820 (1971); A. G. Knudson and L. C. Strong, *J. Natl. Cancer Inst.* **48**, 313 (1972).
5. A. Koufos *et al.*, *Nature* **309**, 170 (1984); S. H. Orkin, D. S. Goldman, S. E. Sallan, *ibid.*, p. 172; A. E. Reeve *et al.*, *ibid.*, p. 174; E. R. Fearon, B. Vogelstein, A. P. Feinberg, *ibid.*, p. 176.
6. B. E. Weissman *et al.*, *Science* **236**, 175 (1987).
7. K. M. Call *et al.*, *Cell* **60**, 509 (1990).
8. M. Gessler *et al.*, *Nature* **343**, 744 (1990).
9. E. A. Rose *et al.*, *Cell* **60**, 495 (1990).
10. P. J. Mitchell and R. Tjian, *Science* **245**, 371 (1989).
11. The methods for total gene synthesis with the use of overlap-extension PCR are described in the following articles: P. J. Dillon, P. Nelblock, A. Perkins, C. A. Rosen, *J. Virol.* **64**, 4428 (1990); P. J. Dillon and C. A. Rosen, *Biotechniques* **9**, 298 (1990); C. Abate, D. Luk, T. Curran, *Cell Growth Differ.* **1**, 455 (1990). Briefly, four overlapping oligonucleotides (120 bp in length) were synthesized, which spanned the zinc finger region of WT33 (*7*). Codon usage was optimized for *E. coli* [see P. M. Sharp *et al.*, *Nucleic Acids Res.* **16**, 8207 (1988)]. A standard PCR reaction that contained the four oligonucleotides was performed for ten cycles. A second PCR reaction run in the presence of short flanking primers gave rise to a 430-bp fragment, which was subcloned into the pDS56 *E. coli* expression vector (*12*). The vector provided the initiator methionine and six histidine residues to permit nickel-chelate affinity chromatography (*12*). The synthetic gene was sequenced on both strands with oligonucleotide primers that were complementary to flanking vector sequence. Log phase *E. coli* that harbored the WT-ZF expression vector were induced with isopropyl-β-D-thiogalactopyranoside (1 mM) and harvested 2 hours later by lysis in 6 M guanidine-HCl, pH 8.0. After nickel-chelate affinity chromatography (*12*), the purified protein was exhaustively dialyzed against binding buffer: 25 mM Hepes-KOH (pH 7.5), 100 mM KCl, 10 μM $ZnSO_4$, 0.1% NP-40, 1 mM DTT, and 5% glycerol. Protein yield averaged 3 to 5 mg per liter of bacteria.
12. E. Hochuli, W. Bannwarth, H. Dobeli, R. Gentz, D. Stuber, *Bio/Technology* **6**, 1321 (1988); C. Abate, D. Luk, R. Gentz, F. J. Rauscher III, T. Curran, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1032 (1990).
13. A. R. Oliphant, C. J. Brandl, K. Struhl, *Mol. Cell. Biol.* **9**, 2944 (1989).
14. The WT-ZF–Sepharose CL-4B affinity column was constructed as follows: pure recombinant WT-ZF (2 mg) was dialyzed against a coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl). Cyanogen bromide–activated Sepharose CL-4B (2 ml) was hydrated and prepared as described by the manufacturer, and incubated with the WT-ZF solution for 24 hours at 4°C. The resin was extensively washed with high and low salt buffers. Coupling efficiency approached 90%. Binding buffer (100 μl) (*11*) that contained double-stranded, degenerate probe (5 μg), poly(dIdC) (20 μg) and bovine serum albumin (0.2 mg/ml) was applied to the column. After consecutive washes with binding buffer that contained 200 and 400 mM KCl, bound oligonucleotides were released by incubating the resin in 0.5 M tris-HCl, 20 mM EDTA, 10 mM NaCl, 0.2% SDS (100 μl) at 50°C for 20 min. Recovered oligonucleotides were purified by phenol extraction-ethanol precipitation and either subjected to additional rounds of chromatography or digested with Xho I and Eco RI and ligated into pGEM-7Zf$^+$ (Promega). Ligations were transformed into competent DH5α bacteria and plated under blue-white selection conditions.
15. Bacterial colonies were inoculated into growth medium (1 ml) and grown at 37°C for 2 hours. Cultures were boiled for 5 min, centrifuged at 20,000*g* for 10 min, and the supernatant (5 μl) utilized for PCR. A standard PCR reaction was performed for 20 cycles except that the extension time at 72°C was reduced to 30 s. The PCR products were purified on preparative polyacrylamide gels (5%) and labelled with T4 kinase and [^{32}P]ATP under standard conditions. The primers used for PCR were complementary to the pGEM-7Zf$^+$ polylinker and were 5′-GCCCGACGTCGCATG-3′ (T7 side) and 5′-ATCGATTTCGAACCC-3′ (SP6 side). DNA binding reactions contained the indicated amounts of WT-ZF protein, poly(dIdC) (2 μg), and 50 to 70,000 cpm of ^{32}P-labeled probe in binding buffer (10 μl) (*11*). Nondenaturing gel electrophoresis was performed as described (*18*).
16. V. Sukhatme *et al.*, *Cell* **53**, 37 (1988); B. Christy and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8737 (1989); X. Cao *et al.*, *Mol. Cell. Biol.* **10**, 1931 (1990); J. Milbrandt, *Science* **238**, 797 (1987); P. Lemaire *et al.*, *Mol. Cell Biol.* **10**, 3456 (1990); R. W. Lim, B. C. Varnum, H. R. Herschman, *Oncogene* **1**, 263 (1987).
17. Oligonucleotides for use in DNA binding assays were synthesized, gel purified, annealed, and labelled as described (*18*). The binding sites used were as follows: EGR-1, 5′-CGCCCTCGCCCCCGCGCCGGG-3′; SP1, 5′-TCCCGCCCCTAACTCCGCCCAT-3′; CRE, 5′-GCGCTCCTTGGCTGACGTCAGAGAGAGAG-3′; MBS 5′-GCTAAAAAACCGTTATAATGTG-3′.
18. F. J. Rauscher III, P. J. Voulalas, B. R. Franza, Jr., T. Curran, *Genes Dev.* **2**, 1687 (1988).
19. J. F. Morris and F. J. Rauscher III, in preparation.
20. F. J. Rauscher III, unpublished data.
21. D. A. Harber *et al.*, *Cell* **61**, 1257 (1990).
22. S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* 77, 51 (1989).
23. T. Curran and B. R. Franza, Jr., *Cell* **55**, 395 (1989); J. I. Morgan and T. Curran, *Trends Neurosci.* **12**, 459 (1989).
24. We thank H. Koprowski for his continuous support; C. Abate for much help and advice on nickel-chelate chromatography and PCR-mediated mutagenesis; R. Gentz for NTA resin and pDS vectors; P. Dillon and D. Luk for advice on PCR-mediated gene synthesis; V. Sukhatme for pBS.Apa I and anti-EGR-1 sera; R. Weinmann for the SP1 binding site; P. Reddy for the myb binding site and many helpful discussions; and G. Rovera, P. Reddy, and B. Knowles for critical reading of the manuscript. Supported by grant CA23413, core grant CA10817, and grant CA0917-15 from the NIH (J.F.M.).

