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Mechanism of Oncogenic Transformation by Rous Sarcoma Virus

I. Intracellular Inactivation of Cell-Transforming Ability of Rous Sarcoma Virus by 5-Bromodeoxyuridine and Light

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Chick embryo fibroblasts brought into stationary phase of growth by maintenance in serum-free Eagle's MEM medium were infected with the Bryan strain of Rous sarcoma virus (B-RSV) and incubated for 18 hr in the presence of 5-bromodeoxyuridine (BUdR). The cells were then allowed to resume growth and deoxyribonucleic acid (DNA) synthesis by addition of an enriched F12 medium containing serum and RSV antibody to prevent spread of viral infection. After 48 hr, the cultures were exposed for various periods to visible light, overlaid with solid culture medium, and observed for the appearance of foci of transformed cells. In cultures treated with BUdR at the time of infection, exposure to light resulted in a suppression of focus formation of from 50 to 90% in various experiments. Treatment with BUdR for 18 hr before infection or on the day after infection, followed by exposure to light, had no effect on focus formation. In cultures in which almost all cells were infected, treatment with BUdR followed by exposure to light did not result in cell death. This suggests that suppression of transformation is not due to selective killing of infected cells by this treatment but rather to the intracellular inactivation of the transforming ability of Rous sarcoma proviral DNA.

The mechanism by which Rous sarcoma virus (RSV) induces oncogenic transformation in vivo and in vitro has not been elucidated. The persistence of virus-specific information in transformed cells is demonstrated by the fact that transformed cells continue to produce infectious virus, transmit this property to progeny cells by an intracellular route (16), and also assume an altered morphology characteristic of the strain of virus (13). According to Temin (14, 15) the viral information present in infected cells is in the form of a deoxyribonucleic acid (DNA)-containing structure called the provirus. Although the provirus is thought to play a role in the production of infectious virus and in the type of morphologic conversion of infected cells, it is not clear whether its function is required to express and maintain the transformed state.

An answer to this problem could come from the inactivation of the provirus within the infected cell. Selective destruction of the provirus, shortly after "fixation" of the transformed state of the cell (10, 15), might result in: (i) development of the transformed condition, which would support the view that a functioning provirus is not required for expression of the malignant state; or (ii) failure to express the transformed condition, which would indicate a direct role of the provirus in malignancy.

An approach to the selective inactivation of proviral DNA is provided by the technique devised by Puck and Kao to select nutritionally deficient mutants of mammalian cells (11). This procedure involves sensitization of DNA to visible or near visible light by incorporation of 5-bromodeoxyuridine (BUdR).

In the experiments reported here, attempts were made to achieve the selective destruction of proviral DNA by this technique. The results obtained show that, in chick embryo cultures infected with RSV in the presence of BUdR and irradiated 2 days later with visible light, formation of foci of transformed cells is suppressed. This suggests that the induction of the malignant state is a provirus function.

MATERIALS AND METHODS

Virus. The Bryan strain of Rous sarcoma virus (B-RSV) supplied by W. R. Bryan (National Cancer Institute, Bethesda, Md.) was used throughout these

experiments. Virus stocks were prepared by removing supernatant fluids from infected chick embryo fibroblast cultures and were stored at −70 C until used.

Cells and cell cultures. Chick embryo fibroblasts (CEF) were obtained from embryonated eggs from a flock of chickens maintained by R. E. Luginbuhl (University of Connecticut, Storrs) and supplied by the Research Resource Program of the National Cancer Institute. All cells were tested for uniform susceptibility to infection with B-RSV as already described (9). Second-passage cultures were used for all light inactivation experiments. First-passage cultures were employed for virus titrations carried out as previously described (8). F12 medium (2) with 10% tryptose phosphate broth, 8% calf serum, 2% normal chicken serum, and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml) was used as growth medium for both first- and second-passage cell cultures. The same medium containing 0.9% agar was used to overlay the focus assay plates which were incubated at 39 C and fed again with the same medium at day 5. At day 9, a few drops of neutral red (0.1% in sterile, distilled water) were added to the plates and the focus-forming units (FFU) were counted the next day. Eagle's Minimum Essential Medium [MEM (1)] without serum was used to bring the cultures to a stationary phase (depletion medium). F12 medium with 5% bovine fetal serum was added to the cultures to permit resumption of growth (enriched medium).

Antiserum. A hyperimmune turkey serum with antibody against B-RSV, kindly supplied by F. J. Rauscher, National Cancer Institute, was added to appropriate culture medium (1:500) to prevent reinfection of cells.

