Effect of Isoleucine Deprivation on Rabbitpox Virus DNA Synthesis in Mouse L Cells

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SUMMARY

Synthesis of rabbitpox DNA was inhibited in mouse L cells deprived of isoleucine. Time-course patterns of incorporation of radiolabelled precursors into viral DNA revealed that synthesis of viral DNA began about 6 h after reversal of the isoleucine-deficient state.

Several researchers have examined the nutritional requirements for poxvirus replication. Holtermann (1968) found that the same amino acids essential for L cell propagation were required for vaccinia virus replication. Studies on the effect of arginine deprivation on synthesis of viral DNA has produced conflicting results. Obert, Tripier & Guir (1971) examined replication of vaccinia virus in HeLa cells deprived of arginine and found that synthesis of viral DNA was reduced to 70% of control. In contrast to these findings, Archard & Williamson (1971) found vaccinia viral DNA synthesis to be practically non-existent in HeLa cells deprived of arginine.

Certain continuous mammalian cell lines, including Chinese hamster ovary cells (line CHO), mouse L cells, and Syrian hamster BHK 21 cells, can be synchronized by depriving the cells of isoleucine (Ley & Tobey, 1970; Tobey & Ley, 1971). The isoleucine deprived cells are arrested in the G1 phase of the cell cycle and are prevented from replicating nuclear DNA. Upon addition of isoleucine the cells resume cell cycle traverse, initiate nuclear DNA synthesis, and subsequently divide in synchrony.

Since isoleucine deficiency prevents nuclear DNA synthesis in L cells (Tobey & Ley, 1971) and apparently synchronizes synthesis of mitochondrial DNA in Chinese hamster cells (Ley & Murphy, 1973), the objective of the present study was to determine the effect of isoleucine deprivation on viral DNA synthesis by infecting isoleucine-deprived L cells with rabbitpox. Our virus results indicate that rabbitpox virus does not synthesize DNA in L cells in the absence of isoleucine and that restoration of isoleucine to these cells allows initiation of viral DNA synthesis.

Earle's strain mouse L-929 cells were grown in suspension culture in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal bovine serum, penicillin, streptomycin, and kanomycin. Cells were routinely cultured for pleuropneumonia-like organisms (PPLO) with the medium of Chanock, Mayflick & Barile (1962) which contained yeast extract prepared as described by House & Waddell (1967). PPLO were not detected during these studies.

L cells were synchronized by suspending exponentially growing L cells in MEM without isoleucine but containing twice the normal concentration of glutamine and 10% dialysed foetal bovine serum. After 36 to 72 h, cells were released from G1-arrest by resuspension in complete MEM.

Rabbitpox virus ATC no. VR-157 (RPV; American Type Culture, Rockville, Maryland, U.S.A.) was first cultivated in cultures of chick embryo fibroblasts (CEF) and then passaged on L cell monolayers. Virus was concentrated by centrifuging and resuspended in isoleucine...
deficient MEM plus dialysed serum. RPV stocks were assayed on CEF monolayers and titres were found to range from 2.0 to 6.5 × 10^7 p.f.u./ml.

Synthesis of RPV DNA was detected by inoculating 2 × 10^7 exponentially growing L cells with 1 to 2 p.f.u. of RPV per cell and exposing infected cells to 41 to 62 μCi [3H]-TdR (16 Ci/mmol; Schwarz/Mann, Division of Becton, Dickinson, and Co., Orangeburg, New York, U.S.A.) for 4 to 6 h. Cells were washed with 0.15 M-NaCl; resuspended in 2 ml of 0.01 M-KCl, 0.005 M-EDTA, 0.01 M-tris-HCl, pH 7.4, and lysed with a Dounce homogenizer. The lysate was centrifuged twice at 900g to remove nuclei and the cytoplasmic extracts were analysed by sucrose density gradient centrifugation by the method of Dahl & Kates (1970). Two ml of cytoplasmic extract were layered on linear sucrose gradients ranging from 25 to 40% sucrose over a 5 ml 80% sucrose cushion and centrifuged in an SW 25.1 rotor (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) for 20 min at 22,000 rev/min. The density gradients were fractionated and the amount of radioactivity in each fraction was determined by liquid scintillation spectrometry.

In certain experiments, cytoplasmic extracts of labelled cells were prepared as described above; however, instead of layering on sucrose density gradients, 0.2 ml of the cytoplasm was placed on Whatman 3MM filter paper discs. The discs were extracted 3 times with 5% TCA, 3 times with ethanol, dried, and the amount of radioactivity determined (Mans & Novelli, 1961).

Density gradient profiles of radiolabelled macromolecules from cytoplasmic extracts of RPV-infected L cells revealed a peak of radioactivity at the top of the gradient, and a second somewhat larger peak at the bottom of the gradient. In contrast, both peaks were absent in the profiles from uninfected exponentially growing cells, and uninfected or infected isoleucine-deprived cells.

These results indicated that we could detect viral DNA in the cytoplasm of infected L cells and that synthesis of RPV-DNA does not occur in isoleucine-deprived cells as measured by continuous labelling with [3H]-TdR. The next objectives were to measure the time-course patterns of viral DNA synthesis after infection and determine if the effect of isoleucine-deprivation on viral DNA synthesis could be reversed by addition of isoleucine.

