*REQUIREMENT FOR THE INTEGRITY OF THE VIRAL GENOME FOR THE INDUCTION OF HOST DNA SYNTHESIS BY POLYOMA VIRUS**

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It has been demonstrated that infection by polyoma virus of contact-inhibited mouse kidney cell cultures leads to the induction of cellular in addition to viral DNA synthesis.[1-3] In this system, polyoma virus replicates with a relatively high efficiency.[4] However, more cellular DNA than viral DNA appears to be made in the cultures.[1, 2]

The experimental conditions used by the authors[1-3] who first described the phenomenon were such that only part of the cell population underwent productive infection. Therefore, the question of whether the induction of cell DNA synthesis takes place in the cells in which the virus multiplies, in a larger fraction of the population, or only in the cells which do not produce virus, remained unanswered. In the following paper, experiments will be described which demonstrate that the induction of host DNA synthesis occurs in the same cells in which polyoma virus replicates.

In view of the possible correlation[1] between this phenomenon and the ability of polyoma virus to transform normal cells into malignant, we have also tested whether the radiation target size of the DNA-inducing ability of polyoma was similar to that of its transforming ability.[5, 6] It was found that this function of polyoma virus is inactivated at the same rate as plaque-forming ability, suggesting that the integrity of the viral genome is required for the induction of cellular DNA synthesis.

Materials and Methods.—Cells: Baby-mouse kidney cultures were prepared according to Winocour[4] and grown in reinforced Eagle's medium (fourfold concentration of amino acids and vitamins) supplemented with either 10% calf or horse serum. They were infected after they had reached confluence (usually 3–4 days after seeding).

Virus: Polyoma virus was grown in mouse kidney cultures and purified by density gradient centrifugation.[4] Both large and small plaque virus were used in the experiments. Virus was assayed for plaque formation by the standard technique on monolayers of mouse embryo secondary cultures.[7]

Infection: Confluent mouse kidney cultures grown in plastic Petri dishes were infected with 0.2 ml of virus suspension/60 mm Petri dish. The virus was allowed to adsorb for 2 hr at 37°C, then the cultures were washed to remove unadsorbed virus, and incubated in Eagle's medium plus 5% horse serum. Control cultures were mock-infected under identical conditions but without virus.

DNA synthesis: The amount of C^{14}-thymidine taken up into DNA was determined from the radioactivity present in TCA-insoluble material. Precipitation was carried out for 1 hr at 4°C, the precipitated material collected on Millipore filters (grade HA), and counted in a Selo low-background counter.

For autoradiography, cultures which had been labeled with H^3-thymidine were washed with a cold buffer, and fixed with acetic acid–70% ethanol (1:9). After a rinse with 95% ethanol, a section of the plates was cut off and mounted in Euparal on a slide, cells facing upward. Autoradiograms were made by the "dipping" method, using NTB2 Kodak emulsion and D19 Kodak developer.

Fluorescent antibody technique: Cells which had been grown on coverslips were washed three times in buffered saline, then fixed with acetone-alcohol (2:1) for 10 min in the cold. The cover-

slips were then stored at −20°C. For staining, the indirect method was used:[8] the coverslips were washed in buffered saline, and incubated for 1 hr at 37°C covered with guinea pig anti-polyoma serum. They were then washed three times and covered with fluorescein-labeled anti-guinea-pig globulin.[9] Following further incubation (1 hr at 37°C), the coverslips were rinsed and mounted in buffered glycerol.

Irradiation technique: The virus was exposed to γ-radiation from a cobalt^{60} source (gamma-cell 200, Atomic Energy of Canada, Ltd.) giving a dose of approximately 1.8×10^5 r/hr. The virus was irradiated when suspended in reinforced Eagle's medium plus 30% serum, in order to minimize the indirect effect of radiations.[6]

DNA extraction: The cells were lysed in NaCl 0.15 *M*, sodium paraminosalicylate 6%, and Tris 0.1 *M* (pH 8).[10] The lysate was then shaken 10 min with an equal volume of 88% phenol, and centrifuged at 5,000 rpm for 10 min. The supernatant was collected and shaken with an equal volume of phenol saturated with NaCl 0.15 *M*, sodium paraminosalicylate 6%, and Tris 0.1 *M* (pH 8). This step was repeated four times. The final supernatant contained highly polymerized DNA and variable amounts of RNA. After dialysis against NaCl 0.14 *M*, Tris 0.1 *M* (pH 8), the solution was used for zonal centrifugation.

