STIMULATION OF DNA SYNTHESIS IN MOUSE CELL LINE 3T3 BY SIMIAN VIRUS 40

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Within the past year, reports from several laboratories have indicated that an induction of cellular DNA synthesis occurs in mouse cells infected with the polyoma virus.[1-3] Most of these virus-infected cells ultimately undergo cytolysis, and cellular transformation is a rare event.

The 3T3 mouse cell-simian virus 40 (SV40) transformation system is particularly well suited to determine whether a similar stimulation of DNA synthesis occurs during the process of cell transformation by an oncogenic DNA virus. After infection with high multiplicities of SV40, the majority of 3T3 cells become infected and form the SV40 specific T antigen, but no cytopathogenic effect occurs and neither infectious virus nor virion antigen is produced.[4] As many as 50 per cent of such cells may become transformed.[4, 5]

The present paper reports results obtained with 3T3 cells infected with SV40. A stimulation of DNA synthesis was found to occur in infected cells. While this work was in progress Gershon *et al.*[3] and Sheinen[6] reported a similar stimulation of DNA synthesis in the rat embryo cell-polyoma virus transformation system.

Materials and Methods.—Cells: The origin and growth characteristics of the 3T3 cell line have been described in detail.[7, 8] Three clones derived from the parent line were used in the present experiments. These clones, 3T3 M, 3T3 MF, and 3T3 M-2, were obtained from Dr. G. Todaro (New York University) and were used at the 19th–25th, the 7th–9th, and the 5th–6th passage levels, respectively. The media and maintenance conditions for the 3T3 cells have been previously described.[4, 9] Cells were grown in 32-oz Brockway bottles and were used from 1 to 5 days after the monolayer became confluent.

Virus: The origin and history of SV40, strain 777 (LLCMK$_2$, AGMK$_5$, BSC-1$_2$) have been given in detail elsewhere.[10] The virus pools used in the present experiments were derived from the second and third passages in a continuous line (BSC-1)[11] of primary African green monkey kidney (AGMK) cells. The virus obtained from the crude lysate of BSC-1 cells was concentrated, treated with sodium deoxycholate and trypsin (final concentrations of 1.0 and 0.01%, respectively), and clarified by methods previously described.[12] From 50 to 75 ml of virus with titers of 1–3 $\times$ 10^{10} tissue culture infectious doses$_{50}$ (TCID$_{50}$) per ml were usually obtained from approximately 2500 cc of crude suspension (titer 1–2 $\times$ 10^9 TCID$_{50}$ per ml).

Ultraviolet irradiation of virus: Three ml of virus were placed in 90-mm glass Petri dishes 16 cm beneath an ultraviolet lamp (Westinghouse Sterilamp G 36T6-L) for 18 min, with agitation every 3 min. This treatment reduced the infectivity of the virus preparations approximately 10^4-fold.

Infection of cultures: Two or 3 days prior to each experiment the medium was changed. At the start of an experiment the medium was removed from confluent monolayer cultures of 3T3 cells and saved; this medium was used later as "conditioned medium." ("Conditioned" or "spent" medium is that medium which had been in contact with 3T3 cells for 2 to 3 days. This treatment has been reported to remove a substance which is present in fresh serum and which stimulates cell division.[13]) The cultures were then infected with 1 or 2 ml of SV40 virus diluted in tris buffered saline, pH 7.2 (TBS) (expts. 1 and 2) or conditioned medium (expts. 3–7) for 2 or 3 hr with frequent gentle agitation. The excess virus was then aspirated, the monolayer rinsed with 10 ml of TBS or "conditioned medium," and 35–40 ml of fresh medium (expts. 1 and 2) or "conditioned medium" (expts. 3–7) were added to each bottle, which was then reincubated at

37°C in a 5% CO_2 atmosphere. Controls were treated similarly after being mock-infected with an equal volume of TBS or "conditioned medium." The phosphate content of the medium was reduced to 2×10^{-5} *M* for the P^{32} experiment.

Isotope incorporation studies: At various intervals following virus infection of the cells 4 μc of uridine-2-C^{14}, 42 mc/mM (New England Nuclear), were added to each of two infected and control bottles. In one experiment 150 μc of P^{32}, (Na_2HPO_4, Oak Ridge, Tenn.) were added to each culture instead of C^{14}-uridine. The cells were exposed to the isotope for 2 hr, after which the cultures were washed 3 times with 15 ml of phosphate-buffered saline, pH 7.2, containing a 100-fold excess of unlabeled uridine. The cells were removed from the bottles by scraping, except that the 4- and 24-hr cultures, which were used for cell count and T antigen determinations, were dispersed with trypsin. The cells were centrifuged at 1200 rpm in a PR-2, International refrigerated centrifuge, at 4°C for 10 min, washed once with buffered saline, and stored as a pellet at −30°C.

