INFECTION AND IMMUNITY, Dec. 1982, p. 834-842
0019-9567/82/120834-09$02.00/0

Vol. 38, No. 3

Restriction Endonuclease Analysis of DNA from Genital Isolates of Herpes Simplex Virus Type 2

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Received 12 April 1982/Accepted 19 August 1982

High-resolution restriction endonuclease analysis of DNA from multiple herpes simplex virus type 2 isolates from 30 patients at a single clinical center has indicated that herpes simplex virus type 2 DNA sequences are highly conserved. However, the use of six endonucleases, *Eco*RI, *Bgl*II, *Hin*dIII, *Kpn*I, *Bam*HI, and *Sst*I, established that each patient was infected by a unique virus. Comparison of virus isolates from the same patient show that the most sequence variation occurred in the terminal and subterminal *Bam*HI fragments. The results suggest that each individual may induce specific variation in the herpes simplex virus type 2 genome and that the results of epidemiological studies based on restriction endonuclease analyses of herpes simplex virus type 2 DNA must be interpreted with care.

Restriction endonuclease cleavage of purified DNA has been shown to have many advantages over conventional biological and immunological procedures for typing herpes simplex virus (HSV) isolates (2, 4, 8, 9). The characteristic patterns of DNA fragments produced by the endonucleases and resolved by agarose gel electrophoresis (22) can type clinical isolates unequivocally as HSV type 1 (HSV1) or HSV2; by referral to published restriction endonuclease cleavage maps (5) the exact position of any variation on the viral genome can be ascertained. Variation of both HSV1 (2, 10) and HSV2 (3) has been examined, although only a few studies have concentrated on isolates from a single center (2, 3, 7, 8), and numbers of patients in these reports were generally small.

We have examined 65 HSV2 isolates for nucleotide sequence variation by using the restriction endonucleases *Eco*RI, *Bgl*II, *Hin*dIII, *Kpn*I, *Bam*HI, and *Sst*I. Isolates were obtained from 30 individuals with well-defined clinical features attending the Sexually Transmitted Diseases Clinic run by the Department of Genito-Urinary Medicine, University of Edinburgh. Considerable variability among isolates from different patients was detected, although with the first four endonucleases (*Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I) certain DNA restriction patterns appeared to predominate, whereas with six endonucleases, an individual virus strain could be assigned to each patient. Some variability was also observed between virus isolates from the same patient.

MATERIALS AND METHODS

Cells and viruses. Human embryo fibroblasts (HEF) prepared in the Department of Bacteriology and a continuous line of Vero cells were grown in Eagle medium (6) with a supplement of 5% newborn calf serum.

Viruses were isolated in HEF cells from specimens submitted in Hanks transport medium (6) from patients attending clinics in the Royal Infirmary of Edinburgh. The viruses were subsequently passed to Vero cells and titrated by plaque titration on Vero cells in microtiter plates (Sterilin M29ART). All isolates were used within passage numbers 5 to 10 for restriction enzyme analyses (within passage numbers 3 to 5 for serotyping) and were maintained by culture at a low multiplicity of infection.

Typing of isolates by immunological tests. The isolates were typed either by a quantal neutralization test (17) or by indirect immunofluorescence. Antiserum for typing was prepared in rabbits by immunization with HSV1 (strain 1657) with an inoculum of 10^5 50% tissue culture infectious doses per ml (12). Nine parts of serum and one part of washed packed Vero cells infected with HSV2 (strain MS) were rotated in a blood mixer for 16 h at 4°C. The cells were deposited at 500 × *g* for 5 min, and a 1:20 dilution of the serum was readsorbed as described above. Finally, the clarified serum was filtered through a 0.22-μm membrane filter (Millipore Corp.). Duplicate samples of Vero cells infected with each isolate were fixed with cold acetone on Teflon-coated slides, treated with either adsorbed or unadsorbed serum, stained with a fluorescent anti-rabbit conjugate (Wellcome), and examined in a Leitz SM LUX microscope with incident UV light.

