[14] Lipsett, M., *Biochem. Biophys. Res. Commun.*, **20**, 224 (1965).

[15] An apparently related phenomenon has been observed at Princeton by Lindahl, Adams, and Fresco, and is reported in the adjacent paper (941–948).

[16] Schleich, T. and, J. Goldstein, these PROCEEDINGS, **52**, 744 (1964); Röschenthaler, R., and P. Fromageot, *J. Mol. Biol.*, **11**, 458 (1965).

---

*INDUCTION OF CELLULAR DNA SYNTHESIS BY POLYOMA VIRUS, III. INDUCTION IN PRODUCTIVELY INFECTED CELLS**

BY MARGUERITE VOGT, RENATO DULBECCO, AND BASIL SMITH†

THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, SAN DIEGO, CALIFORNIA

Communicated February 18, 1966

In cultures of mouse kidney cells, polyoma virus causes a productive infection, which is characterized by viral multiplication and leads to cell death. In crowded cultures, in which there is little DNA synthesis, the virus causes a stimulation of the synthesis of cellular DNA[1–3] and of enzymes involved in DNA synthesis.[4, 5] A similar induction is produced by simian virus 40,[6] but not by viruses that do not produce tumors, hence its considerable interest as a possible factor in cell transformation caused by these viruses.

Induction of cellular DNA synthesis by polyoma virus had so far been observed under conditions where not all cells were productively infected. In contrast with these findings, Sheinin and Quinn[7] showed that in uncrowded mouse embryo cell cultures, in which practically all the cells are productively infected, induction does not occur and cellular DNA synthesis is inhibited. The suspicion thus arises that the induction occurs in cells that are not productively infected, i.e., either in abortively infected cells or, as an indirect phenomenon, in uninfected cells.

In order to clarify the significance of the induction of cellular DNA synthesis, confluent coverslip cultures of mouse kidney cells were infected with polyoma virus, and pulse-labeled with H^3-thymidine at a time at which a high proportion of the DNA synthesized was cellular. The cultures were fixed after an appropriate time of incubation to allow for the synthesis of the viral capsid protein. A combined study of the same cells by radioautography and immunofluorescence showed that at least 90 per cent of the cells synthesizing DNA at the time of the pulse had also made viral capsid antigen at the time of fixation. These results conclusively showed that the induction of cellular DNA synthesis takes place in productively infected cells.

Materials and Methods.—Virus and cell cultures: Polyoma virus of the large plaque type and primary cultures of mouse kidney cells were prepared as described previously.[1] The cells were grown on glass coverslips, 22 × 40 mm in size, inside 60-mm plastic Petri dishes. The coverslips had been boiled previously in medium supplemented with serum to allow a better spreading of the epithelial cells.

Infection of cultures: The coverslip cultures were infected with 0.4 ml of a suspension of purified virus at the appropriate dilution. After 1 hr of incubation at 37°C, the coverslip cultures were washed three times with medium and subsequently incubated at 37°C in a well-humidified incubator flushed with a CO_2-air mixture. All control coverslip cultures were mock-infected under identical conditions using medium instead of virus.

DNA synthesis: The amount of H^3-thymidine incorporated into DNA was determined from the radioactivity in TCA-precipitable material.

SDS-phenol extraction and chromatography of DNA: Two coverslip cultures were combined for each extraction. The method of extraction and the chromatography on a methylated-albumin-kieselguhr (MAK) column were the same as described previously.[1] Since the specific activity of the DNA was found to be the same before and after the process of phenol deproteinization, no preferential degradation of the labeled DNA occurred.

Immunofluorescent technique: The acetone-fixed coverslip cultures were stained with fluorescein-conjugated rabbit gamma globulin containing antibody for the polyoma virus capsid, which was kindly supplied by Dr. R. Sheinin and Dr. M. Fried. The coverslips were mounted onto slides and examined. In each coverslip an average of ten randomly selected fields were photographed in phase contrast to measure the total number of cells and in ultraviolet light to measure the number of cells with nuclei showing immunofluorescence. All cell counts were done on photomicrographs.

Radioautography: The same coverslips which had been stained with fluorescent antibody and photographed were subsequently covered with radioautographic stripping film (Kodak AR 10). After exposure for 4 days at 4°C, the slides were developed; the fields previously photographed were identified and photographed a second time to locate the radioactive nuclei. A comparison of the two sets of photographs allowed counts of nuclei with either immunofluorescence or radioactivity or both.

