from which it was derived, has been studied quite extensively. Sensitivity to the alkylating agent N Methyl N' nitro-N-nitrosoguanidine (MNNG) was studied in 8 tumor lines isolated from patients, 1 normal SV40 transformed line and 1 XP group A SV40 transformed line in tissue culture (Day *et al.*, 1980). In this study, it was shown that the tumor cells and the SV40 transformed cells were unable to reactivate MNNG treated adenovirus type 5 to the same levels that their respective parent cells could. However, they were able to replicate untreated virus to the same levels suggesting that the tumor lines and SV40 transformed lines have a defect in repairing MNNG type damage. At the same time another study was being performed on the same two SV40 transformed lines (Heddle & Arlett, 1980). These cells were shown to have an increased sensitivity to Ethyl Methane Sulphonate (EMS) versus their untransformed counterparts. This sensitivity was assayed for by increased sister chromatid exchange (which is caused by EMS) and decreased colony survival. SV40 transformed lung fibroblasts have also been shown to have altered DNA repair abilities (Squires *et al.*, 1982). A 50 % decrease in the rate in which they are able to perform the initial incision step of excision repair has been observed after UV irradiation when they are compared to their parental cells.

Recently many new SV40 transformed lines have been constructed in order to aid in the elucidation of the excision repair pathway (Daya *et al.*, 1987). Fibroblasts from

complementation groups A, G, XP variant, 2 normal lines (Barbis *et al.*, 1986), group C (Daya *et al.*, 1987) and group F (Yagi *et al.*, 1983) have been transformed with SV40. Studies in which these cells are assayed for unscheduled DNA synthesis rates and colony survivability after UV irradiation show no differences between the transformed cells and the parental cell from which it was derived. This shows that although the repair pathways may be altered total survival can be the same. In general, it has been shown that although total survival of transformed cells can be equivalent to that of their parent they may have altered DNA repair mechanisms and cannot be assumed to be representative of the cells from which they were derived.

The Use Of Viruses In DNA Repair Studies

Bacteriophages have been utilized extensively in the study of DNA repair mechanisms in bacterial cells (for a review refer to Defais *et al.*, 1981). In the same way, mammalian viruses have been used to study DNA repair in mammalian cells (for reviews refer to Defais, 1983 ; Rainbow, 1981). A great deal has been learned about DNA repair deficiencies, such as XP, from these types of studies.

Most of the studies that utilize viruses to observe DNA repair have employed either SV40, human adenovirus or herpesvirus. There are three main types of studies that have been performed. These include HCR, Cellular capacity and

Enhanced reactivation.

Host Cell Reactivation, as its name implies, relies in part on host cell mechanisms to repair damaged viruses. Studies of this nature are performed by infecting cells with virus that has been irradiated or chemically treated and examining the survival of the virus relates to the capacity of the host cell to repair the lesions in the virus. Survival can be assayed by a number of different methods which include ; plaque formation of herpes virus (Ryan & Rainbow, 1986), (Day, 1974 ; Rainbow & Mak, 1973), V antigen formation of adenovirus (Rainbow, 1978), infectious center assays of SV40 (Abrahams & van der Eb, 1976) intranuclear inclusion body formation of adenovirus (Rainbow & Mak, 1972), one cycle herpes virus yields from mass cultures (Coppey *et al.*, 1978) and T antigen formation and transformation of SV40 (Aronson and Lytle, 1970).

Cellular capacity for viral infection is defined as the ability of a certain cell type to support the growth of a particular virus. Experiments, in which cellular capacity is used to observe the cells ability to repair DNA damage, are performed by treating the cells with a DNA damaging agent such as UV irradiation and then infecting the cells with a virus. It is felt that the cell must repair the damage in its genome before it can support viral growth. Therefore, the yield of virus depends on the cells ability to perform this repair (Coohill *et al.*, 1977).

Enhanced reactivation of virus combines the other two

techniques in one assay. In these studies, survival of irradiated or chemically treated virus is used to infect cells which have also been pretreated with some form of DNA damaging agent (Defais, 1983). Survival of the virus is often greater in the treated cells than in the untreated cells (Rainbow, 1981) and it has been reported that in some cases this enhanced reactivation is error prone for HSV 1 (DasGupta & Summers, 1978) and Adenovirus (Bennett and Rainbow, 1988). This increase in survival is similar to Weigle reactivation that is seen in bacteria which is also damage induced and been shown to be error prone (Weigle, 1953).

The Use of Herpes Virus To Study DNA Repair

A decrease in the capacity of cells to support a herpes virus infection has been studied using a wide variety of damaging agents. Such decreases have been observed using monkey kidney cells that have been pretreated with proflavin and light (Lytle and Hestler, 1976), angelicin or 8 - methoxypsoralen and light (Coppey *et al.*, 1979a), or UV light (Bockstahler *et al.*, 1976). As the time between treatment of the cells and infection is increased the decrease in capacity decreases. This is thought to be due to the cell repairing lesions in its DNA (Coppey *et al.*, 1979b).

Studies of capacity using herpes virus have also been performed using normal and XP fibroblasts that have been pretreated with a number of chemical carcinogens and UV

irradiation (for a review refer to Rainbow, 1981). In these investigations, it was found that the decrease in capacity of XP cells is greater than the decrease in capacity for normal cells indicating repair defects in XP cells.

Enhanced reactivation of Herpes virus has been reported for a number of cell lines which include normal and XP fibroblasts. These studies used a variety of treatments such as UV irradiation gamma irradiation and a number of chemical treatments (Rainbow, 1981 ; Coppey *et al.*, 1981 Bubley *et al.*, 1987).

HCR is the most widely used assay when studying DNA repair mechanisms using viruses. In 1969, it was reported that the yield of virus in an infection that was initiated with UV irradiated herpes virus was much lower in XP fibroblasts than in normal fibroblasts (Rabson *et al.*, 1969). Since that time, there have been many studies that have shown that XP cells are unable to repair irradiated herpes virus as well as normal fibroblasts (Takebe *et al.*, 1978 ; Ryan & Rainbow, 1986 ; Bueschleb, 1987). The % HCR value for UV irradiated herpes virus in XP group A fibroblasts (the least repair capabilities) is 18-20 % (calculated by the author) (Lytle *et al.*, 1982) compared to that of adenovirus type 2 which has been shown to be 3-7 % (Rainbow, 1981). One way in which the increase in the % HCR for herpes virus relative adenovirus can be explained is if the herpes virus carries some genes that are involved in DNA repair and does not rely on host cell functions as much as adenovirus does (Rainbow,

1981). It has been shown recently that HSV 1 codes for the DNA repair enzyme, Uracil-DNA Glycosylase (Caradonna et al., 1987) and codes for other enzymes which affected the UV survival of the virus and may be important in DNA repair (Bueschleb, 1987).

Reduced HCR for XP cells compared to normal cells have also been reported for herpes virus treated with N-acetoxy-2-acetylaminoflourene (Selsky & Greer, 1978) but not for formaldehyde (Coppey & Nocetini, 1979), nitrogen mustard (Selsky & Greer, 1977) or X-rays (Lytle *et al.*, 1972).

Herpes virus has also been utilized to study other aspects of DNA repair. In 1982 Lytle and co-workers showed that excision repair is an error free process by infecting XP group A cells (very little excision repair) and normal cells with UV irradiated herpes virus and looked for forward mutations in the thymidine kinase gene (TK) (Lytle *et al.*, 1982). This study showed that the excision repair proficient normal cell did not give a higher rate of mutation than the excision repair deficient XP A cells when normalized to the same number of lethal hits to the virus. Herpes virus was also used to establish convincing evidence that XP cells were equally proficient as normal cells in a repair pathway that utilizes homologous recombination events (Hall & Scherer, 1982).

Herpes Simplex Virus Type 1 (HSV 1) And Its Life Cycle

Viruses from the family Herpesviradae have linear

double stranded DNA, an icosadeltahedral capsid and an envelope that is derived from the hosts nuclear membrane (Roizman & Batterson, 1985). The Herpesviradae family consists of three genera which are the alphaherpesviruses (of which HSV 1 is a member), betaherpesvirus and gammaherpesviruses (Roizman & Batterson, 1985).

HSV 1 is a characteristic alpha virus in the fact that it has a short replication cycle, broad host range and is able to produce persistent infections. The HSV 1 genome is approximately 150 kilobase pairs (Roizman, 1979), that has nicks and gaps in it (Frenkel & Roizman, 1972). The genome is divided into a long (82%) sequence and a short (18%) sequence, each of which has inverted repeats on both sides of it (Varmuza & Smiley, 1985). The order of the sequences in the genome can have four possible arrangements that arise by inversions of the long and short regions with respect to each other and each orientation is found in equimolar amounts in a stock of virus (Hayward *et al.*, 1975). The capsid and envelope contain somewhere between 15 and 35 structural proteins (Cassai *et al.*, 1975). Six of these are the major capsid proteins (Gibson & Roizman, 1972) and seven others are glycoproteins (Ligas & Johnson, 1987).

HSV 1 infections are initiated by proteins in the envelope recognizing cell surface receptors (Fuller & Spear, 1987). Attachment activates a process that is mediated by other viral surface proteins and causes the envelope and the plasma membrane to fuse (Ligas & Johnson, 1987). The end

result of this fusion is the entry of the capsid into the cytoplasm of the cell. Once the capsid is in the cytoplasm it migrates to the nuclear pore and in a viral mediated fashion releases the DNA and possibly some viral proteins into the nucleus (Batterson *et al.*, 1983).

Upon entry of the genome into the host nucleus, DNA synthesis (Auerelian & Roizman, 1965) is shut off, protein synthesis rapidly declines (Roizman *et al.*, 1965) and RNA synthesis is altered (Roizman & Furlong, 1974). At the same time HSV 1 transcripts which will become capped and polyadenylated begin to be transcribed by RNA polymerase II (Stringer *et al.*, 1977). HSV 1 genes fall into three classes which are expressed sequentially in an HSV infection. These classes have been termed ; immediate early (IE), early (E) and late (L) (Roizman & Batterson, 1985).

The category an HSV 1 gene belongs to seems to be dependant on the sequences in its promotor region (Post *et al.*, 1981). IE genes are transcribed in the absence of de novo protein synthesis and may require or be enhanced by the presence of a protein that is brought into the cell in the virion (Campbell *et al.*, 1985). The promotor elements that are thought to be necessary are an enhancer sequence (TAATGARAT, R = purine) (Lang *et al.*, 1984), binding sites for the cellular transcription factor sp1 (Jones & Tjian, 1985) and a TATA box. Protein products of IE genes peak between 2 and 4 hrs. post infection (Honess & Roizman, 1974) and they function in shutting off IE gene transcription and

stimulating transcription of E and L genes (Preston, 1979).

E genes fall into two subclasses ; E1 and E2. Both species reach peak synthesis between 5 and 7 hrs. post infection and code for proteins that are involved in DNA replication such as the thymidine kinase (TK) and DNA polymerase genes (Pol) (Roizman & Batterson, 1985). The promoter regions of two E genes (TK and glycoprotein G (gG)) have been studied. Results from these studies show that they do not possess the enhancer sequence but do have sp1 binding sites, a CAAT box and a TATA box (Jones *et al.*, 1985).

Like the E genes, L genes fall into two categories L1 and L2, however, unlike the E genes they are distinct in their requirement for expression. This is due to the fact that L1 do not require DNA synthesis and are even synthesized in low levels before DNA replication and L2 genes have an absolute requirement of replication for their synthesis (Roizman & Batterson, 1985). Synthesis, for both classes of proteins, peaks between 12 and 15 hrs. post infection and include capsid and nonstructural proteins (Honess & Roizman, 1975). The promoters for L genes are the simplest and appear to require only a TATA box.

After synthesis of E genes the genome of the virus is replicated and can be detected as earlier as 3 hrs. post infection (Roizman and Roane, 1965). There are two origins of replication, one in each of the long and short regions. Replication is thought to occur by the rolling circle method thereby creating large concatamers of full length genomes

(Ben-Porat & Takezewski, 1977).

After production of the late genes empty viral capsids begin to accumulate in the nucleus. Newly synthesized DNA is then packaged into these new capsids after a full length genome has been cleaved from the concatamer (Roizman & Batterson, 1985). The capsid then buds out from the nucleus taking a portion of the nuclear membrane with it that has HSV 1 glycosylated proteins inserted in it (Roizman & Batterson, 1985).

Thymidine Kinase And Its Role In Repair

A cell may synthesize deoxyribonucleotide (dNTPs) precursors in two ways. The first, and most readily used, is by the reduction of ribonucleotides at the diphosphate level except thymidine nucleotides which are produced by deamination and methylation of cytosine nucleotides at the monophosphate level. The second pathway is termed the salvage pathway and involves stepwise phosphorylation of pre-existing (from DNA degradation) nucleosides (Coppey, 1977). The thymidine kinase (TK) gene is involved in the first step of the salvage pathway for pyrimidine biosynthesis. This enzyme phosphorylates thymidine that has been formed during the degradation of DNA to give dTMP. Two more phosphorylated groups are then added to the dTMP molecule and dTTP is formed which is able to be reincorporated into the DNA (Watson *et al.*, 1983).

It has been shown that during normal DNA synthesis both pathways for the formation of dNTPs are active. However, only the salvage pathway appears to be important for precursor synthesis in monkey kidney cells that have been UV irradiated and this appears to be true for both cellular and HSV 1 synthesis (Coppey, 1977). This data, therefore, suggests that the TK gene may play a key role in DNA repair.

Early studies into the importance of the involvement of the TK gene in DNA repair comes from studies using CHO cells. TK minus CHO cells have been shown to have an increased sensitivity to killing by monofunctional alkylating agents and to mutation by EMS when compared to TK CHO plus cells (Meuth, 1981). This finding was further supported by the fact that Friend mouse leukemia TK - cells were shown to have increased sensitivity to killing by UV irradiation, MMS, MNNG and an increase in mutagenesis when treated with mitomycin C compared to TK + (McKenna & Yassen, 1982). Recent investigations have shown that the differences between TK + cells and TK - cells are not observed in cells that are deficient in excision repair (McKenna *et al.*, 1985 ; McKenna & McKelvey, 1986). These results further suggest that the TK gene is important in DNA excision repair.

Upon the introduction of mutagens or the UV irradiation of cells the pools of dNTPs undergo rapid changes (Newman & Miller, 1983). It is felt that the TK gene exerts its influence on repair by balancing these changes in the dNTP pools (McKenna & McKelvey, 1986). Cells with a TK

deficiency would not be able to balance these pools which could lead to increased mutagenesis especially when nucleotides are needed quickly as in the case of repair synthesis.

An enzyme, that has the ability to phosphorylate both thymine and cytosine (ie a thymidine kinase) is detected in cells infected with HSV 1 (Honess & Roizman, 1975). The HSV 1 TK gene has since been cloned and shown to have an open reading frame (orf) capable of coding for a protein of 376 amino acids (Wagner *et al.*, 1981). HSV 1 specific TK proteins have been isolated from cells having molecular weights of 43, 39 and 38 kDa (Marsden *et al.*, 1983). Haar *et al.*, 1985 showed that this was due to the usage of three different in phase initiation codons. All three forms contain the putative ATP binding domain and the substrate binding domain and therefore, retain proper TK activity (Haar & Flatmark, 1987). This enzyme is the target for many antiherpes drugs (Fyfe *et al.*, 1978) which suggests the virus relies on its own TK product rather than one present in the host cell. HSV 1 could then provide a quick method for determining a role for the TK gene in excision repair. Initial studies suggest that UV irradiated TK - virus has reduced survival in Vero cells when compared to UV irradiated TK + virus (Rainbow & Bueschleb, 1987).

DNA Polymerase And Its Role In DNA Repair

DNA polymerase enzymes play a key role in the

excision repair process. It is not known which mammalian polymerase is responsible for the majority of repair synthesis in normal cells but it appears to be either the alpha or delta polymerase.

HSV 1 codes for many genes involved in the replication of its DNA, of which, a DNA polymerase molecule is one (Purifoy *et al.*, 1977). The gene has been cloned from strain 17 (Tsurumi *et al.*, 1987) strain Angeloti (Knopf, 1987) and KOS (Gibbs *et al.*, 1985) and has been shown to map between .413 and .434 map units on the HSV genome. In the case of KOS, it has been sequenced and shown to contain an (orf) of approximately 3705 base pairs. This orf has the potential to code for a protein with a molecular weight of 136,519 Da which is in good accordance with the size of the isolated 136 kDa protein (Gibbs *et al.*, 1985). The polymerase molecule contains a polymerase function as well as a proficient 3' -5' exonuclease function (O'Donnell *et al.*, 1987). It resembles the cellular polymerases alpha and delta in many ways including its nuclear localization (Weissbach *et al.*, 1973), sensitivity to inhibitors such as aphidicolin (Coen *et al.*, 1983) and dideoxythymidine triphosphate (Krokan *et al.*, 1979). The functions that it contains resembles the delta polymerase more than the multifunctional alpha polymerase. In fact, authors suggest that it may be used as a model system for studying eukaryotic DNA polymerases due the great number of similarities (Gibbs *et al.*, 1985).

Like the TK gene, the polymerase gene is one in which

many drugs are directed to inhibit HSV 1 infections and through the use of these drugs many pol mutants have been made which are resistant to these drugs (Gibbs *et al.*, 1985). One mutant pAA^{r}5 is of particular interest here. The polymerase mutant pAA^{r}5 was isolated by passaging virus in the presence of a high concentration of phosphonoacetic acid (Hall *et al.*, 1984). It has been shown that the mutant has a lower frequency of mutation than the wild type molecule from which it was derived (Abbots *et al.*, 1987 ; Hall *et al.*, 1985). In both of these investigations it was shown that the antimutator phenotype was caused by a better selection of nucleotides rather than an increased editing function as was once speculated. Hall *et al* (1985) went further and proposed a model for the increased fidelity of the mutant polymerase. Addition of a base to the growing chain during replication requires recognition of the deoxyribose sugar ring by the polymerase and proper base pairing of the base to its partner on the opposite strand. Energy considerations involved in the recognition of the sugar base and the base pairing determine if the base will be inserted into the growing strand. The wild type polymerase binds very tightly to the sugar ring and therefore, the correct base pairing may not be as important for insertion into the growing strand. The mutant polymerase, however, binds less efficiently to the sugar ring, therefore, proper base pairing will be more important for completion of polymerization. The involvement of the herpes polymerase in excision repair has been studied

initially using HCR in normal and XP fibroblasts (Bueschleb, 1987). These studies indicate that the polymerase mutant strain does not survive as well as the wild type strain in normal cells but survives better in the excision repair deficient XP cells.

HSV 1 As A Vector

Initial studies performed using HSV 1 as vector for studying genes showed that the virus might be able to distinguish foreign promoters from its own (Tackney *et al.*, 1984). However, more recently, it has been shown that normal cellular rabbit beta globin carrying its own promotor expresses properly initiated RNA to high levels when the gene was inserted into the TK locus of HSV 1 (Smiley *et al.*, 1987). The same study was also done with the alpha globin gene and was shown to be expressed RNA to high levels (Barbara Panning, Department of Pathology, McMaster University, personal communication). Functional protein has also been shown to made from the Hypoxanthine Phosphoribosyl transferase gene (HPRT) when inserted into HSV 1 under the TK promotor. These results suggest that HSV 1 is a good vector for studying genes (Palella *et al.*, 1988).

The purpose of this study was to :

1) Construct a herpes virus mutant containing the den V gene at the TK locus in order to ; a) determine if HSV 1 could be used as a suitable vector for studying DNA repair genes

and b) determine if the den V gene functions the same in normal cells as it did in transformed cells.

2) To help elucidate the role of the TK and polymerase genes in UV survival of HSV 1.

3) Determine if differences in HCR values can be detected in Transformed versus nontransformed cells using HSV 1.

MATERIALS AND METHODS

1.0 Cells

1.1 Mammalian Cell Strains And Lines

All normal and SV40 transformed human cell lines were purchased from the NIGMS Human Genetic Mutant Cell Repository (Copewood and Davis St., Camden N.J., 08103).

I. Human Diploid Fibroblasts

1. Normal GM 2803
2. Xeroderma Pigmentosum GM 5509 (XP12BE complementation group A)

II. SV40 Transformed Cell Lines

1. Normal GM 0637B (were made by transforming GM 037A cells with SV40 virus)
2. Xeroderma Pigmentosum GM 4429B (were made by transforming GM5509 cells with SV40)

III. Other Human Cell Lines

1. XP4 :

This cell line was constructed by Dr. A.J. Rainbow by transforming GM 4429B cells with a plasmid containing the muc genes (Andrew Rainbow, Depts. Biology and Radiology, McMaster University, Personal communication). These cells have been shown to have sequences that hybridize to the muc genes. The

reason for using these cells is their rapid growth and they yield large HSV 1 plaques.

2. Human 293 cells :

This cell line was produced by transforming early passage human embryonic kidney cells with the leftward 11% of Adenovirus type 5. (Graham and Smiley, 1977)

IV. Non-Human Cell Lines

1. Vero :

These cells (Yasumura *et al.*, 1963) were the generous gift of Dr. J. Smiley, Department of Pathology, McMaster university. These cells originate from African Green Monkey kidney cells.

1.2 Growth And Passaging Of Mammalian Cells

1.2.1 Media

MEM Alpha medium (Gibco/BRL (BRL), cat.# 410-2000, Gibco Canada Inc., 2270 Industrial Street Burlington, Ontario L7P 1A1) was purchased in powdered form, made in double glass distilled water (ddH_2O) and filter sterilized using a 0.22 micron filter. (Millipore Corporation, Bedford, Massachusetts 01730, USA). This media was used for culturing of all cell lines and strains.

1.2.2 Media Supplements

1. 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.

2. 7.5 % (w/v) $NaHCO_3$ solution (autoclave sterilized) was added to the media in a 1 : 50 dilution.

3. Fetal Bovine Serum (FBS) (BRL, cat.# 200-6140) was added to the media in a 1 : 10 dilution.

Media containing 1 and 2 is termed straight media and media containing 1,2 and 3 is termed complete media.

1.2.3 Solutions Used In Culturing Cells

1. Phosphate-Buffered Saline (PBS) without calcium and magnesium (0.8 g NaCl, 2 g KCl 1.15 g Na2HPO4 and 0.2 g KH2PO4 dissolved in 1 L double distilled water and autoclave sterilized) was used to wash the cell monolayers before trypsinization and before infections.

2. Trypsin (0.25 %) (BRL, cat.# 610-5050) was purchased as a 10x stock and was diluted to 1x with PBS. Trypsin was used in subculturing all cell lines and strains.

1.2.4 Procedure

Cells were grown in 75 cm^2 plastic Falcon flasks (Becton Dickinson and Company, 1950 Williams Dr., Oxnard, CA 93030, USA) in a humidified incubator at 37°C with 5 % CO_2. Upon reaching confluency the media was aspirated off and the monolayers were washed with PBS. The PBS was then aspirated off and 2.5 ml of trypsin was added at which time the flasks were put back into the incubator for 2-10 minutes. An

appropriate amount of complete media was then added to each flask, and cells were pipetted up and down to break up any clumps. The suspension of cells was then divided and added to new flasks for further growth and passaging.

2.0 Viruses

2.1 Viral Strains

The following viruses are all strains of Herpes Simplex Type 1 and except for HDV 1 were the generous gift of Dr. J. Smiley.

KOS : is wild type HSV-1 (Bueschleb, 1987)

PAAr5 : has an altered polymerase gene (Hall *et. al.* 1984)

PTK3B : has a point mutation in the TK locus (Dr. J. Smiley, personal communiction)

L7 14 : has the human B-globin gene inserted at the TK locus (Smiley *et al.*, 1987)

HDV-1 : has the den v gene inserted at the TK locus (constructed in this study)

2.2 Viral Stock Preparations

All viruses used in this study were grown in the following manner. 5 to 10 75 cm^2 flasks of Vero cells were grown to confluency. Once the monolayers were confluent the media was aspirated off and the monolayers were washed with 5 ml of prewarmed (37°C) PBS. The PBS was then aspirated off

and a 2 ml suspension of virus in straight media was added to the flask at a mutiplicity of infection of approximately .01 pfu per cell. The flasks were then incubated at 37°C for 90 min. with manual rocking every 10-15 minutes. 10 ml of straight media containing 2 % FBS was added to the flasks and the flasks were placed in the incubator for about 48 hrs,until all the cells had lifted off or rounded up. At this time the cells were washed off of each flask, pooled together and pelleted in 50 ml Falcon tubes in a clincal bench top centrifuge at setting 3 (International clinical centrifuge, model CL, International Equipment Co., Needham, Mass. USA). The pellets were then resuspended in 0.5 ml per flask of straight media and the suspension was sonicated on ice for 5, 30 s intervals seperated by 30 s using a probe sonicator. 1 ml of the suspension was aliquoted into freezing vials (Nunc Cryotubes, cat.# 3-40711, Nunclon Intermed, Denmark) for storage at -70°C. The stock preparations usually contained 5 x 10^{8} pfu.

2.3 Titre Of Virus

All viruses were titred on Vero cells. Cells from 75 cm^{2} flasks were collected in the same manner as was used for subculturing but in this case complete media was added to bring the total volume to 24 ml. The cells were then seeded into 2, 24 well plates (Nunc, cat.# 1-43982) by adding 0.5 ml of the cell suspension to each well. The plates were then incubated for 24 hrs. to reach confluency. A ten fold

dilution series of virus was then prepared in straight media. The media was then removed from each well and 0.2 ml of the appropriate virus dilution was added to the wells and the plates were incubated for 90 min. with rocking every 10-15 min.. Following this incubation period, 0.5 ml of straight media containing 2 % FBS and 0.05 % human immune serum globulin (16.5 % solution, Connaught, distributed by the Canadian Red Cross Society Blood Transfusion Service, DIN 07280) was added to each well. The plates were then incubated for 72 hrs. The media was then aspirated off and the cells were fixed and stained using 0.2 ml of crystal violet stain (2 g crystal violet dissolved in 20 ml methanol, 144 ml PBS and 36 ml formaldehyde) for 10-20 min. and then washed in cold water. The plaques were then counted and the titre determined.

3.0 Survival Assays

3.1 Preparation Of Cells

Monolayers of the cells of interest were grown in 75 cm^2 flasks until confluent. The cells were collected as described in the passaging section and were plated into 24 well plates as described for titring virus. 24 ml of media was added to flasks of XP 4, 293, GM0637B, and GM 4429 which was then used to seed two 24 plates. But for GM 5509 and GM 069B only 12 ml of media was added and therefore only 1 plate was seeded per flask. The plates were then incubated

overnight.

