JOURNAL OF VIROLOGY, Nov. 1986, p. 807–812
0022-538X/86/110807-06$02.00/0

Vol. 60, No. 2

# Construction and Properties of a Viable Herpes Simplex Virus 1 Recombinant Lacking Coding Sequences of the α47 Gene

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Received 1 April 1986/Accepted 13 July 1986

**The five α genes, α0, α4, α22, α27, and α47, are the first set of herpes simplex virus 1 genes to be transcribed and expressed in productively infected cells. We report here the construction of a viral recombinant from which all of the coding sequences of the α47 gene were deleted. In addition to the α47 protein, infected cell lysates did not contain detectable amounts of two polypeptide bands with apparent molecular weights of 18,000 and 21,000 which could be specified by a gene whose regulatory domain and 5′ transcribed noncoding sequences overlap with the coding sequences of the α47 gene. The α47$^-$ virus grew as well as the wild-type parent virus in Vero, baby hamster kidney, and Rat-1 cell lines.**

The five α genes, α0, α4, α22, α27, and α47, encoded by the herpes simplex virus 1 (HSV-1) genome are the first set of genes that are transcribed and expressed after infection (9, 10, 12, 32). The function of the α genes is not completely understood. The α4 gene specifies a 160,000-apparent-molecular-weight DNA-binding phosphoprotein which is required for the transcription of β and γ genes expressed later in productive infection (3, 14, 25, 37). The α0 gene has been shown to stimulate gene expression in transient expression systems; the function of the gene in lytic infection is not known (6, 22, 23). Analyses of mutants whose lesions map in the domain of the α27 gene suggest that the product of this gene is required for late gene expression (34). By using a novel procedure for the construction of deletion mutants, Post and Roizman (30) reported the isolation of recombinants expressing truncated, unstable α22 proteins (2). These recombinants grew well in some cell lines but not in others. Analyses of virus growth in permissive and restricted cells suggested that α22 specifies a function that complements one or more host functions required for expression of late ($\gamma_2$) genes and that these functions are provided by some cells but not by others (35). Nothing is known about the function of the α47 gene. Recently, this laboratory reported the isolation of a recombinant lacking the internal inverted repeat sequences and several contiguous unique genes located between the gene specifying glycoprotein D and the terminus of the S component (15). These genes include glycoprotein E, α47, and two additional genes located in a transcriptional unit 3′ coterminal with the α47 gene. Because of the extensive deletion, the recombinant is not suitable for analyses of the α47 gene function. To specifically address the function of the α47 gene, we constructed a recombinant from which the coding sequences of the α47 gene were specifically deleted by a modification of the procedure of Post and Roizman (30).

The protocol involved two cloning steps. In the first, a 2.2-kilobase (kb) *Pvu*II DNA fragment from plasmid pRB364 (16) containing the thymidine kinase gene driven by the promoter-regulatory domain of the α4 gene (α4-TK) was inserted into the transcribed noncoding sequences of the α47 gene at the *Nru*I site of the *Sal*I-*Eco*RI subfragment of *Eco*RI-H cloned as pRB421 (22). This fragment contains the intact α47 gene (Fig. 1). In the second step, the α47 coding sequences located immediately adjacent to the TK gene were deleted by digesting the plasmid produced in the first step with *Bst*EII, which cleaves the plasmid DNA at the end of the TK gene and at the 3′ terminus of the α47 gene (Fig. 1). The resulting plasmid (pRB3630), in which the selectable marker, α4-TK chimeric gene, replaced all of the coding sequences of the α47 gene, was then cotransfected with an intact TK$^-$ [HSV-1(F)Δ305] viral DNA and TK$^+$ recombinants were selected among the progeny of the transfection. The TK$^-$ recombinant HSV-1(F)Δ305 contains a 700-base-pair (bp) deletion within the transcribed 5′ noncoding and coding sequences of the TK gene (5, 29). The reason for using a chimeric α-TK gene for the selection was to preclude the recombination of the TK gene contained in the plasmid with the remaining TK gene sequences at the normal gene position in the viral genome, since the α-TK chimeric gene and the partially deleted TK gene resident in the HSV-1(F)Δ305 DNA share only the sequences downstream from the site of the deletion. The selected R3630 recombinant virus had the desired genotype as evidenced by the following experiments.

