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Crossed Immunoelectrophoretic Analysis of Herpes Simplex Virus Type 2 Proteins. Characterization of Antigen-5

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With 7 Figures

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Summary

Herpes simplex virus type 2 proteins extracted from infected cells and analysed by crossed immunoelectrophoresis identified a nonglycosylated antigen named Ag-5. The antigen contained two proteins when extracted from the agarose gel and the molecular weights were 128K and 91K. Both proteins are located in the nucleus of the infected cells and the 128K is identical to ICP-8. The 91K protein is based on the reactivity with monoclonal antibodies most likely the alkaline exonuclease mapped by Preston and Cordingly (25). Our data show that although the proteins ICP-8 and 91K coprecipitate they differ in both peptide compostion and in immunological specificity.

Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) specify more than 50 proteins which are synthetized during a productive infection of tissue culture cells (9, 15). Several of the proteins are processed post translationally by addition of either phosphate, sulfate or by carbohydrate groups (9, 10, 11, 13, 14, 18, 34). We analyzed in previous studies the protein composition of the HSV-1 glycoprotein antigens Ag-11, Ag-8 and Ag-6 and identified the glycoproteins B, D and C respectively (16). The antigenic specificity of these glycoproteins and the glycoproteins E, F (renamed as C of HSV-2), and G were analyzed in several studies and it was observed that the individual glycoproteins specified a characteristic set of antigen determinants not shared with any of the other glycoproteins of the same virus type (1, 18, 20, 22, 29, 31). The HSV-1 and HSV-2 genes specifying gB, gC, gD and gE

map colinear (21, 30) and corresponding proteins of HSV-1 and HSV-2 do share antigenic determinants but do also specify type specific determinant sites (7, 22, 23, 28, 33, 35). It should be noted that no counterpart to gG of HSV-2 has been found in HSV-1 to date (29). Crossed immunoelectrophoretic analysis of HSV-2 infected cell extracts allowed the identification of a glycosylated antigen Ag-4 which was not further characterized (17), and of a nonglycosylated antigen named Ag-5 which is characterized in the present study. We show that Ag-5 is composed of two proteins of molecular weights 128K and 91K both located in the nucleus of the cell. We also show that 91K predominantly carry HSV-2 specific antigen determinants.

Materials and Methods

Cells and Virus Strains

Monolayer cultures of VERO cells were grown in minimal essential medium (MEM) with 10 per cent (v/v) fetal calf serum (FCS). Virus prototype strains HSV-1 (F) and HSV-2 (G) were propagated in VERO cells maintained in MEM with 1 per cent FCS. The HSV-1 × HSV-2 intertypic recombinant strain RH1G48 used in the mapping studies was obtained from Dr. B. Roizman, University of Chicago, U.S.A. (6), and the recombinants Fx9 (5—8), R12-5, RE6, and RH31 were kindly given to me by Dr. Marsden, University of Glasgow, Scotland (4, 5, 14).

Preparation of HSV Proteins

A. Solubilization with non-ionic detergent for immunoprecipitation analysis. VERO cells grown in ROUX bottles were infected with 5 PFU/cell of virus. After absorbtion for 1 hour at 37° C, the virus inoculum was aspirated and the cells were overlaid with 50 ml of maintenance medium (MEM with 1 per cent FCS). Five hours postinfection, the maintenance medium was poured off, and 10 ml of labeling medium was added [10 ml MEM which had 1/10 the normal concentration of isoleucine (I), leucine (L), and valine (V)], supplemented by 1 per cent FCS and 2 μCi/ml medium of each of the three 14-C-labeled aminoacids I (sp. a. 347 mCi/mmol), L (sp. a. 348 mCi/mmol) and V (sp. a. 291 mCi/mmol) (NEN, Dreieich, Germany). The labeling was continued until 18 hours postinfection, when the cell layer was washed once in PBS and the cells collected by scraping into 5 ml of PBS followed by centrifugation. The proteins from the cell pellet were extracted with 0.8 ml of the antigen buffer [0.18 M Tris, 0.06 M Barbital, pH 8.6 with 1 per cent (v/v) Triton X-100, 10^{-5} M TLCK and TPCK]. After sonication, the protein extract was clarified by ultracentrifugation in an SW 60 rotor at 100,000 × *g* for 1 hour at 4° C. The supernatant was the soluble antigen preparation.