3.2 Irradiation Of Virus

An aliquot of stock virus (20-100 ul) was diluted in straight media. 1.6 ml of this suspension was then placed in a 35x10 mm Falcon petri dish (Falcon cat.# 3001) and placed on ice. The lid of the dish was removed and then the suspension was irradiated while being constantly stirred. The irradiation was performed by using a germicidal lamp (FG 596-E., General Electric Company, Nela Park, Clevland, Ohio 44112) which emits 254 nm light. Dose rates given were between 0.5 and 1.0 J/m^2 as determined by a J-225 short wave meter (Cat.# 17456-0, Will Scientific Inc. and subsidiaries Box 1050 Rochester, New York 146033) and the time of irradiation was altered in order to give the desired doses. After each irradiation an aliquot of the suspension was removed and a dilution series was performed in order to determine the titre of the virus.

3.3 Procedure

Cells and virus were prepared as stated above. The media from each well of the plates was aspirated off and the monolayers were washed with straight media. The wash was then removed and 0.2 ml of the appropriate viral suspension was added to each well. In the experiments each dose point had three serial dilutions plated in duplicate. The plates were incubated for 90 min., with rocking every 15-20 min. and then

overlayed as before. After 72 hrs. the plates were fixed and stained as previously described. The titre for each irradiated point was determined by linear regression using least squares analysis.

4.0 Production Of Recombinant Viruses

4.1 Cells

Confluent monolayers of Vero cells grown in 75 cm^2 flasks were collected as described earlier. Complete media was added to the cells in order to bring the final volume up to 25 ml. 5 ml of the cell suspension was added to 60 mm dishes and the cells were incubated overnight. The next day the plates were checked to ensure the cells were 50 % confluent at which time the transfection procedure was performed.

4.2 Transfection Procedure

This procedure has been described elsewhere (Smiley, 1980). 250 ul of 2x hepes (1 g Hepes, 1.6 g NaCl, 0.074 g KCl, 0.02 g Na_2HPO_4, 0.2 g Dextrose added to 100 ml ddH_2O and then filter sterilized through a .22 um Nalgene filter (Nalge , cat.# 120-0020, Nalge Company, Division of Sybron Corporation, Rochester N.Y., USA, 14602)), 10 ug of boiled sonicated salmon sperm DNA, 5 ul of infectious PAAr5 DNA and 0.5 ug of plasmid DNA (all DNAs were passed through a Sephadex G-50 column for sterilization (Pharmacia Fine

Chemicals, cat.# 170043 01, P.O. Box 175, S-751 04 UPPSALA 1, Sweden)) and ddH$_2$O to a final volume of 475 ul was added to a microfuge tube (Biorad, cat.# 223-9501, Biorad Laboratories, 32 nd and Griffith avenue, Richmond, California, USA, 94804). After brief mixing 25 ul of 2.5 M CaCl$_2$ was added and placed at room temperature for 20 min. This mixture was added to the 50 % confluent plates mentioned above.

4.3 Isolation Of TK $^-$ Viruses

The transfected cells from above were incubated until all cells had lifted or rounded (4-5 days) and then they were collected. 1 ml ministocks from each of the 60 mm dishes were made and their titres were determined by the methods previously described. Appropriate dilutions were made and the viral stocks were used to infect confluent 150 mm^2 dishes (NUNC, cat.# 168381) at an moi of 7.5 x 10^3 virus per dish. The reason this mutuplicity is used is because a recombination rate of 1 in 100-1000 is expected. Therefore, the subsequent plates should have between 7.5 and 75 plaques which will be well spread out and therefore easy to pick. Infections and the overlays were performed as previously described with the exception that Acyclovir (Coen *et al.*, 1984) (This nucleoside analogue will only allow tk$^-$ viruses to grow) was included in the overlay medium and the human immune sera was not. These plates were incubated for 3 days or until plaques started to appear at which time the plaques were picked with a wooden stick. The wooden sticks were then

touched into the media of a well in a 24 well plate that had a confluent monolayer of Vero cells in it. These plates were incubated until all cells had lifted or rounded and then ministocks were made from each of the wells.

5.0 Prokaryotic Cell Lines

JM 83 (Viera and Messing, 1982) and HB 101 (Boyer and Rouland-Dussoix, 1969) cells were the gifts of Dr. F. Graham, Department of Biology, McMaster University.

6.0 Recombinant DNA Techniques

6.1 Plasmids

1. pBR322 was purchased from BRL.
2. pUC19 was the generous gift of Dody Bautista (McMaster University, Department of Biology).
3. pTKSB was the generous gift of Dr. J. Smiley.
4. pMCADV was the generous gift of Mike Colicos (McMaster University, Department of Biology)

6.2 Restriction Enzyme Digests

All restriction enzymes were purchased from BRL. All digests included the recommended React buffer provided with the enzyme, 10 units of the appropriate enzyme in a final volume of 20 ul. The digests were carried out in microfuge tubes at 37°C for 3-6 hrs.

6.3 Ligation Of DNA

All ligations were performed using T_4 DNA ligase purchased from Pharmacia (Pharmacia, cat.# 27-0870-01). The ligation reactions were carried out in a final volume of either 20 or 30 ul. The reaction mix consisted of 0.5 ug of each plasmid, 1x recommended buffer, 1mM ATP and 3 units of ligase and ddH_2O to the desired final volume. The reactions were incubated in microfuge tubes overnight at approximately. 14° C.

6.4 Transformation Of Bacteria

DNA mediated transformation of bacteria was performed by the Calcium Chloride technique (Maniatis et al., 1982). Briefly, 100 ml of Luria broth (20 g broth base BRL cat. # M27800 was added to 1L ddH_2O) was inoculated with 1 ml of an overnight culture of bacteria and incubated with shaking for 2-4 hrs.. The culture was then chilled on ice for 10 min. then was placed in 50 ml Falcon tubes in a clinical bench top centrifuge at setting # 7 for 5 min. at 4°C. The cells were then resuspended in half of the original volume in ice-cold 50mM $CaCl_2$ and placed in a 50 ml Falcon tube (Falcon, cat.# 2098) on ice for 15 min.. The cells were then pelleted and resuspended in 1/15 of the original volume in 50mM $CaCl_2$. 0.2 ml of the cells were then aliquoted into prechilled microfuge tubes and placed at 4°C for 12-24 hrs. At this time 10 ul of the ligation reaction mix described above was added to the cells and incubated on ice for 30 min. The tubes were then

transferred to a 42°C water bath for 2 minutes and then 1ml of Luria broth with 25 ug/ml ampicillin (Sigma, cat.# A-9518, Sigma Chemical Company, PO Box 14508, St. Louis, Mo., 63178, USA) was added and the tubes were incubated with shaking at 37°C for 1 hr. The bacteria were then plated on ampicillin or ampicillin/X-gal (Boehringer Mannheim Canada (BMC), cat.# 651745, Boehringer Mannheim Canada, 11450 Cote de Liesse, Dorval, Que., 1T9-1A9) 100 mm plates and incubated at 37°C overnight. The plates were prepared by adding 15g Bacto-Agar (Difco, cat.# 0140-01, Difco Labs Detroit Mich. USA) 20 g LB broth base to 1L of ddH_2O which was then autoclave sterilized. When this had cooled either 25 ug/ml ampicillin or 25 ug/ml ampicillin and 0.05 % X-gal was added to the plating media. The media 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.

6.5 Screening Transformants

6.5.1 Extraction Of DNA

Colonies from the plates produced from transfections were picked with a wooden stick which was then used to inoculate 3 ml of Luria broth with 25 ug/ml of ampicillin in 15 ml plastic snap cap tubes (Falcon, Cat.# 2057). These minicultures were incubated overnight at 37°C with shaking. 1.5 ml of the culture was then placed in a microfuge tube and

centrifuged for 15 s The supernatant was then aspirated off and 100 ul of Lysozyme solution (25 mM Tris.Cl pH 8.0, 50 mM glucose, 10 mM Na$_2$-EDTA and 10 mg/ml Lysozyme (Sigma, cat.# L-6876) was added to the pellet. The pellet was then resuspended and the solution was placed on ice for 10-15 min.. 200 ul of alkali-SDS (0.2 N NaOH, 1 % SDS) was added and incubated on ice for 5 min.. 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 debris was removed by a 5 min. centrifugation. The supernatant was transferred to a new tube and 1 ml of cold 95 % EtOH was added and immediately vortexed and centrifuged for 5 min. The resultant DNA pellet was washed with TE buffer (10 mM Tris.Cl pH 8.0, 1 mM EDTA pH 8.0) and then resuspended in 200 ul cold EtOH and centrifuged for 5 min.. The pellet was then resuspended in 50 ul of TE buffer and placed at 4°C for storage.

6.5.2 Electrophoresis Of Extracted DNA

The DNA extracted from the bacteria was digested with the appropriate enzyme. The digestion products were then electrophoresed to determine fragment sizes. All DNA electrophoresis was performed in Horizontal gel boxes. The gel buffer for all gels was TBE (0.089 M Tris base, 0.089 M boric acid and 0.002 M EDTA pH 8.0) and all gels were 250 ml 0.8 % agarose (BRL cat. # 5510 UB). Included in the gel was .04 mg/ml ethidium bromide in order to visualize the bands. All electrophoresis was performed overnight (12-20 hrs. at 70

Volts. The marker used in all DNA gels was lambda DNA (BRL, cat.# 5250SA) cut with Hind III.

6.5.3 Photographing Gels

All ethidium bromide stained gels were photographd 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)

6.6 Large Scale Plasmid Preparations

Once the plasmids were screened, the ones of interest were grown into large scale plasmid preparations. The method used here for large scale preparations was the LiCl technique described in Maniatis et al, 1982. Briefly, a 500 ml culture was grown under the appropriate conditions overnight. This culture was then added to 500 ml plastic bottles in a JA 10 rotor in a Beckman model 21 C centrifuge (Beckman Instruments 2500 Harbor Boulavard Fullerton, CA, USA, 926321) for 6 min. at 6000 rpm. The cell pellet was resuspended in 10 ml of Lysozyme solution and placed in 30 ml nitrocellulose tubes (Beckman) and left at room temp. for 15 min.. Next, 20 ml of alkali-SDS was added and the mixture was incubated on ice for 10 min. 15 ml of special KOAc (60 ml of 5M KOAc pH 4.8, 11.5 ml glacial acetic acid, 28.5 ml ddH_2O) was then added and the mixture was incubated on ice for 30 min.. The tubes were then placed in an SW 27 rotor and centrifuged in a Beckman L2

65B Ultracentrifuge at 20,000 rpm for 20 min. to pellet the cellular debris. The supernatant was transferred to 30 ml glass corex tubes and 0.6 volumes of isopropanol was added and the tubes were sat at room temperature for 15 min.. The tubes were then centrifuged at room temp. for 30 min. in a JA 20 rotor in a Beckman 21C Centrifuge at 10,000 rpm. to pellet the nucleic acids. These pellets were firstly washed with 70 % and then again with 90 % EtOH, then resuspended in a minimum volume of TE and placed into 15 ml glass corex tubes then an equal volume of 5 M LiCl was added and the tubes were placed on ice for 30 min.. The tubes were placed in a JA 20 rotor and centrifuged at 15,000 rpm. The supernatant was then Etoh precipitated. This precipitation consists of adding 0.5 volumes of 7.5 M NH_4Ace and 2 volumes of Etoh to the aquous solution and then an incubation at -20°C for 30 min.. The tubes were centrifuged again and the DNA pellet was redissolved in a minimum volume of TE (0.5 ml) buffer and placed in microfuge tubes. RNase was then added to a final concentration of 40 ug/ml and incubated at 37°C for 15 min and finally a phenol extraction was performed. This extraction consists of adding an equal volume of phenol to the tubes which were then vortexed and the tubes centrifuged for 5 min. The aquous phase was then placed in a new microfuge tube and an equal volume of chloroform (chloroform 24 : iosamyl alcohol 1) was added and the tubes were again vortexed and centrifuged for 5 min. The aqueous phase was collected and Etoh precipitated as before. At the end of

the incubation the tubes were centrifuged and the pellet was resuspended in TE buffer.

6.7 Isolation Of Infectious Viral DNA

The technique used has been described previously (Enquist et al., 1979). Briefly, 10 75cm^2 flasks of confluent Vero cells were infected as described previously at 10 pfu per cell. After approximately. 48 hr when the cells had lifted they were collected into 50 ml Falcon Tubes and centrifuged at setting # 3 in a clinical bench top centrifuge for 15 min. at 4°C. The cell pellets were then washed with PBS resuspended in lysis buffer (0.2 M EDTA pH 8.0, 0.5 % SDS and 100 ug/ml Proteinase K (BRL, cat.# 5530UA)) and incubated overnight at 37°C overnight. This mixture was gently extracted with phenol 4x ensuring that each transfer was done with a large bore pipet to limit shearing. The resultant supernatant was dialysed against 0.1x SSC for 2-3 days with several changes at 4°C. The dialysate was then collected and stored at 4°C.

6.8 Screening Of Recombinant Viruses

6.8.1 Extraction Of Viral DNA

DNA from minipreps (made during the transfection procedure) was isolated in the following manner. 500 ul of the miniprep was incubated in a microfuge tube at 37°C for 2 hrs with an equal volume of Lysing buffer (.01 M EDTA, 0.1 M

Tris Base, 0.025 % SDS and 1 mg/ml Pronase (BMC, cat.# 165921)). This was then phenol extracted, chloroform extracted and Etoh precipitated overnight. The tubes were then centrifuged in a microcentrifuge and the pellets were dried and resuspended in 50 ul of TE buffer. 1 - 5 ul of the resultant DNA was then restricted and electrophoresed as described previously. The DNA was then transferred to nitrocellulose for southern blotting.

6.8.2 Southern Blotting

All southern blots were performed as described in Manniatis *et al.*, 1982. Briefly, after electrophoresis the gel was U.V. irradiated for approximately. 5 min. using a short wave U.V. light. The gel was then soaked in 1.5 M NaCl and 0.5 M NaOH for 1 hr and then soaked in 1.5 M NaCl and 1 M Tris pH 8.0 for an additional hr. The DNA in the gel was then transferred to nitrocellulose (Mandel, cat.# 53504-416, Mandel Scientific Company, 143 Dennis St. Rockwood Ontario, N0B-2K0) in the following manner. A thin sheet of plastic (saran wrap) was placed on a tray and 4 sheets of Whatman 3MM paper (Whatman, cat.# 303097, Whatman International Ltd., Maidstone England) presoaked in 20x SSC (175.3g NaCl, and 88.2g Na citrate pH adjusted to 7.6) were placed on top of it. Next the gel was placed on top of the Whatman face down. The nitrocellulose presoaked in 2x SSC was then placed on the gel followed by 2 sheets of the whatman paper soaked in the same 2x SSC. One leave of paper towels was then placed on top

followed by a weight. This was allowed to sit at room temp. for approximately. 16 hrs. Following this incubation the membrane was heated at 80°C for approximately. 1.5 hrs.

6.8.3 Labelling 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 labeled dCTP purchased from ICN (ICN, cat.# 33004X, ICN Canada Ltd. 2485 Guennette St. St. Lament, Que., Canada H4R-2H2). In these reactions 50 uCi of the isotope was used to label 1 ug of plasmid DNA. After the incubation period the reaction mix was passed through a Sephadex G 50 column (Pharmacia, cat.# 170043 01) and the eluent was stored at -20°C until the probe was used.

6.8.4 Probing The Nitrocellulose

The membranes were prehybridized overnight in plastic heat sealed bags at 48°C in 20 ml of the following solution, 10x Denhardt's (for a 50x stock to 500ml of ddH_2O add 5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA), 50 % formamide, 5x SSC, 50 mM Tris pH 8.0 and 5 ug of sonicated boiled salmon sperm DNA (Sigma, cat.# D9156 The appropriate radioactive probe was then boiled for 10 min. and injected into the plastic bag containing the membrane and hybridization fluid. This was then incubated at 48°C overnight. At the end of this time period the membranes were

removed from the bag and washed three times. The first wash was 2x SSC and 0.5 % SDS at room temp. for 0.5 hr. The second wash was 2x SSC and 0.1 % SDS again at room temp. for 0.5 hr. and the last wash was 0.1x SSC and 0.1 % SDS at 65°C for 3 hr - 6 hr. The blot was then dried at room temp for 1 hr, wrapped in Saran Wrap and placed next to XAR-5 film (Kodak, Xomat AR Film, Eastman Kodak Company, Rochester N.Y., 14650). The length of exposure and the temperature at which it was carried out depended on the amount of activity on the blot.

7.0 Protein Techniques

7.1 Immunoprecipitations

7.1.1 Antibodies

The den V specific antibodies used in this work were IC3, # 38 (Valerie *et al.*, 1986 ; Valerie *et al.*, 1987b) and were the gifts of Dr. E Henderson, Temple University, Philadelphia.

The antibodies 15BB2 (glycoprotein B specific) (Ligas and Johnson, 1987) and II - 436 (glycoprotein D specific) (Ligas and Johnson, 1987) were the generous gifts of Dr. D.J. Johnson (McMaster University, Dept. of pathology).

7.1.2 Procedure

The expression of den V protein was attempted to be

determined by immunoprecipitations (Johnson and Feenstra, 1987). Vero cells were grown in 35 mm dishes to confluency. These cells were then infected with the virus of interest at an moi. of between 10 and 50 pfu per cell, as described earlier. At various times post infection (2-8 hrs.) the overlay media was aspirated off and the infected cells were washed with 199 -Met media (normal straight media with 2 % FBS and 1 % Hepes pH 7.4 but lacking methionine). Then 2 mls of 199 -Met containing 50 uCi of ^{35}S methionine (ICN, cat. # 312612) was added to the monolayers and the infection was allowed to proceed for varying lengths of time (4-12 hrs.). at which time the proteins were extracted by the following procedure. The media was aspirated off of the cell sheets and 1 ml of RIPA buffer (50 19mM Tris.Cl pH 7.5, 150 mM NaCl, 1 % NP40, 0.5 % DOC and 0.1 % SDS) plus inhibitors (1 mg/ml BSA (Sigma cat. # B-2518)and 1mM PMSF (Sigma, cat.# P-7626)) was added to the dish which was incubated on ice for 5-10 min.. The resultant lysates were then sonicated on ice for 2 30s bursts to disrupt any remaining intact membranes. The membranes were then pelleted by centrifugation at 30,000 rpm for 1 hr. at 4°C. 2-3 ul of antibody was added to 400 ul of the supernatant in an microfuge tube and placed on ice for 1 hr.. At the end of this incubation 100 ul of Protein A beads (0.5 g beads swelled in RIPA for 2 hrs. at 4°C, (Pharmacia, cat.# 170780)) were added and the mixture was mixed end over end for 2 hrs. at 4°C. The microfuge tubes were then centrifuged for 2 min. at room temp. The

supernatant was discarded and the beads were washed in 500 ul of RIPA buffer 5- 10 times with 2 min. centrifugations in between each wash. After the last wash the beads were resuspended in 100 ul of 2x protein loading buffer (4 % SDS, 20 % glycerol, 100 mM Tris pH 6.8, 0.25 % Bromphenol Blue and 2 % Beta Mercaptoethanol). The tubes were then boiled for 5 min., chilled on ice for 5 min. and finally centrifuged for 5 min. The supernatant was then collected.

7.1.3 Polyacrylimide Gel Electrophoresis (SDS PAGE)

15.2 % SDS polyacrylimide gels (18.75 ml of 30 % acrylimide-bis acrylamide solution (Biorad, cat.#'s 1610101,1610200) 14.25 ml of 1 M Tris pH 8.8, 3.75 ml $ddH^{=}O$, degassed for 15 min then add .0.2 ml 20 % SDS, 10% NH_4 persulphate and 0.025 ml Temed (Biorad, cat.# 1610800)) with a 3 % staking gel (1.67 ml of the 30 % solution, 1.25 ml 1 M Tris pH 6.8, 6.7 ml dd H_2O, degassed for 15 min. then add 0.2 ml 20 % SDS, 0.05 ml NH_4 persulphate and 0.015 ml Temed) were used for all protein electrophoresis. The gels were run vertically overnight at 70 volts in Laemli buffer (For a 10x stock 10g SDS, 30.2g Tris base and 144g glycine were dissolved in 1L ddH_2O and the pH was adjusted to 8.8). The protein markers used on all gels was the ^{14}C labelled high molecular weight markers purchased from BRL (Cat.# 6021 SA). 5 ul of the marker and 30 ul of the samples were added per lane.

7.1.4 Post Electrophoresis Processing Of The Gel

All protein gels were Dimethyl sulfoxide (DMSO)-polyphenoloxide (PPO) treated in order to enhance the radioactive signal. Briefly, the gels were first soaked in DMSO used once for 30 min. then transferred into fresh DMSO for 1 hr.. After the hr the gel was transferred to a 20% PPO/DMSO solution for 2-3 hrs. The gel was then washed in running water for approximately. 45 min., dried, and set next to film for exposure.

7.2 Western Blots

Proteins were extracted and electrophoresed by the same methods that were used for immunoprecipitation.

7.2.1 Transfer Of Proteins To Nitrocellulose

After electrophoresis the proteins were transfered to nitrocellulose by the following procedure. The gel was soaked in transfer buffer (25mM Tris, 192 mM Glycine and 20 % Methanol) for 30 min. At this time the proteins were transfered to the nitrocellulose by using a transblot apparatus (Biorad, cat.# Transblot). The blotting procedure was performed at 4°C overnight at 250 Volts. The nitrocellulose was then probed using either an ^{125}I labelled antibody or an alkaline phosphatease conjugated antibody.

7.2.2 Probing The Nitrocellulose Using An ^{125}I Labelled Second Antibody

The nitrocellulose from above was blocked with 3 % BSA in saline (0.9 % NaCl in 10 mM Tris, pH 7.4) for 4 hrs at room temp.. The appropriate antibody was diluted into 3 mls of BSA saline solution and added together with the blocked nitrocellulose into a heat sealed plastic bag. This was then rocked for approximately. 3 hrs. The blot was then rinsed with saline for 1 - 2 hrs with 4 - 5 changes. The ^{125}I labelled antibody was diluted in BSA saline solution and added along with the nitrocellulose into a heat sealed bag and left rocking overnight. The blot was then washed as before and the blot was dried and then exposed to film.

7.2.3 Probing The Nitrocellulose Using An Alkaline Phosphatase Conjugated Second Antibody

The nitrocellulose was placed in a small plastic tray to which 25 ml of Blotto (5 % Skim milk, 0.05 % Tween 20 (Sigma, cat.# P1379) diluted in 1x PBS) was added and rocked for 2 hrs for blocking purposes. The 1^{o} antibody was then added into the mix and rocked for a further 3 hr. The blot was then washed with 5 ml of Blotto 4x. The second antibody was then added and washed in the same way as the first. The blot was then washed for twice for 5 min. with Borate buffer (0.95 g Boric Acid / L, pH9.5). Developing solution (0.1 M

Tris pH 9.2, 0.1 % NBT (Sigma, cat.# N6876), 5 mg/ml BCIP (Sigma, cat.# B8503) and 4 mM $MgCl_2$) was then added until the proteins were visible.

8.0 RNA Techniques

8.1 Extraction Of Viral RNA

The method used to extract viral RNA has been described previously (Berk and Sharp, 1977). Vero cells in 100 mm dishes (Falcon, cat.# 3003) were infected with 10 pfu per cell of the appropriate virus as described earlier. At various times post infection (6- 12 hr) the infected cells were collected in order to extract RNA from them. The cells were washed three times with 5 ml of PBS and then scraped off of the dish with a rubber policeman into 10 ml of PBS. This suspension was then centrifuged in a clinical bench top centrifuge to pellet the cells at setting # 3 for 10 min. The cells were then resuspended in 1 ml of isotonic buffer (0.15 M NaCl, 10 mM Tris.Cl pH 8.0 and 1.5 mM $MgCl_2$, autoclaved and stored at 4°C) and put into a 15 ml snap cap tube. An equal volume of isotonic buffer plus 2 % NP40 (Sigma cat. # 6507) was added and the tubes were incubated on ice for 5 min, vortexed for 5 s and centrifuged at 5,000 rpm at 4°C for 5 min.. The supernatant was transferred to a new tube and an equal volume of Urea/SDS (7 M Urea, 0.35 M NaCl, 0.01 M Tris.Cl pH 7.8, 0.01 M EDTA and 1 % SDS) was added and the tubes were incubated at room temp. for 5 min. Three

successive phenol/chloroform (1:1) extractions were then performed. These consisted of adding an equal volume of phenol/chloroform, then shaking the tubes for 5 min. and centrifugation in a JA 20 rotor for 5 min. at 5000 rpm. After the last extraction the aqueous phase was transferred to a new tube with vol. 95 % Etoh and put at -20°C overnight. The tubes were then centrifuged at 12,000 rpm in a JA 20 rotor for 20 min. The supernatant was discarded and the pellet was washed with 10 ml of RNA wash solution (95 % Etoh / 0.4 M NaAce pH 5.2 (2.5 : 1)). The RNA was centrifuged out between each wash and the final pellet was dissolved in 400 ul of ddH_2O.

8.2 Probing RNA

The RNA harvested above was transferred to a nitrocellulose membrane using a Manifold II Slot Blot System (Schleicher and Schuell, cat.# 44-27570, Scleicher and Schuell Inc., Keene, USA, NH 03431) and was performed using the recommended procedure. Approximately 1 ug of RNA was loaded into each slot. The slot blots were probed using ^{32}P nick translated probes and the results were visualized by audioradiograghy.

8.3 Primer Extension Of RNA

Primer extension experiments were performed on the

RNA extracted in order to determine if any correctly initiated Den V transcripts were being made. The oligomer, 5' CGCGGCAATTCACGATATTCAGCC 3' was purchased from the Institute of Molecular Biology, McMaster University and used in these experiments.

8.3.1 Labelling Of The Oligonucleotide

The oligonucleotide was labelled with ^{32}P by a kinase reaction. The kinase reaction consisted of 35 ul of kinase buffer 1 (20 mM Tris, pH 9.5, 1mM spermidine, 0.1 mM EDTA), 5 ul of kinase buffer 2 (500 mM Tris, pH 9.5, 100 mM $MgCl_2$, 50 mM DTT, and 50 % v/v glycerol), 4 ul ddH_2O, 5 ul ^{32}P ATP (ICN, Cat # 35001), 1ul of the oligomer and 1ul of T4 polynucleotide kinase (Pharmacia, cat.# 27-0734-01). This reaction mix was incubated at 37°C for 1.5 hrs at which time 200 ul of 2.5 M NH_4Ace,3 ul of 5mg/ml t-RNA and 1ml of Etoh was added and put at - 70 for 10 min. At this time the tube was centrifuged and the pellet was dried. The pellet was then resuspended such that there were 1,000,000 cpm / ul.

