

# The *BgIII*-N fragment of herpes simplex virus type 2 contains a region responsible for resistance to antiviral effects of interferon

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Double infection with two interferon (IFN)-sensitive strains of herpes simplex virus (HSV), HSV-1(17syn) and HSV-2(UW268), showed reduced inhibition of virus growth by IFN. Intertypic recombinants with IFN resistance were obtained from the doubly infected cultures. These results indicate that HSV IFN resistance is controlled by at least two genetic regions. Restriction endonuclease analysis demonstrated that the recombinants were similar to HSV-2 in their genomic structure but the *Bam*HI-A, *BgIII*-I and *BgIII*-N fragments of HSV-2 were commonly lost in the recombinants, suggesting that any of these fragments could be associated with HSV-2 IFN resistance. We cloned these fragments and *Bam*HI-

E, which overlaps *BgIII*-N, from an IFN-resistant HSV-2 strain, HSV-2(G), and examined each fragment for its ability to rescue IFN resistance of HSV-2(UW268) by co-transfecting with the HSV-2(UW268) genome. Of the HSV-2(G) fragments, only *BgIII*-N increased plating efficiency of progeny viruses in IFN-treated cells. An IFN-resistant HSV-2 clone was obtained from the *BgIII*-N of HSV-2(G) and HSV-2(UW268) genome co-transfected culture, and a part of *BgIII*-N of HSV-2(UW268) was replaced with that of HSV-2(G) in the HSV-2 clone. Thus, it was concluded that one of the HSV regions encoding IFN resistance is located on the *BgIII*-N fragment of HSV-2.

## Introduction

Interferons (IFNs) have the ability to protect hosts from herpes simplex virus (HSV) infection. Exogenous IFNs reduce the progression of HSV-induced diseases (Pinto *et al.*, 1990; Sanitato *et al.*, 1984) and endogenously produced IFNs play an important role in host defences against HSV infection (Hendricks *et al.*, 1991; Lausch *et al.*, 1991; Stanton *et al.*, 1987; Su *et al.*, 1990). However, the therapeutic effectiveness of IFNs against HSV-induced diseases is considered not as high as expected (Jones *et al.*, 1976; Kaufman *et al.*, 1972; Sugar *et al.*, 1973). In cultured cells, a significant reduction in virus titre by IFNs is observed only when relatively high concentrations are used (Chatterjee *et al.*, 1984; Domke-Opitz *et al.*, 1986; Oberman & Panet, 1988). The ineffectiveness of IFNs is primarily due to the natural resistance of HSV to IFNs (Lausch *et al.*, 1991; Su *et al.*, 1990). This resistance leads to HSV replication and thus to disease development through reduction of anti-HSV effects of IFNs.

Cayley *et al.* (1984) suggested that HSV inhibits the IFN-induced 2'-5'-oligoadenylate synthetase (2-5A)-RNase L pathway by producing a 2-5A analogue. Su *et al.* (1993) reported that one region of the HSV genome containing three partial open reading frames (ORFs), designated UL14, UL15 and UL16, is associated with IFN resistance. However, the mechanism by which HSV abrogates the anti-HSV effects of IFNs is still unclear.

To date, at least two different IFN-induced anti-HSV effects have been reported. One is a reduction of immediate-early (IE) HSV-specific mRNA expression (Klotzbücher *et al.*, 1990; Mittnacht *et al.*, 1988; Oberman & Panet, 1988). The other is a block of synthesis of two HSV glycoproteins (gB and gD) and viral morphogenesis in the late stages of HSV infection (Chatterjee *et al.*, 1985; Chatterjee & Whitley, 1989). These different anti-HSV effects are predictive of at least two HSV genetic regions responsible for IFN resistance.

In the present study, we tested complementation and recombination between IFN-sensitive HSV-1 and HSV-2 strains and determined that at least two genetic regions are associated with HSV IFN resistance. Moreover, we performed marker rescue experiments to physically map one of the coding

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regions and found that the *Bgl*III-N fragment of HSV-2, which was distinct from the region reported by Su *et al.* (1993) contained an IFN-resistance gene. Together with the results of this and previous reports, we would like to discuss the relationship between the HSV-encoded information and IFN resistance.

## Methods

■ **Culture and IFN treatment of cells.** Vero cells were grown in Eagle's minimum essential medium (MEM; Nissui) supplemented with 5% newborn calf serum (NCS; Summit Biotechnology) and maintained in MEM supplemented with 2% NCS at 37 °C. Human retinal pigment epithelium-derived cells, K-1034, (Kigasawa *et al.*, 1994, 1995) were grown in MEM supplemented with 10% foetal bovine serum (FBS; JRH Biosciences) and maintained in MEM supplemented with 5% FBS at 37 °C. K-1034 cells were treated with human IFN- $\beta$  (Toray Industries) by incubating the cell monolayers in the maintenance medium with the desired concentration of IFN from 24 h before infection to the end of the incubation. The specific activity of the IFN was  $8 \times 10^7$  international units (IU)/mg protein.