Results.—Polyoma virus infection of confluent mouse kidney cultures leads to an increase in DNA synthesis.[1-3] Table 1 shows the results of an experiment in which infected and uninfected cultures were labeled with C^{14}- or H^3-thymidine from the 17th to the 29th hour after virus adsorption. Both the total incorporation of thymidine into DNA and the frequency of DNA-synthesizing cells are higher in the infected cultures.

To verify that the observed increase in DNA synthesis was mainly due to cellular DNA synthesis, infected cultures were labeled with C^{14}-thymidine from the 17th to the 29th hour after infection, and the DNA extracted from these cultures was analyzed by band centrifugation.[11] DNA extracted from growing, uninfected mouse kidney cultures, which had been labeled with H^3-thymidine, was added as a marker. Figure 1 shows that about 75 per cent of the radioactivity incorporated by the infected cultures is found in the cellular DNA region, distinct from the sharp peak of the viral DNA; this confirms the increase in cell DNA synthesis demonstrated by other authors.[1-3]

To investigate the cellular response to different multiplicities of infection, dose-response curves for the DNA-inducing ability of the virus were constructed. Mouse kidney cultures were infected at several multiplicities of infection, and after labeling from the 18th to the 29th hour after virus adsorption, the total incorporation of labeled thymidine into DNA, and the number of DNA-synthesizing cells were measured. On a log-log plot the dose-response curve determined from the frequency of DNA-synthesizing cells[12] shows that polyoma virus is quite efficient in producing this effect, which can still be demonstrated at low multiplicities of infec-

TABLE 1

INCORPORATION OF LABELED THYMIDINE INTO POLYOMA-INFECTED AND UNINFECTED MOUSE KIDNEY CELLS

Multiplicity of infection (PFU/cell)	C^{14}-thymidine incorporation (cpm/culture)	Per cent labeled cells (H^3-thymidine)
0	2,200	2.2
20	13,500	49.8
200	18,000	80.0

Mouse kidney cultures were infected with purified polyoma virus (large plaque) as described under *Materials and Methods*. Seventeen hours after virus adsorption 1 μc of 2 C^{14}-thymidine (37 mc/mM) and 1.5×10^{-4} mmoles of thymidine, or 4 μc of H^3-thymidine (7 c/mM) and 1×10^{-5} mmoles of thymidine were added to each culture in 4 ml of medium. The cells were harvested or fixed for autoradiography at the 29th hour.

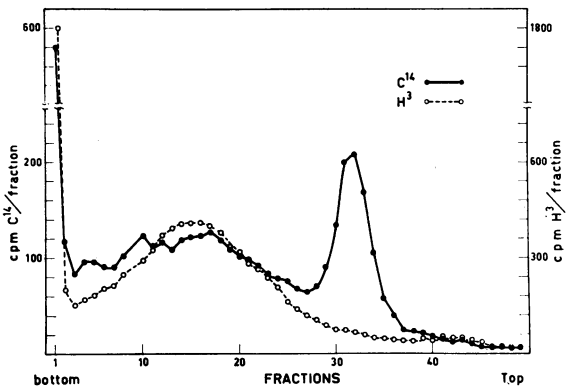

FIG. 1.—Sedimentation analysis (band centrifugation) of the DNA extracted from polyoma-infected cultures (100 PFU/cell) and labeled with 2 C^{14}-thymidine from the 17th to the 29th hour after virus adsorption. DNA extracted from growing uninfected mouse kidney cells labeled with H^3-thymidine added as a marker. Lamella: 0.05 ml of DNA from infected and 0.05 ml of DNA from uninfected cultures. Bulk solution 3 ml CsCl, ρ 1.50 pH 8; 35,000 rpm, 2.30 hr, 20°C. Drops were collected from the bottom of the tube (four drops per fraction), and fractions were counted in a Tri-Carb liquid scintillation spectrometer.

tion (Fig. 2). However, saturation is reached more slowly than expected. The curve may be a composite one, reflecting heterogeneity of the cell population with regard to virus susceptibility. Only at low multiplicities—below 4 PFU/cell—the frequency of labeled cells appears to increase linearly with dose. Similar results are obtained in regard to the incorporation of C^{14}-thymidine into DNA (Fig. 3). This suggests that the average amount of DNA synthesized per stimulated cell does not vary with the multiplicity of infection.