Autoradiography. The autoradiography experiments were carried out with cells grown on 60-mm Falcon plastic petri dishes. At the appropriate time, cultures were pulse labeled with ^{3}H-thymidine [^{3}H-TdR (0.5 μc/ml)] for 30 min, after which they were fixed with Formalin, rinsed several times with distilled water, and treated with acetic acid-methanol (3:1) for 5 min. When the plates were dry, discs were cut from the bottom and attached to slides as described (4). The slides were covered with emulsion (Eastman Kodak NTB2), stored at 4 C for 10 days, and developed. The cells were then stained with Giemsa and the percentage of nuclei labeled was determined by examining about 500 cells for each plate.

Standard procedure for inactivation of proviral DNA. First-passage chick embryo fibroblasts were trypsinized and planted in 60-mm Falcon plastic petri dishes at a density of 5×10^5 cells per plate in growth medium. The following day, fluids were removed and plates were fed with Eagle's MEM (depletion medium). When the stationary-phase condition of the cultures was obtained, each was inoculated with 100 FFU of virus in 0.2 ml of a tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-buffered balanced salt solution. After adsorption for 30 min, plates were fed with depletion medium containing BUdR (10^{-5} M or 5×10^{-6} M). After 18 hr, fluids were removed and the plates were washed and fed with enriched medium containing RSV hyperimmune serum (1:500). At the time indicated, plates with 3 ml of enriched medium with Tris were exposed for various periods of time to the light from two fluorescent lamps (40-w, GE cool-white tubes, 120 cm long) mounted in a fixture with a reflector, where the lamps were approximately 10 cm apart and 10 cm above the dishes. Control plates were kept near to the irradiated ones and shielded from light by an aluminum tray for the maximum period of light exposure. At the end of the irradiation period, the fluids were removed and the plates were overlaid for focus assays as described.

Cell clones. Chick embryo cells were removed from petri dishes with 0.05% trypsin, sedimented in the centrifuge, resuspended in F12 medium, and filtered through a fine-wire gauze to remove cell clumps. Volumes of 0.5 ml containing 200 cells were added to 60-mm plastic petri dishes containing 3.5 ml of "conditioned" F12 medium. This medium consisted of equal volumes of fresh F12 medium and of the same F12 medium which had been exposed to normal chick embryo cells for 4 days. These cultures were incubated for 10 days at 37.5 C in a CO_2 incubator. At this time, the medium was removed and the cells were fixed in 10% Formalin and stained with hematoxylin; the clones were then counted.

Assay of cell sensitivity to infection with virus. Cells were removed from culture plates as described above. Two million cells in F12 medium were planted in each of four 60-mm plastic petri dishes and allowed to attach for 4 hr at 37.5 C. The medium was then removed, and 0.2 ml of virus diluted in Tris buffer was added to each plate. After 60 min at 37.5 C, the cells were overlaid with F12 medium containing agar and were incubated at 39 C for 10 days, at which time the foci of transformed cells were counted.

Cell counts. Cells in a number of randomly picked areas of unit size in plates fixed with 10% Formalin were counted with a phase contrast microscope (magnification, 400). Usually, the cell counts were carried out with plates used also for autoradiography experiments. The reported values, calculated from counts of 1300 to 1600 cells per plate, are expressed relative to cell counts at the beginning of the experiment.

RESULTS

Cell studies. Conditions were studied to provide for the selective incorporation of BUdR into the proviral DNA by exposing cells in stationary phase to the analogue. It was found that the use of medium without serum (depletion medium) caused most cells to stop DNA synthesis in a few days. In several experiments, after 3 days in depletion medium, 1 to 5% of the cells were found to be in S (DNA synthesis) phase when cultures were pulse labeled with ^{3}H-TdR for 30 min. When F12 medium with serum (enriched medium) was added to these stationary-phase cultures, cellular DNA synthesis resumed promptly, providing the conditions required for "fixation" of the transformed state caused by RSV infection (10, 15). In several experiments, the frequency of cells in S phase rose to 20 to 25% 1 day after the addition of enriched medium with a pulse labeling with ^{3}H-TdR for 30 min.

