*THE INDUCTION OF CELLULAR DNA SYNTHESIS BY SIMIAN VIRUS 40 IN CONTACT-INHIBITED AND IN X-IRRADIATED CELLS**

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It has previously been shown that polyoma virus (PV) can induce the synthesis of cellular DNA after cellular DNA synthesis has been repressed by X irradiation,[1] or by contact inhibition.[2-4] In studies on the mechanism of this induction carried out with X-irradiated cells, it was found that the rate of nitrous acid inactivation of the capacity to induce cellular DNA synthesis was similar to that of the capacity to induce cell transformation.[1] This supported the suggestion that the induction of cellular DNA synthesis and transformation are expressions of the same function of the viral genome; a function that is assumed to change the control mechanism for cell replication by inducing a change in the cell surface.[5] It was furthermore shown in these studies that each cell whose DNA synthesis was induced by PV approximately doubled its content of DNA; and that the induction of cellular DNA synthesis can occur in the absence of detectable virus DNA synthesis.[1] It has also recently been reported[6] that in contact-inhibited mouse kidney cells under conditions where up to about half the cells in the culture synthesize virus, cellular DNA synthesis is induced in the virus-yielding cells.

In mouse embryo cell cultures in the logarithmic phase of growth,[7] nearly all the cells can synthesize virus after PV infection at a virus:cell ratio of about 2000 plaque-forming units (PFU) per cell.[7, 8] It has been concluded[7, 9] that an induction of cellular DNA synthesis by PV could not be detected in these exponentially growing mouse embryo cultures infected under these conditions.

The present studies were undertaken in order to determine whether results obtained with PV apply to another DNA tumor virus, simian virus 40 (SV40), and to examine the situation in cultures whose cellular DNA synthesis has been repressed before infection by contact inhibition or by X irradiation, and where nearly all the cells synthesize virus. The present experiments were therefore initiated to determine: (1) whether cellular DNA synthesis can be induced by SV40 after it has been repressed by contact inhibition or by X irradiation, (2) whether such an induction can occur in the absence of detectable virus DNA synthesis, and (3) whether it can be detected in contact-inhibited or X-irradiated cultures in which nearly all the cells synthesize virus. These studies have been carried out with two cell lines, the mouse line 3T3 and the monkey line BS-C-1. Cells of the line 3T3 show a high degree of contact inhibition,[10] are susceptible to transformation by SV40 and by PV,[11] permit the replication of PV,[11] and have been reported as showing little or no replication of SV40.[12] Nearly all the cells in cultures of line BS-C-1[13] can synthesize SV40 even after infection at a low virus:cell ratio,[14] and as reflected by a low incorporation of labeled thymidine (see below), BS-C-1 cells also appear to be well contact-inhibited.

Materials and Methods.—Isotopes: H^3-thymidine (12,300 mc/mM) and C^{14}-thymidine (36 or 38 mc/mM) were obtained from the Radiochemical Centre, Amersham, England.

Cell cultures: Cells of the line BS-C-1 were kindly supplied by Dr. Albert B. Sabin, and cells of the line 3T3F by Drs. George J. Todaro and Howard Green. All the experiments were carried

out in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) supplemented with 10% calf serum. The BS-C-1 line was maintained in the same medium, and the 3T3 line in EM with 10% fetal calf serum. To obtain confluent cultures of BS-C-1 cells, 2×10^6 cells were seeded per 50-mm Petri dish. The 3T3 cells were used in the second to fifth passages after obtaining the line; 5×10^4 cells were seeded per 50-mm Petri dish; and the confluent cultures were used about 2 days after the last mitosis was observed.

Irradiation: For the experiments with X-irradiated cells, nonconfluent cultures of BS-C-1 and 3T3 were X irradiated as monolayers with 5000 r (Type Dermovolt X-ray generator, R. Seifert & Co.), and then trypsinized and seeded at 2.5×10^5 cells per 50-mm Petri dish. The cultures were used 2 days after X irradiation, at which time they were also nonconfluent.

