STIMULATION OF THE THYMIDINE PHOSPHORYLATING SYSTEM IN HeLa CELLS ON INFECTION WITH POXVIRUS

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The nucleic acid of vaccinia virus is DNA, yet it multiplies in the cytoplasm. The question therefore arises whether vaccinia virus DNA codes for any of the enzymes concerned with the synthesis of DNA or its precursors which for some reason, topographical or other, may be lacking in that part of the cell where viral DNA initiates replication. As has been demonstrated for bacteriophage, such enzymes could be identified by their increased activity following infection as well as by their physical properties.

We present here the results of a study of the levels of a number of enzymes converting deoxyribonucleosides to the corresponding triphosphates following infection of exponentially growing HeLa cells with certain poxvirus strains.

Experimental: HeLa S3 cells were grown in spinner culture in Eagle's medium (1959) containing 5% calf serum. The generation time was 24 hours. Cells were infected for 20 minutes at a concentration of 5×10^6 cells/ml; under the conditions used each cell adsorbed about 50 virus particles. The cells were then washed once, and resuspended at 4×10^5 cells/ml and again stirred. Control cells were treated similarly, but no virus was added. At intervals cells were then removed, washed, resuspended in Tris Buffer (0.1M, pH 7.8, containing 0.002 M 2-mercaptoethanol) and disrupted by brief sonication. The sonicates were centrifuged at 100,000g for 30 minutes and the supernatants used immediately for enzyme assays. The results were not significantly different when (a) the cells were disrupted in a glass-steel homogeniser and the supernatant then used, or (b) the whole sonicate was used. The composition of the reaction mixtures for enzyme assays is given in the legends of the

relevant Figures and Tables. The following virus strains were used: cowpox, rabbitpox, and ectromelia. The biological properties of these strains as well as the preparation of heat-inactivated, reactivable virus has been described (Joklik et al, 1960). UV-inactivated virus refers to virus inactivated to 10^{-5} of its initial titre by irradiation with UV-light.

RESULTS

Reaction mechanism. There appear to be two distinct pathways for the conversion of dTMP to dTTP: (a) stepwise phosphorylation via dTDP (Weissman et al, 1960) and (b) pyrophosphorylation of dTMP (Bianchi et al, 1961). It was therefore important to establish which pathway operates in HeLa cells. In preliminary experiments it had been found that even in the initial stages of the conversion of 2-C^{14}-dTMP to 2-C^{14}-dTTP, the number of cpm in dTDP always exceeded the number in dTTP. Further, the rate of formation of dTTP from dTDP was greater than that from dTMP. In order to establish beyond doubt the participation of dTDP, the kinetics of forming C^{14}-dTTP from C^{14}-dTMP in the presence and absence of a "sink" of unlabelled dTDP were compared over short time intervals. As controls, the rates of the reactions dTTP $\rightarrow$ dTDP $\rightarrow$ dTMP and dTDP $\rightarrow$ dTTP were measured. The results are shown in Table 1 and may be summarised thus:

(1) The reaction rates are constant for at least 40 minutes.

(2) Under the conditions used there is no breakdown of dTDP or dTTP.

(3) The accumulation of cpm in dTDP starting with C^{14}-dTMP was independent of the presence of excess unlabelled dTDP.

(4) The accumulation of cpm in dTTP starting with C^{14}-dTMP was greatly decreased by the presence of excess unlabelled dTDP.

(5) The formation of dTTP was faster starting with dTDP than with dTMP.

There is thus no doubt that in HeLa cells the sequence of reactions is dTMP $\rightarrow$ dTDP $\rightarrow$ dTTP.

Comparison of enzyme levels in normal and infected cells. The levels of dT kinase, dTMP kinase and dTDP kinase were determined for normal cells and cells infected with cowpox virus. Assay conditions in all cases were such that there was linearity over a wide range with respect to protein concentration and time; conditions were chosen so as to operate well within this range, so that the amount of substrate phosphorylated was a direct measure of the amount of enzyme present. It should be mentioned here that under our conditions virus

progeny was first detectable at between 6 and 7 hours after infection, and that by 12 hours half the virus yield had been formed. Over 85% of the cells stained with fluorescent antibody to virus and were therefore producing viral protein.

TABLE 1

The role of dTDP as an intermediate in the conversion of dTMP to dTTP.

Substrate	cpm found in	Incubation time (min) 0	5	10	20	40
C^{14}-dTMP	dTDP + dTTP	0	79	165	350	732
	dTTP	0	27	50	108	248
C^{14}-dTMP + excess dTDP	dTDP + dTTP	0	80	166	350	625
	dTTP	0	5	15	24	60
C^{14}-dTDP	dTMP	0	0	0	0	0
	dTTP	0	122	353	753	925
C^{14}-dTTP	dTDP	0	0	0	0	0

Reaction mixtures contained: $MgCl_2$ 30 µmoles, ATP 60 µmoles, Tris Buffer pH 7.8 250 µmoles, cell protein 2 mg, C^{14}-dTMP 0.1 µmole or C^{14}-dTDP 0.5 µmole (30,000 counts/min), excess unlabelled dTDP (where indicated) 0.5 µmole; total volume 2.55 ml. At the times indicated 400 µl of the reaction mixture was withdrawn, chilled rapidly and made 0.5 N with respect to $HClO_4$. After 2 minutes the mixture was neutralized with KOH, centrifuged and 400 µl of the supernatant pipetted into 1.0 ml ice-cold Tris Buffer (0.1 M, pH 7.8). The mixture was then chromatographed on columns of ECTEOLA cellulose (8x1 cm) maintained at 5°. dT, dTMP, dTDP and dTTP were then eluted successively with 50 ml aliquots of H_2O, 0.01 N HCl, 0.05 N HCl and 0.5 N HCl. The eluates were concentrated and counted. The figures represent cpm derived from the 400 µl neutralized aliquots.

