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Induction of DNA synthesis in terminally differentiated myotubes by the activation of the *src* gene of Rous sarcoma virus

(muscle cells/oncogenic virus/class T mutant of Rous sarcoma virus/cellular transformation)

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ABSTRACT **Mononucleated myogenic cells from 11-day-old chicken embryos were infected with tsLA24 or tsNY68, temperature-sensitive transformation mutants of Rous sarcoma virus. The infected mononucleated myogenic cells were incubated at the nonpermissive temperature (41°C) and allowed to develop into multinucleated myotubes. These myotubes have withdrawn from the cell cycle, and no DNA synthesis was observed as long as the cultures were maintained at the nonpermissive temperature. However, when the incubation temperature of these cultures was lowered to the permissive temperature (36°C) for expression of the *src* gene, DNA synthesis was induced in multinucleated myotubes. For this induction of DNA synthesis, cells infected with tsLA24 had to be incubated at the permissive temperature for at least 50 hr, while the induction of DNA synthesis in cells infected with tsNY68 required less than 20 hr. Induction of DNA synthesis was observed by autoradiography as well as by measuring incorporation of [^{3}H]thymidine into the macromolecule fraction in these myotube cultures. For the maintenance of capacity to induce DNA synthesis, constant presence of the *src* gene product is necessary, because when the temperature of these cultures was returned to 41°C, the myotubes lost the capacity to induce DNA synthesis. During the process of DNA induction one biochemical marker of muscle (creatine kinase) remained unchanged.**

In the previous communication in this series and in recent reports from other laboratories (1–9) evidence has been presented that changes due to oncogenic transformation may be related to a change toward a less differentiated state. In addition, it has been possible to induce the transforming activity of Rous sarcoma virus (RSV) in myotubes that have reached the terminal stage of differentiation. In this system, the myogenic cells were infected with a transformation mutant strain of RSV that is temperature sensitive in the ability to maintain cellular transformation. When cells were infected and incubated at the nonpermissive temperature (41°C), the myogenic cells underwent normal differentiation and formed normal multinucleated myotubes. On the other hand, cells that had been incubated at the permissive temperature (36°C) became transformed and few, if any, myotubes were formed. However, when the incubation temperature of these transformed cultures was raised to the nonpermissive temperature, the cells underwent differentiation to form myotubes. In contrast, when infected myotubes that had been incubated at the nonpermissive temperature were shifted to the permissive temperature, a dramatic cellular degradation was observed. Myotubes developed vacuoles and, within 2–3 days, started to detach from the substratum. This effect as a result of expression of the transforming gene product(s) in a cell in which DNA synthesis is completely suppressed due to differentiation appears to be unique to this system. In this communication we report that activation of the *src* gene of RSV can overcome the normal cellular inhibition of DNA synthesis in myotubes, resulting in the induction of DNA synthesis.

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MATERIALS AND METHODS

Preparation of Myogenic Cells. Breast muscle was dissected from avian leukosis virus-free 11-day old chicken embryos (SPAFAS, Lancaster, PA) as described previously (2). The muscle cells were seeded at a concentration of 1×10^6 cells per 35-mm × 10-mm gelatin-coated plastic dish and cultured in 8:1:1 medium [100 ml of Eagle's minimal essential medium, 12.5 ml of horse serum, 12.5 ml of chicken embryo extract, 1.5 ml of Fungizone (250 mg/ml), 1.5 ml of 200 mM glutamine, 1.5 ml of penicillin/streptomycin mixture (penicillin, 10^4 units/ml; streptomycin, 10 mg/ml]. For preparation of chicken embryo extract, 11-day SPAFAS chicken embryos were forced through a 10-cm^3 syringe and incubated overnight at 4°C with an equal volume of Eagle's minimal essential medium. The extract was centrifuged at 100,000 × *g* and the supernatant fluid was filtered through a Millipore filter (0.2 μm). Cells were infected with RSV at the time of plating (approximately 1 focus-forming unit per cell) and cultured at 36°C for 2–3 hr after which the cultures were maintained at either 36°C or 41°C. Forty-eight hours after plating, cytosine arabinonucleoside (cytosine arabinoside), an inhibitor of DNA synthesis, was added to the culture medium at a final concentration of 1 μg/ml. Most of the experiments were carried out with 3-day-old cultures. Medium was changed daily.

