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Virus-Induced Proteins in Pseudorabies-Infected Cells

I. Acid-Extractable Proteins of the Nucleus

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Three basic proteins have been identified in chromatin preparations from pseudorabies virus-infected cells. They appear to be virus specified and are similar in size and charge to host histones; one difference however is that they contain tryptophan. All are produced by 3 h postinfection, and two (IP II and III) seem to be arginine rich. Three similar proteins are also found in herpes simplex MP 17-infected cells, and two of these co-electrophorese with two of the pseudorabies proteins. Partially purified preparations of pseudorabies virus contain low amounts of all three proteins.

The proteins specified by pseudorabies virus in mammalian tissue culture cells have been studied by various research groups (13, 14). They include not only structural viral proteins, but others which, although antigenically distinguishable from host cell proteins, do not eventually become incorporated into the viral particles (3). It has been proposed that various functions carried out by the virus after infection require protein synthesis. These include the inhibition of cellular DNA synthesis and cell-specific mRNA synthesis in nuclei where viral DNA replication and transcription are proceeding at a rapid rate (1, 9). It is therefore of interest to study the virus-induced proteins, both structural and nonstructural, with a view to characterizing the proteins responsible for these effects. In this paper we describe studies on three specific viral proteins which are basic in nature and appear to be associated with partially purified preparations of host chromatin. Use is made of polyacrylamide gel electrophoresis conditions which allow clear separation of these species from host histones.

MATERIALS AND METHODS

Media and solutions. The media and solutions used were as follows: (i) EC10, a modified Eagle medium (2) containing 100 units of penicillin per ml, 100 μg of streptomycin per ml, and supplemented with 10% calf serum; (ii) EC10 ½ AA, as above, only containing 20% of the normal concentration of the amino acid used for labeling; (iii) ETC, as above, only containing 10% tryptose phosphate broth (2.9% in distilled water); (iv) TBS, Tris-buffered saline—0.01 M Tris (pH 7.2), 0.15 M NaCl. 0.01% β-mercaptoethanol (βME). (v) TME, Tris-mercaptoethanol—0.02 M Tris (pH 7.8), 1% β-mercaptoethanol; (vi) PBS, phosphate-buffered saline—0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4).

Cell culture. BHK-21 cells, an established line of hamster fibroblasts (7) were routinely grown in 80-ounce (2.2 liter) roller bottles in EC10. The bottles were gassed to give an atmosphere of 5% CO_2-95% air, and 0.02% phenol red was used to indicate acidity in the medium. Cells were not used beyond a passage number of 15 (12). Continuous checks were carried out for pleuropneumonialike organisms, and if detected the cultures were discarded. Cells were infected in the exponential phase of growth.

Virus. Pseudorabies virus (PrV) was originally derived from a stock preparation (5) and has subsequently been plaque purified three times. Virus stocks were prepared as follows: Monolayer cultures of BHK-21 maintained in ETC were infected at a multiplicity of 1 PFU per 300 cells, in 20 ml of medium. Virus was allowed to adsorb for 1 h and then the innoculum was removed and replaced with 100 ml of ETC. The cultures were rolled at 37 C for 36 h and then harvested aseptically by shaking the bottle to dislodge cells into the medium, transferring them to centrifuge bottles, and spinning at 600 × *g* for 10 min. This procedure pelleted the cells, and the supernatant fluid was then spun at 15,000 × *g* for 2 h to bring down the virus. This supernatant virus was resuspended in PBS (1 ml for every original 80-ounce bottle used), and gently sonically treated to make the suspension uniform. Samples were stored at −70 C and thawed only once before use. Cell-associated virus, prepared by resuspending and sonically treating the cell pellets, gives a low titer and was used only for production of virus stocks. Plaque assays were carried out on monolayer cultures of BHK-21 cells and normally gave titers of 10^9 to 10^{11} PFU/ml.

Preparation of labeled chromatin acid extracts. Cells labeled for the appropriate period of time were scraped from the bottles and centrifuged at 750 × *g* for 5 min. The pellet was washed once in TBS and recentrifuged and then suspended in TME (pH 7.8), and the cells were allowed to swell. They were then disrupted with 3 strokes in a tight-fitting Dounce homogenizer, and whole nuclei were spun down at 1,800 × *g*. The nuclei were washed at least three times in 1% Tween, until they showed no obvious cytoplasmic contamination under a low-powered microscope, and were then burst by taking up in TME and freeze-thawing. The chromatin pellet was spun down at 1,800 × *g*, washed three times in TME and once in 1 mM HCl. After centrifuging, the pellet of chromatin was extracted for 5 h at 0 C with 250 mM HCl-1% β-mercaptoethanol. DNA and residual proteins were spun down at 1,800 × *g*, and in some cases the HCl extract was dialyzed overnight against TME.

