Differential Response of Cycling and Noncycling Cells to Inducers of DNA Synthesis and Mitosis

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ABSTRACT The objective of this study was to determine whether cells in G_0 phase are functionally distinct from those in G_1 with regard to their ability to respond to the inducers of DNA synthesis and to retard the cell cycle traverse of the G_2 component after fusion. Synchronized populations of HeLa cells in G_1 and human diploid fibroblasts in G_1 and G_0 phases were separately fused using UV-inactivated Sendai virus with HeLa cells prelabeled with [^{3}H]ThdR and synchronized in S or G_2 phases. The kinetics of initiation of DNA synthesis in the nuclei of G_0 and G_1 cells residing in G_0/S and G_1/S dikaryons, respectively, were studied as a function of time after fusion. In the G_0/G_2 and G_1/G_2 fusions, the rate of entry into mitosis of the heterophasic binucleate cells was monitored in the presence of Colcemid. The effects of protein synthesis inhibition in the G_1 cells, and the UV irradiation of G_0 cells before fusion, on the rate of entry of the G_2 component into mitosis were also studied. The results of this study indicate that DNA synthesis can be induced in G_0 nuclei after fusion between G_0- and S-phase cells, but G_0 nuclei are much slower than G_1 nuclei in responding to the inducers of DNA synthesis because the chromatin of G_0 cells is more condensed than it is in G_1 cells. A more interesting observation resulting from this study is that G_0 cells differ from G_1 cells with regard to their effects on the cell cycle progression of G_2 cells after fusion. Unlike G_1 cells, G_0 cells upon fusion with G_2 are not able to inhibit the progression of the G_2 nucleus into mitosis. This difference between G_0 and G_1 cells appears to depend on certain factors, probably nonhistone proteins, present in G_1 cells but absent in G_0 cells. These factors can be induced in G_0 cells by UV irradiation and inhibited in G_1 cells by cycloheximide treatment.

The cell fusion studies by Rao and Johnson (15) have identified two characteristics that are associated with cells in G_1 phase of the cell cycle. They are: (*a*) inducibility of DNA synthesis by fusion with S-phase cells and (*b*) the ability of G_1 cells to inhibit the progression of G_2 cells into mitosis in G_1/G_2 heterophasic binucleate cells. These observations indicate that G_1 cells are deficient in the inducers of DNA synthesis but that the G_1 chromatin, unlike that of G_2, can respond to these inducers and, thus, initiate DNA synthesis when fused with S-phase cells. The inhibition of G_2 progression by G_1 cells can be explained as follows. It has been well established that chromatin undergoes progressive decondensation during G_1 and condensation during G_2 (1, 8–12). When G_1 cells are fused with those in G_2, the decondensation factors of the G_1 component seem to neutralize the chromatin condensing factors of the G_2 component and, thus, prevent the G_2 from entering into mitosis. Do G_0 cells have such a capability of inhibiting the progression of a G_2 nucleus into mitosis? In a study of heterokaryons formed by fusion of senescent human diploid fibroblasts (HDF) with an immortal cell line (T98G), Stein and Yanishevsky (18) speculated that a noncycling HDF would prevent a T98G nucleus in G_2 phase from entering into mitosis. To answer this question, we have decided to compare HDF in G_0 phase with those in G_1 with regard to two characteristics, i.e., whether DNA synthesis can be induced in G_0 cells by fusion with S-phase cells as rapidly as in the case of G_1 cells, and whether G_0 cells upon fusion with G_2 can prevent the latter from entering into mitosis. The results of this study indicate that G_0 cells have a 3–4-h lag to respond to the inducers of DNA synthesis and that G_0 cells are unable to block the cell cycle progression of a G_2 component in G_0/G_2 heterokaryons.

MATERIALS AND METHODS

Cells and Cell Synchrony

HeLa cells and HDF (strain no. 78–89) were used in this study. HeLa cells were grown as a monolayer culture at 37°C in a humidifed 5% CO_2 incubator in Eagle's minimal essential medium supplemented with 10% fetal calf serum, sodium pyruvate, glutamine, and antibiotics as previously described (15). These

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cells have a generation time of 22 h , and a G_1 period of 10.5 h, S phase of 7 h, G_2 of 3.5 h, and a mitotic duration of 1 h (14).