Virus. Primary chicken embryo fibroblast cell cultures were prepared and virus was grown and assayed as described by Vogt (10). The class T mutants of RSV used in this study were generous gifts from H. Hanafusa of the Rockefeller University, NY (tsNY68 of the Schmidt–Ruppin strain of RSV) and J. Wyke of the Imperial Cancer Research Fund Laboratories, London (tsLA24 of the Prague strain of RSV).

Assay of Creatine Kinase. Cells were removed from the dish by scraping with a rubber policeman and homogenized with a Dounce homogenizer in 0.3 ml of 50 mM glycylglycine. Creatine kinase was assayed with creatine kinase kit (Sigma no. 45-UV) described by Oliver (11).

Autoradiography. After removal of cytosine arabinonucleoside, cultures were incubated at 36°C for 1 hr with [^{3}H]thymidine (New England Nuclear, 64.7 Ci/mmol) at 5 μCi/ml in 8:1:1 medium (1 Ci = 3.7×10^{10} becquerels). Cultures were then washed three times with Eagle's minimal essential medium and incubated for another hour in cold 8:1:1 medium. The cells were fixed with ethanol and allowed to air dry. A thin layer of Kodak NTB-2 (nuclear track emulsion) was poured onto the cell surface, air dried, and exposed for 7 days at 4°C. The autoradiograms were developed in Kodak D-19, fixed, and stained with hematoxylin and eosin.

[^{3}H]Thymidine Uptake. Cultures were labeled for 1 hr with

Abbreviation: RSV, Rous sarcoma virus.

10 μCi of [^{3}H]thymidine per ml of modified Eagle's medium at 36°C. Cultures were then washed three times with modified Eagle's medium and dissolved in 0.5 ml of 4 M guanidine/50 mM sodium acetate buffer (pH 5.8), and the radioactivity of a 0.1-ml aliquot was determined. The remaining 0.4 ml of this extract was used for quantitation of DNA by the method of Hinegardner (12).

RESULTS

Induction of DNA Synthesis in tsLA24-Infected Myotubes upon Temperature Shift to the Permissive Temperature. During the *in vitro* differentiation of myogenic cells, mononucleated cells fuse to form postmitotic multinucleated myotubes. This cessation of DNA synthesis in myotubes is so complete that nuclei in myotube never reenter S phase (13, 14). We therefore examined whether activation of the *src* gene induces host DNA synthesis in *postmitotic* myotubes. In the experiment illustrated in Fig. 1, myogenic cells that had been infected with a transformation mutant RSV at the time of plating were incubated at the nonpermissive temperature. Normal multinucleated myotubes developed under these conditions. To eliminate contaminating fibroblastic cells, cytosine arabinonucleoside was added 48 hrs after cells were plated. Twenty four hours later, the myotube cultures were shifted to the permissive temperature and, after various times, the cells were exposed to [^{3}H]thymidine in the absence of cytosine arabinonucleoside. Fig. 1 clearly demonstrates that multinucleated myotubes infected with tsLA24 (15) and incubated at 36°C for a period of 65 hr after a temperature shift incorporate radioactive thymidine. It is important to point out that a 40-hr incubation at 36°C prior to a 1-hr pulse of [^{3}H]-thymidine did not give any appreciable labeling of nucleus in myotubes, because this observation demonstrates that these myotubes had indeed stopped DNA synthesis. Thus, the labeled nuclei observed in the myotubes must be the results of *reactivation* of once-stopped DNA synthesis and not due to continued DNA synthesis. In addition, no further fusion took place at the time of the pulse, indicating that labeled nuclei in myotubes did not come from mononucleated cells. Uninfected myotubes did not incorporate any appreciable radioactive thymidine into DNA. The occasional grains observed in the tsLA24-infected myotube culture incubated at 36°C for 40 hr and in the uninfected myotube culture represent incorporation by single cells.

