REACTIVATION OF TK-DEFICIENT HERPES SIMPLEX VIRUS AND HOMOPOLYMER MUTATIONAL HOT SPOTS

by

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ABSTRACT

The majority of herpes simplex virus (HSV) isolates resistant to acyclovir (ACV) have a deficiency of thymidine kinase (TK^D). Such mutants are able to establish latency in animal models but are unable to reactivate which has contributed to the clinical dogma that, following effective eradication, such outbreaks are inevitably followed by ACV-susceptible (ACV^S) reactivations. In addition, genotypic information regarding mutations conferring the ACV-resistant (ACV^R) phenotype onto HSV is very limited.

HSV type 2 strains 1737 and 89-063, ACV^R and uniformly TK^D by all conventional assays, clinically reactivated in AIDS patients in the absence of antiviral drug pressure. Investigation of their neurovirulence and latency characteristics in mouse models, however, yielded neurovirulent TK-wild type (TK^{WT}) and TK-low producer (TK^{LP}) populations. Two further ACV^R reactivated isolates (89-353 and 90-150) contained mixed TK^{WT}/TK^D populations by plaque autoradiography while one (2370) likely exhibited a TK-altered phenotype.

Mutations conferring the ACV^R phenotype were assessed by plaque purification, amplification of the *tk* gene by polymerase chain reaction (PCR) and cycle sequencing. Three of 8 plaque isolates, 1737-14, 90-150-3 and 89-650-5 contained a guanosine insertion within a stretch of 7 guanosines. Correlation by immunoprecipitation and Western blot confirmed the predicted truncated 28 kd protein. Two further isolates, 89-063-1 and 89-353-1, produced truncated proteins 32 and 28 kd in size respectively while one isolate, 90-110-4, produced no detectable product.

Reactivation is dependent on TK activity but may occur with "Ultra-low" levels and may dissociate from the ACV^R phenotype. The likely mechanism is *in vivo* complementation between heterogeneous TK populations containing TK^D or TK^{LP} virus which may be detectable by conventional means or masked. Eradication of TK^D outbreaks does not ensure subsequent reactivations to be ACV^S and alternating antivirals may be required for effective therapy. Mutations in the *tk* gene occur preferentially at homopolymer hot spots and the majority of mutants produce truncated products. Such homopolymer stretches may facilitate reactivation by high reversion frequencies or ribosomal frameshifting and may offer novel targets for detection and therapy of ACV^R HSV isolates.

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LIST OF ABBREVIATIONS

HSV	herpes simplex virus
HIV	human immunodeficiency virus
AIDS	acquired immunodeficiency syndrome
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
TK	thymidine kinase
LAT	latency-associated transcript
ACV	acyclovir
PFA	phosphonoformate (foscarnet)
ACV^R	acyclovir-resistant
ACV^S	acyclovir-sensitive
pol	viral DNA polymerase
TK^D	TK-deficient
TK^A	TK-altered
TK^{WT}	TK-deficient
TK^{LP}	TK-low producer
ts	temperature-sensitive
PCP	pneumocystis carinii pneumonia
ACTG	AIDS Clinical Trials Group
HFF	human foreskin fibroblast
MEM	minimal essential medium
DMEM	Dulbecco's minimal essential medium
HEPES	N-2-hydroxyethylpiperazine-N-2-ethansulphonic acid
FCS	fetal calf serum
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline

CPE	cytopathic effect
ID_{50}	50% inhibitory concentration
^{125}IVaraU	1-ß-D-arabinofuranosyl-*E*-5-(2-[^{125}I]-iodovinyl)uracil
MOI	multiplicity of infection
CO_2	carbon dioxide
^{125}IdC	^{125}Iododeoxycytidine
^{14}C TdR	^{14}C thymidine
NaCl	sodium chloride
NP-40	nonidet P-40
TAE	tris-acetate, EDTA
TBE	tris, boric acid, EDTA
DMSO	dimethylsulphoxide
TEMED	N,N,N',N'-tetramethylethylenediamine
APS	ammonium persulfate
SDS	sodium dodecyl sulphate
BUdR	5-bromo-2'-deoxyuridine

Single letter code for nucleosides: A, adenine; C, cytosine; T, thymidine; G, guanosine

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DEDICATION

To my late father who was not able to see the efforts of his labors bear fruit in his sons and my mother and brother from whom I am separated by thousands of miles.

CHAPTER 1

INTRODUCTION

1.1. HERPES SIMPLEX VIRUS INFECTIONS

Herpes simplex virus (HSV) infections occur worldwide and are one of the most common afflictions affecting mankind. Prevalence is proportional to socioeconomic status, probably due to overcrowding. Recent seroprevalence studies from developed areas, such as Seattle, in the 1970's have demonstrated HSV antibodies in 40% of adults aged 25-29 years old with a 1.5% increase per year thereafter up to age 50. Earlier studies from the 1940's and 1950's reported prevalence rates of up to 90% (1). Type-specific serologies to HSV 2, the most common causal agent of genital herpes, performed in the late 1970's demonstrated seropositivity in 16% of the United States population aged 15-74 years of age, a much larger percentage than generally appreciated (2). A follow-up study by the same group in 1990 demonstrated that HSV 2 seroprevalence had risen to 22% despite the intervening period encompassing the HIV pandemic which heightened awareness about safer sexual practices (3). This illustrates the scope of the growing epidemic. HSV 2 seroprevalence rates begin to rise coincident with the onset of sexual activity. Women, as is the case with other sexually transmitted diseases, are more commonly affected than men. Seroprevalence rates have also been shown to be influenced by socioeconomic status and race (2,4,5).

HSV is a double stranded DNA virus approximately 150 nm in diameter. It is surrounded by a lipid envelope derived from modified cell membrane as it buds from the host cell and from which virus-specific

glycoproteins protrude, these being crucial in mediating viral attachment and entry into the host cell. The membrane surrounds an icosahedral protein capsid composed of 162 capsomers, this in turn housing the genome. Between the capsid and envelope lies an amorphous material known as the tegument which contains a number of viral-encoded proteins. The genome is a linear double-stranded DNA molecule approximately 150 kb long which encodes over 70 protein products. A number of clinical, epidemiological and biological differences allow distinction between 2 HSV types, HSV-1 and HSV-2, and there is a large degree of homology between the genomes of HSV-1 and HSV-2, about 50% of the sequences being highly conserved (4,6).

Infection is initiated by inoculation of virus onto skin with an epithelial breach or mucosal surfaces. The new replication cycle of HSV commences with the attachment of membrane glycoproteins to heparan sulfate; a ubiquitous, normal component of host cells which acts as the cell surface receptor. After attachment, the viral envelope fuses with the host cell plasma membrane and nucleocapsids are liberated into the cytoplasm (7). Next the unenveloped virus migrates to the nucleus where it is uncoated at nuclear pores and the 'naked' DNA enters the nucleus and circularizes. Three sets of viral genes are then expressed in an orderly sequence. The first set of genes, known as alpha or immediate-early, are expressed prior to protein synthesis and their transcription is initiated by a prepackaged transcriptional transactivator, VP16, a constituent of the tegument. Alpha gene products are themselves transcriptional transactivators and induce expression of the beta (early) and gamma (late) genes while simultaneously downregulating cellular gene transcription. Beta genes are expressed prior to and are predominantly involved in DNA replication and include thymidine kinase

(TK). Late genes are expressed after DNA replication and are predominantly structural. The replicated DNA is then cleaved to an appropriate size and packaged. Finally, the virion acquires an envelope as it buds from the inner nuclear membrane and, after glycoprotein modification in the endoplasmic reticulum and Golgi apparatus, leaves the cell as a mature virion able to perpetuate infection in other cells by repeating the cycle of events detailed above. The generation of large numbers of virions in permissive cells is associated with cell death and completes the lytic cycle of the virus (4,8,9).

In a patient naive to HSV a primary infection ensues. This may be symptomatic or asymptomatic. In either case there may be sufficient viral replication to result in infection of terminal branches of sensory endings in which case the nucleocapsid is then transported centripetally inside axons to the cell body in sensory ganglia (10,11). At this stage, a limited degree of local replication occurs in ganglia which may extend to neighboring neuronal and nonneuronal cells, some of which may demonstrate cytopathic effect (12-14). The host immune system eventually controls the mucocutaneous viral replication and epithelial damage, resulting in resolution of the primary infection. Simultaneously, the pattern of gene expression changes, such that all genes responsible for viral replication are shut off, leaving only one region of the genome transcriptionally active to produce a family of transcripts known as latency-associated transcripts (LAT's) (15-17). At this time, infectious virus is undetectable except by ganglionic explantation, surface viral proteins are undetectable and virus is latent, thereby evading both immune attack and currently available antiviral chemotherapies. This latent state underlies our current inability to "cure" patients of HSV infection. As a measure of extent of latent infection, *in situ* hybridization studies show that

0.1-3.0 percent of human neurons contain LAT's (16,17). HSV may be subsequently reactivated from its latent state via certain trigger factors, some defined, others not. Following reactivation, virus travels centrifugally to reinitiate productive mucocutaneous infection at or close to the site of initial infection. The recurrence is modified by the preexisting immune response and, in cases where the primary infection was clinically evident, recurrences are generally briefer in duration and/or less intense (18).

Genital herpes can be caused by either HSV-1 or HSV-2. HSV-1 may account for anywhere between 7-50% of first episode lesions, depending on geographical variation. HSV 1, however, only causes 2% of recurrent genital herpes, suggesting that HSV 2 is better adapted to either exist within and/or reactivate from sacral ganglia (18). Typical lesions of genital herpes may involve any region of the body supplied by the sacral plexus although the vulva and external genitalia are the sites most commonly involved. In male homosexuals and female recipients of anal intercourse, patients may sustain a proctitis and perianal ulceration. Herpetic lesions typically evolve through identifiable and distinct stages which, in temporal order, may include macules, papules, vesicles, ulcers and crusts before eventually healing. On mucous membranes the early stages are not usually apparent and ulcers are often the sole manifestation. Patients who are immunocompromised, particularly those with cell mediated immunity defects such as those with AIDS and transplant recipients, constitute a special subgroup as they may be susceptible to more frequent, more chronic and more severe recurrences and shed virus for more prolonged periods of time. In some such individuals lesions may persist for weeks to months and the prolonged associated viral shedding predisposes to the development of antiviral drug resistance (19-22).

1.2. ANTIVIRAL AGENTS AND ACYCLOVIR

Antiviral therapy includes specific antiviral drugs as well as immune modulators, such as interferon, which augment the host immune system. The viral replicative cycle offers multiple potential sites of intervention when considering the development of effective antiviral therapy. Stages in this cycle include attachment to host cell receptors, penetration into the cell, uncoating of nucleocapsid, replication of genetic material, synthesis and processing of viral-encoded proteins, assembly of new virions and egress out of the cell. Due to their relative simplicity compared to other microorganisms, viruses need to "hijack" variable amounts of host cell machinery to replicate. Such a lack of distinct viral targets has been a major stumbling-block in the development of antiviral agents as few agents have been found to be sufficiently selective in inhibiting virus-specific functions while simultaneously avoiding unacceptable host cell toxicity. Agents directed at several sites of action have entered into clinical practice although the main thrust of established antiviral therapy has been at the interruption of DNA synthesis in herpesviruses and retroviruses. The main class of effective antiviral agents to enter clinical practice has been nucleoside analogues which are analogues of naturally occurring DNA bases and which block viral DNA replication. The prototypic agent, acyclovir (ACV) was released for use in clinical practice in the late 1970's and quickly established a reputation as a safe and effective antiviral agent. ACV is an acyclic analogue of the naturally-occurring DNA base deoxyguanosine (Figure 1.) and is a selective inhibitor of HSV types 1 and 2 as well as varicella-zoster virus where *in vitro* assays show average median effective concentrations of 0.04, 0.10 and 0.50 μg/ml respectively (23). Like all nucleosides, ACV needs to be

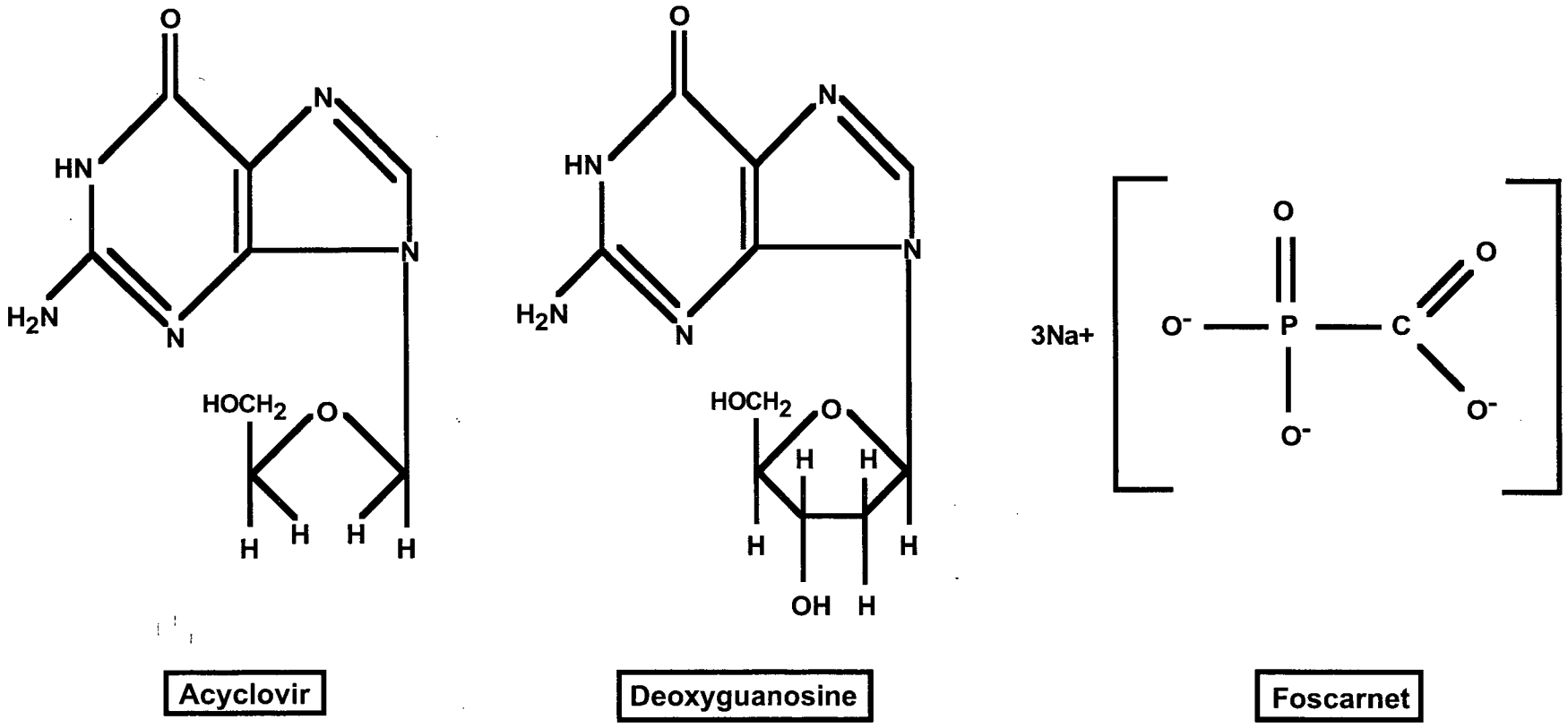


Figure 1. Structures of acyclovir and foscarnet. The diagram depicts the structures of acyclovir, a nucleoside analogue of the related base deoxyguanosine, and foscarnet which is a pyrophosphate rather than a nucleoside analogue and has a completely unrelated structure.

triphosphorylated to enable it to be incorporated into a growing DNA chain. In HSV-infected cells ACV is selectively phosphorylated to its monophosphate by a virus-encoded enzyme, thymidine kinase (TK), a reaction which does not occur in appreciable amounts in uninfected cells. Subsequent di- and tri-phosphorylation is carried out by cellular kinases and ultimately results in the production of ACV triphosphate (ACV-TP) concentrations 40-100 times greater than in uninfected cells (24). Viral DNA polymerase also has a greater affinity for ACV-TP than does host DNA polymerase, resulting in little incorporation of ACV-TP into cellular DNA (25). All these properties confer selectivity, safety and potency on the drug. The mode of action of ACV-TP on viral DNA is threefold. Firstly, it competes with deoxyguanosine triphosphate; secondly, the lack of a 3'OH group results in an inability to form phosphodiester bonds with subsequent nucleotides, resulting in chain termination; thirdly, it acts as a "suicidal inactivator" of the polymerase by forming a tight association with viral DNA polymerase and the terminated DNA to inhibit its function (Figure 2.) (26,27). Since its introduction ACV has established a track record of efficacy and become the standard of treatment and prophylaxis of herpes simplex virus infections in both immunocompetent and immunocompromised hosts (23).

Trisodium phosphonoformate (foscarnet, PFA), a pyrophosphate rather than nucleoside analogue (Figure 1.), also blocks viral DNA replication but does not require viral TK for activation and works by competitively blocking the pyrophosphate binding site of the viral DNA polymerase (28). Its main clinical indication has been as alternative therapy for cytomegalovirus infections. Its independence of the requirement for TK activation, however, offers alternative therapy for patients with ACV-resistant (ACV^R) outbreaks

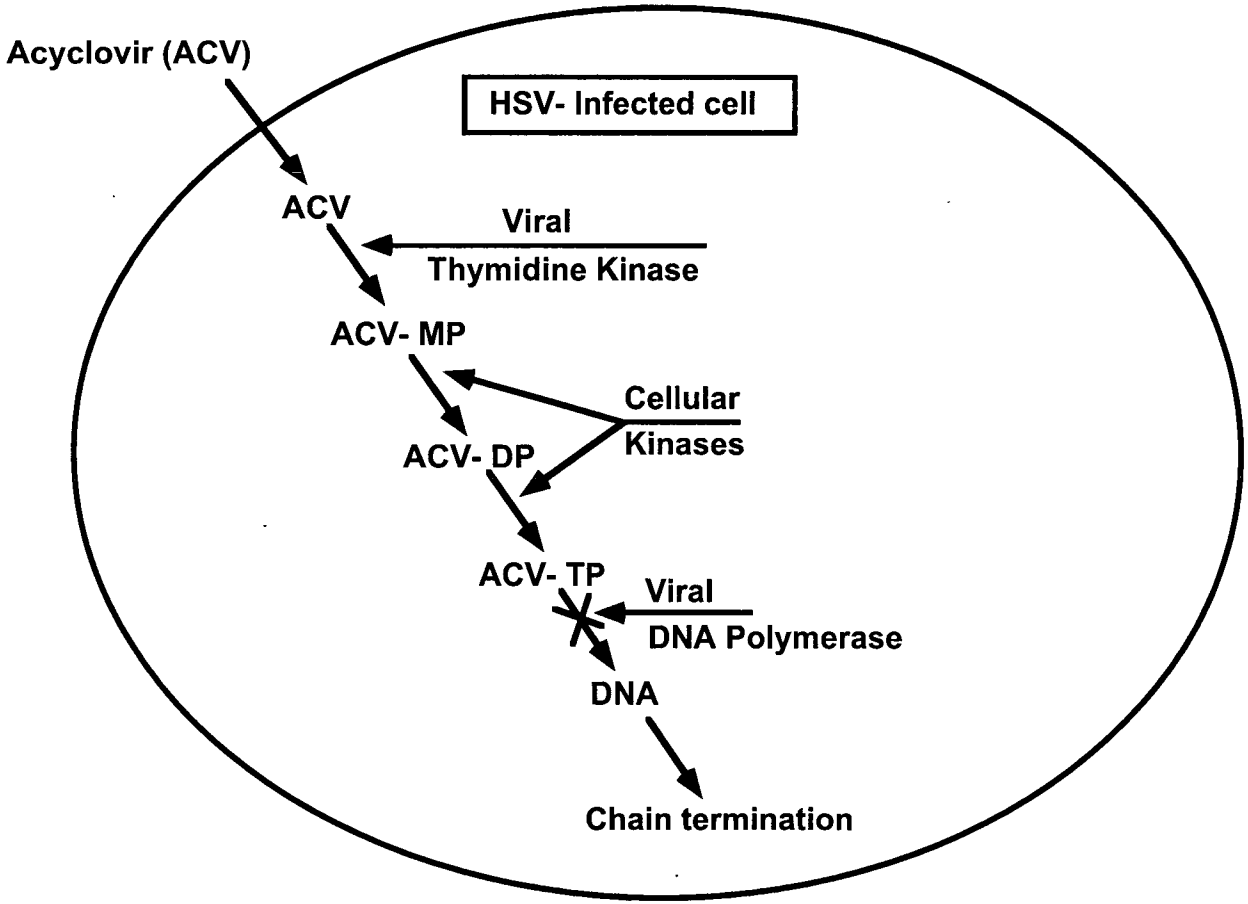


Fig. 2. Pathway of ACV activation and action. ACV is initially phopsphorylated to ACV monophosphate by HSV-encoded thymidine kinase before cellular kinases convert it to ACV triphosphate which results in DNA chain termination and inhibition of viral DNA polymerase.

and controlled trials have demonstrated its efficacy in this setting (29). This less selective mode of action is associated with increased host cell toxicity including renal dysfunction, nausea and vomiting, disturbances in calcium and phosphate homeostasis, anemia, penile ulceration and local thrombophlebitis. In addition, PFA can only be administered intravenously. These drawbacks preclude its use as a first line agent in the treatment of HSV infections (30,31).

