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Herpesvirus Saimiri-induced Proteins in Lytically Infected Cells. I. Time-ordered Synthesis

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SUMMARY

The addition of TPA (phorbol-12-myristate-13-acetate) to cultures during the lytic infection with herpesvirus saimiri led to an enhanced and accelerated production of polypeptides induced by *H. saimiri* and to a rapid shut-down of host cell protein synthesis and allowed a detailed analysis of the protein patterns. Analysis of sequential protein synthesis in owl monkey kidney cells lytically infected with *H. saimiri* 11 permitted the identification of 31 virus-induced polypeptides. The use of the amino acid analogues canavanine (for arginine) and azetidine (for proline) in parallel allowed experiments on the identification of proteins synthesized early and late during lytic infection.

INTRODUCTION

Herpesvirus saimiri is horizontally transmitted and widespread in squirrel monkey populations (*Saimiri sciureus*) without causing overt disease (Meléndez *et al.*, 1968). In several experimental hosts, particularly marmoset species (*Saguinus oedipus*, *S. nigricollis* and *S. fuscicollis*) (Meléndez *et al.*, 1969, 1971; Deinhardt *et al.*, 1974), the virus is highly tumourigenic. *H. saimiri* therefore resembles in some aspects the Epstein-Barr virus (EBV), at present the best candidate for a human tumour virus (Deinhardt & Deinhardt, 1979). Viraemia is, however, rare (Deinhardt & Deinhardt, 1979) and virus replication would, if it happened to a greater extent in the tumour cells, lead to the decay of the tumour mass. *Saimiri sciureus* monkeys, the natural hosts, are lifelong virus carriers following inapparent infection early in life. Virus can be isolated from lymphocytes after co-cultivation *in vitro* with susceptible cells or directly from throat swabs of these animals. There is no evidence that virus is produced in peripheral lymphocytes, suggesting a biological behaviour similar to Marek's disease virus (Calnek *et al.*, 1970) and EBV (Deinhardt & Deinhardt, 1979; Wolf & Bayliss, 1979; Wolf *et al.*, 1981).

Both viruses are lymphotropic and tumourigenic and have specialized target cells within the body of the host, permitting a steady virus production. The suppression of virus replication in carrier cells and in tumour cells therefore seems to be an established fact.

The time-ordered appearance of proteins during lytic infection might suggest their possible regulatory functions. Blocking experiments with translation inhibitors and especially the use of amino acid analogues (Honess & Roizman, 1974; Fenwick & Walker, 1978; Bayliss & Wolf, 1981, 1982) have proved particularly helpful for further characterization of early proteins which control the synthesis of proteins produced later in the cycle. The synthesis of late polypeptides may be controlled via regulatory proteins or simply via the amount of virus DNA available for transcription (Honess & Watson, 1977*a*; Wolf & Roizman, 1978; Wolf & Bayliss, 1979).

METHODS

Cells. Owl monkey kidney cells (OMK) were cultured in 32 oz glass prescription bottles or plastic Petri plates using minimal essential medium (MEM, Earl's salts, Gibco) supplemented with 20 mM-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% heat-inactivated foetal calf serum (Seromed).

Virus. *H. saimiri* strain 11 was originally obtained from B. Fleckenstein and was propagated in OMK cells. Cells were infected with *H. saimiri* 11 at 1 to 2 p.f.u./cell; after an adsorption period of 2 h at room temperature, the cultures were replenished with MEM supplemented with 2% heat-inactivated foetal calf serum and incubated at 34 °C until a pronounced cytopathic effect became visible.

Labelling of proteins synthesized by infected and uninfected cells and preparation of cell extracts. Cells grown in Petri dishes (diam. 3 cm) were infected and labelled at various times after infection with 10 $\mu\text{Ci/ml}$ [^{35}S]methionine (New England Nuclear) in methionine-free MEM. At the end of the labelling period, cells were rinsed three times with cold phosphate-buffered saline (PBS) to stop amino acid incorporation, solubilized in 200 μl of solubilization buffer (0.05 M-Tris-HCl pH 7, 2% SDS, 5% 2-mercaptoethanol, 3% sucrose, bromophenol blue), sonicated for 15 s with a Branson sonifier (microtip at its maximum output), heated for 5 min at 100 °C and stored at -20 °C.