Experiments were then carried out to evaluate

the effect of BUdR treatment and irradiation according to the standard procedure (*see* Materials and Methods) on the growth of exposed cultures. Cultures were treated with BUdR in MEM with or without serum. Half of the plates in each set were exposed to light for 90 min at the end of the BUdR treatment (day 1). Thereafter, these plates and unirradiated controls were fed with enriched medium and studied for several days. The data presented in Fig. 1 show that the number of cells per plate does not increase during exposure to BUdR whether or not the serum is present in the medium during this period. However, after addition of enriched medium, the cells kept in medium with serum during the previous 18 hr multiply to a larger extent than cells kept in MEM without serum. This suggests that a number of cells in cultures with serum entered DNA synthesis during the exposure to BUdR. In the irradiated set of plates (Fig. 1A), cultures treated with BUdR in depletion medium continue to grow at the same rate as cultures not exposed to the analogue. On the other hand, the rate of growth in cultures exposed to BUdR in MEM with serum drops sharply

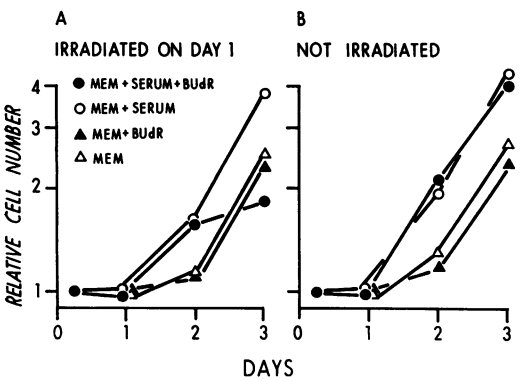

FIG. 1. *Effect of BUdR and light treatment on the growth of CEF exposed to the analogue in the presence or absence of serum. Secondary cultures in stationary phase, prepared as described in Materials and Methods, were exposed to BUdR (10^{-5} M) in either MEM without serum or in MEM with 5% serum. Control plates were fed with either medium, without BUdR. After 18 hr, all the plates were rinsed once with MEM and half of the cultures from each set were exposed to visible light for 90 min, rinsed again, and fed with enriched F12 medium (A). The remaining non-irradiated plates from each set were also fed with enriched F12 medium (B). At different times, plates were fixed with 10% Formalin and air dried. The cells from representative areas of each plate were counted with a phase contrast microscope (magnification, 400). The values represent the number of cells in plates fixed at different times relative to the number of cells at the beginning of the experiment. The data were obtained from counts of 1300 to 1500 cells per plate.*

at day 3. This indicates that a large number of cells in these cultures fail to multiply. Microscopic examination of these cultures shows morphological alterations of cells ranging from vacuolation to formation of multinucleated cells. Figure 2 shows the cell density of cultures at day 3. The cell density of cultures exposed to BUdR in medium with serum is lower than that of cultures exposed to the analogue in depletion medium, and areas of morphologically altered cells are evident.

These experiments show, therefore, that irradiation of cells exposed to BUdR under conditions allowing for incorporation of the analogue in cellular DNA results in a strong inhibition of growth. On the contrary, irradiation of cultures exposed to the analogue in depletion medium does not affect cell growth. Additional evidence on this point is provided by experiments in which cultures were exposed to BUdR in MEM without serum, irradiated on day 1, resuspended with trypsin, and seeded for efficiency of plating assays according to the procedure outlined in Materials and Methods. From the data reported in Table 1, it can be seen that the cloning efficiency of cells treated with BUdR and light is not significantly different from that of control cells or of cells treated only with either BUdR or visible light.

Experiments were also carried out to investigate the susceptibility of cells treated with BUdR and light to infection by RSV. Cultures were treated as in the experiment of Table 1 and assayed for susceptibility to infection as described in Materials and Methods. The data reported in Table 2 indicate that cells treated with BUdR and then irradiated are as susceptible as control cells to the virus.

Virus inactivation studies. After it was determined that the standard treatment with BUdR and light does not affect viability of the cells or their ability to become transformed, a number of experiments were carried out to attempt the inactivation of proviral DNA by using the same basic procedure. Cultures were infected as described in Materials and Methods and were fed with MEM with or without BUdR. Thereafter, all plates were fed with enriched medium to allow the resumption of cellular DNA synthesis and "fixation" of the transformed state. The enriched medium also contained RSV antiserum to prevent secondary spread of the virus. Two days later, the plates were irradiated for various periods and overlaid for FFU assays. The values of Fig. 3 represent the percentage of FFU appearing in irradiated plates treated with BUdR, and in plates not exposed to the analogue, as compared to the number of FFU appearing in plates not exposed to light. It can be seen that irradiation suppresses formation of foci of transformed cells in cultures

