SYN STRAINS HSZP AND ANG OF HERPES SIMPLEX VIRUS TYPE 1 DO NOT CONTAIN MUTATIONS IN THE REGIONS OF UL53 GENE RELEVANT TO SYNCYTIUM FORMATION

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Summary. – Parallel sequencing of UL53 gene of four strains of herpes simplex virus type 1 (HSV-1), two of which (HSZP and ANGpath) were of the syn phenotype while another two (KOS and 17) were of the non-syn phenotype, showed in three strains amino acid mutations unrelated to the already described syn[1] glycoprotein K (gK) mutations (Dolter *et al.*, 1994). The only mutations which altered encoded amino acids were found in strains HSZP (Gln to Arg at position 198) and ANGpath (Val to Ile at position 137). Both mutations were localised outside of the two mutation clusters suspected for affecting syncytium formation. In addition, a CG/GC variation was found at positions 245-246 and 669-670. These compressions affected three codons altering amino acids (aa) 82 (Cys or Ser), 223 (Met or Ile) and 224 (Leu or Val), respectively.

Key words: herpes simplex virus type 1; strain HSZP; strain ANGpath; syncytium formation; UL53 gene; glycoprotein K; nucleotide sequencing

Introduction

Strains HSZP and ANGpath of HSV-1 which cause formation of large polykaryocytes (syncytia), *in vitro* , contain mutations at aa 857 or 858 (HSZP), 854 or 855 (ANGpath), namely in the region II of the cytoplasmic domain of glycoprotein B (gB) (Walev *et al.*, 1994; Lingen *et al.*, 1995; Rajčáni *et al.*, 1996; Saharkiz-Langroodi and Holland, 1997). However, the majority of syn mutations occurs within DNA map coordinates 0.735 – 0.740 (Bond and Person, 1984; Pogue-Geile *et al.*, 1984), later on characterised as the UL53 gene (Debroy *et al.*, 1985; Pertel and Spear, 1996). The latter codes for a multiple transmembrane spanning gK (MacLean *et al.*, 1991; Hutchinson *et al.*, 1992), whose precursor polypeptide (pgK) contains 338 aa with two N-glycosylated Asn at positions 48 and 58 (Ramaswamy and Holland, 1992). gK is expressed in low levels in HSV-1-infected cells. According to Hutchinson *et al.* (1995), gK accumulates in nuclear and endoplasmic reticulum membranes but not in cytoplasmic membranes, and is not processed by the Golgi apparatus. These findings are consistent with those of Ramaswamy and Holland (1992) and Hutchinson *et al.* (1992) quoting that the 40K gK polypeptide contains only immature N-linked mannose core oligosaccharides and is not present in the virion envelope. gK facilitates and/or regulates the nucleocapsid envelopment and, more importantly, the translocation of enveloped particles from the perinuclear space across the cytoplasm to the cell surface. The actively replicating cells can partially compensate for the envelopment of the gK-deficient virus but not for the egress deficiency (Hutchinson and Johnson, 1995; Jayachandra *et al.*, 1997). gB and gK are the only so far known glycoproteins which are affected by syncytial mutations. On the basis of this knowledge, in order to compare the gK nucleotide sequences of syn[3] strains HSZP and ANGpath whose syn phenotype had been assigned to gB (Rajčáni *et al.*, 1996), we sequenced UL53 gene of strains HSZP, ANGpath, KOS and 17 in parallel and compared the obtained sequences.

Abbreviations: aa = amino acid; CPE = cytopathic effect; EDTA = ethylenediamine tetraacetate; gB, gK = glycoproteins B, K; HSV-1 = herpes simplex virus type 1; i.c. = intracerebral; nt = nucleotide; PCR = polymerase chain reaction; pgK = precursor gK polypeptide; SDS = sodium dodecyl sulfate

Materials and Methods

HSV-1 strains. HSZP strain (Szántó, 1960) underwent passaging in rabbit lung ZP, chick embryo and Vero cells. This strain forms extremely large giant cells in culture. Strain KOS originated from Dr. J.L. Melnick, WHO Collaborating Center for Virus Reference and Research, Houston, TX, USA. Strain ANGpath derived from non-pathogenic strain ANG was obtained from the Institute for Virus Research, Cancer Research Center, Heidelberg, Germany. Strain 17 was kindly provided by Dr. V. Preston, MRC Virology Unit, Institute of Virology, Glasgow, UK. Stock preparations of all these strains were obtained by culturing in Vero cells in Eagle's Basal Medium supplemented with 2% calf serum.