HeLa cells were synchronized by the excess ThdR double-block method (14). Synchronized populations of cells in S and G_2 periods were obtained by collecting cells at 1 and 7 h, respectively, after the reversal of the second ThdR block. A pulse labeling with [^{3}H]ThdR revealed a labeling index of 96% in S-phase cells and 15% in G_2 population. The mitotic index was <2% in both S and G_2 populations. Early G_1 population was obtained by collecting the cells at 2 h after the release of a N_2O block after the reversal of a single excess ThdR block (13). The G_1 population had a mitotic index of 5% and a 0% labeling index.

The HDF strain we used was kindly supplied by Dr. Thomas Norwood of the University of Washington (Seattle, Wash.). HDF were grown as monolayers in McCoy's modified 5A medium supplemented with 20% fetal calf serum, glutamine, and antibiotics. HDF were in their 15–18th passages during the period of these experiments. HDF in G_0 were obtained by harvesting at 7 d after cells had reached confluence. To obtain HDF in G_1 phase, the cells were held in confluence for 7 d and then trypsinized and replated at 25% confluence. At 18 h after plating, cells were harvested for fusion. The labeling index was <2% in G_0 and G_1 populations.

Cycloheximide Treatment

Mitotic HeLa cells were obtained by selective detachment from dishes that were exposed to N_2O (80 pounds per square inch) for 10 h. By this method, we can obtain large amounts of mitotic cells of high (98%) purity. These mitotic cells were plated in new dishes in a medium containing cycloheximide (25 μg/ml) and incubated for 8 h, i.e., until the time of fusion. At this concentration of cycloheximide, ~95% of protein synthesis is inhibited in mammalian cells (4). Inhibition of protein synthesis by cycloheximide had no effect on the completion of mitosis and cytokinesis. These cells may be considered to be blocked in early G_1.

UV Treatment

HDF in G_0 were trypsinized and plated into three 60-mm culture dishes in 2 ml of medium. One dish (with the lid off) was exposed to UV for 60 s (21.3 J/M^{-2}/s) from a Sylvania gemicidal lamp (Ultra-Violet Products, Inc. San Gabriel, Calif.) and another dish for 30 s. The third dish, not exposed to UV light, served as a control. Immediately after they were irradiated, the cells were fused with HeLa cells in G_2 phase.

Cell Fusion

The procedure we used for UV-inactivated Sendai virus has been previously described (15). To study the regulation of DNA synthesis, we performed three different fusions. They were: (*a*) HeLa S*/HeLa G_1, (*b*) HeLa S*/HDF G_1, and (*c*) HeLa S*/HDF G_0. (The asterisk indicates the cell population that was prelabeled with [^{3}H]ThdR during the synchronization procedures). Immediately after fusion between a prelabeled and an unlabeled population, each of the fusion mixtures was resuspended in regular medium. About 1 ml of this cell suspension was taken and cells were deposited directly on the slides with a cytocentrifuge (Shandon-Elliot Co., London, England). To the remaining cell suspension, [^{3}H]ThdR (0.1 μCi/ml; sp act, 6.7 Ci/mM) and Colcemid (0.05 μg/ml) were added and plating was immediately done in a number of 35-mm culture dishes. Cell samples were taken at hourly intervals by trypsinizing one of the dishes. The trypsinized cells were deposited on slides as described above, fixed in 3:1 methanol-glacial acetic acid mixture, processed for autoradiography, stained with Giemsa's, and scored for the frequency of labeled nuclei among mono-, bi-, and trinucleate cells. About 500 cells were scored for each time point. The data presented are the averages of three different experiments.

The procedures for estimating the rate of induction of DNA synthesis in G_1 nuclei after fusion between G_1 and S-phase cells have been previously described (15). Briefly, they are as follows. Before fusion, the cells of each population were mononucleate and either labeled (L) or unlabeled (U). After fusion, ~25% of the mixed population consisted of multinucleate cells, i.e., bi-, tri-, and tetranucleate cells. For example, the binucleate cells can be either U/U, L/L, or L/U if two unlabeled, two labeled, or one labeled and one unlabeled cell, respectively, were fused together. When the fused cells are incubated with [^{3}H]ThdR, if the unlabeled nuclei incorporate [^{3}H]ThdR, the frequencies of classes L/U and U/U decrease. The percent decrease in their frequency as a function of time after fusion indicates the rate at which unlabeled nuclei have been changing into labeled nuclei. This change is expressed as a percent increase in the labeling index of the unlabeled nuclei residing either in mono-, bi-, or trinucleate cells.

