*RAPID TRANSFORMATION OF CELLS BY ROUS SARCOMA VIRUS**,†*

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Abstract.—The conditions for synchronous transformation of chick embryo cells by infection with Rous sarcoma virus are studied. Two factors, the treatment of cells with DEAE-dextran and the use of cells which grow rapidly following virus infection, are found to be most important. Under the conditions described, the Schmidt-Ruppin strain of Rous sarcoma virus at a multiplicity of higher than 5 induces morphological alteration in about 90 per cent of the cell population within 24 hours after infection. The alteration in the morphology is accompanied by acquisition of the ability of cells to grow in agar medium and by the increased rate of incorporation of thymidine and uridine into nucleic acids.

Infection with Rous sarcoma virus morphologically alters chick embryo fibroblasts in a short period of time. Since this process is associated with the changes in other cellular properties characteristic of sarcoma cells, the morphological alteration is taken as evidence of the change of a normal cell into a malignant sarcoma cell, and is called cellular transformation. In order to gain insight into the molecular basis of the transformation process, it is highly desirable to have a cell-virus system in which transformation takes place synchronously and with high efficiency following infection. Though RSV can induce transformation with a greater efficiency and speed than any other oncogenic virus, in general only about 25 per cent of the cell population can be infected at one time. There is also a great variation in the time required to transform individual cells. The origin of these difficulties is probably to be found in the condition of both the virus and the host cells. The widely used Bryan strain of RSV may be inappropriate to obtain rapid synchronous infection because it is generally accompanied by an avian leukosis virus that is known to elicit interference with infection by Rous sarcoma virus.[1, 2] The timing of virus infection during the growth cycle of the cells appears to be an important factor. Cells are most susceptible to the virus right after trypsinization,[3] and the synthesis of cell DNA following infection is known to be essential for transformation by Rous sarcoma virus.[4]

These and other factors have been varied and a technique has been developed in which a vast majority of cells can be transformed within 24 hours after infection with Rous sarcoma virus. This paper describes the conditions and some characteristics of the process of this rapid cell transformation.

Materials and Methods.—*Media:* The primary medium consisted of medium 199 with 10% tryptose phosphate broth, 8% calf serum, and 2% chicken serum. The complete medium (CM) used for secondary cultures consisted of Scherer's medium with 10% tryptose phosphate broth and either 2 or 5% calf serum. The agar medium was the same as the 5% serum-CM except that it contained 0.75% agar and 1% beef embryo extract.

Cells: Chick embryo cells were prepared in essentially the same manner as that described by Rubin.[5] Ten-day-old chick embryos from individual eggs (supplied by SPAFAS Co.) were mechanically minced, trypsinized, and incubated at 38°C with the

primary medium. Secondary cultures (c/o type) were prepared from 4- to 6-day-old primary cultures by treatment with trypsin and seeding in 2% serum-CM. Except for the experiments on rapid transformation, about 1.2 million cells were seeded on a plate of 60-mm diameter. On the next day the medium of these cultures was changed to 5% serum-CM or agar overlay medium. Susceptibility of cells of each embryo to RSV variants was determined in the primary cultures.

Virus: The Schmidt-Ruppin stain of RSV (SR-RSV) used in this study was purified by isolation from single foci[6] and proved to be free from avian leukosis virus.[7] Biological properties of this virus were given previously.[7] The purity of virus was tested in each preparation by the terminal dilution method.

The stock of SR-RSV was prepared in tissue culture. Secondary cultures were infected with about 10^7 focus-forming units of SR-RSV with DEAE-dextran in a final concentration of 5 μg/ml. Beginning two days after infection, when most cells had been transformed, the culture fluid was harvested every 12 hr and replaced with fresh 5% serum-CM. The harvest, containing about 2×10^7 focus-forming units of RSV/ml, was kept at 4°C until the virus was concentrated.