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Mapping of Herpes Simplex Virus–1 Neurovirulence to $\gamma_1$34.5, a Gene Nonessential for Growth in Culture

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The gene designated $\gamma_1$34.5 maps in the inverted repeats flanking the long unique sequence of herpes simplex virus–1 (HSV-1) DNA, and therefore it is present in two copies per genome. This gene is not essential for viral growth in cell culture. Four recombinant viruses were genetically engineered to test the function of this gene. These were (i) a virus from which both copies of the gene were deleted, (ii) a virus containing a stop codon in both copies of the gene, (iii) a virus containing after the first codon an insert encoding a 16–amino acid epitope known to react with a specific monoclonal antibody, and (iv) a virus in which the deleted sequences were restored. The viruses from which the gene was deleted or which carried stop codons were avirulent on intracerebral inoculation of mice. The virus with the gene tagged by the sequence encoding the epitope was moderately virulent, whereas the restored virus reacquired the phenotype of the parent virus. Significant amounts of virus were recovered only from brains of animals inoculated with virulent viruses. Inasmuch as the product of the $\gamma_1$34.5 gene extended the host range of the virus by enabling it to replicate and destroy brain cells, it is a viral neurovirulence factor.

APPROXIMATELY ONE IN 250,000 adults per year acquires herpes simplex virus (HSV) encephalitis: an acute, frequently lethal disease with lasting sequelae even in individuals treated promptly with antiviral drugs (*1*). The morbidity associated with the HSV encephalitis has prompted considerable interest in determining which of the more than 70 viral genes impart to the virus the capacity to cause central nervous system (CNS) disease. We report that the protein encoded by the HSV-1 gene designated $\gamma_1$34.5 enables HSV-1 to cause lethal infection of the mouse brain.

The HSV-1 genome, schematically represented in Fig. 1, consists of two sets of unique sequences each flanked by inverted repeats (*2*). The long unique sequence is flanked by 9-kbp inverted repeats designated as *ab* and *b′a′*, respectively (*3*). The observation that the terminal 500-bp *a* sequence acts as a late (γ_1) HSV promoter led to the discovery of the $\gamma_1$34.5 gene. The coding sequences for the $\gamma_1$34.5 gene are located in the adjacent *b* sequences and specify a protein of 263 amino acids with an apparent molecular weight of 44,000 (*4–6*). The remarkable properties of this gene are that it lacks a canonical TATAA box and that its 5′ untranscribed domain, the *a* sequence, is

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GC-rich, contains numerous repeats, and lacks the features characteristic of HSV promoters (*7–9*). The protein contains five to ten repeats of the amino acid sequence Ala-Thr-Pro (*4–6*). Interest in this gene was also kindled by the knowledge that the *a* sequence contains signals for processing of viral DNA. Viral DNA is synthesized as head-to-tail concatemers, and cleavage of unit-length viral DNA for packaging into virus particles occurs in the direct repeat 1 (*8–12*). The transcription of the $\gamma_1$34.5 gene is initiated in this repeat (*4*).