B. Solubilization with SDS and 2-mercaptoethanol for SDS-PAGE and immunoblotting. Monolayer VERO cells grown in 25 cm^2 tissue culture flasks (NUNC, Roskilde, Denmark) were infected as described above, except for the infected cells being overlaid with 5 ml of maintenance medium. For the preparation of unlabeled proteins cells were harvested 18 hours postinfection. After being washed, the cells were solubilized in 0.5 ml of disruption mixture (15), and the extract was sonicated before gel electrophoresis. For the preparation of ILV-labeled proteins these were labeled from 5 to 18 hours postinfection in 2 ml of the labeling medium described above, followed by solubilization in 0.5 ml of disruption mixture.

C. Separation of nuclei and cytoplasm of HSV-2 infected cells. Monolayers of HSV-2 infected VERO cells were used 9 hours postinfection. Fractionation of cells in nuclei and cytoplasm was done according to the procedure of BENECKE and PENMAN (2). 2×10^7 cells were lysed by incubation for 5 minutes in 2 ml of hypertonic buffer [1.6 mM $MgCl_2$, 6 mM KCl, 10 mM Tris pH 8.0, 1 mM dithiotreitol, and 0.5 per cent (v/v) NP-40]. The cell suspension was scraped off, vortexed for 1 minute, and the nuclei were collected by centrifugation for 10 minutes at $480 \times g$ in a Sorwall GLC-2 centrifuge. The cytoplasm (the supernatant) was collected and the nuclear pellet was washed once in PBS before solubilization in disruption mixture. The cytoplasm fraction was adjusted to 2 per cent (w/v) SDS, 5 per cent (v/v) 2-mercaptoethanol and 3 per cent (w/v) sucrose. Both nuclear and cytoplasm extracts were sonicated and boiled before SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation in Gels by Crossed Immunoelectrophoresis

The crossed immunoelectrophoretic analysis of HSV-2 antigens was performed as previously reported (16). HSV-2 proteins were extracted in Triton X-100 containing buffer, separated electrophoretically in a 1 per cent agarose gel (Litex A/S, Glostrup, Denmark) made up in a 0.18 M Tris, 0.06 M barbital buffer pH 8.6, with 1 per cent (v/v) Triton X-100, 10^{-5} M TPCK and TLCK. The HSV-2 proteins were then immunoprecipitated by electrophoresis into a second dimensional gel containing rabbit hyperimmune immunoglobulin made to the HSV-2 proteins. Where indicated in the legends, an intermediate gel was inserted between the first and the second dimensional gel in order to obtain a better separation of the precipitated proteins. The plates were used either for isolation of immunoprecipitates of the antigen designated Ag-5 or for staining by coomassie brilliant blue. The HSV-1 proteins were extracted and analysed analogously but were precipitated into a gel containing rabbit hyperimmune immunoglobulin made to HSV-1 proteins (16).

Rabbit Antiserum

Serum no. 5 was prepared by immunization of rabbits with immunoprecipitates of Ag-5 of HSV-2 cut from agarose gel after crossed immunoelectrophoresis of HSV-2 proteins extracted from HSV-2 infected SIRK cells as previously described (32). The immunoglobulins were purified from the serum according to the method of HARBOE and INGILD (8). The purified preparation was designated serum no. 5.

Preparation of Antibodies to the 91K Protein of HSV-2 by Immunosorption

The 91K protein of HSV-2 was identified on a single nitrocellulose strip cut from transfers prepared as described below and incubated with serum no. 5. The nitrocellulose band carrying only the 91K protein was cut out from the whole transfer sheet and incubated with 10 ml of a 1/100 dilution of serum no. 5 for 1 hour. After the unbound antibodies were washed off with excess of PBS, those specifically bound to the nitrocellulose were eluted by incubation in 1.5 ml of 0.1 M glycine pH 2.8 with 0.01 per cent NaN_3 for 15 minutes at room temperature. The pH was immediately brought up to 7.0 with NaOH, and the eluate was dialyzed against PBS with 0.01 per cent (w/v) of NaN_3 for 24 hours.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The polypeptides of HSV-2 were separated in a 9.25 per cent (w/v) acrylamide gel cross-linked with 0.24 per cent (w/v) N,N-diallyltartardiamide (DATD) (BioRad Laboratories, Richmond, California, U.S.A.). The stacking gel was 3 per cent (w/v) acrylamide, 0.08 per cent (w/v) DATD, and buffer conditions were as described elsewhere (15). The gels were either processed for immunoblotting or stained and dried.