1.3. ACYCLOVIR RESISTANCE

As with all antimicrobial agents, reports of resistance to ACV began to emerge soon after the release of the drug. In vitro resistance of herpes simplex to ACV was first reported in 1980 when it was shown that less sensitive herpes simplex viruses could be selected during exposure to ACV in tissue culture (32). The initial clinical ACV^R isolates were isolated from immunodeficient patients in 1982 (33,34). Mutation at two sites in the HSV genome have been shown to confer resistance; the TK and polymerase (*pol*) genes. Mutations in the TK gene may result in two different phenotypes. They may result in deficiency of enzyme activity to all substrates and these are termed TK-deficient (TK^D). Alternatively, the mutation may cause the enzyme to narrow its normally promiscuous spectrum of nucleoside activity to exclude ACV but still be able to phosphorylate its naturally occurring substrate, thymidine; the latter are termed TK-altered (TK^A). The overwhelming proportion of isolates exhibiting the ACV^R phenotype are due to TK deficiency, TK^A or *pol* mutants being rarely reported (32,35-37). Although occasional studies have demonstrated ACV^R isolates in the normal host prior to and developing during treatment, sensitivity patterns bear no relation to subsequent clinical course and such isolates were rapidly cleared

(38-40). Presumably, in such cases a normal immune system clears virus despite *in vitro* resistance. There has only been one clinically-significant ACV^R isolate reported in an immunocompetent host. This was a TK^A isolate which was unresponsive to ACV and which clearly retained TK activity for thymidine (41). The population almost exclusively subjected to clinically significant ACV resistance has been the immunocompromised host where this has been estimated to occur in 4.7% of such patients (42). Immunocompromised states associated with clinical resistance include organ (particularly bone marrow) transplantation (43,44), AIDS (45-47), hematological malignancy (48,49) and congenital immunodeficiency states (50-52). Such patients suffer chronic mucocutaneous lesions unresponsive to and often progressive on ACV despite high doses. The overwhelming majority of clinically resistant isolates have been of the TK^D phenotype although *pol* and TK^A mutants have occasionally been described (42, 53-57).

The biological behaviour of TK^D isolates *in vitro* and in animal models differs from TK wild type (TK^{WT}) strains. Such mutants are capable of efficient replication in actively dividing cells but not in serum starved or non-dividing cells. They can also replicate efficiently at peripheral sites of inoculation but, although they are able to establish latency, they are less efficient, resulting in a lower incidence of infection and yielding a lower titer of virus. These mutants are also at least one hundred times less neurovirulent in a mouse model and this may reflect a need for TK activity in non-dividing cells where such endogenous activity may be lacking (58-64). Finally, and especially germane to the clinical situation, despite having the ability to establish and maintain latency, these mutants have been repeatedly demonstrated to be unable to reactivate in this animal model (65-70). This

concept has been further reinforced by the demonstrated ability of TK inhibitors to reduce reactivation (71). These findings, together with the dearth of reports of reactivation ability in patients, have contributed to the clinical dogma that TK^D reactivations, once effectively sterilized and in the absence of any ongoing antiviral pressure, will be followed by a ACV-susceptible (ACV^S) reactivation. It has also been shown that the degree of TK activity correlates with the neuropathogenicity, impacting on both virus ganglion titer and encephalitis (63,64). In addition, despite a requirement for TK activity, even low levels of TK activity below 10% are sufficient to allow reactivation in mouse models (72). Finally, it needs to be appreciated that the level of TK expressed by different viruses seems to be a spectrum and strains can be identified which exhibit the ACV^R phenotype during *in vitro* susceptibility testing but demonstrate low but detectable levels of TK, so called TK low producer (TK^{LP}) strains. Such isolates have been arbitrarily defined as having <15% activity by enzyme kinetic experiments. The mechanistic basis underlying these strains is unknown at present (56,63).

1.4. THYMIDINE KINASE

The TK of HSV was first identified in 1963 by Kit and Dubbs (73). Although initially it was thought to exclusively catalyze the phosphorylation of thymidine the enzyme was later subsequently shown to be able to phosphorylate a number of other pyrimidine deoxyribonucleosides. It may thus be more accurately termed a deoxypyrimidine kinase (74-76). In its denatured form, TK has a molecular weight of 40,000-45,000 daltons on polyacrylamide gels (77,78) while in its native form it has a molecular weight of about 82,000 daltons (79). The enzyme exists as a homodimer of identical subunits and one putative mechanism of complementation proposed the

formation of a heterodimer, such that one wild type polypeptide compensates for the lack of activity of its mutated partner (80,81). For some nucleoside analogue antiviral agents (e.g. ACV) the initial phosphorylation in the sequence of triphosphorylation to the active compound is selectively catalyzed by a viral-encoded TK rather than a host-specified kinase, conferring specificity for virus-infected cells. As described above, resistance to pyrimidine analogues, including ACV, is most commonly mediated by alterations in the function of TK and by far the most common such alteration is a deficiency of enzyme activity. Mutations in the gene coding for TK which result in resistance to ACV are usually caused by the alteration in a single base (82). Two types of TK^D mutants have been described. The gene may harbor a base pair substitution, resulting in a missense mutation and producing an intact peptide with negligible enzymatic activity. Alternatively, there may be a base pair insertion or deletion to produce a nonsense mutation in which there is a frameshift resulting in the coding of a premature stop codon to produce a truncated peptide with absent activity. Some mutants do not exhibit a detectable peptide; this may be the result of rapid degradation of the peptide, there may be a very proximal nonsense mutation or there may be mutations in the promoter and/or enhancer elements (83).

HSV 1 and 2 TK genes have been sequenced for strains Cl 101 and 333 respectively. They are located at a collinear position on the HSV genome around map position 0.30. The complete gene, including the coding region and its flanking sequences, is 1656 base pairs in length. There is extensive (80%) homology between the coding regions of HSV 1 and 2, suggesting a common ancestry, the basis of variations between the 2 types being a series of single base changes. The site for initiation of translation is located at

nucleotide 333 which signals the start of an open reading frame of 1128 nucleotides followed by an TAG termination codon, beyond which is a polyadenylation signal. The open reading frame codes for a polypeptide of 376 amino acids (84,85). Three conserved regions have been identified on the basis of altered substrate binding properties in mutants as well as homology studies and these constitute the putative "active centre" of the enzyme. The three sites consist of the binding site for ATP, the phosphate donor, located between amino acids 51 to 63 near the N-terminus of the protein, the nucleoside binding site between amino acids 168-176 located in the middle of the protein and amino acid 336 near the C-terminal end of the protein which participates in both ATP and nucleoside binding (86).

1.5. HETEROGENEITY AND COMPLEMENTATION

Naturally occurring ACV^R mutants can be detected within clinical isolates in normal hosts in the absence of drug pressure even in patients naive to ACV. They occur at a relatively high frequency of 1 in 10^4 pfu, although the clinical isolate as a whole population tests as ACV^S by susceptibility assays. Such subpopulations can be demonstrated in diverse clinical settings including cases of gingivostomatitis, genital herpes, encephalitis and systemic infection. These isolates have been shown to consist of heterogeneous mixtures of ACV^S, TK^D, TK^A and *pol* mutants and the existence of such subpopulations illustrates how selection pressure may ultimately select out a clinically significant ACV^R population (54,87,88). This is supported by animal models which have shown the progressive emergence of ACV^R isolates coincident with a reduction in TK activity using serial passages of a HSV 1 strain in mice treated with suboptimal doses of ACV. The same work also demonstrated the re-emergence of an ACV^S population

when antiviral pressure was removed (89). The relative contributions of passive selection as opposed to increased mutational rates due to such pressure is currently unknown.

HSV has been shown to be capable of complementation, such that a mutant virus can have its defective gene function compensated for by another virus with intact gene function. This phenomenon has been demonstrated between intertypic temperature sensitive (*ts*) HSV mutant pairs, for neuroinvasiveness and for TK activity. Genetic recombination has been demonstrated to occur between some but not all such complementing pairs and likely underlies compensation of function in some cases. In fact, recombinant frequencies of up to 2% have been reported between pairs of TK mutants. Some investigators draw a distinction between recombination and situations where complementation occurs in the absence of demonstrable recombinant events. The relative contribution of recombination and non recombinant complementation in the above settings is unclear. In practice genetic recombinants are often generated when complementation studies are conducted, making the separation of underlying mechanisms difficult. For the purposes of this paper, such a distinction will not be made and the term complementation will include all potential underlying mechanisms including recombination. (81,90-95).

Several lines of evidence support the concept of complementation within ACV^R isolates. *In vitro* work with cells infected with such mutant pairs can be shown to increase TK activity beyond levels of cells infected with each mutant alone while, conversely, the addition of TK^D virus to a TK^{WT} population can reduce TK activity within that population (81). In animal models, studies have demonstrated complementation can occur between

TK^D/TK^D and TK^D/ TK^{WT} pairs to produce increased TK^D titers of virus from mouse trigeminal ganglia. Such experiments showed a correlation between trigeminal ganglion virus titer and percent TK activity and showed that as little as 20% activity could yield virus titers near TK^{WT} levels (63,67). The most striking demonstration of such complementation is shown by the reactivation of a genetically-engineered TK^D virus, having established latency in mouse cervical ganglia, only if ganglia were superinfected with a TK^{WT} strain; so called "superinfection rescue" (65). In the clinical setting complementation has been documented for both TK^D and *pol* mutants. In one report of an isolate from a case of esophagitis from a bone marrow transplant recipient, plaque purification of 10 plaques from the clinical isolate showed that 2 TK^D plaque isolates and 2 *pol* plaque isolates imparted clinically-significant ACV resistance onto the larger 6 ACV^S plaque isolates (54). In another report of a clinically ACV^R throat isolate from an AIDS patient, a subpopulation of 3 of 17 TK^D plaque isolates allowed for clinical resistance in the face of an ACV^S phenotype by susceptibility testing, suggesting that such testing could not always reliably predict clinical outcome (96). In all cases, it seems that the nature of the complementation functions to enhance virus survival.

In view of the principle that TK^D isolates are not able to reactivate, the work described within this thesis primarily set out to determine why a TK^D HSV 2 clinical isolate in an AIDS patient described below exhibited apparently paradoxical behaviour by reactivating in the absence of antiviral drug pressure. The initial hypothesis was that the isolate was inherently different from other TK^D isolates and the work was originally directed at proving this. The first phase of the work involved tissue culture characterization to

exclude TK heterogeneity by currently-available methods followed by animal model studies to determine whether reactivation ability was associated with increased neurovirulence. In the next phase, molecular characterization of this isolate was performed in an attempt to correlate reactivation ability to TK genotype. Finally, the above work was extended to characterize other ACV^R clinical isolates which had also reactivated.

CHAPTER 2

MATERIALS AND METHODS

2.1. PATIENTS

2.1.1. Patient WJ

WJ was a 45 year old mother of two who sustained a probable primary episode of genital herpes in April 1984. She presented with a 3 week history of genital ulceration and a necrotic cervical lesion associated with discharge, both of which yielded positive cultures for HSV 2. The first week of her illness was characterized by marked systemic symptoms and dysuria. Her partner had likely suffered intermittent genital herpes lesions for several years which had never been diagnosed. Her past history included gonorrhea, trichomonas and yeast infections, the removal of a benign cervical polyp, a dilatation and curettage following a threatened abortion, 2 episodes of shingles and a tonsillectomy. In addition, in February 1984 she suffered an undiagnosed illness lasting 1 week and characterized by fever, night sweats and rash. Her partner suffered a similar illness lasting 2 weeks in September 1983. Both these are likely to have been a human immunodeficiency virus (HIV) seroconversion illnesses. She experienced regular (approximately 3 monthly) recurrences of genital herpes which she self medicated with short courses of ACV. In 1985 she was found to be HIV antibody positive following the HIV diagnosis of her bisexual partner. In March 1986 she was admitted following a curtailed vacation to South America where she had been diagnosed with cervical carcinoma and treated by cone biopsy but continued to suffer persistent cervical discharge, lower abdominal tenderness and night sweats. She clinically improved on clindamycin and gentamicin and her CD4

count at this time was 990/mm^3. In May 1988 she suffered a bout of *pneumocystis carinii* pneumonia (PCP) which was successfully treated but by this time her CD4 count had dropped to 30/mm^3 and she was diagnosed as suffering from the acquired immune deficiency syndrome (AIDS). A further episode of PCP followed in May 1989 and was successfully treated.

In September 1989, she was admitted to hospital with a 6 week history of an HSV recurrence unresponsive to 4 weeks of oral ACV at doses of up to 800 mg qid. She was initially treated with intravenous ACV at doses of up to 10 mg/kg 8 hourly for 3 weeks without effect until ACV susceptibility testing demonstrated the clinical isolates from this outbreak (1090,1091,1104,1105 and 1106) to be ACV^R. She was treated with foscarnet (PFA) 60 mg/kg intravenously 8 hourly for 14 days with subsequent rapid clinical and virological resolution of her lesion. She sustained a further brief recurrence in November 1989 which responded to oral ACV (isolate 1201). Further recurrences clinically resistant to ACV soon followed, however, in January 1990 (isolates 1280,1286,1288,1307), April 1990 (isolates 1420,1423,1424) and May 1990 (isolates 1563,1564,1566). The first of these was after attempted self medication with ACV and the other two in the setting of ACV prophylaxis. Each of these episodes also responded rapidly to intravenous PFA as above. Due to the frequency of recurrences, after the fourth episode she was placed on PFA prophylaxis 60 mg/kg twice daily. This was successful until, after weaning her prophylaxis down to every third day, she suffered a further recurrence in July 1990 (no cultures obtained). She self medicated with three doses of 60 mg/kg PFA 8 hourly to good clinical effect as documented at two office visits and decided to cease all antiviral treatment. In August 1990, some

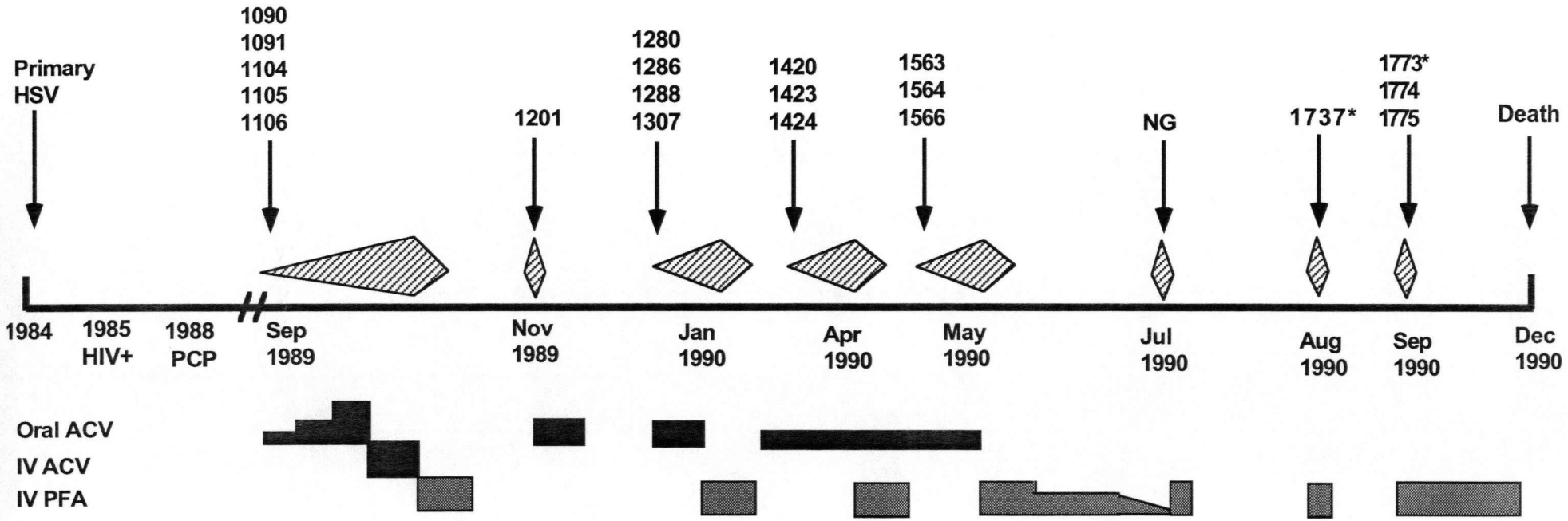


Fig. 3. Timeline for clinical course of patient WJ. This documents clinical outbreaks of HSV with corresponding isolate numbers as well as nature, dose and duration of anti-HSV therapy (NG=no growth). * Represents isolates subsequently characterized further.

five weeks after cessation of antiviral therapy, she was readmitted with neurological complications of HIV infection and was noted to have a further HSV recurrence which was later shown to be ACV^R (isolate 1737). She was treated for only 3 days due to PFA-induced symptomatic hypocalcemia but had a satisfactory clinical and virological response and was discharged on no antiviral treatment. In September 1990 she sustained another recurrence which was found to be ACV^S (isolates 1773,1774,1775) and for which she was discharged home for terminal care on PFA 60 mg/kg q8h. She died at home in December 1990 of causes unrelated to her HSV infection. Post mortem was not performed. A time line depicting these events is outlined in Figure 3.

2.1.2. Clinical isolates derived from ACTG 095

The remaining clinical isolates were graciously provided by Dr. Sharon Safrin from The University of California, San Francisco. They were sourced from a randomized clinical trial comparing PFA with vidarabine (ara-A), another nucleoside analogue but one which does not require activation by TK, in the treatment of resistant HSV in fourteen AIDS patients. This trial was sponsored by the AIDS Clinical Trial Group (ACTG) and was ascribed study number 095 (29). Inclusion criteria required patients to be HIV positive and have a mucocutaneous HSV lesion unresponsive to 2 weeks or more of oral ACV therapy or 10 days or more of intravenous therapy and that clinical isolates from these outbreaks have *in vitro* demonstration of resistance to ACV and susceptibility to PFA and ara-A. After random assignment, patients were administered either PFA 40 mg/kg 8 hourly or ara-A 15 mg per day intravenously for a minimum of 10 days. Patients who had completely healed by day 10 ceased therapy while those with a partial response continued for up to 42 days. Patients who failed treatment or were intolerant were allowed to

cross over to the alternative drug. Following completion of therapy, patients who completely healed were monitored for 3 months for recurrence of HSV lesions during which time the use of antiviral agents was not permitted. The study was terminated after the enrollment of 14 patients due to toxicity and lack of efficacy of ara-A. We initially received 4 pairs of isolates. These consisted of all first clinical reactivations which tested ACV^R and their respective ACV^R clinical isolates which qualified the patients for study entry. These pairs were: clinical isolates 89061/89063, 89353/89350, 90150/90030 and 2370/89660. We later received a further 3 pairs of clinical isolates which consisted of ACV^R isolates qualifying for study entry but in whom first reactivations were ACV^S. These isolates were clinical isolates 89390/89391, 89650/89641 and 90110/3187. These clinical isolates were used for further evaluation to extend the initial findings from isolates originating from patient WJ.

2.2. TISSUE CULTURE

2.2.1. Cell lines

All tissue culture flasks, tubes and dishes were Falcon brand and purchased from Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada. Unless otherwise stated, all media and supplements were purchased from Canadian Life Technologies Inc., (Burlington, Ontario, Canada). African green monkey kidney cells (VERO, Flow Laboratories, Inc., McLean, Va.), and human foreskin fibroblasts (HFF, Clonetics Corp. San Diego, Ca.) were purchased commercially and passaged in 5% MEM [minimum essential medium containing Earle's Salts (MEM), supplemented with 5% inactivated fetal calf serum (FCS), penicillin (100

IU/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), glutamine (2µM), buffered to a pH of 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), and sodium bicarbonate (0.15%)]. Cell line 143B (HOSFTK-; [human osteosarcoma, thymidine kinase-deficient]), was provided by Dr. Silvia Barchetti, McMaster University (Hamilton, Ontario, Canada). 143B cells were maintained in MEM as above except that this was supplemented with 10% FCS (10% MEM), and 50 µg/ml bromodeoxyuridine (Sigma Chemical Company, St. Louis Missouri). Cells were split by trypsinizing with 0.25% trypsin in versene [0.02% EDTA (ethylenediamine-tetraacetic acid) in PBS] and maintained at 37° C in 5% CO_2.

2.2.2. Isolation and Growth of Clinical Isolates of HSV

Clinical isolates were obtained by swabbing lesions with Dacron™ swabs (American Scientific Products, McGraw Park, IL.) by hospital personnel and inserted into vials containing viral transport medium [Medium 199 with Hank's salts and L-glutamine supplemented with 1% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) and buffered to a pH of 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), and sodium bicarbonate (0.075%)]. They were then inoculated into duplicate monolayers of human foreskin fibroblasts (HFF's) (passage <20) in duplicate 16 x 125 mm polystyrene tissue culture tubes seeded with 4 X 10^5 cells containing 5% MEM. After one day growth at 37° in 5% CO_2, they were inoculated with 100 µl of virus media. Cells were then incubated at 37°C in 5% CO_2 and observed daily by microscopy for cytopathic effect (CPE). Infected cells from positive cultures were confirmed and typed by direct immunofluorescence. They were suspended in 200 µl of a 1:1 solution of ethanol and PBS, pH 7.2 and the suspensions were air dried on

microscope slides, fixed with acetone, incubated for 30 min at 37° C with fluorescein isothiocyanate-labelled monoclonal antibodies directed against type-specific antigens of HSV-1 and HSV-2 (Syva, Palo Alto, California) and examined under a mercury vapor fluorescence microscope (Nikon, Canada, Inc., Vancouver, British Columbia, Canada). Viral stocks were grown in confluent VERO cell monolayers in 175 cm^2 flasks at 37° C in 5% carbon dioxide. The monolayer was inoculated with approximately 200 µl of viral transport medium containing the swab and incubated until approximately >70% CPE was seen. The infected monolayer was removed with glass beads, centrifuged at 1000 rpm for 5 min (Beckman J-6B, Beckman Instruments, Palo Alto, Ca, USA) to pellet cells, media removed to leave a residual volume of 4 ml, cells resuspended by vortexing (Vortex-Genie, Scientific Industries, Bohemia, NY, USA), frozen at -70° C and thawed. They were then sonicated (Braunsonic 1510 sonicator, Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada) for 15 s at 150 watts, the cell debris was removed by centrifugation at 1200 rpm for 5 min, the supernatant transferred to a new tube and aliquots removed into 1.8 ml cryotubes (Nunc, Canadian Life Technologies Inc., Burlington, Ontario, Canada). Viral stocks were titered after adsorbing onto confluent VERO cell monolayers in 24 well plates. 200 µl of duplicate serial fivefold dilutions of viral stock in 2% Hanks media [Medium 199 with Hank's salts and L-glutamine, supplemented with 2% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) and buffered to a pH of 7.4 with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), and sodium bicarbonate (0.15%)] were adsorbed onto the monolayer for 1 h and then overlaid with 2% Hanks media containing a 1:750 dilution of anti-HSV-1 rabbit polyclonal antibody (Dakopatts a/s, Dimension Laboratories Inc., Mississauga, Ontario, Canada) to

prevent secondary infection and incubated for 48 h. Media was then removed and cells fixed with 0.5 ml 10% formalin for 10 min, stained with 0.5 ml 0.5% crystal violet for 5 min, washed 3 times with water and air dried. The number of plaques was counted and expressed as plaque forming units (pfu's) per ml.

