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The Herpes Simplex Virus 1 Gene for ICP34.5, Which Maps in Inverted Repeats, Is Conserved in Several Limited-Passage Isolates but Not in Strain 17syn+

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In a previous study, it was reported that herpes simplex virus 1 (HSV-1) strain F contains a transcribed open reading frame situated in the inverted repeats of the L component between the terminal *a* sequence and the open reading frame that encodes the α0 gene (J. Chou and B. Roizman, J. Virol. 57:629–637, 1986). By means of an antibody to repeats of the trimer Ala-Thr-Pro predicted to be specified by the open reading frame, it was shown that the open reading frame specifies a protein (M. Ackermann, J. Chou, M. Sarmiento, R. A. Lerner, and B. Roizman, J. Virol. 58:843–850, 1986). This open reading frame is absent from the reported sequence of HSV-1(17)syn+ (D. J. McGeoch, M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor, J. Gen. Virol. 69:1531–1574, 1988; L. J. Perry and D. J. McGeoch, J. Gen. Virol. 69:2831–2846, 1988). To define the extent of variability in this open reading frame, we compared the sequences of the ICP34.5-encoding open reading frames of the genomes of three strains characterized by limited passage in cell culture with that of the HSV-1(17)syn+ strain. Furthermore, to establish unambiguously that the antibody to the Ala-Thr-Pro repeats reacts with the product of this open reading frame, we inserted a short sequence that encodes a known epitope in frame at the 5′ terminus of the coding domain. Our results indicate that with minor variations, the open reading frame is conserved in the three HSV-1 genomes analyzed but not in HSV-1(17)syn+. Thus, two strains contain an inserted amino acid and one strain, isolated from a case of human encephalitis, lacks a seven-amino-acid sequence. The recombinant virus carrying the foreign epitope expressed a slightly slower-migrating protein which reacted with both the rabbit polyclonal antibody to the Ala-Thr-Pro trimer repeats and the monoclonal antibody to the inserted epitope. The implications of the results are discussed.

The herpes simplex virus 1 (HSV-1) genome consist of two covalently linked components, each consisting of unique sequences flanked by inverted repeats (34, 37). The reiterated sequences flanking the unique long sequences designated as *ab* and *b′a′* are each 9 kilobase pairs, whereas the repeats flanking the unique short sequences are 6.3 kilobase pairs long (15, 19, 37). The shared terminal *a* sequence varies in size; in HSV-1 strain F [HSV-1(F)], the prototype strain used in our laboratory, the *a* sequence is approximately 500 base pairs long (24, 25). It has been known for a long time that the internal inverted repeat sequences contain the coding and regulatory, noncoding sequences of viral genes. Whereas the repeats of the small component each contain the α4 gene which encodes the major regulatory protein designated as infected-cell protein 4 (ICP4) (16, 26), the repeats of the long component contain gene α0, which specifies a protein designated as ICP0 (30) and is reported to be a promiscuous transactivator (9–13, 27–29, 31). As previously reported, several lines of evidence indicate that the internal inverted repeats flanking the long component contain an additional gene transcribed from the terminal *a* sequence toward the transcription initiation site of the α0 gene (Fig. 1). Specifically, (i) a chimeric gene constructed by fusion of the terminal *a* sequence to a reporter gene, the gene for HSV-1 thymidine kinase, expressed and regulated the reporter gene as a late (γ_1) gene (6); (ii) nucleotide sequencing of the region between the *a* sequence and the coding domain of the α0 gene revealed an open reading frame capable of encoding a protein (6); (iii) a polyadenylated transcript appropriate for the predicted protein was readily demonstrable in the infected cells (6); (iv) rabbit polyclonal antibody to a predicted oligopeptide consisting of repeats of the amino acid trimer Ala-Thr-Pro reacted with an infected-cell protein of the predicted electrophoretic mobility (1); and (v) insertional mutagenesis of the viral genome resulting in deletion from the coding sequences of the first 28 amino acids at the 5′ terminus of the gene yielded a protein reactive with the polyclonal antibodies but with faster electrophoretic mobility (1).