■ **Virus.** The 17syn<sup>+</sup> strain of HSV-1 [HSV-1(17syn<sup>+</sup>)] (Brown *et al.*, 1973) and its syncytial variant, HSV-1(17syn), and the G and UW268 strains of HSV-2 [HSV-2(G) and HSV-2(UW268), respectively] (Chiang *et al.*, 1970; Ejercito *et al.*, 1968) were used in this study after plaque purification. Plaque purification and infectivity titration of HSV were carried out using the liquid overlay method (Nii, 1969). Virus yield was assayed by titrating infectivity of cell-associated virus, which accounts for approximately 90% or more of total virus. The infected cells were collected by scraping and centrifugation (180 g, 4 °C, 10 min), sonicated in 1 ml serum-free MEM at 4 °C for 10 s, and centrifuged to remove cell debris (180 g, 4 °C, 10 min). Supernatants were stored at -80 °C for 2 or 3 days and titrated for infectivity.

■ **IFN sensitivity assay for individual HSV strains.** Sensitivity of each HSV strain to IFN was assessed by the dye-uptake assay (McLaren *et al.*, 1983) and/or the virus yield reduction assay (Lausch *et al.*, 1991). In the dye-uptake assay, K-1034 cells in a 96-well microtitre plate were treated with IFN at concentrations of 0, 0.1, 1, 10, 100, or 1000 IU/ml and were infected with each HSV strain at an m.o.i. of 0.25 p.f.u. per cell. After 11 days incubation, retention of pinocytotic activity of the cells in each well was determined by measuring the uptake of neutral red dye (Sigma). Dye uptake was assayed by measuring the optical density (OD) of the cell eluate at 540 nm using a multichannel spectrophotometer (Sanko Junyaku). IFN sensitivity of each HSV strain was expressed as a DU<sub>50</sub> value, i.e. the concentration of IFN producing 50% reduction in the dye uptake by the cells. The mean OD of the IFN-untreated and HSV-infected cells was assigned a value of 0% and the IFN-treated and uninfected cells a value of 100%. IFN treatment alone did not reduce dye uptake. The DU<sub>50</sub> value was calculated as described by Finter (1969).

In the virus yield reduction assay, the IFN-treated and -untreated K-1034 cell monolayers were infected with each HSV strain at an m.o.i. of 0.1 p.f.u. per cell and incubated for 2 or 3 days until the IFN-untreated K-1034 cells exhibited maximum cytopathic effects (CPE). Infectivity titre in the cells was assayed as described above.

■ **Complementation test.** The IFN-treated (1000 IU/ml) K-1034 cells were singly infected with HSV-1(17syn) or HSV-2(UW268) at an m.o.i. of 5 p.f.u. per cell, or doubly infected with both strains at an m.o.i. of 5 p.f.u. per cell, respectively (a total m.o.i. of 10 p.f.u. per cell).

Following an additional 24 h incubation, virus yield in each culture was assayed for comparison as described above. Complementation of IFN resistance between the two HSV strains was shown by determining whether virus yield in the doubly infected cells was apparently higher than that in the singly infected cells.

■ **Isolation and restriction endonuclease analysis of IFN-resistant intertypic recombinants.** After propagating twice in IFN-treated (1000 U/ml) K-1034 cells at an m.o.i. of approximately 0.01 p.f.u. per cell to concentrate IFN-resistant viruses, progeny viruses from the doubly infected cultures in the 'complementation test' were plaque-purified at least twice. Virus particles of each clone were partially purified from the infected cells by a combination of low- (800 g) and high- (8000 g) speed centrifugations. Nucleic acids were extracted by the phenol-chloroform method (McCance, 1996) and electrophoresed in 0.8% agarose gel in Tris-acetate buffer (pH 7.6) at 20 V for 20 h after *Bam*HI digestion. Restriction profiles were visualized by ethidium bromide staining and photographed under ultraviolet light. Intertypic recombinants were identified by differences in viral DNA *Bam*HI profiles between the recombinants and their parental HSV strains. Some intertypic recombinants were further analysed by electrophoresing in 0.4% agarose in Tris-acetate buffer (pH 7.6) at 20 V for 30 h after digestion with *Bgl*III. Sensitivities of the recombinants to IFN were examined by the dye-uptake assay.

