

Latent Phenotype Analysis of Three Deletion Variants of Herpes Simplex Virus Type 1 (HSV-1) in Mouse Model

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Abstract

Latency analysis of three herpes simplex virus type 1 (HSV-1) strain 17 syn+ deletion variants (1704, 1705 and 1706) showed that they established, maintained and reactivated from latency. The kinetics of reactivation of 1705 and 1706 were similar to the parent HSV-1, 17 syn+, in which reactivation occurred 5-6 days post-explantation, but 1704 reactivated with delayed kinetics i.e. on the 12th day post-explantation. Since 1704 has deleted both copies of the latency associated transcripts (LATs) promoter region and one copy of the LAT coding region in internal inverted repeat sequence of long region (IRL), it was concluded that the LATs play a part in latency reactivation of 1704 from dorsal root ganglia (DRG) of spinal cord in mouse model. Restoration of the deleted sequences in the variant 1704 by marker rescue with the wild type BarnHI b fragment resulted in a wild type genotype. This virus was designated as 1704R. Latency studies of 1704R revealed that the rate and frequency of reactivation was intermediate between 17 syn+ and 1704, suggesting a secondary undetected mutation affecting latency phenotype. Isolation of 1704LP-, during the same marker rescue experiment in which both copies of promoter region of the LAT are deleted and reactivation of this virus from latency with delayed kinetics confirms that the LATs play a role in reactivation from latency (JPM 45:99, 1995).

Introduction

Like other members of *Herpesviridae* family, herpes simplex virus type-1 (HSV-1) frequently establishes latent infection in sensory neurons from which it reactivates periodically¹. In humans, invasion of the nervous system leads to latent infection in sensory ganglia and in rare circumstances to fatal encephalitis². Several studies have been carried out to define the role of specific viral genes in latency. Specific ribonucleic acids (RNAs) known as latency associated transcripts (LATs) are abundantly expressed during latent infection of the virus and the molecular basis of this phenomenon has been studied in several animal models and *scm*-positive human cadavers³⁻⁵. At least three transcripts, 2.0, 1.5 and 1.45 kb have been detected by Northern blot hybridization and have been finely mapped. These transcripts are diploid and transcribed complementary to immediate early 1 (IE1) gene transcripts^{6,7}. The proposed promoter region, which is located further upstream than usual at 686 bp from the 5' end of the LATs has proved to be a strong promoter both *in vivo* and *in vitro* and is neuro-specific^{8,9}. The role of LATs in the establishment and maintenance of latency is obscure, but there is evidence that it may affect viral reactivation^{8,10,11}. The variants 1704, 1705 and 1706 had been isolated from a transfection experiment of HSV-1 strain 17 syn+. The deoxyribonucleic acid (DNA) analysis of the isolates exhibited extensive variations in internal inverted repeat sequence of long region/unique sequence of long region (IRL/UL) of the genome. It appeared that they have been closely related to each other and could have arisen from a single progeny molecule, which thereafter underwent several rounds of rearrangement, to give rise to the deletion variants¹². The behaviour of HSV-1 strain 17 syn+ and the variant 1704 has been studied by Steiner et al¹¹ in trigeminal ganglia of mice during acute infection, latent infection and explant reactivation following primary infection of the eye. The variant 1704 replicated in the trigeminal ganglia of infected mice to the same extent as 17 syn+ and

established latency in almost all of the infected animals. However, following trigeminal ganglia explant reactivation kinetics were significantly delayed for 1704 relative to 17 syn+. HSV-1 LATs could not be detected with iZ04 either by Northern blot hybridization or by in situ hybridization, suggesting a role for LATs in reactivation from latency. From the precise sequence analysis of the end points of the deletion of the variants described by Junejo et al¹³ it is evident that in the variant 1704, both copies of the LAT promoter region and one copy of the LAT coding region in the IRL are deleted. In 1705 only the IRL copy of the LATs promoter region and LAT coding region is deleted. The variant 1706 has no deletion in the LAT gene. As a comprehensive analysis of latency we have established a mouse foot pad model system to investigate latency in DRG of the spinal cord in three weeks old BALB/c mice.

Materials and Methods

HSV-1 culture in vitro

Baby hamster kidney 21, clone 13 (BHK-21 C13) cells were propagated in 80 oz roller bottles containing 150 ml of ETC10 [Eagle's medium containing twice the normal concentration of vitamins and amino acids, 100 units/ml of penicillin, 100 µg/ml streptomycin, 0.02 µg/ml of amphotericin B, 0.002% (w/v) phenol red, 5% (v/v) tryptose phosphate broth and 10% (v/v) newborn calf serum] in the presence of 5% (v/v) CO₂ in air. Confluent BHK-21 C13 cells in roller bottle were infected with the viruses (HSV-1 strain 17 syn+, 1704, 1705 and 1706) at a multiplicity of infection (moi) of 0.003 plaque forming units (pfu)/cell and incubated at 31°C for 3-4 days or until extensive cytopathic effect (cpe) appeared. Titres of the virus stocks were determined on the cells and one step growth experiments were carried out as described by Brown and Harland⁴.

Animal inoculation and explant reactivation of DRG

Three weeks old BALB/c mice were anaesthetized with halothane and 0.025 ml of the appropriate virus dilution (approximate 10⁵-10⁷) in phosphate buffered saline (PBS)/calf serum was inoculated into the left foot pad. The virus stocks were always titrated on BHK-21 C13 cells on the day of inoculation to determine the precise quantity of virus inoculated. Mice were killed by deep chloroform anaesthesia, the dorsal lamina of the vertebral column was separated and the spinal cord was then removed. DRG were separated and removed after identification under the dissecting microscope:

Ganglia from the lower thoracic, six lumbar and two sacral vertebrae were dissected out. Each ganglion was placed separately in a round bottom micro-titre plate well containing ESC50 (Eagle's medium containing 50% foetal calf serum).