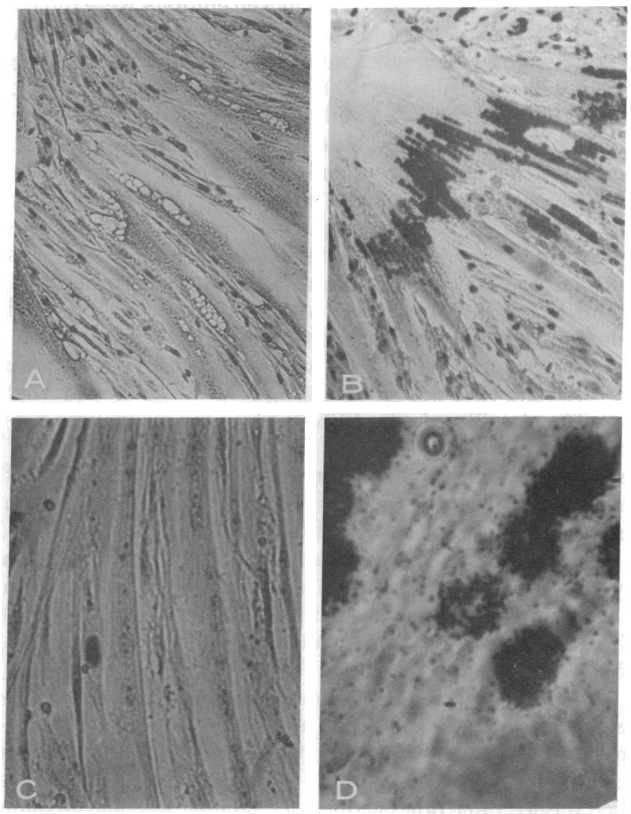

FIG. 1. Induction of DNA synthesis following a shift to the permissive temperature of the myotubes infected by a transformation mutant of RSV. Chicken muscle cells were infected with tsLA24 and cultured at 41°C for 3 days. The incubation temperature was then changed to 36°C. (*A*) Forty hours after temperature shift of tsLA24-infected myotubes. Note that incorporation of [^{3}H]thymidine was not observed in the myotubes, while it was in fibroblasts. (×80.) (*B*) tsLA24-infected myotubes, 65 hr after a temperature shift. Incorporation of [^{3}H]thymidine was observed in multinucleated myotubes. (×80.) (*C*) tsLA24-infected myotubes, cultures at 41°C throughout. (×160.) (*D*) Higher magnification of labeled nuclei under conditions of *B*. (×690.)

Table 1. Time course of induction of DNA synthesis in multinucleated myotubes infected by RSV tsLA24

Time after temperature shift-down, hr	Labeled nuclei/ total nuclei*	Labeled nuclei in myotubes, %
0		0
5		0
10		0
20		0
40		0
50	49/1396	3.5
60	89/1107	8.0
72	117/1106	10.6
80	256/2232	11.5
88	1792/9606	18.7
95	60/725	8.3

RSV tsLA24-transformed myotubes were shifted to 36°C in the presence of cytosine arabinonucleoside and at intervals autoradiography was performed after 1-hr pulse with [^{3}H]thymidine in the absence of cytosine arabinonucleoside.

* Labeled nuclei in multinucleated myotubes and total number of nuclei, including those of mononucleated cells, were counted in at least 20 randomly selected fields.

It is noteworthy that, after a 65-hr incubation at the permissive temperature, the labeled nuclei were found in myotubes. The nuclei in myotubes are easily distinguished from those of fibroblastic contaminants because they are lined up. As described in our earlier publication (2), these myotubes

Table 2. Continued requirement of active *src* gene product(s) for the induction of DNA synthesis

Exposure, hr		Labeled nuclei/	Labeled nuclei
36°C	41°C	total nuclei	in myotubes, %
0	70		0
5	65		0
25	45		0
40	30		0
50	20	23/1280	1.8
60	10	138/2150	6.4
70	0	377/1952	19.3

RSV tsLA24-infected myogenic cells were kept at 41°C for 3 days. At time zero, cells were shifted down to 36°C, and at the times indicated they were shifted back up to 41°C and kept at 41°C for the periods indicated. After these treatments, a 1-hr pulse of [^{3}H]thymidine at 36°C was given, followed by autoradiography. Labeled nuclei in myotubes were counted in at least 20 randomly selected fields.

