Enzyme Induction in Green Monkey Kidney Cultures Infected with Simian Adenovirus

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Thymidine kinase was induced after infection of an established strain of green monkey kidney cells (CV-1) with simian adenovirus SV15. Increased levels of thymidine kinase were first observed 8 to 10 hr postinoculation (PI), and the levels increased four- to eightfold by 16 to 24 hr PI. A transient increase (1.5- to 3-fold) of deoxyribonucleic acid (DNA) polymerase activity was also observed about 18 hr PI, but the level of deoxycytidylic deaminase was not enhanced. The inductions of thymidine kinase and DNA polymerase were not obtained when protein synthesis was inhibited with 10^{-5} M cycloheximide. However, the enzyme increases did take place when infected cultures were treated with 1- β -D-arabinofuranosylcytosine (ara-C), an inhibitor of DNA synthesis and SV15 replication. The incorporation of tritium-labeled thymidine (H^3 -dT) into DNA was also stimulated 8 to 24 hr after infection with SV15.

Thymidine kinase activity was enhanced in green monkey kidney (GMK) cells after infection by simian adenoviruses SV15 and SV32 (8). This communication describes the kinetics of thymidine kinase induction and the effects of inhibitors of protein and deoxyribonucleic acid (DNA) syntheses on thymidine kinase induction. Two other enzymes concerned with DNA biosynthesis, DNA polymerase and deoxycytidylic (dCMP) deaminase, were also studied, as well as the incorporation of tritium-labeled thymidine (H^3 -dT) into DNA of SV15-infected monkey kidney cells.

MATERIALS AND METHODS

Cell cultures. CV-1 cells, an established strain of GMK cells (3), and primary cultures of GMK cells were grown in monolayer cultures as previously described (6).

Virus. Clonal strain (202 A) of simian adenovirus SV15 was used (8). Small, DNA-containing particles were absent from stocks of this virus. SV15 was propagated in monolayer cultures of CV-1 cells in "R5a" medium containing 5 or 10% fetal calf serum (2). SV15 was assayed by plaque formation in monolayer cultures of CV-1 cells. Overlay medium contained "R5a" medium with 0.5% lactalbumin hydrolysate, 0.1% yeast extract (Difco), 2% fetal calf serum, and 1% agar. Neutral red overlay was added 6 days postinoculation (PI), and plaques were counted 7 to 10 days PI.

Growth of SV15 in CV-1 cells. Six-day-old monolayer cultures containing 6×10^6 CV-1 cells in 8-oz

prescription bottles were infected with 5-ml portions of SV15 [334 plaque-forming units (PFU) per cell]. Virus was allowed to adsorb for 2 hr at 37 C, after which extracellular virus was removed by washing the cultures six times with 20-ml portions of Hanks balanced salt solution (BSS). A 20-ml amount of R5a medium containing 5% fetal calf serum was added to each bottle. At the indicated times, two cultures were removed from the incubator and stored at -20 C. The cells and supernatant fluid from each culture underwent sonic treatment for 1 min, and were assayed for virus in CV-1 monolayers.

Infection of cells for biochemical experiments. The culture medium was discarded, and 2 to 6 ml of virus suspension was added to each 8-oz bottle culture of CV-1 cells. The cultures were incubated for 2 hr at 37 C to permit virus adsorption, during which time they were rocked approximately every 20 min. The fluid was discarded, and 20 ml of R5a medium with 10% calf serum was added. The cultures were incubated at 37 C for the indicated times, and then washed with saline-glucose solution. The cells were removed in glucose-saline solution containing 0.05% trypsin (1:300; Nutritional Biochemicals Corp., Cleveland, Ohio) and 0.05% ethylenediaminetetraacetic acid (EDTA). Control bottles were treated similarly, except that 2 to 6 ml of either R5a medium or spent medium (medium removed from 4- to 7-day-old cultures) was substituted for the virus preparation.

