CONSTRUCTION OF AN AD 5 VECTOR CONTAINING THE T4 DEN V GENE

Construction of an adenovirus expression vector containing the T4 den V gene, which can complement the DNA repair deficiency of Xeroderma pigmentosum fibroblasts

by

Michael Adrian Colicos

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

August, 1988

Master of Science (1988)
(Biology)

McMaster University
Hamilton, Ontario

TITLE: Construction of an adenovirus expression vector containing the T4 den V gene, which can complement the DNA repair deficiency of Xeroderma pigmentosum fibroblasts

AUTHOR: Michael Adrian Colicos, B.Sc. (McMaster)

SUPERVISOR: Professor Andrew J. Rainbow

NUMBER OF PAGES: xi, 160

Acknowledgements

I would like to thank Yosef Ahaj-Hamad for teaching me the techniques of molecular biology, Dr. Ken Rosenthal for his encouragement and enthusiasm for the pursuit of original thought, Dr. Frank Graham for chairing my committee, and Dr. Andrew Rainbow for providing the opportunity to obtain my degree. I would also like to thank Suzanne and Bobby for their tolerance and support, and the Academy for making this all possible.

Abstract

This study demonstrates the use of an adenovirus vector system to study the effect of a DNA repair gene on untransformed human fibroblasts. The bacteriophage T4 pyrimidine dimer DNA glycosylase (den V) gene has been inserted into the E3 region of human adenovirus type 5. The resulting recombinant virus Ad Den V was determined to be producing correctly initiated RNA from the RSV 3' LTR promoter used in the den V expression cartridge inserted into the virus. The effect of the den V gene product on human fibroblasts was examined by assaying for the percent host cell reactivation (%HCR) of Vag production for UV irradiated Ad Den V in comparison to that for a control virus. It was shown that the %HCR was significantly greater for Ad Den V as compared to the control virus in xeroderma pigmentosum (XP) cells. UV survival of adenovirus in XP cells exhibited a two component nature. Introduction of the den V gene into XP group A cells increased the D_0 value of the first component of the viral survival curve to a level similar to that of XPC cells, which showed no change in this component irrespective of the presence of the den V gene. It has been suggested that the den V gene is able to partially complement the deficiency in some XP cells because of its small size, allowing it to gain access to the DNA damage site where as the cellular repair enzyme complex can not. Since XPC cells

are proficient in their alteration of DNA secondary structure prior to DNA excision repair, these results are consistant with the hypothesis that the first component of UV viral survival curves reflects the pathway involved in accessing the damaged sites.

The manuscript of a paper has been included as an appendix. The work theorizes on the origin of mammalian immune system diversity and bacteriophage lambda, and their possible relationship to prokaryotic DNA repair genes.

Table of Contents

Page #

List of Figures

List of Tables

List of Abreviations

AP - apurinic-apyrimidinic
BS - Bloom's syndrome
CS - Cockayne's syndrome
ESS - enzyme sensitive sites
FBS - fetal bovine serum
HCR - host cell reactivation
HSV - herpes simplex virus
IHF - intagration host factor
KB - kilo bases
KD - kilo daltons
LTR - long terminal repeat
MMS - methyl methanesulfonate
MN - miccrococcal nuclease
PBS - phosphate buffered saline
PCC - prematurely condensed chromatin
PD - pyrimidine dimers
PFU - plaque forming units
PR - photoreactivation
PRE - photoreactivating enzyme
RSV - rous sarcoma virus
RFLNE - rat fetal liver nuclear extract
RS - recombination signal
TK - thymidine kinase
UDS - unscheduled DNA synthesis
UV - ultra violet
VFU - Vag forming units
VSV - vesicular stomatitis virus
VDJ - variable, diveristy, joining region
XP - xeroderma pigmentosum

Introduction

Maintenance of the integrity of the primary DNA sequence is essential for the transfer of genetic information to successive cell generations. The encoded information supplies the template from which the cell's structure and function will be determined and if this information is lost or altered to a great extent, cell heredity will be compromised. While some changes are essential for the genetic evolution of an organism, and the transposition of long nucleotide sequences an intrinsic step in cellular development, the presence of a mechanism for the repair of damaged DNA is essential.

In a living cell, DNA is constantly subject to alterations by the environment, as well as the spontaneous changes that can occur through mechanisms such as tautomeric shifts and deamination. Ultraviolet light and chemical agents cause a wide variety of changes to the molecular structure of DNA, including alkylation, cross-linking and cyclobutane pyrimidine dimers (Roberts 1978, Ben-Hur and Ben-Ishai 1968). These lesions can affect the integrity of the genetic information by either blocking transcription or replication, or altering genetic function of the sequences involved. To prevent such deleterious consequences, cells have evolved DNA repair mechanisms to remove such lesions and restore the integrity of the sequence. While some fundamental similarities exist, prokaryotic and eukaryotic repair systems appear to differ in enzymology. While the

bacterial DNA repair system has been elucidated in considerable detail, the mammalian DNA repair mechanism is not as well understood. The relationship between a mammalian cell's ability to repair DNA and oncogenesis has been well documented, and investigations into the causal relationship between them has been productive in advancing our understanding, and possible prevention, of cancer (Setlow 1980).

Bacterial DNA Repair Systems

Photoreactivation

One of the simplest and most direct methods of DNA repair in prokaryotic systems is the enzymatic photoreactivation (PR) of pyrimidine dimers. The existence of an enzymatic system of thymine dimer monomerization was first investigated in bacteria and yeast (Rupert et al 1958, Rupert 1960). Subsequent studies have shown the presence of photolyase activity in a large number of plant and animal cell types. PR is accomplished by the binding of an enzyme to the lesion, followed by the absorption of ~300nm light photons and the subsequent monomerization of the dimer (Sutherland 1978). This can also be accomplished by direct photoreversal, in which light photons in the range of 200 - 300 nm can be directly absorbed and monomerize dimers (Setlow

1968), but the enzymatic system has been of greater interest since similar enzymatic activity has been found in higher plants and animals (Rupert 1975).

Excision Repair

The excision repair system in E. coli is one of the best documented and best understood DNA repair systems to date. There are two fundamental excision repair processes in E. coli, base excision repair and repair of bulky lesions (nucleotide excision repair). Briefly, base damage (e.g. alkylation) is repaired by a DNA glycosylase cleaving the damaged base next to the sugar backbone (Hanawalt et al 1979), which in turn is cleaved by a 5′ apurinic-apyrimidinic (AP) endonuclease (Lehmann and Karran 1981). The resulting 5′ deoxyribose-phosphate group can then be excised by either a 3′ AP endonuclease or a 5′-3′ endonuclease (Lindahl 1982, Lindahl 1976). This leaves a gap in one strand of the DNA, which can then be filled in by DNA polymerase and subsequently sealed by DNA ligase.

The majority of damage from UV irradiation that is found to occur in DNA, however, is in the form of bulky base lesions, which are repaired by a system that is different from that of base damage. In E. coli this is accomplished by the uvrABC endonuclease complex (Howard-Flanders et al 1966). The uvrA, uvrB and uvrC genes are under control of

the recA-lexA regulatory system, which has been shown to be inducible by UV light, and controls many other gene complexes, including the SOS repair system (Backendorf et al 1983). The uvrA gene product has a molecular weight of 114,000 KD and has been shown to bind single stranded irradiated DNA with a higher affinity than unirradiated DNA, and exhibits ATPase activity (Seeberg and Steinum 1982). The uvrB and uvrC gene products both have a molecular weight of approximately 70,000 KD (Sancar et al 1981a, Sancar et al 1981b), and neither show a high affinity for DNA binding and have no observed catalytic function. Once complexed, however, uvrA,B and C act as the mechanism of incision at bulky lesions such as pyrimidine dimers in DNA. It is believed that the complex acts as a direct endonuclease rather than a DNA glycosylase - AP endonuclease system (Yeung et al 1983). The current model of their action is as follows. The uvrA protein non-specifically binds to DNA and then complexes with uvrB protein to form a more stable aggregate. The resulting dimer then translocates along the DNA, a process that is apparently ATP dependent, until a lesion is encountered. UvrC protein then joins the complex, which then becomes catalytically active and excises an oligonucleotide from the region of the damage. Excision is accomplished by two nicks made in the deoxyribose-phosphate backbone flanking the dimer, approximately 12-13 bp apart. The resulting gap can then be filled in by DNA polymerase and

sealed by DNA ligase, as with the base excision system (Seeberg and Steinum 1983).

Other Repair Systems

Two other bacterial repair systems are post-replication repair and the SOS error prone repair system. Post replication repair occurs when a lesion has not been removed by the time of DNA replication. During replication the unrepaired lesion will cause the arrest of the polymerase progression, but there will be either a re-initiation downstream from the damage, resulting in a gap in the daughter strand, or trans-lesion synthesis can take place by the use of an error prone polymerase which is able to traverse the dimer (Hanawalt et al 1979). It is believed that the gaps caused by re-initiation can be repaired by the process of bacterial sister chromatid exchange, in which a non- reciprocal recombination event with the intact sister DNA duplex supplies the template for the resynthesis of the deleted strand (Rupp et al 1971). The resulting double helix, which still contains the original lesion, can then be repaired by conventional mechanisms.

Trans-lesion synthesis can also take place. This is inherently error prone, since the information as to the correct base to insert opposite the lesion has been obscured. This is exemplified by the SOS repair system in E. coli. The

SOS repair system, which is inducible by DNA damaging agents, requires the functioning of the recA-lexA repressor system. In this system recA, as well as playing a role as a recombinase itself, can become activated as a protease in the presence of DNA damaging agents. Once activated, it cleaves the lexA repressor, which consequently allows the expression of the genes of the SOS response. RecA itself is regulated by lexA, and is consequently amplified by its own activation (Little and Mount 1982). The repertoire of genes induced by this system include the previously described uvrABC genes and the umuC,D genes (Ellidge and Walker 1983). It has been suggested that trans-lesion synthesis might be facilitated by the inhibition of the 3'-5' exonuclease proof reading function of DNA polymerase III (Villani et al 1978).

Mammalian DNA Repair Mechanisms

While similarities between prokaryotic and eukaryotic DNA repair appear to exist, the differences become apparent from the nature of the DNA itself. With the eukaryotic chromosome possessing a complex secondary structure and an association with DNA binding proteins and the nuclear matrix, access to lesions becomes a more relevant issue than in the case of prokaryotic systems. While present knowledge of the mechanism involved in eukaryotic, and more specifically mammalian systems, is scarce, the subject is best addressed

by the functional stages involved. The first step in excision repair is the recognition and incision of the lesion, in the case of UV damage the pyrimidine dimer. The study of Xeroderma pigmentosum (XP) cells which are deficient in this ability, has assisted in the investigation of the initial processes involved in excision repair. The inability to accomplish this first stage in excision repair was first detected by the reduced unscheduled DNA synthesis (UDS), as assayed by lack of incorporation of ^{3}H thymidine after UV irradiation of the XP cells (Setlow et al 1969). Incision at pyrimidine dimers can also be assayed for by the presence of UV endonuclease sensitive sites (ESS). The bacteriophage T4 den V gene encodes a pyrimidine dimer (PD) DNA glycosylase which can be used to incise at unrepaired dimers, and subsequent alkaline sucrose gradient centrifugation assays the amount of breaks introduced and consequently the amount of unrepaired lesions (Paterson et al 1981). The inability of XP cells to perform the incision step of excision repair has been investigated to determine whether their defect lies in the incision mechanism itself or the proteins involved in the pre-incision steps. It has been demonstrated that crude cell extracts from XP group A cells are able to incise thymine dimers in naked DNA, but are much less efficient in removing dimers from their own DNA (Mortelmans et al 1976). This work was extended to include XP group A,C and G cells, and it was shown that while normal and XP group E cell

extracts could efficiently excise thymine dimers from chromatin in vitro, the XP group A,C and G extracts could not. It was also demonstrated that the XP group A,C and G cell extracts could incise dimers from chromatin that retained the nucleosome structure, but had been depleted of the non-histone proteins (Kano and Fujiwara 1983). This work suggested that the defect seen in certain XP complementation groups lies in the inability of the incision enzyme or enzyme complex to access the damage. This line of investigation has been pursued and comfirmed, with the exception that XP group C cells appear to retain some of the nucleosome rearrangement ability required to access damaged sites (Mathis and Althaus 1986, Smerdon et al 1979, Sidik and Smerdon 1987). The relatively large number of XP complementation groups indicates the complexity of the incision procedure in mammalian cells. This complexity could exist on two levels; in the nature of the actual incision enzyme complex, or in the fact that the secondary structure of the eukaryotic chromosome predicates the requirement of a mechanism to access the lesion before incision.

The relationship between the repair of cyclobutane pyrimidine dimers and cell survival is presently under investigation. It has recently been demonstrated that there is another major form of damage induced by UV irradiation, the pyrimidine(6,4)pyrimidone photoproduct. This product occurs as approximately 20-35% of the total UV damage of all

forms, and is potentially the major cytotoxic lesion induced by UV irradiation (Mitchell 1988). It has also been suggested that the DNA repair mechanisms by which the two forms of lesions are repaired might be different. An XP revertant cell line has been generated that is able to repair the 6-4 photoproduct but is unable to repair thymine dimers. While the revertant cell line post UV cell survival is similar to that of normal cells, it has reduced repair of plasmid shuttle vector DNA post UV irradiation in comparison to normal cells (Cleaver 1988). This also suggests that the repair of thymine dimers is dependent upon the conformation of the DNA that is damaged, and helps to explain why the complementation of DNA repair capacity of XP cells by pyrimidine dimer glycosylases such as the T4 den V gene (Valerie et al 1985, 1987) is only to an intermediate level.

Role of DNA Secondary Structure

Another interesting observation is that antibodies directed against thymine dimers show a more rapid decrease in binding post UV irradiation in comparison to the decrease in ESS. Since the dimers remain viable as substrate for enzymatic cleavage, but antibodies against the dimers lose their specificity, this suggests that there are changes in the conformation of the DNA in the region of the dimer prior to repair (Mitchell et al 1982). Rearrangement of chromatin

structure has been examined in Xeroderma pigmentosum group A cells which were treated with the alkylating agent methyl methanesulfonate (MMS). In both XPA and normal cells it was seen that the repair of the alkylated sites was confined to nuclease sensitive regions of the DNA, and almost completely absent in DNA associated with the core histones. Pulse-chase experiments have indicated that these regions subsequently undergo rapid rearrangements in chromatin structure, suggesting that incision deficient cells are capable of the initial structure changes necessary for DNA repair (Sidik and Smerdon 1987). The alteration in chromatid structure preceding DNA repair is confirmed by <u>in situ</u> observation (Hittelman 1986). Irradiation of quiescent normal human fibroblasts with UV light results in two distinct changes in the chromatin structure in G1 phase cells. The first is the elongation of prematurely condensed chromatin (PCC) and the second is the formation of localized decondensations in the elongated regions. The initial change in structure can be found in cells deficient in the incision step in excision repair as well as normal cells, however the localized decondensation is observed only in normal cells (Hittelman 1986). It has also been shown that these areas of localized decondensation are in the region in which unscheduled DNA synthesis occurs, possibly correlating with the decrease in UDS seen in XP cells. However, the change in conformation does not necessarily indicate access to the dimer. In a

study using photoreactivating enzyme (PRE) extracted from yeast, it was shown that the addition of the enzyme to normal human fibroblasts caused a reduction in the amount of UDS post UV irradiation. This was assumed to occur due to competition for the thymine dimers by the PRE with the excision repair mechanism. This was not observed with XP group A,D,E or H cells, however, indicating some alteration in their ability to access the dimer once the chromatin has decondensed. One notable exception to this was XP group C cells, which retained the ability to allow access to dimers by the PRE (Hoeijmakers 1987).

The complex coiling and association with histones and other proteins of eukaryotic chromatin implies both a heterogeneous susceptibility to DNA damaging agents and the requirement of a mechanism to access the damage once it has occurred. The eukaryotic nucleosome structure has been shown to consist of two molecules of each of histone H2A,H2B,H3 and H4, with 146 bp of the DNA strand wrapped around it. Histone H1 is thought to be associated with the outside of the nucleosome, and each nucleosome is separated by a region of linker DNA approximately 60 base pairs in length (Oudet et al 1975). It has been shown that the chemical mutagenic agent methylmethanesulfonate induces damage almost exclusively in the linker region (Bodell 1977). This is contrasted by the behavior of UV induced pyrimidine dimers, which have been shown to appear evenly throughout the

chromatin structure (Williams and Friedberg 1979). Upon initiation of repair, however, there is an immediate preference for dimers in the linker region, as assayed by the ratio of the repair synthesis in the micrococcal nuclease (MN) sensitive DNA (linker region) to the repair in the MN resistant DNA (nucleosome bound) with time. After 24 hours there is no preference between the two (Smerdon and Lieberman 1978, Smerdon et al 1979). It has been speculated that results such as these along with other evidence (Bodell 1977) indicate that there is a temporary break down of the nucleosome structure in the region of DNA undergoing repair. This implies that the MN sensitive sites which were seen to be preferentially repaired might actually be DNA that was previously associated with nucleosomes, now opened up to allow access of the repair enzymes. However, this has not been substantiated.

The most obvious target of investigation into the cause of secondary structural effects in XP cells is the nucleosome. However this has proved to be unrevealing. Detailed biochemical studies have shown no detectable difference in the nature of histones extracted from normal human fibroblasts and XPA cells (Amari et al 1986). A study into the possible interaction between AP endonucleases and nucleosome structure proved more successful. Two AP endonucleases were isolated, one from normal human fibroblasts and the other from XPA cells. They were assayed

for their efficiency in cleaving a plasmid DNA substrate that had been alkylated by treatment with MMS. Each AP endonuclease was tested with DNA that was non-nucleosomal and DNA that had reconstituted nucleosomal structure by the addition of core histones from either XPA cells or normal human fibroblasts. It was observed that the normal cell extracted AP endonuclease increased its efficiency upon addition of the nucleosomes, but there was no increase in efficiency observed with the XPA AP endonuclease when the DNA was in nucleosome bound form. These results did not change regardless of the source of the histones used (Kaysen et al 1986). This suggests that it might not be the nucleosomes themselves that are altered in XPA cells, but rather the way that enzymes interact with the DNA that is associated with them. However, the effect of higher chromatin structure on DNA repair in mammalian cells is, as of yet, still unclear.

Enzymes Involved in Repair

Nucleases

Although no clear picture of the details of mammalian DNA repair has yet been elucidated, several enzymes that are potentially involved have been characterized. Since only mammalian DNA polymerase δ has been assigned an exonuclease

function (Kunkel et al 1987), the DNA degradation aspect of repair could be performed by accessory enzymes rather than in concert with the polymerase action. One such enzyme that has been isolated is deoxyribonuclease (DNase) IV, partially purified from rabbit bone marrow and lung (Lindahl et al 1969). It has been shown to have preference for exonuclease activity rather than endonuclease action, and is able to utilize UV irradiated DNA as a substrate. Whether or not the ability to excise dimers from incised DNA is present in vivo is not known, but it potentially exists. Another 5'-3' thymine dimer excising enzyme has been isolated from human KB cells. It has been shown to function on UV irradiated DNA that has been pre-incised with the T4 den V gene product (Cook and Friedberg 1978a and 1978b). Other nucleases have been isolated from other sources as well, such as the correxonuclease from human placental tissue and DNase VII from the same source (Doniger and Grossman 1976, Hollis and Grossman 1981). While all these enzymes function in vitro, their role in mammalian DNA repair in vivo is still unsubstantiated.

DNA Polymerases

DNA Polymerase α

There are several mammalian DNA polymerases that have been

identified and implicated in DNA repair. DNA polymerase α is the major polymerase involved in cellular DNA replication during growth of cells, but constitutes only 5 percent of polymerase activity in quiescent cells (Kornberg 1980). Polymerase α has been identified as a holoenzyme complex of 10 separate polypeptides, ranging in molecular weight of approximately 47 to 200 KD (Ottiger et al 1987). There have been multiple forms of DNA polymerase α identified, most of the variation between the forms being in the association of the primase activity with the complex. It appears that the nuclear matrix associated class (7.5 S) possesses only 10% of the primase activity, whereas the matrix independent class (10.5 S) is 95% competent in self priming (Collins 1987). There has as yet been no exonuclease function associated with the holoenzyme complex.

DNA Polymerase ß

When a cell is in the resting state it is assumed that almost 100 percent of the polymerase activity is performed by DNA polymerase ß (Friedberg 1985). There is growing evidence that several polymerase enzymes are involved in DNA repair, but there are differential assignments of roles in the nature of the lesions they repair. DNA polymerase ß has been demonstrated to be more efficient than polymerase α in the repair of short gaps in double stranded DNA and it has also

been shown to extend these gaps beyond the initial site of synthesis (Nowak 1987). DNA polymerase ß has also been shown to exhibit a different substrate specificity than polymerase α, in that DNA synthesis induced by bleomycin damaged DNA is almost exclusively a substrate for polymerase ß rather than polymerase α (Seki 1987).

DNA Polymerase δ

Historically, DNA polymerase ß has been assigned the principle function of the DNA repair. Current research has also implicated DNA polymerase α in the repair of UV irradiated DNA. These studies have been principally based upon results of experiments involving the inhibitory affect of the drug aphidicolin on polymerase α. It has been demonstrated that treatment of HeLa cells with aphidicolin inhibits DNA replication but not DNA repair (Pedrali-Noy and Spadari 1980), suggesting a lack of involvement in repair by DNA polymerase α. Other work, however, has demonstrated that human fibroblasts are sensitized to killing by UV irradiation upon treatment with aphidicolin (Tyrrell and Amaudruz 1987), suggesting that polymerase α is involved in DNA repair. This apparent conflict might be resolved by the recent study of the DNA polymerase δ. DNA polymerase δ is a highly accurate proof-reading polymerase with an associated 3′-5′ exonuclease function (Kunkel et al 1987). It is aphidicolin sensitive,

but differs from polymerase α in that it is highly sensitive to the nucleotide analogue 2',3'-dideoxythymidine 5'-triphosphate (ddTTP) (Wahl et al 1986). It has also been demonstrated that repair synthesis of UV irradiated DNA in ddTTP treated cells was highly inhibited, suggesting that DNA polymerase δ is the aphidicolin sensitive polymerase being implicated in excision repair (Dresler and Kimbro 1987).

Existence of Multiple Nucleotide Excision Repair Pathways

Deficiency in Only One of Two Repair Pathways in XPC Cells

While the role of the individual polymerases in DNA excision repair is still the subject of investigation, the presence of two (and possibly more) nucleotide excision repair pathways is evident. In studies using aphidicolin to block the polymerase α/δ pathway, excision proficient cells were shown to be sensitized to UV irradiation by pretreatment of the cells with the drug. XPC cells showed no change in UV cell survival upon treatment, suggesting the absence of the α/δ pathway. They do, however, retain the ability of partial repair synthesis (Tyrrell and Amaudruz 1987). This suggests that there are two independent pathways of repair, one which is present in XPC cells, and the DNA polymerase α/δ pathway, in which XPC cells are deficient. The existence of two pathways was also suggested in this work by the two

component nature of the UV cell survival curves produced for XP groups A,D and C cells (Tyrrell and Amaudruz 1987). The two component nature of the UV cell survival curves is seen to be enhanced by aphidicolin, and the two components become evident in normal human fibroblasts as well as XP cells. The presence of a pathway in addition to the aphidicolin sensitive polymerase α/δ pathway has been further substantiated by study of DNA damage caused by the chemical carcinogen 4NQO. This damage is repaired by the polymerase α/δ dependent pathway (since aphidicolin blocks its repair in normal cells), yet XPC cells are able to repair 4NQO damage, even in the presence of aphidicolin (Edwards 1987).

Localization of DNA Repair in XPC Cells

Further evidence for the existence of two separate repair pathways exists. XPC cells display a relatively constant degree of repair synthesis capacity as indicated by UDS under varying cell conditions. However, the nature of the region of the genome repaired alters drastically with the condition of the cells. When the XPC cells are in quiescence, excision repair is clustered in pre-existing, non-transient nuclease sensitive sites (Player and Kantor 1987), and the XP cells are as efficient in DNA repair as normal cells in these regions (Cleaver 1987). In proliferating XPC cells, however, the total amount of repair

synthesis is the same as when quiescent, but the repair activity is distributed throughout the genome (Cleaver 1987). Normal cells and XPA cells, in contrast, exhibited uniform repair throughout the genome regardless of the state of the cells. This suggests that the repair taking place in XPC cells during this time is in actively transcribed and consequently essential genes (Player and Kantor 1987). This also means that the pathway deficient in XPC cells (the polymerase α/δ pathway) is targeted for the nuclease resistant regions (Player and Kantor 1987).

The clustering effect in XPC cells was also investigated with respect to the physical localization of repair synthesis at the nuclear scaffold attachment points. It was observed by autoradiography of radiolabelled DNA-matrix structures that the repair synthesis in quiescent XPC cells took place at the chromatid loop attachment point to the nuclear scaffold at 3 - 4 times greater the frequency than the rest of the DNA. This again was different than the situation seen in normal and XPA cells, which showed no preferential repair domains (Mullenders et al 1986).

Nature of the DNA Repair Pathway Deficient in XPC Cells

It has been shown that chromatid structure undergoes rearrangement prior to DNA repair, presumably allowing access to sites of damage (Hittleman 1986). XP cells alter their

chromatid structure yet, with the exception of group C, are unable to allow access to the lesions (Sidik and Smerdon 1987). XPC cells (which are defective in their polymerase α/δ pathway) are able to repair selective regions with an alternate repair pathway, and this pathway functions on more accessible, nuclease sensitive domains.

The clustering effect seen in XPC cells has been shown to be sensitive to aphidicolin (Cleaver 1987). Upon treatment with the drug, XPC cells displayed a random distribution of repair sites throughout the genome of quiescent cells. This implicates DNA polymerase α or δ in the regulation of the distribution of repair in quiescent cells (Cleaver 1987). Polymerase α is functional in XPC cells since replication occurs. Since the UV cell survival and consequently DNA repair seen in XPC cells is not dependent upon the deficient aphidicolin sensitive polymerase α/δ pathway, this suggests that DNA polymerase δ is the deficient repair polymerase in XPC cells.

DNA Repair Deficient Human Disorders

Xeroderma Pigmentosum

Xeroderma pigmentosum is an autosomal recessive genetic disorder that results in patients with a very high photosensitivity and a high incidence of skin cancer (Setlow

1978). There are nine complementation groups known at present (A-I), indicating the complexity of the disorder. While many of the biochemical and cellular phenotypic alterations observed upon exposure of XP cells to chemical mutagens or UV light in culture vary, some manifestations of the disease are common to all groups. After cells are subject to UV irradiation, XP cells are observed to have very little UDS in non-S phase cells, as assayed by radioactive thymidine incorporation (Cleaver 1968). After UV irradiation XP cells also exhibit decreased cell survival, failure to lose DNA PD glycosylase sites, increased sister chromatid exchange, and they also show decreased ability to reactivate damaged virus and enhanced susceptibility to neoplastic transformation (Friedberg 1985). The cells, however, are only moderately sensitive to 137 Cs gamma irradiation, as assayed by ESS post γ irradiation of cells. 50% of the ESS were removed within the first 1.5 hours post irradiation in both normal cells and in all XP groups tested (A,C,D and G) but there was a 10% residual dimer population after 17 hours post irradiation in the XP lines (Fornace et al 1986).

XP variant form cell lines have also been identified, which have been isolated from patients with the clinical manifestations of the disease but the cells isolated showed no apparent defect in nucleotide excision repair (Cleaver 1972). Recent work, however, has suggested that there is an excision repair defect in XP variant cells. Antibodies

directed against thymine dimers have been used to demonstrate that there is a lower rate of loss of antigenicity to the antibodies with time in XP variant cells in comparison to normal human fibroblasts (Roth et al 1987).

Some XP cells also exhibit a decreased capacity for post-replication repair. Post-replication repair is assayed by pulse-chase labelling with ^{3}H thymidine post UV irradiation, and subsequent analysis of the weight-average molecular weight of the newly synthesised DNA. It has been shown that there is a slower increase in molecular weight of newly synthesised DNA post UV irradiation in the XP variant cells in comparison to normal cells (Lehmann et al 1975). This reduced rate of post replication repair is not as great in the classical XP complementation groups. One notable exception is XP group E cells, which appear to have near normal levels of post replication repair as determined by this assay technique (Lehmann et al 1977).

The presence of a photoreactivating enzyme with similar properties to DNA photolyase has been identified in mammalian cells (Sutherland 1974, Sutherland et al 1974). It has been shown that the amount of photoreactivating enzyme in XP cells is lower than that found in normal cells. However there has been no evidence that the enzyme from XP cells itself differs in any way from enzyme isolated from normal cells (Sutherland et al 1975).

The presence of nine known classical XP complementation

groups indicates that there are at least nine genes or domains which are involved in the repair of bulky DNA lesions in human cells. This suggests that the incision step in mammalian DNA repair involves either a multi step pathway or a holoenzyme complex (Friedberg 1985). The evidence for the involvement of secondary structure and multiple DNA nucleotide excision repair pathways implies the potential for defects in steps other than the actual enzymatic incision process.

Cockayne's Syndrome

Cockayne's syndrome (CS) is an autosomal recessive disease believed to include a DNA repair deficiency. Patients are characterized by dwarfism, photosensitivity, deafness, mental deficiency, sunken eyes, large ears and nose (Cockayne 1936). Although cells from Cockayne's syndrome patients show an increased sensitivity to killing by UV irradiation in comparison to normal cells, there is no observed increase in cancer in CS individuals (Schmickel et al 1977). While some studies indicate that there is no decrease in the ability of CS cells to reactivate UV irradiated adenovirus (Hoar and Davis 1979), other studies indicate that there is a reduced percent host cell reactivation (%HCR) of virus (Day and Ziolkowski 1978, Rainbow and Howes 1982). The most prominent defect in CS cells is their delayed recovery of

semiconservative DNA synthesis after UV irradiation (Lehmann and Mayne 1981). Recent study of CS has revealed that the incision of the DNA molecule at thymine dimers is normal, however the rejoining of newly synthesised DNA is delayed, suggesting a defect in the polymerase or ligation system (Schwaiger et al 1986, Squires and Johnson 1983).

Bloom's Syndrome

Bloom's syndrome (BS) is characterized by stunted growth, susceptibility to infection, the development of light induced capillary dilation of facial skin (Bloom 1966), and a high incidence of cancer (Freidburg et al 1979). The DNA repair capacity of Bloom's syndrome cells appears similar to normal cells by most criteria, with the exception of a 10 to 15 fold increase in the number of sister chromatid exchanges (SCE) post UV irradiation (Chaganti et al 1974). This increase in SCE is accompanied by an increased amount of chromosomal abnormalities, such as breaks and rearrangements (German et al 1965). While implication of a defect in the cellular DNA repair mechanism is marginal, strain GM 1492 has been noted as exceptional in that it exhibits a higher sensitivity to UV irradiation (Krepinsky et al 1980). Current research has strongly implicated a defect in the structure of DNA ligase I resulting from a point mutation, as indicated by reduced enzyme activity and which is abnormally heat-labile (Willis

and Lindahl 1987, Chan et al 1987).