■ **Marker rescue experiment.** Virions of HSV-2(UW268) and HSV-2(G) were purified from culture supernatants of the infected Vero cells by sucrose gradient centrifugations (Killington *et al.*, 1996). Intact genomic DNAs were extracted from the purified virions by the phenol-chloroform method. HSV-2(G) DNAs were digested with *Bam*HI or *Bgl*III and were electrophoresed as described above. The *Bam*HI-A, *Bam*HI-E, *Bgl*III-I and *Bgl*III-N fragments of HSV-2(G) were then electroeluted from the agarose gels, cloned into pUC19, and transformed into *Escherichia coli* strain JM109. A plasmid DNA containing each DNA fragment was linearized by *Hind*III digestion and was co-transfected with intact genomic DNA from HSV-2(UW268) into Vero cells using the MBS Mammalian Transfection kit (Stratagene). The transfected cultures were incubated until maximum CPE was observed; progeny viruses in the culture supernatants were propagated twice in IFN-treated cells as described above. Cell-associated virus was prepared from the transfected and propagated cultures, respectively, as described above and the number of plaques produced by each sample was assayed on IFN-treated (1000 IU/ml) and untreated K-1034 cells. Plating efficiency was calculated by dividing the plaque number on the IFN-treated cells by the plaque number on the untreated cells.

■ **Isolation of an IFN-resistant HSV-2 clone.** Progeny viruses in the culture co-transfected with the HSV-2(UW268) genome and the *Bgl*III-N fragment of HSV-2(G) were plaque-purified after concentrating IFN-resistant viruses as described above. Sensitivities of the purified HSV-2 clones to IFN were examined by the dye-uptake assay.

■ **Restriction endonuclease analysis of the DNA sequence between ORFs UL40 and UL41.** The DNA sequence between ORFs UL40 and UL41 of HSV-2 was amplified by nested PCR (nPCR). The *Ava*I, *Cfo*I and *Hae*II profiles of the nPCR-amplified DNAs were examined by electrophoresing the nPCR-amplified DNAs on 6% agarose gels in Tris-acetate buffer (pH 7.6) after digestion with each enzyme.

■ **PCR.** The HSV-2 DNAs were extracted from the purified virions by the phenol-chloroform method, and 2 ng of the viral DNA was added to a PCR mixture solution comprised of: 50 mM Tris-HCl (pH 9.2); 14 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1.75 mM MgCl<sub>2</sub>; 20  $\mu$ M dNTP; 50 mU/ml *Taq* polymerase

(Boehringer Mannheim); and 1 ng/ml outer oligonucleotide primers (5'-TCCATCGTGACTCGGCATATCACC-3' and 5'-ACCGACAAACA-CACCAACTTCTTC-3'). Samples were subjected to 30 cycles of 94 °C for 30 s, 58 °C for 2 min, 72 °C for 3 min after an initial denaturation for 7 min at 94 °C. Amplification products were diluted 1000-fold and 10 µl of the diluent was added to the same PCR mixture solution containing the inner primers (5'-CTGGCTAAATTCTGGGACGAGTAG-3' and 5'-GGGGCCGTCGTCAACGATCTGTGA-3') in place of the outer primers. Samples were subjected to the second amplification of the target DNA sequence under the same conditions as described above.

## Results

### Phenotypic complementation of reduced IFN resistance between HSV-1(17syn) and HSV-2(UW268)

IFN sensitivities of four HSV strains used in this study are shown in Fig. 1. The virus yield reduction assay showed that HSV-1(17syn) and HSV-2(UW268) were IFN-sensitive relative to HSV-1(17syn<sup>+</sup>) and HSV-2(G) (Fig. 1*a*). Replication of HSV-1(17syn) and HSV-2(268) was largely inhibited by IFN in a dose-dependent manner. When 1000 IU/ml IFN was used, reductions in yields of HSV-1(17syn) and HSV-2(UW268) were approximately 10 000- and 1600-fold, respectively. HSV-1(17syn<sup>+</sup>) and HSV-2(G) grew well even in the presence of IFN. Reductions in yield of the strains by IFN were less than one-sixteenth at any of the IFN concentrations tested.

In contrast to HSV-1(17syn), the virus yield dose-response curve for HSV-2(UW268) appears to reach plateau or near plateau levels when treated with IFN at 10 IU/ml. The difference in dose-response curve between the two strains may be due to a difference in IFN-sensitivity mutations between the two strains.

The reduced IFN resistance of HSV-1(17syn) and HSV-2(UW268) was also confirmed by the dye-uptake assay (Fig. 1*b*). The DU<sub>50</sub> values of IFN to HSV-1(17syn) and HSV-2(UW268) were at least 34-fold less than those of HSV-1(17syn<sup>+</sup>) and HSV-2(G). Consistency of the results between the two assays also demonstrated that the dye-uptake assay in this study was accurate.

The two IFN-sensitive HSV strains were subjected to complementation tests for IFN resistance (Fig. 2). IFN caused approximately 32- and 20-fold reductions in yield of HSV-1(17syn) and HSV-2(UW268), respectively, when singly infected. The degrees of the virus yield reductions in this experiment are considerably lower than those in Fig. 1(*a*). This difference does not mean that the magnitude of the IFN antiviral effect is critically controlled by the m.o.i. because virus yield reductions by IFN in the cells infected at an m.o.i. of 2.5 and 10 p.f.u. per cell were similar to those at an m.o.i. of 5 p.f.u. per cell (data not shown). Thus, the difference in virus yield reduction between Fig. 1(*a*) and Fig. 2 seems to be due to the difference between the number of virus replication cycles in cultures infected at high m.o.i. and low m.o.i.