Preparation of labeled virus. Monolayer cultures of BHK-21 cells were infected at 5 PFU/cell and maintained for 16 h in EC10 ⅕ Arg plus 4 μCi of ^{3}H-arginine per ml. The cells were shaken off the glass into the medium, and this was spun at 15,000 × *g* to pellet total virus plus cells. The pellet was taken up in 1.5 ml of EC10 and was sonically treated until a smooth paste was produced. This was layered on top of a 12 to 52% (wt/wt) preformed sucrose gradient and spun for 40 min at 105,000 × *g*. The gradient was then fractionated into 1-ml portions, and 0.1 ml of each was counted for radioactivity. The fractions containing the viral peak were pooled, the sucrose was diluted to 5%, and the virus was pelleted at 100,000 × *g* for 2 h. The pellet was taken up in 1 ml of 250 mM HCl + 1% β-mercaptoethanol, homogenized briefly by hand, freeze-thawed twice, and extracted for 5 h at 0 C. This substance was spun again at 100,000 × *g*, leaving a clear supernatant fluid for gel electrophoresis.

Gel electrophoresis. Gels (7-cm size) contained 15% acrylamide (wt/vol), 0.1% *N,N*-methylene-bis acrylamide, 2% TEMED (tetramethylethylenediamine), and 0.7% ammonium persulfate. They were subjected to preelectrophoresis for at least 30 min in glycine-acetate buffer (pH 4.0) (6) at 3 mA per tube and then run for 4 h at 4 to 5 mA per tube. Samples for electrophoresis were applied to the gels in glycerol. SDS gels (7.5 %) were run according to the method of Weber and Osborn (16).

Gels, frozen in Drikold, were then sliced in 0.5-mm slices by using a Mickle gel slicer. The slices were placed in vials with 0.3 ml of 100-vol hydrogen peroxide and left at 50 C overnight. Scintillant (10 ml) was added, and the radioactivity was measured.

Radiochemicals. Methionine-methyl-^{3}H (specific activity 6.4 Ci/mmol); methionine-methyl-^{14}C specific activity 54 mCi/mmol); ^{3}H-arginine (specific activity 8.9 Ci/mmol); ^{3}H-tryptophan (specific activity 3.2 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Radioactivity measurements. The scintillant used was 0.5% 2,5-diphenyloxazole (Koch Light Lab. Ltd.) in toluene mixed in a ratio of 60:40 with methoxyethanol. A 10-ml amount of this mixture accommodates up to 0.3 ml of aqueous solution. All counting was done in a Phillips liquid scintillation spectrometer.

RESULTS

Basic proteins produced postinfection. Preliminary studies indicated that by 8 h after infection at least two viral-induced proteins, quite distinct from host cell histones, could be extracted with acid from chromatin preparations. In all these experiments, the chromatin was prepared from Tween 80-treated nuclei to minimize cytoplasmic contamination and in addition was well washed prior to acid extraction. It is concluded therefore that any material extracted has a fairly strong affinity for the chromatin.

BHK-21 cells growing exponentially were infected with PrV at a multiplicity of 20 PFU/cell or mock-infected in EC10. At 1 h postinfection, the medium was changed to 50 ml of EC10 ⅕ Met, and 2 h later 200 μCi of methionine-*methyl*-^{3}*H* was added. The cells were harvested at 8 h postinfection, and chromatin was prepared as in Materials and Methods. The basic proteins extractable from washed chromatin were examined on 15% polyacrylamide gels (pH 4.0) both for their staining pattern and their radioactive profile. On staining, both control and infected samples were identical and it was only on slicing and counting the gels that differences were seen [Fig. 1(a) and (b)]. The region to which histones migrate has been indicated on each graph, and the two viral-induced peaks have been marked I and II. Histones are still labeled in the infected sample as their synthesis is not inhibited substantially until 5 h after infection (14). It is clear that larger-molecular-weight material which fails to penetrate the gel is found in the infected samples. We do not discuss this further in the present communication.

