Superinduction of Endogenous Type C Virus by 5-Bromodeoxyuridine from Transformed Mouse Clones

Abstract. *Certain transformed subclones of the mouse cell line BALB/3T3 release 5 to 15 times more type C virus per cell than the parent cell line after treatment with 5-bromodeoxyuridine. Virus release begins within 8 hours and exponentially increases for the first 24 to 48 hours. Superinducibility is associated with the transformed phenotype.*

Treatment with 5-bromodeoxyuridine (BrdU) produces a variety of effects on differentiated functions of animal cells in tissue culture (*1*). One effect is the induction of endogenous type C viruses from virus nonproducing clones of cells derived from several mammalian species (*2*). We now report that certain transformed, tumorigenic subclones derived from the mouse BALB/3T3 cell line produce type C virus much more rapidly and in much larger quantities compared to the parental nontransformed cell line after treatment with BrdU. These superinducible clones have the capacity to generate extracellular virus particles within 8 hours after treatment with the inducing agent. Induction of type C virus from these cell lines constitutes a useful model system for studying the mechnisms that control gene expresssion in vertebrate cells.

BALB/3T3 clone A31 is a contact-inhibited, nontumorigenic cell line (*3*). The following are transformed subclones of A31: S16 Cl-8, S16 Cl-10, S16 Cl-11, and S16 Cl-12 are spontaneous transformants (*4*); KA31 clone 234 is a sarcoma virus transformed nonproducer cell line (*5*); MC5-5 is a subclone transformed in vitro by methylcholanthrene (*6*); and R4 is a radiation-induced transformant (*7*). The cells were maintained in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10 percent calf serum (Colorado Serum). None of the cell lines spontaneously releases detectable levels of infectious type C virus (*8*). Type C virus content of the tissue culture medium was measured by an assay for the viral reverse transcriptase enzyme (*9*) described by Ross *et al.* (*10*). 5-Bromodeoxyuridine (Calbiochem) at a final concentration of 33 μg per milliliter of medium was used to induce virus production. Previous experiments had demonstrated that concentrations of BrdU from 10 to 100 μg/ml yielded approximately the same quantity of induced virus with these cell lines (*4*).

Table 1 shows the results of a series of experiments in which various clone A31 transformants were treated with BrdU for 24 hours during the log phase of growth. All the transformed cell lines release (per flask) more endogenous type C virus than the parent cell line. However, when these values are corrected for the number of cells in each flask, two transformed cell lines, S16 Cl-10 and R4, were roughly equal to clone A31 in virus yield per cell; however, S16 Cl-8, S16 Cl-11, and S16 Cl-12 produced 4 to 5 times more virus per cell; MC5-5 yielded 7 times more, and KA31 approximately 15 times more virus. Since the activated endogenous virus is unable to reinfect the cells that release it, horizontal spread of virus is not a significant factor in these induction experiments (*4*).