Use of TPA and butyric acid. A stock solution containing 2 mg/ml TPA (phorbol-12-myristate-13-acetate, Sigma) was prepared in dimethyl sulphoxide (DMSO) and stored over liquid nitrogen. Butyric acid was prepared as a stock solution of 100 mM. Aliquots of these solutions were diluted in cell culture medium to obtain the concentrations indicated in the experiment.

Use of amino acid analogues. L-Canavanine (Sigma) was diluted in arginine-free cell culture medium to a final concentration of 500 $\mu\text{g/ml}$. Azetidine (Sigma) was used at a concentration of 500 $\mu\text{g/ml}$ in normal MEM (which is proline-free).

Use of inhibitors of DNA synthesis. DNA synthesis was inhibited with three different drugs: hydroxyurea (HU, 4 mg/ml, Sigma), cytosine arabinoside (Ara-C, 50 $\mu\text{g/ml}$, Sigma) and phosphonoacetic acid (PAA, 50 $\mu\text{g/ml}$, a gift from Abbott Laboratories); stock solutions were prepared as 100 \times concentrates in PBS and diluted in MEM to the final concentrations.

TCA precipitation. Aliquots of 5 μl of the cell extracts were spotted on Whatman 3MM filter discs, air-dried, washed twice in cold 5% trichloroacetic acid (TCA) for 10 min and twice in 96% ethanol. After drying at room temperature, filters were placed in vials with Aquasol 2 (New England Nuclear) and counted.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. SDS-PAGE was performed according to the procedure of Laemmli (1970) in a discontinuous buffer system on slab gels. The resolving gel consisted of 10% acrylamide, 0.26% diallyltartardiamide (DATD), 0.1% SDS and 0.375 M-Tris-HCl pH 8.5; the gel length was 15 cm. The stacking gel consisted of 3.1% acrylamide, 0.08% DATD, 0.1% SDS, 0.125 M-Tris-HCl pH 7. Polymerization was initiated by addition of ammonium persulphate to a final concentration of 0.14 mM and *N,N,N',N'*-tetramethylethylenediamine (TEMED) to 0.035%. The upper and lower buffer tanks contained 0.025 M-Tris-HCl, 0.192 M-glycine and 0.1% SDS, pH 8.5. Electrophoresis was carried out at room temperature using a constant current of 5.3 mA/cm² and continued until the leading front reached the end of the gel. The gels were fixed and stained for 1 h in water/acetic acid/isopropanol (4:1:1, by vol.) containing 0.1% Coomassie Brilliant Blue, destained in water/acetic acid/isopropanol (8:1:1, by vol.) and dried on Whatman 3MM filter paper under vacuum. All chemicals for electrophoresis were purchased from Bio-Rad. The following marker proteins were used, with mol. wt. in parentheses. *Escherichia coli* RNA polymerase (a gift from Boehringer, Mannheim, 1 $\mu\text{g/slot}$; the mol. wt. of the α subunit is given in the literature between 40000 and 45000 but for our calculations we used a mol. wt. of 40000, and 155000 and 165000 for β and β' subunits respectively); 0.1 $\mu\text{g/slot}$ of lysozyme (14 000); carbonic anhydrase (30000); bovine serum albumin (68000); phosphorylase b (94000) (all purchased from Sigma). The position of marker bands was indicated with radioactive ink (2000 ct/min/ μl) before the gels were exposed for various times to LKB-Ultro³H-film (for [^{35}S]methionine labels). The molecular weights of the protein bands were calculated using a program for the TI 59 calculator (S. Modrow & H. Wolf, unpublished results) that calculates a best-fitting curve for the marker proteins and derives from them the molecular weights of the protein bands according to their distance of migration.