2 D-Polyacrylamide Gel Electrophoresis

The polypeptides were extracted from 14-C-ILV-labeled HSV-2 infected cells for SDS-polyacrylamide gel electrophoresis and separated in 9.25 per cent polyacrylamide gels as described above. The lane from a first dimensional gel was cut out and layered on top of a 12 per cent polyacrylamide gel made without stacking gel. The proteins in the first dimensional gel were digested by staphylococcus V-8 protease (Miles, Elkart, U.S.A.) by addition of 200 µl enzyme (1 mg/ml) solubilized in 0.125 M Tris-HCl buffer, pH 7.0, with 20 per cent glycerol and 0.1 per cent SDS. The enzyme solution was electrophoresed two third into the first dimensional gel slab at 10 mA. The electrophoresis was stopped for 1/2 hour allowing the enzyme to function. Electrophoresis was thereafter continued under standard conditions (15). The protein peptides were transferred to nitrocellulose paper and reacted with antibodies as described below.

Immunoblotting

Transfer of the proteins from the SDS-PAGE onto nitrocellulose paper (Schleicher & Schüll, 0.45 µm) was done by the procedure of Burnette (3). The transfer was performed at 0.22 A, 60 V for 1½ hours. The nitrocellulose-bound proteins were incubated with rabbit serum diluted 1/50 in PBS with 3 per cent BSA for 1 hour at 37° C. After extensive wash in PBS with 1 per cent BSA, the nitrocellulose strips were incubated for 1 hour with antibodies to rabbit immunoglobulins made in swine and conjugated with peroxidase (DAKO, P-217, Copenhagen, Denmark). The conjugate was diluted 1/40 in PBS with 3 per cent BSA. After wash in PBS with 1 per cent BSA the protein bands were stained with either o-dianisidine or 4-cloro-1-naphthol substrate [0.02 per cent (w/v) o-dianisidine, 0.02 M Tris buffer with 0.02 M NaCl, pH 7.2, and 0.02 per cent (v/v) H_2O_2; 5 mg 4-cloro-1-naphthol dissolved in 1 ml ethanol, 50 ml H_2O, and 0.003 per cent (v/v) H_2O_2]. The staining was stopped when the color was intensive enough. The paper was dried, and when radioactive proteins were transferred for the immunoblotting the autoradiographic image was obtained by exposure on XRP-1 film.

Indirect Immunofluorescence Microscopy (IIF)

VERO cells were grown on glass coverslips and when subconfluent the cells were infected with HSV-2 using 5 PFU/cell. After 1 hour the virus was aspirated and the cells were overlayered with maintenance medium and incubated for 9 hours at 37° C. The cells were fixed in 4 per cent paraformaldehyde and permeabilized with 0.1 per cent NP-40 for 30 minutes (19). After wash in PBS the cells were incubated with either antibodies made to Ag-5 or with antibodies to the 91K protein used in a final dilution of 1/10. The antibodies were bound for 1 hour at room temperature and after wash in PBS fluorescein isothiocyanate (FITC)-coupled swine anti-rabbit IgG (DAKO, Copenhagen) was added. Fluorescence microscopy was done using a Leitz Dialux 20 microscope.

Results

Crossed immunoelectrophoresis of HSV-2 infected cell proteins precipitated into an agarose gel containing immunoglobulin made from HSV-2 hyperimmune serum identified five antigens (Fig. 1). The glycoprotein antigens Ag-11 and Ag-8 correspond to and cross reacts immunologically with the antigens of HSV-1. The Ag-11 and Ag-8 are composed of gB and gD respectively. The Ag-5 is unglycosylated as it does not incorporate