A crude virus concentrate was obtained in the following way. The pooled culture fluid was centrifuged at $8000 \times g$ for 10 min and the supernatant was spun at 19,000 rpm in a type-19 Spinco rotor for 2 hr. The pellet was resuspended in 2% serum-CM and sonicated at 10 kc for 45–90 sec to disperse virus. The suspension was centrifuged at $8000 \times g$ for 10 min and the supernatant was used as a stock of virus after adjustment of pH by a flushing with CO_2. A further purified stock was prepared by the method used by Duesberg *et al.*[8] for the Bryan RSV. The virus was precipitated once by ammonium sulfate, purified by differential and gradient centrifugation, and finally dialyzed overnight against buffered saline. The concentrated stocks prepared by these two methods contained $5\text{–}8 \times 10^8$ FFU of SR-RSV/ml and both gave similar results in the transformation study. The virus was stored at -70°C.

The Bryan high-titer strains of RSV used were B-RSV(RAV-1), B-RSV(RAV-2), and B-RSV(RAV-50). Their titers were about 1×10^7, 2×10^7, and 2×10^7 FFU/ml, respectively. The preparation of B-RSV was described previously.[9]

Virus assay: Generally the virus titer was assayed as described before.[5] Since the attachment of certain RSV variants to cells was enhanced by a factor of 50 to 100 by the treatment of cells with DEAE-dextran,[10] the RSV variants affected by this treatment, i.e., B-RSV(RAV-2), B-RSV(RAV-50), and SR-RSV, were inoculated with DEAE-dextran in a final concentration of 5 μg/ml.

Infection of cells with RSV: The following method was used for relatively synchronous transformation of cells. Secondary cultures were made as described above, except that about 2.4×10^6 cells were seeded in a 60-mm plate, so that cells formed a monolayer within 4 hr after subculture. Then the culture fluid still containing unattached cells was removed and cells were exposed to 0.1 ml of DEAE-dextran (240 μg/ml) and 0.5 ml of SR-RSV in 2% serum-CM. After adsorption of virus for 1 hr, the cultures were washed and reincubated with 5 ml of 5% serum-CM at 39°C.

The effective multiplicity of infection of virus was determined by inoculating higher dilutions of virus in duplicate cultures as described above, overlaying the cultures with agar after an adsorption period, and counting the number of foci on these plates 6 days later.

Determination of infective centers: To determine infective centers, cultures infected with various multiplicities of SR-RSV were trypsinized at 8 hr after infection, and the cells were diluted and seeded with 10^6 normal cells. The cultures were overlaid with agar after incubation for 12 hr at 38°C, and the number of foci in these cultures was scored 6 days later. To prevent the formation of infective centers by either parental or progeny virus, antibody against SR-RSV was added to the cultures at 7 hr after infection and kept present in the assay plates until they were overlaid with agar.

The determination of protein and the rate of incorporation of labeled compounds: The amount of protein in the cells was determined by Lowry's method. The rate of incorpora-

tion of uridine-^{3}H (5-T, 29 c/mM), thymidine-^{3}H (methyl-T, 21 c/mM), or leucine-^{3}H (4, 5-T, 23 c/mM) into the trichloroacetic acid-insoluble fraction of cells was determined by incubating cultures with 2 ml of 5% serum-CM containing these labeled compounds in the concentration of 1 μc/ml. After 2 hr incubation at 39°C, the cultures were washed twice with chilled Tris-saline and kept at −70°. The cells were scraped off with 1 ml of water and washed with 5% trichloroacetic acid and the final acid concentration was adjusted to 5%. The acid precipitate was centrifuged, washed twice with 5 ml of 5% trichloroacetic acid, and then dissolved in 0.1 *N* NaOH, and a 50-μl aliquot was taken for measurement of the radioactivity with a scintillation counter. Another aliquot of the solution was used for determination of protein in the samples.

