The Study of DNA Replication in Vaccinia Virus Through the Characterization of the Temperature Sensitive Mutant ts4149

by A. Kate Millns

A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Master of Science

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Department of Human Genetics
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THE STUDY OF DNA REPLICATION IN VACCINIA VIRUS THROUGH THE CHARACTERIZATION OF THE TEMPERATURE SENSITIVE MUTANT ts4149

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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LIST OF ABBREVIATIONS

AP	-	apyrimidinic or apurinic
DMEM	-	Dulbecco's modified eagle medium
ERCC	-	excision repair cross complementing
FBS	-	fetal bovine serum
kDa	-	kilodalton
MOI	-	multiplicity of infection
NER	-	nucleotide excision repair
OD	-	optical density
ORF	-	open reading frame
PFGE	-	pulsed field gel electrophoresis
PFU	-	plaque forming unit
SFV	-	shope fibroma virus
ts	-	temperature sensitive
UDG	-	uracil DNA glycosylase
UV	-	ultra-violet light

ABSTRACT

Vaccinia virus mutant ts4149 is a temperature sensitive mutant which, at the non-permissive temperature (40°C), displays a DNA-negative phenotype. In agreement with this phenotype, intermediate and late proteins are absent at the non-permissive temperature. Marker rescue and DNA sequencing have localized the mutation to the D4R gene. The mutation is a G→A transition at nucleotide 535 causing a change from glycine to arginine. The D4 gene product shares significant homology with both *E. coli* and mammalian uracil DNA glycosylases. This protein has also been shown to have DNA glycosylase activity (Stuart et al., 1993; Upton et al., 1993). Previous temperature shift experiments (McFadden and Dales, 1980) are compatible with the view that the 25-kDa mutant D4 protein asserts its effect through an assembly defect rather than thermolability. To determine whether the D4 protein is part of a multienzyme complex, and if so, to identify other components of such a complex, we have utilized the yeast two-hybrid system. This system uses the DNA-binding domain of the GAL4 gene or the lexA gene and the GAL4 transcriptional activation region to allow the detection of protein-protein interactions in yeast. Using this technique, we have identified a number of interactions, one of which is an interaction between the D4R protein and the A18R protein.

PART I

CHARACTERIZATION OF THE TEMPERATURE SENSITIVE MUTANT ts4149

INTRODUCTION

Poxviruses

Poxviruses comprise a large family of DNA viruses which can infect both vertebrate and invertebrate hosts. These viruses are characterized by a large complex virion, a linear double stranded DNA genome and by their unique ability to replicate in the cytoplasm of host cells. They were also the first viruses to be observed by light microscopy, and the first to be propagated in tissue culture cells (reviewed in Moss, 1990a).

The most notorious and well known member of the poxvirus family is variola virus, the causative agent of smallpox. Vaccinia virus, the prototypic member of the Orthopoxvirus genus, was used as a vaccine for the prevention of smallpox. Its use also lead to the purposeful eradication of variola virus. This eradication program was initiated by the World Health Organization and began in 1967. During this time, smallpox was endemic in many populations. Due to this program, the last case reported occurred in Somalia in 1977 (Fenner et al., 1988). The origins of the present day strains of vaccinia virus remains obscure. It has been postulated that vaccinia may have evolved from variola virus or cowpox virus through the continual passage in cows and humans, that it may be a hybrid of variola and cowpox or that it may have had a natural host that is now extinct (Fenner et al., 1988; Buller and Palumbo, 1991).

DNA analyses of vaccinia strains shows that they are related to but distinct viruses from variola, cowpox, and other members of this family (Esposito et al., 1985).

Vaccinia virus exists in several different intracellular and extracellular infectious forms. These morphological forms are: (i) the immature virus (IV), (ii) infectious, intracellular mature virus (IMV), (iii) the intracellular enveloped virus (IEV), and (iv) the infectious, extracellular enveloped virus (EEV) which are surrounded by two, two, four and three membranes respectively (Schmelz et al., 1994). The EEV form is believed to be involved in the long distance spread of infections. It has been shown that a mutant containing a deletion of the F13L gene, which encodes for one of the outer envelope proteins, VP37, cannot produce EEV type particles. Infections with this mutant cannot form plaques in the time normally required by the wildtype virus strains. This demonstrates the importance of EEV virus particles in the infection process (Blasco and Moss, 1991). The virions of the mature vaccinia viruses (IMV, IEV, and EEV) are described as being oval or brick-shaped in structure. The viral core, contained inside the virion, is observed to be a biconcave structure with lateral bodies of unknown function in the concavities (Moss, 1990a).

These viruses have a linear DNA genome whose basic features include terminal hairpin loops, an inverted terminal repetition and a highly conserved central region. The length of the genome was initially estimated to be approximately 185kb. Upon sequencing one strain (Copenhagen) it was found that the genome was 191,636bp

with a base composition of 66.6% A+T (Goebel et al., 1990). From the DNA sequence, Goebel et al. (1990) identified 198 "major" open reading frames (ORFs) and 65 overlapping "minor" ORFs for a total of 263 potential genes. Almost 10,000 bp at the terminal regions of the genome comprise an inverted terminal repetition which contains repeated sequences. The number of repeats and the actual sequence of the terminal regions varies between different strains and can also vary within strains. For example, these regions in vaccinia contain a set of thirteen 70bp repeats (in tandem) adjacent to the terminal hairpin followed by a unique sequence of 325bp and then by a second set of eighteen tandem 70bp repeats, two 125bp repeats and eight 54bp repeats (Baroudy et al., 1982). The two complementary DNA strands are covalently cross-linked at their termini by AT-rich hairpin loops. These loops are incompletely base paired and exist in two inverted and mutually complementing conformations called "flip" and "flop" (Baroudy et al., 1982). The majority of essential genes appear to map within the central conserved region and the genes that are non-essential for replication in tissue culture, some of which are involved in host range, are found nearer the ends of the viral genome (Moss, 1990a). The gene density is high with coding regions typically separated by only a small number of bases and relatively few regions of non-coding sequence (Moss, 1990b; Goebel et al., 1990). It appears that each open reading frame (ORF) corresponds directly to a gene in that the genes lack introns (Condit and Niles, 1990). Both strands are transcribed with extensive overlapping of genes being uncommon (Moss, 1990b).

The Life Cycle

The life cycle of vaccinia virus and other members of the poxvirus family proceeds as a complex cascade. To start the cycle, viral and cellular membranes fuse and in the process release the viral core into the cytoplasm. Viral cores initiate the transcription of early genes, which takes place within these cores, producing functional mRNA within minutes of infection (Moss, 1990b). Approximately one-half of the viral genome is made up of these early genes which are transcribed prior to DNA replication. The enzymes required to initiate this process (multi-subunit RNA polymerase, an early transcription factor - VETF, the capping enzyme and a poly(A) polymerase) are already contained in the viral core as they are packaged inside mature virus particles (Moss, 1990b; Gershon & Moss, 1990). Once early transcription is initiated, the viral core undergoes an uncoating step. At this point viral DNA replication begins. Many of the proteins required for this may be early gene products such as the DNA polymerase. The onset of viral DNA replication triggers the next phase of gene expression, the intermediate class of genes (Vos and Stunnenberg 1988). The intermediate transcription factor has not yet been fully identified, although it is known that the mRNA capping enzyme is required (Vos et al., 1991). At least three intermediate genes are required for transcriptional activation of the late class, two of which are part of a transcription factor (Vos and Stunnenberg, 1988; Keck et al., 1990). Following intermediate expression, late proteins are produced. To complete the cascade, two of the late proteins form the early transcription factor (Gershon and Moss,

1990), which helps to activate transcription of early genes during the subsequent round of infection. It is observed that one event must follow the other in order, as each step produces proteins required for the next phase. The final stages of infection are represented by virion morphogenesis involving the packaging of the DNA and required proteins into mature virus particles (see fig.1 for an overview of the cycle).

DNA replication

Replication of the DNA double helix is an elaborate process, which is not fully understood in all organisms. DNA replication is generally thought to involve simultaneous synthesis of both strands, with the leading strand being synthesized continuously, and the lagging strand discontinuously. Much of our present knowledge of the mechanisms of DNA replication has been derived from prokaryotes such as *E. coli* and their bacteriophages. It is known that T4 Bacteriophage, for example, does require and encode the proteins for a DNA polymerase, three accessory proteins, a primase/helicase complex, a single-stranded binding protein, RNaseH and a DNA ligase (reviewed by Nossal, 1992). The mechanism and therefore the proteins required for eukaryotes is thought to be comparable to those of prokaryotic systems. Eukaryotes appear to require the DNA polymerases α, δ, and ϵ, helicase(s), processivity factors PCNA and replication factor C (RF-C), a single-stranded DNA binding protein (also named RPA), topoisomerases I and II, an RNaseH, a primase, and DNA ligase (Thommes & Hubscher, 1990). The mechanism of DNA replication in higher

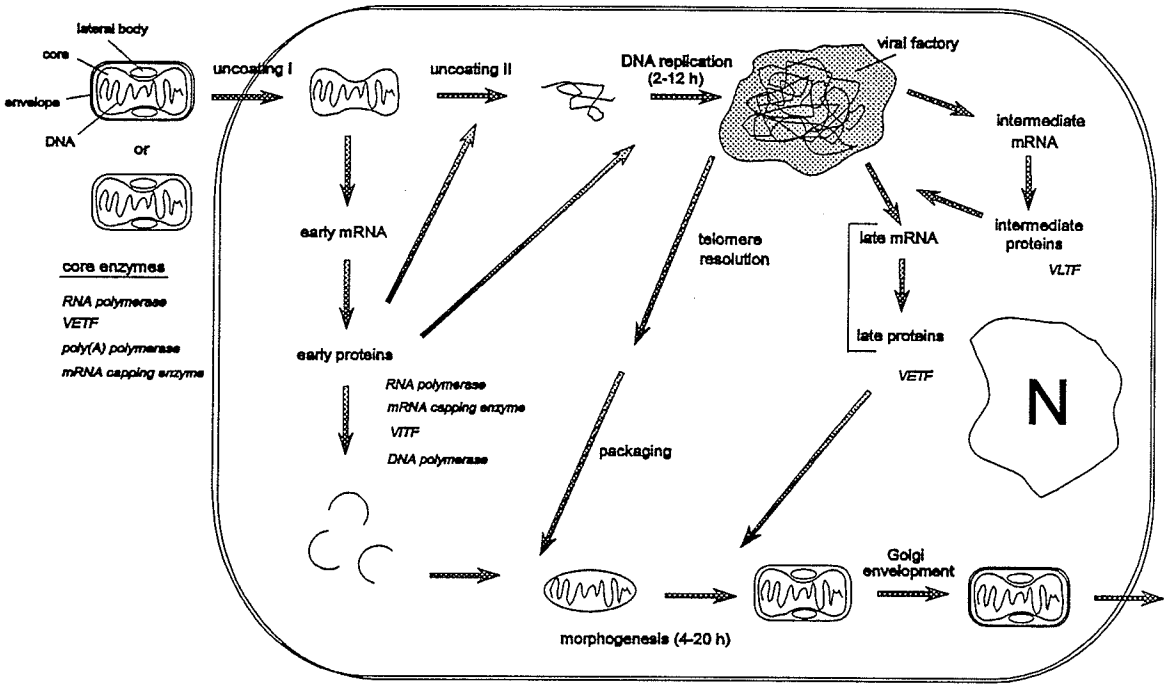


figure 1. The vaccinia virus life cycle. Arrows indicate the order of events. Abbreviations: VETF, VITF, VLTF, vaccinia early, intermediate and late transcription factors; N, cell nucleus. (Modified from Moss, 1990a).

eukaryotes is more difficult to pursue primarily because of the difficulty in obtaining suitable mutants for analysis. DNA viruses that utilize much of the host's cellular replication machinery for their own replication have therefore become the more informative systems to tackle this problem in higher eukaryotes. The small eukaryotic papovavirus (SV40) is particularly useful in this respect. With the exception of the large T antigen, all proteins required to replicate the viral genome are host-derived (Hurwitz et al., 1990). Many of these cellular proteins have been identified using *the in vitro* replication system of Li and Kelly (1984). Vaccinia virus, in contrast to SV40, appears to encode all the proteins needed for the replication of its viral DNA double helix (Traktman, 1990). The powerful combination of genetic and molecular analyses that this virus provides makes it an excellent model system to study mechanisms of DNA replication, recombination and transcription in higher eukaryotes.

DNA replication in vaccinia

DNA replication of vaccinia was first studied by monitoring the cytoplasmic incorporation of exogenously added ^{3}H thymidine into acid-precipitable material to determine when DNA synthesis was initiated and for how long it occurred. With this technique, DNA synthesis was shown to begin at 1-1.5 hours postinfection, reaching maximum levels at 2-2.5 hours, and then decreased sharply. It was thought that more than 90% of the DNA was synthesized by 4-5 hours postinfection (Joklik and Becker, 1964). It is now known that DNA synthesis occurs over a much longer period of time

from experiments based on the method of southern blotting where the DNA is loaded onto nitrocellulose using a microsample filtration manifold (dot blot hybridization) (Ensinger, 1987; Hooda, 1989). This technique allows for the quantitation of the amount of probe bound and therefore gives a better representation of the actual amount of viral sequences present due to DNA replication. Increases in DNA are first detected at 2-3 hours postinfection and sequences then accumulate rapidly and at a constant rate until 10-12 hours postinfection. It should be noted that the initial onset of DNA replication varies depending on the multiplicity of infection and on the host cell type. After 10-12 hours postinfection, synthesis slows down and a plateau in the amount of replicated DNA is reached (Traktman, 1990). It appears that the DNA synthesis detected by thymidine incorporation represents only the earliest hours of DNA replication. The lack of incorporation at later times may reflect repression of thymidine kinase or extensive changes in intracellular nucleotide pools. Inhibition of thymidine kinase in vaccinia (in vivo) at approximately 4 hours postinfection partially explains the observation that incorporation of ^{3}H thymidine into newly synthesized DNA ceases by 5 hours postinfection. The ribonucleotide reductase enzyme may also affect nucleotide precursor pools required for DNA replication and therefore have an affect on ^{3}H thymidine incorporation (Traktman, 1990).

Replication models

A number of different models have been proposed to try to explain the

mechanism of DNA replication in vaccinia, these being (i) self-priming from the DNA strand (Cavalier-Smith, 1974; Bateman, 1975; Moyer and Graves, 1981), and (ii) denovo synthesis of an RNA primer (Pogo and O'Shea, 1978; Baroudy et al., 1982).

(i) self-priming

In the self-priming model (fig. 2; reviewed in Traktman, 1990) the initial event is presumed to be a nick in one of the viral DNA strands near the hairpin termini (Esteban and Holowczak, 1977c; Esteban et al., 1977). This nick creates an available 3'OH primer which is required by the DNA polymerase. The complementary strand is then used as a template for DNA synthesis (self-priming). The actual origin for the initiation of replication has been elusive, as it has been shown that any circular plasmid DNA will replicate in poxvirus-infected cells irrespective of DNA sequence (DeLange and McFadden, 1986). Elongation from this primer then displaces the complementary DNA strand and creates a DNA duplex with one open end and one terminal hairpin. This elongated strand contains an inverted repeat which can fold back on itself and act as a primer for continuing replication. Thus, replication can continue through several of these cycles to form concatemeric DNA molecules. Concatemer formations were first observed during the replication of the orthopoxvirus, rabbitpox (Moyer and Graves, 1981). The high molecular weight concatemers are then post replicatively resolved into mature monomeric linear genomes (reviewed in DeLange and McFadden, 1990).

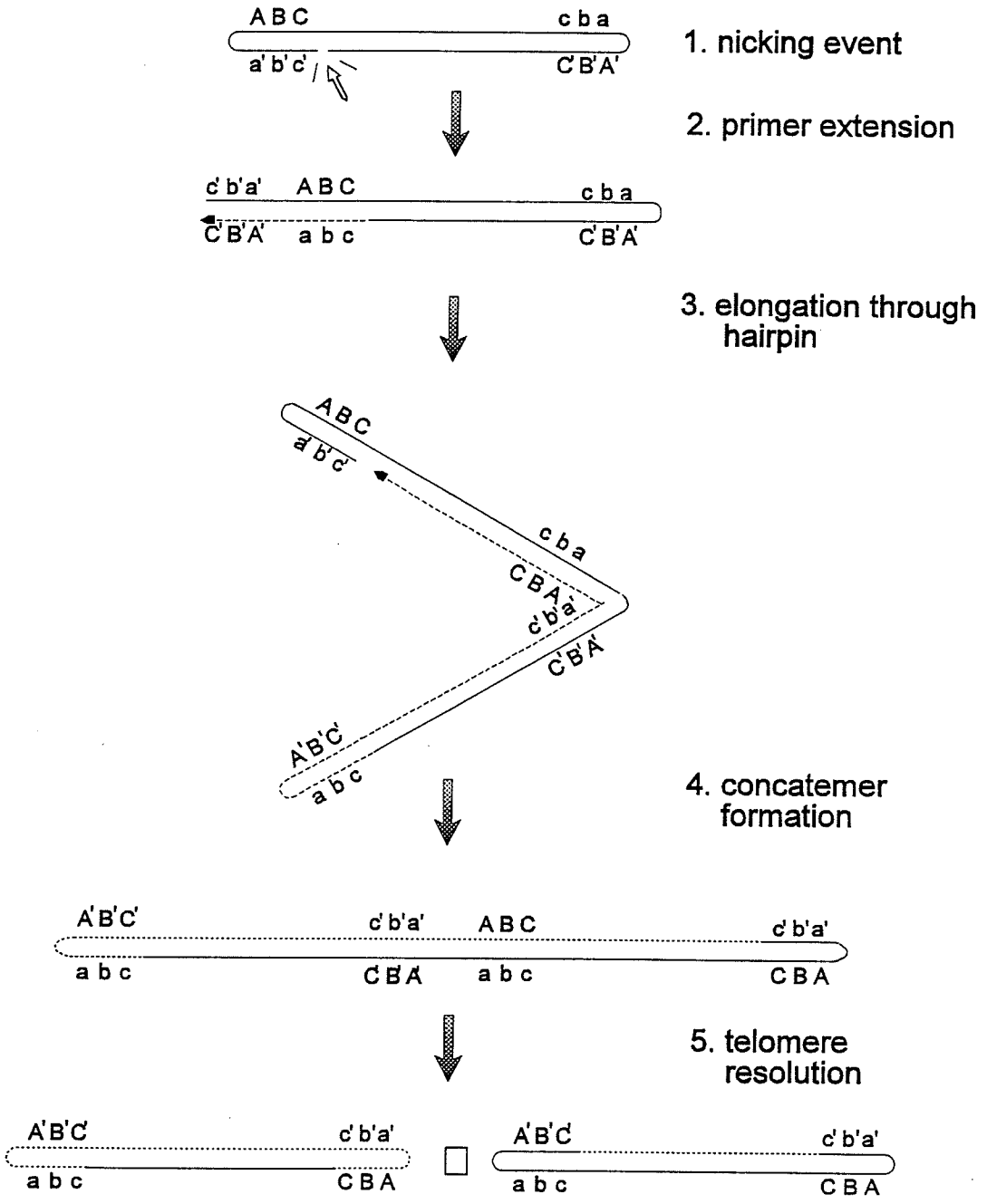


figure 2. Self-priming model for poxvirus DNA replication. The inverted repeat region of the hairpin termini is represented by letters ABC or abc for complementary strands. The termini are not completely identical as extrahelical bases are present in both the flip and flop isomers. These bases are represented by the addition of a prime (′). Dotted lines indicate newly synthesized DNA. Although step 4 only shows a dimer formation, larger concatemers are known to be generated. (Modified from Traktman, 1990).

(ii) *denovo* synthesis

The critical difference between *denovo* synthesis and the self-priming model, involves the initiation of DNA replication. This model invokes RNA primers which supply the 3'OH primer required by the DNA polymerase. The actual initiation of replication has been thought to occur near the terminal areas. Data supporting this demonstrated that nicks occurred in parental molecules at the ends of the genome (Esteban and Holowczak, 1977c). Also, the elimination of the crosslinks was thought to be related to the nicking of the viral DNA and therefore to DNA replication (Pogo, 1977). From electron microscopy images, Esteban et al. (1977) concluded that DNA replication initiated at one end of the genome and proceeded from one end to the other. Pogo et al. (1981) suggested that bidirectional replication occurred, commencing and terminating at both termini. Both models do not require lagging strand synthesis.

Of the two models, the self-priming model is currently the most accepted. It takes into consideration the data on the terminal hairpin nicks with replication being initiated at the hairpin ends and also allows for concatemer formation. This model has no requirement for the production of RNA primers or lagging-strand synthesis. This does not mean that RNA priming and lagging strand synthesis do not occur. It is possible that more than one mechanism is involved in DNA replication.

Based on these models, certain proteins may be required for DNA replication of the viral genome. For the self-priming model, an endonuclease may be required to initiate DNA replication by introducing nicks in the DNA at or near the hairpin termini. Accessory proteins may also be required at this step for specificity. The elongation of the DNA involves the DNA polymerase which may also require some accessory proteins for processivity. A helicase to open the DNA duplex and single-stranded DNA binding proteins for stabilization of the single-stranded DNA may also be required. If both leading and lagging strand synthesis do occur, a primase, an RNaseH and a ligase may be required along with a ribonucleotide reductase, a kinase and a type I topoisomerase specifically for lagging strand synthesis. The resolution of concatemeric structures appears to be a very complex process involving multiple proteins.

Vaccinia virus-encoded enzymes with known or suspected roles in the elongation phase of viral DNA replication include DNA polymerase, topoisomerase I and a DNA ligase (Traktman, 1990). Vaccinia virus also encodes several enzymes with functions in the maintenance of nucleotide pools, such as thymidine kinase, thymidylate kinase, and a heterodimeric ribonucleotide reductase.