Preparation and isolation of ³²P labelled viral DNA in vivo

This is a modification of the method of Lonsdale¹⁵. Nearly confluent BHK-21 C13 cells propagated in PIC medium (Phosphate free Eagle's medium containing 1% calf serum) in Linbro wells were infected at a moi of 10 pfu/ cell and radio-labelled with 5 µCi (micro Curie) of ³²P-orthophosphate for 48 hr at 31°C. Cells were lysed by the addition of sodium dodecyl sulphate (SDS) at a final concentration of 2.5% (v/v) and DNA was extracted once with an equal volume of phenol, precipitated with two volumes of ethanol, dried at 37°C for 15 min and redissolved in sterile water 20 µl of DNA was used for appropriate restriction enzyme analysis and electrophoresed on an agarose gel (0.6-0.8%) for approximately 16 hr at 40-50 volts. Gels were air dried in a hot air oven at 80°C and auto-radiographed using Kodak Xomat S-100 film.

Transfection of virus DNA

Transfection of viral DNA was performed as described by Stow and Wilkie¹⁶. For marker rescue of the deletion in the variant 1704, the wild type fragments spanning the deletion was added to the transfection mix at 5, 10 and 20 molar excess with the intact deletion variant genome i.e if viral DNA is 1 %g then fragment spanning the deletion will be 5, 10 and 20 µg. 0.02-1 µg of viral DNA was mixed with 10 µg of calf thymus DNA as carrier in HEPES buffer (130 mM NaCl, 4.9 mM KCl, 1.6 mM Na₂HPO₄, 5.5 mM

D-glucose, 21 mM HEPES (N-[2-Hydroxyethyl] piperazine-N--[2-ethanesulfonic acid], pH 7.05) and calcium chloride was added to a final concentration of 130 mM. The mixture was allowed to stand at room temperature (20°C) until a fine precipitate developed and was added to 80% confluent monolayers of BHK-21 C13 cells in 50 mm petri dishes from which the medium had been drained. After 45 min of incubation cells were overlaid with ETC5 [Eagles medium containing tryptose phosphate broth 5% (v/v) and calf serum 5% (v/v)]. Four hours post-infection cells were treated with 25% (v/v) dimethyl sulfoxide (DMSO) in HEPES buffer for four min at room temperature (20°C). DMSO was removed gently by washing twice with ETC5 and cells were overlaid with ETC5 and incubated at 31°C for 3-4 days or until cpe appeared. The cells were harvested, sonicated and stored at -70°C. Transfected plate stocks were plated at limiting dilutions and single plaques were prepared for further restriction enzyme analysis of the genome.

Results

Latency analysis of the deletion variants, 1704, 1705 and 1706

Three weeks old BALB/c mice were inoculated separately in the left rear foot-pad with 17 syn+, 1704, 1705 and 1706 weeks post-inoculation nine DRG (last thoracic, six lumbar and two sacral) from the left side of the spinal cord were explanted and transferred to individual micro-titre plates containing ECS50. Screening for release of reactivated virus was carried out by transferring the culture from individual micro-titre wells to flat bottom micro-titre plate wells containing semi-confluent BHK-21 C13 cells and incubated at 37°C. Released virus was detected by the appearance of cpe in the indicator BHK-21 C13 cell. The results for 17 syn+, 1705 and 1706 show that at input doses of 1×10^5 pfu/mouse, 17 syn+ and 1705 infected ganglia started reactivating at five and six days post-explantation and by day ten, 50% of the ganglia from each group of animals had reactivated i.e. 28/54 ganglia for 17 syn+ and 27/54 for 1705 (Figure 1).