Recently, McGeoch and associates (21, 30), in their reports on the complete nucleotide sequence of HSV-1 strain 17syn+ [HSV-1(17)syn+], noted that in that strain the open reading frame ascribed to ICP34.5 "is thoroughly disrupted." The function of ICP34.5 is not known, but because several loci related to virulence map in the vicinity of that gene (4, 18, 23), the function of the gene became of interest and we reexamined the sequence of that gene. HSV-1(F) differs from HSV-1(17)syn+ in several characteristics. HSV-1(F) was isolated from a recurrent facial vesicle (8) and passaged a maximum of four times in cells in culture. HSV-1(F) retains the temperature sensitivity characteristic of many HSV-1 isolates passaged a limited number of times in cells in culture. HSV-1(17)syn+, first reported in 1973 (3), was described as the progeny of a plaque-purified virus. The passage history of HSV-1(17)syn+ has not been described, but it must be considered a multipassage laboratory strain. It

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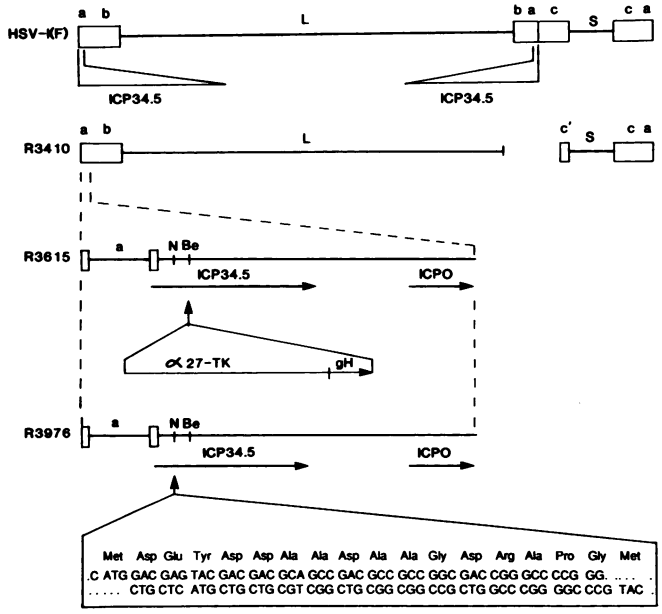

FIG. 1. Schematic representations of the sequence arrangements of HSV-1(F) and recombinant viruses R3410, R3615, and R3976. In the sequence arrangement of HSV-1(F), the *a* sequence identifies the terminal 500-base-pair sequence present in the direct orientation at the two genomic termini and in the inverted orientation at the junction between the L and S components of the genome (34, 37). *b* and *c* are large inverted repeats 9 and 6 kilobase pairs long, respectively, flanking the L and S components of the virus. In the sequence arrangement of recombinant virus R3410, the extent of the deletion of sequences at the L-S component junction is shown by the interrupted line (1). In the sequence arrangement of R3615 showing insertion of an α27-*tk* chimeric gene into the *Bst*EII site of the gene for ICP34.5, the insert contained at its terminus distal from the *a* sequence the regulatory domain, the 5'-transcribed noncoding domain, and the methionine initiation codon of glycoprotein H. The initiation codon was fused in frame with amino acid 27 of ICP34.5, generating a truncated form of this protein (1). In the sequence arrangement and construction of recombinant virus R3976 carrying the epitope encoded by ICP4 amino acids 121 to 135 (17), the sequence that encodes these amino acids was inserted at the *Nco*I site of ICP34.5. At the bottom in the nucleotide sequence and predicted amino acid sequence of the insert that encodes the ICP4 epitope specifically recognized by monoclonal antibody H943 on plasmid pRB3976 that was inserted into recombinant virus R3976 in frame with the gene for ICP34.5.