Virus: SV40, strain 777, was kindly supplied by Dr. Albert B. Sabin, and virus stocks were made after infection of confluent BS-C-1 cells. Stocks of the lysate were prepared as for PV[8] and concentrated as previously described,[15] except that the equilibrium centrifugation in RbCl was omitted. The stocks of PV were derived from the small plaque virus SP2,[16] and they were grown on mouse kidney cells as described previously.[17]

Plaque assay, and immunofluorescent test for nuclear tumor antigen: SV40 was plaque assayed on confluent BS-C-1 monolayers. The plaque assay, with agar in EM plus 10% calf serum, was the same as that used for PV,[8, 18] except that the initial overlay consisted of 8 ml agar medium, followed a week later by 3 ml medium, and the addition of 3 ml agar medium with neutral red on day 14. The plaques were finally scored at 21 days after infection. The immunofluorescent test in infected 3T3 cells, for the nuclear tumor antigen induced by SV40,[14, 19-22] was made by the direct method after acetone-fixation with fluorescein-conjugated anti-SV40 hamster tumor anti-serum. This antiserum, which was kindly supplied by Dr. H. Bernkopf, gave nuclear fluorescence in 3T3 cells infected with SV40 and transformed by SV40, and showed no fluorescence with uninfected 3T3 cells or 3T3 cells infected with PV.

Infection of cultures: To minimize disturbance of contact inhibition by the addition of new medium,[10] the medium used for maintenance of BS-C-1 and 3T3 cells after infection had previously been in contact with cultures of these cells for 1–2 days. After virus infection, 2 hr were allowed for virus adsorption, and the cultures were then washed three times with this medium or with phosphate-buffered saline. Controls were mock-infected and maintained in the same medium.

Rate of DNA synthesis: Labeled thymidine was added to the culture medium in the specified amounts for the specified time periods, and the incorporation of radioactivity into DNA was determined, as in previous experiments.[1]

Chromatography of DNA: Nucleic acids were extracted from the cells by two extractions with sodium dodecyl sulfate-phenol, as in previous experiments,[1, 4] and the preparations were dialyzed against 2× standard saline citrate (0.15 *M* NaCl, 0.015 *M* Na-citrate) (SSC), and then against 0.1 × SSC. To determine if the extracted DNA was of cellular or viral origin, the extracts were fractionated on a 3-layered

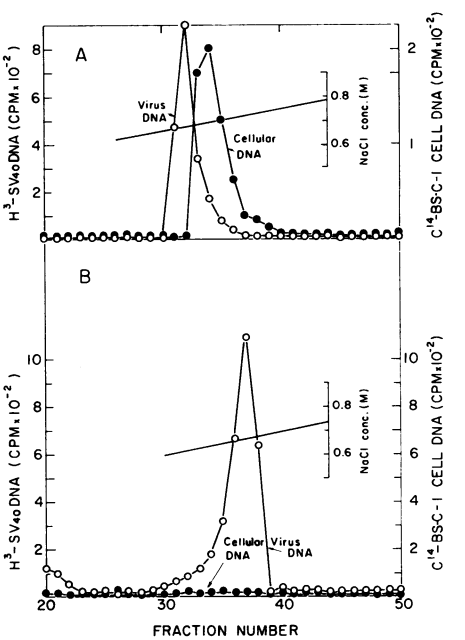

FIG. 1.—MAK column chromatography of DNA from SV40, and from uninfected BS-C-1 cells. (*A*) Without heating. A mixture of C^{14}-labeled BS-C-1 cell DNA and H^3-labeled SV40 DNA was chromatographed on a MAK column as described in *Methods*. The recoveries were: C^{14}, 98%; and H^3, 97%. ● = C^{14}; O = H^3. (*B*) With heating. The same mixture as used in (*A*) was boiled for 5 min in 0.1 × SSC. After quick cooling in an ice bath, the mixture was chromatographed on the MAK column. The recoveries were: C^{14}, zero; H^3, 46%. ● = C^{14}; O = H^3.