Purification and restriction endonuclease cleavage of HSV DNA. For each virus isolate two Roux bottles of

subconfluent Vero cells (approximately 50×10^6 cells) were infected with 0.16 PFU of HSV per cell in 20 ml of medium at 37°C for 1 to 2 h. Eagle medium (50 ml) was then added to each bottle, which was incubated at 37°C for up to 16 h or until a complete cytopathic effect was observed. The infected cells were harvested and washed twice in phosphate-buffered saline and stored as a pellet at −70°C. Pellets were suspended in 3.5 ml of TE (0.01 M Tris–0.01 M EDTA, pH 8.0); then 0.5 ml of 6.6% sodium dodecyl sulfate in TE was added with 0.5 ml of proteinase (20 mg/ml; Calbiochem nuclease-free grade, preincubated in TE for 1 h at 37°C). After incubation for 3 h at 37°C a further 0.5 ml of proteinase was added, and the cell lysates were incubated for a further 16 h. The lysates were prepared for sodium iodide density gradient ultracentrifugation by adjusting the final volume to 8 ml with TE buffer containing 0.66% sodium dodecyl sulfate and 80 μg/ml of ethidium bromide per ml. In this, 80 μl of sodium metabisulfate (50 mg/ml) and 7.5 g of solid anhydrous sodium iodide were added, mixed gently, and centrifuged at $5,000 \times g$ for 10 min. The DNA solution was carefully poured into ultracentrifuge tubes, leaving the protein-sodium dodecyl sulfate pellicle behind, and the DNA solution was centrifuged to equilibrium at 18°C in a 75 Ti rotor at 48,000 rpm (20). The main (cellular) DNA band was first removed with a siliconized Pasteur pipette, and the HSV2 DNA that banded immediately below the cellular DNA band was harvested by side puncture of the tube. The DNA was then extracted extensively and precipitated with isopropanol to remove excess ethidium bromide.

The final precipitate was washed with absolute ethanol, dried under vacuum, and dissolved in 0.01 M Tris–0.001 M EDTA (pH 7.4). Samples of viral DNA, containing approximately 1 μg of DNA, were digested with a 10- to 20-fold excess of the following restriction endonucleases: *Eco*RI and *Bgl*II, prepared on heparin sepharose (1); *Kpn*I (purchased from New England Biolabs); *Hin*dIII and *Sst*I (purchased from Bethesda Research Laboratories); and *Bam*HI (purchased from Boehringer Co. Ltd.). The appropriate buffer as recommended by the suppliers was used for each enzyme.

Gel electrophoresis. Restriction endonuclease fragments of HSV DNA were separated by agarose gel electrophoresis (15) overnight at 100 V on 20- or 40-cm gels of 0.35 to 0.4% agarose in 0.04 M Tris–0.005 M sodium acetate–0.001 M EDTA (pH 7.9) or composite 0.4%–0.8% agarose in the same buffer. The gels were stained with ethidium bromide (0.5 μg/ml) and photographed by UV transillumination with Polaroid film. Permanent records of each DNA fragment pattern were achieved by transferring the DNA from the agarose gels to nitrocellulose filters (18). A Joyce-Loebel densitometer was used for densitometer scans of polaroid negatives.

RESULTS

Sufficient quantities of purified unlabeled DNA from HSV2-infected Vero cells have been obtained to allow analysis of the fragments produced by digestion with six restriction endonucleases. The viral DNA preparations were estimated to be more than 95% free of cellular DNA sequences, after one cycle of sodium iodide gradient ultracentrifugation, as assayed by analytical ultracentrifugation and DNA-DNA hybridization (11, 20). It is impossible to exclude the presence of defective HSV DNA molecules with the same density as HSV DNA, but the higher resolution afforded by the sodium iodide density gradients over cesium chloride gradients excluded the possibility of contamination by defective DNA molecules with higher guanine plus cytosine content. The technique produced an average yield of 10 μg of viral DNA from two Roux bottles of infected Vero cells, which was

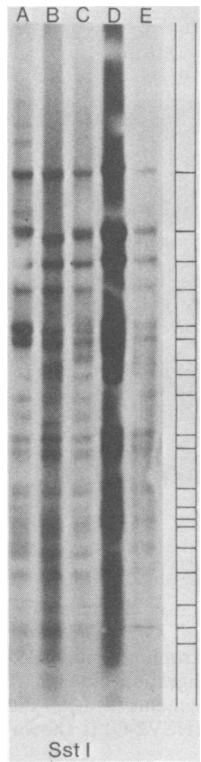

FIG. 1. Visualization of HSV2 DNA fragments by hybridization with ^{32}P-labeled DNA probe. DNA fragments separated by 0.3% agarose gel electrophoresis were transferred to nitrocellulose membrane filters and were hybridized with 5×10^5 cpm of ^{32}P-labeled HSV2 DNA per ml (11, 18). All of the HSV2 DNA was digested with *Sst*I endonuclease. Autoradiographic exposure time was 16 h at −70°C with Kodak intensifier screens. The various tracks chosen show several effects which are observed in such a screening procedure: A, evidence of partial digestion (high-molecular-weight bands above the first common band); B and C, satisfactory digestion of >0.2 μg of HSV2 DNA; D, overloading of gel track, degradation of viral DNA, and contamination with host cell DNA; E, control track containing <0.2 μg of HSV2 DNA. A key to the position of DNA fragments is shown immediately to the right of track E. By comparing autoradiographic intensities the yield of viral DNA in each track may be estimated.

