SV40-SPECIFIC THYMIDINE KINASE*

By MASAKAZU HATANAKA† AND RENATO DULBECCO

THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, SAN DIEGO, CALIFORNIA

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The small DNA-containing tumor-producing viruses, polyoma virus and SV40, cause marked biochemical changes in the cells they infect. In resting cells, cellular DNA synthesis resumes, and the activity of several enzymes connected with DNA synthesis is enhanced.^{1, 2} Among these enzymes are DNA polymerase, dCMP deaminase, and thymidine kinase (TK). The available evidence concerning the dCMP deaminase suggests that it is a cellular enzyme³ whose synthesis is enhanced by infection. The TK activity present after infection was in some cases similar to that present in uninfected cells,³ whereas in other cases, it had different properties.⁴ Infection may therefore cause the synthesis of two types of TK: the cellular type, which is also made in the absence of infection, and a new one, perhaps specified by a viral gene.

The present communication will deal with some properties of the TK activity that appear after infection of African green monkey cells with simian virus 40 (SV40). The results will show that this enzyme activity differs from that present in uninfected cells, both in heat stability and in its response to fluorouridine (FUR), a pyrimidine analogue that is known to affect the composition of messenger RNA.⁵ The enzyme appears to be virus-specific. Some salient characteristics of this enzyme in SV40 and polyoma virus infection will be discussed, and a suggestion will be made concerning the possible significance of the virus-specific thymidine kinase present in cells infected by DNA-containing viruses.

Materials and Methods.—Virus and cell cultures: The methods of preparation and purification of SV40 and the cultivation of either primary African green monkey kidney cell cultures or of cultures of the permanent line of the same cells, called BSC-1, have already been described.¹ 3T3 cultures (a permanent line of mouse cells) were obtained from Dr. H. Green and maintained in Eagle's medium plus 10 per cent calf serum.

Infection of cultures: They were infected with 0.4 ml of a suspension of purified virus dilution. With BSC-1 and AGMK cultures, the input viral multiplicity was 10–40 plaque-forming units per cell (assayed on BSC-1 cells); with 3T3 cells it was 4,000. After one hour incubation at 37°, the cultures were washed once with medium and subsequently incubated at 37° in a well-humidified incubator flushed with a CO₂-air mixture. All controls were mock-infected under identical conditions, using medium instead of virus.

Enzyme assay: Thymidine kinase and DNA polymerase were assayed as described previously.¹ Fluorouridine (FUR) and fluorouracil (FU) were kindly supplied from the Hoffman-LaRoche Company.

Results.—Heat stability of TK: Previous results have shown that in AGMK cells the virus-specific T antigen begins appearing 19–20 hours after SV40 infection, and the activation of TK and other enzymes begins to increase a few hours later.¹ In the present studies, we used cells 42 to 72 hours after infection.

The heat stability of the TK activities present in infected and uninfected cells

was compared by using crude extracts. In order to decrease the sensitivity of the enzyme to differences in the constitution of the environment during heat, TdR in concentration $5 \times 10^{-5} M$ and Mg^{++} at $10^{-3} M$ were added to the enzyme preparations. This concentration of TdR afforded the highest stabilization of TK, without seriously interfering with the enzyme assay.

The results obtained with extracts heated for various periods of time at 73° are given in Figure 1; they show that the TK activity of infected cells is more heat-resistant than that of the uninfected cells. These differences appear to reflect differences in the enzyme proteins, since they persisted under conditions that minimize differences in the composition of the extracts (except for the enzymes themselves) during heating. The following conditions were used. In some experiments, the extracts were passed through columns of Sephadex G-25 in order to remove small molecules; in other experiments, TdR at higher concentrations ($10^{-4} M$), TMP ($10^{-4} M$), or TTP ($2 \times 10^{-4} M$) was added to the extracts. In some experiments, a fresh extract of one type of cells was mixed with an equal volume of the other extract, which had been preheated to destroy most of its TK activity. Under these conditions, the inactivation kinetics at 73° of the TK in the extracts of infected or uninfected cells remained unaltered. It is therefore unlikely that the differences in heat inactivation kinetics result from differences in the environment of the enzymes.