DNA negative mutants of vaccinia virus

To determine the viral proteins involved in DNA replication, several large

collections of temperature-sensitive mutants have been screened. From these collections only three DNA negative complementation groups were initially observed.

The first complementation group has been mapped to the open reading frame (ORF) E9, which encodes the viral DNA polymerase (Traktman et al., 1984; Traktman et al., 1989b). The DNA negative temperature sensitive mutants (ts24, NG26) that were identified in this group were found to contain identical ts lesions upon sequencing (Traktman et al., 1989b). These ts mutants show an essentially complete absence of DNA synthesis at the nonpermissive temperature. ORF E9 was shown to code a 3.4kb mRNA which translates into an approximately 110kDa protein. This protein is found as a monomer (110kDa) which has been shown to possess polymerase activity and which also has an intrinsic 3′ to 5′ exonuclease function (Traktman et al., 1989b). This polymerase requires a primed template, is not highly processive and cannot displace DNA strands or pass through barriers of secondary structure (Traktman, 1990). When its homology was compared to α-like polymerases, including human DNA polymerase-α, it was observed to maintain the six predicted conserved polymerase domains (Wang et al., 1989). DNA polymerase is detected within the first hour of infection, peaking at approximately 2.5 hours postinfection and subsequently declining. This expression pattern is seen to occur even when DNA negative ts mutants are infected at the nonpermissive temperature (McDonald et al,. 1992). In the absence of DNA replication or late gene expression, the synthesis of many early proteins in vaccinia has been shown to persist (Condit and Motyczka, 1981) but DNA polymerase

expression remains transient (McDonald et al., 1992). The transient expression of this DNA polymerase appears to be an intrinsic feature of the early phase of vaccinia virus infection which is unlinked to DNA replication and also to late gene expression.

The second complementation group was mapped to ORF B1R (Traktman et al., 1989a; Rempel et al., 1990) which is known to be transcribed early. This gene was predicted to encode a 34kDa protein with homology to serine/threonine protein kinases (Traktman et al., 1989; Rempel et al., 1990) and in fact has been shown to have this enzymatic activity (Banham et al., 1992; Lin et al., 1992). This enzyme was found to be localized in the viral cytoplasmic factories and is also packaged into virions (Banham et al., 1992). The gene itself, lies outside the highly conserved central region in which previous conditional lethal mutants have been found to map (Remple et al., 1990). Two temperature sensitive DNA negative mutants, ts2 and ts25, have been mapped to this gene (Traktman et al., 1989; Remple et al., 1990) and shown to contain different mutation sites (Traktman, 1990). The extent of their DNA negative phenotype varies dependent on the type of tissue culture cells used, but the exact reason for this remains unknown. There is essentially no synthesis observed in mouse L cells with a less severe defect being noted in BSC40 cells. These mutants display a severe defect at low or high multiplicities of infection in mouse L cells with nonpermissive synthesis being less than 5% of that observed in a permissive infection. In BSC40 cells the defect is less severe, for example, infection with ts25 at a MOI=15 results in significant levels of DNA synthesis (50 to 60% of that observed for

permissive) and 10 to 20% of infectious progeny are produced (Traktman, 1990; Remple and Traktman, 1992). There is a possibility that this protein's affect on DNA replication may be caused by its modifications to other proteins involved in this process (Remple et al., 1990).

The third complementation group was mapped to ORF D5, which codes for a 90kDa protein of unknown function (Evans and Traktman, 1987; Roseman and Hruby, 1987). A number of temperature sensitive mutants were mapped to this gene, these being ts17, ts24, ts69 (Evans and Traktman, 1987; Roseman and Hruby, 1987) derived from WR, and ts6389 (Dales et al., 1978; McFadden and Dales, 1980) which is derived from IHD-W. This protein is required for continued DNA synthesis as it has been shown that replication in the mutants ceases if the temperature is raised from the permissive to the nonpermissive temperature (fast stop mutant) (Dales et al., 1978; McFadden and Dales, 1980; Evans and Traktman, 1987). If this infection is again shifted back down to the permissive temperature from the nonpermissive and new proteins are synthesized, DNA replication can be reinitiated (Pogo et al., 1984; Evans and Traktman, 1987). These mutants also displayed a diminished marker rescue efficiency and were therefore difficult to map to a narrow region as compared to other mutants which have been previously mapped (Evans and Traktman, 1987). The gene is transcribed early, even though the initiating ATG codon is part of the TAAATG motif normally associated with late transcripts (Roseman and Hruby, 1987). Analysis of the mRNA from D5R showed that two transcripts of approximately 3 and 3.7kb were

produced (Evans and Traktman, 1987; Roseman and Hruby, 1987). These transcripts share a 3′ terminus but have a distinct 5′ terminus. It was found that the two transcripts represented the D5R gene and a readthrough transcript from the D4R gene which only translates into the D4R protein. D4 is transcribed early but does not contain the early transcription termination signal TTTTTNT whereas D5 contains two termination signals found downstream of the translational stop for D5R (Evans and Traktman, 1987; Roseman and Hruby, 1987).

Synthesis of the D5 protein peaks at 2.5 hours postinfection and declines by 4.5 hours postinfection (Evans and Traktman,1987). It seems that this regulation, much like that observed for the polymerase protein, persists even when DNA replication and late gene expression are blocked (Evans and Traktman, 1987; McDonald et al., 1992).

The sparsity of DNA negative mutants is somewhat surprising in view of the expectation that all enzymes with a role in viral DNA replication are encoded by the virus. Part of the reason for this apparent discrepancy may lie in the fact that some of these enzymes, including DNA ligase (Kerr and Smith, 1991); (Colinas et al., 1990) and the enzymes involved in maintenance of nucleotide pools such as ribonucleotide reductase, thymidine kinase and thymidylate kinase (Hughes et al., 1991), are non-essential for replication in tissue culture. These enzymes are known to be enzymatically active (Kerr and Smith, 1991; Hughes et al., 1991) and the thymidylate kinase even complements temperature sensitive cdc8 yeast mutants (Hughes et al.,

1991). It is, however, highly unlikely that most replication proteins would be non-essential. The primary goal of this project was to study DNA replication in vaccinia by examining the DNA^- mutant, ts4149

MATERIALS AND METHODS

Materials

All restriction enzymes used were from either Pharmacia, Boehringer Mannheim, Gibco or New England Biolabs. HeLa S3 spinner cells are epitheloid carcinoma (cervix) cells from the American Type Culture Collection (ATCC) #CCL2.2. African green monkey kidney cells (BSC40) were obtained from Dr. Richard Condit. Radioisotopes ^{32}P α-dATP, ^{35}S methionine and ^{35}S α-dATP were obtained from NEN or Amersham.

A) Cell culture

Commonly used tissue culture media and reagents

Dulbecco's modified Eagle medium (DMEM) (Sigma) : Supplemented with 50 IU/ml penicillin (Flow), 50mcg/ml streptomycin (Flow), 2mM L-glutamine (Gibco), 15mg/L Phenol red Na-salt (Gibco) and a specified percentage of fetal bovine serum (FBS)(Gibco)

S-MEM (Sigma) : Supplemented with 2mM L-glutamine, 50 IU/ml penicillin, 50mcg/ml streptomycin and 5% fetal bovine serum (FBS).

Phosphate buffered saline (PBS) : 8g NaCl, 1.14g Na_2HPO_4, 0.2g KCl, 0.2g KH_2PO_4 made up to 1L with water. The pH was adjusted to 7.

PBS-EDTA : Normal PBS containing 1mM EDTA.

Cells

Continuous lines of BSC40 cells were grown as monolayer cultures in DMEM supplemented with 5% FBS. This cell line was adapted to grow and support a productive virus infection at 40°C. HeLa S3 cells were grown in monolayer culture in DMEM supplemented with 10% FBS. For HeLa spinner cultures, S-MEM supplemented with 5% fetal bovine serum was used as a growth medium.

Maintenance of tissue culture cells

BSC40 cells

The media was removed from a T150 (150mm) flask (Corning) containing a confluent monolayer. The monolayer was then washed twice with 10ml of warm PBS-EDTA. 5ml of warm PBS was added to the monolayer along with 0.5ml of 2.5% trypsin (Gibco). The flask was placed at 37°C for approximately 1 minute until the cells appeared to become detached from the flask wall. 5ml of warm DMEM + 5%

FBS was added to the flask to inhibit the action of the trypsin. The cells were then shaken from the flask into solution and pipetted up and down to loosen clumps. At this stage cells can be removed to make plates. An aliquot (0.5ml) was left in the flask to maintain the culture. 50ml of warm DMEM was added to the cells left behind. Carbon dioxide (5%) was briefly blown into the flask. Flasks were then incubated at 37°C in 5% CO_2/95% air.

HeLa cells

These cells were grown both in monolayer cultures and in spinner cultures (Ausubel et al., 1987). For monolayer cultures, cells were given fresh DMEM with 10% FBS as required until a confluent monolayer was reached. They were then treated similarly to the above BSC40 cells for maintenance in monolayer cultures. Cells were also transferred from monolayer cultures, when a confluent monolayer was reached, into spinner cultures. For spinner cultures, cells from a complete monolayer were trypsinized (as above) and the loose cells were pipetted into centrifuge tubes and spun down in a clinical centrifuge. Cells were then resuspended in S-MEM and transferred to 250ml spinner bottles. An aliquot of cells was removed and counted using a haemocytometer. Cell density was then adjusted to 3-4X10^5 cells/ml with S-MEM and culture bottles were incubated at 37°C without CO_2. Initial cultures were monitored every day for 2 to 3 days and were maintained at 3-4X10^5 cells/ml. After 3 days, when the cell density had reached 5X10^5 cells/ml, the cells were diluted to 1.5X10^5 cells/ml with S-MEM and were checked on alternate days to determine if the

cells required fresh media. Spinner cultures were maintained at cell densities between $1.5X10^5$ and $5X10^5$ cells/ml.

Preparation of plates

When flasks of BSC40 cells were split as above, plates were made as required. One T150 flask could make 3 x 150mm plates, 4-6 x 100mm plates, 3 x 6-well plates or 4 x 24-well plates by making the appropriate dilutions.

Virus

Wild-type vaccinia virus strain IHD-W was provided by Dr. G. McFadden. The temperature sensitive (ts) mutant, ts4149 was derived from IHD-W and was provided by Dr. S. Dales. The permissive temperature for mutant ts4149 was 32°C, the nonpermissive temperature was 40°C. A vaccinia strain WR cosmid library was supplied by Dr. R. Condit.

Crude virus stocks were obtained as described previously (DeLange, 1989). Briefly, the virus was passaged at a low multiplicity of infection (0.05 to 0.1 PFU per cell) through BSC40 cells. After 48 hours post infection, infected cells were scraped off the plates. The cells were spun down using a clinical centrifuge. The media was aspirated and a volume of PBS dependent on the size of the plates harvested, was

added to the cells. The virus was then obtained by three cycles of freeze-thawing. The virus stocks were also built up through the use of HeLa spinner cultures to obtain very high titre stocks (Ausubel et al., 1987). 1L of HeLa cells were grown to $5x10^8$ cells/L. These cells were centrifuged in a sorvall at 1800xg for 10 minutes at room temperature. The supernatant was poured off and the cells were resuspended in 50mls S-MEM to give a concentration of approximately $1x10^7$ cells/ml. Cells were transferred to a T150 flask where they were infected with 0.5-1.0 PFU per cell. The infection proceeded for 1.5 hours at room temperature. The cells were then rediluted to 1L and were left to spin for 3 days at either 32°C for ts mutants or 37°C for wild-type strains. After 3 days, the cells were again harvested by centrifugation in a sorvall at 1800xg. The pellet was resuspended in 5mls of PBS and was then subjected to 3 cycles of freeze-thawing after which the stock was sonicated on high for 1 minute using a 4mm diameter tip. Cellular debris was removed before stocks were stored at -70°C.

Plaque purification

This technique was based on the protocol by Mackett et al. (1985). Tissue culture 6-well plates were infected with dilutions such that plaques would be well separated and therefore easy to pick. The cells were infected for 1 hour at 37°C. Equal volumes of 2xDME/FBS and 2% noble agar (held at 40°C and 44°C respectively) were mixed together just prior to aspirating the media from the wells. 1ml of the mix was added directly to the cell monolayer. The plates were quickly swirled to distribute the

agar mix and were then allowed to solidify at room temperature for 5 minutes. The plates were incubated at 32°C, 37°C or 40°C, dependent on what the isolation is for, for 48 hours. For a live stain, 1% noble agar and 0.01% neutral red were used. An equal volume of 2% noble agar and 0.02% neutral red were mixed together, 0.5ml was added to the agar overlay and plates were quickly swirled to distribute the stain. The plates were then incubated for 4 hours at the same temperature as previously, at which time the plaques were visible as clear spots on a red background. Plaques were picked using a pasteur pipette. The plugs were placed directly into 0.5mls of PBS and then frozen. To obtain the virus, the plugs were treated with three cycles of freeze-thawing. These individual plugs were then used to build up new virus stocks.

Titres

Serial dilutions were made of the virus stocks to determine the virus titre. Dilutions from 10^{-5} to 10^{-9} were used to infect 24-well plates (or 6-well plates for more accuracy). These were then incubated at 32°C for 48 hours to determine the virus titre at the permissive temperature for temperature sensitive mutants. Another set of titre plates with dilutions ranging from 10^{-2} to 10^{-4} were also used to determine the terminal dilution and reversion rates of the mutant at 40°C. The terminal dilution only applies to temperature sensitive mutants at 40°C and occurs at the dilution where complete cytopathic effect is observed. After 48 hours, both sets of plates were fixed with neutral buffered formalin and stained with 0.1% crystal violet. The number of plaques

that could be counted per well were used to determine the titre.

B) Experimental techniques

Media for bacterial cultures (Hanahan, 1985)

Luria Bertani (LB) : 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl. Up to 1L water, pH adjusted to 7.5. For LB low salt only 5g NaCl was added. To make agar plates 15g/L agar was added.

SOB : 2g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl, 0.25mls 1M KCl. Make up to 100mls with water. A separate solution of 1M $MgCl_2$ plus 1M $MgSO_4$ was made and filter sterilized. Just before use, a 1/100 dilution of the magnesium stock was added.

SOC : This media was identical to SOB except that it also contained 20mM glucose.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed as described by DeLange (1989). Monolayers of BSC40 cells on 35mm plates were infected at a multiplicity of 20 PFU per cell. At specific

times post-infection, the media was aspirated and the plates were washed twice with ice cold PBS/EDTA. PBS+0.03% trypsin (1.25ml) was added to the plates and left until the cells started to loosen. 1.25ml DMEM+5% FBS was added to the plates to inhibit the trypsin and the cells were scraped off and spun down in a clinical centrifuge. The supernatant was discarded and the pellet was resuspended in 100µl PBS/40mM EDTA prewarmed to 40°C. The pellet was vortexed to resuspend and then incubated at 40°C for 5 minutes. 125µl of 1.4% low-melting-point (LMP) agarose/0.125M EDTA prewarmed to 44°C was added to the cell mixture. The solution was pipetted up and down to mix and was then immediately transferred to 2 pretaped plastic molds (approximately 100µl per mold). The plugs were allowed to solidify for 10 minutes at which time they were transferred into tubes containing 3 ml of proteinase K solution (10mM Tris-HCl pH 7.5, 0.18M EDTA, 1% sarkosyl, 100µg/ml proteinase K) and incubated overnight at 50°C. The plugs were equilibrated with agitation at 4°C using three changes of 0.1xTBE. Plugs could be stored at 4°C in 0.1xTBE. Prior to electrophoresis, plugs were loaded onto 1.5% agarose gels made with 0.1xTBE and sealed using 1% LMP agarose. These gels were initially electrophoresed in a uniform field of 5V/cm for 45 minutes in 0.1xTBE. Gels were then pulsed for 20 hours at 100-s intervals with a hexagonal attachment. Following electrophoresis, the DNA was transferred to nylon. Detection of DNA used a ^{32}P-labelled terminal repeat viral DNA probe, pVT-1, labelled by the random primer method (see below).

Protein labelling

Protein labelling experiments were based on protocols described by Condit and Motyczka (1981) and DeLange (1989). Complete monolayers of BSC40 cells on 35mm plates were infected at a multiplicity of 20 PFU per cell. Virus was adsorbed at 37°C for 30 minutes after which time the infection was aspirated, 1.5ml of prewarmed (32°C or 40°C) DMEM was added and the plates were placed in appropriate incubators. Time points were considered to start at the point of infection. At specific times post infection, the medium was removed, the plates were rinsed with prewarmed PBS and 500µl of PBS containing ^{35}S-Methionine (20µCi/ml) was added. The plates were incubated at the appropriate temperatures for 15 minutes to label the proteins. The label was then removed and 300µl of lysis buffer (30mM Tris-HCl pH 6.8, 5% glycerol, 1% sodium dodecyl sulfate (SDS), 2.5% β-mercaptoethanol, 0.005% bromophenol blue) was added. Approximately 70µl of this mixture was transferred to an eppendorf tube. The tubes were boiled for 4 minutes and then frozen at -20°C. The samples were reboiled for 4 minutes prior to loading 10 µl onto a 12% SDS-polyacrylamide gel. The gels were fixed and stained using a solution of 40% methanol, 10% acetic acid and 0.1% Coomassie blue. Gels were then vacuum dried and autoradiographed.

Viral DNA purification

An adaption of the methods of Ausubel et al. (1987) and Esposito et al. (1981) was used to purify viral DNA. 8 x 150mm plates of BSC40 cells were infected at a multiplicity of 0.1 PFU per cell. The plates were then incubated for two days until a severe (approximately 80%) cytopathic effect was observed. The cells were harvested into 50ml tubes (Corning) and pelleted in a clinical centrifuge. The cells were resuspended into a total volume of 20mls of ice cold isotonic buffer (10mM Tris pH8, 150mM NaCl, 5mM EDTA). They were again pelleted and were resuspended in 9mls of ice cold hypotonic buffer (10mM Tris pH8, 10mM KCl, 5mM EDTA). Cells were left on ice for 10 minutes to allow them to swell. They were then homogenized using a dounce homogenizer - B pestle. This suspension was sonicated and layered onto 28.5mls of 36% sucrose. It was then spun in an ultracentrifuge SW-28 rotor for 80 minutes at 32,900xg (4°C). The virus pellet was resuspended in 1ml of buffer (50mM Tris-Cl pH7.8, 1% SDS, 12% sucrose, 15µl BME, 0.4mls 10mg/ml proteinase K) and incubated at 50°C for 2 hours after which 400µl of 5M NaCl was added to the solution and it was stored overnight at 4°C. The solution was extracted twice with equal volumes of phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (49:1). The viral DNA was then precipitated at -20°C for several hours by the addition of one tenth the volume of 3M sodium acetate and two volumes of absolute ethanol. The DNA was pelleted in a microcentrifuge, rinsed, dried and resuspended in 100µl of TE.

Plasmid construction

Viral DNA was purified as described above. DNA was digested with appropriate restriction endonucleases and electrophoresed in 0.7% agarose gels (w/v). Fragments were purified from agarose gels by electroelution and ligated into the multiple cloning site of the phagemid vector pTZ18R (US Biochemicals) by standard methods (Maniatis et al., 1982). These plasmids were used mainly for the marker rescue protocol and for obtaining fragments to be cloned into other vectors. Recombinant plasmids were used to transform *Escherichia coli* strain DH5α.

Bacterial transformation

$CaCl_2$ method

This method was based on the protocol described by Hanahan (1985). A 10ml culture of DH5-α was grown at 37°C overnight in LB media. The overnight culture (2ml) was used to subculture 25ml of SOB media which was then allowed to grow at 37°C until an absorbance A_{550}=0.5 was reached. The cells (25ml) were chilled on ice for 10 minutes. They were then pelleted in a Sorvall SS-34 rotor at 3000rpm for 5 minutes at 4°C. The supernatant was poured off and the pellet was resuspended in 1/3 the volume of standard transformation buffer, TFB (100mM KCl, 45mM $MnCl_2$, 10mM

$CaCl_2$, 3mM $HACoCl_3$, 10mM K-MES). This was then incubated on ice for 10 minutes. The cells were pelleted again as was done previously and were then gently resuspended in TFB at 1/12.5 of the original culture volume. The solution DnD (1M DTT, 90% v/v DMSO, 10mM K-acetate) was added to 3.5% v/v (70µl of DnD was added). The DnD was pipetted directly into the center of the cells and swirled while being added. This mixture was then placed on ice for 10 minutes. A second equal aliquot of DnD was added as above to give a 7% final concentration. This was again incubated on ice for 15 minutes. 210µl of competent cells were added to chilled Falcon 2059 polypropylene tubes. To this, DNA in a volume of <20µl was added to the cells which were swirled to mix. The reactions were placed back on ice. After 30 minutes, the cells were heat shocked at 42°C for 90 seconds and were then immediately placed on ice to cool for 2 minutes. 800µl of SOC media was added to each tube. The tubes were incubated at 37°C with slight agitation for 40 minutes. Aliquots of cells were spread onto appropriate selective media, typically LB+40µg/ml carbenicillin, and plates were then incubated at 37°C overnight. Transformants were picked the next morning and regrown up in liquid LB/carbenicillin media.