has been used as the parental (temperature-resistant) strain for generation of temperature-sensitive mutants. To establish the extent of variability of the open reading frame that encodes ICP34.5, we compared the nucleotide sequences of three HSV-1 strains passaged a limited number of times outside a human host. We report that the gene that specifies ICP34.5 contains 263 codons conserved in all three limited-passage strains but not in the reported sequence of the HSV-1(17)syn+ strain. To ensure that the antibody to a predicted repeat sequence, Ala-Thr-Pro, reacted with ICP34.5 rather than with a heterologous protein with a similar repeat sequence, we inserted near the 5' terminus of the ICP34.5-coding domain a short sequence of 45 nucleotides that encodes an epitope characteristic of another HSV-1 gene (17). The recombinant virus expressed a protein with an appropriately slower electrophoretic mobility and which reacted with both the monoclonal antibody to the inserted epitope and rabbit antiserum to the Ala-Thr-Pro repeat element.

MATERIALS AND METHODS

Viruses and cells. The isolation and properties of HSV-1(F) have already been described (8). HSV-1(MGH-10) was isolated at Massachusetts General Hospital from a case of HSV encephalitis (14). CVG-2 was an isolate obtained from a patient in a pediatric intensive care unit at Cleveland General Hospital (20). Both viruses were passaged only once since they were received and no more than three times since their isolation from the human hosts. The construction and structure of recombinant virus R3615 was reported elsewhere (1). Transfection of the viral and plasmid DNAs on rabbit skin cells and selection of the recombinant viruses under bromodeoxyuridine on human 143 thymidine kinase-negative cells were done as previously described (32).

Cloning and sequencing strategies of HSV-1 strains. Viral DNAs were prepared from virions that accumulated in the cytoplasm of infected Vero cells as previously described (19). The *Bam*HI SP junction fragments containing the domain of the gene that specifies ICP34.5 were cloned in pUC18 plasmids (36) by using HSV-1(F) sequences as probes in colony blot hybridization. Further subclonings were done as necessary to facilitate sequencing of different regions of the gene. The sequencing was done in part at the Laboratory for DNA Sequencing and Genome Analysis at the University of Nebraska Lincoln School of Biological Sciences by using the dideoxy-chain termination method (33) and in part at our laboratory by using a Sequenase DNA-sequencing kit (United States Biochemical Corp., Cleveland, Ohio) and [α-^{32}P]dATP (Dupont-NEN Research Products, Boston, Mass.). Various primers used in sequencing were synthesized in our laboratory in an Applied Biosystems (Foster City, Calif.) 380D DNA synthesizer. Reactions using dGTP and dITP as substrates in chain elongation and termination as supplied by kit were used to resolve regions of sequences of high G+C content.

Construction of recombinant plasmid pRB3976. An oligonucleotide 50 bases long and its complement sequence designed to end in *Nco*I restriction enzyme cleavage sites (Fig. 1) were synthesized as described above. The two oligonucleotides were then mixed at an equal molar ratio, heated to 80°C, and allowed to anneal by being cooled slowly to room temperature. The annealed DNA was then ligated to a cloned DNA fragment containing the gene for ICP34.5 and cleaved with *Nco*I. In the gene for ICP34.5, the *Nco*I site is located at the methionine initiation codon. The resulting plasmid, pRB3976, was sequenced (data not shown) to confirm the appropriate insertion as shown and then used in transfection experiments with the parental viral DNA, R3615, to generate recombinant virus R3976.