Bacteriophage T4 PD DNA Glycosylase den V

Bacteriophage T4 is a doublestranded DNA coliphage that carries a DNA excision repair enzyme, den V. The existence of a DNA repair enzyme on T4 was first observed when it was noted that T4 had a higher resistance to killing by UV light than other T-phages (Luria 1947). It was then discovered that the den V gene possessed a thymine dimer specific endonuclease activity (Friedberg and King 1971), and the enzyme is now termed the T4 pyrimidine dimer (PD) DNA glycosylase. It is capable of repairing only thymine dimers (Friedberg 1972), and is specific for dimers on one strand only (Simon et al 1975). While the den V gene possesses a PD glycosylase activity that has been demonstrated in vivo, it also possesses an AP endonuclease activity in vitro (McMillan et al 1981). Whether or not this activity is present in vivo is unclear since it has been shown that while the PD glycosylase function cannot be competed for by native, unirradiated DNA, the AP endonuclease can. This is problematic since one would expect an enzyme with such a dual function would perform both activities in a concerted action, rather than the separate nature implied by the competition results observed. Whether or not the AP endonuclease activity is active in vivo is relevant to elucidation of the

mechanism of action. From the available current data it appears that the den V gene first locates and cleaves one of the bases from the dimer, by acting on the bond between the base and the deoxyribose-phosphate backbone. A 3′ AP endonuclease activity then cleaves the backbone between the dimerized bases and the dimer is then monomerized. Endonuclease activity can then excise the damage and surrounding bases, leaving a gap to be filled in by DNA polymerase (Friedberg 1985).

The den V gene has been isolated and cloned from the T4 genome (Valerie et al 1984). The den V polypeptide is approximately 16,000 daltons and has been shown to bind both UV irradiated and unirradiated DNA, and once bound to the DNA it scans the molecule in a processive manner until encountering a thymine dimer (Ganesan et al 1986). The ability of the den V gene to complement the deficiency in XP cells has been extensively investigated. The premise for these investigations is that XP cells are deficient in the incision step of excision repair (Friedberg 1985). Because the den V gene product is able to incise damaged DNA, it was hoped that complementation by the den V gene would lead to the elucidation of a homologous but defective mammalian enzyme responsible for the XP defect. It was shown that macromolecules could be inserted into cells by concomitant treatment with HVJ (Sendai virus) (Okada and Tadokoro 1962). The den V gene product was introduced into XP groups A,B,C,D,

and E cells by this technique and it was shown that DNA repair synthesis as measured by unscheduled DNA synthesis (UDS) could be restored to normal cell levels (Tanaka et al 1975). Further investigation showed that cell survival of XP group A cells could be partially complemented, although not to wild type levels (6.4% restoration, Tanaka et al 1977). Once the den V gene had been cloned (Valerie et al 1984) and shown to express den V gene product in bacteria (Valerie et al 1985a), it was introduced into a Chinese hamster ovary (CHO) DNA repair deficient cell line by DNA mediated transfection (Valerie et al 1985b). It was shown that the established CHO transformant carrying the den V gene had a higher level of UV irradiated DNA repair than normal cells as measured by strand breakage (as determined by alkaline sucrose density centrifugation) and repair synthesis ($[^3H]$ thymidine incorporation). In contrast, UV cell survival was only restored to an intermediate level (56% restoration of normal levels, Valerie et al 1985b). An intermediate level of restoration (24%) of UV cell survival was also seen upon introduction of the den V gene into a DNA repair deficient strain of the yeast Saccharomyces cerevisiae (Valerie et al 1986).

A eukaryotic vector suitable for the expression of the den V gene in mammalian cells was constructed utilizing the Rous Sarcoma Virus (RSV) 3' LTR promoter and SV40 large T poly A+ splice site, as further detailed in Materials and

Methods (Valerie et al 1987a). Using this vector, transient expression was assayed by co-transfection with UV-irradiated pRSVcat (Gorman et al 1982) into SV40-transformed XP group A cells. It was shown that 93% of CAT (chloramphenicol transferase) activity was restored as a result of the presence of den V. Stable complementation of the same cells was also achieved, by transfection of pRSVdenV-SVgpt and subsequent selection in MAX medium (selecting for xanthine-guanine phosphoribosyltransferase, Mulligan and Berg 1981). When stable transformants carrying den V specific DNA sequences incorporated into the genome were assayed for their ability to reactivate UV-irradiated pRSVcat, up to 50% of normal activity was observed. When UV survival of transformants was assayed, a restoration of between approximately 6 to 20% of the decrease in survival seen in XP cells was observed (Valerie et al 1987a). The plasmid pneotk denV (Valerie et al 1985a) has also been used to stably transform an XP group D-Hela hybrid cell line which initially showed no characteristic UV survival deficiency, but spontaneously acquired UV sensitivity with time. The presence of the den V gene was shown to restore between 15% and 23% of the UV survival ability of the cell line, however the true implications of this result are questionable in light of the indeterminate cell line used (Arrand et al 1987).

The Use of Viruses as DNA Repair Probes

The ability of a cell to repair UV irradiation damaged viral DNA has been used as a measure of the cell's DNA repair capacity (Day 1977, Rainbow 1981). This is based on the assumption that the viral DNA is at least partially processed by the same enzyme systems that function on the host cellular DNA (Rainbow 1981). Several approaches have been used, including the host cell reactivation of SV40 DNA (Aaronson and Lytle 1970), herpes simplex virus (Lytle et al 1972, Rainbow and Ryan 1986) and human adenovirus (Day 1977, Day 1974). Extensive research has revolved around the reduced capacity of XP cells to reactivate UV irradiated (Day 1974, Rainbow 1981), and γ irradiated human adenovirus (Rainbow 1979). By comparing UV survival of virus in a DNA repair deficient cell line and survival in a normal cell line, the relative percentage host cell reactivation (%HCR) can be calculated. This gives an indication of the relative decrease in DNA repair capacity of virus for the mutant cell line (Rainbow 1981). UV survival of virus in human fibroblasts can be assayed by detection of Vag formation. Immunofluorescent staining of infected cells with antibodies directed against virion proteins affords an indication of viral function (Rainbow 1978).

Using the technique of assaying for Vag production, the study of the restoration of DNA repair capacity by a DNA

repair gene is possible. By comparing the %HCR of a mutant cell line with the %HCR of that cell line in the presence of the DNA repair gene, an indication of the restoration of DNA repair capacity by that gene can be determined. This can be done by the construction of a virus carrying the repair gene in its genome. The subsequent assay for the percent host cell reactivation of virus with transient expression of the repair gene can then be established and can be compared to the %HCR of the wild type virus. If transient expression of the gene enhances the %HCR, this will be reflected in the enhanced UV survival of the virus. The percentage of restoration of the deficiency by the DNA repair gene can be calculated as compared to normal cells, which can then be related to the percentage restoration of UV cell survival as determined in other research.

The Use of Recombinant Human Adenoviruses as Mammalian Expression Vectors

Adenovirus Structure

Human Adenoviruses are nonenveloped, icosahedron shaped DNA tumor viruses, with linear double-stranded DNA genomes of approximately 36,000 base pairs (Green *et al* 1967). Adenovirus replicates in the cell nucleus, during which time host cell DNA synthesis is reduced by greater than 90% of

normal levels (Horowitz 1971). It is presently believed that DNA polymerase α has no detectable role in adenovirus replication, and that the viral encoded polymerase Ad DNA Pol, along with Ad DNA binding protein (DBP), the preterminal protein (pTP) and at least three host cell replication factors are responsible for the adenovirus replication (Horwitz 1985, Shu et al 1987). Adenovirus DNA has been shown to interact with cellular histones and to adopt a chromatin-like structure (Tate and Philipson 1979), and has been observed to interact with the nuclear matrix (Zhai et al 1987).

Adenovirus Vectors

Adenovirus has a broad host range in vivo and in vitro, making it a suitable vector for study of mammalian gene expression (Haj-Ahmad and Graham 1986). Studies of the packaging constraints of human adenovirus type 5 suggest that even with up to 2.2 kb of additional DNA in the viral genome, the virus can be successfully encapsidated. This is dependent upon the presence of protein IX, a minor component of the adenovirus capsid (Ghosh-Choudhury et al 1987). The total amount of foreign DNA carried by the virus can be increased by the deletion of non-essential adenovirus sequences. It has been demonstrated that the E3 region between the Xba I restriction sites at 78.5% and 84.7% can

be deleted and foreign DNA inserted in its place. The virus is successfully encapsidated and expression of the inserted genes has been demonstrated (Haj-Ahmad and Graham 1986).

By combining the use of adenovirus as a mammalian expression vector and UV viral survival as a measure of DNA repair capacity, the ability of a DNA repair gene to complement a repair deficient cell line can be investigated. In this work an adenovirus recombinant carrying the T4 den V gene in the deleted E3 region was constructed and subsequently assayed for UV viral survival in several mammalian cell types.

Materials and Methods

1 Eukaryotic Cell Lines

1.1 Strains Used

Human Cell Lines

All GM cell lines were obtained from the Human Genetic Mutant Cell Repository (Copewood and Davis St., Camden N.J., 08103) and CRL cell lines were obtained from the American Type Culture Collection (12301 Parklawn Drive Rockville, Maryland 20852), except where noted.

1. GM 5509 Xeroderma Pigmentosum Group A (XP12BE) - Patient shows multiple skin cancers, nervous system involvement.

2. GM 554B Xeroderma Pigmentosum Group A (XP4LO) - Cells show 2% of normal unscheduled DNA synthesis.

3. GM 677 Xeroderma Pigmentosum Group C (XP2BE) - Also ATCC CRL 1166; Patient shows 19% of normal UV induced unscheduled DNA synthesis.

4. CRL 1204 Xeroderma Pigmentosum Group C (XP10BE).

5. GM 2415 Xeroderma Pigmentosum Group E (XP2RO) - Also ATCC CRL 1259; Patient is clinically affected and has

increased incidence of chromatid gaps following X-ray irradiation.

6. GM 1428 Cockayne's Syndrome Group B (CS7SE) - Fibroblasts have markedly reduced post UV light colony forming ability.

7. GM 1492 Bloom's Syndrome - B.S. Registry #44(AbRu); Cells have increased sister chromatid exchange and chromosome breakage, and also increased post UV irradiation unscheduled DNA synthesis.

8. GM 2803 Apparently Normal Fibroblasts

9. GM 969 Apparently Normal Fibroblasts

10. CRL 1229 Apparently Normal Fibroblasts

11. B. Hanes Apparently Normal Fibroblasts - Obtained from Dr. Pat Chang, Department of Pediatrics, McMaster University Medical Center.

12. S6007 Apparently Normal Fibroblasts - Obtained from Dr. Silvia Bacchetti, Department of Pathology, McMaster University.

13. 293 - Human embryonic kidney cells transformed with the left 11% of Human Adenovirus Type 5 (Graham et al 1977). Obtained from Dr. F.L. Graham, Departments of Biology and Pathology, McMaster University.

14. HeLa - Obtained from Dr. F.L. Graham, Departments of Biology and Pathology, McMaster University.

1.2 Growth and Maintenance of Eukaryotic Cell Lines

1.21 Media

All cell lines and strains were maintained in MEM Alpha medium (Gibco-BRL cat. # 410-2000, Gibco Canada Inc., 2270 Industrial Street Burlington, Ontario, L7P 1A1). Media was supplemented with the following:

1. 7.5% $NaHCO_3$ solution (autoclaved, in double distilled H_2O) in a 1:50 dilution.

2. Antibiotic-Antimycotic solution (penicillin 10,000 μ/ml, fungizone 25 mcg/ml and streptomycin 10,000 mcg/ml) (BRL cat. #600-52401) in a 1:100 dilution.

3. Fetal Bovine Serum (FBS, BRL cat. #200-6140) at 15% concentration in complete media, 0% in straight media.

1.22 Growth of Cells

Cells were grown in 75 cm^2 plastic Falcon flasks (Becton Dickinson and Company, 1950 Williams Dr., Oxnard, CA, 93030, USA) at 100% humidity, 5% CO_2, 37°C. During passaging medium was aspirated off and cells washed with Phosphate Buffered Saline (PBS) (8 g NaCl, 20 g KCl, 11.5 g Na_2HPO_4 and 2 g KH_2PO4 dissolved in double distilled water and autoclaved). After aspiration of the PBS, 3 ml of trypsin (0.25% (BRL cat. #610-5050) 10X stock solution diluted to 1X with PBS) was added and the cells returned to 37°C for 5-10 min. Cell aggregates were disrupted by pipeting, and the appropriate amount of medium added. The suspended cells were then divided and transferred to new flasks (~13 ml per 75 cm^2 flask).

2 Viruses

2.1 Viral Strains

The following are all mutants of Human Adenovirus type 5.

1. Ad 5 dlE309 - Figure 1. (Jones and Shenk 1979)

2. Ad 5 SV40 VSV G (Ad VSV) - Constructed from Ad 5 dlE309 by removal of part of the E3 region between the Xba I sites

at 78.5% and 84.7%, and subsequent insertion of a 2.1 kb fragment containing the glycoprotein G from vesicular stomatitis virus (a generous gift of Dr. L. Prevec, McMaster University, Hamilton, Ontario, manuscript in prep.)

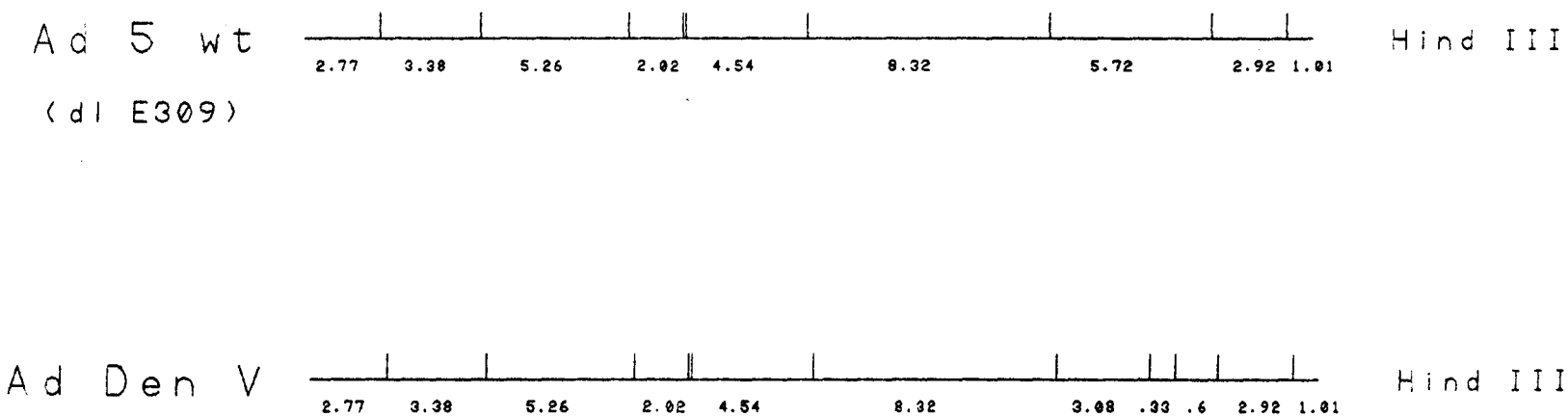
3. Ad Den V - Constructed from Ad 5 dlE309, by removal of part of the E3 region between the Xba I sites at 78.5% and 84.7%, and subsequent insertion of a 1.9 kb fragment containing the Bacteriophage T4 den V gene in an expression cartridge (this study, Figure 1).

2.2 Viral stock preparation

Viral stocks of Ad Den V and Ad VSV were prepared in the following manner. 75 cm^2 flasks of 293 cells were grown to confluency and infected in 1 ml of straight medium for 90 min. after which time 13 ml of complete medium was added. Infection was allowed to proceed until cytopathic effect was observed (~72 hours). Cells were then trypsinized and collected into 50 ml centrifuge tubes (Falcon cat # 2098), spun down for 10 min in an IEC centrifuge (model PR-2, International Equipment Co. (IEC) Limited, Needham Hts., Mass., USA) at 1000 rpm for 10 min. Cells were resuspended in PBS and spun down again. The resulting pellet was resuspended in an appropriate volume of straight medium, and then freeze-thawed 3 times to liberate virus. The cell

Figure 1. Viruses Used

The parental virus used to construct Ad Den V is illustrated showing the Hind III restriction enzyme sites. Distances between sites are in kbp. This can be compared to the Hind III restriction pattern of Ad Den V, shown below.


Ad 5 wt
(dl E309)
2.77
3.38
5.26
2.02
4.54
8.32
5.72
2.92
1.01
Hind III
Ad Den V
2.77
3.38
5.26
2.02
4.54
8.32
3.08
.33
.6
2.92
1.01
Hind III

debris was allowed to settle and the clear supernatant was transferred to freezing vials (Nunc Cryotubes, cat. #3-40711, Nunclon Interned, Denmark) and used as a viral stock for subsequent experiments.

2.3 Titering of Viral Stocks

Ad Den V and Ad VSV were titred for plaque forming units (PFU) on 293 cells. 24 well plates (Nunc, cat. #1-43982) were seeded with 293 cells by trypsinizing one confluent 75 cm^2 flask, resuspending the cells in 24 ml and seeding 2 plates with 0.5 ml per well. The plates were allowed to grow for 24 hrs. before use to allow the cells to reach confluency. Medium was aspirated off the cells and 0.2 ml of straight medium containing varying viral dilutions was added to the wells. Infection was allowed to proceed for 90 min. after which the viral suspension was aspirated off and the cells overlayed with 0.5 % agarose (BRL cat. #5250SA) in 1X F11 medium (Gibco-BRL cat. # 410-1100) which had been mixed at 56°C, and allowed to cool to ~37°C before being added to the cells. After the overlay had set, the plates were returned to the incubator and plaques were visible after 4-6 days. The cells were then fixed by filling the wells with acetone:methanol (1:1) and letting sit for 10 min. The agarose was then removed and the cells 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 min. The cells were then rinsed in cold water, allowed to dry, and then the plaques counted and the titre was determined. A typical titre for Ad den V was 1 x 10^9 PFU/ml and for Ad VSV was 5 x 10^8 PFU/ml. Stocks were also titred for Vag forming units (VFU) on fibroblasts by infecting GM 5509 cells as described under the Survival Assay section. Typical titres obtained were 5 x 10^8 VFU/ml for Ad den V and 5 x 10^6 VFU/ml for Ad VSV.

2.4 Large scale preparation of Viral DNA

Ad 5 dlE309 DNA was prepared for transfection as follows. A 2 litre spinner culture of 293 cells was prepared for infection when the cell density reached 2 x 10^5 cells per ml. This was done by spinning down the culture in 500 ml centrifuge bottles (Nalgene Company, Division of Syborn Corp., Rochester, N.Y. 14602, USA) in an IEC centrifuge (model PR-2, IEC Limited, Needham Hts., Mass., USA) at 1000 rpm for 10 min. and the cells were then resuspended at a cell density of 1 x 10^7 in straight medium. Virus was then added at a multiplicity of infection of 10 PFU/cell. After a 90 min. incubation at 37°C with spinning, the suspension was brought back to the original cell density with complete medium. Cells were allowed to grow and after 4 days inclusion body staining was done to confirm infection.

Inclusion body staining was done by removing 0.1 ml of cells from the suspension culture and then swelling them by the addition of 0.1 ml of 2% sodium citrate and incubation for 1 hour at 37°C. The cells were then spun down, the supernatant aspirated off and the cells fixed by the addition of 1 ml of acetic acid/ethonal (1:3). The cell suspension was then spotted onto a microscope slide and after air drying the cells were covered with 0.1 ml of orcein inclusion body stain (British Drug House, Toronto). Cells were then washed and visualized under a microscope and determined to be infected.

Cells were harvested by centrifugation and resuspended in 10 ml of 0.1 M Tris pH 8.0 with 0.5% Na deoxycholate and incubated on ice for 30 min. The resulting cell suspension was mixed with CsCl (BRL cat. # 5507UB) saturated with 0.1 M Tris pH 7.0 using 1.8 ml CsCl for every 3.2 ml of cell suspension. The mixture was then loaded into Beckman polyallomer Quick-seal tubes (Beckman Instruments, Spinco Division, 1117 California Ave., Palo Alto, CA 94304) and spun at 55,000 rpm in a VTi 60.1 vertical rotor in a Beckman L8M-70 centrifuge at 4°C over night. Virions were collected by puncturing the bottom of the tube and collecting the drips in the region of the visible viral band. The ~1.0 ml sample was dialyzed by placing the suspension in a cellulose dialysis tube, preboiled in double distilled water, and tied off at either end. The tube was placed in 4 l of ice cold

TE (10 mM Tris HCl pH 7.8, 1 mM EDTA pH 8.0) and left spinning slowly over night. The next morning the sample was removed from the bag and mixed with 1.0 ml of 2X protease buffer. Protease (Boehringer Mannheim Canada, Dorval Quebec, H9P 1A9, cat. # 165921) was added at a concentration of 25 mg/ml and the sample incubated over night at 37°C. The sample was then split into 400 µl aliquots and phenol extracted by addition of 400 µl phenol, spun in 1.5 ml eppendorf tubes (Bio-Rad cat. # 2239501) in an eppendorf centrifuge for 10 min. and the upper aqueous phase removed. This was repeated with phenol-chloroform (1:1) and then chloroform. The resulting 400 µl aqueous phase was precipitated by the addition of 200 µl 7.5 M NH_4 acetate and 800 µl of 95% ethanol, chilling at -70°C for 20 min. and centrifugation in an eppendorf centrifuge for 20 min. The resulting DNA pellet was washed with 70% ethanol and resuspended in double distilled autoclaved water at a concentration of ~1.0 µg/µl.

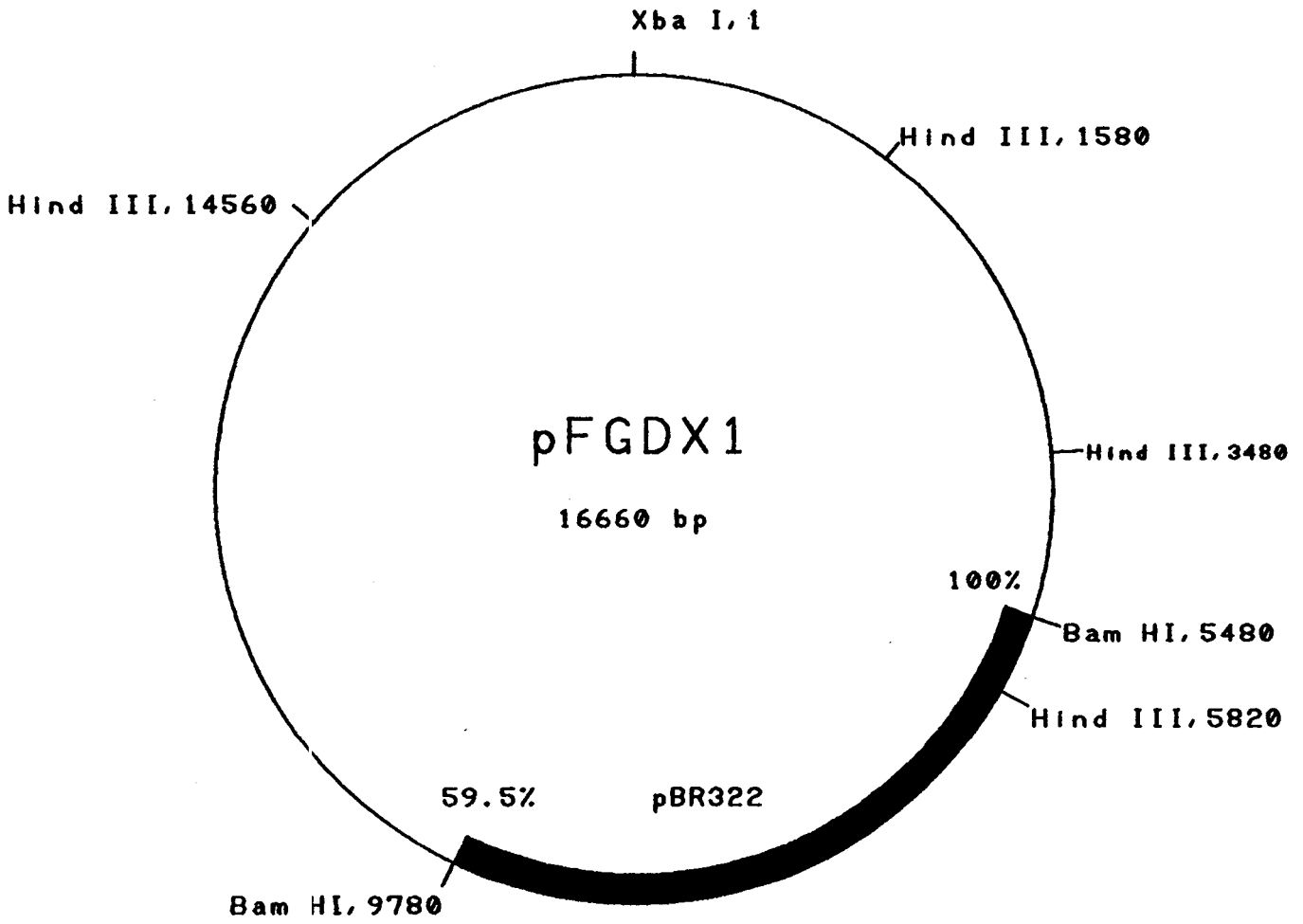
3 DNA Constructs

3.1 Plasmids

1. pFGDX1 - Figure 2. Consists of the right half of Ad 5 dlE309 from the Bam HI site at 59.1%, inserted into the Bam H1 site of pBR322. The Xba I D fragment (78.5%- 84.7%) has

Figure 2. Plasmid pFGDX1.

Plasmid pFGDX1 was constructed by Yosef Haj-Ahmad, Department of Biology, McMaster University, Hamilton, Ont.It consists of the right most 40.5% of wt Ad 5 (dl E309) which was obtained from a Bam HI digest, which was subsequently inserted into the Bam HI site of pBR322. The plasmid also contains a partial deletion in the E3 region, created by digestion with Xba I and subsequent re-ligation. This allows insertion of foreign DNA into the Xba I site and subsequent transfer by co-transfection and recombination, to the adenovirus genome.


Xba I, 1
Hind III, 1580
Hind III, 14560
pFGDX1
16660 bp
Hind III, 3480
100%
Bam HI, 5480
Hind III, 5820
59.5%
pBR322
Bam HI, 9780

been deleted by digestion and religation (Haj-Ahmad and Graham 1986). Plasmid DNA was kindly supplied by Dr. F.L. Graham, Department of Biology and Pathology, McMaster University.

2. pEMBL18/pRSVdenv - Figure 3. Constructed by the insertion of the Den V cartridge (as described later) into pEMBL18 (Dente et al 1983).

3. pMCADV - Figure 4. Constructed by insertion of the Den V cartridge into the Xba I site of pFGDX1 (this study).

3.2 Den V Expression Cartridge

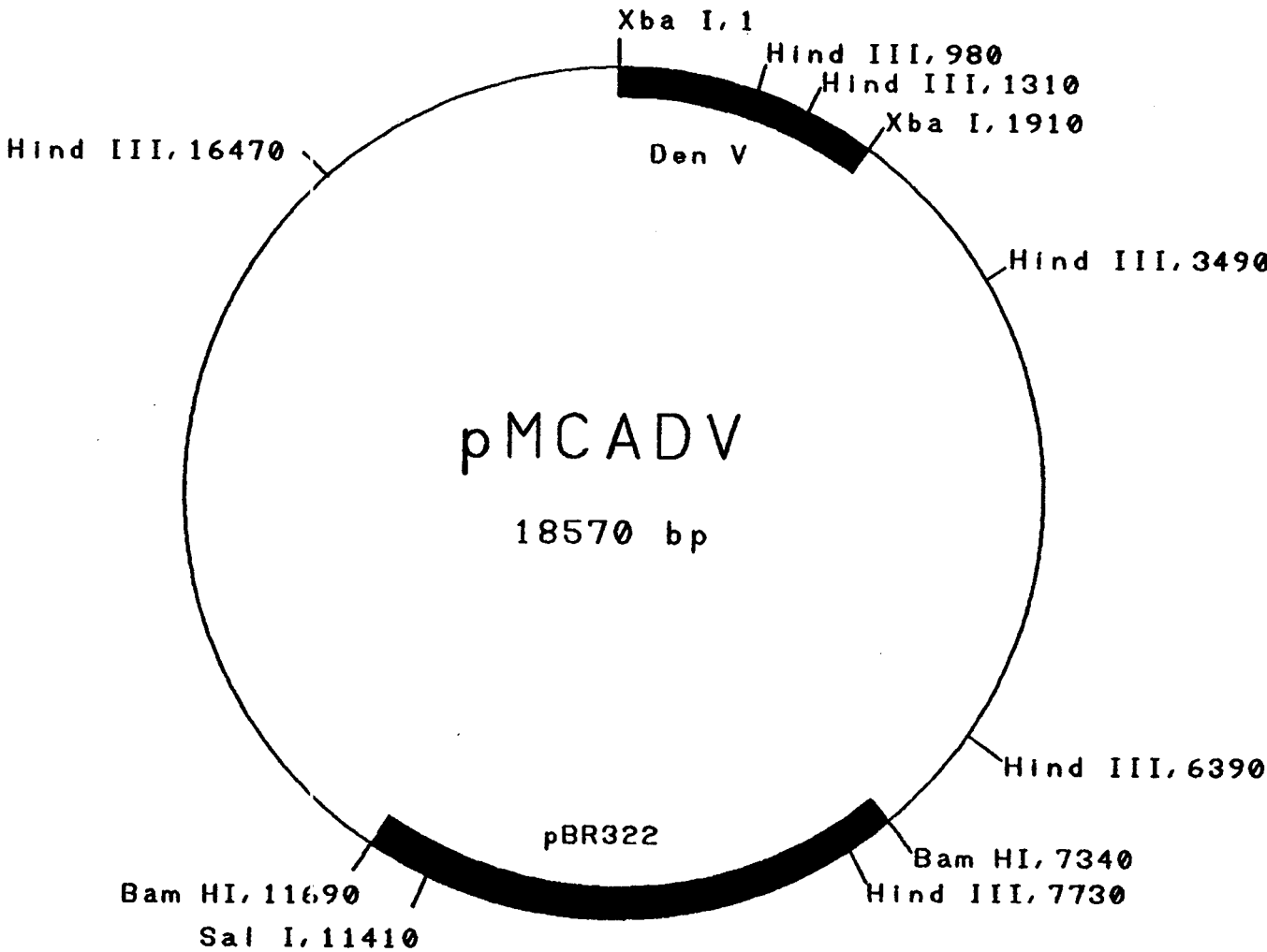
The Den V cartridge was assembled by Dr. Kristoffer Valerie, Department of Molecular Genetics, Smith Kline and French Laboratories, Swedeland, PA 19457. A 524 bp Pvu II - Bst N1 fragment from the 3' LTR of Rous Sarcoma Virus (Gorman et al 1982) was klenowed and ligated to Hind III linkers. It was then digested with Pvu II and Hind III, klenowed and cloned into the klenowed Acc I site of pBR322. An Nde I - Hind III fragment containing the entire RSV sequence plus 50 bp of pBR322 upstream from the Acc I insertion point, followed by 6 bp from the Hind III - Sph I linker of pEMBL19 was blunt end ligated to the Hinf I fragment of T4 phage containing the den V gene (Valerie et al 1985a) which had Cla I linkers

Figure 3. Plasmid pEMBL18/RSVdenV.