Preparation of enzyme extracts. Extracts containing thymidine kinase and dCMP deaminase activities were prepared as described previously (6), except that the extraction buffer contained 0.31 mM deoxycytidine-5'-triphosphate (dCTP) to activate and stabilize the dCMP deaminase. However, for all experiments in which DNA polymerase activity was

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measured, the following procedure was employed for preparing enzyme extracts. Groups of 20 to 50 million cells were harvested by low-speed centrifugation, washed with BSS, and suspended in 7 volumes of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.8) containing 0.003 M 2-mercaptoethanol (about 2×10^7 cells per milliliter). The cells were frozen, thawed, and sonic-treated for 3 min at 4 C with a Raytheon sonic oscillator at 10 kc. The sonic-treated material was centrifuged for 1 hr at 4 C at $34,800 \times g$, and the supernatant fluid was used for enzyme assays. The protein content of the centrifuged extract was determined by the method of Lowry et al. (10).

Modification of previous procedures (6), so as to include the freezing-and-thawing step and the longer sonic-treatment time, was necessary for the optimal release of DNA polymerase activity into the supernatant fraction. When the enzyme extracts were prepared as described above, the supernatant fraction from centrifuged extracts contained all of the DNA polymerase activity present in the crude, sonic-treated material. However, when the freezing-and-thawing step was omitted, about 20 to 50% of the DNA polymerase activity was found in the pellet fraction after centrifugation.

Enzyme assays. Tritium-labeled deoxyuridine (H^3 -dU) was used as nucleoside substrate in the thymidine kinase assay (4). The assays for DNA polymerase (7a) and dCMP deaminase (8a) have been described.

Incorporation of H^3 -dT into DNA. At various times after infection with SV15, replicate cultures (20 ml) were inoculated with 0.2 ml of H^3 -dT (40 μ C and 40 μ g) and incubated for 2 hr at 37 C with frequent agitation. Noninfected cultures were treated similarly. After the 2-hr pulses, the cultures were washed with saline-glucose solution and were trypsinized (0.05% trypsin and 0.05% EDTA). A portion of the resulting cell suspension was used for radioautography, and the specific activity of the DNA was determined on the remainder of the cells (7).

Materials. Tritium-labeled thymidine triphosphate (H^3 -dTTP) and H^3 -dCMP were obtained from Schwarz Bio Research Inc., Orangeburg, N.Y., and H^3 -dT was obtained from New England Nuclear Corp., Boston, Mass. Cycloheximide and 1- β -D-arabinofuranosylcytosine (ara-C) were obtained from Nutritional Biochemicals Corp.

RESULTS

Growth of SV15 in CV-1 cells. The growth of SV15 in CV-1 cells is shown in Fig. 1. The eclipse phase lasted about 14 hr, and then virus titers increased rapidly until 32 hr. Cytopathic changes were apparent 16 to 18 hr after inoculation.

Induction of thymidine kinase activity. Very low levels of thymidine kinase activity were observed in confluent, noninfected cultures of CV-1 cells, and the enzyme level remained low for 24 hr after the medium was changed (Fig. 2). After infection of CV-1 cells with SV15, four- to eightfold increases in thymidine kinase were obtained. In-

FIG. 1. Growth of SV15 in CV-1 cells.

FIG. 2. Effect of SV15 infection on thymidine kinase and DNA polymerase activities of CV-1 cells. Replicate 9-day-old cultures (6.9×10^6 cells per culture) were inoculated with 121 PFU of SV15 per cell. DNA polymerase activity is expressed as micromicromoles of H^3 -dTTP incorporated into DNA per microgram of protein in 30 min at 38 C. Thymidine kinase activity is expressed as micromicromoles of dUMP formed per microgram of protein in 10 min at 38 C.

creases in thymidine kinase activity were first detected 9 to 12 hr PI; the enzyme level increased rapidly until about 15 to 20 hr PI, and then declined (Fig. 2, Table 1).