Electroporation

Both the making of the electrocompetent cells and the electroporation technique are described in the paper by Dower et al. (1988). Electrocompetent cells are required for this technique. Once made, the unused cells can be stored at -80°C

for use at a later time. An overnight culture of DH5-α was grown to stationary phase and was used to inoculate a 1L culture of LB broth (low salt). The cultures were grown to mid-log growth phase measured by checking the absorbance of the culture. Optical density OD_{600} should be between 0.5 to 1.0 (usually around 0.7) for mid-log phase. The cultures were chilled on ice and then pelleted using a Sorvall JA-21 rotor at 5000rpm. The medium was poured off and the pellet was washed twice in ice cold water. The bacteria pellets were then resuspended in 1L of ice cold sterile water. The cells were pelleted as above, the supernatant was poured off and the pellets were resuspended in 500ml of cold water. The cells were again centrifuged and were resuspended in 20ml of 10% glycerol. At this point, the cells were centrifuged in a sorvall SS-34 at 5000rpm and were then finally resuspended in 2ml of 10% glycerol. This mixture was distributed in 50µl aliquots, flash frozen in liquid nitrogen and stored at -80°C.

For transformation, 25µl to 50µl of competent cells were used for each reaction. The cells were thawed at room temperature and then placed on ice. DNA (1µl) in TE was added to the cells and mixed well. The mixture of cells and DNA was transferred to a cold 0.1cm gap cuvette and was then pulsed with the BIORAD Gene Pulser (with pulse controller) set to 25µF capacitance, 1.25kV and 400Ω. The cells were gently resuspended in 1ml of SOC media at room temperature and were then transferred to tubes (12x75mm) for incubation at 37°C for 30 minutes. Aliquots of cells were then plated onto appropriate selective media, typically LB/40µg/ml carbenicillin, and plates

were incubated at 37°C overnight. Tranformants were picked the next morning and regrown up in liquid LB/carbenicillin (20µg/ml) media.

Plasmid DNA preparation

Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (1979). The following protocol was used for a 5ml bacterial culture but can be scaled up dependent on the culture size. A 5ml culture of LB/carbenicillin (20µg/ml) was inoculated from a colony and grown overnight. The culture was transferred to a centrifuge tube and spun at 7000rpm for 5 minutes in a Sorvall SS-34 rotor. The medium was poured off and the bacterial pellet was resuspended in 100µl of Solution I (50mM glucose, 50mM Tris-HCl pH8, 10mM EDTA), transferred to an eppendorf tube and allowed to sit at room temperature for 5 minutes. 200µl of freshly made Solution II (1% SDS in 0.2M NaOH) was added to the tube followed by 150µl of Solution III (3M potassium acetate, 11.5 v/v glacial acetic acid). This was then placed on ice for 15 minutes. After the incubation on ice, the tube was spun in a microcentrifuge for 5 minutes. The supernatant was removed into a new eppendorf tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the liquid. The tube was shaken to mix the two layers and then spun for 2 minutes in a microcentrifuge to separate the layers. The aqueous layer was removed and the DNA

was precipitated by the addition of two volumes of absolute ethanol. The tube sat for 10 minutes at room temperature and was then spun for 10 minutes to pellet the DNA. The supernatant was poured off and the DNA pellet was rinsed with 70% ethanol, dried and suspended in 100µl of TE+RNAase A (10mM Tris-HCl pH 7.5, 0.1mM EDTA, 10µg/ml RNAase A). DNA was stored at 4°C.

Marker rescue

Marker rescue was performed as described previously (Carpenter and DeLange, 1991). DNA was prepared for marker rescue as follows. Plasmid DNA (1µg) containing viral insert was digested with appropriate restriction enzymes. Upon completion, the digested DNA was extracted once with phenol/chloroform and chloroform respectively. The DNA was then ethanol precipitated by the addition of one tenth the volume of 3M sodium acetate and two volumes of absolute ethanol. The tubes were put at -70°C for 5 minutes and then spun at 4°C for 15 minutes to pellet the DNA. The ethanol was poured off and the pellet was rinsed with 70% ethanol, dried and resuspended in 10µl of TE. Prior to the transfection, the calcium-phosphate precipitate was made. The total volume of DNA was added to 140µl of 2XNNH (50mM HEPES [*N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid pH 7.1], 280mM NaCl, 1.5mM Na_2HPO_4). An equal volume (150µl) of 250mM calcium chloride was added dropwise with agitation to the DNA. The precipitate was then left at room temperature for 1 hour.

Marker rescues were routinely performed using 6-well plates (refer to tissue culture section for preparation of plates). Plates were checked before beginning the marker rescue to ensure that there was a healthy monolayer. The virus (stored at -70°C) was thawed, sonicated at maximum for 1 minute and then vortexed. The virus was diluted into Hank's balanced salt solution with 0.1% bovine serum albumin (BSA) or into DMEM+5% FBS to obtain a multiplicity of infection (MOI) of 0.05 plaque forming units (PFU) per cell. The medium was aspirated from the wells and 330µl of the diluted virus was added to the wells for infection. The virus was adsorbed at 37°C for 1.5 hours with rocking every 15 minutes to keep the monolayer moist. After the 1.5 hour adsorption, the infection was aspirated and the monolayer was washed twice with prewarmed (37°C) Hanks solution. The calcium-phosphate-precipitated DNA (300µl) was added to each well and incubated at room temperature for 30 minutes. 3ml of DMEM+5% FBS were added to the wells and they were placed at the non-permissive temperature (40°C). After three hours, the media was aspirated and fresh pre-warmed DMEM+5% FBS was added to the wells. The plates were then incubated for 72 hours after which time the media was aspirated and the wells were fixed with neutral buffered formalin and stained with 0.1% crystal violet.

Southern blotting

DNA samples were electrophoresed on agarose gels dependent on the type of experiment. The DNA from the gels was transferred to zetaprobe by the specifications

of the manufacturer (Bio-Rad) and based on the method of Southern (1975). The gels were stained with a solution of 0.5µg/ml ethidium bromide in TAE or TBE and photographed prior to transfer. The gels were soaked in 0.25M HCl for 10 minutes with shaking, rinsed once with water and were then soaked in 0.2N NaOH, 0.6M NaCl for 30 minutes with shaking. DNA transfer occurred in 10xSSC (1.5M NaCl, 0.15M Na citrate, pH 7) for approximately 12 hours. Following the transfer, membranes were rinsed in 2xSSC for 15 minutes with shaking. The membranes were allowed to air dry and were then baked for 30 minutes at 80°C in a vacuum oven. They were then stored at 4°C until ready to be probed.

DNA Probes

DNA probes were made by the random primer method of Feinberg and Vogelstein (1983). They were purified through NICK columns (Pharmacia) to remove any unicorporated radioactive nucleotides from the probe. Membranes (zetaprobe) for probing were prehybridized and then hybridized with the probe by the standard protocol of the manufacturer (Bio Rad).

DNA Sequencing

Sequencing of wild-type IHD-W clones, mutant ts4149 clones and replacement clones was performed using the Sanger dideoxy chain-termination method (Sanger et

al., 1977). For all sequencing reactions the Sequenase kit (US Biochemicals version 1.0 or version 2.0) was used. The universal and reverse primers were used for sequencing clones in the pTZ18R vector. Templates from double-stranded DNA were prepared by the method of Hattori and Sakaki (1986) prior to sequencing.

RESULTS

The temperature sensitive mutant, ts4149, is one of the seven mutants from the Dales' collection (Dales et al., 1978; McFadden and Dales, 1980) that was initially identified as having a DNA negative phenotype. It was classified as DNA negative based on the reduction in its ability to incorporate ^{3}H thymidine at the nonpermissive temperature in the cytoplasm of mouse L cells. Examining DNA replication by ^{3}H thymidine incorporation suffers one major drawback, namely the fact that this method monitors only the early stages of replication and therefore, a more indepth analysis was required. Pulsed field gel electrophoresis (PFGE) is a more useful method as it facilitates accurate quantitation of DNA and also provides a method to derive qualitative information at all stages of the replication cycle. This, along with protein studies and marker rescue experiments, was used to further characterize ts4149 and to determine the gene containing the mutation which appears to result in the DNA negative phenotype.

DNA synthesis in the mutant ts4149

The initial analysis used to confirm the DNA negative phenotype demonstrated by McFadden and Dales (1980) was by means of pulsed field gel electrophoresis. To further investigate this defect a time course analysis on ts4149 and the wild-type strain IHD-W that it was derived from were performed at both the permissive temperature

(32°C) and at the non-permissive temperature (40°C).

BSC40 cells were infected with either ts4149 or IHD-W at a multiplicity of 20 PFU/cell. At various times postinfection samples were harvested for analysis by PFGE. Wild-type infections showed the characteristic rapid synthesis of DNA, which initially appears as replicating non-linear DNA. This replicating DNA fails to migrate into the gel matrix but is resolved into linear genome-length DNA molecules after a short lag period (fig. 3A). This process was slightly faster at 40°C than at 32°C, and generated mostly linear molecules by 12.5 hours postinfection. In contrast, infection with ts4149 at the permissive temperature resulted in a reduced rate of DNA synthesis and a longer lag period before the DNA was resolved into linear monomers. At the nonpermissive temperature, a very low level of replication is observed coupled with an apparent absence of resolution to linear DNA monomers (fig. 3B). This observation is repeatable giving essentially identical results. Densitometric scans of the Southern blots allowed us to estimate that, compared to wild-type, *ts*4149 synthesized 1-2% of its DNA at the nonpermissive temperature (fig. 4). That synthesis does indeed take place is supported by two findings: (i) there is a small but measurable increase in signal between 2.5 h postinfection (input DNA) and later times postinfection; and (ii) whereas the input DNA migrates as a 185-kb linear species, at later times postinfection most DNA fails to migrate in the gel matrix. It should be noted that, although DNA replication is somewhat delayed and reduced at the permissive temperature, the virus is easily grown to high titre.

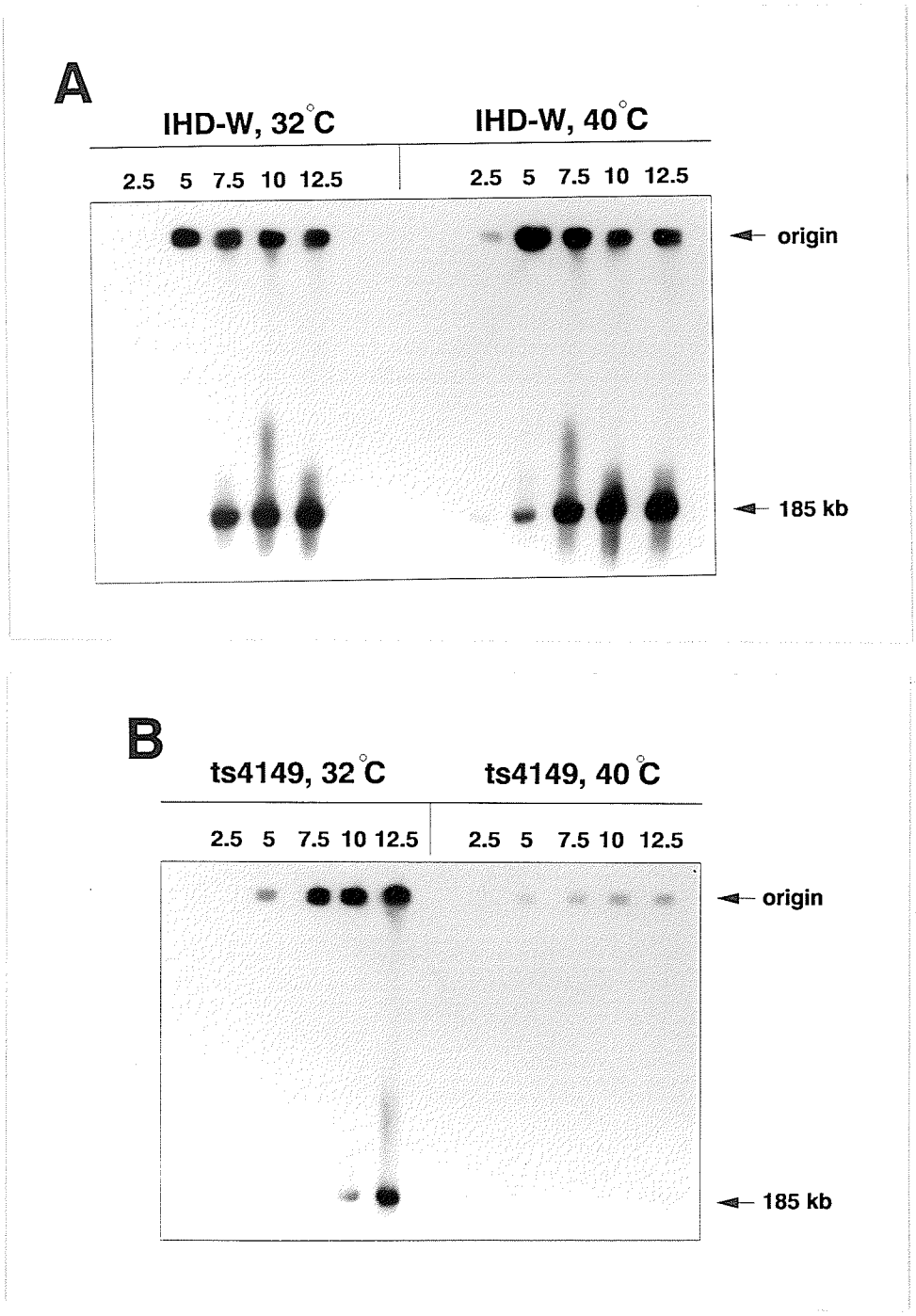


figure 3. Pulsed field gel electrophoresis (PFGE) of vaccinia virus DNA from cells infected with wild-type strain IHD-W (A) or with mutant *ts*4149 (B) at either the permissive (32°C) or the nonpermissive (40°C) temperature. Samples were harvested at specific times postinfection. The location of the well (origin) and the 185-kb mature linear DNA genome are as indicated.

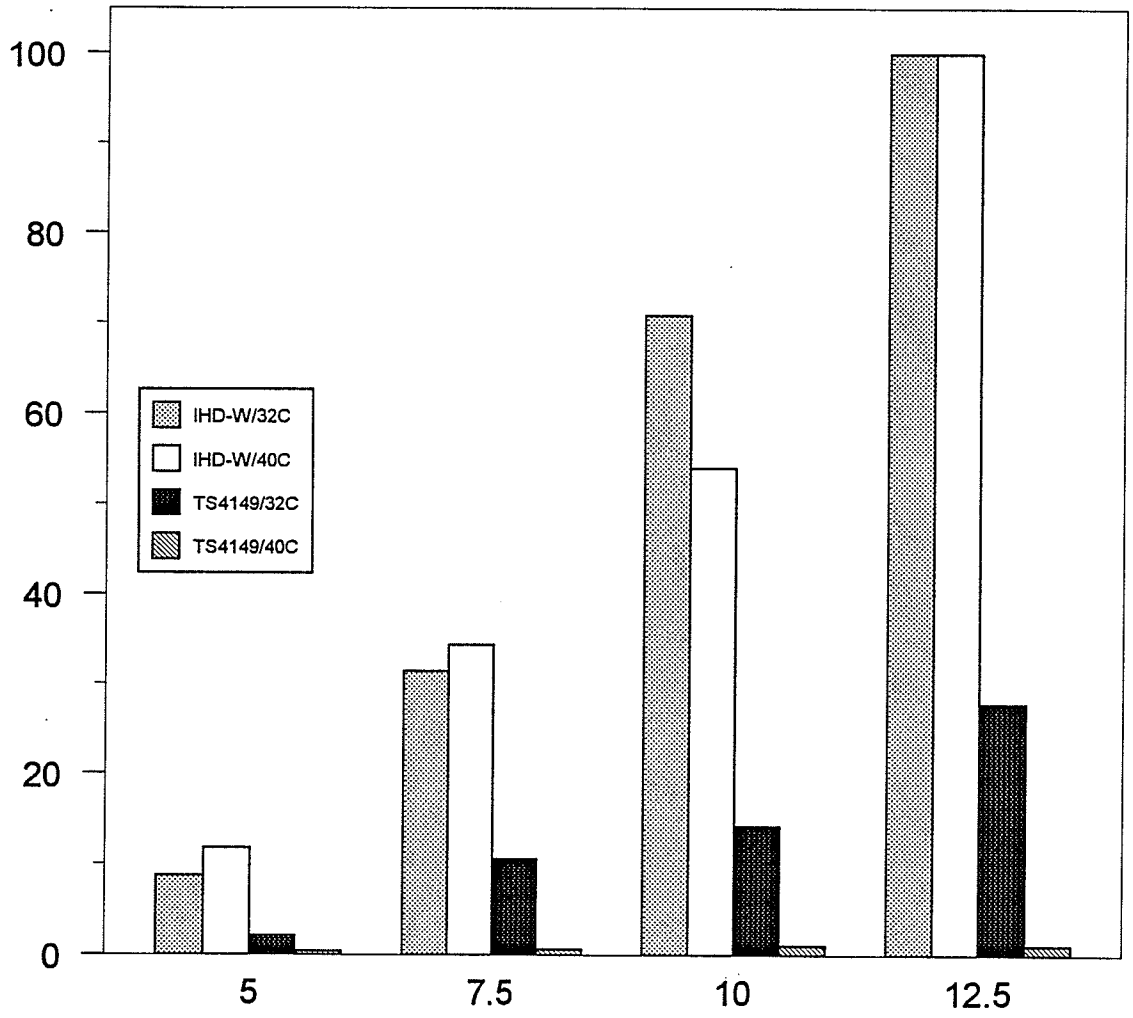


figure 4. Quantitation of DNA synthesis in cells infected with wild-type IHD-W or with mutant *ts*4149. DNA was infected with either IHD-W or *ts*4149, and subjected to PFGE and Southern blotting as described in the Methods section. DNA accumulation was quantitated by scanning of several exposures of the blots. Values represent the average of several measurements in the linear range. OD values were normalized to 100% at the 12.5 h time points of each incubation temperature.

Protein synthesis

The defect in *ts*4149 could be caused directly by a defect during DNA replication or indirectly by a defect prior to DNA replication. For example, a defect in early gene expression would be expected to result in a partial or complete block of viral DNA replication as seen in this mutant. To determine if and how protein synthesis was affected, it was studied at both the permissive and nonpermissive temperatures by pulse-labeling proteins. Monolayers of BSC40 cells were therefore infected with either IHD-W or *ts*4149, and the rate of protein synthesis at different times postinfection by pulse-labeling for 15 min with [^{35}S]methionine at 2.5 hour intervals up to 12.5 hours postinfection was determined (fig. 5). In a normal wild-type infection, prior to DNA replication, both host and early viral proteins were synthesized. Shut-off of host protein synthesis in BSC40 cells occurs shortly after viral DNA replication begins and is somewhat slower at 32°C than at 40°C (Note the characteristic actin bands at approximately 43 kDa in fig. 5B). Early protein synthesis is shut down, DNA replication begins and is then followed by intermediate and late gene expression. Several aspects of the protein synthesis seen in ts4149 differed from those of the normal wild-type infection described above. (1) In the mutant infection, host shut-down was delayed at the permissive temperature and was virtually abolished at the nonpermissive temperature. Significantly, the partial shut-off of host protein synthesis normally observed prior to viral DNA replication failed to materialize in mutant infections (fig. 5; compare the 0 and 2.5 h time points). (2) With the exception

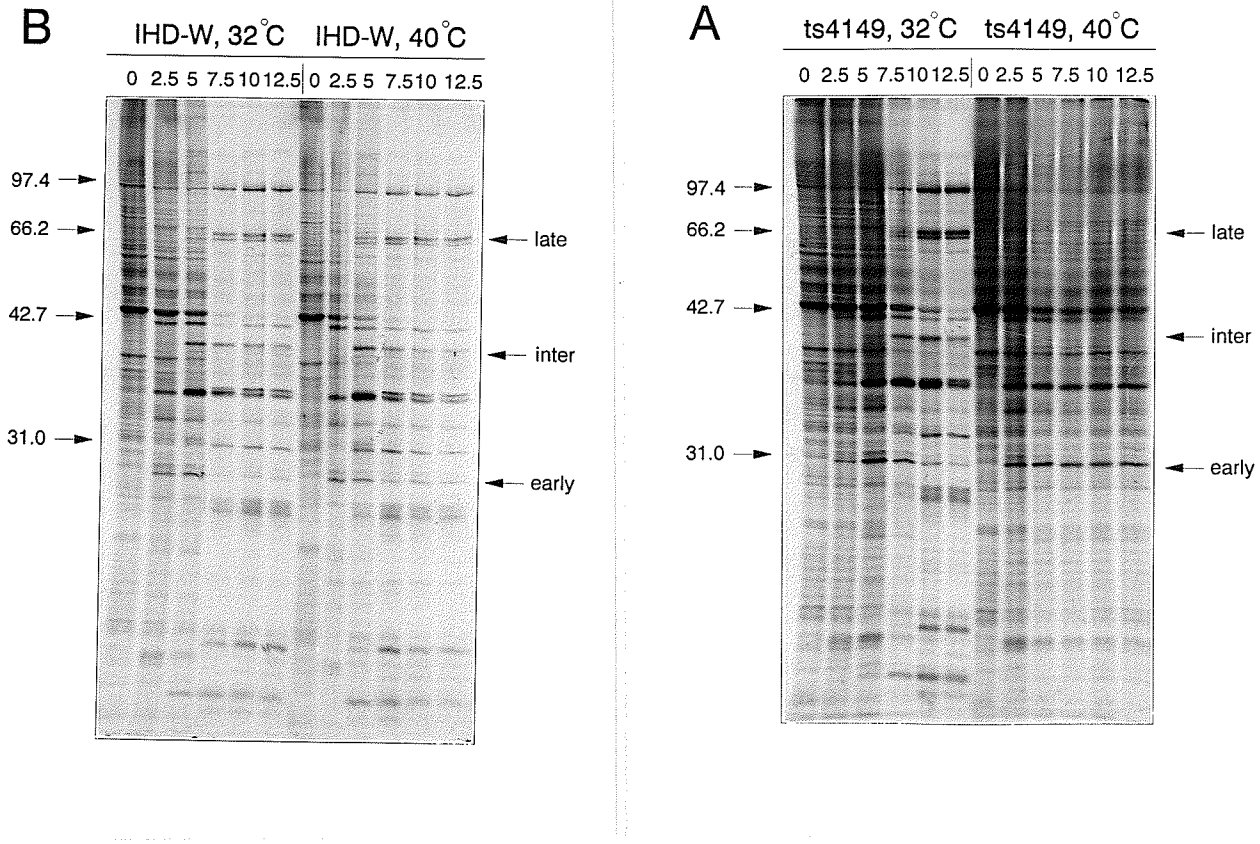


figure 5. Protein synthesis in cells infected with mutant *ts*4149 (A) or wild-type strain IHD-W (B). BSC40 monolayers were infected at a multiplicity of 20 PFU per cell and incubated for 0, 2.5, 5, 7.5, 10 and 12.5 h at 32°C or 40°C, after which total proteins were pulse-labelled with [^{35}S] methionine. Samples were harvested, separated in a 12.5% SDS-polyacrylamide gel, and exposed to X-ray film. Molecular weight masses are given in kilodaltons and representative early, intermediate (inter) and late proteins are indicated.

of a slight delay at the permissive temperature, synthesis of early proteins appeared normal at both temperatures. This was also consistent with the delay in DNA replication at the permissive temperature which was observed by PFGE. (3) As would be expected, the early proteins produced at the permissive temperature were shut down at later times when intermediate and late proteins were produced. At the nonpermissive temperature, both host and early viral proteins continued to be synthesized and neither intermediate nor late viral proteins were detected. The observed protein synthesis in the mutant also helped to confirm the DNA negative phenotype.