methylated-albumin-kieselguhr (MAK) column,[23] with a linear elution gradient from 0.4 to 0.9 *M* NaCl, either with or without prior heat treatment[9] (boiling for 5 min in 0.1 × SSC followed by rapid cooling in ice water). This heat treatment was used since it had been shown for PV[9] that heat-treated virus DNA eluted at the same salt concentration as native virus DNA, whereas there was no elution at this salt concentration of heat-denatured cellular DNA. Chromatography of a mixture of C^{14}-labeled BS-C-1 cellular DNA and H^3-labeled SV40 DNA, with and without the heat treatment (Fig. 1), showed that SV40 DNA behaves in this respect like PV DNA. The radioactive nucleic acids eluted from the columns were precipitated with 10% trichloroacetic acid using 250 μg of bovine serum albumin as carrier material, filtered on Millipore filters, and the radioactivity was counted in the liquid scintillation spectrometer. Where H^3 and C^{14} radioactivity was counted simultaneously, appropriate corrections for channel carry-over were made.

Results.—DNA synthesis after infection of contact-inhibited or X-irradiated BS-C-1 cells: Infection of confluent BS-C-1 cultures with SV40 at a virus:cell ratio of 40 PFU per cell gave a growth curve for total virus (Fig. 2) comparable to that observed previously.[24] At about 5 days post infection (p.i.) there was a complete destruction of the monolayers, as expected from the observation[14] that nearly all the cells in such cultures synthesize virus. The rate of DNA synthesis in confluent cultures was measured by 1-hr pulses with C^{14}-thymidine at different times p.i. (Fig. 2). The low amount of thymidine incorporation in the mock-infected confluent cultures indicates that these BS-C-1 cells were well contact-inhibited. Infection of these contact-inhibited cells with SV40 resulted in a marked increase in the rate of C^{14}-thymidine incorporation. The sharp rise in the rate of C^{14}-thymidine incorporation started between 24 and 36 hr p.i., reached a peak at 72 hr p.i., and then declined. Determinations of the DNA content of the cultures were made by Burton's modification of the diphenylamine reaction.[25] The results indicated a net increase of 31 per cent DNA content in the infected cultures at 72 hr p.i.

Infection of nonconfluent, X-irradiated BS-C-1 cells with SV40 resulted in a

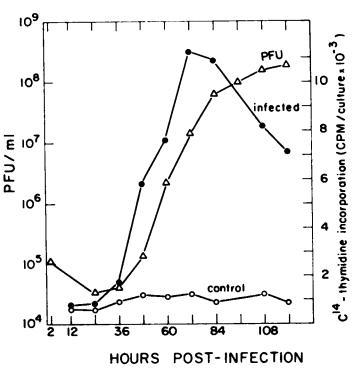

FIG. 2.—Total virus growth curve, and rate of DNA synthesis, in contact-inhibited BS-C-1 cells infected with SV40 at 40 PFU per cell. At different times cultures were labeled for 1 hr with 1 μc C^{14}-thymidine per plate. PFU/ml = PFU per ml of tissue culture fluid; 4 ml per plate.

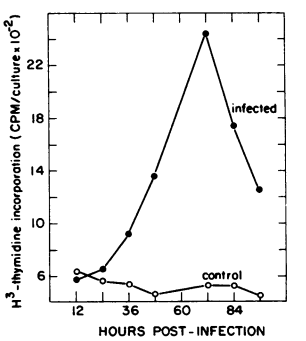

FIG. 3.—Rate of DNA synthesis in noncontact-inhibited X-irradiated BS-C-1 cells infected with SV40 at 40 PFU per cell. Cells were infected 2 days after X irradiation with 5,000 rad. At different times cultures were labeled for 1 hr with 2.5 μc H^3-thymidine per plate supplemented with 0.6 μg/ml unlabeled thymidine.

virus yield and cytopathic effect comparable to that obtained after infection of confluent nonirradiated cultures. One-hr pulses with H^3-thymidine of noncontact-inhibited, X-irradiated cells, showed that there was an increase in the rate of H^3-thymidine incorporation after infection with SV40 (Fig. 3), in a manner similar to that found with the nonirradiated cultures.