Infection and labeling of cells with [^{35}S]methionine and preparation of labeled protein extracts. Monolayer cultures containing 4×10^6 Vero cells were exposed to 5 to 10 PFU of recombinant viruses per cell for 2 h. After adsorption, the inoculum was replaced with mixture 199 supplemented with 1% calf serum and maintained for 12 h. The cells were then labeled with 10 μCi of [^{35}S]methionine (Dupont, NEN Research Products) in the same medium but without methionine for an additional 12 h. The cells were then harvested, washed once with phosphate-buffered saline, pelleted by centrifugation at 4,000 rpm for 5 min, and suspended in disruption buffer which consisted of 0.05 M Tris (pH 7.0), 8.5% (wt/vol) sucrose, 5% (vol/vol) 2β-mercaptoethanol, and

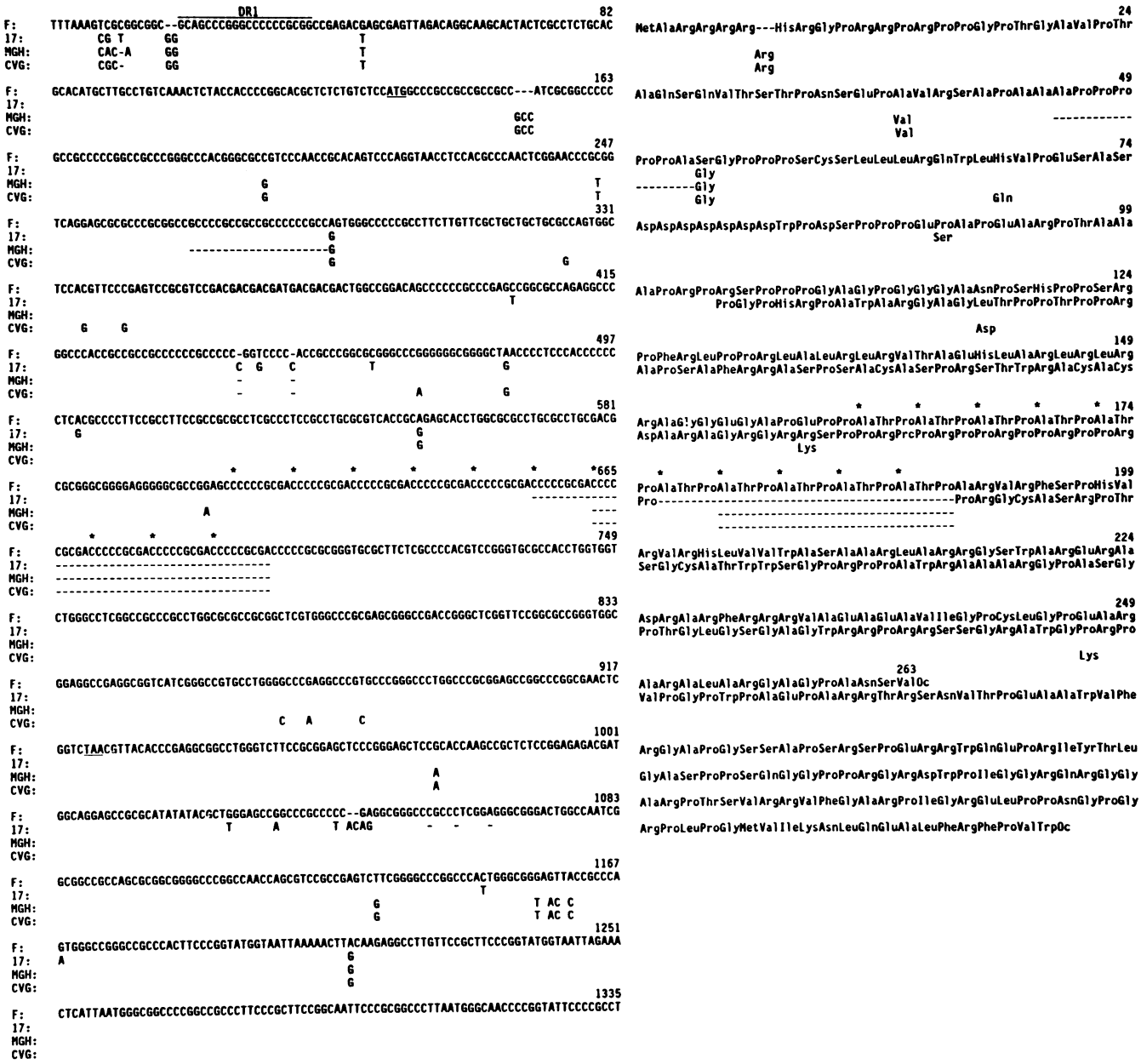

FIG. 2. DNA sequence comparisons of HSV-1 strains F, 17syn+, MGH-10, and CVG-2 in the region of the gene for ICP34.5 (left panel) and the predicted open reading frames for ICP34.5 in these strains (right panel). Unless otherwise indicated by a new base (insertion of A, C, G, or T), a new amino acid (three-letter code), or absence of a base or amino acid (−), the sequences for strains HSV-1(17)syn+, HSV-1(MGH-10), and HSV-1(CVG-2) were identical to the sequence for HSV-1(F). An asterisk indicates initiation of a repeat sequence of nine nucleotides or three amino acids. Direct repeat 1 (DR1) designates the 20-base-pair repeat sequence flanking the *a* sequence. Sequences upstream of direct repeat 1 are contained within the *a* sequence. The number at end of each line indicates the relative position from nucleotide 1 (left panel) or amino acid 1 (right panel). The initiation and termination codons for the HSV-1(F) sequence are underlined.

2% (vol/vol) sodium dodecyl sulfate. Extracts were then boiled for 2 min in water, disrupted by sonication in a Branson Sonifier 200 (SmithKline, King of Prussia, Pa.) three times for 3 s each time, and then subjected to electrophoresis in denaturing polyacrylamide gels.

Polyacrylamide gel electrophoresis and reaction of antibodies to electrophoretically separated polypeptides transferred to nitrocellulose sheets. The polypeptides were separated electrophoretically on denaturing 10% polyacrylamide gels, transferred electrically to nitrocellulose sheets, and reacted with rabbit antiserum R4 or monoclonal antibody H943 by using Vectastain ABC kits specifically for rabbit and mouse antibodies (Vector Laboratories Inc., Burlingame, Calif.) as previously described (1, 2).

RESULTS

Nucleotide sequence and predicted amino acid sequence. Figure 2 shows the nucleotide sequences of the 5′-transcribed noncoding and coding domains of the gene that