2.2.3. Susceptibility testing

Plaque reduction assays (50% reduction; ID_{50}) were performed by adsorbing approximately 50 pfu of titered virus stock in 200 µl 2% Hanks media onto confluent VERO cell monolayers in 24 well plates for 1 h as described above. Wells were then overlaid in duplicate with 0.5 ml of 6 serial dilutions of ACV (Burroughs Wellcome, Research Triangle Park, North Carolina) or PFA (Astra Arcus AB, Sodertalje, Sweden) in 2% Hanks media containing a 1:750 dilution of polyclonal anti HSV-1 antibody and incubated for 48 h. Positive virus control wells without drug and negative virus control wells without drug or virus were included. Monolayers were fixed, stained, washed and dried as described above. Plaques in duplicated wells were counted manually, a mean of the two derived and the subsequent values used to generate a dose-response curve. Statview 512 software (Abacus Concepts Inc., Berkeley, Ca, USA) was then used to derive a linear regression equation from the linear part of the curve. The concentration of ACV or PFA required to reduce plaque formation by 50% compared to drug-free inoculated control wells was calculated from this equation and nominated as the ID_{50}. Concentrations greater than 2µg/ml and 100 µg/ml were taken as the cutoff for resistance to ACV and PFA respectively.

2.2.4. Plaque isolation

Duplicated dilutions of approximately 100, 10 and 1 pfu in 0.5 ml 5% MEM media from titered viral isolates were adsorbed onto confluent VERO cell monolayers in 6 well plates for 1 h. Wells were then overlaid with a mixture of 1.5 ml of 1% agarose and 1 ml double strength 5% MEM at 46° C which was allowed to set and incubated for 48 h. Individual well-separated plaques were picked by drawing up 1 µl using a P20 Gilson Pipetman™ (Mandel Scientific, Guelph, Ontario, Canada) and inoculated into a confluent VERO cell monolayer in 25 cm^2 flasks . When flasks showed >70% CPE, infected monolayers were harvested as described above.

2.2.5. Relative TK activities

This was done by comparing the uptake of 1-ß-D-arabinofuranosyl-*E*-5-(2-[125]-iodovinyl)uracil (^{125}IVaraU) (2200 Ci/mmol) by HFF's infected with the strain of interest compared to HSV 2 strain G (American Type Tissue Collection, Rockville, Maryland). Polystyrene tissue culture tubes (16 x 125 mm) were seeded with approximately $3x10^5$ HFF cells in 2.0 mls 5% MEM and incubated overnight. Media was removed and replaced with 1 ml of 2% Hanks containing virus at a multiplicity of infection (MOI) of 0.1 and $2x10^6$ counts per minute (cpm) of ^{125}IVaraU and incubated for a further 18 h. After inspection of the tubes to confirm the presence of early CPE, the media was then removed, the tubes were washed four times with 4 ml phosphate buffered saline (PBS), pH 7.4 at 37°C, and drained on a paper towel. Cells were trypsinized with 1 ml trypsin in versene as described above and the contents transferred to 12 X 75 mm polystyrene tubes and counted in a gamma counter (LKB-Wallac 1282 Compugamma, Fisher Scientific, Ottawa, Canada).

Uninfected cell background was subtracted and activity expressed as % TK activity relative to strain G.

2.2.6. Plaque autoradiography

The method used for plaque autoradiography was a modification of that described by Martin et. al. (97). All incubations were performed in 5% CO_2 and 37°C. Tissue culture dishes (60 X 15 mm) were seeded with 4 ml 5% MEM containing 5 X 10^5 VERO cells or 10 X 10^5 143B cells per ml and incubated overnight to achieve a confluent monolayer. Approximately 100 pfu of titered virus stocks in 1 ml of 5% MEM were inoculated, adsorbed for 1 h, overlaid with a mixture of 2 ml of 1% methyl cellulose and 1 ml double strength MEM and incubated until adequate sized plaques formed as determined by daily microscopic evaluation. The overlay was then aspirated, 0.5 μCi ^{125}I iododeoxycytidine (^{125}IdC) (2200 Ci per millimole) added to VERO cells and ^{14}C deoxythymidine (^{14}C TdR) (62.8 mCi/millimole) added to 143B cells respectively and incubated for 4 h. Isotopes were purchased from Dupont NEN, Boston MA, USA. Cells were then fixed with 4 ml 10% formalin, stained with 4 ml 0.5% crystal violet as described above, washed twice with 4 ml PBS and air dried. Circumferential rims were then removed, taped onto a paper template and placed in a FBXC 810 autoradiography cassette and exposed to Kodak X-Omat XAR X-ray film for 5 days (Fisher Scientific, Ottawa, Canada). The film was then developed in a Kodak M35A X-Omat processor (Eastman Kodak, Rochester, NY, USA). Plaques on the monolayer were then assessed as to whether they incorporated radiolabelled nucleoside on the basis of the darkness of plaque rims on their corresponding autoradiographs. Dark rims indicated incorporation and demonstrated a TK^{WT} phenotype while plaques lacking corresponding dark rims did not incorporate label and

indicated a TK^D phenotype. Plaque numbers on monolayers and dark rims on autoradiographs were then counted and compared and a % of TK^{WT} plaques generated. Finally, some isolates were seen to have very faint but discernible corresponding rims but tested as ACV^R on susceptibility testing. These were only visible by direct inspection of autoradiographs against a light box and it was not possible to demonstrate these light rims on photographs of the autoradiographs such as those in the text below. These plaques were designated as expressing the TK-low producer (TK^{LP}) phenotype.

2.3. ANIMAL STUDIES

2.3.1. Mouse intranasal neurovirulence model.

The initial animal model studies were designed to characterize the neurovirulence of isolates from patient WJ compared to controls. Therefore, in order to generate a dose-response curve for neurovirulence, the initial mouse studies were conducted using a modified intranasal model described elsewhere (98). Four week old female BALB/c mice (Animal Care Center, University of British Columbia, Vancouver) were lightly anesthetized with methoxyflurane (Pitman-Moore Ltd., Mississauga, Ontario, Canada) using drop anesthesia in a 600 ml beaker with a Perspex™ lid following which the right nostril was inoculated with titered virus stock diluted in 20µl of 5% MEM via a P 20 Pipetman™ fitted with a 0.5 mm diameter 200 ml microflex pipette tip (Island Scientific, Bainbridge, WA, USA). Six mice were inoculated with each virus strain at varying inocula, ranging between 10^1 and 10^6 pfu. Isolates tested were TK^D plaque isolate 1737-14 and TK^{WT} plaque isolate 1773-5 which originated from the same patient and served as an internal control. Other controls included sham-infected mice (inoculated with 5% MEM

alone), strain G (a TK^{WT}, HSV 2 reference strain) and strain ACGr4, a TK^{D}, HSV 1 reference strain (courtesy of Dr. Don Coen, Harvard Medical School, Boston, Massachusetts). Mice were then observed daily for evidence of encephalitis for 21 days. The brains of any TK^{D}-inoculated mice which died were surgically removed. Surgery was performed in sterile 100 X 20 m m tissue culture dishes in a biohazard hood (Nuaire model Nu 425FM-600, Plymouth, MN, USA). Mice were sprayed with ethanol and allowed to dry, the skin was then cut and the skull opened with sterile dissecting scissors and forceps. All surgical instruments were purchased from Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada. The brain was transected at the brainstem, shelled out with a sterile nickel spatula, homogenized in sterile Wheaton 15 ml tissue grinders (Fisher Scientific, Ottawa, Ontario, Canada), and the supernatant inoculated onto confluent VERO cell monolayers in 25 cm^2 flasks. The media was changed after 24 h and, following the development of greater than 70% CPE, infected cells were harvested and stocks prepared as previously described. At 30 days, all surviving mice were sacrificed by administering CO_2 into a mouse cage with a Perspex^{TM} lid (Lab Products Inc., Maywood, NJ, USA). Both trigeminal ganglia of 10 mice inoculated with the highest inocula of 1737-14 (the TK^{D} plaque isolate) were then removed to assess the latency characteristics of this isolate. The brain was removed as described above, the ganglia located and removed with sterile dissecting scissors and forceps. Ganglia were then minced with a 40 mm scalpel blade (Fisher Scientific, Ottawa, Ontario, Canada) and cocultivated on confluent VERO cell monolayers in 25 cm^2 flasks. After the development of >70% CPE, infected cells were harvested and viral stocks were prepared as described above. Isolates obtained from the animal experiments

were recharacterized by susceptibility testing, TK uptake and plaque autoradiography as described above.

2.3.2. Mouse intracerebral neurovirulence model

The aim of subsequent animal model studies was to tease out TK^D subpopulations. Therefore, as opposed to the above intranasal model designed to assess neurovirulence, these were designed using a modification of an intracerebral mouse model previously described to enhance this possibility (99). Animals were also 4 week old female BALB/c mice but, due to lack of availability, were purchased from Charles River (Montreal, Canada) and allowed to settle at the Animal Care Center, University of British Columbia for 48 h after delivery and prior to use. Isolates to be inoculated were diluted and prepared as described above. Isolates to be tested included clinical isolates 1737 and 89-063. Both of these were spontaneously-reactivated TK^D and were shown to be homogeneous by plaque autoradiography, thereby testing as pure TK^D by all conventional criteria Control isolates included strains G, ACGr4 and sham-inoculated animals as described above. Six animals were inoculated via a 100 µl Hamilton syringe (Mandel Scientific, Guelph, Ontario, Canada) with 50 µl of 5% MEM containing 10^4 pfu of each isolate into the right cerebral hemisphere and observed for 21 days as above. Dying or sick animals had brains surgically removed and viral stocks were harvested and prepared as described above. Successfully recovered isolates were characterized by susceptibility testing, TK uptake and plaque autoradiography as described above. The purpose of using 1737 was that, using the parental clinical isolate of 1737-14 combined with the increased sensitivity of an intracerebral model, the observations made originally on 1737-14 could be verified and reproduced with an increased sensitivity.

2.4. MOLECULAR BIOLOGY

2.4.1. Preparation of viral DNA

All reagents and gel apparatus, unless otherwise specified, were purchased from Canadian Life Technologies Inc., Burlington, Ontario, Canada. DNA purification was performed after inoculation of confluent VERO cell monolayers in 175 cm^3 flasks with an MOI of 0.1 and incubated until greater than 70% CPE was seen. The cell monolayer was then washed with 5 ml cold PBS and incubated on ice for 5 min with 1 ml cold lysis buffer (10mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Na-deoxycholate). Cells were harvested with a 25 cm cell scraper (Falcon, Baxter Diagnostics Corporation, Canlab Division, Mississauga, Ontario, Canada) and resuspended in a 15 ml polyethylene snap cap. A further 1 ml of lysis buffer was added to the flask, the flask was scraped again the solution aspirated and this volume was added to the tube which then was topped up to 4 ml with lysis buffer. Nuclei were removed by centrifugation at 3000 rpm for 10 min at 4°C (Beckman J-6B, Beckman Instruments, Palo Alto, Ca, USA) and the supernatant transferred to a new tube and incubated for 2 h at 37°C with 50 µg/ml proteinase K, 5 mM EDTA and 12.5 µg/ml of RNAseA. The solution was deproteinized by 3 rounds of the addition of an equal volume of a 1:1 ratio of phenol and chloroform, mixing by inversion, centrifugation at 5,000 rpm for 5 min at room temperature (Beckman J2-21, JA-2 rotor, Beckman Instruments, Palo Alto, Ca, USA) and transfer of the aqueous supernatant into a fresh tube. Residual phenol was removed by the addition of an equal volume of chloroform, inversion, centrifugation and transfer of the aqueous phase into a fresh tube. DNA was precipitated by the addition of 2.5 volumes of 100% cold ethanol together with 0.3 M Na acetate. The tube was inverted

several times, incubated at -20° C for a minimum of 1 h and centrifuged again at 10,000 rpm for 30 min. The ethanol was removed and the pellet washed with 0.5 ml cold 70% ethanol, air dried for 5 min and resuspended in 100 µl of distilled water. The DNA concentration of the resulting product was estimated by diluting 1 µl in 1 ml distilled water and measuring in a spectrophotometer (Ultrospec II, LKB Biochrom, Cambridge England). DNA yields were consistently approximately 1000 µg/ml. Presence and purity of HSV DNA was confirmed by restriction enzyme digest with Eco R1. Reaction conditions included 5 µl DNA, 2 µl (20 units) of enzyme, 10 µl X10 buffer and distilled water to a total volume of 100 µl in a 1.7 ml eppendorf tube. After incubation for 2 h at 37°C, the digested DNA was ethanol precipitated as described above, resuspended in 10 µl of distilled water, 2 µl of running buffer was added and the total volume run in a 0.8% agarose gel in a Horizon 58 gel apparatus using TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) at 100 volts for 1 h. The gel was removed and bathed in 100 ml distilled water to which 5 µl of a 10 mg/ml solution of ethidium bromide was added. After staining for 15 min, the gel was washed three times and destained for 5 min with distilled water. The gel was then examined for DNA by visualization under ultraviolet light using an ultraviolet transilluminator (Fotodyne Inc., New Berlin, WI, USA) and photographed using a Polaroid MP 4 Land camera (Polaroid Corporation, Cambridge, MA, USA).

2.4.2. Amplification of the TK gene by polymerase chain reaction.

Polymerase chain reaction (PCR) was performed using 2 primers designed to lie external to the TK open reading frame (JS1: CTGATCAGCGTCAGAGCGTT and JS8: CGCTTATGGACACACCACAC) (Oligos Etc. Inc., Wilsonville, OR, USA). Amplification was performed in a

Techne PHC-3 thermocycler (Mandel Scientific, Guelph, Ontario, Canada) and conditions included initial denaturation at 100°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 3 min which was followed by a final extension step at 72° C for 10 min. Reactions were carried out in 0.5 ml eppendorf tubes (National Scientific Supply Co. Inc., San Rafael, CA, USA) and included 2 µl (2 µg) template, 1 µl (50 pmoles) of each primer, 8 µl of 1.25 mM dNTP's, 0.5µl (2.5 Units) of Taq polymerase, 5 µl X10 buffer, 2 µl (4%) dimethylsulphoxide (DMSO) and distilled water to a final volume of 50 µl. TK DNA was separated electrophoretically in 0.8% agarose using a Horizon 11.14 gel apparatus and the band visualized by staining with ethidium bromide as described above. The TK band was cut out with a razor blade and the gel block was placed into a 1.7 ml eppendorf tube. The TK product was then purified using a Sephaglas DNA purification kit (Boerhinger Mannheim Biochemica, Laval, Quebec). After weighing the gel fragment, 1 µl of gel solubilizer was added per mg of agarose, the sample was vortexed and incubated at 60°C for 5 min to dissolve the gel after which 5 µl of sephaglass beads per tube was added. The mixture was vortexed, incubated at room temperature for 5 min, centrifuged at 12,000 rpm for 1 min in a microcentrifuge (Microspin 12, Sorval Instruments, Wilmington, DE, USA) and the supernatant removed to a new tube. The pellet was washed and resuspended in 8 times the volume of wash buffer, centrifuged as above and the supernatant removed. This wash was repeated twice and the resultant pellet was air dried for 10 min and resuspended in 10 µl of elution buffer. After further centrifugation as above the supernatant was removed to a new tube. Finally, 2 µl of the resulting product was electrophoresed in a Horizon 58 gel apparatus together with a Mass ladder, stained with ethidium bromide and visualized under UV light as described

above. This was used to estimate the concentration of DNA template to be used for cycle sequencing; results consistently yielded approximately 30 ng/µl.

2.4.3. Cycle sequencing.

Cycle sequencing was performed by designing 14 overlapping internal primers oriented in both coding and noncoding directions to cover the entire TK open reading frame. Internal primer sequences were as follows; (JS2: CTCATCAGCGTCAGAGCGTT, JS4: TCATTGTTATCTGGGCGCTG, JS5: AATGGCGGACAGCATGGCCA, JS6: TGTCTACGATCTACTCGCCAA, JS7: AATCCAGGACAAATAGATGC, JS9: TACCTCATGGGAAGCATGAC, JS10: CTGCTGCGGGTTTATATAGA, JS11: GTAAGTCATCGGCTCGGGGA, JS12: GGGGAGGCGGCGGTGGTAAT, JS13: GGGTAGCACAGCAGGGAGGC, JS14: GGAACAGGGCAAACAGCGTG, JS15: CACATTTTTGCCTGGGTCTT, JS17: GTTCGGTCAGGCTGCTCGTG, JS18: CAAACGTGCGCGCCAGGTCG, JS19: GTGGGGTCCGTCTATATAAA). Sequencing was also performed using external primer JS8. Reactions were carried out using a dsDNA Cycle Sequencing System (Canadian Life Technologies Inc., Burlington, Ontario, Canada). Sequencing primers were end-labeled with ^{32}P dATP (6000 Ci/mmol, Amersham Life Sciences, Oakville, Ontario). Labeling reaction mixtures included 1 pmol (2 µl) of primer, 2 pmol (1 µl) $\gamma^{32}P$ dATP, 1 unit (1 µl) T4 polynucleotide kinase and 1 µl X5 kinase buffer, incubated at 37°C for 10 min after which the reaction was terminated by incubation at 55°C for 5 min. Sequencing reactions consisted of assembling a pre-reaction mixture in a 0.5 ml eppendorf tube which consisted of 1 pmol (5 µl) of labeled primer, 50 pmol (2 µl) template DNA, 1.25 units (0.5 µl) of 2.5 units/µl Taq polymerase, 4.5 µl of X10 sequencing buffer, 1 µl DMSO and distilled water to a total of 36 µl. For the reaction mixture, 8 µl of the pre-reaction mixture was then added

to each of 4 x 0.5 µl eppendorf tubes containing 2 µl of termination mixes, each containing one dideoxynucleotide as well as a mixture of the four deoxynucleotides. Reactions were then placed in the thermocycler for the sequencing reactions. Reaction conditions included denaturation at 95°C for 3 min, 20 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 70°C for 60 s followed by 10 cycles of denaturation at 95°C for 30 s and extension at 70°C for 60 s. The reactions were then terminated with 5 µl of stop solution. Products were then denatured by heating at 100°C for 5 min and run on a 6% polyacrylamide gel [40.4 g urea, 12.6 ml of 40% acrylamide (19:1 ratio of acrylamide to N,N'-methylenebisacrylamide) 16.0 ml X5 TBE (54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA) and 25.2 ml distilled water and dissolved at 37°C]. The gel was polymerized by the addition of 700 µl of 10% ammonium persulfate (APS) and 40 µl TEMED (N,N,N',N'-tetramethylethylenediamine) and injected using a 60 ml syringe and 16 gauge needle between 2 clean glass plates separated by 0.4 mm spacers and a comb and sealed with bulldog clips and electrical tape. Gels were allowed to polymerize for 1 h, the tape and clips were removed and the gel clipped into a model S2 sequencing gel apparatus with 1X TBE buffer added to the upper and lower reservoirs. The gel was prerun for 1 h at 60 Watts and then 3 µl aliquots of sample were electrophoresed for 1.5 and 3 h at 65 Watts using a Pharmacia ECPS 3000/150 power supply (Pharmacia, Uppsala, Sweden). The gel was then removed onto a double layer of Grade No. 3 mm Chr. Whatman chromatography paper (Fisher Scientific, Ottawa, Ontario, Canada), covered with plastic cling wrap and dried at 80°C for 1 h in model SE 1160 Drygel Sr slab gel dryer (Hoefer Scientific Instruments, San Francisco, CA, USA) After removal of plastic wrap and the outer layer of Whatman paper, baby powder was sprinkled over the gel and it was exposed to Kodak X-Omat X-ray film in

a FB-XC-1417 autoradiography cassette with image intensification (Fisher Scientific, Ottawa, Ontario, Canada) at -70°C. The film was then developed as previously described and the sequence read manually using a light box.