Effect of 5-fluorouridine (FUR): Cultures of uninfected cells kept for 48 hours in the presence of FUR from 10^{-6} to $10^{-4} M$ had no TK activity (Table 1). In contrast, the specific activity of TK of infected cell extracts was almost unaffected by the addition of FUR from the moment of infection. Even more striking were the results obtained by infecting cultures which had already been incubated for two days in the presence of FUR, and had essentially no TK activity left. After 72

TABLE 1
EFFECT OF FUR ON THYMIDINE KINASE ACTIVITY

	Uninfected		Infected		Cells
	Enzyme units	Per cent	Enzyme units	Per cent	
None	688	(100)	10,890	(100)	AGMK, stationary
FUR $10^{-6} M$	25	4	10,700	98	48 hr under FUR
None	2,034	(100)	6,843	(100)	BSC-1, stationary
FUR $10^{-6} M$	37	2	8,703	127	45 hr under FUR
None	1,990	(100)			BSC-1, growing
FUR $10^{-6} M$	54	3			48 hr under FUR

Cultures were infected as described in *Materials and Methods*. After adsorption, the medium with or without FUR was added on cultures. At the times indicated, the cells were collected, homogenized, and centrifuged at $10,000 \times g$ for 10 min at cold temperature. The supernatant solution was used as enzyme. Thymidine kinase activity was assayed at 37° for 5 minutes. Enzyme units are μ mole TdR per mg protein, phosphorylated during 20 min at 37° .

TABLE 2
PRETREATMENT OF BSC-1 CELLS WITH FUR BEFORE SV40 INFECTION

Enzymes	Uninfected unit	SV40-infected unit
$10,000 \times g$ supernate	5	8,840
Sephadex G-25 treated fraction	3	12,250

Crowded BSC-1 cells were incubated with $10^{-6} M$ FUR for 48 hr at 31° . Then the cells were infected with SV40 (40 PFU/cell). Fresh medium with $10^{-6} M$ FUR was added onto the plates after 1 hr virus adsorption and the plates were incubated at 37° for 72 hr. The cells were harvested, homogenized and centrifuged at $10,000 \times g$ 10 min. The supernate (0.5 ml aliquot) was passed through a Sephadex G-25 column (column volume 5 ml). A fraction of large molecules, which contained most TK activity, was used as Sephadex G-25 treated fraction.

FIG. 1.—Thermoinactivation of TK. AGMK cells were infected with SV40 at an input multiplicity of infection of 40 PFU/cell. Extracts were prepared of uninfected cells (●—●) and virus infected cells (○—○) (72 hours post infection). These preparations contained 1,030 and 970 $\mu\text{g}/\text{ml}$ protein, respectively. One-tenth ml of each extract was separately incubated at 73.5° with $5 \times 10^{-5} M$ TdR and $10^{-3} M$ MgCl_2 in Tris-mercaptoethanol (1/5,000 v/v). At the intervals noted, one tube of each extract was removed to an ice-bath. When all the samples were collected, they were assayed for TK at 37° for 5 min.

FIG. 2.—Thermoinactivation of TK. The experimental conditions are the same as described in Fig. 1. SV40-FUR TK was obtained from a culture that had $10^{-4} M$ FUR in the medium after virus adsorption and was harvested 72 hr later. The sample contained 890 μg protein/ml.

hours of further incubation, still in the presence of FUR, these cultures developed a TK activity comparable to that of infected cultures never exposed to FUR (Table 2). The effect of FUR on uninfected cultures was prevented by excess uridine, but not by thymidine (Table 3). Moreover, 5-fluorodeoxyuridine did not affect the TK activity in uninfected cells. The effect observed with FUR in uninfected cells can therefore be attributed directly to the analogue. Passage of extracts of uninfected cells through Sephadex G-25 columns in order to remove possible products of FUR metabolism which may interfere with the enzyme assay did not cause a reappearance of TK activity (Table 2).

