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Proteins Specified by Herpes Simplex Virus

X. Staining and Radiolabeling Properties of B Capsid and Virion Proteins in Polyacrylamide Gels

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Analyses of the structural proteins of herpes simplex virions and of capsids containing viral DNA (B capsids), after electrophoresis in polyacrylamide gels, revealed considerable variability in their properties with respect to: (i) retention of Coomassie brilliant blue (CBB) and fast green stains during destaining, (ii) relative optical absorbance of the CBB-protein complex at different wavelengths, (iii) relative efficiency with which ^{14}C-amino acids are incorporated during early and late periods of the infection cycle, and (iv) capacity to be phosphorylated in vivo. In addition, it was found that protein 22a of B capsids, which does not have an electrophoretically identical counterpart in virions, shares a relatively unique set of staining and radiolabeling properties with virion protein 22, which has a slightly more rapid electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels.

The protein composition of three classes of herpes simplex virus (HSV) particles was described in preceding papers (9, 21) in this series. (i) Enveloped nucleocapsids (virions) contain at least 24 species of proteins, ranging in molecular weight from 25,000 to 275,000. (ii) Intranuclear capsids lacking DNA (A capsids), are composed of one minor (no. 24) and three major (no. 5, 19, and 23) structural proteins. (iii) Intranuclear capsids containing viral DNA (B capsids), in addition to all of the A capsid proteins, contain another major (no. 22a) and at least one more minor (no. 21) protein. All of the B capsid proteins except 22a are present in the virion in approximately the same molar ratios.

The experiments described in this paper focus on a number of staining and radiolabeling properties exhibited by the structural proteins of these virus particles. The data indicate that herpes proteins can be differentiated in sodium dodecyl sulfate (SDS)-polyacrylamide gels on the basis of (i) staining properties, i.e., ability to retain Coomassie brilliant blue (CBB) and fast green (FG) stains, and relative optical absorbance of the CBB-protein complex at different wavelengths, and (ii) radiolabeling properties, i.e., efficiency of ^{14}C-amino acid incorporation during different periods of the infection cycle, and incorporation of $^{32}P_i$ in vivo. An attempt has been made to use these differential properties of the electrophoretically separated proteins to determine whether one of the virion proteins might be related to B capsid protein 22a, which does not have an electrophoretically identical counterpart in the virion.

MATERIALS AND METHODS

Virus and cells. HSV was grown in human epidermoid carcinoma no. 2 (HEp-2) cells. The pertinent properties of the F and G prototypes of HSV-1 and HSV-2 subtypes, respectively, used in these studies and the procedures for their production and assay have been described elsewhere (6, 9, 12, 13, 21). In general, HSV-1 was used in the production of virions and HSV-2 was used in the production of B capsids. It should be emphasized, however, that HSV-1 and HSV-2 B capsid proteins cannot be differentiated with respect to the staining and radiolabeling properties described here.

Infection of cells and labeling of viral proteins. The procedure for infection was as follows. HEp-2 cells in monolayer cultures were exposed to an average of 5 to 10 PFU of virus per cell in mixture 199 (Microbiological Ass. Inc., Bethesda, Md.) containing 10% glucose and 1% calf serum (199-1%). After adsorption for 60 min at 37 C, the inoculum was removed and replaced with 199-1%, and incubation of the infected cultures continued at 37 C.

Viral proteins were radiolabeled using a mixture of ^{14}C-amino acids (NEC-445, 100 μCi/ml, New England Nuclear Corp., Boston, Mass.) in mixture 199 containing the usual amount of arginine but only one-fifth the usual levels of all other amino acids present in the radioactive mixture, and supplemented with 1% dialyzed calf serum (199-1%DC). ^{32}P-orthophosphate (50 mCi/ml, also from New England Nuclear

Corp.) in 199-1% was used to radiolabel phosphorylated proteins. The amount of radioisotope used and the time interval of labeling varied with the experiment and are specified in the text.

Purification of virions and B capsids. The procedures and reagents used in purifying virions and B capsids were previously described in detail (21 and 9, respectively).

Analytical gel procedures. Samples were solubilized and subjected to disk gel electrophoresis in cylindrical 8.5% polyacrylamide gels, essentially as described by Laemmli (14). Details of the techniques used have been published elsewhere (9, 21).

Slab gels (100 mm wide by 120 mm long by 2 mm thick) were prepared essentially as previously described (21), but *N,N'*-diallyl-tartardiamide (DATD, Aldrich Chem. Co., San Leandro, Calif.; see reference 2) was used in place of methylene-bis acrylamide (MBA) as the crosslinking reagent (1.5 g of DATD substituted per g of MBA). DATD linked gels have two advantages: (i) they can be dissolved with 2% periodic acid (2) or 2% sodium periodate (A. R. Hunter, personal communication) at room temperature in 20 to 30 min, and (ii) gels of high acrylamide concentrations (we have tried up to 18% acrylamide) can be dried by the technique of Fairbanks et al. (7) without problems of tearing which generally complicate the processing of MBA-linked gels above 12% acrylamide. Comparisons of electrophoretic migration versus log molecular weight of the standard proteins bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c*, and beta galactosidase (all obtained from Worthington Chemical Co., Freehold, N.J.) in DATD- and MBA-linked gels showed no significant difference in the slope of the curves. A slightly higher (approximately 2%) final concentration of acrylamide, however, was required in DATD-linked gels to retard proteins to the same extent as in MBA-linked gels.