8.3.2 Primer Extension Procedure

The techniques used have described elsewhere (Jones *et al.*, 1985). Briefly, extracted RNA was reprecipitated out of 0.3 M NaAce with ethanol. The pellets were then washed with 70 % Etoh, 95 % Etoh and dried for 1 hr. The RNA pellet was then resuspended in 7 ul of TE buffer. 1 ul of kinased

oligomer (1,000,000 cpm) was added to the RNA. TKE buffer (TE buffer + 1.25 M KCl) was added and the tubes were placed at 60° C for 1hr. 25 ul of reverse transcriptase buffer (20 mM Tris, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 0.33 mM of each dNTP and 10 ug / ml actinomycin D) containing 10 units of the enzyme AMV reverse transcriptase (Joseph Beard, Life Sciences) was added to each tube. This mixture was then incubated at 37° C for 1 hr. 300 ul of Etoh was added and the nucleotides were pelleted by centrifugation. The pellets were washed with Etoh and dried. At this time the pellets were resuspended in 3 ul of ddH_2O and then 9 ul of loading dye was added to each tube. Each tube was then boiled for 2 min. and put on ice until the samples were loaded.

8.3.3 Electrophoresis Of Primer Extension Samples

5 % sequencing gels were used to electrophorese the products from the primer extension experiments. The gels were prepared by dissolving 48 g Urea, 4.75 g acrylamide and 0.25 g bisacrylamide in 53 ml of ddH_2O. 5 mls of 20x TBE was then added and the mixture was filtered through a 0.22 micron nalgene filter and degassed for 5 min. The plates were prepared by coating 1 plate with 5 % repelsilane (Kodak, Cat # 9650) in ethanol and the other with 5 % bindsilane (Aldrich Chemicals, cat# 23,579-2, Aldrich Chemicals, 940 West Saint Paul Ave., Milwaukee, Wisconsin, 53233, USA) in chloroform. The plates were then assembled with the spacers and 60 ml of

the acrylamide solution was poured between them once 50 ul of Temed and 1 ml of 10 % ammonium persulphate was added to it. After the gel had polymerized it was prelectrophoresed for 1 hr at 1800 volts. 4 ul of sample was then loaded into each lane and the gel was electrophoresed for 4 hrs at 2000 volts. The plates were allowed to cool and the gel was fixed to a plate with 50 % methanol and 20 % acetic acid. The gel was rinsed with ddH_2O and dried overnight at which time it was exposed to film.

9.0 Indirect Immunoflourescence Of Cells

9.1 Preparation Of Cells

Indirect immunofluorescence studies were performed on 8 well chamber slides (Lab-Tek Division, Miles Laboratories, Inc., Napierville, Il. 60540, USA). One 75 cm flask of Vero cells was used to seed 8 chamber slides in a volume of 0.5 ml per well. 24 hrs later the slides were infected with the virus in a volume of 40 ul / well. 1.5 hr post infection the wells were overlayed with media containing 2 % FBS. At 8 hrs post infection the slides were processed.

9.2 Processing Of Chamber Slides

The slides were removed from the incubator and the wells were washed three times with 1x PBS for 5 min at each

wash. At this point the cells were fixed to the slide by incubating them in an ice cold 50 % Acetone : 50 % Methanol mixture for 15 min. The slides were then rewashed in 1x PBS. 25 ul of the appropriate dilution of 1° antibody was added to a coverslip which was then gently placed over the fixed cell sheets. The slides were incubated upside down in a humidified incubator at 37°C for 1 hr. The coverslips were then removed and the cell sheets were washed with 1x PBS three times with the last wash being at 37°C for 30 min.. The same procedure was followed with the second, fluorescently labelled antibody. After the slides were air dried the number of fluorescent cells were counted using a fluorescent microscope.

RESULTS

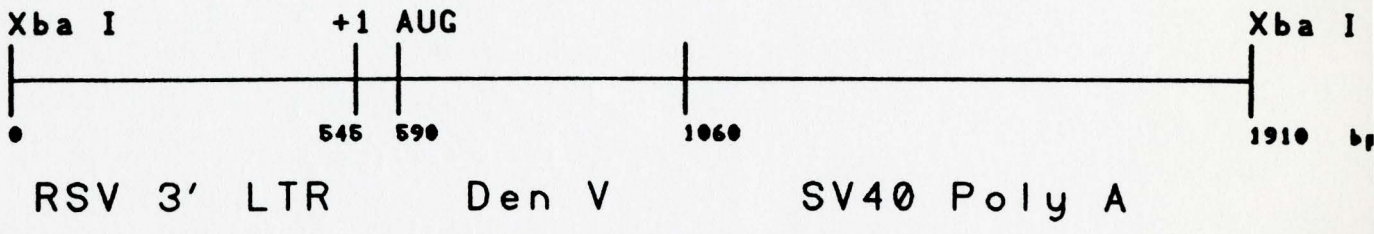
1.0 Construction Of An HSV 1 Mutant Carrying The den V Gene At The Thymidine Kinase Locus

1.1 The den V Eukaryotic Expression Cartridge

The den V cartridge that is seen in figure 1, was constructed by Valerie *et al.* (1987) in the following manner. A 524 bp Pvu II - Bst NI fragment from the 3' LTR of Rous Sarcoma Virus was isolated and the ends were made blunt with klenow. Hind III linkers were then added and the resultant fragment was cut with Hind III and Pvu II. This fragment was inserted into pSV2cat which had been cut with Acc I, blunt ended and then cut with Hind III (Gorman *et al.*, 1982). A Nde I -Hind III fragment containing 50 bp of pBR322 5' of the sequence and 51 bp 3' of the RSV transcription start was isolated from the above plasmid and a 6 bp Hind III - Sph I linker was added to the 3' end and the resultant fragment was cloned into pEMBL (Dente *et al.*, 1983). A Hinf I fragment was isolated from bacteriophage T4 that contained the entire den V gene except for the 1st base of the translation start codon, ATG (Valerie *et al.*, 1985). This base was reconstructed by adding Cla I linkers which were cut and blunt ended to destroy the Cla I site. An 11 bp. Acc I -Bam HI linker was added to the 3' end. The RSV and den V fragments were blunt end ligated together at the Sph I and Acc I ends. This ligation fragment was then inserted into 4.5 kbp Bgl II - Nde I fragment of pSV2dhfr (Valerie *et al.*, 1987). An Nde I - Bam HI, 1.9 kbp fragment, which contained

Figure 1

A cartoon of the Den V Cartridge


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

the RSV & den V sequences plus the SV40 large T splice and polyadenylation signals was isolated and Xba I linkers were added to both ends and blunt end ligated into pEMBL18 to give rise to the plasmid pEMBL18/RSVDenV (Valerie *et al.*, 1987). This plasmid was then cleaved with Xba I and the 1.9 kb fragment was inserted into pFGDX1 at the Xba I site giving rise to the plasmid pMCADV (Colicos *et al.*, manuscript in preparation).

The exact sequence of the cartridge was not available so one was constructed from the sequences in the NIH gene bank and can be seen as figure 2.

1.2 Cloning Strategy

The steps taken to clone the den V gene into the HSV 1 TK locus can be seen as figure 3a. The strategy employed was to clone the den V cartridge into a HSV 1 TK gene that was contained in a plasmid. This plasmid along with infectious HSV 1 DNA was then transfected into cells. This allowed integration of the cartridge into the HSV 1 genome through site specific recombination at the TK locus.

1.3 Plasmid Construction

The ultimate goal of the construction of the virus was to insert the den V cartridge into the TK locus of HSV 1. This was performed by using site specific recombination

Figure 2

The sequence of the Den V cartridge compiled from the sequences in the NIH genebank

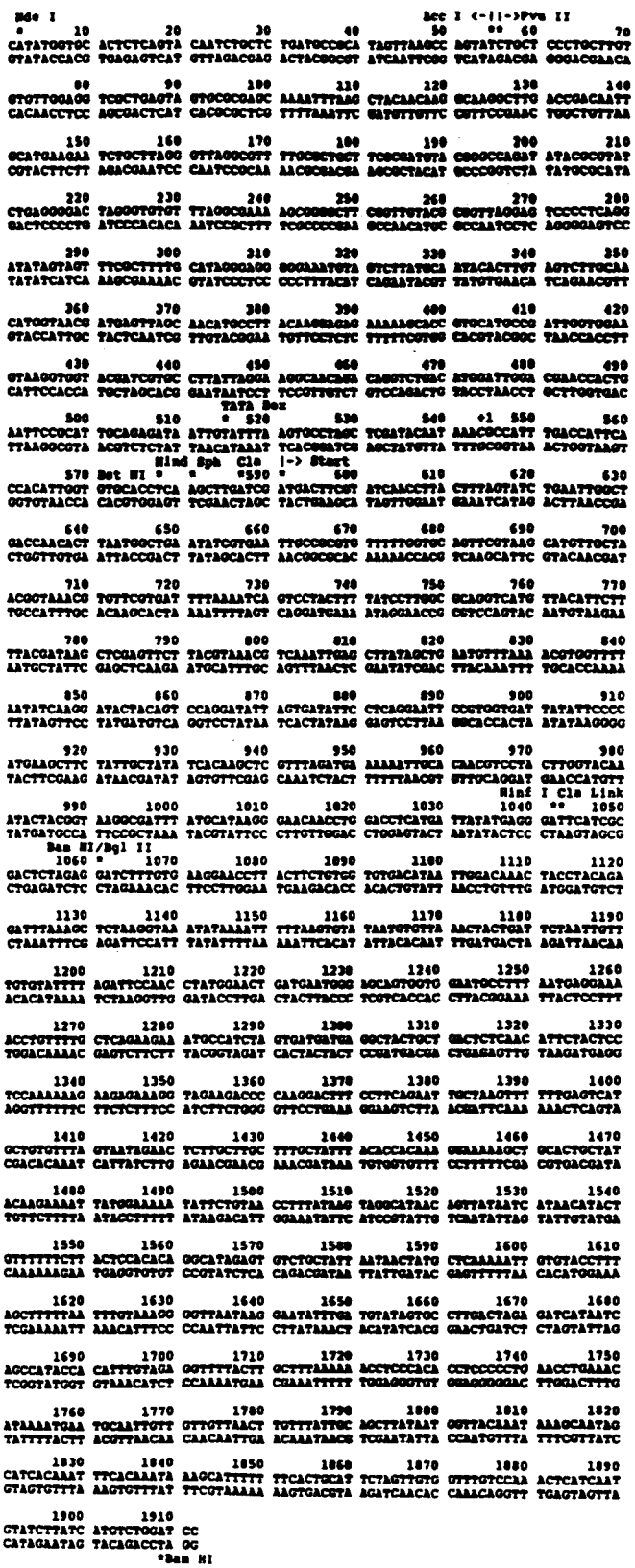
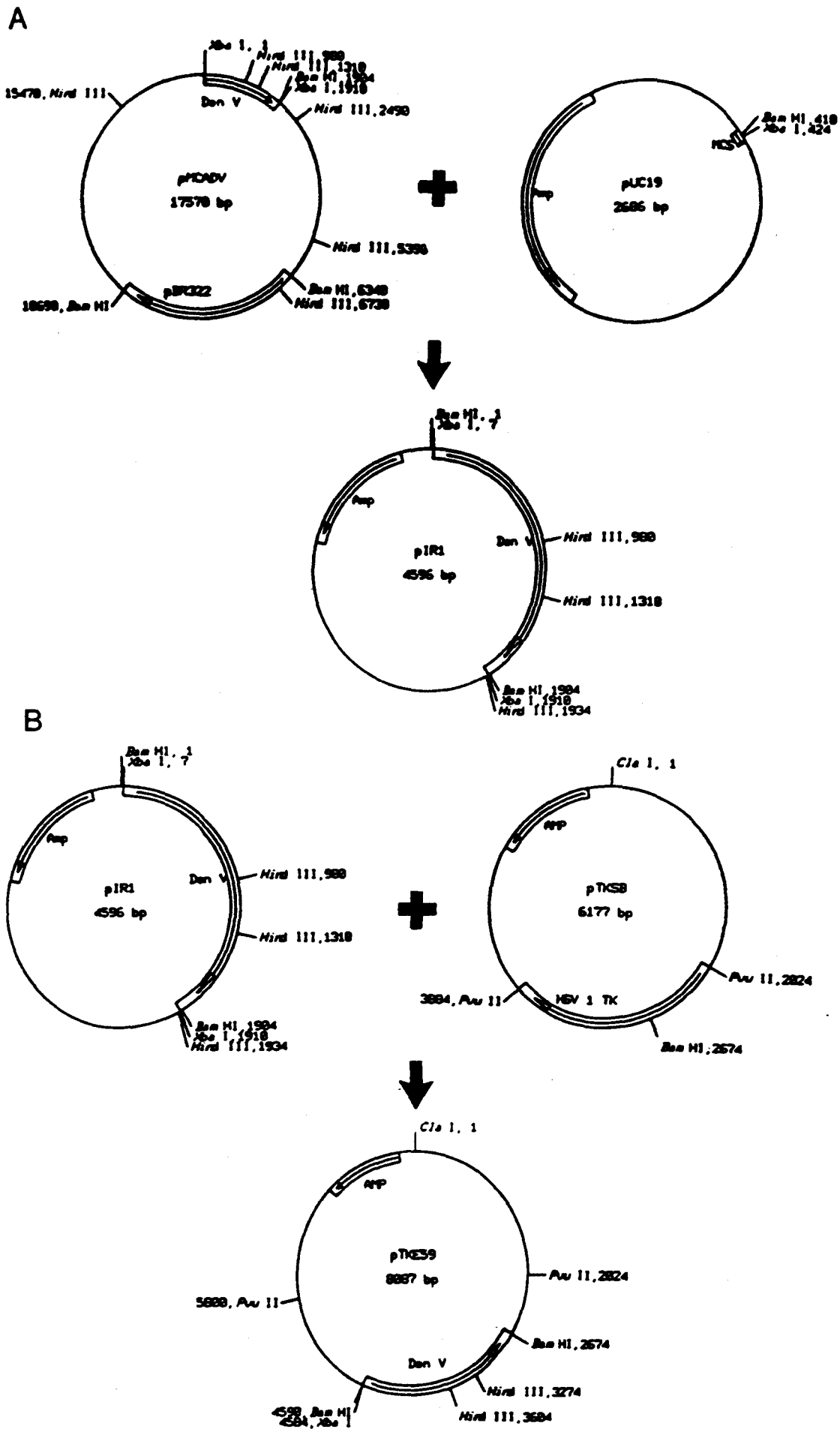

Bde I
Acc I <-||->Pvu II
10 20 30 40 50 60 70
CATATGGTGC ACTCTCAGTA CAATCTGCTC TGATGCCGCA TAGTTAAGCC AGTATCTGCT CCCTGCTTGT
GTATACCACG TGAGAGTCAT GTTAGACGAG ACTACGGCGT ATCAATTCGG TCATAGACGA GGGACGAACA
80 90 100 110 120 130 140
GTGTTGGAGG TCGCTGAGTA GTGCGCGAGC AAAATTTAAG CTACAACAAG GCAAGGCTTG ACCGACAATT
CACAACCTCC AGCGACTCAT CACGCGCTCG TTTTAAATTC GATGTTGTTC CGTTCCGAAC TGGCTGTTAA
150 160 170 180 190 200 210
GCATGAAGAA TCTGCTTAGG GTTAGGCGTT TTGCGCTGCT TCGCGATGTA CGGGCCAGAT ATACGCGTAT
CGTACTTCTT AGACGAATCC CAATCCGCAA AACGCGACGA AGCGCTACAT GCCCGGTCTA TATGCGCATA
220 230 240 250 260 270 280
CTGAGGGGAC TAGGGTGTGT TTAGGCGAAA AGCGGGGCTT CGGTTGTACG CGGTTAGGAG TCCCCTCAGG
GACTCCCCTG ATCCCACACA AATCCGCTTT TCGCCCCGAA GCCAACATGC GCCAATCCTC AGGGGAGTCC
290 300 310 320 330 340 350
ATATAGTAGT TTCGCTTTTG CATAGGGAGG GGGAAATGTA GTCTTATGCA ATACACTTGT AGTCTTGCAA
TATATCATCA AAGCGAAAAC GTATCCCTCC CCCTTTACAT CAGGATACGT TATGTGAACA TCAGAACGTT
360 370 380 390 400 410 420
CATGGTAACG ATGAGTTAGC AACATGCCTT ACAAGGAGAG AAAAAGCACC GTGCATGCCG ATTGGTGGAA
GTACCATTGC TACTCAATCG TTGTACGGAA TGTTCCTCTC TTTTTCGTGG CACGTACGGC TAACCACCTT
430 440 450 460 470 480 490
GTAAGGTGGT ACGATCGTGC CTTATTAGGA AGGCAACAGA CAGGTCTGAC ATGGATTGGA CGAACCACTG
CATTCCACCA TGCTAGCACG GAATAATCCT TCCGTTGTCT GTCCAGACTG TACCTAACCT GCTTGGTGAC
TATA Box
500 510 * 520 530 540 +1 550 560
AATTCCGCAT TGCAGAGATA ATTGTATTTA AGTGCCTAGC TCGATACAAT AAACGCCATT TGACCATTCA
TTAAGGCGTA ACGTCTCTAT TAACATAAAT TCACGGATCG AGCTATGTTA TTTGCGGTAA ACTGGTAAGT
Hind Sph Cla |-> Start
570 Bst NI * * *590 * 600 610 620 630
CCACATTGGT GTGCACCTCA AGCTTGATCG ATGACTTCGT ATCAACCTTA CTTTAGTATC TGAATTGGCT
GGTGTAACCA CACGTGGAGT TCGAACTAGC TACTGAAGCA TAGTTGGAAT GAAATCATAG ACTTAACCGA
640 650 660 670 680 690 700
GACCAACACT TAATGGCTGA ATATCGTGAA TTGCCGCGTG TTTTTGGTGC AGTTCGTAAG CATGTTGCTA
CTGGTTGTGA ATTACCGACT TATAGCACTT AACGGCGCAC AAAAACCACG TCAAGCATTC GTACAACGAT
710 720 730 740 750 760 770
ACGGTAAACG TGTTCGTGAT TTTAAAATCA GTCCTACTTT TATCCTTGGC GCAGGTCATG TTACATTCTT
TGCCATTTGC ACAAGCACTA AAATTTTAGT CAGGATGAAA ATAGGAACCG CGTCCAGTAC AATGTAAGAA
780 790 800 810 820 830 840
TTACGATAAG CTCGAGTTCT TACGTAAACG TCAAATTGAG CTTATAGCTG AATGTTTAAA ACGTGGTTTT
AATGCTATTC GAGCTCAAGA ATGCATTTGC AGTTTAACTC GAATATCGAC TTACAAATTT TGCACCAAAA
850 860 870 880 890 900 910
AATATCAAGG ATACTACAGT CCAGGATATT AGTGATATTC CTCAGGAATT CCGTGGTGAT TATATTCCCC
TTATAGTTCC TATGATGTCA GGTCCTATAA TCACTATAAG GAGTCCTTAA GGCACCACTA ATATAAGGGG
920 930 940 950 960 970 980
ATGAAGCTTC TATTGCTATA TCACAAGCTC GTTTAGATGA AAAAATTGCA CAACGTCCTA CTTGGTACAA
TACTTCGAAG ATAACGATAT AGTGTTCGAG CAAATCTACT TTTTTAACGT GTTGCAGGAT GAACCATGTT
NinI I Cla I Link
990 1000 1010 1020 1030 1040 ** 1050
ATACTACGGT AAGGCGATTT ATGCATAAGG GAACAACCTG GACCTCATGA TTATATGAGG GATTCATCGC
TATGATGCCA TTCCGCTAAA TACGTATTCC CTTGTTGGAC CTGGAGTACT AATATACTCC CTAAGTAGCG
Ban HI/Bgl II
1060 * 1070 1080 1090 1100 1110 1120
GACTCTAGAG GATCTTTGTG AAGGAACCTT ACTTCTGTGG TGTGACATAA TTGGACAAAC TACCTACAGA
CTGAGATCTC CTAGAAACAC TTCCTTGGAA TGAAGACACC ACACTGTATT AACCTGTTTG ATGGATGTCT
1130 1140 1150 1160 1170 1180 1190
GATTTAAAGC TCTAAGGTAA ATATAAAATT TTTAAGTGTA TAATGTGTTA AACTACTGAT TCTAATTGTT
CTAAATTTCG AGATTCCATT TATATTTTAA AAATTCACAT ATTACACAAT TTGATGACTA AGATTAACAA
1200 1210 1220 1230 1240 1250 1260
TGTGTATTTT AGATTCCAAC CTATGGAACT GATGAATGGG AGCAGTGGTG GHATGCCTTT AATGAGGAAA
ACACATAAAA TCTAAGGTTG GATACCTTGA CTACTTACCC TCGTCACCAC CTTACGGAAA TTACTCCTTT
1270 1280 1290 1300 1310 1320 1330
ACCTGTTTTG CTCAGAAGAA ATGCCATCTA GTGATGGTGA GGCTACTGCT GACTCTCAAC ATTCTACTCC
TGGACAAAAC GAGTCTTCTT TACGGTAGAT CACTACTACT CCGATGACGA CTGAGAGTTG TAAGATGAGG
1340 1350 1360 1370 1380 1390 1400
TCCAAAAAAG AAGAGAAAGG TAGAAGACCC CAAGGACTTT CCTTCAGAAT TGCTAAGTTT TTTGAGTCAT
AGGTTTTTTC TTCTCTTTCC ATCTTCTGGG GTTCCTGAAA GGAAGTCTTA ACGATTCAAA AAACTCAGTA
1410 1420 1430 1440 1450 1460 1470
GCTGTGTTTA GTAATAGAAC TCTTGCTTGC TTTCTATTT ACACCACAAA GAAAAAGCT GCACTGCTAT
CGACACAAAT CATTATCTTG AGAACGAACG AAACGATAAA TGTGGTGTTT CCTTTTTCGA CGTGACGATA
1480 1490 1500 1510 1520 1530 1540
ACAAGAAAAT TATGGAAAAA TATTCTGTAA CCTTTATAAG TAGGCATAAC AGTTATAATC ATAACATACT
TGTTCTTTTA ATACCTTTTT ATAAGACATT GGAAATATTC ATCCGTATTG TCAATATTAG TATTGTATGA
1550 1560 1570 1580 1590 1600 1610
GTTTTTTCTT ACTCCACACA GGCATAGAGT GTCTGCTATT AATAACTATG CTCAAAAATT GTGTACCTTT
CAAAAAAGAA TGAGGTGTGT CCGTATCTCA CAGACGATAA TTATTGATAC GAGTTTTTAA CACATGGAAA
1620 1630 1640 1650 1660 1670 1680
AGCTTTTTAA TTTGTAAAGG GGTTAATAAG GAATATTTGA TGTATAGTGC CTTGACTAGA GATCATAATC
TCGAAAAATT AAACATTTCC CCAATTATTC CTTATAAACT ACATATCACG GAACTGATCT CTAGTATTAG
1690 1700 1710 1720 1730 1740 1750
AGCCATACCA CATTTGTAGA GGTTTTACTT GCTTTAAAAA ACCTCCCACA CCTCCCCCTG AACCTGAAAC
TCGGTATGGT GTAAACATCT CCAAAATGAA CGAAATTTTT TGGAGGGTGT GGAGGGGGAC TTGGACTTTG
1760 1770 1780 1790 1800 1810 1820
ATAAAATGAA TGCAATTGTT GTTGTTAACT TGTTTATTGC AGCTTATAAT GGTTACAAAT AAAGCAATAG
TATTTTACTT ACGTTAACAA CAACAATTGA ACAAATAACG TCGAATATTA CCAATGTTTA TTTCGTTATC
1830 1840 1850 1860 1870 1880 1890
CATCACAAAT TTCACAAATA AAGCATTTTT TTCACTGCAT TCTAGTTGTG GTTTGTCCAA ACTCATCAAT
GTAGTGTTTA AAGTGTTTAT TTCGTAAAAA AAGTGACGTA AGATCAACAC CAAACAGGTT TGAGTAGTTA
1900 1910
GTATCTTATC ATGTCTGGAT CC
CATAGAATAG TACAGACCTA GG
*Ban HI

Figure 3a

Stategy for the cloning of the Den V cartridge into a plaasmid containig the HSV 1 TK locus.

A. Plasmid pMCADV and puc 19 were cut with Xba I and ligated together in order to obtain the resultant plasmid pIR1

B. pIR1 and pTKSB were cut with Bam HI and ligated together in order to obtain the resultant plasmid pTKE59


A
Xba I, 1
Hind III, 988
Hind III, 1318
Bam HI, 1984
Xba I, 1918
15478, Hind III
Dan V
Hind III, 2498
pHCADV
17578 bp
Hind III, 5398
pBR322
18698, Bam HI
Bam HI, 6348
Hind III, 6738
pUC19
2686 bp
Bam HI, 418
Xba I, 424
MCS
Amp
Bam HI, 1
Xba I, 7
pIR1
4596 bp
Hind III, 988
Hind III, 1318
Bam HI, 1984
Xba I, 1918
Hind III, 1934
B
Cla I, 1
AMP
pTKSB
6177 bp
3884, Pvu II
HSV 1 TK
Pvu II, 2824
Bam HI, 2574
pTKE59
8087 bp
5888, Pvu II
Dan V
Hind III, 3274
4598, Bam HI
4584, Xba I
Hind III, 3684

between a plasmid carrying the den V cartridge embedded within the TK gene (pTKE59) and infectious viral DNA (Figure 3b).

The den V expression cartridge was obtained within the plasmid pMCADV (Colicos *et al.*, manuscript in preparation). From figure 3a, it can be seen that the cartridge is flanked by Xba I sites and has a Bam HI site immediately 5' to the 3' Xba I site. In order to construct pTKE59 it was necessary to gain a Bam HI site on the 5' side of the cartridge as well. This was performed by the digestion of pMCADV and pUC19 (Figure 3a) with Xba I and ligation of the fragments from the digestion. PUC19 has a Bam HI site adjacent to the Xba I site in the polylinker, therefore, upon ligation in the proper orientation the cartridge would gain a Bam HI site on the 5' side. Figure 4 shows the results of this cloning step. Lane 2 shows pMCADV digested with Xba I resulting in the expected fragment sizes of approximately 15,700 bp and 1,900 bp and lane 3 is pUC19 linearized with Xba I giving the resultant band size of approximately 2,700 bp. The result of the ligation yielded the plasmid seen in lane 4. This plasmid pIR1 was digested with Bam HI and yielded band sizes of approximately 2,700 and 1.900. This shows that the den V cartridge was inserted into pUC 19 in the proper orientation and the cartridge is now flanked by Bam HI sites.