Studies of the identification of the genes associated with neurovirulence have repeatedly implicated DNA sequences located at or near a terminus of the long component of HSV-1 DNA. Thus, Centifanto-Fitzgerald *et al.* (*13*) transferred, by means of a DNA fragment, a virulence marker from a virulent to an antivirulent strain of HSV-1. Deletion of genes located at one terminus of the long component of HSV-1 DNA contributed to the lack of virulence exhibited by a prototype HSV vaccine strain (*14*). In other studies, Javier *et al.* (*15*) and Thompson *et al.* (*16*) demonstrated that an HSV-1 × HSV-2 recombinant virus consisting largely of HSV-1 DNA but with HSV-2 sequences located at one terminus of the long component was avirulent; virulence could be restored by rescue with the homologous HSV-1 fragment. Taha *et al.* (*17, 18*) described a spontaneous deletion mutant lacking 1.5 kbp at both ends of the long component of a HSV-2 strain. Because of heterogeneity in the parent virus population, the loss of virulence could not be unambiguously related to the specific deletion, although the recombinant obtained by marker rescue was more virulent than the deletion mutant. In neither study was a specific gene or gene product identified at the mutated locus, and no gene was specifically linked to a virulence phenotype.

To test the possible role of the product of the $\gamma_1$34.5 gene [infected cell protein (ICP) 34.5], a series of four viruses (Fig. 1) were genetically engineered by the procedures of Post and Roizman (*19*).

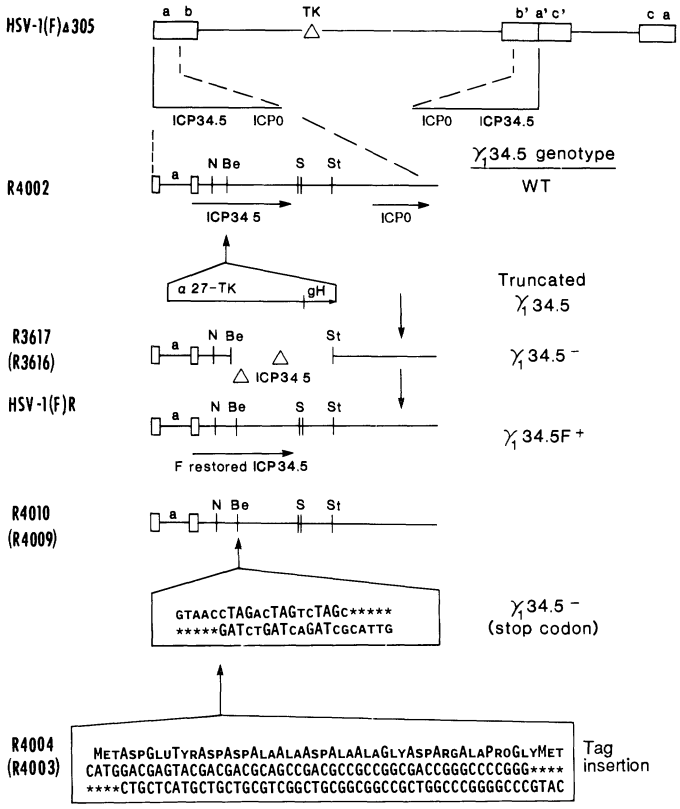

Fig. 1. Sequence arrangements of the genome of wild-type strain HSV-1 strain F [HSV-1(F)] and of recombinant viruses derived from it. Top line, the sequence arrangement of HSV-1(F)Δ305. The rectangles identify the inverted repeats *ab*, *b'a'c*, and *ca* (*3*). The HSV-(F) *a* sequence is present in a direct orientation at the two genomic termini and in the inverted orientation at the junction between the long and short components (*3*). The *b* and *c* sequences are approximately 9 and 6 kbp long, respectively (*3*). The triangle marked TK identifies the position of the *tk* gene and of the Bgl II to Sac I sequence of Bam HI Q fragment deleted from HSV-1(F)Δ305 (*19*). Lines two and three from the top show that the *b* sequences contain the genes specifying ICP34.5 and ICP0 and, since the *b* sequence is repeated in an inverted orientation, there are two copies of these genes per genome. The construction of the α27-*tk* fragment containing portions of the glycoprotein H gene has been described (*4–6*). Line 7 shows a schematic diagram of the insertion of the oligonucleotide containing stop codons in all three reading frames. The plasmids pRB3615 and pRB3976 used in the construction of R4002 and R4004, respectively, were described elsewhere (*4, 6*). To generate pRB3616, plasmid pRB143 (*19*) was digested with Bst EII and Stu I, blunt-ended with T4 polymerase, and religated. The asterisks designate nucleotides from vector plasmid that form cohesive ends with the synthesized oligomers. The insertion of the α4 epitope into the first amino acid of ICP34.5 (line 9) has been described (*6*), except that in this instance the sequence was inserted into both copies of the $\gamma_1$34.5 gene. The *tk* gene was restored in all recombinant viruses tested in mice. HSV-1(F)R (line 6) was derived from R3617 by restoration of the sequences deleted in $\gamma_1$34.5 and *tk* genes. N, Be, S, and St are abbreviations for Nco I, Bst EII, Sac I, and Stu I restriction endonucleases (New England Biolabs), respectively. The numbers in parentheses are the tk^+ version of each construct tested in mice.