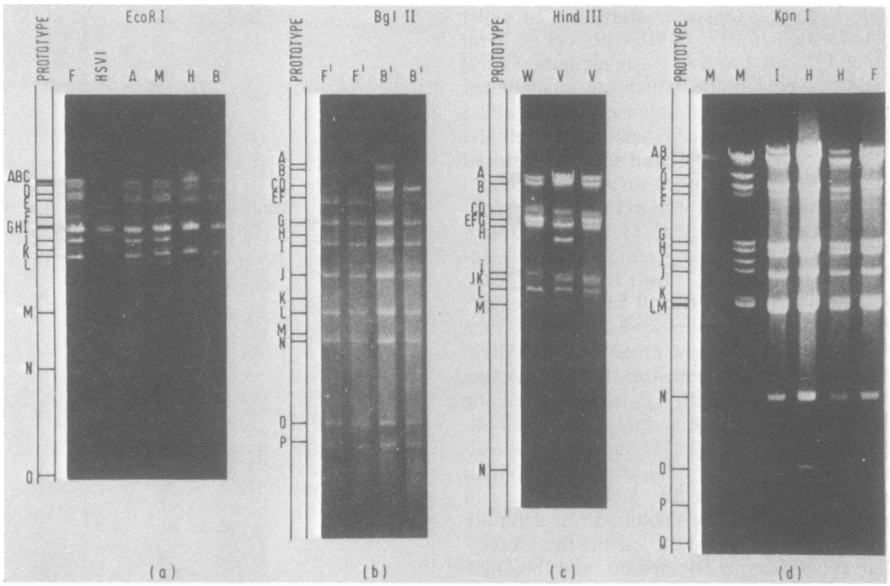

FIG. 2. Restriction endonuclease digestion patterns of HSV2 clinical isolates with endonucleases *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I. Digestions were performed as described in the text, and the DNA fragments were separated by electrophoresis on 0.35% agarose gels. (a) *Eco*RI digestion showing (patients B and H) F-J fragment fusion. (b) *Bgl*II digestion showing (patient F') extra site in C fragment. (c) *Hin*dIII digestion showing (patient V) extra site in E fragment. (d) *Kpn*I digestion showing (patients F, H, and I) D-I fragment fusion. Digestion of the prototype strain (HSV2-G) is shown diagrammatically.

sufficient for at least 10 restriction endonuclease digestions to be performed from the products of a single culture. Yields of both infectious HSV2 and viral DNA varied for different virus isolates, and DNA yields were within the range 4 to 20 μg from two Roux bottles. DNA fragments from the

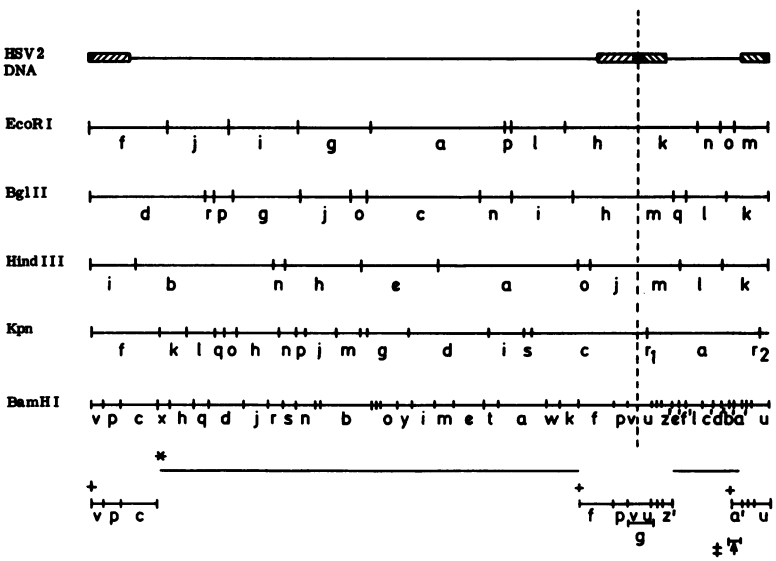

FIG. 3. Map positions of restriction site variations between the genomes of HSV2 clinical isolates. Restriction endonuclease maps after Wilkie et al. (23) and Roizman (14).