Plasmid pEMBL18/RSVdenV was recieved from Dr. Earl Henderson, Temple University School of Medicine, Department of Microbiology and Immunology, Philadelphia, Pennsylvania. It was constructed by extracting the den V expression cartridge from pRSVdenV, liberated by digestion with Nde I and Bam HI, treatment of the fragment with Klenow, ligation to Xba I linkers, and insertion into the Xba I site of pEMBL18 (Dente et al, 1983).

Figure 4. Plasmid pMCADV.

Plasmid pMCADV was constructed in this study to deliver the den V expression cartridge into the E3 region of Ad 5. It consists of plasmid pFGDX1 with the den V expression cartridge inserted into the deleted region between the Xba I sites at 78.5% and 84.7%. The cartridge is inserted with the direction of transcription from the RSV promoter being from right to left with respect to the adenovirus genome.


Xba I, 1
Hind III, 980
Hind III, 1310
Xba I, 1910
Den V
Hind III, 3490
Hind III, 6390
Bam HI, 7340
Hind III, 7730
pBR322
Bam HI, 11690
Sal I, 11410
Hind III, 16470
pMCADV
18570 bp

added which were also filled in. After the den V gene, 11 bp from the Acc I - Bam HI linker of pSP65 (Promega Biotec, Madison, WI) were added and the entire cartridge was inserted into the Bam HI - Nde I fragment of pSV2dhfr (Subramani et al 1981). This resulted in the den V gene being followed by the SV40 small t Ag intron and Large T poly A splice site (Figure 5) (Valerie et al 1987a).

4 Recombinant DNA Techniques

4.1 Restriction Digests

DNA to be digested was brought to a volume of 17 µl with double distilled autoclaved water in a 1.5 ml eppendorf tube. To this was added 2 µl of the appropriate BRL 10X React buffer and then 10 units of the restriction enzyme. The digest was performed at 37°C for 2-4 hours.

4.2 Gel Electrophoresis

4.21 Agarose gel electrophoresis

Agarose gel electrophoresis was used to determine the size of DNA fragments that ranged from 300 - 20,000 bp. Horizontal gels of 0.8% agarose (BRL cat. # 5510 UB) were poured and run in TBE (0.089 M Tris base, 0.089 M boric acid

Figure 5. The Den V Expression Cartridge.

The expression cartridge containing the den V gene from bacteriophage T4 consists of the following elements. A 55 base pair fragment from pBR322 is followed by 524 base pairs from the 3′ LTR of Rous Sarcoma Virus. The TATA box of the RSV promoter can be found at position 510 in the cartridge sequence, and the reported start of transcription at position 545. The den V coding sequence begins at the start codon at position 591, and the TAA opal stop is found at position 1006. After translation termination, there is part of the SV40 small t antigen intron and then the large T poly A splice site, derived from the pSV2 plasmid series. The cartridge used in construction of all plasmids here had the addition of Xba I linker on both ends (not listed in sequence).

```
  Nde I                                                          Acc I 2-||->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 TAGGCTGTGT TTAGGCGAAA AGCGGGGCTT CGGTTGTACG CGGTTAGGAG TCCCCTCAGG
GACTCCCCTG ATCCGACACA AATCCGCTTT TCGCCCCGAA GCCAACATGC GCCAATCCTC AGGGGAGTCC

       290        300        310        320        330        340        350
ATATAGTAGT TTCGCTTTTG CATAGGGAGG GGGAAATGTA GTCTTATGCA ATACACTTGT AGTCTTGCAA
TATATCATCA AAGCGAAAAC GTATCCCTCC CCCTTTACAT CAGAATACGT TATGTGAACA TCAGAACGTT

       360        370        380        390        400        410        420
CATGGTAACG ATGACTTAGC AACATGCCTT ACAAGGAGAG AAAAAGCACC GTGCATGCCG ATTGGTGGAA
GTACCATTGC TACTGAATCG 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
```

```
                                                                  Hinf I Cla Link
      990        1000       1010       1020       1030       1040  **  1050
ATACTACGGT AAGGCGATTT ATGCATAAGG GAACAACCTG GACCTCATGA TTATATGAGG GATTCATCGC
TATGATGCCA TTCCGCTAAA TACGTATTCC CTTGTTGGAC CTGGAGTACT AATATACTCC CTAAGTAGCG
     Bam 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 GAATGCCTTT AATGAGGAAA
ACACATAAAA TCTAAGGTTG GATACCTTGA CTACTTACCC TCGTCACCAC CTTACGGAAA TTACTCCTTT

      1270       1280       1290       1300       1310       1320       1330
ACCTGTTTTG CTCAGAAGAA ATGCCATCTA GTGATGATGA 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 TTTGCTATTT ACACCACAAA GGAAAAAGCT 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
                  *Bam HI
```

and 0.002 M EDTA pH 8.0). A 1:10 dilution of sample loading buffer (0.25% bromophenol blue in 40% w/v sucrose in water) was added to DNA samples from restriction digests, and the samples loaded into the wells. Gels were run at 70 volts overnight. Lambda DNA (BRL cat. #5250SA) was digested with Hind III and used as a molecular weight marker in all gels. Included in the gel was 0.04 mg-ml ethidium bromide, and the gels were photographed subsequent to running using Polaroid 57 film (Hall Photographic, Hamilton, Ont.)

4.22 SDS-PAGE

Proteins were analyzed using SDS-polyacrylamide gel electrophoresis. Gels consisted of the following:

18.75 ml 30% acrylamide
14.25 ml 1 M Tris pH 8.8
3.75 ml D.D. water
Degas 15 min.
Add 0.2 ml 20% SDS
0.15 ml 10% ammonium persulphate
0.030 ml TEMED

The running gel was poured and allowed to set for 1 hour. On top of this a 3 cm stacking gel was poured, into which the comb was inserted. The stacking gel consisted of the

following:

1.67 ml 30% acrylamide

1.25 ml 1 M Tris pH 6.8

6.7 ml D.D. water

Degas 15 min.

Add 0.2 ml 20% SDS

0.05 ml 10% ammonium persulphate

0.020 ml TEMED

After the gel had set it was loaded into a vertical gel box and pre-run in running buffer (6.32 g Tris base, 4.0 g Glycine, 1.0 g SDS in 1 litre). Samples were diluted in 1 volume of 2x protein loading buffer which was prepared as follows:

4 ml H_2O

0.15 g Tris base

pH to 6.8 with HCl

Add

4 ml 10 SDS

1 ml glycerol

20 mg bromophenol blue (0.02%)

0.4 ml B-mercaptoethanol (4%)

bring volume to 10 ml

The samples were boiled for 5 min., cooled on ice and loaded into the wells. Gels were run at 70 volts for 12 hours. After running, the plates were separated and the gel processed as required.

4.23 Sequencing gels

5% sequencing gels were used to accurately determine the length of primer extended oligonucleotides. Acrylamide solution consisted of

53 ml H_2O
48 g urea
4.75 g acrylamide
0.25 g bisacrylamide

Mix until in solution
Add
5 ml 20x TBE

Filter through 0.22 micron Nalgene filter
Degas 5 min.

Plates were prepared as follows:

1. Both plates were washed with soap and water, rinsed with

distilled water, and left to air dry.

2. The long plate was coated with 5 ml of 4% repelsilane (dimethyldichlorosilane, Sigma cat. # D 3879) in 1,1,1 trichloroethane and left to air dry. This was repeated 2 times, polishing with Kimwipes (Kimberly Clark of Canada Ltd. Toronto, Ont.) and 95% ethanol between applications.

3. The procedure was repeated with the short plate, except using 5% bindsilane (3-(trimethoxysilyl)propyl methacrylate, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin 53233 USA, cat. # 23,579-2) in 95% ethanol.

4. The plates and spacers were assembled, and the gel sealed with tape.

5. To 60 ml of acrylamide solution, 50 µl TEMED and 1 ml of 10% ammonium persulphate were added.

6. The gel was poured and the comb inserted.

After setting, the gel was assembled into the apparatus and pre-run at 1800 volts for 1 hour in 1x TBE prior to loading the samples. To each lane 3 µl of boiled sample in sequencing gel loading buffer was added using a glass capillary tube, which had been elongated by heating. The

gel was run for 2-4 hours at 2000 volts, after which time the plates were allowed to cool, separated, and the gel fixed onto the short plate with 50% methanol, 20 % acetic acid in water. Gels were then rinsed with D.D. H_2O and allowed to dry overnight. The next day the gel was exposed to OMAT-AR film and the image visualized within 1 to 4 days.

4.3 Gel Isolation

4.31 Agarose gels

DNA was isolated from agarose gels by the following method:

1. A 0.8% gel was run without ethidium bromide, covered with UV protective plastic. A marker lane and one sample lane was run down one side of the gel, away from the lanes to be isolated.

2. At end of run, a scalpel was used to cut off the marker and one sample lane. This strip was stained by soaking in 0.1 % ethidium bromide for 30 min.

3. The strip was visualized under UV light and marked at the position of the band to be isolated.

4. The band was then cut from the unstained portion of the

gel.

5. Agarose containing isolated DNA was placed in an appropriate size syringe and ejected through an 18 gauge needle. The procedure was repeated until the agarose was disrupted.

6. An equal volume of phenol was added, vortexed extensively and frozen at -70°C for one hour.

7. The sample was placed frozen into an eppendorf centrifuge and spun for 30 min.

8. The upper, aqueous phase was removed and an equal volume of chloroform added to it.

9. After brief centrifugation, the upper, aqueous phase was removed and precipitated by addition of one half volume of 7.5 M ammonium acetate and 2 volumes of 95% ethanol. After centrifugation, the resulting DNA pellet was dried and resuspended as required. No detectable loss of DNA or degradation was observed.

4.32 Polyacrylamide Gels

1. After visualization, the desired band was cut from gel

and mashed in an eppendorf tube.

2. 0.6 ml of elution buffer (500 mM NH_4 acetate, 10 mM MgO acetate, 1 mM EDTA, 1% SDS) was added and the sample incubated overnight at 37°C.

4. The sample was then passed over siliconized glass wool to remove all residual acrylamide and precipitated as in agarose gel isolation.

4.4 Cloning Techniques

4.41 Ligation

Prior to ligation, all DNA used was passed through a G-50 (Pharmacia Fine Chemicals, P.O. Box 175, S-751 04 UPPSALA 1, Sweden, cat. # 17-0043-01) column (Maniatis et al 1982) to remove contaminants and salts. Ligation reactions were carried out using 3 units T4 DNA ligase (Pharmacia, cat. # 27-0870-01), in 30 μl total volume of ligation buffer (300 mM Tris pH 7.4, 100 mM MgCl2, 100mM DTT, 10 mM ATP), with an equal molar amount of DNA ends. The ligation was left at 14°C overnight.

4.42 Transformation of Bacteria

Ligation products or prepared plasmids were used to transform bacteria using the calcium chloride technique (Maniatis et al, 1982). 100 ml of Luria Broth (10 g Bacto-tryptone (Difco Laboratories, Detroit, Michigan, cat. # 0123-01), 5 g Bacto-yeast extract (Difco cat. # 0127-01), 10 g NaCl) was inoculated with an overnight culture of HB101 bacteria and allowed to grow at 37°C with constant agitation for 2-4 hours, until the O.D. was ~0.5. The cells were chilled on ice for 10 min. and then spun down in an IEC model PR-2 centrifuge at 1,500 rpm for 10 min. in 50 ml Falcon centrifuge tubes (Falcon cat. #2098). The cells were resuspended in 1/15th the original volume in an ice cold sterile solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl pH 8.0. DNA was added to 0.2 ml of cells in a 1.5 ml eppendorf tube and set on ice for 1 hour with occasional agitation. The cells were then heat shocked at 42°C for 2 min. and then returned to ice. One ml of LB was added and the cells incubated at 37°C for 30 min. Cells were subsequently plated on LB plates (LB containing 15 g agar per litre (Difco cat. #0140-01, Difco Labs Detroit Mich. USA), in 100 mm petri dishes (Fisher cat. # 8-757-12, Fisher Scientific PO Box 9200 Terminal Ottawa, Ont. K1G 4A9), with 50 μg/ml ampicillin (Sigma cat. #A-9518, Sigma Chemical Company, PO Box 14508, St. Louis, Mo., 63178, USA)).

4.43 Screening of transformants

Ampicillin resistant colonies obtained from transfection were subsequently screened for plasmid DNA. A sterile wooden stick was used to pick the colony, and it was transferred to 5 ml of LB with 50 μg/ml of ampicillin in a 15 ml plastic snap cap tube (Falcon cat. #2057). The tubes were incubated overnight at 37°C with constant agitation. 1.5 ml of the culture was transferred to an eppendorf tube, spun down for 3 min. in an eppendorf centrifuge at room temperature. After the supernatant was aspirated off, the pellet was resuspended in 100 μl of lysozyme solution (25 mM Tris HCl pH 8.0, 50 mM glucose, 10 mM EDTA and 5 mg lysozyme (Sigma cat. #L-6876)) was added and placed on ice for 10 min. 200 μl of freshly made alkali-SDS (0.2 N NaOH, 1% SDS) was then added to the tubes and they were incubated for a further 20 min. Cell debris was precipitated by the addition of 150 μl NaOH pH 5.2 and a further incubation of 1 hour. The samples were spun for 20 min and the supernatant collected and transferred to a new tube. DNA was precipitated by the addition of 1.0 ml of ice cold 95% ethanol and incubation at -70°C for 10 min. The pellet was then spun for 15 min., the supernatant aspirated off and the pellet dried and resuspended in 50 μl of D.D. autoclaved water. Restriction enzyme digestion and agarose gel electrophoresis was then employed to determine the nature of the resident plasmid.

4.44 Large Scale Plasmid Preparation

Large scale preparations of selected clones was performed as described in Maniatis et al 1982, using the Lysis by SDS method. A 500 ml culture of the plasmid bearing bacteria was grown in LB with 75 μg/ml of ampicillin. The cells were spun down in a Beckman centrifuge (model J-21C, JA-10 rotor) at 6,000 rpm for 10 min. and the cells resuspended in 10 ml of 10% sucrose in 50 mM Tris HCl pH 8.0. The suspension was transferred to Oak Ridge tubes and placed on ice. 2 ml of freshly made lysozyme (10 mg/ml in 0.25 M Tris HCl pH 8.0) was added, followed by 8 ml of 0.25 M EDTA. The cells were incubated on ice for 10 min. after which 4 ml of 10 % SDS was added and quickly mixed in. 6.0 ml of 5 M NaCl was then added and the mixture incubated for a further hour. The resulting suspension was spun in a Ti 50 rotor in a Beckman L8M-70 centrifuge at 40°C for 30 min. at 30,000 rpm. The clear supernatant was removed and extracted twice with phenol-chloroform. The DNA was then precipitated with 2 volumes of ethanol and resuspended in 8.0 ml of d.d. H_2O. The DNA was then banded by cesium chloride centrifugation. This was done by first adding one gram of CsCl for each ml of DNA solution and 0.8 ml ethidium bromide (10 mg/ml in H_2O) for each ml of CsCl solution, and then transferring the mixture to a Beckman Quick seal ultracentrifuge tube. The tubes were sealed and spun in a VTi 65 rotor in a Beckman

L8M-70 ultracentrifuge at 55,000 rpm overnight. The lower, plasmid containing band was visualized in the tube by use of a hand held UV source (Fisher Scientific Canada Ltd., model UVS-11), and the band removed by puncturing the tube with an 18 gauge needle and a 1.0 ml syringe. Ethidium bromide was removed from the extracted band by several extractions with 1-butanol, and the DNA precipitated by addition of 3 volumes of H_2O, and then two volumes of 95% ethanol. Plasmid DNA was resuspended in 100 μl of H_2O and the concentration determined by use of a Beckman DU-7 Spectrophotometer.

4.5 DNA Mediated transfection of Eukaryotic cells.

Construction of virus was carried out using the technique of DNA mediated transfection of 293 cells. Plasmid and viral DNA was cleaved with the appropriate restriction enzyme and an aliquot run on an agarose gel to confirm total digestion. Either 5 or 10 μg of each DNA were mixed in all combinations and resuspended in sterile d.d. H_2O. Beforehand, 60 mm diameter cell culture dishes (Falcon cat. # 3002) were seeded with 293 cells and allowed to grow overnight to confluency. The following solution was mixed together, for each transfection:

0.23 ml sterile d.d. H_2O

0.25 ml 500 mM $CaCl_2$

0.02 ml DNA suspension

After mixing, the samples were transferred to sterile 15 ml Corning tubes (Corning Glassworks, Corning N.Y. 14831, cat. # 25319) and aerated with a 1.0 ml pipet connected to a Pipetaid (Drummond Scientific Company, Broomall, P.A.). To the mixture 0.5 ml of 2x PBS-HEPES (16 g NaCl, 0.74 gm KCl, 0.24 g anhydrous Na2HPO4, 2.0 g dextrose, 10.0 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma cat. # H 3375) in 1 litre, pH to 7.15 with NaOH) was added drop wise. A precipitate was allowed to form over 45 min. at room temperature. The medium was aspirated off of the cells and replaced with 1.0 ml of straight medium and then the precipitate was added to the cells, drop wise and evenly dispersed over the entire dish. The cells were then incubated at 37°C for 6 hours, after which time the medium and precipitate was aspirated off and overlayed with 0.5% agarose/F11, as was done in the viral titering procedure. Plaques appeared after 5 days and were picked and screened to analyze viral DNA structure. Screening was done by reinfecting 24 well plates in duplicate, one plate to be kept as a viral stock, the other for viral DNA analysis.

Viral DNA was extracted by first aspirating off the medium and covering the cells with 0.5 ml of protease buffer (0.01M Tris pH 7.8, 0.01 M EDTA, 0.5% SDS). Protease was first prepared by self digestion for 2 hours at 37°C, and then stored at -20°C at a concentration of 20 mg/ml. This was added to the cell extracts to a final concentration of

1 mg/ml and incubated at 37°C overnight. The extract was then phenol extracted, phenol chloroform extracted and finally chloroform extracted to remove cell debris. The resulting aqueous phase was ethanol precipitated and resuspended in 50 μl of d.d. autoclaved H_2O and used for restriction enzyme digest analysis.

4.6 Labelling of DNA

4.61 Nick translation

Both gel extracted fragments and plasmid DNA were labelled using the BRL standard nick translation kit (BRL cat. # 8160SB) and the supplied protocol. α^{32}P dCTP (ICN cat. # 33004X, ICN Canada Ltd. 2485 Guennette St. Lament, Que H4R 2H2) was used as the radioactive isotope and consistently produced probes of ~1 x 10^9 cpm/μg. Unincorporated nucleotides were removed by passage through a G-50 spun column (Maniatis et al, 1982).

4.62 Kinasing

T4 polynucleotide kinasing of 5' ends of DNA oligonucliotides was used in the production of probes for the primer extension assay. The cleaned DNA was heated to 90°C for 2 min. then mixed with 10 units of T4 polynucleotide kinase (Pharmacia

cat. # 27-0734-01), 50 μCi of γ^{32}P ATP in kinase buffer (0.05M Tris HCl pH 7.6, 0.01 M $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) and incubated at 37°C for 1 hour. The labelled fragment was then precipitated with ammonium acetate, resuspended in 0.3 M Na acetate and precipitated, washed 2 times with 70% ethanol and then washed 2 times with 100% ethanol. The resulting pellet was resuspended in d.d. autoclaved H_2O as required.

4.7 Southern Blotting

Southern blotting of DNA run on agarose gels was carried out as described by Maniatis et al, 1982. After electrophoresis, the gel was photographed and UV irradiated on the UV light box (Fotodyne Incorporated, New Berlin, WI USA, model 3-3000) for ~5 min. The gel was then soaked in 1.5 M NaCl and 0.5 M NaOH for 1 hour to denature the DNA. The gel was then neutralized in 1.5 M NaCl and 1.0 M Tris HCl pH 8.0 for one hour. DNA was transferred to nitrocellulose (Mandel cat #53504-416, Mandel Scientific Company, 143 Dennis SAt. Rockwood, Ontario, N0B 2K0) by use of an SSC (175.3 g NaCl, 88.2 g Na citrate pH 7.6, for 20x) gradient (20x - 2x). After transfer the nitrocellulose was soaked briefly in 6x SSC and baked for 2 hours at 80°C. Hybridization was performed by first wetting the nitrocellulose in 6x SSC and then transferring it to a plastic bag containing 10 ml of

hybridization fluid (6x SSC, 0.5% SDS, 5x Denhardt's solution (5g Ficoll (Pharmacia cat. # 17-0400-01), 5 g polyvinylpyrrolidone (Sigma cat. # P 5288), 5 g BSA (Pentax Fraction V, Sigma cat. # A 6793) and water to 500 ml for 50x, and 100 mg/ml denatured salmon sperm DNA (Sigma cat. # D 5139)). Blots were left to pre-hybridize overnight at 45°C, after which the prepared probe was boiled and added to the hybridization fluid. Hybridization was allowed to proceed for ~12 hours, after which the nitrocellulose was removed from the bag and transferred to a tray containing 2x SSC and 0.5% SDS for 5 min. at room temperature. The nitrocellulose was then transferred to a tray containing 2x SSC and 0.1% SDS and incubated with agitation at room temperature for 15 min. The final wash was done at 68°C in a solution of 0.1 % SSC and 0.5% SDS for 3 hours, after which the solution was replaced and allowed to continue washing for an additional hour. The blot was then briefly dried on a piece of Whatman 3mmChr paper at room temperature and then wrapped in saran wrap and exposed to film for the required length of time.

5 RNA Techniques

5.1 RNA Isolation

Confluent monolayers of HeLa cells were grown in 75 cm^2 flasks and infected at a multiplicity of ~30 PFU/cell as

previously described. The cells were then washed with PBS and treated with guanidinium Isothiocynate solution (4M guanidinium isothiocynate (Sigma cat. # G 6639), 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH to 7.0 with NaOH, all reagents and containers RNAse free) at a concentration of 7.5 ml per 100 million cells. The resulting lysate was applied to 2.5 ml of 5.7 M CsCl in a DEPC (diethyl pyrocarbonate, Aldrich cat. # 15,922-0) treated centrifuge tube (boil polyallomer tubes for 10 min. in 0.05% DEPC) per 7.5 ml cell lysate. Tubes were balanced and spun in a SW40 Ti swinging bucket rotor in a Beckman L8M-70 ultra centrifuge at 27,000 rpm overnight at 20°C. Upon removal from buckets, all liquid was removed using an RNAse free pasteur pipet and the inside of the tube dried using a Kimwipe, with care being taken not to touch the RNA pellet at the bottom. The RNA was resuspended in 100 μl 0.5% SDS in sterile d.d. autoclaved RNAse free H_2O and heated to 55°C for 5 min. to fully resuspend all RNA. The sample was spun in an eppendorf centrifuge for 5 min. and the supernatant transferred to a fresh tube. The RNA was precipitated by addition of 40 μl 2 M, pH 5.5, filter sterilized potassium acetate, 260 μl sterile d.d. autoclaved H_2O and 1.0 ml absolute ethanol and incubation at -70°C overnight. The samples were then spun down for 15 min. at 4°C in an eppendorf centrifuge, aspirated, washed with 70% ethanol and dried. The pellets were then resuspended in an appropriate amount of d.d.

autoclaved RNAse free H_2O. Typical yield from the infection of four 75cm^2 was approximately 300 μg of total cellular RNA.

5.2 Primer Extension of RNA

Initiation of transcription of the den V cartridge was determined by primer extension (Jones et al 1985) of RNA harvested from Ad Den V infected cells. RNA was isolated from HeLa cells at both 1 and 22 hours post infection with Ad Den V virus at a multiplicity of approximately 30 PFU per cell as previously described. The RNA was then dehydrated by centrifugation in an eppendorf centrifuge in open top tubes. Approximately 30 μg of RNA was resuspended in 14 μl of 10mM Tris-HCl pH 7.9, 1 mM EDTA which contained approximately 2 million cpm (Cerenkov counts) of kinased primer. The primer used was made by Dinsdale Gooden of the Institute of Molecular Biology and Technology, McMaster University, and corresponded to the complementary sequence from position 669 to 645 in the den V expression cartridge (Figure 5). The sequence (5'-CGCGGCAATTCACGATATTCAGCC-3') was kinased as described previously, and resuspended in an appropriate amount of buffer to give the desired 2 million cpm per 14 μl. Once the RNA was fully resuspended, 4 μl of 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 1.25 M KCl was added. The primer and RNA was allowed to hybridize for one hour at 60°C, then allowed to cool to room temperature. 50 μl of 20 mM

Tris-HCl pH 8.7, 10 mM $MgCl_2$, 5 mM DTT, 0.33 mM of each dNTP, and 10 μg/ml actinomycin D (Sigma cat. # A 1410) with 20 units of AMV (Avian Myeloblastosis Virus) reverse transcriptase (BRL cat. # 8020SB) was then added. After a one hour incubation at 37°C, the sample was ethanol precipitated, washed and run on a 5% sequencing gel.

6 Protein Techniques

6.1 Protein Isolation

Proteins were isolated from eukaryotic cell monolayers. 75 cm^2 flasks were trypsinized and the cells collected into 1.5 ml eppendorf centrifuge tubes. They were then spun down by briefly pulsing the eppendorf centrifuge for a total time of approximately 3 min. The supernatant was then aspirated off and the cells resuspended in PBS and spun down. The cells were then resuspended in 400 μl of RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40 (Sigma cat. # N 6507), 0.5% DOC (deoxycholic acid, Sigma cat. # D 6750), 0.1% SDS) and incubated on ice for 10 min. The suspension was then sonicated with two 30 second bursts, with 30 seconds cooling time between bursts. The resulting solution was spun in an eppendorf centrifuge for 15 min. to remove cell debris and the supernatant transferred to a new tube. The protein was then used within 12 hours, during which time it was stored

at 4°C.

6.2 Immunoprecipitation

For immunoprecipitations, 60 mm cell culture dishes were seeded the night before use and allowed to resume active growth. They were infected with virus at a multiplicity of 20 PFU per cell in 0.5 ml straight medium for 2 hours and then overlayed with 4 ml complete medium. At the desired time of labeling the medium was aspirated off and replaced with 5 ml of 199 -met methionine minus medium containing 50 μCi ^{35}S methionine (ICN). After continued incubation for ~3 hours, the medium was aspirated off and the cells washed with PBS and the proteins extracted. 5 μl of the appropriate Ab was added to 400 μl of the protein extract and incubated at 4°C with constant rotation for ~3 hours. 100 μl of Protein A Sephadex beads (0.5 g protein A sephadex (Pharmacia cat. # 17-0780-01), 20 ml RIPA, mixed with rotation for 2 hours) was then added and incubation continued for 1 hour. The samples were then spun in an eppendorf centrifuge for 5 min. and the supernatant removed by aspiration. The beads were washed by resuspension in 1.0 ml of RIPA and precipitation 5 times. The final precipitation was resuspended in 50 μl of 2x protein loading buffer and boiled for 5 min. 30 μl of the supernatant was loaded in a SDS-PAGE gel and run for 18 hours at 70 volts. After the run the gel was removed from

the plates and DMSO (dimethylsulphoxide) treated in 250 ml for 30 min. with constant rotation in a plastic tray and was then repeated for 1 hour with fresh DMSO. The gel was then soaked in 20% PPO (2,5-diphenyloxazole)-DMSO for 3 hours, following which the gel was washed in running distilled waster for 1 hour. The gel was then dried for 6 hours in a heated gel drier and then exposed to film overnight.

6.3 Western Blotting

Extracted, unlabeled proteins were western blotted by first running them on a SDS-PAGE gel for 18 hours at 70 volts. The gel was then removed from the plates and equilibrated in transfer buffer (25 mM Tris pH 8.3, 192 mM Glycine, 20% Methanol v/v) for 5 min. The gel was then loaded into a transblot apparatus (Bio-Rad Laboratories, Richmond CA 94804) as recommended by the manufacturer. The proteins were transferred to nitrocellulose overnight at 250 m amps at 4°C. The following day the nitrocellulose was removed from the Transblot and the marker lanes cut off and stained with amido black (0.2% in 7% acetic acid). The remaining blot was blocked in blotto buffer (5% Carnation Skim Milk Powder w/v in PBS) in a glass dish for 4 hours at room temperature. This and all subsequent procedures were done with constant agitation. The blocking buffer was then drained and the primary Ab applied in blotto buffer (20 ml with 30-300 μl of

the appropriate Ab) and incubated for an additional 2 hours. The Ab was drained and the blot washed with 4 changes of 0.05% Tween-20 (Sigma cat. # P 1379) in PBS over 30 min. The secondary Ab (goat anti rabbit conjugated to alkaline phosphatase, NCI cat. # 61-275-1, NCI Biomedicals Inc., Costa Mesa, CA 92626, when primary Ab was polyclonal, rabbit anti mouse when monoclonal, NCI cat # 65-125-1) was then applied in 20 ml of blotto buffer and allowed to react for 1 hour, after which the blot was washed as described. When the primary Ab was a monoclonal an additional reaction with the alkaline phosphatase conjugate Ab was performed. After the final wash the nitrocellulose was washed twice in borate buffer for 5 min. The blot was then developed in processing buffer (0.1 M Tris pH 9.2, 0.1% NBT (nitroblue tetrazolium, Sigma cat. # N 6876), 5 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma cat. # B 8503), 2 M $MgCl_2$) for ~1 hour in the dark.

