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Simian Virus 40 Transformation and the Period of Cellular Deoxyribonucleic Acid Synthesis

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The antiviral agents interferon and statolon protected cells of the mouse line 3T3 against the transforming effect of simian virus 40. Loss of ability of these agents to protect when added some time after infection indicated that the transformation was already fixed. The cells of exponentially growing cultures became resistant to the protective effect of interferon at a linear rate after infection; after one cell generation, the whole population was resistant. By use of synchronous cultures, it was shown that, in cells passing through the G-1 period of the growth cycle, the transformation did not pass the interferon-sensitive stage, whereas cells in *S* [the period of cellular deoxyribonucleic acid (DNA) synthesis] readily passed this stage (i.e., became interferon-resistant). An irreversible step in transformation appeared to occur in cells synthesizing DNA, and it seems likely that replicating cellular DNA was the target of the viral action.

In a previous communication (19), it was shown that nondividing cells of the mouse line 3T3 are not susceptible to transformation by simian virus 40 (SV40). Once infected, the cells must grow through a number of generations before they lose their susceptibility to contact inhibition. Of these, the first is necessary to fix the transformed state (i.e., to produce an irreversible change in the infected cell), and several additional generations are required before the transformed phenotype can become fully expressed. It seemed most likely that the fixation of the transformation occurs in a particular period in the division cycle, and it was suggested that this might be the period of deoxyribonucleic acid (DNA) synthesis (the *S* period). The present experiments, in which the kinetics of fixation of transformation were examined by use of the antiviral agent interferon [or statolon, a substance that induces endogenous interferon (7)], support this suggestion and make it probable that cellular DNA in the process of replication is the target of the viral action.

MATERIALS AND METHODS

The cultivation of the mouse fibroblast line, 3T3, and the transformation assay comprising this line and the oncogenic virus, SV40, have been described (17, 18). One pool of SV40 strain 776, titering $10^{8.2}$ TCID/ml, was used for all experiments.

Mouse serum interferon, prepared by intravenous inoculation of Newcastle disease virus, was held at *p*H 2 for 5 days and clarified by centrifugation at 100,000 × *g* for 2 hr. It was kindly provided by Samuel Baron (National Institutes of Health) and titered 3,000 units/ml by vesicular stomatitis virus plaque reduction (1). Control preparations consisted of similarly treated uninfected mouse serum. Statolon (7), a polyanionic polysaccharide from *Penicillium stoloniferum*, also a gift of Dr. Baron, was dissolved immediately prior to use in serum-free medium. Statolon prepared in serum-containing medium failed to show significant antitransforming activity. Petri dish cultures of 3T3 were developed for radioautography by previously described methods (15).

RESULTS

Loss of interferon susceptibility in exponentially growing cultures. Interferon effectively prevents transformation by SV40 when cells are exposed to it before or shortly after infection (16). Within 24 hr after infection of a growing population, however, the addition of interferon has no effect on the transformation frequency; by that time, the transformed state has become fixed. The kinetics of this loss of interferon susceptibility have now been analyzed more closely.

A population of exponentially growing 3T3 cells was infected with SV40 3 days after transfer, when any synchrony with respect to the division cycle had disappeared and the population was randomly distributed around the cycle. At various times after infection of cultures containing 10^5 to 2×10^5 cells, interferon (100 units/ml) was added and allowed to remain in contact with the cells for 3 hr. On the following day, cultures were

diluted for assay of transformation frequency.

Treatment of the cells with interferon just prior to infection reduced the number of transformants by 83% (Fig. 1). When the interferon was added at various times after infection, it lost its ability to prevent transformation in a linear fashion with time; by 24 hr after infection, it had become completely ineffective. Approximately 10 hr after infection of a randomly dividing population, half of the cells had passed the interferon-susceptible stage.

Loss of interferon susceptibility in synchronized cultures. Line 3T3 cells arrested by contact inhibition are all in the G-1 period of the growth cycle (11). When a resting culture is treated with trypsin and transferred with dilution, contact inhibition is removed and the cells prepare for division. Between 2 and 12 hr after transfer, none of the cells has reached the *S* period; between 22 and 32 hr, roughly 80% of the population is synthesizing DNA. (11). Cultures were therefore infected either at 2 or 22 hr after transfer, and the virus was allowed 10 hr to act, after which the cultures were exposed to interferon for 3 hr. On the following day, the cells were replated for assay of transformation frequency; the colonies were scored 14 days later.

