Application of Antibody to Synthetic Peptides for Characterization of the Intact and Truncated $\alpha 22$ Protein Specified by Herpes Simplex Virus 1 and the R325 $\alpha 22^-$ Deletion Mutant

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The $\alpha 22$ protein is one of five proteins synthesized immediately after infection of permissive cells with herpes simplex virus 1 and 2 (HSV-1 and HSV-2). On the basis of the reported nucleotide sequence of the HSV-1 gene, we synthesized two peptides containing the predicted amino acids 12 through 23 (12 residues) and 21 through 36 (16 residues) in two hydrophilic domains near the N terminus of the protein. Rabbit antisera made against these peptides were then used to characterize the $\alpha 22$ protein made by wild-type HSV-1(F) strain and by an HSV-1 mutant, R325, carrying a 500-base-pair deletion within the coding domain of the gene. The results were as follows. (i) Both antisera reacted with HSV-1(F) $\alpha 22$ protein in lysates electrophoretically separated in denaturing polyacrylamide gels and electrically transferred to a nitrocellulose sheet; neither antiserum reacted with the corresponding HSV-2 protein. The protein accumulated at 34 and 39°C in the nucleus of infected permissive HEp-2 and baby hamster kidney (BHK) cells. The protein formed at least five spots differing in charge, mobility, and extent of phosphorylation on two-dimensional electrophoretic separation. (ii) The antisera reacted with a truncated nuclear protein (33,700 apparent molecular weight) in permissive HEp-2 and restrictive BHK cells infected with R325 and incubated at 39°C but not at 34°C. The truncated protein represents, therefore, the product of the undeleted 5' domain of the $\alpha 22$ gene in R325. (iii) The presence of identical as well as slower migrating, reactive proteins in infected BHK cell lysates indicated that wild-type and truncated $\alpha 22$ proteins are processed differently in BHK and HEp-2 cells.

The five α genes of herpes simplex virus 1 (HSV-1), $\alpha 0$, $\alpha 4$, $\alpha 22$, $\alpha 27$, and $\alpha 47$, are the earliest genes to be expressed in lytic infection (2, 8, 17). They are induced by a structural component of the virion (3, 5, 22, 23), and their expression is shut off with the onset of synthesis by β and γ genes expressed later in infection (6, 9, 22; C. Greene and P. Schaffer, 9th International Herpesvirus Workshop, Seattle, Wash., 24 to 29 August 1984). The function of α genes is poorly understood. A functional $\alpha 4$ gene is required for the transition from α to β and γ protein synthesis, and mutants with *ts* mutations in that gene abound (6, 9, 13, 25). $\alpha 0$ and, to a lesser extent, $\alpha 47$ were reported to amplify the expression of β genes in transient expression systems, but viruses with mutations in these genes have not been reported. Preliminary reports have suggested that mutants carrying *ts* lesions in the domain of the $\alpha 27$ gene are affected in late protein synthesis at the nonpermissive temperature (Green and Schaffer, 9th International Herpesvirus Workshop, 1984). The interest of this laboratory in the $\alpha 22$ gene stems from studies on recombinant viruses carrying deletions in that gene. Thus, the recombinants R325 and R328 were constructed by insertion of the TK gene into the domain of the $\alpha 22$ gene and by subsequent deletion of the TK gene along with either 500 (R325) or 100 (R328) base pairs of the coding sequences of the gene (24). In HEp-2 and Vero cell lines, the recombinants grow as well as the wild-type, HSV-1(F) parent (24). Recent studies (26) indicated that the growth of these viruses is considerably reduced in baby hamster kidney (BHK), rat 1, and L cell lines as well as in a

human embryonic lung cell strain. Comparison of the events in the fully permissive Vero cells and restrictive human embryonic lung cells indicated that the R325 virus was affected in the expression of the γ_2 (true γ) subclass of γ genes characterized by stringent dependence on viral DNA synthesis for their expression. Thus, the expression of a γ_2 -TK chimeric gene inserted into the R325 virus was reduced in the restrictive cell lines but was at the same level and accelerated in permissive cells, relative to the expression of the chimeric gene inserted into the wild-type parent. These results suggested that $\alpha 22$ affects the expression of late genes and that a host factor complements fully the $\alpha 22$ gene function in HEp-2 and Vero cells, but less efficiently or less well in the restrictive cell lines.

The $\alpha 22$ protein has been identified solely by its electrophoretic mobility in denaturing polyacrylamide gels, and little is known of its properties other than that it is phosphorylated (22, 29). Of particular interest to us was the observation that cells infected with the recombinants carrying a deletion in the $\alpha 22$ gene contained a new α polypeptide with an apparent molecular weight of 30,000. This polypeptide could have represented the product of the truncated $\alpha 22$ gene from the 5' terminus to the site of the deletion. Because of the unavailability of immunological reagents, the identity of that protein and its synthesis and fate in the infected cells could not be determined.