Alternatively, when IFN-treated cells were doubly infected with the two HSV strains, virus yields in the cultures were

higher than those in the IFN-treated, singly infected cultures and were comparable to those of the IFN-untreated, singly infected cultures. The increase in virus yield is not due to the m.o.i. since almost the same result was obtained from double infection experiments at a total m.o.i. of 5 p.f.u. per cell. The increase in virus yield after double infection suggests complementation of reduced IFN resistance between HSV-1(17syn) and HSV-2(UW268). The result is not conclusive because of low reductions in virus yield by IFN at the high m.o.i., but is suggestive of control of HSV resistance to IFN by different genetic regions.

### Isolation of intertypic recombinants with restored IFN resistance between HSV-1(17syn) and HSV-2(UW268)

Progeny viruses in the cultures doubly infected with HSV-1(17syn) and HSV-2(UW268) must include too many parental type viruses to be subjected to the following analysis with *Bam*HI, since growth suppression of the parental strains by IFN at high m.o.i. (10 p.f.u. per cell in total) is not so strong (Fig. 2). Alternatively, growth suppression of the parental strains at low m.o.i. (0.1 p.f.u. per cell) is sufficient to concentrate IFN-resistant viruses in a mixed population of IFN-resistant and IFN-sensitive viruses although HSV-2(UW268) is less IFN-sensitive than HSV-1(17syn) (Fig. 1*a*). Therefore, we propagated progeny viruses in the cultures doubly infected with the parental strains twice in the IFN-treated K-1034 cells at low m.o.i. This concentration process had no detectable effect on IFN sensitivity of the parental strains as determined by the virus yield reduction assay. Thus, spontaneous generation of IFN-resistant mutations during the process seems to be minimal.

After propagation, 145 virus clones were plaque-purified and examined for their *Bam*HI profiles. Forty of them had *Bam*HI profiles that differed from those of their parental HSV strains and they were divided into 20 groups according to the profiles. The 20 recombinants were then examined for IFN sensitivity, resulting in 15 recombinants with a leaky phenotype for IFN resistance and five recombinants with apparent restoration of IFN resistance.

IFN sensitivities of the five recombinants were assessed by the dye-uptake method. DU<sub>50</sub> values of IFN to the recombinants were found to be more than 1000 U/ml, which was comparable to those of IFN-resistant HSV-1(17syn<sup>+</sup>) and HSV-2(G) and was at least 34-fold higher than IFN-sensitive HSV-1(17syn) and HSV-2(UW268). The *Bam*HI profiles of the recombinants are shown in Fig. 3(*a*). The recombinants resembled HSV-2(UW268) in *Bam*HI profiles but some bands in HSV-2(UW268) were lost in the recombinants. These results again suggest that IFN-sensitive mutations in HSV-1(17syn) and in HSV-2(UW268) are located in different genetic regions although the possibility that IFN-resistant mutations occur outwith the 'intertypic' DNA sequences cannot completely be excluded.

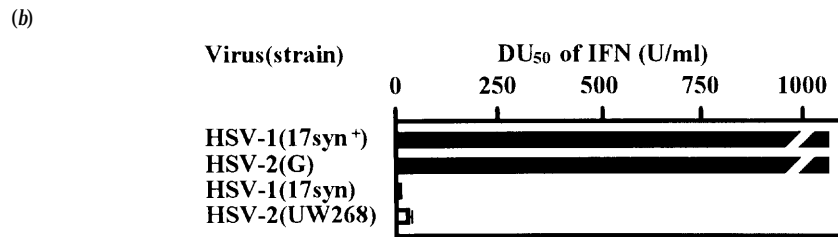
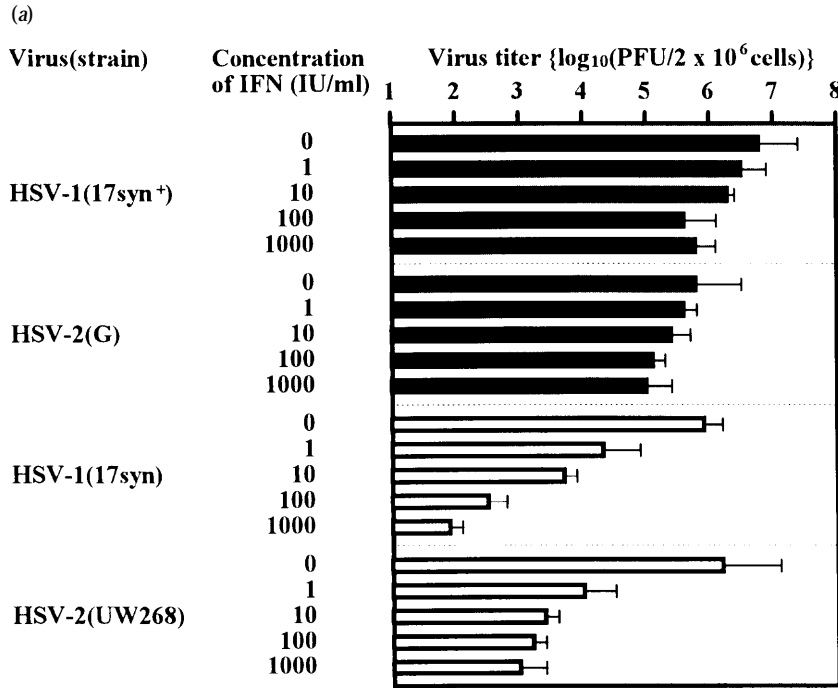