Table 1 shows the results of four separate experiments. Cells infected but not yet synthesiz-

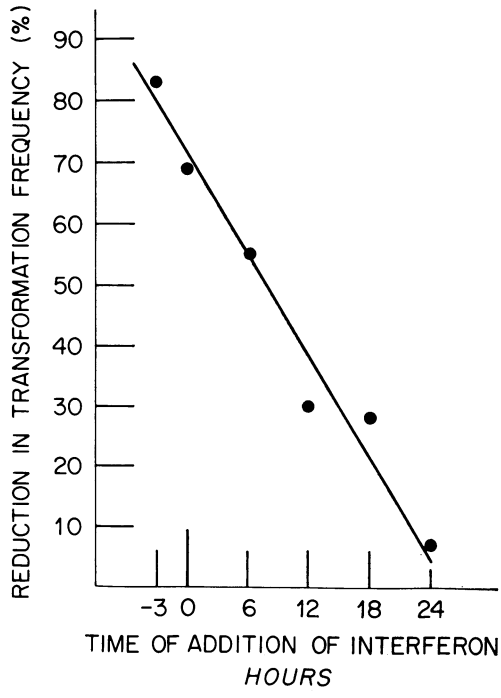

FIG. 1. *Loss of ability of interferon to prevent transformation of exponentially growing cells. Exposure to interferon for 3 hr, beginning at indicated times. Infection at time zero.*

TABLE 1. *Effect of interferon addition 10 hr subsequent to infection of a synchronous cell population*

Expt	Time after plating		Transformation frequency[b]		Reduction in transformation frequency	Loss of interferon inhibition[c]	Nuclei labeled[d]
	Infection[a]	Interferon addition	Without interferon	With interferon			
	hr	*hr*			%	%	%
1	2	12	2.4	0.5			
2			7.2	0.9			
3			3.4	0.5			
4			2.7	0.3			
avg			3.92	0.55	86	0	0
1	22	32	1.8	1.0			
2			1.3	1.2			
3			1.3	1.0			
4			2.2	1.6			
avg			1.65	1.20	27	67	78

[a] Cells infected shortly after trypsin treatment have a higher transformation frequency whether infected in suspension or immediately after reattachment. This may be due to greater uptake of virus, perhaps resulting from removal of material from the cell surface during trypsin treatment.

[b] Expressed as the number of transformed colonies per 100 total colonies.

[c] Relative to exposure to interferon prior to infection (see Figure 1).

[d] Parallel cultures exposed for the same 10-hr interval to 0.1 μc (per ml) of ^{3}H-thymidine. Values obtained are from examination of more than 2,000 nuclei in each case.

ing DNA remained fully susceptible to the action of interferon throughout the 10-hr period; the interferon was as effective as when added prior to infection (86% reduction in transformation frequency). In cultures infected at the beginning of the *S* period and treated with interferon 10 hr later, the ability of the interferon to prevent transformation was greatly diminished (27% reduction in transformation frequency). Parallel 3T3 cultures were infected with SV40 and kept in the presence of 0.1 μc of ^{3}H-thymidine during the two 10-hr periods. The cultures were then fixed and prepared for radioautography. Whereas none of the nuclei exposed to ^{3}H-thymidine from 2 to 12 hr showed labeling, 78% of the cells that had been exposed from 22 to 32 hr after inoculation had labeled nuclei. This may be compared with the 67% decline in the effectiveness of interferon during the same interval.

Essentially the same results were obtained with statolon (50 μg/ml), rather than mouse interferon, as the antiviral agent. For the same 10-hr periods, the inhibition of transformation was 68 and 78% for the cells not synthesizing DNA, as compared to 5 and 32% for the DNA-synthesizing cells. When only 4 hr was allowed for the viral action, the DNA-synthesizing population still largely passed the statolon-susceptible stage.