The question whether the increased incorporation of labeled thymidine in the infected BS-C-1 cultures reflected viral and/or cellular DNA synthesis was studied by MAK fractionation of the nucleic acid extracts with and without prior heat treatment. Accordingly, C^{14}-labeled nucleic acid extracts from infected cells were mixed with a H^3-labeled SV40 DNA marker and fractionated on a MAK column. Results of the chromatographic analysis of heated samples are shown in Table 1, and for comparison, the analysis of heated and unheated samples at 48 hr p.i. is shown in Figure 4. The results of these MAK column analyses of the contact-inhibited cultures can be summarized as follows: (1) At 20 hr p.i. there was no difference in the incorporation of labeled thymidine between infected and uninfected cultures (Table 2, Fig. 2), though it appears that some virus DNA had already been synthesized (Table 1); (2) at 30 hr p.i. there was only a small increase in incorporation of labeled thymidine into the infected cultures, and all of this increased DNA synthesis appeared to be viral; and (3) the marked increase in labeled thymidine incorporation observed at 36 and 48 hr p.i. appeared to be due only to the synthesis of virus DNA. Chromatography of extracts at 72 and 108 hr p.i., tested without heating, and of extracts from X-irradiated, noncontact-inhibited cells labeled from 24 to 72 hr p.i., tested with heating (Table 1), again indicated that the increased DNA synthesis in the infected cultures appeared to be due only to the synthesis of virus DNA.

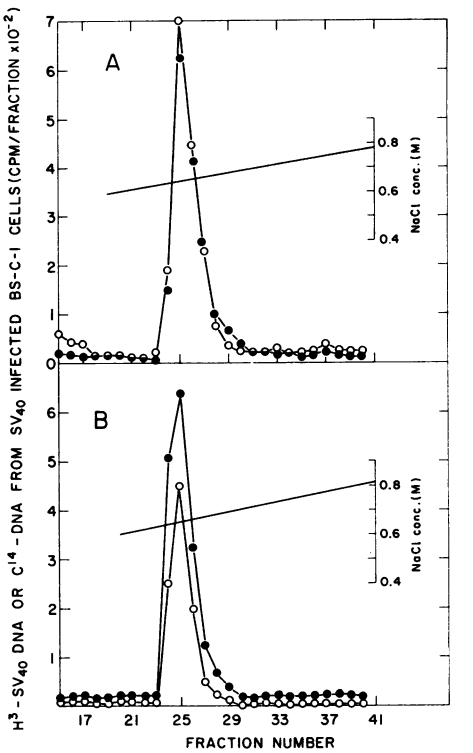

FIG. 4.—MAK column chromatography of DNA from SV40-infected BS-C-1 cells, 48 hr p.i. The infected cultures were labeled for 2 hr with 2.5 μc C^{14}-thymidine per plate. (*A*) Without heating. An aliquot of C^{14}-labeled, infected BS-C-1 cell DNA was mixed with H^3-labeled SV40 marker DNA and chromatographed on a MAK column as described in *Methods*. The recoveries were: C^{14}, 95%; and H^3, 98%. ● = C^{14}; ○ = H^3. (*B*) With heating. The same mixture as used in (*A*) was boiled for 5 min in 0.1 × SSC. After quick cooling in an ice bath, the mixture was chromatographed on the MAK column. The recoveries were: C^{14}, 61%; and H^3, 75%. ● = C^{14}; ○ = H^3. The coincidence of the H^3 and C^{14} radioactivity in (*A*), and the high recovery of C^{14}-radioactivity in (*B*), indicate that about 80–90% of the labeled DNA from the infected BS-C-1 cells is of viral origin.