Fig. 1. Sensitivities of four HSV strains to IFN. The HSV strains were assessed for IFN sensitivity using the virus yield reduction assay (a) and the dye-uptake assay (b). Closed and open columns indicate IFN-resistant and IFN-sensitive HSV strains, respectively. Each assay was independently repeated three times. The values in the virus yield reduction assay and the dye-uptake assay represent geometric means and means of the experiments  $\pm$  SD, respectively.

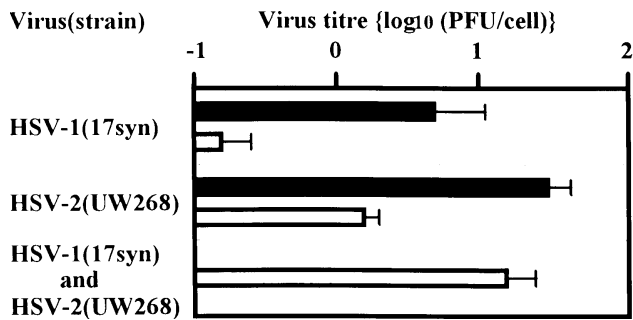


Fig. 2. Phenotypic complementation of reduced IFN resistance between HSV-1 (17syn) and HSV-2(UW268). The IFN-untreated cultures (closed columns) and IFN-treated cultures (open columns) of K-1034 cells were singly infected with HSV-1 (17syn) or HSV-2(UW268), or doubly infected with both strains. Virus yield in each culture was assayed as described in the text. Bars indicate SD of the geometric means of two independent experiments.

**Mapping of an HSV genetic region responsible for IFN resistance**

*Bam*HI analysis demonstrated that five IFN-resistant intertypic recombinants had restriction profiles similar to HSV-2(UW268), but some fragments of HSV-2(UW268) were lost

in the recombinants. Of the fragments, *Bam*HI-A was lost in all five of the recombinants (Fig. 3a).

We further analysed the genomic DNAs of the five recombinants with *Bgl*III (Fig. 3b). *Bgl*III profiles of the recombinants also resembled HSV-2(UW268) but the recombinants lost the *Bgl*III-I fragment of HSV-2 that overlaps the *Bam*HI-A fragment of HSV-2 (Fig. 4). In addition, in two recombinants, designated C4043 and C4083, the MN band of HSV-2(UW268) was not observed (Fig. 3b, lanes 4 and 6). Also, in the remaining three recombinants designated C4023, C4038 and C4054, the density of the MN band of HSV-2 was apparently lower than that of the L band (Fig. 3b, lanes 2, 3 and 5). In the *Bgl*III profile of HSV-2, the density of the MN band is higher than that of the L band (Fig. 3b, lane 7) because the *Bgl*III-M and -N fragments have the same electrophoretic mobility and are half-molar and equimolar to *Bgl*III-L, respectively. Therefore, the low density of the MN band seems to be due to loss of the *Bgl*III-N fragment, which also overlaps the *Bam*HI-A fragment of HSV-2 (Fig. 4). These results suggest that one of the HSV genes responsible for IFN resistance is located in the region containing the *Bam*HI-A, *Bgl*III-I and *Bgl*III-N fragments of HSV-2.

