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Rapid generation of recombinant herpes simplex virus vectors expressing the bacterial lacZ gene under the control of neuronal promoters

Research Article

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Abbreviations: HSV-1, herpes simplex virus type 1; **NSE,** neurone specific enolase; **PNS**, peripheral nervous system; **CNS**, central nervous system; **HCMV,** human cytomegalovirus; **GABA,** γ-amino butyric acid; **LAT**, latency associated;

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Summary

We describe the development of herpes simplex virus type 1 (HSV-1) RL1 negative mutants as vectors expressing the *lacZ* reporter gene under the control of neuronal specific promoters and their expression in neuronal cell cultures. Two neuronal promoters were used in this study 1) the rat neurone specific enolase (NSE) promoter, which is expressed in all neurones and 2) the mouse $GABA_A$ receptor delta (δ) subunit, which is expressed within specific regions of the brain (cerebellum, hippocampus and thalmus). The *lacZ* gene was also placed under the control of two viral promoters: 1) the HCMV IE promoter, a 'universally' strong promoter, and 2) the HSV-1 gD promoter, a strong delayed early lytic promoter. Initial expression experiments with recombinant viruses were carried out on neuronal and non-neuronal cell lines and primary mouse hippocampal cells. The promoter/reporter cassettes were introduced into one of two sites in the HSV-1 genome: firstly a non essential gene in the unique long region; and secondly at the LAT locus in the long terminal repeats just downstream from the LAT P2. Two high frequency methods of generating recombinant HSV are described. The first step for both involves insertion, into a non-essential genomic region, of a unique restriction enzyme site (Pac I), which is used to digest the HSV DNA into 2 arms. In the first method the insert, flanked by Pac I sites is isolated and *in vitro* ligated into the digested HSV vector to generate recombinant virus at a frequency of 10-90%. In the second method the DNA to be inserted is flanked by HSV DNA and co-transfected with the digested viral DNA. *In vivo* recombination across the digested ends of the HSV DNA and through the sequences to be inserted generates recombinants at a frequency of up to 100%.

I. Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotrophic virus that is being increasingly used as a vector for gene delivery to neurones. The virus has a large ds DNA genome (150kbp) that has been fully sequenced and has the capacity to package up to 30kbp of foreign DNA (McGeoch et al, 1988; Glorioso et al, 1995; Longnecker et al, 1988) allowing insertion of entire genes including introns and large promoter elements which might be important in specifying region specific expression in the CNS. Additionally in latently infected neurons of both the peripheral and central nervous systems (PNS and CNS) HSV is stably maintained as an episomal element with transcriptional activity limited to one region of the genome, the latency associated (LAT) region (Glorioso et al, 1995; Stevens, 1989). HSV-1 encodes

approximately 80 genes of which half are deemed 'non-essential' for replication in tissue culture, but may contribute to viral propagation in animal models (Ward and Roizman, 1994). At least some of these 'non- essential' genes could be deleted to provide additional room for inserted sequences, or to modify neuronal tropism without drastically altering the virus growth in tissue culture. Before use as a neuronal gene delivery vector it is essential that the capacity of the virus to undergo a lytic infection in the CNS is abolished: currently we are developing non pathogenic vectors based on deletion of the virulence factor, ICP34.5 encoded by the RL1 gene (MacLean, A. et al, 1991; Coffin et al, 1996; McKie et al, 1998).

Using *lac Z* as a reporter gene, we are interested in studying specificity and duration of neuronal promoter expression in a HSV-1 background in the central nervous system (CNS) of animal models; the ultimate aim being the generation of potentially useful gene therapy vectors for the treatment of neurological disorders such as Huntington's and Alzheimer's disease. Our current studies involve the use of two neuronal promoters: the $GABA_A$ receptor delta (*d*) subunit promoter, which has a specific regionalised expression pattern in the CNS of rodents (cerebellum, hippocampus and thalmus; Laurie et al, 1992); and the neurone specific enolase (NSE) promoter, which is ubiquitously expressed in neurones throughout the CNS (Forss-Peter et al, 1990). In this paper we describe the generation of a number of recombinant viruses and their pattern of expression in both neuronal and non-neuronal tissue culture cells and primary neuronal cells as well as initial expression data *in vivo*.

One major drawback to the use of HSV-1 as a gene delivery vector lies in the labour intensive method of isolating recombinant viruses. The standard method of introducing foreign DNA into HSV-1 is to target insertion into a 'non-essential' gene on the viral genome by homologous recombination (MacLean, C. et al, 1991); firstly by cloning the foreign DNA into a plasmid between flanking HSV-1 sequences of at least 500-1000bp; and secondly by cotransfecting the recombinant plasmid with intact viral DNA onto a cell monolayer to generate progeny virus. The frequency of recombinant viruses within the population varies between 0.1 to 1%, depending upon the efficiency of recombination and the growth characteristics of the mutant virus. If the inserted foreign DNA is a *lacZ* reporter gene under a ubiquitous promoter, recombinant progeny can easily be detected by blue/white plaque staining in the presence of X-gal in the medium overlay (MacLean, C. et al, 1991). If the inserted foreign DNA cannot be used to select for or detect recombinants (as in our case where the promoter driving is only functional in certain non plaquing cell types) the DNA profile of individual plaque isolates requires to be analysed to identify recombinant clones, a labour intensive process (MacLean, A. et al, 1991).