RESULTS

Protein synthesis during infection with H. saimiri 11

Herpesvirus saimiri infection in the OMK cells used in our laboratory is relatively slow. Under standard conditions it takes up to 7 days for all cells to show cytopathic changes. There was no synchronous synthesis of virus proteins during this rather long time interval and the synthesis of host cell proteins ceased late and rather incompletely. Pulse-labelling with [^{35}S]methionine during the course of virus infection allowed only the detection of the late viral proteins, which are the major components of virions. Early viral proteins were hardly visible, due to their presence in low amounts and the high background caused by host protein synthesis. Even very high multiplicities of infection (up to 100 p.f.u./cell) did not change this picture. Only an accelerated and enhanced protein synthesis would allow the direct detection of all classes of virus proteins and avoid the otherwise obligatory use of immunoprecipitation with its disadvantages, namely the danger of missing a number of proteins (Bayliss & Wolf, 1981).

Virus induction with TPA and butyric acid

To accelerate the virus cycle in OMK cells, we used chemicals known to enhance virus production in other virus systems (Hudewentz *et al.*, 1980; Luka *et al.*, 1979). OMK cells (22nd

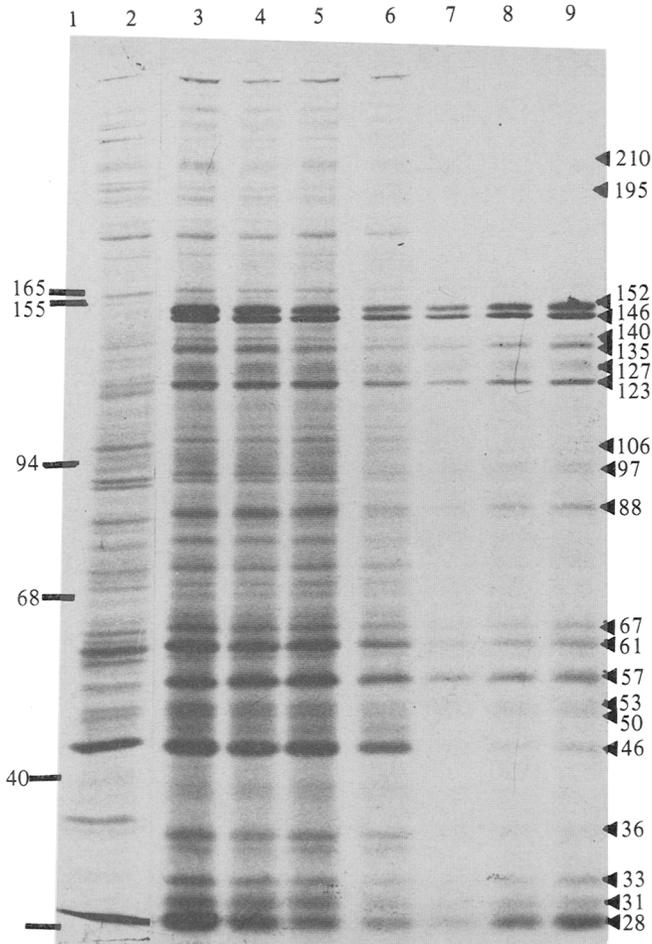


Fig. 1. Effects of TPA and butyric acid on the protein synthesis in cells which were infected with *H. saimiri* 11 (2 to 5 p.f.u./cell). After the adsorption period, medium containing TPA or butyric acid at the concentrations indicated was added, and the polypeptides labelled with [³⁵S]methionine at the time intervals given below. Lane 1, molecular weight marker proteins; lane 2, untreated cells, labelled for 23 to 24 h after mock infection; lane 3, untreated cells, infected with *H. saimiri* 11 and labelled 23 to 24 h after infection; infected cells, treated with 1.5 mM (lane 4), 3 mM (lane 5) and 6 mM (lane 6) butyric acid and labelled from 23 to 24 h after infection; infected cells, treated with 10 ng/ml (lane 7), 20 ng/ml (lane 8) and 40 ng/ml (lane 9) TPA and labelled from 23 to 24 h after infection.