specifies ICP34.5 in HSV-1(F), HSV-1(MGH-10), and HSV-1(CVG-2) along with the reported sequence of HSV-1(17)syn+ (21, 30). As reported previously, the RNA transcribed across the domain of the gene that specifies ICP34.5 is initiated within direct repeat 1 of the *a* sequence (6). Methionine codon 1 in all HSV strains is 90 nucleotides downstream from direct repeat 1 and in three strains, 17syn+, MGH-10, and CVG-2, this sequence is identical in length to that of the F strain and differs from it in only one nucleotide. The differences among the strains within the domain of the gene are as follows. (i) The 5′-noncoding domain of the gene that specifies ICP34.5 includes most of the *a* sequence. The *a* sequences of 17syn+, MGH-10, and CVG-2 contain a dinucleotide insert and vary in the nucleotide sequence in the Ub domain of the *a* sequence. (ii) Whereas both F and 17syn+ contain four Arg residues at amino acids 3 to 6, strains MGH-10 and CVG-2 contain the insert GCC and encode five Arg residues at that position. (iii) MGH-10 lacks 21 nucleotides corresponding to the F strain amino acids (Ala-46–Pro–Pro–Pro–Pro–Pro–Ala-52) present in all other strains. (iv) The strain 17syn+ sequence contains two additional nucleotides not present in other strains. HSV-1(F) and all other strains contain the sequence GCC CCC-GGTCCCC-A at nucleotide positions 423 to 430 (Fig. 2 and 3), whereas the reported sequence for strain 17syn+ is GCCCCCCGGTCCCCCA. The net effects of insertion of the two nucleotides are frameshifts at amino acid positions 104 and 106. In strain 17syn+ there are additional insertions and deletions which occur outside the coding domain of the genes for ICP34.5 of other strains; these occur at positions corresponding to nucleotides 1044 to 1064 of strain F (Fig. 2). (v) With the exception of the missing 21 base pairs predicted to encode seven amino acids in MGH-10 and the additional arginine residue in MGH-10 and CVG-2, the differences among strains MGH-10, CVG-2, and F are minor. MGH-10 has a total of five nucleotide substitutions resulting in three amino acid differences. CVG-2 has 11 nucleotide substitutions resulting in five amino acid differences from F. (vi) The ICP34.5-coding domain in strain F contains 10 repeats of the sequence CCCCCGCGA, which encodes the trimer Ala-Thr-Pro. The numbers of repeats in the coding domains of the other strains are five for 17syn+ and six for strains MGH-10 and CVG-2. Because of the frameshift, the repeat sequence in strain 17syn+ translates as Pro-Arg-Pro. (vii) The open reading frame in HSV-1(F) is predicted to encode 263 amino acids, i.e., less than the previously reported 358 amino acids (6).