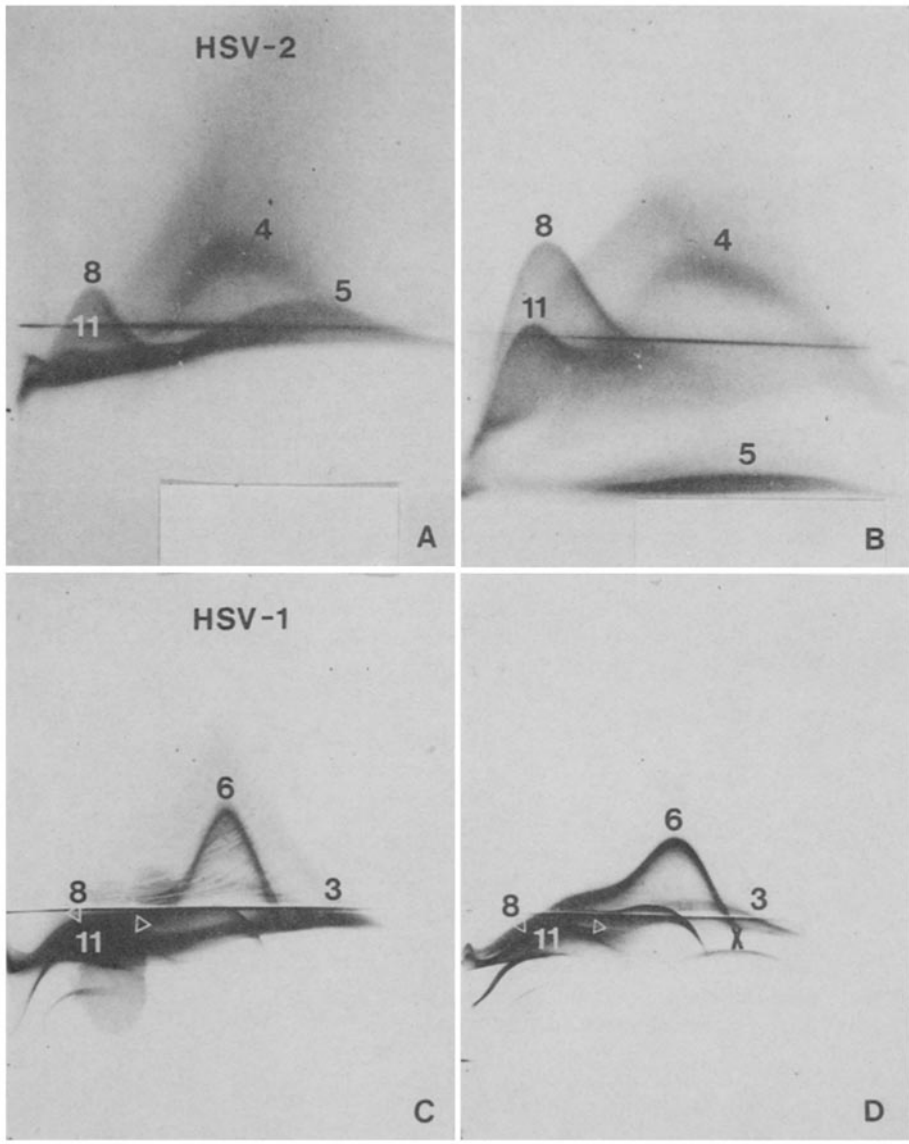

Fig. 1. Precipitation of HSV-1 and HSV-2 proteins in agarose gels by crossed immunoelectrophoretic analysis. *A* 30 µl extract of HSV-2 proteins made in Triton X-100 containing buffer were applied in the first dimensional gel. The proteins were separated at 10 V/cm for 1½ hours. The first and second dimensional gel was separated by a 2 cm wide intermediate gel supplied with 15 µl/cm² of rabbit immunoglobulin prepared from preimmune serum. The second dimensional gel contained 15 µl/cm² of rabbit immunoglobulin made to HSV-2 proteins. The electrophoresis was done at 2 V/cm for 18 hours. *B* As described for A, except for the intermediate gel being supplemented with serum no. 5. *C* 30 µl extract of HSV-1 proteins made from infected cells analysed as described under A, but the second dimensional gel contained rabbit immunoglobulin made to HSV-1 proteins. *D* As described for C, but with serum no. 5 in the intermediate gel. The arrows at C and D identify Ag-8

