Relationship Between Virus-Induced Cellular Deoxyribonucleic Acid Synthesis and Transformation by Simian Virus 40

THOMAS O. FOX AND ARNOLD J. LEVINE

Department of Biochemistry, Princeton University, Princeton, New Jersey 08540

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The fraction of cells in a confluent 3T3 cell monolayer induced by simian virus 40 infection to replicate deoxyribonucleic acid and divide corresponds to those cells which eventually become transformed. Virus-induced cells were partially separated from noninduced cells by sedimentation through Ficoll gradients. Three- to eightfold higher transformation frequencies were obtained with those cells that began to synthesize cellular deoxyribonucleic acid and divide shortly after simian virus 40 infection as compared to noninduced cells.

Confluent monolayer cultures of 3T3 cells are blocked in G-1, the period prior to deoxyribonucleic acid (DNA) synthesis (9). When these cells are infected by simian virus 40 (SV40), they require at least one round of cell division to fix the transformed state as a stable inheritable trait (5, 13). A round of cell division can be obtained either by replating the cells at a lower cell density, as employed by Todaro and Green (5, 13), or by a virus-mediated induction of cellular DNA synthesis followed by cell division (4, 6). In the former case, all cells divide and a fraction of the cells become transformed (5, 13). It is not clear what distinguishes those cells which become transformed from the others.

With high multiplicities of virus infection, cellular DNA synthesis and division are induced by the virus in only some of the cells of a confluent monolayer (4, 6; Fig. 1), and a fraction of the total cell population later becomes transformed. The question is then: are the cells which are induced to divide by virus the same cells in the population which go on to become transformed? It was found that the induced cells had a three- to eightfold higher transformation frequency than those cells, in the same culture, which did not induce.

MATERIALS AND METHODS

Virus. Wild-type SV40 was employed to infect 3T3 mouse cells. The virus was grown on African green monkey cells as described by Levine et al. (7).

Tissue culture. Monolayer cultures of 3T3 mouse cells, originally provided by G. Todaro and H. Green to D. Foster, were grown on plastic flasks (T-75, 250 ml, Falcon) in Dulbecco's modified Eagle medium

(Grand Island Biological Co.) supplemented with 10% calf serum (BBL).

Infection of cells by virus. Three-day confluent monolayers of 3T3 cells were infected at an input multiplicity of 250 plaque-forming units (PFU) per cell in 2 ml of Dulbecco's medium supplemented with 2% depleted (14) calf serum. After 2 hr of adsorption at 37°C, 10 ml of the same medium was added and the incubation was continued.

Radioactive labeling of cells. Cells were grown in the presence of ^{32}P -inorganic phosphate (2 $\mu\text{Ci}/\text{ml}$) for 3 days at which time the cultures became confluent monolayers. The cells were infected with SV40 and then placed in 2% depleted serum medium (14) containing ^3H -thymidine (1 $\mu\text{Ci}/\text{ml}$). Radioisotope measurements were performed as described by Levine (*in preparation*).

Velocity sedimentation of cells. Cells were centrifuged through a 24-ml 7.5 to 15% linear Ficoll gradient. A special cell loading cap (3) containing a 5 to 7.5% Ficoll gradient was employed to maximize cell recovery. Loaded gradients were centrifuged for 11 min at 900 rev/min (International size 2). Ficoll was prepared in Dulbecco's medium containing either 2% calf serum or 10% calf serum. Lower calf serum allowed better efficiencies of ^3H -thymidine counting, whereas higher calf serum gave higher cell plating efficiencies. Cells were collected from the entire gradient, as previously described (3).

Transformation assay. The colony morphology assay (12) was employed to determine the transformation frequency. All colony counts were determined by two investigators, and the results were averaged. Plating efficiencies varied between 5 and 15%, depending on the experiment.