Although a variety of systems exist which allow the selective enrichment of recombinant progeny virus, each of these systems has individual drawbacks. One method is to either reduce the parental progeny population, and/ or select for the recombinant progeny population. By targeting the DNA insertion to the thymidine kinase (TK) locus of HSV-1 and selecting for progeny virus with a TK negative phenotype both criteria can be met (Efstathiou et al, 1989). However, it is not always desirable to have a TK negative virus since such viruses are resistant to acyclovir (the only clinically effective anti-HSV drug) - an undesirable phenotype for a gene therapy vector - and additionally have altered neurovirulence. This method is also restricted to one site in the genome.

Other methods have been developed to maximise the yield of recombinant progeny in the population. The most powerful system is the use of a cosmid system, with cloned overlapping DNA inserts spanning the length of the viral genome (Cunningham and Davison, 1993); this effectively eliminates background parental virus. However, generation of functional overlapping cosmids is a complicated procedure and has the disadvantage that each set is strain/ mutant dependent.

Another technique, widely used in the generation of recombinant viruses, is the P1 phage Cre-loxP system. Here foreign DNA is introduced into the viral genome by cloning the foreign DNA into a loxP bearing plasmid to allow targeted recombination to occur in the presence of the Cre protein between the plasmid and an existing loxP site previously introduced into the parental HSV-1 strain (Gage et al, 1992). Although this procedure is highly efficient at generating recombinant progeny virus, it suffers the drawbacks of firstly having to construct an initial virus mutant with a loxP site and secondly, and, potentially more seriously, the inserted DNA will be flanked by loxP sites, which may affect reporter gene expression.

By taking a genome whose normal XbaI sites had been deleted (MacLean and Brown, 1987), Rixon and McLauchlan (1990) developed a procedure for introducing foreign DNA into a unique XbaI site which had been introduced back into this genome: this was carried out by *in vitro* ligation of foreign DNA with flanking XbaI sites and subsequent transfection of the ligation mixture into cells to generate virus progeny. Huang et al. (1994) improved on this procedure by introducing two unique restriction sites (PacI and SwaI) into the LAT locus of the HSV-1 strain HFEM genome (which contains only a single LAT locus) such that the insert is ligated unidirectionally and re-ligation of viral genome DNA without an insert is significantly impaired because the genomic ends are non-compatible thereby lowering

parental virus background. However, both ligation systems are limited in their ability to be used at any location in the genome and since a lot of work was involved in generating these particular HSV-1 viruses with unique restriction sites these methodologies lack flexiblity because they are again strain/ mutant dependent.

We have extended this latter method to develop two alternative procedures for the creation and isolation of HSV-1 recombinants which cannot easily be detected.

II. Results

A. Generation of recombinant viruses carrying PacI restriction enzyme sites

HSV-1 does not contain a PacI restriction enzyme site and such a site can be introduced at any non-essential site to enable insertion to be specifically targeted to that location on the HSV-1 genome by one of two methods, both relying on digestion at the unique PacI site. Initial studies have targeted insertion to the non-essential UL43 gene (MacLean, C. et al, 1991) in the long unique region of the viral genome. Insertions were carried out into two HSV-1 strain 17 non-virulent mutants, 1716 and 1764. Both mutants are deleted in the RL1 virulence gene (MacLean, A. et al, 1991), while 1764 carries an additional mutation in the Vmw65 immediate early transactivating gene (Ace et al, 1989; Coffin et al, 1996).

A polylinker containing PacI sites was introduced into UL43 at an unique NsiI site at n.p. 94711 in the plasmid p35 containing HSV sequences from a BamHI (n.p. 91610) to EcoRI (n.p. 96751) site (MacLean, C. et al, 1991; **Figure 1**).

A HCMVIE promoter/*lacZ* reporter cassette flanked by PacI restriction sites was inserted into the PacI sites in p35(PacI) and the recombinant plasmid co-transfected separately with HSV-1 1764 and 1716 DNA onto BHK21/C13 cells and recombinant viruses (1780 and 1780R respectively) isolated under X-gal selection. Three subsequent rounds of plaque purification were carried out to ensure the purity of 1780 and 1780R. The structure of both recombinant viruses was confirmed by restriction enzyme analysis and Southern blotting with p35 (data not shown).