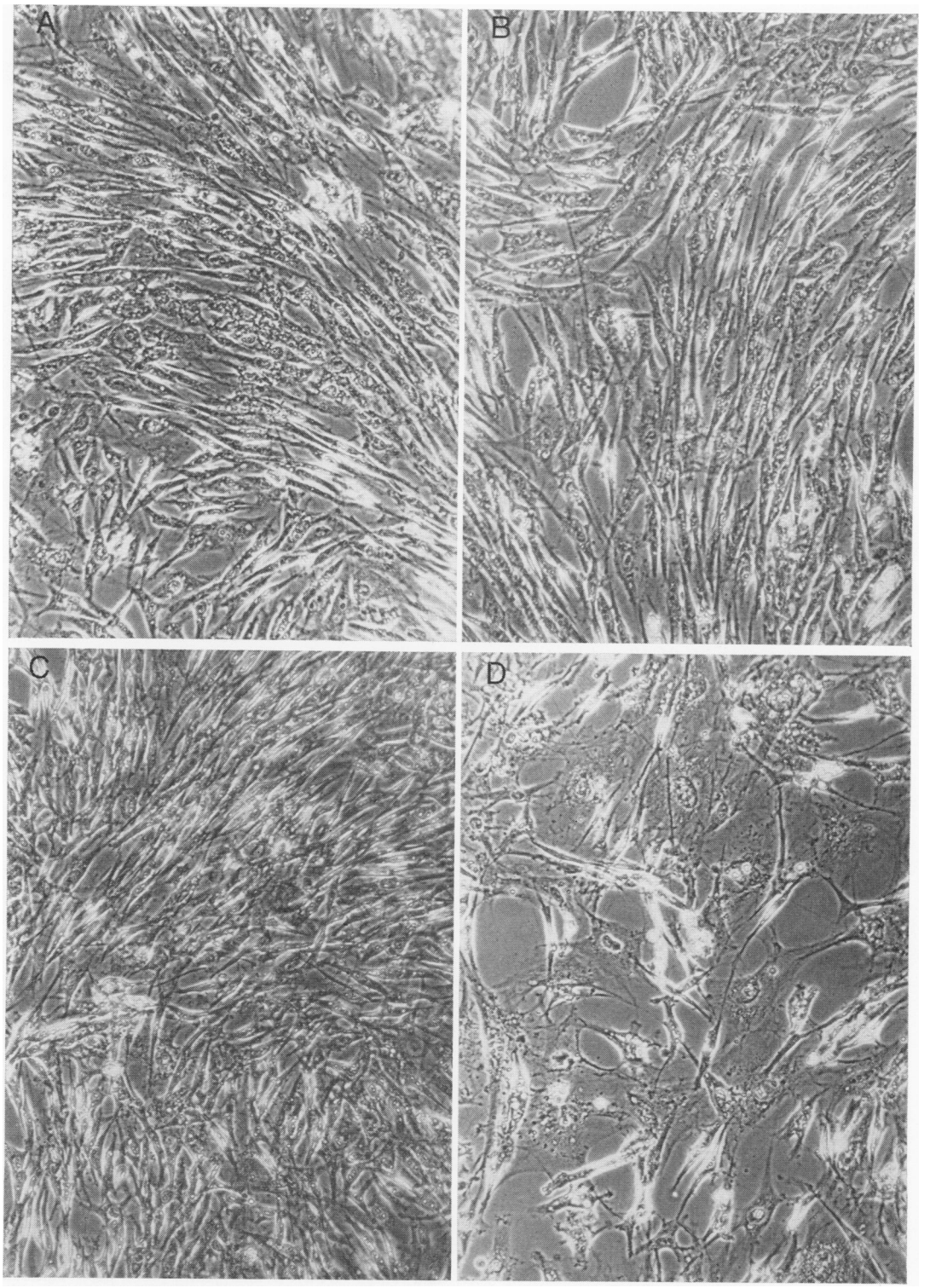

FIG. 2. *Effect of BUdR and light treatment on the morphology and density of cells in cultures exposed to the analogue in the presence or absence of serum. From plates of Fig. 1 (A) at day 3: A, MEM; B, MEM and BUdR; C, MEM and serum; D, MEM, serum, and BUdR.*

TABLE 1. *Cloning efficiency of cells treated with BUdR and light*

Cell treatment[a]		No. of clones for 200 cells	Avg	Per cent efficiency of plating
BUdR (10^{-5} M)	Irradiation for 90 min			
−	−	56, 56, 60, 67	60	30
+	−	56, 58, 60, 63	59	30
−	+	36, 38, 50, 53	44	22
+	+	53, 56, 60, 62	58	29

[a] Stationary-phase cultures were treated with BUdR (10^{-5} M) in depletion medium. Replicate plates were fed with the same medium without BUdR. After 18 hr, all plates were rinsed once with MEM and half of the plates from each set were exposed to visible light for 90 min. Thereafter, the cells from each culture were resuspended with trypsin, diluted in "conditioned" medium (*see* Materials and Methods), and seeded in new plates at a concentration of 200 cells per plate. After 10 days, all plates were fixed with 10% Formalin and stained with hematoxylin, and the clones were counted.