RESULTS

Separation of cells in S from cells in G-1. Ayad et al. (1) fractionated nonsynchronous mouse

lymphoma cells by centrifugation through Ficoll gradients. Cells in the S period were shown to sediment on the heavy side of the total cell distribution. Morris et al. (8) achieved a similar enrichment of S-phase murine mast cells by using 5 to 20% sucrose, 10 to 30% sucrose, and 5 to 20% Ficoll gradients. Fox and Pardee (3) found that Ficoll gradients containing 15% serum separated Chinese hamster ovary cells (CHO cells) on the basis of size, the larger cells sedimenting faster in these gradients. Cells which are induced to synthesize cellular DNA by SV40 (S phase) should be larger in size than cells which are not making DNA (G-1 cells). To test this, ^{32}P -labeled confluent monolayer cultures of 3T3 cells were infected with SV40. After a 2 hr viral adsorption period, depleted serum medium (14) containing ^3H -thymidine was added to the culture. Thus, the total cell population was labeled with ^{32}P , whereas those cells induced by the virus to synthesize cellular DNA were labeled with ^{32}P and ^3H . When the induced cells were in S (24 hr after infection) (4, 10), the cells were removed from the culture surface and sedimented through a Ficoll gradient containing 2% serum as described in the legend to Fig. 1A. It can be seen (Fig. 1A) that the cells which synthesized DNA after SV40 infection (^3H and ^{32}P) sedimented slightly ahead of the cells not making DNA (^{32}P but no ^3H). The ^3H to ^{32}P ratio throughout the gradient varied from a maximum of 3.4 (tube 13) to a minimum of 0.5 (tube 19). In light of the large differences in volume among individual 3T3 cells at confluence (E. Martz, Ph.D. Thesis, Johns Hopkins Univ., Baltimore, 1969), the separation obtained here was about as good as might have been expected.

It might be anticipated that the cells sedimenting fastest are those late in the S period or in G-2 and therefore should contain the highest ^3H to ^{32}P ratio. Tubes 3 to 10 in Fig. 1A, however, did not contain a high ^3H to ^{32}P ratio. Since 3T3 cells in either log phase or in a confluent monolayer have very large size differences (the middle 80% of the cell distribution shows a fourfold range in volume; E. Martz, Ph.D. Thesis, Johns Hopkins Univ., Baltimore, 1969), it was expected that some large cells (^{32}P labeled) that are not induced to synthesize cellular DNA (no ^3H counts per minute) sediment at the leading edge of this Ficoll gradient. The ^3H to ^{32}P ratio should only increase at the position of the majority of virus-induced cells (tubes 11 to 14) and not throughout the faster portion of the gradient.

Induced cells in S at 24 hr after infection (^3H labeled and fast sedimenting) would be expected to divide within the next 24 hr (24 to 48 hr after infection; reference 4). Because individual cells in

a population have widely varying G-1 lengths (3), one would expect that these divided cells would be in all stages of the cell cycle at 48 hr and thus would sediment throughout the Ficoll gradient, rather than in the discrete peak shown in Fig. 1A. To test this, the experiment above was repeated but the infected cells were harvested at 48 hr after infection (after S, G-2, and M) rather than at 24 hr. Figure 1B shows the sedimentation profile of these cells through a Ficoll gradient containing 10% serum. It can be seen that the ^3H and ^{32}P coincided quite well, indicating a randomization in the sizes of cells undergoing S during the first 24 hr after infection and mitosis in the 24- to 48-hr interval. These data strengthen the interpretation that Ficoll gradient centrifugation can separate virus-infected cells in S from noninduced G-1 cells.

It should be noted that in Fig. 1A and 1B the Ficoll gradients contained 2 and 10% serum, respectively. This is because counting efficiencies of ^3H in the trichloroacetic acid-precipitated samples were 5- to 10-fold lower in high serum than at 2% serum. On the other hand, adequate plating efficiencies of cells (5 to 15% plating efficiencies) were obtained only when 10% serum was employed in the Ficoll gradient. Microscopic inspection of 3T3 and CHO cells (using photographs or an ocular reticle) sedimented through Ficoll gradients containing 2 or 10% serum demonstrated that there was no difference in the capability of these gradients to separate cells on the basis of size. For these reasons and because these same results have been obtained in other laboratories (1, 8), it is concluded that the serum concentration in a Ficoll gradient has little or no effect on the distribution of cell sizes obtained in a Ficoll gradient.

Correlation of induced and transformed cells.