Cylindrical gels were fixed and stained with CBB as described by Fairbanks et al. (8). Slab gels were fixed for 2 h at room temperature in 50% trichloroacetic acid, stained 60 min at room temperature in 50% trichloroacetic acid containing 0.1% CBB, and destained against 7% acetic acid-10% methanol until the background was colorless.

For staining with FG (Sigma, St. Louis, Mo.), the gels were first fixed for 18 h in 10% acetic acid-25% 2-propanol. After equilibration in water (pH 3.0), the gels were stained overnight with FG (1% in H_2O, pH 3.0), and then destained for 12 to 24 h in water (pH 3.0).

Absorbance profiles of CBB-stained gels and of autoradiograms were recorded at 550 nm; those of FG-stained gels were recorded at 620 nm. Departures from these techniques are indicated in the text.

RESULTS

Binding of Coomassie brilliant blue stain to HSV B capsid and virion proteins. Prolonged destaining of B capsid proteins separated electrophoretically in polyacrylamide gels and stained with CBB resulted in an unusually rapid and complete removal of the stain from band 22a (Fig. 1A). Although the amount of stain in each band decreased linearly with time, the rate of stain loss from band 22a was twice that of bands 5, 19, and 23 (Fig. 2).

Once destained, the relative intensity of band 22a upon restaining was diminished by 30 to 50%. This reduced ability to bind stain is not due to loss of protein, however, as indicated by the following experiment. Duplicate gels containing electrophoretically separated B capsid proteins, labeled with ^{14}C-amino acids (0.5 μCi/ml) beginning 5 h after infection, were stained with CBB. One gel was destained normally, sliced, and prepared for autoradiography; the second was destained until more than 95% of the CBB had been removed from band 22a, and then it too was sliced and prepared for autoradiography. Comparison of absorbance profiles (not shown) made from the autoradiograms indicated that prolonged destaining reduced the amount of radioactivity in band 22a by only 10%. Thus, the measured loss of labeled protein was three- to fivefold lower than would have been predicted from the decreased stain binding.

Similarly, loss of staining potential does not seem to be due to the removal during destaining of a CBB-binding, nonprotein (i.e., does not incorporate ^{14}C-amino acids during the infection cycle) component. If such a component were present, estimates of the amount of material in band 22a based on staining should exceed those based on autoradiography of ^{14}C-amino acid-labeled preparations. No such discrepancy emerged (compare Fig. 1A and C with Fig. 4A). Both techniques indicated about 36% as much protein 22a present as protein 5 (Table 1, columns 1 and 2). A possible explanation of these results, in light of the experiments presented below, is considered in the Discussion.

Parallel experiments with electrophoretically separated, CBB-stained virion proteins showed that bands 1, 7–8, 17–18, and 22 destained more extensively than the others; the most dramatic loss being from band 22 (Fig. 1B).

Binding of FG stain to B capsid and virion proteins. To examine the affinity of B capsid and virion proteins for other stains, the destaining experiments were repeated with FG stain. This stain is nearly as sensitive as CBB for detecting proteins, conforms to Beer's law over a wide range of protein concentrations, and has an acidic chromophore (10).

Under normal destaining conditions (pH 3.0 for 24 h), FG gave essentially the same absorbance profile for B capsid proteins as CBB (compare Fig. 1A and C). However, further

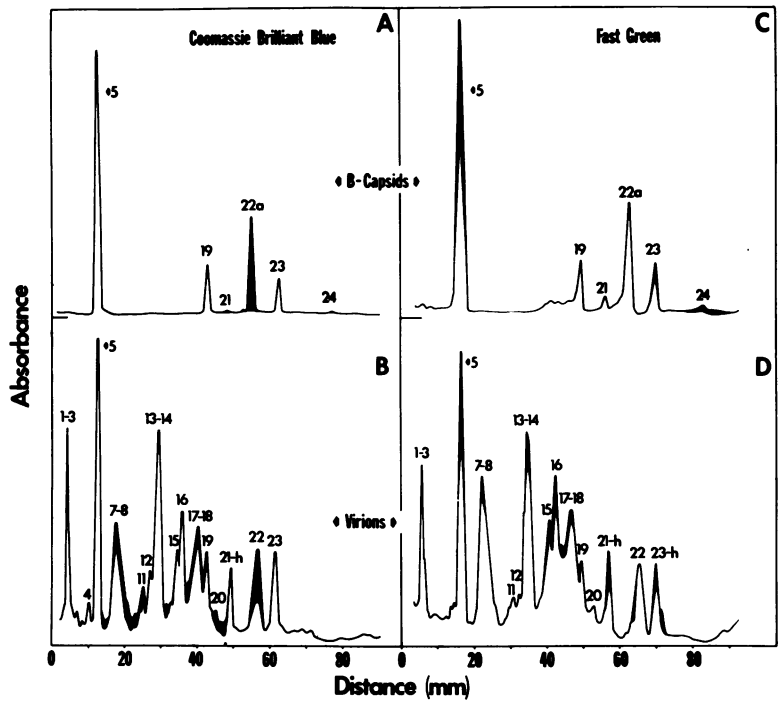

FIG. 1. *Differential loss of CBB and FG stains from B capsid and virion proteins. HSV-2 B capsids and HSV-1 virions were isolated, solubilized, subjected to electrophoresis, and stained, either with CBB or FG, as described in Materials and Methods and previously (9). Shown here are tracings comparing: (A) B capsid proteins stained with CBB and scanned after 2 days and 5 days of destaining (absorbance of peak 5 was 2.50 and 1.00 optical density (OD) units, respectively); (B) virion proteins stained with CBB and scanned after 2 days and 6 days of destaining (absorbance of peak 5 was 1.30 and 0.50 OD units, respectively); (C) B capsid proteins stained with FG and scanned first after destaining at pH 3.0 and again after destaining at pH 6.0 (absorbance of peak 5 was 1.00 and 0.45 OD units, respectively); and (D) virion proteins stained with FG and scanned after destaining at pH 3.0 and again after destaining at pH 6.0 (absorbance of peak 5 was 0.30 and 0.15 OD units, respectively). The shaded portion in each panel represents the amount of stain lost during the second period of destaining. Two host proteins (15) are designated by the letter h. Electrophoresis was from left to right in this and subsequent figures, and the position of the base line in the top panels is indicated by a short line at the extreme left of the profile.*