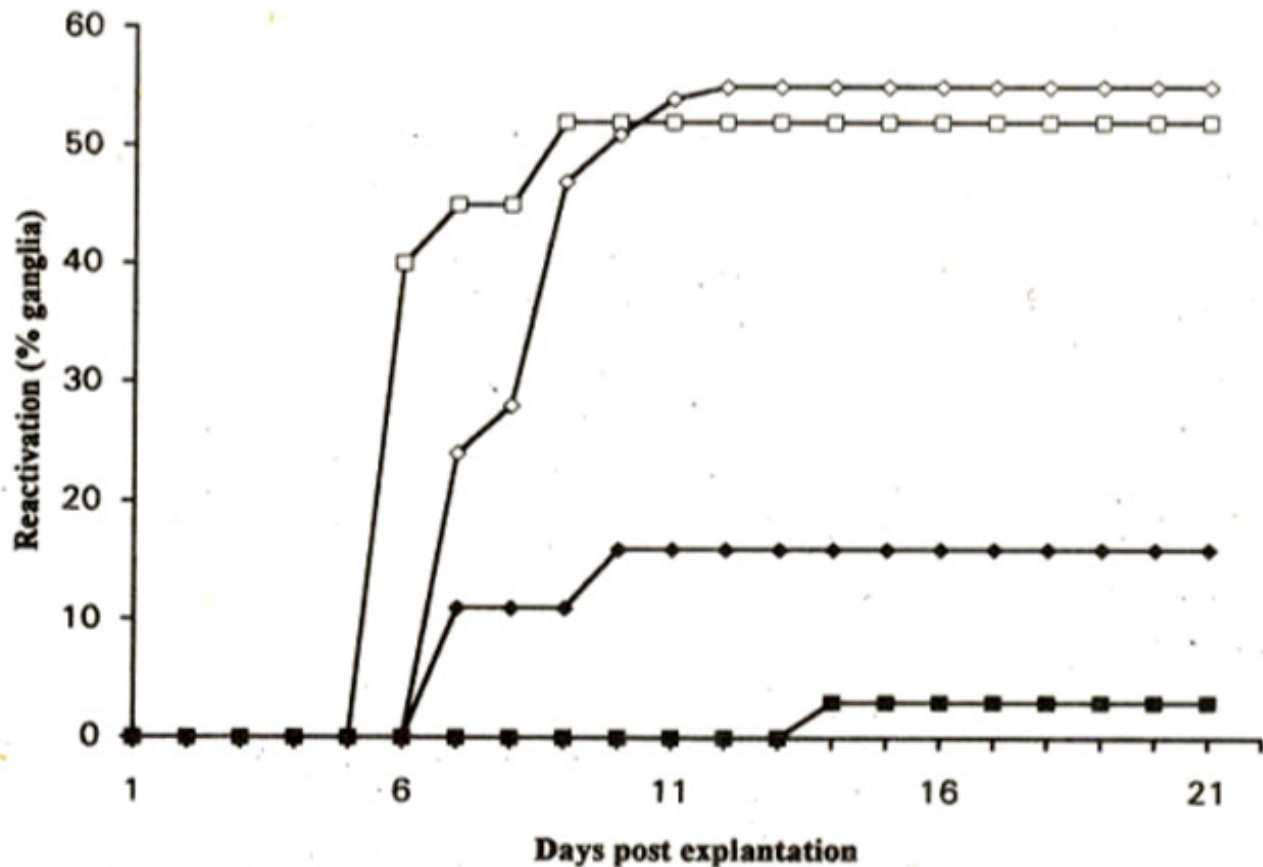


Figure 1. Explant reactivation time course of HSV-1 strain 17 syn+ (□), 1704 (■), 1705 (○) and 1706 (△). Three weeks old BALB/c mice were inoculated into the left rear footpad with 0.025 ml of each virus at a dose of 10^5 pfu/mouse. Data given as percentage of reactivation at each time point.

The ganglia from 1706 infected animals also started to reactivate at about the same time but by the tenth day post-explantation only 16% i.e. 6/36 ganglia were positive for virus release. With animals infected by 1704 at an input dose of 1×10^5 pfu/mouse there was no reactivation until day twelve and even then only 1/36 ganglia (3%) reactivated with no increase in number up to 22 days post explantation.

To determine whether this delay and slow reactivation frequency with 1704 was dose dependent, a group of animals were infected with an input dose of 1×10^7 pfu/mouse. The input dose of 17 syn+ could not be increased as all of the animals would have died. It can be seen that at 1×10^5 pfu input dose 1704 started reactivating on day 7 (4/36 ganglia), plateaued by day 12 and reached a final value of 30% (11/36 ganglia) by day 18 (Figure 2).

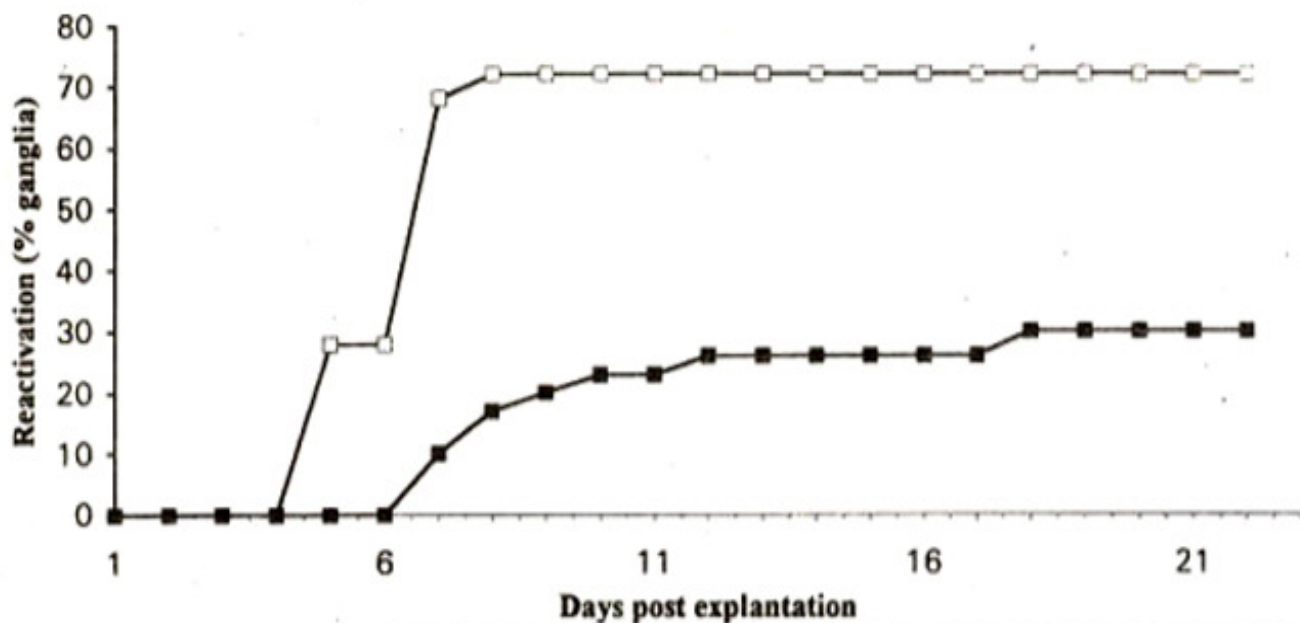


Figure 2. Explant reactivation time course of HSV-1 strain 17 syn+ (□) and 1704 (■). Three weeks old BALB/c mice were inoculated into the left rear footpad with 0.025 ml of virus at a dose of 10^5 pfu/mouse of 17 syn+ and 10^7 pfu/mouse of 1704. Data given as percentage of reactivation at each time point.

The 17 syn+ infected ganglia started reactivating on day five i.e. 5/18 ganglia positive and reached a final value of 72% of ganglia releasing virus (13/18 ganglia) by day 18. Genome analysis of reactivated 1704, 1705 and 1706. To determine if there was any change in the genomic structure of the variant 1704, 1705 and 1706 following inoculation in the mouse foot pad and reactivation from latency, a plaque stock was grown from a representative plaque of each variant, its DNA was radio-labelled with ^{32}P , extracted and subjected to restriction endonuclease digestion with HpaI and BamHI. The DNA profiles of the reactivated variants showed no apparent differences with restriction endonuclease analysis (Figure 3).