TABLE 1. Ranking of HSV2 isolates by restriction endonuclease cleavage patterns

Endonucleases	Restriction pattern[a]	No. of isolates	No. of patients[b]
*Eco*RI, *Bgl*II, and *Hin*dIII	ppp	25	12
	xpp	28	13
	xxp	0	0
	pxp	2	1
	ppx	2	1
	xpx	8	4
	xxx	0	0
	pxx	0	0
*Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I	pppp	12	5
	pppx	13	7
	ppxx	2	1
	pxxx	0	0
	pxpp	2	1
	pxxp	0	0
	pxpx	0	0
	ppxp	0	0
	xppx	28	13
	xpxp	0	0
	xxpx	0	0
	xpxx	8	4
	xppp	0	0
	xxpp	0	0
	xxxp	0	0
	xxxx	0	0

[a] The order of digestions is, reading left to right, *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I. p indicates the prototype (as HSV2G). x indicates the following: for *Eco*RI, loss of F-J site; for *Bgl*II, extra site in C fragment; for *Hin*dIII, extra site in E fragment; for *Kpn*I, loss of D-I site.

[b] The total is 31 patients, since different strains were obtained from patient V.

lower-yielding isolates were visualized by transfer of restriction endonuclease fragments to nitrocellulose membranes (18) and hybridization with standard ^{32}P-labeled HSV2 DNA (11) (Fig. 1).

Analysis of the cleavage patterns of HSV DNA from 30 patients with clearly defined clinical histories (16) by using the endonucleases *Eco*RI, *Bgl*II, *Hin*dII, and *Kpn*I confirmed the immunological typing of the genital HSV2 isolates. Also, it was possible to map any nucleotide base sequence variations between isolates by reference to the published cleavage maps for these endonucleases (5, 14). Typical DNA fragment patterns with endonucleases *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I are illustrated in Fig. 2. The map positions of site variations for each of the four endonucleases are shown in Fig. 3. The most common variation in the *Eco*RI patterns, deletion of the F/J site, occurred in 17 of 30 patients, whereas considerable heterogeneity of the terminal fragments M and K was also observed. The *Eco*RI-O fragment, which is adjacent to the terminal M fragment but lies within the short unique region of the HSV2 genome between map units 93.3 and 94.9, also showed considerable heterogeneity in many of the isolates studied. The *Bgl*II cleavage patterns were the most invariable, apart from two isolates (patient F') which had an extra site in the *Bgl*II-C fragment. Molecular weight heterogeneity (fragments M and K) was also observed in the terminal fragments. Similarly, *Hin*dIII digestion revealed one major variation, the creation of an extra site in the *Hin*dIII-E fragment (present in 5 of 30 patients) in addition to terminal fragment heterogeneity (fragments M and K). *Kpn*I digestion also showed a single major change from the prototype, viz., the deletion of the D-I restriction site in 24 of 30 patients. Terminal fragment size heterogeneity was again observed (C, F, R_1, R_2).

By using the cleavage patterns, a profile of each isolate with respect to a reference virus (prototype taken as HSV2-G) may be built up, and the frequency of occurrence of each virus type is summarized in Table 1. The table expresses frequency as both number of patients and isolates. In general two virus isolates were obtained from each patient except for patients N (four isolates), Q (four isolates), and A' (three isolates). In all cases the DNA fragment patterns were exclusively HSV2 even in cases where the immunological background of the patient was both HSV1 and HSV2 positive. No evidence for in vivo HSV1-HSV2 recombination was therefore detected although such recombinants may be constructed in vivo by using temperature-sensitive mutant viruses (23).