(i) *Bam*HI digests of R3630 DNA contained a new fragment of approximately 3.4 kb (Fig. 2A, band 1, lane 3) as predicted by the insertion of a 2.2-kb *Pvu*II α4-TK fragment into the 1.8-kb *Bam*HI Z fragment and by the deletion of the 0.6-kb *Bst*EII subfragment. This band hybridized with $^{32}$P-labeled *Bam*HI Z (Fig. 2) and TK probes (data not shown), indicating that R3630 *Bam*HI digests contained sequences homologous to both probes. As expected, the *Bam*HI Z probe hybridized to the *Bam*HI N fragment of R3630 and of HSV-1(F) DNA digests (Fig. 2A, lanes 1 and 9) since this fragment shares with *Bam*HI-Z the inverted repeat *c* sequences flanking the unique sequences of the S component (Fig. 1). The significant finding is that R3630 digests did not contain a fragment comigrating with wild-type *Bam*HI-Z that hybridized with the *Bam*HI Z probe.

(ii) The *Bgl*II digests of the R3630 DNA contained a novel submolar band (band 2) of approximately 10.8 kb but lacked the terminal, submolar *Bgl*II L fragment (Fig. 2A, lane 4). The novel band 2 fragment hybridized with both labeled *Bam*HI Z and TK probes, indicating that it contains sequences homologous to *Bam*HI-Z (Fig. 2A, lane 2) and TK DNAs (data not shown). The $^{32}$P-labeled *Bam*HI Z probe also hybridized with the *Bgl*II H fragment, as expected, since both fragments contain the inverted repeat *c* sequence

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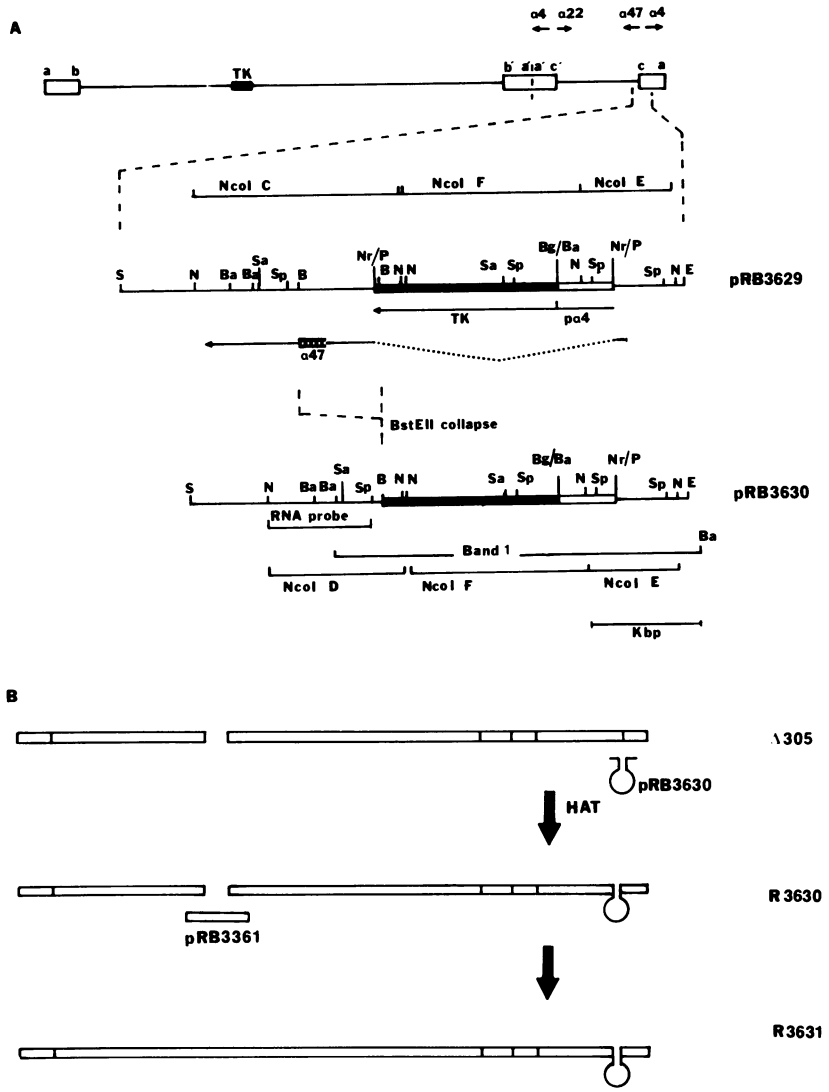