DNA polymerase activity. Noninfected cultures of CV-1 cells displayed low levels of DNA polym-

TABLE 1. *Effect of cycloheximide (10^{-5} M) on the induction of thymidine kinase and DNA polymerase activities in SV15-infected CV-1 cell cultures^a*

Time PI of enzyme assay	Cycloheximide (CH) treatment ^b	Thymidine kinase activity ^c		DNA polymerase activity ^d	
		Noninfected	SV15-infected	Noninfected	SV15-infected
<i>hr</i>					
9	No CH added	0.8	1.3	0.4	0.4
16	No CH added	0.9	3.1	0.6	0.9
	CH added	0.5	0.6	0.3	0.4
18	No CH added	0.8	6.8	0.5	1.4
	CH added	0.4	0.4	0.4	0.4
20	No CH added	1.2	7.6	0.6	1.4
	CH added	0.6	0.5	0.5	0.4

^a Nine-day-old cultures (6.4×10^6 cells per culture) inoculated with approximately 100 PFU of SV15 per cell.

^b Where CH addition is specified, the CH was added at 9 hr PI.

^c Micromicromoles of dUMP formed per microgram of protein in 10 min at 38 C.

^d Micromicromoles of H^3 -dTTP incorporated into DNA per microgram of protein in 30 min at 38 C.

TABLE 2. *Deoxycytidylic deaminase and thymidine kinase activities of SV15-infected monkey kidney cell cultures*

Cell culture ^a	Time PI of enzyme assay	Deoxycytidylic deaminase activity		Thymidine kinase activity	
		Non-infected	SV15-infected	Non-infected	SV15-infected
	<i>hr</i>				
CV-1	18	500 ^b	500 ^b	2.2 ^b	5.2 ^b
	29	510	490	1.7	3.0
GMK	10	240	320	0.7	1.0
	15	310	310	0.8	1.7
	25	320	290	1.0	2.4
	31	360	300	0.7	2.7

^a Seven-day-old CV-1 cells (9×10^6 cells per culture) and 10-day-old primary GMK cell cultures (12×10^6 cells/culture) were inoculated with 36 and 31 PFU of SV15 per cell, respectively.

^b Results expressed as micromicromoles of dUMP formed per microgram of protein in 10 min at 38 C.

erase activity. After infection by SV15, a 1.5- to 3.0-fold increase in the activity of this enzyme occurred about 18 hr PI (Fig. 2, Table 1). This increase was detected about 4 hr later than the initial increase in thymidine kinase activity, and was of short duration. By about 22 to 24 hr after infection, the DNA polymerase activity usually declined to the level found in noninfected cell cultures.

Deoxycytidylic deaminase activity. Enzyme extracts prepared from noninfected CV-1 or GMK cells in buffer containing dCTP exhibited extremely high levels of dCMP deaminase activity. The activity of dCMP deaminase, per unit of

TABLE 3. *Inhibition of SV15 replication in CV-1 cells by 1- β -D-arabinofuranosylcytosine (ara C)^a*

Time PI of enzyme assay	Plaque-forming units per ml		
	No ara-C	5 μ g of ara-C per ml	10 μ g of ara-C per ml
<i>hr</i>			
6	1.4×10^5	1.1×10^5	1.0×10^5
24	2.0×10^7	3.4×10^4	4.5×10^4
48	6.8×10^7	3.3×10^4	2.6×10^4

^a Seven-day-old CV-1 cells (4.8×10^6 per bottle) were inoculated with 26 PFU of SV15 per cell. Ara-C was added to the drug-treated cultures at 2 hr after infection.

protein, was about 300 and 600 times greater, respectively, than that of thymidine kinase and DNA polymerase (Tables 1 and 2). No increase in dCMP deaminase activity was obtained after SV15 infection. For control purposes, the activity of thymidine kinase was measured on the same extracts used to assay dCMP deaminase. Although dCMP deaminase activity did not increase, that of thymidine kinase was stimulated in the SV15-infected cultures (Table 2).

