Identification of a Virus-specified Protein in the Nucleus of Vaccinia Virus-infected Cells

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SUMMARY

A new protein has been detected in the nuclei of vaccinia virus-infected cells. This protein has an apparent mol. wt. of 28000 (VP28) on SDS-polyacrylamide gels and has been detected in Triton X-100-treated nuclei of infected BSC-40, L-929 and CVC cells. Within the infected cells, VP28 was synthesized maximally at 1 to 2 h p.i. in the cytoplasm and accumulated in the nuclei at 4 to 5 h p.i. The appearance of VP28 was not affected by cytosine arabinoside (25 μg/ml), an inhibitor of virus DNA synthesis, or rifampicin (100 μg/ml), an inhibitor of vaccinia assembly, but was inhibited by irradiation of the infecting virions; thus classifying it as an early vaccinia virus gene product. Nuclear-cytoplasmic mixing experiments suggested that the nuclear location of VP28 was not an artefact of the cell fractionation techniques employed. VP28 did not appear to be phosphorylated.

INTRODUCTION

Although it is apparent that most vaccinia virus (VV) replicative steps occur independently within the cytoplasm of infected cells (Portner & Pridgen, 1975), the involvement of one or more host cell-coded functions is necessary for production of infectious progeny (Pennington & Follett, 1974; Hruby et al. 1979a,b). This requirement is consistently expressed as aberrant virus maturation in the absence of an active cell nucleus. Thus, it appears that host nuclear functions play a direct role in VV processes. Conversely, very little is known concerning potential intranuclear alterations due to VV infection. Although hybridization data have been obtained to demonstrate that some VV-specific DNA and RNA synthesis takes place in infected cell nuclei (LaColla & Weissbach, 1975; Bolden et al. 1979), the concomitant nuclear presence of VV-specified proteins to catalyse or modulate these reactions has not been detected.

There is precedence based on studies of another related poxvirus, namely fowlpox, to support the idea that virus-specified or induced nuclear proteins occur in poxvirus-infected cells. Goodpasture & Anderson (1962) originally observed that infection of avian cells with fowlpox elicited the formation of nuclear inclusion bodies. More recently, it has become apparent that a substantial fraction of fowlpox DNA and RNA synthesis occurs in the nucleus (Gafford & Randall, 1976). Analysis of fowlpox-infected cell nuclei at 36 to 48 h p.i. has demonstrated the presence of a single new polypeptide with an apparent mol. wt. of

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36000 (Hardy et al. 1978). While it is not yet clear whether this new protein is virus-specified, induced or causally related to any poxvirus replicative steps, it would seem to be important to study the structure and possible functions of this type of protein in regulating virus–host cell interactions during poxvirus infection.

To this end, the constitutive polypeptides of BSC-40 cell nuclei were carefully examined before and after vaccinia virus infection. Interestingly, a single new protein was detected in the infected nuclei. Data concerning the origin, nature, synthesis and identity of this protein are presented.

**METHODS**

**Cells and virus.** Vaccinia virus (WR strain) was obtained from the American Type Culture Collection and twice plaque purified. Stocks of VV were grown in spinner L-cells and purified by sucrose gradient centrifugation (Hruby et al. 1979a). The VV preparation used in these experiments had a sp. act. of 1:20 (1 x 10^10 p.f.u./ml; 2 x 10^11 particles/ml).

The source and maintenance of BSC-40, L-929 and CVC cells was as previously detailed (Hruby et al. 1979a). For radioactive labelling, VV infections were carried out as described on cells maintained in Eagle's minimum essential medium (minus methionine) supplemented with 5% heat-inactivated foetal calf serum (dialysed) for the duration of the experiment (Hruby et al. 1979a).