TABLE 2. *Susceptibility to RSV of chick embryo cells treated with BUdR and light*

Cell treatment[a]		RSV FFU	Avg	Per cent of control
BUdR (10^{-5}M)	Irradiation for 90 min			
−	−	323, 400, 346, 388	364	100
+	−	422, 428, 342, 268	365	100
−	+	314, 324, 252, 296	296	81
+	+	278, 344, 354, 380	339	93

[a] Stationary cultures were treated with BUdR and light as in the experiment of Table 1. Thereafter, the cells from each set of cultures were resuspended with trypsin and planted at a concentration of 2×10^6 cells per plate. After 4 hr, these cultures were infected with a dilution of B-RSV containing 400 FFU in 0.2 ml. All cultures were overlaid with agar medium after 30 min of adsorption period and foci of transformed cells were counted 10 days later.

treated with BUdR but not in untreated cultures. Treatment with BUdR without irradiation did not reduce the number of foci as compared to control cultures, indicating that BUdR alone at this concentration has no effect on transformation. Furthermore, the expected number of foci developed in these cultures. This indicates that the experimental system was adequate for focus assays.

To investigate the time relationship of BUdR treatment to suppression of focus formation, experiments were carried out in which different sets of plates were exposed to BUdR for 18 hr either before infection, immediately after infection, or the day after infection and were irradiated 2 days later. The results observed with this type of experiment are presented in Fig. 4. Light inactivated focus formation only in plates which had been exposed to BUdR immediately after infection. These results show that to be effective BUdR has to be present at the time of proviral DNA synthesis. Treatment of cells with BUdR before or after this critical period does not render the focus-forming activity susceptible to light inactivation.

These experiments, however, do not exclude the possibility that suppression of transformation is a result of selective killing of infected cells by the experimental procedure. It is possible, in fact, that infection with RSV might stimulate cellular DNA synthesis, with resulting incorporation of BUdR into cellular DNA. This would render the cells sensitive to irradiation with suppression of focus formation. Two lines of evidence were investigated relative to this point.

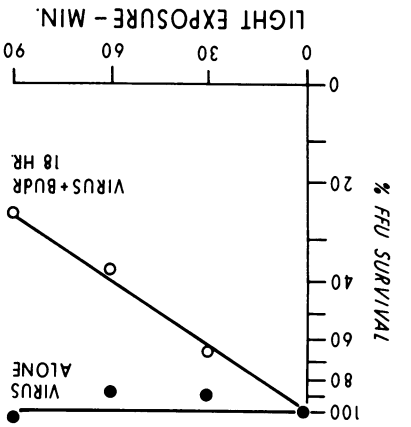

FIG. 3. *Effect of visible light on focus formation in plates infected with B-RSV in the presence of BUdR. Secondary cultures in stationary phase were infected with a dilution of B-RSV containing 100 FFU in 0.2 ml for 30 min at 37 C. At the end of the adsorption period, half of the plates were fed with MEM containing BUdR (5×10^{-6} M); the remaining plates were fed with MEM without BUdR. After 18 hr of incubation at 37 C, the fluid in all plates was removed and the plates were rinsed once with MEM and fed with enriched medium containing antiserum against B-RSV to prevent secondary infection of cells. Two days later, one set of plates treated with BUdR and one set without BUdR were exposed to visible light for the time indicated and then overlaid for FFU assay. Three control and three BUdR-treated plates were kept shielded from light and also overlaid. Data are expressed as percentages of FFU in irradiated plates compared to numbers of FFU in plates not exposed to light. Each point is the average of the counts of three plates.*

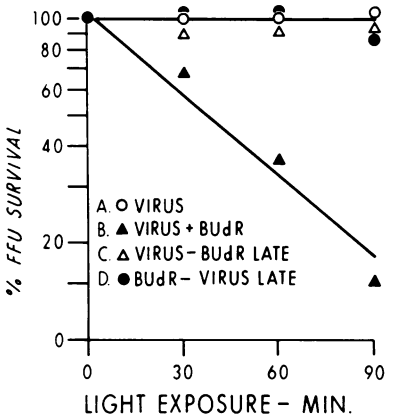

FIG. 4. *Effect of BUdR treatment before, during, and after infection with B-RSV. Three sets of secondary cultures in stationary phase were infected with 100 FFU of RSV (A, B, C). After adsorption for 30 min, two sets (A and C) were fed with MEM without serum; the other set (B) was fed with the same medium containing BUdR (5×10^{-6} M). A fourth set of non-infected plates (D) was also fed with MEM with BUdR. After 18 hr, plates of sets A, B, and C were rinsed and fed with MEM containing RSV antiserum; set D plates were fed with MEM only. At 24 hr after beginning of infection, set C received MEM with antiserum and BUdR and set D was infected with B-RSV. After 30 min of adsorption, this set was also fed with MEM without serum. After 18 hr, all plates were rinsed and fed with enriched medium plus RSV antiserum. After 2 days, all plates were exposed to visible light for the time indicated, overlaid with agar medium, and assayed for FFU. Results are expressed as percentages of FFU in plates not exposed to light. The values are the averages of two plates per point.*

