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Replacement of Glycoprotein B Gene Sequences in Herpes Simplex Virus Type 1 Strain ANG by Corresponding Sequences of the Strain KOS Causes Changes of Plaque Morphology and Neuropathogenicity

By K. WEISE,[1] H. C. KAERNER,[1] J. GLORIOSO[2] AND C. H. SCHRÖDER[1]*

[1]*Institute for Virus Research, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, F.R.G. and* [2]*Unit for Laboratory Animal Medicine and Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109, U.S.A.*

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SUMMARY

DNA sequences encoding glycoprotein B (gB) derived from herpes simplex virus type 1 (HSV-1) strain KOS321 were transferred to HSV-1 ANG. In cotransfection experiments the cloned HSV-1 KOS *Bam*HI G fragment served as donor, and genomic DNA of two ANG variants as recipients. One of these variants, HSV-1 ANG path, expresses gC and the other, C18, was a spontaneous gC-negative mutant. Both ANG strains are of the syncytial (syn) phenotype whereas HSV-1 KOS321 is non-syncytial (syn^+). Recombinants were identified by means of a monoclonal antibody which selectively recognizes gB^{KOS}. Among the HSV-1 ANG path/gB^{KOS} recombinants, the majority displayed an altered plaque morphology, i.e. they were of the syn^+ phenotype. In contrast all of the C18/gB^{KOS} recombinants were of the syn phenotype. The possibility that the mutant C18 carries a syn mutation not present in the parental strain could be excluded. Marker transfer experiments involving subfragments of the gB gene mapped the syn mutation of HSV-1 ANG path to a locus within the gene that has been previously termed syn 3. Subclones of HSV-1 ANG path were established either directly or after intermittent transfection or cotransfection with the KOS *Bam*HI G fragment. The pathogenicity in mice of these clones was compared. The data obtained indicated that at high frequency, the *Bam*HI G fragment confers apathogenicity.

INTRODUCTION

Fresh isolates of herpes simplex virus (HSV) usually do not promote the fusion of infected cells *in vitro* (Spear, 1984). They produce non-syncytial (syn^+) plaques characterized by rounding and aggregation of cells. Mutations in several different viral genes may result in conversion of this phenotype to a syncytial (syn) phenotype characterized by cell fusion. There is evidence that in addition to viral factors, host cell factors also determine plaque morphology.

The location of some HSV-1 syn mutations have been mapped. One mutation, designated syn 1, has been mapped to genome coordinates 0·735 to 0·74 (Bond & Person, 1984; Pogue-Geile *et al.*, 1984) and confers the capacity to fuse Vero cells (Pogue-Geile *et al.*, 1984). A second mutation, designated syn 2, maps close to the syn 1 locus but outside the map coordinates mentioned above and confers the capacity to fuse Vero and HEp-2 cells when present together with the syn 1 mutation (Ruyechan *et al.*, 1979; Pogue-Geile *et al.*, 1984). Another locus involved in syncytium formation, syn 3 (Ruyechan *et al.*, 1979), can be assigned to the glycoprotein B (gB) gene. Specifically the syn 3 mutation is located close to the C terminus of gB, which represents the interior domain of this transmembrane protein (DeLuca *et al.*, 1982; Kousoulas *et al.*, 1984).

There are also mutations in other loci affecting the syn phenotype, to which a syn designation has not yet been formally assigned. Pertinent to our study is the observation that a number of fusion-inducing mutants are also defective in the synthesis of gC (Heine *et al.*, 1974; Cassai *et al.*, 1976; Spear, 1984).

It can be assumed that glycoproteins not only define biological properties of the virus *in vitro* but also its pathogenic properties. However, changes in syn phenotype or gC expression of individual HSV strains do not result in consistent alterations of the pathogenicity phenotype in an animal model system (Wheeler, 1964; Cassai *et al.*, 1976; Shimizu *et al.*, 1978; Dix *et al.*, 1983). Recently, a genetic function that contributes to a difference in neuroinvasiveness between two wild-type strains of HSV-1 has been localized to the genome region defined by the *Hin*dIII A fragment (0·25 to 0·53 map units; Thompson *et al.*, 1986). One of the proteins encoded by this fragment is gB (Ruyechan *et al.*, 1979; Wagner, 1985).

In this report we show that the syn phenotype of HSV-1 strain ANG path (Kaerner *et al.*, 1983) is governed by the syn 3 locus. We present evidence that the absence of gC expression under certain genetically defined conditions is not compatible with the syn$^+$ phenotype. We further provide evidence that the *Bam*HI G fragment derived from the peripherally apathogenic HSV-1 KOS strain may confer apathogenicity to a pathogenic strain.

METHODS

Virus and cells. HSV-1 strains were propagated in African green monkey kidney cells (RC-37; Italdiagnostic Products, Rome, Italy) as described (Schröder *et al.*, 1976). For transfection BSC-1 cells (American Type Culture Collection) were used (Hennes-Stegmann & Schröder, 1982).