2.4.4. Immunoprecipitation

Identification of TK protein was performed by immunoprecipitation and Western blot using an anti-HSV 2 TK mouse monoclonal antibody, TK 30/4 (kindly provided by Dr. Ken Powell, Burroughs Wellcome, Beckenham, United Kingdom). For immunoprecipitation, 60 x 15 mm tissue culture dishes were inoculated with an MOI of 0.1 and adsorbed for 1 h. The infected monolayer was washed three times with 1 ml of methionine/cysteine-free DMEM without L-glutamine (ICN Biomedicals Canada Ltd, St. Laurent, Quebec, Canada), overlaid with a 5 ml mixture containing a 9:1 ratio of methionine/cysteine-free to regular DMEM, 4% dialyzed fetal calf serum and 50 µCi/ml Trans^{35}S label (1101 Ci/mmol ICN Biomedicals, St. Laurent, Quebec, Canada) and incubated overnight. The monolayer was then washed with 2 ml ice cold PBS, the cells lysed with 1 ml cold lysis buffer for 15 min on ice, removed with a cell scraper, aspirated and transferred to a 1.7 ml eppendorf tube. The nuclei were removed by centrifugation at 15,000 rpm for 5 min at 4°C. The supernatant was incubated overnight on ice with 100 µl of a mixture of 10% NP-40, 10% Na-deoxycholate and 1% sodium dodecyl sulfate (SDS) together with a 1/100 dilution of TK antibody. Immune complexes were collected by adding 100 µl of a 10% suspension of *Staphylococcus aureus* cells (Sigma Chemical Company, St. Louis, MO, USA), rocking at 4°C for 2 h and centrifugation at 12,000 rpm for 1 min. The pellet was washed sequentially with 0.5 ml of the following solutions; wash buffer #1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40), wash buffer #2 (20 mM Tris-HCl pH 8.8, 150

mM NaCl, 1% NP-40, 0.2% SDS) and wash buffer #3 (20 mM Tris-HCl pH 6.8, 150 mM NaCl, 1% NP-40, 0.2% SDS). The pellet was then resuspended in 50 µl of SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 10% glycerol and 0.001% bromophenol blue), denatured at 100°C for 5 min and electrophoresed. Electrophoresis was carried out in a model SE 400 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, USA) with 1.5 mm wide spacers. After clamping, a 10% resolving gel was poured [10 ml of 30% acrylamide, 7.5 ml of X4 resolving buffer (1.5 M Tris base, 0.4% SDS, pH 8.8) 12.5 ml distilled water, 200 µl 10% APS and 40 µl TEMED], covered with a layer of distilled water and allowed to polymerize for 1 h, after which the water was poured off and a 6% stacking gel was poured over the resolving gel [3 ml 30% acrylamide, 3.8 ml of X4 stacking buffer (0.5 m Tris base, 0.4% SDS, pH 6.8), 8.2 ml distilled water, 100 µl 10% APS and 20 ml TEMED], a comb inserted and the gel allowed to polymerize for 20 min. The comb was then removed and wells flushed with running buffer (X10 = 0.25 M Tris base, 1.92 M glycine and 1% SDS). Samples were loaded into wells, running buffer added to upper and lower chambers and samples were electrophoresed at 25 mA for 4 h. Gels were removed and then fixed in 40% methanol with 5% acetic acid for 1 h. The gel was then dried in a gel dryer, exposed to X-ray film and developed as described above.

2.4.5 Western blot analysis

Inoculation and adsorption of isolates for Western blot analysis was performed as described for immunoprecipitation except that an MOI of 3 was inoculated and the infected monolayers were subsequently incubated overnight with regular DMEM. Cells were washed and lysed and nuclei removed as described above. Protein was then precipitated from the

supernatant by adding 4 volumes of 100% acetone, incubated at -20°C for a minimum of 1 h, centrifuged at 15,000 rpm for 10 min at 4°C, washed with 80% acetone. The resulting pellet was dried and resuspended in 50 µl SDS sample buffer. The samples were boiled and electrophoresed as described above. The gel was then removed and incubated in transfer buffer (56.5 g glycine, 12.0 g Tris base and 800 ml methanol made up to 4000 ml with distilled water) for 20 min. The gel was then transferred onto a BA-S NC nitrocellulose transfer and immobilization membrane (Schleicher and Schuell, Keene, NH, USA) using an electroblotting transfer apparatus at 1 Amp for 1 h filled with transfer buffer and stirred with a magnetic stir bar (Bio-Rad Laboratories, Mississauga, Ontario). The membrane was removed and transfer of protein confirmed by staining for 5 min with Ponceau S [X10 =2% Ponceau S (Sigma Chemical Company, St. Louis, MO, USA), 30% trichloroacetic acid, 30% sulfosalicylic acid) followed by 3 washes in distilled water. Antibody incubations and detection was performed using a LumiGLO HRP Western blot kit (Kirkgaard and Perry Laboratories, Gaithersberg, Maryland, USA). The membrane was blocked with 0.5% milk block for 1 h at room temperature, incubated with a 1/5000 dilution of anti- HSV 2 TK monoclonal antibody (primary antibody) in 0.1% milk block for 1 h at room temperature, washed 3 times in wash buffer, incubated with 60 u/ml secondary antibody (goat anti-mouse, Boehringer Mannheim Biochemica, Laval, Quebec, Canada) in 0.1 % milk block and washed 3 more times. Chemiluminescent substrates were then applied to the membrane for one minute which was then covered with plastic cling wrap. The membrane was then exposed to X-ray film in an autoradiograph cassette, removed and developed as described above.

CHAPTER 3

RESULTS

3.1 THE MECHANISM OF REACTIVATION OF 1737.

3.1.1. Characterization of clinical isolates from patient WJ

All available clinical isolates were characterized. Patient WJ sustained 7 clinical episodes of genital herpes between September 1989 and September 1990. Twenty clinical isolates were obtained from these episode and all were shown to be HSV 2. Characterization of these isolates by ACV susceptibility testing and TK uptake studies demonstrated 5 of these outbreaks to be TK^- deficient and ACV-resistant while 2 were ACV-susceptible. All isolates sourced from the same clinical outbreak were consistent in their phenotype (Table 1). The TK^D clinical isolate which reactivated spontaneously, 1737, was further characterized together with a clinical isolate from the next outbreak which was ACV-susceptible, 1773, and which served as an internal control.

3.1.2. Plaque isolation of clinical isolates 1737 and 1773.

Prior cases have been reported where ACV^R subpopulations within heterogenous mixtures of viral phenotypes were able to impart properties, in this case antiviral resistance, to the population as a whole (54,96). Our initial aim was to determine whether heterogeneous viral populations containing TK^{WT} subpopulations may have explained the apparently paradoxical activity of isolate 1737 by providing sufficient TK activity to the population as a whole to enable reactivation while simultaneously maintaining the ACV^R phenotype. Initial investigation of heterogeneity was performed by plaque

Table 1. ACV susceptibilities and thymidine kinase uptakes for all clinical isolates available for patient WJ. Isolates are grouped according to the outbreak they were sourced from.*Denotes spontaneously-reactivated ACV^R isolate of interest (1737) and wild type internal control (1773).

Date	Clinical Isolate	ID_{50} (μg/ml)	TK Activity (%)
September 1989	1091	5.4	11.8
	1090	7.4	10.5
	1104	7.8	6.4
	1105	6.5	8.6
	1106	8.4	0.6
November 1989	1201	0.08	55.8
January 1990	1280	6.0	0
	1286	6.5	0
	1288	5.7	0
	1307	5.6	6.8
April 1990	1420	13.4	6.1
	1423	6.8	3.4
	1424	7.4	0
May 1990	1563	8.1	10.1
	1564	5.1	3.8
	1566	7.6	2.8
August 1990	*1737	6.6	9.7
September 1990	*1773	0.11	198.6
	1774	0.08	137.4
	1775	0.09	68.6

purification of individual plaque isolates from clinical isolates and recharacterization of these to identify TK^{WT} plaques. Nineteen plaque isolations were successfully regrown from 1737 and twenty from 1773. All plaque isolates derived from 1737 was ACV^{R} with ID_{50}'s to ACV ranging from 2.1 to 17.1 μg/ml and all plaque isolates derived from 1773 were ACV^{S} with ID_{50}'s ranging between 0.06 and 0.56 μg/ml (Table 2.), suggesting homogeneity within both clinical isolates. Because of this apparent homogeneity, an individual plaque isolate sourced from clinical isolate 1737, 1737-14 (ACV ID_{50} 11.8 μg/ml; TK activity 5.4%), was selected for further neurovirulence studies. Another plaque isolate, 1773-5 (ACV ID_{50} 0.14 μg/ml; thymidine kinase activity 134.7%), sourced from clinical isolate 1773 (obtained from the reactivation following successful sterilization of 1737 with PFA and which was ACV^{S}), was chosen as an internal control. Both plaque isolates underwent PFA susceptibility testing and 1737 was thus proven not to harbor a *pol* mutant (PFA ID_{50}'s: 1737-14= 30.9 and 1773-5= 34.0 μg/ml respectively). In view of the poor sensitivity of TK uptake studies in discriminating between low and absent TK levels, subsequent assessment of TK phenotype was performed by plaque autoradiography of viral populations and demonstrated both 1737 and 1737-14 to be homogeneous TK^{D} while 1773 and 1773-5 were homogenous TK^{WT}. This also excluded the possibility of a TK^{LP} phenotype. In addition, similar results using ^{14}C TdR, the natural substrate for TK, excluded the possibility of a TK^{A} phenotype. This technique also enabled the sampling of a larger number of approximately 100 plaques to more comprehensively exclude TK heterogeneity (Figures 4a. and 4b.).

Table 2. ACV susceptibilities for plaque isolates derived from clinical isolates 1737 and 1773. * Denotes plaque isolates selected for further characterization. DNG = did not grow.

1737	ACV ID_{50} (μg/ml)	1773	ACV ID_{50} (μg/ml)
1	3.7	1	0.08
2	17.1	2	0.23
3	2.1	3	0.27
4	13.5	4	0.39
5	6.2	5*	0.14
6	13.3	6	0.30
7	4.1	7	0.19
8	9.3	8	0.27
9	5.1	9	0.49
10	3.2	10	0.06
11	12.6	11	0.54
12	DNG	12	0.55
13	13.7	13	0.14
14*	11.8	14	0.17
15	13.4	15	0.17
16	11.7	16	0.19
17	8.5	17	0.16
18	7.9	18	0.24
19	13.6	19	0.23
20	2.7	20	0.12

Figure 4a. ^{125}IdC plaque autoradiographs from WJ isolates. Strain G is a TK wild type control and strain ACGr4 is a TK-deficient control. The upper panels demonstrate plaques grown and the lower segments demonstrate corresponding plaques either exhibiting dark-rims and thereby expressing TK activity or lacking dark rims due to TK deficiency. This figure shows results using 0.5 µCi [^{125}I]-iododeoxycytidine (^{125}IdC) as the thymidine kinase probe in VERO cells and demonstrates uniformity of the TK^D plaques within clinical isolate 1737 and plaque isolate 1737-14.

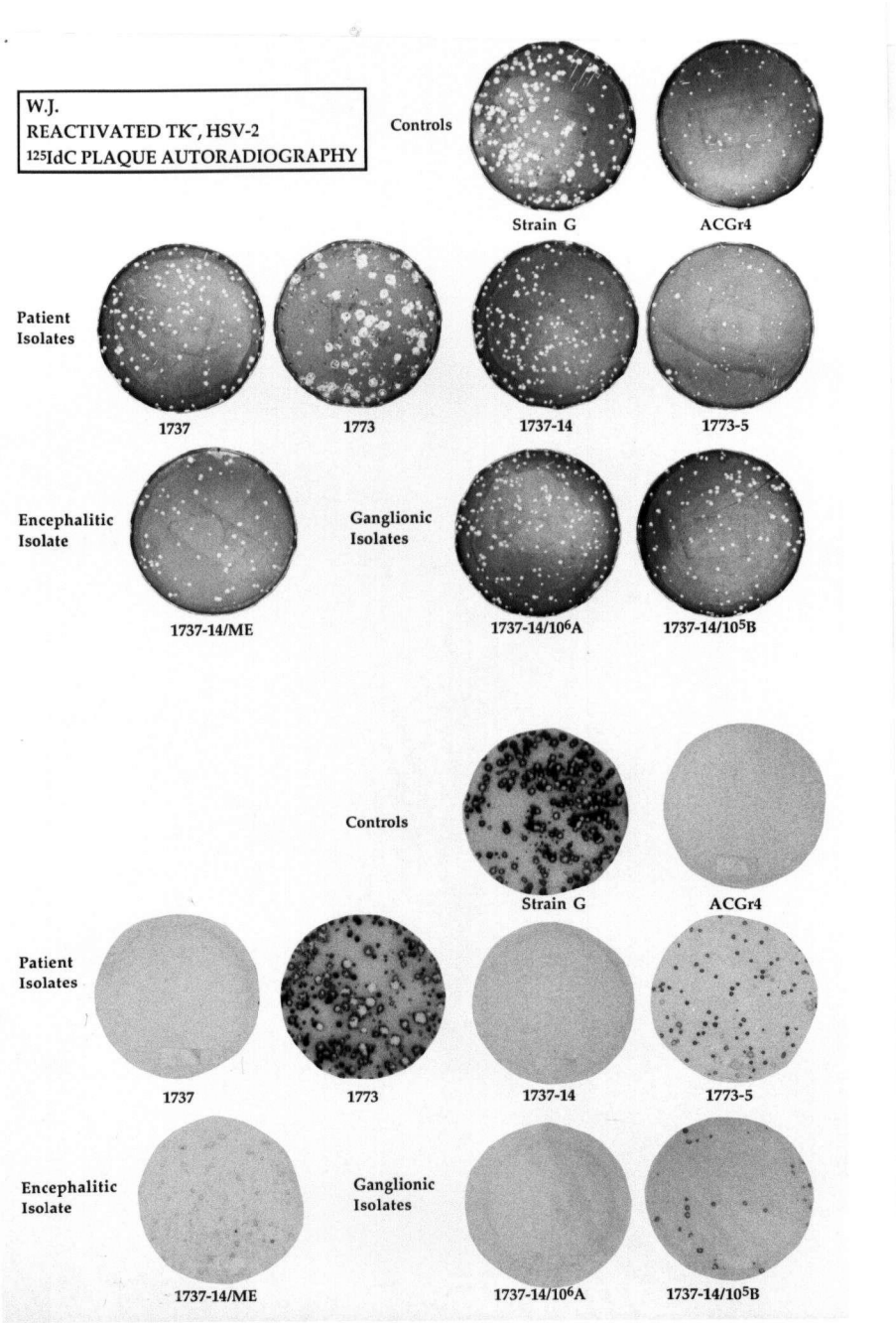
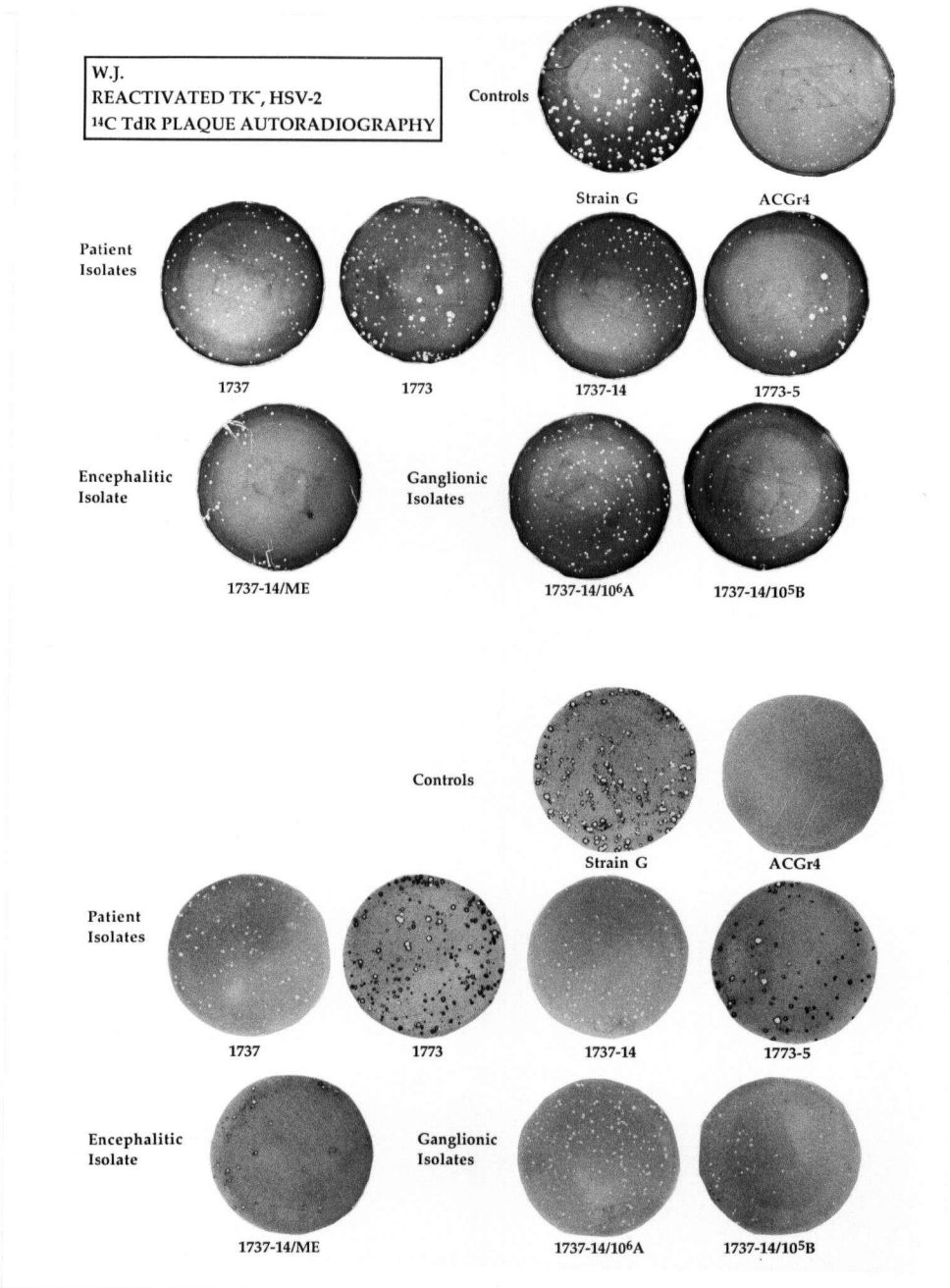


Figure 4b. ^{14}C TdR plaque autoradiographs from WJ isolates. Plaque autoradiography are in similar arrangement to Figure. 4a. and are interpreted in a similar fashion. Results using 0.5 μCi ^{14}C thymidine (^{14}C TdR) in a thymidine kinase-deficient cell line (143B) again confirm the homogeneity of TK^D plaques within clinical isolate 1737 and plaque isolate 1737-14.



3.1.3. Mouse neurovirulence studies of 1737-14

Because clinical isolate 1737 did not harbor a subpopulation of TK^{WT} virus when this was sought by all conventional methods, the neurovirulence of the isolate was assessed to determine whether the unusual reactivation ability of the isolate was associated with other phenotypic markers which were also atypical for TK^{D} isolates and which might offer further clues to reactivation mechanisms. As already alluded to, TK^{D} isolates typically have reduced neurovirulence. Accordingly, plaque isolate 1737-14 was assessed for neurovirulence and latency characteristics in a mouse model. Reference TK^{WT} strain G and internal TK^{WT} control, 1773-5, both demonstrated marked neurovirulence as predicted by their phenotype with no mice surviving beyond an inoculum of 10^2 pfu. In contrast, reference TK^{D} strain ACGr4 was demonstrated to be non-neurovirulent, also as predicted, with no resultant mouse deaths. Animals inoculated with plaque isolate 1737-14, however, yielded an interesting and unexpected result. The isolate overall showed reduced neurovirulence as predicted from its phenotype but surprisingly, 1 of 6 mice at the highest inoculum (10^6 pfu) died from encephalitis (Table 3.). Two explanations were possible for this finding; either the *in vivo* assay was demonstrating the lower threshold of the reduced neurovirulence of the isolate or it was detecting unexpected neurovirulence. In view of this finding, the isolate resulting in neurovirulence was grown from the brain of the dying animal. The output encephalitic isolate, 1737-14ME, was characterized and surprisingly was shown to be fully ACV^{S} (ID_{50} 0.12 μg/ml; TK activity 154.9%), demonstrating a complete change in phenotype from the input strain. Simultaneously, ganglia were extracted from surviving mice inoculated with 1737-14 and attempts to cocultivate virus were successful in

Table 3. Mouse intranasal neurovirulence studies of WJ isolates. The table shows the number of surviving mice out of a total of 6 inoculated at each inoculum of each strain. (* Only 5 mice were inoculated with 1737-14 at this inoculum).

Inoculum (pfu)	G	ACGr4	1773-5	1737-14	Sham
10^6	0	6	0	5	
10^5	0	6	0	5*	
10^4	0	6	0	6	
10^3	0	6	0	6	
10^2	1	6	4	6	
10^1	6	6	5	6	
0					6

2 mice. Characterization of these 2 ganglionic strains demonstrated one, 1737-14/10^6A, to be a similar ACV^R, TK^D phenotype to the input strain (ACV ID_{50} 9.0 μg/ml; TK activity 8.9%; PFA ID_{50} 11.8 μg/ml). In contrast, the other isolate, 1737-14/10^6B, despite being ACV^R, demonstrated a surprisingly high level of TK activity (ACV ID_{50} 15.2 μg/ml; TK activity 53.5%; PFA ID_{50} 9.7 μg/ml). In view of these apparently ambiguous results, plaque autoradiography was performed on these isolates. This demonstrated 1737-14ME, despite the input strain being homogenous TK^D, to consist of a homogeneous TK^{WT} population. Strain 1737-14/10^6A, as expected, was a homogeneous TK^D population similar to the input strain. Strain 1737-14/10^5B, however, was a heterogeneous mixture of almost equal populations of TK^D and TK^{WT} plaques (Figures 4a. and 4b.), thus explaining its unexpectedly high TK activity. PFA susceptibility excluded a viral DNA *pol* mutant phenotype. The derivation of the abovementioned isolates is depicted in Figure 5.

