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Nonproductive Infection and Induction of Cellular Deoxyribonucleic Acid Synthesis by Bovine Adenovirus Type 3 in a Contact-Inhibited Mouse Cell Line

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Bovine adenovirus type 3 (BAV-3), which has been reported to produce tumors in newborn hamsters, induced cellular deoxyribonucleic acid (DNA) synthesis in a contact-inhibited mouse kidney cell line (C3H2K). In this system, the virus did not multiply, whereas virus-specific tumor antigen (T antigen) was detected in nearly all cells. Replication of viral DNA could not be detected by DNA-DNA hybridization on membrane filters. The cellular DNA synthesis induced by BAV-3 did occur in the absence of added serum. Extent of induction of cellular DNA synthesis was closely correlated with the multiplicity of infection. Cells activated to synthesize DNA in the serum-free medium by the virus infection progressed to cell division without noticeable cell killing.

Several agents have been found to induce cellular deoxyribonucleic acid (DNA) synthesis after it has been repressed by contact-inhibition. For example, introduction of oncogenic viruses (8-10, 14, 15, 20, 21, 25, 30, 32) or of various other agents, i.e., serum factors (16, 35, 38), proteolytic enzymes (29), ribonuclease, hyaluronidase, or digitonin (36), into the culture system has been reported to activate cells to enter S phase from the G_1 phase of the cell cycle.

In this paper, we present results of experiments undertaken to find a system in which nearly all the cells (C3H2K cells) can be activated to enter S phase and to divide by the serum factor as well as by an oncogenic virus (BAV-3). The characteristic feature of our system compared to the previously reported ones (17, 19, 27, 28, 31, 34) is that almost all the cells thus activated can divide without any appreciable cell killing.

Detailed experiments concerning the character of abortive infection in the system are also described.

MATERIALS AND METHODS

Cells and media. The C3H2K cell line derived from newborn C3H/He mouse kidney was kindly supplied by H. Yoshikura of the National Institute of Health, Tokyo, Japan, and was grown as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum ("growth medium"). Trypsin-dispersed primary calf kidney cells (2) were grown in Hanks salt solution supplemented with 0.5% lactalbumin hydrolysate and 10% calf serum.

Virus. BAV-3 (4), originally passaged from material possessed by J. H. Darbyshire, was cloned three times by limiting dilution technique and then was passaged by Y. K. Inoue of our institute. Virus stocks were prepared by infecting the virus to primary or secondary calf kidney cells maintained in a medium consisting of Hanks salt solution supplemented with 0.5% lactalbumin hydrolysate and 2% calf serum. After the appearance of a typical cytopathic effect (CPE), infected cells were harvested. They were suspended in one-tenth volume of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.1, after which they were frozen and thawed three times. They were then titrated on calf kidney cells, adequately diluted with phosphate-buffered saline (PBS), and used for the "seed virus."

Chemicals. Sources of chemicals were as follows: Pronase (highly purified) from Seikagaku Kogyo Co., Tokyo, Japan, was heated at 80 C for 15 min in 1 M NaCl, 0.01 M Tris buffer, *p*H 8.1, before use. Deoxyribonuclease I (ribonuclease free) was purchased from Worthington Biochemical Corp., Freehold, N.J. Pancreatic ribonuclease I-A, from Sigma Chemical Co., St. Louis, Mo., was heated at 80 C for 10 min in 0.15 M NaCl and 0.1 M phosphate buffer, *p*H 7.0, before use. Calf thymus DNA was purified (18) in our laboratory. Fluorocarbon (Daifron S3) and phenol from commercial sources were distilled before use. ^{3}H-thymidine and ^{32}P-orthophosphate were purchased from

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Japan Isotope Corp., Osaka, Japan, and the former was purified by using Dowex-1 (OH^-) column chromatography.

Miscellaneous. The serum of hamsters intraperitoneally immunized with transplanted tumor extract produced by BAV-3 (method of Gilden et al.,) (11) were kindly supplied by Y. K. Inoue of our institute (24) and were used for fluorescent antibody test to detect T antigen.

^{32}P-labeled and unlabeled Pt_{190} phage DNA species were given by M. Imai of our laboratory.