RNA and DNA were separated by the Schmidt-Thannhauser procedure.[14] Aliquots of the two fractions were assayed for radioactivity in a liquid scintillation counter, and for UV absorption at 260 mμ. The counting efficiency for C^{14} was 50% in a dioxane-phosphor scintillation mixture.[15] P^{32} was counted on 2.5-cm stainless steel planchets in a gas-flow, low-background beta counter. The samples were counted for a sufficient length of time to give a statistical accuracy of ±4%. The results are expressed as a function of the interval from addition of virus to the mid-point of the labeling period.

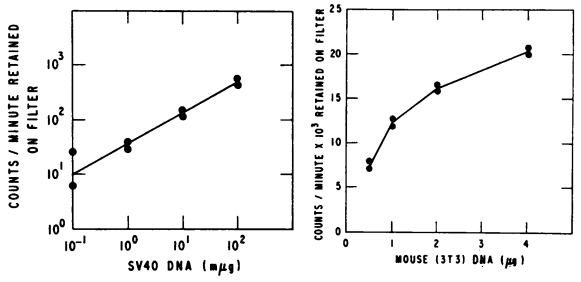

FIG. 1.—Calibration curve for SV40 DNA and SV40 radioactive RNA obtained by reacting serial dilutions of purified DNA with 140,000 cpm of SV40 RNA (*left*). Similar reactions of serial dilutions of mouse (3T3) DNA with 195,000 cpm of mouse RNA were used to determine the mouse calibration curve (*right*).

Counting of cells and determination of T antigen: Two samples of the trypsinized cells from each of two replicate control and infected cultures were obtained at 4 and 24 hr after infection and counted to determine the extent of cell multiplication during the experiment.

Aliquots of the cell suspensions from the 4-hr samples were planted in plastic Petri dishes containing glass coverslips. These were fixed in acetone 24 and 48 hr later and stained for the SV40-specific T antigen by the indirect fluorescent antibody procedure previously described.[16]

Nucleic acid homology studies: The cells used for DNA extraction were infected with SV40 in the same manner as for the isotope incorporation studies. The cells were harvested at 4, 16, 20, and 32 hr following infection and their DNA was extracted by a previously described modification of the Marmur method.[17, 18] DNA was obtained from control 3T3 cells in the same manner.

The nucleic acid homology studies were performed using a modification of the membrane filter method of Nygaard and Hall.[19, 20] Aliquots corresponding to 4 μg of the DNA, extracted from virus-infected and mock-infected control cultures, were reacted with 140,000 cpm of radioactive RNA complementary to SV40 DNA, or 190,000 cpm of RNA complementary to mouse cell (3T3) DNA. Duplicate determinations were made with each RNA-DNA combination. As part of the same experiments calibration curves were determined using serial dilutions of purified DNA solutions and the same amounts of radioactive RNA. From these curves (Fig. 1) the amount of SV40 DNA (mμg) extracted per μg of mouse DNA was determined.

TABLE 1

SUMMARY OF ISOTOPE INCORPORATION EXPERIMENTS

Expt. no.	Cell line and passage no.	Isotope label		Cell Counts (× 10⁶)* 0**	Cell Counts (× 10⁶)* 24**	Multiplicity of infection	Per cent cells with T antigen*,†	Specific Activity of DNA*,‡ 16**	Specific Activity of DNA*,‡ 24**	Specific Activity of DNA*,‡ 32**
1	3T3 M(19)	C^{14} uridine	Control	8.7	9.2	—	—	214§	—	64
			Virus	9.3	9.5	500	60	920	1000	368
2	3T3 M(25)	P^{32}	Control	14.0	12.8	—	—	2700	2700	1630
			Virus	14.2	13.6	500	80	9200	6200	2800
3	3T3 M-2(6)	C^{14} uridine	Control	18.5	19.5	—	—	410	110	50
			Virus	16.4	22.2	400	90	1040	300	250
			UV virus	15.7	23.2	0.04	0.04	410	80	50
4	3T3 MF(8)	C^{14} uridine	Control	7.0	6.9	—	—	95	115	—
			Virus	6.7	8.0	500	40	258	230	246
5	3T3 MF(7)	C^{14} uridine	Control	8.6	12.5	—	—	170	185	—
			UV virus	8.9	12.6	0.02	0.1	220	225	91

* Each value is the average of two determinations.
† Determined at 24 (expt. 1) or 48 hr after infection.
‡ Specific activity is expressed as cpm divided by optical density at 260 mμ (cpm/OD).
§ Control value for 4-hr time point.
** Hr after infection.