First, in a series of experiments, DNA synthesis in infected cells was studied by autoradiographic techniques. Cultures were infected with undiluted preparation of RSV (titer 2×10^7 FFU/ml) to insure near-simultaneous infection of the majority of the cells. As control, replicate cultures were inoculated with undiluted preparations of virus inactivated at 60 C for 1 hr. This inoculum, therefore, contains the same amount of serum as the infectious virus preparation. Evidence for the high level of infection in cultures exposed to infectious virus was provided by the massive transformation of replicate cultures overlaid with agar medium. Similar cultures inoculated with the heat-treated virus failed to reveal a single focus of transformed cells. Both infected and mock-infected cultures were exposed to BUdR in depletion medium for 18 hr, irradiated for 90 min on day 1, and fed with enriched F12 medium. At various times during the course of the experiment, cultures were pulse labeled for 30 min with ^{3}H-TdR, and the percentage of cells in S phase was measured. (Fig. 5). It can be seen that the frequency of pulse-labeled cells does not increase during the first 18 hr after infection or mock infection. In experiments with continuous labeling for 18 hr in the presence of BUdR, the percentage of S-phase cells in infected cultures is not higher, and in some experiments is lower than that of mock-infected cultures. Moreover, the average grain count per cell is lower in the infected cultures than in the mock-infected ones. This suggests an inhibition rather than a stimulation of cellular DNA synthesis by virus infection (Fig. 6). This inhibition, however, could be due to some toxic factor in the untreated inoculum. After irradiation and addition of enriched medium, the frequency of cells in S phase increases to about 12 to 14% in both infected and mock-infected cultures. The percentage of cells synthesizing DNA remains at approximately the same value at day 3 for both sets of cultures. At day 4, the level of cells in S phase drops to about 10% in mock-infected plates, whereas it persists at a 15% level in the infected cultures in coincidence with morphological evidence of massive transformation in these cultures. These results clearly indicate that RSV infection does not stimulate DNA syn-

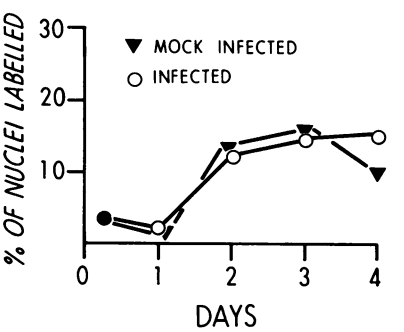

FIG. 5. *Effect of B-RSV infection on DNA synthesis in CEF. Stationary-phase cultures were prepared as in previous experiments. One set of plates was infected with 0.2 ml of undiluted B-RSV (titer 2×10^7 FFU/ml). Replicate plates were mock-infected with the same preparation of virus inactivated at 60 C for 1 hr. After 30 min of adsorption, both sets of plates were exposed to BUdR (10^{-5} M) in MEM without serum. After 18 hr, all plates were rinsed once with MEM and irradiated with visible light for 90 min. Thereafter, all plates were rinsed again and fed with enriched medium. At various times, plates were pulse labeled with ^{3}H-TdR (0.5 μc/ml) for 30 min, fixed with 10% Formalin, and processed for autoradiography as described in Materials and Methods. The reported values are percentages of cells with labeled nuclei at various times in the course of the experiment. Percentage values were calculated on counts of at least 500 cells per plate.*

thesis in stationary phase cells during exposure to BUdR in depletion medium, or within 2 days thereafter.

A second, direct approach to the problem of BUdR incorporation in infected cells, and its possible effect on cell viability and ability to multiply, was a study of cell growth in heavily infected cultures. Cultures were exposed to RSV at high multiplicity of infection. As control, replicate cultures were inoculated with the same preparation of virus inactivated at 60 C for 1 hr. Both infected and mock-infected cells were exposed to BUdR in depletion medium and were irradiated for 90 min 18 hr later. Thereafter, all cultures were fed with enriched medium. Cell growth in these plates was measured at various times, and cultures were examined for morphological alteration of the cells. The results of these experiments are reported in Fig. 7. It can be observed that the growth of infected cultures does not differ significantly from that of mock-infected cultures in either the irradiated cultures or in the nonirradiated ones. No appreciable growth occurs during exposure to BUdR. After addition of enriched medium, growth resumes promptly. No inhibition of growth takes place on day 3 in the irradiated cultures. This is in contrast with what was observed with noninfected cultures exposed to BUdR in the presence of serum, when incorporation of BUdR in cellular DNA had presumably occurred (*see* Fig. 1A). Also the density and the morphology of cells in infected plates treated with BUdR and light are similar to those of mock-infected cultures.

