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Identification of a Subset of Herpesvirus Saimiri Polypeptides Synthesized in the Absence of Virus DNA Replication

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The effects of phosphonoacetic acid on the synthesis of herpesvirus saimiri-specific polypeptides in productively infected cells were examined. At concentrations that inhibited virus DNA synthesis (≥150 μg/ml), phosphonoacetic acid prevented the synthesis of the majority of virus-specific polypeptides while allowing the synthesis of a subset of virus proteins (i.e., 110,000 [110K], 76K, 72K, 51K, 48K, 29K, 24K, and 20K or 21K) and the protracted synthesis of host-specified polypeptides. Other inhibitors of DNA synthesis (e.g., cytosine arabinoside) showed the same selective inhibition of late virus protein synthesis and identified the same resistant subset of early virus-specific polypeptides. This DNA synthesis-independent subset included the 51K phosphoprotein, which, together with the 110K, 48K, and 31K polypeptides, accumulated in the nuclear fraction of infected cells.

Events coupled to the synthesis of virus DNA represent important regulatory steps in the control of herpesvirus gene expression (2, 4, 10, 13). Studies of several herpesviruses, including herpes simplex virus, cytomegalovirus, and Epstein-Barr virus, showed early groups of DNA synthesis-independent proteins and late groups whose normal synthesis is dependent on virus DNA replication (4, 6, 10, 12). These studies were facilitated by the use of phosphonoacetic acid (PAA), a drug that has been shown to selectively inhibit herpesvirus DNA replication and whose site of action, at least for herpes simplex virus, was shown by biochemical and genetic analysis to reside in the virus DNA polymerase (7, 8, 11).

We recently identified more than 30 virus-induced polypeptides in cells productively infected with herpesvirus saimiri (HVS), the majority of which satisfied multiple criteria for virus specificity (11a). We also showed that HVS replication is highly sensitive to PAA, confirming the results of Barahona et al. (1), and extended these observations with the demonstration that HVS DNA synthesis was selectively inhibited by the drug. Thus, in the presence of >100 μg of PAA per ml, plaque formation was reduced to 0.001% of the control value and the onset of virus DNA synthesis during high-multiplicity infections was inhibited. In addition we provided evidence for the synthesis of a novel PAA-sensitive DNA polymerase during HVS infection and isolated a PAA-resistant (P^r) mutant that plaques efficiently in the presence of about 100 μg of PAA per ml and shows increased drug resistance of the virus-induced DNA polymerase in vitro (P. O'Hare, Ph.D. thesis, Council for National Academic Awards, London, England, 1982; P. O'Hare et al., submitted for publication). In this paper we used PAA to examine the relationship between virus DNA replication and protein synthesis during a productive infection with HVS. The results showed that synthesis of the majority of HVS-specified polypeptides was sensitive, whereas that of a subset of virus-specific proteins was resistant, to concentrations of PAA (≥150 μg/ml) that inhibit the onset of virus DNA replication.

High-multiplicity infections (5 PFU/cell) of permissive cultures of owl monkey kidney (OMK), green monkey kidney (Vero), and marmoset kidney (MAK) cells were performed with the attenuated strain of HVS (11Att) (11a). Where appropriate, different concentrations of PAA were added immediately after removal of the virus inoculum (1.5 h after infection). Conditions for radiolabeling of infected and mock-infected cells with [^{35}S]methionine and methods for cell fractionation and one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis are given elsewhere (11a) or in the figure legends.

Figure 1 shows the effects of different concentrations of PAA on protein synthesis in Vero cells infected with HVS. Clearly PAA had a