passage) in Petri dishes were infected with low (2 to 5 p.f.u./cell) and high (80 to 100 p.f.u./cell) multiplicities of *H. saimiri* 11. After 1 h, unadsorbed virus was removed and the cultures were re-fed with medium containing various amounts of TPA (10 ng/ml, 20 ng/ml and 40 ng/ml) or butyric acid (1.5 mM, 3 mM and 6 mM). At 7, 23 and 32 h after infection the medium was removed and replaced by methionine-free medium, containing 10 μ Ci/ml [³⁵S]methionine and the same concentration of TPA and butyric acid as before. The culture dishes were incubated for an additional hour, harvested and analysed as described in Methods (Fig. 1). Cells treated with TPA during infection showed an accelerated synthesis of virus-induced proteins. The synthesis of early proteins was especially enhanced by the addition of TPA (data not shown). With 20 ng/ml of TPA, maximal synthesis of viral proteins was observed and the virus cycle was complete by 24 h after infection. When the cultures were labelled at later times only virus structural proteins were observed. Fig. 1 shows the effect of TPA: it drastically reduced the host background and yields a clear profile of late virus-induced proteins. The multiplicity of infection did not appear to have any effect in these experiments within the range of 2 p.f.u./cell to 80

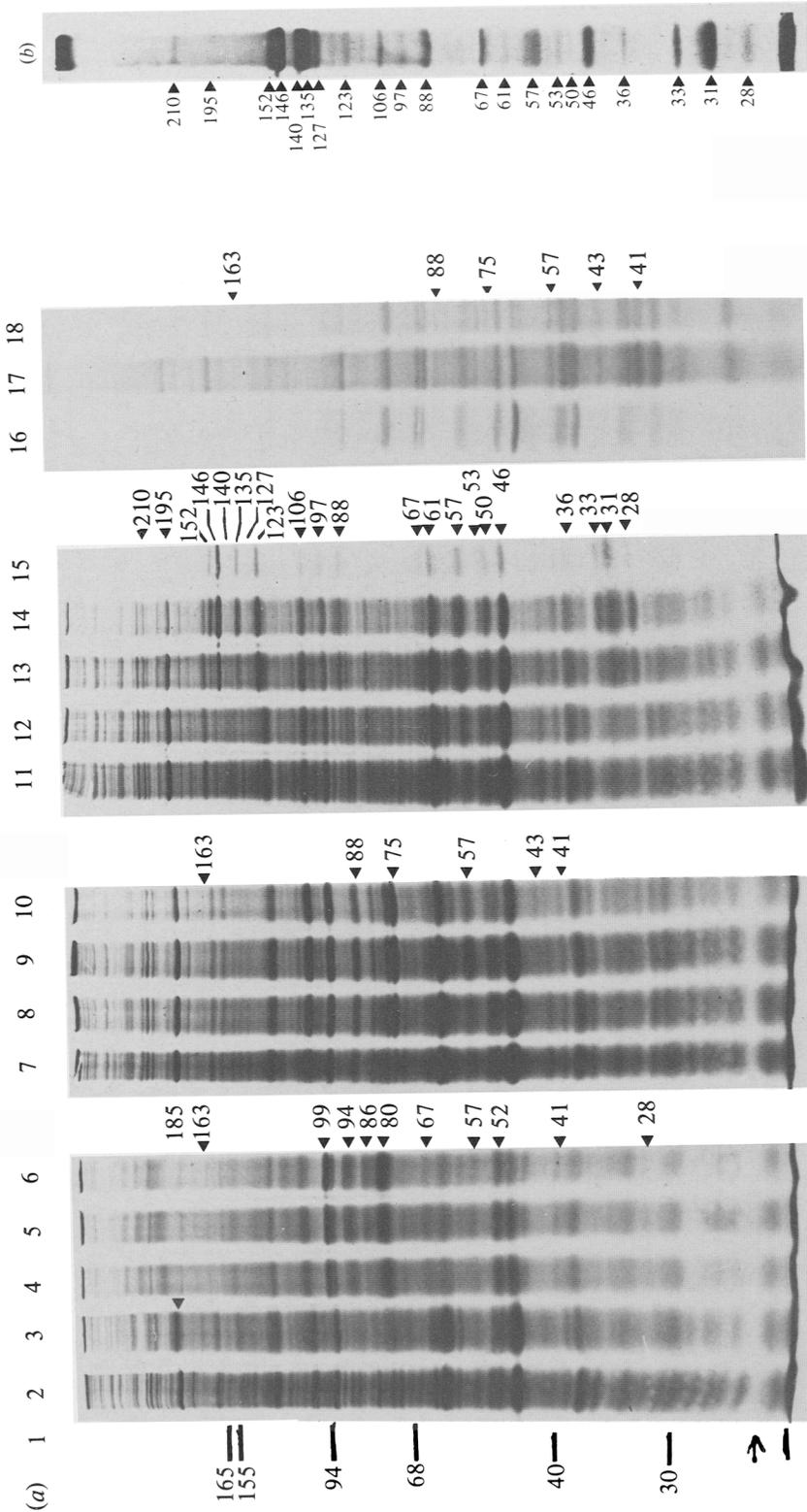


Fig. 2. (a) Protein profiles from *H. saimiri* 11-infected cells (2 to 5 p.f.u./cell) at various times after infection. Samples of 20000 ct/min were applied per slot. Lane 1, molecular weight marker proteins; lane 2, mock-infected cells, labelled 24 to 26 h after mock infection; virus-infected cells, treated with canavanine and