Synchronization of C3H2K cells by medium change and infection of C3H2K cells with virus. C3H2K cells were suspended in the growth medium in a concentration of 5×10^4 cells/ml. Each suspension of 2 ml was seeded into glass petri dishes (diameter, 2.9 cm) in a CO_2 incubator (5% CO_2-95% air). Each petri dish contained a cover slip (diameter, 2.2 cm). After formation of confluent monolayers of C3H2K cells on the cover slip, the growth medium was replaced with a maintenance medium (Eagle's MEM without serum), and cells were maintained at 37 C for 3 days. Cultures were then infected with BAV-3 at the input multiplicity indicated below. After adsorption of virus for 2 hr at 37 C, cultures were washed with PBS and maintained at 37 C in fresh growth medium or fresh maintenance medium. Mock infection was carried out under identical conditions without virus.

Determination of rate of DNA synthesis. One hour prior to harvest, 0.1 ml of ^3H-thymidine (5 Ci/mmole, 0.15 μCi/dish) was added to each culture dish containing 2 ml of medium. After pulse-labeling at 37 C for 1 hr, cells on the cover slip were washed five times with 0.85% NaCl and then were fixed with a 200-fold diluted solution followed by fixation with an undiluted solution consisting of three parts ethanol and one part acetic acid. Extraction with 0.4 N perchloric acid (PCA) five times at 0 C was done, and the material was then dried by washing in methanol and broken into several pieces. These were put in vials to which 10 ml of toluene scintillator solution was added and were counted in a Nuclear-Chicago Mark I liquid scintillation spectrometer.

Autoradiography. Autoradiographic examination of cells in the DNA synthetic phase was carried out as follows. Pulse-labeled and washed cells (described above) were fixed for 3 min with methanol, followed by extraction with 0.4 N PCA two times at 0 C for 5 min. Cells on the cover slip were then dried by washing in methanol, coated with Sakura NR-M2 liquid emulsion, and exposed at 4 C for 12 days. Autoradiographs were developed for 5 min in Konidol X, and then were dipped for 1 min in 0.5% acetic acid, fixed for 15 min in Konifix, and washed in running water for 30 min.

Cells on the cover slips were stained with a hematoxylin staining solution for 5 min, and nuclei containing more than five silver grains were counted to estimate per cent of labeled nuclei. At least 1,000 cells were counted.

Estimation of mitotic index. Stained cover slips used for the autoradiography were also used to estimate the mitotic index. Cells from late prophase to late telophase were counted. At least 1,000 cells were counted.

Cell counting. Cells were counted by using a hemocytometer.

Purification of BAV-3. "Seed virus" (described above) was further purified by the method of Green and Piña (12). To prepare labeled virus, ^{32}P-orthophosphate was added (2 μCi/ml) to cultures after virus infection. The labeled virus was purified in the same manner.

Preparation of viral DNA. Unlabeled and ^{32}P-labeled viral DNA were purified by the method of Green and Piña (13) with the following modifications. To the purified and diluted virus preparation, sodium dodecyl sulfate (SDS), Pronase, and ethylenediaminetetraacetate (EDTA) were added to final concentrations of 0.5%, 2 mg/ml, and 0.01 M, respectively, and the whole was incubated at 37 C overnight. It was then shaken with an equal volume of phenol [saturated with a buffer of 0.15 M NaCl, 0.1 M EDTA, and 0.1 M Tris buffer, (pH 8.0)] for 10 min at 4 C. They were treated as described previously (13). ^{32}P-labeled viral DNA was purified from ^{32}P-labeled virus in the same manner.

Preparation of cellular DNA. Harvested C3H2K cells were centrifuged at $900 \times g$ for 5 min, washed two times with 0.85% NaCl, and suspended in a buffer consisting of 0.15 M NaCl, 0.1 M EDTA, and 0.1 M Tris buffer (*p*H 8.0). SDS and Pronase treatment, as well as phenol extraction, were carried out as described for viral DNA. The final aqueous layer was shaken five times with an equal volume of ether to remove phenol. After ether was removed from the resulting solution by gentle bubbling at 37 C for 30 min, preheated ribonuclease was added to a final concentration of 50 μg/ml. The mixture was kept at 37 C for 4 hr and extracted three times with phenol to remove ribonuclease. Ethanol and potassium acetate were added to the final aqueous phase to final concentrations of 66% and 0.2 M, respectively. This solution was kept at -20 C overnight, followed by centrifugation at $2,000 \times g$ for 30 min. The pellet was washed once with a solution consisting of 66% ethanol and 0.2 M potassium acetate and then was dissolved in a solution consisting of 0.01 M Tris buffer (*p*H 8.1), 0.01 M EDTA, and 0.05 M NaCl. The whole then was centrifuged at 40,000 rev/min for 20 min in the no. 40 rotor of a Spinco centrifuge. The supernatant solution was used as cellular DNA.