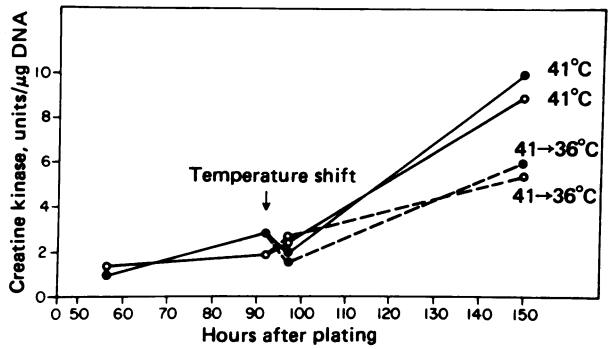

FIG. 2. Lack of effect of a temperature shift on the level of creatine kinase in tsLA24-infected myotubes. Chicken embryo myogenic cells were infected with tsLA24 and cultured at 41°C for 3 days. After the formation of myotubes, the culture was shifted down to 36°C and creatine kinase was assayed. ○, tsLA24-infected myotubes; ●, noninfected myotubes.

eventually deteriorate and detach from the substratum. To determine exactly how long an incubation at the permissive temperature was required for the induction of DNA synthesis, the time of incubation at the permissive temperature was varied. As shown in Table 1, the minimum length of incubation at the permissive temperature that resulted in the induction of DNA synthesis was approximately 50 hr in the case of myotubes infected by tsLA24. To explore the possibility that an exposure of less than 50 hr to 36°C may be enough if cells are returned to 41°C, experiments were performed as indicated in Table 2. Cells were incubated at 41°C for 3 days so that the myotubes were allowed to develop. The cultures were then incubated at 36°C for from 5 to 70 hr and returned to 41°C. At the end of 70 hr all the cells were examined for their [^{3}H]thymidine incorporation. As shown in this table, only after the exposure of the tsLA24-infected myotubes to 36°C for 50 hr was the induction of DNA synthesis observed. These results indicate that the transforming gene has to be functional for a period of 50 hr or more in order to bring about the induction of DNA synthesis.

Lack of Influence of the Temperature on the Level of Creatine Kinase. In the preceding communication from this laboratory we reported that when myogenic cultures were infected with RSV tsLA24 and incubated at 36°C (permissive temperature) very little synthesis of creatine kinase, a marker enzyme of myotubes, was observed (2). It was of interest, therefore, to examine the level of this enzyme in myotubes that undergo deterioration due to the activation of the transforming gene product. The data presented in Fig. 2 represent the time course of the change in creatine kinase in tsLA24-infected myotubes after the shift to the permissive temperature. As noted in this figure, the level of creatine kinase kept increasing even after the temperature shift. Although the increase of the enzyme level was less with the cultures that had been placed at 36°C, this difference is a simple temperature effect on the level of this enzyme, because a similar temperature effect was observed with uninfected myotubes. Thus, despite the deterioration of infected myotubes after the shift to 36°C, there was still some increase in the enzyme activity relative to DNA amount. It should be pointed out that the presence of the transforming gene product(s) in the mononucleated myogenic cells may interfere with *de novo* synthesis of creatine kinase during the differentiation of the myogenic cells (2), but once creatine kinase is expressed in the differentiated cells, activation of transforming gene product(s) does not inactivate the preexisting creatine kinase nor does it inhibit the increase in enzyme activity. It should be added that the data presented in this section by no means contradict the data in our previous publication, which revealed an increase in creatine kinase activity during the temperature shift-up of the transformed myogenic cells.