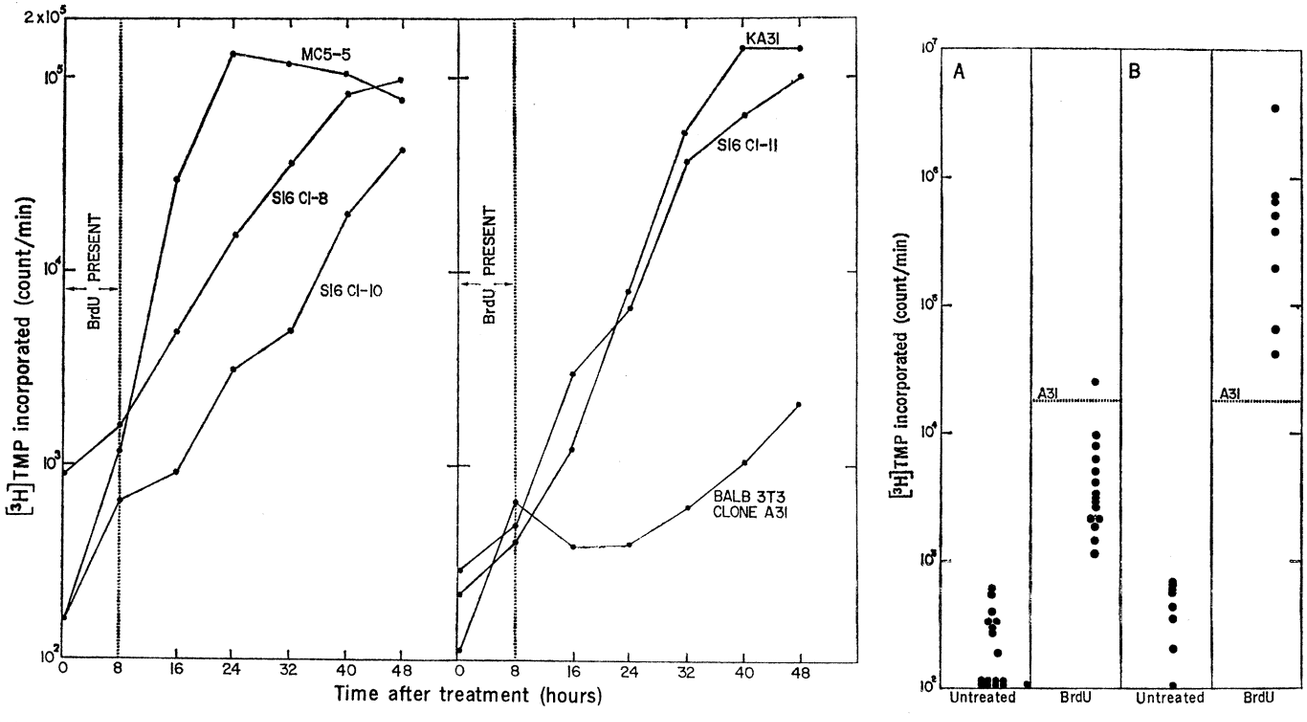

Fig. 1. Kinetics of BrdU-induced production of endogenous type C virus. Twelve duplicate flasks of each cell type were set up. When the cell monolayer was about 50 percent confluent, treatment with BrdU (33 μg/ml) was carried out for 8 hours. The media had been changed 24 hours before induction. At each time point, media from two flasks (which had been media changed 8 hours previously) were assayed for reverse transcriptase as described in Table 1, and the two results were averaged. Fig. 2. Induction of type C virus with BrdU from transformed and nontransformed clones of BALB/3T3. Twenty-four hours after media change, clone A31, and 15 flat nontransformed subclones (A) and the 7 transformed cell lines identified in Table 1 (B) were treated at 50 percent monolayer confluence with BrdU (33 μg/ml) for 24 hours. Media were collected from induced cells and untreated controls 4 days later for the reverse transcriptase assay as described in Table 1.

The results of a series of experiments analyzing virus production during the first 48 hours after an 8-hour application of BrdU are presented in Fig. 1. The transformed cell lines begin to release virus particles within the first 8-hour period after the initiation of chemical treatment; the amount of virus then increases exponentially. Moreover, the subclones that produce the most virus per cell have the highest initial rate of virus release. The chemically transformed subclone MC5-5 has consistently given the most rapid response, with a maximum rate of virus production achieved within 24 hours after induction. Each of the transformed subclones, however, responds considerably more rapidly than the nontransformed A31 cell line (*11*).

Figure 2A shows the results of BrdU treatment of 15 nontransformed single cell subclones of clone A31, isolated with the use of macrotiter plates (*12*); these subclones were selected from among 50 subclones isolated because of their flat morphology, low saturation density, and the polyhedral shape of the individual cells. These features contrast with the "heaped-up" growth and fibroblastic shape typical of the transformants, and occasionally seen in indivdual variant cells in clone A31 mass cultures. When tested 4 days after induction (at the time of maximum virus production), all 15 subclones show a relatively low level of induciblity; 14 of 15 are less inducible than the parental mass culture of A31 (*13*).

Treatment with BrdU for 30 minutes is sufficient to induce detectable amounts of virus (*4*). We can now add that the transformed cell lines begin exponential release of completed virus particles within 8 hours after exposure to the nucleoside. Ninety-six hours after BrdU treatment, these cell lines are producing 5- to 15-fold more virus than the parent cell line on a per cell basis. This rapid and high level production of type C virus has not been observed with either A31 or with nontransformed subclones derived from A31. The spontaneous production of endogenous type C viruses by spontaneously transformed clones of A31 after hundreds of virus-free cell generations has been described (*4, 14*); A31 itself has never been observed to release type C virus spontaneously. "Superinducibility" also appears associated with the transformed phenotype. Since the transformed cells, in addition, will grow to a saturation density approximately 10 to 20 times higher than BALB/3T3, and since such dense cultures remain fully inducible, yields of induced virus can be increased 50- to 300-fold per flask relative to clone A31 (Fig. 2B). Virus production by the superinducible clones is comparable in magnitude to productive infections with exogenously added laboratory strains of murine leukemia virus; however, the latent period is markedly shorter than that found with exogenous infection by RNA tumor viruses.