Nucleotide sequence variation was further investigated with endonucleases *Sst*I and *Bam*HI, which cleave the HSV2 genome considerably more frequently than the other endonucleases used. The complex fragment patterns produced by digestion of HSV2 DNA with *Bam*HI and *Sst*I are illustrated in Fig. 4 and 5. The 30 to 40 fragments were resolved with 40-cm agarose gels of either uniform 0.35 to 0.4% agarose (Fig. 4b and d) or composite 0.4%–0.8% agarose (Fig. 4a and c). Some stacking of DNA fragments at the 0.4%–0.8% boundary was occasionally observed, but in general the composite gels permitted retention of the very small DNA fragments as discrete bands while resolving considerably larger fragments. This technique has also proved useful in the analysis of cytomegalovirus DNA fragments (N. Maitland and H. Hart, unpublished observations) and is capable of resolving, on the same gel, fragments within the molecular weight range 0.25×10^6 to 25×10^6. Comparison of *Bam*HI and *Sst*I digestion patterns from different patients indicated that only two patients (A and W) possessed identical (prototype) viruses. On extension of the rank-

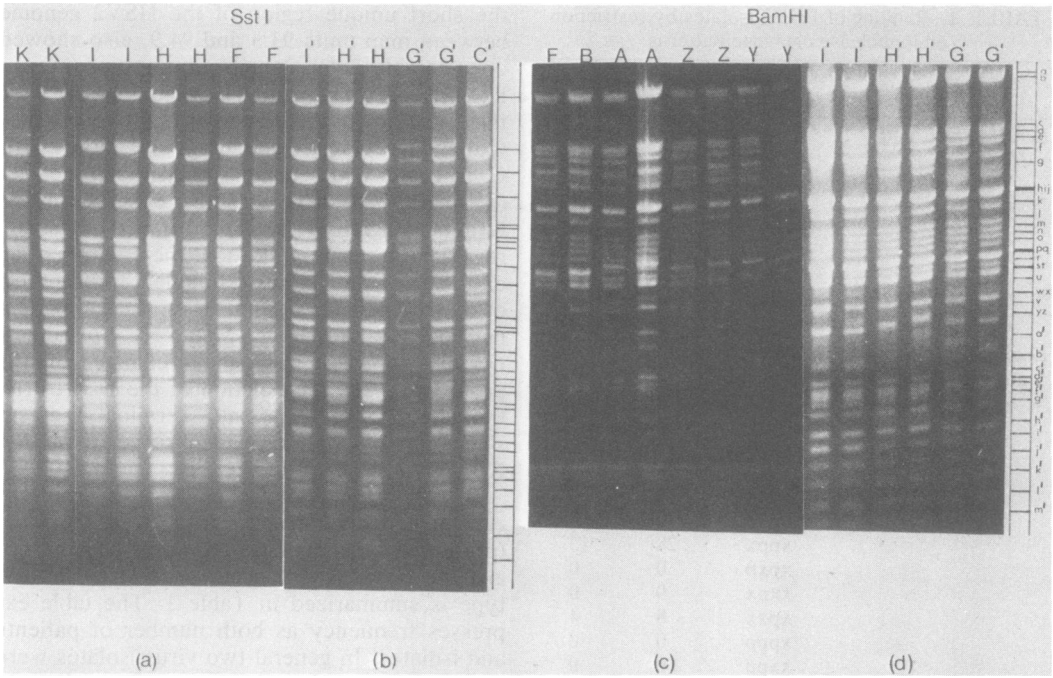

FIG. 4. Restriction endonuclease digestion patterns of HSV2 clinical isolates with endonucleases *Bam*HI and *Sst*I. Digestions were performed as described in the text, and the DNA fragments were separated by electrophoresis on 0.4% (b and d) or 0.4%–0.8% discontinuous (a and c) agarose gels. Digestion of prototype strain is shown diagrammatically. (a and b) Digested with *Sst*I; (c and d) digested with *Bam*HI. Patient codes are indicated at the top of each lane.

ings of HSV2 isolates in Table 1 for four endonucleases to six endonucleases it was apparent that all 30 patients possessed their own unique virus (data not shown) since patients A and W differed in their *Kpn*I digestion pattern. It is therefore possible to define an individual virus by cleavage of the DNA with six restriction endonucleases. However, since it is difficult to unambiguously identify the various fragments in *Bam*HI or *Sst*I digests or the source of the variation (i.e., addition or deletion of restriction sites, addition or deletion of short sequences) we define the individual strains by the DNA cleavage patterns with *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I.

Reisolations of HSV2 on subsequent visits to the clinic by each of the 30 patients permitted the study of HSV variation in vivo. The results of these studies are shown in Table 2 for endonucleases *Bam*HI and *Sst*I. The results with *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I indicated that in 29 of 30 patients no difference was apparent between isolates from the same patient, whereas with patient V both the *Hin*dIII and *Eco*RI digestion patterns varied. This implied that patient V had been reinfected with a completely new strain of HSV2 subsequent to his first infection. However, with the multicut endonucleases *Bam*HI and *Sst*I considerable variability was observed between isolates from the same patients: eight patients showed variation in *Bam*HI digestion patterns, and nine showed variation with *Sst*I; only three patients showed variation with both endonucleases. By referral to the *Bam*HI map of HSV2 (23) the fragment variations were assigned, if possible, to specific DNA fragments as shown in Fig. 3, from which it is clear that both terminal and subterminal fragments from the L and S regions of the genome are hypervariable. Since no restriction endonuclease map for *Sst*I is available these variations have not yet been assigned to specific map locations.