Purification of viral DNAs. When Vero cell cultures infected with the HSV-1 strains of concern showed an extensive cytopathic effect (CPE), the cells were scraped off, pelleted by centrifugation at 800 x g for 10 mins, and the resulting supernatant was centrifuged at 60,000 x g for 3 hrs at 4°C. The obtained pellet contained the virus. The cells pelleted by the first centrifugation were treated with an hypotonic buffer containing Nonidet P-40 at ambient temperature for 15 mins and the cell-released virus was pelleted by ultracentrifugation. Both virus pellets were pooled and subjected to linear sucrose density gradient (5 – 55%) centrifugation at 80,000 x g for 1 hr at 4°C. The obtained virus band was resuspended in TE buffer (10 mmol/l Tris.HCl and 1 mmol/l ethylenediamine tetraacetate (EDTA), pH 8.0) and treated with 200 μg/ml proteinase K and 1% sodium dodecyl sulfate (SDS). Virus DNA was isolated by phenol/chloroform extraction and ethanol precipitation.

Amplification and nucleotide sequencing. A 1303 bp fragment spanning from nucleotide (nt) 111,955 to nt 113,258 (McGeoch *et al.*, 1988) was amplified by polymerase chain reaction (PCR) from 10 ng DNA of each strain using the primers SP21 (downstream) and SP24 (reversed upstream) (Table 1). The reaction mixture (100 μl) contained 0.2 mmol/l each of dNTPs (Perkin Cetus Elmer), 0.5 mmol/l each of two primers, 1.5 mmol/l $MgCl_2$ and 2.5 U of Taq DNA polymerase (Boehringer Mannheim). After denaturation at 95°C for 7 mins, the mixture was cycled 35 times (95°C for 2 mins, 52°C for 1.5 min and 72°C for 2 mins with a 3 sec prolongation at each cycle). The PCR products were purified with a Wizard™PCR Preps DNA Purification System (Promega) and sequenced (Sanger *et al.*,1977) using a TAQuence Cycle Sequencing Kit (USB). The primers designed for bidirectional sequencing were end-labelled with [gamma-^{32}P]dATP (3,000 Ci/mmol, Amersham) and used in sequencing reaction with 30 cycles (95°C for 40 secs, 55°C for 40 secs and 72°C for 90 secs) in the presence of 7-deaza-dGTP. All sequences were aligned and compared with the database sequence of strain 17 (Acc. No. K 03541) using DNASIS software programme (Pharmacia).

Results and Discussion

Out of five mutations found in the HSZP UL53 gene as compared with the same gene of the prototype strain 17, only one (A to G at nt 112,771) caused an amino acid change (Gln to Arg at aa 198) in the putative polypeptide (Table 2). This mutation was HSZP-specific, i.e. it did not occur in any other strain compared. Out of six mutations in the ANGpath UL53 gene, only one (G to A at nt 112,587) caused an amino acid change (Val to Ile at aa 137) in the putative polypeptide. Again, this mutation was ANGpath-specific. UL53 genes of strains HSZP and ANGpath differed from that of strain KOS in four nucleotides. Mutations of T (strains HSZP, ANGpath and 17) to C (strain KOS) at nt 112,480, and of G (strains HSZP, ANGpath and 17) to A (strain KOS) at nt 113,103 caused changes of coding properties of codons 101 (Met to Thr) and 309 (Val to Met), respectively. Both these mutations were KOS-specific.