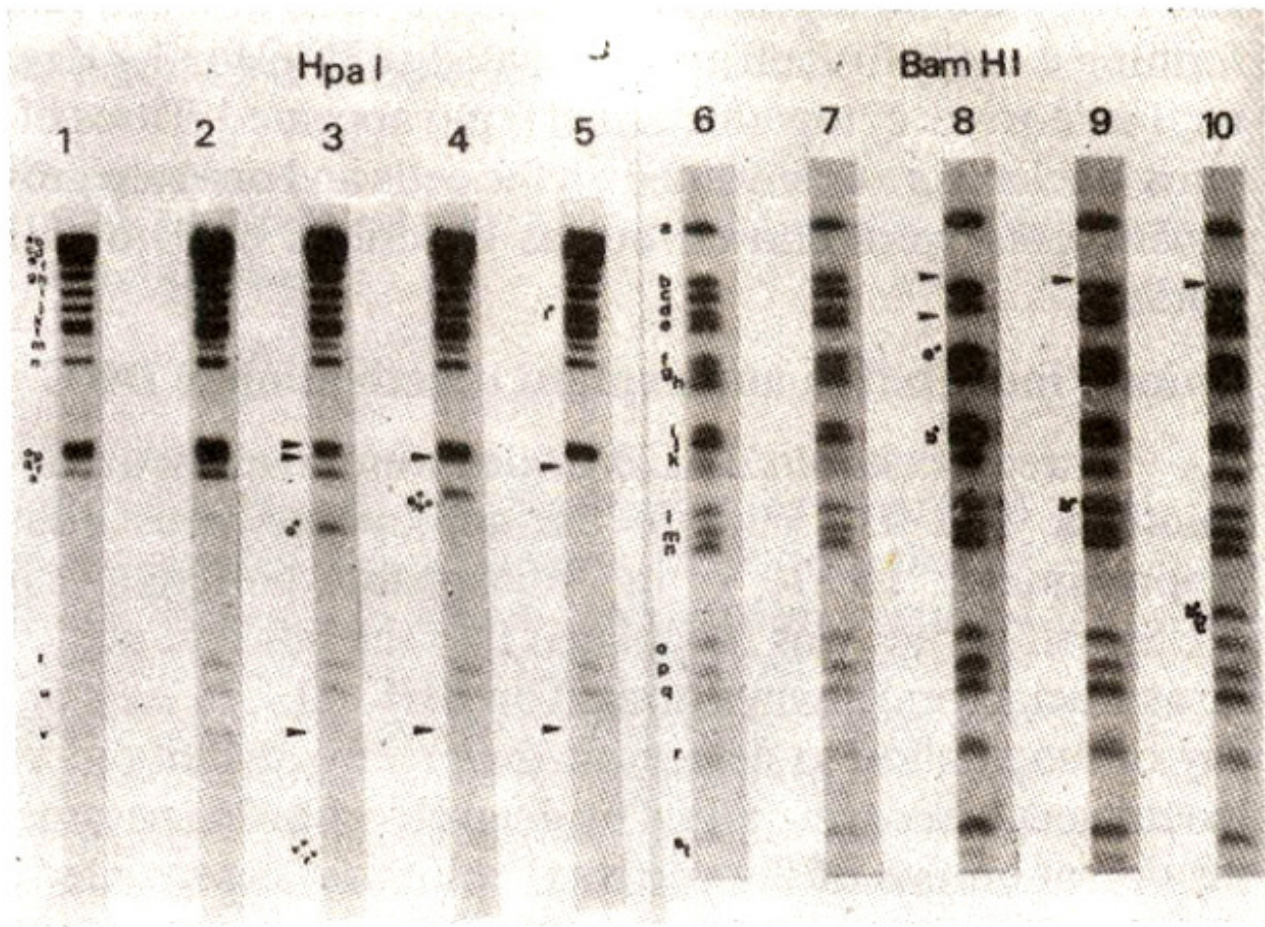


Figure 3. Autoradiograph of restriction enzyme digest of viral DNA, ^{32}P labelled *in vivo* of reactivated virus recovered from DRG of BALB/c mice. *HpaI* (lanes 1 to 5) and *BamHI* (lanes 6 to 10) of 17 syn+ (lanes 1 and 6), reactivated 17 syn+ (lanes 2 and 7), reactivated 1704 (lanes 3 and 8), reactivated 1705 (lanes 4 and 9) and reactivated 1706 (lanes 5 and 10). Letters refers to specific fragments, arrowheads indicate the position where the fragments are missing and stars indicate novel fragments.

Correction of the deletion in the variant 1704

To determine whether the phenotype of 1704 was entirely due to the deleted sequences, it was necessary to correct the deletions interterminal repeat sequence of long region (TRL) and IRL. It was also necessary to know whether the phenotype of the resultant recombinant was that of the parental virus. To do this, unit length 1704 genomes and the Bam HI b restriction endonuclease fragment spanning the deletion from the 17 syn+ genome were co-transfected onto BHK-21 C13 cells. The deletion in 1704 was shown to be within HpaI o in TRL and within JJpaI v and r in UL/IRL12. The Barn HI b fragment covers the HpaI s, r, v and m fragments (Figure 4).