DISCUSSION

Although SV40 is unable to multiply in the mouse cell line 3T3, it is quite efficient in transforming the cells (3, 20). This transformation can be prevented by treating the cells with interferon (16). The experiments described above show that, in a randomly growing population of 3T3 cells, interferon progressively loses its ability to prevent transformation with time after infection. This decline of effectiveness begins soon after infection and is linear until, by 24 hr or roughly one cell generation after infection, virtually all of the cells are resistant. Such kinetics are most easily explained by the random entry of cells into a period of the growth cycle during which they become susceptible to the action of the virus and thereafter resistant to the action of interferon; all cells would have been expected to have passed through this period within one cell division after infection. Nongrowing cultures of 3T3 that are arrested in the G-1 phase of the cycle, in contrast, remain interferon-susceptible for at least several days after infection (19).

The suggestion that cells are susceptible to transformation only during a discrete period of the cycle is supported by the data obtained from study of synchronous cultures. A cell population that is passing through the *S* period is highly susceptible to transformation (rapidly becomes interferon-resistant), whereas the same population is not susceptible to the virus when passing through G-1 period. These results most probably mean that transformation requires an interaction between viral genetic material and replicating cellular DNA. The experiments do not, however, distinguish between susceptibility to transformation throughout the *S* period and susceptibility only at a specific time during this period, perhaps corresponding to the time of replication of a particular chromosome or chromosomal segment.

Both of the oncogenic DNA viruses that have been well studied in culture, polyoma and SV40, have been shown to persist intracellularly for many days after infection (4, 19), and this persistence is probably responsible for the phenomenon of "delayed transformation" (13). It has therefore been uncertain in experiments involving infection at different phases in the cell cycle at what time the fixation of the transformation actually occurs. The use of interferon and statolon makes it possible to define this time, since these substances elicit the production of an antiviral protein (5, 8, 14) that effectively prevents the formation of new transformants. The antiviral state induced by interferon begins very quickly, and viral functions are very effectively suppressed in less than 4 hr (9).

Basilico and Marin (2), studying polyoma virus transformation of the hamster cell line BHK21, concluded that all periods of the cell cycle were susceptible to transformation, but that infection of cells in the G-2 period led to twice the transformation frequency obtained at the other times during the cycle. They suggested that this increased susceptibility might be due to the fact that the cells in G-2 contain twice the complement of DNA on which the virus could act. This interpretation would require that the virus become uncoated and interact with the cellular DNA within the first 2 hr after infection (the time span of G-2), and that later interactions do not contribute to the transformation rate. An alternative explanation would be that, in these experiments, maximal effectiveness of the virus for action during *S* is achieved by infection during the preceding G-2 period.

There is recent evidence from experiments of Marcus and Salb (10) and Joklik and Merigan (6) that interferon blocks translation of viral messenger ribonucleic acid (mRNA), thus preventing the synthesis of virus-specific proteins. Joklik and Merigan concluded from studies with vaccinia-infected L-cells that viral RNA is

produced at a normal or elevated rate in the interferon-treated cell, indicating that viral DNA can function, at least in transcription, and is presumably not affected by the interferon. If this is also true for SV40 infection, then an interaction involving only viral DNA and the host genome could not account for the interferon susceptibility of the transformation process. However, if this interaction depended on the prior synthesis of a viral protein, then of course the process would be interferon-susceptible. Another possibility is that viral mRNA is itself the effective agent in the interaction with the cellular DNA (Fig. 2). This interpretation is consistent with the fact that the degree of inhibition of SV40 transformation by interferon is similar to the degree of inhibition of SV40 T-antigen synthesis (12). An interaction of viral mRNA with replicating cell DNA could perhaps result in permanent change of cellular function through specific derepression. If this model were correct, labeled viral DNA should not become incorporated into cellular DNA, and it might be possible to transform cells with RNA extracted from SV40-infected monkey kidney cells, a lytic system in which the viral genes are readily transcribed.

The time following infection when interferon can no longer prevent cellular transformation represents the time when the information that produces the transformed state can no longer be recognized as viral. If transformation depended on a permanent alteration in the expression of host cell genes, then, of course, the continuing function of those genes would not be sensitive to interferon. If, however, the transformed state requires the persistence of viral genes, whether integrated or not, then these or their products must somehow be modified so that the interferon-induced antiviral system is no longer able to recognize them.