labelled from 0 to 4 (lane 3), 4 to 9 (lane 4), 9 to 15 (lane 5) or 15 to 25 (lane 6) h after infection; virus-infected cells, treated with azetidine and labelled from 0 to 4 (lane 7), 4 to 9 (lane 8), 9 to 15 (lane 9), 15 to 24 (lane 10) h after infection; virus-infected cells labelled without analogues from 0 to 4 (lane 11), 4 to 9 (lane 12), 9 to 15 (lane 13), 15 to 24 (lane 14), and 24 to 26 (lane 15) h after infection; lane 16, mock-infected cells, treated with azetidine and labelled 24 to 26 h after mock infection; virus-infected cells, treated with azetidine and labelled 8 to 19 (lane 17) or 24 to 26 (lane 18) h after infection. (b) Protein pattern of purified *H. saimiri* 11 virions. OMK cells grown in 32 oz glass prescription bottles were infected with *H. saimiri* 11 (2 to 5 p.f.u./cell). After the adsorption, the cultures were replenished with medium containing 20 ng/ml TPA and [³⁵S]methionine (1 μCi/ml) and incubated for 4 days. Virus was purified from the supernatant using a sucrose gradient (10 to 30% w/w), solubilized, heated for 5 min at 100 °C and electrophoresed on 10% SDS-polyacrylamide gels.

p.f.u./cell. It is therefore not necessary to use high virus concentrations to accelerate virus replication in the presence of TPA.

Addition of butyric acid in the concentrations used did not accelerate or enhance viral protein synthesis to the extent seen with TPA. Though the quantity of virus-specified proteins in butyric acid-treated samples (Fig. 1) seems to be higher than in the TPA-treated samples, this merely reflects the fact that the TPA-treated cells are already at an advanced stage of the lytic cycle with a number of infected cells already lysed.