Effect of inhibition of protein synthesis on the enzyme increases. Incorporation of C^{14} -labeled amino acids into the proteins of GMK cells was inhibited by 95% within 2 hr after the addition of cycloheximide (10^{-5} M) to the cultures. This inhibition persisted for at least 26 hr. To learn whether protein synthesis was essential for induction of thymidine kinase and DNA polymerase activities, SV15-infected cultures were incubated for various periods of time with 10^{-5} M cyclohexi-

TABLE 4. Effect of ara-C (10 µg/ml)^a on the induction of thymidine kinase and DNA polymerase activities in SV15-infected CV-1 cell cultures

Expt ^b	Time PI of enzyme assay	Treatment of cells	Thymidine kinase activity ^c		DNA polymerase activity ^d	
			Noninfected	SV15-infected	Noninfected	SV15-infected
I	2	none				0.3
	16	none	0.7	1.9	0.6	0.6
		ara-C	1.3	2.3	0.8	0.6
	18	none			0.7	1.1
		ara-C			0.8	1.3
	20	none			0.6	1.3
II		ara-C			0.7	1.3
	2	none		0.8		0.3
	24	none	0.9	2.6	0.6	0.7
		ara-C	3.1	7.2	1.0	1.6

^a Ara-C was added to cultures at 2 hr PI.
^b Experiment I: 9-day-old CV-1 cultures (6.4 × 10⁶ cells per culture) were inoculated with 88 PFU of SV15 per cell; experiment II: 7-day-old CV-1 cultures (6.8 × 10⁶ cells per culture) were inoculated with 57 PFU of SV15 per cell.
^c Micromicromoles of dUMP formed per microgram of protein in 10 min at 38 C.
^d Micromicromoles of H³-dTTP incorporated into DNA per microgram of protein in 30 min at 38 C

FIG. 3. Incorporation of H³-dT into DNA of SV15-infected CV-1 cells. Replicate 7-day-old cultures (9.2 × 10⁶ cells) were inoculated with 152 PFU of SV15 per cell and were incubated at 37 C. At the indicated times, H³-dT (2 µc and 2 µg per ml) was added, and the cultures were incubated for an additional 2 hr at 37 C.

mid. When added at 9 hr PI, cycloheximide inhibited the increases in enzyme activities normally obtained 16 to 20 hr PI (Table 1). These results indicated that protein synthesis was required for the increases in thymidine kinase and DNA polymerase activities to occur.

Effect of inhibition of DNA synthesis on the enzyme increases. Ara-C (5 to 20 µg/ml) has been shown to inhibit by 90% or more the incorporation of H³-dT into DNA of GMK cells (6). Ara-C also inhibited SV15 replication (Table 3). However, ara-C did not prevent the inductions of thymidine kinase, dihydrofolate reductase, and DNA polymerase in SV40-infected GMK

cells (2a, 6, 7a), or the induction of thymidine kinase and dihydrofolate reductase in polyoma-infected mouse kidney cells (5; Frearson et al, *in press*). To learn whether DNA synthesis was essential for stimulations of thymidine kinase and DNA polymerase activities in SV15-infected CV-1 cells, infected cultures were treated at 2 hr after infection with ara-C (10 µg/ml).

Ara-C has been shown previously to increase thymidine kinase activity in noninfected CV-1 cultures (6). In the present experiments, a similar increase in thymidine kinase (three- to fourfold), as well as a small increase in DNA polymerase activity, was observed in noninfected CV-1 cultures at 24 hr PI (Table 4). Ara-C, however, did not prevent the expected increases in either thymidine kinase or DNA polymerase activities after infection of CV-1 cultures with SV15 (Table 4). Hence, DNA synthesis was not essential for induction of these enzymes.