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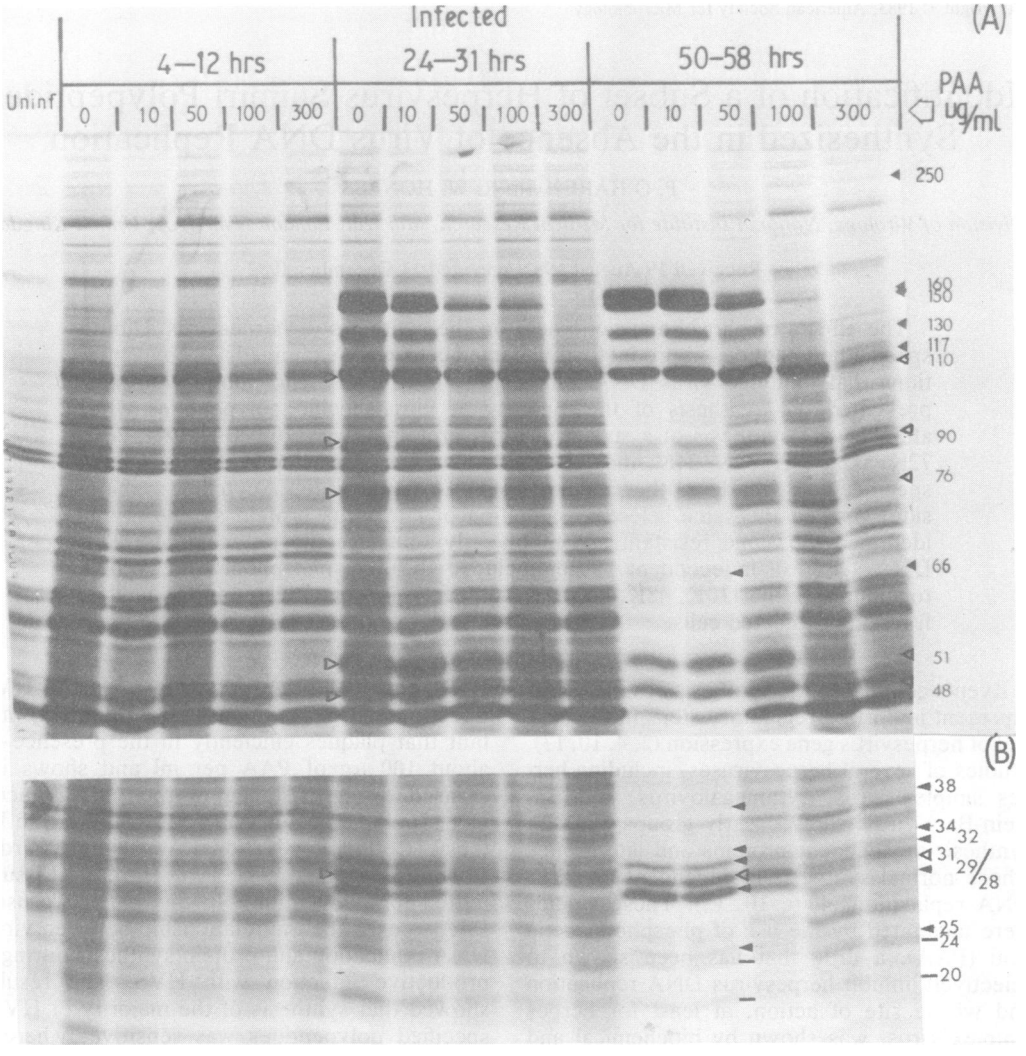

FIG. 1. Autoradiogram of 10 (A) and 15% (B) polyacrylamide gel slabs containing electrophoretically separated proteins from lysates of Vero cells labeled with 5 μCi of [^{35}S]methionine per ml from 4 to 12, 24 to 31, and 50 to 58 h after infection with 5 PFU of HVS (11Att) per cell. Infected cultures were maintained in the presence of 0, 10, 50, 100, or 300 μg of PAA per ml. Filled and open arrows, Virus-induced polypeptides whose synthesis was relatively sensitive or resistant, respectively, to PAA; short bars, proteins whose sensitivity could not be unambiguously determined due to comigrating host components during one-dimensional electrophoresis. Selected virus-specific polypeptides are indicated by their apparent molecular weights (in thousands) as previously described (11a).

profound and selective effect on the pattern of infected cell protein synthesis, with virus-induced proteins differing markedly in their sensitivity to the drug. During the 4- to 12-h labeling interval (when virus DNA synthesis in control cultures is less than 10% of its maximal rate [3; submitted for publication]), the only virus-induced polypeptide detected at this time, 110,000 (110K), was resistant to concentrations of PAA up to 300 μg/ml. The synthesis of most of the major virus-induced polypeptides was detected between 24 and 31 h, at which time rates of virus DNA synthesis were maximal in control cultures; of these polypeptides, at least six (110K, 90K, 76K, 51K, 48K, and 31K) were resistant to PAA concentrations up to 100 μg/ml, while the majority of virus proteins were clearly sensitive. For example, the synthesis of the 160K and 150K polypeptides was considerably reduced in the presence of 50 μg of PAA per ml (compare the sensitive synthesis of the 160K and 150K proteins with the resistant synthesis of the 110K