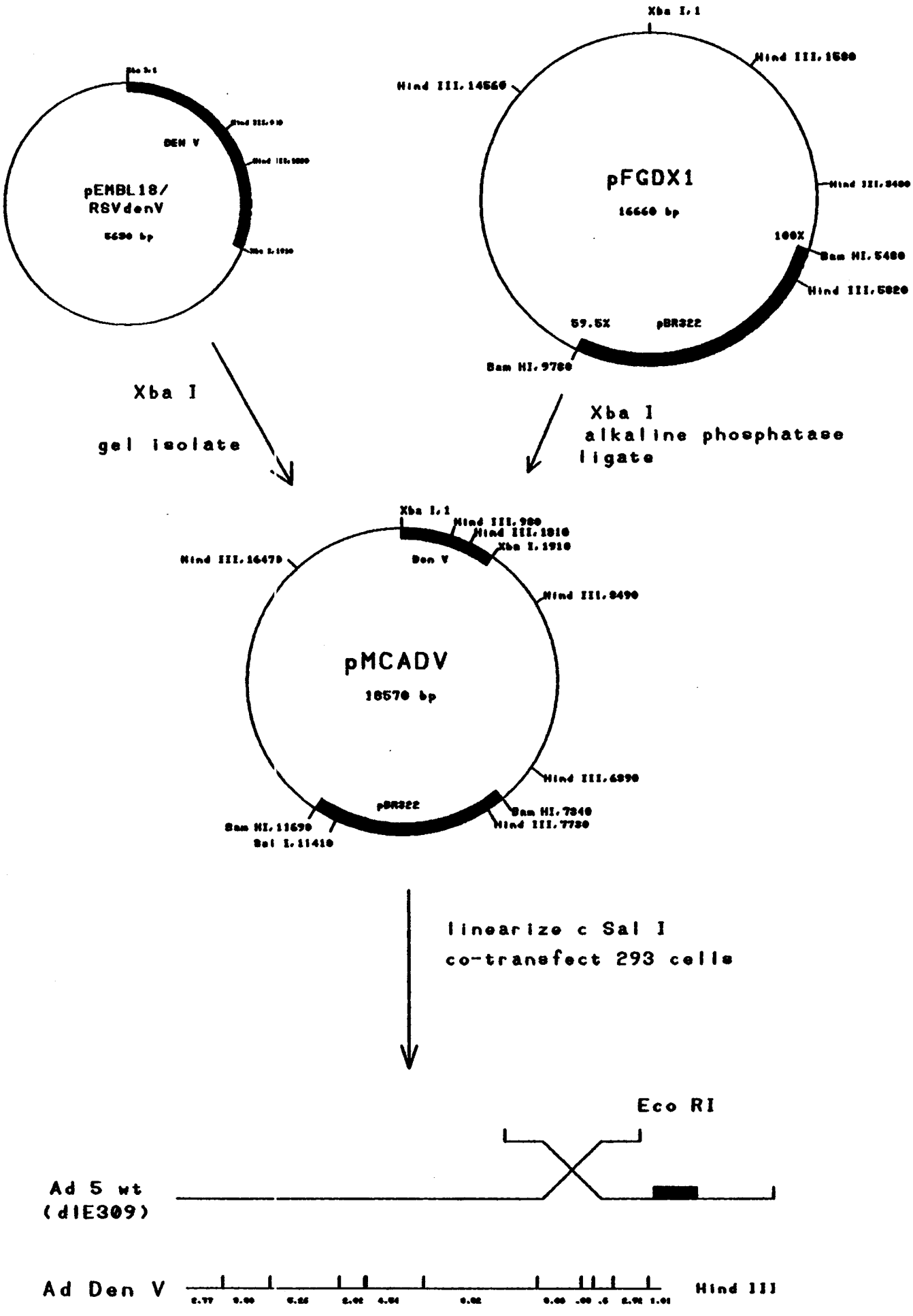
7 Virus Construction Strategy

The den V gene was obtained from plasmid pEMBL18/pRSVdenV by digestion with Xba I, followed by gel isolation of the 1.91 kb band from a polyacrylamide gel. This fragment contains the den V gene in an expression cartridge consisting of the RSV LTR promoter and the poly signal sequence from SV40, as previously described. Plasmid pFGDX1, which consists of the

Ad 5 genome from 59.5% to 100% cloned into the Bam HI site of pBR322, was digested with Xba I, phosphatased, and ligated with the gel isolated fragment. The ligation mixture was used to transform E. coli HB101 as described, and colonies screened by restriction digest for insertion of fragment. Of the two orientations obtained, the one which transcribed den V in the opposite direction as the resident Ad 5 major late promoter was chosen, namely pMCADV. This was done to ensure transcription initiation from the RSV promoter and minimized longer readthrough transcription from E3. Plasmid pMCADV consists of pBR322 and the right 40% of the Ad 5 genome, with the E3 region from 76.4% to 84.6% deleted (by collapsing two Xba I sites) and the den V gene inserted into the single remaining Xba I site. 10 μg of pMCADV was linearized with Sal I and mixed with Eco RI digested wild type Ad 5 (dl E309) after heat inactivation of the restriction enzymes. The mixture was used to co-transfect 293 cells by the calcium chloride method (Graham and van der Eb 1973), after which the cells were overlaid with 0.5% agarose and left for 5 days to form viral plaques. Since the wild type virus was cleaved at 75.9%, this left 16.4% of the genome overlapping with pMCADV in which recombination could occur. The only other source of viable virus would be from uncut input wt Ad 5 or by re-ligation of the Eco RI fragments. The resulting virus (Ad Den V) is illustrated at the bottom of Figure 6.

Figure 6. Virus Construction Strategy.

The virus Ad den V was constructed as illustrated. Plasmid pEMBL18-RSVdenV was digested with Xba I and the 1.9 kbp fragment containing the den V expression cartridge was isolated by extraction from a polyacrylamide gel. The fragment was then ligated to plasmid pFGDX1 which had been digested with Xba I and treated with alkaline phosphatase. The ligation mixture was use to transform E. coli strain HB101, and ampicillin resistant colonies were screened for those bacteria containing plasmids with inserts. The resulting plasmid, pMCADV, was then linearized with Sal I, and mixed in varying ratios with wild type Ad 5 (dl E309) which had been digested with Eco RI, and the mixture was used to transfect human 293 cells. Since pMCADV contains the Adenovirus genome from 59.5% to 75.9% in common with the left hand end of Eco RI digested Ad 5, there was 16.4% homologous overlap in which recombination could occur. Recombinant viruses were selected for since only background undigested Ad 5 or re-ligated Eco RI fragments could produce viable virus independently. Of 24 viral plaques arising from the transfection screened, 3 were recombinants, one of which was designated Ad Den V and the Hind III digestion pattern for this virus can be seen at the bottom of the figure.


pEMBL18/
RSVdenV
5690 bp
DEN V
pFGDX1
16660 bp
Xba I.1
Hind III.1600
Hind III.14560
Hind III.5400
Bam HI.5480
Hind III.5820
59.5%
pBR322
Bam HI.9780
Xba I
gel isolate
Xba I
alkaline phosphatase
ligate
pMCADV
18570 bp
Xba I.1
Hind III.900
Hind III.1810
Xba I.1910
Hind III.16470
Den V
Hind III.8490
Hind III.6890
pBR322
Bam HI.7340
Hind III.7730
Bam HI.11690
Sal I.11410
linearize c Sal I
co-transfect 293 cells
Eco RI
Ad 5 wt
(dlE309)
Ad Den V
Hind III

8 Assay for UV Survival of Virus

8.1 Seeding of Chamber Slides

8 well glass chamber slides (Lab-Tek Products, Naperville, Ill, cat. # 4808) were seeded with human fibroblasts to be studied. One confluent 75 cm^2 flask of cells was trypsinized and resuspended in 25 ml of complete medium and 0.4 ml of the cell suspension added to each well. Cells were allowed to elongate and reach confluency for a minimum of 24 hours.

8.2 Irradiation of Virus

Viral stocks were diluted appropriately in straight medium, and 1 ml of viral suspension was irradiated in 35mm-diameter cell culture dishes (Falcon Plastics cat. # 3001) and kept on ice with constant swirling during irradiation. Dosimetry of the UV germicidal lamp (G8T; General Electric Company, Cleveland, Ohio) was determined using a J-225 short-wave UV meter (Ultra-Violet Products Inc., San Gabriel, Calif.). Incident dose rate was determined to be 1 $J/m^2/sec$ for doses in the range 0-600 J/m^2. For higher doses, an incident dose rate of 2 $J/m^2/sec$ was used.

8.3 Infection of Chamber Slides

Chamber slides were infected with 20 µl of appropriately diluted viral suspension and infection allowed to proceed for 90 min. The cells were then overlayed with 0.4 ml of complete medium and incubated for 48 hours before staining.

8.4 Vag Staining of Cells

Non-irradiated and irradiated virus was assayed for ability to form Vag in various human fibroblasts. After 48 hrs of infection the slides were fixed with acetone:ethanol (50:50) for 10 min, the wells and gaskets removed and the slides allowed to air dry. After preincubation for 30 min. in PBS at 37°C, the slides were incubated at 37°C for 1 hr in the presence of rabbit Ad 2 antiserum (Rainbow and Jeeves 1983) by applying 50 µl of a 1:20 dilution of Ab in PBS underneath a coverslip. After the incubation the coverslips were removed and the cells washed in PBS for 30 min. at 37°C. The cells were then incubated with fluorescein-conjugated sheep anti-rabbit globulin (No. 660-352; Grand Island Biological Company) in the same manner as the first Ab. After the secondary Ab, the cells were washed for 1 hour in PBS at 37°C, and then air dried. Infected cells were visualized under a Leitz indirect fluorescent microscope and the number of Vag producing cells per well counted. All slides were

stained concurrently and scored by a single observer for Vag production.

8.5 Mathematical Analysis

8.51 Surviving Fractions

Each slide was infected with three two-fold serial dilutions of the viral sample to be titred, each dilution infected in duplicate, and the remaining two wells used as mock infected controls. The Vag positive counts for the duplicate wells of the three dilutions were used as independent points and subject to linear regression. Surviving fraction was calculated by comparison to the unirradiated control to determine the surviving fraction of the virus at each dose point.

8.52 D_0 Values

D_0 values were calculated and used as a quantitative measure of viral survival in the specific cell line under investigation. In a one component, one hit, one target system the D_0 is numerically equivalent to the D_{37} value, which is the dose required to reduce survival of the input virus to 37%. The D_{37} value for normal cells and a repair deficient line can be compared to give an indication of the

fraction of DNA repair capability present. This is termed the Percent Host Cell Reactivation (%HCR) of the cell, and is calculated by the division of the cell line's D_{37} by that of normal cells. The D_0 in these experiments was calculated by use of pooled results for all experiments done with one virus on one cell type, each survival point being used as a separate point for the linear regression and the error determined using the standard error of the mean. Analysis of the survival curves produced was done in two components, with the turning point being estimated from the graph, usually at the lowest UV dosage given. Normal and Bloom's syndrome cells did not exhibit a two component nature, and were analyzed as single component curves. Each experiment was done with a separate irradiation, with the exception of one group experiment which was performed with the same irradiated virus in order to establish consistency in the difference between cell lines, between experiments. The ability of the presence of the den V gene in the virus to enhance the DNA repair capacity of the cell was quantitatively calculated as the percentage of the cells inherent decrease in %HCR that is restored by the den V gene.

1 Construction of the Ad Den V Viral Vector

The use of adenovirus as a vector to deliver a foreign gene into mammalian cells was demonstrated by Haj-Ahmad and Graham (1986). A study of the packaging constraints of adenovirus type 5 showed that up to approximately 2 kb of additional DNA can be inserted into the genome and the virus is still successfully packaged. Packaging is dependent upon the presence of protein IX, a minor component of the adenovirus capsid (Goutam Ghosh-Choudhury et al 1987). An adenovirus recombinant (Adtk) was constructed by insertion of the herpes simplex virus (HSV) thymidine kinase gene into the E3 region which had been deleted between the Xba I sites at 78.5% and 84.7%. It was inserted in the opposite orientation to the E3 promoter and was shown to express at a low level (Haj-Ahmad and Graham 1986). The construction technique utilized in the production of Adtk was used to construct a recombinant adenovirus virus carrying the T4 den V gene.

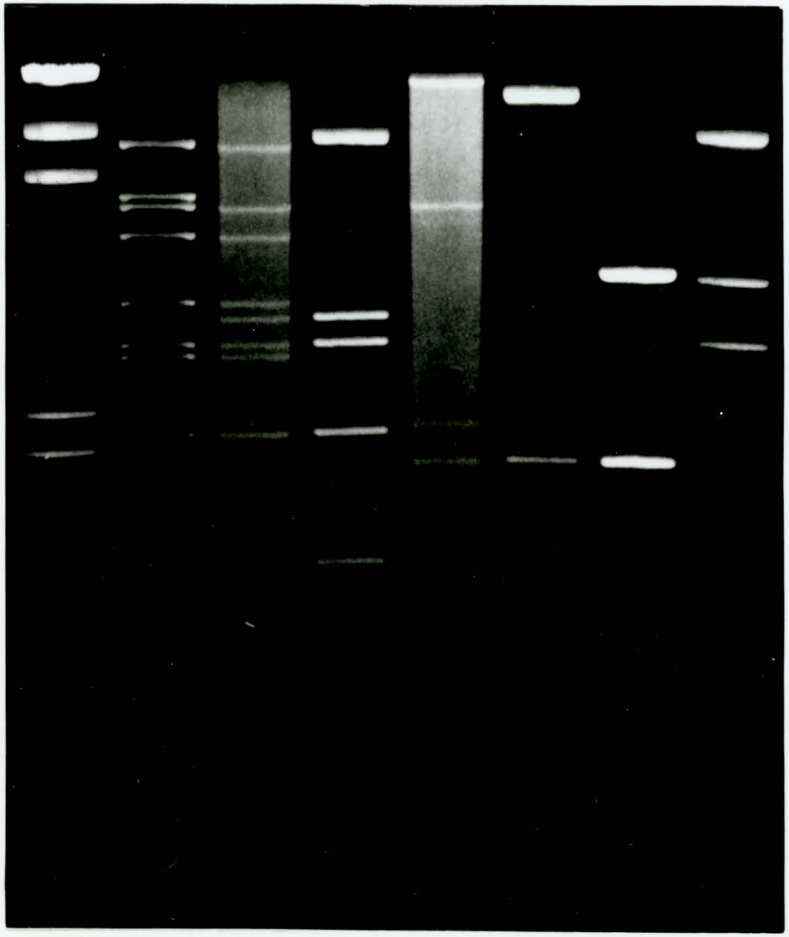
Ad Den V was constructed as outlined in materials and methods and Figure 6. Figure 7 shows confirmation of the cloning steps involved in the construction. Digestion of pEMBL18/RSVdenV (Figure 2) with Xba I liberated a 1.91 kb fragment containing the den V expression cartridge (Figure 1), which was gel isolated and inserted into pFGDX1 (Figure 4). The resulting plasmid, pMCADV (Figure 5) consisted of the right 40% of the adenovirus genome, with the den V gene

Figure 7. Southern Blot Analysis of Ad Den V.

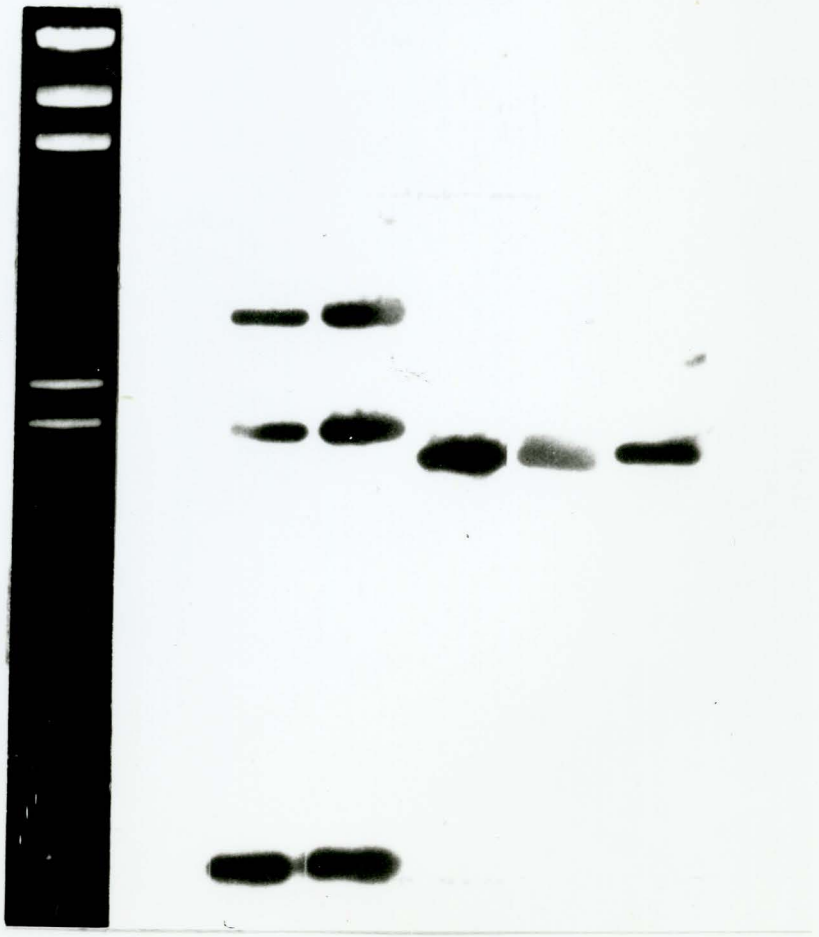
Figure 7a shows various digests of DNA used in this study. The DNA was digested and run on a 0.8% agarose gel, which was subsequently transferred to nitrocellulose and probed (Figure 7b). The probe used was the 1.9 kb Xba I fragment containing the den V expression cartridge, which was gel isolated from a digestion of pEMBL18-RSVdenV with Xba I. 200 ng of the fragment was nick translated to a specific activity of ~2 x 10^9 cpm/μg. The marker used was lambda DNA digested with Hind III (lane 1), which produces bands of 23130, 9416, 6557, 4361, 2322, 2027, 564, and 125 base pairs. Lane 7 shows the parental plasmid pEMBL18/RSVdenV digested with Xba I, releasing the den V expression cartridge, which can be seen to hybridize to itself in the blot. This can be compared to pFGDX1 in Lane 8, which shows no hybridization to the probe. Lane 6 contains pMCADV digested with Xba I, liberating the 1.9 kb expression cartridge, as shown by the hybridization in the blot. Lane 5 contains the recombinant virus Ad Den V digested with Xba I, which can also be seen to liberate the 1.9 kb fragment. Lane 4 contains pMCADV digested with Hind III, which can also be compared to the Hind III digestion pattern of Ad Den V (lane 3), confirming insertion of the den V fragment. Lane 2 contains the parental virus Ad 5 dl E309 digested with Hind III, and comparison to the recombinant show the disappearance of the B fragment and the appearance of a 3.1 kb, 2.0 kb, and 0.4 kb band.

Results

A



B



inserted in the E3 region. This was then linearized and cotransfected with Eco RI digested wt Ad 5 (dl E309, Figure 2) DNA, and in vivo recombination between the overlapping 16% produced the recombinant Ad den V virus. Figure 7a shows the digestion and Figure 7b the southern blot analysis of vectors used during construction. The blot has been probed with the 1.91 kb den V expression cartridge which was obtained by digestion of the parental plasmid pEMBL18/RSVdenV with Xba I and subsequent gel isolation and nick translation. In lane 7 the parental plasmid pEMBL18/RSVdenV digested with Xba I can be seen to release the 1.91 kb fragment containing the den V gene, leaving the remainder of the 5.6 kb vector. This fragment was then transferred to the Xba I site of plasmid pFGDX1. Plasmid pFGDX1 can be seen in lane 8 digested with Hind III, producing an 9.1 kb, 3.54 kb, 2.9 kb and 1.34 kb band. The resulting plasmid, pMCADV can be seen to release the 1.95 den V band upon Xba I digestion (lane 6) and its homology to the den V expression cartridge confirmed by the blot. The Hind III digestion pattern of pMCADV is illustrated in lane 4, and can be compared to the Hind III digestion pattern of pFGDX1 (lane 8), which upon insertion of the fragment has lost the 3.54 kb band (which contains the Xba I site) and has additional bands of 3.1 kb, 2.0 kb, and 0.33 kb, due to the 2 internal Hind III sites in the cartridge. All three additional bands can be seen to contain den V specific sequences as illustrated by the blot. The

orientation of the insert can be ascertained from the position of the Hind III sites within the cartridge, which are at position 580 and 914 (Figure 1). Since the distance between the Xba I site and the first Hind III site left toward 59.5% in the adenovirus genome is 2.1 kb, the addition of a 3.1 kb band indicates that there is ~1.0 kb before the first Hind III site in the cartridge. From this it can be concluded that the cartridge is inserted with the RSV promoter transcribing from right to left (100% -> 0%) with respect to the adenovirus genome.

To confirm the transfer of the den V cartridge to the recombinant virus, the Hind III and Xba I digestion patterns of Ad Den V (lanes 3 and 5, respectively) and the source plasmid pMCADV (lanes 4 and 6, respectively) can be compared in the gel and the blot. The recombinant virus and the parental virus Ad 5 dl E309 (lane 2 digested with Hind III) can be compared. The disappearance of the Hind III B fragment and the addition of the den V specific 3.1 kb, 2.0 kb, and 0.33 kb bands further confirm the construction of the desired recombinant. From this evidence it can be seen that a recombinant adenovirus vector has been constructed, containing a deleted E3 region, and which has the den V gene inserted in the place of the deletion.

2 Expression of the den V gene in Ad Den V

In order to determine if the constructed Ad Den V virus was suitable for testing the biological effect of the den V gene product, viral expression of the inserted gene was investigated. This was done by attempting to detect both RNA transcription and protein expression from the den V expression cartridge.

2.1 Protein Expression

The den V gene codes for a ~16 KD polypeptide (Valerie et al 1984) which has been isolated to physical homogeneity (Higgins and Lloyd 1987). Polyclonal and monoclonal antibodies have been raised to den V-Protein A fusion protein (Valerie et al 1987b), and have been demonstrated to react with purified and partially purified den V gene product extracted from prokaryotic systems. These antibodies were provided by Dr. Earl Henderson (Department of Immunology and Microbiology, Temple University, PA), and several attempts were made to utilize them to detect den V protein in crude mammalian cell extracts.

2.11 Immunoprecipitation

Expression of the den V protein by Ad den V was assayed by

immunoprecipitation with both monoclonal and polyclonal Ab raised against a den V-Protein A fusion protein. 293 human cells were radiolabeled from 6 to 9 hours post infection, infecting with either Ad Den V, Ad VSV or mock infecting. Total cellular protein was then harvested and immunoprecipitated with either polyclonal or monoclonal antibodies. The most satisfactory results were obtained using 5 μl of Ab and the results are shown in Figure 8. It can be seen that a band is visible in the Ad den V infected, monoclonal Ab precipitated lane at ~16 KD that is not as prominent in quantity in the Ad VSV or mock infected lanes, however there is an abundance of other bands present as well. The repeated occurrence of this result prompted two possible explanations. Either there was a highly homologous cellular protein which possessed several multimeric forms, or the Ab supplied had low affinity for the den V gene product. In order to investigate the existence of a true cellular homolog, several uninfected cell extracts from normal and XP human fibroblasts were tested by immunoprecipitation with the monoclonal Ab. In Figure 9 it can be seen that there is a predominance of bands in all cells, although the effect is observed most prominently in XP rather than normal cells. While many bands are observed, most relevant is the dominant species that appears at ~16,000 D. The presence of this band in uninfected cells makes identification of virally produced den V gene product

Figure 8. Immunoprecipitation with den V specific monoclonal and polyclonal antibodies.

Antibodies received from Dr. Earl Henderson, Temple University School of Medicine, Philadelphia, Pennsylvania, were tested for their ability to immunoprecipitate den V protein from Ad Den V infected cells. Lanes 2 and 3 are Ad Den V infected 293 cells, immunoprecipitated with polyclonal and monoclonal antibodies, respectively. Lanes 4 and 5 are wt Ad 5 infected 293 cells, immunoprecipitated with polyclonal and monoclonal antibodies, respectively. Lanes 6 and 7 are mock infected 293 cells, immunoprecipitated with polyclonal and monoclonal antibodies, respectively.

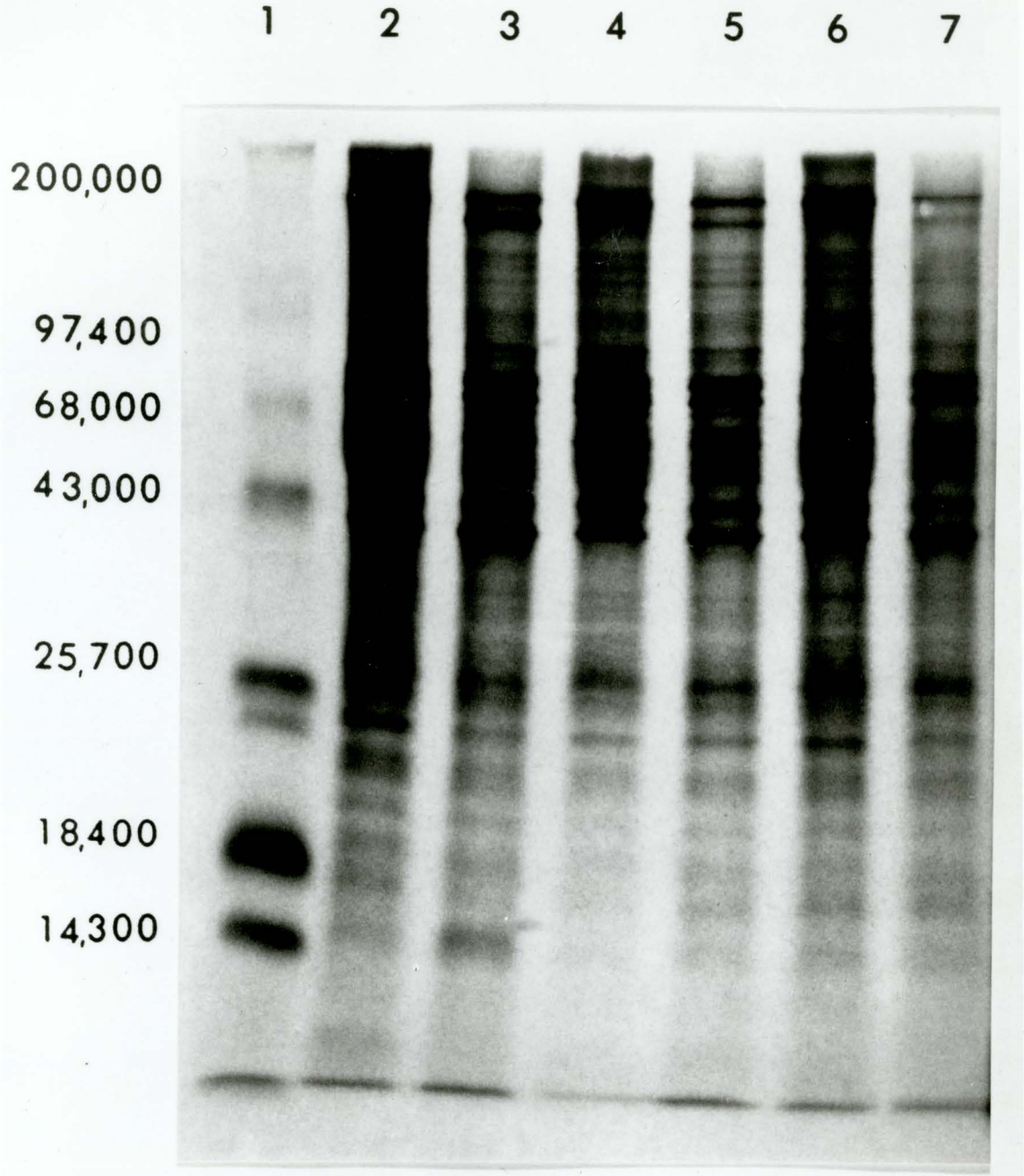

1
2
3
4
5
6
7
200,000
97,400
68,000
43,000
25,700
18,400
14,300

Figure 9. Immunoprecipitation of Cellular Proteins with den V Monoclonal Antibodies.

Immunoprecipitation of 4 XP cell lines and 3 normal human fibroblasts with den V specific monoclonal antibodies.

Lane 2 - GM 544B XPA (XP4LO)

Lane 3 - CRL 1204 XPC (XP10BE)

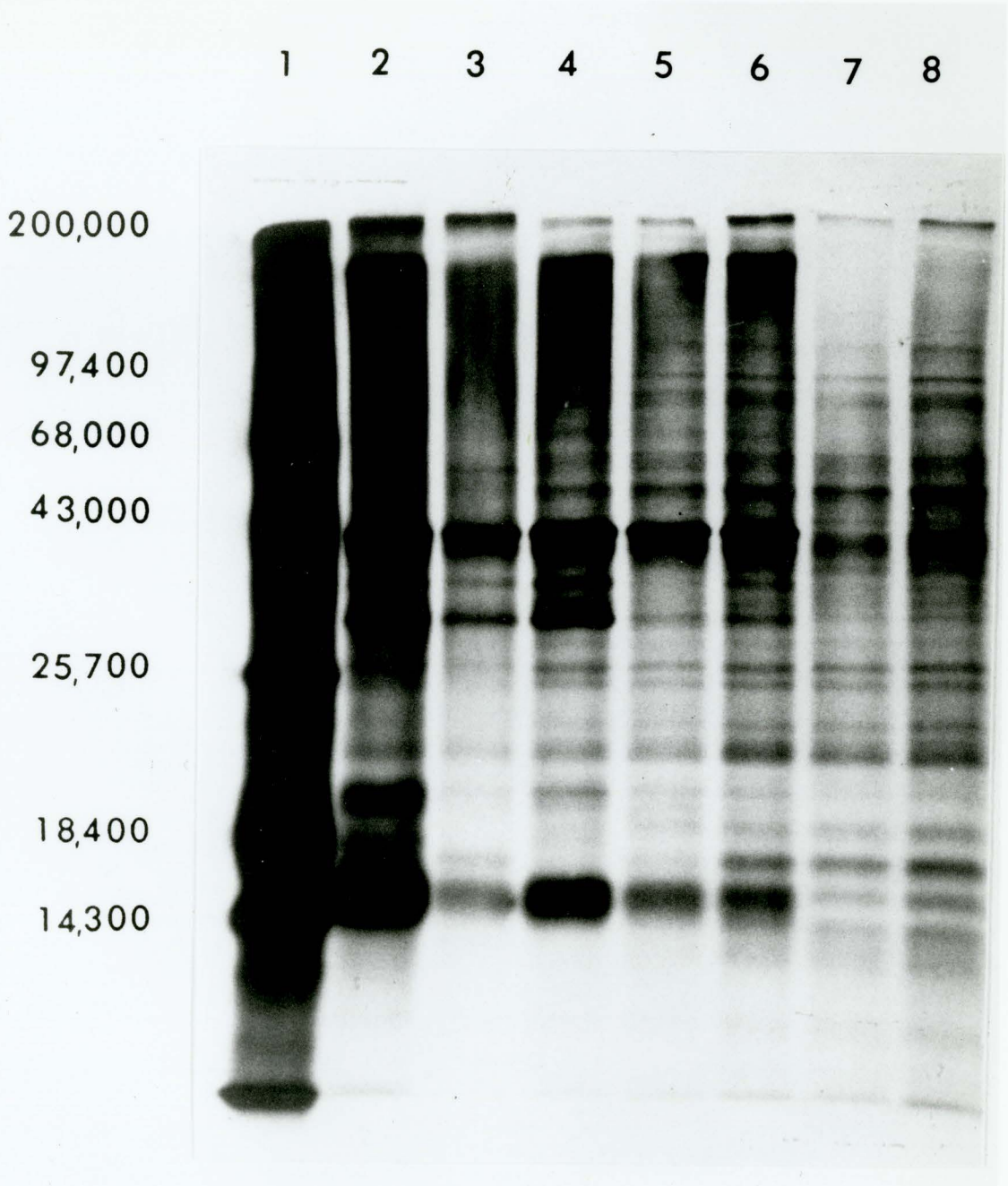
Lane 4 - CRL 1259 XPE (XP2RO)

Lane 5 - GM 5509 XPA (XP12BE)

Lane 6 - GM 969 Normal

Lane 7 - CRL 1229 Normal

Lane 8 - S6007 Normal


1
2
3
4
5
6
7
8
200,000
97,400
68,000
43,000
25,700
18,400
14,300

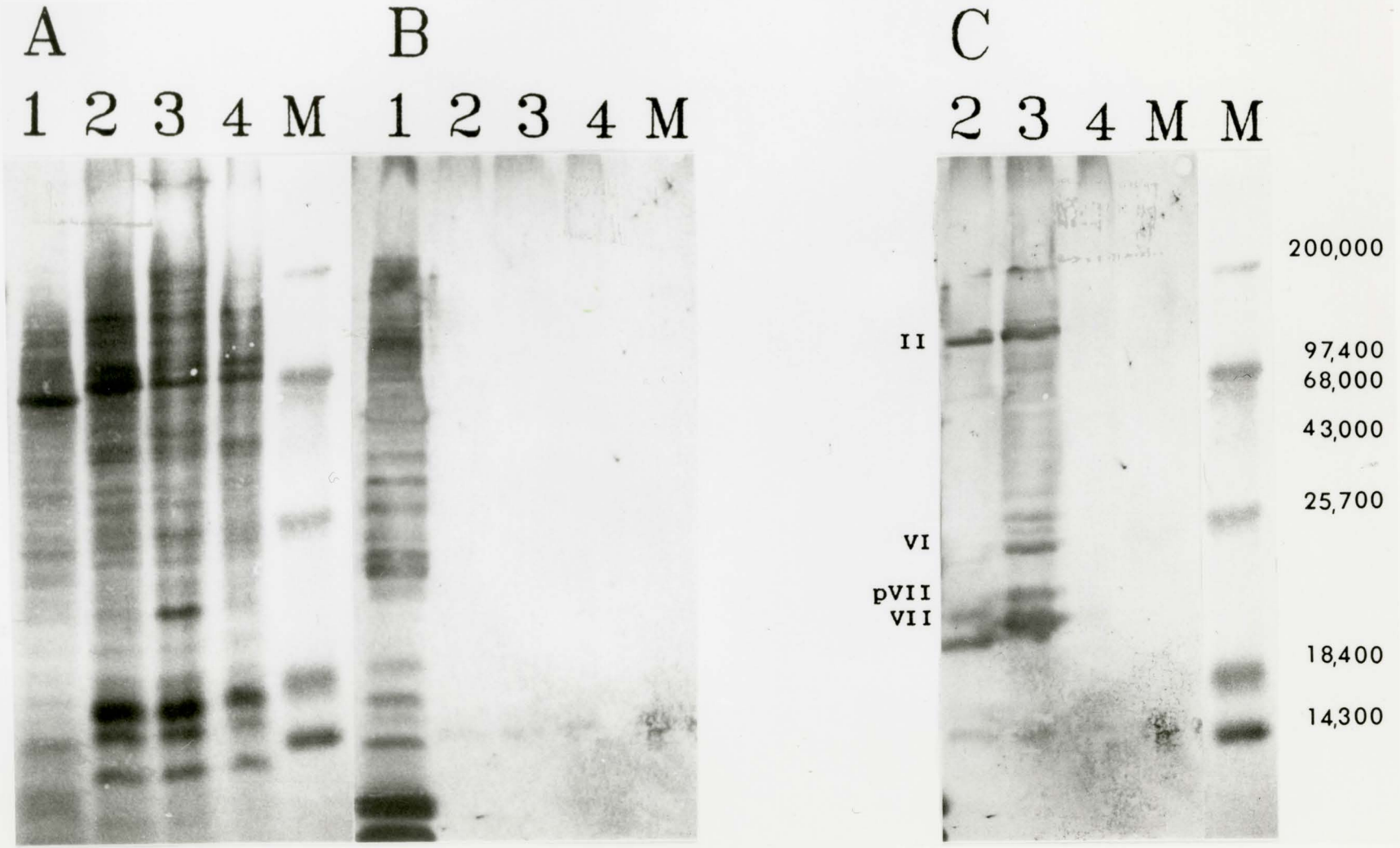
impossible by one dimensional SDS-PAGE due to the cellular background.