FIG. 1. (A) Schematic diagram of sequence arrangment of wild-type and recombinant HSV-1 DNAs and of plasmids constructed for deletion of the α47 gene. The top line shows the sequence arrangement of HSV-1(F) DNA and the locations of the TK, α4, α22, and α47 genes. The TK gene maps in the unique sequences of the L component flanked by the inverted repeat sequences *ab* and *b'a'* (depicted as open boxes), whereas the coding sequences of the α22 and α47 genes map in the unique sequences of the S component flanked by the inverted repeats *c'a'* and *ca* (also boxed). The expanding portion represents the *Eco*RI-*Sal*I fragment cloned into pRB421, which contains the entire α47 transcriptional unit. pRB3629 is derived from pRB421 by insertion of the α4-TK chimeric gene from pRB364 into the unique *Nru*I site of pRB421. ■, *Bgl*II-*Pvu*II fragment of the TK gene. □, α4 promoter-regulatory sequences. Direction of transcription of the inserted α4-TK chimeric gene is the same as that of the α47 gene. Restriction enzyme cleavage sites are abbreviated as follows: E, *Eco*RI; N, *Nco*I; Sp, *Sph*I; Ba, *Bam*HI; Sa, *Sac*I; B, *Bst*EII; S, *Sal*I; Nr/P, fusion of fragments at *Nru*I and *Pvu*II cleavage sites; Bg/Ba, fusion of fragments at *Bgl*II at *Bam*HI cleavage sites (19). *Nco*I C, D, F, and E, *Nco*I fragments shown in Fig. 2B. Band 1, the novel *Bam*HI fragment shown in Fig. 2A. (B) Schematic representation of the procedures for the selection of recombinant viruses. Parental HSV-1(F)Δ305 virus has a deletion in the TK gene. Cotransfection of HSV-1(F)Δ305 and pRB3630 DNAs, followed by plating of the progeny virus in medium containing hypoxanthine, methotrexate, and thymidine (7, 29, 33), yielded the R3630 recombinant virus. Cotransfection of intact R3630 viral DNA with a *Bam*HI Q subfragment DNA (pRB3361) yielded the R3631 recombinant virus in which the deletion in the TK gene in *Bam*HI-Q has been restored.

of the S component. Both the TK and the *Bam*HI Z probes hybridized, as expected, to the L-S component junction fragments indicated in Fig. 2.

(iii) To verify that the deletion in R3630 DNA corresponds to the deletion in the plasmid, electrophoretically separated *Nco*I digests of appropriate plasmid and viral DNAs were hybridized to the $^{32}$P-labeled *Bam*HI Z probe (Fig. 2B). The *Nco*I DNA fragments were designated C, D, E, F, and G (Fig. 1). These fragments contain the α47 gene (*Nco*I-C), the TK gene (*Nco*I-F), the α47 regulatory sequences (*Nco*I-E), and the pUC8 vector (*Nco*I-G). *Nco*I-D contains the *Nco*I-C sequences but without the *Bst*EII subfragment. The *Nco*I C, D, and E fragments hybridized, as expected, with the labeled *Bam*HI Z probe. The low intensity of the *Nco*I-D band was caused by the small stretch of DNA sequences (150 bp) shared with the probe. The *Bam*HI Z probe hybridized with two fragments in electrophoretically separated *Nco*I digests of HSV-1(F)Δ305 viral DNA, i.e., with fragment B of

