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Origin of the Thymidine Kinase Induced by Polyoma Virus in Productively Infected Cells

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Cells of the 3T3 mouse line efficiently supported the multiplication of polyoma virus, and the infectious process was accompanied by a marked increase in thymidine kinase (TK) activity. Two lines of 5-bromodeoxyuridine-resistant 3T3 cells have been isolated. As expected, these cells incorporated practically no exogenous thymidine into their deoxyribonucleic acid (DNA) and contained negligible TK activity. Like the parental 3T3 cells, TK$^-$ lines were susceptible to productive infection by polyoma virus, but infection did not lead to an increase in TK activity. Since kinase activity did appear after infection with another virus (vaccinia) known to contain the gene(s) for that enzyme, it is concluded that TK is not one of the gene products of polyoma virus. As induction of cellular DNA synthesis by polyoma virus occurs normally when the TK$^-$ cells are infected in the stationary phase, TK cannot play a role in the determination of this phenomenon.

The genome of polyoma virus (molecular weight about 3×10^6) contains information sufficient to code for 5 to 10 proteins. The identification of these proteins is of considerable importance, since at least one of them must be responsible for the viral ability to carry out neoplastic transformation of cells. The viral nucleic acid is assumed to contain the genes for the viral coat proteins, the nuclear T antigen, the virus-specific transplantation antigen, and the defective protein of the ts-a mutant (for a review, *see* reference 5).

It has also been suggested that the enzyme thymidine kinase (TK) may be a viral product, since polyoma or simian virus 40 (SV40) lytic infection of stationary cells leads to a several-fold increase in TK activity in the infected cultures (7, 8, 10, 12, 16). TK induction is accompanied by activation of other enzymes of the deoxyribonucleic acid (DNA) synthetic pathways and induction of cellular DNA synthesis (6, 9, 20, 22). The TK induced by polyoma virus was found to differ in some properties (heat lability, K_m and K_s' values) from the enzyme found in normal, uninfected cells (7, 16, 17). Similarly, SV40-induced TK differed from the host cell enzyme in K_i and K_m (12), heat lability (8), immunological specificity (3), and response to fluorouracil (8). These findings led to the suggestion that TK might be one of the gene products of polyoma virus or SV40, and that it may play an important role in either transformation or viral multiplication. When baby hamster kidney (BHK) cells lacking TK activity were infected by polyoma virus, the cells were transformed but did not acquire the enzyme, either in the early stages of infection or after transformation was evident (14). Thus, the presence of this enzyme is not necessary for viral transformation. However, BHK cells do not support multiplication of polyoma virus, and the possibility remained that viral genes for this enzyme existed, but could be expressed only in cells supporting a lytic infection.

Polyoma virus multiplies rapidly and to high titers in wild-type 3T3 cells. Recently, we have been able to isolate cellular variants of the 3T3 mouse line which virtually lack TK activity. Although viral multiplication proceeds normally in the TK$^-$ 3T3 cells, there is no detectable increase in TK activity. These findings support the conclusion that TK is not one of the gene products of polyoma virus.

MATERIALS AND METHODS

Growth medium. All cultures were grown in the Dulbecco and Vogt modification of Eagle's medium supplemented with 10% calf serum.

Cells. The 3T3 mouse cell line (18) and two 5-bromodeoxyuridine (BUDR)-resistant derivatives, designated C2 and C2F, were used in the experiments.

Virus. The virus used in most of the experiments was the small-plaque variant of polyoma virus, but experiments performed with large-plaque virus gave essentially the same results. Virus was grown in mouse

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kidney or 3T3 cultures and for some experiments was purified by density gradient centrifugation (21).

Virus titrations. Hemagglutination assays were carried out as described (1). Plaque assays were performed on confluent monolayers of 3T3 M cells, with the standard technique developed for mouse embryo cultures (1). The efficiency of plating of polyoma virus on 3T3 cells is about twofold higher than that on mouse embryo cells. Time of appearance of plaques, morphology, etc. do not differ significantly.

Infection. Cell monolayers in 60-mm petri dishes were infected with 0.2 ml of virus suspension. After adsorption for 2 hr at 37 C, the cultures were washed to remove unadsorbed inoculum and were incubated in medium containing 5% horse or calf serum. Control cultures were mock-infected under identical conditions, but without virus.