To prepare labeled cellular DNA, ^3H-thymidine (5 Ci/mmole, 0.5 μCi/ml) was added to the culture bottle at the time of the seeding of C3H2K cells, and cells were incubated at 37 C. After formation of a complete monolayer, cells were collected. DNA from these was prepared in the same manner.

Preparation of DNA induced by BAV-3 in contact-inhibited C3H2K cells. After formation of confluent monolayers of C3H2K cells, the growth medium was replaced with a maintenance medium, and cultures were maintained at 37 C for 3 days. Then, cultures were infected with BAV-3 [20 median tissue culture infective doses ($TCID_{50}$) per cell]. After 2 hr of adsorption, 2 ml of fresh maintenance medium, including ^3H-thymidine (5 Ci/mmole, 0.5 μCi/ml), was added. Cells were harvested 48 hr after infection. Induced DNA was prepared in the same manner as was cellular DNA.

Estimation of DNA content. DNA content was estimated by using the diphenylamine reaction (3) with calf thymus DNA as standard.

Purity and integrity of purified DNA species. Purities of the DNA species prepared were checked by using deoxyribonuclease I. When DNA species were incubated with deoxyribonclease I (20 μg/ml) at 37 C for 120 min, more than 90% of the DNA species became acid-soluble. The molecular integrity of DNA species was examined by centrifugation, using a 5 to 20% sucrose density gradient with ^{32}P-Pt_{190} DNA as a marker. Molecular weights were roughly estimated to be larger than 10^6 daltons.

Hybridization of DNA species on membrane filter. The nature of the DNA species was analyzed by using a modification of the hybridization technique for DNA on membrane filters devised by Warnaar and Cohen (37). Hybridization was carried out in a screw-capped vial containing, unless stated otherwise, 1.0 ml of 2.5× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate), *p*H 8.1, containing 0.1 M Tris, 1 mg of SDS, 0.25 μg of labeled heat-denatured DNA, and a filter containing 5 μg of immobilized, unlabeled DNA.

After incubation at 60 C for 17 hr, each side of the filter was washed two times with 15 ml of 2× SSC. The filter was then dried, and its radioactivity was counted. A filter with no DNA in a hybridization reaction was included in each test to check nonspecific adsorption of labeled DNA ("noise"). The "noise" was always less than 0.5% of the given count. The amount of DNA hybridized was expressed as the percentage of DNA (counts/min) trapped on the filters as compared to the input DNA (counts/min).

Measurement of radioactivity. Aqueous and dried samples were counted by adding Bray's or toluene scintillator solution, respectively. Radioactivity was measured in a scintillation spectrometer (Beckman LS100 or Nuclear-Chicago Mark I).

Absorbance. Absorbance was measured in a Shimazu QV-50 spectrophotometer.

Detection of T antigen of BAV-3. A nearly confluent monolayer of C3H2K cells was infected with BAV-3 (200 $TCID_{50}$ per cell). Cells on the cover slip were washed with PBS 48 hr after infection and then were fixed with CCl_4 at room temperature for 30 min and stored at −20 C until further treatment. Before staining, cells were again treated with CCl_4 for 120 min at 37 C. Fluorescein-conjugated hamster serum, immunized with transplanted tumor extract produced by BAV-3, was applied on the cover slip, and cells were allowed to stand overnight at 4 C. After being washed with PBS, cells were observed by fluorescent microscopy.

RESULTS

Cell system used for virus infection. A confluent monolayer was made by seeding the cells and changing the medium to the maintenance medium as described above.

At this stage, C3H2K cells which had been held in the maintenance medium for 3 days (Fig. 1, from day 6 to day 9) neither died nor detached from the glass surface (note that the number of cells at day 6 was not reduced at day 9, as shown in Fig. 1). These results present a striking contrast to Dulbecco's finding that, in the absence of serum, cell detachment and accumulation of dead cells in the medium were observed (7). This discrepancy seems to reflect the difference in the stability of cells in the absence of serum.

After incubation for 3 days without serum, the maintenance medium was aspirated off and growth medium was added to the culture. At various times after the addition of growth medium, the rate of DNA synthesis, number of cells, percentage of labeled cells, and mitotic indexes were determined as described above.