3.2 MECHANISM OF REACTIVATION OF ACTG 095 ISOLATES

3.2.1. Characterization of clinical isolates.

In view of the initial findings seen in patient WJ and to determine whether other spontaneously-reactivated TK^D HSV 2 isolates also harbored TK^{WT} subpopulations, we sought to detect TK heterogeneity within clinical isolates sourced from other patients which had exhibited similar behaviour. We were fortunate to receive a gracious donation of 4 isolate pairs which were part of a clinical trial. The trial, as already described, was ACTG protocol number 095 and compared the efficacy of foscarnet to vidarabine for the treatment of ACV^R genital HSV infections in AIDS patients. Inclusion criteria

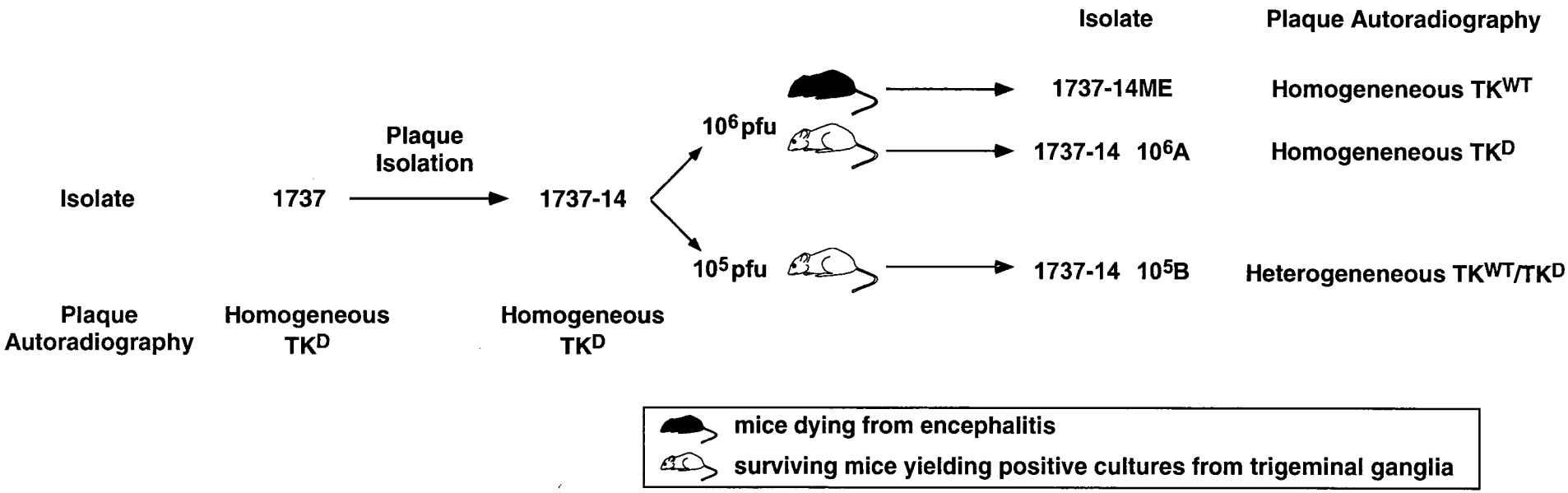


Fig. 5. Derivation of input and output WJ neurovirulence strains. The diagram depicts how reactivated TK^D clinical isolate, 1737, underwent plaque purification to produce plaque isolate 1737-14, both of which appear as homogeneous TK^D populations by plaque autoradiography. Following the inoculation of 1737-14 into a mouse model, neurovirulent brain-derived output strain, 1737-14ME, was homogeneous TK^{WT} while latent virus strains successfully recovered from the trigeminal ganglia of 2 surviving mice, 1737-14/ 10^6A and 1737-14/ 10^5, were shown to consist of a homogeneous TK^D population and a heterogeneous TK^{WT}/TK^D population respectively.

required the demonstration of an HSV isolate clinically unresponsive to ACV. Following eradication of the outbreak by therapy, the patients were prohibited from further antiviral therapy until a subsequent reactivation. Four post-treatment reactivated isolates which were ACV^R (clinical isolates 89-063, 89-353, 90-150 and 2370) were sent to us for further characterization together with the corresponding initial ACV^R isolates which qualified these 4 patients for enrollment into the trial (clinical isolates 89-061, 89-350, 90-030 and 89-660). All isolate pairs underwent confirmatory ACV susceptibility testing and TK uptake studies. All isolates were confirmed to be ACV^R while TK uptake studies demonstrated pretreatment clinical isolates to have negligible activity while reactivated isolates, although having low activity, showed a trend to increased activity. In addition, 3 post-treatment reactivated isolates which were ACV^S (clinical isolates 89-391, 89-641 and 3187) and the respective pre-treatment isolates (clinical isolates 89-390, 89-650 and 90-110) also underwent ACV susceptibility testing and were found to be ACV^S and ACV^R respectively (Tables 4a. and 4b).

As an initial assessment of TK heterogeneity, all ACV^R reactivated clinical isolates underwent plaque autoradiography to determine whether this, on its own, might demonstrate TK heterogeneity without the need for neurovirulence studies. Three of the 4 reactivated isolates (clinical isolates 89-353, 90-150 and 2370) demonstrated heterogeneity on autoradiography. The fourth (clinical isolate 89-063) was homogeneously TK^D as originally described for 1737 from patient WJ. As a result 89-063 was selected for assessment in a mouse neurovirulence model to detect "masked heterogeneity" as performed for 1737.

Table 4a. ACV susceptibilities and TK activities of ACTG 095 ACV^R reactivated isolate pairs. The table depicts characteristics of ACV^R clinical isolates which qualified for enrollment to ACTG 095 and their corresponding spontaneously-reactivated, ACV^R first-reactivation clinical isolates.

Enrollment Isolates			Reactivation Isolates		
Isolate	ACV ID_{50} (μg/ml)	TK (%)	Isolate	ACV ID_{50} (μg/ml)	TK (%)
89-061	9.5	1.3	89-063	12.8	4.2
89-350	10.5	0	89-353	6.2	11.2
90-030	10.2	0	90-150	3.7	23.6
89-660	11.1	0	2370	2.1	20.1

Table 4b. ACV susceptibilities and TK activities of ACTG 095 ACV^S reactivated isolate pairs. The table depicts ACV characteristics of clinical isolates which qualified for enrollment to ACTG 095 and corresponding first reactivations which were ACV^S.

Enrollment Isolates		Reactivation Isolates	
Isolate	ACV ID_{50} (μg/ml)	Isolate	ACV ID_{50} (μg/ml)
89-390	6.0	89-391	0.23
89-650	10.0	89-641	0.15
90-110	8.7	3187	0.30

The 3 reactivated clinical isolates which demonstrated TK heterogeneity on plaque autoradiography were then assessed to determine whether there was an increase in TK^{WT} plaques on plaque autoradiography from the enrollment ACV^{R} isolates to the reactivated ACV^{R} isolates. The plaque autoradiographs on these isolate pairs demonstrated an increase in the proportion of TK^{WT} plaques between enrollment and reactivation when both ^{125}IdC and ^{14}C TdR were used as the probe. As the percentage of TK^{WT} plaques was at all times greater by ^{125}IdC than by ^{14}TdR (the natural substrate) this excluded the emergence of a TK^{A} phenotype (Figures 6a. and 6b. and Tables 5a. and 5b.). Isolate 2370 is of particular interest because by ^{125}IdC it already shows the majority of plaques demonstrating the TK^{WT} phenotype despite it being ACV^{R}. This suggests the presence of either a TK^{A} phenotype or a mutation in the viral DNA polymerase locus.

3.2.2. Mouse intracerebral inoculation of 89-063 and 1737.

Due to the detection of a TK^{WT} subpopulation within clinical isolate 1737 despite an apparent homogeneous TK^{D} population by plaque purification and plaque autoradiography we attempted to confirm the initial findings. On this occasion the primary focus of the work was not to assess neurovirulence but rather to specifically use this animal model to tease out such a TK^{WT} subpopulation. Accordingly, an intracerebral inoculation model was used and inoculated at a high MOI of 1×10^{4} pfu. In addition, as the previous assessment of clinical isolate 1737 was via its plaque isolate, 1737-14, we inoculated the parent clinical isolate. Both these strategies were aimed at increasing the sensitivity of the assay. In addition, because clinical isolate 89-063 was phenotypically identical, we also inoculated it in an attempt to detect TK^{WT} strains. Input strains 1737 and 89-063 were shown to be uniformly TK^{D}

Figure 6a. ^{125}IdC plaque autoradiography of ACTG 095 isolates. The upper panel demonstrates the total number of inoculated plaques after fixing and staining of monolayers while the lower panel demonstrates the corresponding autoradiograph after exposure to X ray film, showing TK^{WT} plaques which are identified by those which produce dark rims. There is an increase in TK wild type plaques from the time at which isolates which ACV^R isolates qualified patients for inclusion into ACTG 095 to the time of first-reactivation isolates which were ACV^R, indicating an increase in TK activity coincident with reactivation.

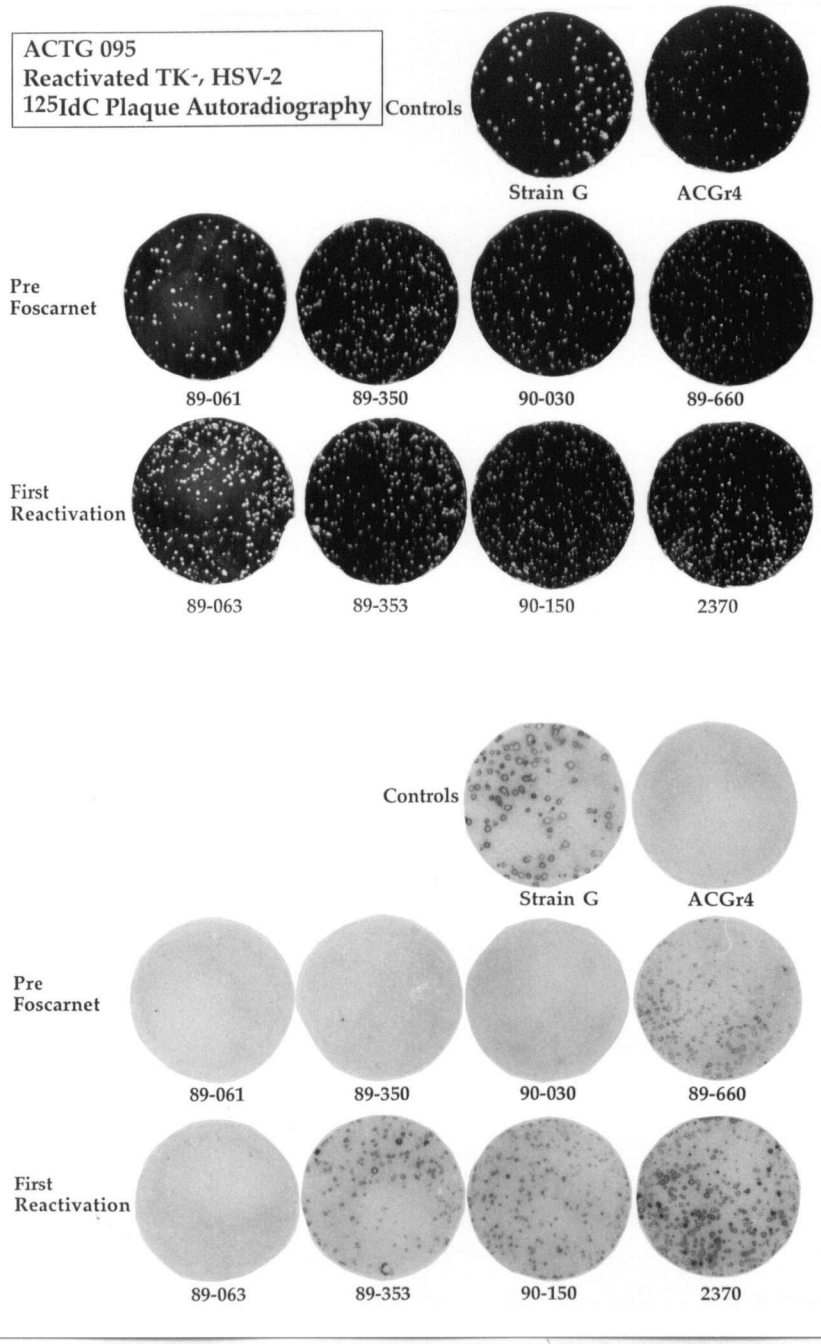


Figure 6b. ^{14}C TdR plaque autoradiography of ACTG 095. The figure again demonstrates an increase in TK wild type plaques between isolates which developed ACV^R to qualify for inclusion into ACTG 095 and first-reactivation isolates which were ACV^R.

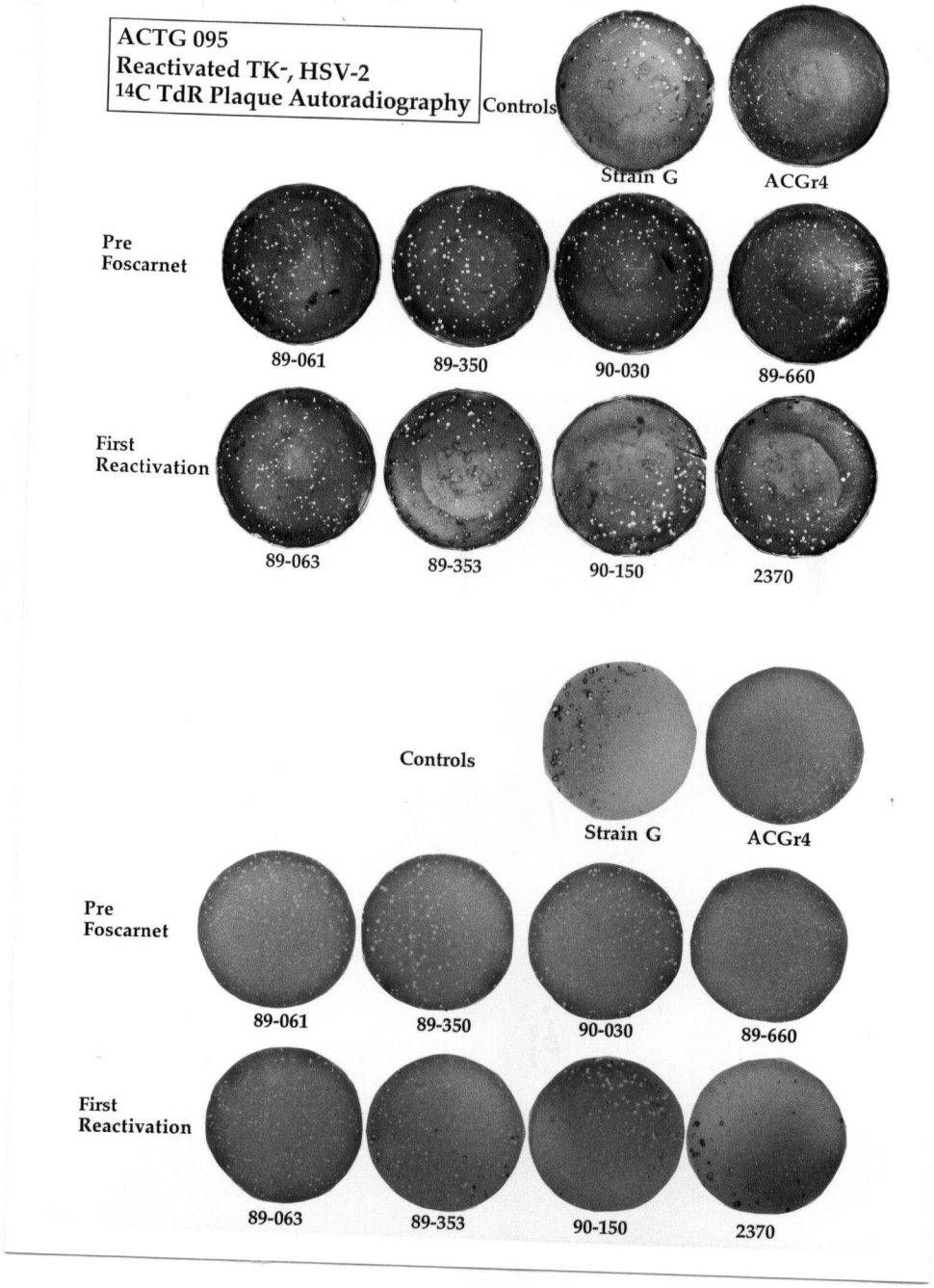


Table 5a. Percentage of TK^{WT} plaques within ACTG 095 isolates by ^{125}IdC plaque autoradiography The table compares the percentages of TK^{WT} plaques within ACV^{R} clinical isolates which qualified for inclusion into ACTG 095 and corresponding first reactivations which were ACV^{R}. An increase in the proportion of wild type (dark rim) plaques is noted in reactivated isolates for all pairs of isolates except for 89-061/89-063 which were homogeneously TK^{D}.

Pre-Foscarnet		First reactivation	
Isolate	% TK Wild Type	Isolate	% TK Deficient
89-061	0	89-063	0
89-350	0	89-353	48.0
90-030	0	90-150	58.5
89-660	84.2	2370	100.0

Table 5b. Percentage of TK^{WT} plaques within ACTG 095 isolates by ^{14}C TdR plaque autoradiography The table lists percentages of TK^{WT} plaques as depicted in Table 5 a. but using ^{14}C TdR plaque autoradiography. This also demonstrates an increase in the proportion of TK wild type plaques between original ACV^R isolates and first reactivations which were ACV^R for all pairs of isolates except for 89-061/89-063.

Pre-Foscarnet		First reactivation	
Isolate	% TK Wild Type	Isolate	% TK Wild Type
89-061	0	89-063	0
89-350	0	89-353	16.3
90-030	0	90-150	18.3
89-660	0	2370	51.5

by plaque autoradiography (Figures 4a. and 4b. and Figures 6a. and 6b.). At the end of the experiment on day 21, all 6 mice inoculated with 1737 and 5 of 6 mice inoculated with 89-063 had died. All mice inoculated with TK^{WT} reference strain G and no sham-infected mice or mice inoculated with TK^{D} reference strain ACGr4 had died as expected (Table 6.). Virus was recovered from all dying mice inoculated with 89-063 or 1737. Characterization of these output strains demonstrated them to be all ACV^{R} with negligible TK levels except for 1737 F which exhibited 23.7% TK activity (Table 7.). Plaque autoradiography of 1737 output strains using ^{125}IdC demonstrated a very small subpopulation of dark-rimmed TK^{WT} plaques in addition to a population of plaques with faint rims consistent with TK^{LP} phenotype. Output strains derived from 89-063-inoculated animals, in contrast, demonstrated the presence of a uniform population of TK^{D} plaques. Plaque autoradiography using ^{14}C TdR demonstrated that 1737 output strains harbored a similar subpopulation of TK^{WT} plaques as seen by ^{125}IdC but faint rims suggestive of the TK^{LP} phenotype were not seen. Output strains derived from 89-063, however, demonstrated a significant population of faint rim plaques suggestive of the TK^{LP} phenotype (Figures 7a. and 7b.), suggesting that the selection of this phenotype was the major mechanism responsible for the contribution of TK activity and reactivation ability of this isolate. Attempts to recover virus from the brain of the single surviving mouse inoculated with 89-063 was unsuccessful.

Table 6. Intracerebral mouse neurovirulence studies of 1737 and 89-063. The table depicts the number of surviving mice at 21 days. Six mice were inoculated with each strain at an inoculum of 10^4 pfu.

Isolate	Number Surviving
G	0
ACGr4	6
1737	0
063	1
Sham	6

Table 7. ACV susceptibilities and TK uptakes of 1737 and 89-063 neurovirulent output strains. The table depicts characterization of strains derived from the brains of mice dying after intracerebral inoculation with clinical isolates 1737 and 89-063.

Isolate	ACV ID_{50} (μg/ml)	% TK
1737 A	5.3	0
1737 B	11.3	0
1737 C	9.5	3.3
1737 D	11.4	2.5
1737 E	8.2	2.7
1737 F	9.4	23.7
063 A	14.1	2.7
063 B	12.5	0
063 C	12.0	1.3
063 D	10.1	0
063 E	14.9	0

Figure 7a. ^{125}IdC plaque autoradiography of 89-063 and 1737 intracerebral output strains. The figure depicts isolates recovered from mouse brains after intracerebral inoculation with strains 89-063 and 1737. This demonstrates the recovery of a subpopulation of TK^{WT} plaques with dark rims in 1737-derived output isolates. These isolates also contain a population of faint-rimmed plaques suggestive of a TK^{LP} phenotype which is not evident on the photographs below but is only visible on direct autograph visualization against a light box. Output strains derived from 89-063-inoculated animals demonstrate a lack of rims by any method of inspection, suggesting a uniform TK^{D} population.