Attempts to produce monoclonal antibodies specific for the α proteins yielded antibodies reactive with $\alpha 4$, $\alpha 0$, and $\alpha 27$ (1), but not $\alpha 22$ proteins. To obtain suitable immunological reagents, we took advantage of the published sequence of the $\alpha 22$ (18, 20) gene to synthesize two peptides repre-

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senting the predicted amino acid sequence of the hydrophilic domains near the N terminus of the protein. In this paper, we report on the properties of the wild-type and truncated $\alpha 22$ proteins accumulating in cells infected with the wild-type parent and R325 $\alpha 22$ deletion mutant, respectively.

MATERIALS AND METHODS

Viruses and cells. The properties of HSV-1(F) and HSV-2(G) were reported elsewhere (7, 11, 27). Recombinant R325 derived from HSV-1(F) contains a 500-base-pair deletion in the $\alpha 22$ gene (24). All virus stocks were made in HEp-2 cells. Growth medium for cells consisted of Dulbecco modified Eagle minimum essential medium supplemented with 10% calf serum. The maintenance medium 199-V consisted of mixture 199 supplemented with 1% calf serum. Infected cell lysates for the electrophoretic separation of polypeptides were made from HEp-2- or BHK-infected cells as indicated in the text.

Buffers and solutions. Disruption buffer consisted of 0.05 M Tris (pH 7.0), 8.5% (wt/vol) sucrose, 5% (vol/vol) 2-mercaptoethanol, and 2% (vol/vol) sodium dodecyl sulfate. Hypotonic buffer used for the separation of nuclei from cytoplasm (1, 4) consisted of 1.6 mM $MgCl_2$, 6 mM KCl, 10 mM Tris (pH 8), 1 mM dithiothreitol, and 0.5% (vol/vol) Nonidet P-40. Solution A consisted of phosphate-buffered saline (PBS; 8.2 mM $Na_2 HPO_4$, 1.5 mM KH_2PO_4 , 0.14 M NaCl, 2.5 mM KCl) supplemented with 1% (vol/vol) Nonidet P-40, 1% sodium deoxycholate, 10^{-5} M sodium- α -tosyl-L-lysine chloromethyl ketone, and L-1-tosylamide-2-phenylethyl chloromethyl ketone. For electrophoretic separation by isoelectric focusing, infected cell lysates were solubilized in 2D buffer consisting of 0.5% L- α -phosphatidylcholine-dipalmitoyl, 9 M urea, 4% Nonidet P-40, and 4% ampholines (pH 3.5 to 9.5; LKB, Gaithersburg, Md.).

Infection and labeling of cells with radioactive precursors. Monolayer cultures containing 4×10^6 cells were exposed to 20 PFU of virus per cell. After 1 h of adsorption, the inoculum was replaced with maintenance medium, and the cells were further incubated at 34 or 39°C as indicated in the text. Cells were labeled with [^{35}S]methionine or $^{32}P_i$ at various times after infection as indicated in Results. For labeling with [^{35}S]methionine, the cells were overlaid with maintenance medium containing only 10% of the usual amount of methionine but supplemented with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) to a final concentration of 25 $\mu Ci/ml$ (1.260 Ci/mmol). Labeling with $^{32}P_i$ was done as described by Wilcox et al. (29). The cells were preincubated in phosphate-free medium for 3 h before the addition of $^{32}P_i$ (carrier free; New England Nuclear). The cells were maintained in the presence of the label from 4 to 10 h postinfection before being harvested.

Preparation of labeled protein extracts. The cells were washed three times with PBS, scraped off, and pelleted by centrifugation at $150 \times g$ for 5 min. Total cell, nuclear, and cytoplasmic extracts were prepared as described previously (1, 4). Nonidet P-40-soluble and -insoluble proteins were prepared by solubilization of cells in solution A. The cell lysates were disrupted by sonication. After centrifugation at 25,000 rpm for 1 h at 4°C in an SW 50.1 rotor, samples of the supernatant fluids and pellets were solubilized in disruption buffer and subjected to electrophoresis in denaturing polyacrylamide gels.