Thymidine incorporation into DNA. The amount of radioactive thymidine incorporated into DNA was determined from the radioactivity present in acid-insoluble material. Cultures which had been exposed to radioactive thymidine were chilled and were washed three times with cold tris(hydroxymethyl)aminomethane (Tris)-buffered saline. The cells were then taken up in 1 N NaOH. The solution was then chilled and the *p*H was brought to neutrality with the addition of HCl; then trichloroacetic acid was added to a concentration of 10%. After 1 hr at 4 C, the precipitate was collected on membrane filters (grade HA, Millipore Corp., Bedford, Mass.) and was counted in a liquid scintillation spectrometer.

TK activity. For the preparation of enzyme extracts, the cells were harvested, washed twice in cold tris-buffered saline, and resuspended at a concentration of approximately 10^7 cells/ml in 10^{-2} M Tris (*p*H 8.0) containing mercaptoethanol (1:5,000). The cell suspension was subjected to ultrasonic disintegration for about 10 sec and was centrifuged for 45 min at 17,000 × *g*; the supernatant fluid was used as a crude enzyme preparation. In most of the experiments, the enzyme extracts were immediately frozen at −90 C and were kept at this temperature until assayed. Storing at −90 C, even for long periods of time, did not lead to any decrease in enzymatic activity.

The assay conditions were similar to those described previously (14). The reaction mixture contained 20 mM $MgCl_2$, 55 mM Tris (*p*H 8.0), 5 mM sodium phosphoglycerate, 7.4 mM adenosine triphosphate, 20 μg of bovine serum albumin per ml, and 0.1 mM ^{3}H- or ^{14}C-thymidine. Incubation was carried out for 10 or 15 min at 37 C, and the reaction was stopped with the addition of trichloroacetic acid. The phosphorylated thymidine derivatives were separated from the substrate by high-voltage paper electrophoresis.

Protein determinations. Protein determinations were made according to the method of Lowry et al. (15).

RESULTS

Isolation of TK-deficient 3T3 cells. TK-deficient cells are unable to phosphorylate BUDR and therefore are protected against the lethal effect resulting from the incorporation of this analogue into DNA. TK-deficient 3T3 cells were therefore isolated by virtue of their resistance to BUDR.

For this purpose, 3T3-4 cells were plated in the presence of 5 μg of BUDR per ml. The great majority of the cells did not survive, but a few colonies appeared. Two of these, designated C2 and E, were isolated and propagated in medium containing 5 μg of BUDR per ml. After a number of transfers, the drug concentration was increased to 30 μg/ml and the surviving cells were used as main lines. The E line exhibited a low plating efficiency and a definite reversion frequency and was not used in the present experiments. The experiments described in this paper were carried out with the C2 line and with a subclone of this line, designated C2F.

C2 and C2F cells are resistant to high concentrations of BUDR. For example, in a typical experiment, the plating efficiency of C2 cells was 39% in the absence of BUDR, 44% in the presence of 30 μg of BUDR per ml, and 39% in the presence of 100 μg of BUDR per ml. They had a generation time of about 20 hr, either in the presence or absence of BUDR. The C2 line is rather insensitive to contact inhibition of growth, in comparison with the wild-type 3T3 cells, and for this reason the C2F subline was employed whenever stationary cultures were needed. The saturation density of C2F was about twice as high as that attained by the parental 3T3 cells, but once this density had been achieved, mitotic activity and DNA synthesis occurred at the very low rate characteristic of 3T3 cells.

Extracts of C2 and C2F cells were prepared from exponentially growing cultures and were found, as expected, to contain very little TK activity (Table 1). The deficiency in TK was reflected in vivo by the negligible ability of growing C2 or C2F cells to incorporate radioactive thymidine into their DNA (Table 2).

To detect whether the deficiency in kinase activity of these lines could have been due to the presence of inhibitors of enzymatic activity, extracts from TK^+ and TK^- cells were combined and the mixture was tested for kinase activity. The results of such an experiment showed that an extract of TK^- cells did not inhibit the enzymatic activity of an extract of TK^+ cells (Table 3). This finding is consistent with the fact that somatic cell hybrids obtained by co-cultivating the TK^- 3T3 sublines with BHK cells display TK activity.