The effect of TPA on virus replication was less pronounced when OMK cells of low passage number (around 15) were used. In these cells, virus infection was more effective than in OMK cultures with a high passage number (around 60). Optimal conditions for protein induction experiments were found to be OMK cells with not more than 30 passages, infected at 90% confluency, and the addition of 20 ng/ml TPA. These conditions were used in all further experiments. TPA addition to mock-infected cultures had no effect (not shown).

The staggered synthesis of virus-induced proteins

To study the course of protein synthesis during the lytic cycle of virus replication, infected cell cultures were pulse-labelled at various times after infection. Almost confluent cultures of OMK cells were infected with *H. saimiri* 11 (2 to 5 p.f.u./cell). After adsorption at room temperature, excess virus was removed and the cultures were re-fed with medium containing 20 ng/ml TPA and incubated at 37 °C. At the times indicated in the figures, the medium was replaced by medium containing [³⁵S]methionine at 10 µCi/ml; the cultures were incubated for additional time intervals (indicated in Fig. 2*a*), harvested and analysed as described.

In Fig. 2*a*), lanes 11 to 15 show the sequential synthesis of viral proteins labelled with [³⁵S]methionine. Twenty-one of these were synthesized starting at 9 to 10 h post-infection and are most likely late proteins, since their synthesis increased further and they could also be identified in purified virions (Fig. 2*b*). Early proteins could be detected in trace amounts only. The use of inhibitors should be of help in enriching these early polypeptides and facilitate their detection and distinction from late proteins.

Use of amino acid analogues for the identification of early viral proteins

OMK cells were infected with *H. saimiri* 11 as described before. The medium added to the cells after adsorption contained 500 µg/ml canavanine or 500 µg/ml azetidine; cells were labelled with [³⁵S]methionine (10 µCi/ml medium) at the times shown in Fig. 2*a*), lanes 2 to 10, harvested and analysed as described. Eleven virus-induced proteins were synthesized in the presence of canavanine (p185, p163, p99, p94, p86, p80, p67, p57, p52, p41, p28); addition of azetidine allowed six proteins to be synthesized (p163, p88, p75, p57, p43, p41), three of them (p163, p57, p41) being identical to those obtained after addition of canavanine. Three of the proteins synthesized in the presence of the analogues are also found in the virions: p88, p57 and p28. The presence of the analogues allowed the detection of several polypeptides which were not found in untreated cells. When canavanine and azetidine were added to the cultures simultaneously, the protein pattern appeared indistinguishable from the one obtained with canavanine alone (not shown).

To demonstrate that the proteins produced in the presence of amino acid analogues are indeed virus-specified and not artefacts of the blocked cell cycle, uninfected OMK cells were treated with canavanine, azetidine and TPA. This effect is shown in Fig. 2*a*), lanes 16 to 18: in uninfected cells some polypeptides accumulated in the presence of azetidine. These proteins had, however, molecular weights different from those synthesized in *H. saimiri*-infected cells. Addition of canavanine to mock-infected cells gave similar results (not shown).

Dependence of virus-induced protein synthesis on DNA replication

OMK cells were infected with *H. saimiri* 11 as described before. After adsorption, cells were rinsed and replenished with medium containing Ara-C, HU or PAA at the concentrations given in Fig. 3. Proteins were labelled with 10 µCi/ml [³⁵S]methionine at the times indicated, harvested and analysed as described. All late proteins were found to be dependent on prior DNA

