

# Effects of Cycloheximide and Puromycin on Synthesis of Simian Virus 40 T Antigen in Green Monkey Kidney Cells

R. V. GILDEN<sup>1</sup> AND R. I. CARP

*Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania*

## ABSTRACT

GILDEN, R. V. (Wistar Institute, Philadelphia, Pa.), AND R. I. CARP. Effects of cycloheximide and puromycin on synthesis of simian virus 40 T antigen in green monkey kidney cells. *J. Bacteriol.* **91**:1295–1297. 1966.—Synthesis of the simian virus 40 (SV40) T antigen in primary African green monkey kidney cells was abolished when cycloheximide was added up to 10 hr postinfection. In contrast, puromycin, another inhibitor of protein synthesis, did not suppress antigen production. The basis of this differential effect was the inability of puromycin to inhibit protein synthesis in the cells used. This was shown by the failure of the drug to depress the incorporation of labeled amino acid into protein and also failure to inhibit poliovirus synthesis. The puromycin preparation used was very effective in inhibiting poliovirus synthesis in HeLa cells. Thus, appearance of the SV40 T antigen is dependent on protein synthesis in infected cells.

A number of compounds which appear to inhibit distinct biosynthetic events selectively are currently in wide use. However, variations in cellular susceptibility to these "standard" compounds often occur, thus necessitating control experiments to verify at least the gross action of any drug used in new situations. The present report concerns the synthesis of the non-virion complement-fixing antigen induced by simian virus 40 (SV40; 1) in African green monkey kidney cells (GMK) and the differential effects of two inhibitors of protein synthesis, puromycin and cycloheximide (4), on this process. The term T (tumor) antigen is synonymous with ICFA (induced complement-fixing antigen) used in other publications from the Wistar Institute, and is used here in the interest of uniform nomenclature.

## MATERIALS AND METHODS

Primary African green monkey kidney cells were planted in 60-mm plastic petri dishes containing 11 by 22 mm cover slips, and were incubated in an atmosphere of 4% CO<sub>2</sub> in air. The growth medium consisted of Earle's salt solution containing Eagle's medium plus 10% calf serum. After reaching confluence, cultures were infected with SV40 (strain RH-911, courtesy of A. J. Girardi) at a multiplicity of 3 to 10. After virus adsorption (2 hr at 37 C), the

inoculum was replaced with maintenance medium (Earle's salt solution containing Eagle's medium plus 2% calf serum).

Immunofluorescent staining was carried out by the direct method by use of a fluorescein isothiocyanate-labeled  $\gamma$ -globulin preparation obtained from hamsters immunized with extracts of SV40-induced transplant tumors (virus-free). Methods of washing, fixing, mounting, and examination have been described (5). The staining characteristics of this reagent do not differ from those obtained by the indirect technique (6, 8).

The protein inhibitors, puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio) or cycloheximide (Acti-dione, The Upjohn Co., Kalamazoo, Mich.), were incorporated into the maintenance medium at various concentrations, either immediately after virus adsorption or at specified times thereafter.

Protein synthesis was estimated by the incorporation of C<sup>14</sup>-valine into material insoluble in cold 5% trichloroacetic acid. Precipitates were collected on glass-fiber filters (type E; Gelman Instrument Co., Ann Arbor, Mich.) under vacuum, washed with 5% trichloroacetic acid, dried, and then analyzed for radioactivity by use of a liquid scintillation counter (Packard Tricarb).

The kinetics of viral deoxyribonucleic acid (DNA) synthesis were inferred from infectious virus yields obtained in 72-hr cultures which had been exposed to 5-fluorodeoxyuridine (FUDR) at 50  $\mu$ g/ml for varying periods of time. The plaque assay method for SV40 has been described (2).

The efficacy of the puromycin preparation was

<sup>1</sup> Present address: Flow Laboratories, Rockville, Md.

tested by its ability to inhibit the synthesis of poliovirus (type 1, Mahoney strain) in HeLa cells (10). At specified times, cells were scraped into the medium. The cell suspension was frozen and thawed twice and then was titrated for poliovirus by a plaque assay method (3) in Rhesus monkey kidney cells.