The syn strains HSZP and ANGpath, whose UL53 gene sequences are presented here, share one common mutation (G to A at nt 112,757) which, however, does not result in an amino acid change. The abovementioned ANGpath-specific mutation was located just at the end of the second hydrophobic domain (Ramaswamy and Holland, 1992). The HSZP-specific mutation mentioned above which altered the encoded amino acid was located between the two hydrophobic domains far from any of the UL53 syn[1] mutations described (Dolter *et al.*, 1994). The latter authors found eight mutations (at aa 33, 40, 85, 99, 111, 121, 304 and 310) in syn strains derived from KOS strain. The mutation at aa 40 was detected

Table 1. Primers used for sequencing of UL53 gene

Primer	Nucleotide position[a]	Sequence
SP 21	111 955 – 111 975	5'-TGG GTC CTC CTA CAG CTA GTC-3'
gK F2	112 272 – 111 292	5'-AGT CCG CTG CAC CGA TGT ATT-3'
gK F3	112 565 – 112 585	5'-GTG GTT CCT GTA TCT GGC GTT-3'
gK F4	112 871 – 112 891	5'-CAT CGT CGG CAC CGC TTT CAT-3'
SP 24	113 258 – 113 238	5'-CCA AGA CAG GAC AGT TTC CAA-3'
gK R5	112 971 – 112 951	5'-TCA GGC CGA TGG TGG AGA CAA-3'
gK R6	112 701 – 112 793	5'-GGA ACA CGC TCG ATA CGA TGC-3'
gK R7	112 427 – 112 407	5'-GTA GCA GAT ATG GGC GTG GTT-3'

[a]According to McGeoch *et al.* (1988).

Table 2. Mutations of UL53 gene of HSV-1 strains HSZP, ANGpath and KOS as compared to the reference strain 17[a]

HSV-1 strain	Nucleotide change	Nucleotide number	Codon change	Amino acid change	Amino acid position
HSZP	G to A	112 757			
	A to G	112 771	CAG to CGG	Gln to Arg	198
	A to G	112 973			
	G to T	113 057			
	C to T	113 075			
ANGpath	T to C	112 472			
	G to A	112 587	GTC to ATC	Val to Ile	137
	G to A	112 757			
	G to T	112 898			
	G to T	113 057			
	C to T	113 075			
KOS	T to C	112 472			
	T to C	112 480	ATG to ACG	Met to Thr	101[b]
	C to T	113 019			
	C to T	113 075			
	C to G	113 070			
	G to A	113 103	GTG to ATG	Val to Met	309[b]

[a]The sequence of reference strain 17 according to McGeoch *et al.* (1988).
[b]The same amino acids described by Dolter *et al.* (1994).

also in UL53 gene of MP strain (Pogue-Geile and Spear, 1987), the prototype strain of the syn[1] phenotype.

Comparing the gK database nucleotide sequences of strain KOS, its syn mutants and strain MP, we were struck by discrepancies at positions 245-246 and 669-670 of the UL53 gene sequence. As a result, different amino acids may occur at the positions 82 (Cys or Ser corresponding to codons TGC or TCG, respectively), 223 (Met or Ile corresponding to codons ATG or ATC) and 224 (Leu or Val corresponding to codons CTA or GTA, respectively) of pgK. Alteration of the putative amino acids at positions 223-224 might influence the hydrophobicity of a relative long part of gK polypeptide and consequently influence its function. However, we have noticed that in the reported UL53 gene sequences, in case they had been obtained by parallel sequencing at the same time and in an identical manner, the nucleotides at the two particular positions had the same order (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987; Dolter *et al.*, 1994; Pertel and Spear, 1996). The only exceptions were recombinants R15 and R19 (Ben-Hur *et al.*, 1987; Moyal *et al.*, 1992). Because we have sequenced UL53 gene of strains HSZP and ANGpath along with that of strains KOS and 17 using deaza-dGTP known to provide a better band distinction, we have seen in our sequencing gels all the four nucleotide bands in question in comparable positions (Figs. 1 and 2). The nucleotide order in the downstream strand (in the sense of transcription) could be easily determined at positions 245-246 from the forward primer F2 (Fig. 1A) as well as at positions 669-670 from the forward primer F3 (Fig. 2A) as G, C in accord with the published sequence of strain 17 (McGeoch *et al.*, 1998). In the complementary strand, the bands of these