protein in Fig. 1). Similarly, the synthesis of the 32K and 28K proteins was reduced in the presence of 50 μg of PAA per ml and undetectable at 300 μg/ml, at which concentration the synthesis of the 31K polypeptide was readily detected during the 24- to 31-h labeling interval. The synthesis of the 110K, 76K, 51K, 48K, and 31K virus-induced proteins was also differentially resistant to PAA in infected cultures of OMK and MAK cells (Fig. 2). Moreover, similar experiments with other inhibitors of DNA synthesis (e.g., cytosine arabinoside, 50 μg/ml) identified the same subset of virus-induced proteins whose synthesis was relatively insensitive to inhibition of virus DNA synthesis (data not shown). Two minor proteins of 73K and 62K, which are induced early after infection of OMK cells, and a 90K polypeptide detected early after infection of Vero cells were also synthesized in the presence of high concentrations of PAA (150 μg/ml). However, the 73K and 62K polypeptides were not detected in infections of Vero or MAK cells, and the 90K polypeptide was not detected in OMK or MAK cells; these polypeptides are presently regarded as virus-induced host cell proteins (11a).

Two additional features of protein synthesis in cells infected with sensitive strains of the virus should be noted. First, although PAA had no direct effect on protein synthesis in uninfected cultures, doses that selectively inhibited virus-specific protein synthesis also delayed the virus-induced inhibition of host cell-specific protein synthesis normally observed at late times in infected cultures (Fig. 1). Second, although PAA clearly differentiated between relatively resistant and sensitive classes of polypeptides, the synthesis of members of the resistant class was reduced by high concentrations of the inhibitor. For example, it is apparent that at 300 μg of PAA per ml, the synthesis of the 110K protein was reduced in comparison with synthesis in control samples (Fig. 1). The reduction in synthesis of polypeptides of the resistant subset by high inhibitor concentrations was not a specific effect of PAA; similar results were obtained with cytosine arabinoside (data not shown).

Confirmation of the identity of the major PAA-resistant virus-specific polypeptides and the recognition of some additional minor species were achieved by analyzing the proteins separated by equilibrium and nonequilibrium (9) two-dimensional gel electrophoresis (Fig. 3). Twenty-three virus-specific polypeptides were detected in the infected control cultures, and the synthesis of eight of these (Fig. 3, large arrows) was relatively resistant to PAA. The increased resolution afforded by equilibrium (not shown) and nonequilibrium two-dimensional separations added some virus-specific species to our

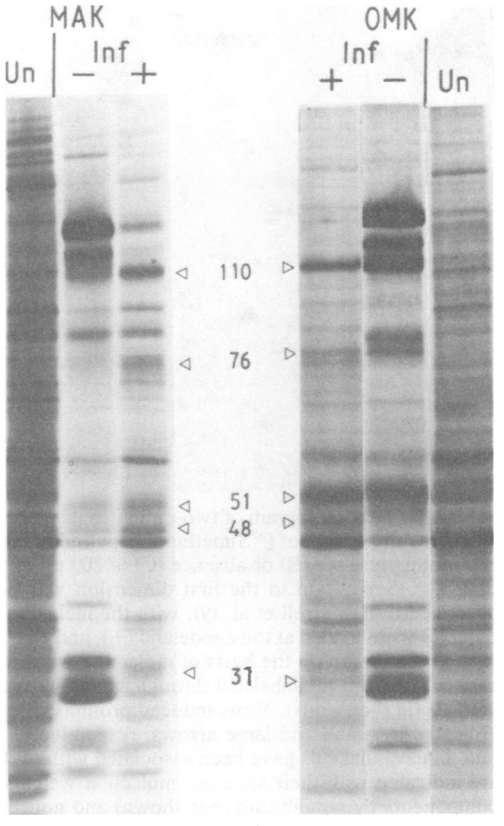

FIG. 2. Autoradiogram of 12% polyacrylamide gels containing electrophoretically separated proteins from lysates of MAK and OMK cells labeled with 5 μCi of [^{35}S]methionine per ml from 20 to 43 (MAK) and 20 to 60 h (OMK) after infection with 5 PFU of HVS per cell. Infected cell cultures (Inf) were maintained without (−) or with (+) 200 μg of PAA per ml. Uninfected cell cultures (Un) were labeled in parallel with the corresponding infected cultures without drug. Virus-induced polypeptides whose synthesis was resistant to PAA are indicated with their apparent molecular weights (in thousands).