The results of these two sets of experiments support the view that no significant incorporation of BUdR in cellular DNA occurs as a consequence of RSV infection and that infected cells are not harmed by irradiation. The overall evidence from these experiments, therefore, indicates that inactivation of focus formation is not due to selective killing of infected cells. It rather suggests that proviral DNA, sensitized by BUdR, is selectively inactivated by visible light and that infected cells survive without expressing the transformed state.

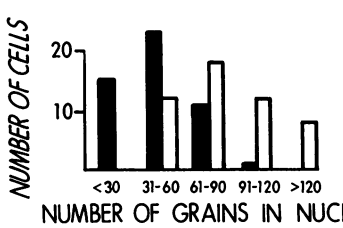

FIG. 6. *Frequency distribution of cells with the number of grain per nucleus indicated in the abscissa. Counts of grains per nucleus were carried out on autoradiographic preparations of plates continuously labeled for 18 hr with ^{3}H-TdR (0.5 μc/ml) from the beginning of the experiment of Fig. 5 to the time of irradiation (day 1). Data are based on counts of 100 nuclei. Solid columns, infected; open columns, mock-infected.*

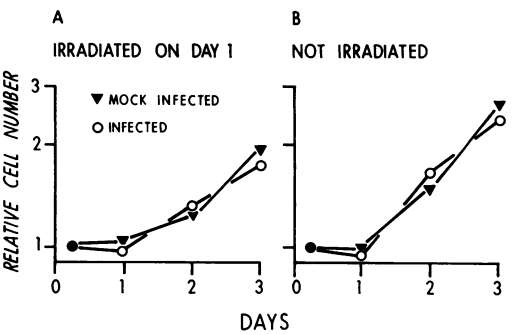

FIG. 7. *Effect of BUdR and light treatment on the growth of RSV-infected CEF. Stationary-phase cultures, prepared as in previous experiments, were infected with 0.2 ml of undiluted B-RSV (titer 2 × 10^7 FFU/ml). Replicate plates were mock-infected with the same preparation of virus inactivated at 60 C for 1 hr. After 30 min of adsorption, both sets of plates were exposed to BUdR (10^{-5} M) in MEM without serum. After 18 hr, all plates were rinsed once with MEM and irradiated with visible light for 90 min. Thereafter, all plates were rinsed again and fed with enriched medium. At various times, plates were fixed with 10% Formalin and air dried. The cells from representative areas of each plate were counted with a phase contrast microscope (magnification, 400). The values represent the numbers of cells in plates fixed at different times, relative to the numbers of cells at the beginning of the experiment. The data were obtained from counts of 1300 to 1500 cells per plate.*

DISCUSSION

The experiments reported here represent an approach to the definition of the role of viral genetic information in the expression of the malignant state in transformed cells and in their progeny. The approach investigated here is the selective destruction of intracellular viral information of cells infected with B-RSV, a strain which transforms chick embryo fibroblasts with high efficiency. In the case of RSV, the critical event in oncogenic transformation of the cell is the synthesis of a DNA-containing provirus (14, 15). The use of BUdR to make this proviral DNA susceptible to inactivation by visible light has been explored in this study, and the conditions worked out which appear to permit a selective incorporation of the analogue into proviral DNA. Under the conditions of metabolic deprivation of cells maintained in MEM without serum, cellular DNA synthesis occurs in less than 5% of the cells. However, susceptibility to viral infection and synthesis of proviral DNA are not affected. In this situation, incorporation of BUdR appears to

occur in proviral DNA which is rendered sensitive to visible light in a selective manner, whereas the cell viability and susceptibility to infection are apparently unharmed. The exact time of proviral DNA synthesis has not been established, but it appears to occur to a large extent within 18 hr after infection. The addition of serum in an enriched medium after this period allows resumption of cellular DNA synthesis with "fixation" of the virus-induced transformed state of the cell (10, 15). When cultures containing RSV-infected cells treated with BUdR are exposed to visible light and then assayed for development of FFU, inhibition of focus formation is observed. This inhibition proceeds with one-hit kinetics, suggesting the inactivation of one target per cell.