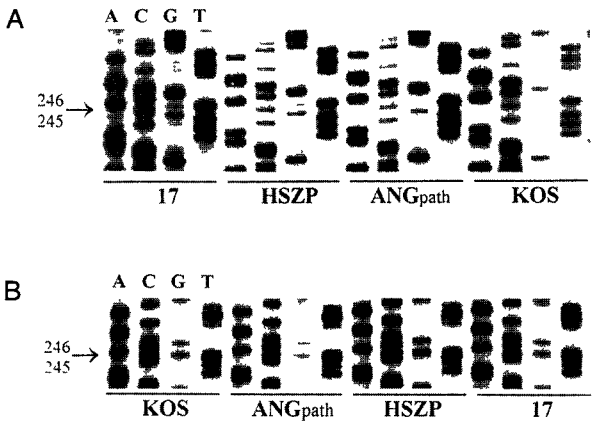

Fig. 1
Compressions which might influence reading of the UL53 gene nucleotide sequence at positions 245 and 246 with consequent changes in the amino acid sequence
Reading from forward primer F2 (A) and reverse primer R6 (B).

nucleotides read from the reverse primers R6 (Fig. 1B) and R5 (Fig. 2B) were very close to each other making their distinction really difficult and thus favouring the reading of the opposite nucleotide order. From these reasons we considered more correct that order of nucleotides which was in accord with the sequence of strains 17 and KOS as determined by Dolter *et al.* (1994).

The differences in amino acid sequence of pgK at positions 82, 223 and 224 were considered responsible for differ-

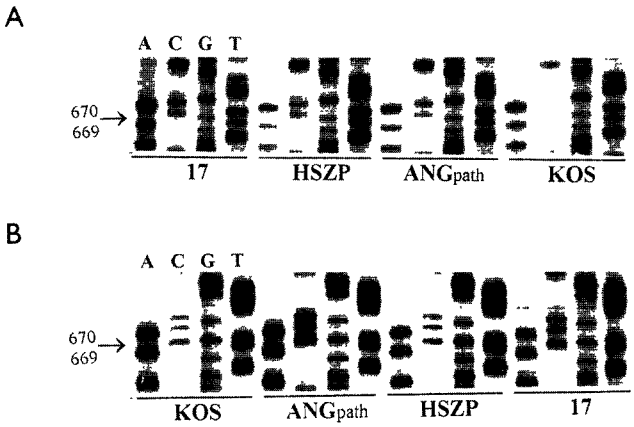

Fig. 2
Compressions which might influence reading of the UL53 gene nucleotide sequence at positions 669 and 670 with consequent changes in the amino acid sequence
Reading from forward primer F3 (A) and reverse primer R5 (B).

ent neuroinvasivity of recombinants R15 and R19 after intracerebral (i.c.) inoculation to mice. The connection between these putative amino acid differences and the i.c. pathogenicity, which arises from comparison of amino acids in these positions in strains R15, R19, 17 and KOS, and from comparison of pathogenicity of these strains after i.c. administration (Moyal *et al.*, 1992), should be reconsidered in the light of the abovementioned interpretation. In case of recombinants R15 and R19, it is difficult to accept a conclusion that they differ from each other in amino acid in question without simultaneous sequencing of all sequences of concern (including controls) on the same gel, because sequencing artefacts are frequent in the abovementioned positions.

Summing up, the observed alterations in UL53 gene of syn^3 gB mutants HSZP and ANGpath are irrelevat to those so far found in the syn^1 gK mutants at nt 33-121 and 304-310. Their possible relationship to the syn phenotype or gK function remains to be established.

The UL53 nucleotide sequences of strains HSZP and ANGpath are accessible at the GenBank Database under Acc. Nos. Z 82036 and Z 82037, respectively.

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