Time course of production of viral proteins. HCl extracts were prepared at various times after infection to see if the induced proteins are "early" or "late" as defined by Hamada and Kaplan (3). For this, exponential BHK-21 cells were infected as before, and one control and one infected bottle were labeled for each of the time periods 1 to 3, 3 to 5, 5 to 7, 7 to 10, and 10 to 12 h with methionine-*methyl*-^{3}*H*. The results is shown in Fig. 2; only one control is shown as all were identical.

With methionine-*methyl*-^{3}*H* as label, induced protein (IP) I is the first to appear at 1 to 3 h postinfection, whereas IP II is not observable until 3 to 5 h, and in some experiments, in fact, could not be seen until after 5 h.

Note that a third peak can be seen in the

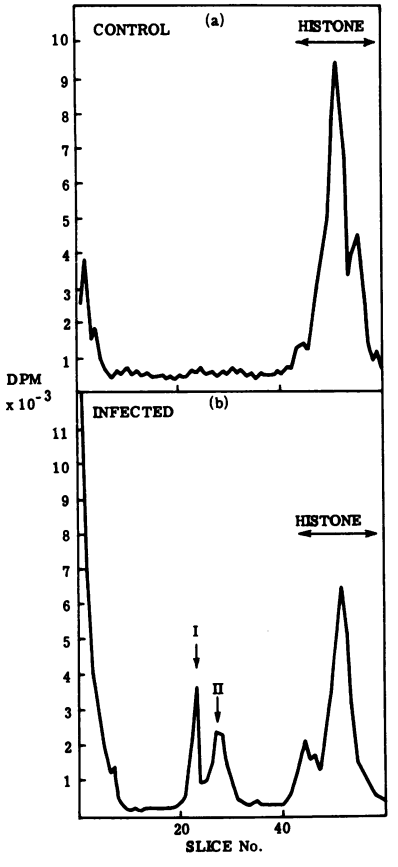

FIG. 1. *Electropherogram of radioactively labeled proteins extracted with 0.25 N HCl from partially purified preparations of chromatin from pseudorabies virus (PrV)-infected (b) and mock-infected (a) BHK-21 cells. The cells were labeled with methionine-methyl-^3H 3 to 8 h postinfection. The region of the gels where histones run is marked, and the viral-induced proteins are designated I and II.*

histone region. At first this was thought to represent incomplete inhibition of histone synthesis, but the fact that it was still being synthesized at 10 to 12 h suggested that a third viral protein was present. This has since been shown to be the case.

Prelabeling of cells. To check that the viral-induced proteins were not derivatives of host cell proteins altered in some way postinfection, a prelabeling experiment was carried out. BHK-21 cells were labeled for 5 h in the early exponential phase with methionine-*methyl*-^3H, the radioactive medium was then replaced with normal EC10, and the ^3H label was diluted out by growing for a further 18 h. The cells were then infected with PrV and labeled 3 to 8 h after infection with methionine-*methyl*-^{14}C. Chromatin was prepared and extracted as usual, and none of the host ^3H-labeled proteins are converted to viral-induced peaks (Fig. 3). This experiment also shows very clearly the presence of IP III in the histone region of the gels.

Arginine label. Arginine deprivation is known to be a particularly important factor in determining the infectivity of several viruses —herpes simplex (15), adenovirus (11), polyoma (A. L. Winters and R. A. Consigli, Bacteriol. Proc., 1969, p. 182), and pseudorabies (8). In the case of pseudorabies, the effect of arginine depletion has been proposed by Kaplan (8) to be due to inhibition of synthesis of a particularly arginine-rich nuclear protein which causes the condensation of viral structural proteins into viral particles. It was therefore considered important to study the incorporation of labeled arginine into IP I, II, and III. The results of a 1- to 5-h label are shown in Fig. 4. It is interesting to note that IP II is the major labeled

