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Control of Thymidine Kinase Synthesis in IHD Vaccinia Virus-Infected Thymidine Kinase-Deficient LM Cells

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The synthesis of vaccinia virus-induced thymidine kinase is normally arrested several hours after infection. In thymidine kinase-deficient LM cells infected with IHD strain of vaccinia virus, arrest occurs whether or not viral DNA synthesis is inhibited. With virus inactivated by UV irradiation, enzyme synthesis takes place, but arrest is abolished. It is suggested that an early viral genetic function is responsible for the cessation of thymidine kinase synthesis.

Poxviruses induce the synthesis of a number of proteins in infected cells. These proteins are classified into two broad groups: early, which appear before the onset of viral DNA synthesis, and late, which require prior DNA synthesis. Thymidine kinase (EC 2.7.1.75) belongs to the first group (4, 9). However, its synthesis, which proceeds linearly during early infection, is stopped after about 5 h by a process that depends on viral DNA replication (9) and also requires RNA (3, 10) and protein (10) synthesis. It was therefore proposed that the arrest of thymidine kinase production is caused by a late viral protein(s) acting as repressor (2, 10).

This information was obtained with HeLa cells infected with rabbit and cowpox vaccinia strains. Virus-induced thymidine kinase has also been studied in another system, in which thymidine kinase-deficient LM cells [LM-(TK$^-$)] are infected with vaccinia virus IHD strain (6, 7). Here too, enzyme synthesis ceases at a certain stage of the viral cycle (12). However, this system responds differently to actinomycin D. The differences between these two systems are as follows. The inhibitor administrated before infection suppresses both RNA and thymidine kinase synthesis in poxvirus-infected HeLa cells. When administrated 2 to 4 h after infection, the drug abolishes RNA synthesis, while the enzyme synthesis now continues unabated beyond the time at which it is normally stopped. In contrast, virus-induced RNA synthesis in IHD-infected LM(TK$^-$) cells is not completely suppressed, and cells treated with actinomycin D before infection are still able to produce about 40% of the normal amount of thymidine kinase, as assayed 7 h after infection or earlier (8, 13), but no precise kinetic data have been reported.

We have investigated this point in more detail and found that in contrast to other systems described earlier (9, 10), the arrest of IHD-induced thymidine kinase synthesis does not require viral DNA replication. Data presented in this communication suggest that the expression of an early genetic function could be responsible for arrest of the enzyme synthesis.

LM(TK$^-$) cells and the IHD strain of vaccinia virus, clone C11, were kindly provided by S. Kit (Baylor University College of Medicine, Houston, Texas), and HeLa cells and the WR strain of vaccinia virus by E. Katz (Hadassah Medical School, Hebrew University, Jerusalem, Israel). Cells were grown in monolayers in minimal essential medium supplemented with 0.3% tryptose phosphate broth, 0.35% glucose, 10% calf serum and antibiotics. Stock cultures of LM(TK$^-$) cells were routinely grown in the presence of 25 μg of 5-bromo-2'-deoxyuridine (Sigma). IHD was propagated in LM(TK$^-$) and WR in HeLa cells. Both viruses were purified before use (1). One optical density unit at 260 nm of purified virus corresponded to about 10^8 PFU. Plaque assays were performed on BSC-1 or L-929 cells, with 0.75% agar or agarose, 2% calf serum, and Eagle medium as an overlay. Plaques were demonstrated by staining with 0.005% neutral red and counted on the day 5 of incubation.

Thymidine kinase was assayed directly on 1-cm DEAE paper squares according to Munyon et al. (11), with the following modifications: the supernatant (cytoplasmic extract) used as enzyme source was prepared at 12,000 $\times$ *g* instead of 150,000 $\times$ *g*, and the amount of [^{3}H]thymidine applied per square was 6 $\times$ 10^4 to 7 $\times$ 10^4 counts/min in 0.02 ml of assay buffer. The reaction was linear with time up to 20 min and with the amount of enzyme. The reaction on paper was quantitatively similar to that in solution. Cytoplasmic extracts were prepared from the cells from a 35-mm petri dish, which

were frozen and thawed in 1 ml of assay buffer, sonicated for 3 min at 1A (Raytheon sonic oscillator, model DF 101), and centrifuged for 2.5 min at 12,000 × g. The cytoplasmic extracts were diluted 10 times with assay buffer, and 0.02 ml was taken for assay.

RNA and DNA synthesis was measured after 15-min pulses with 5 μCi of [^{3}H]uridine per ml (40 Ci/mmol, The Radiochemical Centre, Amersham, England) or [^{3}H]thymidine (16 Ci/mmol, from the same source), respectively. Trichloroacetic acid-insoluble radioactivity was determined either in cytoplasmic extracts (to measure rates of virus-induced nucleic acid synthesis) or in whole cells (to estimate the inhibitory action of the drugs). Trichloroacetic acid precipitates were collected on membranes (Millipore Corp.) or fiber glass filters, washed with 6% trichloroacetic acid and ethanol, dried and counted in a Tricarb scintillation spectrometer, Packard, model 3003, using as a scintillation liquid 2,5-diphenyloxazole, 4 g; 1,4-bis-(5-phenyloxazolyl)-benzene, 0.05 g; toluene, 1,000 ml.