In-frame insertion of an epitope into the sequence of the gene that specifies ICP34.5. The evidence that the open reading frame between the terminal *a* sequence and the open reading frame that encodes α0 specifies a protein and that this protein is made in lytically infected cells was based in part on the reactivity of infected-cell extracts with a polyclonal antibody to the synthetic oligonucleotide (Ala-Thr-Pro)$_{10}$–Cys. Inasmuch as the divergence in the nucleotide sequence of strain HSV-(17)syn+ occurs before the predicted repeat Ala-Thr-Pro in HSV-1(F), it could be argued that the antibody to the trimer repeat reacted with a different protein containing such repeats. It could also be argued that truncation of the open reading frame in recombinant virus R3615 was coincidental with the selection of a variant virus containing fewer Ala-Thr-Pro repeats. To demonstrate unambiguously that the rabbit polyclonal antibody to the repeat Ala-Thr-Pro reacts with the protein predicted to be encoded by the nucleotide sequence of HSV-1(F), we inserted an oligonucleotide that encodes the amino acids 121 to 135

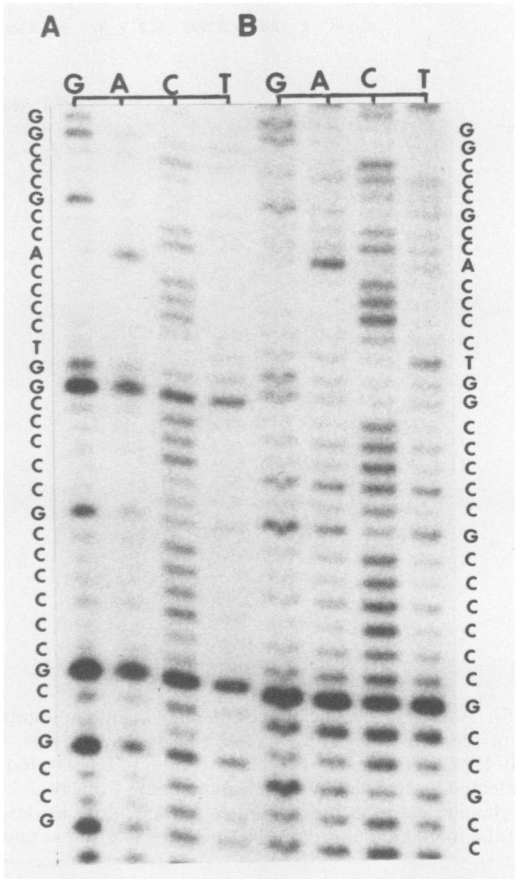

FIG. 3. Autoradiographic image of a DNA-sequencing gel showing sequences from nucleotide positions 424 to 458 (Fig. 2, left panel) of strain F. Reactions using dITP (A) and dGTP (B) as substrates for chain elongation as supplied in the sequencing kit were run side by side to resolve sequences of high G+C content and to improve accuracy. G, A, C, and T indicate the lanes in which the respective dideoxy triphosphates were used as termination substrates in the sequencing reactions (33).

containing an epitope of ICP4 in frame with the open reading frame at the *Nco*I site, as illustrated in Fig. 1. Previous studies (17) have shown that the synthetic polypeptide that encodes this sequence reacts with monoclonal antibody H943 to ICP4. The recombinant virus containing this epitope was designated R3976.