Results.—Conditions for rapid transformation: When secondary cultures were inoculated with SR-RSV at a multiplicity higher than 5, and within 6 hours after the seeding of the cells, morphological alteration became visible in a small fraction of cells by 14 hours after infection. After 16 hours this fraction had increased, and by 24–26 hours more than 90 per cent of the cells had been altered. Changes in the infected cultures were estimated by direct observation of at least 500 cells under the microscope. The results are shown in Figure 1. Since the alteration

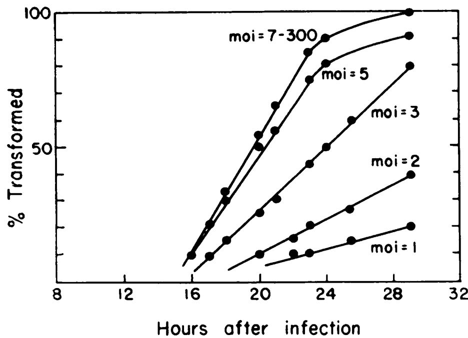

FIG. 1.—Rate of morphological alteration of RSV-infected cells.

Secondary cultures were infected with various concentrations of SR-RSV, and the fraction of morphologically altered cells was estimated under the microscope at various times after infection.

of individual cells does not take place instantaneously, the estimate during transformation could be relatively inaccurate. Morphology of normal and transformed cells is shown in Figure 2. Two types of cells, spindle and round, can be seen in infected cultures, and both of them can be distinguished easily from normal fibroblast cells by the shape, the refractile appearance, and the smooth cell surface. These two types of cell morphology are not caused by heterogeneity of

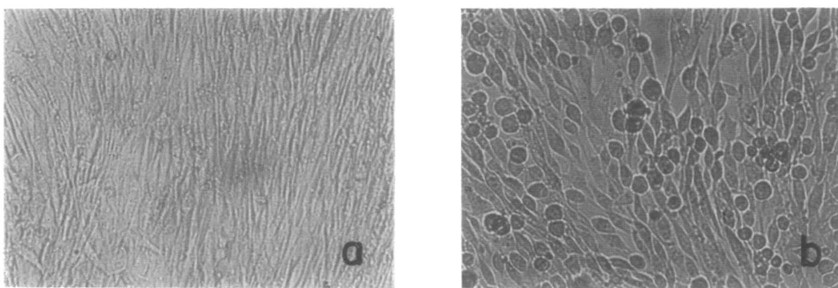

FIG. 2.—Photomicrograph of (*a*) normal and (*b*) SR-RSV-infected cells at 28 hr after infection.

virus, because both types can be induced in single cultures infected by any clonal isolate of the virus, and both can be seen in each focus of transformed cells produced by diluted virus. They are at least partially interconvertible and seem to differ in the degree of attachment to a solid surface. The morphologically altered cells rapidly acidify the culture medium, but their continuous growth can be maintained by frequent changes of the medium.

To confirm the significance of the morphological alteration observed under the microscope, the fraction of infected cells was determined by the infective center assay. To check the efficiency of attachment of plated cells, one control culture that had been infected with RSV (m.o.i. 10) 48 hours earlier was trypsinized and plated in the same manner. As is shown in Table 1, the plating efficiency of cells was about 94 per cent, if it is assumed that all cells in the control-transformed culture were infected. In the experimental group, more than 90 per cent of cells were registered as infective centers with a multiplicity of infection of 10 or higher.

(1) *Multiplicity of infection:* The effect of multiplicity of SR-RSV on cell alteration was examined by varying the multiplicity of RSV infection from 0.1 to 300. As seen in Figure 1, infection at a multiplicity greater than 7 gave essentially the same results in the rate of transformation. In accord with the results of the infective center assay, these results indicate that, unlike some Bryan RSV stocks,[2] SR-RSV when inoculated in large excess does not result in autointerference. The results also suggest that not more than three infectious particles are required for the rapid transformation of a single cell.