Plasmids. The plasmids pLH60 containing the *Bam*HI G fragment (Holland *et al.*, 1983*b*) and pFH50 containing the *Sal*I fragment T (Holland *et al.*, 1984*b*) cloned in pBR322 were kind gifts from M. Levine. Both restriction endonuclease fragments had been derived from HSV-1 strain KOS321 (Holland *et al.*, 1983*a*). Subfragments of KOS *Bam*HI G containing parts of the gB gene were cloned in the plasmid vector pUC19 (pUC19K1 and pUC19K2, Fig. 1).

Monoclonal antibodies. A pool of monoclonal antibodies directed against gC consisted of the antibodies C4, C11, C13 and C16. The antibodies B3, B4, B5 and B6 directed against gB were used individually. The procedure used for the production of hybridomas secreting neutralizing antibody specific for HSV-1 strain KOS321 has been described in detail elsewhere (Holland *et al.*, 1983*a*).

Mice. DBA/2J mice were obtained from Deutsche Gesellschaft für Versuchstierkunde, Hannover, F.R.G. Males of 6 to 8 weeks were used throughout these studies. The mice were infected intraperitoneally (i.p.) with HSV-1 ANG path, with subclones of HSV-1 ANG path and with subclones derived from ANG path in marker transfer experiments. Dead mice were recorded daily. Experiments were terminated after 3 weeks.

Transfection assay. Rescue experiments were carried out via cotransfection of genomic HSV-1 DNA (Hennes-Stegmann & Schröder, 1982) with individual restriction endonuclease fragments cloned in pBR322 or pUC19. Prior to cotransfection, the plasmids were cleaved with appropriate restriction endonucleases in order to separate viral sequences from vector sequences. The amount of genomic and of plasmid DNA (vector plus insert) used in the individual experiments is indicated in the tables presented.

For transfection the calcium phosphate technique essentially as described by Graham *et al.* (1973) was used except that cells were transfected in suspension. Briefly, BSC-1 cells, at 6 to 8 days after subcultivation, were trypsinized and suspended in HEPES buffer. Samples of 2×10^6 cells were pelleted, resuspended with 1 ml of the individual DNA–calcium phosphate precipitates usually containing 0·5 μg virus DNA plus 10 μg/ml of calf thymus DNA and shaken for 45 min at 37 °C. Subsequently, 5×10^6 BSC-1 indicator cells, trypsinized and suspended in 20 ml MEM supplemented with 10% foetal calf serum, were added. The resulting mixture of transfected BSC-1 cells and BSC-1 indicator cells was seeded into four 60 mm Petri dishes. After 8 h, the medium was changed to fresh medium containing 1% carboxymethylcellulose in two of the cell cultures. Plaques on plates containing carboxymethylcellulose were analysed directly 3 to 4 days after transfection. Virus replication in the two other cultures was stopped by freezing at −70 °C 2 days after transfection and the infectious virus progeny were assayed on monolayers of RC-37 indicator cells.

Black plaque assay. The transfer of glycoprotein markers was monitored in the black plaque assay as described by Holland *et al.* (1983*b*). This assay allows the recognition of glycoproteins exposed at the surface of infected cells in two cycles of antibody treatment. In the first step, monolayers with virus plaques are exposed to monoclonal antibodies with specificity for certain antigenic domains of glycoproteins. In the second step bound antibody is recognized by an anti-IgG antibody coupled to horseradish peroxidase. Staining of plaques is achieved by the addition of chloronaphthol as a substrate for the enzyme.

Plaque purification. Establishing virus subclones involved at least three consecutive plaque purification steps. Enrichment of glycoprotein variants to homogeneity was accomplished by direct analysis of the individual purification steps in the black plaque assay. In this case the monolayers of cells containing individual virus plaques were stained without glutaraldehyde fixation.

Labelling of glycoproteins. Cultures (RC-37, $1{\cdot}5 \times 10^6$ cells/60 mm Petri dish) were infected at an m.o.i. of 5 and

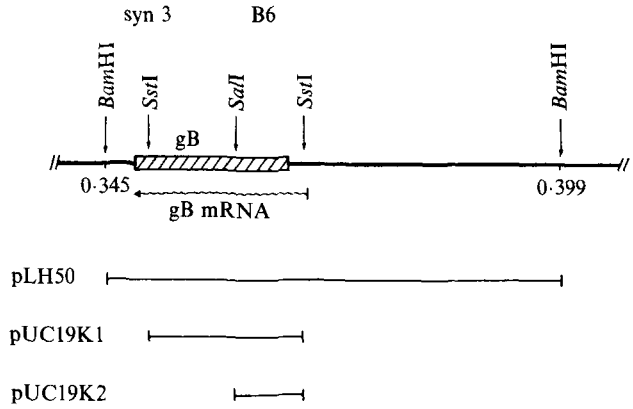

Fig. 1. Genomic location of the gB gene and of cloned restriction fragments. The plasmids pUC19K1 and pUC19K2 were derived from the HSV-1 KOS restriction fragment *Bam*HI G cloned in pBR322 (Holland *et al.*, 1983*b*). The syn 3 locus (Ruyechan *et al.*, 1979; DeLuca *et al.*, 1982; Kousoulas *et al.*, 1984), the B6 epitope, and the gB mRNA (Holland *et al.*, 1984*a*; Bzik *et al.*, 1984; Pellett *et al.*, 1985) are indicated.