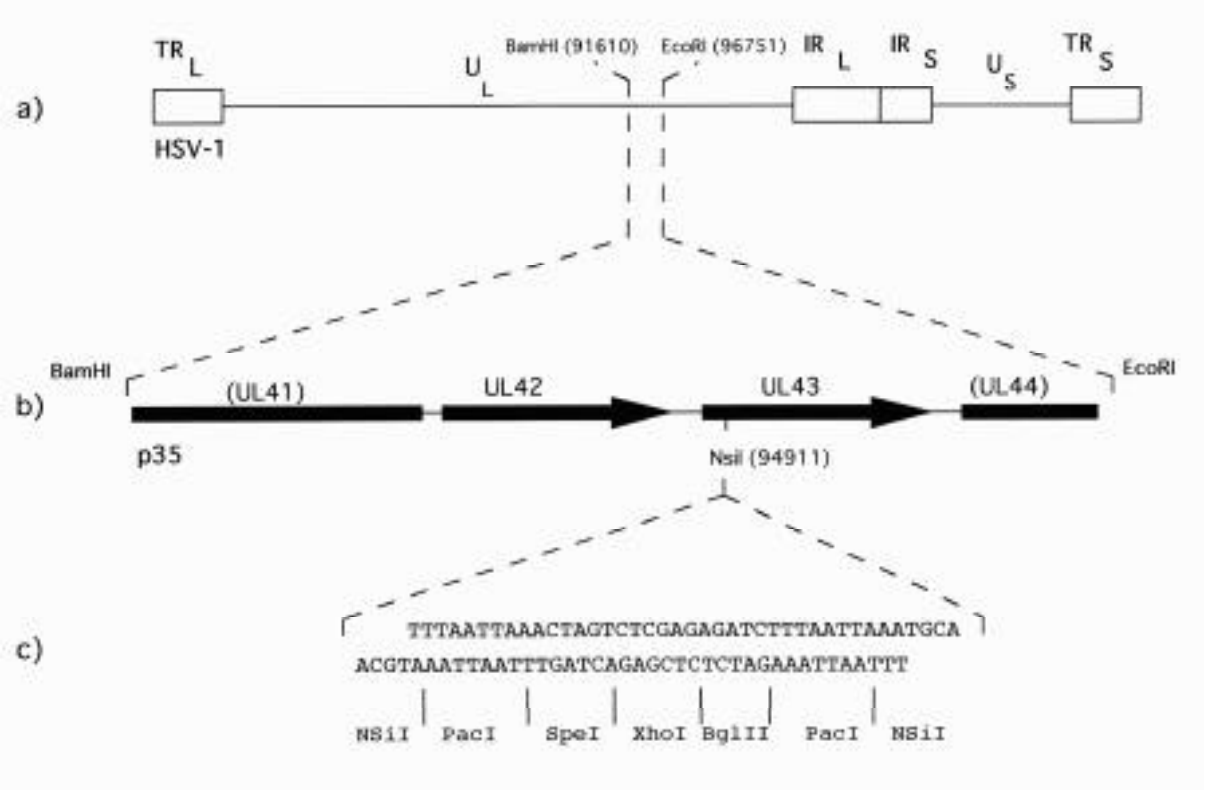

Figure 1.
a) A diagram of the HSV-1 genome with the region spanning UL43 expanded. The n.p. of the BamHI and EcoRI sites used to construct p35 are marked MacLean, C. et al., 1991).
b) An expansion of the HSV-1 sequence cloned in p35 with the n.p. of the flanking BamHI and EcoRI sites and the NsiI site used for insertion marked.
c) The sequence and sites present in the polylinker inserted into the NsiI site to generate p35(Pac).
All expression cassettes were inserted into UL43, via the introduced PacI site, at the position of the unique NsiI site.

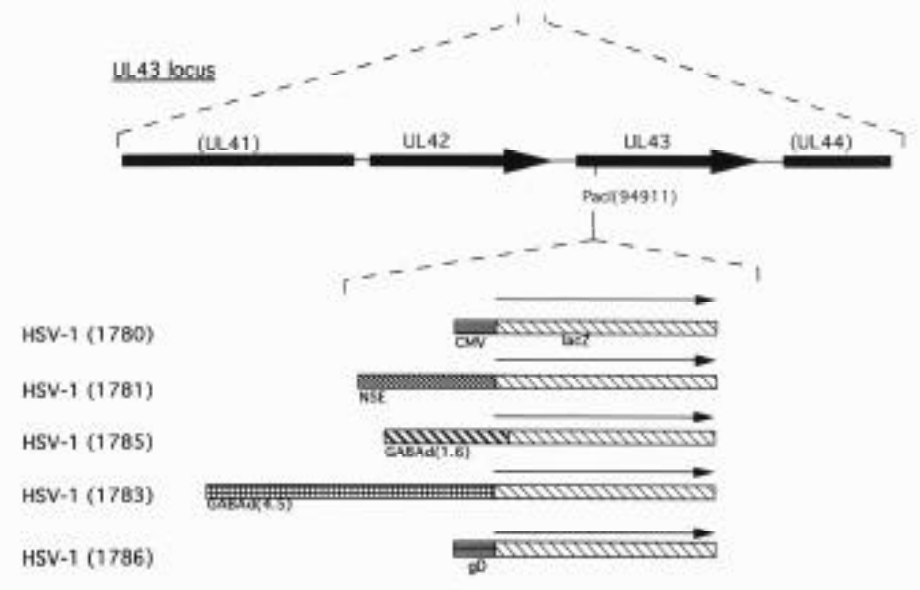

Figure 1d) The *lacZ* expression cassettes inserted into the UL43 locus.

DNA prepared from 1780 and 1780R was digested with PacI and viral DNA re-ligated to remove the *lacZ* cassette and produce a virus with a single PacI site in the UL43 locus (mutants 1784 and 1784R respectively; **Figure 2**). 1784 and 1784R were isolated under X-gal staining by isolating white plaques, which were subjected to three subsequent rounds of plaque purification. The structure of 1784 and 1784R was confirmed by BamHI restriction enzyme analysis and Southern blotting with p35 (data not shown).