To study mitotic regulation, we fused HeLa cells synchronized in G_2 period and prelabeled with [^{3}H]ThdR separately with six different cell populations. The fusions were: (*a*) HeLa G_2/HeLa G_1, (*b*) HeLa G_2/HeLa G_1 treated with cycloheximide, (*c*) HeLa G_2/HDF in G_1, (*d*) HeLa G_2/HDF in G_0, (*e*) HeLa G_2/HDF G_0 exposed to UV for 60 s, and (*f*) HeLa G_2/HDF G_0 exposed to UV for 30 s.

The cells were resuspended in a medium containing Colcemid (0.05 μg/ml) immediately after fusion and were plated in a number of 35-mm plastic culture dishes. Cell samples were taken at regular intervals by trypsinizing one of the dishes and processed for autoradiography as described above. Mitotic indices (MI) were scored for mono- and binculeate cells and plotted as function of time after fusion.

RESULTS

Induction of DNA Synthesis in G_0 cells

Because the data obtained from the fusion experiments involving G_1-phase cells of either HDF or HeLa cells are identical in all respects, only the data from the HeLa G_1 fusions with S-, and G_2-phase cells are presented in this study. The rate of initiation of DNA synthesis in the nuclei of G_1 HeLa and G_0 HDF as a result of fusion with S-phase HeLa cells is shown in Fig. 1. DNA synthesis was rapidly induced in G_1 nuclei located in G_1/S binucleate cells. In these cells, a labeling index (LI) of 50% was reached by 1.5 h after fusion compared with 8.5 h in the G_1 mononucleate cells. The LI in the mononucleate G_0 cells remained <2% throughout this experiment. However, the G_0 nuclei located in G_0/S binucleate cells started to incorporate [^{3}H]ThdR ~3 h after fusion and the LI in these cells reached a 50% level at ~4.5 h after fusion.

If the slower response of G_0 nuclei to the S-phase inducers were caused by the absence of any inducer molecules in G_0 cells relative to those of G_1, one would expect a rapid induction of DNA synthesis in G_0 nuclei by increasing S-phase component in the fused cells. To find out whether increasing the ratio of S:G_0 would alter the kinetics of initiation of DNA synthesis in G_0 nuclei, we scored trinucleate cells containing 1 S:2 G_0 or 2 S:1 G_0 nuclei for labeling index (Fig. 2). These data indicate that increasing the ratio of S:G_0 by a factor of two advanced the entry of the G_0 nuclei into S phase by only 0.5 h. When this ratio was reversed, i.e., 1 S:2 G_0, the entry of both the G_0

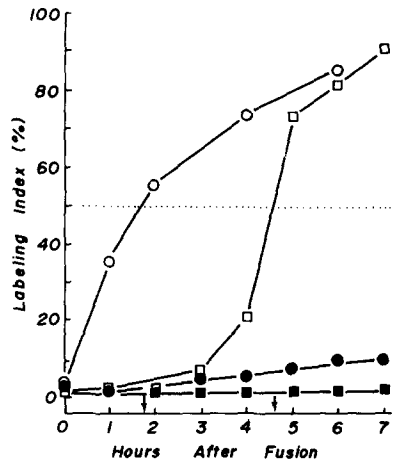

FIGURE 1 The kinetics of initiation of DNA synthesis in the G_0 and G_1 nuclei residing in G_0/S and G_1/S heterophasic binucleate cells. HeLa cells in G_1 and HDF in G_0 phase were separately fused with ([^{3}H]ThdR) prelabeled S-phase HeLa cells. The incorporation of label into the G_1 or G_0 nuclei was measured as the LI. The procedures for calculating the LI were previously described (15). (○), G_1 nuclei residing in G_1/S dikaryons; (□), G_0 nuclei in G_0/S dikaryons; (●), mononucleate G_1 phase HeLa cells; (■), mononucleate HDF in G_0 phase. The data from fusions involving HDF-G_1 and S-phase HeLa are not presented because they are similar to those of HeLa G_1 and HeLa S fusion. The dotted line indicates the 50% level.

nuclei into S phase was delayed by ~30 min. However, some asynchrony was observed with regard to the initiation of DNA synthesis in the G_0 nuclei of the trinucleate (1 S:2 G_0) cells. In ~25% of the cases, one of G_0 nuclei incorporated [^{3}H]ThdR whereas the other one did not. Within the next 30 min, the second nucleus also became labeled.