**Cell fractionation.** Uninfected and VV-infected cells were fractionated according to the methods of LaColla & Weissbach (1975) with certain modifications. At indicated times after infection, triplicate monolayers of BSC-40 cells (1 x 10^6 to 3 x 10^6 cells/60 mm dish) were loosened with a rubber policeman, rinsed into conical centrifuge tubes and pelleted for 3 min at 2000 rev/min at 4 °C in an IEC-6000 centrifuge. Cells were then washed twice with 0.32 M-sucrose, 2 mM-MgCl₂ and 1 mM-potassium phosphate, pH 7.3 (buffer 1). Cellular pellets were resuspended in 2 ml buffer 2 (10 mM-NaCl, 5 mM-EDTA, 1 mM-potassium phosphate, pH 7.3) and put on ice for 10 min to swell. Cells were then disrupted with 10 strokes of a Dounce homogenizer. The crude nuclei were pelleted from the homogenate by centrifugation as above. The supernatant fraction was removed and saved for analysis as the 'cytoplasmic fraction'. The nuclear pellet was resuspended in 2 ml 0.32 M-sucrose, 1 mM-potassium phosphate, pH 7.3, and 0.3% Triton X-100 (buffer 3), and re-homogenized. The nuclei were again pelleted and the supernatant fraction discarded. The nuclei were resuspended in 2 ml buffer 3, dispersed by mild homogenization and gently layered over 25 ml 1 M-sucrose in buffer 3 and centrifuged at 3000 rev/min for 7 min at 4 °C in an SS-34 rotor in the RC2-B centrifuge. The nuclei were resuspended in 0.5 ml buffer 2, transferred to a conical microfuge tube and re-pelleted at 8000 rev/min for 10 min at 4 °C in an SS-34 rotor. This final nuclear pellet was resuspended in 50 μl water and frozen at -20 °C until subsequent gel analysis.

**Protein analysis.** Appropriate volumes (10 to 100 μl) of proteins were precipitated with 4 vol. of cold acetone for 10 min, the precipitates collected by centrifugation, dried, dissolved in 25 μl sample buffer and boiled for 5 min. Samples were then electrophoresed on 1 mm 12.5% SDS–polyacrylamide (PAGE) slab gels for 4 h at 100 V (Studier, 1973). Gels were stained for 1 h with 0.25% Coomassie brilliant blue, then destained with methanol: acetic acid (5:7.5%) in water and dried using a Bio-Rad gel dryer. At this point the positions of non-radioactive marker proteins were noted. Autoradiography of dried films was carried out on Kodak RP Royal X/Omat medical X-ray film and the film later developed in a Kodak Rapid-Process automatic developing unit. For quantification, films were traced using a Joyce-Loebl microdensitometer.

**Materials.** ³⁵S-methionine (647 Ci/mmol) was purchased from New England Nuclear,
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Boston, Mass., U.S.A. Cytosine arabinoside and rifampicin were from Sigma, St Louis, Mo., U.S.A. Marker proteins were obtained from Gallard-Schlesinger Chemical Manufacturing Corp., New York, N.Y., U.S.A. (14,300 to 71,500) and Pharmacia, Piscataway, N.J., U.S.A. (bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome c). All gel electrophoresis reagents came from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

RESULTS

Analysis of nuclear proteins

To compare the constitutive polypeptides of uninfected vs. VV-infected BSC-40 cells, infections were carried out in the presence of [35S]-methionine, and the nuclear and cytoplasmic fractions were isolated and purified at 4 h p.i. Fig. 1 shows the SDS-PAGE analysis of the radioactively labelled proteins: Fig. 1(a) shows the typical pattern of vaccinia virion proteins, including the major capsid proteins 4a and 4b (mol. wt. of 63,000 and 58,500, respectively). In Fig. 1(b), it is apparent that VV infection inhibited the cytoplasmic synthesis of cellular proteins and directed the expression of a variety of early virus genes. In contrast, the protein content of nuclei isolated from VV-infected cells was very similar to uninfected cell nuclei (Fig. 1 c) except that a single new major protein species appeared, with an apparent mol. wt. of 28,000 (VP28). Interestingly, proteins migrating at a similar position to VP28 were also present in the infected cell cytoplasm and vaccinia virions (Fig. 1 a, b).