(2) *Timing of infection:* The susceptibility of cells to RSV at various times after subculture was examined by measuring the efficiency of focus formation by diluted RSV as well as the efficiency of rapid transformation by high titers of RSV. In cultures infected at 12 and 24 hours after cell seeding, the efficiency of focus formation by SR-RSV was about 65 and 25 per cent, respectively, of that in cultures infected at 4 hours, and rapid transformation was found in only a small fraction of cells. The high susceptibility of cells to RSV shortly after cell seeding may be due either to the modification of cell surface by trypsin or to a partial synchronization of cell division as a result of reseeding, or to both. The last-named possibility is supported by the success of rapid cell transformation, even at 48 hours after cell seeding, under the following conditions of cell growth: Secondary cultures were prepared as described above and incubated at 38°C with a medium containing 20 mM thymidine, which is known to inhibit cell DNA synthesis.[11] Twenty-four or 48 hours later the thymidine-containing medium was replaced with the regular medium, and then the cultures were infected with RSV. In these cultures, presumably synchronized by this treatment, the morphological transformation by RSV appeared with the same speed as in the freshly prepared secondary cultures.

(3) *Transformation with the Bryan strain of RSV:* Cell alteration by B-RSV was studied under these same conditions using freshly prepared secondary cultures. Round, transformed cells induced by B-RSV first appeared at about 15 hours and then their proportion in the cultures increased linearly to 24 hours as shown with SR-RSV. With the same multiplicity of infection (10), about 60 per cent of the cells were transformed by 24 hours with B-RSV (RAV-1)

or B-RSV (RAV-2), and about 90 per cent with B-RSV(RAV-50). The relatively low efficiency of the first two RSV variants may be a result of the strong interfering effect of the associated virus (RAV-1 or RAV-2) in their respective RSV stocks,[2] but this question was not examined. There was a noticeable difference in the cytotoxicity of B-RSV and of SR-RSV. After transformation (usually after 30 hours), some cell deterioration such as the formation of giant cells and the appearance of apertures between cells was often found in cultures infected by B-RSV. The deterioration could have been exaggerated by a cytotoxic effect of associated leukosis viruses. On the contrary, no appreciable cytopathic effect was found in transformed cells induced by SR-RSV.

Growth of cells and virus: A relatively rapid multiplication of SR-RSV was found in the rapidly transformed cultures. The RSV-infected cultures (m.o.i. 10) were washed five times at the end of one hour of adsorption and twice at four hours after infection. The medium of the cultures that were to be kept longer than 24 hours was replaced every 12 hours. Virus released into the medium was harvested at various times and assayed with DEAE-dextran. As seen in Figure 3, the titer of virus increased rapidly after 8 hours and reached its highest level at 36 hours. The virus titer was maintained at this level for a week by changing the medium every 12 hours.

Growth of cells during and after the transformation process was measured under three different conditions: in fluid medium either (*a*) changed every 12 hours or (*b*) unchanged or (*c*) under agar. When cells were kept in 5 per cent serum-CM without further changes of medium, both uninfected and infected cells increased in number approximately four times in the first 30 hours; thereafter, they multiplied very little (Fig. 4*A*). There is no significant difference of normal and infected cells either in growth rate or in final population density except for a slight delay in replication of infected cells between 12 and 20 hours. Also, no difference in cell growth was observed when normal and infected cells were allowed to continue to grow by repeated change of fluid medium. As seen in Figure 4*A*, the final population density of cells was about 2×10^7 cells per 60-mm plate in both cultures by the end of three days. During this period, infection produced no significant change in cell size or in the amount of protein per cell.

A difference in the growth characteristics between normal and infected cells was apparent, however, when the cultures were kept in the medium containing 0.5 per cent agar (Fig. 4*B*). To maintain the pH of culture neutral, an additional 2 ml of agar was added to both groups of cultures at 36 hours. While no further growth was observed in the normal cells after 40 hours, the transformed cells continued to multiply for 72 hours. At three days the cell density of the transformed cultures was about three times that of the normal cultures.