The cartridge was then cloned into the HSV 1 TK gene that is carried on the plasmid pTKSB (Varmuza and Smiley,

Figure 3B

Recombination Between pTKE59 and HSV 1 DNA : Integration Site Of The Den V sequences

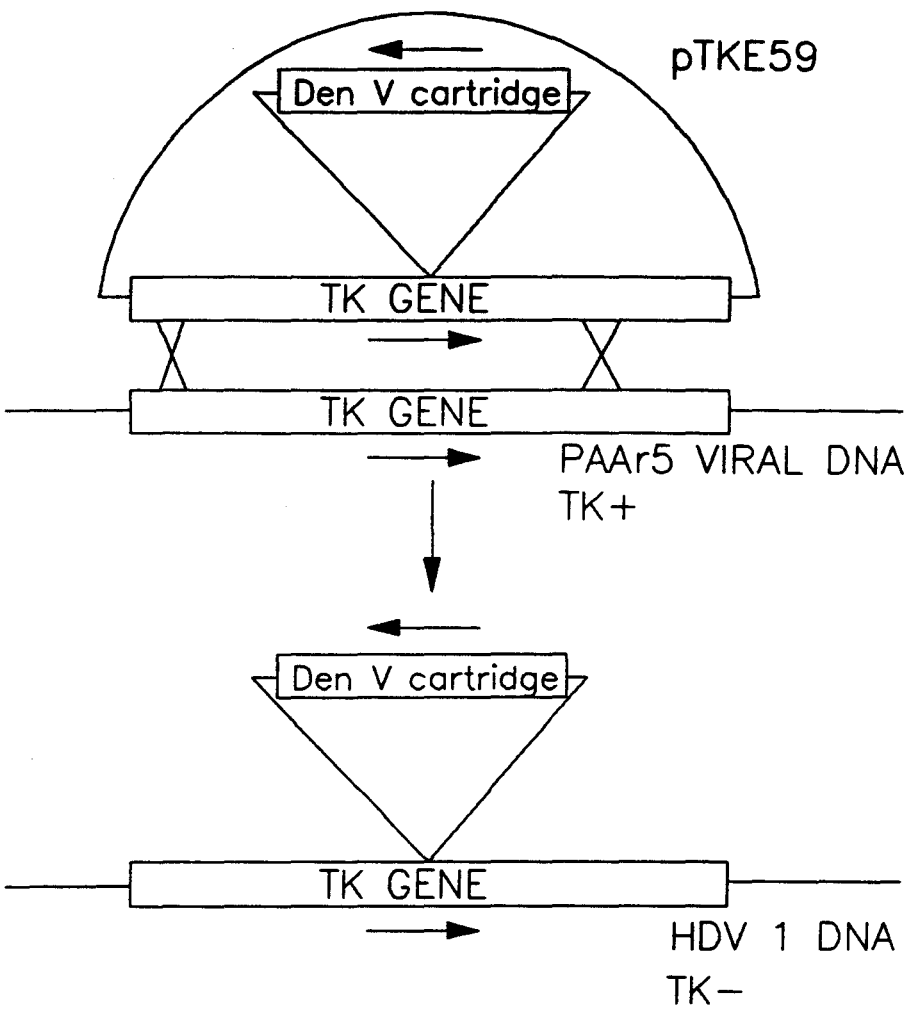
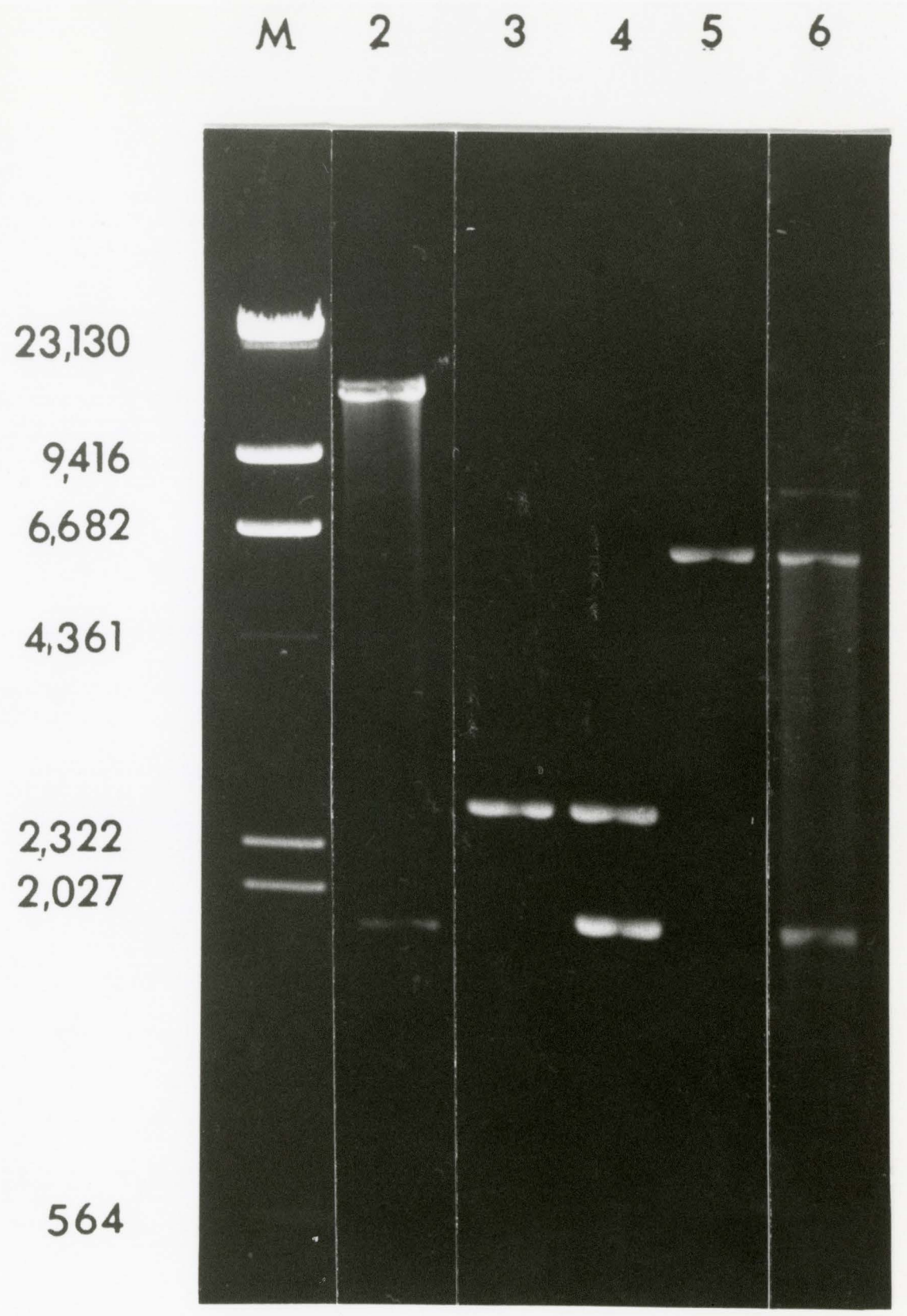

pTKE59
Den V cartridge
TK GENE
TK GENE
PAAr5 VIRAL DNA
TK+
Den V cartridge
TK GENE
HDV 1 DNA
TK−

Figure 4

Restriction enzyme analysis of plasmids.

Plasmid DNA from large scale plasmid preparations were digested with the approproate enzyme for 4 hr and electrophoresed overnight in a 0.8 % agarose gel.The gel was then stained with EtBr and photographed.

Lane M, Lambda DNA cut with Hind III as a marker. This gives size fragments of 23,130 bp, 9,416 bp, 6,682 bp, 4,361 bp, 2,322 bp, 2027 bp, 564 bp and 125 bp which is not seen. Lane 2, pMCADV digested with Xba I. Lane 3, pUC 19 digested with Xba I. Lane 4, pIR1 digested with Bam HI Lane 5, pTKSB digested with Bam HI and lane 6, pTKE59 digested with Bam HI.


M
2
3
4
5
6
23,130
9,416
6,682
4,361
2,322
2,027
564

1985). This plasmid has the Pvu II A fragment of the Bam HI Q fragment of HSV 1 inserted into the Pvu II site of pBR322. This fragment contains the whole TK gene as well as some sequences both upstream and downstream of the gene. During construction of the plasmid a Bam HI site was inserted at about 600 bp from the 5' side of the gene and also during this construction about 100 bp were deleted. The Bam HI site was also removed from the pBR322 sequences in order to allow easy insertion into the Bam HI site within the TK gene. PTKSB and pIR1 were digested with Bam HI and the fragments were ligated together (Figure 4). Lane 4 shows pIR1 digested with Bam HI resulting the expected fragments of approximately 2,700 bp and 1,900 bp and lane 5 is pTKSB linearized with Bam HI yielding a band of approximately 6,100 bp.. Lane 6 shows the resultant plasmid from the ligation pTKE59 digested with Bam HI which yielded fragment sizes of 6,100 bp and 1900 bp. This shows that the den V cartridge was inserted into pTKSB. Lane 6 also shows a band at approx 8,000 bp which is undigested pTKE59.

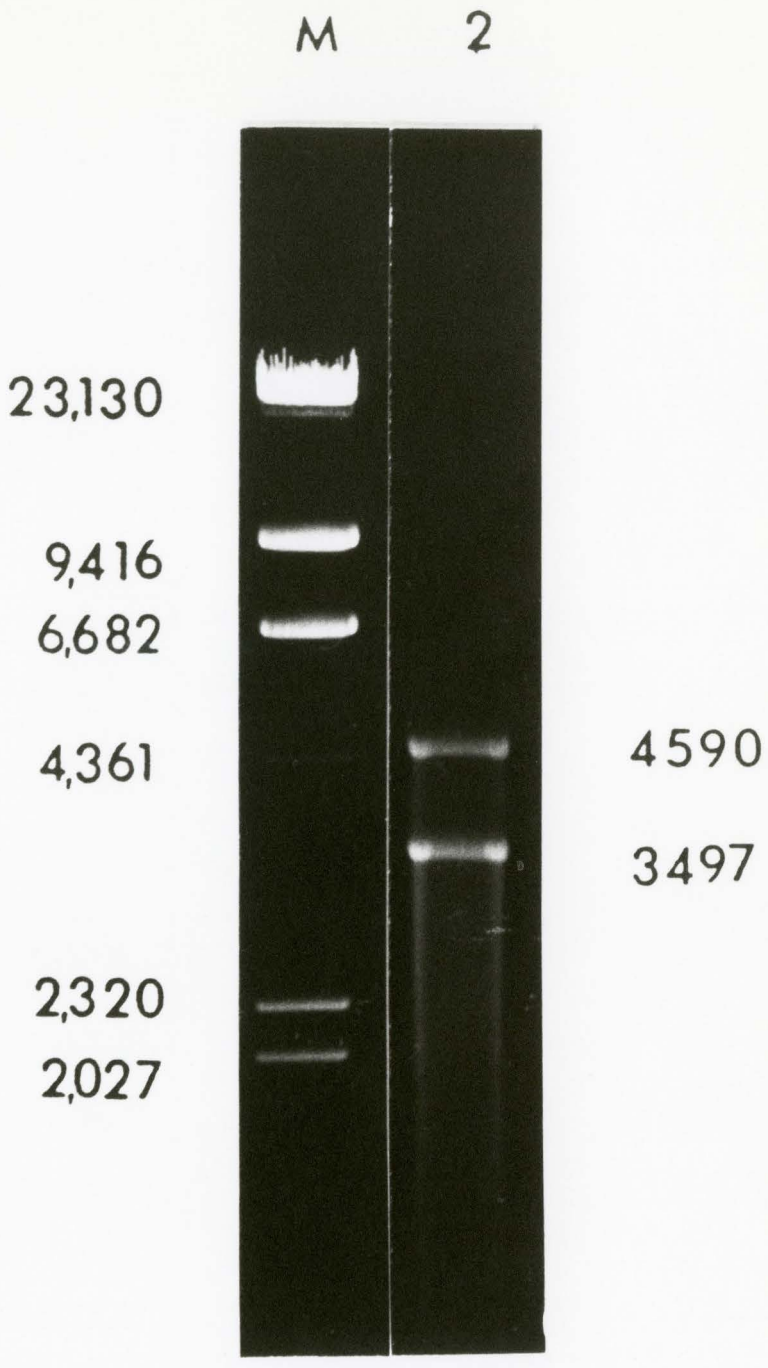
The orientation of the cartridge, within pTKE59, was determined by a Cla I, Xba I double digest. Fragment sizes of approx 4,600 bp and 3,500 bp were obtained (Figure 5). This shows that the den V cartridge in pTKE59 was inserted in the opposite orientation to that of the TK gene. A plasmid containing the cartridge in the other orientation was also isolated and named pTKE55 (data not shown).

Figure 5

Determination of the orientation of the Den V sequences in pTKE59

Plasmid pTKE59 was digested with Xba I and Cla I for 4 hr then electrophoresed overnight in a 0.8 % agarose gel. The gel was then stained with EtBr and photographed.

Lane M, Lambda DNA marker. Lane 2, pTKE59 digested with Cla I and Xba I.


M
2
23,130
9,416
6,682
4,361
2,320
2,027
4590
3497

1.4 Virus Construction

Infectious viral PAAr5 DNA and pTKE59 were co-transfected into Vero cells in order to allow site specific recombination to integrate the den V cartridge at the TK locus. The initial transfection plates were allowed to go to until complete c.p.e. had been reached and stocks were made from these plates. These stocks were then plated with the drug Acyclovir in order to select for TK - viruses. Plaques from these plates were then picked and screened for the den V insertion.

Screening was performed by extracting viral DNA and digesting it with Pvu II and electrophoreseing the products. Pvu II releases a 2,040 bp TK specific fragment from the parental virus and a recombinant virus would yield a 3,850 (1940 + 1910) bp fragment if the den V sequences had been integrated into the genome. Southern blotting and probing of such a gel (Figure 6) with pTKSB shows hybridization to a 2,000 bp fragment in the parental lane (lane 7) and a 3,800 bp fragment in the recombinant virus lanes (lanes 2 - 5, 8 - 10). Hybridization to a 3,800 Pvu II fragment from pTKE59 as well as hybridization to the pBR322 backbone was also seen. This shift in the TK gene size shows the virus of interest has been constructed. One of these viruses HDV 1 was chosen for study in greater detail.

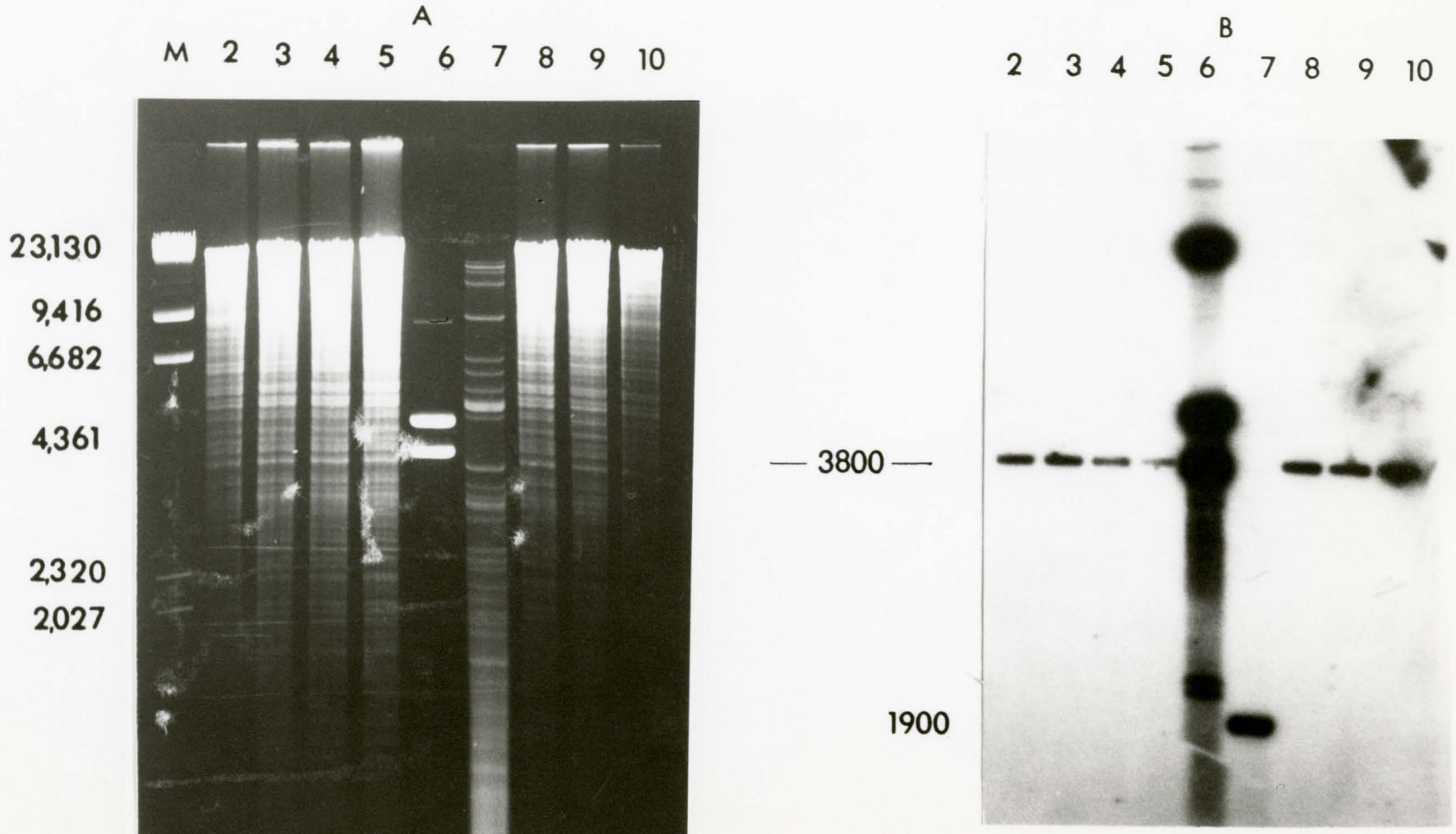
Figure 6

Screening of transfection stocks for recombinant viruses

DNA from viral stocks produced by cotransfections were digested with Pvu II and electrophoresed overnight at 70 volts in a 0.8 % agarose gel and was then stained with EtBr and photographed. At this time the gel was then southern blotted and probed with pTKSB.

A. EtBr stained gel. Lane M, Lambda DNA marker. Lanes 2 -5 and lanes 8 -10 are transfection produced viruses digested with Pvu II. Lane 7 is DNA from PAAr5 digested with Pvu II and lane 6 is pTKE59 digested with Pvu II.

B. Autoradiogram of the southern blot of the gel in A probed with pTKSB.


A
M 2 3 4 5 6 7 8 9 10
23,130
9,416
6,682
4,361
2,320
2,027
B
2 3 4 5 6 7 8 9 10
3800
1900

1.5 Further Characterization OF HDV 1

To ensure that the sequences were integrated into the genome at the TK locus and were of den V origin the following experiments were performed.

HDV 1 DNA and pBR322 DNA were digested with PVU II. The products were electrophoresed and transfered to nitrocellulose by southern blotting. This was then probed with pBR322. Figure 7B shows that the pBR322 probe hybridized only to the pBR322 band in lane 1 and not to any sequences in HDV 1 (Lane 2). This shows that the sequences integrated into the HSV 1 genome are not of pBR322 origin.

HDV 1 DNA, PAAr5 DNA, pTKE59 and pTKSB were digested with Pvu II and electrophoresed. Figure 8A shows the ethidium bromide stained gel. From this, the appearance of a 3,800 bp band in the HDV 1 lane (lane 4) that is not in the PAAr5 lane (lane 3) can be seen. This band comigrates exactly with the den V containing Pvu II fragment from pTKE59 (Lane 5). Also, in the PAAr5 lane there is a band at approximately 2,000 bp which is not seen in the HDV 1 lane. This size is in good accordance with where the wild type Pvu II fragment containing the TK gene should migrate. The Pvu II fragment containing the den V gene from pTKSB migrates at about 100 bp lower than the band in the PAAr5 lane (lane 2). This is expected due to the 100 bp deletion made during the construction of the plasmid. This gel was then southern blotted and probed with pIR1. This plasmid will only

Figure 7

Characterization Of The Sequences Inserted Into The HSV 1 Genome In HDV 1

DNA from HDV 1 and PBR322 were digested with PVU II and electrophoresed overnight in a 0.8 % gel. The gel was then stained with EtBr photographed, southern blotted and probed with pBR322.

A. EtBr stained gel. Lane .M1, Lambda DNA Marker. Lane 1, pBR322 digested with PVU II. Lane 2, HDV 1 DNA digested with PVU II.

B. Autoradiogram of the southern blot of the gel in a probed with pBR322.

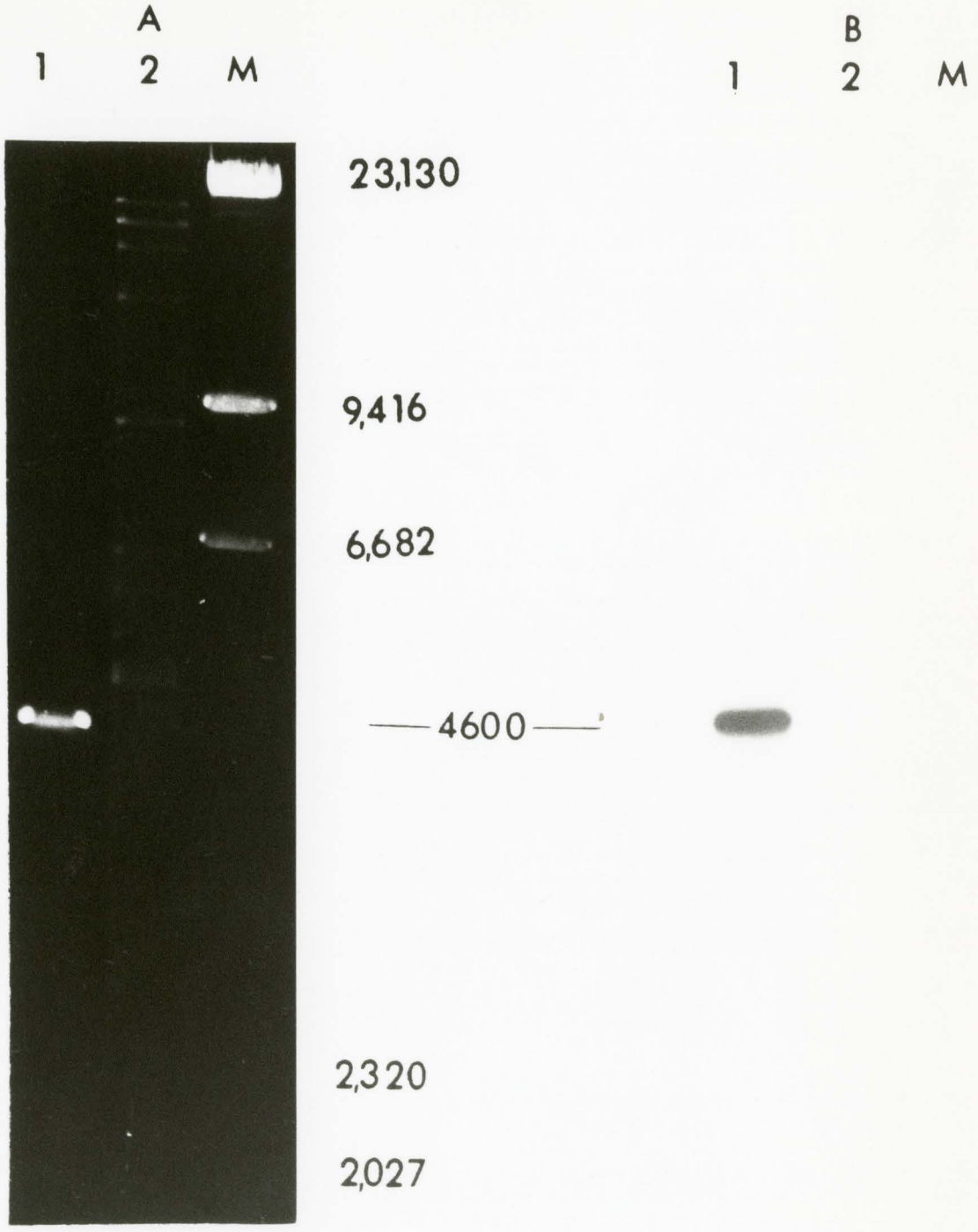

A
1
2
M
B
1
2
M
23,130
9,416
6,682
4600
2,320
2,027

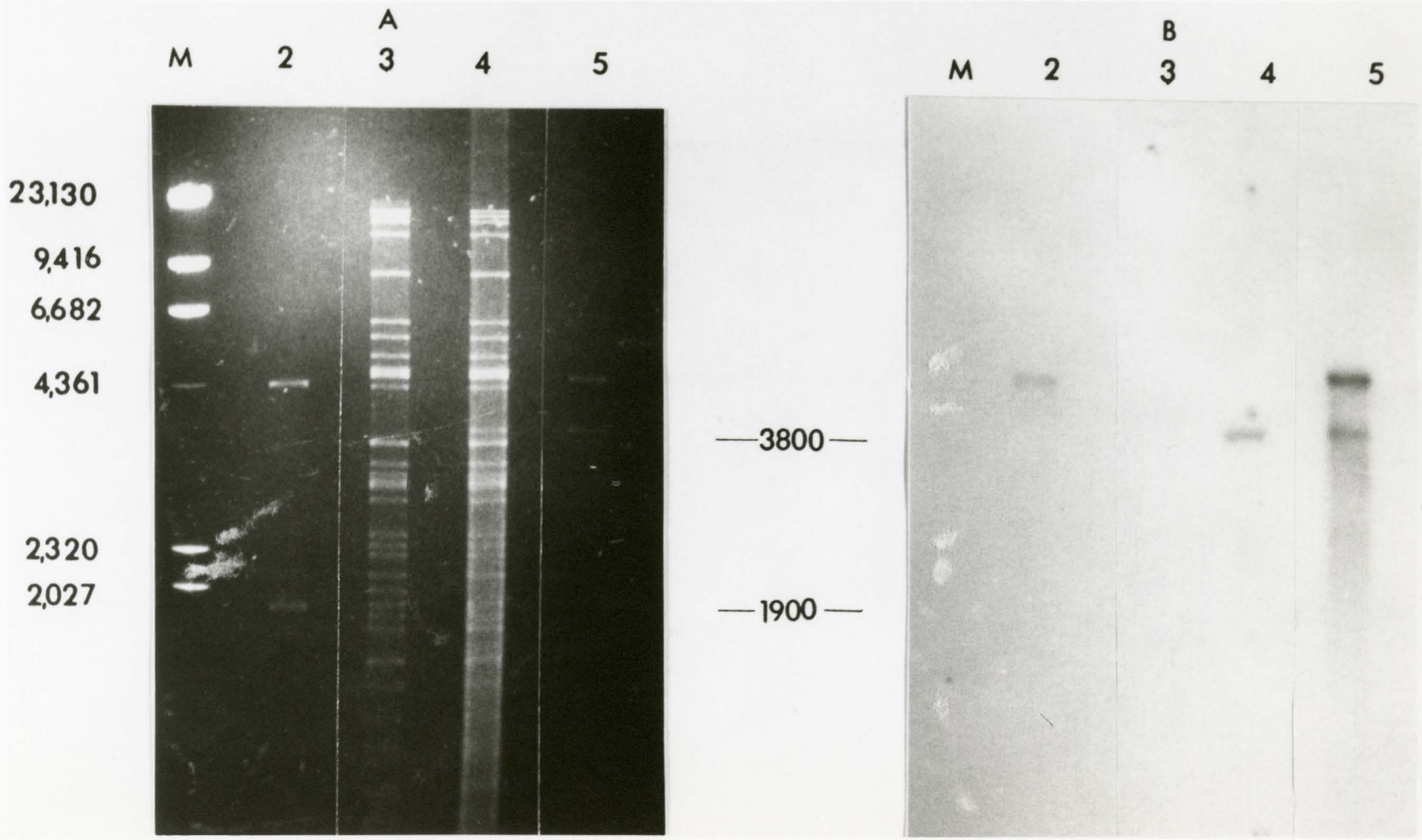
Figure 8

Further analysis of the sequnces inserted into the recombinant virus HDV 1

Viral and plasmid DNA were digested for 4 hr with Pvu II and electrophoresed overnight at 70 volts. The gel was stained with EtBr, photographed, Southern blotted and probed with pIR1.