Mapping of the mutation in ts4149

The region of the ts4149 mutation was quickly localized by marker rescue using a set of overlapping cosmids (Thompson and Condit, 1986). Two cosmids, pWR67-98 and pWR74-111 which map to the central region of the vaccinia genome, were found to rescue the ts phenotype efficiently. The DNA from cosmid pWR67-98 was then used as a probe to screen a vaccinia strain WR random DNA library of partial-*Mbo*I fragments cloned into the *Bam*HI site of pTZ18R. The positive clones that were found were then screened for their ability to rescue the ts phenotype of ts4149. Plasmid pJB319 was thus shown to rescue with high efficiency (see fig. 6). This clone contains ORFs D2, D3, D4, and parts of D1 and D5. To narrow down the region, a 1.8kb *Kpn*I-*Kpn*I fragment which contained all of ORF D4 and part of ORFs D3 and D5 was

cloned into *Kpn*I digested pTZ18R to produce the subclone pJB319KK. This plasmid was obtained by cloning a 1.8kb *Kpn*I fragment from pJB319 into *Kpn*I digested pTZ18R. Marker rescue with this clone was as efficient as with pJB319. It should be noted that pJB319 was derived from a WR library, and WR does not contain the *Kpn*I site in ORF D4 as is the case with IHD-W. PJB319KK was easily obtained from WR-derived DNA, but we were unable to generate the same clone from IHD-W DNA. A positive rescue with a subclone containing an *Eco*RI-*Sal*I fragment (pJB319ES), which contained all but the first codon of ORF D4 and part of D5, ruled out ORF D3. Plasmid pJB319ES was obtained by cloning the *Eco*RI-*Sal*I fragment of pJB319 into pTZ18R. The reduced efficiency of marker rescue with this latter clone suggested that the mutation site was in ORF D4. This conclusion was corroborated by the finding that ts4149 failed to complement mutant ts6389 (McFadden & Dales, 1980) which is known to harbour a mutation in ORF D5 (Evans and Traktman, 1987). In an attempt to further localize the mutation, the D4 ORF was divided at the *Pst*I site (see fig. 6) to create two more subclones. However, neither the left (pJB319PP), nor the right (pJB319P) fragment were able to rescue above background (fig. 6B). A different strategy was then adopted, namely the construction of "replacement" clones. To allow direct comparison of ts4149 with the wild-type IHD-W, the *Hin*dIII-*Sal*I fragments containing viral genes D1 to D4 and part of D5 were ligated into similarly digested pTZ18R DNA. Plasmids pKM1wt and pKM1ts were derived from IHD-W and ts4149 respectively. In the replacement clones, the *Pst*I-*Mlu*I fragments of these clones were switched (fig. 6C). Plasmid pKM1r1 was obtained by replacing the 482bp *Pst*I-*Mlu*I

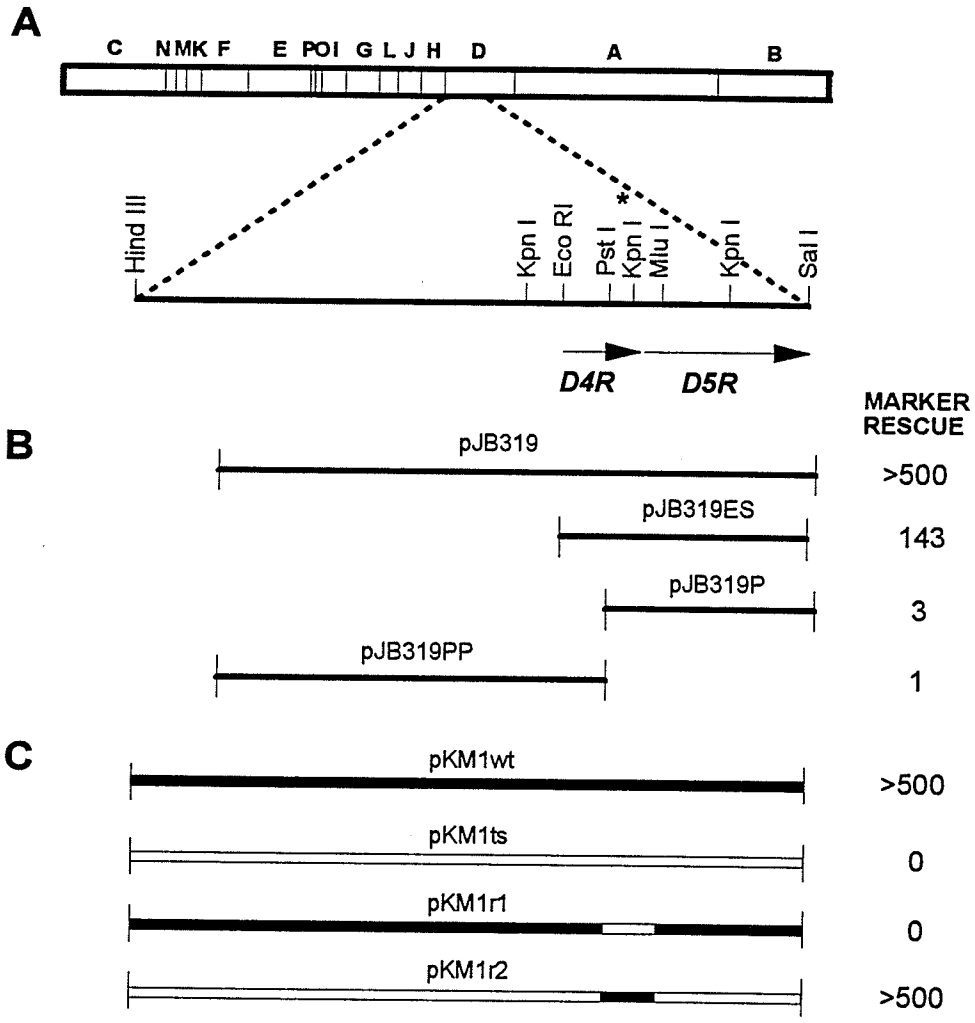


figure 6. Marker Rescue of mutant *ts*4149. Monolayers of BSC40 cells were infected with *ts*4149 at a multiplicity of 0.05. For each rescue, 10 ug of linear viral DNA was added to the infected monolayers. The monolayers were then incubated for 72 h and rescued plaques visualized by staining with crystal violet. (A) Restriction map of vaccinia virus. The letters A to P represent the *Hin*dIII fragments of vaccinia virus WR. The location of genes D4 and D5 and the direction of transcription are as indicated (the *Hin*dIII-*Sal*I fragment contains a truncated D5 ORF). Note that the starred *Kpn*I site is present in DNA from IHD-W or *ts*4149 but not from WR. (B) The insert in plasmid pJB319 is of WR origin. The other three plasmid are derivatives of pJB319. (C) *Hin*dIII-*Sal*I fragments from wild-type strain IHD-W (pKM1wt) or *ts*4149 (pKM1ts) were cloned. pKM1r1 and pKM1r2 represent clones in which the *Pst*I-*Mlu*I fragments have been switched. The results of the marker rescue experiment are shown at the right. The values shown indicate the number of plaques per monolayer of 3×10^6 cells.

fragment, which contains the junction of ORFs D4 and D5, from pKM1wt with that of pKM1ts. Similarly, pKM1r2 was derived by replacement of this 480-bp fragment from pKM1ts with that of pKM1wt. Marker rescue with these DNA clones placed the mutation within the this region. To facilitate sequencing with the universal and reverse primers for pTZ18R, each of the four plasmids (pKM1wt, pKM1ts, pKM1r1, and pKM1r2) were digested with *Mlu*I+*Sal*I, to allow sequencing from the *Mlu*I end, or with *Hin*dIII+*Pst*I, to allow sequencing from the *Pst*I end. These digests were then ligated and the resultant clones were used in sequencing reactions. The identity of each of these clones was confirmed by DNA sequencing. DNA sequencing also allowed us to determine the mutation site within ORF D4 (fig. 7). The sequence of wild-type strain IHD-W differs from that of ts4149 by a single base change, namely a G → A transition at nucleotide 535 of ORF D4R. This mutation results in a change from the amino acid glycine to arginine at position 179, which significantly reduces the hydrophobicity of a hydrophobic region near the carboxyl terminus of the D4R protein (fig. 8). The IHD-W sequence was also compared with homologous sequences from two other strains of vaccinia virus, Copenhagen and WR. When comparing IHD-W with WR, there are 5 base differences located at nucleotides 504, 534, 561, 577 and 623, all in ORF D4. Interestingly, Copenhagen differs at 9 positions namely nucleotides 561, 577, 727, 748, 754, 757, 781, 809 and 823, 7 of which are in ORF D5. Note that the base difference at position 623 is the cause of the restriction fragment polymorphism at the *Kpn*I site alluded to previously. Finally, the amino terminal half of ORF D4R has also been sequenced and found to be identical to that

of strain WR.

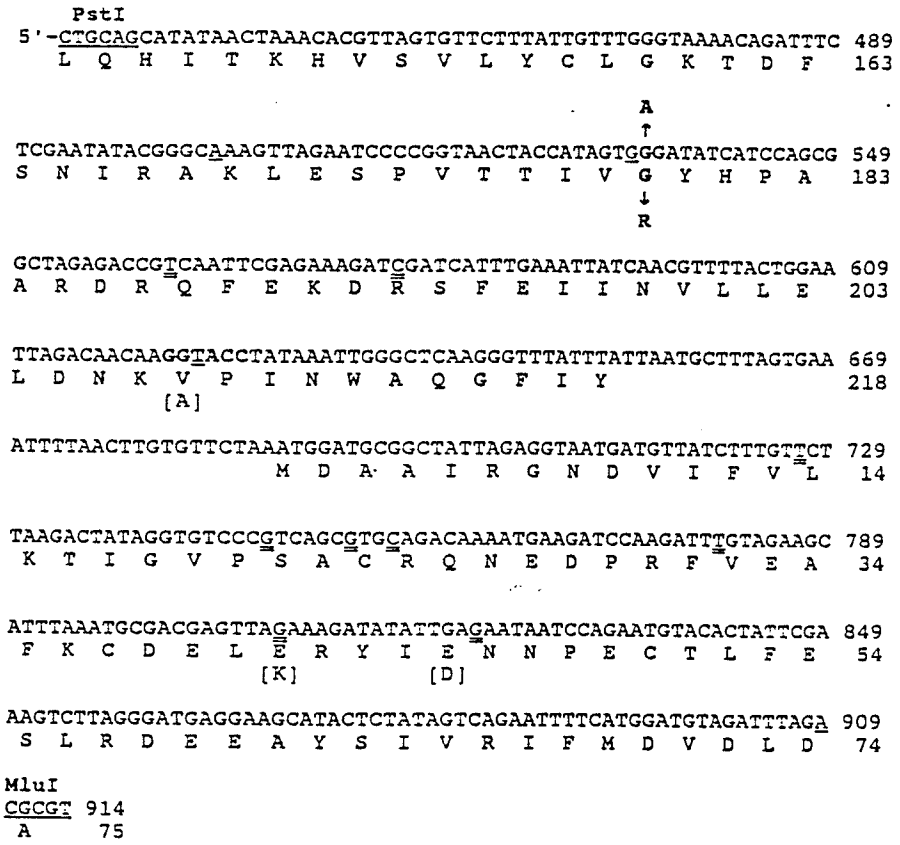


figure 7. The DNA sequence of the 482-bp *PstI-MluI* fragment capable of rescuing *ts*4149. This 482-bp region contains the 3′ end of ORF D4 and the 5′ end of ORF D5. The sequence of wild-type strain IHD-W differs from that of ts4149 by a single base change, namely a G → A transition at nucleotide 535 of ORF D4 (the numbering starts at the beginning of ORF D4, for DNA, and at ORF D4 or D5 at the amino acid level). This mutation causes a change from glycine to arginine at position 179. The IHD-W sequence was also compared with homologous sequences from two other strains of vaccinia virus, Copenhagen and WR. The nine nucleotide differences between IHD-W and Copenhagen are doubly underlined; IHD-W and WR differ at five positions, all in ORF D4 (singly or doubly underlined). Differences in amino acids are shown in brackets.

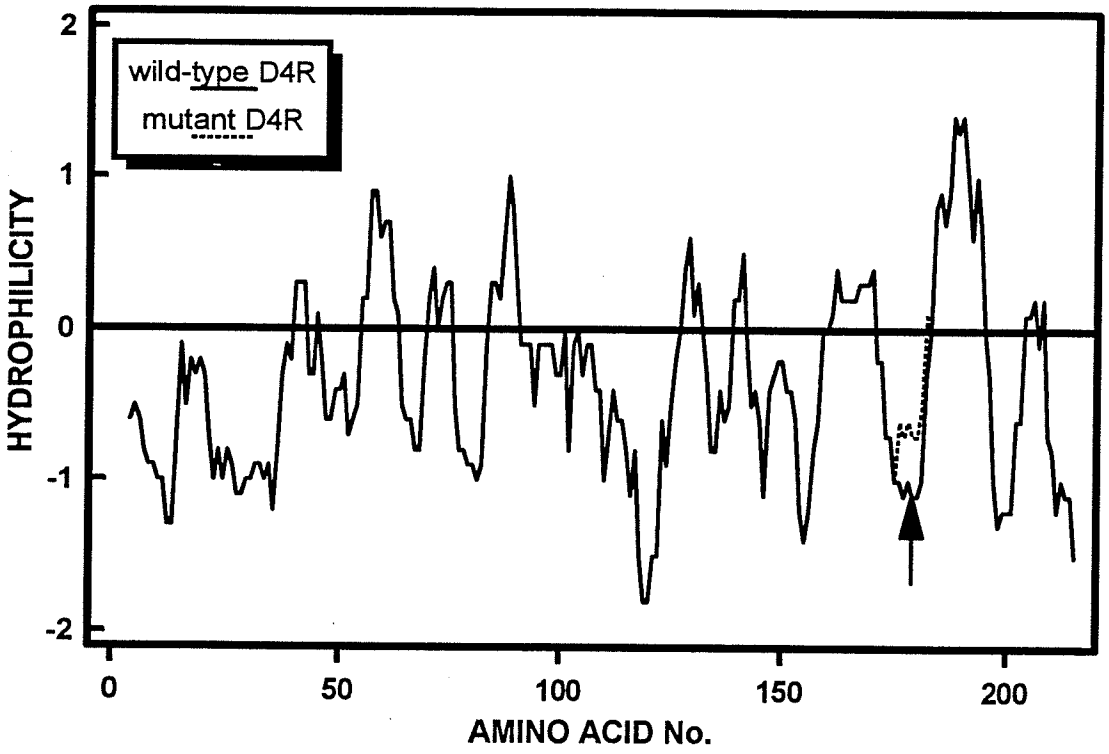


figure 8. Hydrophilicity plot of the wild-type and mutant D4 gene product using the Hopp and Woods algorithm (Hopp and Woods, 1981). Positive values indicate hydrophilicity while negative values indicate hydrophobicity. The arrow indicates the location of the *ts* lesion in mutant *ts*4149. Dashed lines correspond to the predicted structural change the mutant sequence would have on the D4 protein.

DISCUSSION

*ts*4149 is a temperature-sensitive derivative of the IHD-W strain of vaccinia virus. It was previously classified as DNA$^-$ on the basis of a marked reduction of the incorporation of [^{3}H]thymidine at the nonpermissive temperature. We have now shown that the defect in DNA replication is both quantitative and qualitative: the amount of DNA replicated at the nonpermissive temperature was about 1% of that in wild-type infections, and the little DNA that replicated failed to convert to mature linear DNA molecules. We have also shown that the defect in DNA replication is not caused by a general defect in early gene expression. Furthermore, as might be expected by this severe defect in DNA replication, intermediate and late gene expression could not be detected. At the permissive temperature, we observed a reduced rate of DNA synthesis and a marked delay in the conversion to linear monomers. We have mapped the point mutation that is responsible for this ts DNA$^-$ defect to the C-terminus of ORF D4R. This ORF has recently been shown to code for a 25-kDa protein with uracil DNA glycosylase activity (Stuart et al., 1993; Upton et al., 1993).

The D4 gene is expressed early and its transcript has the unique property of being co-terminated with the downstream D5 gene (Lee-Chen et al., 1988). Mutants in the D5 gene have an absolute defect in DNA elongation similar to that observed for DNA polymerase mutants. In contrast, some residual DNA replication takes place in

the D4 mutant described. In this respect, mutants in the B1R gene, which encodes a serine/threonine protein kinase and whose defect results in defective DNA replication in mouse L cells (Rempel and Traktman, 1992) seems to resemble the D4 mutant. It is not yet known whether the observed residual replication is caused by leakiness of the mutant protein or whether it reflects the function of the wild-type protein. It should be noted that the existence of mutants with a much reduced level of DNA synthesis is not without precedent. For example, such a phenotype is also displayed by conditional mutants in the gp32 and gp41 genes of bacteriophage T4, which encode a helix-destabilizing single-stranded DNA binding protein and a helicase respectively (reviewed in Benson and Kreuzer, 1992). Other replication fork proteins of bacteriophage T4 are absolutely required for viral DNA synthesis, and conditional mutants in genes coding for those proteins display the tight DNA negative phenotype also observed in vaccinia mutants within the DNA polymerase or D5 genes.

Though the most apparent defect of *ts*4149 is at the level of DNA replication, a less obvious defect was observed prior to the onset of DNA replication. Specifically, shut-off of host proteins, which is normally observed prior to the onset of DNA replication in wild-type infections (2-2.5 hours postinfection), failed to materialize in cells infected with the mutant virus. The efficiency and kinetics of the shut-off of host proteins is known to be dependent on cell type (reviewed in Moss, 1990a). Infections of some cell lines cause a rapid and virtually complete shut-off which does not require viral gene expression. In other cell lines, the initial shut-off is incomplete, and the

ultimate complete shut-off takes place after the onset of DNA replication and late gene expression. In our hands, BSC40 cells fall into the latter class. The highly variable degree of the pre-replicative shut-off of host protein synthesis in different cell lines does not significantly affect the rate of subsequent viral DNA replication. We therefore believe that the failure to shut-off protein synthesis in *ts*4149 has no direct bearing on the DNA negative phenotype of this mutant. This belief is further supported by two additional observations. First, early shut-off in *ts*4149 is defective at both the permissive and the nonpermissive temperature. Second, at least one other mutant in which the rate of viral DNA synthesis is unaffected shows a comparable host shut-off defect (Carpenter and DeLange, 1991).

The vaccinia D4 protein and its homolog of the Leporipoxvirus SFV have limited homology to a highly conserved class of uracil DNA glycosylases (Tomilin and Aprelikova, 1989; Upton et al., 1993). Members of this class of enzyme have been detected in organisms as evolutionarily distinct as *E. coli* (Varshney et al., 1988), yeast (Percival et al., 1989), herpes simplex virus types 1 and 2 (Worrad and Caradonna, 1988; Mullaney et al., 1989), and humans (Olsen et al., 1989). That this homology reflects a functional equivalence was most dramatically illustrated by the ability of the human uracil DNA glycosylase to substitute for the *E. coli* enzyme (Olsen et al., 1991). Biochemical assays of the recombinant vaccinia D4 protein and its SFV homolog have demonstrated that these poxvirus proteins, like other members of this homology group, possess uracil DNA glycosylase activity (Stuart et al., 1993; Upton et al., 1993). It is

interesting that, whereas uracil DNA glycosylase-negative mutants of *E. coli* (Duncan, 1985), yeast (Percival et al., 1989), and herpes simplex virus (Mullaney et al., 1989) are viable, similar mutants of the vaccinia enzyme are inviable (Stuart et al., 1993). It thus appears unlikely that a mere failure to remove uracil residues from DNA would cause the observed block in DNA replication. Why then is the D4 protein essential for the completion of viral DNA replication and of the viral life cycle?