FIG. 2.—Specific activity of DNA extracted by the Schmidt-Thannhauser procedure from mock-infected control (O—O), UV-irradiated virus-infected (■—■), and virus-infected cells (▲—▲). The data in this figure correspond to expt. 3 in Table 1.

FIG. 3.—Ratios of specific activities of DNA's from virus-infected and mock-infected control cells (V/C = ●), and UV-irradiated virus and mock-infected cells (UV/C = ▲).

Results.—Stimulation of DNA synthesis in SV40-infected 3T3 cells: A representative pattern of the incorporation of uridine-C^{14} into the DNA of virus-infected and control 3T3 mouse fibroblasts is shown in Figure 2. In this experiment there was a striking stimulation of isotope incorporation in the virus-infected cells at 16 hr following infection when compared with the control cells. The absolute level of isotope incorporation in the infected cultures later declined but remained significantly higher than that of the control cultures. The relatively high level of isotope incorporation at early time points is probably related in some way to manipulation of the cultures at the time of infection and emphasizes the necessity for mock-infected control measurements. There was no consistent difference in isotope incorporation into DNA between the mock-infected controls and cultures infected with UV-inactivated virus.

As summarized in Table 1, the stimulation of DNA synthesis in the infected cells occurred in three different clones derived from the 3T3 cell line and in both early and late passage cells. This stimulation was observed when contact inhibition was achieved (expts. 1 and 4) and also when some cell division occurred during the course of the experiment (expt. 3). Also, the effect was noted with both P^{32} and C^{14}-uridine. The percentage of cells which formed T antigen varied between experiments despite a relatively constant multiplicity of infection. This may be due to slight variations in conditions of cell culture and infection, and to the use of different cell populations in the several experiments. The ratios of the specific activities of the DNA synthesized in infected and control 3T3 cells from the five experiments listed in Table 1 are summarized in Figure 3. During the first 12 hr there was no difference between mock-infected control and virus-infected cultures. At 16 hr, however, the virus-infected cultures consistently showed a two- to threefold increase in the specific activity of the DNA when compared to the controls. This increase was usually maintained through the 24-hr point. Although the total amount of isotope incorporated declined after the 24-hr point, the relative increase in specific activity of DNA from the infected cultures was greatest at 32 and 48 hr. In two experiments (nos. 3 and 5, Table 1) where UV-inactivated virus was used to infect the cells as an additional control, there was no change in specific activity of the DNA when compared with mock-infected control cells.

There was no consistent difference in the specific activity of the RNA extracted from mock-infected and virus-infected cultures in the experiments listed in Table 1. In addition, analysis in a linear sucrose gradient (5–20%) revealed no difference in sedimentation pattern of the radioactive RNA extracted from control and infected cells after 40, 120, or 240 min exposure to tritiated uridine.

Nucleic acid homology studies: The results of the nucleic acid homology studies are given in Table 2. In the two experiments with a high multiplicity of infection the SV40 DNA persisted in the infected cells and the data indicate a 1.5- to 2.5-

TABLE 2

NUCLEIC ACID HOMOLOGY EXPERIMENTS (MμG SV40 DNA/μG MOUSE DNA)

Expt. no.	Multiplicity of infection		Hours Postinfection 4	16	20	32
6	500	Control	0	0	0.08	0
		Virus	1.35	2.40	1.02	1.06
7	700	Control	0		0	
		Virus	1.70		4.11	

fold increase in the amount of SV40 DNA detected at 16 hr (expt. 1) and 20 hr (expt. 2) when compared to the values at 4 hr after infection. In a third experiment where the multiplicity of infection was approximately 50, we did not detect any SV40 DNA in the virus-infected cultures.