Measurements based on the frequency of the DNA-synthesizing cells, however, were found to be more accurate and reproducible. Therefore, this parameter was used in most of the experiments. It should also be mentioned that the same dose

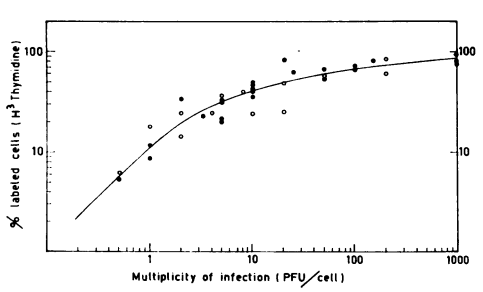

FIG. 2.—Dose-response curve of the DNA-inducing ability of polyoma virus as determined from the frequency of cells induced to synthesize DNA (corrected for control values as described in the text) in cultures infected at different input multiplicities and labeled with H^3-thymidine from the 18th to 29th hour after virus adsorption. Pooled data from several experiments. Small plaque polyoma virus, ●; large plaque, O. For each point at least 1000 cells were scored.

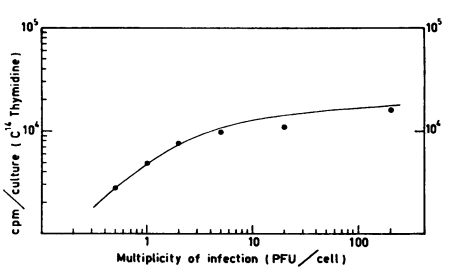

FIG. 3.—2 C^{14}-thymidine incorporation in cultures infected with large plaque virus at different input multiplicities and labeled from the 18th to the 29th hour after virus adsorption. Results from a typical experiment. Values represent the difference in C^{14}-thymidine incorporation between the infected and control uninfected cultures. For each point three cultures were used and pooled.

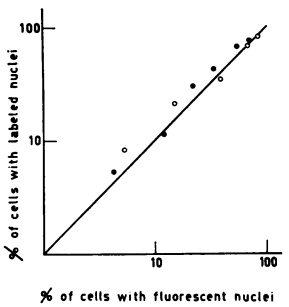

FIG. 4.—Correlation between the frequencies of DNA-synthesizing cells (corrected for control values as described in the text) in cultures infected at different multiplicities (0.5, 1, 5, 10, 100, 1000 PFU/cell), and the frequencies of virus-producing cells at the corresponding multiplicities. DNA-synthesizing cells were determined autoradiographically after labeling with H^3-thymidine. Virus-producing cells were determined by the fluorescent-antibody test (see text for details). Data from two independent experiments. For each point 1000–2000 cells were scored.

response is obtained if the cells are pulse-labeled with radioactive thymidine for 1-hr periods, between the 21st and the 25th hour after infection.

To determine whether the cells which synthesized DNA were the same cells in which virus multiplied, cells which had been grown on coverslips were infected at different virus/cell ratios, and exposed to H^3-thymidine from the 18th to the 30th hour after virus adsorption. At this time the cells were fixed and several duplicate coverslips were scored for frequency of DNA-synthesizing and virus-producing cells. The criterion for identification of virus-producing cells was the presence of viral antigen in their nucleus, as revealed by the fluorescent antibody test.

In Figure 4 the per cent of H^3-thymidine-labeled cells obtained at various multiplicities of infection is plotted against the per cent of cells with fluorescent nuclei obtained at the corresponding multiplicity. It is clear that a good correspondence exists between the frequencies of DNA-synthesizing and virus-producing cells, i.e., the dose-response curve shown in Figure 2 would apply as well for virus-producing cells. This finding suggests that cellular DNA is induced to replicate only in the cells in which virus multiplies.