destaining at pH 6.0 resulted in a pattern of differential destaining that contrasted strikingly with the analogous CBB profile. Specifically, protein 22a as well as proteins 19 and 21 preferentially retained FG, whereas proteins 5, 23, and perhaps 24 destained more rapidly (Fig. 1C). Stain loss from band 5 was rapid during the first 12 h but very slow thereafter. Proteins 23 and 24 destained continuously, and more gradually than did protein 5.

Absorbance profiles of virion proteins stained with FG and then destained at pH 3.0 and 6.0 show that of the major virion proteins, no. 19 and 22 retained the stain most avidly. It is of interest to note that those proteins which bound CBB most avidly (i.e., no. 5, 13–14, 15–16, 21-h, and 23) retained FG stain least well (compare Fig. 1B and D).

Fluorescence of HSV structural proteins. B capsid band 22a and virion bands 13, 14, and 22 exhibited a pink fluorescence when stained normally with CBB and examined under intense illumination directed either from the side or the end of the gel. None of the other major capsid or virion proteins fluoresced under these conditions.

Although the fluorescence intensity of the CBB-protein complex was not measured directly, the unusual nature of the interaction could be demonstrated by a relatively increased optical absorbance at longer wavelengths. This phenomenon is illustrated in Fig. 3 with a gel containing virion proteins. Comparisons of the absorbance profiles recorded at 550 and 725 nm (Fig. 3) show that, relative to the fluorescing bands 13–14 and 22, the absorbance of all other virion protein bands was diminished at 725 nm. Further, the proportionality of bands 13 -14 and

22 was approximately constant at both wavelengths, suggesting that the percentage of the total number of bound CBB molecules contributing to the fluorescence must be the same in each band.

Incorporation of ^{14}C-amino acids into B capsid and virion proteins. During the course of these experiments some variability was noted in the amount of ^{14}C-amino acids incorporated into B capsid protein 22a, depending on the time of addition of the labeled amino acids and the duration of the labeling interval. This observation prompted us to compare the efficiency with which ^{14}C-amino acids are incorporated into B capsid and virion proteins early and late in the replicative cycle of the virus.

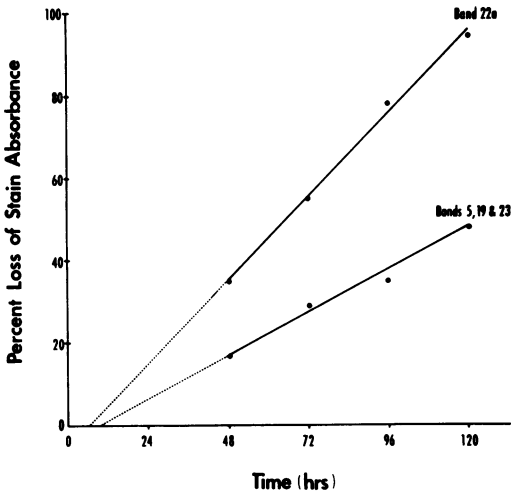

FIG. 2. *Kinetics of CBB stain loss from HSV-1 B capsid proteins. An 8.5% polyacrylamide gel containing B capsid proteins was stained with CBB and then destained. At 48, 72, 96, and 120 h after staining, absorbance profiles of the gel were recorded and the areas beneath peaks 5, 19, 22a, and 23 were calculated. A small scratch made in the gel served to normalize each tracing. The plot labeled "Brands 5, 19 & 23" represents the average of the three; variation was less than 5% from this average. The dotted line is an extrapolation of the data points and intercepts the abscissa at about the time that protein bands first became noticeable above background staining (i.e., 12 h).*

B capsids were isolated 20 h after infection from cells labeled continuously with ^{14}C-amino acids (1 μCi/ml) beginning either 2 (early) or 5 (late) h after infection. Both capsid preparations were solubilized and subjected to electrophoresis in polyacrylamide gels, after which the gels were stained with CBB, scanned, and then further analyzed by autoradiography. As summarized in Table 1, measurements based on absorbance profiles of the stained gels and their autoradiographic images showed the following. (i) Staining and autoradiographic quantitation techniques gave similar estimates of the relative amounts of proteins 5, 19, and 22a in B capsids labeled beginning 2 h after infection (Table 1, column 4). (ii) The relative amounts of proteins 5, 19, and 23, as measured by autoradiography, were essentially the same in both preparations (Table 1, column 5; Fig. 4A and B). Peak 22a, however, was 26% smaller in the preparation labeled beginning at 5 h after infection. Since measurements based on staining indicated no significant quantitative differences between the proteins in the early and late labeled preparations, this reduction must derive from a diminished incorporation of the radioisotope during the late labeling, interval. It is not known, however, whether this is due to a reduction in the amount of this protein made late or the accumulation of a large pool made before label

TABLE 1. *Comparison of staining and autoradiographic measurements of the relative proportions of HSV-2 B-capsid proteins*[a]