A. EtBr stained Gel. Lane M, Lambda DNA marker. Lane 2, pTKSB cut with Pvu II. Lane 3, pAA-5 cut with Pvu II. Lane 4, HDV 1 cut with Pvu II and lane 5, pTKE59 cut with Pvu II.

B. Autoradiogram of the southern blot of the gel in A probed with pIR1.


A
M
2
3
4
5
23,130
9,416
6,682
4,361
2,320
2,027
3800
1900
B
M
2
3
4
5

hybridize to the den V cartridge or pBR322 sequences. Figure 7B shows that pIR1 hybridized to a 3,800 bp band in both the HDV 1 lane and in the pTKE59 lane where it also hybridized to the 4,600 bp backbone fragment. There was no hybridization in the PAAr5 lane and hybridization only to the 4,600 backbone fragment in the pTKSB lane. This result, combined with the results of probing HDV 1 with pBR322, shows that the sequences integrated are of den V origin.

This same blot was stripped and then probed with pTKSB in order to show that the sequences that hybridized with the first probe contain TK sequences and also to show that the TK fragment had shifted from 2,000 bp to 3,800 bp. Upon probing with pTKSB, the 3,800 bp fragment in the HDV 1 and pTKE59 lanes hybridized as they did with the pIR1 probe and this time a 2,000 bp fragment from the PAAr5 lane and a 1900 bp fragment from the pTKSB lane hybridized as well (Figure 9). The combination of all three results show that the den V cartridge was inserted intact into the TK locus of HSV 1.

2.0 Expression Of The den V Gene In HDV 1

Both monoclonal and polyclonal antibodies raised against the den V gene were available to aid in the detection of den V expression in HDV 1. However, before they were used RNA slot blots were performed on RNA harvested from infected cells and probed with pIR1 to determine if any den V RNA was

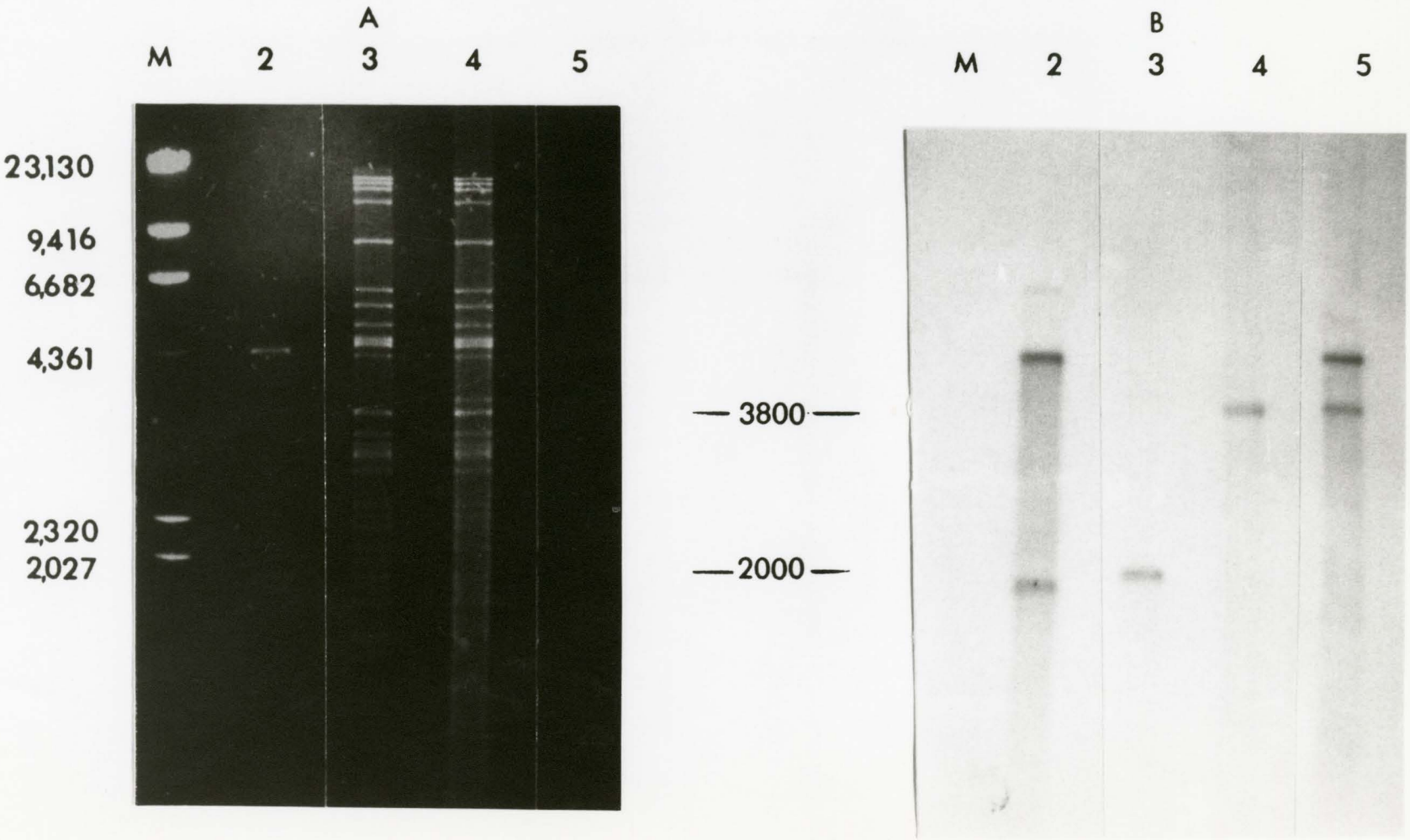
Figure 9

Characterization of the origin of insertion of the Den V sequences in HDV 1

Viral and plasmid DNA were digested for 4 hr with Pvu II and electrophoresed overnight at 70 volts. The gel was stained with EtBr, photographed, Southern blotted and probed with pIR1.

A. EtBr stained gel. Lane M, Lambda DNA marker. Lane 2, pTKSB cut with Pvu II. Lane 3, PAAr5 cut with Pvu II. Lane 4, HDV 1 cut with Pvu II and lane 5, pTKE59 cut with Pvu II.

B. Autoradiogram of the southern blot of the gel in A probed with pTKSB.


A
M
2
3
4
5
23,130
9,416
6,682
4,361
2,320
2,027
3800
2000
B
M
2
3
4
5

present in the cells. The control virus used in all studies was L714 (Smiley *et al.*, 1987). This virus like HDV 1 has a 2 kb insertion at the TK locus and was also made through recombination using PAAr5 as the parental virus. The results show the control probe, pSS17GD (a probe for glycoprotein D and I), hybridizes to RNA from both L714 and HDV 1 infected cells. But the pIR1 probe only hybridizes to RNA from HDV 1 infected cells. This indicates that there is den V specific RNA in the HDV 1 infected cells (Figure 10).

An attempt to detect den V protein from HDV 1 infected cells was performed by immunoprecipitations (IP) and western blots. IPs were done using both the monoclonal antibody IC3 (Valerie et al., 1986) and the polyclonal antibody # 38 (Valerie et al., 1987b). Both antibodies yielded the same results so only the results for the polyclonal antibody have been presented. Figure 11a shows typical results obtained from IP experiments using the polyclonal antibody. Lanes 2, 3 and 4 show that the proteins recognized in mock infected cells, L714 infected cells and HDV 1 infected cells are all the same. The den V protein is 16 kD and, therefore, a band was expected to migrate to this spot on the gel. A band at approximately this size is seen in all of the lanes which might indicate that there is a homologous cellular protein that the antibody recognizes. The results presented show the proteins extracted and immunoprecipitated from cells 6 hrs. post infection. Proteins were extracted from cells from 2 - 12 hrs. post

Figure 10

Detection of Den V specific RNA from HDV 1 infected cells

RNA was extracted from infected cells and transferred to nitrocellulose through a slot blot apparatus. The same amount of RNA was loaded per slot and each sample was loaded in duplicate. The nitrocellulose was then cut to seperate the duplicate samples and one set was probed with the gD plasmid pSS17GD and the other set probed with pIR1.

A. Autoradiogram of the samples probed with pSS17GD. Slots 1 & 2, mock infected cells at 4 & 8 hr post infection respectively. Slots 3 & 4, L714 infected cells at 4 & 8 hr post infection respectively. Slots 5 & 6, HDV 1 infected cells at 4 and 8 hr post infection respectively.

B. Autoradiogram of the samples probed with pIR1. Slots are the same as those in A

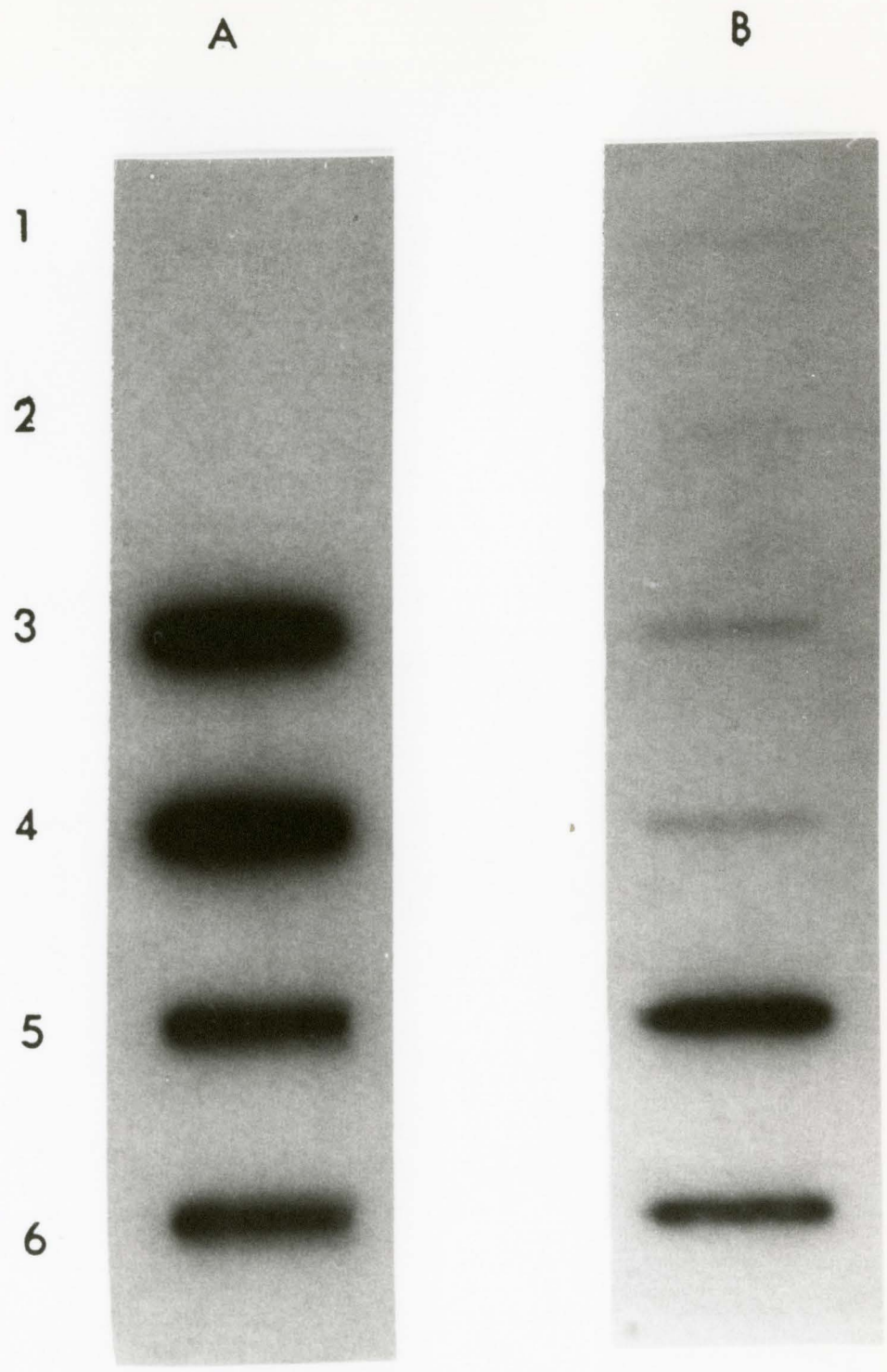

A
B
1
2
3
4
5
6

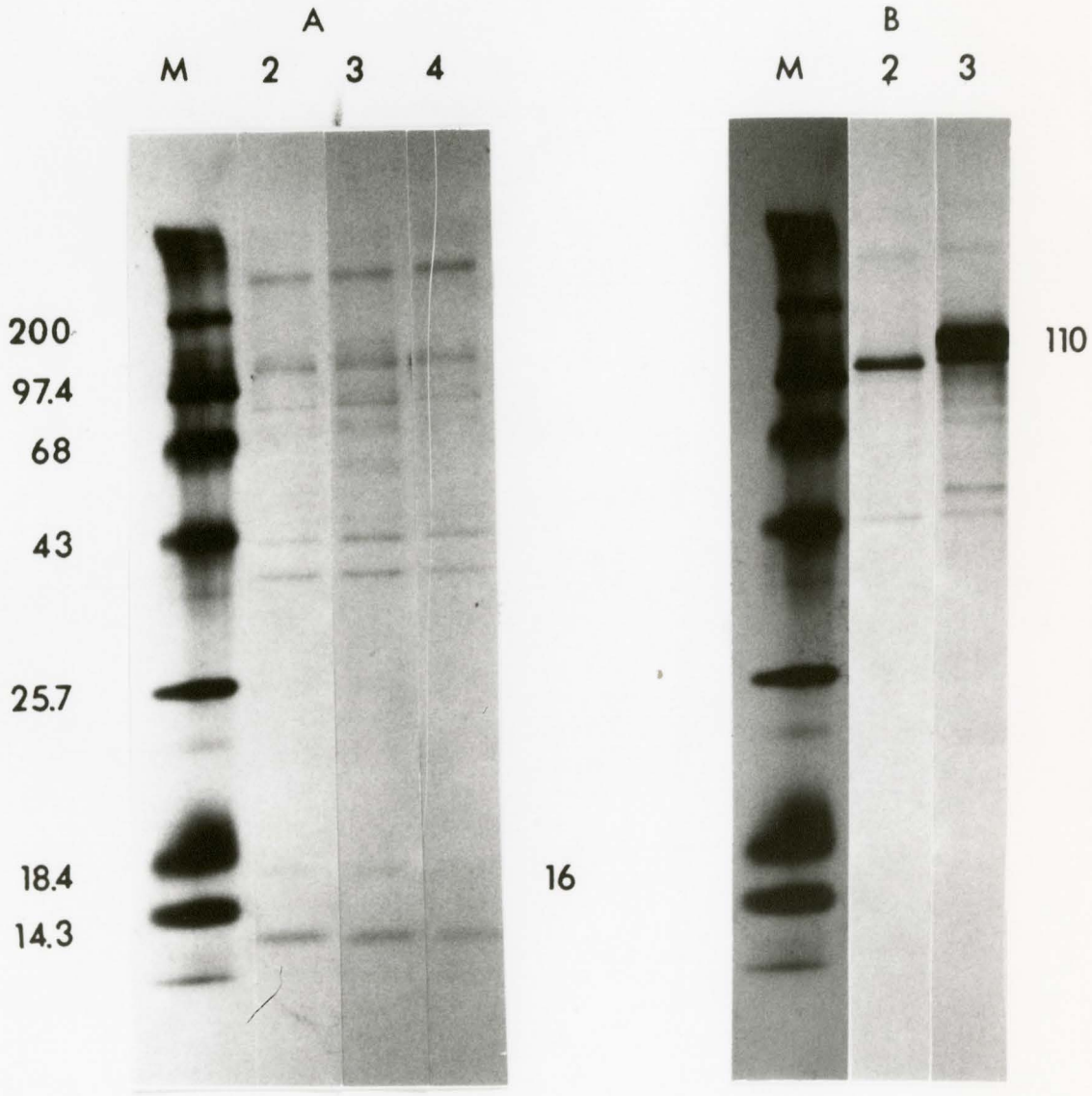
Figure 11

Detection of Den V protein from HDV 1 infected cells by immunoprecipitations

Protein was labelled with ^{35}S methionine, extracted from infected Vero cells or bacteria, immunoprecipitated with the poyclonal antibody # 38 or the monoclonal antybody 15BB2 and electrophoresed overnight on a 15.2 % SDS PAGE gel.

A. Autoradiogram of proteins immunoprecipitated with #38. Lane M, Marker purchased from BRL with Molecular Weight sizes of 200,000, 97,400, 68,000, 43,000, 25,700, 18,400 and 14,300. Lane 2 mock infected cells. Lane 3, L714 infected cells at 6 hr p.i. Lanes 4, HDV 1 infected cells at 6 hr p.i.

B. Autoradiogram of proteins precipitated by 15BB2. Lane M, Marker purchased from BRL. Lane 2, Mock infected cells. Lane 3 HDV 1 infected cells.


A
M
2
3
4
200
97.4
68
43
25.7
18.4
14.3
16
B
M
2
3
110

infection. All times yielded the same results and, therefore, only one time point was chosen to present here.

Mock and HDV 1 infected samples were immunoprecipitated using the antibody 15BB2 raised against glycoprotein B. This was done to ensure that the protein extraction and IP techniques employed were working properly and that the bands seen in the Figure 11a were not just background proteins that are termed sticky and bind to most antibodies. Figure 11b shows that a protein that is specific to the HDV 1 lane migrates at approx 105 - 110 kD which is the reported size of the gB precursor protein (Claesson-Welsh and Spear, 1986). There is also a band that migrates at approximately 95 kD in both the HDV 1 lane and the mock infected lane which is a cellular protein that is recognized by the gB antibody (Mike Ligas, Dept. of Biology, McMaster University, personal communication). This shows that the techniques employed were working properly. The fact that the proteins that migrated at approximately 16 kD in IPs done with #38 were not in the IPs done with 15BB2 indicates that these protein bands may be specifically recognized by the den V protein.

A bacterial strain, carrying the plasmid pTac-Den V, was to be used as positive control for den V protein. This plasmid contains the den V gene which can be induced to overproduce den V protein upon the addition of IPTG to the media (Valerie et al., 1987b). Figure 12 shows an immunoprecipitation of proteins from bacteria containing pTac

Figure 12

Detection of the Den V protein from Bacterial cells containing the plasmid pTac - Den V.

An overnight culture of bacterial cells was diluted and grown into log phase. IPTG was then added to the media and 3 hrs post induction the cells were labelled with ^{35}S methionine. Proteins were then collected at four hrs post induction and immunoprecipitated using antibody # 38.

Lane M, Marker purchased from BRL. Lane 2, protein extracted from LE392 cells. Lane 3, protein extracted from cells carrying pTac - Den V.


M
2
3
68
43
25.7
18.4
14.3

- den V (Lane 3) and LE392 bacteria as a control (Lane 2). As can be seen in the figure, many proteins were immunoprecipitated by antibody # 38 and none were specific to the pTAC -Den V lane. This shows that the positive control does not work with the antibodies used.

Western blots, using an ^{125}I conjugated second antibody, were attempted to show the expression of the den V protein (Figure 13). The antibody did not bind to anything specific in the HDV 1 infected lanes and hybridization was seen to high molecular weight proteins and to a less extent to a band at approximately 16 kD in all lanes including uninfected cells. Before the blot was probed, a marker lane from the right hand side of the gel was cut and stained with immido black in order to visualize it (data not shown).

Western blots were also performed using an alkaline phosphatase conjugated second antibody (Figure 14). As in the case of the immunoprecipitations no den V specific bands were seen in the HDV 1 lanes (Lanes 5 and 6, 4 and 8 hrs. post infection respectively) and there was a 16 kD band seen in all of the lanes as in the immunoprecipitations.
The bacterial lane (7) also gave the same result as the immunoprecipitations in that the antibody hybridized to many proteins. Before the blot was probed, a marker lane was cut and stained with immido black in order to visualize the marker (lane M).

Valerie *et al.*, (1987) reported expression of the den V gene in mammalian cells by using indirect

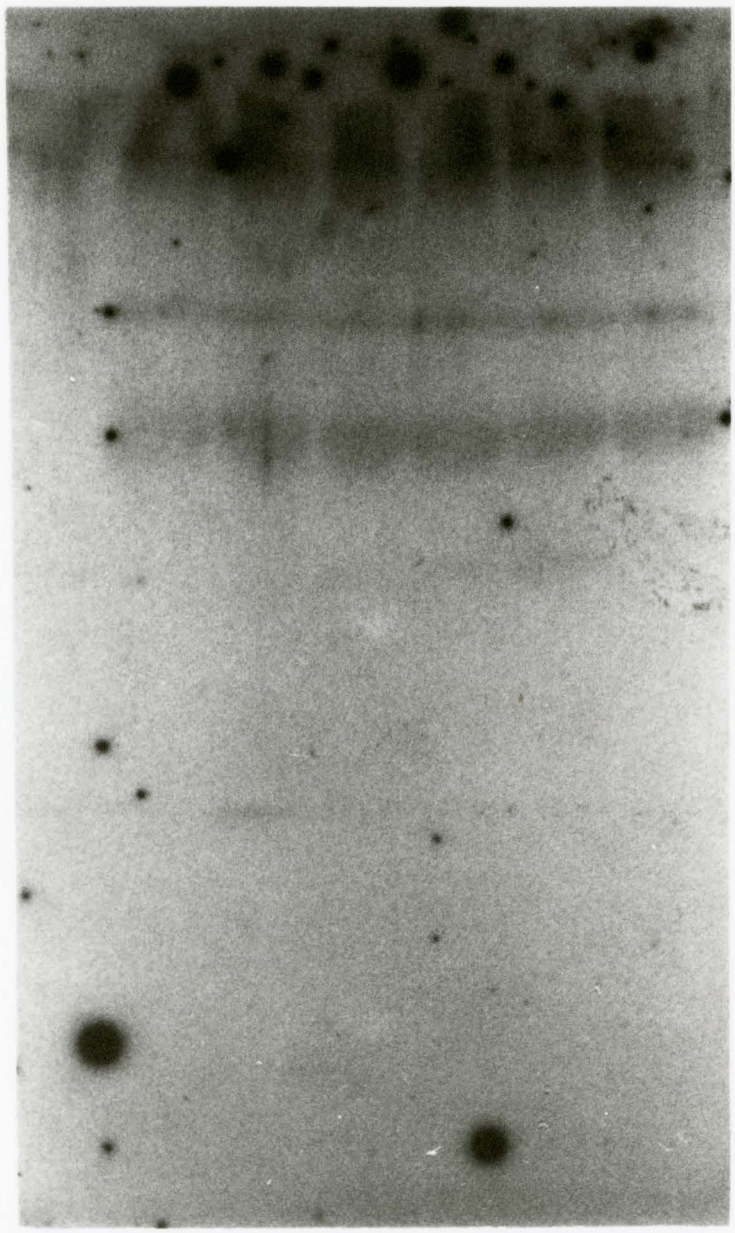
Figure 13

Detection of Den V protein from HDV 1 infected cells by Western Blot using an ^{125}I conjugated second antibody.

Protein was extracted from infected cells or bacteria and electrophoresed on a 15.2 % SDS PAGE gel. The protein was then transfered to nitrocellulose using a trans blot apparatus and then probed with the polyclonal antibody #38.

Autoradiogram of the gel. Lane M, BRL Marker. Lane 2, Mock infected cells. Lanes 3 & 4, protein from L714 infected cells at 4 and 8 hr p.i. respectively. Lanes 5 and 6, protein from HDV 1 infected cells at 4 and 8 hr p.i. respectively.

M 2 3 4 5 6 7



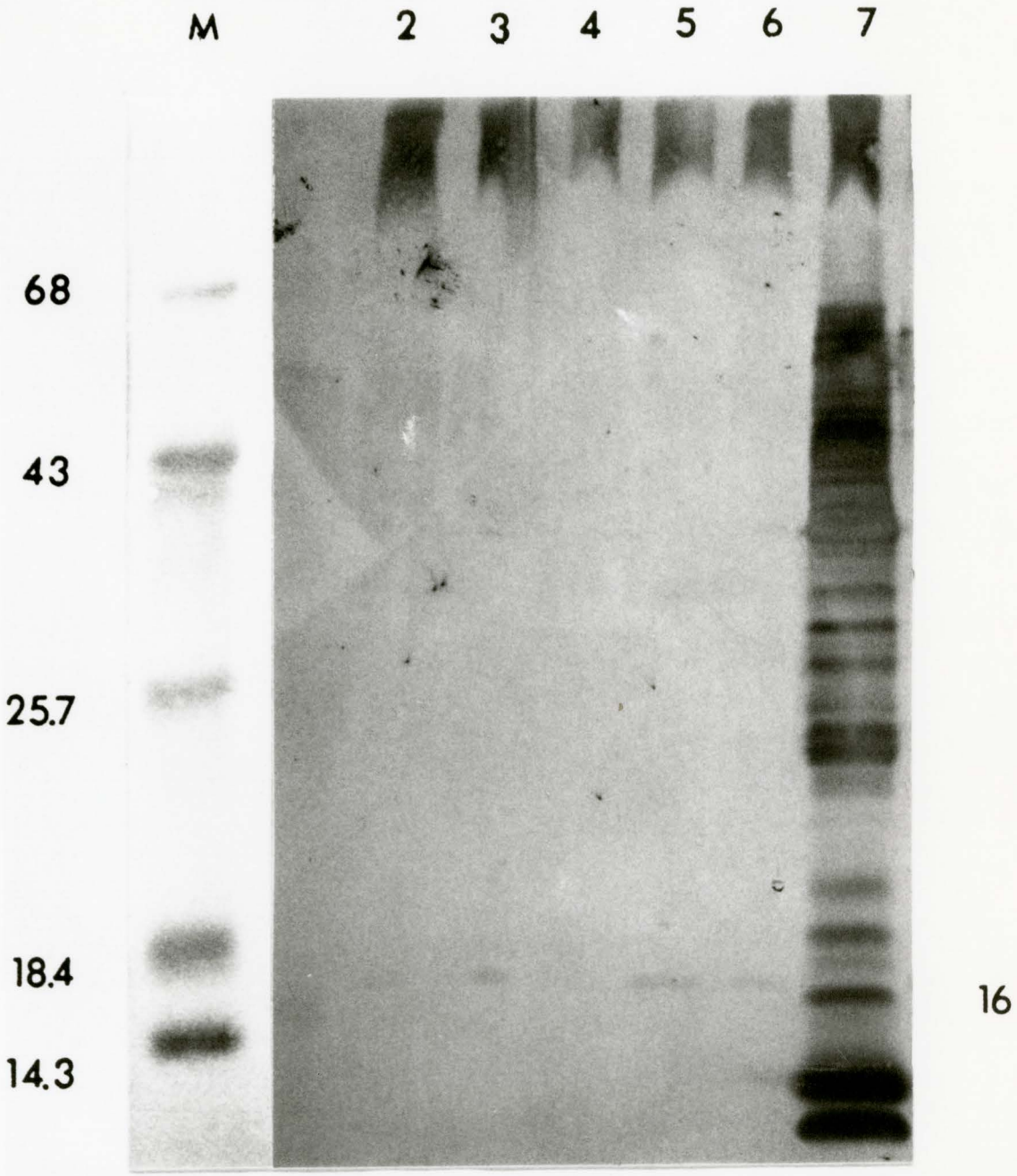
16

Figure 14

Detection of Den V protein from HDV 1 infected cells by Western Blot using an alkaline phosphatase conjugated second antibody.