As shown in Fig. 1 and as reported by others (38), C3H2K cells were largely in G_1 phase of the cell cycle before the medium was changed. After the medium was changed to growth medium, almost all of the cells were activated to enter cell division. In this case, more than 60% of cells were activated synchronously.

DNA synthesis in contact-inhibited C3H2K cells after infection with BAV-3. A confluent monolayer was made as described in Fig. 1 and above. Then cells were infected with BAV-3 at an input multiplicity of 18 $TCID_{50}$/cell (Fig. 2a) or 10 $TCID_{50}$/cell (Fig. 2b), and control cells were mock-infected with PBS as described above. At various times after infection, the rate of DNA synthesis was examined as described above. The serum-free medium showed little inducing effect on DNA synthesis in mock-infected cells (Fig. 2a, MO). Cells, mock-infected and changed to the fresh growth medium (Fig. 2a and 2b, M10), showed higher rates of DNA synthesis. The same observation is shown in Fig. 1, and these effects correlate well with the reported effect of the serum factor (16, 35, 38; *see below*).

Virus-infected cultures showed higher rates of DNA synthesis than did M10 (Fig. 2a and b). This phenomenon occurred irrespective of the presence (Fig. 2a and b, V10) or absence of serum (Fig. 2a, VO). At these multiplicities of infection, the maximum rate was observed around 24 hr after virus infection. In this step, however, it was not clear whether the DNA synthesized in infected cultures consisted of only induced cellular DNA, only viral DNA, or both DNA species.

Morphological change and virus replication in C3H2K cells after virus infection. In virus-infected C3H2K cells, a morphological change resembling a CPE was observed, i.e., the rounding of cells. However, this change in cell morphology differed from the characteristic CPE observed in calf kidney cells productively infected with BAV-3 and was not accompanied with cell aggregation or cell death and dropping off from the glass surface. To be sure that this morphological change did not

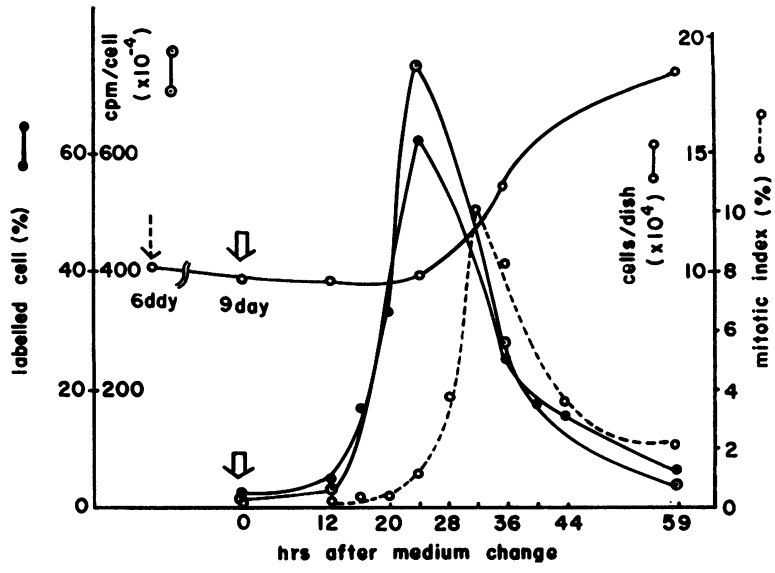

FIG. 1. *Synchronization of C3H2K cells by a medium change. Six days after the C3H2K cells were seeded, when a confluent monolayer of cells had been formed, the growth medium was replaced with the maintenance medium, and the whole was incubated for an additional 3 days at 37 C. Nine days after the cells were seeded, the maintenance medium was replaced by the growth medium. At various times after the medium change, some cultures were harvested and the cells were counted. Some cultures were pulse-labeled with ^{3}H-thymidine for 1 hr from the indicated time, and incorporation into the acid-insoluble fraction was counted; or after pulse-labeling, cells were fixed and prepared for radioautography and for counting mitotic cells. Abscissa: hours after medium change to growth medium. Ordinate: O—O, number of cells per dish (×10⁴ cells/dish). Average of three experiments for each point. ⊙—⊙, Incorporation of ^{3}H-thymidine into the acid-insoluble fraction (counts per min per cell × 10⁻⁴). Average of three experiments for each point. ●—●, Per cent of cells having grains in nuclei on autoradiographic observation. Average of two to three experiments for each point. At least 1,000 cells were counted. O---O, Per cent of cells in mitotic phase. Average of two to three experiments for each point. At least 1,000 cells were counted. Dashed arrow, medium change to maintenance medium; open arrows, medium change to growth medium.*