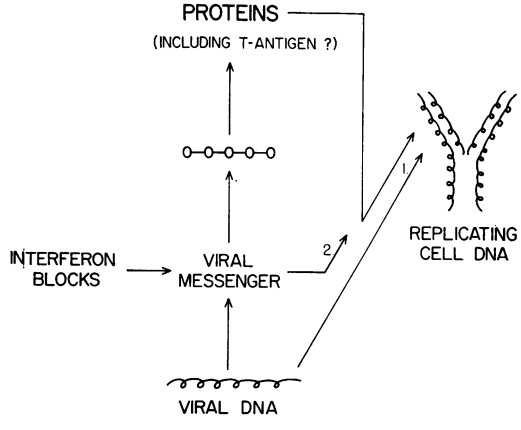

FIG. 2. *Site of action of interferon in preventing the fixation of transformation. Two major possibilities are considered: a joint interaction involving viral DNA and a viral specific protein, perhaps the T antigen; and a directed action of viral RNA on the replicating cellular DNA. More intricate models, consistent with the available data, can also be constructed.*

LITERATURE CITED

1. BARON, S., AND C. E. BUCKLER. 1963. Circulating interferon in mice after intravenous injection of virus. Science **141**:1061–1062.
2. BASILICO, C., AND G. MARIN. 1966. Susceptibility of cells in different stages of the mitotic cycle to transformation by polyomavirus. Virology **28**:429–437.
3. BLACK, P. H. 1966. Transformation of mouse cell line 3T3 by SV40: dose response relationship and correlation with SV40 tumor antigen production. Virology **28**:760–763.
4. FRASER, K. B., AND E. M. CRAWFORD. 1965. Immunofluorescent and electron-microscopic studies of polyoma virus in transformation reactions with BHK21 cells. Exptl. Mol. Pathol. **4**:51–65.
5. FRIEDMAN, R. M., AND J. A. SONNABEND. 1964. Inhibition of interferon action by p-fluorophenylalanine. Nature **203**:366–367.
6. JOKLIK, W. K., AND T. C. MERIGAN. 1966. Concerning the mechanism of action of interferon. Proc. Natl. Acad. Sci. U.S. **56**:558–565.
7. KLEINSCHMIDT, W. J., J. C. CLINE, AND E. B. MURPHY. 1964. Interferon production induced by statolon. Proc. Natl. Acad. Sci. U.S. **52**:741–744.
8. LEVINE, S. J. 1964. Effect of actinomycin D and puromycin dihydrochloride on action of interferon. Virology **24**:586–588.
9. LOCKART, R. Z., JR., AND B. HORN. 1963. Interaction of an interferon with L cells. J. Bacteriol. **85**:996–1002.
10. MARCUS, P. I., AND J. M. SALB. 1966. Molecular basis of interferon action: inhibition of viral RNA translation. Virology **30**:502–516.
11. NILAUSEN, K., AND H. GREEN. 1965. Reversible arrest of growth in G-1 of an established fibroblast line (3T3). Exptl. Cell Res. **40**:166–168.
12. OXMAN, M. M., AND P. H. BLACK. 1966. Inhibition of SV40 T antigen formation by interferon. Proc. Natl. Acad. Sci. U.S. **55**:1133–1140.
13. STOKER, M. 1963. Delayed transformation by polyoma virus. Virology **20**:366–371.
14. TAYLOR, J. 1964. Inhibition of interferon reaction by actinomycin. Biochem. Biophys. Res. Commun. **14**:447.
15. TAYLOR, J. H. 1960. Nucleic acid synthesis in relation to the cell division cycle. Ann. N.Y. Acad. Sci. **90**:409–421.
16. TODARO, G. J., AND S. BARON. 1965. The role of

interferon in the inhibition of SV40 transformation of mouse cell line 3T3. Proc Natl. Acad. Sci. U.S. **54**:752–756.

17. TODARO, G. J., AND H. GREEN. 1964. Enhancement by thymidine analogues of susceptibility of cells to transformation by SV40. Virology **24**:393–400.
18. TODARO, G. J., AND H. GREEN. 1964. An assay for cellular transformation by SV40. Virology **23**:117–119.
19. TODARO, G. J., AND H. GREEN. 1966. Cell growth and the initiation of transformation by SV40. Proc. Natl. Acad. Sci. U.S. **55**:302–308.
20. TODARO, G. J., AND H. GREEN. 1966. High frequency of SV40 transformation of mouse cell line 3T3. Virology **28**:756–759.