Infection of TK-deficient 3T3 cells with polyoma virus. Wild-type 3T3 cells were very susceptible to infection by polyoma virus and supported viral multiplication. The burst size was on the order of 2,000 plaque-forming units (PFU). Kinetics of virus multiplication were similar to those obtained in mouse kidney cells (2), but the growth

TABLE 1. *Thymidine kinase activity of 3T3 sublines*

Cells	Enzyme units[a]	Per cent
3T3	1,310	100
C2	25	1.9
C2F	49	3.7

[a] Picomoles of thymidine phosphorylated per milligram of protein in 10 min at 37 C.

TABLE 2. *Thymidine incorporation into DNA of 3T3 sublines*[a]

Expt	Cells	Radioactivity per 10^6 cells per hr	Per cent
		counts/min	
I	3T3	837	100
	C2	15	1.8
II	3T3	1,945	100
	C2F	18	0.92

[a] In experiments 1 and 2, respectively, exponentially growing cells were exposed to ^{3}H- or ^{14}C-thymidine (5×10^{-6} M, 0.25 μc/ml) for variable lengths of time and then were treated as described under Materials and Methods.

TABLE 3. *Thymidine kinase activity of mixed extracts from TK⁻ and TK⁺ 3T3 cells*[a]

Source of enzyme	Enzyme units[b]	Per cent
3T3 (TK^+)	1,212	100
C2 (TK^-)	26	2.1
3T3 (0.5 volume) + C2 (0.5 volume)	588	48.5

[a] 3T3 and C2 cell extracts containing the same amount of protein were combined and kept at 4 C for 15 min. The mixture was then assayed for enzymatic activity.

[b] Picomoles of thymidine phosphorylated per milligram of protein in 10 min at 37 C.

cycle was somewhat faster, especially when growing, rather than stationary cultures, were infected.

The TK-deficient 3T3 sublines remained highly susceptible to polyoma virus infection. As in the case of wild-type 3T3 cells, the frequency of productively infected cells in a single-step growth cycle varied with the multiplicity of infection and was generally about 50 to 70% at a multiplicity of 50 to 100 PFU/cell. Viral yields were also comparable to those of wild-type 3T3 cells. In a typical experiment, the infectious virus yield of a culture containing 2×10^6 C2 cells and infected at a multiplicity of 50 was 7×10^6 PFU at 18 hr (eclipse phase), 3.5×10^8 PFU at 27 hr, and 7.5×10^9 PFU at 48 and 72 hr after infection.

Polyoma virus infection of stationary wild-type 3T3 cells led to a marked increase in the incorporation of radioactive thymidine into DNA, which resulted from the induction both of DNA synthesis and of TK activity. The incorporation reached its maximal rate between 15 and 30 hr after infection. If infection of TK^- 3T3 cells by polyoma virus induces the appearance of kinase activity, we would expect to find a considerable incorporation of ^{3}H-thymidine into the DNA of the infected TK^- cultures. Accordingly, C2 cells were infected with polyoma virus and exposed to ^{3}H-thymidine for 5-hr periods, from 0 to 30 hr after infection (Table 4). There was no increase in thymidine incorporation in the infected cultures over the negligible level of the uninfected cultures, suggesting that the infection did not lead to an induction of TK activity.

In another series of experiments, the TK activity of extracts of infected and uninfected cells was assayed in vitro. As described for mouse kidney cells (7), infection by polyoma virus of stationary 3T3 cells led to a marked increase in TK activity (Table 5). Under our experimental conditions, a 5- to 20-fold increase in TK activity took place in TK^+ 3T3 cells after polyoma infection. Maximal levels of enzyme activity were reached at about 25 hr after infection. In the case of C2 and C2F cells, extracts of infected cells did not reveal any increase in enzymatic activity over those of noninfected cultures. This was the case when the infection took place in stationary or growing TK^- cells. The results, therefore, show that polyoma virus is unable to induce TK activity in enzyme-deficient cells.