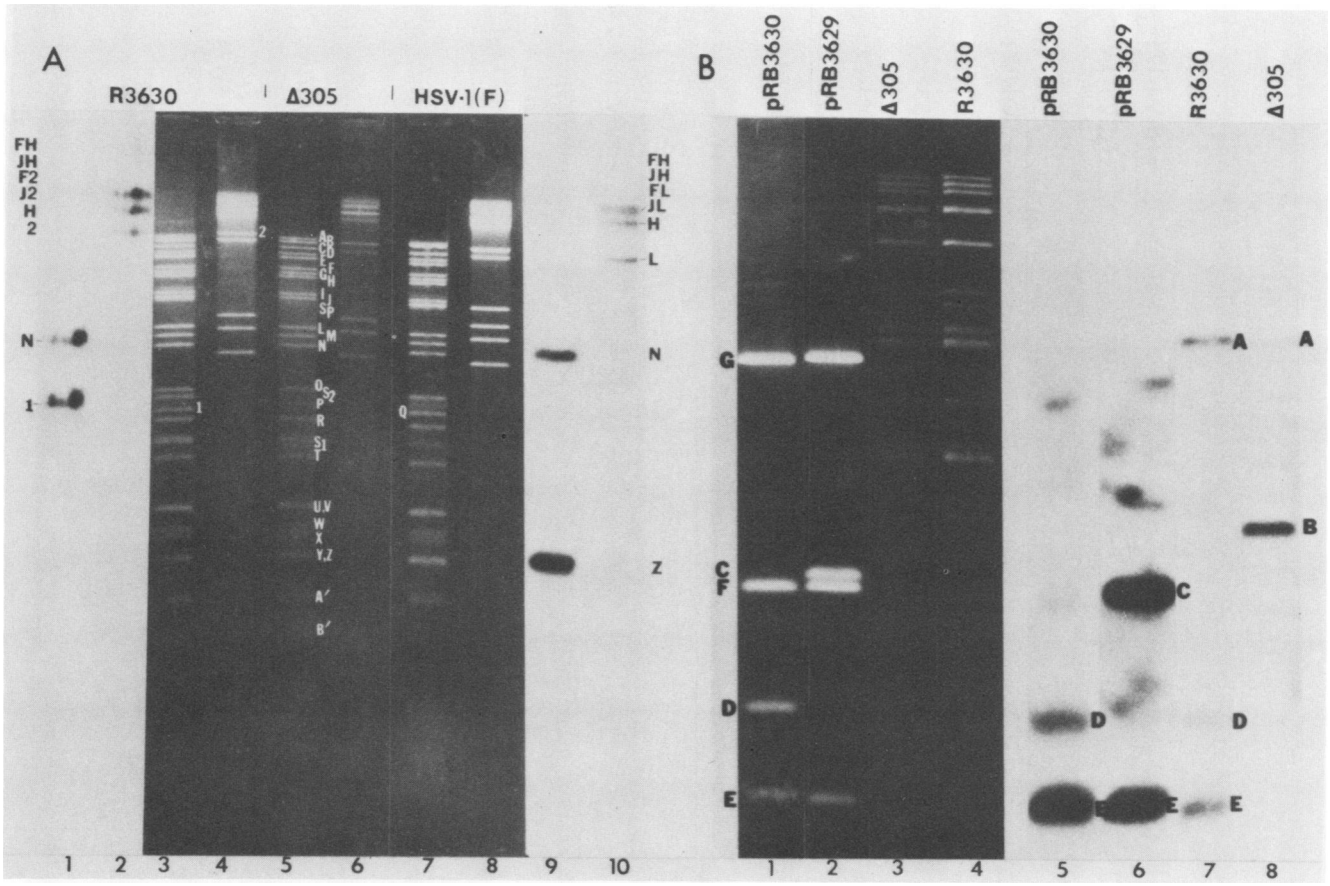


FIG. 2. Photographs and autoradiograms of electrophoretically separated *Bam*HI and *Bgl*II restriction enzyme digests of recombinant R3630, parental Δ305, and HSV-1(F) viral DNAs. (A) Analyses for α47 gene sequences of *Bam*HI and *Bgl*II digests. The digests were electrophoretically separated in a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to the $^{32}$P-labeled *Bam*HI Z probe, an *Sph*I-*Sph*I fragment from pRB421 (22) containing approximately 800 bp from the *c* sequence, the coding sequences, and 150 bp of the 3′ noncoding transcribed sequences of the α47 gene (19). Lanes: 1 and 2, autoradiographic images of lanes 3 and 4; 3 to 8, ethidium bromide-stained gel; 9 and 10, autoradiographic image of lanes 7 and 8. Bands: 1, novel fragment containing *Bam*HI-Z sequences and schematically represented in Fig. 1; 2, the novel *Bgl*II L terminal fragment. Fragments: FH, JH, F2, J2, and H at the left of the panel, the *Bgl*II H and the *Bgl*II L-S junction fragments of R3630 that hybridize to the probe; N, the *Bam*HI N fragment; Letters at the right of lane 5, the *Bam*HI fragments of parental HSV-1(F)Δ305; (left of lane 7), the *Bam*HI Q fragment of HSV-1(F). FH, JH, FL, JL, H, and L, (right of the panel), the *Bgl*II fragments of HSV-1(F) homologous to the probe; N and Z, the *Bam*HI N and Z fragments. (B) Analyses for α47 sequences of *Nco*I digests. The DNA fragments were electrophoretically separated, transferred to nitrocellulose, and hybridized with the *Bam*HI Z probe as described above. Lanes: 1 to 4, ethidium bromide gel; 5 to 8, autoradiographic images of lanes 1 to 4. Fragments: C, F, D, and E, fragment identification as in the legend to Fig. 1; G, the *Nco*I fragment of plasmids pRB3629 and pRB3630 containing the pUC8 vector; A, the 3.9-kb *Nco*I fragment contained in the *Bam*HI N fragment; B, the 2.1-kb *Nco*I fragment contained in the *Bam*HI Z fragment. The procedures for cloning, electrophoretic separation and transfer to nitrocellulose, labeling of probes, hybridization, and autoradiography were as previously described (15, 17, 20, 28, 36).