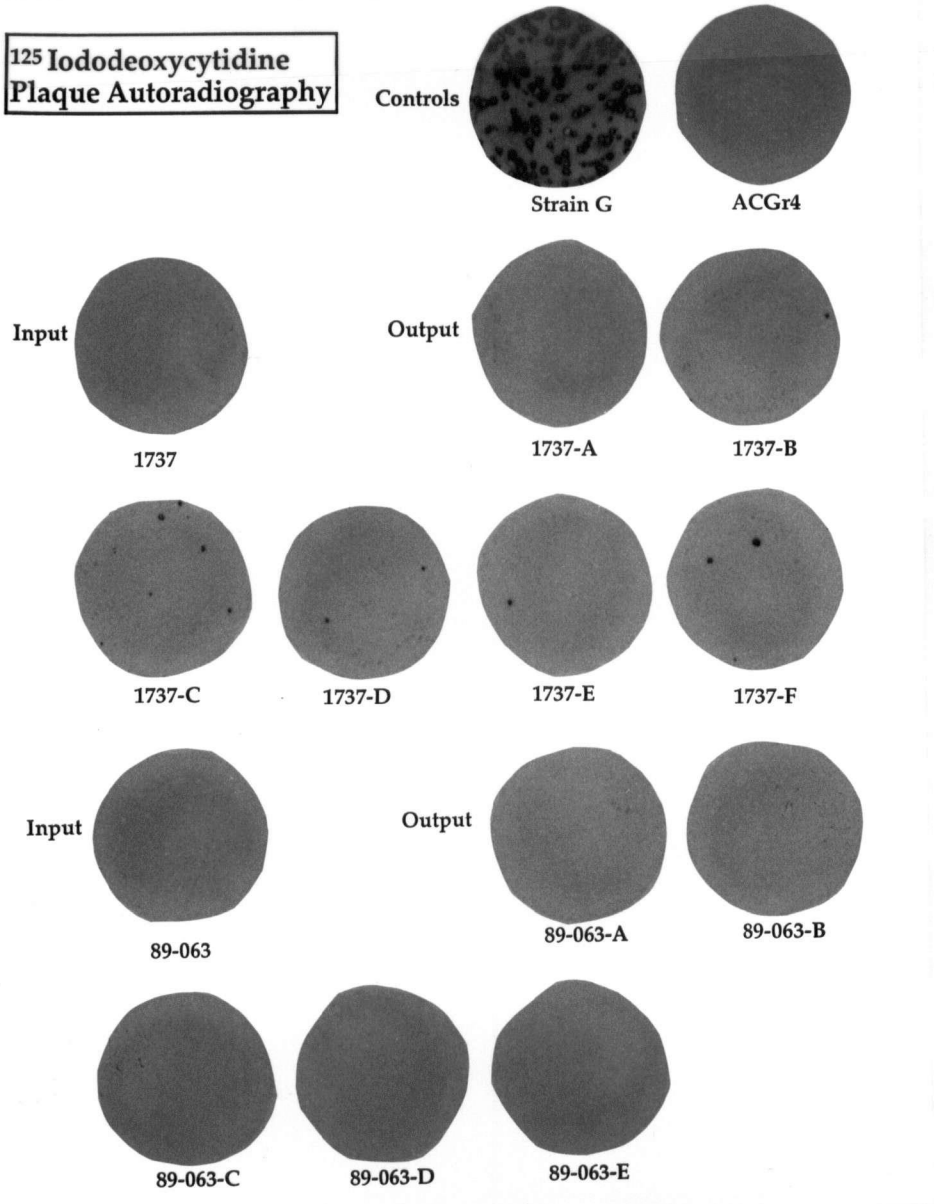
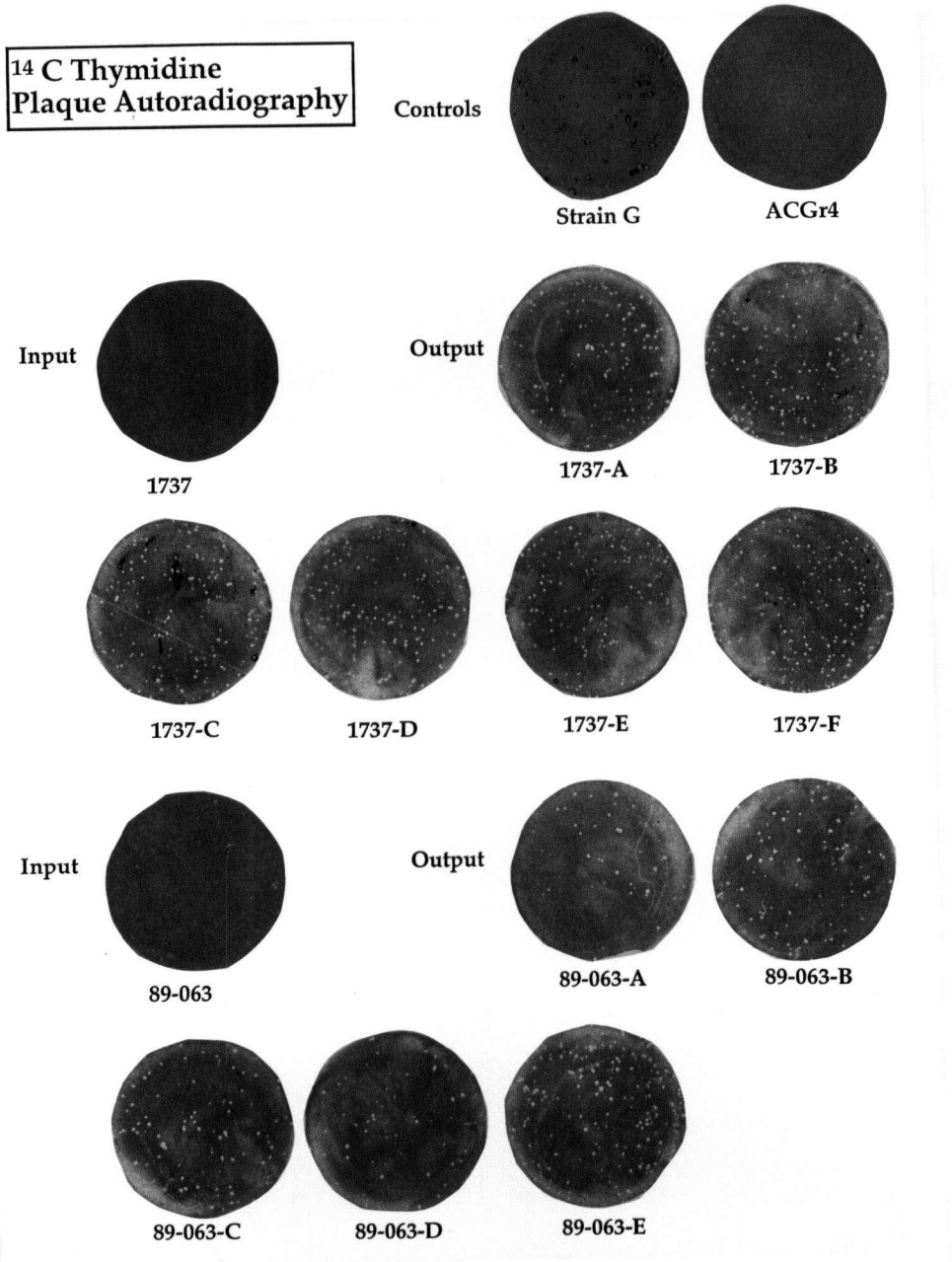


Figure 7b. ^{14}C TdR plaque autoradiography of 89-063 and 1737 intracerebral output strains The figure is similar in outlay to figure 6a and depicts plaque autoradiography using ^{14}C TdR. This again demonstrates the recovery of a subpopulation of TK^{WT} with dark rimmed plaques in 1737 although the faint rims suggestive of a TK^{LP} seen by ^{125}IdC are not visible even with direct visualization against a light box. In contrast, 89-063-derived plaques do demonstrate faint rims by light box examination although this is not evident on the photograph below, again suggesting a TK^{LP} population.

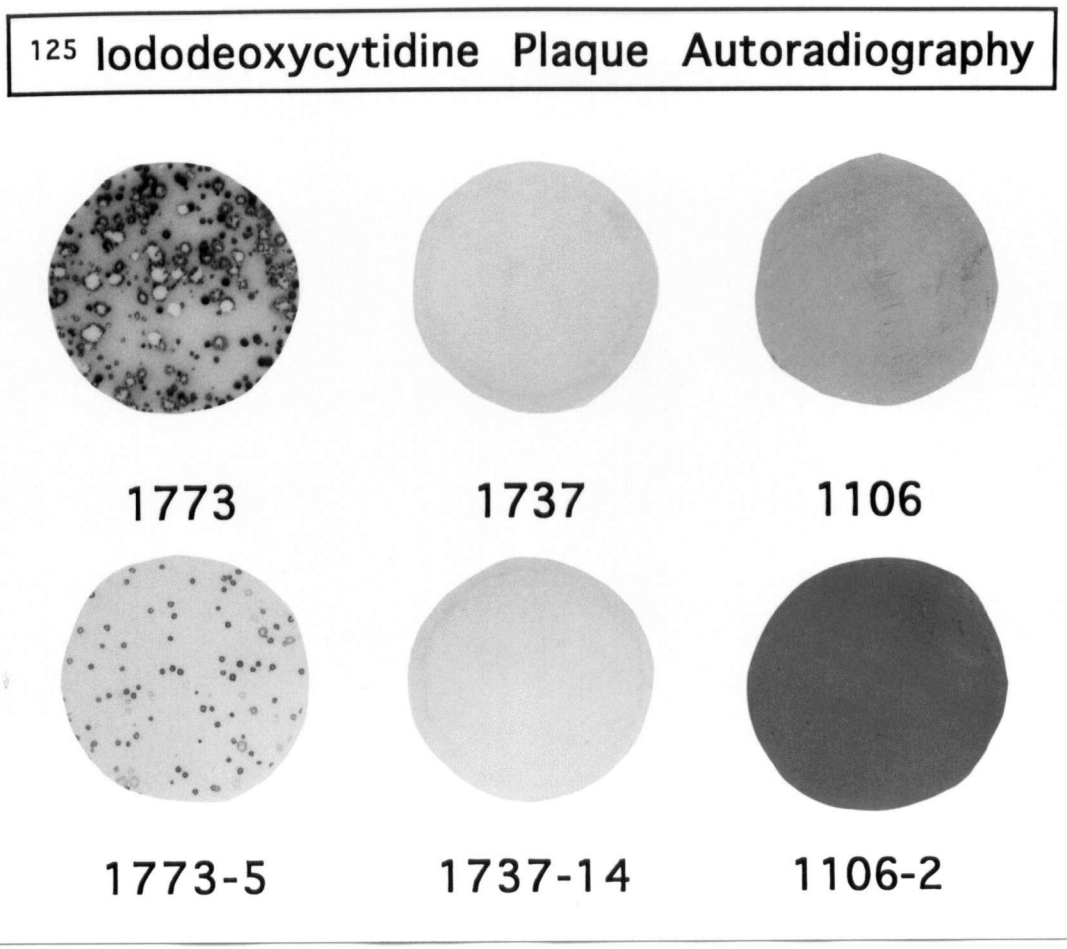


3.3. MUTATION CONFERRING THE TK^D PHENOTYPE ONTO 1737

3.3.1. DNA sequencing of isolates from patient WJ.

To determine the genetic lesion conferring the TK^D phenotype onto clinical isolate 1737 and to determine whether this may be mechanistically implicated in the ability of 1737 to reactivate, DNA sequencing of the TK gene was performed. Specifically, sequencing was performed on plaque isolate 1737-14, the plaque isolate originating from 1737, as this was the strain which resulted in mouse neurovirulence and yielded output strain 1737 10^6ME as previously described. The homogeneity of the mutation within the viral population was suggested by the uniform plaque autoradiography of 1737 (Figures 4a. and 4b.), the uniform ACV^R phenotype of the 20 plaque isolations derived from 1737 (Table 2.) and the detection of the same mutation in another plaque isolate (1106-2) originating from a different TK^D clinical isolate from the same patient (1106) which was also uniformly TK^D by plaque autoradiography (Figure 8.). A TK^{WT} plaque isolate (1773-5) originating from clinical isolate 1773 also obtained from patient WJ was used as an internal control with the expectation that the difference in TK DNA sequence between the two strains should only differ from 1737-14 by the mutation conferring the TK^D phenotype. The sequence of 1773-5 was compared to published HSV-2 TK^{WT} sequence of strain 333 and demonstrated 4 base pair differences (85). These were; adenosine for guanosine at position 79 of the open reading frame, guanosine for thymidine at position 85, guanosine for adenosine at position 232 and thymidine for cytosine at position 476. All four base pair changes were non-conservative but none of the amino acid substitutions occurred in the three regions which comprise the putative active center as

Figure 8. ^{125}IdC plaque autoradiography of WJ-derived clinical and plaque isolates. The figure depicts all plaque isolates derived from WJ which underwent DNA sequencing of the TK gene. Isolates include internal control clinical and plaque isolates, 1773 and 1773-5 respectively, to be uniformly TK^{WT} with dark-rimed plaques while clinical isolates, 1737 and 1106, and their respective plaque isolates, 1737-14 and 1106-2, demonstrate plaques without rims consistent with uniform TK^{D} populations.



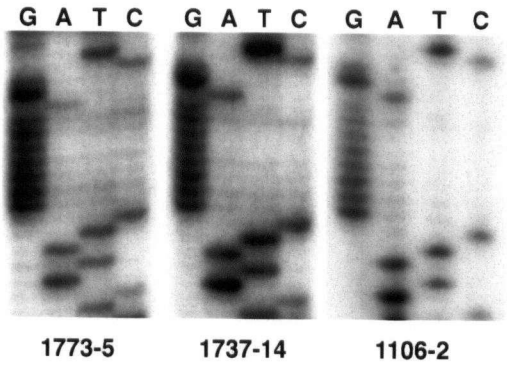
previously described (86). The TK DNA sequence of 1737-14 differed from strain 333 by the same 4 base pair substitutions as 1773-5 but differed from 1773-5 by a single G addition in a homopolymer string of 7 G's residing between positions 433 and 439 of the open reading frame (Figure 9.), suggesting both to have a common origin. Primer JS 12, which spanned the region containing the string of 7 guanosines, was then used to sequence the same region of 1106-2. This was demonstrated to have the same guanosine addition as 1737-14 (Figure 9.). Plaque isolate 1106-2 was sourced from clinical isolate 1106 which also exhibited the TK^D phenotype but, unlike 1737, it was followed by the expected TK^{WT} reactivation. This demonstrated that, even within the same patient, this mutation could spontaneously reactivate as a TK^D isolate or be followed by a TK^{WT} reactivation, suggesting that such a mutation does not confine the isolate to one mode of reactivation behaviour.

The predicted consequences of this guanosine insertion were to produce a frameshift which would result in a premature stop codon at position 684 of the open reading frame and which would result in a truncated protein of 228 amino acids of approximately 28 kd molecular weight compared to the wild type 376 amino acids of 40 kd molecular weight. It would also alter every amino acid in the putative nucleoside-binding site and eliminate the combined nucleoside and ATP-binding site. Such a protein should theoretically be devoid of TK activity and confer the TK^D phenotype (Figures 10a. and 10b.).

3.3.2. Thymidine kinase protein studies of isolates from patient WJ.

Having predicted the translational consequences of the guanosine addition which conferred the TK^D phenotype onto isolates from patient WJ,

Figure 9. TK mutations within WJ isolates. The figure depicts DNA sequencing gels of the TK genes of TK^{WT} control plaque isolate 1773-5 and TK^{D} plaque isolates 1737-14 and 1106-2 originating from patient WJ. These show 1773-5 to have a stretch of 7 G's while both 1737-14 and 1106-2 have an identical G insertion mutation to produce a stretch of 8 G's.



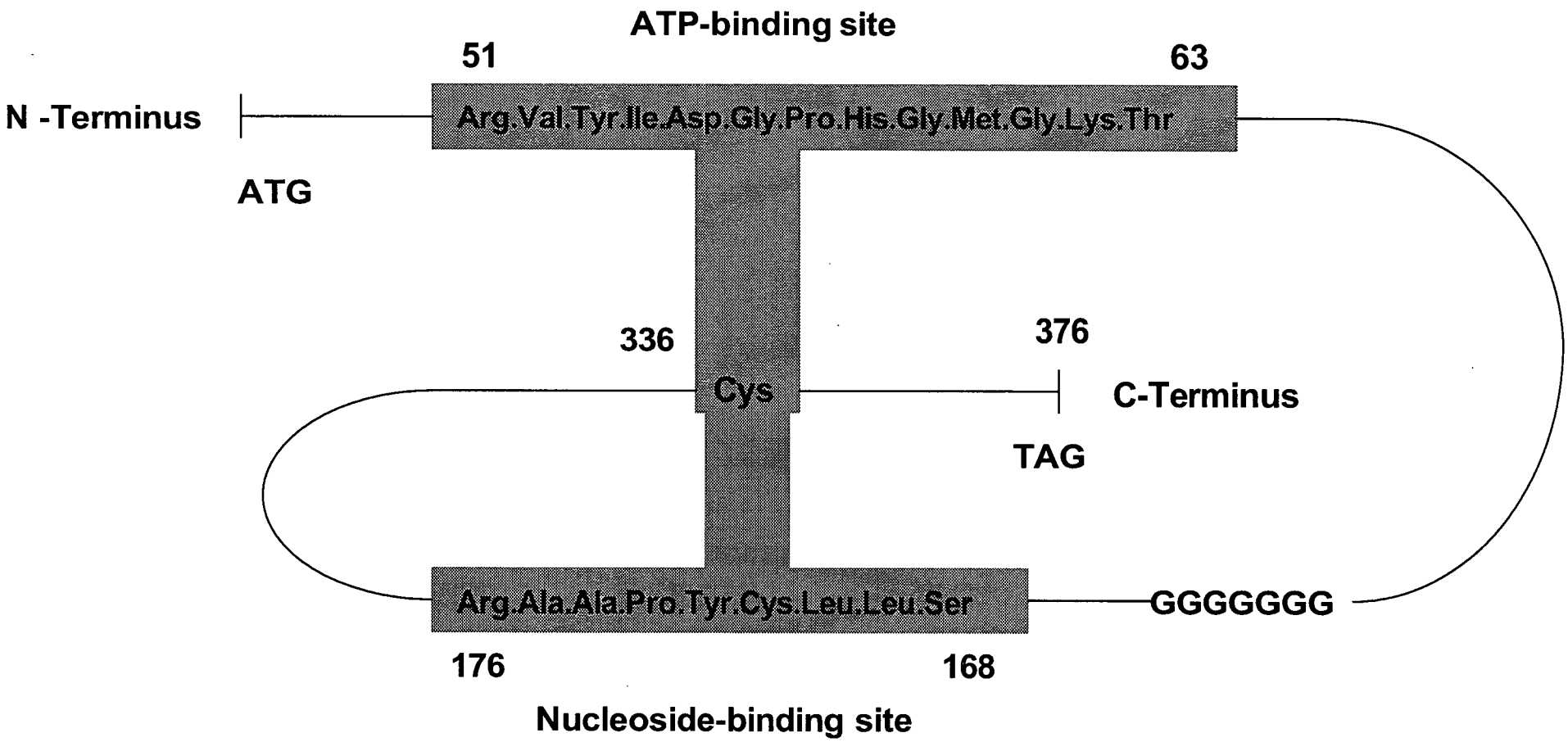


Figure. 10a. Wild type TK. The diagram depicts the full length TK protein of 376 amino acids predicted to be produced by TK^{WT} plaque isolate 1773-5. The amino acids comprising the three "active centers" are highlighted and the string of seven guanosines, which functions as a mutational hot spot, is also shown.

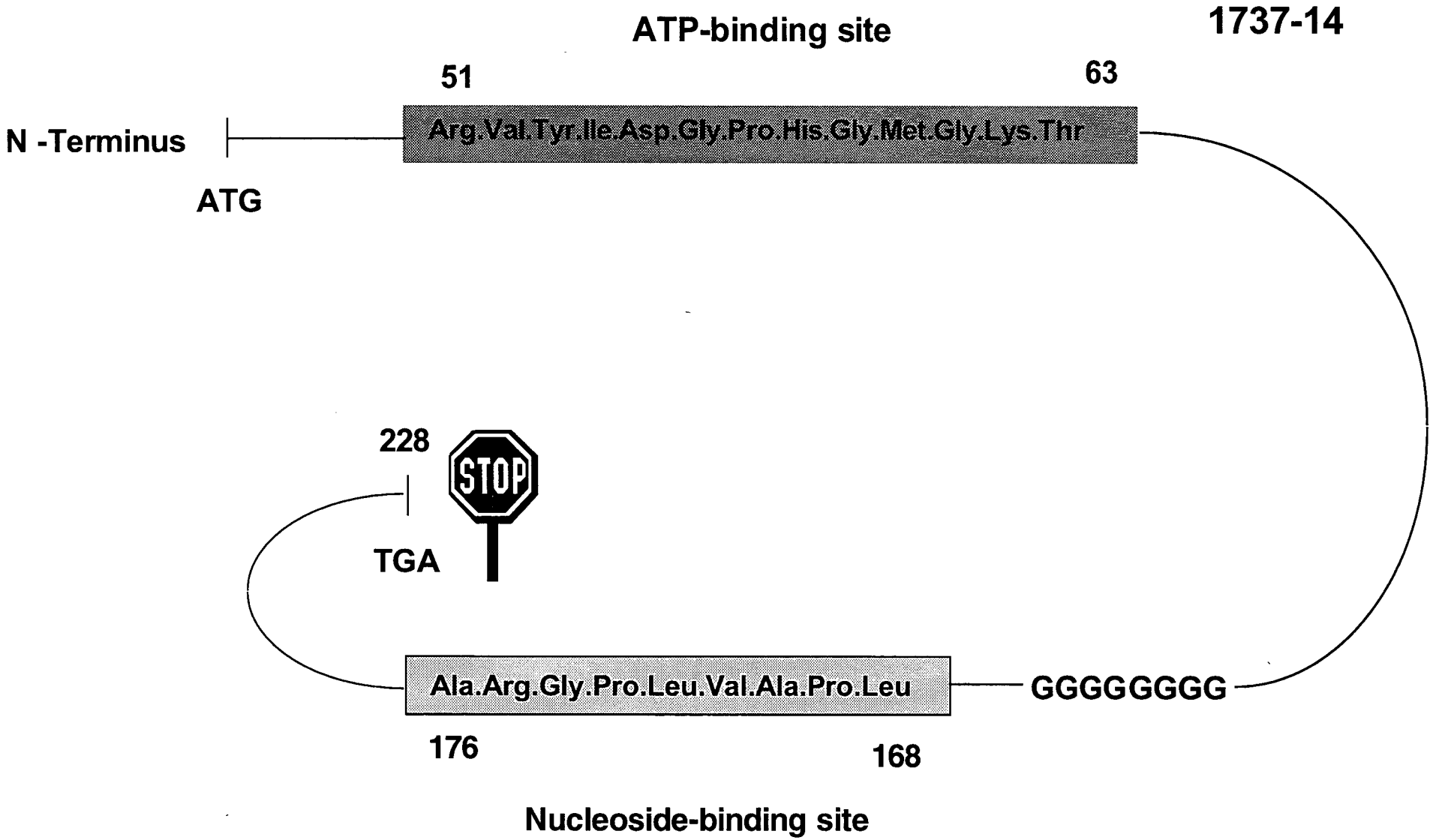


Figure. 10b. Mutation within 1737-14. The diagram depicts the truncated TK protein of 228 amino acids predicted to be produced by 1737-14 and highlights the intact ATP-binding site, the altered nucleoside-binding site produced by the guanosine insertion within the string of seven guanosines and the absent combined ATP/nucleoside-binding site.

the presence of the predicted truncated TK protein product was sought by protein studies. Initial studies by immunoprecipitation using ^{35}S methionine metabolic labeling and an anti-HSV-2 monoclonal antibody demonstrated 1773-5 to produce the expected full length 40 kd protein while both 1737-14 and 1106-2 produced the predicted truncated 28 kd protein product (Figure 11.). The technique of Western blot analysis using the same antibody was then perfected and, due to its superior specificity and the lack of a requirement for radioisotopes, was adopted in favor of immunoprecipitation. Western blot analysis also confirmed 1773-5 to produce a full length 40 kd protein and 1737-14 and 1106-2 to produce a 28 kd truncated protein product as predicted from the amino acid sequence resulting from the mutation and seen by immunoprecipitation (Figure 12.)