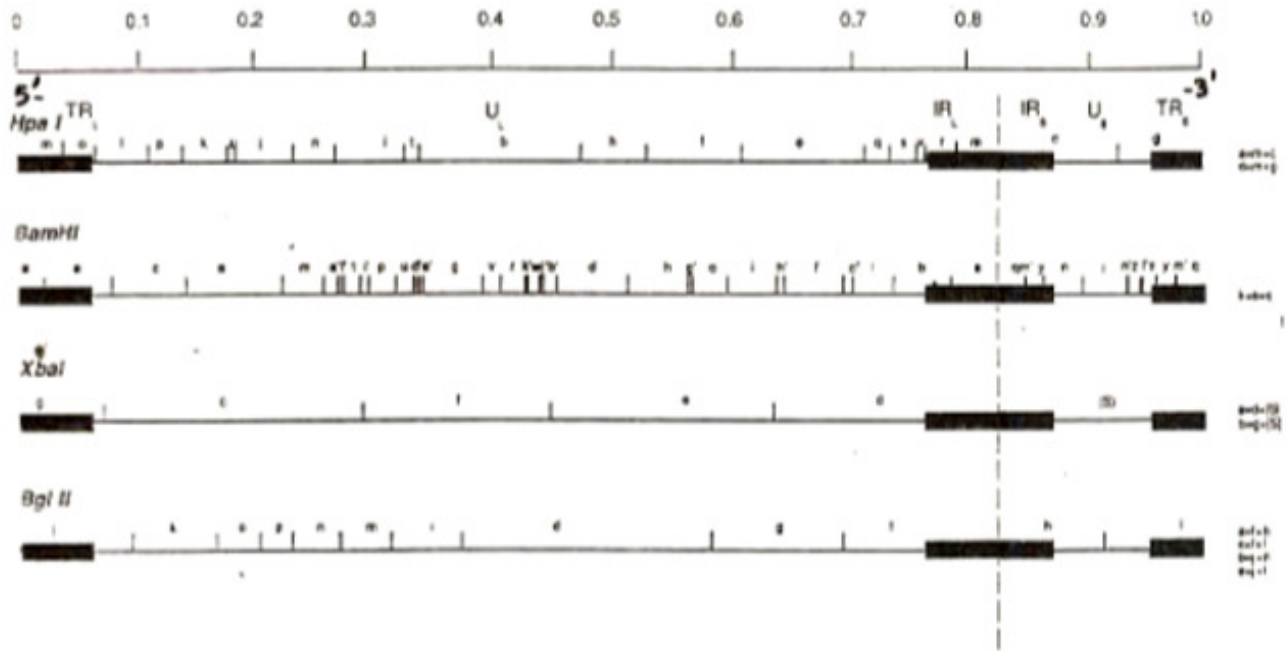


Figure 4. *HpaI*, *BamHI*, *XbaI* and *BglII* restriction enzyme map of DNA of HSV-1 strain 17 syn+. Top bar indicates scale in map units (0.1 map unit = ~15.2 kb). The genome is divided in U_L and U_S regions bounded by terminal repeat sequences, TR_L , IR_L , IR_S and TR_S (thick black bands). Long and short regions of the genome are divided by vertical dotted line. The restriction endonuclease fragments are given a specific name of alphabet (small letters) according to the size of the fragment i.e. largest fragment is represented by *a* and smallest by *z*. If there are more than 26 fragments they will represent *a'*, *b'* and so on. Intramolecular and intermolecular recombination events in the terminal repeat sequence can generate inversion of the U_L and/or U_S leading to four equimolar isomers. As a result of which restriction fragments may be the combination of smaller fragments from the repeat sequences, represented on the right end of each restriction profiles.

The selection of this particular fragment for marker rescue experiments was based on the assumption that if recombination occurs in IR_L the deletion would also be corrected in TR_L as the sequence at the termini of long region of the genome are homologous with opposite orientation. Seventy plaques were picked and the DNAs of 66 of them were analyzed with *BglII*. Recombination between *BamHI* b and intact 1704 was demonstrated on *BglII* digestion by the appearance of the f band in the wild type position, the return of the j band to its normal position and also the reappearance of fragment a ~7' +j) (Figure 5).

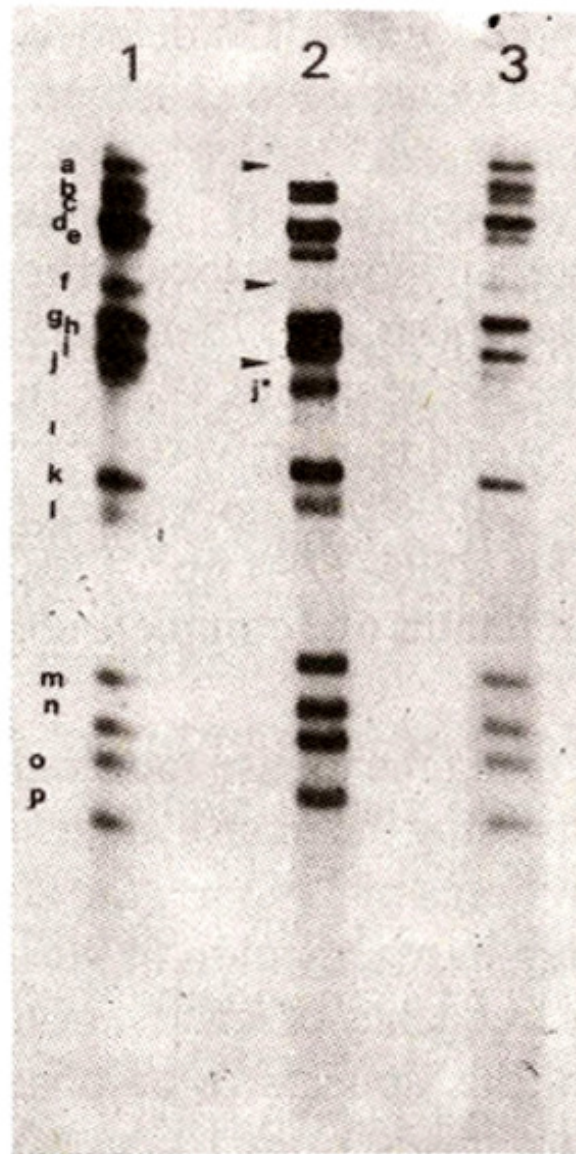


Figure 5. Autoradiograph of *Bg/II* digest of viral DNA ^{32}P labelled *in vivo* of 17 syn+ (lane 1), 1704 (lane 2) and 1704R (lane 3). Letters refer to specific fragments, arrowheads indicate the position where the fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.6% agarose gel.