We postulate that the poxvirus uracil DNA glycosylase has acquired a novel function or property that makes it essential for sustained viral DNA replication. One of the ways to become multi-functional is through association with one or more other proteins. The existence of such a multiprotein complex is supported by several lines of evidence. (i) Temperature-shift experiments with mutant *ts*4149 have shown that the defect of this mutant is not thermolabile at the level of DNA synthesis (McFadden and Dales, 1980), an observation that is compatible with the view that this is an assembly-type mutant. (ii) Even though D4 shares homology with the highly conserved class of uracil DNA glycosylases, this homology is significantly lower than that between any other members of this homology group.

The idea that a repair enzyme such as a uracil DNA glycosylase may exist in a multienzyme complex is not novel. Besides the highly conserved uracil DNA glycosylase alluded to above, human cells harbour at least two other enzymes with this activity. One is equivalent to the 37-kDa subunit of the homomultimeric glycolytic

enzyme glyceraldehyde-3-phosphate dehydrogenase (Meyer-Siegler et al., 1991); the second shares significant similarity to A and B type cyclins (Muller and Caradonna, 1993). Evidence is emerging that suggests that cellular uracil DNA glycosylases are likely present in high molecular weight complexes (Seal and Sirover, 1986; Slupphaug et al., 1991; Muller and Caradonna, 1993).

What would the function of such a complex be in the case of the vaccinia D4 protein? First, considering the known repair capability of this enzyme it is plausible that it is part of a repair complex. This is not a unique idea as nucleotide excision repair complexes exist in prokaryotes where incision, synthesis and ligation are coupled to function as a 'repairosome' complex (Caron et al., 1985). In the case of the base excision repair enzyme uracil DNA glycosylase, one would predict such a complex to remove uracil residues and repair the generated apyrimidinic (AP) sites. As mentioned above, failure to remove uracil bases should in itself not cause a block in DNA replication. The observed replication block might be explained in one of two ways. (1) If the mutant D4 protein has normal glycosylase activity but fails to coordinate the down-stream repair processes, the DNA polymerase might be unable to synthesize past this lesion. In fact, failure to remove AP sites is expected to affect DNA replication, since high-fidelity replicative DNA polymerases have a tendency to stall at such sites (Schaaper et al., 1983). (2) The observation that AP sites inhibit both nuclear and mitochondrial uracil DNA glycosylases suggests that these enzymes may bind to AP sites (Domena et al., 1988). It is therefore plausible that the vaccinia uracil

DNA glycosylase similarly binds with high affinity to AP sites. Such an association might indeed form the basis for subsequent repair steps. Assuming that the mutant uracil DNA glycosylase is capable of generating AP sites but incapable of coordinating subsequent repair of these sites, the enzyme might then stably associate with the AP sites it generated, and thereby form a physical block for movement of DNA polymerase. What constitutes repair of AP sites in poxvirus DNA is an unresolved question. One of the ways that the virus might cope with or even take advantage of AP sites is by using such sites as initiation points for recombination. Such a repair system could be somewhat analogous to the post-replication recombination-repair system in *E. coli* (Ganesan, 1974). In such a scenario, the mutant D4 protein would again have normal uracil DNA glycosylase activity but would be defective in its interaction with one or more components of a putative recombination-repair multienzyme complex and thereby fail to promote strand exchange and recombination.

A second possible role for the D4 protein is in a DNA replication complex. It has been shown previously that one of the human uracil DNA glycosylases is associated with DNA polymerase alpha (Seal and Sirover, 1986). Furthermore, at least one uracil DNA glycosylase is physically associated with replicating DNA (Krokan, 1981; Lee and Sirover, 1989), and synthesis of at least two uracil DNA glycosylases peaks just prior to DNA synthesis (Mansur *et* al., 1993; Muller and Caradonna, 1993). If the poxvirus uracil DNA glycosylase exists in a DNA replication complex, the DNA

replication defect in mutant *ts*4149 could be the result of the inability to form a functional replication complex.

To establish the role(s) of this repair enzyme during viral DNA replication and/or recombination, a combination of genetic and biochemical approaches needs to be used.

PART II

GLYCOSYLASES AND MULTIENZYME COMPLEXES

INTRODUCTION

DNA repair

DNA is a target for numerous physical and chemical agents which can cause damage to the DNA strands and may result in deleterious mutations that adversely affect the organism. These lesions may also hinder other cellular processes such as DNA replication and transcription (Hoeijmakers, 1991). Examples of such agents include heat which causes deamination of bases and base loss by glycosylic hydrolysis; UV radiation which produces pyrimidine dimers and 6-4 photoproducts; ionizing radiation which results in ring opening, base fragmentation, single and double stranded breaks; and chemicals that range from activated oxygen species to inorganic and organic electrophiles such as metals, alkylating agents and polycyclic aromatic hydrocarbons (Sancar and Sancar, 1988). In order to maintain the integrity of the DNA sequences and for protection from these agents, DNA repair mechanisms have evolved. One of the most important repair mechanisms for all organisms is excision repair which includes nucleotide excision repair and base excision repair.

i) nucleotide excision repair

Nucleotide excision repair (NER) is a general repair mechanism that is capable of dealing with an array of structurally unrelated lesions. These include the various UV

induced photoproducts such as the pyrimidine dimers and 6-4 photoproducts as well as chemical adducts and certain types of cross-links. In all organisms, NER consists of five basic steps, these being, 1) damage recognition 2) incision of the damaged strands on both sides of the lesion and some distance from it 3) excision of the lesion containing oligonucleotide 4) synthesis of new DNA using the undamaged strand as a template 5) ligation of the new strand (Hoeijmakers, 1993a).

Most of our understanding of this repair mechanism has been derived from prokaryotes such as *E.coli.* It is felt that this is a valid model for eukaryotic systems as both types of organisms share the basic steps of NER, appear to have similar substrate recognition and both include preferential repair of the transcribed DNA strand (Hoeijmakers, 1993a). However, it is believed that the eukaryotic mechanism is more complex largely due to the significant increase in the number of proteins that are involved. In *E. coli,* there are four major proteins necessary for this process (UvrA, B, C and D) along with two auxiliary factors (Phr and Mfd). The Mfd protein confers repair specificity for the transcribed strand (Selby and Sancar, 1993). In the yeast *Saccharomyces cerevisiae,* the collection of proteins currently includes 11-12 members that comprise the *rad3* epistasis group. This is also believed to be an incomplete list (Hoeijmakers, 1993a). At this point, no overall sequence homology between the Uvr proteins of *E. coli* and the RAD proteins of yeast has been observed, suggesting that the two have diverged substantially. However, between yeast and humans there is a striking overall homology (Hoeijmakers, 1993b).

While far less is known about eukaryotes, there have been a number of important advances recently concerning the eukaryotic proteins involved in NER. In yeast, the RAD3 protein which is known to be involved in excision repair has also been found to be essential for the transcription of genes by RNA polymerase II (Guzder et al., 1994). This protein is homologous to the human ERCC2 protein. In humans, it was found that ERCC3 which is involved in NER is also part of the transcription factor TFIIH (Schaeffer et al., 1993). These findings allow for new models in which the NER proteins are involved in two different aspects of DNA metabolism, transcription and repair (Bootsma and Hoeijmakers, 1993).

ii) base excision repair

Unlike NER, base excision repair involves the removal of very specific types of abnormal or damaged bases that do not belong in the DNA strands. This repair mechanism is carried out by glycosylases which are generally small enzymes of approximately 20 to 30 kDa in size. They are known to have a narrow substrate specificity and also have no cofactor requirements. Glycosylases have been purified from both prokaryotic and eukaryotic sources. Enzymes that have been found include uracil DNA glycosylase, hypoxanthine DNA glycosylase, 3-methyladenine DNA glycosylase I and II, and formamidopyrimidine DNA glycosylase (Sancar and Sancar,1988).

All glycosylases catalyze the hydrolytic cleavage of the N-glycosylic bond that

links altered bases to the sugar phosphate backbone. Upon hydrolysis of this bond, an apurinic or apyrimidinic (AP) site is produced. The original sequence is then restored by the consecutive action of an AP endonuclease, DNA polymerase and DNA ligase (Sancar and Sancar, 1988).

Uracil DNA glycosylases

Uracil DNA glycosylases (UDG) specifically remove uracil residues from DNA strands by the mechanism described above (refer to fig. 9). These enzymes are apparently inhibited by free uracil and also by the presence of AP sites in the DNA (Sancar and Sancar, 1988; Domena et al., 1988). The uracil residues found in DNA may have resulted from either the misincorporation of dUTP by DNA polymerase or by the deamination of cytosine. The incorporation of uracils into DNA is not necessarily a mutagenic event that requires repair. The deamination of cytosine however, is mutagenic as it results in a GC to AT transition if not corrected.

As stated in the previous discussion, the vaccinia D4 protein has a limited homology to a highly conserved class of uracil DNA glycosylases (Tomilin and Aprelikova, 1989; Upton et al., 1993) and is known to have DNA glycosylase activity (Stuart et al., 1993). Members of this class of enzyme have been detected in a diverse array of organisms such as *E. coli* (Varshney et al., 1988), yeast (Percival et al., 1989), herpes simplex virus types 1 and 2 (Worrad and Caradonna, 1988; Mullaney et al.,

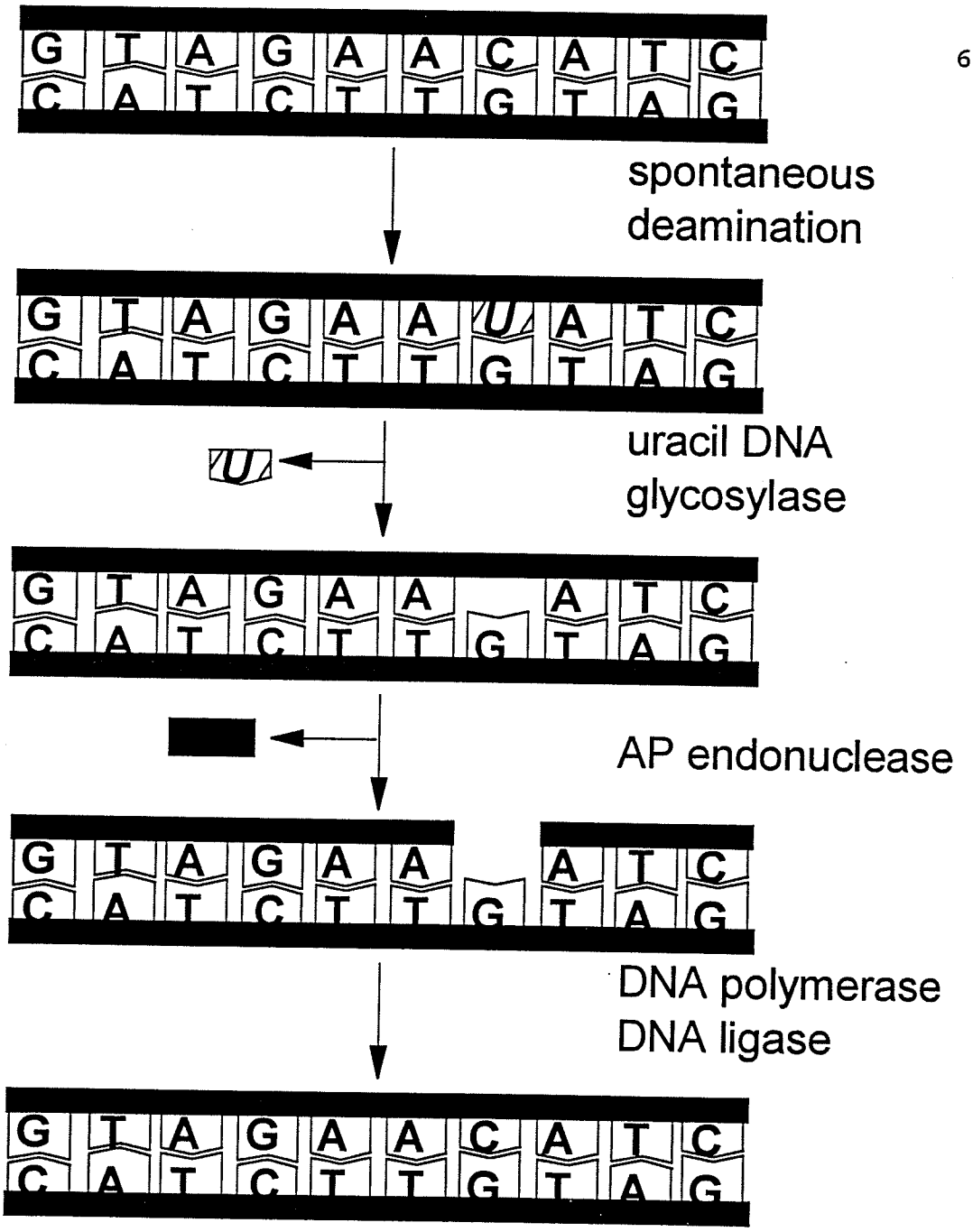


figure 9. Base excision repair invoving the uracil DNA glycosylase enzyme. Spontaneous deamination of cytosine results in the presence of uracil in the DNA strand. This is recognized by the UDG enzyme which removes the uracil residue by cleaving the N-glycosylic bond joining the base to the sugar and leaving an AP site. An AP endonuclease recognizes this site and removes the sugar-phosphate backbone. DNA polymerase adds the correct base and DNA ligase then seals the nick (modified from Alberts et al., 1989).

1989), and humans (Olsen et al., 1989). As this enzyme catalyzes a very specific repair mechanism, it is intriguing that mutants of *E. coli* (Duncan, 1985), yeast (Percival et al., 1989), and herpes simplex virus (Mullaney et al., 1989) are viable, whereas the mutation in *ts*4149 leads to a lethal DNA replication defect. It therefore appears unlikely that a failure to remove uracil residues from DNA would be the main cause of the phenotype observed for *ts*4149.

It was also postulated in the previous discussion that the glycosylase protein of vaccinia has acquired novel properties, possibly through protein interactions, that make it distinct from the other glycosylases in this group. The idea that the repair enzyme, UDG, may exist in a complex is not new. It has been found that human cells harbour at least two other enzymes with this glycosylase activity and these particular proteins may be present in high molecular weight complexes (Seal and Sirover, 1986; Slupphaug et al., 1991; Muller and Caradonna, 1993). One has been identified as the 37-kDa subunit of the homomultimeric glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Meyer-Siegler et al., 1991) while the second shares a significant similarity to A type cyclins (Muller and Caradonna, 1993).

Considering the known repair capability of this enzyme it is reasonable to assume that the D4 protein is part of a repair complex. This is not a unique proposition, as nucleotide excision repair complexes exist in prokaryotes where incision, synthesis and ligation are coupled to function as a 'repairosome' complex

(Caron et al., 1985). In the case of the base excision repair enzyme uracil DNA glycosylase, it could be predicted that such a complex would remove uracil residues and repair the generated AP sites.

A second possible role for the D4 protein is in a DNA replication complex, based on the DNA$^-$ phenotype of *ts*4149. It has been shown previously that one of the human uracil DNA glycosylases is associated with DNA polymerase α (Seal and Sirover, 1986). Furthermore, at least one uracil DNA glycosylase is physically associated with replicating DNA (Krokan, 1981; Lee and Sirover, 1989), and synthesis of two other uracil DNA glycosylases peaks just prior to DNA synthesis (Mansur et al., 1993; Muller and Caradonna, 1993). If the poxvirus uracil DNA glycosylase exists in a DNA replication complex, the DNA replication defect in the mutant *ts*4149 could be the result of an inability to form a functional replication complex possibly due to a lost interaction. To try to determine if such a complex exists, a system for detecting interacting proteins in yeast was utilized.

The two-hybrid system

The two-hybrid system is an *in vivo* genetic assay which allows for the detection of protein-protein interactions between any two polypeptides in yeast (Fields and Song, 1989; Chien et. al., 1991). This assay is based on the reconstitution of the yeast GAL4 transcriptional activator. Under normal conditions the GAL4 protein is

required for the expression of genes encoding the enzymes for galactose utilization. This protein consists of two separable and functionally essential domains: the N-terminal domain which binds to specific DNA sequences (UAS_G); and the C-terminal domain which contains the acidic regions necessary to activate transcription. Plasmids are constructed containing the DNA-binding domain of GAL4 fused to a known protein and the GAL4 transcriptional activation domain fused to protein sequences encoded by a library of DNA fragments. The library must be constructed in the activation domain plasmid to avoid detecting random sequences that can activate transcription when fused to a DNA-binding domain (Chien et al, 1991). These plasmids are transformed into a specific yeast strain, where interaction between proteins encoded by the two plasmids leads to the transcriptional activation of a reporter gene, β-galactosidase (fig. 10).

For these studies, the protein of interest is the vaccinia virus uracil DNA glycosylase (D4) which is mutated in *ts*4149. If this protein does exist in a complex, it may be possible for us to determine more about the mechanism of DNA replication in vaccinia by observing what interactions occur.

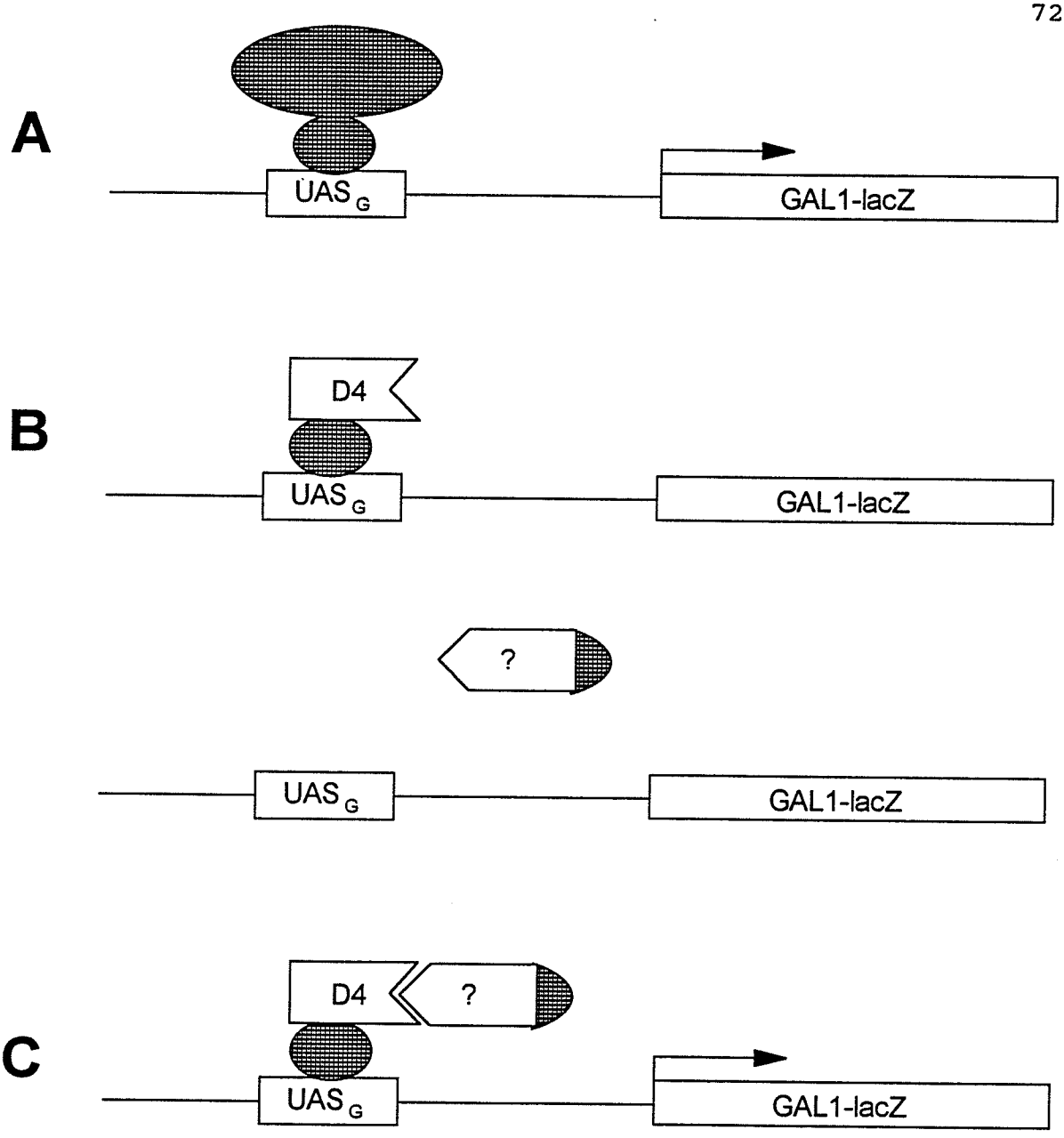


figure 10. Diagrammatical representation of the two hybrid system. (A) Transcriptional activation by the native GAL4 protein. (B) Represents how the two domains are separable; the DNA-binding domain of GAL4 (amino acids 1-147) fused to the protein of interest (D4) cannot activate transcription nor can hybrids containing the transactivating domain of GAL4 (768-881). (C) A protein-protein interaction between the D4 fusion protein and a GAL4 (768-881) fusion protein, will bring the DNA-binding domain and the transactivating domain close together, resulting in transcription of the reporter gene lacZ (modified from Fields et al., 198).

MATERIALS AND METHODS

A) The two-hybrid system

The two-hybrid system is an *in vivo* genetic assay which allows for the detection of protein-protein interactions in yeast (Fields and Song, 1989; Chien et al., 1991). Requirements for this system include plasmids containing the DNA-binding domain of GAL4 fused to DNA fragments of interest, a random DNA library fused to the GAL4 transcriptional activation domain and the specific yeast strains used for the transformation of these plasmids.

media (Sherman,1987)

YPAD : 6g Bacto-yeast extract, 12g Bacto-peptone, 500ml water, 10g agar (for plates). The amount of adenine sulfate added was dependent on liquid or solid medium. For plates 48mg/600ml was added, for liquid 24mg/600ml was added. The dextrose was in a solution of 10g in 100ml of water which was added separately after both the "yeast extract, peptone, adenine sulfate" and "dextrose" solutions were autoclaved.