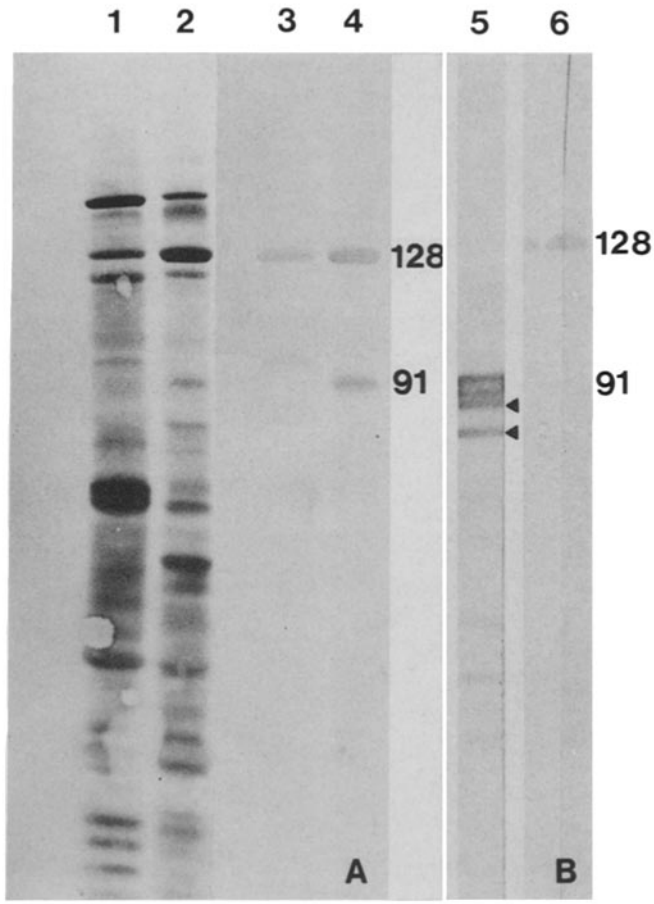

Fig. 2

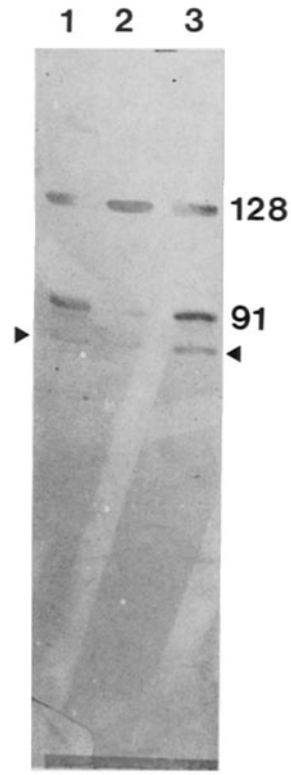

Fig. 3

Fig. 2. Immunoblotting of HSV-1 and HSV-2 proteins reacted either with serum no. 5 or with immunosorbent purified 91K and 128K specific antibodies. *Panel A: 1* An autoradiographic image of 14-C-ILV labeled HSV-1 infected cell proteins electrophoretically separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. *2* An autoradiographic image of 14-C-ILV labeled HSV-2 infected cell proteins separated and transferred as described for the HSV-1. *3* and *4* The immune reactions of the proteins shown in slots 1 and 2 after incubation with rabbit serum no. 5 followed by peroxidase coupled swine anti-rabbit immunoglobulin and o-dianisidine substrate. *Panel B: 5* Incubation of the transferred proteins with immunosorbent purified 91K binding antibodies. The arrowheads mark two proteins which are most likely degradation products of the 91K. *6* Incubation of the transferred proteins with immunosorbent purified 128K binding antibodies

Fig. 3. Immunoblotting of HSV-2 proteins extracted from nuclei and cytoplasm fractions prepared from 9 hours infected cells. *1* Immunoblotting of a total cell extract made 9 hour postinfection and reacted with serum no. 5. *2* The nuclear proteins reacted with serum no. 5. *3* The corresponding cytoplasm protein reacted with serum no. 5. The arrowheads in slots 1 and 3 mark a protein which might be a proteolytic degradation product derived from the 91K protein

14-C-glucosamine (data not shown). The antigen is further characterized by the use of antibodies made to the antigen cut from the agarose gel as detailed in Materials and Methods. The specificity of the antiserum to Ag-5 (serum no. 5) was tested in crossed immunoelectrophoresis by the intermediate gel technique and only the Ag-5 was precipitated by the serum