In addition to these experiments with cultures infected at a virus:cell ratio of 40 PFU per cell, contact-inhibited, and X-irradiated noncontact-inhibited BS-C-1

TABLE 1

RECOVERY IN MAK COLUMN CHROMATOGRAPHY OF HEATED DNA FROM BS-C-1 CELLS INFECTED WITH SV40*

DNA sample	Hours postinfection	Percentage recovery from the MAK column†
Infected BS-C-1 cells	20	9.0
SV40 marker	—	61.0
Infected BS-C-1 cells	30	59.0
SV40 marker	—	76.0
Infected BS-C-1 cells	36	75.0
SV40 marker	—	78.2
Infected BS-C-1 cells	48	61.0
SV40 marker	—	75.0
Infected X-irradiated BS-C-1 cells	24 to 72	91.0
SV40 marker	—	83.5

* DNA samples from C^{14}-labeled BS-C-1 cells, at different times after infection with SV40, were mixed with H^3-labeled SV40 virus DNA (as marker) and boiled for 5 min in 0.1 × SSC. After quick cooling in ice water, the material was subjected to MAK column chromatography as described under *Methods*. Infected X-irradiated cultures were labeled with 10 μc C^{14}-thymidine per plate. Cultures at 20 hr p.i. were labeled for 2 hr with 10 μc C^{14}-thymidine; at other times for 2 hr with 2.5 μc C^{14}-thymidine per plate.

† Calculated with respect to the total radioactivity applied to the column. As shown in Fig. 1, the percentage recovery of heated DNA from uninfected BS-C-1 cells was essentially zero.

TABLE 2

SPECIFIC ACTIVITY OF DNA FROM CONTACT-INHIBITED, SV40-INFECTED, AND CONTROL BS-C-1 CULTURES, AT DIFFERENT TIMES POST INFECTION*

Hours post infection	Cpm per OD Unit	
	Infected cultures	Control
20	237	242
30	570	310
36	1420	388
48	4810	323

* Cultures at 20 hr p.i. were labeled for 2 hr with 10 μc C^{14}-thymidine, at other times for 2 hr with 2.5 μc C^{14}-thymidine per plate; DNA was extracted as described under *Methods*.

cells were infected with SV40 at 400 PFU per cell and labeled for 2 hr with 5 μc C^{14}-thymidine per plate at 36 hr, and at 48 and 72 hr p.i., respectively. Chromatography of the nucleic acid extracts indicated that also in this case, the increased incorporation of labeled thymidine in the infected cultures appeared to be all due to the synthesis of virus DNA.

The increase in DNA synthesis in the contact-inhibited and X-irradiated BS-C-1 cells infected with SV40 thus appeared to be entirely due to the synthesis of virus DNA, and there was no detectable induction of cellular DNA synthesis in these cells.

DNA synthesis after infection of contact-inhibited or X-irradiated 3T3 cells: The rate of DNA synthesis after infection of contact-inhibited 3T3 cells with SV40 or PV was measured by 1-hr pulses of H^3-thymidine (Fig. 5*A*). As also reported by Todaro and Green after infection with SV40 at a virus:cell ratio of 30:1,[12] no induction of DNA synthesis was observed after infection at 40 PFU per cell. However, after infection with SV40 at 100 or 500 PFU per cell, there was a marked induction of DNA synthesis with a peak at 24 hr p.i., followed by a decline. When tested at 30 hr p.i., 8 per cent of the cells showed fluorescence for the nuclear tumor antigen after infection at 40 PFU per cell, and 53 per cent after infection at 500 PFU per cell. Induction of DNA synthesis in the contact-inhibited 3T3 cells was also found after infection with PV (Fig. 5*A*).