1) Recombinant virus R4002 (Fig. 1, lane 3) contained the insertion of a thymidine kinase (*tk*) gene driven by the promoter of the α27 gene (α27-*tk*) in both copies of the ICP34.5 coding sequences. It was constructed by cotransfecting rabbit skin cells with intact DNA of HSV-1(F)Δ305, a virus from which a portion of the *tk* gene was specifically deleted (*19*), with the DNA of plasmid pRB3615, which contains the α27-*tk* gene inserted into the $\gamma_1$34.5 gene contained in the Bam HI S fragment. Recombinants that were tk^+ were then selected on human 143 thymidine kinase minus (TK^-) cells. The fragment containing the α27-*tk* gene contains downstream from the *tk* gene: the 5′ untranscribed promoter, the transcribed noncoding sequence, and the initiating methionine codon of the glycoprotein H gene (*4*). The Bst EII site into which the α27-*tk* fragment was inserted is immediately upstream of the codon 29 of the $\gamma_1$34.5 open reading frame. As a consequence, the initiating codon of glycoprotein H was fused in frame and became the initiating codon of the truncated open reading frame of the $\gamma_1$34.5 gene (Fig. 1, line 3). The recombinant selected for further study, R4002, was shown to contain the α27-*tk* gene insert in both copies of $\gamma_1$34.5 gene (Fig. 2, lanes 4) and specified only the predicted truncated product of the chimeric $\gamma_1$34.5 gene (Fig. 3, right panel, lane 3). The amounts of the native ICP34.5 protein detected in these and previous studies have been generally low (*5*). The chimeric genes formed by the fusion of the 5′ transcribed noncoding region and the initiating codon of glycoprotein H in frame with the truncated $\gamma_1$34.5 gene were expressed far more efficiently than the native genes.

2) The recombinant virus R3617 (Fig. 1, line 5 from the top) lacking 1 kb of DNA in each copy of the $\gamma_1$34.5 gene was generated by cotransfecting rabbit skin cells with intact R4002 DNA and the DNA of plasmid pRB3616. In this plasmid, the sequences containing most of the coding domain of $\gamma_1$34.5 had been deleted (Fig. 1, line 5 from top). The tk^- progeny of the transfection was plated on $143TK^-$ cells overlaid with medium containing bromodeoxy uridine (BrdU). This procedure selects tk^- viruses, and since the *tk* gene is present in both copies of the $\gamma_1$34.5 gene, the selected progeny of the transfection could be expected to contain deletions in both copies. The selected tk^- virus designated as R3617 was analyzed for the presence of the deletion in both copies of the $\gamma_1$34.5 gene (*20*). For assays of neurovirulence, the deletion in the native tk^- gene of R3617, which traces its origin

from HSV-1(F)Δ305, had to be repaired. This was done by cotransfection of rabbit skin cells with intact R3617 DNA and Bam HI Q fragment containing the *tk* gene. The virus selected for tk^+ phenotype in 143TK$^-$ cells was designated R3616. This virus contains a wild-type Bam HI Q fragment (Fig. 2, right panel, lane 6) and does not make ICP34.5 (Fig. 3, right panel).

3) To ascertain that the phenotype of R3616 indeed reflects the deletion in the $\gamma_1$34.5 gene, we restored the deleted sequences by cotransfecting rabbit skin cells with intact R3617 DNA, the HSV-1(F) Bam HI Q DNA fragment containing the intact *tk* gene, and the Bam HI SP DNA fragment containing the intact $\gamma_1$34.5 gene in the molar ratios of 1:1:10, respectively. Viruses that were tk^+ were then selected in 143TK$^-$ cells overlaid with medium containing hypoxanthine, aminopterin, and thymidine. The tk^+ candidates were then screened for the presence of wild-type *tk* and $\gamma_1$34.5 genes. As expected, the selected virus designated HSV-1(F)R (Fig. 1, line 6) contained a wild-type (tk^+) Bam HI Q fragment (compare Fig. 2, right panel, lanes 2 and 7), a wild-type terminal long component fragment (compare Fig. 2, left panel, lanes 2, 7, and 8), and expressed ICP34.5 (Fig. 3, right panel, lane 6).