previous (11a) catalog (e.g., 72K, 47K, two additional species of 28K to 31K, and one additional species of 20K to 25K), and it was clear that the 72K polypeptide, one polypeptide of 20K to 21K (close to host polypeptide M), and one of the 24K to 25K polypeptides were members of the PAA-resistant class. Although large fractions of the 110K and 76K species remained at the origin of the nonequilibrium electrofocusing gel, they were clearly PAA resistant, as were the 51K polypeptide and two proteins in the 28K to 31K region. One of these latter proteins comigrated with the 31K polypeptide observed on one-dimensional gels (large arrow on cathode side of host protein j, Fig. 3B, and next to the 32K protein, Fig. 3C). The other species comi-

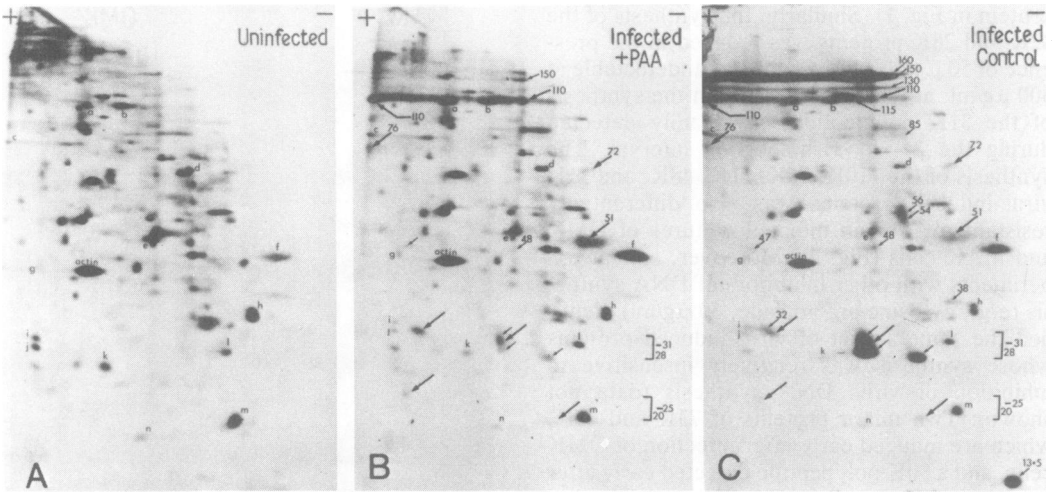

FIG. 3. Autoradiogram of two-dimensional electrophoretic separations of proteins from lysates of MAK cells labeled with 100 μCi of [^{35}S]methionine per ml of medium from 8 to 26 h after infection with 20 PFU of HVS per cell in the presence (B) or absence (C) of 200 μg of PAA per ml and from 8 to 26 h after mock infection without drug (A). Separation in the first dimension was performed by nonequilibrium electrofocusing essentially as described by O'Farrell et al. (9), with the inclusion of 0.5% sodium dodecyl sulfate in the sample preparation. Samples were loaded at the anode end (+), and electrophoresis was performed at 400 V for 3 h. Separation in the second dimension on the basis of molecular weight was performed in 12% polyacrylamide gels. Corresponding host cell proteins are labeled a through m, and virus-induced proteins are indicated by arrows and their molecular weights (in thousands). Virus-induced proteins whose synthesis was relatively sensitive or resistant to PAA are indicated by small and large arrows, respectively. In cases where the virus-specific polypeptides detected on one-dimensional gels have been associated with proteins separated by nonequilibrium electrofocusing, the spots are indicated with their apparent molecular weights. In the ranges 20 to 25K and 28 to 31K we detected more components by equilibrium (not shown) and nonequilibrium than by one-dimensional gel electrophoresis. The relationships between some of these additional components and those previously characterized (11a) are not yet certain, and they were left without specific labels.

grated with the 29K polypeptide characterized on one-dimensional gels (11a) and was not separated from a triplet of virus-induced polypeptides (Fig. 3B and C above host protein n on the cathode side of host protein k) on the nonequilibrium gels illustrated, but was resolved as a completely PAA-resistant subcomponent on the basic side of this group by equilibrium electrofocusing (not shown).