DISCUSSION

In this study we have analyzed the DNA sequence complexity of 65 HSV2 isolates from 30 patients. Despite the isolation of HSV1 from 20% of patients with herpetic genital infection in Edinburgh (13) no evidence for HSV1-HSV2 intra-typic recombination was detected. Six restriction endonucleases were used, for five of which HSV2 DNA cleavage maps have been published (5, 14, 23), thus allowing the mapping of DNA sequence variation to particular sites on the HSV2 genome. By employing multicut endo-

TABLE 2. Variations among DNA HSV2 isolates from the same patient with multicut endonucleases

Patient	Variation in digestion with the following:			Serological status of patient at first isolation[a]
	*Eco*RI, *Bgl*II, *Hin*dIII, *Kpn*I digestion pattern[b]	*Sst*I	*Bam*HI	
A	pppx	+	−	+
B	xppx	−	−	−
D	pppp	+	+	−
F	ppxx	−	−	−
H	xppx	−	−	+
I	xppx	−	−	+
J	xppx	+	−	−
K	pppx	+	−	+
L	pppx	−	−	+
M	pppp	−	−	+
N	pppp	−	+	−
Q	xppx	+	−	−
R	xppx	−	−	+
S	xppx	−	−	+
T	pppp	+	−	+
U	xppx	−	−	+
V	pppx xpxx	+	+	+
W	pppp	−	−	+
X	xpxx	−	−	−
Y	xppx	−	+	−
Z	pppx	−	−	+
A′	xpxx	−	−	+
B′	xpxx	−	−	−
C′	xppx	−	−	+
D′	xppx	+	−	+
E′	xppx	−	+	−
F′	pxpp	−	−	+
G′	pppx	+	+	+
H′	pppx	−	+	−
I′	xppx	−	+	ND

[a] +, Antibody present in serum; −, no antibody present in serum; ND, not determined.
[b] See footnote *a* of Table 1 for details.

nucleases such as *Bam*HI and *Sst*I it was possible to increase the sensitivity of the analysis of nucleotide base sequence variation from the previous 1 to 2% of the HSV2 genome (2, 3, 7, 8) to between 5 and 6%. This is still relatively low resolution but if the technique is to be used for epidemiology it should attempt to balance sensitivity with the ability to analyze large sample numbers. In the latter respect the use of ^{32}P-labeled DNA (9) is probably more useful, although our technique, if used routinely, permits the detailed analysis in 1 week of between 20 and 30 isolates of HSV2, free of high-density defective DNA molecules as also shown by Halpern et al. (7) and with no requirement for radioisotopic labeling of the DNA as used in other previous studies (2, 9).

In the population from which the patient sample was drawn, HSV2 DNA showed relatively infrequent variation in the cleavage sites for *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I (one site deletion or addition with each endonuclease). If the frequency of site variation was a function of the number of restriction sites probed then a greater site variation on digestion with *Bam*HI and *Sst*I would be expected. Certainly greater variation was observed, but principally as fragment size heterogeneity, although there may be at least one site deletion or addition present in several isolates on digestion with *Sst*I. By referral to the *Bam*HI cleavage map (23) these variations were mapped to the terminal and subterminal fragments (Fig. 3) and probably consist of sequence insertions, deletions, and rearrangements in these positions. However, variations also occurred in the *Eco*RI-O fragment, which maps in the short unique region (Fig. 3) and in *Bam*HI fragments C and F which extend into the long unique region. Similar variation has been reported in the terminal fragment of HSV1 DNA (19) and HSV2 DNA (3). Some of these regions have also been shown to encode immediate-early RNA in HSV1 (21), although whether the sequence variations occur in translated portions of the mRNA is not known. The map positions of variations detected by *Sst*I cleavage are also unknown at present.