3.4 MUTATIONS CONFERRING TK^D PHENOTYPES ONTO ACTG 095 ISOLATES.

3.4.1. DNA sequencing of ACTG 095-derived isolates

3.4.1.1. Spontaneously-reactivated isolates.

In view of the genetic lesion detected in isolates 1737-14 and 1106-2, the genetic lesions in other TK^D spontaneously-reactivated clinical isolates were evaluated. Accordingly, ACV^R isolates derived from clinical trial ACTG 095 which exhibited such behaviour were sequenced to determine whether there was a common genetic basis for this ability. In an attempt to improve isolate purity, 5 plaque isolates were picked from these clinical isolates and retested for ACV resistance (Table 8.). These plaque isolates were screened for the guanosine addition identified in 1737-14 and 1106-2 by sequencing the region covering this mutation using the primer spanning this region (JS 12).

Figure 11. Immunoprecipitation of WJ isolates. The figure depicts immunoprecipitation using ^{35}S methionine labeling and anti-HSV 2 TK monoclonal antibody. The left hand lane is a VERO cell extract which was mock infected and shows labelled cellular protein. G strain is a TK^{WT} control while 1773-5 is a TK^{WT} plaque isolate from patient WJ; both demonstrate a full length 40 kd protein. KpnΔ333 is a control TK^{D} deletion mutant and demonstrates a truncated TK protein at approximately 35 kd. Plaque isolates 1737-14 and 1106-2 are TK^{D} and originate from patient WJ; both demonstrate an identical truncated product at 28 kd.

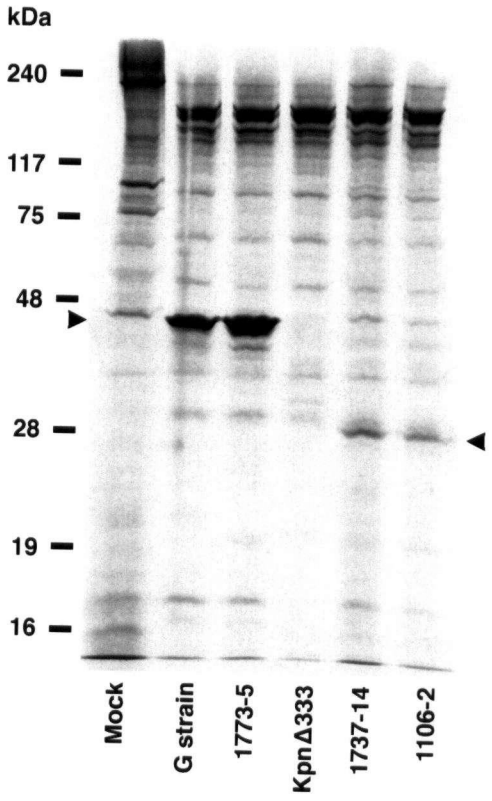


Figure 12. Western blot analysis of WJ isolates. The figure depicts plaque isolates derived from patient WJ clinical isolates. The left lane is mock infected and shows no signal. Reference TK^{WT} strain G and plaque isolate 1773-5 demonstrate full length 40 kd proteins. Plaque isolates 1737-14 and 1106-2 show a truncated 28 kd product.

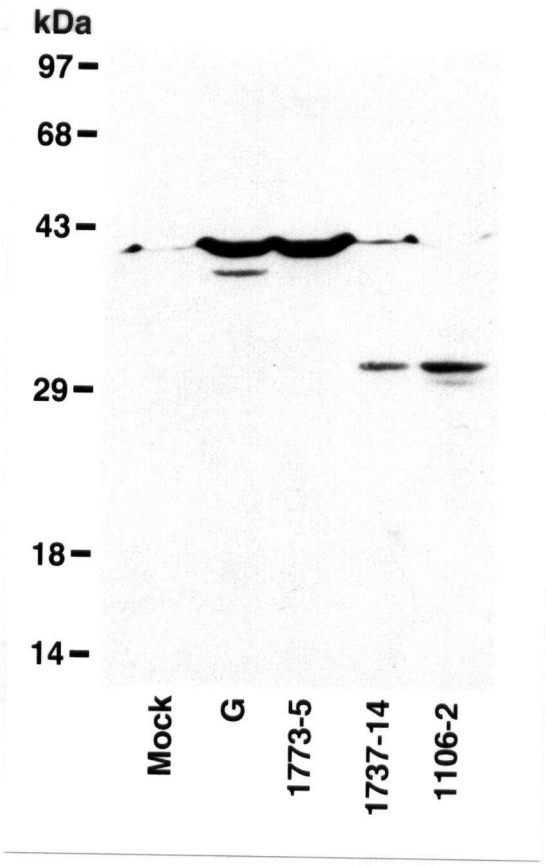


Table 8. ACV susceptibilities of ACTG-095 plaque isolates. The table depicts characterization of plaque isolates originating from ACTG-095 clinical isolates and demonstrates all to be ACV^R.

Reactivated ACV^R		Reactivated ACV^S	
Plaque Isolate	ACV ID_{50} (μg/ml)	Plaque Isolate	ACV ID_{50} (μg/ml)
89-063-1	19.2	390-4	9.5
89-353-1	21.8	650-5	10.1
90-150-3	23.7	110-4	9.5
2370-2	19.5		

Sequence screening demonstrated 1 of 4 of these ACTG 095-derived plaque isolates (90-150-3) to have the identical G insertion as 1737-14 and 1106-2 (Figure 13.).

3.4.1.2. Isolates followed by an ACV^S reactivation

To see whether the G insertion only occurred in ACV^R isolates which were able to spontaneously reactivate or whether they also occurred in isolates which were followed by the expected ACV^S reactivation, we also screened for the mutation in 3 such isolates sourced from ACTG 095. Of these, one plaque isolate, (89-650-5), also demonstrated the identical guanosine insertion as seen in 1737-14 and 1106-2 (Figure 14.), suggesting that such mutations can result in reactivations which could either spontaneously reactivate as a TK^D phenotype or be followed by the expected ACV^S phenotype.

3.4.2. Protein studies of ACTG 095-derived isolates.

3.4.2.1. Spontaneously-reactivated isolates.

Western blot analyses of ACTG 095-derived plaque isolates also confirmed a 28 kd truncated TK product for both 90-150-3 and 89-650-5 which corroborated the predictions of the sequencing data. Of other ACTG 095-derived plaque isolates, 2370-2 produced a full length 40 kd protein, 89-353-1 demonstrated a truncated product identical in size to the 28 kd product associated with the above reported guanosine insertion while 89-063-1 demonstrated a slightly larger truncated product of approximately 32 kd (Figure 15.). Plaque autoradiography using ^{125}IdC performed on these plaque isolates demonstrated 90-150-3 to consist of 89% TK^D plaques. Of particular interest was plaque isolate 2370-2 which produced a full length product. It, as

Figure 13. ACTG 095 ACV^R mutational screen. The figure depicts DNA sequence screening of the gene region covering the stretch of 7 guanosines in plaque isolates derived from ACTG 095 spontaneously-reactivated ACV^R clinical isolates. Of 4 isolates screened, plaque isolate 89-150-3 has the identical guanosine insertion within the stretch of 7 guanosines as was demonstrated in 1737-14 and 1106-2.

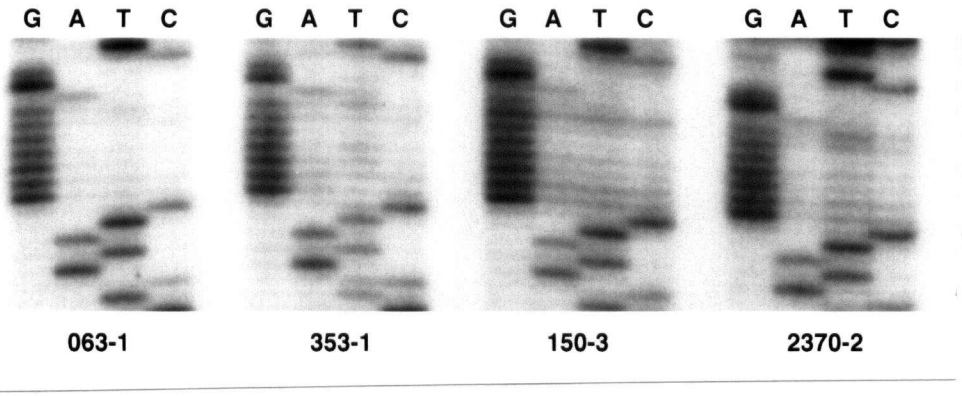


Figure 14. ACTG 095 ACV^S mutational screen. the figure depicts DNA sequences screening of the gene region of the 7 guanosines in plaque isolates derived from ACTG 095 which were followed by the expected ACV^S reactivations. Of 3 isolates screened, plaque isolate 89-650-5 also has the identical guanosine insertion within that stretch as was demonstrated in 1737-14 and 1106-2.

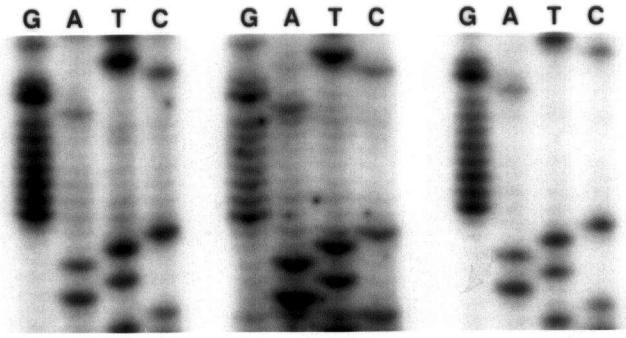
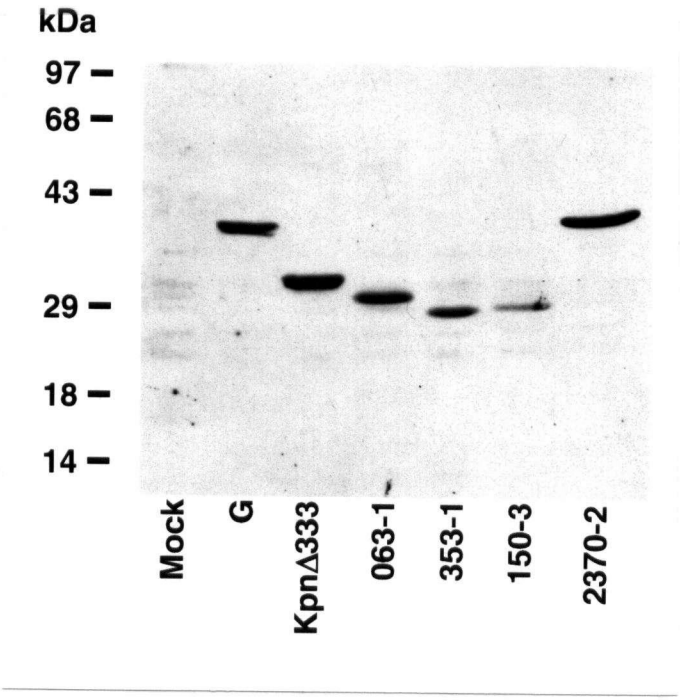


Figure 15. Western blot analysis of ACTG 095 ACV^R plaque isolates. The figure depicts proteins produced by plaque isolates derived from clinical isolates which were ACV^R and spontaneously reactivated. Reference TK^{WT} strain G and plaque isolate 2370-2 demonstrate full length 40 kd proteins. Plaque isolate 90-150-3 produces a truncated 28 kd product identical to 1737-14 and 1106-2. Reference TK deletion mutant KpnΔ333 demonstrates a truncated product of approximately 35 kd. Plaque isolate 89-353-1 demonstrates a truncated product identical in size to the 28 kd product associated with the guanosine insertion described while plaque isolate 89-063-1 demonstrates a slightly larger truncated product of approximately 32 kd.



well as it parent clinical isolate 2370, despite testing ACV^R by susceptibility testing and having low TK uptake by ^{125} IVaraU, demonstrated most plaques to have an almost uniform population of plaques exhibiting a high level of TK activity by both ^{125} IdC and ^{14} C TdR (Figures 16a. and 16b.). This suggested that the basis of its ACV resistance was due to mechanisms other than TK deficiency.

3.4.2.2. Isolates followed by an ACV^S reactivation.

Western blot analysis of plaque isolates from ACV^R clinical isolates sourced from ACTG 095 which were followed by the expected ACV^S reactivation also confirmed a 28 kd truncated TK product identical to 1737-14 and 1106-2 for 89-650-5. Of other isolates, 89-390-4 produced a full length 40 kd protein while 90-110-4 did not produce a detectable product (Figure 17.). Plaque autoradiography using ^{125}IdC performed on these plaque isolates demonstrated 89-650-5 to be a homogeneous TK^D population. Also of interest was plaque isolate 89-390-4 which also produced a full length 40 kd product and which, by plaque autoradiography was shown to consist of plaques with a faint rim, suggestive of a TK^{LP} phenotype (Figures. 16a and 16b.). Further characterization of both 2370-2 and 89-390-4 is ongoing.

Figure 16a. ACTG 095 plaque isolate ^{125}IdC plaque autoradiography. The figure depicts ^{125}IdC plaque autoradiography of plaque isolates sourced from clinical isolates from ACTG 095. Plaque isolate 89-650-5 is uniformly TK^D while 90-150-3 is 89% TK^D. Also of note is the almost uniform marked TK activity of plaques within 2370 and 2370-2. The barely perceptible faint rims produced by 89-390-4 are not evident on the photograph below but are visible by direct inspection against a light box

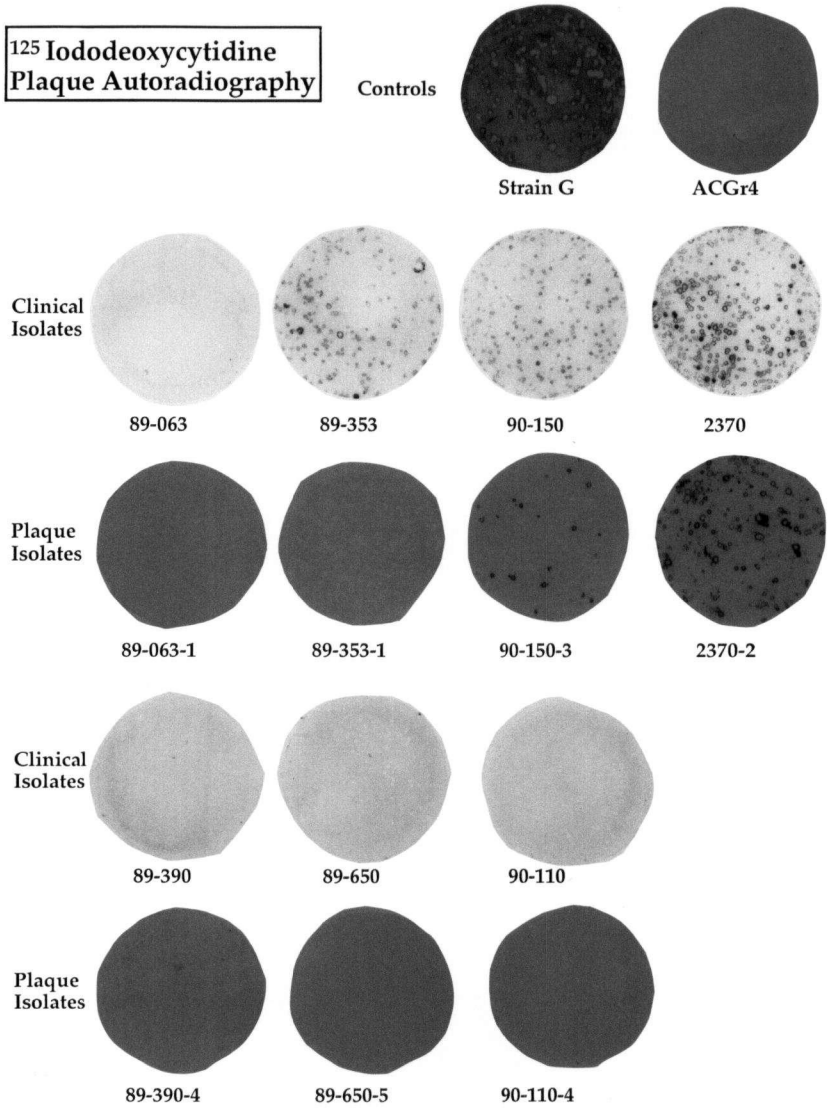


Figure 16b. ACTG 095 plaque isolate ^{14}C TdR plaque autoradiography. The figure depicts ^{14}C TdR plaque autoradiography of plaque isolates sourced from clinical isolates from ACTG 095. Plaque isolate 89-650-5 again is uniformly TK^D while 90-150-3 is mostly TK^D. Again the marked TK activity of plaques within 2370 and 2370-2 is apparent.

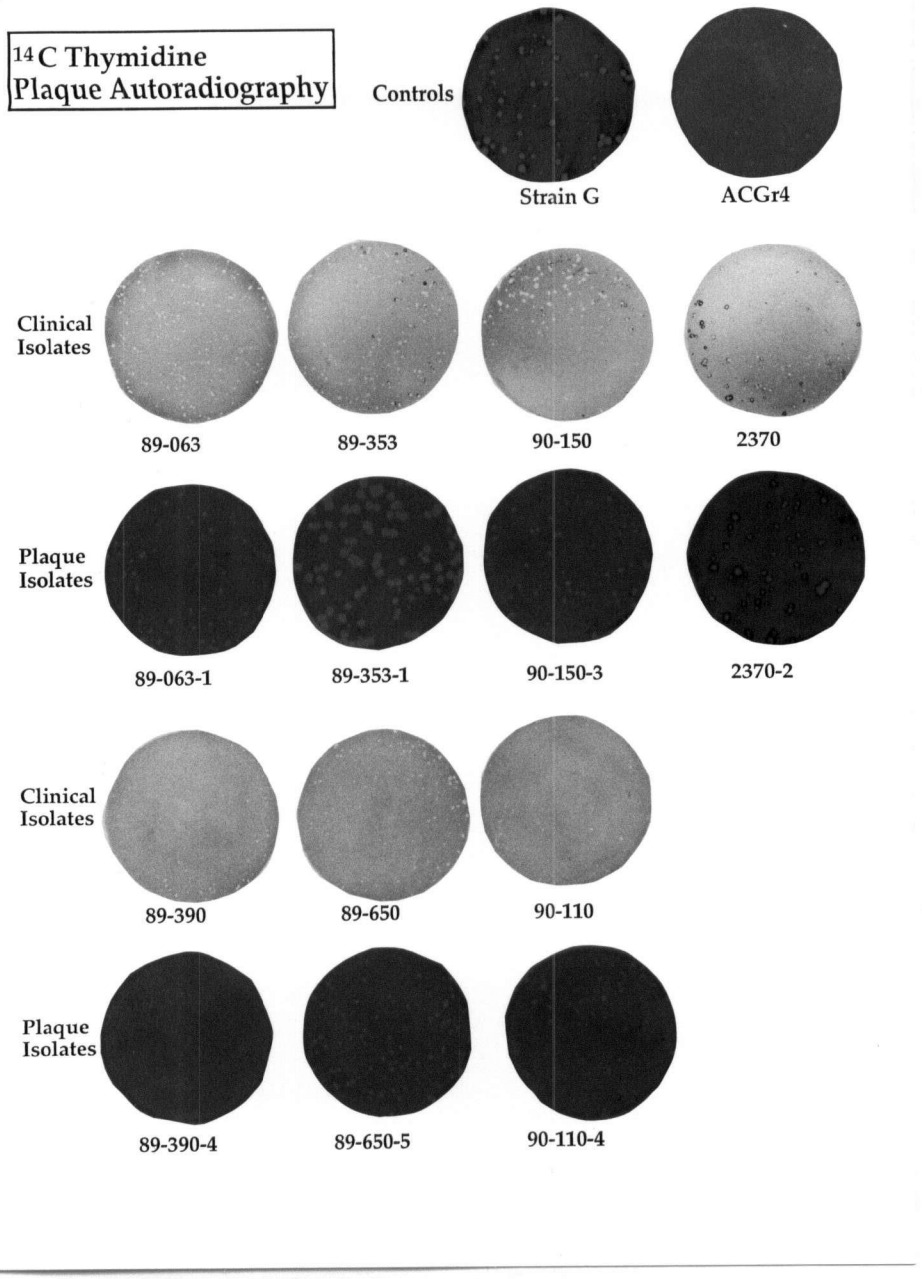
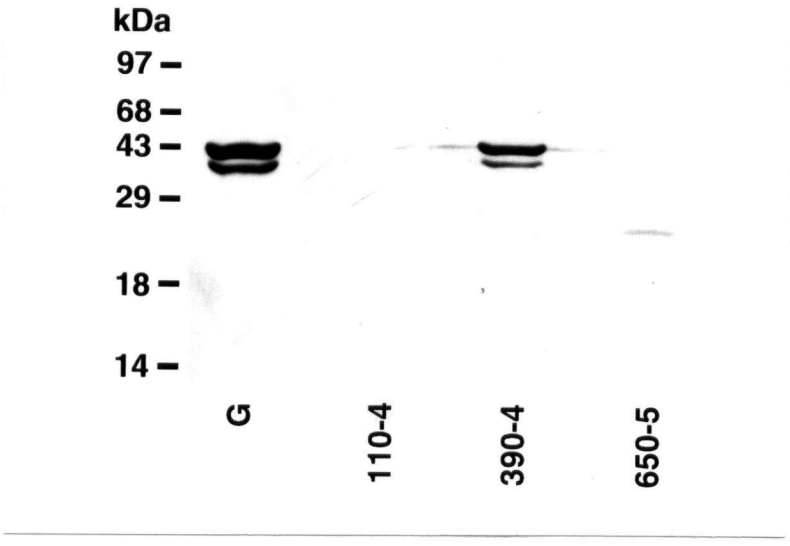


Figure 17. Western blot analysis of ACTG 095 ACV^S plaque isolates. The figure depicts proteins produced by plaque isolates derived from clinical isolates which were ACV^R and which were followed by the expected ACV^S reactivations. Plaque isolate 89-390-4 produces a full length 40 kd protein identical to reference TK^{WT} strain G. Plaque isolate 89-650-5 produces an identical 28 kd protein as was demonstrated in 1737-14 and 1106-2 while plaque isolate 90-110-4 did not produce a detectable protein product.