Lysates of cells mock infected or infected with the three viruses, i.e., parental HSV-1(F) and recombinant viruses R3615 and R3976, were electrophoretically separated in denaturing gels, electrically transferred to nitrocellulose, reacted with monoclonal antibody H943 or rabbit polyclonal serum R4, and subjected to autoradiography. The results of the analyses of these viruses are shown in Fig. 4. We have elected to present this figure because the bubbles which interfered in part with the transfer of some proteins to nitrocellulose allowed unambiguous alignment of the autoradiographic images with the bands formed by the reaction of the anti-immunoglobulin G antibody coupled to peroxidase with the protein transferred to nitrocellulose. Lysates of R3976-infected cells exhibited a band which reacted with both monoclonal antibody H943 and polyclonal rabbit serum R4 (Fig. 4). This band migrated more slowly than the band formed by lysates of HSV-1(F)-infected cells which reacted with R4 rabbit serum only. As expected on the basis of

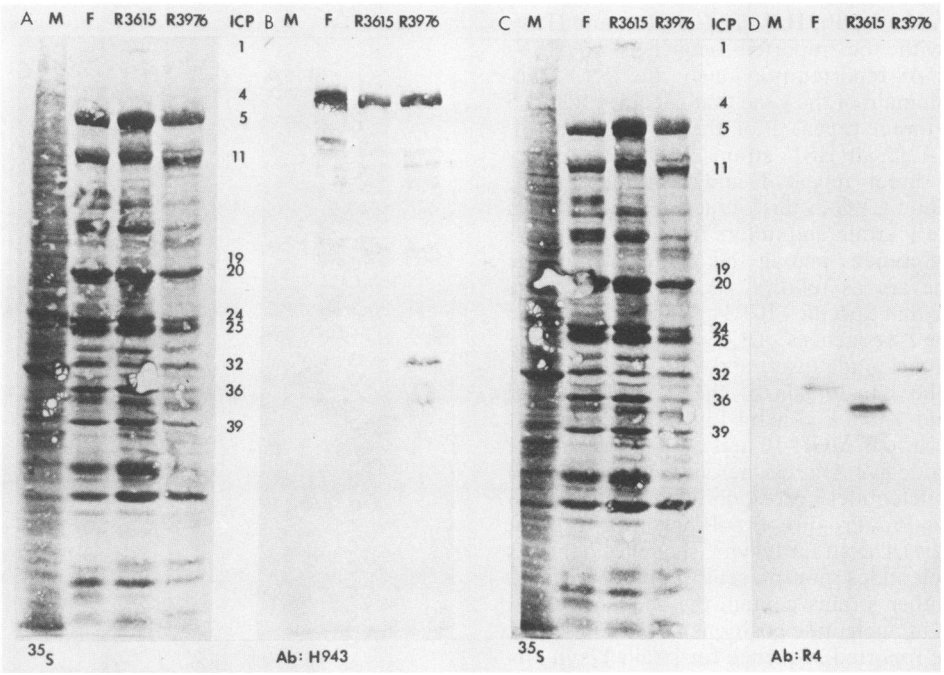

FIG. 4. Autoradiographic images (A and C) and photographs of lysates of cells mock infected or infected with HSV-1(F) or recombinants that were separated electrophoretically in denaturing gels, transferred electrically to a nitrocellulose sheet, and stained with monoclonal antibody H943 (B) or rabbit polyclonal antibody R4 (D). Replicate cultures of Vero cells were infected with HSV-1(F), R3615, or R3976 or mock infected and labeled with [^{35}S]methionine from 14 to 24 h postinfection. Lysates were subjected to electrophoresis in denaturing 10% polyacrylamide gels and transferred to nitrocellulose as described in Materials and Methods. The numbers to the right of panels A and C indicate the infected-cell protein designations of Honess and Roizman (16) and Morse et al. (26).