Protein was extracted from infected cells or bacteria and electrophoresed on a 15.2 % SDS PAGE gel. The protein was then transfered to nitrocellulose using a trans blot apparatus and then probed with the polyclonal antibody #38.

Photograph of the Gel. Lane M, BRL Marker. Lane 2, Mock infected cells. Lanes 3 & 4, protein from L714 infected cells at 4 and 8 hr p.i. respectively. Lanes 5 and 6, protein from HDV 1 infected cells at 4 and 8 hr p.i. respectively. Lane 7 protein from pTac-Den V at 4 hr post induction.


M
2
3
4
5
6
7
68
43
25.7
18.4
14.3
16

immunofluorescence. This technique was attempted and the results are reported in table 1. The same number of cells fluoresced in HDV 1 infected and uninfected slides when number 38 antibody was used as the primary antibody. (914.5, 12.67 and 935 $\pm$ 20.08 respectively). However, when II- 436 (gD antibody, Ligas and Johnson, 1987) was used as the primary antibody the HDV 1 infected slides had over 20 times more fluorescent cells. This again suggests that either the den V antibodies are recognizing a cellular protein or are not specific enough to detect den V protein.

It is not possible to tell from these results whether or not the virus is expressing the den V protein.

2.1 Primer Extension Experiments

Due to the inability to show expression of den V protein, primer extensions were performed in order to show that correctly initiated RNA was produced upon infection with HDV 1. The primer 5' CGCGGCAATTCACGATATTCAGCC 3' runs from base 668 to base 644 (Fig 3). This, therefore, should yield a product of 123 bp when extended if properly initiated RNA was present in HDV 1 infected cells. Figure 15 shows that a product of this length was detected in HDV 1 infected cells (Lane 5), but, not in mock infected cells (Lane 4). This result shows that properly initiated den V specific RNA was made in HDV 1 infected cells.

The RNA was extracted at 6 hrs. post infection. The

Table 1

Indirect immunofluoresence of Cells : Detection of Den V Protein

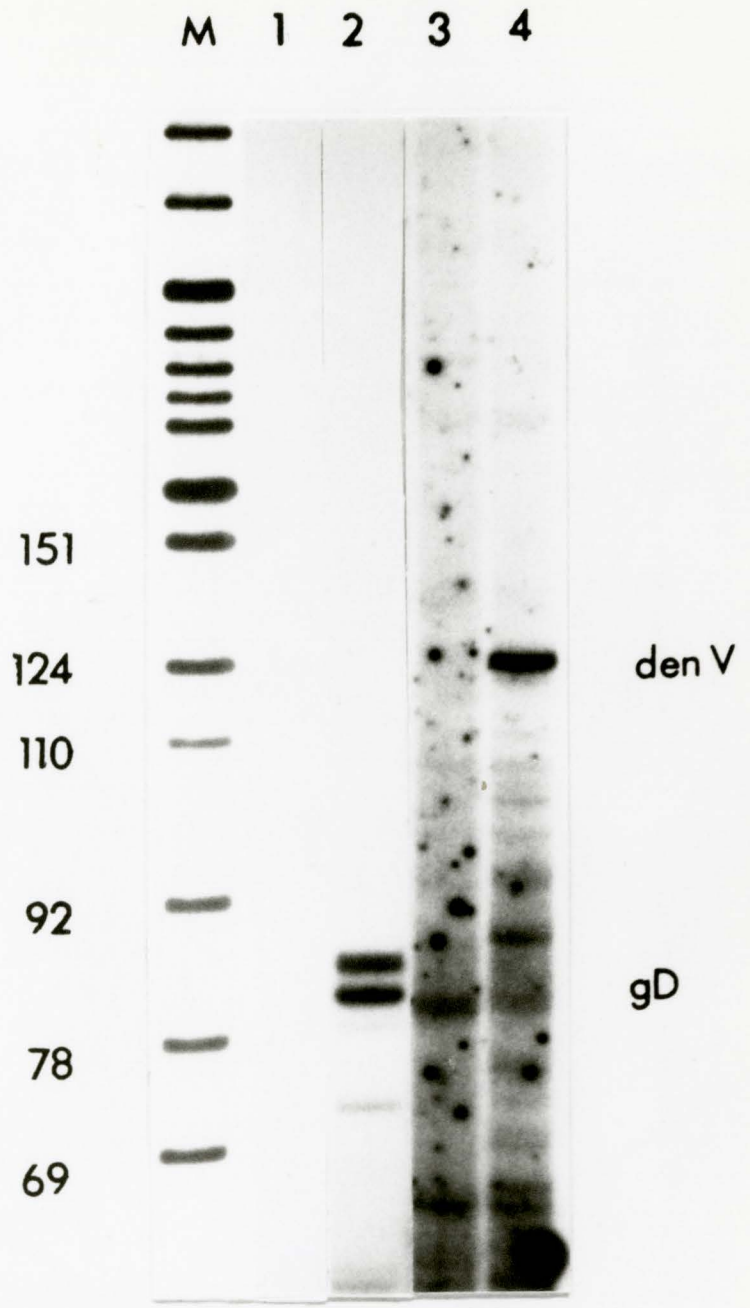
1° Antibody	Ave. # Of Flourescent Cells	
	Uninfected Cells	HDV 1 Infected Cells
# 38	935.0 ± 20.08	914.5 ± 12.67
15BB2	30.0 ± 7.48	723.0 ± 10.45

Figure 15

Detection of properly initiated den V RNA using primer extension

RNA was isolated from mock infected and HDV 1 infected cells 6 hr. post infection. The RNA was then annealed to either a gD or den V primer and the primers were then extended using reverse transcriptase. The products were then electrophoresed on an 8 % sequencing gel.

Autoradiogram of the gel. Lane M, marker lane. Lane 2 and 4, mock infected cells primmed with gD and den V primers respectively. Lanes 3 and 5, HDV 1 infected cells primmed with gD and den V respectively.


M
1
2
3
4
151
124
110
92
78
69
den V
gD

amount of product extended equals,approximately, the amount of product extended using the gD oligomer (lane 3) which suggests the two may be in the same kinetic class and, therefore, expressed at the same time.

3.0 Host Cell Reactivation Studies

Host cell reactivation of the plaque forming ability of several strains of HSV 1 was used to study whether certain virally encoded genes are important in DNA repair of the virus. It was also used to determine if the integrated den V gene could increase the survival of UV irradiated HDV 1 and therefore, show whether or not HSV 1 can be used as a suitable vector for studying DNA repair genes. Lastly, it was used in order to help elucidate the possible differences between normal and transformed cells.

These experiments were performed by seeding cells of interest into 24 well plates 24 hrs. prior to virus infection. Each experiment consisted of irradiating a suspension of virus with 3 doses and three two fold dilutions of the irradiated suspension for each dose was plated in duplicate. A control of unirradiated virus also had three two fold dilutions plated in duplicate. Plaques were scored after 3 days and titers for the irradiations were determined by linear regression using the least squares method. 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. Plotting the log of the surviving fraction vs dose given to the virus results in a straight line if looking at 1 component or 2 straight lines if the doses are over a great enough range to cover both components. The negative inverse of the slope is termed the Do value and is the dose required to reduce the level of survival of any point along the straight line to 0.37 of that level. Do. values were determined by pooling the data from all experiments and, through least squares analysis, the best fit line through all of the points was determined and the Do. was found from this line. HCR was then determined by dividing the Do. for the virus in the cell of interest by the Do. of the virus in normal cells.

3.1 Determination Of Inflection Points For HSV 1 Survival Curves

The point at which the second component of the survival curve begins is termed the inflection point and was determined for each virus on each cell line (table 2). This was done by first determining the mean value at each dose. After this was done the mean values were used to determine which points gave the highest correlation coefficients for a straight line.

3.2 UV Survival Of Kos And PAAr5

The survival curves for the mean value at each dose

Table 2

Points Of Inflection for HSV 1 UV Survival Curves

Virus	Cells	Inflection Point J/m2
Kos	293	30-50
	GM 637	30-50
	GM 2803	30-50
	XP 4	5-10
	GM 5509	5-10
	GM 4429	5-10
PAAr5	293	30-50
	GM 637	30-50
	GM 2803	30-50
	XP 4	5-10
	GM 5509	5-10
	GM 4429	5-10
HDV 1	293	10-20
	GM 637	10-20
	GM 2803	10-20
	XP 4	5
	GM 5509	5
	GM 4429	5
L714	293	10-20
	GM 637	10-20
	GM 2803	10-20
	XP 4	5
	GM 5509	5
	GM 4429	5
pTK3B	293	10-20
	GM 637	10-20
	GM 2803	10-20
	XP 4	5
	GM 5509	5
	GM 4429	5

point for Kos and PAAr5 in 293 cells (normal) and XP 4 (XP) cells can be seen as figures 16 and 17 respectively.

The Do values for Kos in 293 cells are 15.27 J/m^2 for the first component and 62.50 J/m^2 for the second component. These values can be seen in tables 3a and 3b and are reported with 1 standard error as are all Do values for all viruses. Tables 4a and 4b show that the Do value for the first component for PAAr5 is 12.17 J/m^2 and 48.01 J/m^2 for the second component. These data indicate that Kos survives better than PAAr5 in normal cells.

From tables 3a and 3b it is seen that the Do values for Kos in XP 4 cells are 3.00 J/m^2 for the first component and 7.97 J/m^2 for the second component. Tables 4a and 4b show that the Do values for PAAr5 are 4.55 J/m^2 for the first component and 13.07 J/m^2 for the second component. This data shows that PAAr5 survives better than wild type Kos in XP cells.

The differences between the two viruses are seen to a greater extent when the HCR values for the two are observed (Table 7). The HCR values for the first component are 19.65 % for Kos and 37.39 % for PAAr5. Second component values are 12.75 for Kos and 27.13 for PAAr5 and the differences in HCR in both components are significant.

3.3 UV Survival OF pTK3B

The mean value of each dose point for pTK3B and Kos

Figure 16

Survival of plaque forming ability of UV irradiated HSV 1 strains Kos and PAAr5 in 293 cells.

# Of Experiments		Virus
1st Component	2nd Component	
5	3	○ PAAr5
5	3	□ Kos

Monolayers of 293 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

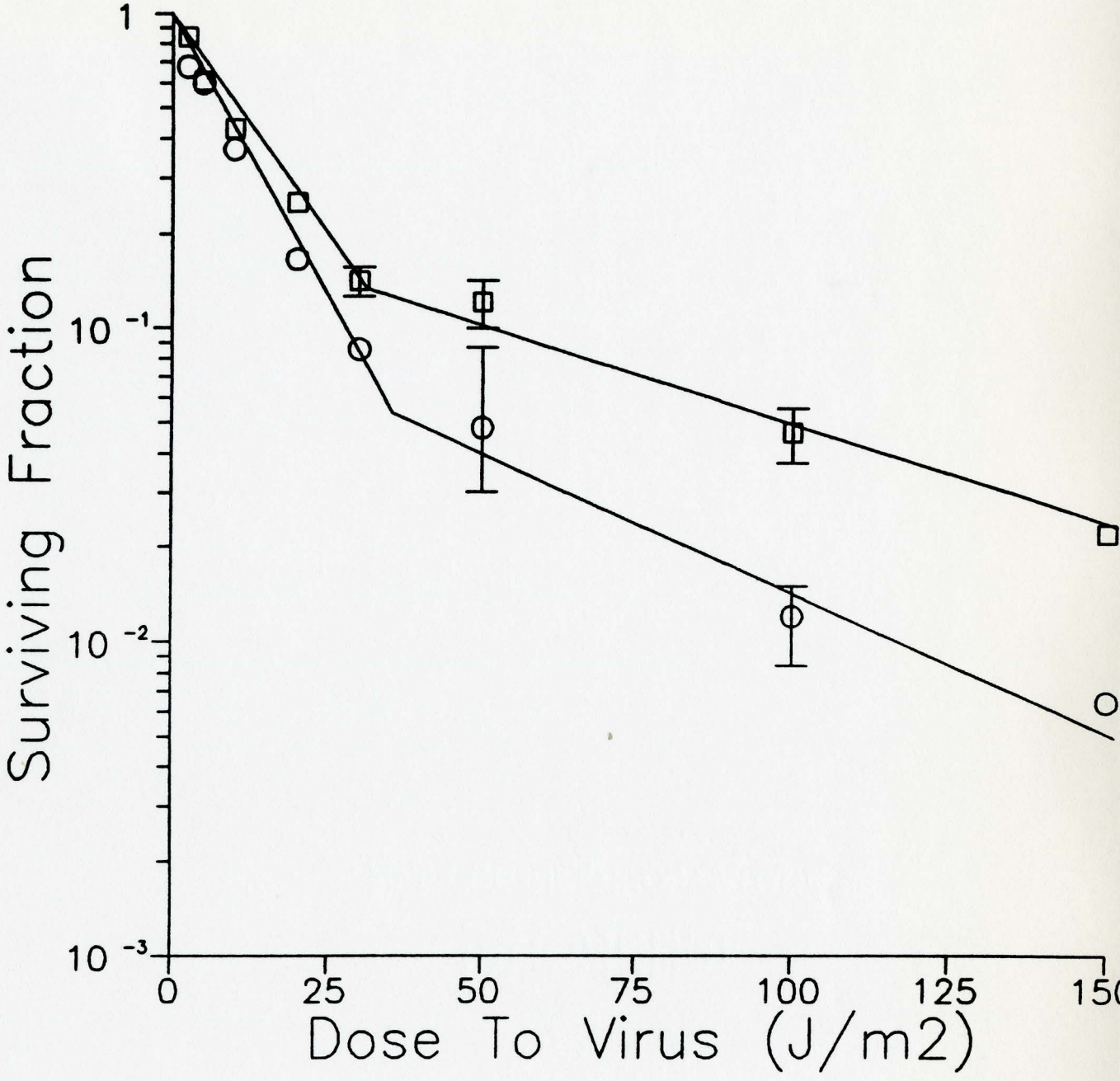

1
10 -1
10 -2
10 -3
Surviving Fraction
0
25
50
75
100
125
150
Dose To Virus (J/m2)

Figure 17

Survival of plaque forming ability of UV irradiated HSV 1 strains Kos and PAAr5 in XP 4 cells.

# Of Experiments		Virus
1st Component	2nd Component	
3	4	PAAr5
3	5	Kos

Monolayers of XP 4 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

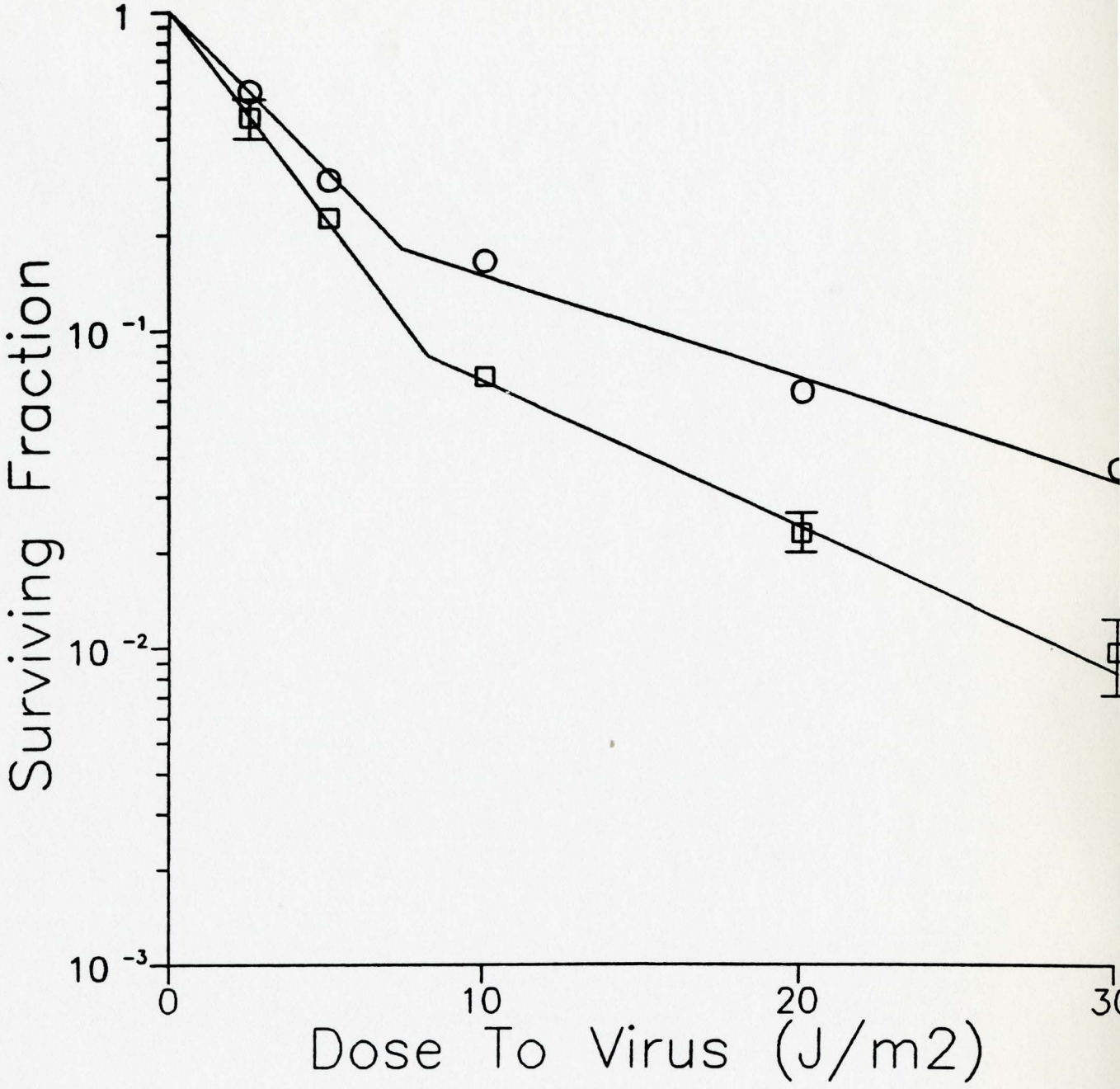

1
10 -1
10 -2
10 -3
Surviving Fraction
0
10
20
Dose To Virus (J/m2)

Table 3a

Do values for 1st Component of UV irradiated HSV 1 Strain Kos

Cell	Do ± S.E. (J/m^2)	# of Experiments
293	15.27 ± 0.70	5
GM 637	14.79 ± 1.11	3
XP 4	3.00 ± 0.30	3

Table 3b

Do values For 2nd Component of UV irradiated HSV 1 Strain Kos

Cell	Do± S.E. (J/m^2)	# of Experiments
293	62.50 ± 7.81	3
GM 637	47.62 ± 6.76	3
GM 2803	50.00 ± 5.00	2
XP 4	7.97 ± 0.53	5
GM 4429	9.26 ± 0.52	3
GM 5509	8.70 ± 0.45	3

Table 4a

Do values for 1st Component of UV irradiated HSV 1 Strain PAAr5

Cell	Do± S.E. (J/m^2)	# of Experiments
293	12.17 ± 0.52	5
GM 637	12.99 ± 1.18	3
XP 4	4.55 ± 0.19	4

Table 4b

Do values For 2nd Component of UV irradiated HSV 1 Strain PAAr5

Cell	Do± S.E. (J/m^2)	# of Experiments
293	48.01 ± 4.62	3
GM 637	46.29 ± 6.94	3
GM 2803	43.45 ± 4.30	3
XP 4	13.07 ± 0.84	4
GM 4429	12.69 ± 1.61	3
GM 5509	14.40 ± 1.98	3

Table 8

% Host Cell Reactivation Of HSV 1 Strains Kos, PAAr5, pTK3B, L714 and HDV 1 in 293 and XP 4 cells

Virus	1st Component % HCR	2nd Component % HCR
Kos	19.65 ± 2.84	12.75 ± 2.45
PAAr5	37.39 ± 3.12	27.13 ± 4.48
pTK3B	37.28 ± 5.44	25.21 ± 3.86
L714	37.01 ± 6.54	25.45 ± 4.39
HDV 1	34.97 ± 4.69	23.02 ± 3.63

has been plotted as a function of dose for both 293 cells and XP 4 cells (Figures 18 and 19 respectively).

The Do values for the first and second components of pTK3B in 293 cells are 7.94 J/m^2 and 31.50 J/m^2 respectively (Table 5a and 5b). These values are both significantly lower than the values of 15.27 J/m^2 and 62.50 J/m^2 reported for Kos.

However, the Do values for both components for pTK3B in XP 4 cells are essentially the same as those for Kos (Tables 5a and 5b). pTK3B has values of 2.96 J/m^2 for the first component and 7.94 J/m^2 for the second compared to the Kos values of 3.00 J/m^2 and 7.94 J/m^2.

Tables 5a, 5b, 6a, 6b, 7a and 7b show that all the TK minus viruses (pTK3B, L714 and HDV 1) have similar Do values for both components. Therefore, the comparison of Kos with pTK3B applies for all of the TK minus viruses.

The % HCR value for pTK3B is 37.20 % as seen in table 8. This value, within error, is the same as the value obtained for PAAr5. It should be noted that the reason due to a large % HCR value in this case is due to a poor survival in normal cells and not an increase in survival in XP cells as was seen for PAAr5.

3.4 UV Survival Of HDV 1 And L714

HDV 1 and L714 were assayed for their UV survival on 293 and XP 4 cells. The mean values, at each dose point, were

Figure 18

Survival of plaque forming ability of UV irradiated HSV 1 strains Kos and pTK3B in 293 cells.

# Of Experiments		Virus
1st Component	2nd Component	
3	5	△ pTK3B
3	5	□ Kos

Monolayers of 293 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

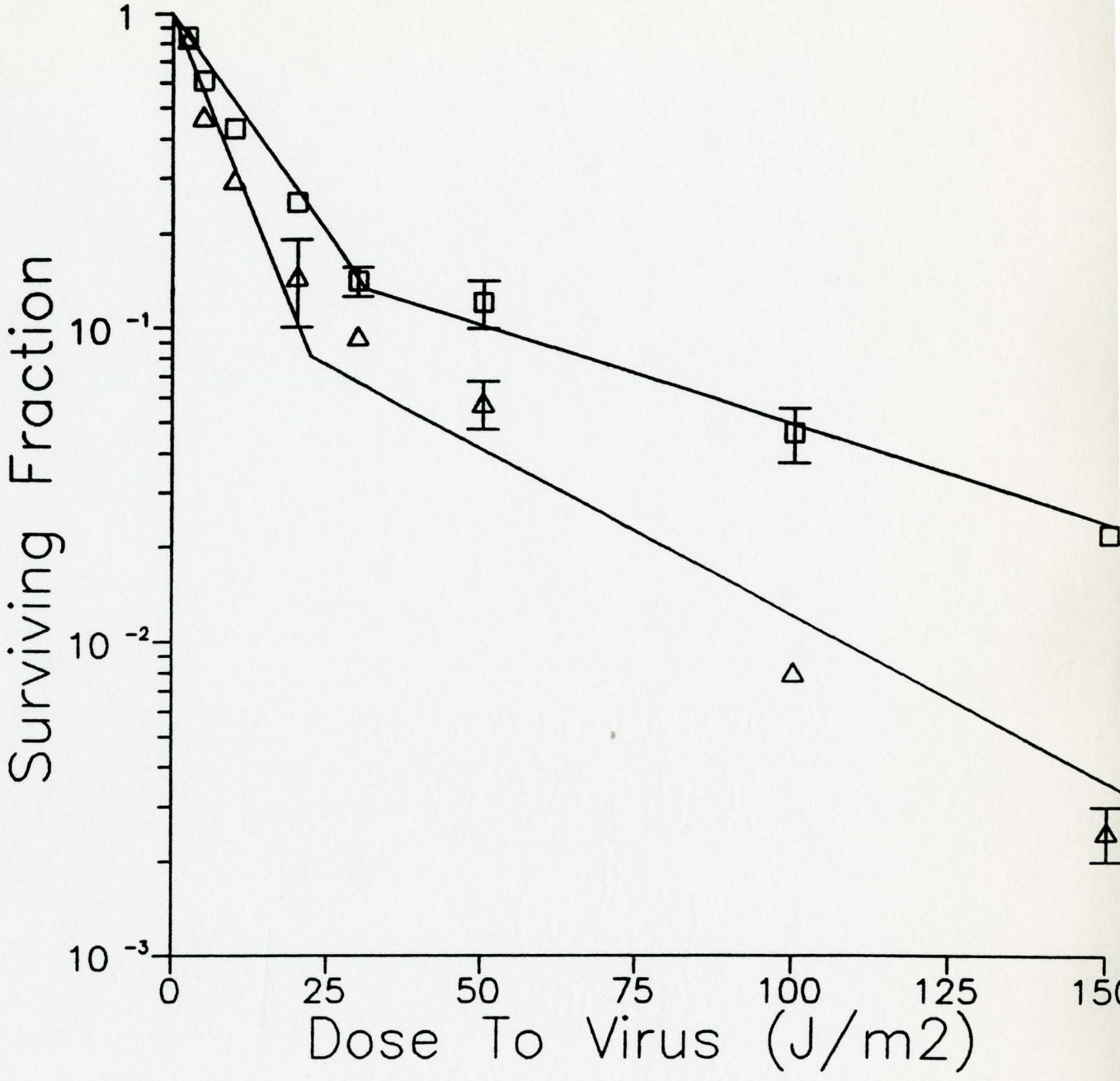

Surviving Fraction
1
10 -1
10 -2
10 -3
0
25
50
75
100
125
Dose To Virus (J/m2)

Figure 19

Survival of plaque forming ability of UV irradiated HSV 1 strains Kos and pTK3b in XP 4 cells.