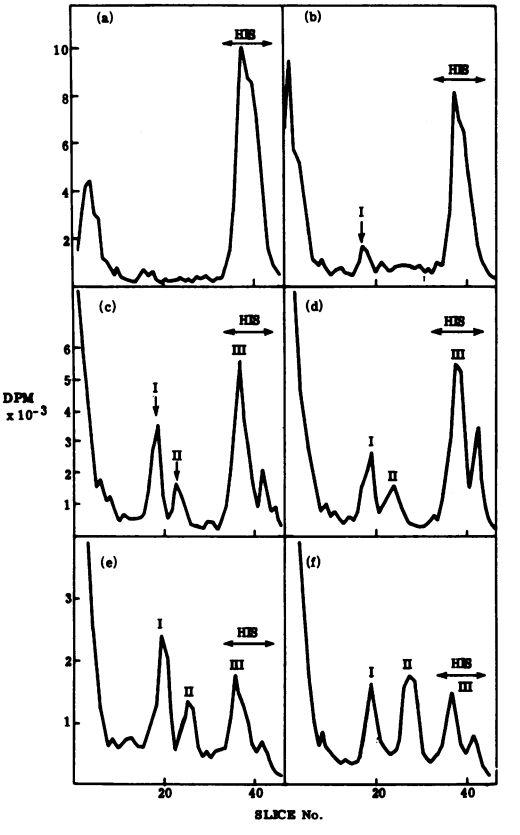

FIG. 2. *Time course of production of induced proteins (IP) postinfection. Control and infected 80-ounce bottles were labeled with methionine-methyl-^3H for the time periods indicated below. The acid-extractable material from chromatin was then run on 15% polyacrylamide gels to give the patterns shown above. Viral proteins are indicated I, II, and III. (a), Control 1 to 5 h; (b), 1 to 3 h; (c), 3 to 5 h; (d), 5 to 7 h; (e), 7 to 10 h; and (f), 10 to 12 h.*

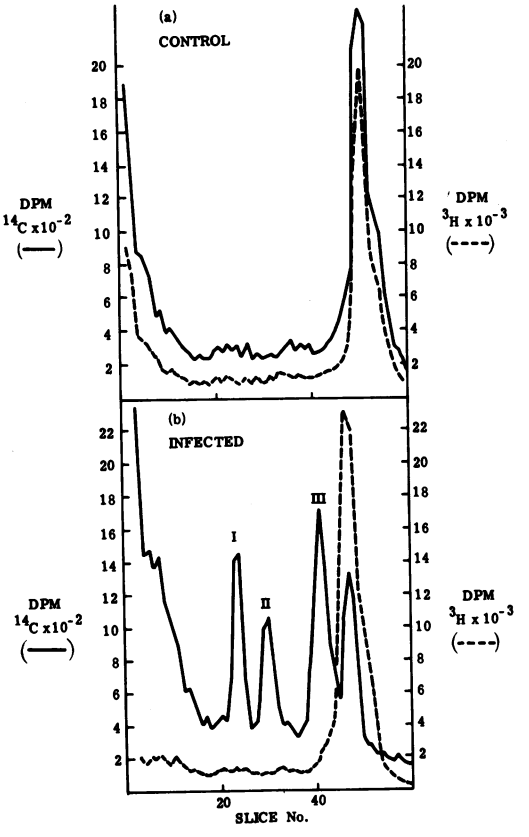

FIG. 3. *Pre-labeling of cells. BHK-21 cells were labeled for 5 h in the early exponential phase with methionine-methyl-^{3}H, and then this was diluted out overnight with EC10. After infection with PrV or mock-infection with EC10, the cells were further labeled with ^{14}C-methionine. Those proteins which are ^{14}C-labeled and contain no ^{3}H counts are therefore viral proteins produced postinfection. (a), Control; (b), infected.*

protein under these conditions, and it has subsequently been shown that it is highly labeled in a 1- to 3-h pulse of ^{3}H-arginine (unpublished results). An examination of the effect of arginine deprivation on the synthesis of all three proteins is currently under way.

Tryptophan label. The absence of tryptophan is one of the characteristics used to identify histones in normal cells. ^{3}H-labeled tryptophan was therefore used to see if the proteins were viral-induced histones by this definition. The result of a 1- to 4-h label is shown in Fig. 5; a 4- to 8-h label gave a very similar picture.

IP II and IP III contain quite considerable amounts of tryptophan, whereas IP I is almost unobservable and must contain very little, if any, tryptophan; it is therefore the most histone-like in this respect.

Purification of PrV. To determine whether the three induced proteins found in host chromatin were structural viral proteins, attempts were made to purify the virus from host cell material. Pseudorabies virus, labeled with methionine-*methyl-^{3}H* was centrifuged on sucrose gradients as described in Materials and Methods. This method has been used by Jacquemont (Abstr. Annu. Rep. Int. Agency for Res. Cancer, 1972, p. 58) to purify preparations of the herpesvirus from nasopharyngeal carcinoma.