One- and two-dimensional polyacrylamide gel electrophoresis and reaction of the electrophoretically separated polypeptides transferred to nitrocellulose with antibody. The

polypeptides were separated on 11% (vol/vol) polyacrylamide gels containing sodium dodecyl sulfate and cross-linked with *N,N'*-diallyltartardiamide as described by Morse et al. (19). Two-dimensional electrophoresis was performed as described previously (1). Briefly, the cells were solubilized in 2D buffer and disrupted by sonication. The polypeptides were separated by nonequilibrium pH gradient electrophoresis for a total of 10,000 V · h and run from base to acid. After equilibration in a solution containing sodium dodecyl sulfate, the tube gel was placed horizontally over a stacking gel for electrophoresis in the second dimension. The stacking gel was molded to contain wells for polypeptide samples to be separated in one dimension in the same gel as that used for the second dimension. After the electrophoresis, the polypeptides were transferred electrically to a nitrocellulose sheet and reacted with the rabbit antisera as previously described (1, 4). Briefly, the nitrocellulose sheet was incubated for 1 h at 37°C in PBS containing 3% (wt/vol) bovine serum albumin and for 3 h at the same temperature with rabbit serum diluted 1:100 in 1% bovine serum albumin. The nitrocellulose sheet was then incubated in PBS containing 1% bovine serum albumin and 5 μl of horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) per 3 ml of PBS. After being washed with PBS, the nitrocellulose sheet was transferred to a substrate solution containing 0.01% (wt/vol) 4-chloro-1-naphthol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 0.015% H_2O_2 . Autoradiographic images of the labeled bands were made on Cronex X-ray film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

Synthesis of peptides. Divinylbenzene resin (1% cross linked) substituted with 0.5 meq/g of BOC-L-proline and all other BOC-L-amino acids were purchased from Vega (Tucson, Ariz.). Dichloromethane, trifluoroacetic acid, methyl alcohol, *N,N*-dimethylformamide, and 1,3-dicyclohexylcarbodiimide were obtained from Aldrich. Keyhole limpet hemocyanin (KLH) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester were from Calbiochem-Behring (La Jolla, Calif.).

Two overlapping peptide sequences (residues 12 through 23 and 21 through 36) were selected, based on local hydrophilicity as predicted by the method of Hopp and Woods (10). The peptides were synthesized by the solid-phase method of Merrifield (28) with a Vega Coupler 1000 peptide synthesizer. A cysteine was added to the N terminus of the peptide (residues 21 through 36) to facilitate coupling to KLH; residue 12 of the other peptide (residues 12 through 23) was a cysteine. The reason for having a cysteine at the N terminus instead of at the C terminus was to eliminate from the KLH coupling reaction those peptide chains which may have terminated early during the synthesis. After the synthesis, the peptides were cleaved from the resin with hydrogen fluoride (Union Carbide, Danbury, Conn.), eluted with diethyl ether, and lyophilized.

Coupling of peptide to KLH. The peptides were conjugated to KLH via the N-terminal cysteine residue essentially as described by Liu et al. (15). Thus, 1 ml of a 1 mg/ml concentration of KLH dialyzed against PBS was activated with 0.5 ml of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (5 mg/ml) in *N,N*-dimethylformamide by slowly adding the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester-*N,N*-dimethylformamide solution to the KLH-PBS solution while stirring at room temperature. The solution was left stirring an additional 30 min at room temperature and was then passed through a Sephadex G-25 column at 5°C. Eluted KLH-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester

FIG. 1. Hydropathic analysis of the predicted amino acid sequence of the $\alpha 22$ gene. The published nucleotide sequence of the $\alpha 22$ gene (18, 20) was translated, and the predicted amino acid sequence was analyzed with the aid of an HP9000 computer as described elsewhere (21).

was adjusted to a final concentration of 3 mg/ml (assuming $A_{280}/2.02 = \text{mg/ml}$), and 1 ml was added at room temperature slowly while stirring to 1 ml of a 3 mg/ml concentration of peptide dissolved in PBS. The pH was adjusted to 7.2, and the solution was left stirring an additional 3 h at room temperature. The sample was dialyzed against PBS overnight to remove peptide chains which had either terminated early in the synthesis or which had not coupled to KLH. Coupling efficiency, as determined by using radiolabeled peptide, was 60%.

Immunization of rabbits. New Zealand White rabbits (2 kg) were immunized intradermally on the back or subcutaneously in each hind limb with 200 μg of peptide coupled to KLH. The first injection was done in Freund complete adjuvant. All subsequent immunizations were done with Freund incomplete adjuvant. Immunizations were done regularly at 2-week intervals until specific antisera were detected by the horseradish peroxidase assay on electrophoretically separated, electrically transferred, infected cell lysates at a 1:100 dilution of the antiserum.