The isolate showed a *Bg/II* profile identical to that of wild type 17 syn+, indicating that recombination correcting the deletion in 1704 had taken place. The recombinant was designated as 1704R. The gel electrophoresis of the DNA of the plaques digested with *Bam*HI and *Hpa*I confirms their structure. Digestion of 1704 DNA with *Bam*HI (Figure 6 panel A)

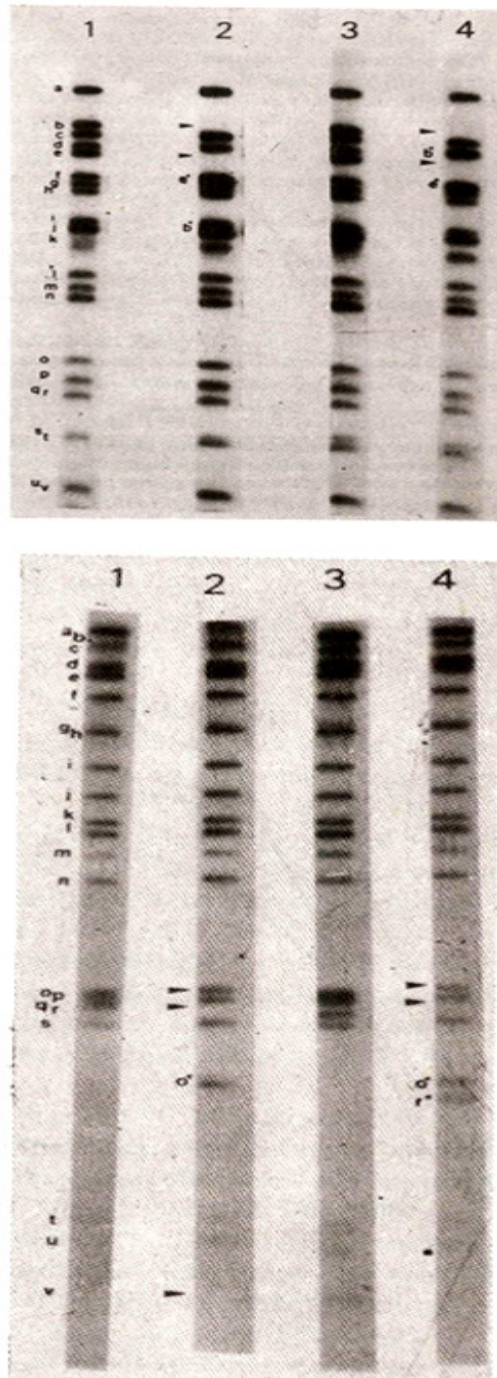


Figure 6. Autoradiograph of *Bam*HI (Panel A) and *Hpa*I (Panel B) digests of viral DNA ³²P labelled *in vivo* of 17 syn+ (lane 1), 1704 (lane 2) and 1704R (lane 3) and 1704LP- (lane 4). Letters refer to specific fragments, arrowheads indicate the position where the fragments are missing and stars indicate novel fragments. The DNA products were separated on a 0.8% agarose gel.

shows that the b fragment is reduced by 2.5×10^6 M and e fragment by 0.7×10^6 M. In 1704R the b fragment was electrophoresing at the wild type position as was the e fragment. On *Hpa*I digestion 1704R *Hpa*I v, r and o were all electrophoresing in the wild type position, indicating that the deletion both in UL/IRL and TRL had been corrected (Figure 6 panel B). During the process of screening for the isolation of 1704 rescuant, another variant designated as 1704LP- was isolated (Figure 6). In this variant, the deletion in the parent 1704 had been corrected fully except for the promoter region of LAT gene (Results not shown, manuscript in preparation).

Latency analysis of 1704R and 1704LP

Three weeks old BALB/c mice were inoculated separately via the left rearfoot pad with 10^5 pfu/mouse of 17 syn+, 1704, 1704R and 1704LP-. The results are shown in Figure 7.