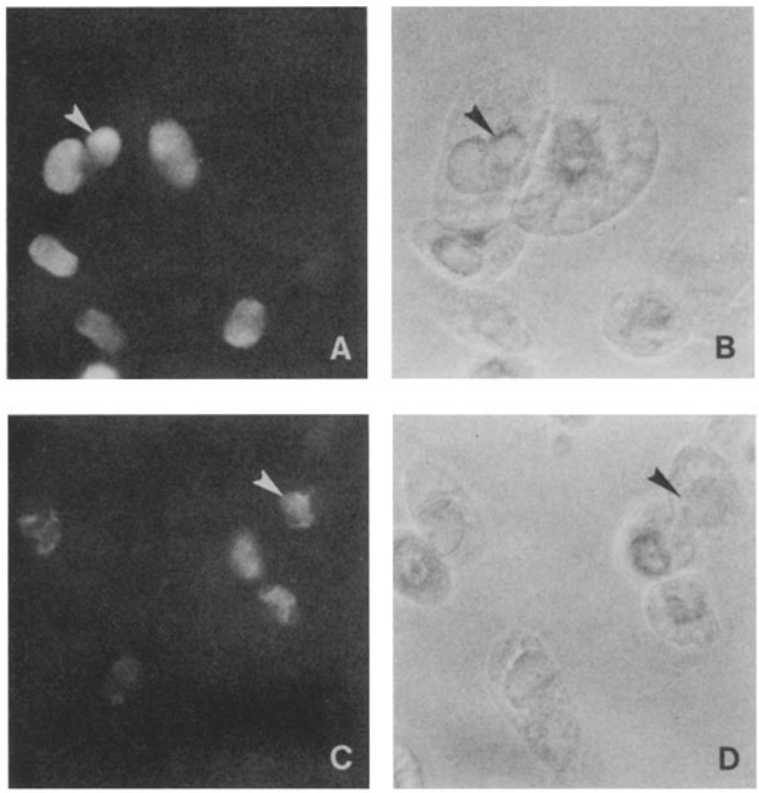

Fig. 4. Indirect immunofluorescence staining (IIF) of HSV-2 infected cells fixed 9 hours postinfection. *A* IIF staining with serum no. 5 used in a dilution of 1/10. *B* Phasecontrast microscopy of the same cells. *C* IIF staining with antibodies specific for the 91K protein diluted 1/10. The meshlike staining should be noted. *D* Phasecontrast microscopy of the cells in C. The arrowheads mark identical cells shown in IIF and in phasecontrast microscopy

(Fig. 1, A, B). The serum reacted only slightly with an antigen Ag-x of HSV-1, an antigen which has not been identified before (Fig. 1, C, D). Ag-4 has not been characterized yet as several attempts to extract the protein have failed either because of limited protein content or because of instability.

Characterization of the Proteins of Ag-5

The proteins of Ag-5 of HSV-2 were identified by their reactivity with the specific antiserum no. 5 by use of immunoblotting analysis. Extracts of

HSV-2 proteins electrophoretically separated and transferred to nitrocellulose were incubated with serum no. 5. Two proteins of molecular weights 128K and 91K showed strong binding of the antibodies (Fig. 2, slot 4). The 91K protein was identified as the alkaline exonuclease with monoclonal antibodies obtained from Dr. Powell, Leeds (data not shown). Analysis of the immunological cross reactivity of serum no. 5 with HSV-1 proteins showed that two proteins 128K and 99K were reactive, but the binding to the 128K protein less strong and the 99K protein was hardly detectable

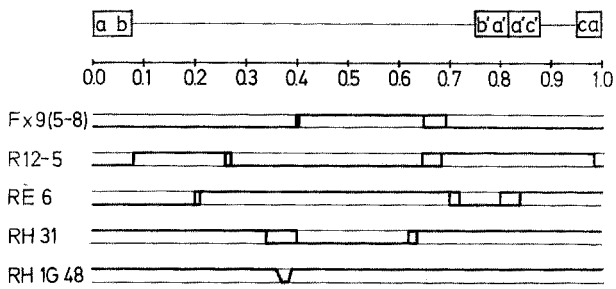

Fig. 5. The genetic map of the HSV-1 × HSV-2 intertypic recombinants used for mapping the 91K gene. The upper line drawn for each recombinant represents the HSV-1 sequences, the lower line the HSV-2 sequences. The DNA present in the recombinants is shown by the heavy line

(Fig. 2, slot 3). The 128K protein of both HSV-1 and HSV-2 had an electrophoretic mobility identical to that of ICP-8 as shown after X-ray exposure of the antibody stained transfer (Fig. 3, slot 1, 2).

Intracellular Location of the Ag-5 Proteins

HSV-2 infected cells were fractionated into nuclei and cytoplasm 9 hours postinfection. ICP-8 was evenly distributed between the nuclei and the cytoplasm, but the 91K protein was released into the cytoplasm (Fig. 3, slots 2, 3). The protein marked with an arrow might be a proteolytic degradation product of 91K although the inhibitors TLCK and TPCK were present in the buffer used for cell fractionation.

Indirect immunofluorescence staining of HSV-2 infected cells fixed 9 hours postinfection showed that serum no. 5 stained exclusively the nuclei (Fig. 4, A, B). Antibodies made specifically to the 91K protein by immunosorption of serum no. 5 on nitrocellulose bound 91K also stained the nuclei of infected cells but in a more meshlike pattern (Fig. 4, C, D). The purity of the 91K specific antibodies as tested in immunoblotting is shown in Fig. 2, slot 5. The two minor bands indicated by the arrows are most likely degradation products of the 91K protein detected with the selected and

concentrated antibody preparation. The immunosorbent purified antibodies made to the 128K protein did not cross-react with the 91K protein (Fig. 2, slot 6). The SDS-disruption mixture did not contain protease inhibitors.