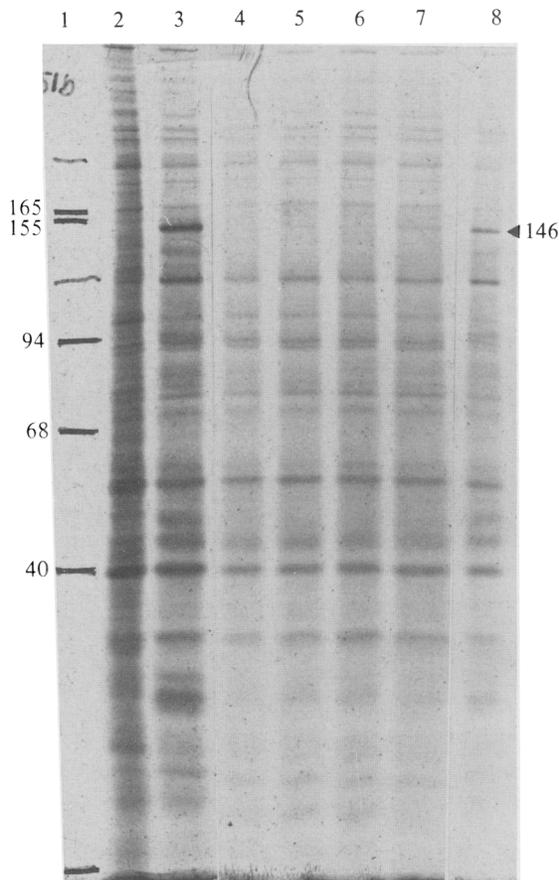


Fig. 3. Effect of different inhibitors of DNA synthesis and of simultaneous addition of the analogues on *H. saimiri* 11-infected cells. The cultures were labelled with [35 S] methionine (10 μ Ci/ml) from 24 to 26 h after infection. Samples of 20000 ct/min were applied per slot. Lane 1, mol. wt. marker proteins; lane 2, mock-infected untreated cells; *H. saimiri*-infected cells: lane 3, untreated; lane 4, treated with Ara-C (50 μ g/ml); lane 5, treated with HU (4 mg/ml); lane 6, treated with PAA (500 μ g/ml); lane 7, with 200 μ g/ml; lane 8, with 50 μ g/ml.

synthesis. With 200 μ g/ml PAA, only one polypeptide (p146) was synthesized, and at 500 μ g/ml the synthesis of this protein was inhibited as well.

DISCUSSION

Repression of the lytic cycle is essential for the manifestation of an oncogenic potential, since replication of herpesviruses invariably causes the death of the host cells. It seems reasonable to conclude from observation with other members of the herpesvirus group that the expression of viral genes follows a regulated pattern. It would be of great interest to know where this cascade of regulated expression is interrupted in the tumour cell (Wolf & Bayliss, 1979) and eventually understand the mechanisms responsible for this regulation. Whereas the highly lytic herpes simplex virus allowed detailed analysis of the lytic cycle (Hones & Roizman, 1974, 1975; Morse *et al.*, 1978; Wolf & Roizman, 1978), it is extremely difficult to obtain results for EBV, where an efficient lytic system is not known (Kallin *et al.*, 1979; Müller-Lantzsch *et al.*, 1979; Feighny *et al.*, 1981; Kawanishi *et al.*, 1981; Bayliss & Wolf, 1981, 1982). In order to study the regulation of gene expression, we examined the course of protein synthesis in lytically infected cells. The analysis of proteins by *H. saimiri* in OMK cells during the lytic infection has been hampered by its slow replication in connection with a weak shut-down of host cell protein synthesis. The

addition of TPA during virus replication leads to a considerably accelerated and enhanced production of virus polypeptides and to a rapid and effective shut-down of the synthesis of host cell proteins. This enhanced virus production of *H. saimiri* is analogous to other members of the herpesvirus group, especially EBV (zur Hausen *et al.*, 1978), where TPA increases the number of virus-producing cells as well as the amount of viral DNA synthesized per individual cell (Hudewentz *et al.*, 1980). TPA is likely to induce the replication of *H. saimiri* DNA and thus accelerate the infective cycle of the virus in OMK cells. This effect is more pronounced in high- than in low-passage OMK cells, which are already more favourable for virus replication. The enhancement of virus protein synthesis by TPA was essential for the analysis of virus-induced proteins. Analysis of electrophoretically separated polypeptides revealed that, during the course of infection, at least 31 virus-induced proteins are synthesized. The polypeptides could be characterized and classified according to their molecular weights and the times of their appearance during the lytic cycle.