More direct evidence for this was obtained by combining autoradiography and fluorescent antibody technique. Mouse kidney cells grown on coverslips were infected and labeled with H^3-thymidine from the 18th to the 30th hour. Then they were fixed with acetone-alcohol, stained with fluorescent antibodies, and mounted on a slide, with cells facing upward as it was found that such air-dried preparations still allow a satisfactory distinction between fluorescent and nonfluorescent nuclei. Several microscopic fields were examined and photographed. Autoradiograms were then made, and the same microscopic fields were observed and photographed to detect tritium-labeled cells. Comparison of the slides showed clearly the identity of the cells which have been labeled by radioactive thymidine with the cells which show nuclear fluorescence, at any multiplicity of infection (an example is given in Fig. 5).

Particularly at low PFU/cell ratios, some DNA-synthesizing but nonfluorescent cells are seen. However, these could be accounted for by cells which were synthesizing DNA independently of virus infection (control values). In addition, at low multiplicities of infection the appearance of the viral antigen is delayed. Thus, it was found that at input multiplicities from 100 to 1000 PFU/cell the relative highest frequency of fluorescent cells obtained within the first virus cycle was reached about 26 hr after virus adsorption; at lower multiplicities (5–100) the maximum was reached at 30 hr; at multiplicities on the order of 0.5–1.0 PFU/cell the maxi-

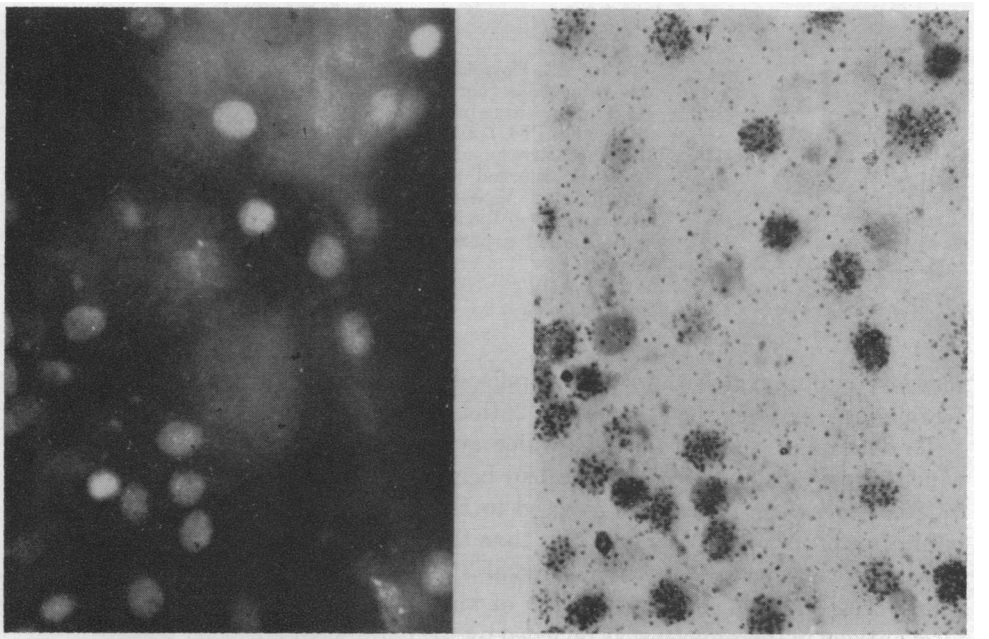

FIG. 5.—A microscopic field from cultures infected at 10 PFU/cell and labeled with H^3-thymidine after staining with fluorescent antibodies (*left*) and after autoradiography (*right*) (see text for other details). The cells with fluorescent nuclei and the cells with labeled nuclei are the same.

mum was at about 35 hr. Therefore, at low multiplicities, the frequencies of fluorescent cells determined at 30 hr are slightly underestimated (20–30%).

To determine whether the integrity of the entire polyoma genome was necessary for the induction of DNA synthesis, or whether only a part of it was involved, as has been shown for the transforming ability,[5, 6] the γ-ray inactivation curve of the polyoma plaque-forming ability was compared to that of the ability to induce DNA synthesis, as measured from the frequency of DNA-synthesizing cells.

As expected from the shape of the dose-response curve shown in Figure 2, exponential inactivation is obtained only when multiplicities of infection on the order of 5 PFU/cell or lower are used. At higher multiplicities the inactivation curves show progressively broader shoulders (Fig. 6). Therefore, the relative target sizes of the DNA-inducing ability and of the plaque-forming ability were determined at low multiplicities of infection. Figure 7 shows the results of a typical experiment. The DNA-inducing ability of polyoma virus is inactivated exponentially, following one-hit kinetics, at the same rate as the plaque-forming ability.