Incorporation of nucleosides and an amino acid: The rate of incorporation of thymidine, uridine, and leucine into the trichloroacetic acid-insoluble fraction was determined with normal and RSV-infected cells. The cultures were kept in fluid with unchanged medium until they were incubated with the labeled precursors, so that the number of cells and their protein content were almost identical in a set of normal and infected cultures. A representative result is shown in Figure 5. In the cultures of normal cells, both thymidine and uridine incorpora-

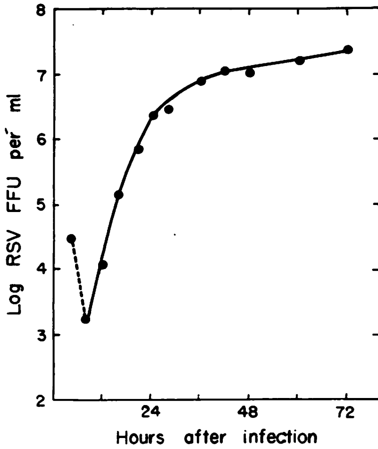

FIG. 3.—Growth of SR-RSV. Cultures were infected with RSV (m.o.i 10) and the titer of virus released into medium was assayed.

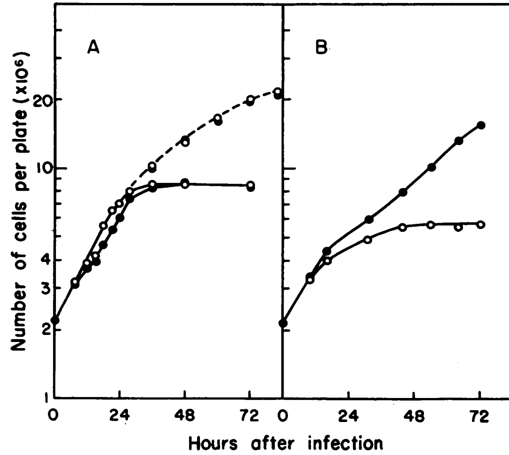

FIG. 4.—Growth of normal and infected cells. (*A*) Cultures were kept in fluid with (*broken line*) or without (*solid line*) change of medium every 12 hr.

(*B*) Cultures were overlaid with 0.5% agar medium and an additional 2 ml of agar was added at 36 hr. (O) Uninfected and (●) SR-RSV-infected cultures.

tion declined with time, reflecting the cessation of cell replication. However, their incorporation into infected cells rose to a peak at about 24 hours, and then declined at a slower rate. At 36 and 48 hours the uptake was consistently two to three times higher in infected cells. No significant change was found in the incorporation of leucine accompanying the transformation. The uptake of thymidine and uridine was not stimulated by infection with the same high multiplicity RAV-2, a nontransforming avian leukosis virus, despite the fact that RAV-2 multiplies at a rate similar to SR-RSV (Fig. 5).

Discussion.—These experiments have demonstrated that under the conditions described here, the RSV infection of chick embryo cultures results in morphological transformation in about 90 per cent of the cells within 24 hours. Among various factors examined, two were found most critical: (1) the treatment of cells with DEAE-dextran to enhance the efficiency of attachment of SR-RSV to the cells and (2) the conditions assuring the active replication of host cells immediately following infection. The latter condition was achieved either by the infection of secondary cells shortly after the subculture or by the treatment of cells with an excess of thymidine removed just before infection. Both techniques produce a partial synchrony in cell division and most of the cells are in the *S* period following infection. The choice of the Schmidt-Ruppin RSV is based principally on the absence of contaminating avian leukosis virus that is generally present in high concentrations in stocks of the Bryan RSV. The leukosis virus is unable to induce morphological change in infected cells but is very similar to RSV in its structure and in many of its biological properties.[12] Therefore, in the analysis of transformation by B-RSV, one must consider the effects of the superinfecting