Protein no.	Peak area (cm^2) determined by			Ratio of corresponding peak areas	
	Staining (1)[c]	Autoradiography[b]		Staining/autoradiography (early) (4)	Autoradiography (early)/autoradiography (late) (5)
		Early[d] (2)	Late[e] (3)		
5	6.33	6.33	6.33	1.00	1.00
19	0.88	0.86	0.82	1.02	1.05
22A	2.36	2.21	1.63	1.07	0.74
23	0.85	1.38	1.36	0.62	1.02

[a] The procedure for isolating B capsids and the conditions of electrophoresis, staining, and autoradiography are described in Materials and Methods.
[b] Values have been normalized to the peak area of protein 5 as determined by staining (column 1).
[c] Column number.
[d] ^{14}C-amino acids added beginning 2 h after infection.
[e] ^{14}C-amino acids added beginning 5 h after infection.

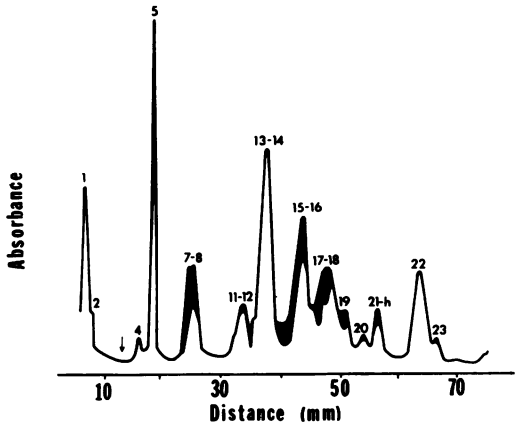

FIG. 3. *Increased relative absorbance of several CBB-stained herpes virion proteins when scanned at longer wavelengths. Virion proteins were separated electrophoretically and stained with CBB as described in Materials and Methods, with the exception that DATD was used as the cross-linking reagent in place of methylene-bis acrylamide. This substitution is without effect on the fluorescence of HSV proteins. Absorbance profiles of the destained gel were recorded at 550 and 725 nm, and normalized such that the area of peak no. 22 was approximately the same in both. The shaded areas indicate a decreased absorbance at 725 nm, relative to that at 550 nm. The arrow between peaks two and four denotes the position to the left of which there was strong absorbance at 725 nm in the absence of visible CBB staining or fluorescence. Absorbance of peak 5 was 1.30 OD units at 550 nm and 0.10 OD units at 725 nm.*

was added. (iii) Autoradiographic estimates of the amount of protein 23 in B capsids showed 38% more radioactivity, in both early and late labeled preparations, than would be predicted from the relative intensity of that band when stained with either CBB or FG (Table 1, column 4; Fig. 1A and C). As discussed below, we suspect that the discrepancy between the two techniques reflects a staining peculiarity of band 23, in which case our earlier estimate (7) of the amount of protein 23 in A and B capsids may be 5% too low. Further analyses using independent techniques will be required to resolve this question.

To examine the efficiency of incorporation of ^{14}C-amino acids into virion proteins, virions were isolated 20 h after infection from two cell cultures (5×10^8 cells each) labeled with ^{14}C-amino acids (1.0 μCi/ml)—one during the interval 2 to 4 h after infection, the other during the interval 8 to 20 h after infection. Both preparations were solubilized, subjected to electrophoresis, and then analyzed by staining and autoradiography.

Absorbance profiles recorded from autoradiograms of these preparations, shown in Fig. 4C and D, indicate that the virion proteins can be reasonably well fitted into one of three classes, with respect to efficiency of ^{14}C-amino acid incorporation during early (2 to 4 h after infection) and late (8 to 20 h after infection) times in the infection cycle. The first class includes those proteins whose relative autoradiographic intensities were approximately the same in both preparations (i.e., no. 5, 7, 11, 12, 15, 16, 17, 18, and probably also no. 2 and 19). The second class includes proteins 4, 6, 21, and 22, whose relative autoradiographic intensities were significantly greater in the preparation labeled from 2 to 4 h after infection. And the third class includes proteins 1, 8, 13, 14, and possibly 24, whose relative autoradiographic intensities were strikingly increased in the preparation labeled from 8 to 20 h after infection.

Incorporation of $^{32}P_i$ into B capsid and virion proteins. B capsids and virions were isolated 18 h after infection from cells labeled with $^{32}P_i$ (5 μCi-ml) beginning 2 h after infection. Both preparations were then solubilized, subjected to electrophoresis, stained, and processed for autoradiography.

As shown in Fig. 5B, a significant portion of the ^{32}P radioactivity in the B capsid preparation comigrated in the gel with protein 22a. Heat disruption of the capsid followed by treatment with DNase and venom phosphodiesterase greatly reduced the amount of radioactivity trailing into the gel from the origin (Fig. 5C). Elmination of this material, presumably viral DNA, revealed the following. (i) In vivo phosphorylation of the major B capsid proteins is restricted almost exclusively to protein 22a, although a significant amount of the radioisotope is also present in band 19. (ii) The ^{32}P radioactivity present in band 22a was completely resistant to DNase and phosphodiesterase as judged by the undiminished autoradiographic absorbance of that band after nuclease treatment (compare Fig. 5B and C). (iii) The low-molecular-weight material migrating in the gel near the ion front may also contain phosphorylated, non-DNA components, since the ^{32}P radioactivity in that region of the gel was not appreciably diminished by the nuclease treatment.