incubated for 24 h in the presence of 2 μCi/ml ^{14}C-labelled glucosamine. At the end of the labelling period, the cells were rinsed with ice-cold phosphate-buffered saline, scraped off (5×10^6 cells/ml) and lysed by heating at 80 °C in 0·06 M-Tris–HCl pH 6·8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol for electrophoresis on SDS–polyacrylamide gels (10%) using the system described by Thomas & Kornberg (1975).

RESULTS

Characterization of a spontaneous gC⁻ mutant (C18) derived from HSV-1 ANG path

Viable gC$^-$ mutants were isolated from virus progeny obtained by transfection with genomic HSV-1 ANG path DNA and selection with neutralizing anti-gC monoclonal antibodies (Holland *et al.*, 1984*b*). One individual gC$^-$ clone (C18) was established which induced cell fusion like the parental strain. In the black plaque assay described by Holland *et al.* (1983*b*) plaques induced by the C18 mutant were not recognized by the same pool of monoclonal antibodies that were used for selection. In order to confirm the gC$^-$ character of the mutant, parallel cell cultures were infected with the mutant or with the parental strain. The cultures were labelled with [^{14}C]glucosamine. Proteins were extracted 24 h after infection and electrophoretically separated on a polyacrylamide gel. An autoradiogram of the gel is shown in Fig. 2 and demonstrates the absence of a protein migrating like gC in cells infected with the gC$^-$ mutant. Revertants to the gC$^+$ phenotype can be detected in virus stocks of C18 only after serial passages without intermittent recloning. These data indicated that reversion is a rare event. The rare occurrence of spontaneous revertants suggests that the mutation which causes the gC$^-$ phenotype is a point mutation.

Genomic location of the C18 mutation

A cotransfection was carried out with genomic DNA of the gC$^-$ mutant C18 and cloned 3·7 kb *Sal*I T fragment derived from HSV-1 KOS (Holland *et al.*, 1984*b*). This 3·7 kb fragment contains an almost complete gC gene except for 260 non-coding base pairs at the 3′ end of its mRNA (Frink *et al.*, 1983). Of the plaques formed on the monolayer by the transfected cells (transfection plaques) 22 to 37% were recognized by a pool of monoclonal antibodies against gC in the black plaque assay. In contrast, a smaller fraction of positively staining plaques was obtained when the virus progeny of the same cotransfection experiment was titrated on preformed monolayers of indicator cells and the resulting plaques (progeny virus plaques) were analysed (Table 1). This discrepancy demonstrates a genotypic heterogeneity of virus contained in individual transfection plaques induced by conglomerates of infectious DNA (calcium