Studies on Myotubes Infected with tsNY68. All the preceding experiments were performed with tsLA24. In the preceding communication, we reported that myotubes infected with tsLA24 underwent distinct morphological changes after a shift to the permissive temperature (vacuolation and degeneration of the tubes). Similar experiments performed with tsNY68 (16) revealed marked differences in the morphological changes observed, as shown in Fig. 3. When the incubation temperature was lowered, myotubes infected by tsNY68 were converted into a series of smaller, thin myotube-like cells that contained a few nuclei. The exact reason for the difference between the effect of the two transformation mutants is unknown. It remains obscure whether the resulting smaller myotubes are capable of replication at 36°C. It has been observed, however, that the tsNY68-infected cells deteriorate with time and that the number of cells per dish decreases dramatically after the shift to 36°C. It was noted that the morphological response to the temperature shift of myotubes infected by tsNY68 was more rapid than that of tsLA24-infected myotubes. The morphological change illustrated by Fig. 3 can be observed as early as 6–7 hr after the temperature shift. On the other hand, it takes more than 24 hr for the morphological changes to appear in myotubes infected with tsLA24. Because of the rapidity of the activation of the *src* gene of tsNY68, one can observe the

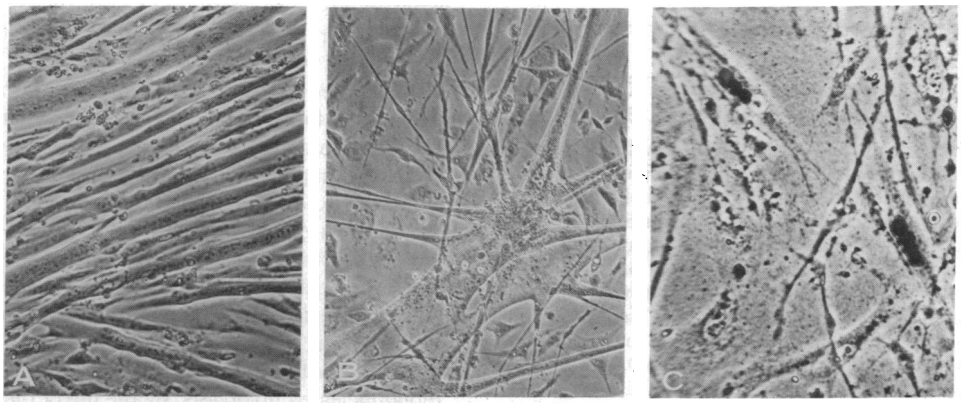

FIG. 3. Morphological change of tsNY68-infected myotubes after a shift to the permissive temperature. Myotubes infected with tsNY68 were shifted to 36°C. Pictures were taken 20 hr after the temperature shift-down. Transformed myotubes became thinner and smaller but no appreciable vacuolization was observed. (All ×160.) (*A*) Control myotubes. (*B*) tsNY68-infected myotubes, 20 hr after the temperature shift. (*C*) Autoradiography of tsNY68-infected myotubes, 20 hr after the cultures were shifted to 36°C.

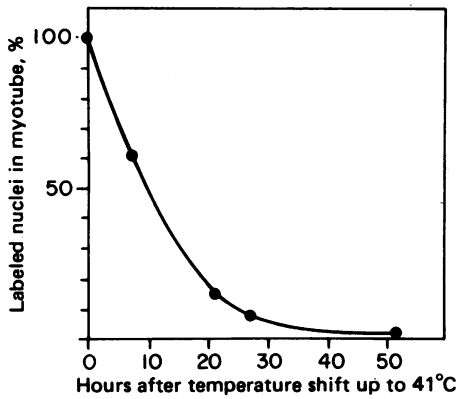

FIG. 4. Requirement of *src* gene product(s) for the maintenance of capacity to induce DNA synthesis in myotubes. tsNY68-infected myotubes were incubated at 41°C for 3 days. The resulting myotube cultures were shifted to 36°C for 30 hr, then shifted back to 41°C. Cultures were kept in cytosine arabinonucleoside from day 2 on. Autoradiography was performed at the time indicated in the figure after a 1-hr pulse with [^{3}H]thymidine at 36°C in the absence of cytosine arabinonucleoside. The number of labeled nuclei in the myotubes was counted and expressed as % of the control that was kept at 36°C for the same period after the original temperature shift to 36°C. In the control cultures which were kept at 36°C for 80 hr, the capacity to induce DNA synthesis was not more than in those cultures exposed to 36°C for 30 hr.