Figure 1 shows that IHD and WR viruses grow similarly in HeLa cells (Fig. 1a), that in LM(TK$^-$) cells the growth curve of IHD is not markedly changed, and that thymidine kinase production in those cells stops when only 10% or less of the IHD viral progeny has been formed (Fig. 1b).

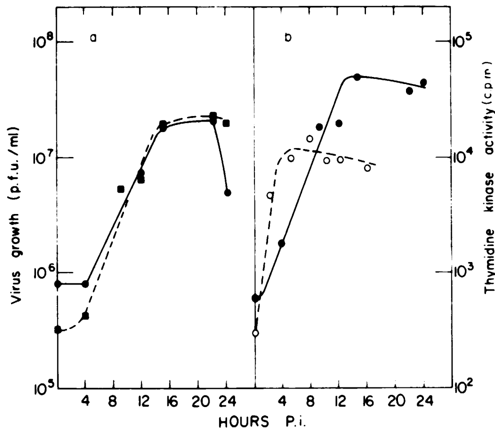

FIG. 1. *One-step growth of WR and IHD vaccinia viruses in HeLa cells (a) and IHD growth and thymidine kinase induction in LM(TK$^-$) cells (b). Monolayers of HeLa or LM(TK$^-$) cells in 35-mm Petri dishes (in duplicates) were infected with 5 × 10^6 PFU of WR or IHD (in 0.05 ml). After 60 min of absorption at 37 C monolayers were washed three times to remove the unabsorbed virus and 1.5 ml of minimal essential medium containing 2% calf serum was added to each plate (zero time). At the times indicated, plates were put into a −70 C refrigerator either with (for infectivity assay) or without (for thymidine kinase assay) maintenance medium and kept frozen until being tested. Symbols: (■) WR; (●) IHD; (O) thymidine kinase activity.*

Figure 2 illustrates some effects obtained with actinomycin D. At 5 μg/ml (Fig. 2a) the inhibitor, when administrated 30 min after infection, had little effect on thymidine kinase production, which reached the same level and was arrested as in the absence of the drug. The same result was obtained when the drug was added later (not shown). When administrated 60 min before infection, this concentration of actinomycin caused a marked inhibition (about 50 to 70%) of enzyme synthesis. Here, arrest of synthesis did not occur before the experiment was terminated. However, it cannot be concluded that arrest was abolished; it might have been delayed in parallel to the slowing down of enzyme synthesis. When the actinomycin concentration was lowered to 2 μg/ml in an attempt to abolish the arrest under conditions where the rate of enzyme synthesis was not inhibited, arrest was not abolished (Fig. 2b).

The remaining experiments reported here were performed to examine the relationships between nucleic acid synthesis and thymidine kinase synthesis and its subsequent arrest. Table 1 shows that under the conditions tested actinomycn D permits about 60% of the initial RNA synthesis but strongly inhibits it at 2 h after infection. The drug is also a fairly effective inhibitor of DNA synthesis in IHD-infected LM(TK$^-$) cells, whereas cytosine arabinoside (ara C, Nutritional Biochemicals) stops DNA synthesis virtually completely. McAuslan (9) has shown that in WR-infected HeLa cells the

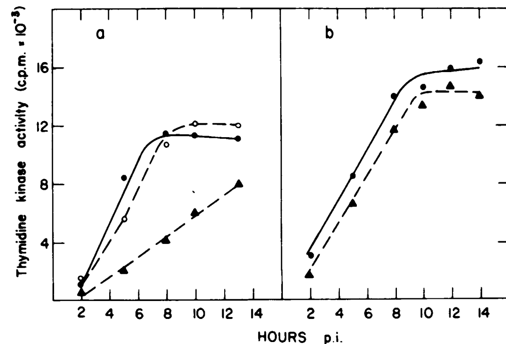

FIG. 2. *The effect of actinomycin D on IHD-induced thymidine kinase synthesis. LM(TK$^-$) cells were infected as in Fig. 1. Cells were treated with 5 μg/ml (a) or 2 μg/ml (b) of actinomycin D. Symbols: (●) untreated control; (▲) 60 min before infection and throughout the infection; (O) 30 min after infection.*

TABLE 1. *The effect of actinomycin D and cytosine arabinoside on RNA and DNA synthesis in IHD-infected LM(TK⁻) cells*[a]

Experiment no.	Inhibitor	Inhibitor concn (μg/ml)	Time assayed (h after infection)	Incorporation into trichloroacetic acid precipitate			
				[³H]uridine		[³H]thymidine	
				Counts/min	%	Counts/min	%
1	Actinomycin D	0	0	3,434	100	240	
		5	0	2,019	58.8	195	
		0	2	9,121	100		
		5	2	731	8		
		0	5			2,001	100
		5	5			315	15.7
2	Actinomycin D	0	5			8,734	100
		2	5			2,248	25.7
		5	5			522	5.9
3[b]	Cytosine arabinoside	0	2			11,975	100
		10	2			239	1.9
		0	5			25,404	100
		10	5			201	0.6

[a] One hour before infection the monolayers were pretreated with the appropriate drug, and inhibitors were present until experiments were terminated. Cells were infected as in Fig. 1.