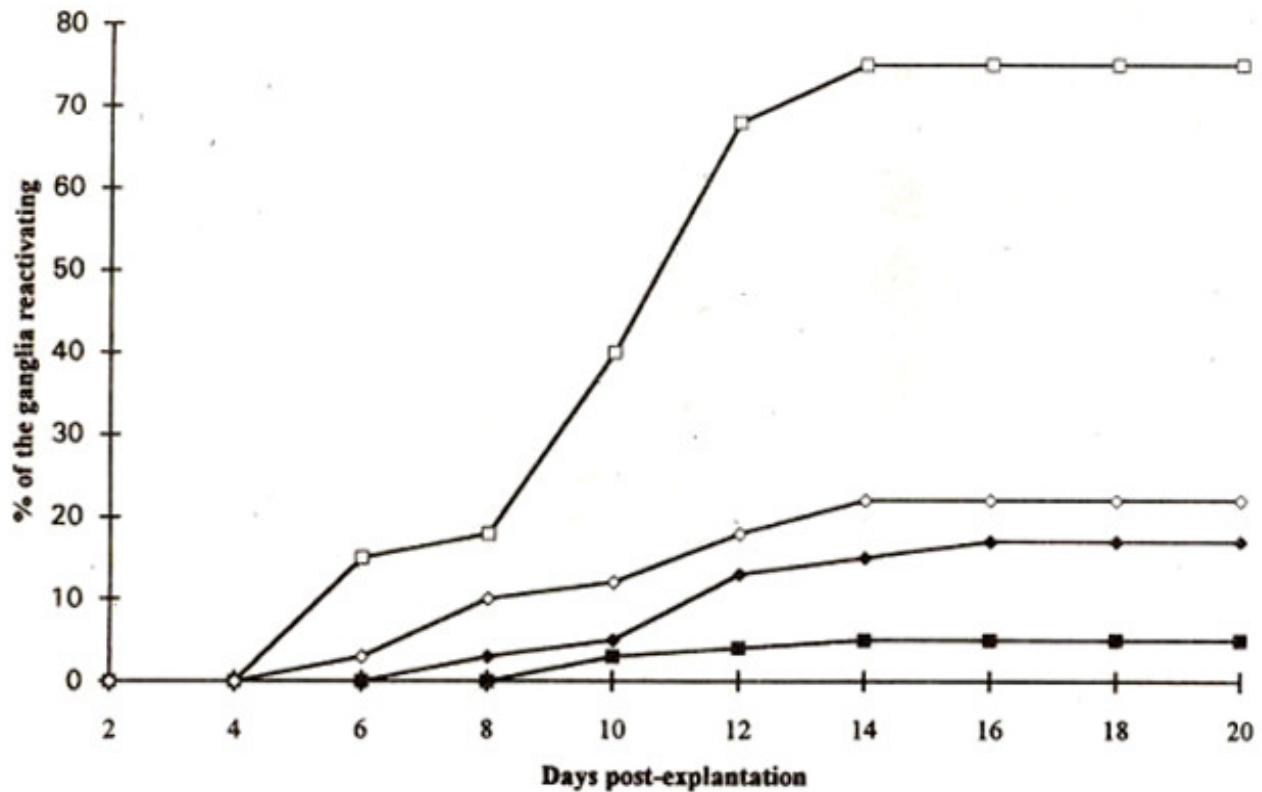


Figure 7. Explant reactivation time course of HSV-1 strain 17 syn+ ($\square$), 1704 ($\blacksquare$), 1704R ($\diamond$) and 1704LP- ($\blacklozenge$). Three weeks old BALB/c mice were inoculated into the left rear footpad with 0.025 ml of each virus at a dose of 10^5 pfu/mouse. Data given as percentage of reactivation at each time point.

Detection of 1704R on the sixth day post explantation demonstrated that the kinetics of reactivation of 1704R had returned to that of 17 syn+ suggesting that the sequences deleted in the 1704 confer the slow phenotype. However, the frequency of reactivation of 1704R remained poor indicating that this virus had not reverted fully to wild type behaviour. The kinetics of reactivation of 1704LP- on the other hand mimicked those of 1704. The variant 1704LP- fails to make detectable LATs by Northern blotting (N.W. Fraser, personal communication).

Growth properties of 1704R and 1704LP- in vitro

To see whether the replication efficiency of 1704R and 1704LP- is as competent as that of their parent, their growth pattern was analyzed in BHK-2 1 C13 cells over a 24 hr period at 37°C (Figure 8).