Exposure to FUR, however, is not without effect on the TK of infected cells, since it changes its heat inactivation kinetics. As shown in Figure 2, the TK activity of infected cells exposed to FUR is much more heat-sensitive than that of infected cells not exposed to the analogue. In order to minimize the effect of differences in the environment of the enzymes, the heat inactivation of the mixture of extracts of FUR-treated and untreated cultures (both infected) was studied. The residual activity of the mixture after heating corresponded to the sum of the activities remaining when the two extracts were heated separately. Thus, the difference between FUR-treated and untreated extracts cannot be due to the presence of inhibitors or activators of the enzyme.

TABLE 3
FUR AND FUDR EFFECTS ON THE ACTIVITY OF THYMIDINE KINASE

Addition	Uninfected		Infected		Cells
	Enzyme unit	Per cent	Enzyme unit	Per cent	
None	4,097	(100)	13,696	(100)	BSC-1, stationary 45 hr under FUR
FUR 10^{-5} M	74	2	17,405	127	
FUR 10^{-5} M plus TdR 10^{-4} M	0	0	—	—	
FUDR 5×10^{-7} M	4,483	109	20,939	153	
FUDR 5×10^{-6} M	5,484	134	22,940	167	BSC-1, stationary 44 hr under FUR
None	5,480	(100)	—	—	
FUR 5×10^{-7} M	62	1	—	—	
FUR 5×10^{-7} M ₄ plus UR 5×10^{-4} M	5,120	94	—	—	

The experimental conditions are the same as described in Table 1.

TABLE 4
EFFECTS OF FUR ON THE ACTIVITIES OF THYMIDINE KINASE AND DNA POLYMERASE

Addition	Uninfected TK		Infected TK		Uninfected DP		Infected DP		Cells
	Enzyme unit	Per cent	Enzyme unit	Per cent	Enzyme unit	Per cent	Enzyme unit	Per cent	
None	688	(100)	10,890	(100)	74	(100)	179	(100)	AGMK, stationary 48 hr under FUR
FUR 10^{-5} M	25	4	10,700	98	63	85	131	73	
None	491	(100)	4,530	(100)	77	(100)	161	(100)	AGMK, stationary 72 hr under FUR
FUR 10^{-5} M	22	5	4,610	102	58	75	100	62	

The experimental conditions are the same as described in Table 1. DNA polymerase activity (DP) was assayed at 37° for 1 hr. Enzyme units are μ moles dTTP incorporated into acid insoluble fraction during 20 min at 37°.

TABLE 5
EFFECT OF FU ON THYMIDINE KINASE ACTIVITY

Addition	Uninfected		Infected		Cells
	Enzyme units	Per cent	Enzyme units	Per cent	
None	1,905	(100)	13,000	(100)	BSC-1, stationary 60 hr under FUR
FUR 5×10^{-5} M	18	1	19,600	151	
FU 5×10^{-5} M	433	23	31,400	242	
FU 10^{-4} M	30	2	32,600	251	
FU 2×10^{-4} M	0	0	28,400	219	

The experimental conditions are the same as described in Table 1.

The effect of FUR on the heat-stability of the TK of infected cells can be taken as evidence that FUR is taken up by the infected cells. It would be important, however, to show that it is taken up equally by both cells, in order to exclude the possibility that the differences derive from differences in the effective intracellular concentration of the analogue. Since we were unable to measure incorporation of FUR in the cellular RNA, for lack of a radioactive form of the analogue, two different approaches were followed.

In one group of experiments, we studied the effect of FUR on another enzyme, DNA polymerase, in infected and uninfected cells. As shown in Table 4, the activity of this enzyme was affected in a nearly equal way in the two cases. In a second group of experiments, we studied the effect of fluorouracil (FU). As shown in Table 5, FU acts on TK activity of infected and uninfected cells in much the same way as FUR. Since radioactive FU was available, we studied its incorporation into the RNA of both infected and uninfected cells. As shown in Table 6, incorporation was higher in the RNA of infected cells.