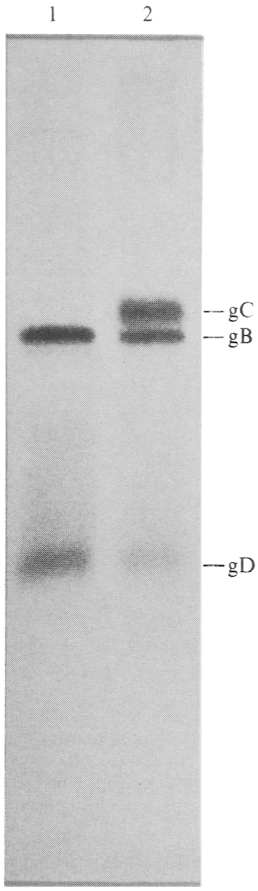

Fig. 2

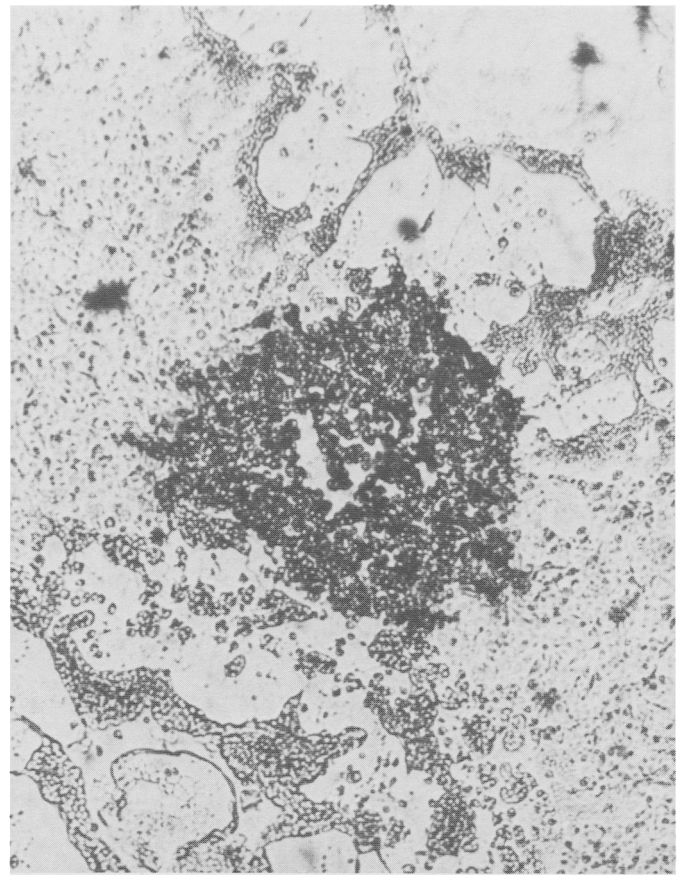

Fig. 3

Fig. 2. Autoradiogram of an SDS-polyacrylamide gel slab showing the electrophoretic separation of [^{14}C]glucosamine-labelled glycoproteins made by HSV-1 ANG path (lane 2) and mutant C18 (lane 1).

Fig. 3. Photomicrograph of a B6$^+$ syn$^+$ plaque flanked by two B6$^-$ syn plaques. The plaques were stained by the black plaque assay procedure using the monoclonal antibody B6 (Holland *et al.*, 1983*b*).

phosphate–DNA particles). In an analysis of individual transfection plaques that appeared to be gC-positive, an excess of gC$^-$ virus could be demonstrated directly (data not shown). In a control transfection with genomic C18 DNA alone the gC$^+$ phenotype was also obtained but at a lower frequency than in cotransfection with the *Sal*I T fragment. The detection of this phenotype was most easily achieved when transfection plaques were analysed. From the data given in Table 1 it can be concluded that the mutation responsible for the gC$^-$ phenotype maps to the gC structural gene and that in transfection experiments the mutant displays a measurable reversion frequency which appears to be higher than observed in infection experiments.

The monoclonal antibody B6 specific for gB of HSV-1 strain KOS does not recognize gB of HSV-1 ANG path

Virus-neutralizing monoclonal antibodies specific for gB of HSV-1 strain KOS (Holland *et al.*, 1983*a*) were tested for their ability to bind to HSV-1 strain ANG path. In the black plaque assay all of the gB-specific antibodies except for one, termed B6, bound both to cells infected with the KOS strain and cells infected with the ANG path strain. Failure to express the gB epitope that is recognized by the monoclonal antibody B6 appeared to be a stable property of HSV-1 ANG

Table 1. *Transfer of gC to mutant C18*

	C18 DNA (μg)	pFH60 DNA (μg)	gC-positive plaques (%)* Induced by transfection	Induced by progeny virus
Expt. 1	0·6	–	2·4 (168)	0·4 (265)†
	0·3	2·0	25 (190)	2 (175)
	0·6	2·0	37 (450)	11 (400)
Expt. 2	0·6	–	3·2 (62)	0·11 (880)
	0·3	2·0	22 (27)	3 (200)
	0·6	2·0	22 (26)	3·4 (207)

* Figures within parentheses indicate the number of plaques that were analysed for the presence of gC.
† An average value of 0·4% was determined in six independent transfection experiments.

Table 2. *Transfer of the HSV-1 KOS B6 epitope to HSV-1 ANG path**

	B6 donor fragment	Total number of plaques analysed	B6$^+$ plaques syn	B6$^+$ plaques syn$^+$
Expt. 1	*Bam*HI G (pLH50)	1050	22	71
2	*Bam*HI G (pLH50)	1090	49	35
3	*Bam*HI G (pLH50	1110	12	20
Expt. 1	*Sst*I (pUC19K1)	2775	20	0
2	*Sst*I (pUC19K1)	1000	6	0
Expt. 1	*Sst*I–*Sal*I (pUC19K2)	1000	4	0

* In all experiments recipient HSV-1 ANG path DNA (0·5 μg) was cotransfected with 3 μg of plasmid DNA. Plaques induced by progeny virus of the transfection were analysed by the black plaque assay using the monoclonal antibody B6.

path. In numerous experiments involving transfection of genomic DNA of this virus strain, no spontaneous variant was detected that would bind the B6 antibody. This antibody can be used as a tool to monitor the exchange of gB gene sequences between the KOS and the ANG strains.