In Table 1 the 65 isolates taken from the 30 patients are ranked according to their digestion patterns with three (*Eco*RI, *Bgl*II, and *Hin*dIII) or four (*Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I) endonucleases. It can be seen that certain patterns predominate and that digestion with a panel of four endonucleases would not be able to differentiate unique isolates of HSV2. Another interesting finding relates to the subdivision of isolates as grouped in Table 1. Isolates previously ppp became either pppx (13 of 25 isolates) or pppp (12 of 25 isolates) with equal frequency, whereas all of the isolates ranked as xpp became xppx (28 of 28 isolates) and all of the isolates ranked as xpx became xpxx (8 of 8 isolates). This may simply reflect the fact that 51 of 65 isolates were *Kpn*I x structure (i.e., D-I site deleted), but the linkage of the *Eco*RI x structure (i.e., F-J site deleted) with the *Kpn*I x structure may be important in the derivation of new HSV2 DNA restriction patterns in Edinburgh. Studies of greater numbers of patients should confirm this observation. Arrangement of the data in this way does indicate that certain configurations of HSV2 predominate in the Edinburgh population and that the gross variations previously described for American HSV2 isolates (3) were rarely encountered in our study. It should also be noted that the two most common Edinburgh patterns were also found in the limited American studies (8). Equally, no clustering of the primary isolation dates of one particular restriction pattern of HSV2 was observed from the clinical

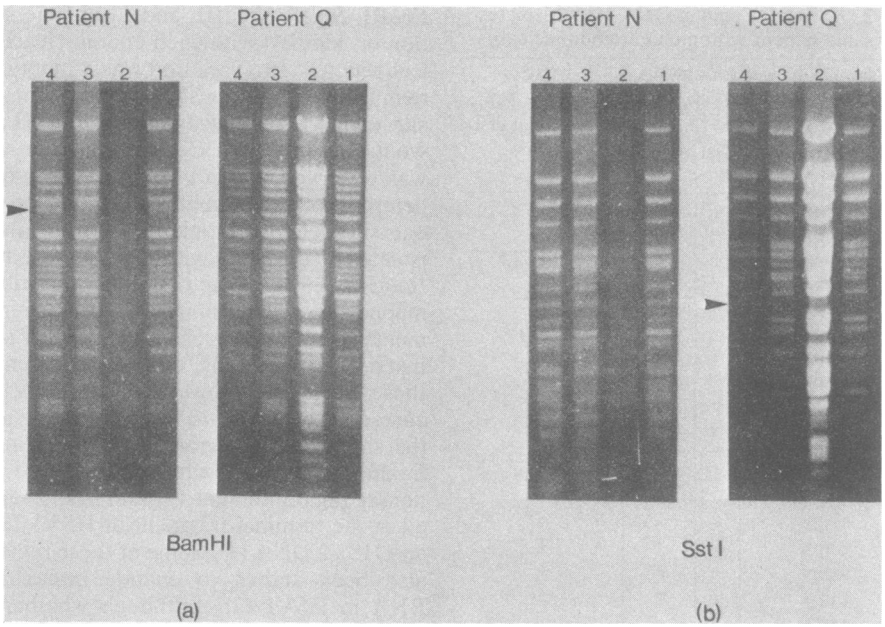

FIG. 5. *Bam*HI and *Sst*I, restriction endonuclease digestion patterns of successive HSV2 clinical isolates from two patients. Digestions were performed as described in the text, and the DNA fragments were separated by electrophoresis on 0.4% agarose gels. (a) *Bam*HI digests of four successive isolates (numbered 1 through 4) from each patient; (b) *Sst*I digests of the same isolates. Variations detected are shown by large arrows (patient N, *Bam*HI isolate 4; patient Q, *Sst*I isolate 4).

records. Thus all of the most common restriction patterns of HSV2 DNA are in constant circulation in the Edinburgh population.

The four endonucleases we have used to define virus strains (viz., *Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I) were chosen for ease of interpretation of results. First, the restriction endonuclease cleavage maps with these endonucleases are relatively simple (5); and second, the DNA fragment patterns on 0.4% agarose gels permit positive identification of new DNA fragments on the basis of mobility, without recourse to extensive mapping. (However, with the new cleavage in *Bgl*II fragment C the map position was confirmed by excising one of the two new fragments from the agarose gel, nick translating the DNA, and hybridizing the resultant probe to a *Bam*HI digest of HSV2 DNA.) The endonucleases *Xba*I and *Hpa*I, whose restriction maps have been published for HSV2 DNA (5), were also employed, but these enzymes produced relatively few, highly clustered, large-molecular-weight DNA fragments which proved difficult to resolve adequately on 0.4% agarose gels. The greater resolution afforded by *Bam*HI and *Sst*I was sufficient to define substrains of HSV2, which allowed the virus isolated from each patient to be unambiguously defined.