We previously identified two major phosphoproteins specific to HVS-infected cells with molecular weights of 59K and 51K (11a). The 51K phosphoprotein comigrated with the 51K polypeptide, whose synthesis we have shown here to be resistant to PAA. An analysis of phosphorylation in infected cells in the presence and absence of the drug (Fig. 4) showed that the phosphorylation of 51K was relatively resistant, whereas that of 59K was undetectable in the presence of 200 μg of PAA per ml. Furthermore, the 51K phosphoprotein was shown in cell fractionation experiments to partition almost exclusively with the nuclear fraction of cells infected in the presence of the drug (Fig. 5). The 110K and a significant fraction of the 31K polypeptides were also present in the nuclear fraction,

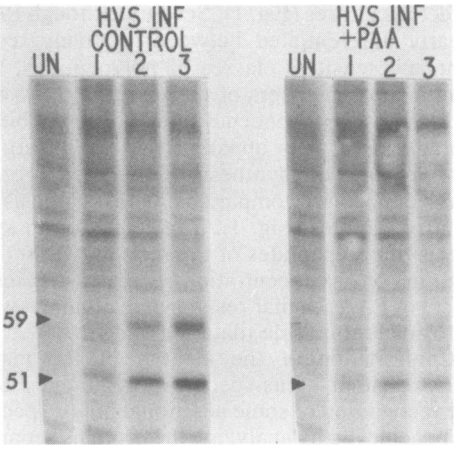

FIG. 4. Autoradiogram of an 8% polyacrylamide gel slab containing electrophoretically separated proteins from lysates of OMK cells labeled with 20 μCi of $^{32}P_i$ from 4 to 13, 13 to 22.5, and 23 to 33 h after infection (lanes 1, 2, and 3, respectively) with 10 PFU of HVS per cell and from 4 to 13 h after mock infection (lane UN). Infected and mock-infected cultures were maintained in the presence of 200 μg of PAA per ml (+PAA) or in the absence of the drug (control).

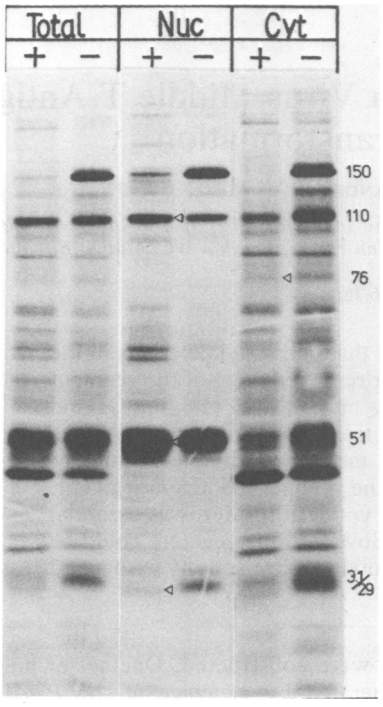

FIG. 5. Autoradiogram of a 12% polyacrylamide gel slab containing electrophoretically separated proteins from unfractionated infected cell lysates (Total) and from nuclear (Nuc) and cytoplasmic (Cyt) fractions of Vero cells labeled with 5 μCi of [^{35}S]methionine per ml from 6 to 26 h after infection with 5 PFU of HVS per cell. Infected cultures were maintained with (+) or without (−) 250 μg of PAA per ml. At the end of the labeling interval, cultures were removed, washed with cold phosphate-buffered saline, and suspended in 1 mM phosphate buffer (pH 7.4) containing 0.5% Nonidet P-40. A sample of the washed cell suspension was withdrawn for analysis (Total), and the remainder was disrupted by Dounce homogenization and separated into nuclear and cytoplasmic fractions by sedimentation (3,000 rpm for 10 min).

whereas the 76K protein was found mainly in the cytoplasm. Although not resolved in Fig. 5, the 48K polypeptide also partitioned with the nuclear fraction of infected cells in the presence and absence of PAA.

Thus, we have identified a subset of HVS proteins whose synthesis is resistant to concentrations of PAA that dramatically inhibit the synthesis of the majority of HVS proteins. We have also shown that of this resistant subset, the 51K phosphoprotein and the 110K, 48K, and 31K polypeptides are located in the nucleus, whereas the 76K protein is found mainly in the cytoplasm of cells infected in the presence of the drug. Moreover, previous results showed that in infected cells the 51K and 31K proteins are transported rapidly to the nucleus, being found quantitatively in this fraction within 60 min of synthesis (11a). The subset of PAA-resistant proteins corresponds directly with the subset classed as early proteins on the basis of their kinetics of synthesis during a productive infection with HVS (11a; unpublished data). Taken together, these results indicate that the 110K, 76K, 72K, 51K, 48K, 31K, 29K, 24K, and 20K or 21K polypeptides represent an early, DNA synthesis-independent group of proteins likely to be involved in HVS DNA replication and the regulation of virus gene expression.

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