induction of DNA synthesis within 20 hr after the shift to permissive temperature (Fig. 3C). It is therefore more convenient to use this virus to examine whether or not the once-induced capacity to synthesize DNA exists even after a return to the nonpermissive temperature. RSV tsNY68-infected myotubes were incubated for 30 hr at 36°C after the temperature shift from 41°C, so that capacity to synthesize DNA in the myotubes could be induced, then the cultures were shifted back to 41°C. All cultures were kept in cytosine arabinonucleoside during these periods (except for the time of [^{3}H]thymidine pulse). Fig. 4 shows the relative number of pulse-labeled nuclei in myotubes at various times after the temperature was shifted back to 41°C. It is clear from this figure that a progressive decrease in the number of labeled nuclei occurs after a shift to 41°C. These results indicate that the continued presence of the *src* gene product(s) is required for maintenance of capacity to induce DNA synthesis.

To support the conclusion that activation of the *src* gene induced DNA synthesis in multinucleated myotubes, total DNA synthesis in the culture was examined. In this experiment tsNY68-infected myoblasts were allowed to develop into normal multinucleated myotubes at the nonpermissive temperature. These cultures were then shifted down to the permissive temperature. At the various times indicated in Fig. 5, cultures were pulse labeled (1 hr) with [^{3}H]thymidine. It is clear that an exposure to the permissive temperature of approximately 10 hr was necessary for induction of DNA synthesis. However, [^{3}H]thymidine incorporation reached a maximum approximately 30 hr after the temperature shift.

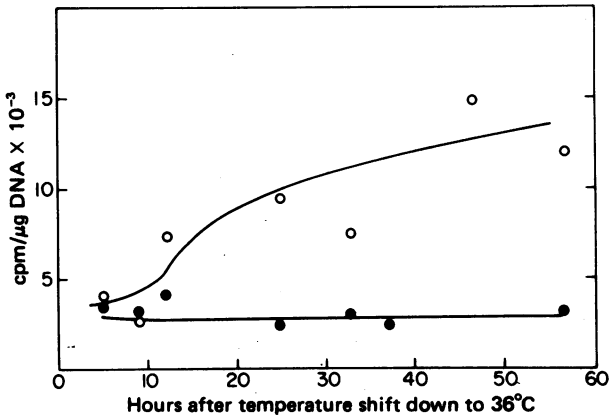

FIG. 5. [^{3}H]Thymidine uptake in the myotube culture after activation of the *src* gene. Myogenic cells were plated and infected with tsNY68. Cultures were incubated at 41°C for a total of 4 days. Cytosine arabinonucleoside was included in the culture medium at a final concentration of 1 μg/ml, beginning 2 days after plating. The temperature of the cultures was lowered to 36°C and, at the times indicated in the figure, cytosine arabinonucleoside was removed. Cultures were then pulse labeled with [^{3}H]thymidine at 10 μCi/ml in Eagle's minimal essential medium for 1 hr, washed three times with cold Eagle's minimal essential medium, and dissolved in 0.5 ml of 4 M guanidine containing 50 mM acetate buffer, pH 5.8. This extract was used to determine [^{3}H]thymidine incorporation. Values presented are averages of two independent experiments. o, Cultures with temperature shift-down to 36°C; ●, cultures with temperature kept at 41°C.