4) To eliminate the possibility that the phenotype of R3616 reflects deletion in cryptic open reading frames, we constructed a virus (R4010, Fig. 1, line 7 from the top) that contains translational stop codons in all three reading frames in the beginning of the ICP34.5 coding sequence. The 20-base oligonucleotide containing the translational stop codons and its complement sequence (Fig. 1) were made in an Applied Biosystems 380D DNA synthesizer, mixed at equal molar ratio, heated to 80°C, and allowed to cool slowly to room temperature. The annealed DNA was inserted into the HSV-1(F) Bam HI S fragment at the Bst EII site. The resulting plasmid pRB4009 contained a stop codon inserted in the beginning of the ICP34.5 coding sequence. The 20-nucleotide oligomer DNA insertion also contained a Spe I restriction site, which allowed rapid verification of the presence of the insert. To generate the recombinant virus R4010, rabbit skin cells were cotransfected with the intact DNA of R4002 and the pRB4009 plasmid DNA. Recombinants that were tk^- were selected in 143TK$^-$ cells in medium containing BrdU. The tk^+ version of this virus, designated R4009, was generated by cotransfection of intact tk^- R4010 DNA with HSV-1(F) Bam HI Q DNA fragment, and selection of tk^+ progeny. The virus selected for neurovirulence studies, R4009, contained the Spe I restriction endonuclease cleavage site in both Bam HI S and SP fragments (compare Fig. 2, left panel, lanes 9 and 10) and did not express ICP34.5 (Fig. 3, right panel, lane 7).

5) R4004 (Fig. 1, last line) was a recombinant virus produced by insertion of a sequence encoding 16 amino acids. This sequence had been shown to be the epitope of the monoclonal antibody H943 reactive with a viral protein designated as ICP4 (*21*). The virus was generated by cotransfecting intact R4002 DNA and the DNA of plasmid pRB3976 containing the insert, and the selected tk^- progeny was analyzed for the presence of the insert. For neurovirulence studies, its *tk* gene was restored (recombinant virus R4003) as described above. The DNA sequence was inserted in frame at the Nco I site at the initiating methionine codon of the $\gamma_1$34.5 gene. The insert regenerated the initiating methionine codon and generated a methionine codon between the epitope and the remainder of ICP34.5. Because of the additional amino acids, the protein migrated more slowly in denaturing polyacrylamide gels (Fig. 3, right panel, lane 4).

Plaque morphology and size of all of the recombinants were similar to those of the wild-type parent, HSV-1(F) when plated on Vero, 143TK$^-$, and rabbit skin cell lines. Whereas HSV-1(F)R and R4003 replicated as well as the wild-type virus in replicate cultures of Vero cells, the yields of R3616 and R4009 were reduced to one-third to one-fourth the amount of the wild type. Although ICP34.5 was not essential for growth of HSV-1 in cells in culture, the results of the studies shown in Table 1 indicate that the deletion or termination of translation of the $\gamma_1$34.5 had a profound effect on the virulence of the virus. Thus, all of the mice inoculated with the highest