The phosphorylated components present in virions are shown in Fig. 6E. For purposes of identification, herpes simplex virions radiolabeled with ^{14}C-amino acids beginning 6 h after infection were subjected to electrophoresis in the same gel (Fig. 6F). Comparison of autoradiograms in Fig. 6E and F reveals the following. (i) All of the major bands of ^{32}P radioactivity in

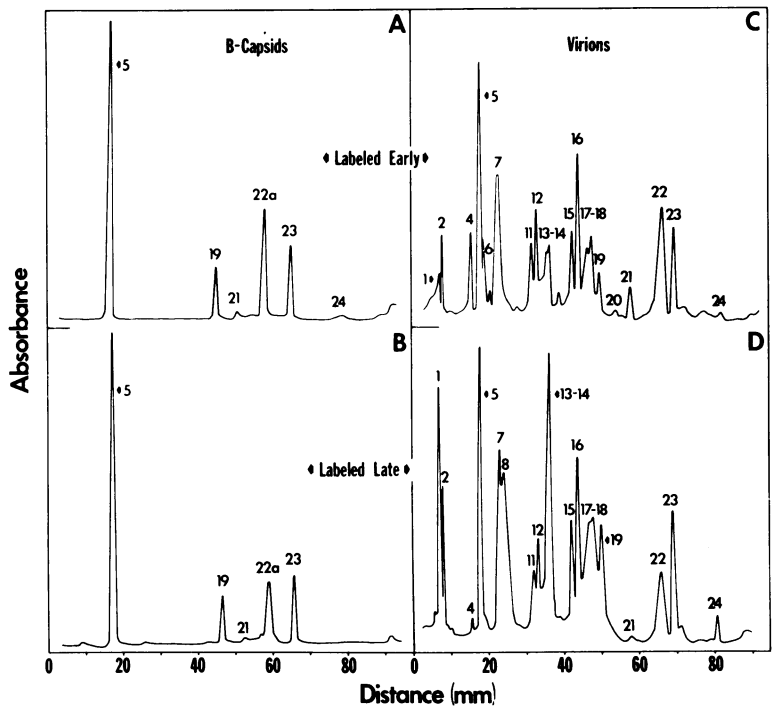

FIG. 4. *Electropherograms of B capsid and virion proteins labeled with ^{14}C-amino acids at different times during the reproductive cycle. HSV-2 B capsids were isolated from cells labeled with ^{14}C-amino acids (1 μCi/ml) beginning at 2 or 5 h after infection. HSV-1 virions were isolated from cells labeled with ^{14}C-amino acids (1 μCi/ml) during the interval 2 to 4 h after infection, and from cells labeled during the interval 8 to 20 h after infection. These preparations were solubilized and subjected to electrophoresis, and autoradiograms were made from each. Absorbance profiles of the autoradiograms are shown as follows: (A) B capsids labeled beginning at 2 h after infection; (B) B capsids labeled beginning 5 h after infection; (C) virions labeled 2 to 4 h after infection; and (D) virions labeled 8 to 20 h after infection. The absorbance of peak 5 in these tracings was: (A) 0.60; (B) 1.30; (C) 1.40, and (D) 2.00 OD units.*

the virion preparation correspond exactly with known protein components of the virion. (ii) Proteins 5 and 23 which did not contain ^{32}P radioactivity in the B capsid preparation were also unlabeled in the enveloped virus particle. Conversely, protein 19 which was radiolabeled in B capsids, also contained a small but significant amount of ^{32}P radioactivity in the virion preparation. (iii) Protein 22 exhibited the greatest level of ^{32}P radioactivity among the virion proteins; however, bands 6, 13, 14, and 16 also gave strong ^{32}P autoradiographic images and detectable amounts of radioactivity were present in bands 7, 12, 18, and 19.

The stringent conditions of protein solubilization, fixation, and staining used in analyzing these virus preparations suggest that the ^{32}P radioactivity comigrating in these gels with the viral proteins is convalently attached. Results of the following experiment support this possibility. Virion band 22 was sectioned from a fixed, stained, and dried-down gel. The gel slice was dissolved in 2% periodic acid at 23 C for 2 h, and the protein was precipitated, in the presence of 100 μg of bovine serum albumin, with 10% trichloroacetic acid (0 C for 16 h). After dissolving the precipitate in 0.2 M NH_4HCO_3, the protein was reprecipitated in 90% acetone at 4 C for 2 h. After this treatment, 65% of the ^{32}P radioactivity present in the original gel slice (500 count/min, Cerenkov radiation) was recovered in the final acetone precipitate.

To determine whether these phosphorylated virion components could be detected in infected cell lysates, autoradiographic profiles of the nuclear and cytoplasmic fractions of infected and uninfected cells labeled with $^{32}P_i$ (5 μCi/ml) were compared (Fig. 6A–D). The following was observed. (i) There was an apparent stimulation of phosphorylation in both the nuclear and cytoplasmic fractions of infected cells as compared with the corresponding fractions from uninfected cells. (ii) The cytoplasmic fraction of infected cells contained four major bands of ^{32}P radioactivity, three of which have electrophoretic mobilities indistinguishable from virion