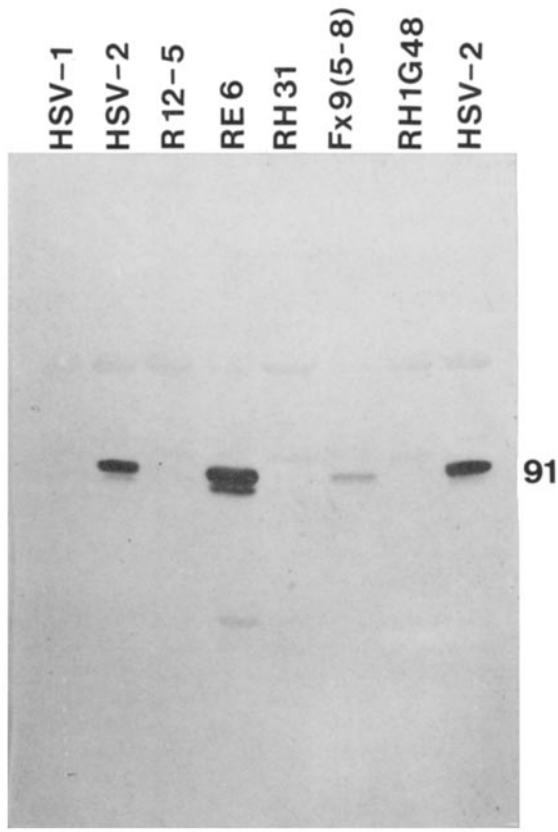

Fig. 6. Mapping of the 91K protein gene by the use of intertypic recombinants. The infected cell proteins extracted from the recombinants were transferred to nitrocellulose and reacted with 91K specific antibodies

Mapping of the Structural Gene for the 91K Protein

The 91K protein of HSV-2 was mapped by the use of the series of HSV-1 × HSV-2 intertypic recombinants listed in Fig. 5. Immunoblotting and incubation of the transfer with 91K specific antibodies showed that the recombinants Fx9 (5—8) and RE6 were positive for 91K whereas RH31 and R12-5 were negative (Fig. 6, slots 3—6). The weaker reaction obtained by Fx9 (5—8) is due to less viral antigen on the transfer. When the left end of the long unique sequences was derived from HSV-2 the recombinants were positive for 91K. An exchange of the HSV-2 sequences from 0.089 to

0.20 map units by HSV-1 derived sequences resulted in a failing reaction with the 91K antibodies (R12-5). It should be noted that the sequences between 0.7 and 0.83 are exchanged at the same time and the coordinates for the 91K protein is therefore either within 0.089—0.2 or 0.7—0.83 map units. The recombinant RH1G48 was included as an extra control for the separate map locations for ICP-8 and 91K. The recombinant carried only HSV-2 sequences in the ICP-8 gene (6) which has the map coordinates 0.361—0.417 (27).