The nuclear location of VP28 in infected cells was probably not an artefact of isolation for several reasons. First, the nuclear isolation procedure used here incorporated several detergent washing steps, a procedure known to minimize cytoplasmic contamination (Birnie, 1978). Furthermore, phase-contrast light microscopy of purified nuclei showed them to be clean and free of visible cytoplasmic remnants. Secondly, if damage to nuclear membrane integrity during isolation was responsible for the nuclear appearance of VP28, then the presence of other virus proteins, reflective of their cytoplasmic concentrations, might also be expected. This was not observed. In fact, there was very little leakage of major cytoplasmic proteins, such as actin, into the infected nuclei. Finally, in vitro mixing of radioactively labelled VV-infected cytoplasm with unlabelled nuclei, followed by the usual nuclei isolation protocol, did not result in the selective nuclear localization of VP28 as seen in vivo (data not shown).

It was not clear from the experiments reported in Fig. 1 whether VP28 represented a virus-specified protein or induction of previously quiescent host genetic information (Hightower & Smith, 1978; Kelley & Schlesinger, 1978). Two independent lines of research suggested that VP28 was a vaccinia protein. First, isolation of nuclei from VV-infected L-929, CVC and BSC-40 cells showed that, although there were qualitative and quantitative differences in the profile of host proteins, VP28 was present and apparently identical in all three cases. Fig. 2 demonstrates the second line of evidence. Five types of VV infections were carried out in BSC-40 cells: no virus; untreated virus; u.v.-irradiated virus incapable of expressing any viral genetic information; in the presence of 25 μg/ml of cytosine arabinoside (araC), an inhibitor of virus DNA synthesis which allows continued expression of early but not late VV proteins; and in the presence of 100 μg/ml rifampicin, an inhibitor of virus assembly which enhances the expression of both early and late VV genes. It is obvious from Fig. 2 that VP28 was present in nuclei from infected, infected araC-treated and infected rifampicin-treated cells. This indicates that VP28 was a virus-specified early protein, that is its synthesis does not require virus DNA synthesis, but does require expression of early VV genetic information.
Fig. 1. SDS–PAGE analysis of vaccinia virus-specified proteins from (a) virions, (b) cytoplasm and (c) nuclei in BSC-40 cells. The cells were infected with vaccinia virus at a m.o.i. of 50 with 5 $\mu$Ci/ml $^{35}$S-methionine present from 1 to 4 h p.i. At 4 h, nuclear and cytoplasmic fractions were prepared from infected (---) and control uninfected (——) cells as described. Equivalent amounts of radioactive material from each sample were subjected to SDS–PAGE analysis. The gel was dried, applied to X-ray film and the subsequent autoradiograph scanned using a Joyce-Loebl microdensitometer. Apparent mol. wt. were calibrated by co-electrophoresis of marker proteins of known mol. wt. The arrow indicates a mol. wt. of 28,000.
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Fig. 2. Effect of various inhibitors of vaccinia virus replication on VP28 synthesis. Vaccinia virus infections (m.o.i. of 50) of BSC-40 cells were carried out under the following conditions: no virus (control), untreated vaccinia virus (infected), u.v.-irradiated vaccinia virus (u.v.), 25 μg/ml of cytosine arabinoside (araC) and 100 μg/ml of rifampicin (rif). Proteins were labelled from 1 to 4 h with 5 μCi/ml \textsuperscript{35}S-methionine. At 4 h, nuclear and cytoplasmic fractions were prepared and the radioactive polypeptides then analysed by SDS-PAGE. Lane 1, virion proteins; lane 2, vaccinia early proteins; lane 3, control cytoplasm; lane 4, infected cytoplasm; lane 5, u.v. cytoplasm; lane 6, araC cytoplasm; lane 7, rif cytoplasm; lane 8, control nuclei; lane 9, infected nuclei; lane 10, u.v. nuclei; lane 11, araC nuclei; lane 12, rif nuclei; lane 13, vaccinia late proteins; lane 14, mol. wt. markers located by Coomassie blue staining. Note: u.v.-irradiation was at a dose sufficient to cause a $10^{-5}$ decrease in infectious titre.