approximately 2.1 kb (the predicted size of the *Nco*I fragment contained in *Bam*HI-Z) and with fragment A of 3.9 kb (the predicted size of the *Nco*I fragment in *Bam*HI-N that contains the inverted repeat *c* sequences) (19). Fragment B, as expected, was absent from the *Nco*I digests of R3630. Instead, two novel fragments which comigrated with *Nco*I-D and -E hybridized to the *Bam*HI Z probe. Additional support for the proposed structure of the R3630 DNA emerged from the observation that the *Sac*I fragments hybridizing with the labeled *Bam*HI Z probe were of the expected size (data not shown).

To investigate the phenotype of the α47 deletion mutant, the 700 bp of the TK gene originally deleted from HSV-1(F)Δ305, the parent of R3630, were restored by cotransfecting intact viral R3630 and plasmid pRB3361 DNAs into rabbit skin cells. Plasmid pRB3361 carries the 2-kb *Pvu*II fragment containing the TK gene of HSV-1(F) cloned into pUC9 (22). Individual plaques were picked from infected Vero cell monolayers, and the DNA of these progeny was screened by restriction enzyme analyses for the presence of the wild-type *Bam*HI Q fragment containing the *Bgl*II cleavage site missing in R3630 and HSV-1(F)Δ305 DNAs. The TK$^+$ derivative virus, R3631, was used in two series of experiments.

In the first, Vero cells were treated with cycloheximide (50 μg/ml) for 30 min before infection, during infection, and for 5 h postinfection. The drug was then removed, and the infected cells were labeled with [$^{35}$S]methionine for 30 min, solubilized in denaturing buffer containing sodium dodecyl sulfate, and subjected to electrophoresis in denaturing polyacrylamide gels as described elsewhere (1, 21). Autoradiographic images of the electrophoretically separated proteins