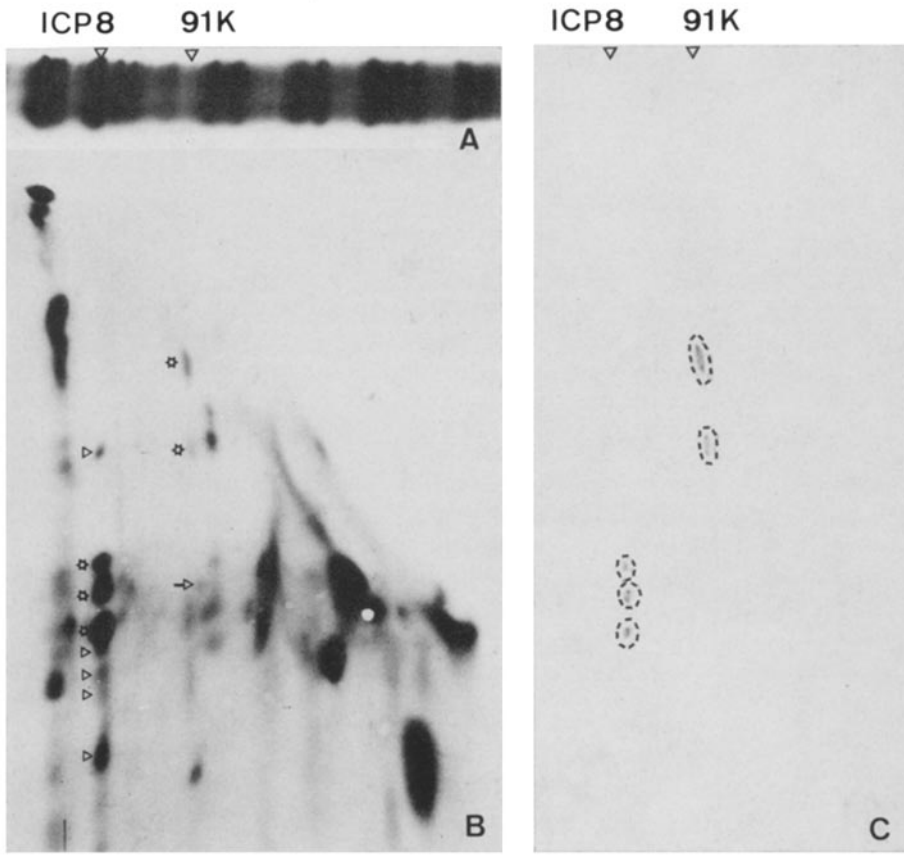

Fig. 7. Two-D polyacrylamide gel analysis of HSV-2 proteins and immunoblotting of the oligopeptides. SDS-polyacrylamide gel electrophoresis of ILV-labeled HSV-2 infected cell proteins in 9.25 per cent polyacrylamide gel followed by staphylococcus V-8 protease degradation and separation of the oligopeptides in 12 per cent polyacrylamide gel. The oligopeptides were transferred to nitrocellulose and the autoradiographic immage is shown in *A*. The 128K and the 92K proteins are marked with arrows and the different oligopeptide pattern obtained from the two proteins should be noted. *B* The immunoreactivity of the transferred HSV-2 oligopeptides after incubation with serum no. 5. The reactive spots are encircled

Peptide Composition of 128K and 91K

HSV-2 proteins electrophoretically separated in 9.25 per cent polyacrylamide gels were analysed in a second dimensional gel after proteolytic degradation with staphylococcus aureus V8. ICP-8 was degraded into 8 and 91K into 5 peptides of different molecular weight. Antiserum no. 5 identified 3 of the ICP-8 peptides and 2 of the 91K ones (Fig. 7 A, B). It should be noted that there is a protein with a peptide pattern very similar to that of ICP-8 but which migrate a little faster than the 91K. This protein is not recognized by the antiserum (Fig. 7 A).

Discussion

The analysis of HSV-1 and HSV-2 infected cell proteins by the use of crossed immunoelectrophoresis was initiated in order to understand the correlation between immunospecifity and protein composition of viral antigens. The glycoprotein antigens received most attention. They were of special interest as they were located on the surface of both the viral envelope and the plasma membrane of infected cells, and the glycoproteins were thus likely to play a role in the pathogenesis of HSV (for review see reference 18). The special interest in Ag-5 of HSV-2 was based on the observation that the protein was abundant in HSV-2 infected but not in HSV-1 infected cells.

Serum no. 5 made to Ag-5 reacted only slightly with an antigen named X of HSV-1. The antibodies identified two proteins of HSV-2 in immunoblotting, the 128K and the 91K proteins, of which the 128K was identified ICP-8 based on the electrophoretic mobility of the radioactively labeled protein (Fig. 2) and on the nuclear location in the immunofluorescence test (12, 15, 24). The antibodies reacted also with ICP-8 of HSV-1 but the second band with MW of 99K was hardly visible in the immunoreaction although the protein labeled well with the 14-C-aminoacids (Fig. 2). The 91K protein of HSV-2 which coprecipitated with ICP-8 in gels was also located in the nucleus of infected cells. The protein was apparently not associated physically to the ICP-8 as cell fractionation into nuclei and cytoplasm by use of an NP40 containing buffer extracted the 91K protein into the cytoplasm. If the protein is associated to the nuclear membrane or just leaks out of the nuclei remains unclear.

The 2-D-gel analysis showed that the ICP-8 and the 91K proteins were different and the 91K was therefore not a cleavage product of ICP-8. The mapping data also showed that the map coordinates for the two proteins differed. ICP-8 has been mapped within the coordinates 0.361—0.417 (27) and we estimated the 91K coordinates to be either 0.089—0.2 or 0.7—0.83 based on the analysis of intertypic recombinants. Our data show that although the two proteins coprecipitate they differ both by peptide analysis and by immunological specificity, but it is not known if their function in

the nuclei is coordinated. ICP-8 of HSV-1 associates to the nuclear matrix and are apparently translocated from prereplicative to replicate acompartments within the nucleus (26). It should be noted that Quinlan *et al.* (26) identify a 85K protein of HSV-1 which crossreacts immunologically with ICP-8 as demonstrated by immunoprecipitation with a monoclonal antibody. This protein might very well be identical to the 99K protein which we find bind antibodies of serum no. 5 to a limited extent.

We were unable to identify the exact map coordinates of the 91K gene with the recombinants available, but the 91K specific antibodies purified by the immunosorbent technique reacted in immunoblotting with the same protein which reacted with monoclonal antibodies made to the alkaline exonuclease, antibodies which were kindly given by Dr. K. Powell, University of Leeds. Based on this observation we can conclude that the 91K protein is most likely the HSV-2 counterpart to the exonuclease 85K which is mapped within the coordinates 0.080—0.185 (25).

The analysis of the possible binding of the 91K protein to the nuclear matrix is under investigation.

Acknowledgement

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