### RESULTS

In the absence of inhibitors, the maximal percentage of T antigen-positive cells (80 to 100%) was reached by 40 to 48 hr postinfection. The effects of the two protein synthesis inhibitors were evaluated on cover slips removed at 48 hr, and the results are expressed as a percentage of the untreated 48-hr value. As shown in Fig. 1, cycloheximide completely inhibited T antigen production when added up to 10 hr postinfection. Synthesis of antigen appeared to begin at 10 to 12 hr and continued to a maximum at about 40 hr. The synthesis of viral DNA, based on FUDR-insensitive virus production, began at 22 to 24 hr postinfection, and thus lags behind T antigen synthesis by 10 to 14 hr. Since the complete inhibition of virus production obtained with FUDR

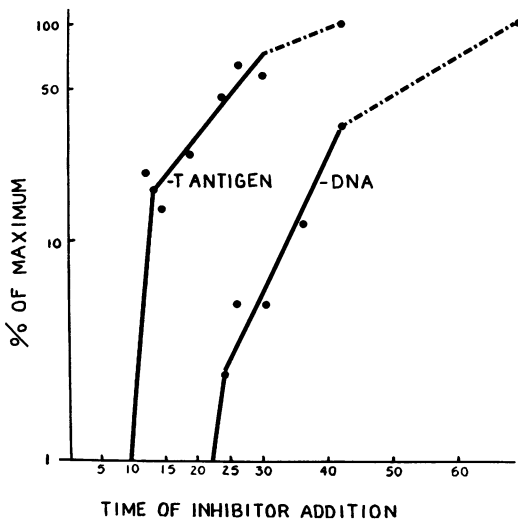


FIG. 1. Synthesis of SV40 T antigen and virus DNA in cycloheximide and FUDR-treated GMK cells. Cycloheximide (5  $\mu\text{g}/\text{ml}$ ) was added to SV40-infected GMK cultures at various times after infection. At 48 hr postinfection, cover slip cultures were analyzed for SV40 T antigen positive cells as described in the text. Other cultures were treated with FUDR (50  $\mu\text{g}/\text{ml}$ ) at various times postinfection. At 72 hr after infection, the infected cells were scraped into maintenance medium, and then frozen and thawed three times prior to plaque titration for infectious virus. The results are expressed as a percentage of the values obtained in untreated cultures at the same times of harvest.

up to 22 hr postinfection was reversible with thymidine, a direct action on viral DNA synthesis is assumed. As previously reported (5, 7), T antigen synthesis proceeded at the maximal rate in the presence of FUDR.

In contrast to the cycloheximide results, puromycin at 10  $\mu\text{g}/\text{ml}$  had no effect on T antigen synthesis, even when added immediately after virus adsorption. Similar results have recently been reported (7). However, incorporation of labeled valine into protein was determined (Fig. 2), and it was found that puromycin did not to any detectable extent suppress protein synthesis in GMK. Comparable results were obtained whether or not virus was present. In contrast, cycloheximide was rapidly effective in reducing protein synthesis by 90 to 95%.

The efficacy of the puromycin preparation was checked by following the synthesis of poliovirus in both GMK and HeLa cells. The results (Fig. 3) show that, whereas 2  $\mu\text{g}/\text{ml}$  of puromycin substantially inhibited poliovirus synthesis in HeLa cells, at least 100  $\mu\text{g}/\text{ml}$  of the drug was required to achieve comparable inhibition in GMK. The latter concentration is extremely toxic for GMK. As expected, cycloheximide at 5  $\mu\text{g}/\text{ml}$  completely prevented poliovirus replication in GMK. It thus appears quite clear that puromycin, at subtoxic doses, is ineffective as an inhibitor of protein synthesis in GMK, whereas cycloheximide is very efficient in this regard.