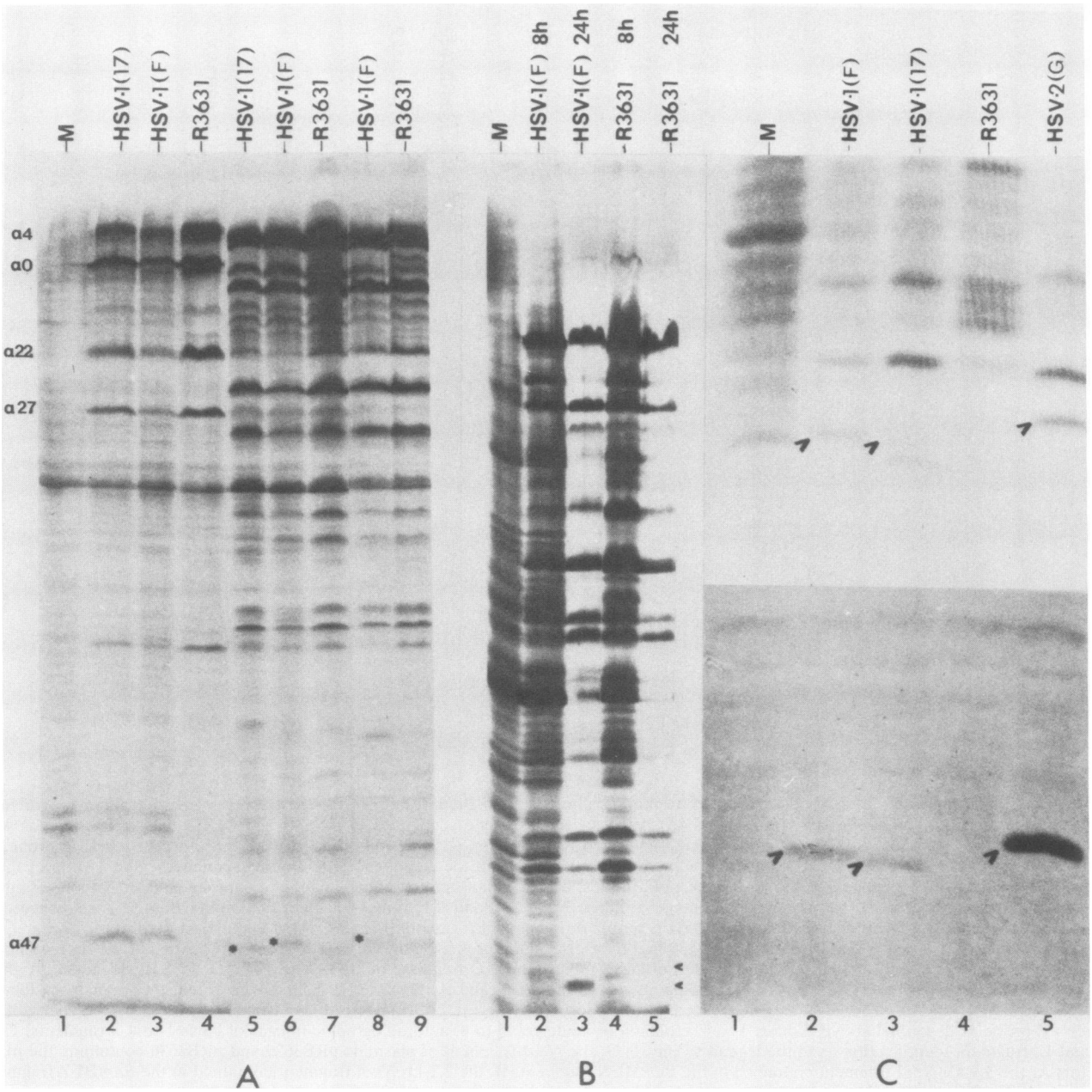


FIG. 3. Autoradiograms of [$^{35}$S]methionine-labeled polypeptides from lysates of Vero cells infected with wild-type and recombinant viruses. (A) Autoradiographic image of lysates separated in denaturing 17% polyacrylamide gels. Lanes: 1, mock-infected cells; 2 to 4, virus-infected cell polypeptides labeled with [$^{35}$S] methionine after cycloheximide treatment as described in the text; 5 to 7, cells labeled at 5 to 7 h postinfection; 8 and 9, cells labeled at 10 to 12 h postinfection. The α proteins were identified on the basis of previous studies (9, 10, 30). *, α47 protein made at 5 to 7 and 10 to 12 h postinfection. (B) Autoradiogram of [$^{35}$S]methionine-labeled polypeptides from lysates of Vero cells infected with HSV-1(F) and R3631 electrophoretically separated in denaturing 12% polyacrylamide gels. Lanes: 1, mock-infected cells; 2 and 4, cells labeled at 6 to 8 h postinfection; 3 and 5, cells labeled at 22 to 24 h postinfection. Arrowheads indicate proteins with apparent molecular weights of 18,000 and 21,000 missing from R3631-infected cells. (C) Autoradiograms and immunoreactivity of polypeptides from infected Vero cells eletrophoretically separated in denaturing 17% polyacrylamide gel and electrically transferred to nitrocellulose sheets. Vero cells were infected with the indicated viruses and labeled from 5 to 7 h postinfection. Electrophoretically separated polypeptides were transferred to nitrocellulose and reacted with rabbit serum R973. Top: autoradiographic image of the lower portion of the gel. Bottom: immunoreactivity of rabbit R973 serum with the proteins shown in the top lanes. Procedures for labeling of proteins, electrophoresis in denaturing gels and electric transfer to nitrocellulose, autoradiography, and staining with antibody were as described elsewhere (1). Procedures for preparation of the synthetic peptide and of antiserum were similar to those previously described and will be detailed elsewhere (M. Ackermann, M. Sarmiento, and B. Roizman, manuscript in preparation).

indicated that a protein migrating with an apparent molecular weight of 12,000, corresponding to the electrophoretic mobility of the α47 protein (18, 24), was not present in lysates of cells infected with R3631 (Fig. 3A, lane 4). In addition, Vero cells were labeled with [$^{35}$S]methionine for 2 h at 5 and 10 h (Fig. 3A) and at 6 and 22 h (Fig. 3B) postinfection, and the lysates were electrophoretically separated in denaturing polyacrylamide gels. As in the case of other α proteins (1, 2),