CHAPTER 4

DISCUSSION

4.1 MECHANISMS OF REACTIVATION OF TK^D ISOLATES .

The original aim of this work was to define the mechanisms underlying the apparent paradoxical behaviour of a clinical isolate which was TK^D but reactivated. At the outset our working hypothesis was that the reactivation potential of such isolates was due to a fundamental phenotypic difference from other TK^D isolates. Subsequent work, as detailed above, disproved this. Although reactivation by TK^D isolates had been previously noted (98), the work with isolate 1737, originating from patient WJ, is the first report which documents, fully characterizes, and suggests a mechanistic basis for the spontaneous clinical reactivation of a truly TK^D HSV isolate. Resistance was acquired during the treatment of previous episodes and then the TK^D isolate reactivated without selection pressure by continuing antiviral administration. Complete eradication of HSV from the affected area between episodes was well-documented, demonstrating that latency of the TK^D strain had been established. This was further supported by experimental data demonstrating the ability of the isolate to establish latency within mouse trigeminal ganglia. The reactivation of this isolate in the patient appeared paradoxical in the face of current dogma suggesting that TK^D HSV should not be able to reactivate. This isolate was homogeneously TK^D by all conventional tests including plaque isolation and plaque autoradiography. Although TK uptake studies may have suggested a low level of activity, this assay has proved insensitive in discriminating between low and absent activity and its true phenotype is much more accurately defined by the

homogeneously TK^D plaques shown by plaque autoradiography as well as the failure to produce full length product by protein studies. Despite this apparent absence of TK activity, inoculation of the TK^D isolate into a mouse model by either intranasal or intracerebral routes was able to both result in encephalitis with a pure TK^{WT} population as well as establish trigeminal ganglia latency with a mixed populations of both TK^D and TK^{WT} strains. In addition, the intracerebral neurovirulence model was able to demonstrate 1737 to harbor an additional population suggestive of a TK^{LP} phenotype, suggesting yet another possible mechanism of furnishing TK activity.

Further work characterizing clinical isolate 89-063 also identified it as a reactivated truly TK^D isolate by all conventional means. Mouse intracerebral inoculation of this isolate also extracted virus bearing a TK^{LP} phenotype but, on this occasion, without a demonstrable TK^{WT} population. The nature of the TK^{LP} phenotype is very poorly understood at present; such isolates may constitutively express low levels of TK or, alternatively, ribosomal frameshifting may play a role in their generation. Whether only the intracerebral model is capable of teasing out TK^{LP} subpopulations is uncertain at present. Regardless of the underlying mechanism, it is very likely that the TK activity demonstrated in the neurovirulence studies also enabled these isolates to reactivate in the clinical setting and further enhances the principle that TK activity is required for reactivation of HSV. The intracerebral model, given its ability to detect TK activity in nearly all inoculated mice, clearly proved itself to be a more sensitive assay in the detection of low levels of TK activity than the intranasal model.

It has been previously documented that the TK activity of a clinical isolate correlates well with the proportion of TK^{WT} virus within a mixed

population (97) and that mutants with low levels of TK activity can reactivate from latency (72). The above work suggests that very sensitive assays challenge old phenotypes and demonstrate that the levels of TK required for reactivation are quantitatively much lower than previously appreciated. Indeed, these data demonstrate that TK activity may fall below the threshold of conventional assays and, in this case, TK activity was amplified using passage in the animal model. Herein we have designated such strains as "ultra-low" producers. This is the first clinical demonstration that 'ultra low' level thymidine kinase expression, predicated on masked heterogeneity, is sufficient for reactivation of clinically-significant ACV^R disease. The distinction between isolates with very low levels of TK as opposed to absolute deficiency remains confused and we define the latter as being a pure TK^D population. Our data suggest that the TK activity of isolates represent a continuum, based on the proportion of TK^{WT} virus, with the threshold for reactivation yet to be determined and hence, such pure TK^D populations may, indeed, be unachievable from clinical isolates.

There are two possible explanations for the emergence of TK^{WT} virus within apparently pure populations; either a TK^{WT} population, present in such low numbers as to be undetectable by conventional means, became unmasked due to the exquisite sensitivity of the animal model in amplifying such small numbers of preexisting TK^{WT} virus or, alternatively, *in vivo* reversion to TK^{WT} took place from a pure TK^D population during passage in the animal model. In either case, the resulting TK^{WT} virus would have a virulence advantage and would explain the recrudescence of the neurovirulent phenotype. The frequency of reversion of the thymidine kinase gene has been estimated at 10^{-4} to 10^{-6}, (87) although mutational hot

spots with much higher frequencies may exist within the viral genome and a later discussion regarding the genetic lesion in this isolate will further address this issue. Which of the two mechanisms operated in these cases is still unresolved and it would require further plaque purifications followed by reintroduction into an animal model to determine whether TK^{WT} emergence could be reduced, thereby suggesting the washout of a preexisting population. In addition, at present the relative contributions of TK^{WT} and TK^{LP} subpopulations to TK activity and therefore reactivation is also unresolved. Regardless of the underlying mechanisms, if one extrapolates from the experience of the animal model, the demonstrated TK heterogeneity suggests that reactivation of both 1737 and 89-063 was due to small subpopulations with intrinsic TK activity within a larger TK^{D} population and that such subpopulations were able to impart their phenotypic TK expression onto the larger TK^{D} population and enable it to reactivate; this may be another example of *in vivo* complementation. This is yet another example whereby a larger virus population can widen the repertoire of its phenotypic expression by utilizing those of a much smaller subpopulation. The virus is thus able to retain ACV resistance while extending its phenotypic expression to include reactivation potential, such a strategy offering a survival advantage in the face of antiviral pressure.

Further work on clinical isolates sourced from ACTG 095 which also reactivated as ACV^{R} showed that 3 of 4 reactivated isolates, (89-353, 90-150 and 2370) harbored subpopulations of virus with the TK^{WT} phenotype demonstrated simply by performing plaque autoradiography and without the need to resort to very sensitive mouse neurovirulence studies as was done for 1737 and 89-063. Characterization of the pre-reactivation ACV^{R} clinical

isolates from these 3 patients demonstrated an increase in the number of TK^{WT} plaques from the initial pretreatment ACV^{R} isolate to the corresponding ACV^{R} isolate which reactivated, showing that, in cases where TK activity is demonstrable within reactivated isolates by conventional means, the ability to reactivate is associated with an emergence of the TK^{WT} population and a corresponding increase in TK activity. In any case, these experiments conclusively demonstrate that supposed TK^{D} clinical isolates which reactivate retain some TK activity, further enhancing the principle that TK activity is essential for reactivation. It also demonstrates that clinical isolates can selectively retain some phenotypic expression of thymidine kinase activity, thereby retaining the characteristics of reactivation and neurovirulence, while still testing as ACV^{R} in susceptibility assays, . This is the first demonstration that there can be a dissociation between these characteristics.

The ability of a TK^{WT} subpopulation to impart its characteristics onto a larger TK^{D} population to enable it a wider phenotypic expression by reactivating suggests *in vivo* complementation. There have been multiple prior reported examples of such interactions within HSV viral populations. Viral heterogeneity with possible complementation has been previously demonstrated to be an important component of clinical disease progression within ACV^{R} clinical isolates bearing both the TK^{D} and *pol* phenotypes (54,96). In addition, evidence for *in vivo* complementation has been documented in pairs of temperature sensitive mutants (90,91), neuroinvasive mutants (93) and between pairs of thymidine kinase strains, both TK^{WT}/TK^{D} and TK^{D}/TK^{D} (63,67,81). There are three possible mechanisms by which complementation may occur. First, there may be genetic recombination and at

least some of the above mentioned complementations were shown to be due to this mechanism, although there were many complementation pairs where no such evidence could be detected, suggesting the possibility of other mechanisms (90,91,93). Secondly, as the thymidine kinase gene exists as a homodimer, it has been proposed that a heterodimer may form where a TK^{WT} polypeptide may in some way combine with a TK peptide lacking activity, thereby compensating for its deficient activity (81). Finally, local exchange of phosphorylated nucleotide pools may occur, such that TK^{WT} virions may phosphorylate nucleosides which, due to their small size may enter cells infected with TK^{D} virions, bypassing the need for TK activity. The contribution of theses 3 putative mechanisms is at present unclear.

Clinical isolate 89-063 was uniquely interesting in that, even using an animal model, a phenotypically TK^{WT} subpopulation could not be extracted. Rather, it yielded a population of viruses consistent with a TK^{LP} phenotype on plaque autoradiography This phenotype would also test as ACV^{R} on susceptibility assays and produce minimal TK activity. Arbitrarily, such isolates have been defined as expressing 1-15% TK activity based on their thymidine phosphorylating ability (56). Again this data strengthens the principle that TK activity, even if in only small amounts, is essential for reactivation but suggest a second mechanism by which the virus could generate it.

The issue of whether thymidine kinase activity can be dissociated from neurovirulence has also been recently debated. There have been 2 reports of neurovirulent TK^{D} HSV isolates which have supposedly retained neurovirulence. Chatis and Crumpacker reported a plaque-purified TK^{D} HSV isolate from an AIDS patient which, due to a single base substitution,

produced a full length protein and retained full neurovirulence (98,100). Plaque autoradiography in those studies, however, was conducted in a TK-producing cell line using a ^{14}C-thymidine overlay, potentially reducing assay sensitivity in demonstrating heterogeneity or a TK-low producer phenotype. In addition, the combination of a full length TK polypeptide and a base substitution which is not in any of the three described conserved binding domains, calls into question the significance of this mutation. The second report by Tanaka et al. (101) performed only a single plaque purification and omitted plaque autoradiography altogether. In addition, although the identified TK mutation was close to the putative nucleoside binding site, again it was not within it and this was not corroborated with protein studies. These discrepancies raise the possibility that masked heterogeneity, as described in our report, or a low TK producer phenotype may have provided sufficient TK activity for neurovirulence while testing as TK^D. Contrary to this, the isolates from the above work demonstrated neurovirulence to be directly linked to TK activity, a finding which supports conventional wisdom. It thus seems likely that TK activity in the above isolates may have been left sufficiently intact to allow for neurovirulence and the proof of a dissociation between neurovirulence and TK activity has not yet been convincingly proven.

The clinician will need to appreciate that effective clinical and virological eradication of mucocutaneous viral shedding through foscarnet (or alternative) therapy, followed by no antiviral treatment whatsoever, may still eventuate in a subsequent TK^D HSV reactivation episode. Accordingly, the use of ACV may, in some cases, fail even for treatment of a new and unchallenged recurrence. Despite the documentation of TK^D recurrences,

they are rare and TK^{WT} activity prevailed in the vast majority of untreated reactivations. One possible explanation for this is that clones expressing TK are more efficient reactivators. That withdrawal of ACV treatment pressure in this patient eventually led to an TK^{WT} reactivation further supports this hypothesis. Alternatively, TK^{WT} reactivations may have originated from different latently-infected ganglionic sites altogether which had not been subjected to the development of ACV resistance. Regardless of the underlying mechanism, for patients with refractory disease, this reversion of herpes simplex virus over time to an ACV^S population may provide hope for a useful clinical alternative, such as alternating antiviral interventions.

4.2 TK^D MUTATIONS AND REACTIVATION.

The second aim of this work was to identify whether specific genotypic lesions were associated with reactivation potential. Initially, work was directed at identification of the mutation in the TK gene of the original reactivated TK^D HSV 2 isolate, 1737, and identification of its TK product. The mutation in 1737 was identified as a guanosine insertion in a stretch of 7 guanosine bases. The effects of this mutation would be to alter every amino acid in the nucleoside-binding site, and to introduce a premature stop codon which would eliminate the combined ATP/nucleoside-binding site (Figs. 10a and 10b). Such a protein should theoretically be devoid of TK activity.

Given that this was the longest homopolymer stretch of bases in the gene and that the gene is particularly G-C rich, it is likely purely by chance, that such a long stretch would be composed of G/C pairs. Alternatively, it would be tempting to speculate that ACV, by being a guanosine analogue, may cause the viral DNA polymerase to selectively slip or stutter in a region

containing a stretch of guanosines and induce such a mutation. ACV is an obligate chain terminator of viral DNA, hence any such mutational influence it exerted would have to influence viral DNA polymerase without incorporation. Such mechanisms remain intellectually attractive but speculative at this stage.

Although only a single plaque isolate from 1737 was sequenced, the clinical isolate was uniformly TK^D by plaque autoradiography and another plaque isolate originating from an independent prior outbreak in the same patient whose parent clinical isolate was also uniformly TK^D by plaque autoradiography, contained the same mutation. This suggests that the guanosine insertion was a homogeneous mutation. Reports of mutations in the TK gene of other TK^D HSV isolates is very limited. It has been well documented that the majority of TK^D HSV 1 mutants produced truncated TK proteins, suggesting that frameshift mutations are common in this setting, although this early work was not correlated with DNA sequencing of the TK gene (83). Of available genotypic data, Kit et al. (102) has previously described a deletion mutation in the same string of guanosines in a laboratory-derived 5'-bromodeoxyuridine (BUdR)-resistant HSV 2 isolate and Hwang et al. (103) have described the same G addition as 1737-14 in a plaque-purified TK^D HSV 1 clinical isolate. In addition, screening of ACTG 095-derived plaque isolates has identified the same G insertion in 1 of 4 other reactivated ACV^R HSV 2 plaque isolates and 1 of 3 ACV^R HSV 2 clinical isolates which were followed by an ACV^S reactivation. These data identify this stretch of 7 G's as a mutational hot spot for the HSV TK gene and are in marked distinction to the varicella-zoster *tk* gene where mutations are widely distributed throughout the gene (104)

Homopolymer stretches have been shown to be particularly susceptible to frameshift mutation in other biological systems. Consecutive runs of single nucleotides have been shown to also be mutational hotspots for the lysozyme and rII genes in T4 bacteriophage (105,106), the T antigen gene in polyoma virus (107), as well as for eucaryotic cells such as mouse immunoglobulin heavy chain locus (108). This suggests homopolymer nucleotide stretches as general hot spot mutational mechanisms distributed throughout diverse biological systems. Such hot spots can be susceptible to both spontaneous and mutagen-induced mutations and the likelihood of mutation is directly related to the number of bases within the homopolymer stretch. The putative mechanistic model is of local misalignment of base pairs within homopolymer stretches which provide frequent sites for misaligned but complementary base pairing (105). Such hot spots resulting in frameshift mutations may not only occur in reiterations of single base pairs but also within more complex repeats, raising the possibility of such sequences also operating within HSV TK (109,110).

As well as forward mutation, homopolymer base stretches have also been shown to have an increased reversion frequency to wild type (111). This may offer a putative explanation for the emergence of heterogeneity within a seemingly pure population as a high rate of reversion at such hot spots may potentially generate TK^{WT} subpopulations producing sufficient TK activity to allow reactivation in animal models and in the clinical situation as described in the initial characterization of 1737. An alternative potential explanation has been reported by Hwang et al. (103). They have demonstrated an *in vitro* net +1 translational frameshifting by an HSV isolate harboring the same guanosine insertion described above; previously a phenomenon only

reported in RNA viruses. Whereas such strains produce a truncated product in the majority of translations, occasional frameshifts may produce full length protein in 2% of translations. We have already demonstrated that TK activity required for reactivation is very small and such mechanisms may allow production of a full length TK protein in sufficient quantity to allow reactivation. The frequency of such frameshifts are homopolymer length-dependent (Dr. Don Coen, personal communication), again suggesting that the string of 7 guanosines is the most likely site of such events. Such low level TK production may explain the neuropathogenic faint-rimmed plaques seen in output strains from mice inoculated with 89-063 and may play a role in the genesis of the TK^{LP} phenotype in at least some isolates. The relative contributions of the 2 genetic mechanisms is unclear. The demonstration of TK^{WT} heterogeneity within the majority of TK^{D} isolates suggests that such subpopulations are important in most instances. In these cases a high reversion frequency at such homopolymer sites may be crucial to generate such subpopulations. In the case of 89-063, however, where no such subpopulations can be demonstrated but reactivated faint plaques are identified, frameshifting may well be the dominant mechanism. Frameshifting may also contribute to TK activity within homopolymer TK^{D} isolates already harboring TK^{WT} subpopulations, as suggested by 1737 where both TK^{WT} and TK^{LP} subpopulations can be identified. Only two reports of TK^{D} HSV TK gene sequences have documented single base substitutions (100,101). Interestingly, these isolates were the isolates described above with retained neurovirulence. By contrast, the Western blot results from the above work indicated truncated products in 5 of 8 isolates (1737-14, 90-150-3, 89-650-5, 89-063-1 and 89-353-1). Two isolates (2370-2 and 89-390-4) produced full length proteins while the remaining isolate (90-110-4) failed to produce a detectable

product. This, together with the literature cited above, suggests that frameshift mutations constitute the majority of TK^D isolates. Full sequencing of isolates not containing the G insertion is in progress to identify any other potential hot spots; specifically at other homopolymer sites.

4.3 CLINICAL IMPLICATIONS AND APPLICATIONS

These data have multiple implications for the management of patients. Firstly they reinforce the observation that, as well as within the context of selection of resistant subpopulations during antiviral therapy, that TK^D reactivations can occur in the absence of any therapy. Hence, the long held belief that, following successful virological sterilization of a TK^D lesion, the next reactivation would be ACV^S no longer applies and the clinician can no longer invariably rely on the efficacy of ACV. This may mandate the use of different management strategies such as alternating a TK-dependent antiviral, such as ACV, with one independent of TK, such as PFA.

In addition, the identification of a limited number of hot spots allows for the design of specific probes to detect common mutations, thereby allowing for easier detection of resistant isolates. Protein probes to detect truncated proteins are feasible and may be used to screen large populations of viruses within clinical specimens and are currently under development. The current proposed strategy is to produce one polyclonal antibody directed at the N-terminus of the TK protein which would detect all truncated proteins while another antibody directed at the C-terminus would only detect full length TK proteins. If the cell monolayer was grown and inoculated with the clinical isolate on a membrane and the proteins detected using a Western blot system, a subtraction film analogous to plaque autoradiography would allow

the detection of such truncated proteins within a large heterogeneous clinical population.

This work also has therapeutic implications. Homopolymer hot spots may also offer a novel mechanism of antiviral attack, perhaps by permitting targeting of mutational sites either to stabilize such homopolymer stretches or inactivate established mutations. One way this could conceivably be implemented would be via antisense strategies. These goals are further away from realization but may be attainable in the future. Further work to better define the frequency and distribution of such mutations is needed.

Finally, the strengthening of the principle that TK is required for reactivation increases the likelihood that strategies to neutralize TK may be useful in preventing reactivation altogether. Pharmacological TK inhibitors exist and are currently undergoing development. They will likely enter clinical trials in the not too distant future. Alternatively, TK inactivation could be achieved through a different mechanism; i.e. immunologically via an anti-TK vaccine which offer the prospect of long-lasting inhibition.

4.4 THE FUTURE

This work begun here will be extended. Firstly, the mutations conferring resistance on the remaining clinical isolates need to be identified to determine whether there are any other hot spots within the gene. The development of a protein probe to detect truncated proteins within entire populations of clinical isolates has already been discussed above. In addition, the detected mutations need to be repaired using site-directed mutagenesis and the resultant virus recharacterized to prove that the identified mutation is indeed responsible for the observed mutant phenotype. This work is

currently ongoing. The mechanism of the low TK producer needs to be identified; specifically the promoter region needs to be sequenced and mRNA detected by Northern blot to determine whether mutations within control elements outside the coding sequence are responsible for lower expression of TK. In addition, many more resistant isolates need to be sequenced to strengthen the data presented here and more fully describe the proportions of different type of mutations conferring the ACV^R phenotype. Integral to this would be a commitment to sequence a large bank of TK^{WT} isolates to help define whether base substitutions seen may be a phenomenon of strain variation or whether they may confer the ACV^R phenotype, given that such mutations have not yet been convincingly proven to do so. Finally, further plaque purifications of isolates 1737 and 89-063 need to be performed and reintroduced into animal neurovirulence models to discriminate whether the appearance of TK^{WT} output strains is due to small preexisting subpopulation or *in vivo* genetic reversion.

CHAPTER 5

CONCLUSION

This thesis set out to prove the hypothesis that a truly TK^D virus can reactivate and disproved it. The present work elucidated the mechanisms whereby a clinical isolate which tests as ACV^R by susceptibility testing and can even appear as a uniform TK^D population by conventional assays can defy existing dogma and reactivate. The work still supports the principle that TK activity is necessary for reactivation but defines ways in which HSV can circumvent such restrictions by genetic reversions at hot spots in the TK gene or ribosomal frameshifting such that TK activity is present. By doing so, the work also demonstrates that the level of TK activity required to reactivate is far lower than previously appreciated and defines the mouse neurovirulence model as a more sensitive assay for teasing out such low levels of TK activity in virus populations where conventional techniques fail to identify it and defines such isolates as having "ultra low" TK activity. Furthermore, the work demonstrates for the first time that the ACV^R phenotype can be dissociated from the ability to reactivate.

The other major finding of this work was to identify a homopolymer string of guanosines within the TK gene as a mutational hot spot. This may provide a mechanism for reactivation by allowing the generation of TK activity by reversion or frameshifting but it also provides the opportunity for the development of diagnostic tests for the detection of TK^D virus within clinical isolates. In the distant future such information may also ultimately allow for the development of antiviral strategies for TK^D virus populations. In many ways this work has fundamentally changed the ways in which we

think about the behaviour of these isolates and has opened up new diagnostic and therapeutic options.

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