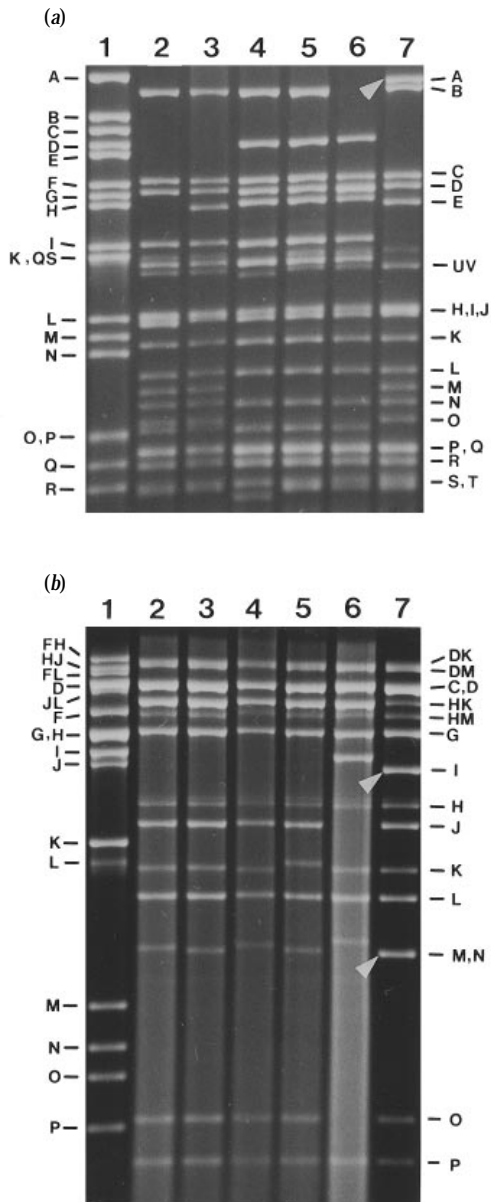


Fig. 3. *Bam*HI and *Bgl*III profiles of five intertypic recombinants between HSV-1 (17syn) and HSV-2 (UW268). The recombinants, IR(C4023) (lane 2), IR(C4038) (lane 3), IR(C4043) (lane 4), IR(C4054) (lane 5) and IR(C4083) (lane 6), were analysed with *Bam*HI (a) and *Bgl*III (b) to compare the recombinants with parental HSV-1 (17syn) (lane 1) and HSV-2 (UW268) (lane 7) as described in the text. The designation of HSV-1 and HSV-2 fragments is referred to in previous reports (McGeoch *et al.*, 1985, 1986, 1988; Morse *et al.*, 1977; Perry & McGeoch, 1988; Wilkie *et al.*, 1978). Triangles indicate the fragments of HSV-2 (UW268) that were not observed in the profiles of the five recombinants.

We then cloned these DNA fragments and *Bam*HI-E, which overlaps *Bgl*III-N (Fig. 4), from IFN-resistant HSV-2(G) and tested their abilities to rescue reduced IFN-resistant HSV-2 (UW268) (Fig. 5). When progeny viruses in the co-transfected culture were examined, plating efficiencies of co-transfected groups were 9- to 34-fold less than those of HSV-2(G) genome-transfected (positive control) groups. There was no

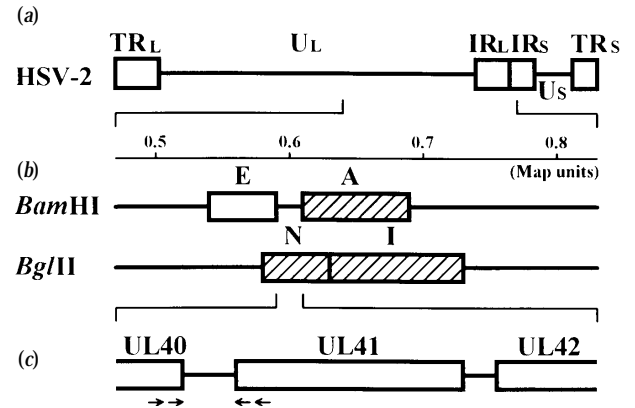


Fig. 4. Schematic representation of four HSV-2 restriction fragments used in this study and three ORFs included in the *Bgl*III-N fragment of HSV-2. (a) Structure of HSV-2 genome. Lines show the unique sequences of the L component ( $U_L$ ) and the S component ( $U_S$ ). Boxes represent the inverted repeats.  $U_L$  is flanked by the terminal and internal repeats of the L component ( $TR_L$  and  $IR_L$ ) and  $U_S$  is flanked by the terminal and internal repeats of the S component ( $TR_S$  and  $IR_S$ ). (b) Four HSV-2 DNA fragments used in the marker rescue experiment. Boxes show DNA fragments that were cloned from HSV-2(G) and used for co-transfection with HSV-2 (UW268) genome. Hatched boxes show the HSV-2 DNA fragments commonly lost in IFN-resistant intertypic recombinants between HSV-1 (17syn) and HSV-2 (UW268). (c) The three ORFs in the genetic region that are included in the *Bgl*III-N but not in *Bam*HI-A and *Bam*HI-E fragments of HSV-2. The position of primers for nPCR to amplify the DNA sequence between ORFs UL40 and UL41 is indicated by arrows.

significant difference in plating efficiency between co-transfection groups and HSV-2 (UW268) genome-transfected (negative control) groups. However, plating efficiency in the co-transfection groups with the *Bgl*III-N fragment of HSV-2 (G) increased approximately 2-fold after first propagating the progeny viruses in IFN-treated K-1034 cells and 4- to 12-fold after a second propagation, although plating efficiencies in the groups co-transfected with any of the other fragments and the negative control groups remained at similar levels even after propagation. These results suggest that the *Bgl*III-N fragment of HSV-2 contains the region responsible for IFN resistance.