results published earlier (1), the band containing the truncated ICP34.5 protein reacted only with rabbit R4 serum and migrated faster than the authentic protein specified by HSV-1(F).

DISCUSSION

Discovery of the gene that encodes ICP34.5 followed the finding that the terminal *a* sequence in juxtaposition to a reporter gene was able to promote expression (6). The evidence that an open reading frame exists and is expressed was painstakingly collected for several reasons. (i) The sequence that encodes ICP34.5 is difficult to sequence because it is G+C rich and contains long stretches of guanines and cytosines. (ii) The 5′-untranscribed domain of the gene does not contain a TATAA box and is contained entirely within the terminal *a* sequence that also contains numerous repetitive G+C-rich elements, unlike most HSV-1 genes (6). (iii) If the open reading frame was expressed, its close proximity and overlap with the terminal *a* sequence could portend the possibility that its gene product is involved in one of the several functions of the terminal *a* sequence (5, 7, 24, 25, 35). (iv) Several laboratories (4, 18, 23) have reported the mapping of a locus which attenuated the ability of HSV-1 to cause central nervous system disease in mice at or near the inverted repeat region. The evidence cited in the introduction strongly suggests that the open reading frame exists and is expressed. In this report, we show that in fact the reading frame is conserved in three strains of HSV-1 characterized by limited passage outside a human host. We also provide definitive evidence supporting the existence of the open reading frame by showing that insertion of a foreign epitope into the gene in frame resulted in expression of both the predicted trimer Ala-Thr-Pro and the foreign epitope in the same protein. The repeated trimer must be in ICP34.5 and not in some other protein.

One hypothesis to explain the nonconservation of the open reading frame that specifies ICP34.5 in the reported sequence of HSV-1(17)syn+ (21, 30) is that the sequenced virus has accumulated mutations since its original isolation. Consistent with this view is the report by Taha et al. (34a) from the same institution that a variant of HSV-2 strain HG52 lacks 1.5 kilobase pairs covering precisely the domain of the gene that specifies ICP34.5. The virus grows but is completely avirulent (50% lethal dose, $>10^7$ PFU) in mice inoculated intracerebrally. We should stress that sequencing of the HSV-1 genome (21, 22, 30) has been an important and difficult accomplishment. The results presented in this report, however, document the existence of the gene that specifies ICP34.5 and show that the nucleotide sequence of that gene in three HSV-1 strains, i.e., HSV-1(F), HSV-1(CVG2), and HSV-1(MGH10), differs significantly from the reported sequence of the corresponding HSV-1(17)syn+ gene. The data raise the possibility that additional mutations have accumulated in other genes of HSV-1(17)syn+ and underscore the need to sequence regions of particular interest in more than one virus strain to ensure that the sequence approximates that of a wild-type virus and does not reflect accumulated mutations of serial passages outside a human host.

The role of the gene for ICP34.5 in determining the ability of a virus to multiply and destroy central nervous system tissue is of particular interest. Of the viruses examined in this study, two have been extensively tested for virulence in mice. HSV-1(F), isolated from a recurrent facial lesion, is of

moderate virulence, with a 50% lethal dose of approximately 100 PFU by intracerebral inoculation of mice. In contrast, MGH-10, derived from a case of human encephalitis, has a 50% lethal dose of 1 to 5 PFU by the same route in identical mouse strains (23). We cannot ascribe the differences in virulence between the two strains to the differences in the nucleotide sequences of the ICP34.5 genes, but this hypothesis is being tested.

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