One-hr pulses of H^3-thymidine after infection of X-irradiated, noncontact-

inhibited 3T3 cells also showed no detectable induction of DNA synthesis after infection with SV40 at 40 PFU per cell, but there was a marked induction after infection with SV40 at 500 PFU per cell, or after infection with PV (Fig. 5*B*).

Chromatography of the nucleic acid extracts from contact-inhibited 3T3 cells infected with PV at 200 PFU per cell, and labeled from 12 to 36 hr p.i. with 10 μc C^{14}-thymidine per plate, showed that the induced DNA synthesis was both cellular and viral. However, chromatography of the nucleic acid extract from contact-inhibited 3T3 cells infected with SV40 at 100 PFU per cell, and labeled from 24 to 72 hr p.i. with 10 μc C^{14}-thymidine per plate, indicated that all the induced DNA was cellular. These results thus indicate that induction of cellular DNA synthesis by SV40 in 3T3 cells can occur in the absence of detectable virus DNA synthesis.

After infection of contact-inhibited 3T3 cells with SV40 at 500 PFU per cell or with PV at 200 PFU per cell, an induction of mitoses was observed starting at about 20–24 hr p.i. Such an induction of mitosis was not observed in the mock-infected controls of contact-inhibited 3T3 cells, X-irradiated 3T3 cells infected with SV40 or PV, SV40-infected BS-C-1 cells, or in the PV-infected X-irradiated mouse and rat embryo cells used in the previous studies.[1]

Discussion.—The results have indicated that at a high multiplicity of infection, SV40 can induce the synthesis of cellular DNA in contact-inhibited and in X-irradiated 3T3 cells, and that this induction can occur, as in the case of PV,[1] in the absence of detectable virus DNA synthesis. With both viruses, induction results in a sharp rise of synthesis of cellular DNA followed by a decline, and as most clearly seen from the PV-infected rat cells[1] and SV40-infected 3T3 cells, this decline is not due to cell lysis. It is probable that this "shutoff" after induction is due to the normal cellular regulatory mechanism for DNA synthesis that brings about a similar "shutoff" of synthesis during the normal mitotic cycle. The induction of mitoses observed after infection of contact-inhibited 3T3 cells, but not after

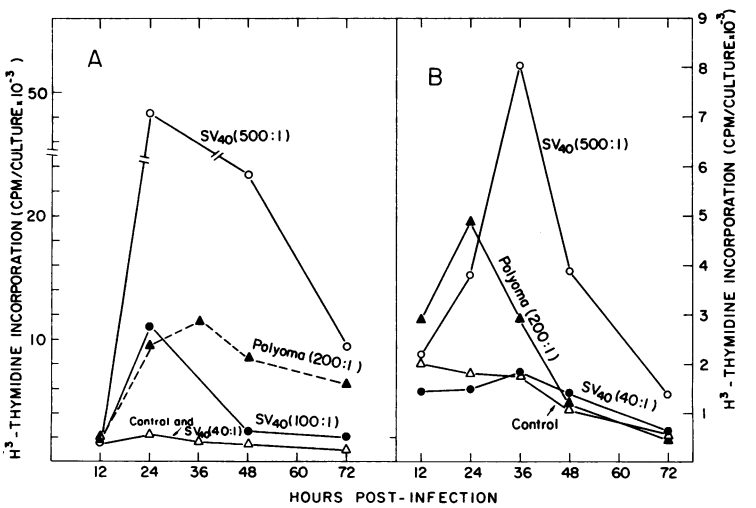

FIG. 5.—Rate of DNA synthesis in contact-inhibited (*A*) and in noncontact-inhibited, X-irradiated (*B*) 3T3 cells infected with polyoma virus or SV40. In (*B*) the cells were infected 2 days after X irradiation with 5,000 rad. At different times cultures were labeled for 1 hr with 10 μc H^3-thymidine per plate. (40:1), etc. = PFU per cell used for infection.