Regulation of Mitosis in the Fused Cells

HeLa cells in G_2 period (prelabeled with [^{3}H]ThdR) were fused separately with G_1 HeLa and G_1 or G_0 population of HDF, and the rate of mitotic accumulation in the presence of Colcemid was determined for the mono- and binucleate cells. The kinetics of mitotic accumulation in different types of binucleate cells were compared (Fig. 3). The mono- and binucleate G_2 cells were the first to enter mitosis and reach a MI of 50% by 5.5 h after fusion. However, the G_1/G_2 heterodiakaryons were delayed significantly in their entry into mitosis and their MI remained <2% during the course of this experiment.

In contrast, the rate of entry into mitosis of G_0/G_2 heterodikaryons was intermediate between those of G_2/G_2 and G_1/G_2 binucleate cells. In this case, the G_2 component entered normal mitosis whereas the G_0 nuclei underwent premature chromosome condensation. The G_0/G_2 binucleate cells had a MI of 46% at 8 h as compared with the 70% MI of the G_2/G_2 binucleate cells. This indicates that G_0/G_2 binucleate cells are some what slower than the G_2/G_2 binucleate cells in their rate of entry into mitosis. These findings demonstate a functional difference between the noncycling G_0 and cycling G_1 cells, i.e., the G_1 component inhibits the progression of the G_2 component in a fused cell from entering into mitosis, whereas the G_0 component lacks this ability. However, when HeLa cells arrested in G_1 phase (by treating mitotic cells with cycloheximide) were fused with G_2 cells, ~20% of G_1/G_2 binucleate cells entered mitosis within 8 h as compared with 2% or 3% in the control (Fig. 4).

Because UV irradiation of mammalian cells is known to induce decondensation of chromatin (6, 17, 19), we wanted to investigate the effects of UV irradiation of G_0 cells and their subsequent fusion with HeLa G_2 cells on the rate of entry of G_0/G_2 binucleate cells into mitosis. In these experiments, we have observed that the exposure of G_0 cells to UV light before fusion retarded the progression of the G_2 component into mitosis (Fig. 4). The higher the dose of UV irradiation, the slower is the rate of entry of G_0/G_2 binucleate cells into mitosis.

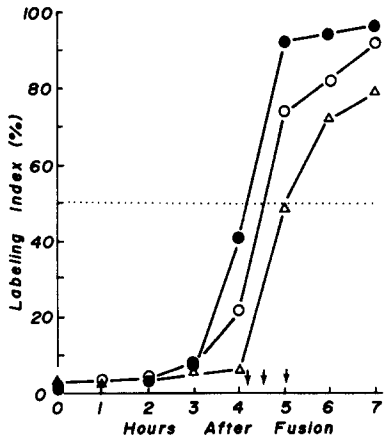

FIGURE 2 Effect of S:G_0 ratio in the trinucleate cells on the kinetics of labeling of G_0 nuclei. HDF in G_0 were fused with prelabeled HeLa cells in S phase. The LI of G_0 nuclei residing in trinucleate cells consisting of 1 S:2 G_0 or 2 S:1 G_0 were compared with those in the binucleate (1 S:1 G_0) cells. (○), G_0 nuclei in binucleate (1 S:1 G_0) cells; (●), G_0 nuclei in trinucleate (2 S:1 G_0) cells; (△), G_0 nuclei in trinucleate (1 S:2 G_0) cells. The arrows indicate the time required for each class of cells to achieve a 50% LI. The dotted line indicates a 50% level.