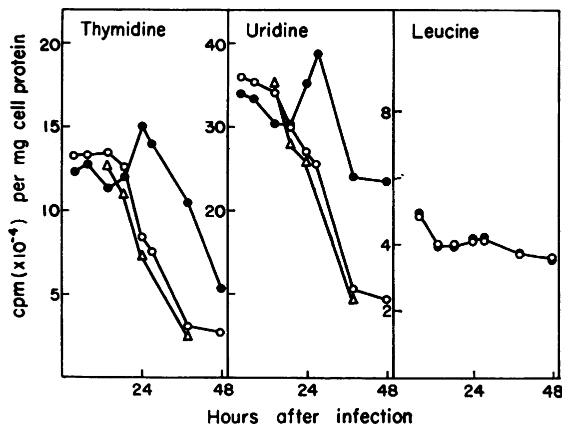

FIG. 5.—Incorporation of labeled thymidine, uridine, and leucine. Cultures infected with SR-RSV or RAV-2 at multiplicity of infection of 10 were incubated with 1 μc/ml of the H^3-labeled compounds for 2 hr and the radioactivity in trichloroacetic acid-insoluble material was determined. Each point represents the radioactivity incorporated during 2 hr, beginning at the time indicated.

(O) uninfected, (●) SR-RSV-infected, and (Δ) RAV-2-infected cells.

leukosis virus. From a practical point of view, the leukosis virus would reduce the efficiency of infection by RSV and thereby interfere with the RSV in simultaneous infection. The cytotoxicity of the Bryan strain also makes it less favorable for the studies on transformed cells.

The changes in cell morphology have been regarded as an important index of cell transformation. Cells morphologically altered by B-RSV were proved malignant in the animals even when they were not producing a virus infectious to the host.[9] The morphology of cells altered *in vitro* was indistinguishable from that of cells obtained from sarcomas. Alteration in cell morphology was accompanied by the acquisition by the cells of the capacity to grow under agar that is a common characteristic of transformed cells induced by many oncogenic viruses.[13-15] The earliest alterations were observed approximately 14 hours after infection, suggesting that the reaction critical for neoplastic change could have taken place before 14 hours. The possibility of such a reaction in the early steps of virus-cell interaction is suggested by the demonstration that adenovirus DNA associates with host cell chromosomes within 21 hours after infection.[16]

It seems likely that the change in nucleic acid synthesis observed after in-

TABLE 1. *Percentage of cells registered as infective centers.*

	Multiplicity of Infection of SR-RSV					Transformed* cells
	30	10	6	3	1	
Per cent cells registered as infective centers	97	94	87	75	47	94

Cultures were infected with dilutions of SR-RSV and the number of infective centers was determined at 8 hr after infection by plating cells on normal cells.

* A completely transformed culture was treated and plated in the same manner to estimate the efficiency of attachment of plated cells.

fection with RSV relates to the process of cell transformation. The stimulation of uptake of the nucleosides occurred soon after the completion of cell transformation, and a nontransforming leukosis virus (RAV-2) failed to induce such changes in infected cells. Rubin and Colby[17] have reported the increased uptake of thymidine by RSV-infected cells; however, under the conditions they used, only infected cells were able to continue to grow, so that the increased uptake could be explained by the differential cell growth. In the present study there was no difference in cell growth between normal and infected cultures, and the stimulated incorporation of nucleosides must be explained by other mechanisms. It remains to be established whether the stimulation is caused by the increase in the net synthesis of DNA and RNA or by drastic changes in the intracellular pools of these nucleosides.

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† Abbreviations used: RSV, Rous sarcoma virus; CM, complete medium; SR-RSV, Schmidt-Ruppin Rous sarcoma virus; FFU, focus-forming unit; m.o.i., multiplicity of infection.

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