Further evidence supporting this conclusion was gained by comparing the yields of infectious

TABLE 4. *^{3}H-thymidine incorporation into DNA of TK^- and TK^+ 3T3 cells after infection*[a] *with polyoma virus*

Duration of pulse[b] (hr after infection)	Radioactivity (counts per min per 10^6 cells)					
	C2 (TK^-)[c]		3T3 (TK^+)[c]		3T3 (TK^+) stationary	
	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
0–5	36	39				
5–10	32	29				
10–15	55	34			1,426	253
15–20	25	39	4,630	1,880	3,537	238
20–25	28	64	5,470	1,850	6,566	153
25–30	28	42			6,270	150

[a] Multiplicity of infection = 100 PFU/cell.

[b] A 1.5-μc amount of ^{3}H-thymidine (10 c/mmole) and 25 nmoles of unlabeled thymidine were added to each culture.

[c] Slowly growing.

virus obtained by infecting TK^+ or TK^- cells in the presence of drugs which specifically interfere with DNA synthesis or template activity (Table 6). BUDR, at concentrations as low as 5 μg/ml, practically suppressed the production of infectious virus in TK^+ cells. In these cells, BUDR is phosphorylated and incorporated into viral DNA, thereby exerting its lethal effect. On the other hand, BUDR had no effect in cells which lack the phosphorylating enzyme (TK), since the drug is not incorporated. Comparison of the yields of infectious virus of TK^+ and TK^- 3T3, in the presence of BUDR (30 μg/ml), showed that BUDR greatly reduces the infectious yield by TK^+ cells but is without effect on the yield by TK^- cells. On the other hand, virus production in TK^- cells is drastically reduced when the cells are exposed to medium containing aminopterin, hypoxanthine, and thymidine (HAT; 13). Aminopterin blocks the endogenous synthesis of thymidylic acid, and DNA synthesis can proceed only by utilization of the thymidine present in the medium. Since this can be performed only when the cells contain TK, viral infection evidently did not lead to production of the enzyme. As expected, wild-type 3T3 cells possessed sufficient TK to yield normal amounts of virus under these conditions.

If the ability to lead to synthesis of the enzyme were a property of only a small minority of the virus population, their existence would have escaped detection by these methods. This possibility was tested by plating up to 10^4 PFU of polyoma virus on TK^- 3T3 cells in the presence of HAT medium. No plaques were obtained, although, as expected, HAT medium did not decrease the efficiency of plating of the virus in TK^+ 3T3 cells. The ability to induce TK synthesis is therefore not possessed by even a small minority of the virus population (10^{-4}). In these experiments, it was also confirmed that BUDR does not change the efficiency of plating of the virus on TK^- cells and abolishes the efficiency of plating on TK^+ cells.

It was considered of interest to test whether the infection of TK-deficient 3T3 cells by vaccinia virus, which is known to possess the genes for TK (4), would lead to the appearance of kinase activity. As expected, infection of C2F cells with vaccinia virus (strain WR) led to a considerable induction of enzymatic activity, which reached values of over 1,600 enzyme units between 4 and 6 hr after infection. (The activity of uninfected cells was 50 enzyme units.)

Induction of cellular DNA synthesis by polyoma virus in TK^- cells. Infection by polyoma virus of contact-inhibited mouse kidney cell cultures, which have a very low DNA-synthesizing activity, leads to the induction of both cellular and viral DNA synthesis (2, 6, 20, 22). The same phenomenon could be observed when stationary 3T3 cells were infected by polyoma virus.

To determine whether TK^- 3T3 cells would also show an induction of cellular DNA synthesis upon infection by polyoma virus, confluent cultures of C2F cells were infected at a multiplicity of 100 PFU/cell. DNA synthesis was then meas-

TABLE 5. *Thymidine kinase activity of wild type and TK^- 3T3 after infection with polyoma virus*

Cells	Time after infection	Enzyme units	
		Uninfected cells	Infected cells
	hr		
Wild-type 3T3 stationary[a]	18	184	605
	26	239	1,080
Wild-type 3T3 stationary[b]	25	114	1,916
C2F stationary[b]	25	60	59
C2 growing[a]	17	13	8.1
	25	11.8	14

[a] Multiplicity of infection, 100 PFU/cell.
[b] Multiplicity of infection, 150 PFU/cell.