(A) dT kinase. In Fig. I the activities of dT kinase in extracts of uninfected and infected cells are plotted against the time post-infection. Enzyme activity increased markedly between 4 and 6 hours after infection, reaching a plateau at about 9 hours. This increase amounted to 10 - 15-fold.

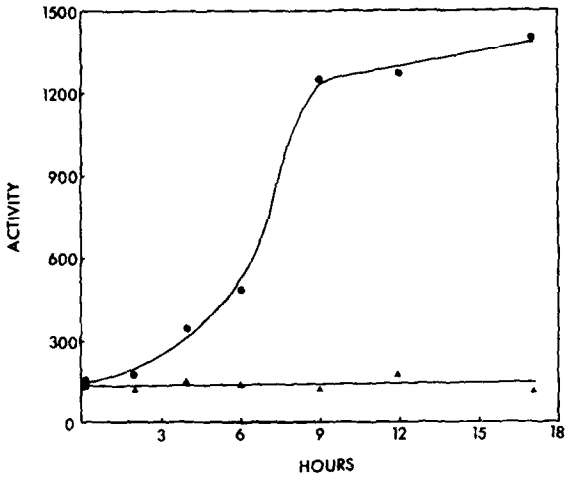
(B) dTMP kinase and dTDP kinase. There was no increase in the level of either of these two enzymes during the first 9 hours postinfection. Table 2 gives the results obtained with extracts of normal cells and cells infected for 6 hours.

The following observations are relevant:

(1) The increase in the activity of dT kinase following infection is not obtained in the presence of 0.001 M FPA (fluorophenyl alanine), which under our

FIGURE I

Activity of dT kinase in extracts of normal cells and cells infected with cowpox virus.



Assay conditions: $MgCl_2$ 1.5 μmole, ATP 1.5 μmole, Tris Buffer (pH 7.8) 25 μmoles, 2-C^{14}-dT 0.007 μmole (2500 cpm), cell protein 300 μg, total volume 250 μl. After 30 minutes incubation at 37° the reaction mixtures were chilled rapidly and treated as described in the legend to Table 1. Activity is expressed as cpm in dTMP + dTDP + dTTP. Triangles: uninfected cells; circles: infected cells.

conditions inhibited incorporation of amino acids into cellular protein by approximately 90%. On removal of the FPA and replacement by phenylalanine, the increase promptly occurred. Protein synthesis is therefore essential for the increases to take place. However, this cannot be taken as proof that infection results in the de novo synthesis of new enzyme protein since it has been found that an enzyme "uncoating" viral DNA is synthesised after infection (Joklik, 1962) and it may be that it is the synthesis of this protein, the induction of which is essential if the information stored in viral DNA is to be available, which is blocked by FPA.

(2) Increased levels of dT kinase are also found after infection with rabbitpox and ectromelia virus, though the increases here are somewhat smaller (4 - 5-fold). dT kinase formation is also induced by infection with UV-inactivated virus (known to be "uncoated") but not by heat-inactivated, reactivable

TABLE 2.

Phosphorylation of dT and dTMP by extracts of normal cells and cells infected with rabbitpox virus

Cell extract	Compound phosphorylated	Percent conversion to dTMP	dTDP	dTTP
Normal Cells	2-C^{14}-dT	6	3	1.5
Infected Cells	2-C^{14}-dT	50	9	5
Normal Cells	2-C^{14}-dTMP	-	10	10
Infected Cells	2-C^{14}-dTMP	-	11	10

Cells had been infected for 6 hours. Assay for phosphorylation of dT: $MgCl_2$ 1.5 µmoles, ATP 1.5 µmoles, Tris Buffer (pH 7.8) 25 µmoles, C^{14}-dT 0.007 µmole (2500 cpm), cell protein 400 µg, total volume 250 µl. Assay for phosphorylation of dTMP: as for dT but ATP 6 µmoles, dTMP 0.01 µmole (2500 cpm).

virus (known not to be "uncoated", Joklik, 1962). The induction is thus caused only by virus particles the genetic information of whose DNA becomes available within the cell. This strengthens the case for supposing that the code for the induced dT kinase resides in viral DNA.

(3) Hanafusa et al (1960) reported that extracts of infected cells showed a higher rate of incorporating H^3-dT into DNA than did extracts of normal cells. The above results suggest that this was primarily due to the presence of higher amounts of dT kinase in these extracts (Table 2). We have tested the levels of DNA polymerase in our cell system but find that its level is increased by no more than 50% following infection.

(4) A number of other enzymes has also been tested. There is no increase following infection in the levels of dAMP and dCMP kinases. dC kinase could not be tested owing to the presence of a powerful dC deaminase. However, extracts of infected cells formed 3 - 5 times as much dUMP and dUDP from dC as extracts from non-infected cells. This is being investigated.

(5) All experiments have been carried out with host cells growing exponentially, and therefore containing maximal amounts of kinases and DNA polymerase. Any increased amounts of enzyme formed as a result of the presence of viral DNA thus have to be detected against large background enzyme levels. Experiments are currently being conducted with stationary phase cells in which any increases due to infection may be more easily detectable.

The significance of the heightened phosphorylation of dT is not obvious at the moment. Evidence is accumulating however that dT exercises some regulatory control over DNA synthesis.

Summary: Infection of exponentially growing HeLa cells with any of 3 poxvirus strains results in the induced formation of enzyme(s) capable of phosphorylating dT and dU (increases of up to 10 - 15-fold). No increases in dTMP, dTDP, dAMP and dCMP kinases were found. DNA polymerase levels rose by less than 50%. Only particles which could be "uncoated" within the cells caused the formation of the enzymes.

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