infection of X-irradiated cells, supports the suggestion that damage by the X irradiation to other synthetic processes required for mitosis may have played a part in the lack of an observed concomitant induction of histone synthesis after PV infection of X-irradiated cells.[1] The finding of an induction of cellular DNA synthesis in 3T3 cells infected with SV40 at 100 or 500 PFU per cell but not after infection at 40 PFU per cell indicates that the detectable induction of DNA synthesis in nonvirus-yielding cells required infection with a considerable number of PFU per cell. In nonvirus-yielding cells there thus appears to be a low frequency of expression per inoculated virion for the property of cellular DNA induction. Evidence has previously been presented[1] in favor of the assumption that the induction of cellular DNA synthesis and transformation are expressions of the same function of the viral genome.[1, 5] Further studies are in progress to determine what percentage of cells induced to synthesize cellular DNA and to undergo mitosis can form colonies of transformed cells.

Contact-inhibited 3T3 cells are arrested in stage G1 of the cell cycle.[10] It will be of interest to determine whether the block in DNA synthesis after X irradiation that can be overcome by PV and SV40 is the same block at G1, and/or at some other stage of the cell cycle. It will also be of interest to determine whether the cellular DNA that appears to be included in the PV particle[26] plays a role in the induction of cellular DNA synthesis in cells repressed by contact inhibition or by X irradiation.

The results with BS-C-1 cells, where nearly all the cells yield virus, indicated that in contrast to contact-inbibited and X-irradiated mouse cells infected with PV where the major fraction of the induced DNA synthesis can be attributed to cellular DNA,[1-4, 6] there was no detectable induction of cellular DNA synthesis after infection of BS-C-1 cells with SV40 at 40 or 400 PFU per cell. This indicates that not all types of virus-yielding cells are necessarily induced to synthesize cellullar DNA after it has been repressed by contact inhibition or by X irradiation. It also appears that SV40 DNA synthesis in BS-C-1 cells can occur in the absence of a detectable induction of cellular DNA synthesis. It should, however, be noted, that although the major fraction of the labeled DNA in the SV40-infected BS-C-1 cells behaved like viral DNA on the MAK column, the presence of a small amount of labeled cellular DNA (of the order of about 10–20%), cannot be excluded by the techniques used.

Regarding the possible function of the induction of cellular DNA synthesis, it was suggested from the results obtained with cell transformation by carcinogenic hydrocarbons[27] that the expression of transformation requires a process associated with cell replication. Experiments on transformation induced by X irradiation[28] and by SV40[12] then indicated that fixation of the transformed state as a hereditary property of the cell requires that the process associated with cell replication has to occur soon after treatment with the transforming agent. An efficient transforming agent would thus be one that can induce both the change in control mechanism in the cell required for transformation, and the fixation of the transformed state. Since the requirement for fixation of PV- and SV40-induced transformation could be the replication of cellular DNA, the ability of these viruses to induce the synthesis of cellular DNA could be one of the factors that accounts for their high carcinogenic potency.[29]

Summary.—It has been shown that SV40 can induce the synthesis of cellular DNA in contact-inhibited and in X-irradiated 3T3 cells. This induction was detected at a high multiplicity of infection (100 or 500 PFU per cell), but not at a lower multiplicity (40 PFU per cell). In contact-inhibited or X-irradiated BS-C-1 cells, where nearly all the cells synthesize virus, no induction of cellular DNA synthesis was detected after SV40 infection at either the higher or the lower multiplicity. The results indicate that SV40 can induce the synthesis of cellular DNA in 3T3 cells in the absence of detectable virus DNA synthesis; and that in contrast to mouse cells infected with polyoma virus where the major fraction of the induced DNA synthesis can be attributed to cellular DNA, there was no detectable induction of cellular DNA synthesis by SV40 in the virus-yielding BS-C-1 cells.

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