2.12 Western Blots

Previous work done with the antibodies provided have shown detection of purified and partially purified den V gene product isolated from bacterial systems (Valerie et al 1987b). This was done using the Western Blotting technique (Johnson et al 1984 and Towbin et al 1979). This technique was tested to determine if the Ab performed better in such a system than under the conditions of immunoprecipitation. Figure 10 shows typical results of such analysis. Figure 10a shows protein from bacteria carrying a den V producing plasmid, Ad Den V infected HeLa cells, Ad VSV infected cells and mock infected cells, stained with amido black to show total protein. After destaining the blot, the protein was then reacted with den V polyclonal antibody, and it can be seen in Figure 10b that although the den V infected cells produced a band of the appropriate size for den V expression, a similar band was evident in the Ad VSV and mock infected lanes. There was also an abundance of bacterial proteins shown to react with the den V Ab. To confirm that the western blot technique was functioning properly, the same blot was then reprocessed by the same procedure except using the Vag antibodies used in the

Figure 10. Western Blot Analysis of Protein with Den V Specific Antibodies.

Total cellular protein from Ad Den V, Ad VSV and Mock infected Hela cells was isolated 4, 36 and 36 hours after infection, respectively, and run on a 15% SDS-polyacrylamide gel. Lane 1 contains proteins extracted from HMS174 E. coli carrying pDENV52, lane 2 is Ad Den V infected cells, lane 3 Ad VSV infected cells and lane 4 mock infected cells. After transfer to nitrocellulose the blot was stained with amido black to reveal all transferred proteins (Figure 10a). The blot was then destained and reacted with anti-den V protein polyclonal antibodies, and visualized by the alkaline phosphatase technique (Figure 10b). The antibody can be seen to react with a protein of ~16,000 MW in lanes 2-4, and with a large proportion of the bacterial proteins in lane 1. To confirm the viability of the technique the same blot was then reacted with Vag to visualize the adenovirus proteins in lanes 2 and 3 (Figure 10c). Lane 2 (4 hours post infection) shows the detection of the input virion proteins, most notably hexon (II) and core (VII). Lane 3 (36 hours post infection) shows the addition of protein VI and possibly pVII. Lane 4 (mock infected) shows no detectable additional proteins, illustrating the specificity of the technique.


A
1 2 3 4 M
B
1 2 3 4 M
C
2 3 4 M M
II
VI
pVII
VII
200,000
97,400
68,000
43,000
25,700
18,400
14,300

survival curve assays. It can be seen in Figure 10c that no non-specific antibody binding occurred in the mock infected lane, while several adenovirus specific proteins were identified in lanes 2 and 3. Since the Ad Den V infected cells were harvested 4 hours post infection, the major bands visualized are the hexon (II) and core (VII) proteins, probably from input virus. The Ad VSV infected cells were harvested at 36 hours, and additional proteins can be seen, hexon associated protein (VI) and possibly pVII, the core protein precursor. The differential response of the Vag antibody to different time points in infection also confirms the authentic results of the western blot technique. From these results it was concluded that using the antibodies provided it was not possible to demonstrate expression of den V protein by the Ad Den V virus.

2.2 Transcription of RNA from Ad Den V

Due to inconclusive results obtained with the den V Ab provided, molecular evidence of expression of the den V gene by Ad Den V was sought by detection of the production of correctly initiated RNA transcripts. Previous studies have utilized the production of correctly initiated RNA transcripts as an indication of gene expression (Smiley et al 1987), and this strategy was employed to similarly study expression from the Ad Den V virus. The technique of primer

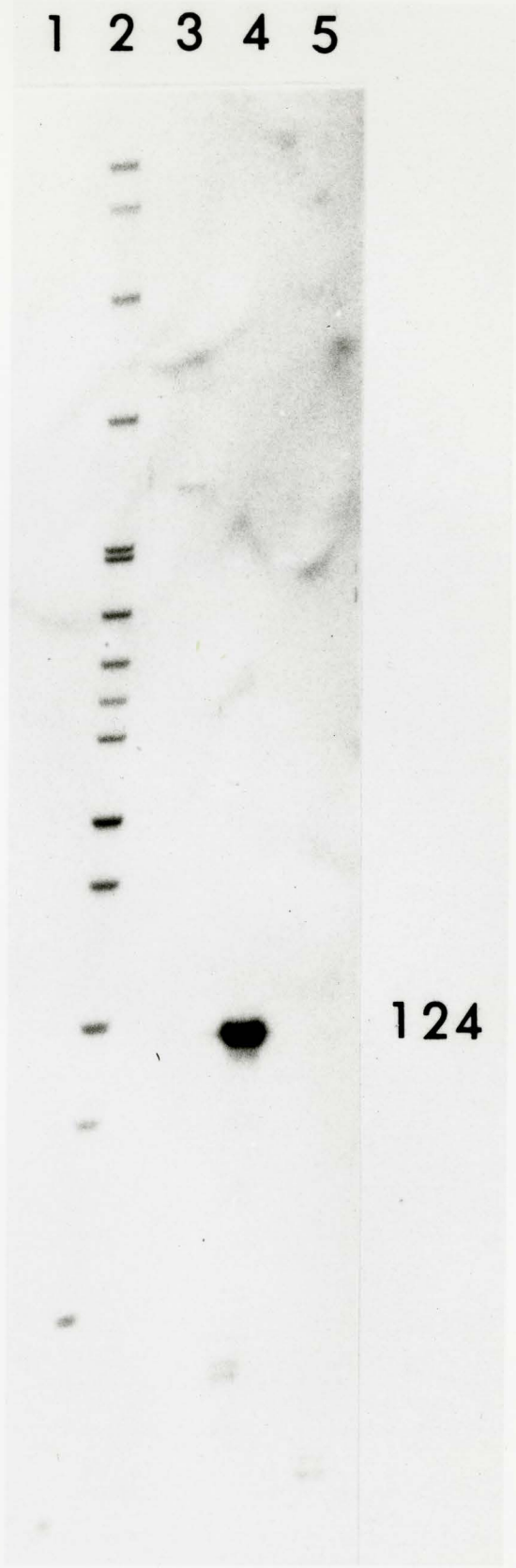
extension (Jones et al 1985) was performed using RNA extracted from Ad Den V infected HeLa cells at two time points, 1 hour post infection and 22 hours post infection. Cells were infected at 30 PFU to insure maximum RNA yield, and 30 μg of each RNA sample used for each extension assay. The 24 mer oligonucleotide synthesized was homologous to the den V expression cartridge complementary to position 669 to 645, and approximately 2 million cpm of labelled probe was used for each reaction. It can be seen in Figure 11 that while no appreciable background was observed in either the purified primer (lane 1) or in reaction with uninfected HeLa cell RNA (lane 5), or HeLa RNA 1 hour post infection (lane 3) a strong signal appears at approximately 124 bases upon infection with Ad Den V at the 22 hour sample (lane 4). This corresponds to an RNA transcript that has been initiated at position 546 in the cartridge sequence, which corresponds exactly to published reports of the start of transcription of the RSV 3' LTR promoter.

3 Survival of UV Irradiated Ad Den V in Human Fibroblasts

Since the results of the primer extension assay suggested that the den V cartridge was being correctly transcribed, the UV survival of the Ad Den V virus was examined in human fibroblasts. Previous studies have shown that the introduction of the den V protein into XP group A cells

Figure 11. Primer Extension of Ad Den V infected HeLa Cell RNA.

A 24 base oligonucleotide (5'-CGCGGCAATTCACGATATTCAGCC- 3') corresponding to the den V expression cartridge sequence (complementary strand) from position 669 to 645 was synthesized. The oligonucleotide was kinased and used as a primer for reverse transcription of total cellular RNA harvested from HeLa cells 1 hour and 22 hours post infection with Ad Den V virus at a multiplicity of ~30 PFU per cell, as well as RNA from uninfected HeLa cells as a control. ~50 μg of each RNA sample was reverse transcribed and run on an 8% sequencing gel for ~2.5 hours at 2000 volts. Marker (lane 2) is Hpa II digested pBR322, klenowed with radiolabelled nucleotides and strand separated. Sizes are as follows: 623, 528, 405, 310, 243, 239, 218, 202, 191, 181, 161, 161, 148, 148, 124, 111, 91, 77, 68, 35, 35, 27, 27, 16, 10, and 10 bases. It can be seen that a large amount of transcription appears in Ad Den V infected cells at the 22 hour time point at approximately 124 bases (lane 4) there is no such extension occurring from the cellular RNA (lane 5). This corresponds to position 545 in the den V expression cartridge sequence, which is the published start of transcription for the RSV 3' LTR promoter. No appreciable transcription is observed at 1 hour post infection (lane 3). The input primer is shown in lane 1.


1 2 3 4 5
124

by membrane permeablization by Sendai virus (Tanaka et al 1977), and by transfection of the den V gene (Valerie et al 1987a) can partially restore DNA repair capacity as measured by increased cell survival post UV irradiation. In these studies restoration of cell survival has ranged from 6.4% up to 30% of normal levels. XP cells have been noted to have a reduced ability to reactivate UV damaged adenovirus (Day 1974, Rainbow 1981), and the quantitative measure of the host cells ability to reactivate damaged virus reflects the cell's DNA repair capacity (Day 1977). The survival of UV irradiated Ad Den V in comparison to a control virus was determined in several human fibroblast cell lines in order to investigate the ability of the den V gene to enhance DNA repair capacity.

It has been previously shown that the UV survival of adenovirus in human fibroblasts can be determined using the expression of Vag formation (Rainbow 1978, Rainbow 1981). Figure 12 shows a photograph of adenovirus infected human fibroblasts stained by the Vag technique, showing how clear identification of positive cells was possible (Rainbow 1980). Figure 13 shows typical results for actual Vag counts for Ad VSV and Ad Den V at two dose points. Counts generally ranged from 20 to 1000 Vag positive cells per 1 cm^2 well and were proportional to the dilution of virus used. Counts were used to calculate slope by regression through the origin. This result gives a virus titer at each dose point, which can

Figure 12. Vag Staining of Adenovirus Infected Cells.

Shown is a photograph of Vag stained adenovirus infected cells. Cells were first fixed using acetone:methanol 50:50 for 10 min. and then incubated in PBS for 30 min. The slides were then dried and 50 μl of polyclonal antibodies against adenovirus type 2 coat proteins was then added to each slide in a 1:20 dilution in PBS, and the slide covered with a coverslip and incubated for 1 hour at 37°C. The slides were washed in PBS at 37°C for 30 min. and incubated with sheep anti-rabbit conjugated antibodies for an additional hour at 37°C. The slides were then washed in PBS for one hour at 37°C, dried and observed under a fluorescent microscope. Vag positive cells were clearly distinguishable, as can be seen in the photograph (photograph taken by M. Howes, Departments of Radiology and Biology, McMaster University).

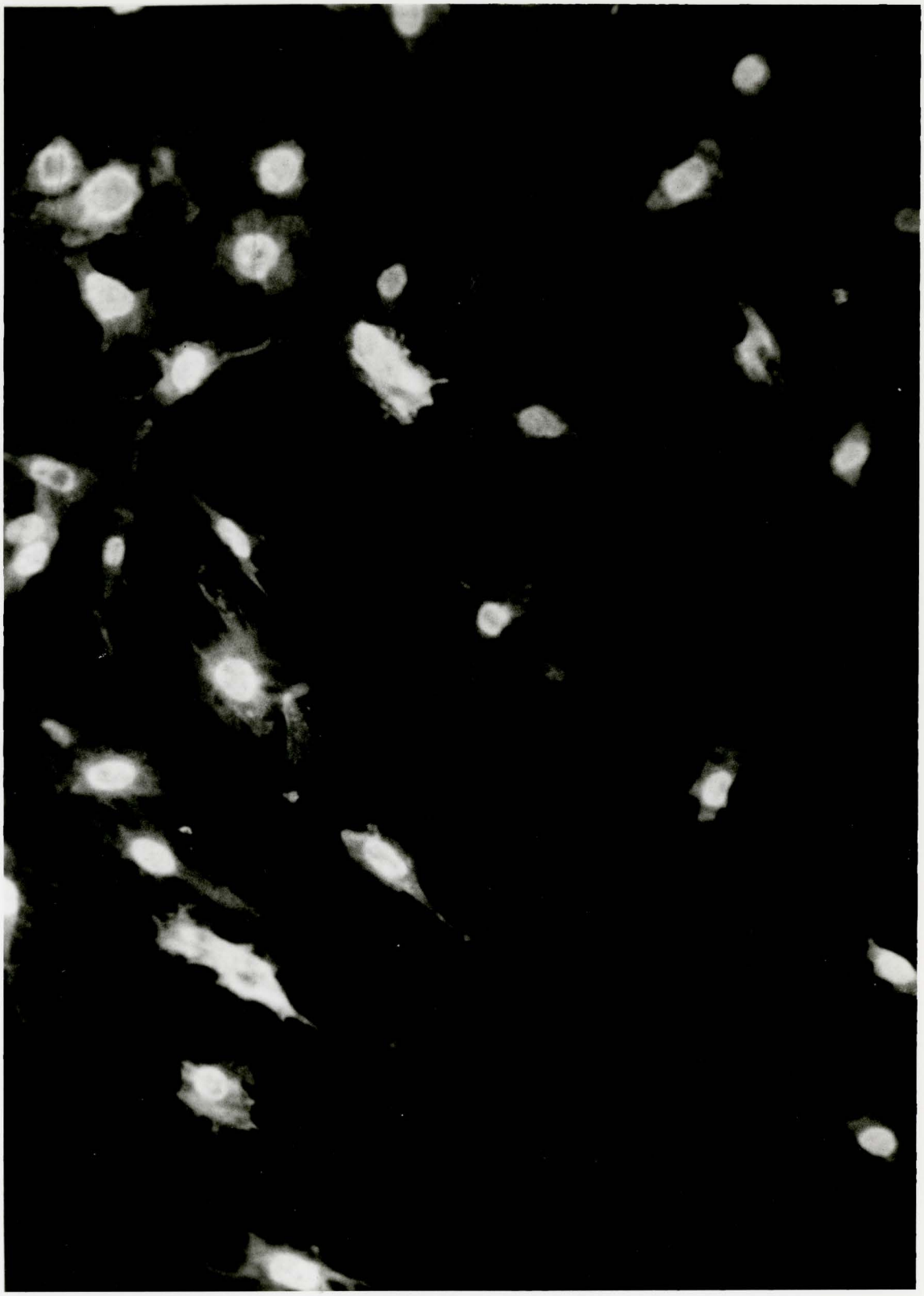
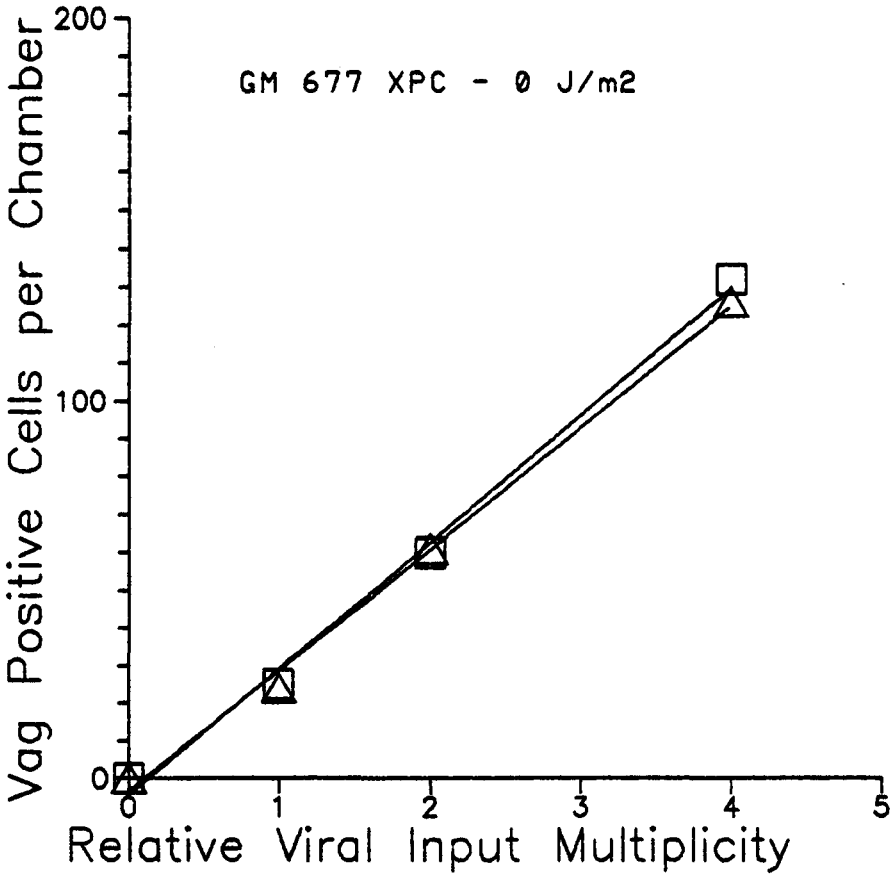
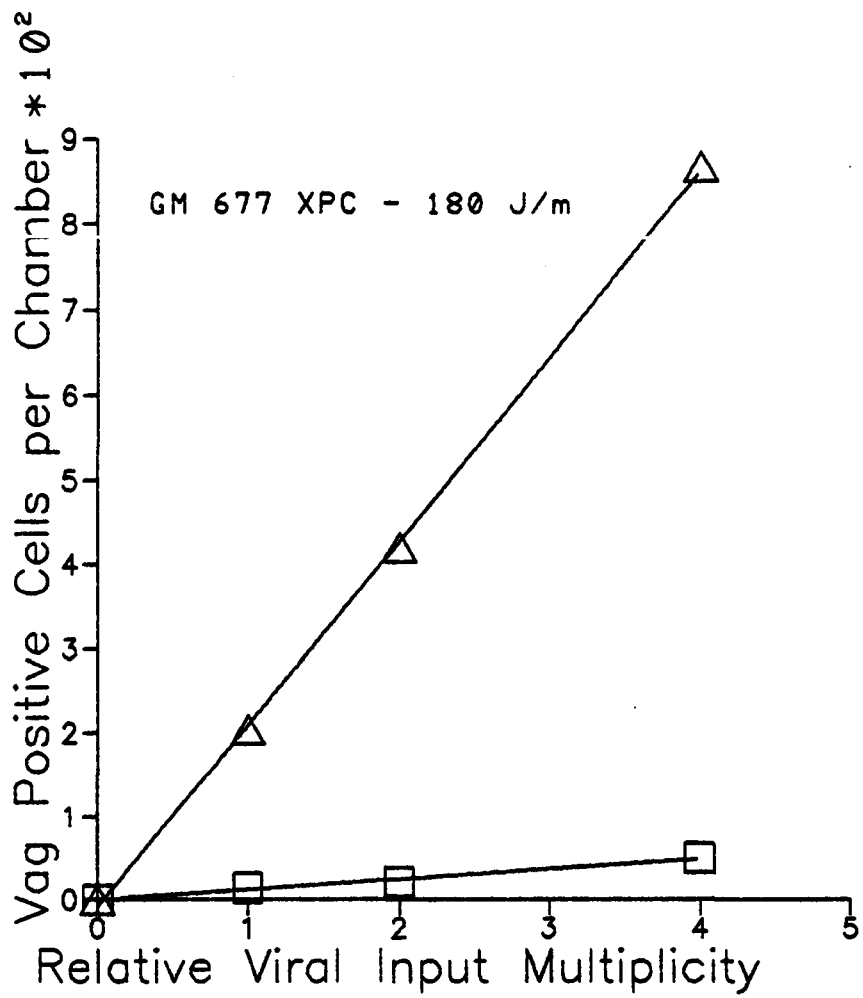


Figure 13. Relationship Between Vag Positive Cells and Viral Input Multiplicity.

The number of surviving viruses at a given dose point was calculated as follows. 8 well chamber slides were seeded with the appropriate cell type and allowed to reach confluency overnight. Three dilutions of the irradiated sample were used to infect 6 of the wells, and the other two wells were mock infected as controls. Vag counts generally ranged from 20 to 1000 infected cells, and the relationship between viral dilution and Vag positive cells is shown on the graph. Two examples of typical results are shown, illustrating data from survival on GM 677 (XP2BE) XPC cells at two dose points, 0 and 180 J/m^2. Triangles represent Ad Den V data, squares represent Ad VSV. Each point shown is the average of two wells, the difference between counts not exceeding ~15%. Titre at each dose point is numerically calculated by linear regression through the origin, and surviving fraction calculated by the division of the titre at the dose point by the control sample (0 J/m^2), after correction for dilution.


GM 677 XPC - 0 J/m2
Vag Positive Cells per Chamber
200
100
0
0
1
2
3
4
5
Relative Viral Input Multiplicity


GM 677 XPC - 180 J/m
Vag Positive Cells per Chamber *10²
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Relative Viral Input Multiplicity

then be used to calculate surviving fraction at each dose. The resulting survival curve was graphed and D_0 values for each component calculated by semi-log regression of the surviving fraction at each dose.

3.1 UV Survival of Ad Den V in XP Group A Cells

Figure 14 shows the results of 3 experiments assaying survival of Ad Den V in comparison to Ad VSV in GM 5509 XPA cells. Both curves exhibit a two component nature and it can be seen that both components show increased survival in the presence of the den V gene in the viral genome. This suggests that there is partial complementation of the DNA repair deficiency by the den V protein in XP group A cells. Tables 1 and 2 show a summary of the D_0 and %HCR for each component. From the survival curves it can be seen that there is an 11% restoration in the first component and a 26% restoration in the second component, which is comparable to the restoration of cell survival as previously discussed.

3.2 UV survival of Ad Den V in Normal Human Fibroblasts

When survival is assayed in normal cells (GM 2803) there is no significant difference in viral survival as determined by Vag expression (Table 1 and 2, Figure 15), with the D_0 values of the two viruses being within one standard error of

Figure 14. Survival of Ad Den V and Ad VSV on GM 5509 (XP12BE) XPA Cells.

Shown are the results for three separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D0 values calculated in Table 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension.

Figure 15. Survival of Ad Den V and Ad VSV on GM 2803 Normal Human Fibroblasts.

Shown are the results for four separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D_0 values calculated in Tables 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension.

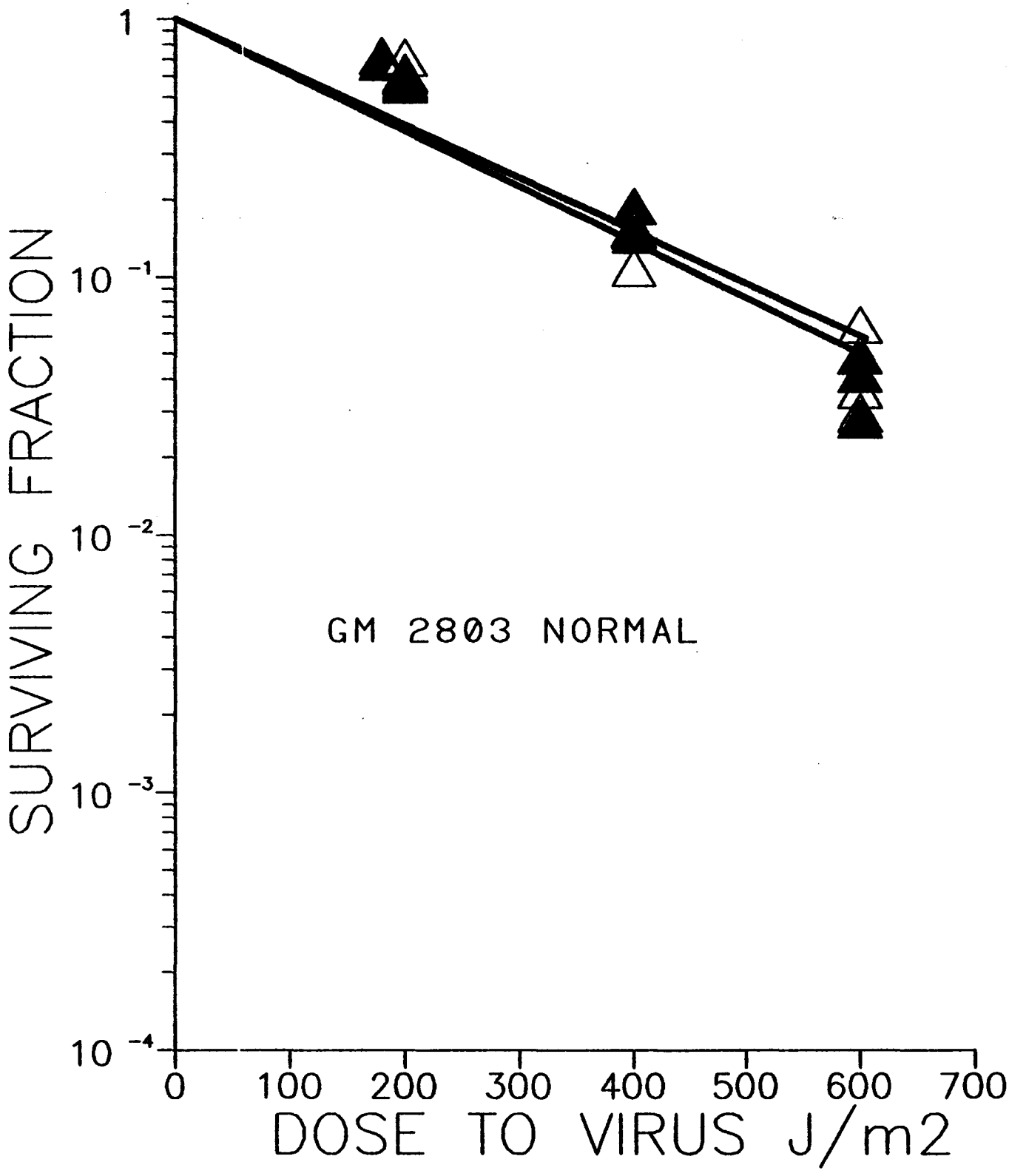

SURVIVING FRACTION
1
10 -1
10 -2
10 -3
10 -4
GM 2803 NORMAL
0
100
200
300
400
500
600
700
DOSE TO VIRUS J/m2

Table 1. UV Survival of Vag Production of Ad VSV

Cell Line	First Component		Second Component		# of Expts.
	D_0	%HCR	D_0	%HCR	
Normals					
GM 2803	175 ± 12				4
B. Hanes	206 ± 10				3
Average	190.5				
XP					
GM 5509 (XP12BE)	16 ± 0.3	8.4	38 ± 2	19.9	3
GM 2415 (XP2RO)	28 ± 5	14.7	53 ± 3	27.8	4
GM 677 (XP2BE)	30 ± 2	15.7	36 ± 2	18.9	4
Cockayne's					
GM 1428	109 ± 8	57.2			3
Bloom's					
GM 1492	116 ± 4	60.9			1

Table 2. UV Survival of Vag Production of Ad Den V

Cell Line	First Component		Second Component		# of Expts.
	D_0	%HCR	D_0	%HCR	
Normals					
GM 2803	178 ± 13				4
B. Hanes	207 ± 13				3
Average	192.5				
XP					
GM 5509 (XP12BE)	35 ± 3	18.2	79 ± 18	41.0 ± 12.1	3
GM 2415 (XP2RO)	60 ± 5	31.2	199 ± 9	103.4	4
GM 677 (XP2BE)	34 ± 2	17.7	156 ± 24	81.0	4
Cockayne's					
GM 1428	103 ± 6	53.5			3
Bloom's					
GM 1492	109 ± 3	56.7			1

Table 3. Percentage of Deficiency Restored By Ad Den V

Cell line	First Component	Second Component
XP GM 5509 (XP12BE)	10.7	26.3
GM 2415 (XP2RO)	19.4	100
GM 677 (XP2BE)	2.4	76.6

each other as determined from a total of 7 separate experiments in 2 different normal cell lines (see also Figure 19). This indicates that there is no intrinsic enhanced survival of the Ad Den V virus as prepared in a DNA repair proficient background.

