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

Masaya Narita,1,2 Yasutaka Ando,1,2 Sumi Soushi,1 Takeshi Kurata1 and Yujiro Arao1

1 Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan
2 Department of Ophthalmology, Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo 160, Japan

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 BamHI-A, BglII-I and BgII-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 BamHI-

**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 (Hendrick 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; Müllenhoff 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 regions responsible for resistance to antiviral effects of interferon.
regions and found that the BgIII-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-β (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 × 10^7 international units (IU)/mg protein.

**Virus.** The 17syn strain of HSV-1 (HSV-1(17syn))(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 a m.o.i. of 0.25 p.f.u. per cell. After 11 days incubation, retention of pinocytic 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_{50} 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 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_{50} 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 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 BamHI digestion. Restriction profiles were visualized by ethidium bromide staining and photographed under ultraviolet light. Intertypic recombinants were identified by differences in viral DNA BamHI 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 BglIII.

Sensitivity 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 BamHI or BglII and were electrophoresed as described above. The BamHI-A, BamHI-E, BglII-I and BglII-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 HindIII 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 BglIII-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 AvaI, CfoI and HaelI 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 (NH₄)₂SO₄; 1.75 mM MgCl₂; 20 μM dNTP; 50 μM/ml Taq polymerase
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) + and HSV-2(G) (Fig. 1a). Replication of HSV-1(17syn) and HSV-2(UW268) 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 1000- and 1600-fold, respectively. HSV-1(17syn) 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. 1b). The DU_{50} values of IFN to HSV-1(17syn) and HSV-2(UW268) were at least 34-fold less than those of HSV-1(17syn) + 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. 1a. 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. 1a 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 BamHI, 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. 1a). 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 BamHI profiles. Forty of them had BamHI 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_{50} 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) + and HSV-2(G) and was at least 34-fold higher than IFN-sensitive HSV-1(17syn) + and HSV-2(UW268). The BamHI profiles of the recombinants are shown in Fig. 3a. The recombinants resembled HSV-2(UW268) in BamHI 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 be completely excluded.
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 ± 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**

BamHI 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, BamHI-A was lost in all five of the recombinants (Fig. 3a).

We further analysed the genomic DNAs of the five recombinants with BglII (Fig. 3b). BglII profiles of the recombinants also resembled HSV-2(UW268) but the recombinants lost the BglII-I fragment of HSV-2 that overlaps the BamHI-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 BglII profile of HSV-2, the density of the MN band is higher than that of the L band (Fig. 3b, lane 7) because the BglII-M and -N fragments have the same electrophoretic mobility and are half-molar and equimolar to BglII-L, respectively. Therefore, the low density of the MN band seems to be due to loss of the BglII-N fragment, which also overlaps the BamHI-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 BamHI-A, BglII-I and BglII-N fragments of HSV-2.
We then cloned these DNA fragments and BamHI-E, which overlaps BglII-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 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 BglII-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 BglII-N fragment of HSV-2 contains the region responsible for IFN resistance.

**Demonstration of recombination between the HSV-2(UW268) genome and the BglII-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 BglII-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 BglII-N fragment (Fig. 4), to determine whether the IFN-resistant HSV-
Fig. 5. Rescue of reduced IFN resistance of HSV-2(UW268) with the BglII-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. AvoI, CfoI and HaeII 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 AvoI (lanes 1–3), CfoI (lanes 4–6) and HaeII (lanes 7–9).

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 BglII-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 BglII-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 BamHI-A and -E fragments of HSV-2(G), which overlap BglII-N, did not improve plating efficiencies. Thus, the genetic region included in BglII-N, but not BamHI-A and BamHI-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 & McMenamin, 1984; Mayman & Nishioka, 1985; Read & Frenkel, 1983). Many reports on the anti-HSV effects of IFN have concluded that IFN reduces HSV IFN resistance or the region reported by Su et al. (1991). Evidence for novel inhibitors of the HSV coding region for human IFN resistance

References


Received 18 August 1997; Accepted 11 November 1997