[b] We used 60-mm petri dishes instead of 35-mm plates, and the radioactivity was determined in whole cells.

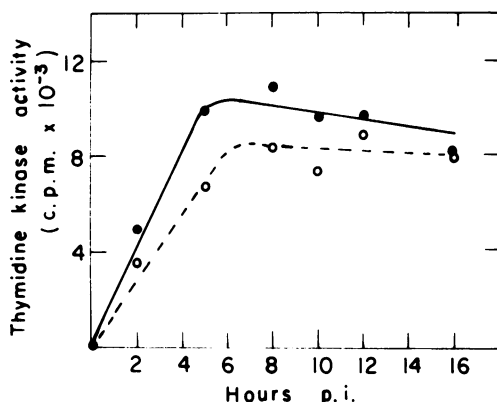

FIG. 3. *The effect of cytosine arabinoside on IHD-induced thymidine kinase synthesis. LM(TK⁻) cells were pretreated with 10 μg of ara C per ml (60 min before infection and throughout the infection) and infected as in Fig. 1. Symbols:* (●) *untreated control;* (O) *ara C.*

inhibition of DNA synthesis abolishes the arrest of thymidine kinase synthesis, so that enzyme production continues past the time when it normally ceases, and we have confirmed this observation. A different result was obtained with IHD virus. Both in HeLa (now shown) and in LM(TK⁻) cells (Fig. 3), the arrest of enzyme synthesis occurred normally whether ara C was present or absent. The same results were obtained when another DNA synthesis inhibitor, fluorodeoxyuridine, was used in the experiments. This indicates that the arrest of thymidine kinase synthesis in IHD-infected cells does not depend on viral DNA replication, in contrast to the apparent situation in WR-infected cells.

Vaccinia virus DNA synthesis can also be inhibited by UV irradiation of the virus (14). This treatment has, in addition, been reported to abolish the arrest of thymidine kinase production (9, 10), and the effect has been ascribed to the inhibition of DNA synthesis. As with other vaccinia viruses, UV irradiation of IHD virus has been shown to abolish the arrest of enzyme synthesis in a spinner culture of infected LM(TK⁻) cells (5). If the same pattern occurs in monolayer cells, which we used, then our results (Fig. 3, Table 1) indicate that this cannot be due solely to the inhibition of DNA synthesis. The experiment of Fig. 4 is a further examination of this point. The result showed a clear arrest of enzyme synthesis in the presence of cytosine arabinoside (i.e., in the absence of DNA synthesis), whereas UV irradiation abolished the arrest. Thus, UV irradiation would appear to abolish the arrest by an effect other than its effect on DNA synthesis. A not unlikely explanation might be that the arrest of IHD-induced enzyme production is caused by an early genetic function (i.e., expressed before DNA replication) which is more sensitive to UV

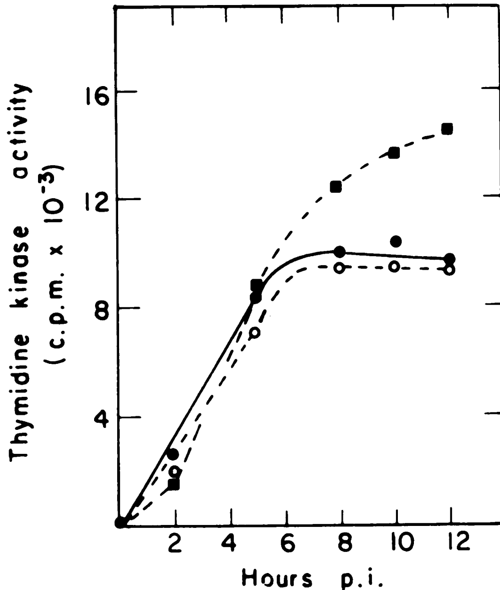

FIG. 4. *The effect of ultraviolet irradiation on IHD-induced thymidine kinase synthesis. Purified virus was diluted to 10^8 PFU/ml and 1 ml of this dilution was irradiated with ultraviolet light (200 $\mu W/cm^2$, Philips TUV 15-W lamp) for 30 s resulting in a 10^3-fold drop in the titer. LM(TK⁻) cells were infected as in Fig. 1. Pretreatment with ara C was as in Fig. 3. Symbols: (●) infected control; (○) ara C; (■) ultraviolet-inactivated virus.*

irradiation than enzyme synthesis itself. If this is so, the IHD-LM(TK^-) system may provide a valuable supplement to other systems in studying the control of thymidine kinase synthesis.

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