Figure 6 shows the distribution of ^{3}H label in

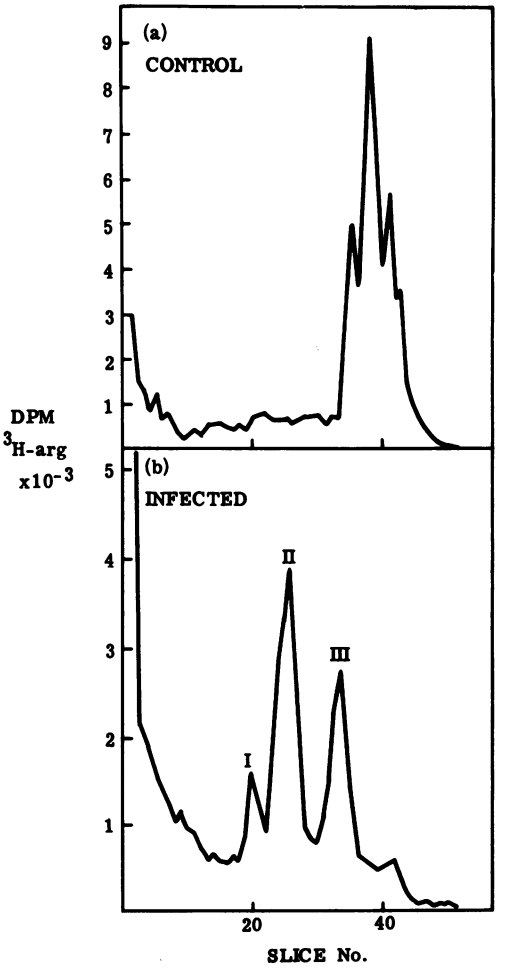

FIG. 4. *BHK-21 cells were labeled with ^{3}H-arginine 1 to 5 h postinfection or, in the case of the control, after mock-infection. The basic proteins were examined on 15% polyacrylamide gels, and the radioactive profile of these is shown. (a), Control; (b), infected.*

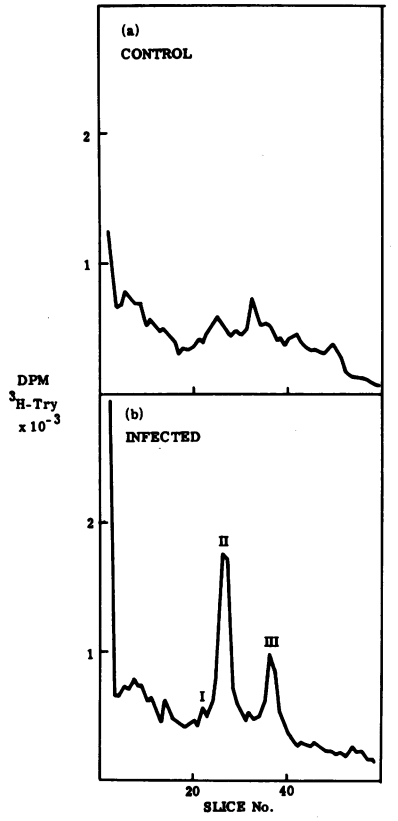

FIG. 5. *Tryptophan label. Exponential BHK-21 cells were labeled with ^{3}H-tryptophan 1 to 4 and 4 to 8 h after infection with PrV or mock-infection with EC10. Chromatin acid extracts were run on 15% gels, and the radioactive profile for the 1- to 4-h pulses is shown above. The 4- to 8-h label gave a very similar picture (not shown). (a), Control; (b), infected.*

a 12 to 52% sucrose gradient after sedimentation for 40 min at 105,000 × *g*. Subsequent experiments have shown the coincidence of thymidine activity with peak I in infected cells labeled with both ^{3}H-methionine and ^{14}C-thymidine.

The viral peak was spun down by high-speed centrifugation, and the pellet was suspended in 250 mM HCl-1% β-mercaptoethanol, briefly treated sonically and then freeze-thawed three times. Basic proteins were extracted for 5 h at 0 C and then the extracted material was examined on 15% polyacrylamide gels as before.

Figure 7(a) shows that all three proteins appear to be present in the viral preparation although in very low amounts. The similarity between the two sets of proteins was shown by a co-electrophoresis of ^{3}H-arginine-labeled proteins from purified virus and a ^{14}C-arginine-labeled chromatin extract from a 3- to 8-h label [Fig. 7(b)]. The three IP appear to co-electrophorese exactly with the viral proteins prepared from partially purified PrV.