The presence of two PacI sites flanking the HCMVIE*lacZ* cassette gives a significantly lower parental virus background, and thus a higher percentage of recombinants, after PacI digestion of viral DNA: for this reason 1780/1780R were the parental strains used in most PacI viral selection system experiments. A detailed methodology for each of the PacI systems is given below. As a prerequisite for both methods, to ensure a minimum level of parental virus background, it is essential to confirm complete digestion of the HSV DNA with PacI by checking the transfectibility of the digested compared to undigested DNA. Following digestion there should be a knockdown of at least 1000-fold in plaque numbers.

Introduction of foreign DNA at the inserted unique PacI site was either by 1) a modification of the ligation/ insertion strategy developed by Rixon and McLauchlan (1990); or alternatively 2) a homologous recombination virus rescue protocol using a parental virus digested into 2 arms at the site of insertion by PacI. Although novel to HSV-1, this method is similar to one employed in the generation of recombinant baculovirus (Kitts et al, 1993). The latter procedure is achieved by firstly cloning the foreign DNA into a vector with flanking HSV sequences homologous to the region surrounding the PacI site in the HSV-1 viral genome (**Figures 1 and 2A**). The recombinant HSV plasmid and PacI digested HSV-1 viral DNA are co-transfected onto BHK21/C13 cells by calcium phosphate precipitation (Stow and Wilkie, 1976) allowing recombination to take place *in vivo* to generate progeny virus. The PacI recombination protocol, although lower in efficency at generating virus compared to the *in vitro* ligation strategy, produces a high proportion of recombinant progeny (up to 100%) that have unidirectional insertions, thus enabling rapid purification of HSV-1 recombinants: this method should be especially useful for purifying recombinant viruses with a selective growth disadvantage. A summary diagram illustrating both the PacI ligation and recombination strategies is illustrated in **Figures 2A and B**.

B. PacI ligation protocol

The PacI ligation protocol was carried out as follows. Firstly, viral DNA was digested overnight with an excess of PacI enzyme, the DNA phenol/ chloroform extracted, ethanol precipitated, washed with 70% ethanol, air dried and carefully resuspended at a concentration of 0.1ug/ul in water overnight. Secondly, a plasmid containing a promoter/ reporter cassette flanked by PacI sites was digested with PacI and gel purified. Two ug of viral DNA was ligated with up to 5ug of purified PacI fragment DNA in a total volume of 40ul with 8 units of T4 DNA ligase in ligation buffer and 1mM ATP and incubated overnight at 16^{o}C.