#### Demonstration of recombination between the HSV-2 (UW268) genome and the *Bgl*III-N fragment of HSV-2 (G) in an IFN-resistant HSV-2 clone isolated from the culture co-transfected with the two DNAs

In order to confirm the results of marker rescue experiments, we tried to isolate an IFN-resistant HSV-2 clone from progeny viruses in the culture co-transfected with HSV-2 (UW268) genome and *Bgl*III-N of HSV-2 (G). After propagation in the IFN-treated K-1034 cells, we plaque-purified seven HSV-2 clones from the progeny viruses and examined them for IFN-sensitivity. One HSV-2 clone, HSV-2 (C5001), had IFN resistance comparable to that of HSV-2 (G) and the other clones had the same IFN sensitivity as HSV-2 (UW268).

We analysed the HSV-2 (C5001) DNA sequence between ORFs UL40 and UL41, which were contained in the *Bgl*III-N fragment (Fig. 4), to determine whether the IFN-resistant HSV-

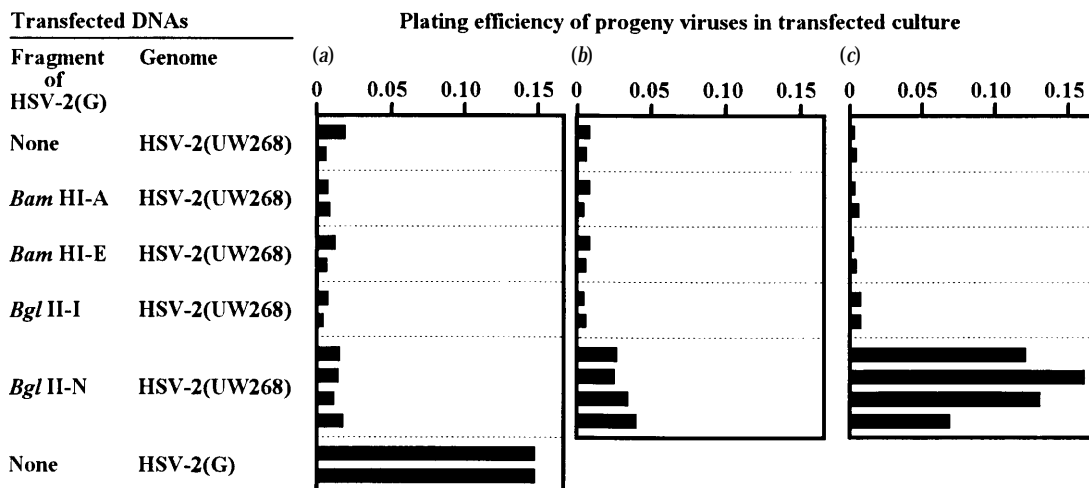


Fig. 5. Rescue of reduced IFN resistance of HSV-2(UW268) with the *Bgl*II-N fragment of HSV-2(G). The ability of each HSV-2(G) fragment to rescue reduced IFN resistance of HSV-2(UW268) was determined as the plating efficiency of progeny viruses in transfected cultures. The plating efficiency was assayed before propagation (a), after a first propagation (b) and after a second propagation (c) in IFN-treated K-1034 cells.

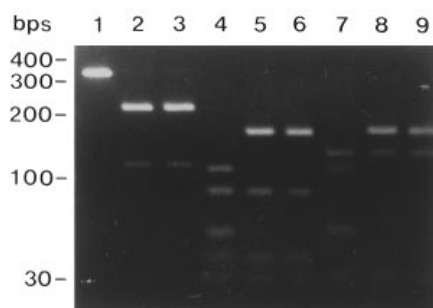


Fig. 6. *Ava*I, *Cfo*I and *Hae*II profiles of the HSV-2(C5001) DNA sequence between ORFs UL40 and UL41. The DNA sequence between ORFs UL40 and UL41 was amplified from HSV-2(UW268) (lanes 1, 4 and 7), HSV-2(C5001) (lanes 2, 5 and 8) and HSV-2(G) (lanes 3, 6 and 9) by nPCR and was analysed electrophoretically after digestion with *Ava*I (lanes 1–3), *Cfo*I (lanes 4–6) and *Hae*II (lanes 7–9).

2 clone was an intratypic recombinant between HSV-2(UW268) and the *Bgl*II-N fragment of HSV-2(G). The HSV-2(G) and HSV-2(UW268) differ in *Ava*I, *Cfo*I and *Hae*II profiles between ORFs UL40 and UL41 (Fig. 6). Therefore, identical HSV-2(C5001) and HSV-2(G) restriction profiles are clear evidence for recombination between the HSV-2(UW268) genome and the *Bgl*II-N fragment of HSV-2(G). Restriction profiles of nPCR-amplified HSV-2(C5001) products were identical to HSV-2(G) but not to HSV-2(UW268) (Fig. 6). Thus, it is strongly suggested that the IFN-resistant HSV-2(C5001) resulted from replacement of the mutated IFN-resistant gene of HSV-2(UW268) with the normal one of HSV-2(G) and not by spontaneous reversion in HSV-2(UW268).