To determine if cells in S during the first 24 hr of infection yield a higher transformation rate than cells in G-1, the experiment described in Fig. 1 was repeated (with 10% serum in the Ficoll gradient), and samples of each gradient fraction (1:10 and 1:100 dilutions) were plated to determine transformation frequencies by the colony morphology assay. The results of several experiments of this type are presented in Table 1. For cells harvested at 24 hr after infection, the faster sedimenting fraction yielded transformation frequencies of 40 to 57% whereas only 7 to 12% of the more slowly sedimenting cells were transformed.

Determination of transformation frequencies by this assay requires several cell divisions for expression of transformation (5, 13), and therefore all of the cells in the original culture proceeded through the cell cycle after the first 24

FIG. 1A. Sedimentation of 3T3 cells through a Ficoll gradient 24 hr after infection. ^{32}P -labeled monolayer cultures of 3T3 cells were infected with SV40 and 2 hr later ^3H -thymidine was added. At 24 hr after infection, the culture was washed twice with warm phosphate-buffered saline (Mg^{2+} - and Ca^{2+} -free) containing 0.05% trypsin and $3.5 \times 10^{-3} \text{ M}$ ethylenediaminetetraacetic acid. The cells were removed from the dish by incubation in this same solution at 37°C for 5 to 10 min. A total of 400×10^3 to 500×10^3 cells were sedimented through a Ficoll gradient. Symbols: ○, ^{32}P counts per minute, total cell population; ●, ^3H counts per minute, cells synthesizing DNA after infection; □, ^3H counts per minute to ^{32}P counts per minute ratio. The dashed line represents the sum of all of the ^3H counts per minute/the sum of all of the ^{32}P counts per minute in the gradient. FIG. 1B. Sedimentation of 3T3 cells through a Ficoll gradient 48 hr after infection. ^{32}P -labeled 3T3 cells were infected with SV40 and 2 hr later ^3H -thymidine was added. At 48 hr after infection, the cells were removed from the petri dish surface and sedimented as described in Fig. 1A. For this experiment, Ficoll was made up in Dulbecco's medium containing 10% serum. This was done to obtain better plating efficiencies. Because Fig. 1A employed 2% calf serum in the Ficoll gradient, the sedimentation profiles of the ^{32}P counts per minute in Fig. 1A and 1B are not strictly comparable. Symbols: ○, ^{32}P counts per minute, total cell population; ●, ^3H counts per minute, cells synthesizing DNA after infection; □, counts per minute ratio. The dashed line represents the sum of all of the ^3H counts per minute/the sum of all of the ^{32}P counts per minute in the gradient.

hr of infection. (All of the cells divide to yield colonies in the cultures dishes employed for the transformation assay.) This is why transformants appeared, albeit at a lower frequency, in the slower sedimenting fractions of these gradients. A second experiment was performed in which the cells were harvested at 46 to 48 hr after infection, by which time the cells in S during the first 24 hr had divided and had the random sedimentation pattern (Fig. 1B). As expected, transformation frequencies of the cells in both the faster and slower sedimenting fractions of the gradient now were about equal (Table 1).

Effects of Ficoll and tritium on relative plating efficiencies of normal and transformed cells. For these results to be valid, normal and transformed cells must have the same plating efficiencies across the Ficoll gradient. Figure 2 presents additional data on this point, obtained from the experiment described in Fig. 1B (cells harvested 48 hr after infection). The total viable cells (colony assay) and the total cell number (^{32}P -counts per minute) in each fraction of the Ficoll gradient are plotted. There was a 1.5- to 2-fold increase in cell viability near the top of the Ficoll gradient (tubes 20 to 26). This can be seen in the ratio of total colonies per

TABLE 1. *Time after infection for sedimentation of cells*^a

Expt	24 Hr					48 Hr				
	Fast sedimenting peak		Slow sedimenting peak		Ratio	Fast sedimenting peak		Slow sedimenting peak		Ratio
	No. of colonies counted	Per cent of transformation	No. of colonies counted	Per cent of transformation		No. of colonies counted	Per cent of transformation	No. of colonies counted	Per cent of transformation	
1	108	57	557	9	6.3					
2	108	57	210	7	8.1	568	9	614	8	1.1
3	470	40	1,028	12	3.3	421	26	457	32	0.8

^a Transformation frequencies of cells obtained either 24 or 48 hr after infection and sedimenting fast (in S period) or slowly (in G-1 period) through a Ficoll gradient. The ratio is the fast sedimenting per cent transformation over the slow sedimenting per cent transformation. No correction for plating efficiencies has been introduced into these values.