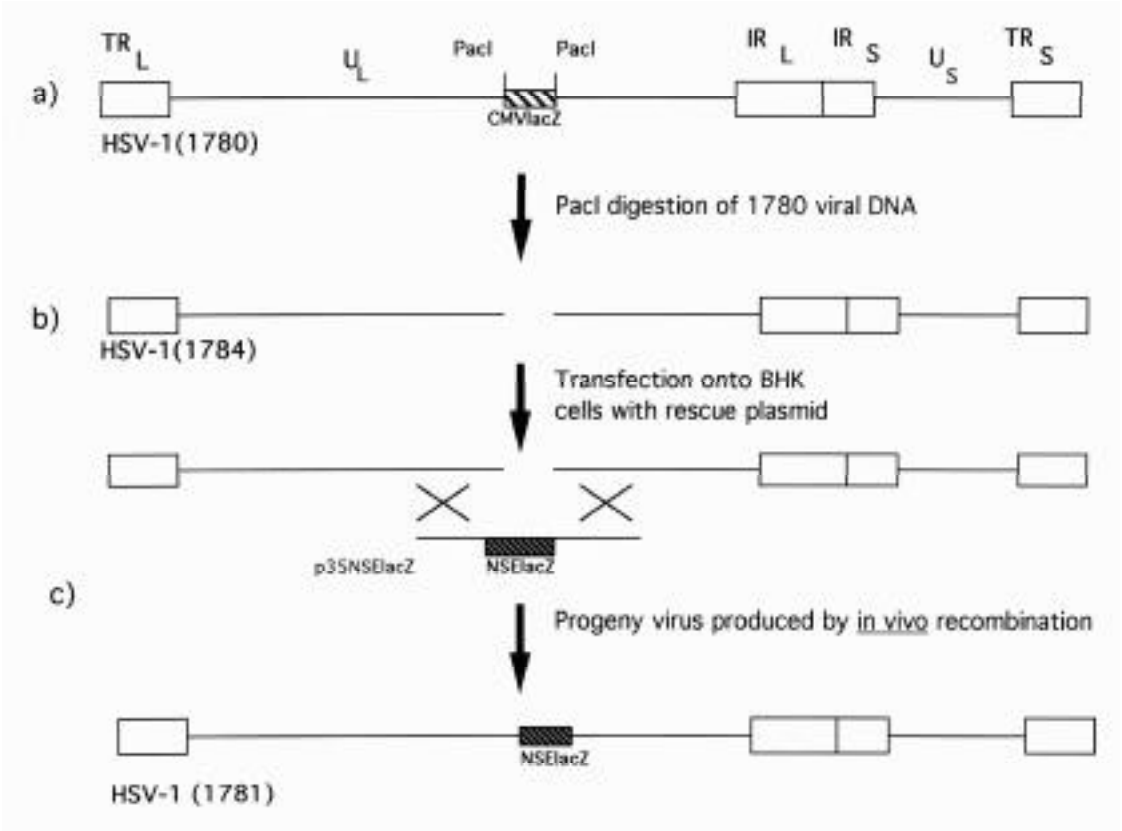

Figure 2A. A diagrammatic illustration of the PacI *in vivo* recombination selection system used to generate recombinant virus at the UL43 locus. a) 1780 with a CMVIE/lacZ insert flanked by 2 PacI sites in UL43. b) 1784 with the CMVIE/*lacZ* insert removed and containing an unique PacI site in UL43. Digestion of 1784 with PacI separates the genome into two arms. c) Cotransfection of the two arms with a HSV plasmid spanning UL43 and containing an insert allows *in vivo* recombination to generate recombinant virus at high frequency. The example given is NSE*lacZ*.

The ligation reaction was transfected by the calcium phosphate precipitation/ DMSO boost protocol (Stow and Wilkie, 1976). Once complete cpe was established on the transfected monolayer (approximately 3-4 days), the plates were harvested and recombinant virus isolated by the standard procedures of DNA analysis of individual plaque isolates or limiting dilution (Rixon and McLaughlan, 1990).

C. PacI Recombination protocol

The PacI recombination protocol was carried out as follows. Firstly, viral DNA was digested with PacI, purified and resuspended at 0.1ug/ml as described above. Five - ten ug of recombinant p35(PacI) plasmid, containing a promoter/ reporter cassette, was linearised by digestion with XmnI, which cuts once in the ampicillin resistance gene of the plasmid backbone. The plasmid DNA was phenol/ chloroform extracted, ethanol precipitated, washed with 70% ethanol, air dried and resuspended at a concentration of 0.5ug/ul in water. Five ug of PacI digested viral DNA and 2-10 ug XmnI linearised plasmid DNA were mixed together and co-transfected onto BHK21/C13 cells using the standard calcium phosphate/ DMSO boost protocol (Stow and Wilkie, 1976). Infected monolayers were harvested 7-8 days post transfection regardless of whether complete cpe had been obtained. Recombinant virus was isolated by a similar procedure used in the PacI ligation protocol.

Using these two procedures, a number of recombinant 1716 and 1764 based viruses in UL43 was isolated (**Figure 3; Table 1**). By the ligation method: NSE *lacZ* and GABA 1.6kbp *lacZ*; and by the recombination method, GABA 1.6kbp *lacZ* and 4.5kbp *lacZ*.. The percentage of recombinant virus generated by the PacI ligation sytem varied between 10 to 90% of the total viral progeny and recombinant virus generated via the PacI recombination protocol constituted up to 100% of the total viral progeny.

Equivalent viruses in the LAT locus were isolated by standard cotransfection (**Figure 4; Table 1**).