That clones of "virus-free" vertebrate cells can release an endogenous type C virus either spontaneously, or when treated with appropriate chemical or physical agents, has been demonstrated by workers in several laboratories (*2, 4, 14, 15*). The rapidity with which virus particles can be secreted from such cells after treatment with BrdU suggests that the viral information is present in the cells in a form that allows for prompt expression. Such a process is conceptually analogous to the switching on of a previously repressed differentiated cellular function. As demonstrated above, the mechanisms regulating the expression of endogenous type C virus can be modified by the process of transformation; such transformed cells appear less able to control normally repressed viral genetic information.

Table 1. BrdU induction during the log phase of growth. Duplicate flasks of 3×10^5 cells were set up 24 hours prior to the addition of BrdU. One flask of each cell line was treated with BrdU (33 μg/ml) for 24 hours. Four days after treatment, the cells were harvested with 0.1 percent trypsin and counted with a hemocytometer. The 15 ml of medium from each tissue culture flask was clarified by centrifugation at 12,000*g* for 10 minutes in a type 30 rotor (Beckman); the virions were then centrifuged through 20 percent glycerol at 105,000*g* for 90 minutes at 4°C. The pellet was suspended in 0.1 ml of a buffer (0.05*M* tris-HCl, *p*H 7.8, 0.10*M* NaCl) containing 0.1 percent Triton X-100; 0.01 ml of this solution was added to the standard reaction mixture containing 220 pmole of [methyl-^{3}H]thymidine triphosphate (New England Nuclear). Results are expressed as counts per minute of [methyl-^{3}H]thymidine monophosphate (TMP) incorporated during a 60-minute incubation at 37°C. The specific activity of the thymidine triphosphate used was 40,000 count/min per picomole.

Cell line	Supernatant reverse transcriptase assay		
	Untreated (10^3 count/min)	Induced (10^3 count/min)	Count/min per 10^3 cells
A31	0.29	13.6	6.04
S16 Cl-10	0.84	111.8	5.10
R4	0.32	40.7	7.40
S16 Cl-8	0.36	142.9	27.2
S16 Cl-11	0.21	336.5	28.7
S16 Cl-12	0.49	121.5	22.2
MC5-5	0.19	515.9	40.3
KA31	1.16	731.6	94.3

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References and Notes

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8. Each subclone was cultivated with equal numbers of mouse NIH/3T3 cells. Such cells readily support replication of endogenous BALB/c type C viruses [S. A. Aaronson, J. W. Hartley, G. J. Todaro, *Proc. Nat. Acad. Sci. U.S.A.* **64**, 87 (1969)], and, when tested by the reverse transcriptase assay 2 weeks later, the cell mixtures were not producing type C virus. All the cell lines used in our study (including the NIH/3T3, which has not been observed to produce complete type C virus) generally show some incorporation (100 to 1000 count/min above background) when tested by the standard reverse transcriptase assay. This could represent a low level type C virus release not detected by the present mixed cell cultivation–infectivity assays.
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11. To test the possibility that differential rates of uptake of BrdU from the medium might account for the variable levels of inducibility, incorporation of [6-^{3}H]BrdU (New England Nuclear) into the DNA of A31 and its transformants was studied. The cells were treated with BrdU (33 μg/ml; specific activity, 50 mc/mmole) for 24 hours while in the log phase of growth. Less than a twofold variation in the amount of radioactive nucleoside incorporated per cell into DNA was observed among all the cell lines, except for MC5-5, which incorporated only 25 percent as much [^{3}H]BrdU per cell. Thus, no direct correlation was found between the amount of BrdU incorporation and the inducibility. In a control experiment, a cell line lacking thymidine kinase incorporated 2 percent of the radioactive nucleoside per cell compared to its wild-type parental cell line.
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13. Clone A31 should not be considered an example of a "normal cell." While this cell line is relatively contact inhibited and nontumorigenic even at large cell doses, it is also aneuploid, clones with high efficiency, and appears to have an infinite life-span in vitro.
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16. We thank Janis Koci for technical assistance. Supported in part by the Special Virus Cancer Program of the National Institutes of Health.

20 February 1973; revised 28 March 1973