SC minimal media: 3g ammonium sulfate, 1g yeast nitrogen base without amino acids and ammonium sulfate (Difco) and 12g of dextrose was dissolved

in 600ml water. 0.35g of appropriate amino acid mix was added to the solution. This mix contains the required amounts of adenine, arginine, homoserine, isoleucine, lysine, methionine, phenylalanine, tyrosine, valine and may also contain histidine, leucine, tryptophan and/or uracil dependent on the type of ommission media. For plates, 10g of agar is also added.

X-gal minimal media : 3g ammonium sulfate, 1g yeast nitrogen base without amino acids and ammonium sulfate and 12g of sucrose was dissolved in 600ml of water. 0.35g of the correct amino acid mix was added along with 10g of agar. After autoclaving the media was allowed to cool to approximately 50°C. At this time, 35ml of 1M potassium phosphate pH 7 and 1ml of 60mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal) (Diagnostic Chemicals Limited) in dimethyl formamide was added before pouring the plates.

Yeast strains

Two different yeast strains are required for the library screenings. The strain GGY1::171 whose genotype is leu2-3,112 hisΔ200 gal4 Δgal80 ΔURA3::GAL1-lacZ (Gill and Ptashne, 1987) was used with the pMA424 constructs while the strain CTY10-5d (MATa ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ) was required for use with the pBTM116 constructs.

Plasmid construction

Vectors used to construct plasmids were pMA424 (Ma and Ptashne, 1987); pDP4, pDP7 and pDP12 from Dr. R.D. Gietz; pGAD424 and pBTM116 from Dr. S. Fields (fig. 11 and fig. 12). The pMA424 and pBTM116 plasmids contain the DNA binding domain of GAL4 while the pGAD424 and library plasmids contain the acidic domain. The multiple cloning sites of pMA424, pBTM116 and pGAD424 are identical in sequence and in reading frame. The viral gene of interest, D4R, was initially cloned into the pMA424 vector (Ma and Ptashne, 1987) which contains the DNA binding domain of the yeast GAL4 gene. An *EcoRI* to *MluI* fragment from pKM1wt containing all of D4R (except the first codon) and the 5′ end of D5R was blunt end cloned into the *BamHI* site of pMA424 creating the plasmid pMAD4. This put the gene in frame, creating a fusion protein containing the DNA binding domain and the D4R protein. For later screens the D4R gene was cloned into the pBTM116 vector which contains the lexA DNA binding domain of *E. coli*. This was accomplished by digesting pMAD4 with *EcoRI* and *SalI* and cloning this fragment into a similarly digested pBTM116 vector to create pBD4. The D4R gene was also similarly cloned into the pGAD424 vector. The plasmid pBD4ts was made by digesting pKM1ts with *EcoRI* and *MluI*. This fragment was then blunt end cloned into the *BamHI* site of pBTM116. 3′ end deletion clones of the D4R gene were constructed using pBD4 as a base. pBD4 was digested with *KpnI* and *SalI* (in the MCS) and the larger fragment produced was then recircularized to form pBD4K. Similarly, pBD4 was digested with *PstI* (one of the sites

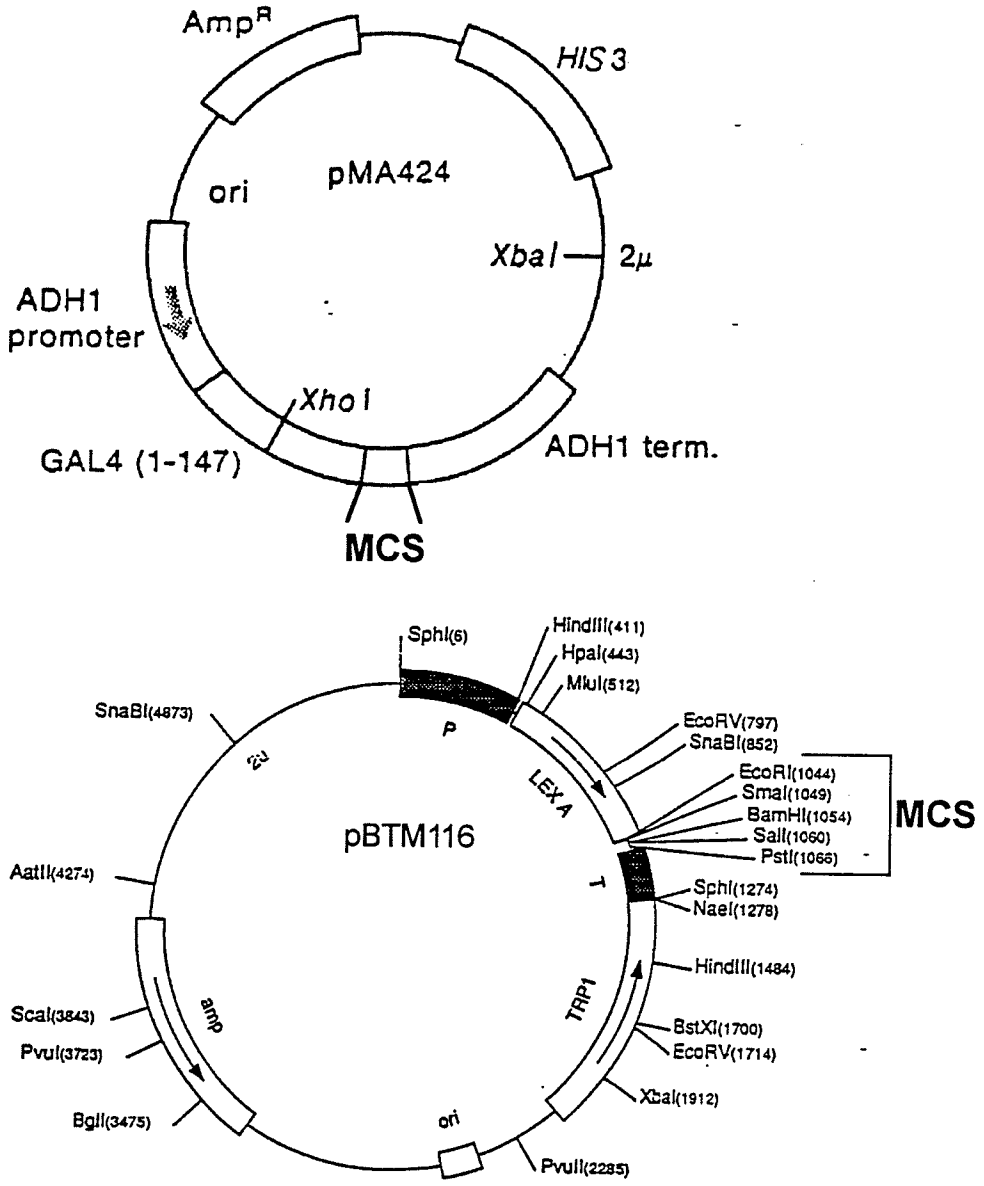
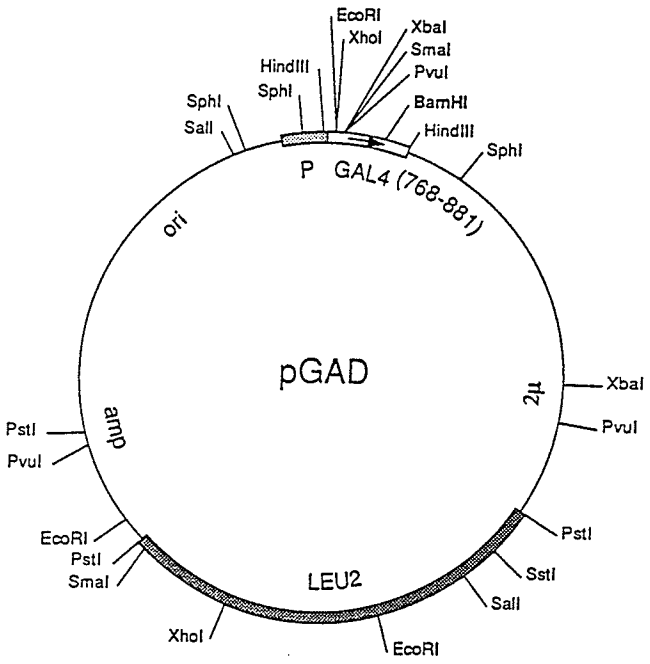


figure 11. Plasmids pMA424 and pBTM116 for use in the two hybrid system. The plasmid pMA424 contains amino acids 1-147 from the GAL4 DNA binding domain followed by the multiple cloning site GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC to generate fusion proteins (Ma and Ptashne, 1987). pBTM116 contains the lexA DNA binding domain (amino acids 1-202) followed by the identical multiple cloning site (S. Fields).



Vector	Reading Frame
pDP4	GGA TCC XXX
pDP7	XGG ATC CXX
pDP12	XXG GAT CCX

figure 12. Diagram of the two hybrid system library plasmids, pDP4, pDP7 and pDP12. These plasmids represent the three possible reading frames using the *BamHI* site as the cloning site. All plasmids contain amino acids 768-881 of the transactivating domain of the GAL4 protein (from Chien et al., 1991).

is in the MCS) and then recircularized to make the pBD4P clone. The replacement clones pBD4r1 and pBD4r2 were constructed by digesting both pBD4ts and pBD4 with *PstI* and exchanging the 500bp *PstI-PstI* fragments produced. pBD4r1 contains the wild type IHD-W fragment while pBD4r2 contains the ts4149 fragment with the mutation site. All clones are visually represented in figure 17 in the results section. Plasmids containing the A18R sequence were constructed by amplifying the required gene using the polymerase chain reaction (PCR), purifying the PCR product from an agarose gel and cloning it into the desired vector by standard techniques. pGA18M was made using the PCR primers A18M and A18L. It contains the A18R sequence starting at the first *MboI* site to mimic the clone pB3 that was initially found to interact with pMAD4 in the first two-hybrid screen. pGA18 contains the full length A18R sequence amplified by PCR using primers A18U and A18L and cloned into the *BamHI* site of pGAD424. The plasmid, pBA18 was similarly made by cloning the full length PCR product of A18R into the pBTM116 vector. pGA18 was digested with *HincII* and *SphI* and the fragment was then blunt-end ligated into a pGAD424 vector cut with *EcoRI* and briefly digested with S1 nuclease to make the pGA18H clone. pGA18H-1 was made from pGA18H digested with *BglII* and *PstI* to make a deletion of the 3′ region of A18. Similarly, pGA18H-2 was made from pGA18H digested with *EcoRI* and *PstI* to create a 3′ deletion after the *EcoRI* site. The clone, pGA18E contains a 5′ deletion of the A18R gene to the *EcoRV* site. It was made by initially digesting pGA18 with *SmaI* and then partially digesting it with *EcoRV*. The fragment containing the 3′

region of A18R was blunt end ligated. Refer to figure 18 in the results section for a diagrammatical representation of the above A18 clones. Recombinant plasmids were used to transform the *E. coli* strain DH5α. The reading frames of the above clones were confirmed by sequencing.

Polymerase Chain Reaction (PCR)

The protocol used for PCR reactions was described in Ausubel et al. (1987). Cloned *pfu* polymerase (Stratagene) was used in these reactions. The A18R gene of vaccinia virus was amplified both in full length and with the 5′ end deleted. The primers used were A18U=GGTTGGATCCTGTCACTATTAAAGATGGAG and A18L=GCTAGGATCCACTATGAGGGGTAATACACT for the full length gene and A18M=GGTTGGATCCCCTTTAAGTTTCTGATAC in combination with the A18L primer for the amino-terminal deletion. All three primers have *BamHI* sites added to allow for easier in frame cloning into the pGAD424 and pBTM116 vectors. Annealing temperatures of 54°C and 25 cycles were used for these primers.

DNA Sequencing

Sequencing of putative positive clones from the two-hybrid system was performed using the Sanger dideoxy chain-termination method (Sanger et al., 1977). For all sequencing reactions the Sequenase kit (US Biochemicals version 1.0 or version

2.0) was used. Primers were made for sequencing from pDP4, pDP7, pDP12, pGAD424, pMA424 and pBTM116. Templates from double-stranded DNA were prepared by the method of Hattori and Sakaki (1986) prior to sequencing.

Viral DNA libraries

WR vaccinia virus libraries were prepared by Dr. R. D. Gietz. These libraries were constructed by a series of digests involving partially digesting WR viral DNA with the restriction enzyme *MboI*. Fragments were size selected in that a group of approximately 1-3kb and a group made up of 3-6kb fragments were selected for cloning. These groups were then cloned into the *BamHI* site of vectors pDP4, pDP7 and pDP12 which contain the acidic transactivating domain of the yeast GAL4 gene. The three separate vectors with differing cloning sites were used to account for all three reading frames.

High efficiency yeast transformation

The high efficiency transformation of yeast uses the method described by Gietz et. (1992). This method was used for all library screening. Briefly, the yeast strain to be transformed was grown in 10ml of YPAD (or selective medium if required) overnight until the cell density is $>1X10^7$ cells/ml. This culture was subcultured into 100ml prewarmed YPAD to a density of $5X10^6$ cells/ml and incubated at 30°C until the

cell density reached $2X10^7$ cells/ml. The cells were collected by centrifugation in a sorvall SS-34 rotor (5000rpm, 5 minutes), washed twice with sterile water and resuspended in 1ml of sterile water. The suspension was then transferred to an eppendorf tube, washed twice with 1ml of TE-lithium acetate (10mM Tris, 1mM EDTA, 100mM lithium acetate) and resuspended in TE-lithium acetate to a density of $2X10^9$ cells/ml. Plasmid DNA, carrier DNA (boiled salmon sperm DNA) and 50µl of the cell suspension were used in the transformation reaction. To this, 300µl PEG/lithium acetate (40% PEG 3500, 100mM lithium acetate) was added and vortexed briefly to mix. This mixture was incubated at 30°C for 30 minutes and then heat shocked at 42°C for 20 minutes. The cells were pelleted, the liquid was removed and the cells were then resuspended in 1ml of sterile water by pipetting the cells up and down. Appropriate dilutions were plated onto SC minimal medium with selection for both transformed plasmids (-His -Leu for GGY1::171 and -Trp-Leu for CTY10-5d). The plates were incubated at 30°C.

Colony yeast transformation

This method was based on the protocol by Gietz, et al. (1992). The yeast strain (either GGY1::171 or CTY10-5d) was streaked out onto -URA medium from frozen stocks. The plate was placed at 30°C for two days and then subcultured onto YPAD for overnight growth. The yeast cells were scraped off the plate and resuspended in 1ml of sterile water. They were then pelleted and the water removed. An equal

volume of TE/lithium acetate (10mM Tris-HCl pH7.5, 1mM EDTA, 100mM lithium acetate) was then added to the pellet. This was vortexed to resuspend. For the transformation reaction, 50µl of the yeast solution was used with 5µl of carrier DNA and 5µl of each plasmid to be transformed into the yeast strain. 300µl of PEG/TE/lithium acetate solution (40%PEG, 10mM Tris-HCl pH7.5, 1mM EDTA, 100mM lithium acetate) was added to the above solution. This mixture was incubated at room temperature for 30 minutes after which it was heat shocked at 42°C for 15 minutes. The cells were briefly pelleted and the supernatant was removed. 400µl of sterile water was used to resuspend the cells. 200µl of this was then plated onto minimal medium with selection for the plasmids that were transformed in. The plates were incubated at 30°C until transformants appeared.

Segregation and selection of interacting plasmids in the two-hybrid system

Library screening plates were replica-plated onto the corresponding x-gal minimal medium which was then incubated at 30°C until blue colonies were observed. Colonies corresponding to the blue transformants were then picked from the replicated master plate and grown overnight in 2ml of synthetic complete media with selection, (either -Trp-Leu or -His-Leu) dependent on which DNA binding domain plasmid had been used in the screen. The number of cells per ml were counted using a haemacytometer and between 500 to 1000 cells were plated onto SC -Trp-Leu or SC -His-Leu plates (dependent on plasmid). Colonies were grown for 2 to 3 days and

were then replica-plated onto corresponding x-gal media. In this way pure blue colonies containing both interacting plasmids were obtained. The yeast strain with both plasmids was frozen in 20% glycerol. The transformant was also grown up in liquid -Leu medium to obtain the interacting plasmid. The cells were counted and 500 to 1000 cells were plated onto -Leu media. Once colonies had grown, the plates were replica-plated onto -Leu x-gal media and onto -Trp-Leu plates. These were incubated at 30°C for 2 days (or longer) while the blue colour developed. To select only the interacting pGAD library plasmid, colonies that grew on SC -Leu medium, but not on SC -Trp-Leu medium and were white on -Leu x-gal plates were picked. This also ensures that both plasmids were required for colour development and for transcriptional activation. The plasmid was then isolated from yeast, after which it was transformed back into the yeast strain containing the DNA binding domain plasmid fused to D4R, and the interaction was tested by replication to X-gal medium based on colour development.

Isolation of plasmid DNA from yeast

Plasmid DNA was isolated from yeast for transformation into *E. coli* using the the method of Hoffman and Winston (1987). 1.5 ml cultures were grown at 30°C for 1 day in SC minimal medium with appropriate selection to maintain the plasmid. The culture was poured into a sterile eppendorf tube and centrifuged briefly in a microfuge to pellet the cells. The liquid was removed and the pellet was vortexed to resuspend

the cells in any remaining liquid. 200 µl of yeast cracking buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA), 300mg of glass beads (425-600 microns, acid washed, Sigma # G-8772) and 200µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture was vortexed for 2 minutes followed by centrifugation for 1 minute to separate the two phases. The aqueous phase was removed into a new eppendorf tube and re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The plasmid DNA was precipitated by the addition of 0.1 volumes of 3M sodium acetate, pH 5.2 and two volumes of ice cold absolute ethanol. The nucleic acids will pellet after incubation at -20°C for 1 hour and centrifugation for 10 minutes at 4°C. The pellet was rinsed with 70% cold ethanol, dried, dissolved in 50µl of TE and stored at -20°C. For transformation into DH5-α, 1µl of DNA was sufficient to transform 25µl of electrocompetent cells using the electroporation protocol from methods I.

Sequence analysis

Identification of viral sequences obtained from the two-hybrid system were determined with the use of the BLAST network service, the blastn program, from the National Center for Biotechnology Information (Altschul et al., 1990). This service was accessed via the e-mail address blast@ncbi.nlm.nih.gov.

B) Experimental techniques

Replication assay

This assay was based on experiments by DeLange and McFadden (1986). The plasmid, pTZ18R was grown up in either the bacterial strain DH5-α or in CJ236 (Kunkel et al., 1987) which has the genotype dut1, ung1, thi1, relA1/pCJ105 (Cm^r) and will therefore incorporate some uracil nucleotides into the plasmid DNA. Plasmid DNA was prepared by the alkaline lysis method. Prior to the transfection, the calcium-phosphate-precipitated DNA was prepared. 1μg (1μl) of plasmid was made up to a volume of 250μl in 250mM calcium chloride. An equal volume (250μl) of freshly prepared 2xNNH was immediately added to the DNA mix with agitation. The precipitate was then left at room temperature for 1 hour.

Separate 35mm tissue culture plates were prepared the day before the experiment. Plates were checked on the day of the assay to ensure a healthy monolayer was present. The cells were infected at 37°C for 1 hour with DMEM+5% FBS containing virus (either IHD-W or ts4149) at a multiplicity of 2 PFU/cell. After the 1 hour incubation period, the infection was aspirated and the monolayer was washed with prewarmed PBS. DMEM supplemented with 10% FBS (2.5ml), prewarmed to 37°C or 40°C, was added to the monolayer. The precipitated DNA (0.5ml) was added directly into the medium and the plates were put at their respective temperatures for 4 hours. The media was replaced after the incubation and the plates

were again put at their respective temperatures until 24 hours post-infection. After 24 hours the plates were harvested. The cells were scraped off into the media, transferred to 15ml corning tubes and the plates were washed with PBS to make sure all the cells had been removed. The cells were pelleted in a clinical centrifuge, rinsed once with PBS, centrifuged again and finally resuspended in 500µl of 10mM Tris-HCl pH7.5, 0.1mM EDTA, 1% sarkosyl, 100µg/ml proteinase K. These tubes were incubated overnight at 50°C. The samples were then transferred to eppendorf tubes where they were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The DNA was precipitated by the addition of 1/10 the volume of 3M sodium acetate and two volumes of ethanol. Samples were left overnight at -20°C. The DNA was pelleted and resuspended in 25µl TE/RNAase A.

To determine if the plasmid had been replicated, 5µl of each sample were digested with either the restriction endonuclease *DpnI* alone or with a combination of *DpnI* and *EcoRI*. *DpnI* cleaves input methylated plasmid DNA but will not cut DNA that has been replicated in mammalian cells as it is unmethylated. The enzyme *EcoRI* will cleave both methylated and unmethylated DNA and will therefore cause any replicated DNA to appear as a linear form. The digests were run out on a 0.7% agarose gel overnight, Transferred to nylon (zetaprobe), probed with the plasmid pTZ18R which had been labelled by the random primer method and autoradiographed.

RESULTS

In the first discussion, we postulated that the vaccinia virus uracil DNA glycosylase may have acquired a novel function or property, possibly through an association with one or more proteins. Evidence for the existence of such a multiprotein complex is supported by several facts. (i) Previous temperature-shift experiments with mutant *ts*4149 have shown that the defect of this mutant is not thermolabile at the level of DNA synthesis (McFadden and Dales, 1980), implying that it may be an assembly-type mutant. (ii) The D4 protein possesses uracil DNA glycosylase activity (Stuart et al., 1993; Upton et al., 1993) but has only a limited homology to this highly conserved class of enzymes. (iii) Vaccinia virus uracil DNA glycosylase mutants are inviable, whereas similar mutants of *E. coli* (Duncan, 1985), yeast (Percival et al., 1989) and herpes simplex virus (Mullaney et al., 1989) are viable. To try to identify possible components of such a complex, the two-hybrid system (Fields and Song, 1989; Chien et. al., 1991) was utilized.