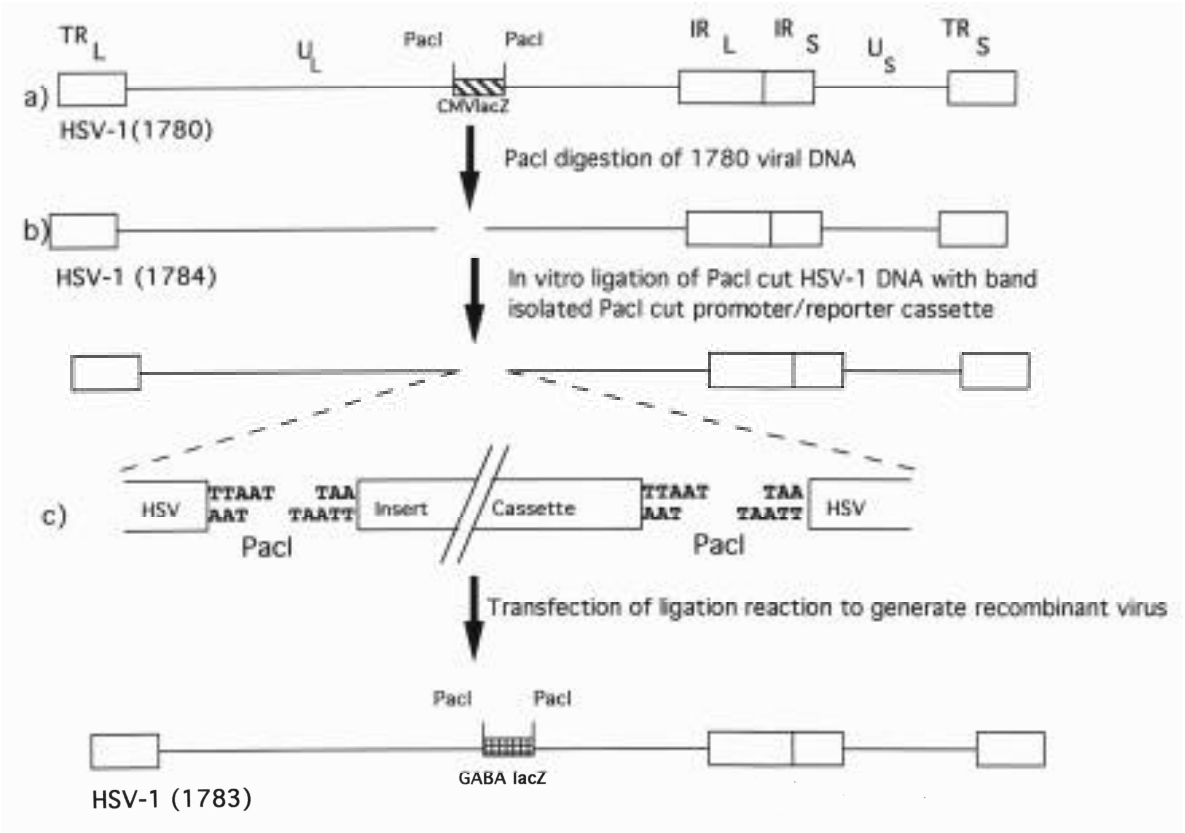

Figure 2B. A diagrammatic illustration of the PacI *in vitro* ligation selection system used to generate recombinant virus at the UL43 locus. a) 1780 with a CMVIE/lacZ insert flanked by 2 PacI sites in UL43. b) 1784 with the CMVIE/*lacZ* insert removed and containing an unique PacI site in UL43. Digestion of 1784 with PacI separates the genome into arms. c) Ligation of the two arms *in vitro* in the presence of an excess of an insert with PacI ends generates a mixture of recombinant virus and 1784. The example given is 4.5kbp $GABA_A$ *d lacZ*.

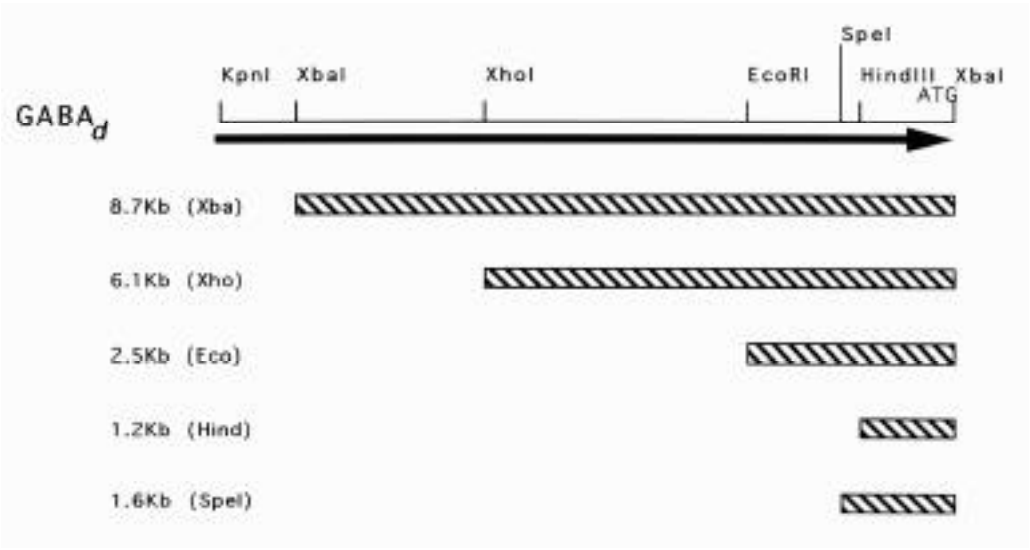

Figure 3 Diagram of the $GABA_A$ *d* promoter and the fragments, with their sizes, used to generate *lacZ* expression cassettes. The initiating ATG is also marked. Restriction enzyme sites used in the cloning are marked.