RESULTS

Selection of sequences for synthetic peptide synthesis. The hydropathic analysis of the amino acid sequence of the $\alpha 22$ protein predicted from the published nucleotide sequence (18, 20) is shown in Fig. 1. Because the objective of this study was to prepare a serum reactive with the $\alpha 22$ protein made by wild-type virus and the truncated protein made by the R325 recombinant, we selected two hydrophilic domains near the N terminus for synthesis as synthetic peptides. Peptide 1 consisted of amino acids 12 through 23, i.e., of Cys-Val-Lys-Ala-Arg-Arg-Pro-Ala-Leu-Arg-Ser-Pro, whereas peptide 2 consisted of amino acids 21 through 36, i.e., of Cys-Arg-Ser-Pro-Pro-Leu-Gly-Thr-Arg-Lys-Arg-Lys-Arg-Pro-Ser-Arg-Pro. The synthesis of the peptides and the procedure for immunization of rabbits with these peptides are described in Materials and Methods. The sera selected for these studies were R77 made against peptide 1 and R10 made against peptide 2. As described below, antisera to either peptide reacted with an HSV-1(F) protein

with an electrophoretic mobility corresponding to $\alpha 22$ electrically transferred from denaturing polyacrylamide gels to nitrocellulose. The R10 antiserum was the stronger of the two, but neither antiserum effectively precipitated undenatured $\alpha 22$ protein from lysates of HSV-1(F)-infected cells (data not shown).

Specificity of the reactivity of rabbit sera prepared against synthetic peptides. In this series of experiments, HEp-2 cells were labeled with [^{35}S]methionine (25 $\mu\text{Ci/ml}$) from 18 to 24 h postinfection with HSV-1(F), HSV-2(G), or mock infection. The cells were then harvested and solubilized in disruption buffer, and the lysates were electrophoretically separated in a denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with the rabbit antisera. With R77 antibody, both R77 and R10 antisera reacted specifically with an HSV-1(F) protein migrating with an apparent molecular weight of 72,000 (Fig. 2), i.e., the reported apparent molecular weight of the $\alpha 22$ protein in polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide (17, 19).

Solubility of the $\alpha 22$ protein. Previous studies on $\alpha 4$, $\alpha 0$, and $\alpha 27$ (1) have revealed considerable variability in the solubility of these proteins. In contrast with the $\alpha 4$ and $\alpha 27$ proteins, the $\alpha 22$ protein was soluble in solution A and remained in solution after centrifugation for 1 h at $72,000 \times g$ (Fig. 3).

Synthesis, accumulation, and reactivity of the truncated $\alpha 22$ protein specified by the R325 recombinant carrying a deletion in the $\alpha 22$ gene. As noted above, the HSV-1(F) mutant R325 carries a 500-base-pair deletion in the $\alpha 22$ gene. Autoradiographic images of the α proteins specified by R325 revealed the absence of the wild-type $\alpha 22$ and the presence of a novel 30,000-molecular-weight protein that could have represented the translated product of an mRNA transcribed from the intact, 5' domain of the gene (16, 24). Attempts to detect this product in lysates of cells harvested 24 h after infection with R325 and incubation at permissive temperatures (34 or 37°C) were not successful. Because previous studies have shown that HSV-1(F) is temperature sensitive at 39°C and infected cells tend to accumulate α proteins at that temperature (1,

FIG. 2. Autoradiographic images and immunoreactivity of polypeptides from infected and mock-infected cells electrophoretically separated in denaturing polyacrylamide gels and then transferred to a nitrocellulose sheet. Replicate cultures of HEP-2 cells were infected with HSV-1(F) or HSV-2(G) or mock infected (mock) and labeled with [^{35}S]methionine (^{35}S) from 18 to 24 h postinfection. Cell lysates were subjected to electrophoresis and then transferred to nitrocellulose and reacted with rabbit serum R77 (Ab) as described in Materials and Methods. Numbers on the left of the figure indicate the infected cell protein (ICP#) designation as described by Honess and Roizman (8, 9) and Morse et al. (19). The numbers on the right side indicate the apparent molecular weight ($\text{MW} \times 10^3$). Lanes 1 to 3. Autoradiographic images of electrophoretically separated polypeptides in lysates of infected and mock-infected cells. Lanes 4 to 6. Reactivity of rabbit serum R77 with electrophoretically separated polypeptides transferred to a nitrocellulose sheet. The 72,000-apparent-molecular-weight polypeptide band of HSV-1(F) is indicated by an arrow. A, Actin.

FIG. 3. Autoradiographic images and immunoperoxidase reactions of $\alpha 22$ protein in lysates of infected HEP-2 cells. HEP-2 cells were infected with HSV-1(F) and incubated from 18 to 24 h postinfection in medium containing [^{35}S]methionine (^{35}S). The cells were then harvested and solubilized in solution A. After treating cell lysates as indicated for each lane separately, polypeptides were separated on sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose, and reacted with rabbit serum R77 (Ab). The numbers on the left of panels refer to infected cell protein designations. Lanes 1 to 3 show the reactivity of R77 with electrophoretically separated, transferred polypeptides in immunoperoxidase reactions. The polypeptide bands reacting with the antiserum are indicated by arrows. Lanes 4 to 6 show the autoradiographic images of lanes 1 to 3. Lane 1, Immunoreactivity of total cell lysates. Disruption buffer was added before electrophoresis. Lane 4, Autoradiographic image of lane 1; lane 2, immunoreactivity of the electrophoretically separated, transferred polypeptides in the supernatant fluid obtained after centrifugation of the cell lysates for 1 h at $72,000 \times g$; lane 5, autoradiographic image of lane 2; lane 3, immunoreactivity of the electrophoretically separated, transferred polypeptides contained in the solubilized pellet obtained after the centrifugation described above; lane 6, autoradiographic images of lane 3.