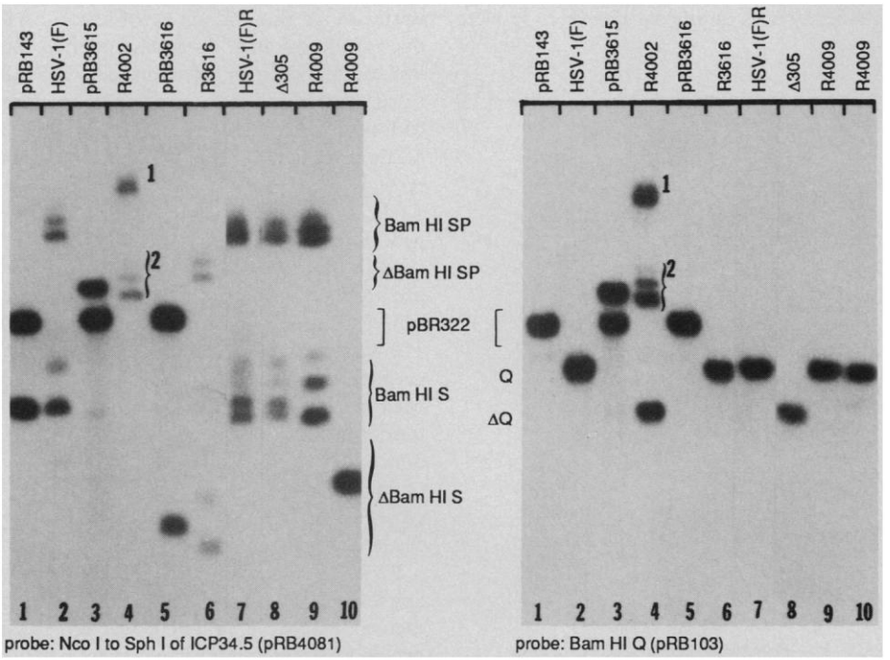

Fig. 2. Autoradiographic image of electrophoretically separated digest of plasmid, wild-type, and mutant virus DNAs, transferred to a solid substrate and hybridized with labeled probes for the presence of $\gamma_1$34.5 and *tk* genes. The plasmids or viral DNAs shown were digested with Bam HI or, in the case of the R4009 shown in lanes 10, with both Bam HI and Spe I. The hybridization probes were the fragment Nco I to Sph I contained entirely within the coding sequences of $\gamma_1$34.5 (left panel) and the Bam HI Q fragment of HSV-1(F) (right panel). The probes were labeled by nick translation of the entire plasmid DNAs with [α-^{32}P]deoxycytidine triphosphate and reagents provided in a kit (Du Pont Biotechnology Systems). The DNAs that were limit digested with Bam HI (all lanes) or both Bam HI and Spe I (left panel, lane 10) were electrophoretically separated on 0.8% agarose gels in 90 mM trisphosphate buffer at 40 V overnight. The DNA was then transferred by gravity to two nitrocellulose sheets sandwiching the gel and hybridized overnight with the respective probes. $\gamma_1$34.5 maps in Bam HI S and SP fragments, which form a characteristic ladder of bands at 500-bp increments. The ladders are a consequence of a variable number of *a* sequences in the repeats flanking the unique sequences of the long component. Inasmuch as Bam HI cleaves the viral genome within the inverted repeats but not at the junction between the long and short components, Bam HI S is the terminal fragment of the viral genome at the terminus of the long component, whereas Bam HI SP is a fragment formed by the fusion of the terminal Bam HI S fragment with Bam HI P, the terminal Bam HI fragment of the short component. Bands of Bam HI S, SP, and Q and their deleted versions, ΔBam HI S, ΔBam HI SP, and ΔBam HI Q (ΔQ), respectively, are indicated. Band 1 represents the 1.7-kbp α27-*tk* insert into the Bam HI SP fragment in R4002, and therefore this fragment reacted with both labeled probes (lanes 4). Band 2 represents the same insertion into the Bam HI S fragment.

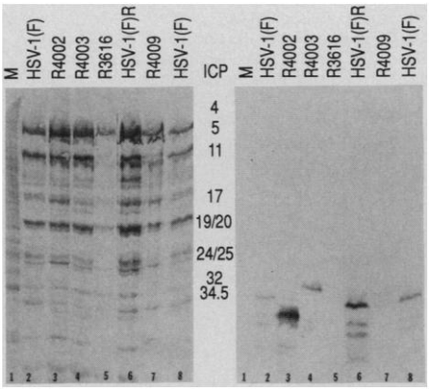

Fig. 3. Autoradiographic images (left panel) and photograph of lysates of cells mock infected (M) or infected with HSV-1(F) and recombinant viruses (right panel) separated electrophoretically in denaturing polyacrylamide (10%) gels, transferred electrically to a nitrocellulose sheet, and stained with rabbit polyclonal antibody R4 described elsewhere (*5, 6*). Replicate cultures of Vero cells were infected and labeled with [^{35}S]methionine (Du Pont Biotechnology Systems) from 12 to 24 hours after infection, and equivalent amounts of cell lysates were loaded in each slot. The procedures were as described (*5, 6*) except that the bound antibody was made apparent with the alkaline phosphatase substrate system supplied by Promega, Inc. Infected cell proteins were designated by number according to Honess and Roizman (*24*). The chimeric ICP34.5 specified by R4003 migrated more slowly than the protein produced by other viruses because of the increased molecular weight caused by the insertion of the epitope. The reactivity of the inserted epitope with monoclonal antibody H943 was reported elsewhere (*6*) and is not shown here.

Table 1. Comparative ability of wild-type and recombinant viruses to cause death after intracerebral inoculation of mice. The neurovirulence studies were done on female BALB/C mice obtained at 21 days of age (weight ± SD, 9.4 ± 1.8 g) from Charles River Breeding Laboratories in Raleigh, North Carolina. The viruses were diluted in minimum essential medium containing Earle's salts and 10% fetal bovine serum, penicillin, and gentamicin. The mice were inoculated intracerebrally in the right cerebral hemisphere with a 26-gauge needle. The volume delivered was 0.03 ml, and each dilution of virus was tested in groups of ten mice. The animals were checked daily for mortality for 21 days. The LD_{50} was calculated with the aid of the "Dose effect Analysis" computer program from Elsevier Biosoft, Cambridge, United Kingdom.

Virus in the inoculum	Genotype	PFU/LD_{50}
HSV-1(F)	Wild-type parent virus	420
R3616	1000-bp deletion in the $\gamma_1$34.5	>1,200,000
HSV-1(F)R	Restoration of $\gamma_1$34.5 and *tk*	130
R4009	Stop codon in $\gamma_1$34.5	>10,000,000
R4003	Monoclonal antibody epitope inserted at the NH_2 terminal	4,200

concentration [1.2×10^6 plaque-forming units (PFU)] of R3616 survived. In the case of R4009, only three of ten mice died as a result of inoculation with the highest concentration of virus (~10^7 PFU). In comparison with other deletion mutants, R3616 and R4009 rank among the least pathogenic viruses we have tested (*14, 22*). The virus in which the $\gamma_1$34.5 gene was restored exhibited the virulence of the parent virus.

The wild-type virus and all of the recombinants have identical surface glycoproteins necessary for attachment and penetration into brain cells. Injection of 10^6 PFU into the brain should result in infection and death of a significant number of the brain cells. Death after intracerebral inoculation results from viral replication, spread from cell to cell, and cell destruction before the immune system has a chance to act. Titrations of brain tissue suspended in minimal essential medium containing Eagle's salts and 10% fetal bovine serum showed that the brains of animals inoculated with the viruses that failed to make ICP34.5 contained very little virus. Thus, for the R3616 and R4009 viruses, the recovery was 120 and 100 PFU per gram of brain tissue, respectively. Given the amount of virus in the inoculum (highest concentration tested), it is not clear whether the small amounts of recovered virus represent a surviving fraction of the inoculum or newly replicated virus. In contrast, the amounts of virus recovered from mice inoculated with HSV-1(F)R and R4003 were 6×10^6 and 4×10^6, respectively. These results indicate that the failure of the two recombinant viruses to cause death must be related to poor spread of virus in neuronal tissue as a consequence of the inability of mutant viruses to replicate in the CNS, reflecting a reduction in their host range.