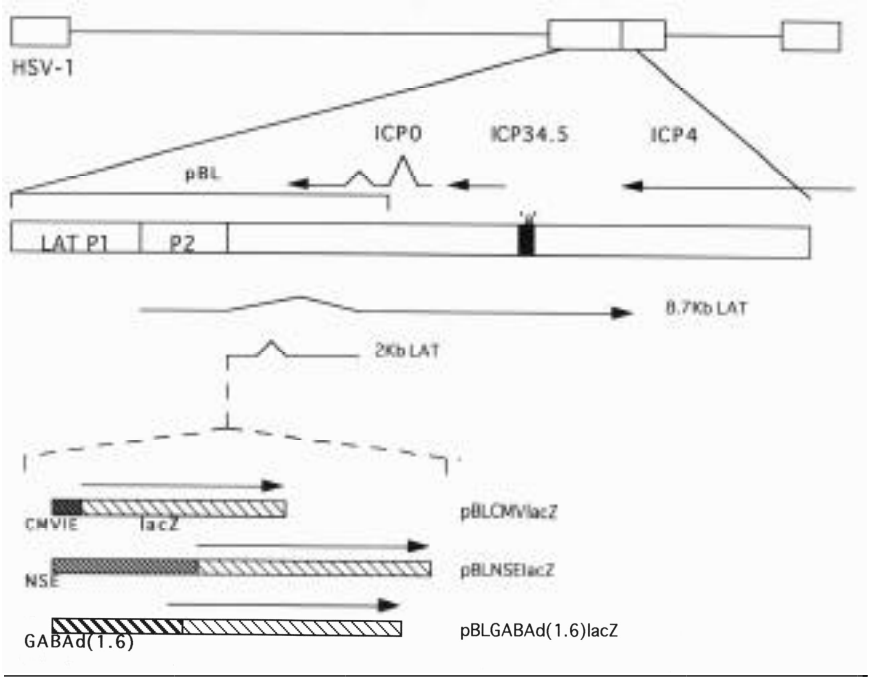

Figure 4 A diagram of the HSV-1 genome with IR_L expanded showing the location of the identified LAT transcripts and promoters and other genes (ICP0, ICP34.5, ICP4) encoded in the repeats. Expression cassettes were inserted downstream of LAP2 at the 5' end of the 2kb LAT. Below the line are illustrated the three *lacZ* expression cassettes inserted into the LAT locus (CMVIE, NSE, 1.6 GABAd). An equivalent arrangement is found in TR_L.

UL43 locus	**LAT locus**
4.5 GABA 1.6 GABA	1.6 GABA
NSE	NSE
HSVgD HCMVIE	HCMVIE

Table 1. 1716 and 1764 recombinant viruses.

D. Construction of GABA*d*/*lacZ* expression cassettes

A 10kbp upstream fragment of the GABA*d* promoter was used as the starting plasmid and a range of fragments deleted from the 5' end constructed to generate 5 *lacZ* expression cassettes with promoter fragments ranging in size from 1.6-10.1 kbp (**Figure 5**). All five were inserted into the SpeI site of p35(Pac) and the 1.6 and 4.5 kbp promoter fragment constructs used to generate recombinant viruses. In addition the 1.6kbp NSE promoter was cloned in front of the *lacZ* gene, inserted into p35(Pac) and used to generate recombinant virus. The 1.6 kbp GABA*d* and NSE promoter/*lacZ* constructs were also inserted into a plasmid carrying RL sequences just downstream of LAP2 and recombined into virus by the standard calcium phosphate/ DMSO boost protocol (Stow and Wilkie, 1976).

E. Expression of *lacZ* recombinant viruses

1. *In vitro* expression

Initially *lacZ* expression from the recombinant viruses was assayed in a range of neuronal (ND7 and PC12) and non-neuronal (BHK21/C13) tissue culture and primary hippocampal cells as outlined in **Table 2**. As no significant differences in expression levels were observed between either 1716 or 1764 or either genomic locus, only 1 set of results is given. Varying multiplicities of infection (0.01-10 pfu/cell) were used and samples were fixed 48 h post infection: the numbers of positive cells increased with multiplicity, but expression levels were independent of multiplicity. Both the HCMVIE and gD promoters expressed strongly in the neuronal and non-neuronal cell lines, but slightly less in the primary neuronal cells. NSE expressed weakly in both the neuronal cell lines and primary hippocampal cells, whereas expression from the GABA *d* 1.6kb promoter was only faintly detected in the neuronal cell lines. No expression was detected from the GABA *d* 4.5kb delta promoter in any cell type used.

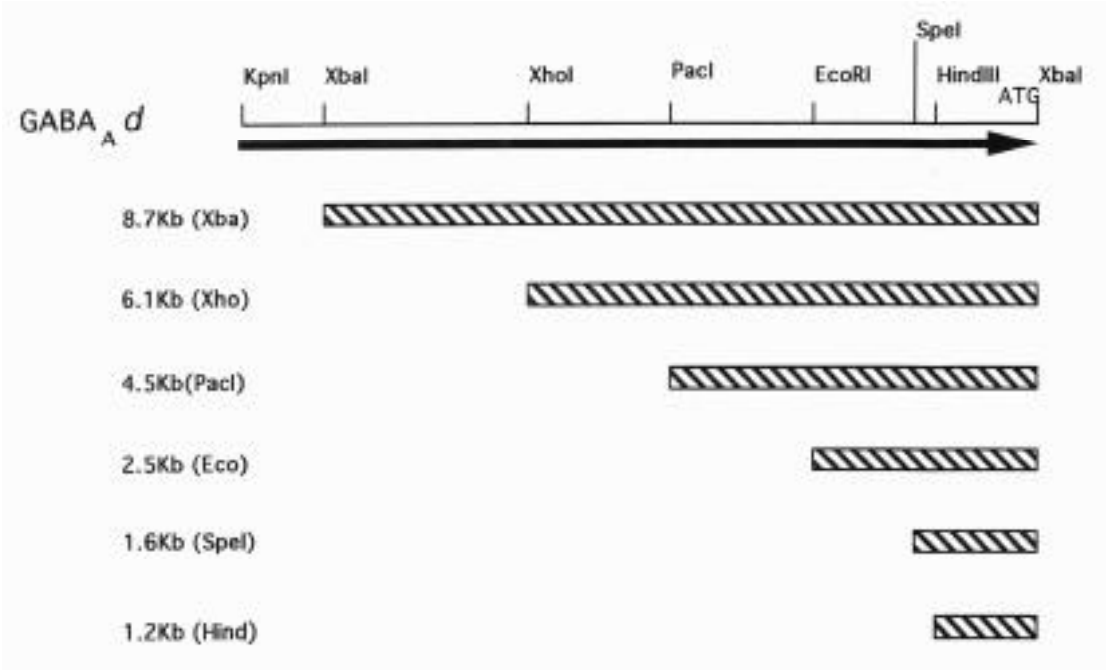

Figure 5. A restriction map of the GABA$_A$ δ upstream region (5' UTR), showing the sizes of fragments used to generate *lacZ* expression cassettes.