An important question which arises is whether the reduction in FFU is caused by the destruction of infected cells exposed to BUdR. This could occur if RSV in these conditions stimulates cellular DNA synthesis, with resulting incorporation of BUdR. The experiments presented here demonstrate that most cells can be infected when a large dose of virus is employed, an observation which confirms results obtained by others (3). However, a study of cellular DNA synthesis in stationary cultures infected at high multiplicity or mock-infected with heat-inactivated virus in depletion medium did not reveal any stimulation of DNA synthesis. These results are in agreement with data obtained by Temin in similar conditions (H. M. Temin, *personal communication*).

Recent reports by others (5–7) on stimulation of DNA synthesis by RSV infection do not contradict our data, since they are based on results obtained with different experimental conditions. Moreover, the stimulation observed may actually be due to some factor, other than the virus, present in the inoculum (7) or it may reflect an early release from inhibition of DNA synthesis as a consequence of virus-induced transformation (12). In addition to the failure to detect stimulation of DNA synthesis in our nutritionally deprived conditions, the examination of BUdR-treated cultures in which the majority of cells are infected reveals neither inhibition of growth rate after exposure to light nor morphological alterations of cells. It does appear, therefore, that the cells themselves are not damaged. This in turn suggests that the proviral DNA is selectively destroyed in the infected cell. As a consequence, the cell fails to become transformed and to produce infectious virus able to transform surrounding cells.

The results of these experiments, therefore, indicate a requirement for an intact provirus for the expression of the malignant state.

Temin recently reported [*In* R. Barry and B. Maky (ed.), *The Biology of Large RNA Viruses*, Academic Press Inc., *in press*] preliminary results obtained by Boettiger on the inactivation of foci formation by the B77 strain of RSV exposed to 500 mg of BUdR per ml and light. The interpretation of the phenomenon offered by these authors involves the inactivation of newly formed DNA which has incorporated BUdR and is in agreement with the conclusions of the present study.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–433.
2. Ham, R. G. 1965. Clonal growth of mammalian cells in a chemically defined synthetic medium. Proc. Nat. Acad. Sci. U.S.A. **53**:288–290.
3. Hanafusa, H. 1959. Rapid transformation of cells by Rous sarcoma virus. Proc. Nat. Acad. Sci. U.S.A. **63**:318–325.
4. Hare, J. D. 1966. A simplified sampling technique for studying radio-isotope labelling of cells in culture. Proc. Soc. Exp. Biol. Med. **121**:774–777.
5. Kara, J. 1968. Induction of cellular DNA synthesis in chick embryo fibroblasts infected with Rous sarcoma virus in culture. Biochem. Biophys. Res. Commun. **32**:817–824.
6. Lee, H. H., M. E. Kaighu, and J. D. Ebert. 1968. Induction of thymidine-^{3}H incorporation in multinucleated myotubes by Rous sarcoma virus. Int. J. Cancer **3**:126–136.
7. Macieira-Coelho, A., I. J. Hiu, and E. Garcia-Giralt. 1969. Stimulation of DNA synthesis in resting stage human fibroblasts after infection with Rous sarcoma virus. Nature **222**: 1172.
8. Morgan, H. R. 1965. Antibodies for Rous sarcoma virus in fowl, animal and human populations of East Africa. I. Antibodies in domestic chickens and wild fowl. J. Nat. Cancer Inst. **35**:1043–1045.
9. Morgan, H. R. 1968. Ultrastructure of the surfaces of cells infected with avian leukemia-sarcoma viruses. J. Virol. **2**: 1133–1146.
10. Nakata, Y., and J. P. Bader. 1968. Studies on the fixation and development of cellular transformation by Rous sarcoma virus. Virology **36**:401–410.
11. Puck, T. T., and F. Kao. 1967. Genetics of somatic mammalian cells. V. Treatment with 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants. Proc. Nat. Acad. Sci. U.S.A. **58**:1227–1234.
12. Rubin, H., and C. Colby. 1968. Early release of growth inhibition in cells infected with Rous sarcoma virus. Proc. Nat. Acad. Sci. U.S.A. **60**:482–488.
13. Temin, H. M. 1960. Control of cellular morphology in embryonic cells infected with Rous sarcoma virus in vitro. Virology **10**:182–197.
14. Temin, H. M. 1964. The participation of DNA in Rous sarcoma virus production. Virology **23**:486–494.
15. Temin, H. M. 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and for cell division. J. Cell. Physiol. **69**:53–66.
16. Temin, H. M., and H. Rubin. 1959. A kinetic study of infection of chick embryo cells in vitro by Rous sarcoma virus. Virology **8**:209–222.