result from a cytotoxic effect due to virus replication, virus yields were investigated on calf kidney cells at various times after infection (Table 1). The table clearly shows that no virus multiplication occurred in C3H2K cells. To examine a possible cytotoxic effect of the virus, the colony-forming ability of cells harvested, both from virus-infected cultures (5 $TCID_{50}$ per cell, 3 days after infection) and from mock-infected cultures, was examined. The efficiencies of colony formation of cells from these cultures did not differ significantly. In addition, cells from virus-infected cultures (400 $TCID_{50}$ per cell, 48 hr after infection) showed no difference in the ability to exclude trypan blue compared with cells from mock-infected cultures.

The morphological change and some altered characters of the infected cells were transmitted to some progeny cells (*manuscript in preparation*). [In summary, virus-infected cells were subjected to moderate but definite morphological alteration. The cells thus altered could form colonies in a methocell medium in high proportion, whereas the original cells could not. A transformed clone keeping these characters was obtained. BAV-3-specific surface antigen was also detected in this clone. (K. Nakajima, *personal communication*).]

Induction of T antigen. As described above, BAV-3 could not multiply in C3H2K cells. The induction of DNA synthesis by virus infection suggests that some early genome of the virus might be expressed. Therefore, we checked whether the T antigen of the BAV-3 was expressed. A nearly confluent cell layer of C3H2K was infected with 200 $TCID_{50}$ of the virus per cell. Forty-eight hours after infection, cells were fixed and treated with immunofluorescein-conjugated hamster serum, immunized with transplanted tumor extract produced by BAV-3 (*see above*). As shown in Fig. 3, every cell examined contained T antigen in its nucleus.

Homology test for induced DNA by virus in contact-inhibited C3H2K cells. To see whether the activation of DNA synthesis observed in virus-infected cells (as shown in Fig. 2a and b) includes replicated viral DNA or only induced cellular DNA, DNA from virus-infected cells was hy-