The significance of the results reported in this paper stems from several considerations. First, most deletion mutants in coding sequences generated and tested to date (*14, 22*) have a reduced capacity to cause disease in experimental animals. None of the HSV-1 mutants that carry a deletion in a single gene, however, exhibited as drastic a decrease in ability to cause death in experimental animals as those reported in this study. Second, the loss of capacity to replicate in the CNS and cause death is not due to rearrangement of DNA as a consequence of the deletion. Identical loss of capacity to replicate in the CNS was obtained by insertion of the stop codons. Furthermore, the loss of virulence by the mutants containing the stop codons was not a consequence of some rearrangement of the DNA sequences at the terminus of the genome, since insertion of the ICP4 epitope, a much larger insert, into the coding sequence had a marginal effect on the ability of the virus to replicate in the CNS. These data also indicate that the modification of the NH_2 terminus of the protein did not grossly debilitate the capacity of the protein to function in the mouse brain. Lastly and more significantly, while the function of ICP34.5 is not known, it is not essential for growth in cells in culture. The slight decrease in replication of this virus in cells in culture is not concordant with the loss of its ability to multiply and to destroy the CNS in mice. The failure to recover virus from CNS suggests that brain cells, unlike cells grown in culture, do not express genes whose products can substitute for $\gamma_1$34.5 gene product and complement the deletion mutants. ICP34.5 extended the host range of HSV-1 and enabled the virus to replicate in CNS. From this point of view, the protein is necessary for the dissemination of the virus from cell to cell and destruction of brain tissue characteristic of human encephalitis.

Gross deletions in the viral genome that result in decreased capacity to cause death in experimental animals have been previously reported (*13, 14*). In this report, we have excluded rearrangements in the viral genome, insertion of nonhomologous DNA, or deletion of cryptic open reading frames as the cause of the loss of neurovirulence. The results directly relate the capacity to replicate in CNS, the hallmark of neurovirulence, to the synthesis and expression of a specific protein made in relatively moderate amounts and not essential for replication in cultured cells. By its properties, the product of the $\gamma_1$34.5 gene comes closest of all known viral proteins to the definition of an HSV neurovirulence factor.

REFERENCES AND NOTES

1. R. J. Whitley, in *Virology*, B. N. Fields *et al.*, Eds. (Raven, New York, ed. 2, 1990), pp. 1843–1887.
2. P. Sheldrick and N. Berthelot, *Cold Spring Harbor Symp. Quant. Biol.* **39**, 667 (1975).
3. S. L. Wadsworth, R. J. Jacob, B. Roizman, *J. Virol.* **15**, 1487 (1975).
4. J. Chou and B. Roizman, *ibid.* **57**, 629 (1986).
5. M. Ackermann, J. Chou, M. Sarmiento, R. A. Lerner, B. Roizman, *ibid.* **58**, 843 (1986).
6. J. Chou and B. Roizman, *ibid.* **64**, 1014 (1990).
7. E. S. Mocarski and B. Roizman, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7047 (1981).
8. ———, *Cell* **31**, 89 (1982).
9. J. Chou and B. Roizman, *ibid.* **41**, 803 (1985).
10. L. P. Deiss, J. Chou, N. Frenkel, *J. Virol.* **59**, 605 (1986).
11. S. L. Varmuza and J. Smiley, *Cell* **41**, 793 (1985).

12. D. A. Vlazny, A. D. Kwong, N. Frenkel, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1423 (1982).
13. Y. M. Centifanto-Fitzgerald, T. Yamaguchi, H. E. Kaufman, M. Tognon, B. Roizman, *J. Exp. Med.* **155**, 475 (1982).
14. B. Meignier, R. Longnecker, B. Roizman, *J. Infect. Dis.* **158**, 602 (1988).
15. R. T. Javier, R. L. Thompson, J. G. Stevens, *J. Virol.* **65**, 1978 (1987).
16. R. L. Thompson, S. K. Rogers, M. A. Zerhusen, *Virology* **172**, 435 (1989).
17. M. Y. Taha *et al.*, *J. Gen. Virol.* **70**, 705 (1989).
18. ———, *ibid.*, p. 3073.
19. L. E. Post and B. Roizman, *Cell* **25**, 227 (1981).
20. J. Chou and B. Roizman, unpublished data.
21. J. Hubenthal-Voss, R. A. Houghten, L. Pereira, B. Roizman, *J. Virol.* **62**, 454 (1988).
22. B. Meignier, R. Longnecker, P. Mavromara-Nazos, A. E. Sears, B. Roizman, *Virology* **162**, 251 (1987).
23. L. E. Post, A. J. Conley, E. S. Mocarski, B. Roizman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4201 (1980).
24. R. W. Honess and B. Roizman, *J. Virol.* **12**, 1346 (1973).
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Calcium Mobilization and Exocytosis After One Mechanical Stretch of Lung Epithelial Cells