Properties of TK of SV40-transformed cells: SV40 causes transformation of cells

TABLE 6

INCORPORATION OF BASES FLUOROURACIL AND URACIL INTO RNA OF CELLS INFECTED BY SV 40

Isotope	Time Hour	Uninfected (cpm/culture)		Infected (cpm/culture)		Cells
		Total	RNA	Total	RNA	
FU-6H ³	0-24	149,180	19,780	193,120	22,130	AGMK, stationary
3.1 c/mmole	24-48	98,240	9,410	222,260	25,480	
U-6H ³	0-24	63,160	4,940	112,642	12,600	AGMK, stationary
15 c/mmole	24-48	30,440	2,610	115,100	8,570	

Confluent cultures were infected as described in *Materials and Methods*. During the times indicated, 50 μ c of fluorouracil-6H³ (3.1 c/mmole), or of uracil-6H³ (15 c/mmole) was added to infected and uninfected cultures (5 ml of medium per plate). Cultures were removed at 24 and 48 hr after infection, washed 5 times with Tris-saline and homogenized with 1 ml of Tris-saline, then a 0.05-ml aliquot was counted in 10 ml of Bray's liquid scintillation solution. This value is shown as total incorporation into the cells. 0.8-1.0 ml of the homogenate was added to 1 ml of saline-containing SDS and trichloroacetate; nucleic acids were extracted with phenol as described previously.¹ Extracted nucleic acids were precipitated with ethanol, then dissolved in 0.5 ml of Tris. 0.4 ml of the sample was incubated with 30 μ g of DNase and 10⁻³ M MgCl₂ for 1 hr at 37°. After DNase digestion, 5 ml of 5% TCA was added and the precipitates were collected on an HA filter after washing 4 times with 5 ml of 5% TCA. The filters were dried and counted. The counts are incorporated in RNA.

of the 3T3 line of mouse cells, although the virus does not multiply in these cells. It is already known, however, that infection with SV40 causes a transient increase in TK activity, as well as in DNA synthesis.⁶ Under our experimental conditions, a 5- to 25-fold increase of the TK activity took place in 3T3 cells, following SV40 infection. The enzyme activity, both in infected and uninfected cells, was found to be much more heat-sensitive than in AGMK or BSC-1 cells, so that inactivation had to be performed at 53° rather than at 73°. The activation of extracts of both infected and uninfected cells showed similar kinetics; they inactivated quickly at first, with similar rates, and then both left a residual activity of 10-20 per cent, which resisted further inactivation. Similar results were obtained with a line of 3T3 cells transformed by SV40.

FUR has a small effect on the TK activity of 3T3 cells when they are in stationary (confluent) cultures, as shown in Table 7. The degree of inhibition in uninfected, SV40-infected or transformed cells is the same. In growing 3T3 cultures, FUR is considerably inhibitory for TK activity; the effect is similar on 3T3 cells and on their transformed derivative.

Discussion.—The data presented here reveal profound differences between the properties of the TK activity of uninfected and SV40-infected AGMK cells. The two activities respond in very different ways to heat treatment and to incorporation of FUR in cellular RNA's. Extensive controls tend to exclude the possibility that these differences derive from environmental differences. The different sensitivity to FUR indicates either that the two enzymes differ in primary structure, or that the infected cell enzyme is specified by a messenger made before addition of FUR (which in most experiments occurred at the time of infection). The latter possibility is very unlikely because cells exposed to FUR for two days before infection and

TABLE 7

EFFECT OF FUR ON THYMIDINE KINASE ACTIVITY

Addition	3T3, Uninfected		3T3, Infected		3T3, Transformed by SV40		Cells
	Enzyme units	Per cent	Enzyme units	Per cent	Enzyme units	Per cent	
None	307	(100)	2,180	(100)	2,630	(100)	Crowded,
FUR 10 ⁻⁵ M	214	70	1,740	80	1,724	66	42 hr under FUR
None	288	(100)			2,840	(100)	Growing,
FUR 5 $\times$ 10 ⁻⁷ M	58	20			480	17	46 hr under FUR

The experimental conditions are the same as described in Table 1.

kept under FUR for another three days after infection still had an almost normal rise in TK activity (Table 2). Within the two days preceding infection, a considerable fraction of the pre-existing normal mRNA molecules would have decayed.⁷

We conclude that, in all likelihood, the enhancement of TK activity after infection is due to a new enzyme. We shall refer to this enzyme as SV40-specific TK, in order to distinguish it from enzymes whose activity is increased owing to induction of cellular syntheses.