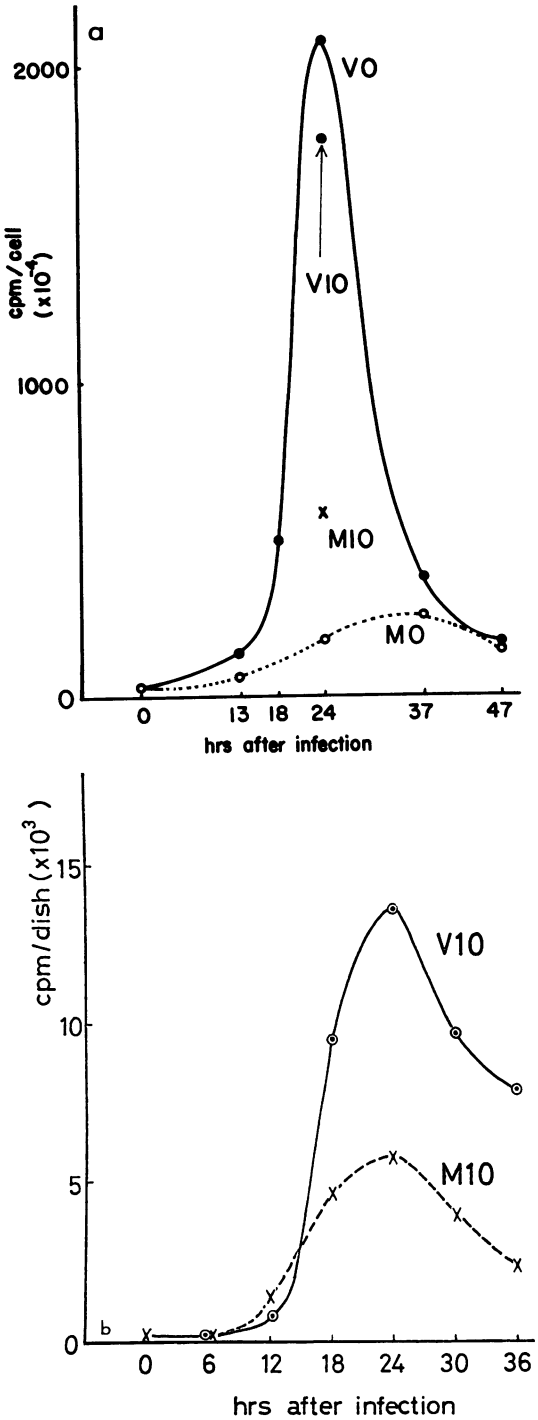

FIG. 2. *DNA synthesis in contact-inhibited C3H2K cells after infection with BAV-3. At 9 days after the cells were seeded as described in Fig. 1, monolayer cells which formed were infected with BAV-3 at 18 $TCID_{50}$/cell (a) or at 10 $TCID_{50}$/cell (b). After adsorption for 2 hr and subsequent washing, the cells were incubated in growth medium (V10- ⊙—⊙) or in maintenance medium (V0, ●—●). After mock infection was carried out using PBS instead of virus solution, the cells were incubated in growth medium (M10, ×---×) or in maintenance medium (MO, ○---○). At various times after infection, ^{3}H-thymidine was added to the culture medium and the whole was incubated, from the indicated time, for 1 hr. The material was harvested, and the acid-insoluble fraction was counted as described in the text. Each point is the average of three experiments.*

TABLE 1. *Replication of BAV-3 in C3H2K cells*[a]

Time after infection (hr)	Intracellular titer[b] ($TCID_{50}$/cell)	Extracellular titer[b] ($TCID_{50}$/cell)
0	1.4×10^{-2}	ND[c]
24	6.0×10^{-5}	ND
48	2.0×10^{-5}	$< 4 \times 10^{-6}$
72	1.4×10^{-4}	ND
96	2.0×10^{-5}	$< 4 \times 10^{-6}$

[a] Multiplicity of infection was median tissue culture infective doses (110$TCID_{50}$) per cell.

[b] After adsorption for 2 hr, cell layers were washed with PBS five times, and then a fresh growth medium was added and the whole was incubated at 37 C.

[c] Not done.

bridized with cellular DNA or with viral DNA (Table 2). In our DNA preparation, as shown in Table 2, about 10% homology between homologous cellular DNA species, about 30% homology between homologous viral DNA species, and about 60% homology between homologous bacteriophage Pt_{190} DNA species, were observed. No homology was detected between cellular DNA and Pt_{190} DNA in control experiments.

As shown in Table 2, no homology was detected between viral DNA and DNA prepared from virus-infected C3H2K cells which hybridized well with the cellular DNA. The extent of homology was comparable to that between homologous cellular DNA species. Amount of virus-infected C3H2K DNA hybridized to immobilized viral DNA did not increase even when the ratio of the former to the latter in the incubation mixture was increased from 1:20 to 1:2. We concluded from these results that the DNA synthesis induced by virus infection in contact-inhibited C3H2K cells was entirely cellular with no detectable synthesis of viral DNA.

Relationship between multiplicity of infection and extent of induction in cellular DNA synthesis. Contact-inhibited cultures were infected with virus preparation in a range from 0.001 to 53 $TCID_{50}$ per cell. After virus adsorption, the medium was changed to the maintenance medium.

TABLE 2. *Hybridization of DNA in BAV-3-infected C3H2K cells with cellular and viral DNA and some control experiments*

DNA on a filter[a]	Input DNA[b]	Counts/min incubated	Counts/min bound to filter	Net counts/min bound[c] (% of input)	Average (% of input)
C3H2K 5γ	^{3}H-C3H2K 0.25γ	4,780	652	13.3	10.3[d]
			393	7.9	
			477	9.7	
C3H2K 5γ	^{3}H-C3H2K 0.25γ	14,390	1,516	10.3	10.4[e]
			1,548	10.4	
C3H2K 5γ	^{3}H-C3H2K 0.25γ	4,110	450	10.5	11.8[f]
			556	13.1	
Virus[g] 2γ	^{32}P-virus[g] 0.2γ	440	103	23.2	26.2[d]
			126	28.2	
C3H2K 5γ	^{32}P-Pt$_{190}$ 0.25γ	1,300	8	0.4	0.2[e]
			3	0	
Pt$_{190}$ 5γ	^{3}H-C3H2K 0.25γ	14,390	250	1.5	1.0[e]
			105	0.5	
Pt$_{190}$ 5γ	^{32}P-Pt$_{190}$ 0.25γ	4,920	3,427	69.0	56.5[h]
			2,154	44.0	
C3H2K 5γ	^{3}H-(virus[g]-infected)[i] C3H2K 0.25γ	813	114	13.7	15.5[d]
			119	14.3	
			131	18.6	
C3H2K 5γ	^{3}H-(virus[g]-infected)[i] C3H2K 0.25γ	7,446	1,213	15.9	15.0[f]
			1,081	14.1	
Virus[g] 2.5γ	^{3}H-(virus[g]-infected)[i] C3H2K 0.25γ	813	8	0.7	0.3[d]
			0	0	
			1	0	
Virus[g] 5γ	^{3}H-(virus[g]-infected)[i] C3H2K 0.25γ	7,446	46	0.2	0.1[f]
			9	0	
Virus[g] 2.5γ	^{3}H-(virus[g]-infected)[i] C3H2K 0.5γ	14,892	14	0	0[f]
			34	0	
Virus[g] 5γ	^{3}H-(virus[g]-infected)[i] C3H2K 2.5γ	74,460	277	0	0[f]
			118	0	