Similar to other members of the herpesvirus group (Rakusanova *et al.*, 1971; Honess & Roizman, 1974, 1975; Morse *et al.*, 1978; Bayliss & Wolf, 1981), the function of early proteins is essential for the synthesis of polypeptides produced later in the lytic cycle. The amino acid analogues canavanine (for arginine) and azetidine (for proline) are incorporated into the polypeptide chains and replace the corresponding amino acids. In this way, they affect the function of proteins without detectably changing their molecular weights. The addition of these analogues to uninfected cells leads to accumulation of certain proteins. These proteins are not enriched when cells are infected. In this case, the production of early polypeptides continues for more than 24 h after infection and the synthesis of late proteins is not initiated.

In the presence of azetidine, six different virus proteins were synthesized: p163, p88, p75, p57, p43 and p41. With canavanine added to the cultures, 11 polypeptides were produced (p185, p163, p99, p94, p86, p80, p67, p57, p52, p41 and p28), three of them being identical to those synthesized in the presence of azetidine: p163, p57 and p41. This suggests that two groups of proteins may belong to a first class of early proteins. Depending on their arginine and proline content and participation in active groups in the polypeptide chains, their function may be destroyed by the incorporation of either of the amino acid analogues and stop the synthesis of a second class of proteins.

One of the proteins synthesized in the presence of canavanine (p185) seems to change its mol. wt. during the course of infection towards a higher value; this may be due to a post-translational modification. The protein does not seem to contain phosphate or glucosamine groups (S. Modrow & H. Wolf, unpublished results); it may contain other sugars or sulphate groups. Some of the proteins synthesized in the presence of analogues (p185, p163, p99, p94, p86, p80, p75, p52, p43, p41) were not found in untreated cells. An explanation for this effect may be that their enrichment in cells with the analogues is essential for their detection. Alternatively an altered post-translational modification could be responsible for these proteins. Experiments involving the simultaneous addition of canavanine and azetidine suggest, by the inhibition of the synthesis of proteins which normally appear in the presence of azetidine alone, that the proteins p88, p75 and p43 are controlled by proteins synthesized in the presence of canavanine and are not part of an independent control group. Peptide mapping or sequencing will have to be used to answer that question. Twenty-one polypeptides could be identified as late proteins whose synthesis started about 9 to 10 h post-infection (p210, p195, p152, p146, p140, p135, p127, p123, p106, p97, p88, p67, p61, p57, p53, p50, p46, p36, p33, p31 and p28).

Some of these late proteins are also synthesized in the presence of amino acid analogues (p88, p57 and p28) and possibly are polypeptides whose synthesis is required during the whole cycle.

Inhibitors of DNA synthesis were used to examine whether DNA replication is necessary for the synthesis of certain proteins. PAA inhibits the replication of EBV DNA (Yajima *et al.*, 1976) but not that of host cells whereas HU prevents the replication of cellular DNA (Mele *et al.*, 1974; Honess & Watson, 1977b); it is believed that HU does not inhibit the replication of DNA of superinfecting EBV (Wolf & Bayliss, 1979; Lamon & Lidin, 1979). Ara-C is known to be a rather effective inhibitor of total DNA synthesis. All these drugs were found to be effective on *H. saimiri* replication: all late virus proteins were dependent on prior DNA synthesis. Only one

the detection of the corresponding proteins. Alternatively, DNA replication may allow the opening of special initiation sites and by this allow the transcription of these genes.

Table 1 summarizes all the data obtained on H. saimiri-induced proteins. Black bars give the time intervals during which a protein is synthesized; hatched bars represent protein bands found in uninfected cells as well as in infected cells and virions. These bands may represent either closely comigrating cellular and virus proteins or may be parts of the virus envelope originating from the membrane of the cell nucleus.

Altogether, the sequential synthesis of 31 virus proteins during lytic infection was observed. Twenty-one of these were found late in the cycle; the others were synthesized only at early times after infection.

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