Inactivation of the virus plaque-forming ability sometimes proceeded at a faster rate than described previously.[6] This was due to the use of purified virus preparations, to which the addition of 30 per cent serum does not give the same degree of protection against the indirect effect of radiation as to unpurified virus. If unpurified virus preparations are used, the inactivation proceeds at the same rate previously shown[6] (D_0 5.3×10^5 r). In both conditions, however, the inactivation of the virus ability to induce DNA synthesis proceeds at the same rate as that of the plaque-forming ability. These data therefore show that the viral ability to induce

host cell DNA synthesis is lost upon γ-ray irradiation at the same rate as infectivity, suggesting that the integrity of the viral genome is required for the expression of this function.

Additional support of these data was obtained by comparing the γ-ray inactivation of the virus infectivity to that of its DNA-inducing ability by means of determining the number of virus-producing cells and that of DNA-synthesizing cells obtained in the cultures infected with irradiated virus. Also in this experiment the frequencies of virus-producing and DNA-synthesizing cells decreased at the same rate and, as expected, showed similar absolute values.

Discussion.—These experiments indicate that the induction of cellular DNA synthesis, which occurs in polyoma-infected mouse kidney cultures, takes place only in the cells in which the virus replicates.[13] This finding rules out the possibility that the phenomenon might be ascribed to nonspecific mechanisms, such as removal of contact inhibition in the uninfected fraction of the cell population, as a result of loss of viability of the neighboring infected cells. In addition it suggests that virus replication and cell DNA replication may be causally related, or at least that when in mouse kidney cells host DNA synthesis is induced, a crucial step of the virus growth cycle has taken place, so that from there on, the virus has practically 100 per cent probability of completing its growth.

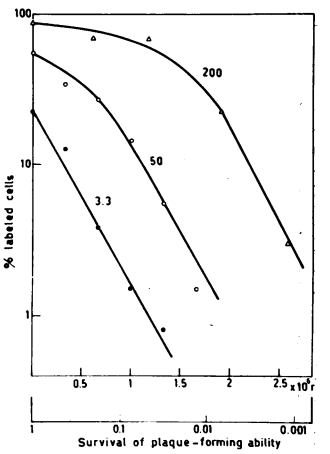

FIG. 6.—γ-Radiation survival curves of the DNA-inducing ability of polyoma virus determined at different multiplicities of infection (●, 3.3; O, 50; Δ, 200 PFU/cell at 0 dose). The DNA-inducing ability is expressed as the percentage of cells labeled with H^3-thymidine from the 18th to the 29th hour after virus adsorption (corrected for control values as described in the text). The log scale in abscissa represents the γ-radiation survival of plaque-forming ability of the same virus preparation.

The inactivation experiments suggest that the integrity of the viral genome is necessary for the induction of DNA synthesis. In fact, the DNA-inducing ability of the virus is inactivated at the same rate as plaque-forming ability. Assuming the entire viral genome to be necessary for the plaque-forming ability, which involves virus replication, it would appear that the integrity of the whole viral genome is required also for the expression of the DNA-inducing ability. The situation appears to be different from that demonstrated for transformation. In fact, in our conditions, the relative target of the transforming ability appears to be about half the size of that of the plaque-forming ability.[6]

Different results were obtained by Gershon *et al.*[14] using nitrous acid-inactivated virus. These authors found a relative target size for the DNA-inducing ability corresponding to 20 per cent that of the plaque-forming ability. However, it should be pointed out that they used a different cell system, consisting of heavily X-irradiated mouse embryo cells. It seems unlikely that the repression of DNA synthesis due to

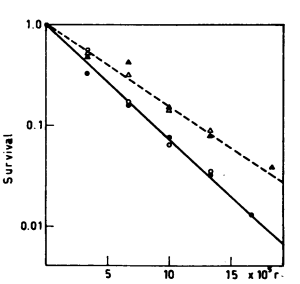

FIG. 7.—γ-Radiation survival of polyoma virus, assayed for plaque-forming (●, ▲) and DNA-inducing ability (O, Δ). Purified virus, ●, O; unpurified virus, ▲, Δ.

contact inhibition and that obtained by X irradiation are achieved by the same mechanisms.