3.3 UV Survival of Ad Den V in Cockayne's Syndrome Cells

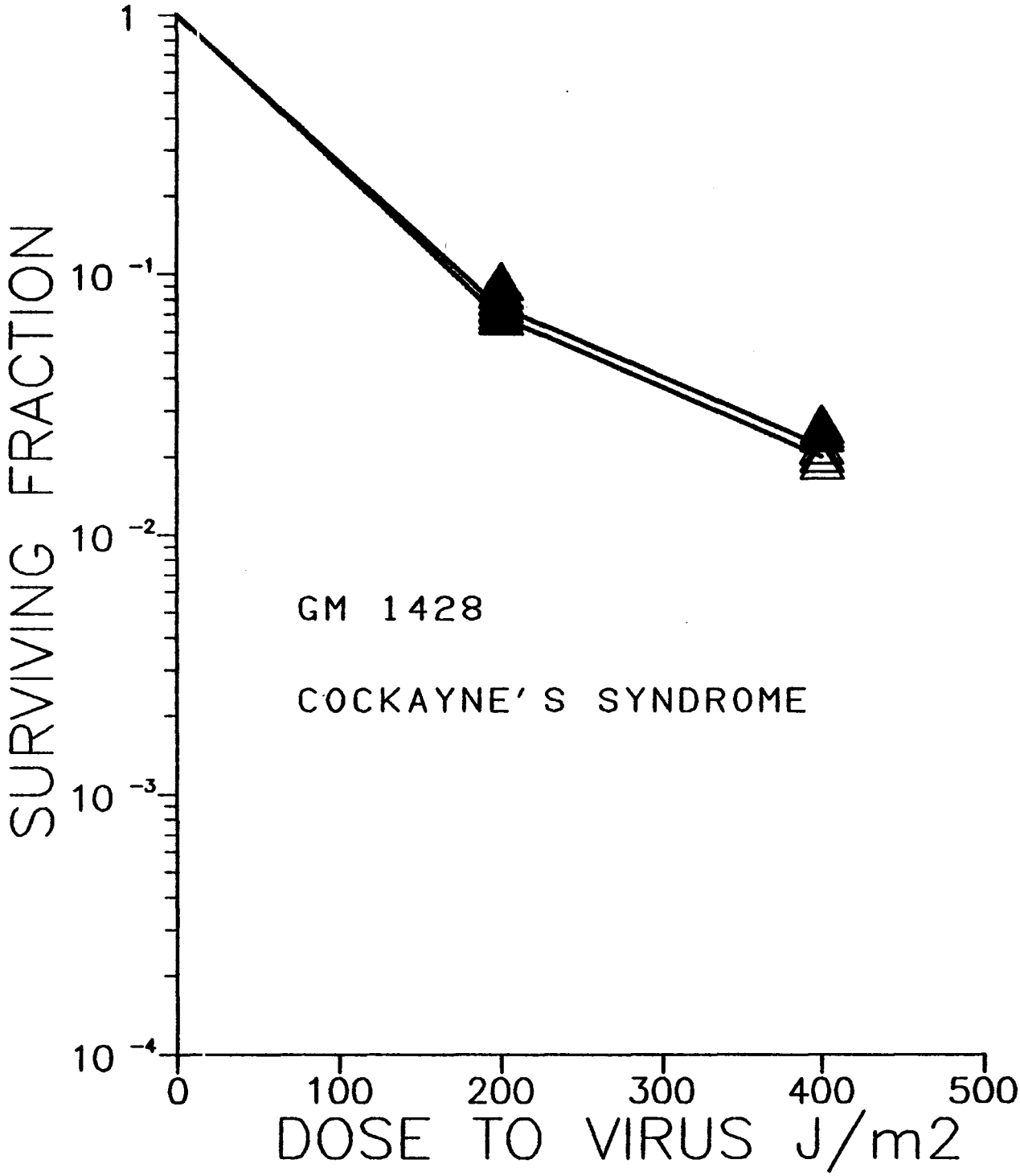
Previous studies have shown that UV damaged adenovirus shows decreased survival in Cockayne's syndrome cells in comparison to normal human fibroblasts (Day and Ziolkowski 1978, Rainbow and Howes 1982). It is currently held that Cockayne's syndrome cells are competent in the incision stage of excision repair but deficient at a latter step, possibly the ligation of the newly repaired region (Schwaiger et al 1986). UV survival of Ad Den V in GM 1428 (Cockayne's syndrome) cells is shown in Figure 16. It can be seen that there is no significant difference in viral repair capacity in CS cells with or without the den V gene, suggesting that the complementation seen in Group A XP cells is specific for defects in the incision step in excision repair.

3.4 UV Survival of Ad Den V in Other Cell Lines

While there are nine XP complementation groups known at present, UV cell survival in the presence of the den V gene

Figure 16. Survival of Ad Den V and Ad VSV on GM 1428 Cockayne's Syndrome Cells.

Shown are the results for three separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D_0 values calculated in Tables 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension.


SURVIVING FRACTION
1
10 -1
10 -2
10 -3
10 -4
GM 1428
COCKAYNE'S SYNDROME
0
100
200
300
400
500
DOSE TO VIRUS J/m2

has only been reported for group A (Valerie et al 1987a, Tanaka et al 1977) and group D - like cell line (Arrand et al 1987). The construction of the viral vector carrying the den V gene in this study facilitates the investigation of the affect of DNA repair genes in a wide variety of human cell types. Utilizing this ability, UV survival of Ad Den V was assayed in two other XP complementation groups, as well as in another DNA repair deficient cell line, GM 1492 (Bloom's Syndrome). Four experiments were performed with one cell line from Group C (GM 677) and 4 experiments with one cell line from Group E (GM 2415). The results are summarized in Tables 1 to 3 and in Figures 17 and 18. Group E cells exhibited a two fold increase in restoration in the first component in comparison to XPA cells, and the second component can be seen to be fully restored to normal levels (Table 3). XPC cells, however, showed almost no change in the first component and a large degree of restoration of the second component (77%, Table 3).

An additional 3 experiments were done with the clinically isolated normal human fibroblast cell line B. Hanes. This was done in order to confirm that the first normal cell line tested (GM 2803) did not give anomalous viral UV survival results. It can be seen from Figure 19 and Tables 1 and 2 that similar results were obtained with both normal cell lines.

One experiment with Bloom's Syndrome cell line GM 1492

Figure 17. Survival of Ad Den V and Ad VSV on GM 677 (XP2BE) XPC Cells.

Shown are the results for four separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D_0 values calculated in Tables 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension. XPC data is represented by triangles, and the average survival of both viruses in GM 2803 normal cells at one dose point is shown (diamonds) for comparison.

Figure 18. Survival of Ad Den V and Ad VSV on GM 2415 (XP2RO) XPE Cells.

Shown are the results for four separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D_0 values calculated in Tables 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension. XPE data is represented by circles, and the average survival of both viruses in GM 2803 normal cells at two dose points is shown (diamonds) for comparison.

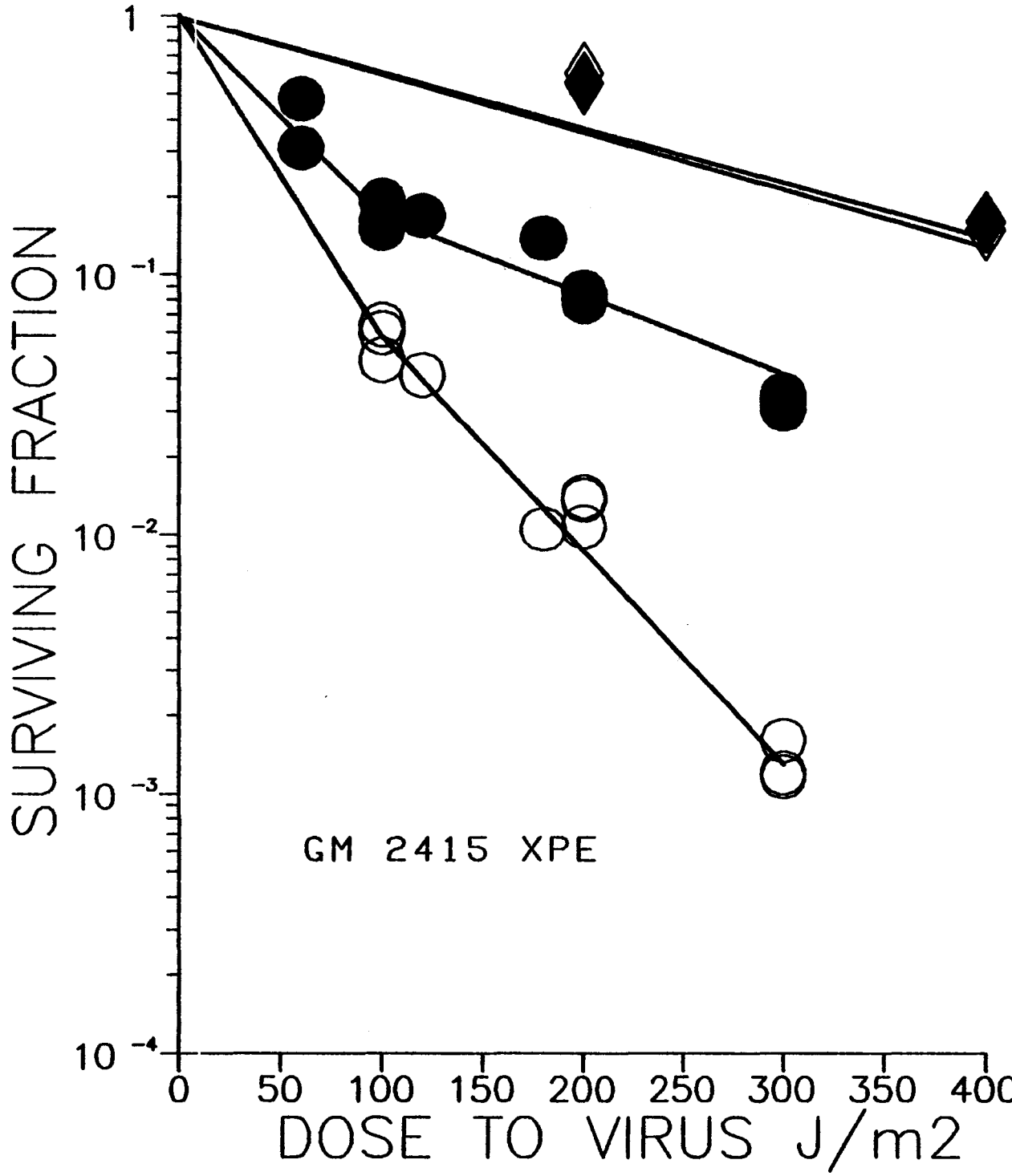
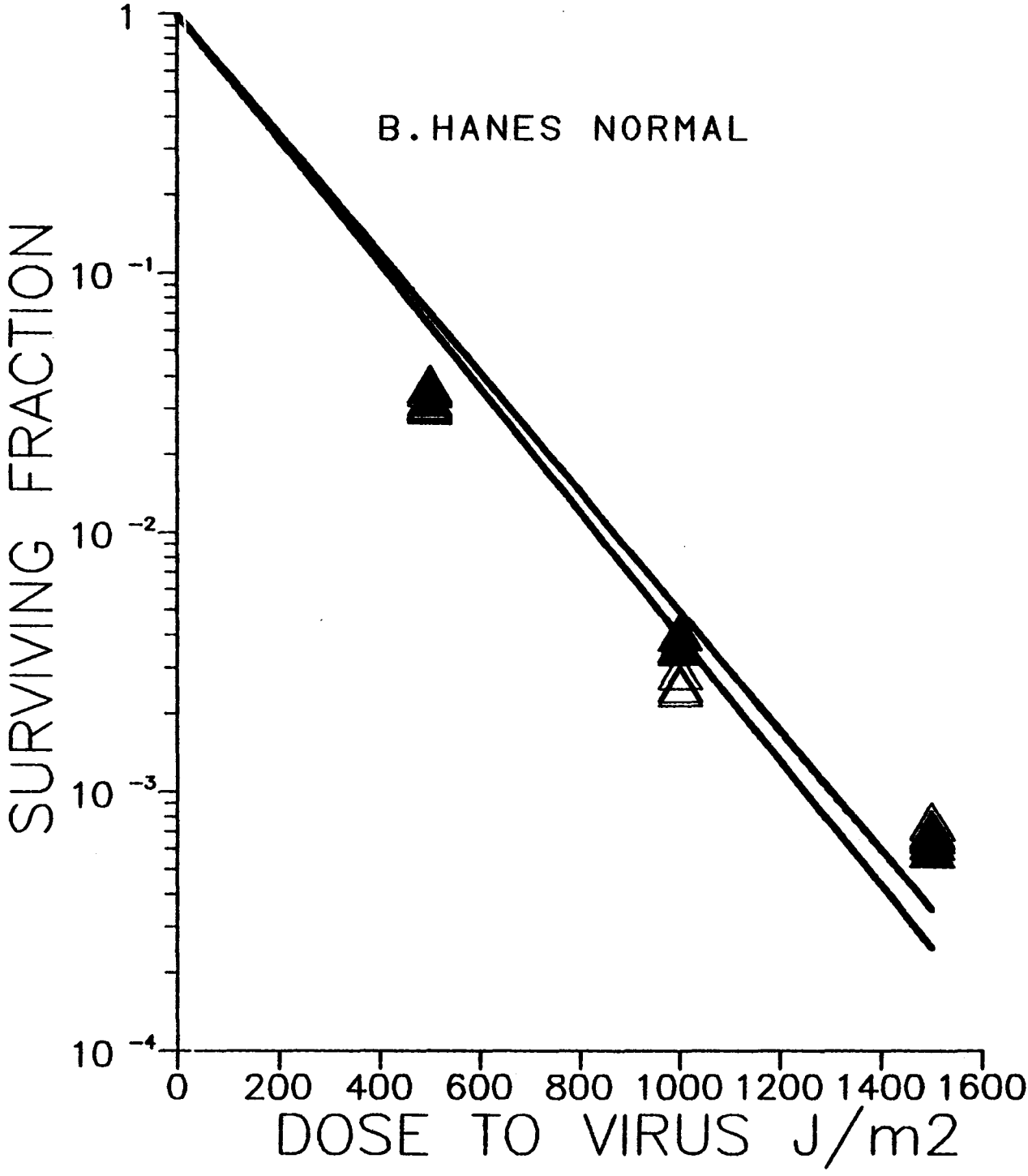

SURVIVING FRACTION
1
10 -1
10 -2
10 -3
10 -4
0
50
100
150
200
250
300
350
400
DOSE TO VIRUS J/m2
GM 2415 XPE

Figure 19. Survival of Ad Den V and Ad VSV on Normal Human Fibroblasts Cells from B. Hanes.

Shown are the results for three separate experiments. Dark symbols are Ad Den V, open symbols are Ad VSV. Lines are drawn from D_0 values calculated in Tables 1 and 2. Error bars for individual experiments are within the symbol size. Surviving fraction was calculated as the titre of surviving virus at the dose point divided by the titre of virus in the unirradiated input virus suspension. The cells used in this experiment were a primary culture obtained from a patient at McMaster University Hospital by Dr. Pat Chang.


B. HANES NORMAL
SURVIVING FRACTION
1
10 -1
10 -2
10 -3
10 -4
0
200
400
600
800
1000
1200
1400
1600
DOSE TO VIRUS J/m2

is also included in the tables. Bloom's Syndrome cells appear similar in most criteria to normal cells, with the exception of a 10 to 15 fold increase in the number of sister chromatid exchanges post UV irradiation, in comparison to normal human fibroblasts (Chaganti et al 1984). This is accompanied by an increased amount of chromosomal abnormalities, such as breaks and rearrangements (German et al 1965). While implication of a defect in the cellular DNA repair mechanism is marginal, strain GM 1492 has been noted as exceptional in that it exhibits a higher sensitivity to UV irradiation (Krepinsky et al 1980). It has recently been demonstrated that the ligase I activity in Bloom's syndrome cells is decreased in comparison to normal cells, and this has been suggested to be a contributing factor to the deficiency (Willis and Lindahl 1987, Chan et al 1987). When UV survival of Ad Den V and the control virus was assayed, survival was seen to be similar for both viruses (Tables 1 and 2), as was the case in Cockayne's Syndrome cells (Figure 18). This suggests the deficiency in Bloom's Syndrome cells is not in the incision step of excision repair.

Discussion

Overview

This study demonstrates the use of an adenovirus vector system for the study of a DNA repair gene in untransformed human cell lines. To date, DNA mediated transfection of transformed cells has been the most viable technique available for the study of DNA repair genes (Thompson et al 1987, Green et al 1987). However, this technique has several drawbacks. The DNA repair profile of transformed cells has been shown to be altered in comparison to their untransformed counterparts (Squires et al 1982) and therefore one might not be looking at gene function in a normal environment. A number of adenovirus vectors have recently been developed which could be utilized to overcome this problem (Ghosh-Choudhury et al 1987, Haj-Ahmad and Graham 1986). One such vector is Ad 5 dlE3 (Haj-Ahmad and Graham 1986).

In this work the bacteriophage T4 den V gene has been inserted into the E3 region of Ad dlE3. This vector was then used to deliver and examine the effect of the den V gene product on UV viral survival in human XP fibroblasts. The effect of the den V gene was examined by assaying for the host cell reactivation of Vag production for UV irradiated Ad Den V and comparing it to that for Ad VSV. It was shown that the relative %HCR was significantly greater for Ad Den V as compared to Ad VSV in XP cells but there was no significant difference in normal or Cockayne's syndrome

cells.

Using this method a partial complementation of the DNA repair deficiency in XP cells upon the introduction of the den V gene was demonstrated. Unlike the restoration of post UV irradiation unscheduled DNA synthesis to normal levels seen by using the technique of microinjection of the den V protein (Hoeijmakers 1987), survival of UV irradiated virus did not reach levels obtained in normal fibroblasts. These results are similar to those where biological restoration of colony forming ability was examined in transformed XP cells after UV irradiation (Tanaka et al 1977, Valerie et al 1987a, Arrand et al 1987) in that in all cases the introduction of the den V gene did not result in the restoration of normal levels of UV resistance. This suggests the restoration of unscheduled DNA synthesis does not necessarily lead to complete biological restoration of DNA repair to normal levels (Valerie et al 1987a, Arrand et al 1987). It has been suggested that the den V's ability to restore DNA repair is not due to a direct complementation of a deficient gene, but rather by the substitution of an alternate system of initial dimer incision, since normal repair levels are not obtained (Arrand et al, 1987). The 6-4 photoproduct of UV irradiation is a form of damage that den V gene product does not recognize (Mitchell et al 1982). It has recently been demonstrated that the 6-4 photoproduct plays a determinate role in UV cell survival, possibly explaining the inability

of the den V gene product to restore UV survival to normal levels (Cleaver et al 1988, Mitchell 1988). It has also been suggested that some DNA repair deficiencies involve an inability to alter the chromatin secondary structure and allow access to the damage of the repair enzymes (Hoeijmakers 1987). The two component nature of the UV survial curves for adenovirus seen in this study suggest the existence of two processes occuring during mammalian nucleotide excision repair. The results of this study suggest that one of the two processes might be representative of the mechanism of structural modification to allow access of repair enzymes.

The Ad Den V vector used in this study illustrates the advantage of an adenovirus vector system over the DNA mediated transfection method in the study of the effect of DNA repair genes in mammalian cells. To further extend the possible modes of investigation using viral vectors with DNA repair genes, the construction of an E1 deleted mutant of Ad Den V, which can be grown in 293 cells (Graham et al, 1977) but results in non-lytic infection of HeLa cells and human fibroblasts, would be of value. Infection of primary human fibroblasts with such a construct would allow a study of the repair capability of the cells themselves by an examination of colony survival after UV irradiation.

Construction of Ad Den V Viral Vector

The construction of the Ad Den V recombinant adenovirus viral vector was based on the previous construction of similar vectors that successfully expressed a foreign gene upon infection of mammalian cells (Haj-Ahmad and Graham 1986). A study of the packaging constraints of adenovirus type 5 showed that up to approximately 2 kb of additional DNA can be inserted into the genome and the virus still is successfully packaged (Ghosh-Choudhury et al 1987). As expected, the insertion of the 1.91 kb den V expression cartridge in the E3 region of adenovirus type 5 in the deleted region between the Xba I sites at 78.5% and 84.7%, was successfully packaged and the virus produced was found to be infective. The den V expression cartridge was inserted in the opposite orientation to the E3 promoter and was shown to express RNA and was biologically active. This parallels previous observations of the Adtk virus, which was also shown to express the inserted gene under the control of the HSV tk promoter (Haj-Ahmad and Graham 1986). The construction technique employed in the production of Adtk was used in this work to construct a recombinant adenovirus virus carrying the T4 den V gene (Ad Den V).

Ad Den V was constructed as outlined in materials and methods and Figure 6. The den V expression cartridge was received in the plasmid pEMBL18/RSVdenV on a 1.91 kb

fragment. The successful transfer of this fragment to plasmid pFGDX1 has been demonstrated, as can be seen in the gel and subsequent Southern Blot analysis of that gel in Figure 7. The plasmid produced by this insertion, pMCADV, was then used to cotransfect 293 cells along with Eco RI digested Ad 5 dlE309. While only 3 resulting viruses out of 24 screened proved to be recombinants carrying the den V sequences, this was a similar result to previous experiments in which the same system was used to construct recombinant adenoviruses with different inserts of approximately the same size (Ghosh-Choudhury, personal communication). The recombinant viruses were plaque purified three times, and after initial biological testing it was found that there was no difference in activity between the three isolates (data not shown). One of the isolates was used for a large scale viral preparation which was used in all subsequent experiments (Ad Den V).

Protein Expression

In order to determine if the constructed recombinant virus was successfully producing den V protein, monoclonal and polyclonal antibodies directed against the den V gene were obtained from Dr E. Henderson (Temple University, Department of Immunology and Microbiology, Pennsylvania). The antibodies provided were directed against a den V-Protein A

fusion protein, and shown to be able to detect purified and partially purified den V gene product isolated from bacteria cells (Valerie et al 1987b). They have also been demonstrated to detect den V gene product from yeast cell extracts (Valerie et al 1986) and have been used in detection of den V protein by immunoflourescent staining of SV40 transformed XPA cells transfected with den V sequences (Valerie et al 1987a). The antibodies have not, however, been demonstrated to precipitate den V protein from crude protein extracts from mammalian cells. In order to detect the presence of den V protein in Ad Den V infected cells, protein extracts from numerous cell lines, at different infection times and multiplicities of infection were assayed by immunoprecipitation and Western blots. The results of an immunoprecipitation can be seen in Figure 8, in which it is possible to speculate a preferential binding to Ad Den V infected cellular proteins precipitated with monoclonal antibodies. However, the result becomes dubious in light of the background reaction with other cellular proteins in all lanes. In order to investigate this, and the potential of a cellular homolog of the den V gene, several uninfected cell protein extracts were immunoprecipitated in a similar manner. Figure 9 shows the results of one such experiment, in which various XP and normal human fibroblasts were immunoprecipitated with monoclonal den V antibodies. The cross reacting proteins, including a predominant species at

~16 KD, can be seen, perhaps more prominently in the XP cell lines. This effectively negates the usefulness of the antibody in distinguishing virally produced den V gene production comparison to uninfected and Ad VSV infected cells.

Since the original work done with the antibody utilized the Western blot technique, the same method was also attempted with Ad Den V infected cells. The results of this can be seen in Figure 10. Figure 10a shows the amido black stained protein transfer, which was subsequently washed and used in the alkaline phosphatase reaction with polyclonal anti-den V antibodies in Figure 10b. While results very similar to published work were obtained in the Ad Den V lane (reaction with a ~16 KD protein), similar results were also obtained in the control lanes, again negating the validity of the result. In order to demonstrate the viability of the technique, the protein transfer was subsequently reacted with the Vag antibody serum used in the viral survival assay. It can be seen in Figure 10c that there was specific reaction with adenovirus proteins, presumably hexon (II) and core (VII), confirming the functionality of the protocol. Further investigation of protein production was abandoned in light of these observations, as the antibodies provided appeared to give inconclusive results.

RNA Expression

The technique of primer extension of RNA has been previously used to determine the initiation site of RNA transcription from a gene under investigation (Jones et al 1985). Correct initiation of RNA transcription has also been used as an indication of gene expression (Smiley et al 1987). Since assaying for protein production was not feasible, correct initiation of transcription from the den V expression cartridge in Ad Den V was demonstrated. It can be seen from Figure 11 that RNA extracted from Ad Den V infected HeLa cells 22 hours post infection is capable of extending a 124 base fragment from the primer sequence. This indicates that initiation of transcription is at position 584 in the den V expression cartridge. This is the site of transcription initiation from the RSV promoter as previously reported (Yamamoto et al 1980). The transcription of correctly initiated RNA suggests that the den V expression cartridge in Ad Den V is functional upon infection of mammalian cells.

UV Survival of Ad Den V

Infectivity and Vag Production from Ad Den V

It has previously been shown that the production of Vag during adenovirus infection can be used as an assay for DNA

repair in human fibroblasts (Rainbow 1974). In this work the technique was utilized to determine the UV survival of Ad Den V in comparison to the control virus Ad VSV in several different cell lines. The linearity of the frequency of Vag positive cells with respect to relative viral input multiplicity is illustrated in Figure 13, and is comparable to previous results (Rainbow 1980). It can be seen from Figure 12 that the immunoflourescent staining for Vag production was effective, as previously demonstrated (Rainbow 1980). Based on this, UV survival of Vag formation for the recombinant and the control was used as a measure of the DNA repair capacity of human fibroblast cells. The comparison of the survival of each virus was taken as an indication of the relative increase in DNA repair capacity afforded the cell by the transient expression of the den V gene.

UV Survival of Ad Den V in Normal Human Fibroblasts

It can be seen from Figure 15 and 19 and from tables 1 and 2 that there is no significant difference between the survival of Vag production of Ad Den V and AD VSV in either normal cell strain. The average D_0 for Ad VSV was 190.5 $\pm$ 12 J/m^2 and 192.5 $\pm$ 13 J/m^2 for Ad Den V. This is comparable to previously reported values of UV survival of adenovirus type 2 plaque formation in normal human fibroblasts (220 $\pm$ 20 J/m^2, Day 1977). Previous work measuring survival of Vag

formation of adenovirus type 2 in normal human fibroblasts have reported D_0 values of 205 ± 23 J/m^2 (Bueschleb 1987), 320-370 ± 50 J/m^2 (Krepinsky et al 1980) and 380-410 ± 50-80 J/m^2 (Rainbow 1980). This suggests that there is no anomalous enhanced survival of the Ad Den V recombinant in a repair proficient cell line. The average D_0 values quoted were then used to calculate the percentage host cell reactivation (%HCR) of virus in repair deficient cell lines. By comparing the UV survival of a virus in normals cells and a mutant cell line, the percentage of DNA repair capacity remaining in the mutant cell line can be calculated. This value can be compared to other DNA repair assays, such as UDS, DNA repair synthesis and cell survival.

UV Survival of Ad Den V in XP Group A Cells

Xeroderma pigmentosum fibroblasts are deficient in the incision stage of excision repair. They exhibit many phenotypic variations from normal, including reduced ability to reactivate UV irradiated human adenovirus (Day et al 1974, Rainbow 1981). Due to the fact that the UV survival of adenovirus type 5 in XP cell lines in this study exhibited two components, two D_0 values were calculated for each XP UV survival curve. The D_0 values obtained in this work for the UV survival of Ad 5 in XP12BE (group A) cells were 16 ± 0.3 J/m^2 for the first component and 38 ± 2 J/m^2 for the second

component. Typical D_0 values obtained in other studies for the reactivation of UV irradiated adenovirus type 2 are in the range of 20 J/m^2 (Day 1977) to 27 J/m^2 (Rainbow 1980). Previous studies have reported %HCR of adenovirus type 2 virus for XP group A cells in the range of 3.4-6.8% (Day 1975) and at 6% (Rainbow 1981, Rainbow 1980) and a %HCR of 13% for SV40 DNA (Abrahams and van der Eb 1975). Ratios of the D_0 values of the post UV cell survival of XP cells in comparison to normal cells have reported in the range of 15% (Arrand et al 1987) to 17% (Andrews et al 1978). When post UV irradiation UDS was investigated, XP group A cells were seen to have 0-10% of normal cell levels (Friedburg et al 1978). The results for UV survival of the control virus in XP12BE group A in this study cells show a %HCR of 8.4 for the first component and 19.9 for the second (Table 1), comparable to previously reported findings.

UV survival of Ad Den V in XP group A cells indicates a D_0 value for the first component of 35 ± 3 J/m^2 and 79 ± 18 J/m^2 for the second component. When compared to Ad Den V UV survival in normal cells, a %HCR value for the first component of 18.2 and 41.0 for the second (Table 2) is calculated. This suggests that the presence and transient expression of the den V gene in Ad Den V enhances the cells DNA repair capacity, as reflected in the statistically significant increase in %HCR value (95% confidence). From this value, the percentage restoration of the DNA repair

deficiency in XP group A cells can be calculated. Previous studies have shown that the deficiency in UV cell survival in XP group A cells can be restored by the introduction of the den V gene protein by between 6 and 20% (Tanaka et al 1977, Valerie et al 1987a). The percentage restoration of UV viral survival seen here is comparable to this, 10% for the first component and 26% for the second (Table 3).

UV Survival of Ad Den V in Bloom's Syndrome and Cockayne's Syndrome Cells

A recent study has revealed that the incision of the DNA molecule at thymine dimers during nucleotide excision repair is normal in CS cells post UV irradiation, as determined by sucrose gradient centrifugation behaviour of post UV irradiated cellular DNA from CS and comparison to normal cells (Schwaiger et al 1986). Repair synthesis after the incision step of excision repair, as measured by autoradiography of ^{3}H thymidine incorporation, was found to be reduced in CS cells in comparison to normal cells after 4 hours post UV irradiation. This suggested that the defect in CS cells is possibly in the polymerization or ligation of newly synthesised DNA during the nucleotide excision repair process. It was also found in this study that the rejoining of the UV damaged DNA in CS cells was accelerated by the addition of extracellular NAD+ (Schwaiger et al 1986), a

requirement for the activation of the ligase enzyme by the ADP-ribosylation process (Creissen and Shall 1982). Similar work was previously done examining the accumulation of long lived DNA breaks in a CS cell line, also suggesting a defect in the polymerization or ligation step of excision repair (Squires and Johnson 1983). Work done on cells from Bloom's syndrome patients has demonstrated that DNA ligase I enzyme extracted from BS cells has reduced activity (Willis and Lindahl 1987). Mammalian DNA ligase I has been implicated as the ligase involved in DNA replication and not repair (Messina et al 1984). This suggests that the defect in CS and BS cells is at a later stage in DNA repair or it does not involve the DNA excision repair mechanism at all. Since incision in CS and BS cells is normal, the presence of the den V gene product would not be expected to enhance DNA repair capacity of the cells. This result was observed as can be seen in Figure 16 and Tables 1 and 2. This suggests that the complementation observed in the DNA repair deficient XP cell lines is specific for the type of mutation complemented. It appears that the den V gene is functioning to enhance UV host cell reactivation of Vag production of adenovirus by a mechanism specific to its enzymatic function.

UV Survival of Ad Den V in Other Cell Lines

The usefulness of the adenovirus vector system in the study

of DNA repair genes is demonstrated by the rapid testing of several other cell lines for complementation by the den V gene. The first such cell line investigated was XP group C cells, GM 677. It can be seen from Figure 17 that results quite different from those obtained with XP group A cells were observed. The UV survival of Ad Den V in comparison to Ad VSV showed a dramatic increase in the second component, while the first component remained relatively unchanged. This differential response to complementation by den V might reflect the previously observed differences between XP complementation groups in other assays (Edwards 1987, Sidik and Smerdon 1987, Cleaver 1987).

It can be seen from Figure 18 that when UV survival was assayed in XP group E cells, the second component was restored to wild type levels, while the first component showed a moderate increase, similar to the response seen in XP group A cells. This demonstrates the differential response between XP complementation groups that can be detected by this assay.