FIG. 4. Autoradiographic images and immunoperoxidase reactions of lysates of cells infected with the R325 recombinant. Replicate cultures of HEp-2 and BHK cells were infected with R325 and incubated at 34 and 39°C, respectively, before being harvested in disruption buffer at 24 h postinfection. HEp-2 cells were also infected with HSV-1(F) or mock infected and incubated in the presence of [35 S]methionine from 18 to 24 h postinfection. The polypeptides in the cell lysates were separated in denaturing polyacrylamide gels and electrically transferred to nitrocellulose before being reacted with rabbit serum R10. Lane 1, Autoradiographic images of HSV-1(F)-infected (F) cell lysates used as markers; lane 2, autoradiographic images of mock-infected (M) HEp-2 cell lysates; lane 3, immunoreactivity of lane 1; lane 4, antibody reactivity of mock-infected HEp-2 cells corresponding to lane 2; lane 5, immunoreactivity of R325-infected BHK cells incubated at 34°C; lane 6, immunoreactivity of R325-infected BHK cells incubated at 39°C; lane 7, immunoreactivity of HEp-2 cells infected with R325 and maintained at 34°C; lane 8, same as lane 7, except that infected cells were incubated at 39°C. Numbers on the left of the figure represent infected cell protein (ICP#) designation. A, Actin. Numbers on the right of the figure indicate the apparent molecular weight (MW $\times 10^3$). The arrows point to bona fide (lane 3) and truncated $\alpha 22$ protein made in BHK cells (lane 6) and HEp-2 cells (lane 8), respectively. The short arrowheads point to slower migrating, reactive polypeptide bands present in lysates of R325-infected BHK cells. Note that this antibody and many others, both polyclonal and monoclonal, have been found to bind nonspecifically to ICP5. The binding is nonspecific and varies from one preparation to another.

12), the experiment was repeated with HEp-2 and BHK cells harvested 24 h after infection and incubation at 39°C. The R10 antiserum reacted with a 33,700-apparent-molecular-weight polypeptide present in the lysates of infected HEp-2

cells and with several bands migrating with apparent molecular weights ranging from 33,700 to 39,000 present in lysates of infected BHK cells (Fig. 4). The $\alpha 22$ truncated protein was not detected in replicate infected cultures incubated at 34°C.

Accumulation and compartmentalization of wild-type and truncated $\alpha 22$ protein in infected HEp-2 and BHK cells. In this series of experiments, replicate cultures of HEp-2 and BHK cells were either infected with HSV-1(F) and incubated at both 34 and 39°C or infected with R325 and incubated at 39°C. The cultures were labeled from 10 to 24 h postinfection and then harvested and fractionated into nuclear and cytoplasmic fractions. The solubilized fractions were then subjected to electrophoresis in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and reacted with the R10 rabbit serum. The salient features of the results (Fig. 5) were as follows.

(i) The HSV-1(F) $\alpha 22$ protein accumulated largely in the nucleus, although trace amounts of $\alpha 22$ protein are readily detected in the cytoplasm. The amounts of the main 72,000-apparent-molecular-weight protein accumulating at 34 and 39°C were roughly equivalent. However, slower migrating bands were present in lysates of infected HEp-2 cells incubated at 34°C and in infected BHK cells incubated at 39°C.

(ii) The truncated $\alpha 22$ protein specified by R325 accumulated in nuclei, and only trace amounts of the protein were detected in the cytoplasm. As in the studies presented in Fig. 4, the truncated $\alpha 22$ protein accumulated in lysates of infected HEp-2 cells formed a single band, whereas that accumulating in lysates of infected BHK cells formed multiple bands migrating at the same and slower rates than the truncated $\alpha 22$ protein made in HEp-2 cells.

(iii) Although both infected HEp-2 and BHK cells were maintained in the same incubators under identical conditions, the 24-h lysates of infected HEp-2 cells incubated at 39°C contained α proteins labeled between 10 and 24 h postinfection, whereas the infected BHK cells did not. At 39°C, the multiplication of both HSV-1(F) and R325 was arrested later in the reproduction cycle in BHK cells than in the HEp-2 cells. This is reflected in the presence of late γ_2 proteins (e.g., ICPs 11, 29) in the profile of infected BHK cell protein from lysates of cells incubated at 39°C.