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Deep inflation of the lung stimulates surfactant secretion by unknown mechanisms. The hypothesis that mechanical distension directly stimulates type II cells to secrete surfactant was tested by stretching type II cells cultured on silastic membranes. The intracellular Ca^{2+} concentration was measured in single cells, before and after stretching. A single stretch of alveolar type II cells caused a transient (less than 60 seconds) increase in cytosolic Ca^{2+} followed by a sustained (15 to 30 minutes) stimulation of surfactant secretion. Both Ca^{2+} mobilization and exocytosis exhibited dose-dependence to the magnitude of the stretch-stimulus. Thus, mechanical factors can trigger complex cellular events in nonneuron, nonmuscle cells and may be involved in regulating normal lung functions.

EVIDENCE FROM EXPERIMENTS WITH whole animals or isolated lungs suggests that deep inflation of the lung augments secretion of pulmonary surfactant, the phospholipid-rich, chemically heterogeneous material that lowers surface tension at the air-liquid interface within the lungs (*1*). The mechanism by which an increase in lung volume apparently stimulates surfactant secretion is unknown. Because alveolar surface area may increase by as much as 80% during inflation to total lung capacity (*2*), one hypothesis is that mechanical distension of the alveolus causes surfactant to be secreted by alveolar type II epithelial cells. (Surfactant components are synthesized by the type II cell, stored in intracellular organelles, and secreted by exocytosis.) We found that a single stretch of alveolar type II cells cultured on elastic membranes caused a transient increase in cytosolic Ca^{2+}, after which surfactant secretion persisted for 30 min. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in single cells before and after stretching. Calcium was mobilized from intracellular rather than extracellular stores. Thus, mechanical factors can trigger complex cellular events in a nonneuron, nonmuscle cell and may be of general importance in regulating cellular functions.

Rat alveolar type II cells were cultured for 20 to 24 hours on one of two types of circular elastic silicone membrane dishes (*3*). In experiments designed to study surfactant secretion, cells were incubated during this period with [^{3}H]choline (1 μCi/ml) to label phosphatidylcholine (PC), the major component of surfactant (*4*). Release of lactic acid dehydrogenase (LDH) into the media was measured as one indicator of cellular damage (*5*).

Membranes were stretched by applying hydrostatic pressure beneath the membranes (*6*). To imitate deep inflation of the lung, we distended membranes once and then returned them to the relaxed position. Secretion from cells on stretched or unstretched membranes was then measured. Changes in membrane surface area for each type of membrane were determined by measuring the height of membrane displacement and calculating the increase in surface area from the formula for the area of a sphere (*7*). We compared the increase of measured two-dimensional cellular surface area (CSA) (*8*) to the increase in calculated membrane surface area (MSA) by photographing living cells on membranes in stretched and relaxed states and digitizing cellular surface area (Fig. 1). For cells cultured on both types of membranes, stretch-induced increases in secretion relative to unstretched controls correlated well with increases in CSA rather than with increases in MSA.

We measured secretion of [^{3}H]phosphatidylcholine ([^{3}H]PC) over 1 hour from cells on silastic sheeting and Sylgard membranes (Table 1). One hour after a single stretch that increased mean CSA by 16% (silastic sheeting membranes) or 17% (Sylgard membranes), [^{3}H]PC secretion by cells on both types of membranes (Table 1 and Fig. 2) was significantly increased when compared to control membranes studied concurrently in the same device.

There was a direct correlation between the magnitude of stretch (as measured by an

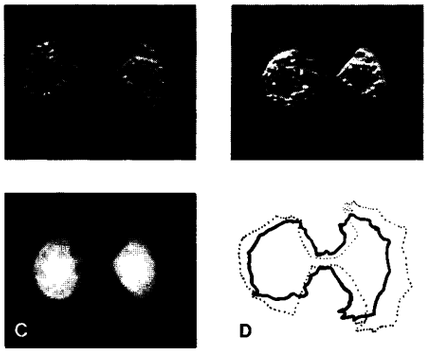

Fig. 1. Living rat alveolar type II cells cultured for 22 hours on Sylgard membranes. Photographs were taken at equal magnification on a Leitz Orthoplan microscope with differential interference contrast optics (×40 lens). Cells were loaded with fluorescein diacetate (50 μM for 1 hour) and the membrane was held and stretched in a modified Sykes-Moore chamber (Bellco Glass) with a no. 1 cover slip bottom. (**A**) Two connecting cells shown in the stretched state. The calculated increase in MSA was 52%. The measured increase in two-dimensional CSA was 23% for the cell on the left and 25% for the cell on the right compared to the relaxed state (**B**). (**C**) Staining with the vital dye fluorescein acetate indicates viability of the two cells. (**D**) Overlaid outlines of the two cells, showing the difference in shape and area. Cells were covered with a thin layer of medium (MEM with 25 mM Hepes buffer with Krebs salts).

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