Initial testing of the two-hybrid system

Initially, we decided to screen three vaccinia viral proteins of interest to the ongoing work in the lab with the hybrid GAL4 activating domain - vaccinia DNA library. The three proteins were the ligase (A50L), the small subunit of the mRNA capping enzyme (D12L) and the uracil DNA glycosylase protein (D4R). These

preliminary screenings were performed by Dr. R.D. Gietz. The ligase protein is not known to interact with any other specific protein(s), nor is the uracil DNA glycosylase. There is an interaction between the small subunit of the capping enzyme (D12L) and the large subunit, D1R. This interaction has been characterized previously (Martin et. al., 1975). We were interested in knowing if this interaction (D12/D1) could be found using this system and were also interested in any other interactions that may be discovered.

For the preliminary screen with the D12 protein, approximately 14,000 colonies were analyzed for activation of the reporter gene. Seven putative interactions were picked. Upon sequencing the library plasmids from these interactions, six were shown to contain identical D1R fragments (data not shown). The seventh putative positive did not turn blue when rechecked against the D12L plasmid. This proves that the system is capable of detecting known interactions but did not give us any new information for this particular protein.

Screening with D4R

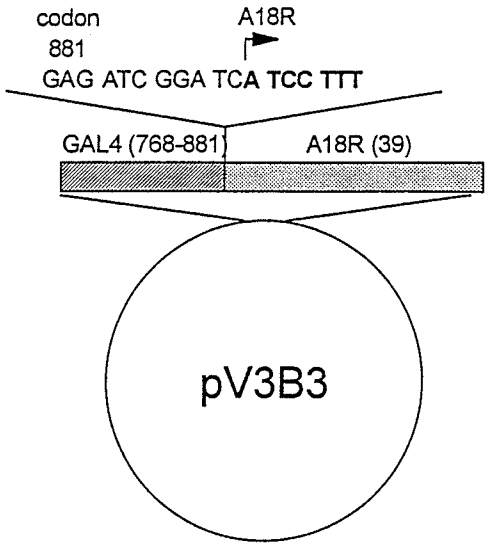
The D4R gene was of interest to the lab and particularly to me as this is the protein that is affected in the virus mutant *ts*4149. We had previously suggested that the D4 protein may have aquired a novel function through its association with one or more proteins thereby leading to an essential role in sustained viral DNA replication.

To try to determine what protein(s) may interact, the two-hybrid transactivation system was used. A fusion protein containing the DNA binding domain of GAL4 and the D4 protein was produced (pMAD4). This plasmid was transformed into the yeast strain GGY1::171 along with the DNA library. For this test a total of about 11,000 colonies were screened to determine if any fusion proteins produced from the library plasmids were interacting with the GAL4/D4 fusion protein. Four blue colonies were obtained from this screen (Table 1). The same fragment containing the D4R gene was then cloned into the pBTM116 vector which contains the lexA DNA binding domain instead of the GAL4 DNA binding domain. This plasmid, pBD4, was screened through the library again with approximately 100,000 transformants being analyzed. From this screen 26 blue colonies were isolated (Table 1). Library plasmids containing the interacting fusion proteins were segregated from the DNA binding domain plasmids and were then sequenced and analyzed using the blastn program (Altschul et al., 1990) which will find similar or identical sequences and give the gene and organism it is from. Many of the putative interactions were considered to be false based on the orientation of the reading frame in the library plasmid as compared to that for the known virus sequence (Goebel et al., 1990). These negative clones were not pursued further. Of the blue transformants initially observed, only a few were determined to be possible interactions, these being A18R, D6R, O1L and B5R (figs. 13-16). The A18 protein was the only interaction found in the initial screen with pMAD4. As the second screen with pBD4 was performed approximately six months later, it was the interaction between D4 and A18 that was studied further.

Table 1: Result of Library Screens with D4

screen	number of putative positives	false positives	positives
pBD4 with *MboI* library	26	22	4
pMAD4 with *MboI* library	4	3	1

A



B

```
          A18R>
129321 attaaaaaat gtcactatta aagatggagt ataatcttta tgccgaacta aaaaaaatga
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       gatcatcctt taagtttctg atacctaagg gattttacgc ctctccttcc gtaaagacga
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       ggaaaatgat cgaatcaaaa cgtcctctat acattactct tcacttggcg tgtggatttg
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       ataagatatc catagatgga gtaagtagtc tattaaagga actaaagact caaagtccgg
       atgtattaat agtagtcagt agacatctga caaacgatgc cttttgtaaa tatatcaata
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       cagttacaag atttttagcg tattatcctc cgatgatgtg ttatttttta actgctacac
       ctagaccagc taaccgaatt tattgtaaca gtattattaa tattgccaag ttatccgatc
       taaaaaaaac tatctatgcg gtagatagtt tttttgagcc atattccaca gataatatta
       gacatatgat aaaacgatta gatggaccat ctaataaata tcatatatat actgagaagt
       tattatctgt agacgagcct agaaatcaac ttattcttga taccctggta gaagaattca
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       tctacaaacg attattagat cttttcggac cagaggttgt atttatagga gacgcccaaa
       atagacgtac tccagatatg gtcaaatcaa tcaaggaact aaatagattt atattcgtat
       ccaccttatt ttattccggt actggtttag atattcctag tttggattcg ttgttcattt
       gctcggcagt aatcaacaat atgcaaatag agcaattact agggagggta tgtcgagaaa
       cagaactatt agataggacg gtatatgtat ttcctagcac atccatcaaa gaaataaagt
       acatgatagg aaatttcatg caacgaatta ttagtctgtc tgtagataaa ctaggattta
       aacaaaaaag ttatcggaaa catcaagaat ccgatcccac ttctgtatgt acaacatcct
       ccagagaaga acgtgtatta aatagaatat ttaactcgca aaatcgttaa gaagtttaag
```
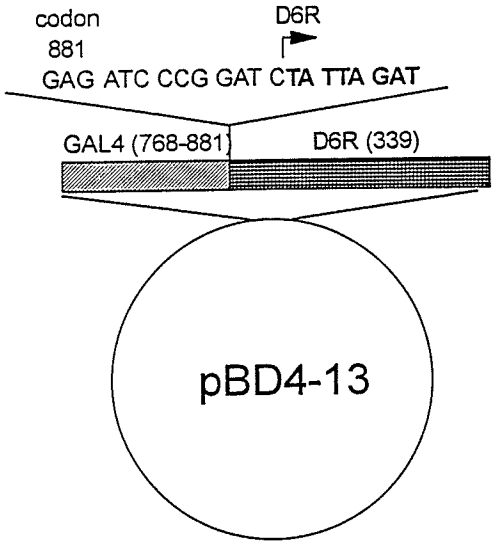

figure 13. The interacting plasmid, pV3B3 contains the A18 protein in the library plasmid pDP4. (A) Shows the reading frame in the library plasmid. The A18 fusion protein begins at amino acid 39. (B) The DNA sequence of A18R. Bold type represents where the fusion protein starts. Underlined sequences show the starting ATG and terminating codons.

A



B

```
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       gtataccaac tatattacct catcagttag ctactctaga ttatctagtt agaactatca
       tagatgagaa cagaagcgtg ttattgttcc atattatggg atcaggtaaa acaataatcg
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       atgaggcaca taatatcttt gggaataata ctggagaact tatgaccgtg ataaaaaata
       aaaacaagat tccttttcta ctattgtctg gatctcccat tactaacaca cctaatactc
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       tacagacact caacggaaaa cattttatat acttttctaa ttctacatat ggtggattgg
       taattaaata tatcatgctc agtaatggat attctgaata taatggttct cagggaacta
       atccacatat gataaacggc aaaccaaaaa catttgctat cgttactagt aaaatgaaat
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       gactttccta cgaaccattt gtttggggag ttaactttcg taaagaatat aacgtggtat
       cttctccata aaactgatga gatatataaa
```
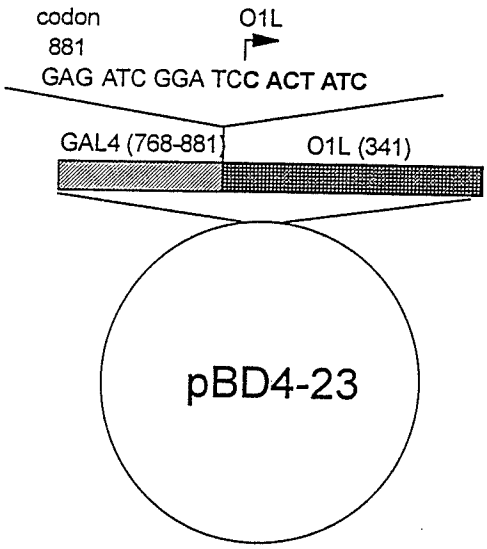

figure 14. The interacting plasmid, pBD4-13 contains the D6 protein in the library plasmid pDP12. (A) Shows the reading frame in the library plasmid. The D6 fusion protein begins at amino acid 399. (B) The DNA sequence of D6R. The bold type represents the fusion protein start. Underlined sequences show the starting ATG and terminating codons.

A



B

```
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      ttccaagtca ctgtgtgtaa gaagattata ttctatcata agcttgacta catttggtcc
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      cctaacattg agaatgtcgg agttaatagc ttccagatac agtgattttg gcaatagtcc
      gtgtaatcca taatccagta acacgagctg gtgcttgcta gacacctttt caatgtttaa
      ttttttttgaa ataagctttg ataaagcctt cctcgcaaat tccggataca tgaacatgtc
                                                                  <O1L
```
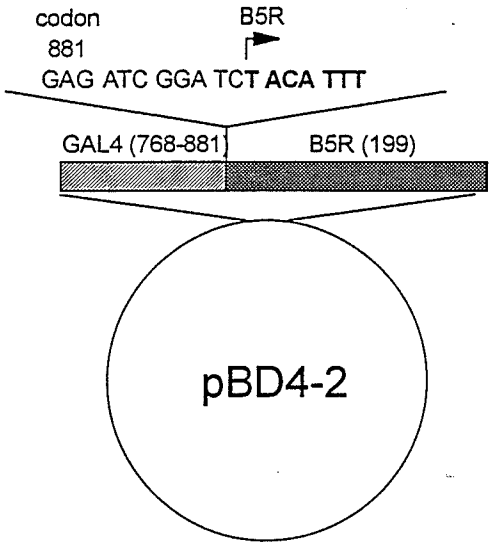

figure 15. The interacting plasmid, pBD4-23 contains the O1 protein in the library plasmid pDP4. (A) Shows the reading frame in the library plasmid. The fusion protein begins at amino acid 341. (B) DNA sequence of O1L. Bold type represents the fusion protein start. Underlined sequences show the starting ATG and terminating codons.

A



B

```
                                B5R>
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       aatctccgtt atagtattag tttgttcctg tgacaaaaat aatgaccaat ataagttcca
       taaattgcta ccgtaa
```

figure 16. Interacting plasmid pBD4-2 contains the B5 protein in the library plasmid pDP4. (A) Shows the reading frame in the library plasmid. The B5 fusion protein begins at amino acid 199. (B) DNA sequence of B5R. The bold type represents where the fusion protein starts. Underlined sequences show the starting ATG and terminating codons.

The D4/A18 interaction

The A18R sequence was found in both the pMAD4 and the pBD4 screens. The colony that was isolated from the pBD4 screen appeared to have undergone a deletion that made it impossible to determine the reading frame. This deletion may have occured when the yeast was stored at 4°C prior to the isolation of the library plasmid. After this storage it was difficult to grow this colony and isolate the library plasmid.

Deletions of both the D4R and A18R genes were made to try to determine what regions of the genes were coding for the interacting protein domains. The strongest interaction between the A18R clones and the full length pBD4 occurred with the original clone that was isolated, pB3, and also with the PCR clone that was created to mimic pB3, which is pGA18M (figs. 18, 19 and 20B). The clone, pGA18E contains a deletion of the first putative helicase motif that corresponds to the nucleotide binding site. This clone (pGA18E) combined with pBD4 does interact, but the color of this interaction is lighter than the original pB3 interaction with pBD4 (figs. 18, 19 and 20B). This may imply that the interaction is not as strong as that between pBD4 and pB3. The full length pGA18 and the deletions that originated at the HincII site of A18 (pGA18H, pGA18H-1, pGA18H-2) do not appear to strongly interact (figs. 18, 19 and 20B). Deletions made in the D4R sequence corresponding to the carboxy terminal end of the protein could not interact with the original A18R isolate, pB3, or with any other A18R clones (figs.17A,19and 20A).

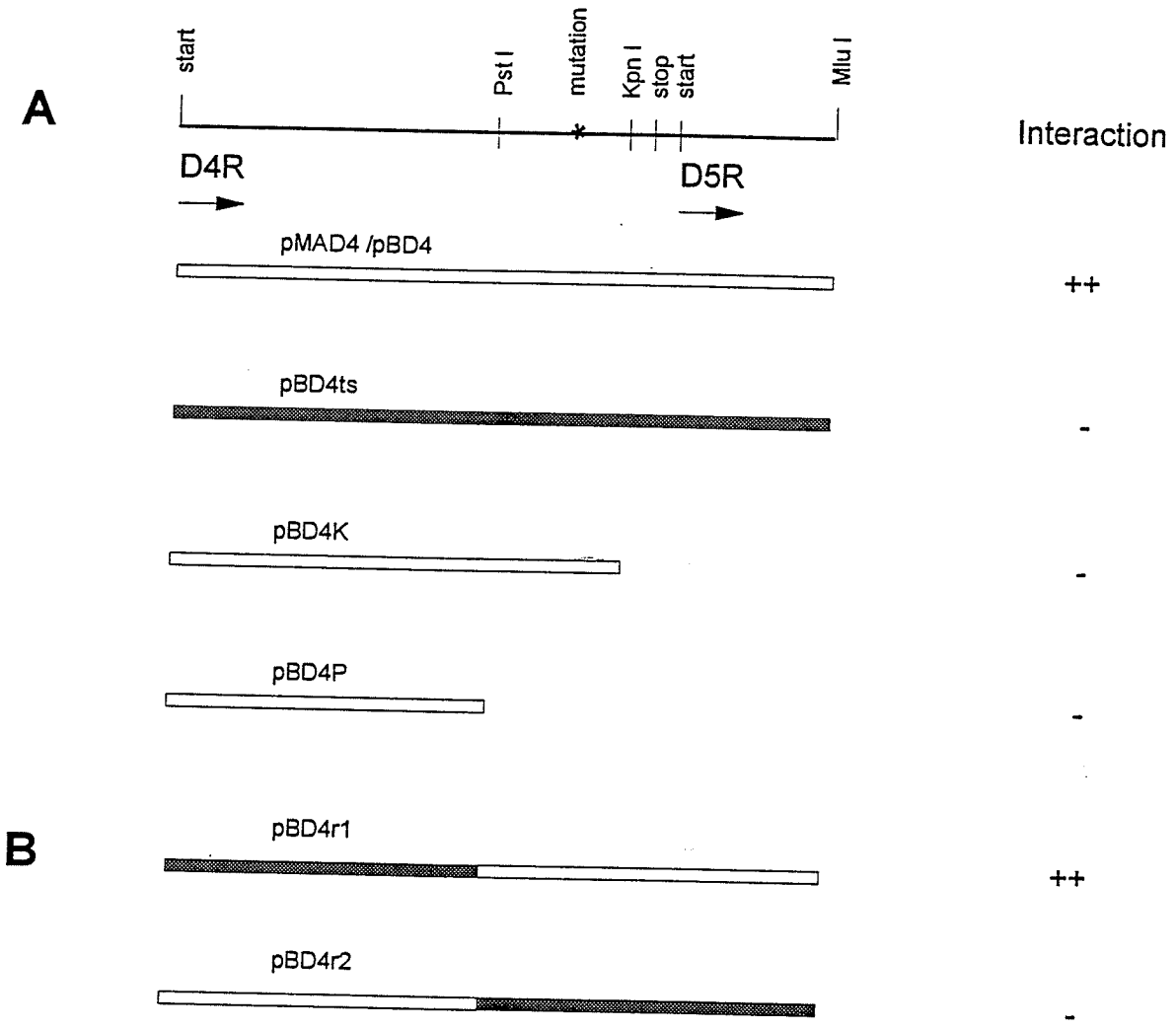

figure 17. All inserts are in the pMA424 or pBTM116 vectors. (A) Diagrammatical representation of the D4 clones created for use in the two-hybrid system. (B) D4 and *ts*D4 replacement clones. The degree of interaction between the D4 clones and pB3 is shown.

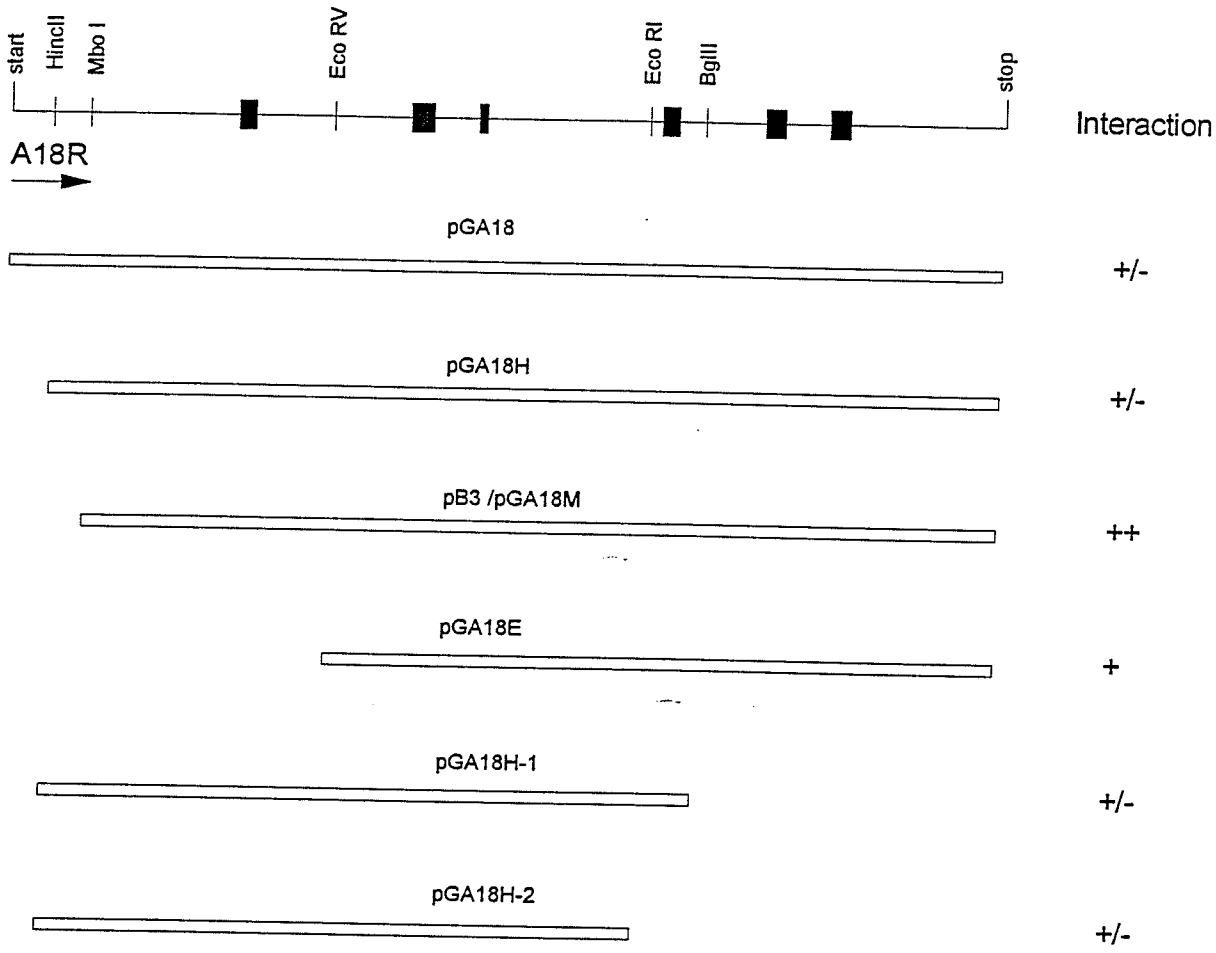

figure 18. Representation of the A18 clones for use in the two-hybrid system. Boxes show the sites of the helicase domains within the A18R sequence. The degree of interaction between pBD4 and A18 is shown.

figure 19. Control plates for the two-hybrid system after four days of growth. Left plate contains negative control: pB3 Middle plate, positive controls: top - Snf1/Snf4, bottom - D12/D1. Right, negative controls: top - pBD4ts, bottom - pBD4.