Results.—Proportion of cellular DNA synthesis in cells infected under different conditions: To determine the amounts of cellular and viral DNA synthesized under different conditions of infection, primary mouse kidney cells at different stages of growth were infected with several multiplicities of polyoma virus and pulse-labeled with H^3-thymidine at various times after infection. The DNA was subsequently extracted and chromatographed on a MAK column together with P^{32}-labeled viral DNA as marker. The proportion of newly synthesized cellular and viral DNA, estimated from the elution pattern,[1] was found to vary with the density of the culture, the timing of the H^3-thymidine pulse, and the multiplicity of infection. As

TABLE 1

PROPORTION OF NEWLY SYNTHESIZED CELLULAR AND VIRAL DNA UNDER VARIOUS CONDITIONS OF INFECTION

Expt. no.	Density of cultures	Virus input, PFU per culture[a]	Duration of pulse[b] (hr after infection)	TCA-Precipitable Counts per Minute per Culture[c]		Counts Incorporated per Culture[d] into		Incorporation[d] into cellular DNA, %
				Infected	Mock-infected control	Cellular DNA	Viral DNA	
1	30% covered	4×10^9	20–24	35,320	24,005	4,300	7,015	38
	30% covered	4×10^8	20–24	30,380	23,970	—	6,410	—[e]
	Confluent	4×10^8	20–24	13,260	1,400	8,780	3,080	74
2	Confluent	4×10^9	12–16	4,000	1,955	1,740	305	85
			16–20	14,485	2,305	9,990	2,190	82
			20–24	34,900	1,475	23,065	10,360	69
			24–28	37,180	1,995	21,815	13,370	62
			28–32	34,900	1,430	18,075	15,395	54
			32–36	40,900	1,545	18,495	20,860	47
		8×10^7	20–24	7,620	1,475	5,470	675	89
			24–28	15,830	1,995	11,620	2,215	84
			28–32	23,310	1,430	14,880	7,000	68
			32–36	27,520	1,545	16,365	9,610	63
			36–40	29,110	1,850	14,720	12,540	54

[a] Coverslip cultures, which were used in expt. 1, contain approximately 2×10^6 mouse kidney cells when confluent, whereas Petri dish cultures, which were used in expt. 2, contain approximately 5×10^6 mouse kidney cells when confluent.

[b] 50 μc of H^3-thymidine (1.9 c/mmole) + 10^{-5} M cold thymidine were added to each culture.

[c] Each number represents the average of two cultures.

[d] These values were calculated using only incorporated counts above those in the mock-infected controls; no correction was made for possible inhibition of the regular cellular DNA synthesis in the infected cells.

[e] The counts incorporated into cellular DNA equalled those of the mock-infected control.

shown in Table 1, the percentage of counts incorporated into cellular DNA reached a value of 50 per cent or more only in confluent cultures, in which the pulse of H^3-thymidine was given early during the rise period of DNA synthesis. The highest value was observed at the lowest multiplicity of infection.

In the studies combining radioautography and immunofluorescence, most confluent cultures were therefore infected with lower multiplicities of the virus and the pulses of H^3-thymidine given 16–24 hr after infection, i.e., at a time when a high proportion of the newly synthesized DNA could be expected to be cellular.

The data in Table 1 show that the counts incorporated into cellular DNA at the lower input multiplicity are fewer than at the high multiplicity. The ratio of the counts corresponds roughly to the ratio of the proportions of infected cells at these multiplicities (35–40% at the higher and 24–27% at the lower multiplicity; that a large decrease in input multiplicity causes only a small change in the proportion of infected cells is a known fact with this system[8]). Thus the rate of induced synthesis of cellular DNA is the same per infected cell, irrespective of multiplicity.

Relationship between induction of cellular DNA synthesis and synthesis of capsid protein in individual cells: As previously shown,[1] the synthesis of the viral capsid protein starts several hours after the onset of DNA synthesis in infected mouse kidney cells. Therefore, in order that the synthesis of viral antigen be detected in cells that were synthesizing DNA at the time of the pulse, a time interval must be allowed between the pulse and the fixation of the cultures. It was found that a suitable interval is 6–10 hr, the precise interval depending upon the multiplicity of infection. If the interval was too short (fixation 2–6 hr after the pulse), many cells showed DNA synthesis but no viral antigen; in addition, cells with the most grains showed only a weak immunofluorescence. With the optimal interval, cells with a strong incorporation of radioactive label showed also a strong immunofluorescence, as would be expected. The results are shown in Table 2.