The mutation determining the syn phenotype of HSV-1 ANG path maps to the gB gene

HSV-1 ANG path causes cell fusion, in contrast to HSV-1 KOS which is of the syn$^+$ phenotype. If the mutation determining the syn phenotype of HSV-1 ANG path is a syn 3 mutation (Spear, 1984) replacement of ANG gB sequences by corresponding KOS gB sequences should result in a reversion of the plaque phenotype. In order to test this possibility, cotransfections were carried out with genomic HSV-1 ANG path DNA and the cloned *Bam*HI G restriction fragment derived from HSV-1 KOS. The monoclonal antibody B6 was used to screen the virus progeny for B6$^+$ recombinants, i.e. for the transfer of HSV-1 KOS gB sequences to the ANG path strain. In the results listed in Table 2, 3 to 9% of the virus progeny carried the KOS-specific epitope recognized by the B6 antibody. The majority of these B6$^+$ recombinants displayed the non-fusing (syn$^+$) phenotype (Fig. 3). It can be concluded that the mutation leading to the syn phenotype of HSV-1 ANG path is located in the gB gene.

The fact that there are other B6$^+$ recombinants which still display the capacity to fuse cells (syn phenotype) can be explained by assuming that in these cases only limited parts of the gB gene specifying the B6 epitope were transferred. It is known that in HSV-1 HFEM *ts*B5, the syn 3 locus maps to the gB gene region encoding the C-terminal domain of the gB polypeptide, which extends into the cytoplasm of infected cells. The B6 epitope can be assumed to be located in the N-terminal domain which is exposed to the exterior of infected cells. Hence, it is reasonable to expect that not all of the B6$^+$ recombinants would be of the syn$^+$ phenotype.

The separation on the gB gene of sequences coding for the B6 epitope and for the syn 3 locus could be demonstrated using defined subfragments of *Bam*HI G. Cotransfection with a DNA

Table 3. *Transfer of the HSV-1 KOS B6 epitope to the gC⁻ mutant C18**

	Total number of plaques analysed	B6⁺ plaques	
		syn	syn⁺
Expt. 1	577	32	0
Expt. 2	750	25	0
Expt. 3	534	14	0

* C18 recipient DNA (0·5 μg) was cotransfected with pLH50 DNA (3 μg). Progeny virus was used for the induction of plaques.

fragment containing essentially the complete coding sequence for gB except for part of its cytoplasmic domain at the C terminus resulted in the generation of B6⁺ syn recombinants (Table 2). The fragment used was a cloned *Sst*I subfragment derived from the KOS *Bam*HI G fragment (Fig. 1). These data indirectly confirm the location of the syn 3 locus which so far rests on studies involving HSV-1 HFEM and derivatives of this strain (Ruyechan *et al.*, 1979; DeLuca *et al.*, 1982; Kousoulas *et al.*, 1984).

In other cotransfection experiments, a *Sal*I subfragment of the *Sst*I fragment was used which contains sequences coding for the N-terminal signal peptide and for 292 amino acids at the N terminus of gB (Fig. 1). As expected, only B6⁺ recombinants which displayed the parental syn phenotype were obtained (Table 2). It can be concluded that alterations within the 292 amino acid stretch, which is part of the surface domain of gB (696 residues; Bzik *et al.*, 1984; Pellett *et al.*, 1985), influence the recognition of gB by the monoclonal antibody B6.

Transfer of gB^{KOS} sequences to the HSV-1 ANG path gC⁻ mutant C18

Cotransfection of genomic C18 DNA with the same cloned *Bam*HI G fragment used in cotransfection with genomic DNA of the gC-positive parental strain also resulted in the generation of B6⁺ recombinant virus. However, all recombinants were still capable of inducing cell fusion (Table 3). By comparison with the above experiments with the gC⁺ HSV-1 ANG path DNA, one would expect that about two of three B6⁺ recombinants would carry sequences specifying both the B6 epitope and the C-terminal domain of the KOS gB polypeptide. One possible explanation for the absence of syn⁺ recombinants among the B6⁺ recombinants would be that absence of gC expression somehow antagonizes expression of the syn⁺ phenotype (Manservigi *et al.*, 1977). In order to test this hypothesis, five individual B6⁺C⁻ recombinants were converted to the gC⁺ phenotype both by spontaneous reversion and by marker transfer involving the *Sal*I T fragment which carries the KOS gC gene. Results indicated no concomitant conversion to the gC⁺ phenotype and the syn⁺ plaque morphology. In summary, a function of gC that would suppress syncytium formation was not detected.

Is there a second mutation in the C18 mutant affecting the syn phenotype?

The failure to obtain B6⁺gC⁻ syn⁺ recombinants following cotransfection of genomic gC18 DNA with the KOS *Bam*HI G fragment could also be explained by a second mutation in C18 that affects plaque morphology. However, by reversing the sequence of genetic manipulations described so far, it could be shown that this is not the case. First, two types of gC-positive revertants were derived from C18: (i) spontaneous revertants and (ii) revertants arising in marker transfer experiments involving cotransfection of genomic C18 DNA with the HSV-1 KOS *Sal*I T fragment. In the following step genomic DNA of individual revertants was co-transfected with the KOS *Bam*HI G fragment. In all cases syn⁺ and syn B6⁺ recombinants were obtained in a ratio similar to that observed in cotransfection with genomic HSV-1 ANG path DNA (Table 4). Therefore, it can be concluded that C18 does not carry a mutation relevant for the syn phenotype which is absent from the parental strain.