DISCUSSION

Possible induction of DNA synthesis in myotubes has been studied in four separate laboratories (17–21), and it has been claimed that subjecting myotubes to wild-type RSV induces DNA synthesis (17). Our attempts to induce DNA synthesis in myotubes with RSV under similar conditions have so far been unsuccessful. Because the myotube cultures usually have mononucleated myoblast contaminants, the possibility exists that infected mononucleated myoblasts are responsible for the induction of DNA synthesis in myotubes after they are fused. Indeed this is apparently believed to be the cause for the DNA synthesis induction in myotubes by polyoma virus infection. Thus, Yaffe and Gershon (18) state "infection of the differentiated cells [myotubes] appears to occur via the introduction of nuclei of pre-infected myoblasts into the growing fibres [myotubes]." They believe that some infected mononucleated myoblasts would fuse into myotubes with concomitant stoppage of DNA synthesis. The viral genome in myotubes would then induce DNA synthesis, resulting in mitotic nuclei in myotubes. The experiments of Fogel and Defendi (19) indicated that fully differentiated myotubes are insensitive to DNA tumor virus, and no induction of DNA synthesis was observed upon infection with DNA tumor virus. Furthermore, DNA synthesis was not observed when polyoma virus was injected into myotubes, while synthesis of T-antigen in myotube was observed (21). In our preceding communication we reported that DNA synthesis in myotubes could not be observed in infected cultures. These experiments were carried out with the following conditions. Cultures were infected with wild-type RSV or RSV tsLA24 and incubated at 36°C or 41°C, and on days 3, 4, 5, or 6 the cultures were exposed to [^{3}H]thymidine for a 1-hr pulse. At 41°C myotubes were formed and, as we have shown in the present paper, no DNA synthesis was observed. At 36°C we found no labeled myotubes simply because there were very few, if any, myotubes under our experimental conditions. It should be pointed out that the preceding experiments did not include temperature shift-down experiments as reported in this paper.

We felt that we could obtain a clearer picture of possible DNA synthesis in myotubes under the influence of tumor virus by activating the *src* gene product in the myotubes that had been infected with the T class mutants of RSV at the stage of mononucleated myoblasts. By the use of these mutants we were able to eliminate the possibility that DNA synthesis in myotubes may come from fusing myoblasts. Because the culture had been kept at 41°C, all the infected myoblasts that could fuse were

already fused at the time of the experiment. It was also possible to establish that fused myotubes have indeed stopped DNA synthesis because we did not observe any labeled nuclei in myotubes before the temperature shift or within 50 hr of temperature shiftdown. From these considerations, we believe that we have demonstrated the *induction* of DNA synthesis in myotubes.

Induced DNA synthesis does not appear to be repair synthesis of DNA [as reported by Stockdale and O'Neill (22) after UV irradiation of myotube] because of the large quantity of radioactivity incorporated. To establish this point, however, further experiments are necessary. It should be pointed out that the present results do not contradict the negative results on myotube DNA synthesis we reported in the preceding communication, which did not involve temperature shift-down experiments. Whatever the nature of the block of DNA synthesis in myotubes may be, this is different from the DNA synthesis block in contact inhibited fibroblasts because T-antigen can stimulate DNA synthesis in contact-inhibited 3T3 cells (23) while it does not stimulate myotube DNA synthesis as discussed above. The block may not be the lack of DNA polymerase as previously thought (24, 25) because one report indicates that the level of DNA polymerase in the myotube was comparable to that of mononucleated cells (26). Although we do not have direct evidence that nuclear DNA is replicated, it seems likely that activation of the transforming gene product results in the induction of *cellular* DNA synthesis. In support of this concept, our preliminary experiment using radioactive viral complementary DNA showed no large increase of viral DNA during the temperature shift-down. We do not yet know how far nuclear DNA synthesis would proceed before detachment of myotubes from the substratum. Nor do we know whether any of the myotube nuclei would undergo mitosis under our experimental conditions.

Activation of the transforming gene product has been the subject of intensive studies (27–36). These studies demonstrated that activation of the transforming gene results in cellular expression of tumorgenic characteristics. When differentiating cells are infected with transformation mutants of RSV, incubation of the cells under permissive conditions usually leads to a loss of differentiated characteristics (1–9, 37–39). For example, the synthesis of sulfated proteoglycan, a biochemical marker of chondrocytes, decreases markedly (6). In addition, hyaluronic acid synthesis appears to increase after activation of the transforming gene product in chondrocytes (1), as well as in myogenic cells (unpublished observation). The present observation adds yet another important result of the *src* gene action: induction of DNA synthesis in terminally differentiated cells. The molecular elucidation of the present observation would contribute toward an understanding of molecular nature of the "terminally differentiated state" as well as of the *src* gene action.

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