Homogeneity and phosphorylation of $\alpha 22$ protein. Lysates of 24-h HSV-1(F)-infected HEp-2 cells maintained at 39°C were mixed with trace amounts of lysates of cells infected in the presence of cycloheximide, maintained in the presence of the drug for 5 h, and labeled with [35 S]methionine for 1 h immediately after cycloheximide reversal. The cells were further incubated at 37°C without radioactive precursors until 24 h after cycloheximide withdrawal before being harvested in 2D buffer. The artificial mixture was electrophoretically separated by isoelectric focusing from base to acid in the first dimension and in a denaturing gel in the second dimension and then transferred electrically to a nitrocellulose sheet and reacted with the R77 antiserum. The results (Fig. 6) indicate that the antibody reacted with at least five spots formed by the HSV-1(F) $\alpha 22$ protein. The distribution of the $\alpha 22$ protein spots indicates that the variability is reflected in both net charge and electrophoretic mobility. The $\alpha 22$ protein appeared to be the most charged in comparison with other α proteins identified in the autoradiogram on the basis of the results obtained in the earlier study (1).

In the second series of experiments, the lysates of 24-h HSV-1(F)-infected HEp-2 cells were mixed with trace amounts of lysates of HEp-2 cells labeled with 32 P between

FIG. 5. Autoradiographic images and immunoreactivity of electrophoretically separated and transferred infected cell polypeptides. Replicate monolayers of BHK and HEp-2 cells were infected with HSV-1(F) (F) or R325, respectively. Replicate cultures of HSV-1(F)-infected cells were incubated at 34 and 39°C, respectively, whereas R325-infected cells were incubated only at 39°C. The cultures were labeled with [35 S]methionine from 10 to 24 h and then harvested and fractionated into nuclear and cytoplasmic fractions. The electrophoretically separated and transferred polypeptides in the cell lysates were reacted with rabbit serum R10. Lanes 1 to 12 show autoradiographic images of the [35 S]methionine-labeled polypeptides, whereas lanes 12 to 24 show the immunoreactivity of the proteins in those lanes. The headings identify the nuclear (N) or cytoplasmic (C) fractions from the same infected cell culture, the cell line HEp-2 or BHK, the temperature of incubation (34 or 39°C), and the virus HSV-1(F) or R325. Numbers on the left of the figure indicate infected cell protein designation. The arrows point to the full-size and truncated forms of α 22 protein reacting with rabbit serum R10.

4 and 10 h postinfection. The artificial mixture was electrophoretically separated in two dimensions, electrically transferred to a nitrocellulose sheet, and reacted with the R10 rabbit serum as described above. The results (Fig. 7) indicate that α 22 is phosphorylated and suggest that forms 2 to 4 are more extensively phosphorylated than forms 1 and 5 of the protein. It is also noteworthy that, of the α proteins, α 22 was the most extensively labeled with 32 P under the labeling conditions of this study.

DISCUSSION

The salient features of the results presented in this report are as follows.

(i) Both synthetic peptides containing the amino acids corresponding to the predicted sequence of hydrophilic domains near the 5' terminus of the α 22 protein induced antibody which reacted specifically with the α 22 protein of HSV-1(F) but not that of HSV-2(G), suggesting that the

corresponding proteins may differ in the amino acid sequences of these domains. In both permissive HEp-2 and BHK cells, the α 22 protein accumulated in the nucleus. Larger amounts of the protein were processed to a higher apparent molecular weight in infected BHK cells than in HEp-2 cells. Like the α 4, α 0, and α 27 proteins, the α 22 protein appeared to be heterogeneous. The migration of HSV-1(F) protein in two-dimensional gels suggests that it is more highly charged than the α 0, α 4, and α 27 proteins and that it is heterogeneous both with respect to charge and apparent molecular weight.

(ii) Earlier studies on the R325 recombinant carrying a 500-base-pair deletion in the coding sequence of the α 22 gene suggested that a novel α protein with an apparent molecular weight of approximately 30,000 represented the product of the 5' terminus of the α 22 gene (24). The results obtained with the antisera made against the synthetic polypeptides are consistent with this hypothesis. It is noteworthy, however,