TABLE 6. *Growth of polyoma virus in TK^- and TK^+ 3T3 in the presence of selective inhibitors*[a]

Expt	Host cell	Multiplicity of infection (PFU/cell)	Virus yield (PFU/culture)		
			Standard medium	BUDR[b] medium	Aminopterin[c] medium
I	C2 (TK^-)	100	1.6×10^9	1.3×10^9	4×10^7
II	C2 (TK^-)	20	1.5×10^8	1.5×10^8	6×10^6
	3T3 (TK^+)	20	4.5×10^8	4×10^6	5×10^8

[a] Infected cells were overlaid with medium containing the desired drugs at the end of the adsorption period. Yield was determined at 32 hr after infection in experiment I, and at 36 hr after infection in experiment II. The virus content of the cultures at 16 hr (eclipse phase), in experiment II, was 8×10^6 PFU.
[b] A 30 μg/ml amount.
[c] Aminopterin (10^{-5} M), thymidine (4×10^{-5} M), hypoxanthine (10^{-4} M), glycine (10^{-5} M).

ured by determining the amount of $^{32}PO_4$ or ^{3}H-cytidine incorporated into DNA. A considerable induction of DNA synthesis was observed, the rate reaching levels of 20 to 30 times the values of uninfected cultures. Pulsing the cells at different times after infection showed that the kinetics of induction were similar to those observed in TK$^+$ 3T3 cells (induction was first noticeable at about 12 to 14 hr after infection and reached its maximal rate at about 25 hr; *see* also Table 4).

Band centrifugation (19) of the DNA synthesized in infected resting C2F cultures showed that it consisted predominantly of cellular DNA (78% cellular DNA and 22% viral DNA in cells labeled from 22 to 28 hr after infection). Therefore, the situation does not differ from that observed in TK$^+$ 3T3 or in mouse kidney cells (2, 6, 20, 22). Since no increase in kinase activity appeared in these experiments, TK is not required for the induction of host DNA synthesis by polyoma virus.

DISCUSSION

As shown in this paper, it was possible to isolate 3T3 cell variants which are deficient in TK activity. These lines were isolated on the basis of their resistance to BUDR, as has already been done for TK$^-$ variants of other mammalian cells (11). They contain a very low TK activity and have an even lower rate of incorporation of radioactive thymidine into DNA.

These cell variants were used to answer the question of whether the TK induced by polyoma virus in infected cells is coded for by the viral or by the host cell genome. As mentioned before, experiments performed with TK$^-$ BHK cells and polyoma had shown quite conclusively that these cells did not acquire the enzyme when infected and transformed by the virus (14). There were two possible interpretations of these experiments: (i) polyoma does not code for TK; (ii) TK synthesis is a viral function not expressed in the transforming interaction. The second interpretation found some support when other authors demonstrated that a specific enzyme was induced by polyoma (16, 17) and SV40 (8, 12) only in productively infected cells. Since the TK-deficient 3T3 lines retained the high susceptibility of the parental cells to productive infection by polyoma virus, we would have expected that, if the enzyme were coded for by the viral genome, infection of either stationary or growing TK$^-$ 3T3 cells would have led to the appearance of demonstrable kinase activity. None appeared, although in most of the experiments the majority of the cells were productively infected and showed, when infected in the stationary phase, the characteristic induction of cellular DNA synthesis produced by the virus.

A possibility which had to be considered was that the TK$^-$ cell lines possessed inhibitors of enzyme synthesis or activity. Since this was ruled out by the mixing experiments and the experiments with vaccinia virus, the results strongly support the hypothesis that the "polyoma specific" TK is not coded for by a viral gene. Thus, the induction of TK activity which is found in polyoma-infected TK$^+$ cells probably reflects the increased synthesis of a cellular enzyme, perhaps modified to some extent by a viral product.

In conclusion, it should be added that, since the experiments reported in this paper were performed with polyoma virus, the possibility remains that the situation could be different for SV40. However, the TK$^-$ 3T3 can be transformed by SV40 and, as in the polyoma-BHK system (14), TK is not acquired by the transformed cells.

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