The percentage of nuclei showing both radioactivity and immunofluorescence was 90 per cent or more, independent of the proportion of newly synthesized cellular

TABLE 2

RELATIONSHIP BETWEEN INDUCTION OF DNA SYNTHESIS AND SYNTHESIS OF CAPSID PROTEIN IN INDIVIDUAL CELLS

Expt. no.	Virus input, PFU per culture	Duration of pulse (hr after infection)	Interval between pulse and fixation in hours	Proportion of radioactive nuclei, %*	Proportion of radioactive nuclei that show fluorescence, %	Ratio of TCA-precipitable counts, infected/mock-infected control	Proportion of counts incorporated into cellular DNA, %
1	4×10^9	20–25	6	37.4 (2834)†	91.8 (885)	6.8	41
		17–25	6	36.9 (1604)	91.0 (544)	5.3	
2	4×10^8	16–20	10	24.9 (3251)	90.7 (226) 92.4 (184)	5.6	80
3	8×10^7	20–24	10	27.2 (3434)	95.6 (367) 97.4 (502)	4.7	71

Confluent coverslip cultures were infected as described in *Materials and Methods*. At the times indicated, 50 μc of H^3-thymidine (11 c/mmole) and 10^{-6} *M* thymidine were added to each culture. At the end of the pulse, the cultures were washed with medium containing cold thymidine and the cells incubated in medium containing 5×10^{-6} *M* cold thymidine for a further 6–10 hr. The coverslips were then fixed with acetone, stained with capsid-specific fluorescein-labeled antibody, photographed, coated with radioautographic stripping film, and again photographed. Total cell counts and counts of nuclei with either fluoresence or radioactivity or both were done from the two sets of microphotographs, which represent ten randomly selected fields from each coverslip.

The determination of TCA-precipitable counts and the elution profile of the extracted DNA from the MAK column were done on parallel coverslip-cultures collected at the end of the pulse.

* Proportion of radioactive nuclei in mock-infected controls varied from 4 to 7% in the three experiments.

† The numbers in parentheses refer to the total number of nuclei counted.

DNA. The 90 per cent coincidence cannot be due to chance, since the proportion of radioactive and fluorescent nuclei in the total cell population was small. At the higher multiplicity the chance coincidence would have been 14 per cent, and 6 per cent at the lower. The few nuclei which exhibited grains but no fluorescence had less than an average grain density. Therefore, since at the lower multiplicities 70–80 per cent of the newly synthesized DNA was cellular, we must conclude that induction of cellular DNA synthesis occurs in productively infected cells.

Stability of newly synthesized DNA: The conclusion that cellular DNA synthesis is induced in productively infected cells assumes that the pulse-labeled cellular DNA is stable during the time interval that elapses between the pulse of H^3-thymidine and the fixation of the cells. The correctness of this assumption was shown in two ways.

(*a*) In experiments 2 and 3 of Table 2, the proportion of nuclei incorporating H^3-thymidine was determined both in cultures fixed at the end of the pulse and in cultures fixed 10 hr later; in both cases similar values were found.

(*b*) If the radioactivity incorporated into the cellular DNA were unstable, the addition of an excess of cold thymidine would be expected to chase these counts. To test this possibility, H^3-thymidine (5×10^{-7} *M*) was added to the cultures 16 hr after infection. At 20 hr after infection, the radioactive supernatants were removed from half of the cultures and the cell sheets were washed with medium containing cold thymidine. These cells were incubated for 10 hr in medium containing 5×10^{-5} *M* cold thymidine, and then extracted with SDS and phenol. The other half of the cultures were extracted at the end of the pulse. Both extracts were fractionated on MAK columns. The number of TCA-precipitable counts found in the viral and cellular peaks is summarized in Table 3. At 30 hr after infection, very little progeny virus had been released into the culture supernatant, as shown by the small number of TCA-precipitable counts. It is clear that there was little loss of TCA-precipitable counts during the chase period and no transfer of counts from cellular to viral DNA, as would have been expected if the one were the precursor of the other.