FIG. 6. Autoradiographic images and immunoreactivity of $\alpha 22$ protein separated in two dimensions. Cells were infected with HSV-1(F) and maintained at 39°C before being harvested. Before separation in the first dimension, 1/10 volume of [^{35}S]methionine (^{35}S)-labeled cell lysate was added to the sample. Labeled cell lysate was prepared by infecting HEP-2 cells in the presence of cycloheximide (50 $\mu\text{g}/\text{ml}$). After 4 h of incubation at 37°C, the drug was removed, and the cells were incubated at 37°C in medium containing [^{35}S]methionine for 1 h. The cells were further incubated at 37°C without radioactive precursors until 24 h after cycloheximide withdrawal. The direction of migration in the first dimension was from base to acid. The electrophoretically separated polypeptides in the second dimension were transferred to a nitrocellulose sheet and reacted with rabbit serum R77. Panel A, Autoradiographic image; panel B, immunoreactivity of the separated polypeptides in panel A. Lane 1, Mixture of labeled and unlabeled cell lysates separated only in the second dimension; lane 2, mixture of mock-infected labeled

FIG. 7. Autoradiographic images and immunoreactivity of infected cell polypeptides separated in two dimensions. HEP-2 cells were infected with HSV-1(F) and maintained at 39°C for 24 h before being harvested. Immediately before the separation in the first dimension, 1/10 volume of ^{32}P -labeled cell lysate was added to the sample. The infected cells were labeled with ^{32}P from 4 to 10 h postinfection by the addition of ^{32}P to the medium. The direction of migration in the first dimension was from base to the acid. Panel A, Autoradiographic images of ^{32}P -labeled and transferred infected cell lysates. Lane 1, ^{32}P -labeled material separated only in the second dimension; lane 2, [^{35}S]methionine-labeled, mock-infected cell lysates separated only in the second dimension; lane 3, 18- to 24-h [^{35}S]methionine-labeled infected cell lysates, separated in the second dimension and used as marker. Panel B, Immunoreactivity of the separated polypeptides shown in panel A with the R10 anti-serum. The large numbers in panel A indicate the infected cell protein designation. Arrows point to $\alpha 22$ protein. Small numbers in panel B indicate $\alpha 22$ forms heterogeneous with respect to net charge and apparent size.

and unlabeled cell lysates separated only in the second dimension; lane 3, HSV-1(F)-infected cell lysate used as a marker lane and separated only in the second dimension. Large numbers in panel A indicate infected cell protein designation. The arrows point to $\alpha 22$ protein. Small numbers in panel B indicate $\alpha 22$ protein differing in net charge and electrophoretic mobility.

that the truncated $\alpha 22$ protein with the apparent molecular weight of 33,700 was detected only in infected cultures incubated at 39°C and not at 34°C. In contrast, the bona fide 72,000-apparent-molecular-weight product of the wild-type HSV-1(F) $\alpha 22$ gene accumulated at both temperatures. These results could be interpreted to indicate that the $\alpha 22$ truncated protein was less stable at the permissive temperature than the wild-type $\alpha 22$ protein. The failure of the antisera to immunoprecipitate the $\alpha 22$ protein precluded us from carrying out pulse-chase experiments to establish whether this is in fact the case.

(iii) The purpose of the incubations at 39°C was to accumulate larger amounts of the α proteins than was possible at 34 and 37°C. HSV-1(F), like many other HSV-1 isolates passaged a limited number of times in cell culture, grows well in the 33 to 37°C range and very poorly at 39°C. At the higher temperature, the infected cells readily accumulated α proteins, although depending on the stringency of the temperature control, some β and γ_1 proteins may also accumulate. This is readily seen in the accumulation of several α proteins in the electrophoretic profiles of infected HEP-2 cell proteins (Fig. 5) and in the studies reported earlier (1, 12). We have not detected a significantly greater accumulation of $\alpha 22$ protein at 39°C than at 34°C. An interesting and significant finding, however, was that the lysates of BHK cells, infected, labeled, and maintained at 39°C under the same conditions and at the same time as the infected HEP-2 cells, failed to accumulate significant amounts of labeled α proteins. Moreover, at 39°C the reproductive cycle of HSV-1(F) progressed further in BHK cells than in HEP-2 cells. These results suggest that α protein synthesis was turned off earlier in infected BHK cells than in HEP-2 cells and that the temperature sensitivity of HSV-1(F) is cell dependent and reflects either differential folding or processing of viral HSV-1 proteins in HEP-2 cells at the nonpermissive temperature. Cell-type dependence of temperature sensitivity of herpesvirus mutants has been reported (14).

(iv) A major objective of these studies was to determine whether differential processing or translocation of the putative truncated $\alpha 22$ protein detected in earlier studies could account for the excellent growth of R325 recombinant in HEP-2 cells and rather poor growth in BHK cells. The truncated $\alpha 22$ protein accumulating in both HEP-2 and BHK cells contains the recognition sequence for transport into the nucleus. The accumulating truncated $\alpha 22$ protein is processed to a higher molecular weight just as is the bona fide $\alpha 22$ protein, suggesting that the signal for processing is in the N-terminal half of the $\alpha 22$ protein. However, the patterns of accumulation of the truncated $\alpha 22$ protein in the permissive HEP-2 cells do not differ significantly from those seen in the restrictive BHK cells. In contrast to the wild-type bona fide $\alpha 22$ protein, there was no appreciable accumulation of the truncated $\alpha 22$ protein in either infected HEP-2 or BHK cells at the permissive temperature. These results do not support the hypothesis that the excellent growth of R325 $\alpha 22$ deletion mutant in HEP-2 cells reflects higher stability or differential translocation of the truncated $\alpha 22$ protein.