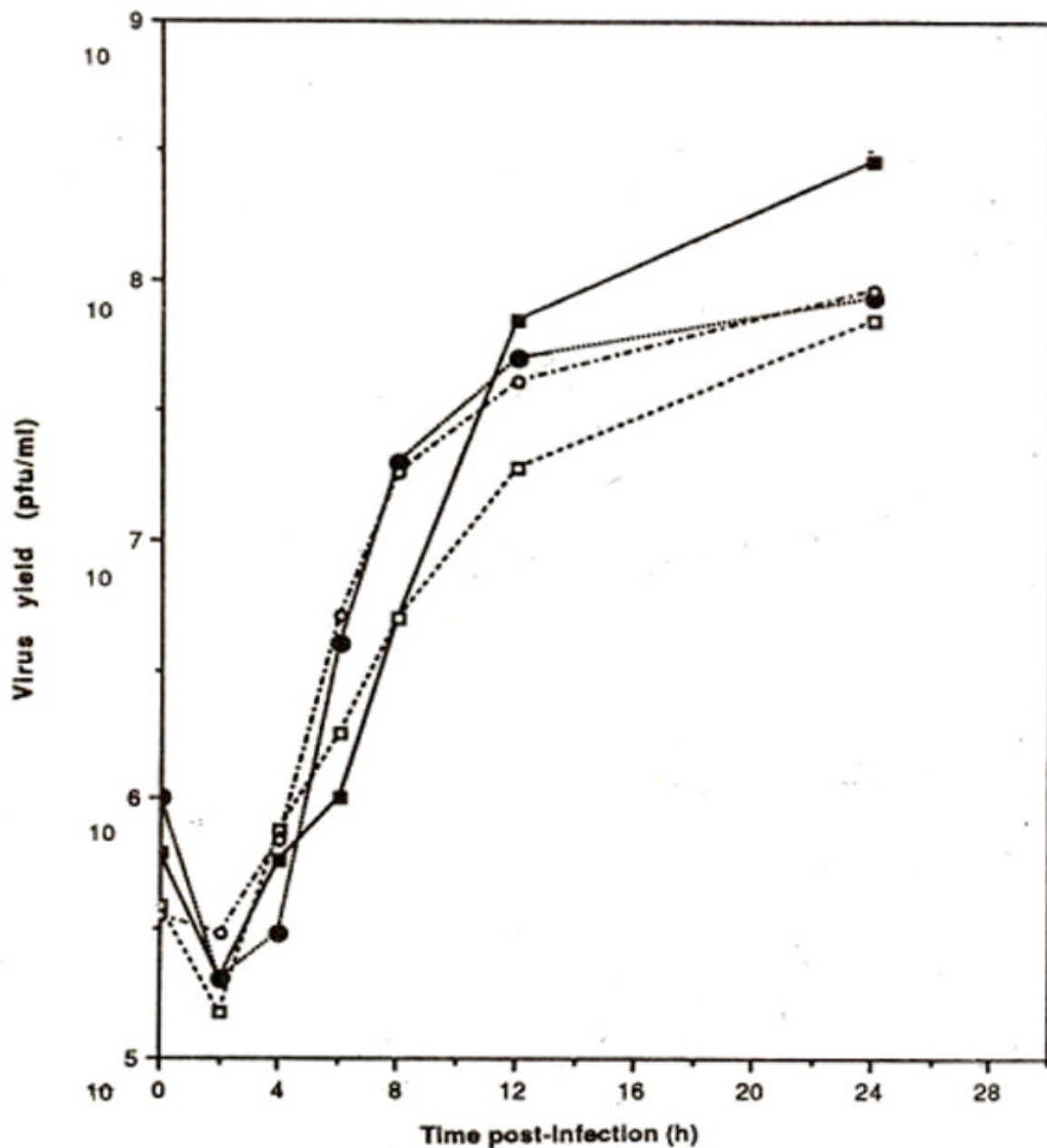


Figure 8. One step growth curves of HSV-1 strain 17 syn+ (■), 1704 (●), 1704R (□) and 1704LP- (○) in BHK-21 C13 cells. Cells were infected at a multiplicity of 5 pfu/cell, the monolayers washed twice with PBS/calf serum, overlaid with ETC10 and incubated at 37°C. Plates were harvested at 0, 2, 6, 8, 12 and 24 hours post infection and titrated as normal.

It has been previously shown that 1704 grows at a slightly slower rate than the wild type virus and produces a lower 24 hr yield¹². Although 1704LP- grows similarly to the parent, 1704R was slightly impaired in its rate of growth and gave a 24 hr yield similar to 1704.

Discussion

When Steiner et al¹¹ demonstrated that 1704 failed to make LATs as detected by Northern blotting and in situ hybridization in addition to reactivating slowly from latency, the precise extent of the deletion

with respect to the LATs and their promoter region had not been determined; the assumption was made that the absence of detectable LATs could be due to only the deletion affecting the transcripts or promoter region. Sequence analysis of 1704 has shown that in UL/IRL, 170 bp of UL56 is retained and the deletion did not effect IEL gene whose 3' end is at nucleotide position (np) 120882. The 5' end of the LATs is at np 119461 which means that 799 bp of the transcripts have been deleted¹³. Wechsler et al⁷ showed that the LAT promoter region was located between 662-940 bp upstream of its 5' end (np 118575-118775) and is therefore totally absent in the IRL region of 1704. In the TR1 the LAT transcripts whose 5-end is at np 6910 is not removed but the deletion between 7202-8144 np completely removes the promoter region, i.e. 7596-7796 np. 1704 has therefore no LAT promoters but retains one complete copy of LAT and 2/3 of other copy¹³. The finding using the mouse foot-pad model of latency confirms the results using the mouse model eye system in that at equivalent input doses of 17 syn+, the absence of LATs in 1704 causes a delay in reactivation in vitro. In addition the foot-pad model the frequency of reactivation was significantly reduced. However, this appears to be dose dependent. On increasing the input doses from 10⁵ to 10⁷ pfu/mouse, the kinetics of reactivation are akin to those of wild type. The percentage of ganglia reactivating (30%) is much higher than at an input dose of 10⁵ pfu/mouse (3%) but only about half the value of 17 syn+ infected ganglia reactivating. At an input dose of 10⁷ pfu/mouse compared to 10⁵ pfu/mouse it is assumed that either (1) latency is established in more neurons/ganglia or (2) more 'virus molecules/neurons establish latency or that (1) plus (2) pertain. Similar observations were found in HSV-I mutant in 1814 in which the percentage of LAT positive neurons increased with increasing dose of the virus¹⁷. Low neurovirulence and neuro invasiveness are also the main factors affecting virus reactivation from latency in vivo¹⁸. This would suggest that the process may be independent of the presence or absence of the LAT sparse but could be dependent on the gene dose on one or more other HSV genes. On the other hand if reactivation is dependent on a cellular factor initiating transcription and this process is facilitated by the LATs, although they are not an absolute requirement then the higher the number of genomes present, the greater chance of reactivation occurring and virus then becoming detectable. The rate of the reactivation of 17 syn+ in our set of experiments varies between 52% to 72%. Various factors affect the outcome of in vivo experiments including seasonal variation in animal house temperature and the resultant stress this produces in the animals. It has been shown that reactivation from latency and peripheral replication of virus is more efficient when the temperature of the animal house does not go below 70°F (L. Robertson, personal communication). Other factors such as age and route of inoculation have already been discussed^{19,20}. The isolation of 1704R, the rescuant with a rate of reactivation and frequency intermediate between that of 17 syn+ and 1704, would suggest that 1704R has a secondary undetected mutation precluding its reverting fully to wild type behaviour. Similar results have also been found with 1704R in the mouse eye model of explant reactivation (N.W. Fraser, personal communication). However, when 1704R was compared to 1704 and 17 syn+ in vivo reactivation using the eye model, it was found that 17 syn+ and 1704R behaved similarly, whereas frequency and kinetics of reactivation of 1704 were significantly impaired²¹. These apparently contradictory results are not immediately explicable. It will be necessary to isolate another 1704 rescuant and test its phenotype before definitive conclusion can be drawn. The latency result of 1705 mimics that of 17 syn+. This could be due to one complete spare copy of LATs in IRL. Although 1706 infected ganglia had similar reactivation kinetics to 17 syn+ and 1705, the percentage of ganglia reactivating was considerably reduced (16% compared to 50%). As the deletion/insertion in 1706 does not effect the LATs or LAT promoter, it is assumed that 1706 is less efficient possibly due to its growth impairment. In vitro at low moi (1:1000), 1706 is 12-24 hr delayed in growth compared to 17 syn+ and 1705 over a 72 hr period¹². This impairment is also maintained in vivo and hence there is an effect on latency reactivation. The effect of gene duplication (UL1, 2,3 and 4) and rearrangement of 1706 may be responsible for its altered

biological behaviour, but this needs further investigation. It is possible that diploid genes in 1706 result in over expression of their protein products which may be detrimental to other protein products essential for the virus growth.

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