If we assume that the induction of cell DNA synthesis results from the synthesis of an "inducer" coded for by the virus, it seems likely that the induction would only require the integrity of the part of the viral genome responsible for the synthesis of the "inducer" and at least should not require the portion of the viral genome devoted to code for coat proteins. In such a case a possible explanation of our results is suggested by the studies of Harriman and Stent[15] of the effect of P^{32} decay on cistron functions of T4 phage. These authors demonstrated the occurrence, among the types of damages produced by P^{32} decay, of "long-range hits" (presumably double-strand breaks of the DNA molecule) which inactivate the function of the entire genome. This suggested that the macromolecular continuity of the viral DNA complement is a necessary condition for the functional expression of any of its parts. Breaks which interrupt the continuity of the DNA molecule would therefore interfere with the function of any part of it, perhaps through alteration of the transcription process.[16] The results obtained for polyoma-transforming ability[5, 6] do not necessarily contradict this hypothesis, since it could be assumed that in the transformation process the specific part of the viral genome responsible for the transforming ability might be integrated into the cellular DNA in such a fashion that its function, unless directly damaged, would be restored.

On the other hand, the similar target sizes of plaque-forming and DNA-inducing ability might indicate that viral DNA replication is needed for the latter activity, since it seems likely that any damage to the DNA molecule would affect its ability to replicate in a normal fashion. Viral DNA replication might be involved in determining the induction of cellular DNA synthesis through two mechanisms: (*a*) a dosage effect, many copies of viral DNA being required to make the necessary amount of "inducer" to elicit cellular DNA synthesis; (*b*) the "inducer" being a kind of "late" protein, that can be coded for only by progeny viral DNA.

Summary.—In confluent cultures of mouse kidney cells, infected by polyoma virus, induction of cellular DNA synthesis occurs only in the cells in which the virus multiplies. In addition, the virus ability to induce DNA synthesis is inactivated by γ radiation at the same rate as its infectivity, suggesting that the integrity of the whole viral genome is required for the induction of cellular DNA synthesis.

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[1] Dulbecco, R., L. H. Hartwell, and M. Vogt, these PROCEEDINGS, **53**, 403 (1965).

[2] Weil, R., M. R. Michel, and G. K. Ruschmann, these PROCEEDINGS, **53**, 1468 (1965).

[3] Winocour, E., A. M. Kaye, and V. Stollar, *Virology*, **27**, 156 (1965).

[4] Winocour, E., *Virology*, **19**, 158 (1963).

[5] Benjamin, T. L., these PROCEEDINGS, **54**, 121 (1965).

[6] Basilico, C., and G. di Mayorca, these PROCEEDINGS, **54**, 125 (1965).

[7] Dulbecco, R., and G. Freeman, *Virology*, **8**, 396 (1959).

[8] Coons, A. H., *General Cytochemical Methods*, ed. J. F. Danielli (New York: Academic Press, 1958), vol. 1, p. 399.

[9] Kindly provided by Dr. R. Stevenson, National Institutes of Health, Bethesda, Md.

[10] Kirby, K., *Biochem. J.*, **66**, 495 (1957).

[11] Vinograd, G., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, **49**, 902 (1965).

[12] The frequency of DNA-synthesizing cells in uninfected cultures usually did not exceed 2–3%. However, the frequency of cells induced to synthesize DNA by viral infection (p_v) was determined by correcting the total frequency of H^3-labeled cells observed in the infected cultures (p_t) for the

values observed in control uninfected cultures (p_c). Correction was made assuming that normal and virus-induced DNA synthesis were independent events, according to the formula $p_v = \frac{p_t - p_c}{1 - p_c}$.

[13] When this manuscript was in preparation, an article by Vogt, M., R. Dulbecco, and B. Smith was published [these PROCEEDINGS, **55**, 956 (1966)]. These authors also concluded that induction of cellular DNA synthesis occurs in productively infected cells.

[14] Gershon, D., P. Hausen, L. Sachs, and E. Winocour, these PROCEEDINGS, **54**, 1584 (1965).

[15] Harriman, P. D., and G. Stent, *J. Mol. Biol.*, **10**, 488 (1964).

[16] Hayashi, M., M. N. Hayashi, and S. Spiegelman, these PROCEEDINGS, **51**, 351 (1964).