[a, b] Nonradioactive and radioactive DNA species were prepared, denatured, hybridized, washed, and counted as described in the Materials and Methods.

[c] "Noise" (counts/min) was subtracted from the value (counts/min) bound to filters.

[d, e, f, h] Letters indicate different sets of experiments.

[g] BAV-3.

[i] Infected at an input multiplicity of 20 median tissue culture infective doses/cell.

Twenty-four hours after virus infection, ^{3}H-thymidine was added to the culture and pulse-labeled for 1 hr. The rate of DNA synthesis then was examined as described. As shown in Fig. 4, the extent of induction for cellular DNA synthesis in a virus-infected culture was closely correlated with the multiplicity of infection. Induction of cellular DNA synthesis began to rise from 0.1 $TCID_{50}$ per cell, and the plateau level was obtained at about 1 $TCID_{50}$ per cell under the experimental conditions described above.

Fate of C3H2K cells activated to synthesize DNA by virus infection. To examine whether mitosis is arrested after virus infection, as has been

reported (17, 19, 27, 28, 31, 34), or whether cells activated to enter the S phase can progress to division, the change of number of cells was examined after virus infection. G_1-arrested monolayer cells were made as shown in Fig. 1, and cells were counted 48 and 72 hr after virus infection (10 $TCID_{50}$/cell).

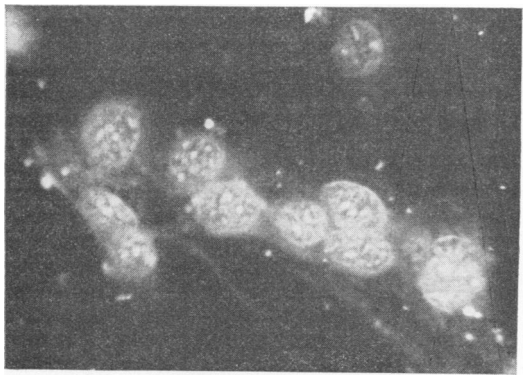

FIG. 3. *Production of T antigen of BAV-3 in virus-infected C3H2K cells (200 $TCID_{50}$/cell). At 48 hr after infection, cells were fixed, treated with fluorescent antibody, and then observed by using fluorescent microscopy (× 600).*

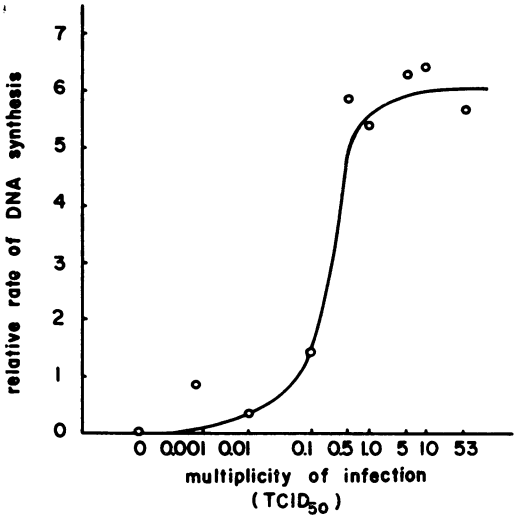

FIG. 4. *Relationship between multiplicity of infection and extent of induction in cellular DNA synthesis. Experimental procedures and abbreviations for experimental conditions are the same as for Fig. 2. After adsorption for 2 hr with virus at the input multiplicity indicated on the abscissa, the medium was changed to a maintenance medium (VO). At 24 hr after virus infection, ^3H-thymidine was added to the culture, which was then incubated for 1 hr. The acid-insoluble fraction was counted as described in the text. As control experiments, mock infections (MO) were carried out. Abscissa: input multiplicity of infection ($TCID_{50}$/cell). Ordinate: [(counts/min of experiment VO) − (counts/min of experiment MO)]/(counts/min of experiment MO). Each point is the average of three experiments.*