Time-course patterns of viral DNA synthesis as a function of time were measured by exposing cells to [3H]-TdR at hourly intervals after infection. Incorporation of [3H]-TdR into viral DNA began in exponentially growing cells 1 to 2 h after infection and the rate continued to increase until it peaked at 3 to 4 h. (Fig. 1a). Incorporation decreased sharply after that to almost the original level, but a slight increase in synthesis was seen at 5 to 6 h. In contrast to these data, there was no peak of radiolabel incorporation into the cytoplasm of infected, isoleucine-deprived cells (Fig. 1b). The time-course patterns of viral DNA synthesis in cells released from G1-arrest by the addition of isoleucine is shown in Fig. 2. The unreleased, uninfected cells showed little evidence of incorporation of [3H]-TdR into the cytoplasm when exposed continuously to labelled thymidine, whereas, in cells infected and released from G1-arrest, incorporation of radiolabel began at about 6 h after release and continued to increase up to at least 14 h after infection.

Data presented here indicate that L cells which have been deprived of isoleucine are unable to support poxvirus DNA replication. After restoration of isoleucine, poxvirus DNA synthesis commences at about 6 h. Evidence from our experiments indicated that viral DNA synthesis is inhibited in cells deprived of isoleucine since [3H]-TdR incorporation was not incorporated into TCA precipitable material in the cytoplasm from isoleucine deficient, infected cells. Our results are in accord with Archard & Williamson (1971) who found that viral DNA synthesis was virtually non-existent in vaccinia-infected cells deprived of arginine.
The validity of using incorporation of radiolabelled precursors into cytoplasmic DNA as an indication of viral DNA replication has been previously established. Joklik & Becker (1964) found that newly synthesized TCA-precipitable material isolated from the cytoplasm of vaccinia virus-infected HeLa cells contained DNA. They demonstrated that the cytoplasmic DNA was viral DNA by comparing the caesium chloride equilibrium density gradient profiles of newly replicated cytoplasmic DNA, host cell DNA, with authentic viral DNA.
isolated from highly purified virus. The cytoplasmic DNA coincided exactly with the authentic DNA while the host cell DNA was heavier than viral DNA. They also found that newly synthesized viral DNA exists in large aggregates which can be analysed by sucrose density gradient centrifugation. Other workers (Dahl & Kates, 1970; Polisky & Kates, 1972) determined that the viral DNA aggregates were actually DNA-protein complexes which were absent in uninfected cells. In addition, our time-course patterns of $[^{3}H]$-TdR incorporation into viral DNA from exponentially growing cells agree with those of Joklik & Becker (1964).

The mechanism by which isoleucine deprivation prevents poxvirus DNA synthesis is not known. One obvious possibility would be that cellular protein synthesis is impaired by the limiting amino acid. This would be consistent with the observation that continuous protein synthesis is required for replication of the poxvirus genome (Joklik, 1964; Joklik & Becker, 1964; Dales, 1965; Kates & McAuslan, 1967a, b). However, Enger & Tobey (1972) found that although synthesis of nuclear DNA decreased almost immediately after CHO cells were deprived of isoleucine, both RNA and protein syntheses were much less affected. By 30 h after isoleucine deprivation, RNA synthesis was still 55 to 70% of that measured in exponentially growing cells and protein synthesis was 60 to 70% of control. Because the strain of

Fig. 2. Time-course patterns of viral DNA synthesis in cells after reversal of G1-arrest by restoration of isoleucine. Isoleucine-deprived cells were resuspended in complete MEM and inoculated with 1 p.f.u. of RPV/cell. After adsorption for 1 h, 1.5 μCi $[^{3}H]$-TdR/ml medium were added and $2.5 \times 10^6$ cells were taken at 2 h intervals for determination of radioactivity as described in the text: •, released, infected cells; •, released, uninfected cells.
L cells used in the present study can continue cell cycle traverse and accumulate in G₁ after isoleucine deprivation (Tobey & Ley, 1971) this may be taken as evidence that, as with CHO cells, protein synthesis continues at a reduced rate. The reason cells deprived of isoleucine pile-up in G₁ is unknown, but a recent study by Ley (1975) has indicated the existence of a 80000 mol. wt. cytoplasmic protein that is synthesized by synchronized CHO cells prior to initiation of nuclear DNA synthesis. Synthesis of this protein was not detected during G₁-arrest induced by isoleucine deprivation, but was detected 5 to 6 h after release of cells from G₁-arrest. It may be that this or some other cellular protein is necessary for viral DNA synthesis to proceed. That mitochondrial DNA appears to be synchronized in G₁ cells produced by isoleucine deprivation (Ley & Murphy, 1973) may be further evidence for the existence of a protein required for DNA synthesis but not synthesized in cells deprived of isoleucine.

Based on these preliminary observations, it is not possible to rule out inhibition of a viral protein required for viral DNA synthesis. Viral proteins have been described in adenovirus-infected cells that bind to viral DNA and may be involved in viral DNA replication (Van der Vliet & Levine, 1973; Yamashita & Green, 1974). However, it is interesting to note that synthesis of poxvirus DNA in the present study did not begin until 6 h after the readdition of isoleucine. One might expect that if isoleucine deficiency prohibited synthesis of a viral protein required for DNA synthesis that the lag period after addition of isoleucine would be about the same as infection of exponential cells, i.e. 1 to 2 h (Fig. 1a). Further studies of isoleucine deprivation and its effect on poxvirus DNA synthesis are needed to determine which processes prior to initiation of viral DNA synthesis are blocked by isoleucine deficiency.

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