TABLE 1. Growth of HSV-1(F) and R3631 in Vero, BHKtk⁻, and Rat-1 cells

| Multiplicity of infection (PFU/cell) | Cell line | Titer of: | |
|---|---|---|---|
| | | HSV-1(F) | R3631 |
| 0.01 | Vero | $1 \times 10^6$ | $2.4 \times 10^6$ |
| | BHKtk⁻ | $2.4 \times 10^6$ | $3.4 \times 10^6$ |
| | Rat-1 | $1.4 \times 10^4$ | $1.1 \times 10^4$ |
| 1.0 | Vero | $2.4 \times 10^6$ | $5 \times 10^6$ |
| | BHKtk⁻ | $9 \times 10^6$ | $2.2 \times 10^7$ |
| | Rat-1 | $3.6 \times 10^5$ | $9.6 \times 10^5$ |

the synthesis of the 12,000-molecular-weight protein, identified as α47, was detected at least until 10 h postinfection in the HSV-1(F)- and HSV-1(17)-infected cells (Fig. 3A). Of interest, however, was the observation that two late proteins of apparent molecular weights of 18,000 and 21,000 were absent from lysates of Vero cells infected with R3631 (Fig. 3B, lane 5). These polypeptides may represent two different forms of the infected cell protein 48 (ICP48) or the 21K protein, predicted to be encoded downstream from the α47 gene (19, 31) since the deletion in the R3631 virus genome eliminated both the promoter and 5′ transcribed noncoding sequences of this gene. With these exceptions, the patterns of protein synthesis of R3631- and HSV-1(F)-infected Vero cells were similar at those times postinfection.

To verify that the missing 12,000-apparent-molecular-weight protein was indeed α47, we used a rabbit antiserum (R973) made against the synthetic peptide representing amino acids 30 to 43 of the α47 protein, as predicted from the nucleotide sequence of the gene (19). Vero cells were labeled with [$^{35}$S]methionine for 5 to 7 h postinfection with HSV-1(F), HSV-1(17), R3631, and HSV-2(G), and the lysates were electrophoretically separated in denaturing polyacrylamide gel, electrically transferred to nitrocellulose, and reacted with the rabbit antiserum R973. Antiserum R973 reacted with a protein comigrating with the α47 protein in both HSV-1(F)- and HSV-1(17)-infected cell lysates (Fig. 3C). This protein band was absent from R3631- and mock-infected cell lysates. Moreover, antiserum R973 also reacted strongly with a slower-migrating protein in the HSV-2(G) cell lysates, which may correspond to the α47 protein produced by HSV-2(G). Consistent with the results described above, the HSV-1(17) protein reacting with this serum migrated faster than did the α47 protein specified by HSV-1(F) (Fig. 3C).