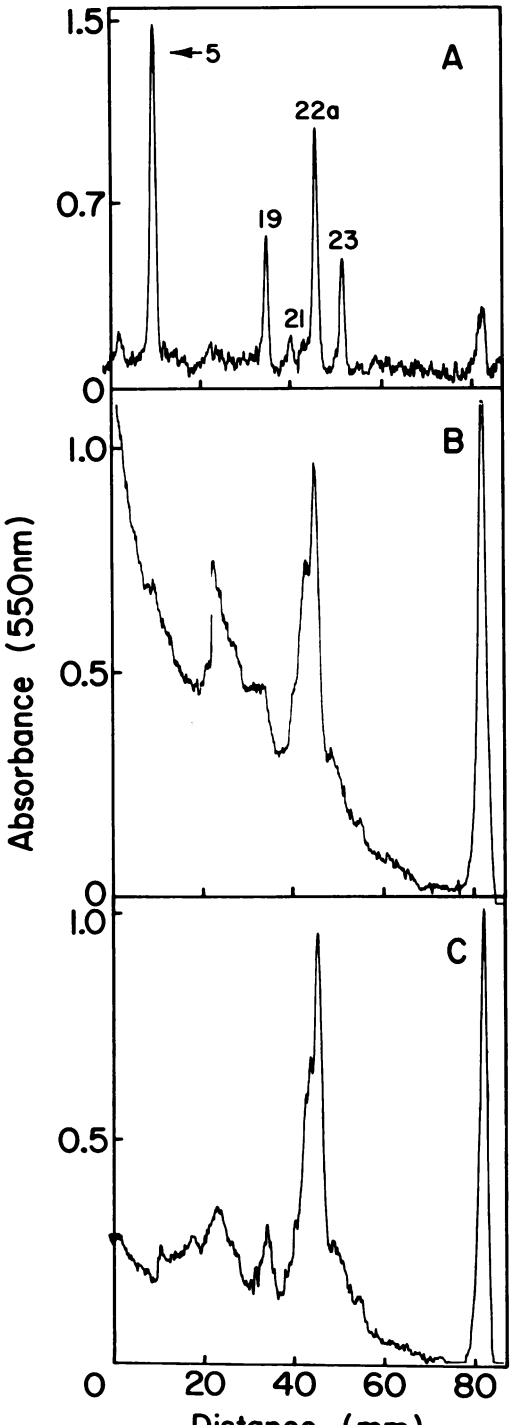

FIG. 5. *Electropherograms showing the comigration of ^{32}P radioactivity with structural proteins of HSV B capsids. B capsids were isolated 18 h after infection from 5 × 10^8 cells infected with HSV-2 and grown in the presence of $^{32}P_i$ (5 μCi/ml, added 2 h after infection). Two samples, each containing 100 to 200 μg of protein, were heated (90 C for 30 min in 0.01 M sodium phosphate buffer, pH 7.2, 0.1 M NaCl, and 25% [wt/wt] sucrose) to weaken or dissociate the capsid structure. The samples were then incubated at 37 C for 2 h—one in the presence of DNase (50 μg/ml, electrophoretically purified, Worthington Biochemical Corp., Freehold, N.J.) and venom phosphodiesterase (2 × 10^{-1} units/ml, phosphodiesterase I, also from Worthington); the other without any enzymes added. Immediately after incubation, both samples were solubilized and subjected to electrophoresis; the gels were then stained with CBB, and processed for autoradiography. Shown here are absorbance profiles of: (A) CBB-stained proteins in the untreated B capsid preparation; (B) an autoradiogram prepared from the untreated B capsid preparation whose CBB-stained profile is shown in A; and (C) an autoradiogram of the DNase-phosphoidesterase-treated B capsid preparation. To provide coincidence between tracings of the stained gel and its autoradiographic image (panels A and B, respectively), the stained gel profile shown in A was recorded by scanning directly through the filter paper onto which the gel had been dried in preparation for autoradiography.*

proteins 6, 12, and 16. The fourth migrated in the gel just slower than protein 16 and was equally as intense. The band corresponding in electrophoretic mobility with virion protein 12 appears to have a counterpart in the uninfected cell cytoplasm; however, closer examination both of this gel and others revealed that the infected cell band migrates slightly slower. (iii) The nuclear fraction of infected cells contained all four major cytoplasmic bands and two additional bands. One of these additional bands corresponds in electrophoretic mobility with virion protein 22; the other was not detectable in the virion preparation. (iv) The relative intensities of the ^{32}P-labeled virion bands does not directly reflect the general distribution of ^{32}P radioactivity among infected cell proteins.

DISCUSSION

In this paper we are presenting results of experiments designed to examine the staining and radiolabeling properties of HSV B capsid and virion proteins. Two significant findings emerged from these studies. First, there is appreciable variability in the capacity of HSV proteins to (i) retain CBB and FG stains, and (ii) incorporate ^{14}C-amino acids and $^{32}P_i$ during the replicative cycle of the virus. Second, a protein (22a) present in B capsids, but without an electrophoretically identical counterpart in virions, shares several relatively unique properties with virion protein 22.

Staining of HSV proteins in polyacrylamide gels. An assumption inherent in the procedure of using protein-binding dyes to quantitate