By increasing the number of nucleotide bases probed for variation from 1 to 2% to 5 to 6%, it might be expected that more base changes would be detected. As discussed above this was not the case, and the major problem when using restriction endonuclease digestion as an epidemiological tool at this level of resolution was the variation in *Bam*HI and *Sst*I digestion patterns between isolates from the same patient (Table 2, Fig. 4 and 5). If one discounts patient V who displayed gross differences with four of the six enzymes and probably suffered from reinfection with a new strain of HSV2, the variations detectable with *Bam*HI and *Sst*I probably represent in vivo changes in the HSV2 genome. Were similar changes to occur upon transmission of HSV2 between individuals, then the use of endonucleases *Bam*HI and *Sst*I would produce false-negative results in postulating direct transmission, whereas the use of only four endonucleases (*Eco*RI, *Bgl*II, *Hin*dIII, and *Kpn*I) would produce false-positive results in a population with little gross strain variation. Our present series of studies on HSV isolates from patients and their proven consorts should provide further information on the optimum panel of restriction endonucleases for epidemiological studies.

If it is assumed that a negative serological reaction at the time of the first HSV isolation indicates a primary infection (Table 2), then the analysis of isolates from the same patient, on digestion with *Bam*HI and *Sst*I (again excluding

patient V), shows the following: subsequent isolations after a primary infection showed restriction pattern variation in 7 of 11 patients, whereas those from nonprimary infections varied only in 5 of 17 cases. Although the larger numbers of patients now under study will be required to verify this hypothesis it appears that adaption or selection of HSV2 within a new host may occur preferentially during or after the first period of virus latency.

The biological consequences of this sequence variation are apparent in the various yields of HSV2 detected by plaque titration of the various isolates, although in our hands the virus yield variability is less than that found with HSV1, which also shows greater sequence variation (14). It has been reported that in vivo passage of HSV1 in mouse brain (H. C. Kaerner et al., personal communication) results both in HSV sequence variation of the types we have observed in human HSV2 isolates and in alterations of in vivo pathogenicity. We have presently no evidence to suggest that a similar situation occurs in humans. The in vivo variation described above could arise either by selection of a particular variant with increased growth capabilities from a heterogeneous primary infection or by adaption in the patient of an initially homogeneous HSV population to permit selection of the faster-growing variant. By either hypothesis the selective pressure must vary between patients, or all isolates of HSV2 would tend toward an identical consensus strain. The first hypothesis is supported by the finding of heterogeneity between single plaques of HSV1 cultures (10, 19). In this laboratory, extensive passage over 4 years of an uncloned clinical strain (8300) at low multiplicity did not select for a faster-growing HSV2 variant. No variation in the restriction endonuclease cleavage patterns with six endonucleases was observed in the virus DNA over this period of time. Ten passages at high multiplicity of infection in vitro did not give rise to the multiple DNA bands of similar molecular weight on restriction digests of NaI gradient-purified HSV2 DNA (multiple DNA bands are often characteristic of a mixed or defective population of virus). High-multiplicity passage, like low-multiplicity passage, did not produce a new variant of HSV2 8300. However, 12 months after the isolation of strain 8300 the same patient (Q) yielded a virus which (Table 2 and Fig. 5) varied in its *Sst*I digestion pattern. These in vitro growth results suggest that the variability of HSV2 DNA restriction patterns are a consequence of the in vivo growth cycle.

We have, therefore, demonstrated two classes of sequence variation in wild-type isolates of HSV2, viz., gross variation, corresponding to the deletion or addition of restriction endonuclease sites which identify individual strains of HSV2, and minor variation, corresponding to the deletion or addition of short DNA sequences to the same HSV2 strain. The gross variation is sufficient to identify the most common naturally occurring strains of HSV2, but the low degree of variability within a small population may limit the usefulness of restriction endonuclease analysis of HSV2 isolates as an epidemiological tool. The minor variation, occurring as it does between isolates which are otherwise identical from the same patient, are reflected in different in vitro growth properties of the clinical isolates which may be analogous to in vivo growth properties. The highly conserved nature of the HSV2 genome (14), apart from the hypervariable regions, when compared with HSV1 and other human DNA viruses has also been confirmed by our analysis.

ACKNOWLEDGMENTS

This work was supported by a grant to K.W.J. by the Cancer Research Campaign.

We thank J. G. Collee for his critical reading of the manuscript and Alison Jardine and Ann Clarkson for their technical assistance.

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