To investigate further whether synthesis of virus DNA occurred in these cells, the following experiment was performed. The 3T3 cells were infected with SV40 at a multiplicity of 700 $TCID_{50}$ per cell. Sixteen hours after infection, 5-bromodeoxyuridine was added to a final concentration of 3×10^{-5} *M* and the cells were incubated for four additional hours. The DNA was extracted from the harvested cells and then fractionated by isopycnic banding in a preparative cesium chloride gradient, pH 8.0, for 48 hr at 35,000 rpm in a Spinco SW 39 rotor. The gradient was collected in 0.125-ml fractions and assayed for optical density at 260 mμ. The DNA was resolved into two components ($\rho = 1.698$ and $\rho = 1.752$) corresponding, respectively, to unsubstituted DNA and DNA with *ca.* 50 per cent of the thymine replaced by bromodeoxyuridine (semiconservatively replicated DNA). The component containing bromodeoxyuridine accounted for approximately 15 per cent of the total DNA applied to the gradient. Each fraction of the gradient was reacted with radioactive mouse and SV40 complementary RNA. Appropriate calibration curves were constructed in the same experiment (see Fig. 1). The results demonstrated the presence of 0.07 mμg of SV40 DNA per μg of mouse DN in the unsubstituted DNA and 0.06 mμg of SV40 DNA per μg of mouse DNA the bromodeoxyuridine-substituted DNA.

Discussion.—These studies demonstrate that there is a stimulation of DNA synthesis in 3T3 cells infected with SV40. The highest level of isotope incorporation was observed 16–24 hr after infection. Isotope incorporation in the infected cultures remained significantly higher than in the control cultures until the experiments were terminated at 32 or 48 hr. Functional viral DNA is necessary for this stimulation phenomenon to occur, for when UV-inactivated virus was employed as the infecting agent, no significant increase in isotope incorporation was observed.

In considering the nature of the newly synthesized DNA, nucleic acid homology studies of bromodeoxyuridine-substituted DNA demonstrated that approximately 99.9 per cent of the semiconservatively replicated DNA was of host cell origin. Nevertheless, the virus DNA persisted in the infected cells up to 32 hr after infection and comprised 0.06 per cent of the bromodeoxyuridine-substituted DNA. This indicates that a very limited replication of the viral genome occurred.

This stimulation of host cell DNA synthesis occurred in a transformation system in which neither infectious virus nor virion antigen is synthesized as a result of infection with SV40. Two other recent reports have shown stimulation of host cell DNA synthesis in a virus-cell system in which no cytopathogenic effect occurs and in which cell transformation takes place, i.e., in rat embryo cells infected with polyoma virus.[3, 6] Todaro and Green[21] did not observe a stimulation of DNA synthesis in 3T3 cells using SV40 at a multiplicity of 30 $TCID_{50}$ per cell. In their study the absence of stimulation may have been due to the low multiplicity of virus used, since in our studies employing the same cell system the multiplicity of infection was 13–17 times greater. It is not known from the studies reported here or from the studies of Sheinen[6] and Gershon *et al.*[3] whether the DNA stimulation occurs only in the cells which will ultimately become transformed. Preliminary evidence

from autoradiographs using a 4-hr pulse of thymidine-H^3 indicates that approximately 10 per cent of the infected cells are involved in DNA synthesis. This proportion of cells is roughly equivalent to the percentage of cells which can be expected to undergo transformation following infection with multiplicities of virus used in these experiments.[4] Although considerably more cells form the T antigen than become transformed or undergo stimulation of DNA synthesis, it would appear that the latter event may be more intimately related to the transformation process than is T antigen formation.[4] Gershon *et al.*[3] have deduced from nitrous acid inactivation curves that the portion of the genome which causes stimulation of DNA synthesis may be responsible for cell transformation.

In the 3T3 transformation system a late protein, the virion antigen, is not produced and there is a block in the replication of viral DNA. In other cell-virus systems in which the host does not support effective production of infectious virus the block may occur at several different levels. Thus, in host-restricted phage infection of bacteria one may get rapid degradation of input virus in the restrictive host.[22] In adenovirus 7 infection of AGMK cells viral DNA replicates effectively but there is a block in the production of late structural viral proteins.[18] In this system the host cell is killed. Therefore, the presence and nature of the block may well determine whether a virus-cell interaction results in disappearance of the infecting viral genome, lytic interaction, or cell transformation.

After completion of this work, it has come to our attention that Gershon *et al.* have observed a similar stimulation in the same virus-cell system reported here. By fractionation of the newly synthesized DNA on a methylated-albumin-kieselguhr column, it was concluded that this DNA was of host cell origin.[23]

Summary.—We have investigated nucleic acid production during the early stages of transformation of 3T3 cells by SV40 virus. A stimulation of DNA synthesis was observed at 16–24 hr after infection. The nucleic acid homology studies demonstrated that: (1) most of the newly synthesized DNA was of host cell origin, and (2) viral DNA persisted up to 32 hr after infection and replicated to a very limited extent.

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