Two Component Nature of Mammalian Nucleotide Excision Repair

The two component nature of UV cell survival has been previously noted in XP group A,D and C cells, and can be observed in normal human fibroblasts upon treatment with drugs which selectively block repair pathways. It has been

suggested that this is indicative of two nucleotide excision repair processes taking place (Tyrrell and Amaudruz 1987).

Individual polymerase species found in mammalian cells have also been associated with different aspects of these repair processes (Friedberg 1985). While it has been suggested that the two independent pathways arise from independent polymerase action (Tyrrell and Amaudruz 1987), the present data on the role of secondary structure in repair (Sidik and Smerdon 1987, Cleaver 1987) suggests that access to the damage could also be a fundamental stage in the nucleotide excision repair process.

DNA Secondary Structure and Repair

The alteration of chromatin structure preceding DNA repair has been experimently substantiated (Hittelman 1986). Just as DNA replication or RNA transcription require an unwinding or loosening of the chromatin structure of the eukaryotic chromosome to allow access by enzymes, so would a DNA repair mechanism. This has been observed both directly (Hittelman 1986) and biochemically (Mitchell et al 1982, Sidik et al 1987). This change appears to consist of two stages, first the elongation of prematurely condensed chromatin and second the local decondensation in the elongated regions (Hittelman 1986). While the initial stage is apparently functional in XPA cells (Sidik et al 1987), the localized decondensation

is not observed (Hittelman 1986).

This observation correlates with the fact that XP cells are proficient in the incision of thymine dimers from naked DNA. If the DNA is in a chromatin structure however, only XP group D cells have been shown to be able to perform the incision (Sidik and Smerdon 1987, Mortelmans et al 1976, Kano and Fujiwara 1983). It has also been shown that the ability of XP cell extracts to incise thymine dimers is dependent upon the removal of non-histone proteins only (Kano and Fujiwara 1983). XP cells have near normal levels of repair of DNA damage caused by MMS (Friedburg et al 1979, Andrews 1983) and this repair is thought to reflect the action of DNA glycosylases and AP endonucleases (Lindhal 1982). This suggests that excision repair in XP cells can be initiated by much smaller enzymes, such as glycosylases. From this it can be concluded that the method of complementation of the DNA repair deficiency in XP cells by the den V gene is probably through the ability of the small enzyme to gain access to the dimers. This can occur irrespective of the XP cells' deficiency in the alteration of chromatin structure accompanying DNA repair in normal cells.

The Affect of den V on XP Group C Cells

There are several noteworthy differences between XP group C cells and other XP complementation groups. Results from the

study of XPC cells have indicated the following characteristics of XPC cells. DNA repair in quiescent XPC cells is localized in essential genes (Cleaver 1987), which are located in nuclease sensitive regions (Player and Kantor 1987), at the attachment point to the nuclear scaffold (Mullenders et al 1986). XPC cells are proficient in repair of these regions, but deficient in the aphidicolin sensitive DNA polymerase α/δ pathway which is responsible for DNA repair in the rest of the genome (Player and Kantor 1987). It has been suggested that the partial DNA repair capacity in vivo of XPC cells is due to a retention of at least some of the chromatin decondensation activity (Sidik and Smerdon, 1987). This is substantiated by the fact that the nucleosome rearrangements that occur during DNA repair are present in this complementation group (Smerdon et al 1979).

The results presented here demonstrate that the den V gene is able to enhance the second component of the UV viral survival curve in XPC cells, but has no observed effect on the first component. This result correlates with the evidence that XPC cells are deficient in only one of the two repair processes. The lack of change in the first component could indicate that the den V gene is unable to complement this aspect of the deficiency, however this is unlikely since the first component was affected in group A and E cells. It more likely reflects the fact that the deficiency in the first component of UV viral survival in XPC cells can not be

enhanced beyond that already observed (a D_0 of 34 J/m^2, Tables 1 and 2). This value is similar to the increase seen in XP group A cells upon the addition of the den V gene product, upon which the first component is increase to a D_0 value of 35 J/m^2. It has been suggested that XPC cells possess at least partial ability to make the chromatid rearrangements necessary for excision repair (Sidik and Smerdon 1987). It has also been previously suggested that the den V gene is able to partially complement the deficiency in XP cells as a result of its small size, gaining access to thymine dimers which the host cell repair enzymes can not (Lindahl 1982). If the increase in UV viral survival seen in the first component of XPA cells is due to den V action as proposed, and since XPC cells apparently do not suffer the same access deficiency as XPA cells, one would expect the level of increased survival seen in XPA cells to equal the level observed in XPC cells with or without the den V gene product, and this is what was observed in this work. The fact that neither cell line was restored to normal levels of UV viral reactivation for this component suggests that even with the effective removal of thymine dimers from the genome, normal levels of UV viral survival can not be achieved. This correlates with the recent observation that major form of damage caused by UV irradiation is the 6-4 photoproduct (Cleaver 1988, Chan et al 1988). This form of damage can not be repaired by the den V gene, possibly accounting for the

remainder of the deficiency seen in the first component.

The results presented here suggest that the first component of UV viral survival curves at least in part reflects the component of DNA nucleotide excision repair that is involved in accessing the lesion to be repaired. Other observations, such as the two component nature of herpes simplex virus host cell reactivation, also contributes to the hypothesis that two mechanisms are involved in the viral repair process (Ryan and Rainbow 1986). However, the relationship, if any, between the two processes indicated by the two component nature of UV viral survival in XP cells and the two pathways suggested by Tyrrell and Amaudruz is uncertain.

The Affect of the den V gene on XPE Cells

To date, XP group E cells have not been investigated in detail. XPE cells exhibit a high level of post UV unscheduled DNA synthesis in comparison to other XP complementaion groups (69-84% of normal cells (Kondo et al 1987)). They also have a higher level of removal of UV endonuclease sensitive sites post UV irradiation (60% of normal cell levels (Paterson et al 1987)). The results presented in this work demonstrate an increase in both components of UV viral survival, and the restoration to wild type levels in the second component following introduction

of the den V gene. This result suggests that XP group E cells are more effectively complemented by the den V gene, correlating with previous results in that the observed DNA repair deficiency is initially not as severe as in other XP groups. It is possible to suggest, in light of the XPC results presented here, that while the first component of UV viral survival might reflect the accessibility of the repair enzymes to the damaged sites and is more strongly influenced by the repair of 6-4 photoproducts, the second component might be more influenced by the repair of thymine dimers since this component can be increased to normal levels in the XP group E strain. However, XP groups A and C did not obtain D0 values for the second component similar to normal cell levels. The full restoration seen in XPE cells might be due to the fact that XP group E cells have been observed to possess near normal levels of post-replication repair (Lehmann et al 1977).

Conclusions

It would appear that a fundamental deficiency in XP cells is the inability of the required enzymes to gain access to UV induced lesions. The den V gene is able to complement this defect, due to the fact that it can substitute a novel thymine dimer specific endonuclease which is small enough to function in the absence of the chromatin relaxation activity

seen in normal cells. The evidence of the existence of two steps in the incision stage of DNA excision repair pathways is suggested by the reported initial change in secondary structure before the actual repair process. This work suggests that the first of these two steps could be reflected in the first component of the UV viral survival curves in XP cells. The results from UV survival of virus in XPA and XPC cells suggest that the first component seen in viral survival is also potentially influenced by 6-4 photoproducts. The ability of the second component to achieve normal levels of viral reactivation upon introduction of the den V gene product into XPE cells might reflect the increased role of the process of enzymatic repair of thymine dimers in this component and the involvement of post-replication repair.

It has also been demonstrated that an adenovirus vector can be effectively used to study the effect of DNA repair genes in a variety of untransformed mammalian cell types. The use of the RSV 3′ LTR promoter appears to be an effective choice since abundant transcription occurs from this promoter during infection by the virus.

References

Aaronson, S. A. and Lytle, C. D. (1970). Decreased host cell reactivation of irradiated SV40 virus in xeroderma pigmentosum. Nature **228**, 359.

Abrahams, P. J. and Van der Eb, A. J. (1976). Host-cell reactivation of UV-irradiated SV40 DNA in five complementation groups of xeroderma pigmentosum. Mutat. Res. **35**, 13.

Amari, N. M., Lambert, W. C. and Lambert, M. W. (1986). Comparison of histones in normal and xeroderma pigmentosum lymphoblastoid cells. Cell Biol. Int. Rep. **10(11)**, 875.

Andrews, A. D., Barrett, S. F., Yoder, F. W. and Robbins, J. H. (1978). XP neurological abnormalities correlate with colony forming ability after UV irradiation. Proc. Natl. Acad. Sci. USA **75**, 1984.

Arrand, J. E., Squires, S., Bone, N. M. and Johnson, R. T. (1987). Restoration of UV-induced excision repair in Xeroderma D cells transfected with the denV gene of bacteriophage T4. EMBO J. **6**, 3125.

Backendorf, C. M., van den Berg, E. A., Brandsma, J. A., Kartasova, T., van Sluis, C. and van de Putte, P. (1983). In vivo regulation of the UVR and SSB genes in Escherichia coli. In Cellular responses to DNA damage, E.C. Friedberg and B.A. Bridges, eds, New York: Alan Liss. 161.

Ben-Hur, E. and Ben-Ishai, R. (1968). Trans-syn thymine dimers in ultraviolet-irradiated denatured DNA: Identification and photoreactivability. Biochem. Biophys. Acta. **166**, 9.

Bloom, D. (1966). The syndrome of congenital telangiectatic erythema and stunted growth: observations and studies. J. Pediat. **58**, 103.

Bodell, W. J. (1977). Nonuniform distribution of DNA repair in chromatin after treatment with methylmethane sulfonate. Nucleic Acids Res. **4**, 2619.

Bueschleb, A. B. (1987). Reactivation of UV-irradiated type 2 and herpes simplex virus type 1 in mammalian cells. M.Sc. thesis, McMaster University, Department of Health and Radiation Physics.

Chaganti, R. S. K., Schonberg, S. and German, J. (1974). A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc. Natl. Acad. Sci. USA **71**, 4508.

Chan, J. Y. H., Becker, F. F., German, J. and Ray, J. H. (1987). Altered DNA ligase I activity in Bloom's syndrome cells. Nature **325**, 357.

Cleaver, J. E. (1986). DNA repair in human xeroderma pigmentosum group C cells involves a different distribution of damaged sites in confluent and growing cells. Nucleic Acids Res. **14(20)**, 8155.

Cleaver, J. E. (1987). Relative importance of incision and polymerase activities in determining the distribution of damaged sites that are mended in xeroderma pigmentosum group C cells. Cancer Res. **47**, 2393.

Cleaver, J. E., Cortes, F., Karentz, D., Lutze, L. H., Morgan, W. F., Player, A. N., Vuksanovic, L. and Mitchell, D. L. (1988). The relative biological importance of cyclobutane and (6-4) pyrimidine-pyrimidone dimer photoproducts in human cells: Evidence from a xeroderma pigmentosum revertant. Photochem. Photobiol. **48(1)**, 419.

Cleaver, J. E., Cortes, F., Lutze, L. H., Morgan, W. F., Player, A. N. and Mitchell, D. L. (1987). Unique DNA repair properties of a Xeroderma Pigmentosum revertant. Mol. Cell. Biol. **7**, 3353.

Collins, J. M. and Chu, A. K. (1987). Binding of the DNA polymerase alpha-DNA primase complex to the nuclear matrix in HeLa cells. Biochemistry **26(18)**, 5600.

Cook, K. H. and Friedberg, E. C. (1978a). Multiple thymine dimer excising nuclease activities in extracts of human KB cells. Biochemistry **17**, 850.

Cook, K. H. and Friedberg, E. C. (1978b). Partial purification and characterization of three thymine dimer excising activities from human KB cells. In DNA repair mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York. 301.

Creissin, D. and Shall, S. (1982). Regulation of DNA ligase activity by poly(ADP-ribose). Nature **296**, 271.

Day, R. S. III. (1975). Caffeine inhibition of the repair of UV-irradiated adenovirus in human cells. Mutat. Res. **33**, 321.

Day, R. S. III. (1977). Human adenoviruses as DNA repair probes. In DNA Repair Processes: Cellular Senescence and Somatic Cell Genetics, eds W.W. Nichols and D.G. Murphy. **1**, 119.

Day, R. S. III. (1978). Viral probes for mammalian cell DNA repair: results and prospects. In DNA Repair Mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York. **9**, 531.

Day, R. S. III. (1974). Studies on repair of adenovirus 2 by human fibroblasts using normal, xerderma pigmentosum, and xeroderma pigmentosum heterozygous strains. Cancer Res. **34**, 1965.

Day, R. S. III and Ziolkowski, C. (1978). Studies on UV-induced viral reversion, Cockayne's syndrome and MNNG damage using adenovirus 5. In DNA Repair Mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York. **9**, 535.

Dente, L., Cesareni, G. and Cortese, R. (1983). pEMBL: a new family of single stranded plasmids. Nucleic Acids Research **11**, 1645.

Doniger, J. and Grossman, L. (1976). Human correxonuclease. Purification and properties of a DNA repair exonuclease from placenta. J. Biol. Chem. **251**, 4579.

Dresler, S. L. and Kimbro, K. S. (1987). 2',3'-dideoxythymidine 5'-triphosphate inhibition of DNA replication and ultraviolet-induced DNA repair synthesis in human cells: evidence for involvement of DNA polymerase delta. Biochemistry **26(10)**, 2664.

Edwards, S., Fielding, S. and Waters, R. (1987). The response to DNA damage induced by 4-nitroquinoline-1-oxide or its 3-methyl derivative in xeroderma pigmentosum fibroblasts belonging to different complementation groups. Carcinogenesis **8(8)**, 1071.

Elledge, S. J. and Walker, G. C. (1983). Proteins required for ultraviolet light and chemical mutagenesis in Escherichia coli: identification of the products of the umuC locus of Escherichia coli. J. Mol. Biol. **164**, 175.

Fornace, A. J. . Jr., Dobson, P. P. and Kinsella, T. J. (1986). Repair of gamma-ray-induced DNA base damage in xeroderma pigmentosum cells. Radiat. Res. **106(1)**, 73.

Friedberg, E. C., Ehmann, U. K. and Williams, J. I. (1979). Human diseases associated with defective DNA repair. Adv. Rad. Biol. **8**, 85.

Friedberg, E. C. (1972). Studies on the substrate specificity of the T4 excision repair endonuclease. Mutat. Res. **15**, 113.
Friedberg, E. C. and King, J. J. (1971). Dark repair of ultraviolet-irradiated deoxyribonucleic acid by bacteriophage T4: purification and characterization of a dimer-specific phage-induced endonuclease. J. Bacteriol. **106**, 500.

Friedberg, E. C. (1985). DNA Repair. W.H. Freeman and Company, Ron Newcomer and Associates, San Francisco.

Ganesan, A. K., Seawell, P. C., Lewis, R. J. and Hanawalt, P. C. (1986). Processivity of T4 endonuclease V is sensitive to NaCl concentration. Biochemistry **25**, 5751.

German, J., Archibald, R. and Bloom, D. (1965). Chromosomal breakage in a rare and probably genetically determined syndrome of man. Science **148**, 506.

Ghosh-Choudhury, G., Haj-Ahmad, Y. and Graham, F. L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. The EMBO Journal **6**, 1733.

Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I. and Howard, B. H. (1982). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**, 6777.

Gorman, C., Padmanabhan, R. and Howard, B. H. (1983). High Efficiency DNA-Mediated Transformation of Primate Cells. Science **221**, 551.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**, 59.

Graham, F. L. and Van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**, 456.

Green, M. H. L., Lowe, J. E., Arlett, C. F., Harcourt, S. A., Burke, J. F., James, M. R. and Lehmann, A. R. (1987). A gamma-ray-resistant derivative of an ataxia telangiectasia cell line obtained following DNA-mediated gene transfer. J. Cell Sci. Suppl. **6**, 127.

Green, M., Pina, M., Kimes, R., Wensink, P. C., MacHattie, L. A. and Thomas, C. A. (1967). Adenovirus DNA. I. Molecular weight and conformation. Proc. Natl. Acad. Sci. USA **57**, 11302.

Haj-Ahmad, Y. and Graham, F. L. (1986). Development of a Helper-Independent Human Adenovirus Vector and Its Use in the Transfer of the Herpes Simplex Virus Thymidine Kinase Gene. J. Virol. **57**, 267.

Hanawalt, P. C., Cooper, P. K., Ganesan, A. K. and Smith, C. A. (1979). DNA repair in bacteria and mammalian cells. Ann. Rev. Biochem. **48**, 783.

Higgins, K. M. and Lloyd, R. S. (1987). Purification of the T4 endonuclease V. Mutat. Res. **183**, 117.

Hittelman, W. N. (1986). Visualization of chromatin events during DNA excision repair in XP cells: deficiency in localized but not generalized chromatin events. Carcinogenesis **7(12)**, 1975.

Hoar, D. I. and Davis, F. (1979). Host-cell reactivation of UV-irradiated adenovirus in Cockayne syndrome. Mutat. Res. **62**, 401.

Hoeijmakers, J. H. (1987). Characterization of genes and proteins involved in excision repair of human cells. J. Cell Sci. (Suppl) **6**, 111.

Hoeijmakers, J. H. J., Zwetsloot, J. C. M., Vermeulen, W., De Jonge, A. J. R., Backendorf, C., Klein, B. and Bootsma, D. (1983). Phenotypic correction of xeroderma pigmentosum cells by microinjection of crude extracts and purified proteins. In Cellular responses to DNA Damage, E.C. Friedberg and B.A. Bridges, eds, New York: Alan Liss. 173.

Hollis, G. F. and Grossman, L. (1981). Purification and characterization of DNase VII, a 3'-5' directed exonuclease from human placenta. J. Biol. Chem. **256**, 8074.

Horwitz, M. S. (1971). Intermediates in the replication of type 2 adenovirus DNA. Virology **8**, 675.

Horwitz, M. S. (1985). Adenoviruses and their replication . In Fields Virology, eds B.N. Fields, R.M. Chanock, J.L. Melnick, B. Roizman, R.E. Shope. Raven Press, New York.

Howard-Flanders, P., Boyce, R. P. and Theriot, R. (1966). Three loci in Escherichia coli K-12 that control the excision of thymine dimers and certain other mutagen products from host or phage DNA. Genetics **53**, 1119.

Jeeves, W. P. and Rainbow, A. J. (1986). An aberration in gamma-ray-enhanced reactivation of irradiated adenovirus in ataxia telangiectasia fibroblasts. Carcinogenesis **7(3)**, 381.

Johnson, D. A., Gautsch, J. W., Sportman, J. R. and Elder, J. H. (1984). Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. **1**, 3.

Johnson, R. T., Squires, S., Elliott, G. C., Rainbow, A. J., Koch, G. L. E. and Smith, M. (1986). Analysis of DNA repair in XP-HeLa hybrids; lack of correlation between excision repair of UV damage and adenovirus reactivation in an XP(D)-like cell line. Carcinogenesis **7**, 1733.

Jones, K. A., Yamamoto, K. R. and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**, 559.

Jones, N. and Shenk, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **16**, 683.

Kano, Y. and Fujiwara, Y. (1983). Defective thymine dimer excision from xeroderma pigmentosum chromatin and its characteristic catalysis by cell-free extracts. Carcinogenesis **4**, 1419.

Kaysen, J. H., Amari, N. M. and Lambert, M. W. (1986). Enhancement of two apurinic/apyrimidinic endonuclease activities from normal but not xeroderma pigmentosum lymphoblastoid cells by nucleosome structure. Mutat. Res. **165(3)**, 221.

Kondo, S., Satoh, Y. and Kuroki, T. (1987). Defect in UV-induced unscheduled DNA synthesis in cultured epidermal keratinocytes from xeroderma pigmentosum. Mutat. Res. **183(1)**, 95.

Kornberg, A. (1980). DNA Replication. W.H. Freeman and Company, San Francisco.

Krepinsky, A. B., Rainbow, A. J. and Heddle, J. A. (1980). Studies on the ultraviolet light sensitivity of Bloom's syndrome fibroblasts. Mutat. Res. **69**, 357.

Kunkel, T. A., Sabatino, R. D. and Bambara, R. A. (1987). Exonucleolytic proofreading by calf thymus DNA polymerase delta. Proc. Natl. Acad. Sci. USA **84(14)**, 4865.

Lehmann, A. R. and Karran, P. (1981). DNA repair. Int. Rev. Cytol. **72**, 101.

Lehmann, A. R., Kirk-Bell, S. and Arlett, C. F. (1977). Post-replication repair in human fibroblasts. In Research in Photobiology, ed A. Castellani, New York, Plenum, 293.

Lehmann, A. R. and Mayne, L. (1981). The response of Cockayne syndrome cells to UV-irradiation. In Chromosome damage and repair, eds E. Seeberg and K. Kleppe, New York: Plenum, 367.

Lindahl, T. (1976). New class of enzymes acting on damaged DNA. Nature **259**, 64.

Lindahl, T. (1982). DNA repair enzymes. Ann. Rev. Biochem **51**, 61.

Lindahl, T., Gally, J. A. and Edelman, G. M. (1969). Deoxyribonuclease IV: a new exonuclease from mammalian tissues. Proc. Natl. Acad. Sci. USA **62**, 597.

Little, J. W. and Mount, D. W. (1982). The SOS regulatory system of Escherichia coli. Cell **29**, 11.

Luria, S. E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. Proc. Natl. Acad. Sci. USA **33**, 253.

Lytle, C. D., Aaronson, S. A. and Harvey, E. (1972). Host-cell reactivation in mammalian cells II. Survival of herpes simplex virus and vaccinia virus in normal human and xeroderma pigmentosum cells. Int. J. Radiat. Biol. **22(2)**, 159.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Press, New York.

Mathis, G. and Althaus, F. R. (1986). Periodic changes of chromatin organization associated with rearrangement of repair patches accompany DNA excision repair in mammalian cells. J. Biol. Chem. **261**, 5758.

McMillan, S., Edenberg, H. J., Radany, E. H., Friedberg, R. C. and Friedberg, E. C. (1981). denV Gene of bacteriophage T4 codes for both pyrimidine dimer-DNA glycosylase and apyrimidinic endonuclease activities. J. Virology **40**, 211.

Mirkovitch, J., Mirault, M. E. and Laemmli, U. K. (1984). Organization of the higher-order chromatin loop: Specific DNA attachment sites on nuclear scaffold. Cell **39**, 223.

Mitchell, D. L. (1988). The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem. Photobiol. **48(1)**, 51.

Mitchell, D. L., Nairn, R. S., Alvillar, J. A. and Clarkson, J. M. (1982). Loss of thymine dimers from mammalian cell DNA. The kinetics for antibody binding sites are not the same as that for T4 endonuclease V sites. Biochim. Biophys. Acta **697**, 270.

Mortelmans, K., Friedberg, E. C., Slor, H., Thomas, G. and Cleaver, J. E. (1976). Evidence for a defect in thymine dimer excision in extracts of xeroderma pigmentosum cells. Proc. Natl. Acad. Sci. USA **73**, 2757.

Mullenders, L. H. F., van Kesteren, A. C., Bussmann, C. J. M., van Zeeland, A. A. and Natarajan, A. T. (1986). Distribution of UV-induced repair events in higher-order chromatin loops in human and hamster fibroblasts. Carcinogenesis **7**, 995.

Mulligan, R. C. and Berg, P. (1980). Expression of a Bacterial Gene in Mammalian Cells. Science **209**, 1422.

Mulligan, R. C. and Berg, P. (1981). Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**, 2072.

Nowak, R., Kulik, J. and Siedlecki, J. A. (1987). The ability of DNA polymerase beta to synthesize DNA beyond the gap with displacement of the non-replicated strand. Acta Biochim. Pol. **34(2)**, 205.

Okada, Y. and Tadokoro, J. (1961). Analysis of giant polynuclear cell formation caused by HVJ from ehrlich's ascites tumor cells. Experimental Cell Research **26**, 108.

Ottiger, H., Frei, P., Hassig, M. and Hubscher, U. (1987). Mammalian DNA polymerase alpha: a replication competent holoenzyme form from calf thymus. Nucleic Acids Res. **15(12)**, 4789.

Oudet, P., Gross-Bellard, M. and Chambon, P. (1975). Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. Cell **4**, 281.

Paterson, M. C., Bech-Hansen, N. T. and Smith, P. J. (1981). Heritable radiosensitive and DNA repair deficient disorders in man. In Chromosome damage and repair, eds E. Seeberg and K. Kleppe, New York: Plenum, 335.

Paterson, M. C., Middlestadt, M. V., MacFarlane, S. J., Gentner, N. E. and Weinfeld, M. (1987). Molecular evidence for cleavage of intradimer phosphodiester linkage as a novel step in excision repair of cyclobutyl pyrimidine photodimers in cultured human cells. J. Cell Sci. Suppl. **6**, 161.

Pedrali-Noy, G. and Spadari, S. (1980). Aphidicolin allows a rapid and simple evaluation of DNA repair synthesis in damaged human cells. Mutat. Res. **70**, 389.

Player, A. N. and Kantor, G. J. (1987). The endogenous nuclease sensitivity of repaired DNA in human fibroblasts. Mutat. Res. **184(2)**, 169.

Rainbow, A. J. (1978). Production of viral structural antigens by irradiated adenovirus as an assay for DNA repair in human fibroblasts. In DNA Repair Mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York. **9**, 541.

Rainbow, A. J. (1980). Reduced capacity to repair irradiated adenovirus in fibroblasts from xeroderma pigmentosum heterozygotes. Cancer. Res. **40**, 3945.

Rainbow, A. J. (1981). Reactivation of viruses. From Short term tests for chemical carcinogens. ed H.F. Stich, 20.

Rainbow, A. J. and Howes, M. (1979). Decreased repair of gamma-irradiated adenovirus in xeroderma pigmentosum fibroblasts. Int. J. Radiat. Biol. **36**, 621.

Rainbow, A. J. and Howes, M. (1982). A deficiency in the repair of UV and gamma ray damaged DNA in fibroblasts from Cockayne's syndrome. Mutat. Res. **93**, 235.

Roberts, J. J. (1978). The repair of DNA modified by cytotoxic, mutagenic and carcinogenic chemicals. Adv. Rad. Biol. **7**, 211.

Roth, M., Muller, H. and Boyle, J. M. (1987). Immunochemical determination of an initial step in thymine dimer excision repair in xeroderma pigmentosum variant fibroblasts and biopsy material from the normal population and patients with basal cell carcinoma and melanoma. Carcinogenesis **8(9)**, 1301.

Rupert, C. S. (1960). Photoreactivation of transforming DNA by an enzyme from baker's yeast. J. Gen. Physiol. **43**, 573.

Rupert, C. S. (1975). Enzymatic photoreactivation: overview. Molecular mechanisms for repair of DNA, eds P.C. Hanawalt, F.C. Friedberg, C.F.Fox, New York, Plenum, 73.

Rupert, C. S., Goodgal, S. H. and Herriott, R. M. (1958). Photoreactivation in vitro of ultraviolet inactivated Hemophilus influenzae transforming factor. J. Gen. Physiol. **41**, 451.

Rupp, W. D., Wilde, C. E. III, Reno, D. L. and Howard-Flanders, P. (1971). Exchanges between DNA strands in ultraviolet-irradiated Escherichia coli. J. Mol. Biol. **61**, 25.

Ryan, D. K. G. and Rainbow, A. J. (1986). Comparative studies of host-cell reactivation, cellular capacity and enhanced reactivation of herpes simplex virus in normal xeroderma pigmentosum and Cockayne syndrome fibroblasts. Mutat. Res. **166**, 99.

Sancar, A., Clarke, N. D., Griswold, J., Kennedy, W. J. and Rupp, W. D. (1981). Identification of the uvrB gene product. J. Mol. Biol. **148**, 63.

Sancar, A., Wharton, R. P., Seltzer, S., Kacinski, B. M., Clarke, N. D. and Rupp, W. D. (1981). Identification of the uvrA gene product. J. Mol. Biol. **148**, 45.

Schmickel, R. D., Chu, E. H. Y., Trosko, J. E. and Chang, C. C. (1977). Cockayne syndrome: A cellular sensitivity to ultraviolet light. Pediatrics **60**, 135.

Schwaiger, H., Hirsch-Kauffmann, M. and Schweiger, M. (1986). DNA repair in human cells: in Cockayne syndrome cells rejoining of DNA strands is impaired. Eur. J. Cell. Biol. **41(2)**, 352.

Seeberg, E. and Steinum, A. L. (1982). Purification and properties of the uvrA protein from Escherichia coli. Proc. Natl. Acad. Sci. USA **79**, 988.

Seeberg, E. and Steinum, A. L. (1983). Properties of the uvrABC endonuclease from Escherichia coli. In Cellular responses to DNA damage, E.C. Friedberg and B.A. Bridges, eds, New York: Alan Liss. 39.

Seki, S., Mori, S. and Oda, T. (1987). Bleomycin-induced DNA synthesis in a cell-free system using a permeable mouse sarcoma cell extract. Acta Med. Okayama **41(5)**, 195.

Setlow, R. B. (1968). The photochemistry, photobiology and repair of polynucleotides. Prog. Nuc. Acids Res. Mol. Biol. **8**, 257.

Setlow, R. B. (1978). Repair deficient human disorders and cancer. Nature **271**, 713.

Setlow, R. B. (1980). Damages to DNA that result in neoplastic transformation. Adv. in Biol. Med. Phys. **17**, 99.

Setlow, R. B., Regan, J. D., German, J. and Carrier, W. L. (1969). Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA . Proc. Natl. Acad. Sci. USA **64**, 1035.

Shu, L. M., Horwitz, M. S. and Engler, J. A. (1987). Expression of enzymatically active adenovirus DNA polymerase from cloned DNA requires sequences upstream of the main open reading frame. Virology **161(2)**, 520.

Sidik, K. and Smerdon, M. J. (1987). Rearrangement of nucleosome structure during excision repair in xeroderma pigmentosum (group A) human fibroblasts. Carcinogenesis **8(5)**, 733.

Simon, J. J., Smith, C. A. and Friedberg, E. C. (1975). Action of bacteriophage T4 ultraviolet endonuclease on duplex DNA containing one ultraviolet-irradiated strand. J. Biol. Chem. **250**, 8748.

Smerdon, M. J., Kastan, M. B. and Lieberman, M. W. (1979). Distribution of repair-incorporated nucleotides and nucleosome rearrangement in the chromatin of normal and xeroderma pigmentosum human fibroblasts. Biochemistry **18**, 3732.

Smerdon, M. J. and Lieberman, M. W. (1978). Distribution of UV-induced DNA repair synthesis in human chromatin. In DNA repair mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York. **9**, 327.

Smiley, J. R., Smibert, C. and Everett, R. D. (1987). Expression of a cellular gene cloned in herpes simplex virus: rabbit beta-globin is regulated as an early viral gene in infected fibroblasts. Journal of Virology **61(8)**, 2368.

Squires, S. and Johnson, R. T. (1983). UV induces long-lived DNA breaks in Cockayne's syndrome and cells from an immunodeficient individual (46BR): defects and disturbance in post incision steps of excision repair. Carcinogenesis **4(5)**, 565.

Subramani, S., Mulligan, R. and Berg, P. (1981). Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. Mol. Cell. Biol. **1**, 854.

Sutherland, B. M. (1974). Photoreactivating enzyme from human leucocytes. Nature **248**, 109.