# Of Experiments		Virus
1st Component	2nd Component	
3	5	△ pTK3B
3	5	□ Kos

Monolayers of XP 4 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

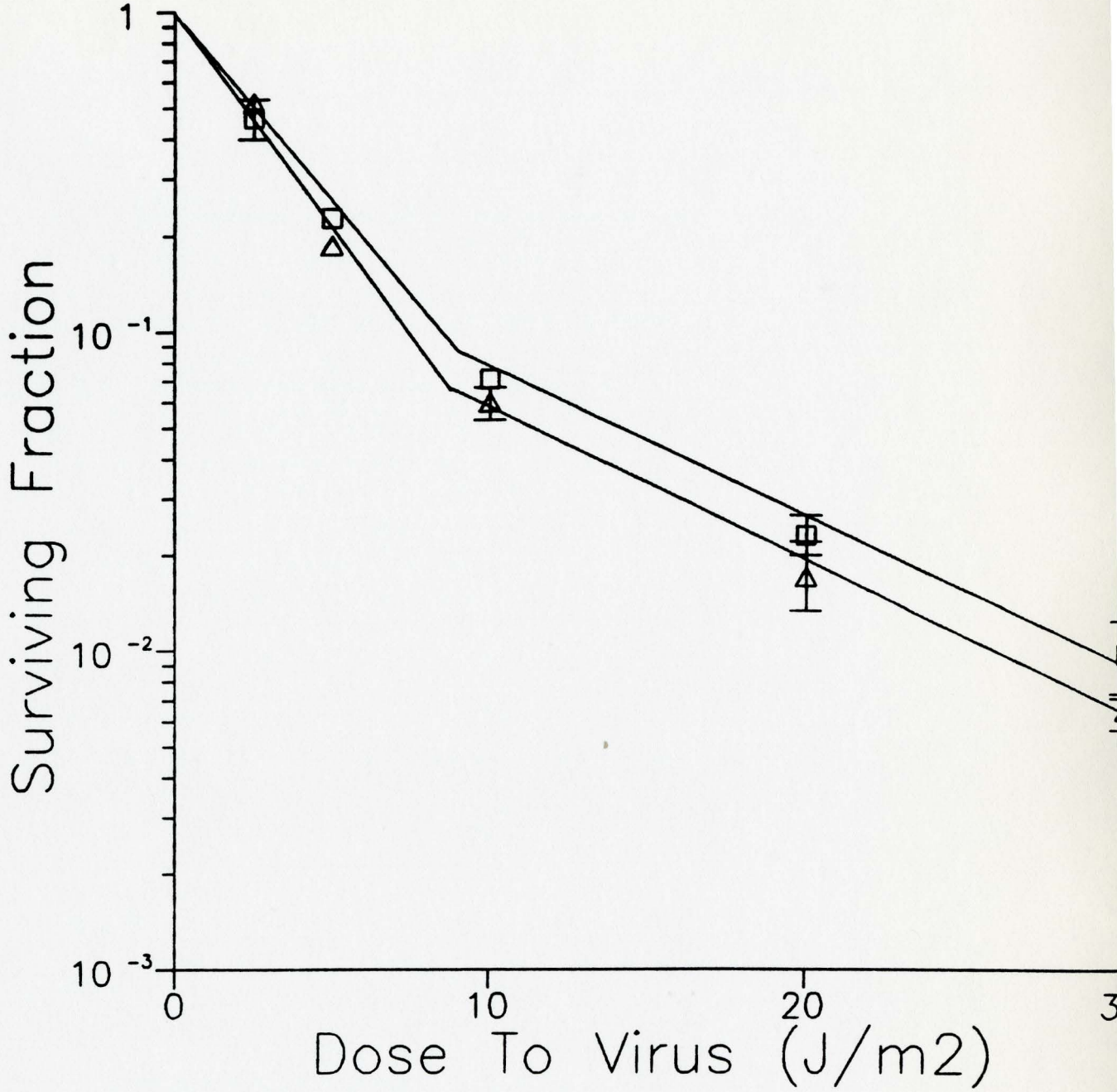

1
10 -1
10 -2
10 -3
Surviving Fraction
0
10
20
Dose To Virus (J/m2)

Table 5a

Do values for 1st Component of UV irradiated HSV 1 Strain pTK3B

Cell	Do± S.E. (J/m^2)	# of Experiments
293	7.94 ± 0.60	3
XP 4	2.96 ± 0.21	3

Table 5b

Do values For 2nd Component of UV irradiated HSV 1 Strain pTK3B

Cell	Do± S.E. (J/m^2)	# of Experiments
293	31.50 ± 2.09	5
GM 2803	27.77 ± 3.05	3
XP 4	7.94 ± 0.69	5
GM 4429	9.17 ± 0.44	3
GM 5509	7.09 ± 0.66	3

Table 6a

Do values for 1st Component of UV irradiated HSV 1 Strain L714

Cell	Do± S.E. (J/m^2)	# of Experiments
293	6.89 ± 0.68	5
XP 4	2.55 ± 0.20	3

Table 6b

Do values For 2nd Component of UV irradiated HSV 1 Strain L714

Cell	Do± S.E. (J/m^2)	# of Experiments
293	27.86 ± 2.49	3
GM 2803	32.26 ± 3.65	3
XP 4	7.09 ± 0.59	5
GM 4429	6.21 ± 0.62	3
GM 5509	6.54 ± 0.73	3

Table 7a

Do values for 1st Component of UV irradiated HSV 1 Strain HDV 1

Cell	Do± S.E. (J/m^2)	# of Experiments
293	7.32 ± 0.49	7
XP 4	2.56 ± 0.17	4

Table 7b

Do values For 2nd Component of UV irradiated HSV 1 Strain HDV 1

Cell	Do± S.E. (J/m^2)	# of Experiments
293	29.70 ± 3.04	4
GM 2803	25.91 ± 3.68	3
XP 4	6.85 ± 0.38	7
GM 4429	7.01 ± 0.42	3
GM 5509	7.30 ± 0.44	3

used to plot survival curves for the two viruses on these cells (293 Figure 20 and XP 4 Figure 21). This type of experiment could also provide a functional assay to determine whether the den V gene in HDV 1 can increase the survival of the virus.

The Do values for the two viruses are taken from tables 6a, 6b, 7a and 7b. The first component Do values for HDV 1 are 7.32 J/m^2 on 293 cells and 2.56 J/m^2 on XP 4 cells and for L714 the Do values are 6.89 J/m^2 and 2.55 J/m^2.

The second component values for HDV 1 are 29.76 J/m^2 on 293 cells and 6.85 on XP 4 cells. The 293 value for L714 is 27.86 J/m^2 and for XP 4 the value is 7.09 J/m^2.

The HCR values taken from table 8 show that HDV 1 has HCR values of 34.97 % and 23.02 % for the 1st and 2nd components respectively and L714 has values of 37.01 % and 25.45 %.

In terms of survival the two viruses are identical, which suggests either the den V gene is not expressed or it is unable to help the UV survival of HSV 1 under the conditions employed.

3.5 UV Survival Of HSV 1 In Transformed And Nontransformed Cells

There have been reports that a difference in UV survival of Adenovirus can be detected when transformed cells are compared to their normal counterparts (Rainbow, 1984). It

Figure 20

Survival of plaque forming ability of UV irradiated HSV 1 strains HDV 1 and L714 in 293 cells.

# Of Experiments		Virus
1st Component	2nd Component	
7	4	☆ HDV 1
5	6	◆ L714

Monolayers of 293 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

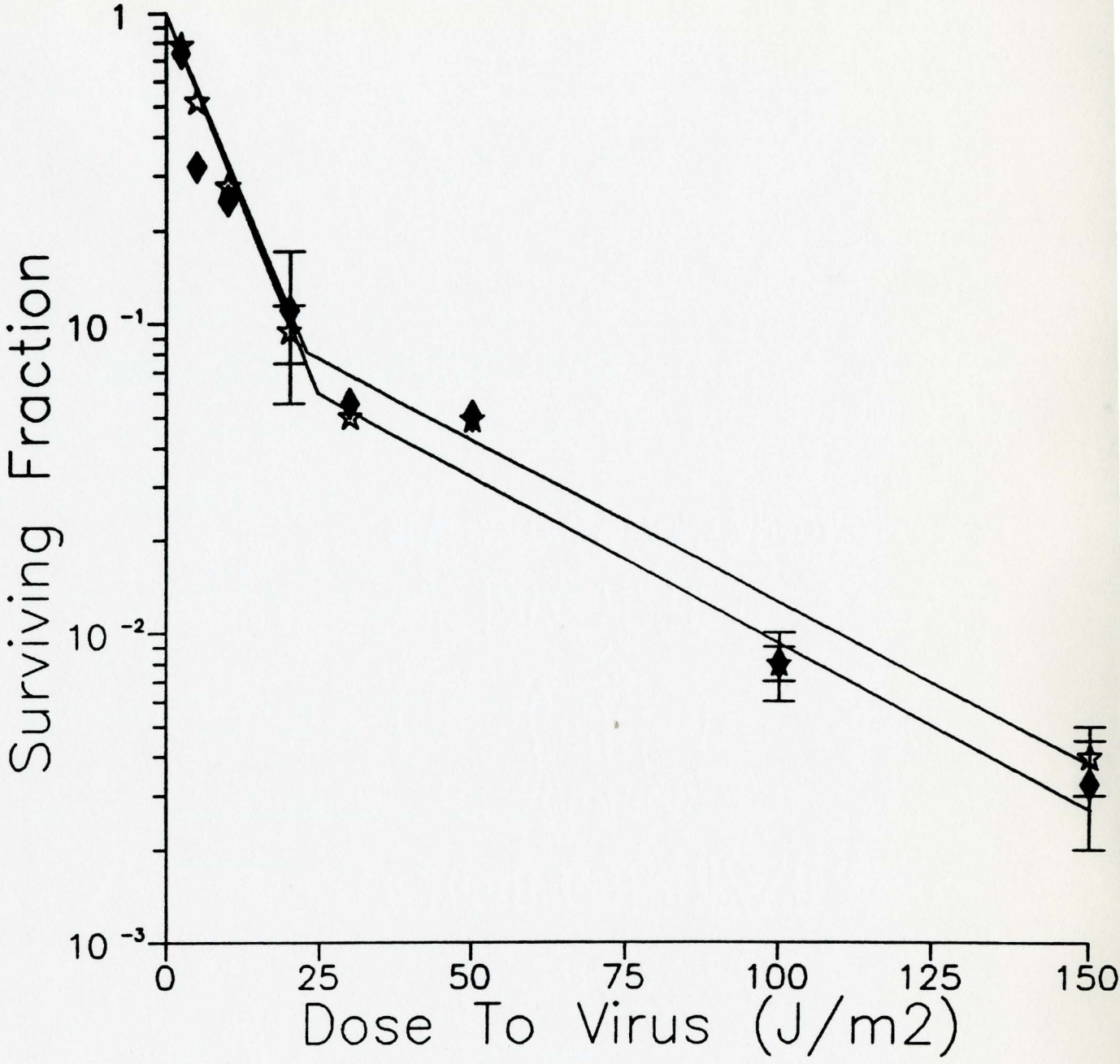

1
10 -1
10 -2
10 -3
Surviving Fraction
0
25
50
75
100
125
150
Dose To Virus (J/m2)

Figure 21

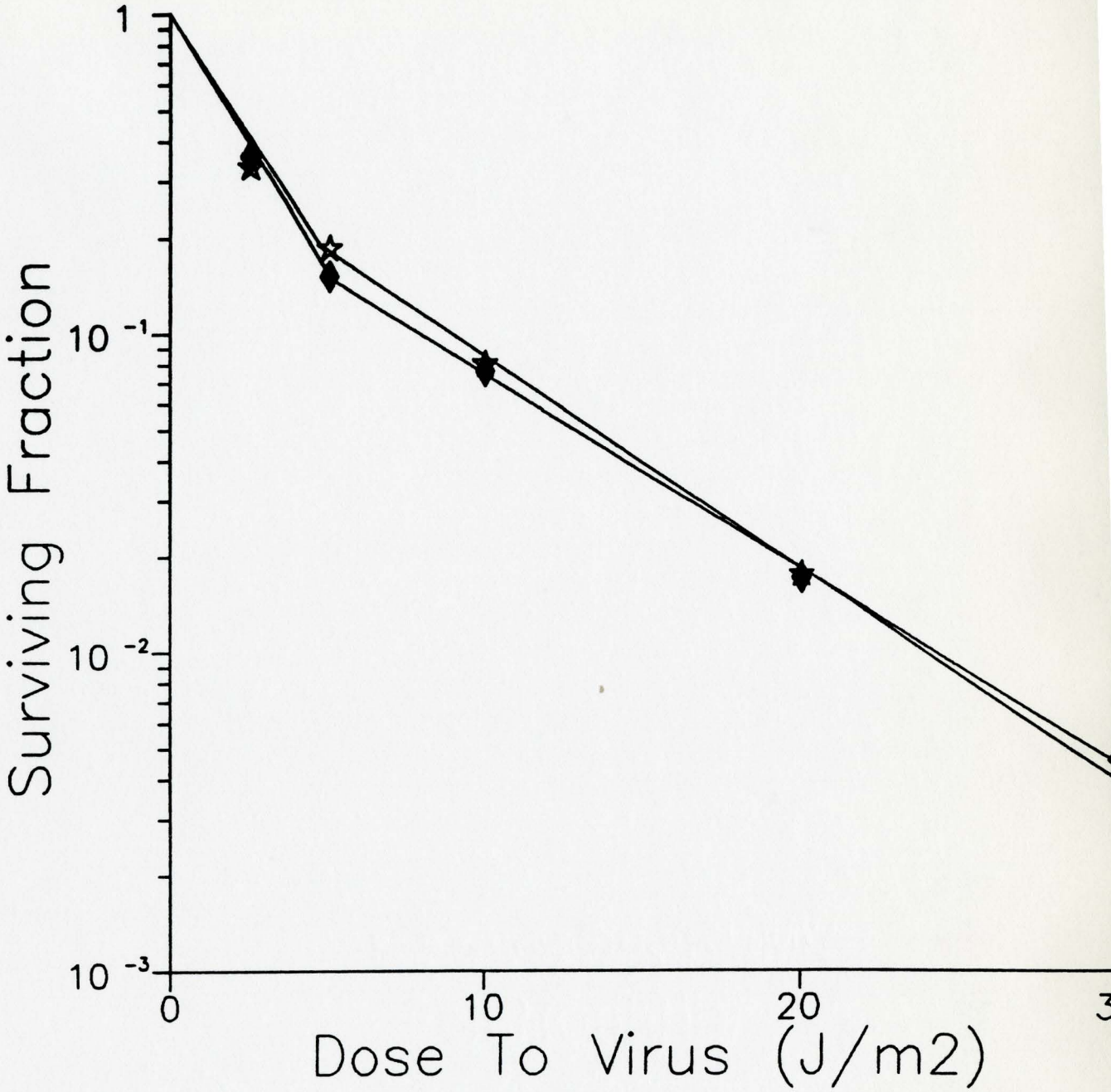
Survival of plaque forming ability of UV irradiated HSV 1 strains HDV 1 and L714 in XP 4 cells.

# Of Experiments		Virus
1st Component	2nd Component	
4	7	☆ HDV 1
3	5	◆ L714

Monolayers of XP 4 cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.


Surviving Fraction
1
10 -1
10 -2
10 -3
0
10
20
Dose To Virus (J/m2)

was, therefore, of interest to see if these differences could be detected using HSV 1.

In order to investigate this UV survival studies were performed on 293 cells (Adenovirus type 5 transformed normal cells), GM 637 cells (SV40 transformed normal cells) and GM 2803 cells (untransformed normal cells). The UV survival of Kos on these three cell types is seen as figure 22. From tables 3a and 3b it is seen that the Do for the first component for 293 and GM 637 are the same within error, 15.27 J/m^2 and 14.79 J/m^2 respectively and the values for all 3 cell lines are the same within error for the second component , 293 62.50 J/m^2, GM 637 47.62 J/m^2 and 50.00 J/m^2 for GM 2803 cells.

The same type of study was performed using 3 XP lines (Figure 23). GM 4429 are SV40 transformed group A cells, XP 4 cells are GM 4429 cells transformed with the muc genes and GM 5509 are nontransformed XP cells. Only XP 4 cells were observed in the first components so comparisons must be kept to the second component. The Do values for the second component as in the first component are all the same within the error stated. The XP 4 Do for Kos is 7.97 J/m^2, for GM 4429 it is 9.26 J/m^2 and 8.70 J/m2 for GM 5509. These experiments indicate that there is no detectable difference between transformed cells and their untransformed counterparts for reactivating UV irradiated HSV 1.

Further support for this is seen in tables 3a - 7b where for each individual virus all normal cells have the

Figure 22

Survival of plaque forming ability of UV irradiated HSV 1 strain Kos in 293, GM 637 cells and GM 2803 cells.

# Of Experiments		Cells
1st Component	2nd Component	
5	3	□ 293
3	3	● GM 637
	2	△ GM 2803

Monolayers of each of the cell lines cells were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.

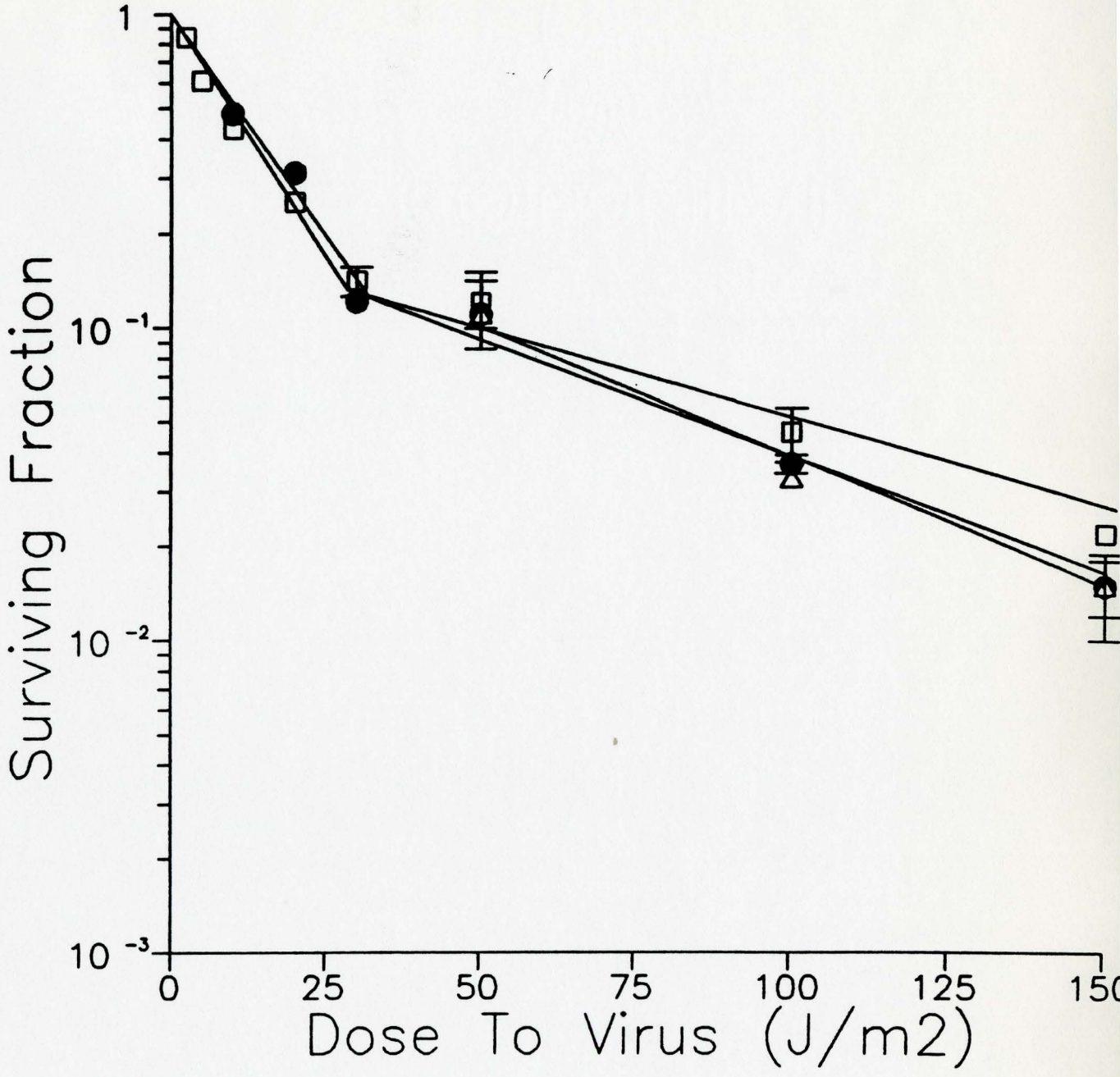

Surviving Fraction
1
10 -1
10 -2
10 -3
0
25
50
75
100
125
150
Dose To Virus (J/m2)

Figure 23

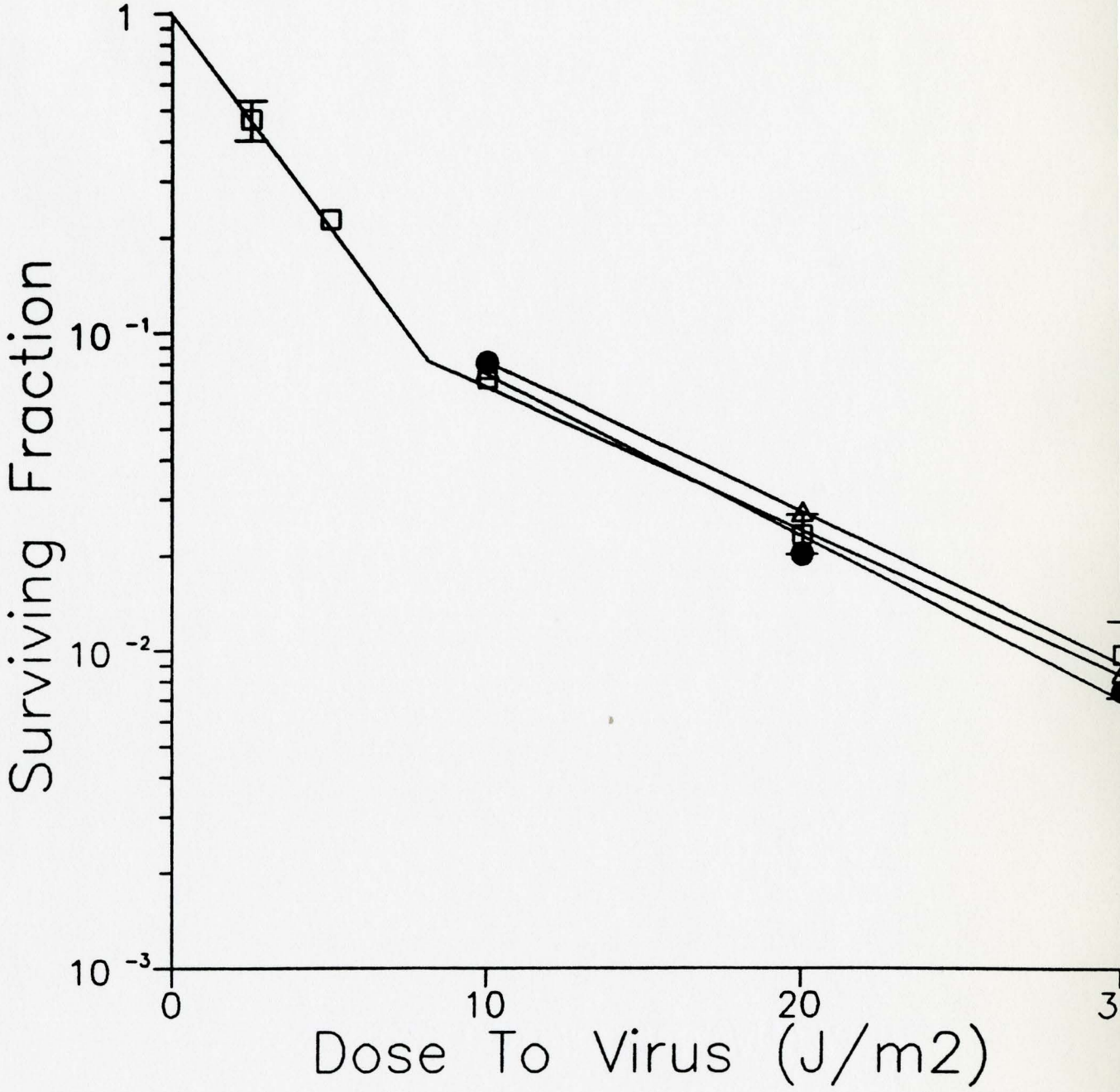
Survival of plaque forming ability of UV irradiated HSV 1 strain Kos in XP 4, GM 4429 cells and GM 5509 cells.

# Of Experiments		Cells
1st Component	2nd Component	
3	5	XP 4
	3	GM 4429
	3	GM 5509

Monolayers of each of the cell lines were plated into 24 well plates 24 hr. prior to infection. UV irradiations and plaquing of virus was performed as described in materials and methods. Plaques were stained and counted 48 hours post infection and were used to determine the titre of the virus. The surviving fraction was determined by dividing the titre of irradiated virus by the titre of the unirradiated control virus. These values were then plotted as a function of the UV dose given to the virus.

Each point represents the logarithmic mean of the pooled data at each dose point. The points were fitted to two straight lines by hand. The inflection points were determined by using the points which yielded the highest correlation coefficients in the least squares analysis.

The error bars on each point represent one standard error. If no error bar is present on a point then the size of the symbol is greater than 1 standard error.


Surviving Fraction
1
10 -1
10 -2
10 -3
0
10
20
Dose To Virus (J/m2)

same Do within error and so do all the XP cells.

DISCUSSION

The main objectives of this research were : a) to construct a HSV 1 mutant virus containing the den V gene inserted into the thymidine kinase locus in order to determine if HSV 1 can be used as a suitable vector to study DNA repair genes b) to determine if a difference in DNA repair abilities can be detected between SV40 transformed cells and their untransformed counterparts using HCR studies of HSV 1 and c) to perform HCR studies on various HSV 1 mutant strains in order to elucidate which virally encoded genes may play a role in the repair of damaged virus.

Construction Of HDV 1

Several HSV 1 viruses were constructed that contain DNA sequences inserted at the TK locus of the HSV 1 genome (Fig. 6). One of these viruses, HDV 1 was further characterized to show that the sequences inserted at the TK locus were of den V origin (Fig. 7,8,& 9).