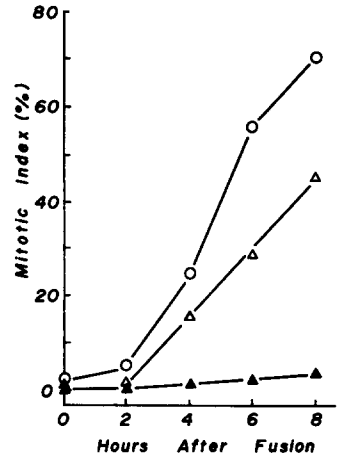

FIGURE 3 The rate of mitotic accumulation in G_0/G_2 and G_1/G_2 fusions. HeLa cells in G_1 and HDF in G_1 and G_0 phases were separately fused with prelabeled HeLa cells synchronized in G_2. The MI were scored for the mono- and binucleate populations and plotted as a function of time. Data involving HDF- G_1/HeLa G_2 fusion are not presented because they were identical to the data from HeLa G_1/HeLa G_2 fusion. (○), Homophasic binucleate cells, G_2/G_2; (△), heterophasic binucleate cells, G_0/G_2; (▲), heterophasic binucleate cells, G_1/G_2. The MI for mononucleate G_0, G_1 cells, and the homophasic binucleate cells, i.e., G_1/G_1 and G_0/G_0, were <2% and, hence, are not included in the figure.

In this study indicate that DNA synthesis can be induced in G_0 nuclei after fusion between G_0- and S-phase cells, but that G_0 nuclei are much slower than G_1 nuclei in

DISCUSSION

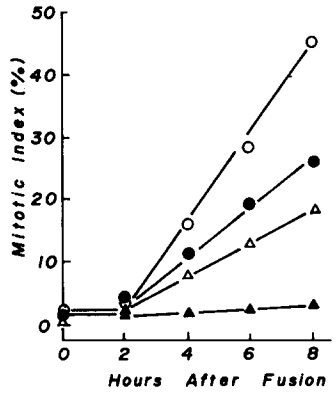

FIGURE 4 Effects of the inhibition of protein synthesis in G_1 and UV irradiation of G_0 cells before fusion on the cell cycle traverse of the G_2 component in G_0/G_2 and G_1/G_2 dikaryons. HeLa cells arrested in G_1 by treating mitotic cells with cycloheximide were fused with prelabeled HeLa G_2. HDF in G_0 were UV irradiated for 60 s and then fused with HeLa G_2. The rate of mitotic accummulation of the binucleate cells in the presence of Colcemid was determined as a function of time. (○), HeLa G_2/HDF − G_0 untreated; (●), HeLa G_2/HDF − G_0 UV irradiated for 60 s; (△), HeLa G_2/HeLa G_1 arrested by cycloheximide treatment; (▲), HeLa G_2/HeLa G_1 untreated.

responding to the inducers of DNA synthesis (Fig. 1). After fusion between a "quiescent" and an S-phase population of 3T3 cells, Brooks (3) observed that the rate of induction of DNA synthesis in the quiescent (G_0) nuclei residing in the heterophasic (G_0/S) binucleate cells was remarkably slow. The G_0 nuclei became labeled only in 10% of the G_0/S binucleate cells at 4 h after fusion. This had risen to 51% at 8 h and to 76% at 12 h (3). Even though Brooks has referred to these quiescent cells as G_1 cells, in light of this study, it would appear that he was actually dealing with G_0 cells. His results could be explained by assuming that the quiescent cells were in a state of deeper G_0 and, hence, would take a longer time to respond to the inducers of DNA synthesis. The slow response of the G_0 nuclei observed by us and by Brooks could be caused by one of the following reasons. (*a*) Because the cycling G_1 cells are progressing towards S phase, they are likely to contain relatively more molecules of the inducers of DNA synthesis than are the noncycling G_0 cells. This difference could result in the early onset of DNA synthesis in G_1 nuclei after fusion with S-phase cells. (*b*) The conformational pattern of chromatin of G_0 cells is different from that of G_1 chromatin. It is evident from the literature that the chromatin of G_0 cells is more condensed than it is in G_1 cells (2, 7). Because the G_0 chromatin is more condensed, it takes ~2–3 h after fusion with S-phase cells to become decondensed and be able to initiate DNA synthesis (Fig. 1). In light of these data, the second possibility appears to be more likely than the first (Fig. 2). If the absence of inducer molecules in G_0 cells is the cause of delayed initiation of DNA synthesis in G_0 nuclei of G_0/S binucleate cells, one would expect a rather rapid initiation by doubling the ratio of S-phase components to the G_0 component. This expectation is based on the model for nonconcentration dependent cooperative initiation of DNA synthesis proposed by Fournier and Pardee (5) and later confirmed by Rao et al. (16). A 50% LI in 1 G_0:2 S trinucleate cells was reached at 4.25 h compared with 4.45 h in the case of 1 G_0:1 S binucleate cells (Fig. 2). Therefore, doubling the number of inducer molecules, as in the case of 2 S:1 G_0 trinucleate cells, did not result in a significant advancement in the rate of entry of the G_0 nucleus into S phase. Reversing this ratio to 2 G_0:1 S caused only a very small delay (<30 min) in the entry of these cells into S phase. From the foregoing discussion, it appears that differences in the conformation of chromatin in G_0 and G_1 cells may be a cause of their differential response to inducers of DNA synthesis.