The design of the second series of experiments took into account the observation that the α22 deletion mutants, as well as the wild-type parent virus, grew in HEp-2 and Vero cells but that the recombinants failed to grow in rodent cell lines (35). The inability to grow in these cell lines was especially manifest at lower multiplicities of infection and was ultimately shown to be caused by decreased production of late ($\gamma_2$) proteins. At the time of isolation of the R3630 and R3631 viruses, it was evident that both grew in Vero cells. To measure the ability of R3631 to grow in rodent cell lines, BHKtk⁻ and Rat-1 cell lines were infected at 1 and 0.1 PFU per cell. The results (Table 1) indicate that, in contrast to the R325 recombinant, R3631 grew as well as the parental HSV-1(F) in all cell lines at the multiplicities of infection tested.

The salient features of the results presented in this paper are as follows. (i) The procedure we used to delete the α47 gene is similar to that described by Post and Roizman (30) except that, instead of first inactivating the target gene by inserting the TK gene and then deleting both the TK and the adjacent target sequences, the procedure described here simply replaced the target gene with the TK gene in one step.

(ii) The design of the deletion protocol was to remove all but the last codon of the α47 gene. The absence of the α47 gene from the recombinants constructed in this study was confirmed by analyses of the DNA fragments containing the gene, by analyses of proteins labeled in infected cells, by the absence of reactivity of the electrophoretically separated infected cell polypeptides with a polyclonal antiserum made against a synthetic peptide representing a hydrophilic domain of the α47 amino acid sequence predicted from the nucleotide sequence, and by analyses of the viral cytoplasmic RNA (data not shown).

(iii) Studies carried out in this laboratory have focused on the two genes mapping in the S component of HSV-1 DNA, i.e., α22 and α47. In earlier studies and in this study, it has been shown that recombinants lacking either of these genes are capable of growing in at least some cells in culture. In contrast to the α22 deletion mutants which grew well in HEp-2 and Vero cells but not in rodent cell lines, the α47⁻ recombinant grew well in all cell lines tested. These studies suggest that the α47 gene product is not required for growth in the cell lines tested and, by analogy to the α22⁻ recombinant virus, the cells in culture supply factors missing in α47 deletion mutants that may be required for virus growth.

(iv) In the past several years, many viral genes and sequences of as yet unidentified function have been deleted from viral genomes without affecting the ability of these viruses to grow in at least unmodified HEp-2 and Vero cells. These genes include one copy each of the α4, α0, and $\gamma_2$34.5 genes (11, 26, 27) and the unique genes specifying the glycoproteins C (8, 13) and E (15), the α22 (30) and α47 genes, the TK genes (4, 29), the two genes located 3′ to the α47 gene, and the genes located between the α27 and the α0 genes (R. Longnecker and B. Roizman, unpublished results). For heuristic reasons, if not in principle, viruses must not be regarded as carrying unnecessary genes or DNA sequences. In the case of HSV, the existence of these seemingly unessential genes may be rationalized on the basis of the behavior of the virus in its natural host. A biologic characteristic of HSV is its ability to grow in a wide variety of cells in culture, to establish latency, and to become reactivated. It is conceivable, by analogy with the studies done on the α22⁻ mutant, that the function of many of these genes is to complement functions missing in some cells infected by HSV in its natural host. The function of the α47 gene remains to be determined. Further studies in both cell culture and experimental animals may yield a system in which the growth of this virus is restricted.

We thank Nancy Michael and Denise Simmons for expert technical assistance.

These studies were aided by Public Health Service grants CA-08494 and CA-19264 from the National Cancer Institute and by American Cancer Society grant MV-2R. P.M.N. is a Damon Runyon postdoctoral trainee (DRG767).

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