Cell line/ Promoter	HCMV IE	gD	NSE	GABA 1.6kbp	GABA 4.5kbp
BHK21/C13	+++	+++	-	-	-
PC12	++	++	+	+	-
ND7	++	++	+	+	-
hippocampal cells	+	+	+	-	-
hippocampal neurones	+	+	+	-	-

Table 2. Tissue culture expression of recombinant viruses. The number of + refers to strength of blue staining in individual cells. Primary hippocampal cells were established from rat embryos with neurone enriched cultures being derived by treatment with ara C to kill the dividing cells (Kennedy et al., 1995).

2. *In vivo* expression

All viruses were assayed for expression in the PNS and CNS of Balb/C mice.

To detect expression in the PNS, 4 week old Balb/C mice were infected with 10^7 pfu/mouse in the footpad to allow acute replication/ establishment of latency in the dorsal root ganglia (DRG) of the PNS as previously described (Robertson et al, 1992). In the PNS *lacZ* expression could only be detected from the gD and HCMVIE promoters. As previously observed a larger number of *lacZ* positive neurones were detected with 1764 based viruses, possibly due to the higher particle numbers used to give an equivalent multiplicity (Ace et al, 1989; Coffin et al, 1996). Expression peaked at 7 days post inoculation, was declining rapidly by day 21 post inoculation and was undetectable 35 days post inoculation.

To detect expression in the CNS, 3 week old female Balb/C mice were infected with 10^5 pfu/mouse as described by MacLean, A. et al. (1991). *LacZ* expression could only be detected with the gD based constructs for the first 7 days post inoculation, presumably due to expression during the abortive lytic infection observed with ICP34.5 negative viruses.

III. Discussion

One of the practical difficulties in using HSV as a gene therapy vector is the inability to rapidly isolate recombinants. Standard methods generate a low frequency of recombinants and involve time consuming analysis of a large number of individual plaque isolates (MacLean, A. et al, 1991). Both methods described in this paper generate recombinants at significantly increased frequency, thus reducing the number of isolates which have to be screened. The recombination method, while requiring subcloning of the sequence to be inserted into a HSV flanking plasmid generates recombinants at nearly 100% in the desired orientation. The ligation method has the advantage that

the sequences to be inserted do not require to be first subcloned into a plasmid with flanking HSV sequences, but produces a lower frequency of recombinants (5-90%) in random orientations. The isolation of a parental virus with PacI sites surrounding *lacZ* under the control of a strong ubiquitous promoter, HCMVIE, is non labour intensive as recombinant viruses can be readily detected under X-gal staining. Construction of one plasmid and recombinant viruses allows insertion into the same site of all mutants of the one HSV-1 strain, as demonstrated by insertion into 1716 and 1764 in this paper.

Using these techniques a range of recombinant viruses have been constructed and are described in **Figure 4**.

One of the major problems of using HSV as a gene delivery vehicle is obtaining long term expression from the latent genome, preferably in a cell type dependant manner. Towards this aim we are particularly interested in studying neuronal promoter expression in a HSV-1 background in the CNS/PNS of animal models. Our current studies involve the use of two neuronal promoters: the $GABA_A$ receptor *d* subunit promoter, which has a specific regionalised expression pattern (cerebellum, hippocampus and thalmus) in the CNS of rodents; and the neurone specific enolase (NSE) promoter, which is ubiquitously expressed in neurones throughout the CNS. Both the level and specificity of expression from these promoters in a HSV-1 vector is being analysed using beta-galactosidase as a reporter gene. As controls we are using 2 ubiquitous strong promoters: the HCMV IE and HSV gD promoters. Our inability to detect expression *in vivo* from either the NSE or $GABA_A$ *d* constructs suggests any expression is low level and below the limits of detection by Xgal staining and we may require to detect expression by the more sensitive method of RT-PCR, or use of a more sensitive reporter gene such as green fluorescent protein (GFP). This hypothesis is supported by the low level of expression in tissue culture (**Table 2**).

In the case of the $GABA_A$ *d* 1.6kbp promoter both transcriptional and translational fusion cassettes were generated in an effort to maximise reporter gene expression: no difference was detected. However, to obtain maximal/ regional specific levels of expression of a reporter gene may require the presence of further upstream sequences (6.5 to 10kbp 5' UTR): we have constructed such lac Z expressing cassettes and are currently generating recombinant viruses using the PacI recombination system, as we anticipate viruses contaning such large inserts 10-14kbp will be at a significant growth disadvantage. Recently, it has been shown with the rat tyrosine hydroxylase promoter a 9kbp flanking sequence is required for regional specific expression of a *lacZ* reporter gene in transgenic mice (Min et al, 1994). Future work will be concentrated on constructing recombinant viruses using the full length 10kbp $GABA_A$ *d* fragment to drive *lacZ* expression; increasing the sensitivity of our assays; and determination of whether *lacZ* expression obtained in the CNS under $GABA_A$ *d* promoter control can be region specific.

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