The SV40-specific TK could be coded for either by a viral or a cellular gene. In the latter case, the gene must be totally inactive in uninfected cells, because in them no TK is left after FUR treatment. The problem of genetic control of the SV40-specific TK, however, cannot be resolved by the available data, but only by the study of viral or cellular mutants when they will become available.

A striking relationship is noticed by comparing the heat-stability of the SV40-specific TK, described here, and that of the polyoma virus-specific TK, studied by Sheinin.⁴ In both cases, the virus-specific enzyme, although more heat-stable than the cellular enzyme, falls in the same stability range of the latter. The SV40 enzyme, like that of AGMK or BSC-1 cells, has measurable inactivation at 73°; for the polyoma enzyme, like that of mouse cells, inactivation is measurable at 63°. This relationship suggests a relatedness between the virus-specific enzyme and the enzyme of the cells that serve as the usual host for the virus. For instance, the gene of the virus-specific enzyme, whether it is incorporated in the viral or in the cellular genome, may have arisen from a mutated duplication of the gene of a normal cellular enzyme; or the virus-specific and the normal cellular enzymes are made up by several subunits, of which some are identical in the two enzymes. The studies now in progress on the purification of the enzymes may clarify these points.

Lack of SV40-specific TK (with the characteristics found in infected AGMK cells) in abortively infected or transformed mouse cells could mean either that in mouse cells SV40-specific TK does not differ from regular mouse TK, or that a virus-specific TK is not made and the increased activity observed in abortive infection is an aspect of the process of induction of cellular syntheses.

The finding of a virus-specific TK in cells lytically infected by either SV40 or polyoma virus raises the question of the significance of the enzyme. It does not appear to be relevant for cell transformation, since TK-less cells can be transformed by polyoma virus, and they do not develop TK.⁸ The problem is clarified by the fact that a virus-specific TK is produced in cells infected by DNA-containing animal viruses of many types.^{4, 9} TK could be important, therefore, for viral multiplication. Although the reaction catalyzed by TK is not essential for DNA synthesis, it may be important under the conditions or multiplication of these viruses in nature. In the animal body in which they have evolved, these viruses multiply in cells that are usually in a resting stage. The replication of the viral DNA requires precursors made by cellular enzymes, which in resting cells have little activity. The role of TK may be to activate these enzymes. This result can be obtained by phosphorylating TdR taken up from the blood to TMP, which is then phosphorylated to TTP by cellular enzymes. TTP is the most general activator of the reductive enzymes responsible for the production of the diphosphates of the other three deoxynucleosides from the corresponding ribonucleoside diphosphates.¹⁰

TK would have the role of starting and possibly maintaining production of these essential precursors. A virus-specific TK is required for this role, because resting cells have a low TK activity.

SV40 and polyoma virus have, in addition, the ability of stimulating the synthesis of cellular TK (together with other enzymes); they choose either one or the other device, under different circumstances. The mechanism of this choice is still not understood.

Summary.—SV40-infected African green monkey kidney cells contain a virus-specific TK, which differs from the host cell enzyme in heat inactivation kinetics and response to FUR. The TK of abortively infected or SV40-transformed mouse cells is similar to the host enzyme in both properties. The possible role of TK in viral infection is discussed.

Abbreviations used: TK = deoxythymidine kinase; FU = fluorouracil; FUR = 5-fluorouridine; TdR = thymidine; DP = DNA polymerase; FUDR = 5-fluorodeoxyuridine; AGMK = African green monkey kidney; UR = uridine.

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† Present address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

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