As shown in Fig. 5, increases by about 120 and 40% were observed 48 hr after virus- and mock-infected cells, respectively (in another experiment, 130 and 20% increases, respectively). In other words, it is likely that the number of cells in the virus-infected culture nearly doubled 48 hr after virus infection, while the mock-infected culture showed little increase.

DISCUSSION

As is shown in Fig. 1, C3H2K cells used for these experiments were largely in G_1 phase of the cell cycle before virus infection. In these cells, almost all of the cells were activated to enter cell division via S and G_2 phase with more than 60% synchrony by serum factor.

Data obtained by using this system clearly demonstrate that infection of a mouse cell line, C3H2K, with BAV-3 resulted in the induction of T antigen and host cell DNA synthesis followed by cell division. As shown in Fig. 5, even in the absence of serum, many, if not all, of the cells proceeded to cell division.

In experiments M10 and VO (*see above*), cells were largely in G_1 phase before medium change or virus infection, after which most of the cells divided finally both in M10 and VO (Fig. 1 and 5). The patterns (time course) of the rate of DNA synthesis both in M10 and VO were very similar

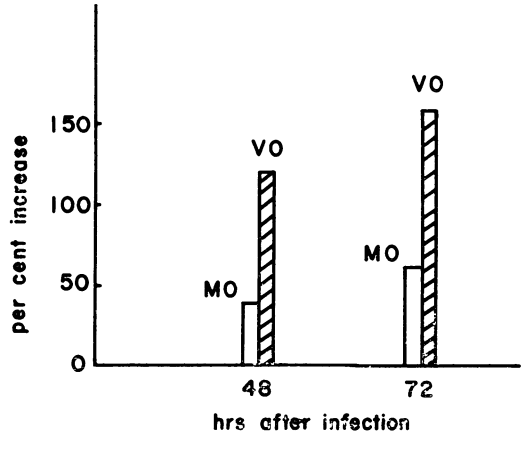

FIG. 5. *Increase of number of cells after virus infection. At 9 days after the cells were seeded as described in Fig. 1., monolayer cells were infected with BAV-3 (10 $TCID_{50}$/cell). Cells were counted 48 and 72 hr after infection. Open bars, MO; cross-hatched bars, VO (abbreviations are given in Fig. 2). Abscissa: hours after infection. Ordinate: per cent of increase in number of cells. Each point is the average of four experiments.*

to each other (Fig. 2). In addition, a higher rate of DNA synthesis was observed in VO than in M10 (Fig. 2). Considering these results and that more than 60% of cells are activated synchronously in M10 (Fig. 1), it is expected that a higher degree of synchronization may be occurring in VO.

Furthermore, it should be noted that neither cell killing nor mitotic arrest (17, 19, 22, 23, 26 27, 28, 31, 34) occurred in this virus-cell interaction. In addition, it is also unlikely that cellular DNA induced in virus-infected cells has unusual character, as has been reported (1), for the activated cells could divide normally in our system.

However, a remarkable morphological alteration was observed, which was transmitted to some progeny cells and was similar to that seen in malignantly transformed cells (a transformed clone which was keeping the altered character and also keeping BAV-3-specific surface antigen was observed; *manuscript in preparation*).

In other words, it is suggested that BAV-3 induces division of fairly high proportion of C3H2K cells, which have been arrested in G_1 phase, with a concomitant morphological alteration characteristic to transformed cells. It is also noteworthy that these inductions in virus-infected cells occurred in medium lacking serum.

The same cell line could also be induced by serum factor(s) to enter cell division with normal morphology.

Employing the latter system (with serum factor) as a normal control, one can utilize the former system (with BAV-3 in medium lacking serum factor) to analyze a series of events occurring in cells which are being activated from normal to transformed state. Studies along this line are in progress.

Recently, as the present investigation was nearing completion, similar activation of cell division by virus infection was reported in BALB/c-3T3 cells infected with polyoma Ts-a (6) and also in BALB/3T3 cells infected with SV40 (33).

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