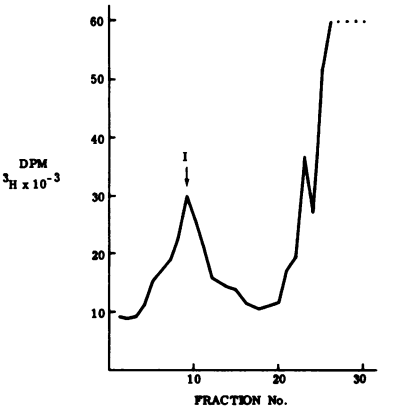

FIG. 6. *Sucrose gradient of PrV. Sonically treated preparations of cell-associated labeled PrV were layered on to a 12 to 52% sucrose gradient and spun for 40 min at 105,000 × g. The distribution of methionine-methyl-^{3}H activity in the gradient is shown above. I marks the viral peak.*

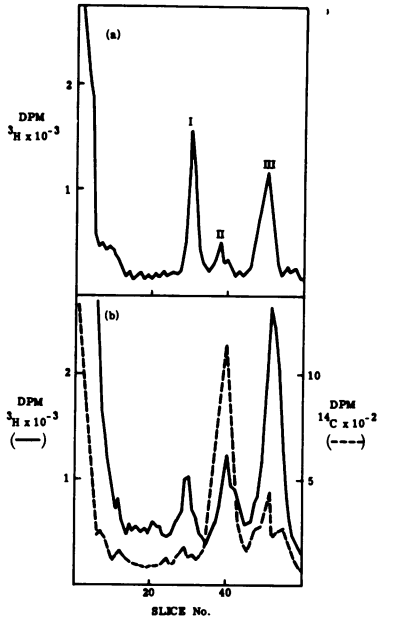

FIG. 7. *(a), 15% polyacrylamide gel of the acid-extractable material from partially purified PrV. (b), Co-electrophoresis of a ^{14}C-labeled chromatin extract postinfection with the ^{3}H-labeled extract of purified virus [Fig. 7(a)]. Note that peak I has diminished greatly in size relative to II and III, probably due to breakdown on storage of the material.*

The fact that the counts found in pseudorabies preparations are low may indicate that they represent contamination with host chromatin material and are not viral constituents at

all. However, we have evidence that the viral preparations contain a high proportion of empty particles, and, if the induced proteins are core proteins, as might be expected from their small basic nature, this may explain why only a small amount is found.

The high counts in chromatin make it unlikely that the induced proteins are present as part of contaminating virions, although electron micrographs of partially purified chromatin preparations do show the presence of some viral particles. We do not rule out the possibility that a virus-synthesising complex exists in the chromatin network within which the induced proteins may function in the production of progeny virus.

Comparison of PrV with herpes simplex MP17. To determine whether the presence of basic viral proteins in host chromatin was specific for PrV, or possibly a more general phenomenon in herpesviruses, an experiment was carried out infecting exponential BHK-21 cells with herpes simplex MP17 under identical conditions to those for pseudorabies. The pattern of basic proteins on polyacrylamine gel electrophoresis is shown in Fig. 8(a). Three induced peaks are again apparent, although IP I and II of herpes simplex appeared to have travelled further relative to IP III than in the case of PrV. To test this, a ^{3}H-arginine 4- to 8-h chromatin extract of herpes simplex virus-infected cells was subjected to co-electrophoresis with a similar ^{3}H-arginine-labeled extract of cells infected with PrV [see Fig. 8(a), (b), and (c)]. It appears that IP II of PrV co-electrophoreses with IP I of herpes simplex virus under these conditions. IP III of both viruses also seem to co-electrophorese, though this is more difficult to substantiate since host histones run in the same region. IP I of PrV and IP II of herpes simplex virus seem to be virus specific.

SDS gels of PrV-infected material. To compare these three virus-induced proteins with those identified by other workers (13, 14) the usual HCl-extracted material from partially purified host chromatin was run on 7.5% SDS-polyacrylamide gels. In addition, HCl extracts of whole nuclei (14) were made and analyzed on 15% (pH 4.0) and 7.5% SDS-polyacrylamide gels. The results are shown in Fig. 9 and 10.

The pattern obtained for extracts of whole nuclei on SDS gels agrees very well with that found by Stevens et al. (14), who showed the presence of five induced proteins in nuclear acid extracts. On 15% gels these same extracts give three or four virus-induced peaks (Fig. 10) although their separation is possibly better than in the SDS system. In comparison, the usual washed chromatin extracts give a much clearer picture on SDS gels than whole nuclear extracts, and it appears that substantial purification has been obtained.