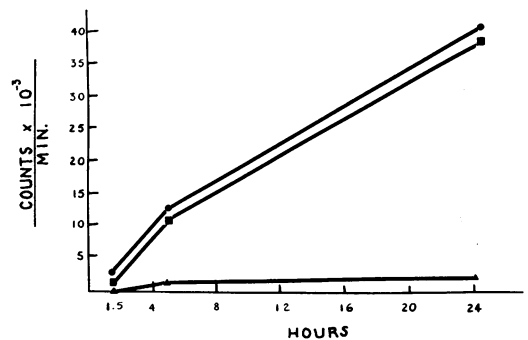


FIG. 2. Effect of puromycin and cycloheximide on protein synthesis in GMK. Parallel cultures containing puromycin (10  $\mu\text{g}/\text{ml}$ ), cycloheximide (5  $\mu\text{g}/\text{ml}$ ), or no drug were incubated in the presence of  $C^{14}$ -valine (1.5  $\mu\text{C}$  per plate). At 1.5, 5, and 24 hr, cultures were washed two times with phosphate-buffered saline and harvested by scraping into phosphate-buffered saline. After freezing and thawing, the incorporation of radioactivity into protein was determined as described in Materials and Methods. Symbols: ●, control; ■, puromycin; ▲, cycloheximide.

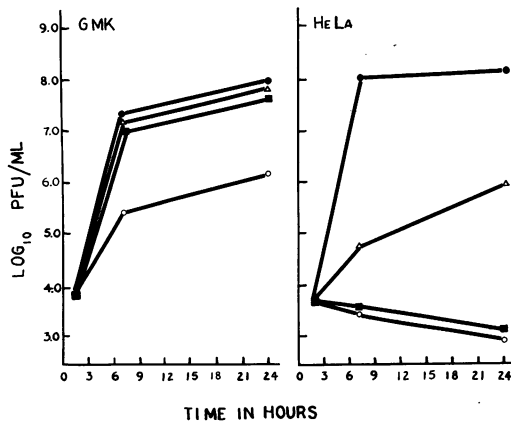


FIG. 3. Effect of puromycin on poliovirus synthesis in GMK and HeLa cells. Parallel cultures were infected with poliovirus (type 1, Mahoney strain). After adsorption, 1 hr at 37 C, the specified concentration of puromycin was added in maintenance medium. At 1.5, 7.5, and 24 hr, cultures were harvested and virus titrated as described in *Materials and Methods*. Symbols: ●, control; △, 2 µg of puromycin per ml; ■, 10 µg of puromycin per ml; ○, 100 µg of puromycin per ml.

#### DISCUSSION

The data clearly show that the appearance of SV40 T antigen in newly infected cells is dependent on protein synthesis. Along with other data on antigen production showing (i) the need for integrity of SV40-DNA (Carp and Gilden, *Virology*, *in press*) (ii) the need for DNA-dependent ribonucleic acid synthesis (7; Gilden and Carp, *in preparation*), and (iii) the occurrence of the antigen in a wide variety of cells infected with SV40 regardless of species or even class (9), this finding strongly supports the hypothesis that T antigen is a virus-related product synthesized *de novo* in infected cells. This hypothesis indicates the retention of at least that part of the viral genome concerned with T antigen synthesis in tumors and transformed cells which retain antigen but have been virus-free for extended periods of time. For example, one line of human transformed cells maintained in this Institute retains antigen but has been virus-free for nearly 3 years. The functional nature of T antigen remains unknown, and thus its role in SV40 replication and carcinogenesis remains open to speculation.

The inability of puromycin to inhibit protein synthesis in GMK was unexpected because of the well-known activity of the drug in other mammalian cells. Whether the mechanism of drug resistance is cellular "detoxification" or simply

membrane impermeability in this case is not yet known.

In view of the inactivity of puromycin in GMK, the suggestion that T antigen may be a low molecular weight protein or even a small polypeptide because of insensitivity to this inhibitor (7) is unwarranted. In addition, based on gel filtration and sucrose gradient centrifugation experiments, the T antigen appears to be of relatively high molecular weight (5). Confirmation and extension of these studies will be necessary before a definitive structure can be specified.

#### ACKNOWLEDGMENTS

We are grateful to Elizabeth Miller and James McMunn for technical assistance.

This investigation was supported by research grant E-89 from the American Cancer Society.

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