A

B

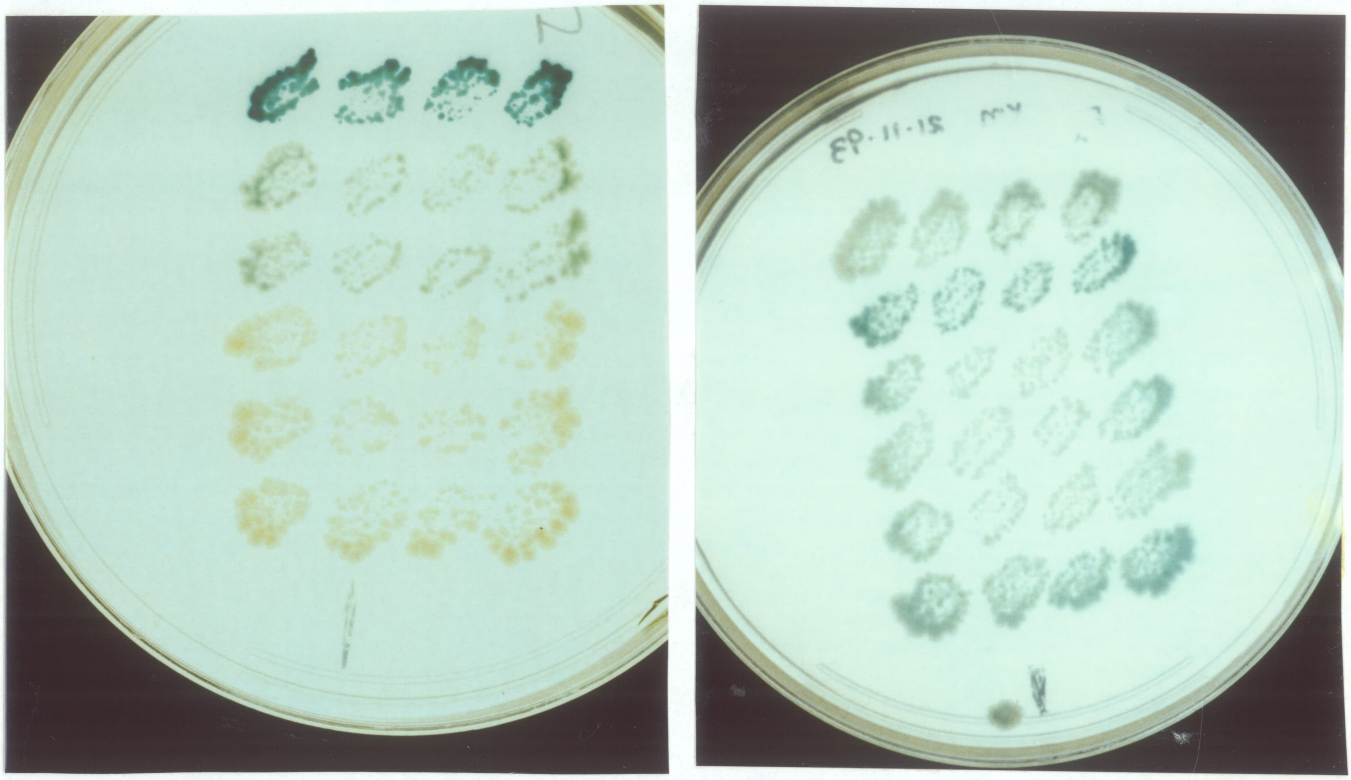

figure 20. (A) The interaction of D4 deletion clones with pB3. From top to bottom: D12/D1 control, pBD4/pGA18M, pBD4/pB3, pBD4ts/pB3, pBD4P/pB3, pBD4K/pB3. (B) Interaction of deletion clones of A18 with pBD4. From top to bottom: pBD4/pGA18, pBD4/pB3, pBD4/pGA18H, pBD4/pGA18H-1, pBD4/pGA18H-2, pBD4/pGA18E. The blue color observed represents the activation of the reporter gene due to two interacting proteins after four days of growth.

Interactions with the mutant *ts*4149

The D4R gene of the mutant *ts*4149 was cloned into the pBTM116 vector to create the clone pBD4ts. Both plasmids pBD4ts and pB3 were transformed into CTY10-5d and grown at 30°C until transformants were observed. These plates were replica-plated onto x-gal minimal media with selection for the plasmids and were then incubated at either 30°C or 40°C to determine if the *ts*-phenotype observed in tissue culture would also be observed in this system. The wild type D4R gene in pBTM (pBD4) was also transformed in with the A18R plasmid pB3 as a control. Transformants were replica-plated and were also grown at both 30°C and 40°C. The wildtype interaction turns blue at both temperatures whereas the mutant protein did not appear to interact at either temperature as no blue color was detected.

To determine if the only difference was the *ts* mutation, recombinants were created. In these plasmids a PstI-PstI fragment containing the 3′ region of the D4R gene, including the mutation site and the 5′ region of D5R gene, were switched (figs. 17B, 19 and 20A). The clone pBD4r1 containing the wild type PstI-PstI fragment interacted with pB3 but the other recombinant plasmid containing the mutation could not interact. Upon sequencing of the PstI-PstI fragment of both plasmids, only the mutation seen in *ts*4149 was observed. This mutation therefore affects the interaction between the D4 and A18 proteins in this system.

Screening with A18R

Similarly to the screening with the D4R gene, the A18R gene was also screened through the GAL4 acidic domain fusion library. The full length A18R gene was cloned into the plasmid pBTM116 to create pBA18. This clone, along with the *MboI* WR DNA library was transformed into the CTY10-5d strain. Approximately 80,000 transformants were screened for putative positives of which none were found. Possible explanations for this may be found in the D4/A18 interactions. It was found that the full length A18R plasmid, pGA18, could not interact with pBD4. Also, a full length A18 fusion protein in pBTM116 (pBA18) was made and it could not interact with the D4 protein fused to the transactivating domain of GAL4 (pGD4). Other possibilities include the introduction of one or more mutations into the pGA18 and pBA18 clones by PCR. A better screen may have utilized a pBTM116 clone that started at the same site as the pGA18M clone.

The replication assay

The D4R gene is known to encode a 25kDa protein with uracil DNA glycosylase activity (Stuart et al., 1993; Upton et al., 1993). Uracil DNA glycosylases are not essential for the viability of some organisms but, vaccinia virus is inviable when this protein is mutated. It was postulated that this protein might be involved in a multienzyme complex of which some evidence for its capability of interaction with

other proteins has been observed using the two-hybrid system. There are a number of hypotheses to try to explain why the uracil DNA glycosylase is essential to vaccinia. (i) If the mutant D4 protein has normal glycosylase activity such that it recognizes lesions and binds to them but fails in its ability to complete the repair processes, the DNA polymerase might be unable to synthesize past these lesions. Failure to remove the apyrimidinic (AP) sites produced during repair is expected to affect DNA replication, since DNA polymerases have a tendency to stall at such sites (Schaaper et al., 1983). Also, it was observed that AP sites inhibit both nuclear and mitochondrial uracil DNA glycosylases suggesting that these enzymes may bind these sites (Domena et al., 1988). Therefore, the vaccinia uracil DNA glycosylase may bind to AP sites as well. Assuming that the mutant uracil DNA glycosylase is capable of generating AP sites but incapable of completing the repair, the enzyme might not be able to disassociate from the generated AP sites it produces, and therefore form a physical block on the DNA. In this way, the enzyme is not directly involved in DNA replication but may still have a direct affect on this process. (ii) The D4 protein may be directly involved in a DNA replication complex, possibly having a role in the initiation of replication or an involvement just after. The mutant, *ts*4149, may have difficulty with the transition from initiation to elongation. Support for this may be suggested by the PFGE data, as it is observed that *ts*4149 is capable of replicating approximately 1% of its DNA at 40°C (figs. 3, 4). This may imply that the mutant virus is constantly having to reinitiate replication as replication consistently fails at some point following this step. At this point the actual initiation process is not understood.

It is known that an origin for the initiation of replication has not been found in vaccinia as the virus has been shown to replicate any plasmid DNA without requiring the presence of viral sequences (DeLange and McFadden, 1986). (iii) Finally, there is a question about transcription and the possible role it may play in this mutant phenotype. From the two-hybrid system data, the D4 protein was found to interact with the vaccinia protein A18. The human homologue to the A18 protein is ERCC3 which is known to be part of the transcription factor TFIIH and is also involved in excision repair (Buratowski, 1993; Schaeffer et al., 1993). The "replication assay" was designed to try to clarify the D4 protein role in DNA replication. All results thus far are preliminary.

Previous experiments performed by Dr. A.M. Delange (DeLange and McFadden, 1986) showed that vaccinia virus could replicate any type of plasmid DNA and that viral sequences were not required for this replication to be initiated. The "replication assay" was based on this procedure. The plasmid pTZ18R was used for the assay to see if the mutant virus was capable of replicating a naked non-viral DNA template. This plasmid was also replicated in the bacterial strain CJ236 which results in a plasmid containg uracil nucleotides. In this way it was also designed to try to determine if the uracil DNA glycosylase activity is the actual cause of the DNA negative phenotype.

Results from this assay showed that the wild-type IHD-W virus could replicate

the plasmid pTZ18R both at 37°C and at 40°C (fig. 21A and B, lanes 2). The plasmid grown up in the bacterial strain CJ236 was designated as pTZ18R(uracil) to indicate that it contained uracils. The wild-type virus was also capable of replicating the uracil containing plasmid at both temperatures, though apparently at a lower level of synthesis compared to the pTZ18R (fig. 21). For IHD-W at 40°C (fig. 21B lane 4), it is difficult to see the replicated pTZ18R(uracil) DNA band. This may be partially due to the pTZ18R(uracil) plasmid itself but, may also be caused by the visible underloading in this lane.

At 37°C, *ts*4149 can replicate the normal plasmid, but it has been difficult to ascertain if and to what extent *ts*4149 can replicate the uracil containing plasmid at this temperature (fig. 21A). Since the wild-type virus replicates the uracil-containing DNA at a lower level it is reasonable to assume that *ts*4149 may also replicate this DNA at a lower level. It has also been shown that *ts*4149 does have some difficulty replicating its DNA at the permissive temperature as compared to wild-type strains (fig. 3) which may also be the observation here. At this time the results are inconclusive and there has been no determination of *ts*4149's ability to replicate the uracil-containing plasmid. It is observed that replication of neither plasmid occurs at the non-permissive temperature (fig. 21). This is consistent with the DNA^- phenotype observed by PFGE.

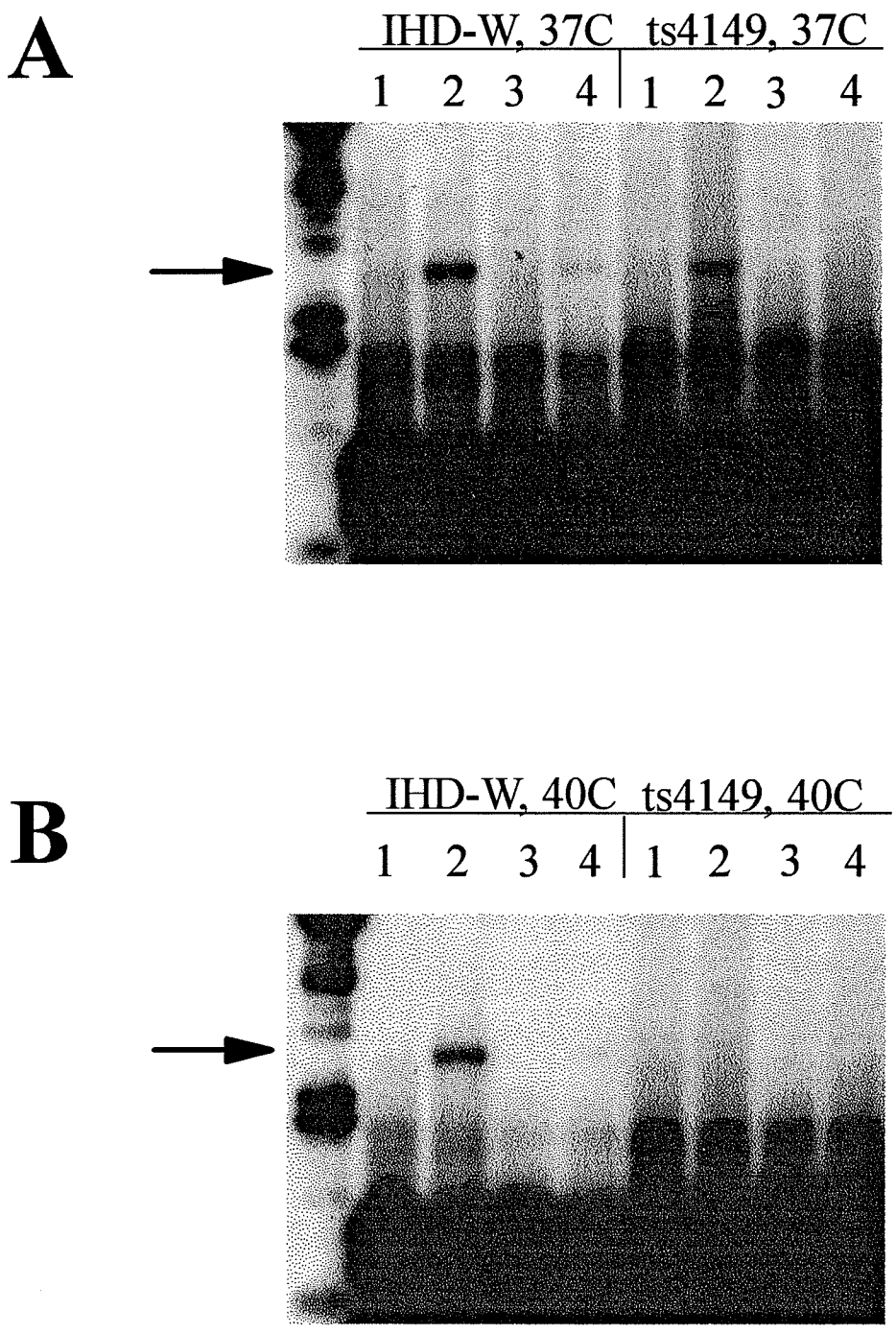

figure 21. (A) Replication assay performed at 37C. (B) Assay at 40C. For both (A) and (B): lane 1 represents the pTZ18R plasmid digested with DpnI; lane 2 is pTZ18R digested with both DpnI and EcoRI to show the replicated plasmid DNA; lane 3 is the pTZ18R (uracil) plasmid digested with DpnI; lane 4 represents pTZ18R (uracil) digested with both DpnI and EcoRI. Arrows indicate replicated plasmid DNA.

DISCUSSION

Two-hybrid system

Although the biological significance of the data obtained from the two-hybrid system remains to be verified in virus infected mammalian cells, it does lend support to the existence of a multienzyme complex. It is feasible that the two-hybrid system is detecting transient interactions due to the stability of the β-galactosidase protein (Chien et al., 1991). By using this system, four possible interacting proteins were discovered, these being A18, D6, O1 and B5. The D6 gene is transcribed at late times during infection. Its protein encodes the small subunit of the virion early transcription factor (Lee-Chen and Niles, 1988; Christen et al., 1992). O1L is a protein of unknown function that contains a leucine zipper motif (Johnson et al., 1993). B5 is a glycoprotein found in the extracellular enveloped form of vaccinia (Isaacs et al., 1992; Martinez-Pomares et al., 1993). The A18R gene is transcribed both early and late and its protein may function either directly or indirectly in viral transcription (Pacha et al, 1990; Bayliss and Condit, 1993). It is the A18 interaction with D4 that was studied in more detail.

It is difficult to ascertain how much information can be obtained from the two-hybrid system concerning interacting domains. For instance, The full length A18

protein does not interact very well (if at all) with D4 whereas, the deletions at the first *MboI* site result in a protein that will give a positive interaction. It may be that the PCR product used to clone the full length gene contained a mutation. If so, this mutation would have to be past the *EcoRV* site as the gene was sequenced this far. Also, deletions up to the *EcoRV* site (clone pGA18E) do lead to a positive result but the colouring is much lighter than that of the initial interaction. This could be the result of a lower level of interaction. It is known that interactions that reconstitute only a low level of GAL4 function will produce a detectable signal as the β-galactosidase protein is stable and will accumulate over time (Chien et al., 1991). The lack of a signal is not strong evidence against a specific interaction. Two proteins may interact *in vivo* but may not be observed to interact in this system for a number of reasons. These include instability or improper folding of the hybrid proteins, the hybrid proteins may not include the residues necessary for the interaction or the GAL4 domains might occlude a site of interaction (Chien et al., 1991).

Deletions of the carboxy terminal end of the D4 protein did not result in activation of the reporter gene. As the mutation in the D4 protein of *ts*4149 occurs in this region at amino acid 179, it appears that this area may be directly involved with the observed interaction between D4 and A18. This also correlates to the data obtained for the interaction between the mutant D4 protein and A18. The two proteins in yeast do not display a temperature sensitive phenotype since they do not interact at a permissive temperature of 30°C or at a nonpermissive temperature of 40°C. Just

what does this tell about the interaction if it occurs in virus infected cells? It is conceivable that the two proteins do not interact well at either temperature in infected cells which may explain why ts4149 is delayed at the permissive temperature. There may be a problem with this assumption since what we see in yeast may not necessarily be true in virus infected cells. It should be noted that similar experiments were performed using the mutant D12 protein from *ts*9383 and its interacting protein, D1. A temperature sensitive phenotype is also not observed as these proteins do not interact at any temperature in the two-hybrid system (Luke DeLange, personal communication). This data is not supported by immunoprecipitation analysis which showed that the mutant D12 protein can interact with D1 at a permissive growth temperature in virus infected cells (Mike Carpenter, personal communication).

It is interesting that the A18 protein of vaccinia shares homology to a eukaryotic nucleotide excision repair enzyme. This viral protein is homologous to the NER protein ERCC3 (Koonin and Senkevich, 1992) which is a known helicase and is also part of the transcription factor TFIIH (Schaeffer et al., 1993). The A18 protein in vaccinia is a DNA dependent ATPase that binds double-stranded DNA (R. Condit). Temperature sensitive mutants in this gene display an abortive late phenotype. The infection is normal as early, intermediate and late protein synthesis is initiated at the appropriate times. At approximately eight hours post-infection, the breakdown of viral mRNA and host rRNA occurs accompanied by the cessation of all protein synthesis (Pacha et al., 1990). These mutants were found to contain an increased amount of

double-stranded RNA, inducing the cellular 2-5A pathway and resulting in the RNA breakdown. This increased amount of dsRNA is believed to be caused by aberrant transcription implicating the A18 protein in the transcription process (Bayliss and Condit, 1993).

What could the function of the D4 and A18 complex be? There does not appear to be any correlation between the phenotypes of vaccinia virus carrying mutations in the D4 or A18 proteins. It may be that the A18 protein has two functions that act at different times in the life cycle as A18 is transcribed both early and late. The phenotype of *ts*4149 still appears to represent a defect in DNA replication therefore the two proteins may play a role in this process. The A18 protein may be required for the unwinding of the DNA helix perhaps for the initiation of replication. There is also a possibility that the two proteins function together in a repair complex as homologues to them are known to be involved in excision repair in other organisms. Mutations in the human homologue to A18, for example, are known to result in Xeroderma Pigmentosum and a form of Cockayne syndrome. Human cell lines from affected individuals are deficient in NER involving both preferential repair and all over genome repair (Hoeijmakers, 1993b). A18 also invokes transcription involvement though why this would lead to the phenotype for *ts*4149 is unknown. The mechanisms of DNA replication, repair and transcription may be more intertwined than has previously been thought.

Replication assay

This assay is only a starting point for further studies. The main information gained was that *ts*4149 cannot even replicate a naked DNA template at the nonpermissive temperature. This would definitely appear to support a DNA replication defect. It is unknown if the virus is capable of replicating a uracil containing template. If this is determined, we might know if the glycosylase is fully functional or if it is deficient either in binding to the uracil in DNA or in removing itself from the AP site produced. If it remains bound to the produced AP site, it may be a physical block to both DNA replication and transcription. Where then, do the other proteins enter the picture? Is there conceivably a link between early transcription and DNA replication that has not yet been determined? Why does a repair enzyme result in this type of defect? At this time there is no answer to these questions. Further experimentation is required to understand the real defect in *ts*4149 and what role, if any, the interactions found in the two-hybrid system play in DNA metabolism and the virus life cycle.

Future directions

There are still a large number of unanswered questions pertaining to the role of a uracil DNA glycosylase in vaccinia virus replication. As a small amount of the DNA is actually replicated in the mutant at the nonpermissive temperature, it might be a

good idea to try to determine what part of the DNA is replicated at 40°C. Are certain sequences more prevalent than others thereby leading to a plausible explanation for the role of this protein in the replication of vaccinia? For example, if the majority of the DNA is from the ends of the genome it may imply that initiation can occur but the problem is the transfer from initiation to elongation. Thus it may show that D4 has a role in the initiation of replication.

The interaction of D4 and A18 should also be examined by other methods to prove or disprove the two-hybrid interaction in the virus. Possible experiments could include antibody work (immmunoprecipitations) or the use of other systems that will detect interactions (expression vectors and purification of the two proteins). As the A18 protein may be involved in transcription it may be worthwhile to determine if there is a less obvious defect in transcription that has been overlooked. For instance, there appears to be a slight delay in early gene expression at the permissive temperature. Whether or not this is a consequence of the mutation in ts4149 has yet to be determined.

DNA replication, repair and transcription are processes that are not yet fully comprehended. In vaccinia, the finding of a new complementation group supplemented with further studies involving the mutant *ts*4149 and the normal protein may lead to a better understanding of these mechanism.

SUMMARY

*ts*4149 is a temperature sensitive mutant of vaccinia virus which, at the nonpermissive growth temperature of 40°C, displays a DNA negative phenotype. In the virus, this phenotype is characterized by a lack of DNA replication and also by an observed deficiency in intermediate and late gene expression. Three previous DNA negative complementation groups have been identified which contain mutations in the DNA polymerase gene, B1R which encodes a serine/threonine protein kinase and D5R which has an unknown function. *ts*4149 represents a fourth group.

The defect in ts4149 was mapped to the D4R gene and is a G to A transition resulting in an amino acid substitution from glycine to arginine. This gene encodes a 25kDa protein with uracil DNA glycosylase activity (Stuart et al., 1993). Further studies using the two-hybrid system revealed a possible interaction between the D4 protein and A18 which shares homology to a number of helicase proteins.

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