DISCUSSION

Three virus-induced proteins have been shown to be associated with chromatin from 2 to 3 h postinfection. They are small basic proteins which, however, differ from host histones in the case of IP II and III in containing appreciable amounts of tryptophan. They are associated with host chromatin from the early hours of infection and appear to have a strong affinity for

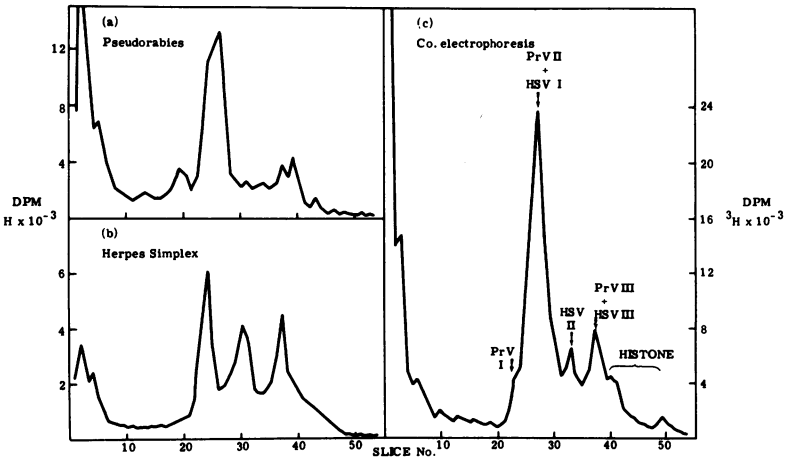

FIG. 8. *15% gels of ^{3}H-arginine-labeled extracts of chromatin after PrV infection and a similar preparation of identically labeled extract after Herpes simplex (MP17) infection. (a) and (b), The pattern for each extract alone; (c), a co-electrophoresis of (a) and (b).*

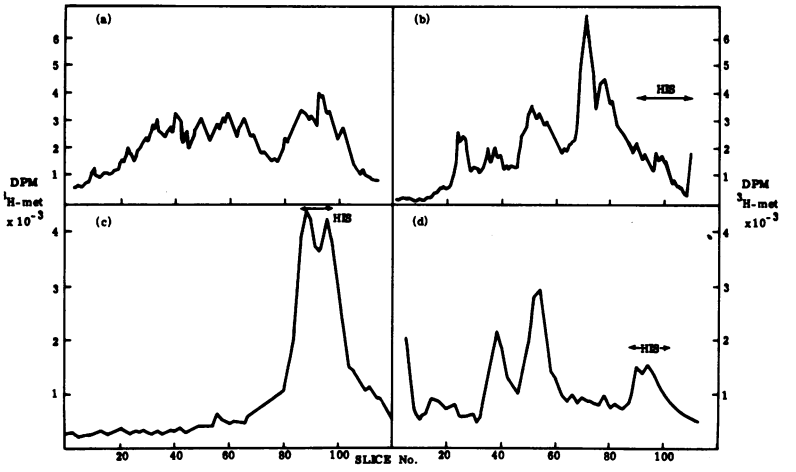

FIG. 9. *SDS gels (7.5%) of acid extracts labeled 3 to 8 h postinfection with methionine-methyl-^{3}H. (a), Whole nuclei, control; (b), whole nuclei, infected; (c), chromatin, control; (d), chromatin, infected.*

the nucleoprotein as they are not removed by fairly rigorous washing procedures. This includes dispersion of the chromatin in 0.35 M NaCl, which is claimed by Johns (4) to remove nuclear proteins which are nonspecifically adsorbed to the chromatin during extraction. None of the proteins are removed by this treatment (unpublished results). Likewise, sonic treatment of chromatin, a procedure which would be expected to dislodge viral particles from the nucleoprotein, does not cause a reduction in peak I and III, although peak II almost disappears.

The presence of the induced proteins in chromatin at a time when viral inhibition of host DNA and mRNA synthesis is occurring may indicate that they are involved in these regulatory processes. Alternatively, they may function within a virus-synthesising complex caught up in the chromatin network, e.g., as Kaplan's condensing protein (8), allowing transport of structural viral proteins from cytoplasm to nucleus, or as assembly proteins altering the conformation of viral DNA to a suitable form for encapsulation. Finally, the possibility that they are simply structural proteins which become associated with chromatin merely due to their basic nature cannot be ignored.