FIG. 2. Distribution of viable cells and total cells throughout a Ficoll gradient. The conditions of this experiment are given in Fig. 1B. Plating efficiencies were about 10%. Symbols: ●, total colonies per fraction, viable cells; △, ³²P counts per minute per fraction, total cells; ○, total colonies/³²P counts per minute; □, transformed colonies/³²P counts per minute.

³²P counts per minute (Fig. 2, top graph). The ratio of transformed colonies to ³²P counts per minute also increased at this point in the gradient. It is clear from this experiment that, although plating efficiencies were better near the top of a Ficoll gradient, there was no difference in the relative plating efficiencies of normal and transformed cells throughout the gradient.

A possible differential effect of tritium decay on colony formation by normal and transformed cells was negligible, since the same results presented in Table 1 were obtained when ³H-thymidine was not employed in these experiments.

DISCUSSION

Todaro and Green (5, 13) have shown that at least one round of cell division is required to fix the transformed state as a heritable trait. In their experiments, virus-directed induction of cellular DNA synthesis did not play a major role in the transformation event. The differential between cells undergoing one round of cell division after viral infection and nondividing cells was achieved by replating cells from two different cultures at a cell density that permitted one or no rounds of cell division.

The experiments presented in this paper employ

a virus-directed induction of cellular DNA synthesis to stimulate cell division in a maximum of about 60% of the cells (see the area under the ^3H -counts per minute curve in Fig. 1A). In this case, the cells undergoing DNA synthesis shortly after infection come from the same cell culture as the cells not replicating their DNA in the first 24 hr of infection. Thus, the comparison of transformation frequencies in this experiment is done with cells obtained from a single culture. Mock-infected control cultures have been employed to show that cellular DNA synthesis, monitored by ^3H -thymidine incorporation, is virus-directed and not due to a few cells in the confluent monolayer that are synthesizing DNA (background values; reference 10).

Cellular DNA synthesis and cell division can be initiated in confluent monolayer cultures of most cell lines with SV40 (4, 10) or by the addition of fresh serum to the culture medium (14). Confluent monolayer cultures of BSC-1 cells (one particular monkey cell line) can be stimulated to synthesize appreciable amounts of cellular DNA (about a twofold increase in DNA content; A. J. Levine, *in preparation*) by the addition of fresh serum to the culture medium (14). Infection of these same cells (BSC-1) with SV40 results in a productive lytic infection (4, 10) but little or no increase in the rate of incorporation of ^3H -thymidine into cellular DNA (4, 10) or the amount of cellular DNA synthesized (A. J. Levine, *in preparation*). These data imply that serum-mediated induction of cellular DNA synthesis is different from virus-directed induction of DNA synthesis (10). In the case of polyoma, a virus-directed induction of cellular DNA synthesis requires at least one viral coded function (W. Eckhardt et al., Proc. Nat. Acad. Sci. U.S.A., *in press*).

The experiments presented in this paper demonstrate that cellular DNA synthesis during the first 24 hr after infection of 3T3 cells (when viral genomes are present in large quantities) is necessary for an efficient transformation event. This is in good agreement with the results of Todaro and Green (5, 13). The necessity of one round of cellular DNA synthesis for fixing the transformed state (5) could be due to the possibility that the viral genome is integrated into the host cell DNA during cellular DNA replication. Alternatively, there might be a cellular or viral gene product, made only during or after (G-2 or M) the induced S period, which is required for a transformation event. In this connection, several different laboratories have now shown that cellular DNA synthesis is required for the alteration of the cell surface observed in all transformed

cells by using the wheat germ glycoprotein agglutinin assay (2; W. Eckhardt et al., Proc. Nat. Acad. Sci. U.S.A., *in press*; J. Sheppard et al., *in preparation*). Although integration of the viral genome into the cellular DNA appears to occur in transformed cells (11, 15), the second alternative is now equally reasonable.

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