TABLE 3

STABILITY OF THE NEWLY SYNTHESIZED CELLULAR DNA IN A CHASE EXPERIMENT

Set of plates	Duration of pulse (hr after infection)	Duration of chase, hr	Time of collection (hr after infection)	TCA-Precipitable Counts per Plate in: Viral DNA	Cellular DNA	Supernatant
Pulse only	16–20	—	20	1,250	46,000	0
Pulse and chase	16–20	20–30	30	1,050	44,000	150

Discussion.—Under the experimental conditions used, induction of cellular DNA synthesis by polyoma virus undoubtedly takes place in productively infected cells. Thus, induction is the expression of a viral function in the infected cells, as was also suggested on different grounds by Gershon *et al.*[9] The lack of induction in actively multiplying mouse embryo cells therefore cannot be attributed to the different proportion of productively infected cells. It seems more likely that it is the consequence of the different physiological state of the cells at the moment of infection. In fact, even in mouse kidney cells induction of cellular DNA synthesis may fail to occur if the cultures are sparse and slowly growing. This is seen in experiment 1 of Table 1. Similar results have been obtained with simian virus 40.[6, 10]

It therefore appears that the expression of certain viral functions is dependent upon the physiological state of the cell at the time of infection. That the effect of a tumor virus on its host cell depends upon the operation of certain cellular functions has been previously shown by Temin[11] and Bader[12] for the RNA-containing Rous tumor virus.

Summary.—A combined study by radioautography and immunofluorescence showed that the induction of cellular DNA synthesis in polyoma-infected cultures of mouse kidney cells occurs in productively infected cells.

The competent technical assistance of Mr. Paul Holdy and Mrs. Mildred Barnes is gratefully acknowledged.

* This work was supported by grant no. CA 07592 from the National Cancer Institute, National Institutes of Health.

† Postdoctoral fellow of the U.S. Public Health Service, # 1 F2-AI-30, 286-01 from the National Institute of Allergy and Infectious Diseases.

[1] Dulbecco, R., L. H. Hartwell, and M. Vogt, these PROCEEDINGS, **53**, 403 (1965).
[2] Weil, R., M. R. Michel, and G. K. Ruschmann, these PROCEEDINGS, **53**, 1468 (1965).
[3] Winocour, E., A. M. Kaye, and V. Stollar, *Virology*, **27**, 156 (1965).
[4] Hartwell, L. H., M. Vogt, and R. Dulbecco, *Virology*, **27**, 262 (1965).
[5] Sheinin, R., *Virology*, **28**, 47 (1966).
[6] Hatanaka, M., and R. Dulbecco, unpublished.
[7] Sheinin, R., and P. A. Quinn, *Virology*, **26**, 73 (1965).
[8] Weisberg, R. A., *Virology*, **23**, 553 (1964).
[9] Gershon, D., P. Hausen, L. Sachs, and E. Winocour, these PROCEEDINGS, **54**, 1584 (1965).
[10] Sauer, G., H. Fischer, and K. Munk, in preparation.
[11] Temin, H., *Virology*, **20**, 577 (1963).
[12] Bader, J. P., *Virology*, **22**, 462 (1964).

TEMPLATE ACTIVITY OF URIDYLIC ACID-DIHYDROURIDYLIC ACID COPOLYMERS

BY FRITZ ROTTMAN* AND PETER CERUTTI†

NATIONAL HEART INSTITUTE AND NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated by Seymour S. Kety, February 21, 1966

Dihydrouridylic acid (H_2Up) has been described as a minor constituent of alanine-sRNA[1] and serine-sRNA[2] isolated from yeast. An RNA fraction obtained from pea bud nucleohistones also contains dihydrouridylic acid, in this instance, representing 27 per cent of the total nucleotide composition.[3] Earlier work has demonstrated the enzymatic incorporation of dihydrouridine-5′-monophosphate into RNA[4] and more recently the DNA-dependent incorporation of dihydrouridine-5′-triphosphate into RNA.[5]

Due to its shortened π-electron system, H_2Up occupies a unique position within the minor nucleotides of natural RNA. Since this may well be reflected in its functional role, studies of the coding properties of polymers containing H_2Up are of special interest. The discovery of a convenient photochemical method of preparing partially reduced poly U (poly U/H_2U) of high molecular weight[6] has made these