Michaelis constants (K_m) of thymidine kinase partially purified from SV15-infected CV-1 cells. Thymidine kinase from SV15-infected CV-1 cell cultures was purified 4- to 12-fold compared with crude-enzyme extracts as described previously (6), except that the ammonium sulfate precipitation step was performed after the negative calcium phosphate gel-adsorption step, and the protein fraction precipitated by 20 to 32% saturation of the solution with ammonium sulfate was collected. The K_m value for deoxyuridine of the thymidine kinase partially purified from SV15-infected cells was 5.2 ± 0.1 × 10⁻⁵ M, whereas

TABLE 5. *Radioautographic study of incorporation for 2 hr of H^3 -dT into the nuclei of SV15-infected CV-1 cells^a*

Hr PI	Percentage of nuclei labeled	
	Noninfected	SV15-infected
2-4	5.8	8.0
4-6	5.8	3.3
6-8	4.7	7.1
8-10	8.9	13.8
10-12	13.0	73.8
12-14	18.3	84.3
14-16	20.6	89.9
16-18	24.8	87.8

^a Seven-day-old CV-1 cells (9×10^6 cells per culture) were inoculated with 152 PFU of SV15 per cell. At the times indicated in the table, 0.2 ml of H^3 -dT (containing $2 \mu\text{C}$ and $2 \mu\text{g}$ of dT per ml) was added to each bottle for 2 hr.

the K_m value of the enzyme from noninfected CV-1 cells was $2.8 \pm 0.2 \times 10^{-5} \text{ M}$. It has previously been shown that there was an increase in the K_m value of thymidine kinase obtained from cell cultures infected with poxvirus and from papovirus (4, 6). The present data showed that an increase in the K_m value of thymidine kinase also occurred after adenovirus-SV15 infection.

Incorporation of H^3 -dT into DNA. To learn whether DNA synthesis was stimulated in confluent cultures of CV-1 cells after infection with SV15, "pulse"-labeling experiments were performed by use of H^3 -dT as substrate (Fig. 3). When CV-1 cultures were incubated for 2-hr pulses with H^3 -dT, the incorporation of H^3 -dT into DNA was increased between 8 and 24 hr after inoculation with SV15, coincident with the increased activities of thymidine kinase and DNA polymerase. Radioautographic experiments indicated that, during a 2-hr pulse with H^3 -dT, less than 10% of the cells in noninfected CV-1 cultures were synthesizing DNA during the first 10 hr. However, the percentage of cells in noninfected cultures displaying labeled nuclei increased to 25% by 16 to 18 hr. In the SV15-infected cultures, the percentage of cells with labeled nuclei began to increase 8 hr PI, and by 16 to 18 hr PI about 90% of the cells were incorporating H^3 -dT into the nuclei (Table 5).

DISCUSSION

This investigation demonstrated that a marked increase in thymidine kinase activity occurred in confluent cultures of monkey kidney cells infected with simian adenovirus SV15. This increase

preceded the formation of infectious virus. A transient increase in DNA polymerase activity was also observed at about the time infectious SV15 maturation began. Along with these enzyme increases, the infected monkey kidney cells exhibited an enhanced incorporation of H^3 -dT into DNA.

Neither the thymidine kinase nor the DNA polymerase activities increased when protein synthesis was inhibited by cycloheximide (Table 1). Also, the enhancement of thymidine kinase activity by SV15 did not take place when ribonucleic acid (RNA) synthesis was inhibited by actinomycin D (8). The thymidine kinase that was induced has been partially purified and shown to have a K_m value for deoxyuridine which is altered in comparison with the K_m value of the enzyme from noninfected cells. Thus, adenovirus infection causes changes in the enzymology of cell cultures analogous to those previously described for vaccinia and papovaviruses (4-6, 7a, 8).

In a study of primary cultures of monkey kidney cells infected with human adenovirus type 2, Ledinko (9) has observed an enhancement of thymidine kinase, but not of DNA polymerase, activity. Ledinko (9) also reported a 1.6- to 2.0-fold increase in dCMP deaminase activity late in the infectious cycle, that is, at 40 to 60 hr after infection.