Pathogenicity of B6⁺ recombinants

Glycoproteins in general can be regarded as factors determining the pathogenicity of a given virus strain. They elicit immune responses and in turn are targets for the induced responses. It is

Table 4. *Transfer of the HSV-1 KOS B6 epitope to gC⁺ derivatives of mutant C18**

Recipient DNA	Total number of plaques analysed	B6⁺ plaques	
		syn	syn⁺
C18 (gC⁺) rec1†	1500	5	21
C18 (gC⁺) rec2	2538	13	64
C18 (gC⁺) rev1	1435	22	27
C18 (gC⁺) rev2	1070	12	12

* The indicated recipient DNA was cotransfected with the HSV-1 KOS *Bam*HI G fragment. Plaques induced by progeny virus were analysed.

† Two spontaneous gC^{KOS} gC⁺ recombinants (rec) and two revertants (rev) of mutant C18 served as a source of genomic DNA.

Table 5. *Pathogenicity of virus clones derived from HSV-1 ANG path**

No.	Subclones	Transfection clones	Cotransfection clones†	
			B6⁻	B6⁺
1	0/4‡	0/4	0/4	0/4 (syn⁺)
2	4/4	0/4	0/4	0/4 (syn⁺)
3	2/4	2/4	0/4	0/4 (syn⁺)
4	4/4	1/4	3/4	0/4 (syn⁺)
5	4/4	4/4	1/4	0/4 (syn⁺)
6	4/4	3/4	1/4	0/4
7	1/4	2/4	1/4	0/4
8	4/4	3/4	4/4	1/4
9	4/4	3/4	1/4	2/4
10	4/4	3/4	3/4	1/4
11	4/4	2/4		
12	4/4	2/4		
13	4/4	4/4		
HSV-1 ANG path control	4/4	3/3	4/4	

* The pathogenicity of individual clones was determined. Groups of four DBA/2 mice were infected i.p. at a dose of 2×10^6 p.f.u./animal.

† Virus clones were established from the *Bam*HI G cotransfection experiments listed in Table 2.

‡ No. of dead mice per group.

therefore conceivable that the transfer of a glycoprotein gene from an apathogenic strain to a pathogenic strain might result in a concomitant change of pathogenicity. In the mouse model, the two HSV-1 strains, ANG path and KOS, differ markedly in pathogenicity (Kaerner *et al.*, 1983; Schröder *et al.*, 1983). HSV-1 ANG path had been selected for its capacity to cause lethal encephalitis when injected i.p. By the same route of infection HSV-1 KOS is apathogenic even when administered at the highest doses applicable. The possibility that gB derived from strain KOS might confer apathogenicity to the ANG path strain was tested by i.p. infection of DBA/2 mice with individual B6⁺ recombinant clones that were obtained in the above marker transfer experiments. Infection of the animals was carried out at a dose of 2×10^6 p.f.u. which is considerably higher than the established LD_{50} for the pathogenic ANG strain (about 1×10^2 p.f.u.; Kaerner *et al.*, 1983). Out of 10 B6⁺ recombinants tested, seven proved to be apathogenic (Table 5). This group of 10 B6⁺ recombinants was compared to the following control groups: (i) 13 subclones derived directly from HSV-1 ANG path, (ii) 13 subclones derived from the progeny of a transfection with HSV-1 ANG path DNA, and (iii) 10 B6⁻ clones derived from the cotransfection of HSV-1 ANG path DNA with the cloned *Bam*HI G fragment. The number of clones that proved to be apathogenic at the virus dose used was one of 13 for the subclones established directly, two of 13 for the transfection subclones, and three of 10 for the B6⁻ cotransfection clones (Table 5, columns 1, 2 and 3). A similar increase in number is found when clones of reduced pathogenicity are compared. It can be concluded that spontaneous