**Synthesis of VP28**

Although VP28 could be grossly classified as an early protein, there exist many different kinetic modes of early vaccinia gene expression (Pennington, 1974) so that this preliminary designation was unsatisfactory. Therefore, more precise information concerning the timing of VP28's cytoplasmic synthesis and rate of nuclear accumulation was needed (Fig. 3). Within the cytoplasm of VV-infected cells VP28 was translated at a maximum rate at 1 to 2 h p.i. and then shut off abruptly. VP28 accumulated in the nuclear compartment somewhat later, at 4 to 5 h p.i. and then decreased in amount slowly.

**Identification of VP28**

Nuclear VP28 co-migrated with proteins found in the VV-infected cell cytoplasm and vaccinia virion, but it was not clear if these three proteins were one and the same. To answer this question, use was made of the partial proteolysis–gel electrophoresis peptide
Fig. 3. Kinetics of cytoplasmic synthesis and nuclear accumulation of VP28. Cytoplasmic synthesis (●) of VP28 was measured by pulsing vaccinia virus-infected cells with $^{35}$S-methionine for 15 min at the indicated times and then isolating the cytoplasmic fraction. Nuclear accumulation (○) of VP28 was measured by labelling infected cells from 1.5 to 2 h p.i. and then purifying nuclei at the indicated times. In both cases, equal amounts of radioactive polypeptides were analysed by SDS-PAGE and autoradiography. The autoradiographs were traced using a Joyce-Loebl microdensitometer and the area under the VP28 peak calculated with an electronic graphics calculator.

The pattern of partial products derived by S. aureus V8 protease digestion of the nuclear, cytoplasmic and virion proteins was similar at high V8 protease concentration (0.5 µg), suggesting that they may all represent the same basic polypeptide. Although direct comparisons between the patterns of early VV proteins and virion proteins described here and previous work (Moss, 1974; Pennington, 1974; Opperman & Koch, 1976) are difficult due to different gel systems, different mol. wt. standards and a confusing nomenclature, it is likely that the protein designated here as VP28 is the same as vaccinia protein previously designated as polypeptide 8 (Moss, 1974). This is based on the size of VP28 and its migration relative to other vaccinia polypeptides.

When the proteins made in VV-infected cells in the presence of $^{32}$PO$_4$ were examined on gels, there was no radioactive label associated with VP28. However, two other VV proteins known to be phosphorylated, namely polypeptide 11 (Rosemond & Moss, 1973; mol. wt. 11000) and FP 11 (Nowakowski et al. 1978b; mol. wt. 34000) were apparent. The status of other potential protein modifications such as acetylation or glycosylation has not yet been determined.

DISCUSSION

The data reported here describe the detection of a novel protein, designated VP28, found within the nuclear compartment of vaccinia virus-infected cells (Fig. 1). VP28 was shown to be an early vaccinia virus gene product whose expression was under strict temporal regulation, being synthesized at 1 to 2 h p.i. and accumulating in the nucleus at 4 to 5 h p.i. (Fig. 2, 3). These kinetics are unusual in that although VP28 is synthesized very early in infection, it is also packaged as a major virion constituent, tentatively identified as polypeptide 8, late in infection.

Whether the nuclear location of VP28 is due to a special case of adventitious binding, which is doubtful for reasons previously discussed, or is indicative of its in vivo function remains to be established. It is interesting to note two previous reports of 28000 mol. wt. vaccinia virus proteins isolated from infected cell cytoplasm (Soloski et al. 1978) or vaccinia replicative ‘factories’ in the cell (Nowakowski et al. 1978a). Both of these proteins have a strong affinity for DNA. If VP28 were synonymous with either or both, it is possible then
that its function within the cytoplasm, nucleus and virion might involve interaction with host or virus DNA sequences.

At this point it is premature to discuss the possible function(s) of VP28. It is, however, worthwhile to note the previous reports of nuclear involvement in vaccinia virus replication (Hruby et al. 1979a, b), and speculate how VP28 might be involved with induction of the specific host activities required for a productive vaccinia infection. In any case, the discovery of a VV-specified nuclear protein is obviously exciting and warrants further studies into its nature and function as it is a good candidate for involvement in a number of previously unexplained virus–host cell phenomena.

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