ACKNOWLEDGMENTS

We thank Philip E. Pellett for the computer analysis, Howard Tager for permission to use the Vega Coupler 1000 synthesizer, and Anthony J. Conley and William Wald for advice.

These studies were aided by Public Health Service grants CA08494 and CA19264 from the National Cancer Institute and grant MV-2T from the American Cancer Society. M.A. is a fellow of the Swiss Nationalfond.

LITERATURE CITED

1. Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman. 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. *J. Virol.* **52**:108–118.
2. Anderson, K. P., R. H. Costa, L. E. Holland, and E. K. Wagner. 1980. Characterization of herpes simplex virus type 1 RNA present in the absence of de novo protein synthesis. *J. Virol.* **34**:9–27.
3. Batterson, W., and B. Roizman. 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. *J. Virol.* **46**:371–377.
4. Braun, D. K., L. Pereira, B. Norrild, and B. Roizman. 1983. Application of denatured, electrophoretically separated, and immobilized lysates of herpes simplex virus-infected cells for detection of monoclonal antibodies and for studies of the properties of viral proteins. *J. Mol. Biol.* **46**:103–112.
5. Campbell, M. E. M., J. Palfreyman, and C. M. Preston. 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J. Mol. Biol.* **180**:1–19.
6. Dixon, R. A. F., and P. A. Schaffer. 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP 175. *J. Virol.* **36**:189–203.
7. Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. *J. Gen. Virol.* **2**:357–364.
8. Honess, R. W., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14**:8–19.
9. Honess, R. W., and B. Roizman. 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc. Natl. Acad. Sci. USA* **72**:1276–1280.
10. Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**:3824–3828.
11. Kieff, E. D., S. L. Bachenheimer, and B. Roizman. 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. *J. Virol.* **8**:125–132.
12. Knipe, D. M., W. Batterson, C. Nosal, B. Roizman, and A. Buchan. 1981. Molecular genetics of herpes simplex virus. VI. Characterization of a temperature-sensitive mutant defective in the expression of all early viral gene products. *J. Virol.* **38**:539–547.
13. Knipe, D. M., W. T. Ruyechan, R. W. Honess, and B. Roizman. 1979. Molecular genetics of herpes simplex virus. IV. The terminal sequences of the L and S components are obligatorily identical and constitute a part of the structural gene mapping predominantly in the S component. *Proc. Natl. Acad. Sci. USA* **76**:4534–4538.
14. Koment, R. W., and F. Rapp. 1975. Temperature-sensitive host range mutants of herpes simplex virus type 2. *J. Virol.* **15**:812–819.
15. Liu, F.-T., M. Zinnecker, T. Hamaska, and D. H. Katz. 1979. New procedures for preparation and isolation of conjugates and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* **18**:690–697.
16. Mackem, S., and B. Roizman. 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of α genes. *Proc. Natl. Acad. Sci. USA* **77**:7122–7126.
17. Mackem, S., and B. Roizman. 1981. Regulation of herpesvirus macromolecular synthesis: temporal order of transcription of α genes is not dependent on the stringency of inhibition of protein synthesis. *J. Virol.* **40**:319–322.
18. McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J. Mol. Biol.* **181**:1–13.
19. Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer. 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified

- by HSV-1 $\times$ HSV-2 recombinants. J. Virol. 26:389-410.
20. Murchie, M. J., and D. J. McGeoch. 1982. DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). J. Gen. Virol. 62:1-15.
21. Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman. 1985. The anatomy of the herpes simplex virus 1 strain F glycoprotein gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. 53:243-253.
22. Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman. 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology 77:733-749.
23. Post, L. E., S. Mackem, and B. Roizman. 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell 24:555-565.
24. Post, L. E., and B. Roizman. 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell 25:227-232.
25. Preston, C. M. 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *tsK*. J. Virol. 29:275-284.
26. Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus 1 mutant deleted in the α 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. 55:338-346.
27. Spear, P. G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9:143-159.
28. Stewart, J. M., and J. D. Young. 1969. Solid phase peptide synthesis. W.H. Freeman & Co., New York.
29. Wilcox, K. W., A. Kohn, E. Sklyanaskaya, and B. Roizman. 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. 33:167-182.