Table 6. In vitro *replication of cotransfection clones*

B6⁻ clone no.*	Time after infection (h) 16	24	32	B6⁺ clone no.*	Time after infection (h) 16	24	32
(1)	$3{\cdot}8 \times 10^7$	$9{\cdot}8 \times 10^7$	$1{\cdot}4 \times 10^8$	(1)	$7{\cdot}2 \times 10^7$	$1{\cdot}7 \times 10^8$	$2{\cdot}0 \times 10^8$
(2)	$6{\cdot}9 \times 10^7$	$1{\cdot}1 \times 10^8$	$2{\cdot}3 \times 10^8$	(2)	–	$6{\cdot}3 \times 10^7$	$1{\cdot}0 \times 10^8$
(3)	$1{\cdot}3 \times 10^7$	$1{\cdot}3 \times 10^7$	$2{\cdot}3 \times 10^7$	(3)	$3{\cdot}4 \times 10^7$	$9{\cdot}0 \times 10^7$	$9{\cdot}0 \times 10^7$
4	$1{\cdot}0 \times 10^8$	$1{\cdot}1 \times 10^8$	$2{\cdot}1 \times 10^8$	(4)	$1{\cdot}2 \times 10^8$	$2{\cdot}7 \times 10^8$	$2{\cdot}9 \times 10^8$
5	$6{\cdot}6 \times 10^7$	$1{\cdot}2 \times 10^8$	$2{\cdot}5 \times 10^8$	(5)	$1{\cdot}0 \times 10^8$	$2{\cdot}0 \times 10^8$	$2{\cdot}3 \times 10^8$
6	$5{\cdot}2 \times 10^7$	$8{\cdot}8 \times 10^7$	$1{\cdot}2 \times 10^8$	(6)	$1{\cdot}0 \times 10^8$	$1{\cdot}3 \times 10^8$	$1{\cdot}7 \times 10^8$
7	$4{\cdot}0 \times 10^7$	$5{\cdot}4 \times 10^7$	$8{\cdot}8 \times 10^7$	(7)	$1{\cdot}6 \times 10^7$	$6{\cdot}4 \times 10^7$	$1{\cdot}2 \times 10^8$
8	$8{\cdot}6 \times 10^7$	$9{\cdot}0 \times 10^7$	$1{\cdot}6 \times 10^8$	8	$1{\cdot}2 \times 10^8$	$1{\cdot}4 \times 10^8$	$2{\cdot}3 \times 10^8$
9	$3{\cdot}1 \times 10^7$	$5{\cdot}0 \times 10^7$	$8{\cdot}4 \times 10^7$	9	$1{\cdot}0 \times 10^8$	$1{\cdot}4 \times 10^8$	$2{\cdot}0 \times 10^8$
10	$6{\cdot}6 \times 10^7$	$1{\cdot}2 \times 10^8$	$1{\cdot}8 \times 10^8$	10	$7{\cdot}0 \times 10^7$	$1{\cdot}3 \times 10^8$	$1{\cdot}7 \times 10^8$
HSV-1 ANG path control	$1{\cdot}3 \times 10^8$	$2{\cdot}0 \times 10^8$	$2{\cdot}9 \times 10^8$				

* The numbering of clones is the same as that used in Table 5. (Apathogenic clones in parentheses.)

† Cultures of 2×10^6 RC-37 cells in 60 mm Petri dishes were infected with the individual virus clones at a m.o.i. of 4 p.f.u./cell. Infectious titres were determined after two cycles of freezing and thawing.

apathogenic variants can be derived from HSV-1 ANG path and that the frequency at which such variants arise is increased by the transfection procedure, which is known to be mutagenic (Calos *et al.*, 1983). The difference between transfection and B6⁻ cotransfection clones (the latter being on average less pathogenic) is puzzling. Considering a transfer frequency of the B6 marker of less than 10%, it seems unlikely that the comparatively low pathogenicity of B6⁻ cotransfection clones could be attributed solely to the transfer of some other *Bam*HI G sequences not specifying the B6 epitope. In summary, however, the data of Table 5 indicate that the KOS *Bam*HI G fragment may carry a determinant of peripheral pathogenicity of HSV-1.

In order to exclude poor growth as a trivial explanation for apathogenicity all virus clones established after cotransfection of HSV-1 ANG path DNA with the cloned KOS *Bam*HI G fragment were assayed for their capacity to replicate *in vitro*. Progeny virus yields (intra- and extracellular virus) obtained 16, 24 and 32 h after infection are listed in Table 6. The values display a similar distribution both for the apathogenic and for the pathogenic clones with an average mean at 32 h p.i. of $1{\cdot}5 \times 10^8$ and $1{\cdot}8 \times 10^8$ p.f.u./ml respectively. Differences in the growth properties of individual clones therefore do not appear to reflect the differences in pathogenicity.

DISCUSSION

In cotransfection experiments with genomic HSV-1 ANG path DNA and the 8·0 kb *Bam*HI G fragment derived from HSV-1 KOS we obtained ANG/gB^{KOS} recombinants. These were screened for their ability to react with antibody B6 which selectively recognizes gB^{KOS} but not gB^{ANG}. Concomitantly, the majority of B6-positive recombinants displayed the syn$^+$ phenotype. It can be concluded that the syn phenotype of HSV-1 ANG path results from a mutation in the syn3 locus. No other syn mutations have been mapped to the *Bam*HI G fragment (Spear, 1984). The B6-positive recombinants may have taken up either the complete gB (KOS) gene or only that part to which the B6 epitope maps. The finding that a minority of B6-positive recombinants still display the syn phenotype can therefore be explained by assuming that in these cases only external parts of the glycoprotein gene carrying the B6 epitope were exchanged and not the part at the C terminus known to comprise the domain responsible for syncytium formation (Ruyechan *et al.*, 1979; DeLuca *et al.*, 1982; Kousoulas *et al.*, 1984).