## Discussion

The aim of the present study was to discover genetic regions of HSV controlling IFN resistance that were distinct from the region reported by Su *et al.* (1993). As expected,

phenotypic complementation of reduced IFN resistance was observed between IFN-sensitive HSV-1 and HSV-2 strains, and intertypic recombinants with restored IFN resistance were produced by double infection with the two strains. These results and the mapping of one of the IFN-resistant genetic regions in the HSV genome provide additional clues to the mechanism of IFN resistance of HSV.

Transfer of the DNA sequence contained in the *Bgl*II-N fragment of IFN-resistant HSV-2(G) could impart to IFN-sensitive HSV-2(UW268) the information necessary to counteract the anti-HSV effects of IFN. The results clearly indicate that one of the genes responsible for IFN resistance is located within the fragment. In marker rescue experiments, the *Bgl*II-N fragment of HSV-2(G) increased plating efficiency in the IFN-treated cells when co-transfected with IFN-sensitive HSV-2(UW268). However, the *Bam*HI-A and -E fragments of HSV-2(G), which overlap *Bgl*II-N, did not improve plating efficiencies. Thus, the genetic region included in *Bgl*II-N, but not *Bam*HI-A and *Bam*HI-E, encodes IFN resistance in HSV. This region contains two intact ORFs designated UL40 and UL41 and one partial ORF named UL42.

ORFs UL40, UL41 and UL42 have been reported to code for the small subunit of ribonucleotide reductase, virion host shut-off (vhs) protein and the accessory protein of DNA polymerase, respectively (Ward & Roizman, 1994). Of these gene products, vhs protein is the most likely to play an important role in HSV IFN-resistance for two reasons. First, vhs protein has the ability to degrade mRNA (Zelus *et al.*, 1996), inhibit accumulation of host cell mRNA (Fenwick & McMenamin, 1984; Mayman & Nishioka, 1985; Read & Frenkel, 1983) and block the resulting host cell protein synthesis (Fenwick & Walker, 1978; Nishioka & Silverstein, 1978). This might include IFN-induced anti-HSV proteins. These functions are consistent with HSV IFN resistance since

inhibition of anti-HSV protein synthesis is an effective means by which to abolish the IFN-induced anti-HSV effects. Previously, Cayley *et al.* (1984) reported the ability of HSV to produce a 2-5A analogue that inhibits the IFN-induced (2-5A)-RNase L pathway. vhs protein is not likely to be directly associated with the 2-5A analogue, and the protein and 2-5A analogue may work independently to abrogate the anti-HSV effect of IFN.

The second reason for vhs protein involvement in HSV IFN resistance is that vhs protein functions before the onset of HSV IE gene expression (Fenwick & McMnamin, 1984; Mayman & Nishioka, 1985; Read & Frenkel, 1983). Many reports on the anti-HSV effects of IFN have concluded that IFN reduces HSV IE gene expression (Domke-Opitz *et al.*, 1986; Klotzbücher *et al.*, 1990; Mittnacht *et al.*, 1988; Muñoz & Carrasco, 1984; Oberman & Panet, 1988; Feduchi *et al.*, 1989; Straub *et al.*, 1986). Therefore, the IFN-induced anti-HSV effects must act before or during HSV IE gene expression. This period coincides with the stage when vhs protein functions.

It is difficult to consider ribonucleotide reductase and the accessory protein of DNA polymerase as inhibitors of HSV IE gene expression and/or HSV glycoprotein synthesis. Therefore, both ORFs UL40 and UL42 are unlikely to be coding regions for IFN resistance.

In this study, we could not assign IFN resistance to ORF UL41 alone although more precise marker rescue experiments were performed. This may be due to an inadequate experimental system for identification of IFN-resistant progeny viruses in the marker rescue experiments or a low frequency of recombination between the intact genomic DNA of an IFN-sensitive HSV strain and a small DNA fragment from an IFN-resistant HSV strain. Further experiments are in progress to assign HSV IFN resistance to ORF UL41.

In summary, we have shown that HSV IFN resistance is associated with at least two genetic regions of HSV and one of these can be physically mapped to the BgIII-N fragment of HSV-2, which is distinct from the region previously reported by Su *et al.* (1993). Whether IFN-sensitive mutation(s) in HSV-1(17syn) are located in a third genetic region responsible for IFN resistance or the region reported by Su *et al.* (1993) remains to be determined. Further experiments are currently under way to determine genetic region(s) of the IFN-sensitive mutation(s) in HSV-1(17syn).

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