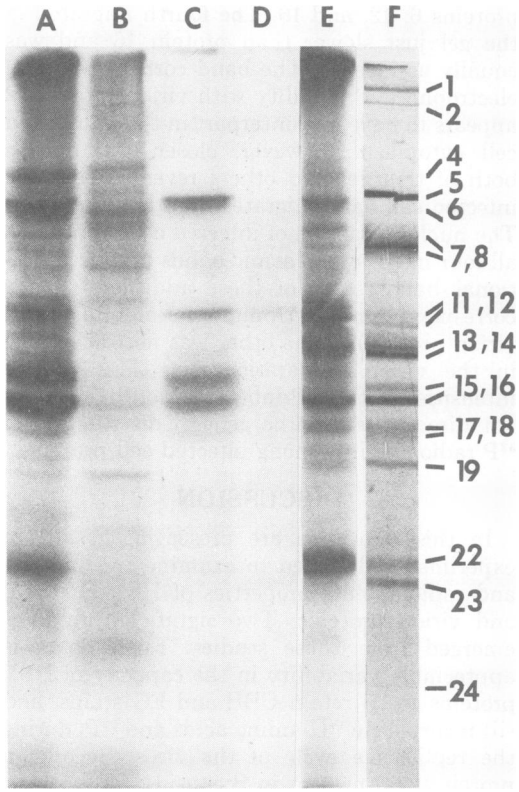

FIG. 6. *Photograph of an autoradiogram showing the cellular and virion components radiolabeled in vivo with $^{32}P_i$. The cellular fractions were prepared as follows. One cell culture (5×10^7 cells) was infected with HSV-1; another (5×10^7 cells) was left uninfected. Both were grown in the presence of $^{32}P_i$ (5 μCi/ml, added beginning 1 h after infection), harvested 20 h after infection, and separated into nuclear and cytoplasmic fractions with Nonidet NP-40 (0.5% final concentration, as previously described, reference 9). The total volume of the nuclear and cytoplasmic fractions was 0.5 and 1.0 ml, respectively; 20 μliters of each sample was subjected to electrophoresis. Preparation of ^{32}P radiolabeled virions was as follows. Virions were isolated 18 h after infection from 2×10^8* cells infected with HSV-1. Radiolabeling with $^{32}P_i$ (5 μCi/ml) *was begun 2 h after infection. In preparation for gel electrophoresis, the purified virions were pelleted out of suspension by centrifugation at 26,000 rpm and 4 C for 3 h in a Spinco SW27 rotor. Virions radiolabeled with ^{14}C-amino acids (1 μCi/ml) were prepared similarly; the radioisotope was added 6 h after infection. After solubilization, the samples were subjected to electrophoresis in a 10% acrylamide slab gel and cross-linked with DATD; the gel was then fixed, stained, and processed for autoradiography. The gel channels contained: (A) infected cell nuclear fraction; (B) uninfected cell nuclear fraction; (C) infected cell cytoplasmic fraction; (D) uninfected cell cytoplasmic fraction; (E) virions grown in the presence of $^{32}P_i$; and (F) virions grown in the presence of ^{14}C-amino acids. This photograph is a composit of three exposures of the same autoradiogram—one exposed for 1 day (channels A, B, C, and D); the second for 5 days (channel E); and the third for 4 weeks (channel F).*

the amount of protein present in analytical polyacrylamide gels is that the amount of stain remaining bound to the protein after destaining is proportional to the quantity of protein actually present. With few exceptions, this procedure applied to herpesvirus proteins works well, as judged by a close correspondence between stain and radioactivity profiles (see reference 15, Fig. 6A and B). The exceptions, as documented in this report, are several proteins which destain at a faster rate than the others. These are of interest from two points of view. First, since differential rates of destaining can affect estimates of the amount of protein present, attention is focused on the need for both alternative methods of quantitation as well as careful control of the destaining process. Second, as discussed below, the unusual staining properties of these proteins provide a useful means for their identification.

Although there is insufficient data to determine what specific feature(s) of these polypeptides is responsible for their rapid destaining, the results suggest that it may relate to their electronegativity. Thus, virion proteins 1–3, 7–8, 17–18, 22, and in particular B capsid protein 22a, which lost CBB more rapidly than other capsid and virion proteins, bound FG more avidly (Fig. 1). Since FG has an acidic chromophore and would therefore be expected to bind most strongly to basic molecules, the implication is that these proteins, especially 22a, are comparatively more basic. By extension then, the proteins which bound FG poorly, most notably no. 23, are relatively more acidic. Consistent with this hypothesis are results from double-labeling experiments which indicate that the ratio of [^{14}C]lysine to [^{3}H]leucine is highest for protein 22a (0.24), lower for proteins 19 (0.12) and 5 (0.08), and small (to the point of vanishing) for protein 23 (0.002). Further, since the highly acidic protein pepsin (isoelectric point 1.0) binds CBB poorly (8), the discrepancy noted between autoradiographic and staining estimates of the amount of protein 23 present in B capsids (Table 1, column 4) is also compatible with this hypothesis. We do not know why protein 5 in both the capsid and virion preparation showed a rapid initial loss of FG but then appeared refractory to further destaining; it may be that this major capsid component is electrostatically binary, having an

acidic region that would rapidly lose FG and a basic region that would not.

Since avid binding of FG does not necessarily correlate with unusually rapid loss of CBB, as demonstrated by protein 19 which binds both stains well (Fig. 1A and C), some further consideration should be given to the striking loss of CBB from band 22a on prolonged destaining. Under normal conditions, both staining (Fig. 1A and C) and autoradiographic (Fig. 4A) techniques indicated the same relative amount of material present in B capsid band 22a. Complete removal of CBB from band 22a by prolonged destaining, however, reduced its capacity to bind CBB on restaining without appreciably diminishing the amount of radiolabeled protein present. One interpretation of this observation is that prolonged exposure to the destaining solution (pH 2.5) may result in an in situ denaturation of amino acid residues which normally bind CBB. Parenthetically, deamination of glutamine and asparagine can occur at this pH (3) and, if these residues were present in sufficiently high amounts, deamination could cause the net charge of the protein to become more acidic. Such a modification could render the protein less stainable while leaving the amount of ^{14}C-amino acid radioactivity unaffected. The fact that a similar loss of FG stain from band 22a was not observed at approximately the same pH (3.0) may reflect a difference in the binding specificities of CBB and FG, or it may indicate that FG protects a normally acid-labile moiety from denaturation.