Similar marker transfer experiments carried out with genomic DNA of the spontaneous gC$^-$ ANG path mutant (C18) also yielded B6-positive recombinants; however, no change in the syn phenotype was observed. All recombinants displayed the fusion-inducing phenotype of strain ANG. The possibility was excluded that the failure to detect B6$^+$ gC$^-$ syn$^+$ recombinants is due to a second syn mutation present in the mutant C18 but not in the parent HSV-1 ANG path strain. A more complex explanation implies a functional interaction between gB and gC so that

gB plays a critical role in the promotion of cell fusion whereas gC can act to inhibit this process. This hypothesis had been originally suggested for HSV-1 (MP), a strain which is gC^- and syn, but was refuted later (Manservigi *et al.*, 1977; Lee *et al.*, 1982; Spear, 1984). If gC possesses a fusion-suppressing activity, reversion of $B6^+gC^-$ recombinants to the gC^+ phenotype should have resulted in a concomitant change to the non-fusing phenotype in some cases. However, this was not the case. Furthermore, the application of the gB–gC interaction hypothesis to our system would imply that gB^{KOS} in the absence of gC expression possesses the capacity to fuse cells. This possibility appears unlikely. In a detailed analysis of gC^- mutants derived from the KOS strain carried out by Holland *et al.* (1984*b*) and Homa *et al.* (1986) 101 of 103 mutants still displayed the parental syn^+ phenotype.

The fact that the C18 mutant cannot be converted to the syn^+ phenotype could also be explained by an alternative hypothesis which, like the gB–gC interaction hypothesis, implicates gB and gC plus additional, undefined viral gene products. In our hypothesis, in a certain genetic background that has yet to be defined, some forms of gB cannot be tolerated in the absence of gC expression. This hypothesis assumes that $B6^+$ recombinants are formed upon cotransfection of C18 DNA with the KOS *Bam*HI G fragment but that these are not viable. Recombinants carrying only the B6 epitope and other parts of the KOS gB surface domain would be viable whereas those ANG/gB^{KOS} recombinants carrying the surface domain and the internal domain of gB^{KOS} would not be viable.

It is interesting to look at the above $gB–gC^-$ incompatibility hypothesis from another point of view i.e. the transition from the gC^+ to the gC^- phenotype. Our hypothesis would imply that under certain genetically defined conditions the loss of gC expression is a lethal event. Its loss would only be tolerable provided a certain configuration at the C-terminal domain of gB is present. If only the cell-fusing function of this domain is relevant, other syn loci may also counteract the potential lethality of the absence of gC expression. The concomitant loss of gC expression and conversion to the syn phenotype which had been found in the analysis of a number of spontaneous syn variants (Heine *et al.*, 1974; Cassai *et al.*, 1976; Spear, 1984) thus could be explained by a selection pressure towards the syn phenotype.

The question whether individual disease patterns induced by different HSV-1 strains are influenced by the ability to fuse cells or by gC expression has been addressed by a number of laboratories. However, no consistent change in neurovirulence has been found when phenotypic variants were compared to the respective parental strains (Wheeler, 1964; Cassai *et al.*, 1976; Shimizu *et al.*, 1978; Dix *et al.*, 1983). The HSV-1 ANG path strain used in this study is pathogenic when injected i.p. Its development and selection from the apathogenic parental strain HSV-1 ANG was accomplished by enforced animal passages (Kaerner *et al.*, 1983).

The observation reported here that one of 13 subclones of HSV-1 ANG path was apathogenic and two displayed a reduced pathogenicity is difficult to explain by the argument that only one gene determines pathogenicity. Several genes may determine pathogenicity and an alteration in only one of these genes may be sufficient to reduce neurovirulence. Consistent with this hypothesis, the transfection procedure itself, which is known to induce mutations, resulted in the occurrence of even more apathogenic subclones. At the highest frequency, however, apathogenic subclones were found among B6-positive recombinants resulting from the exchange of gB^{ANG} sequences with gB^{KOS} sequences mediated by the HSV-1 KOS *Bam*HI G fragment. This conversion to apathogenicity apparently did not reflect a reduced capacity of the subclones to replicate in infected cells. It could be concluded that there are domains of gB^{KOS} that determine the apathogenicity of HSV-1 KOS and the HSV-1 ANG path/gB^{KOS} recombinants. However, functions other than gB specified by the *Bam*HI G fragment may also be considered to determine pathogenicity.

In a different approach with an apathogenic HSV-1 strain as recipient for virulence functions from a pathogenic strain, Thompson *et al.* (1986) identified the *Hin*dIII A fragment (map units 0·25 to 0·53) as a genome region that can confer an enhanced peripheral neurovirulent character. The identification of part of *Hin*dIII A as a region carrying pathogenicity determinants could indicate that in both systems the same gene functions are involved. From our results we tend to assume that gB is one determinant of peripheral neurovirulence of HSV-1.

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