Determination Of den V Expression In HDV 1

The expression of HSV 1 genes are grouped into three main categories. These are the immediate early, early and late classes. Both the early and immediate early classes reach peak synthesis prior to DNA replication whereas although the late genes begin synthesis prior to DNA replication do not reach peak synthesis until after the

genome has been replicated (Roizman and Batteson, 1985). The class to which a gene belongs appears to be determined by the sequences in its promotor (Lang et al., 1984).

The class to which the den V gene would belong to if expressed was a very important consideration here. In prokaryotes the den V gene is involved in the incision step of excision repair of damaged DNA. Therefore if it works the same in eukaryotes as previous studies show it does it must be expressed before DNA replication in order to aid in repair. This would make the gene either an early or immediate early gene.

Sequencing of the actual promotor inserted into the virus was not performed. However, a sequence of the LTR from the same strain that was used to construct the cartridge was available in the NIH gene bank and can be seen as figure 2. The start of transcription is at base number 545. 28 bases upstream from this is a TATA box starting at 515 to 522. At position number 483 (63 bases upstream from +1) there is a very close to consensus CAAT box. Both upstream and downstream from the CAAT box sequence there are G rich sequences which could act as binding sites for the transcriptional elongation factor SP1. The above three elements are thought to be the necessary requirements for a gene to behave as a early gene (Jones et al., 1985). The additional sequence that is thought to be important for a gene to be an immediate early gene is the sequence TAATGARAT, which is thought to be a HSV 1 enhancer element (Lang et al.,

1984). There are two sequences in the LTR that could be used as this enhancer sequence. They are at 324 and at 275. Upon this examination of the LTR it was felt that the gene would probably be expressed as an early gene at the latest and therefore, construction of the virus was worthwhile.

The first report of a foreign gene inserted into the HSV 1 genome was by Tackney *et al.* (1985). The adenine phosphoribosyl transferase gene was inserted at the TK locus and it was shown that the gene was not transcribed upon infection. Since the report of Tackney *et al.* (1985) several other viruses have been constructed. Smiley *et al.* (1987) inserted the rabbit beta globin gene into the HSV 1 genome and showed that properly initiated transcripts were produced upon infection. The gene had the kinetics of the immediate early class. The same results were also obtained for the alpha globin (Barbara Panning, Department of Pathology, McMaster University, personal communication). Recently, it was shown that upon introduction of the HPRT gene under the TK promotor into the HSV 1 genome the HPRT protein can be isolated from HSV 1 infected cells (Pallela *et al.*, 1988).

The expression of den V transcription in HDV 1 was determined by primer extension of RNA extracted from HDV 1 infected cells. The primer used was 5' CGCGGCAATTCACGATATTCAGCC 3' which runs from 668 to 644 (Fig. 2). The expected extension product length was 123 bp which was seen in HDV 1 infected cells but not control cells. This shows that the that properly initiated RNA for den V

transcription was produced in HDV 1 infected cells (Fig. 15). The RNA in this experiment was extracted at 6 hr. and it is seen from the figure that the amount of the den V extension product was similar to the amount of the products produced in the primer extension using an oligomer for gD. This result suggests that the den V gene was expressed in a similar manner as gD in terms of the time transcription is initiated (ie an early protein).

Protein Detection Problems

Immunoprecipitations, Western blots and indirect immunofluorescence of cells were the techniques employed to detect den V protein from HDV 1 infected cells. All of these techniques rely on antibodies that are specific for den V protein. Both the monoclonal IC3 and polyclonal # 38 were used and yielded the same results which were inconclusive as to whether den V protein was made or not.

The monoclonal antibody IC3 was reported to be able to detect den V protein from yeast cells transfected with den V DNA by western blot analysis (Valerie *et al.*, 1986).

Valerie *et al.* (1987b) showed that # 38 antibody could detect a fusion protein between protein A and den V from bacteria that overproduce this fusion protein by western blot analysis. However, upon closer examination of these results it was noted that the protein preparations were put through several purification steps in order to greatly enrich

the den V protein. The reason for the purification steps is not mentioned in the reports and it is felt that one possible reason could be that the antibody shows very high background binding as was seen in the results found in this study.

In both the western blot analysis and the immunoprecipitation experiments a protein was detected at 16 kD (den V size). This protein was detected in all lanes including the mock infected cells which suggests that there could be a protein in the cell that the antibody recognizes. This protein may just be cross reacting with the antibody or it could possibly be a eukaryotic homologue of the den V protein.

Further support for the fact that there is a protein in the cell that is recognized by the antibodies comes from the indirect immunofluorescence experiments. Valerie *et al.* (1987) showed that den V protein could be detected in XP A cells transfected with the den V gene by indirect immunofluorescence. When similar studies were performed in this work, the antibodies again detected a protein from mock infected cells. It is felt that the den V protein may not have been detected from HDV 1 infected cells due to either the low affinity of the antibody for the den V gene or due to the presence of a protein in the cells that the antibodies recognize.

UV Survival Studies Of Various HSV 1 Strains

Survival curves of all strains of HSV 1 in all cells exhibited two components (Figures 16 - 23). These types of curves have been reported by other workers for the survival of HSV in mammalian cells (Bueschleb, 1987 ; Ryan and Rainbow 86 ; Lytle *et al.* 1982 ; Hall *et al.*, 1980 and Lytle and Benane 1974). The reason for the two components is not known but it has been shown that multiplicity reactivation and heterogeneity in the virus population are not the cause (Lytle, 1971). Infection of subpopulations of cells at different stages in the cell cycle has also been ruled out as a cause for the two components (Lytle & Schmidt, 1981). Although the majority of workers have reported two component curves some reports have shown single component curves (Takebe *et al.*, 1974 ; Selsky & Greer, 1978).

HCR Of Transformed and Untransformed Cells

Many reports suggest that there is a change in DNA repair capacity associated with cellular transformation and tumorigenisis. 17 of 88 cell strains that have been isolated from human tumors have been shown to be defective in the ability to repair MNNG treated Adenovirus type 5 (Day and Ziolkowski, 1979). The same decrease in repair ability was shown in 7 of 11 SV40 transformed cell lines tested (Day *et al*, 1980). Also, transformed cells have been shown to be more

sensitive to DNA damaging agents then their untransformed counterparts (Heddle & Arlett, 1980 : Simon et al., 1981). All these results suggest that there is an alteration in the DNA repair of transformed cell. This was further supported when an incision defect was found in UV irradiated SV40 transformed cells (Squires et al., 1982). In this study they showed that the DNA incision rate of UV irradiated fibroblasts was much lower in SV40 transformed and tumor cells versus their normal counterparts. Elliot & Johnson (1983) showed that SV40 transformed murine cells also have an incision defect when compared to their normal counterparts. Most recently Rainbow (1984) reported that 5 human tumor lines and 1 SV40 transformed cell line have reduced HCR of UV irradiated Ad 2. It was, therefore, of interest to determine if these differences can be seen using HSV 1.

Several strains of HSV 1 were used in this study to determine if a difference in HCR could be detected in transformed versus untransformed cells. The normal fibroblast line, GM 2803, the SV40 transformed normal fibroblast line, GM 637 and Adenovirus type 5 transformed embryonic kidney line, 293 were tested. Figure 22 shows the survival curves for the pooled data of Kos in these cell lines. GM 637 and 293 cells were examined in the first component. The Do for GM 637 was 14.79 $\pm$ 1.11 J/m^2 and the Do for 293 cells was 15.27 $\pm$ 0.70 J/m^2 (Table 2a). This shows that no difference can be detected in the two virally transformed cell lines. All three cell lines were studied for their second component.

The Do values for 293, GM 637 and GM 2803 were 62.50 ± 7.81 J/m², 47.62 ± 6.76 J/m² and 50.00 ± 5.00 J/m² respectively (Table 2b). These values show that within experimental error no difference can be detected between the transformed and untransformed cells. This finding is further supported by the results of the other 4 viruses tested which also show no significant difference between any of the cell lines looked at in either component (Tables 3a -6b).

XP cell lines were also tested to see if a difference in HCR values could be detected in transformed cells. All XP cells in this study were either XP12BE (XP group A) or cells derived from this line. GM 4429 is an SV40 transformed fibroblast that was derived from GM 5509 (XP12BE) cells. The other line used was XP 4 cells. These cells were made by transforming GM 4429 cells with the muc genes. Muc gene sequences have been detected in these cells by southern blot but expression of the genes has not been determined (A.J. Rainbow, Departments of Radiology and Biology, McMaster University, personal communication). The survival curve for the pooled data of Kos on these three cell lines can be seen as figure 23. Only XP 4 cells were tested for their ability to reactivate UV irradiated Kos for the first component, therefore, only the second component can be discussed. The Do values obtained for Kos for the second component are 7.97 ± 0.53 J/m², 9.26 ± 0.52 J/m² and 8.70 ± 0.45 J/m² for XP 4, GM 4429 and GM 5509 respectively. This shows that no significant difference in HCR could be detected between the

transformed and untransformed cells. This again is further supported by the fact that the other 4 viruses tested gave the same results (Tables 3a - 6b).

The reason that no UV survival differences are seen between transformed cells and their untransformed counterparts using HSV 1 when there is a difference observed when using Adenovirus is not known. This result is in line with other results which show that differences are seen between HSV 1 and Adenovirus when repair of these viruses are studied (Buoschleb, 1987).

The HCR of all strains of HSV 1 tested showed that no difference could be detected between the three normal strains or the three XP strains tested. Therefore, one line from each group was chosen to perform the bulk of the experiments. XP 4 cells and 293 cells were chosen due to the speed at which they double, their plating efficiency and because they were very easy to maintain. Therefore, most of the data discussed hereafter will be data that was collected from these two cell lines but the results can be extrapolated to include all the lines tested.

UV Survival Of Kos In Normal And XP Cells

The UV survival of HSV 1 Kos on 293 cells can be seen in figure 16. The Do for the first component was 15.27 $\pm$ 0.70 J/m^2. This value is within the range of Do values that have been reported for the survival of HSV in normal human cells.

The values for first component that have been reported previously are 21 ± 1 J/m² (Bueschleb, 1987), 28.2 ± 1 J/m² (Ryan & Rainbow, 1986), 8.9 ± 1.5 J/m² (Lytle et al., 1982) and 10 -19 J/m² (Lytle & Benane, 1974).

The Do for the second component lies within the previously reported values as well. Table 2 shows that the Do value obtained from the pooled data is 62.50 ± 7.81 J/m². The Do values previously reported are 83 ± 16 J/m² (Bueschleb, 1987), 84.4 ± 4.0 J/m² (Ryan and Rainbow, 1986), 30.6 ± 0.3 J/m² (Lytle et al., 1982) and Do values ranging from 32 - 53 J/m² (Lytle and Benane, 1974).

The reason that the Do values are different in all of these studies could be due to a combination of factors. The first of these is that different viral strains were employed in the different studies. Ryan and Rainbow (1986) used HSV 2 in their studies whereas Lytle et al. 1982 and Lytle and Benane (1974) used the HSV 1 macroplaque strain. This study and Bueschleb (1987) employed HSV 1 strain Kos. Inherent differences in the virus strain could cause differences in survival. Each study used a different cell line or lines which could also be a factor effecting the survival of the virus. For example Bueschleb (1987) used CRL 1121, Ryan and Rainbow (1986) used GM 969, this work used GM2803 and Lytle et al. (1982) used another normal human fibroblast cell line. Irradiation conditions and the time at which plaques were scored were also different in each of the reports. In this study the virus was irradiated in straight

alpha - mem and all plaques were scored at three days post infection. Lytle (1982) and Lytle and Benane (1974) irradiated the viral suspensions in PBS and scored plaques from unirradiated virus at 3 days and their irradiated virus plaques at 4 days. Bueschleb (1987) and Ryan and Rainbow (1986) irradiated the viral suspension in straight alpha - mem and the plaques were scored at two days. The results obtained here lie within the range of experiments previously reported. The Do for the second component was 4.1 x greater than the Do for the first component. This is in close agreement to the value of 4 that Lytle and Benane (1974) reported and 4.05 that Bueschleb reported. All these values are somewhat larger than the 3.2 value reported by Ryan and Rainbow. This could be due to the fact that they used HSV 2 in their study.

The HCR of Kos in an XP group A fibroblast was also studied (Figure 17). The Do for the first component of the pooled data was 3.0 $\pm$ 0.30 J/m^2 (Table 2a). This is close to the value of 1.8 $\pm$ 0.1 J/m^2 reported by Lytle et al. (1982) for another group A and quite a bit lower than the value of 12.1 J/m^2 reported by Ryan and Rainbow (1986) for XP25RO. The Do value obtained for the second component was 7.97 $\pm$ 0.53 J/m^2 (Table 2b). This value is higher than 5.5 $\pm$ 1.2 J/m^2 reported by Lytle et al. (1982) and significantly lower than the values of 23.5 J/m^2 and 17 $\pm$ 1 J/m^2 reported by Ryan and Rainbow (1986) and Bueschleb (1987) (XP4LO) respectively. The reasons for the differences in survival of the virus in

XP cells could be due to the same factors as discussed for normal cells.

The Do for the second component was 2.66 times larger than the first component. This value is higher than the value of 1.9 reported by Ryan and Rainbow (1986) but lower than the value of 3.05 reported by Lytle *et al.*, 1982.

It was found that the % HCR for the first component was 19.65 ± 2.84 % and 12.75 ± 2.95 % for the second component when the HCR of Kos in the XP group A, XP 4 cells was compared to the 293 normal cells (Table 7). Percentage HCR values that have been reported for the first component are 20.2 % (Lytle *et al.*, 1982) (calculated by the author), and 43.8 % (Ryan and Rainbow, 1986). Second component % HCR values that have been reported are 18.01 % (Lytle *et al.*, 1982), 27.8 % (Ryan and Rainbow, 1986) and 10.8 % (Bueschleb, 1987). Therefore, the values obtained during the HCR studies in this work are in reasonable agreement when compared to work that has been previously reported.

UV Survival Of PAAr5 In Normal And XP Cells

Survival curves for PAAr5 on 293 and XP 4 cells can be seen as figures 16 and 17. From table 3a and 3b it can be seen that the Do values from the pooled data for the first component for 293 cells was 12.17 ± 0.52 J/m^2 and 48.01 ± 4.62 J/m^2 for the second component. The only other reported Do value for PAAr5 in XP cells comes from Bueshcleb (1987).

This value is 54 ± 5 J/m² and is reported for the second component in XP4LO. It is seen that this value and the one obtained in this study are not different within experimental error.

The Do values obtained for the two components for the survival of PAAr5 on XP 4 cells were 4.55 ± 0.19 J/m² and 13.07 ± 0.84 J/m² for the first and second component respectively. Bueschleb (1987) reported a Do value for the second component of 17 ± 1 J/m² which is significantly higher than the value obtained in this study.

It is felt that the HSV 1 DNA polymerase gene might play a role in the repair of UV damaged virus (Bueschleb, 1987). Therefore, one might expect to see a difference between the survival of Kos and PAAr5. The Do values for Kos were 15.27 ± 4.58 % J/m² for the first component and 62.50 ± 12.50 % J/m² for the second component on 293 cells. These values can be compared with 12.17 ± 4.26 % J/m² for the first component and 48.01 ± 4.62 J/m² for the second on 293 cells for PAAr5. The values for the first component were significantly different and show that Kos survives better than PAAr5 upon UV irradiation. Considering this data with the fact that in every experiment Kos survived better than PAAr5 it can be said that in normal cells Kos survives better than PAAr5. This is further supported by the data of Bueschleb (1987) which shows that Kos survives significantly better than PAAr5 in the second component.

PAAr5 carries a polymerase gene that differs from the

wild type gene found in Kos in that it is an antimutator and is resistant to PAA (Hall et al., 1984). Thus one would expect that PAAr5 could correctly repair damage better than Kos and lead to a higher survival than Kos. The opposite of this is true and the reason why it actually has lower survival is not known. It has been postulated that the polymerase might be slower than the Kos polymerase and therefore if the plaques were scored late enough the survival of PAAr5 could turn out to be the same as Kos (Bueschleb, 1987). The rate of replicative DNA synthesis was observed and shown to be no different between PAAr5 and Kos (Hall et al. 1984). However, there could still be differences between the rates of repair synthesis.

The same type of comparison can be made for the Do values obtained for the two viruses on XP 4 cells. Kos had Do values of 3.00 $\pm$ 0.70 J/m^2 for the first component and 7.97 $\pm$ 0.53 J/m^2 for the second component. The first component value for PAAr5 was 4.55 $\pm$ 0.19 J/m^2 and 13.07 $\pm$ 6.43 J/m^2 was obtained for the second component. These values show that PAAr5 survives significantly better than the Kos in XP 4 cells in both components. This observation is the reverse of what was seen for normal cells where Kos survived better.

The differences between Kos and PAAr5 is further exemplified when the % HCR for XP versus normal cells is examined. The % HCR for the first component for PAAr5 was 37.39 $\pm$ 3.12 % and the value for the second component was

27.13 ± 4.48 %. This value is within error when compared to 31.4 % which was reported by Bueschleb (1987) for the second component. When the % HCR values are compared to the values for Kos of 19.65 ± 2.84 % for the first component and 12.75 ± 4.48 % for the second component it is seen that PAAr5 is reactivated to a much greater extent in the XP cells.

The reason for this change in relative survival to Kos is not known. One possible explanation that would lead to a higher survival XP 4 cells while having lower survival in 293 cells is if the polymerase mutation can in some way directly complement the defect in XP cells. The defect in XP cells is in the initial incision step of excision repair, therefore, the mutation may complement this defect. One way in which this could be accomplished is if the mutation produced a endonuclease within the polymerase gene. This is highly unlikely due to the way in which the viral strain was isolated.

A more feasible method of complementation involves the polymerase in the nicking complex. XP group A (XP 4) cells have the lowest amount of repair of all the XP groups and this has been shown to be due to the least amount of incision. It has also been shown that the polymerase involved in DNA repair may be essential for not only repair synthesis but also for the initial incision step as well (Tyrell and Amaudruz, 1987). This, therefore, might suggest that the PAAr5 polymerase might be able to interact with the cellular machinery better and cause an increase in the nicking

activity and thereby help in the survival of the virus. The reason that this increase wouldn't be seen in normal cells is because normal cells have proper nicking activity and any additional nicking by the PAAr5 polymerase would not be seen within the high cellular background.

Bueschleb (1987) reported that the same increase in UV survival relative to Kos was seen for PAAr5 when the viruses were used to infect Cockayne syndrome cells. These cells have a defect in removing dimers but are not deficient in the incision step of excision repair (Bueschleb, 1987). This suggests that the polymerase mutant may not be complementing an incision defect in the cells. However, these results were based upon 2 experiments that were performed on only one Cockayne cell line. Upon close examination it was found that the pooled data for the two experiments show that the differences between Kos and PAAr5 are not significant within experimental error and should be repeated.

At some point after infection the virus will start to replicate whether or not the genome has been repaired. If the genome isn't repaired, as could be the case in XP A cells, the genome will have to be repaired by post replication repair. In this type of repair the genome is replicated and then the damage is repaired during or after replication.

XP A, B, C, D are all slightly deficient in PRR whereas the XP variant is markedly deficient in this pathway (Lehmann, 1972). Studies in which aphidicolin was used to

inhibit alpha and delta polymerases have shown that PRR is not dependant on the polymerases speculated to conduct the major portion of repair (Tyrell and Amadruzm, 1987). In this study it was shown that aphidicolin greatly sensitized these cells to U.V. irradiation. This shows that the aphidicolin sensitive polymerase dependant repair pathway is working in the cells and since there is little post replication repair in these cells aphidicolin sensitive polymerase is not involved in PRR.

The HSV 1 polymerase has been shown to be similar to the cellular alpha polymerase and delta polymerases (Gibbs et al., 1985). It would, therefore, seem that the HSV 1 polymerase might not be involved in post replication repair like the aphidicolin sensitive cellular polymerase. However, HSV 1 only codes for one polymerase molecule and this molecule could be responsible for all types of replication and repair of the genome. The reason why the mutant polymerase might be better at PRR than the wild type polymerase is unclear but this possibility can not be ruled out.

Another point to note is that the increase in survival seen for the polymerase mutant is dependant on the presence of the TK gene. This is seen when the Do values for Kos and either L714 or HDV 1 are compared. Both L714 and HDV 1 carry the mutant polymerase but also lack a functional TK gene. These viruses survive the same as Kos in XP cells which suggests that the polymerase effect is abolished when

UV Survival Of PTK3B In Normal And XP Cells

The survival curves for the pooled data of PTK3B can be seen as figures 18 and 19 for 293 and XP 4 cells respectively. From tables 4a and 4b it can be seen that the Do values for the first and second components were 7.94 ± 0.60 J/m² and 31.50 ± 2.09 J/m² respectively. The second component value can be compared to the value of 67 ± 9 reported by Bueschleb (1987).

When the Do values for PTKSB are compared to the Do values for the first component (15.27 ± 0.70 J/m²) and second component (62.50 ± 7.81 J/m²) for Kos it can be seen that PTKSB survives significantly lower than Kos in 293 cells. In fact the Do values for Kos are two times higher than the values for PTKSB.

However, when the Do values are observed for XP cells the viruses survive the same. The Do values for PTK3B for the first and second component were 2.96 ± 0.21 J/m² and 7.94 ± 0.69 J/m² respectively. These values are almost identical to the values of 3.00 ± 0.30 J/m² and 7.97 ± 0.53 J/m² obtained for Kos.

This data correlates well with the observations of McKenna and McKelvey (1986). They reported that TK + normal cells survive better than TK - cells when these cells are exposed to a DNA damaging agent. However, when excision repair deficient TK + and TK - cells are assayed no difference in their survival is seen.

The TK gene has been shown to be important in normal cellular repair and replication (Coppey, 1977). When the virus enters the cell it shuts down the host mechanisms and therefore, shuts down de novo nucleotide synthesis. Thus the salvage pathway and hence the TK gene would be expected to be even more important for viral repair and replication.

When PTK3B was in an excision repair proficient background it did not survive nearly as well as the wild type virus Kos after UV irradiation. The only difference between these two viruses is that PTK3B has a point mutation in the TK gene and therefore, this gene is not expressed upon infection. This suggests that without the TK gene normal levels of viral excision repair are not able to be conducted. This idea is further supported by the fact that when the virus is in XP group A cells which have little or no excision repair capabilities it survives just as well as Kos. This shows that when no excision repair is being conducted the TK gene is not important for the survival of the virus.

Viral strains L714 and HDV 1 are both TK minus viruses. Both viruses have an insertion at the TK locus which interrupt the expression of the TK gene. These viruses differ from PTK3B not only in the way in which their TK genes have been interrupted but also in the fact that they have the mutant PAAr5 polymerase. From the earlier discussion one would expect these viruses survival to be lower than that of Kos in normal cells but to be much higher than Kos in XP cells based upon the fact that they carry the mutant

polymerase. However when their survival was assayed in normal and XP cells the Do values (Tables 5a -6b) show that the two viruses do not survive significantly different than PTK3B. This shows that the lack of the TK gene in the viruses do not allow the increased survival expected by having the mutant polymerase.

The data from these experiments suggest that the TK gene is important for the excision repair of the virus and that the PAAr5 mutant shows increased UV survival in XP cells due to some enhancement of excision repair. The mechanisms used for viral repair are thought to reflect the repair of the cell, therefore, the TK gene may be important for excision repair in general.

UV Survival Of HDV 1 And L714 In normal And XP Cells

Initially, when experiments were conducted on these viruses, it was felt that although details about the expression of HDV 1 could not be detected by protein isolation, HCR studies would provide a functional assay to determine if the RNA that was being transcribed was being translated into functional den V protein.

Survival curves for the pooled data of the two viruses can be seen as figures 20 and 21 for 293 and XP 4 cells respectively. The Do values for L714 on 293 cells were 6.89 $\pm$ 0.68 J/m^2 for the first component and 27.86 $\pm$ 2.49 J/m^2 for the second component. When these are compared to the

values of 7.32 ± 0.49 J/m² for the first component and 29.76 ± 3.04 J/m² for the second component for HDV 1 it is seen that the viruses do not survive significantly different. This was expected whether the den V gene is expressed in HDV 1 or not because as was seen by Arrand *et al.* (1987) the den V gene did not affect the survival of normal cells.

The Do values for the first component of survival in XP cells were 2.55 ± 0.20 J/m² for L714 and 2.56 ± 0.49 J/m² for HDV 1. The second component values were 7.09 ± 0.59 J/m² and 6.85 ± 0.38 J/m² for L714 and HDV 1 respectively.

The data shows that L714 and HDV 1 were indistinguishable when they were assayed for their UV survival in normal or XP cells. This would suggest that the functional den V protein was not expressed in HDV 1 or the lack of TK in HDV 1 does not allow the protein to complement the excision repair defect in the XP cells. In light of the fact that properly initiated RNA was detected in HDV 1 infected cells it is felt that the second of the two possibilities is more probable.

During the course of this study strong evidence was gathered that suggests the TK gene is important for repair of the virus in normal human cells. This idea could be further supported if the TK gene could be restored into one of the TK -viruses. This could be accomplished by inserting the TK locus into another area of the genome but this is rather impractical. Another way in which this could be accomplished is by transfecting cells with the HSV 1 TK gene. Upon

infection of these cells with HSV 1 the gene might be transcribed and therefore the HSV 1 TK protein would be present in the cells. The quickest and easiest method would be to use the virus ADTK (Haj Amad *et al.*, 1987). This virus is an E1 mutant of Ad 5 that carries the HSV 1 TK gene and has been shown to be stimulated to transcribe TK upon HSV 1 superinfection. The way in which these studies could be conducted would be to preinfect XP cells with ADTK to ensure that each cell has at least one copy of the virus in it. The next step would be to superinfect cells with either PAAr5 or L714. Proof of the importance of the TK genes involvement would be generated if the survival of L714 was brought up to the level of PAAr5 in cells that were preinfected with ADTK as compared to the proper controls. If this method were to work it may be also possible to show a complementation of the XP defect in HDV 1 infected cells.

The den V gene has been inserted into Ad 5, shown to make properly initiated RNA and increase the survival of the virus (Colicos *et al.*, Department of Biology, McMaster University, personal communication). This indicates that the gene is able to affect the UV survival of a virus and therefore the reason that an effect isn't seen in HDV 1 isn't because the gene cannot act on viral DNA but is more likely due to the concomitant loss of TK activity in HDV 1.

Using herpes virus as a vector has advantages over adenoviruses of which a quicker life cycle and broader host range are a couple. Recently a new system has been developed

for inserting genes into the Herpes virus genome (Ligas and Johnson, 1987). In this system the gene of interest is inserted into the nonessential glycoprotein I. Therefore, in this system genes that are important for the excision repair pathway (polymerase and TK) of the virus would not be disrupted and the effect of an inserted DNA repair gene would be amenable for study. It is thus felt that HSV may still be a good choice to use as a vector for studying DNA repair genes provided an appropriate construct is used.

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