Another staining property characteristic of several virion (no. 13, 14, and 22) and B capsid (no. 22a) proteins was fluorescence of the CBB-stained protein under intense illumination. Clayton and Haselkorn (4), using slightly different fixation and staining techniques, recently described a similarly selective fluorescence among proteins of bacterial photosynthetic membranes. This phenomenon is not uncommon as a number of other proteins, including lysozyme, cytochrome *c*, structural protein band(s) 4 (molecular weight 23,000), and the histone-like proteins of polyoma virus, also fluoresce when stained with CBB (Gibson, unpublished observations). Why CBB fluoresces in association with only a few proteins is not clear. However, our observation that cytochrome *c* and lysozyme (isoelectric points 10 and 11, respectively) fluoresce with CBB, whereas ovalbumin and bovine serum albumin (isoelectric points 4.6 and 4.8, respectively) do not fluoresce, suggests that this property may also relate to the electronegativity of the protein.

Radiolabeling characteristics of HSV structural proteins. Two general observations emerged from the series of radiolabeling experiments with B capsid and virion proteins. First, the efficiency of ^{14}C-amino acid incorporation into proteins which end up in virions and B capsids is not constant throughout the infection cycle. Thus, as shown in Fig. 4, ^{14}C-amino acids were incorporated into proteins 4, 6, 22a, and 22 relatively more efficiently early (2 to 4 h after infection) in the infection cycle, whereas proteins 1, 8, 13–14, and possibly 24 were more efficiently labeled at later times (8 to 20 h after infection). These differences proved useful for comparative purposes, as discussed below. However, since they result from the interaction of a number of parameters, they may not accurately reflect the overall pattern of synthesis of virion proteins.

Second, $^{32}P_i$ incorporation into herpesvirus proteins was highly selective in vivo. Phosphorylation of the major B capsid proteins was limited almost exclusively to protein 22a (Fig. 5C), and phosphorylation of virion proteins was limited principally to no. 6, 13, 14, 16, and 22 (Fig. 6E). Although examples of both selective (17, 18, 20) and nonselective (22) in vivo phosphorylation of viral structural proteins have been reported, the significance of these differences is not known.

Experiments with $^{32}P_i$-labeled infected and uninfected cells, summarized in Fig. 6, showed the following. (i) Those host components phosphorylated in uninfected cells were no more extensively phosphorylated in infected cells. (ii) At least four of the six major ^{32}P-labeled bands present in infected cells appear to correspond with structural proteins of the virion. (iii) The distribution of ^{32}P radioactivity in infected cells differed from that in the virion. (iv) Two of the major phosphorylated components in infected cells partitioned specifically with the nuclear fraction (band 22, and a second band electrophoretically slower than 5).

Although these results do not bear directly on the genetic origin (host or viral) of the protein kinase activity observed here, or its functional significance, they do indicate that in infected cells it appears to be specific for components not significantly phosphorylated in uninfected cells (components presumably specified or induced by the virus). Whether the enzyme involved in phosphorylating these infected cell proteins is the same as that present (15, 16) in the virion is unknown.

Finally, it should be emphasized that although (i) most of the radioactivity present in the structural protein region of the gels was

exactly coincident with HSV protein bands (Fig. 6E and F), and (ii) 65% of the ^{32}P-counts per minute present in virion band 22 were recovered with the protein after trichloroacetic acid and acetone precipitations, these data do not constitute rigorous proof that phosphate is covalently attached to these proteins. Conclusive evidence will require identification of the phosphorylated amino acid residue(s).

Comparative properties of capsid and virion proteins. Comparison of the staining (Fig. 1) and radiolabeling (Fig. 4, 5, and 6) properties of HSV structural proteins revealed that corresponding B capsid and virion proteins (i.e., 5, 19, 21, 23, and 24) behaved similarly. This finding was not surprising and probably indicates that there are no extensive chemical alterations (i.e., deamination, phosphorylation) of these proteins during envelopment of the B capsid.

More interestingly though, these results also show that two electrophoretically separable (9) proteins, 22a and 22 of B capsids and virions, respectively, share in common a set of characteristics which differentiates them from all other HSV structural proteins thus far identified. Specifically, both proteins (i) retained CBB poorly but FG avidly (Fig. 1), (ii) fluoresced when stained with CBB (Fig. 3), (iii) were most efficiently labeled with ^{14}C-amino acids at early times during the infection cycle (Fig. 4), and (iv) exhibited the highest level of ^{32}P radioactivity among the protein components of their respective virus particles (Fig. 5C and 6E). Although a number of other proteins exhibited some of these same properties, only no. 22a and 22 shared all of them in common. These observations lead us to speculate that virion protein 22 may be a processed (perhaps cleaved or more extensively phosphorylated) form of B capsid protein 22a. If it is the case that 22 is derived from 22a, then the putative modification must presumably alter its ability to bind to the capsid since protein 22 is readily removed from the virion by detergents which do not remove 22a from B capsids (9).

This hypothesis is compatible with (i) the circumstantial evidence presented in this report, (ii) the absence in virions of a protein with the same electrophoretic mobility as that of 22a (9), and (iii) increasing evidence with T4 (14, 19), vaccinia (18), and adenovirus (1) that maturational processing (modification and/or removal) of proteins may be a general phenomenon, at least among DNA-containing viruses. Definitive evidence for the relatedness of B capsid protein 22a and virion protein 22, however, will require additional evidence based on analyses of tryptic digests.

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