In our laboratory, it was found that the dCMP deaminase activity [expressed as micromicromoles of deoxyuridylic acid (dUMP) formed per microgram of protein in 10 min at 38 C] of either noninfected CV-1 or noninfected primary monkey kidney cells was extremely high, provided that enzyme extracts were prepared in buffer containing dCTP to stabilize the enzyme (8a). Increases in the activity of dCMP deaminase of monkey kidney cells were not detected after SV15 infection. The enzyme activities reported by Ledinko (9) were expressed as counts per minute of product formed per microgram of protein and the specific activities of the substrates were not given, so that comparisons with the present data are difficult to make. If dCMP deaminase extracts are prepared in the absence of dCTP, relatively low enzyme activities are obtained and an apparent increase may be seen after virus infection, possibly due to an intracellular stabilization of the enzyme. Perhaps this is the reason that Ledinko (9) observed an increase in the activity of this enzyme in the adenovirus-infected GMK cell cultures.

It is to be emphasized that the increases in thymidine kinase and DNA polymerase activities observed in this study took place even when DNA synthesis, cell growth, and virus replication were

inhibited by ara-C treatment. In this respect, the increases differ from the enhancement of aspartic transcarbamylase activity in HeLa cells infected with human adenovirus type 5 reported by Consigli and Ginsberg (1). These investigators observed that aspartic transcarbamylase activity did not increase when DNA synthesis was prevented.

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LITERATURE CITED

1. CONSIGLIA, R. A., AND H. S. GINSBERG. 1964. Activity of aspartate transcarbamylase in uninfected and type 5 adenovirus-infected HeLa cells. *J. Bacteriol.* **87**:1034-1043.
2. DUBBS, D. R., AND S. KIT. 1965. The effect of temperature on induction of deoxythymidine kinase activity by herpes simplex mutants. *Virology* **25**:256-270.
- 2a. FREARSON, P. M., S. KIT, AND D. R. DUBBS. 1966. Induction of dihydrofolate reductase activity by SV40 and polyoma virus. *Cancer Res.* **26**:1653-1660.
3. JENSEN, F. C., A. J. GIRARDI, R. V. GILDEN, AND H. KOPROWSKI. 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. *Proc. Natl. Acad. Sci. U.S.* **52**:53-59.
4. KIT, S., AND D. R. DUBBS. 1965. Properties of deoxythymidine kinase partially purified from noninfected and virus-infected mouse fibroblasts. *Virology* **26**:16-27.
5. KIT, S., D. R. DUBBS, AND P. M. FREARSON. 1966. Enzymes of nucleic acid metabolism in cells infected with polyoma virus. *Cancer Res.* **26**:638-646.
6. KIT, S., D. R. DUBBS, P. M. FREARSON, AND J. L. MELNICK. 1966. Enzyme induction in SV40-infected green monkey kidney cultures. *Virology* **29**:69-83.
7. KIT, S., D. R. DUBBS, AND T. C. HSU. 1963. Biochemistry of vaccinia-infected mouse fibroblasts (strain L-M). III. Radioautographic and biochemical studies of thymidine- H^3 uptake into DNA of L-M cells and rabbit cells in primary culture. *Virology* **19**:13-22.
- 7a. KIT, S., D. R. DUBBS, L. J. PIEKARSKI, R. A. DE TORRES, AND J. L. MELNICK. 1966. Acquisition of enzyme function by mouse kidney cells abortively infected with papovavirus SV40. *Proc. Natl. Acad. Sci. U. S.* **56**:463-470.
8. KIT, S., D. R. DUBBS, R. A. DE TORRES, AND J. L. MELNICK. 1965. Enhanced thymidine kinase activity following infection of green monkey kidney cells by simian adenoviruses, simian papovavirus SV40 and by an adenovirus-SV40 "hybrid." *Virology* **27**:453-457.
- 8a. KIT, S., R. A. DE TORRES, AND D. R. DUBBS. 1966. Arabinofuranosylcytosine-induced stimulation of thymidine kinase and deoxycytidylic deaminase activities of mammalian cultures. *Cancer Res.* **26**:1859-1886.
9. LEDINKO, N. 1966. Changes in metabolic and enzymatic activities of monkey kidney cells after infection with adenovirus 2. *Virology* **28**:679-692.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the folin-phenol reagent. *J. Biol. Chem.* **193**:265-275.