All three appear to be present in partially purified preparations of PrV, although the small amounts found may indicate that this is an artifact due to contamination with nuclear material. However, the similar finding of two minor components in highly purified preparations of herpes simplex virus (10), which are identified on 14% SDS-polyacrylamide gels, is of interest in this respect.

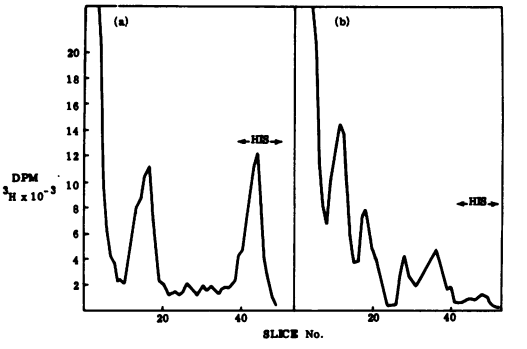

FIG. 10. *Polyacrylamide gels (15%) of the acid-extractable material from whole nuclei. (a), Control; (b), infected.*

Comparison of the results for pseudorabies with those obtained for the related herpes simplex virus indicate that the presence of virus-induced proteins in host chromatin may be a general phenomenon in herpesviruses. It is also of interest that two of the proteins from PrV and herpes simplex virus appear to co-electrophorese, suggesting further similarities between the two viruses.

Finally, a comparison of the material examined here with results obtained by other groups on whole cell or nuclear extracts, run on SDS gels [Fig. 9(a) (d)], indicates that substantial purification has been obtained. The extra proteins present in whole nuclei, as compared with chromatin, may well be inner-nuclear membrane proteins as they are not found in the nucleoplasm, and this possibility is currently under study.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Ben-Porat, T., and A. S. Kaplan. 1965. Mechanism of inhibition of cellular DNA synthesis by pseudorabies virus. Virology **25**:22-29.
2. Busby, D. W. G., W. House, and J. R. Macdonald. 1964. *In* Virological techniques. Churchill, London.
3. Hamada, C., and A. S. Kaplan. 1965. Kinetics of synthesis of various types of antigenic proteins in cells infected with pseudorabies virus. J. Bacteriol. **89**:1328-1334.
4. Johns, E. W., and S. Forrester. 1969. Studies on nuclear proteins. The binding of extra acidic proteins to deoxyribonucleoprotein during the preparation of nuclear proteins. Eur. J. Biochem. **8**:547-551.
5. Kaplan, A. S., and A. E. Vatter. 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7**:394-407.
6. McAllister, H. C., Y. C. Wan, and J. L. Irvin. 1963. Electrophoresis of histones and histone fractions on polyacrylamide gels. Anal. Biochem. **5**:321-329.
7. Macpherson, I. A., and M. G. P. Stoker. 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**:147-151.
8. Mark, G. E., and A. S. Kaplan. 1971. Synthesis of proteins in cells infected with herpesvirus. VII. Lack of migration of structural viral proteins to the nucleus in arginine-deprived cells. Virology **45**:53-60.
9. Rakusanova, T., T. Ben-Porat, M. Himeno, and A. S. Kaplan. 1971. Early functions of the genome of herpesvirus. 1. Characterisation of the RNA in cycloheximide-treated infected cells. Virology **46**:877-889.
10. Robinson, D. J., and D. N. Watson, 1971. Structural proteins of Herpes simplex virus. J. Gen. Virol. **10**:163-171.
11. Rouse, N. C., V. N. Bonifas, and R. W. Schlesinger. 1963. Dependence of adenovirus replication on arginine and inhibition of plaque-forming by PPLO. Virology **20**:357-365.
12. Sheddon, W. I. H., and P. Wildy. 1966. Regular supply of large numbers of standard continuous line cells. Nature (London) **212**:1068-1069.
13. Shimono, H., T. Ben-Porat, and A. S. Kaplan. 1969. Synthesis of proteins in cells infected with herpesvirus. I. Structural viral proteins. Virology **37**:49-55.
14. Stevens, J. G., G. J. Kado-Boll, and C. B. Haven. 1969. Changes in nuclear basic proteins during pseudorabies virus infection. J. Virol. **3**:490-497.
15. Tankersley, R. W. 1964. Amino acid requirements of Herpes simplex virus in human cells. J. Bacteriol. **87**:609-613.
16. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.