Sutherland, B. M. (1978). Enzymatic photoreactivation of DNA. In DNA repair mechanisms, eds P.C. Hanawalt, E.C. Friedberg and C.F. Fox, Academic Press, New York, 113.

Sutherland, B. M., Rice, M. and Wagner, E. K. (1975). Xeroderma pigmentosum cells contain low levels of photoreactivating enzyme. Proc. Natl. Acad. Sci. USA **12**, 103.

Sutherland, B. M., Runge, P. and Sutherland, J. C. (1974). DNA photoreactivating enzyme from placental mammals. Origin and characterization. Biochemistry **13**, 4710.

Tanaka, K., Hayakawa, K., Sekiguchi, M. and Okada, Y. (1977). Specific action of T4 endonuclease V on damaged DNA in xeroderma pigmentosum cells in vivo. Proc. Natl. Acad. Sci. USA **74**, 2958.

Tanaka, K., Sekiguchi, M. and Okada, Y. (1975). Restoration of ultraviolet-induced unscheduled DNA synthesis of xeroderma pigmentosum cells by the concomitant treatment with bacteriophage T4 endonuclease V and HVJ (Sendai virus). Proc. Natl. Acad. Sci. USA **72**, 4071.

Tate, V. E. and Philipson, L. (1979). Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. Nucleic Acids Res. **6**, 2769.

Thompson, L. H., Salazar, E. P., Brookman, K. W., Collins, C. C., Stewart, S. A., Busch, D. B. and Weber, C. A. (1987). Recent progress with the DNA repair mutants of Chinese Hamster Ovary cells. J. Cell Sci. Suppl. **6**, 97.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**, 4350.

Tyrrell, R. M. and Amaudruz, F. (1987). Evidence for two independent pathways of biologically effective excision repair from its rate and extent in cells cultured from sun-sensitive humans. Cancer Res. **47**, 3725.

Valerie, K., De Riel, J. K. and Henderson, E. E. (1985b). Genetic complementation of UV-induced DNA repair in Chinese hamster ovary cells by the denV gene of phage T4. Proc. Natl. Acad. Sci. USA **82**, 7656.

Valerie, K., Fronko, G., Henderson, E. E. and De Riel, J. K. (1986). Expression of the denV gene of coliphage T4 in UV-sensitive rad mutants of Saccharomyces cerevisiae. Mol. Cell. Biol. **86**, 3559.

Valerie, K., Fronko, G., Long, W., Henderson, E. E., Nilsson, B., Uhlen, M. and De Riel, J. K. (1987a). Production and detection of coliphage T4 endonuclease V polyclonal and monoclonal antibodies using staphylococcal protein-A hybrid proteins. Gene **58**, 99.

Valerie, K., Green, A. P., De Riel, J. K. and Henderson, E. E. (1987b). Transient and stable complementation of ultraviolet repair in xeroderma pigmentosum cells by the denV gene of bacteriophage T4. Cancer Res. **47**, 2967.

Valerie, K., Henderson, E. E. and De Riel, J. K. (1984). Identification, physical map location and sequence of the denV gene from bacteriophage T4. Nucleic acids Res. **12**, 8085.

Valerie, K., Henderson, E. E. and De Riel, J. K. (1985a). Expression of a cloned denV gene of bacteriophage T4 in Escherichia coli. Proc. Natl. Acad. Sci. USA **82**, 4763.

Villani, G., Boiteux, S. and Radman, M. (1978). Mechanism of UV-induced mutagenesis: Extent and fidelity of in vitro DNA synthesis on irradiated templates. Proc. Natl. Acad. Sci. USA **75**, 3037.

Wahl, A. F., Crute, J. J., Sabatino, R. D., Bodner, J. B., Marraccino, R. L., Harwell, L. W., Lord, E. M. and Bambara, R. A. (1986). Properties of two forms of polymerase delta from calf thymus. Biochemistry **25**, 7821.

Williams, J. I. and Friedberg, E. C. (1979). Deoxyribonucleic acid excision repair in chromatin after ultraviolet irradiation of human fibroblasts in culture. Biochemistry **18**, 3965.

Willis, A. E. and Lindahl, T. (1987). DNA ligase I deficiency in Bloom's syndrome. Nature **325**, 355.

Yamamoto, T., de Crombrugghe, B. and Pastan, I. (1980). Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus. Cell **22**, 787.

Yeung, A. T., Mattes, W. B., Oh, E. Y. and Grossman, L. (1983). Enzymatic properties of the purified Escherichia coli uvrABC complex. In Cellular responses to DNA damage, E.C. Friedberg and B.A. Bridges, eds, New York: Alan Liss., 77.

Zhai, Z. H., Nickerson, J. A., Krochmalnic, G. and Penman, S. (1987). Alterations in nuclear matrix structure after adenovirus infection. J. Virol. **61(4)**, 1007.

Appendix

Mechanism of Immunoglobulin Recombination: Possible Origin in Prokaryotic SOS Regulated muc and Bacteriophage Lambda Genes

A Paper

Submitted

June 23, 1987

Abstract

The plasmid borne genes mucA,B (Mutagenesis, UV and Chemical), are homologous to and can complement the Escherichia coli umuC,D mutations. They are part of the S.O.S. repair system, which is recA dependent and inducible by DNA damaging agents. Activation results in error prone repair and consequently mutation. While there is implicit involvement of recombination in repair, the muc genes have demonstrated the recombinagenic nature of their primary DNA structure, independent of their gene products possible role. This work reports an analysis of the structure of the muc genes with respect to their homology with immunoglobulin sequences and their DNA recombination signals, and with the bacteriophage lambda integrase gene. It is shown that the putative VDJ signal sequences found in mucB can function as a recognition site for nuclear enzymatic activity as has been demonstrated with known immunoglobulin signals in vitro. The implications of a parallel prokaryotic recombination system in the genesis of lambda phage and immune system diversity is discussed.

The mucA,B Operon

The *E. coli* umuD,C genes are part of the error-prone DNA repair process which is referred to as the S.O.S. system. UmuD,C are under coordinate control of the recA-lexA repressor system (Little 1982) and mutants in the umuD,C locus are nonmutable by UV irradiation (Kato and Shinoura 1977). Mutations in the umuD,C locus can be complemented by the plasmid borne mucA,B genes, which have been sequenced and shown to be 52% homologous to the umuD,C operon. Both operons are polycistronic and contain two overlapping reading frames producing polypeptides of approximately 16 and 46 KD (Perry *et al* 1985).

While there has been considerable research done into the role of the umuD,C and mucA,B gene products in mutagenesis, there have also been observations regarding the nature of the primary DNA structure of the mucA,B operon. In the clinically isolated plasmid R46, which carries the muc genes, it can be seen that they, along with a region involved in replication, are framed by inverted repeats giving a transposon-like structure (Langer *et al* 1981). Recombinational instability of the primary DNA structure of the muc genes has been suggested by results of cloning attempts in *Haemophilus influenzae*. It was consistently observed that a specific fragment of the mucB coding region was spontaneously spliced out of a constructed plasmid and was subsequently found to

have been inserted in the bacteria genome upon transformation (Balganesh and Setlow 1985). The inserted fragment containing mucB coding sequences had consistently integrated proximal to a promoter, as implied from the fact that all clones were mucB+ as shown by complementation studies.

Recombination in Mammalian Immunoglobulin Genetics

Recombination events in the mammalian genome are an integral part of cellular development. While the genetics of immunoglobulin rearrangement has been well documented, the precise mechanism of the splicing involved has yet to be elucidated. Of interest here is the phenomenon of V-D-J joining. In the creation of antibody diversity one of multiple variable (V) regions is linked to the 5' end of the final immunoglobulin gene (Tonegawa 1983). An initial step in this process is the recognition of a signal sequence at the end of the V region, consisting of a 7mer followed by either 11 or 23 random bases (corresponding to one or two twists of the double helix) followed by a 9mer consensus sequence. The downstream target site has the complementary sequence, and several models for their interaction have been suggested (Early et al 1980, Tonegawa 1983). Investigation into the biochemical nature of this process has resulted in the detection of enzymatic activity in the nuclear extract of fetal mouse liver and B-lymphoid lines, which is able to

recognize and cleave this switch signal in vitro, suggesting its putative involvement in recombination (Desiderio and Baltimore 1984, Hope et al 1986).

Recombination in Bacteriophage Lambda

The bacteriophage lambda also undergoes site specific recombination during its lysogenic insertion and excision from the bacterial genome. This occurs at the phage att site, which is the point of cleavage of the circularized genome (Mizuuchi et al 1981). Enzymatically this is accomplished by the integrase gene product (gpint) along with a host factor (IHF) (Kikuchi and Nash 1978, Nash et al 1977). The int gene product has been shown to bind to specific bacterial and att sequences, namely P and P', and to bind and cleave at the core sequences B,B' and C,C' (Ross and Landy 1983, Ross and Landy 1982). Two P-type integrase binding sites are located at the very end of int transcription, and are therefore possibly involved in gene regulation. The binding site of the host factor has been identified (Craig and Nash 1984), and it has been suggested that IHF is involved in the site specific recombination process during lambda phage integration (Eisenstein et al 1987).

It has been speculated that the eukaryotic immunoglobulin site specific recombination system was 'borrowed' from

prokaryotes rather than invented (Shimizu and Honjo 1984). This is implied by similarities found between the two systems. It has been suggested that immunoglobulin recombination is regulated by accessibility of the DNA to recombinases (Yancopoulos et al 1986), which is similar to the proposed action of prokaryotic recombination enhancers (Kahmann et al 1985). Also, the upstream Ig enhancer is homologous to sequences found in several bacterial genes (Falkner et al 1986). By looking at the structural and functional relationship between the mucA,B operon and bacteriophage lambda, and also between the mucA,B operon and immunoglobulin recombination, parallels between the systems become evident.

Homology Analysis of the mucA,B Operon

1. Homology with Lambda Phage

Previous studies have reported that the mucA polypeptide is homologous to the bacteriophage lambda repressor protein cII (Perry et al 1985). In this study, computer analysis of the mucB protein sequence revealed a region of homology with the bacteriophage lambda integrase gene, which showed 68% homology to the mucB polypeptide over 92 amino acids (Figure 1). Homology analysis of the mucA,B operon DNA sequence revealed two major features. As mentioned, the lambda integrase protein binds to the P and P′ sites during the integration of the phage into the bacterial genome. Sites

Figure 1

Homology Between mucB and The Lambda Phage Integrase Gene

Listed is the homology between the mucB protein (top strand) and the lambda integrase gene (bottom strand). Homology is 62% conservation (.) and 25% identity (:) over 92 amino acids. Numbers refer to postion in the protein sequence.

```
145' AKLCNHAAKTWPATGGVVALDDGARLKKLMSILPVAEVWGVGHRTEKALATMGIK-TVLD
                                                .: :: ::..::: ..:
 39" RDRRIAITEAIQANIELFSGHKHKPLTARINSDNSVTLHSWLDRYEKILASRGIKQKTLI

204' LARADTRLIRKTF-GVVLER-TVRELRGEACFSLEENPPAKQQIVVSRSFGQRVETLTDM
      ..... ::... .. ::  :..:. .     ..:. .:..... :   ..  :....
 99" NYMSKIKAIRRGLPDAPLEDITTKEIAAMLNGYIDEGKAASAKLIRSTLSDAFREAIAEG

262' QQAVTGFAARAAEKLRNERQYCRVISVFIRTSPYSVRDTQYANQATEKLTVATQDSRTII
     . ...  ::  :.:
159" HITTNHVAATRAAKSEVRRSRLTADEYLKIYQAAESSPCWLRLAMELAVVTGQRVGDLCE
```

homologous to the P′ binding site (greater than 70% homology) are found flanking the start of transcription and also flanking the stop codon for the mucB gene. The integration process has been shown to also involve the binding of IHF to a 14mer consensus sequence (Craig and Nash 1984). A 12/14 match to this sequence is found immediately following the ATG of mucB.

2. Homology with Immunoglobulin Genes

As mentioned, there is a frameshift between the mucA and mucB reading frames, and a second region of high homology on the amino acid level was detected in the mucB coding sequence in the mucA reading frame. This region of homology was to an immunoglobulin variable region precursor gene and extended over 63 amino acids with 62% homology (Figure 2). It was also observed that the last 10 amino acids of this region were 100% homologous to a human Ig heavy V-III chain variable region (data not shown). When the mucA,B DNA sequence was analyzed, it was found that immediately following the homology to the immunoglobulin genes there was a 7mer and 9mer, separated by 11 base pairs, homologous to the VDJ recombination signal in the immunoglobulin system (FIgure 3). Two other 7mer/9mer signals were also found in the mucB sequence, and are also listed in Figures 4 and 5.

Figure 2 Homology with Ig Kappa chain V Region Precursor - Rabbit

Listed is the homology found between the translated muc sequence (top strand) and an Ig kappa chain V region precursor fragment (bottom strand). Homology is 62% conservation (.) and 32% identity (:) over 63 amino acids. Numbering of the muc sequence represents amino acid position relative to the start of mucA transcription. Amino acids were deduced from the nucleotide sequence in the mucA reading frame.

Figure 3

Analysis of muc sequence homology to VDJ recombination signals: muc signal at position 880

The first homologous signal (at position 880 in the mucA,B DNA sequence) is shown in the line beginning at position 858, and the consensus 7 mer and 9 mer sequences are listed above for comparison. A 5-7 match to the consensus 7 mer can be seen, with an 11 base spacer followed by a consensus 9mer. The 3' flanking region adjacent to the 7 mer is also homologous to the flanking region of the 7 mer found in the V heavy chain germline sequence, as indicated in the figure. It can also be seen that in both sequences listed there is a succession of TG dinucleotides in the region of the 7 mer.

Figure 4

Analysis of muc sequence homology to VDJ recombination signals: muc signal at position 1180

The second homologous signal (at position 1180 in the mucA,B DNA sequence) is shown in the line beginning at position 1159. It can be seen the the 9 mer in both sequences are identical. While there is only limited homology to a 7 mer in the muc sequence, the presence of TG dinucleotide pairs is again evident (underlined in both sequences). The presence of TG dinucleotides has been reported to increase recombination at such switches, and it has been suggested that they play a functional role in recombination (8).

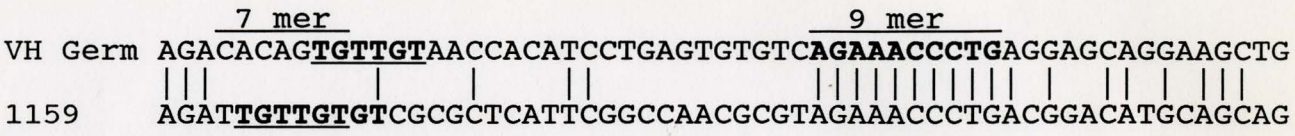
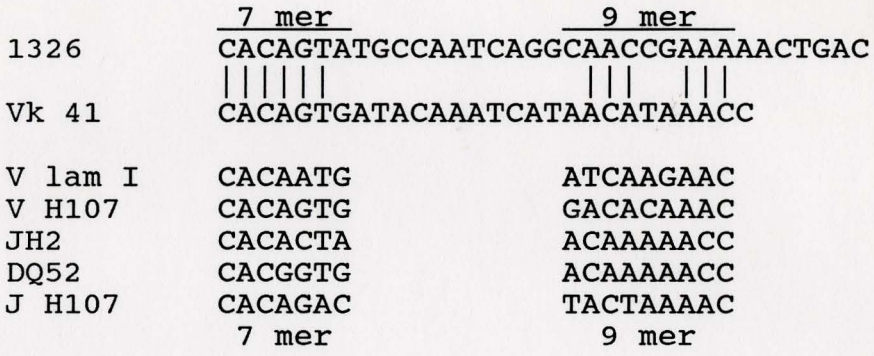

7 mer
9 mer
VH Germ AGACACAGTGTTGTAACCACATCCTGAGTGTGTCAGAAACCCTGAGGAGCAGGAAGCTG
1159 AGATTGTTGTGTCGCGCTCATTCGGCCAACGCGTAGAAACCCTGACGGACATGCAGCAG

Figure 5

Analysis of muc sequence homology to VDJ recombination signals: muc signal at position 1340

The third VDJ recombination signal (at position 1340 in the mucA,B DNA sequence) is listed, showing the presence of a 6/7 match to the consensus 7 mer and rough homology to the Vk 41 9 mer (Tonegawa 1983). Examples of reported 7 mers and 9 mers are listed below the Vk 41 sequence to illustrate the diversity found in actual switch signals. The V lam I, V H107 and J H107 sequences are examples of VDJ recombination signals as assembled from Tonegawa 1983. The JH2 and DQ52 recombination signals were from Sakano et al 1981.

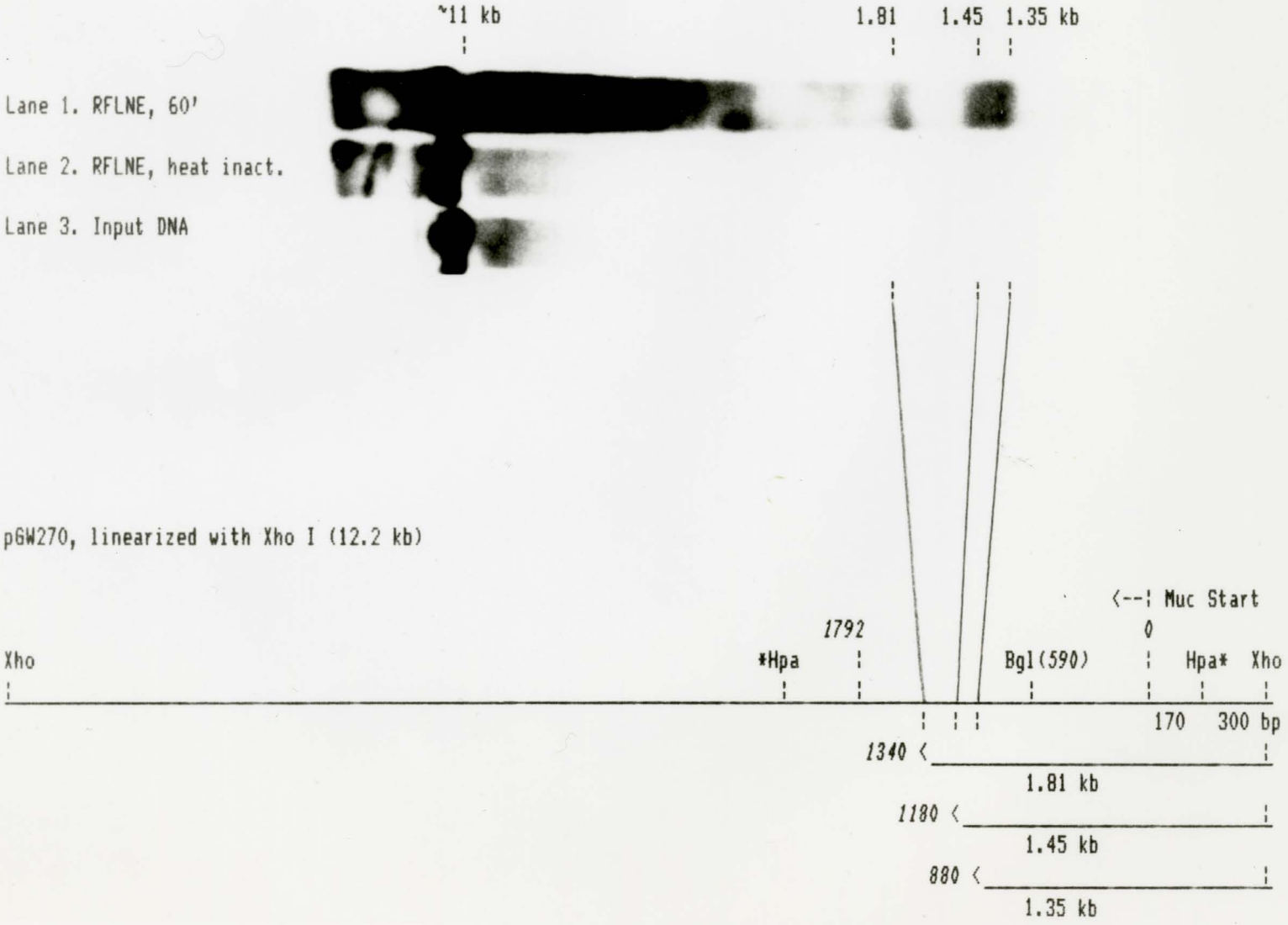

7 mer
9 mer
1326
CACAGTATGCCAATCAGGCAACCGAAAAACTGAC
Vk 41
CACAGTGATACAAATCATAACATAAACC
V lam I
CACAATG
ATCAAGAAC
V H107
CACAGTG
GACACAAAC
JH2
CACACTA
ACAAAAACC
DQ52
CACGGTG
ACAAAAACC
J H107
CACAGAC
TACTAAAAC
7 mer
9 mer

Functional Homology with the Enzymatic Immunoglobulin Recombination Mechanism

The homologous VDJ recombination signals found in the mucA,B sequence were then assayed for functional homology with the eukaryotic signals. This was done by testing their viability as substrate for endonucleolytic cleavage in the same manner as previously reported with known immunoglobulin recombination signals (Hope *et al* 1986). Figure 6 shows the results of digestion of mucA,B DNA sequences with rat fetal liver nuclear extract. Sites of specific cleavage can be seen, and the position of these sites correspond to the three putative VDJ recombination signals listed in Figures 3,4 and 5. More precise fragment size analysis will be required to determine the exact points of cleavage of the mucA,B DNA by the cellular extract. Although not conclusive, this result suggests that there is an enzyme in the nuclear extract able to recognize the muc sequences and that they can be cleaved *in vitro* in a similar way as has been shown with the eukaryotic switch sequences. It should be noted that although the 1340 cleavage site does not have a highly homologous 9mer, it has been shown that the 7mer TACTGTG can be functionally recognized during VDJ joining irrespective of the presence of a 9mer (Besmer *et al* 1986).

Figure 6.

In vitro cleavage of mucA,B DNA with rat fetal liver nuclear extract (RFLNE) detected by Southern blot hybridization. 10 μg of pGW270 was linearized with Xho I and divided into three alliquots after heat inactivation of the enzyme (75° C, 10'). Nuclear extract was prepared from 16 day rat fetus livers as previously published Hope et al 1986), with the exception that no PMSF or DMSF was added. The linearized plasmid was incubated with 10 l of extract and restriction enzyme buffer for 60' at 37° C (lane 1), the control DNA incubated with heat inactivated extract (80° C, 10', lane 2) and untreated digest was run in lane 3. Italic numbers refer to position in the muc sequence. Along with previously reported chemical and non-specific degradation, two distinct (position 1340 and 880) and one diffuse band (position 1180) can be seen in the enzymatically active digest. The longer remaining fragments appear close to the parental band (~11 kb). DNA was run on a 0.8% agarose gel and probed after transfer to nitrocellulose using the 2.1 kb Hpa I fragment (gel isolated, nick translated). Sizes were calculated using the David Mount Cubic Spline Fit program in comparison to Lambda Hind III and Lambda Bst EII digests.


~11 kb
1.81
1.45
1.35 kb
Lane 1. RFLNE, 60'
Lane 2. RFLNE, heat inact.
Lane 3. Input DNA
pGW270, linearized with Xho I (12.2 kb)
<--- Muc Start
1792
0
Xho
*Hpa
Bgl(590)
Hpa*
Xho
170
300 bp
1340
1.81 kb
1180
1.45 kb
880
1.35 kb

Summary of Homology Found in the mucA,B Operon

Figure 7 gives an overview of the regions of homology found in the mucA,B sequence. The mucA coding region is dominated by a long stretch of homology with lambda repressor, as has been previously reported (Potter et al 1984). In the mucB coding sequence there is the region of homology with an Ig kappa variable region, in the same reading frame as mucA. Following this there is the first immunoglobulin recombination switch (position 880 in the mucA,B DNA sequence). After the 880 switch, the mucB polypeptide contains a region of homology to the transposase from Tn3, immediately followed by homology to the lambda integrase gene. These two regions of homology might be indicative of the functional domain of the protein. The position of the two other recombination switches, as well as the integrase and IHF binding sites are also listed in Figure 7.

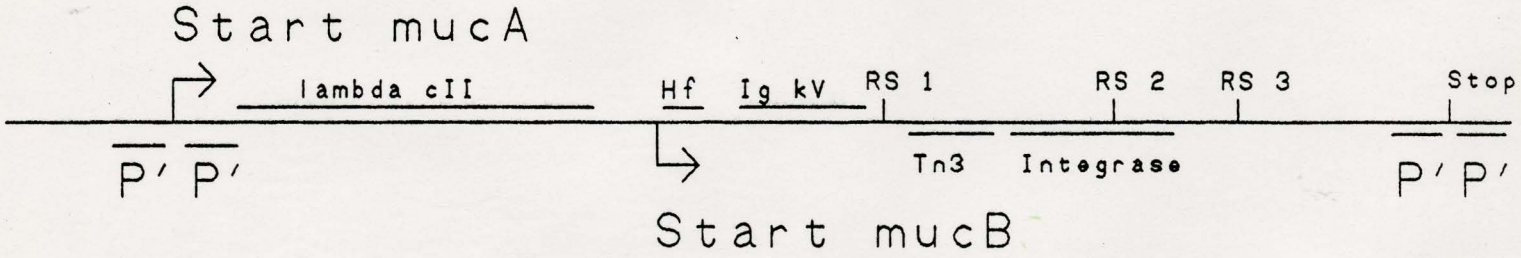
The Relationship Between the mucA,B Operon and Immunoglobulin Variable Regions

In terms of the evolutionary origin of the diversity of the immune system, the muc genes have the potential of having served as a recombinagenic nucleus for the production of V regions. The region of mucB that inserted in the Haemophilus influenza genome includes the homology with the Ig kappa V

Figure 7

Overview of Homology Found in mucA,B Cistron

Regions of homology to the mucA,B DNA sequence are indicated by a line above the central axis and include the integrase binding sites (P'), the Host factor binding sites and the immunoglobulin recombination signals (RS 1-3). Regions of protein homology in the mucA reading frame are indicated by a line above the central axis and include the lambda phage cII repressor (lambda cII) and the Ig kappa variable region precursor (Ig kV). Regions of protein homology in the mucB reading frame are indicated by a line below the central axis, and include the transposase from Tn 3 (Tn3) and the lambda phage integrase gene (Integrase).


Start mucA
lambda cII
Hf
Ig kV
RS 1
RS 2
RS 3
Stop
P' P'
Tn3
Integrase
P' P'
Start mucB

region and the sequences 3′ of this point (Perry et al 1985), which would include the recombination signals. Upon insertion of this fragment in a recipient genome, a similar structure to modern variable regions would be generated. Variability would be generated by the site of insertion, which would contribute to the 5′ region of the gene.

The Relationship Between The mucA,B Operon and Lambda Phage

The significance of the homology found between the mucB polypeptide and the lambda integrase gene must be considered in light of several other facts. Flanking the start of transcription and the end of translation are tandem integrase binding sites (inferred from homology to P′). This parallels the situation in lambda in which the binding sites are tandemly linked, and in that two such sites appear at the end of transcription of the integrase gene itself. It is also known that the lambda cII repressor binds and regulates integrase transcription, relevant due to the fact that mucA is dominated by homology to cII. Although functional domains of gpint are not yet documented, a system of self regulation for the mucA,B cistron can be easily speculated.

Irrespective of transcriptional regulation, the potential for a system to regulate the recombinagenic nature of the mucA,B cistron also becomes evident. In lambda, initiation of site specific recombination is governed by the level of cII

repressor, which functions in part by regulating levels of gpint and gpxis (which is responsible for excision). Lysogenic induction of phage has also long been known to be related to the SOS functions and can be controlled through the recA-lexA repressor pathway. If the mucA,B operon is as recombinagenic as it appears, it could constitute a 'miniature lambda phage'. The mucA,B operon is inducible by recA, produces a cII and int-like polypeptide, contains att site features, and has the ability to integrate into a bacterial genome. These characteristics are considered to be among the requirements for a 'primordial phage' (Boltstein and Campbell 1983). It is interesting that the att site PP' and BB' common sequences are also homologous to the internal resolution site of Tn3 (Boltstein and Campbell 1983), considering the adjacent a.a. homology in mucB to the transposase from Tn3, and the overall transposon-like structure.

The mucA,B Operon, Lambda Phage and Immunoglobulin Recombination

One major element is missing in the parallel between lambda phage and the muc genes, however, and that is a distinct site of DNA cleavage. Recalling the fact that immunoglobulin signal sequences exist in mucB, an interesting correlation can be seen. At the 1180 cleavage site in mucB we see the deviant sequence TGTTGTGT in the position of a 7mer

suggests a mechanism for protein evolution.

The implications of a DNA damage inducible mobile genetic element in the process of evolution are self-evident. The parallel elements of the site specific recombination systems in both prokaryotes and eukaryotes suggests the structural and functional diversity of the genomic spectrum is not as prodigious as it might appear.

References

Alt, F. W. Nature 322(6082), **772** (1986).

Balgenesh, M. & Setlow, J.K. Proc. Natl. Acad. Sci. **82**, 7753-7756 (1985).

Besmer, P et al. J. Vir. **60(1)**, 194-203 (1986).

Campbell, A. and Boltstein, D. in Lambda II, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, p.366 (1983).

Conrad, B. and Mount, D.W. Nucleic Acids Res. **10**, 31-38 (1982).

Craig, N. L. and Nash, H. A. Cell 39, 707-716 (1984)

Desiderio, S. & Baltimore, D. Nature **308**, 860-862 (1984).

Early, P. et al. Cell **19**, 981-992 (1980).

Eisenstein, B. I. et al Proc. Natl. Acad. Sci. 84, 6506-6510 (1987).

Falkner, F.G., Moccikat, R. and Zachau, H.G. Nuc. Acids Res. **14(22)**, 8819-8829 (1986).

Hope, T.J. et al. Science **231**, 1141 (1986)

Kahmann, R., Rudt, F., Koch, C., Mertens, G. Cell **41**, 771-780 (1985).

Kato, J. and Shinoura, Y. Mol. Gen. Genet. 156, 121 (1977)

Kikuchi, Y. and Nash, H.A. J. Biol. Chem. **253**, 7149 (1978).

Langer, P.L., Shanabruch, W.G. & Walker, G.C. J. Bac. **145**, 1310-1316 (1981).

Leder, P., Battey, J., Lenoir, G. Science **222**, 765-771 (1983).

Little, J. W. Biochimie 64, 585 (1982).

Mizuuchi, K. et al. Cold Spring Harbour Symp. Quant. Biol. **45**, 429 (1981).

Nash, H.A. et al. in DNA insertion elements, plasmids and episomes (ed. A. Bukhari et al.), Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, p.363 (1977).

Perry, K.L. et al. Proc. Natl. Acad. Sci. USA **82**, 4331-4335 (1985).

Potter, A.A., Nestmann, E.R., Iyer, V.N. Mut. Research **131**, 197-204 (1984).

Ross, W. and Landy, A. Proc. Natl. Acad. Sci. **79**, 7724 (1982).

Ross, W. and Landy, Cell **33**, 261 (1983).

Sakano, H. et al. Nature **290**, 562-565 (1981).

Shimizu, A. and Honjo, T. Cell **36**, 801-013 (1984).

Tonegawa, S. Nature **302**, 575-581 (1983).

Tsujimoto, Y. et al. Science **229**, 1390-1393 (1985).

Weisberg, R. and Landy, A. in Lambda II, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York, p.222 (1983).

Yancopoulos, G. D. et al. Cell **44**, 251-259 (1986).