A more interesting observation resulting from this study is that G_0 cells differ from G_1 cells with regard to their effects on the cell cycle progression of G_2 cells after fusion. The fusion between G_1 and G_2 cells inhibited the G_2 component's entry into mitosis in G_1/G_2 dikaryons (Fig. 3). In contrast, in G_0/G_2 dikaryons, the G_0 component caused only a slight delay in the entry of the G_2 nucleus into mitosis and the consequent premature chromosome condensation of the G_0 nucleus (Fig. 3). This difference between G_0 and G_1 appears to depend on certain factors, perhaps nonhistone proteins, present in G_1 cells but absent in G_0 cells. In earlier studies (16), we have shown that there is a progressive decondensation of chromatin during G_1 that is associated with accumulation of inducers of DNA synthesis. The proteins synthesized during G_1 period may be responsible for the decondensation of chromatin, whereas those synthesized during G_2 may be responsible for chromatin condensation. Hence, it is possible that, in a binucleate cell formed by the fusion of G_1 and G_2 cells, the condensation factors of the G_2 component are neutralized by the decondensation factors of the G_1 component and, thus, the cell cycle progression of G_2 nucleus is delayed until G_1 nucleus completes DNA synthesis. This suggestion is further supported by the fact that G_1 cells, in which protein synthesis was inhibited, were not so effective in blocking the progression of the G_2 component as the control G_1 cells (Fig. 4).

In this study, we have also demonstrated that the influence of the G_0 component on the rate of entry into mitosis of G_0/G_2 dikaryons can be modified by UV irradiation of G_0 cells before fusion (Fig. 4). The fusion of UV-irradiated G_0 HDF with HeLa cells in G_2 resulted in a significant retardation in the rate of entry of G_0/G_2 dikaryons into mitosis. This appeared to be dose dependent (the data for a 30-s exposure to UV are not presented). UV irradiation is known to induce substantial unscheduled DNA synthesis in G_1 and G_2 nuclei, which reflects repair replication of UV-damaged DNA. Waldren and Johnson (19) have shown that G_1 chromosomes of cells irradiated with UV in G_1 phase are elongated and attenuated and appear to be very similar to the prematurely condensed chromosomes of S-phase cells. Further studies by Schor et al. (17) have revealed a close correlation between the degree of chromosome decondensation and the amount of unscheduled DNA synthesis induced by UV irradiation during G_1 and mitosis. UV-irradiated mouse fibroblasts were shown to incorporate more acridine orange in their nuclei than the unirradiated controls (6). The amount of an intercalating dye, such as acridine orange, bound to DNA has been shown to be directly proportional to the degree of chromatin decondensation (12). Therefore, a significant change in the UV-irradiated G_0 cells would be the decondensation of chromatin and the activation of the DNA repair synthesis. In light of these facts, we suggest that the factors induced by UV irradiation that cause chromatin decondensation may counteract the condensation factors present in the G_2 component and, thus, delay the entry of G_0/G_2 dikaryons into mitosis. We have made a similar suggestion earlier to explain the inhibition of progression of the G_2 component into mitosis in G_1/G_2 or S/G_2 binucleate cells (15). However, the exact molecular basis for this phenomenon remains to be elucidated.

In conclusion, this study shows that cells in G_0 phase are functionally distinct from those in G_1 phase with regard to their ability to respond to inducers of DNA synthesis, and to inhibit the progression of G_2 nuclei into mitosis.

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Note Added in Proof: The report by W. E. Mercer and R. A. Schlegel (1980, Exp. Cell Res. 128:431–438), published while our paper was in press, indicates that there is a lag in the initiation of DNA synthesis in quiescent (G_0) nuclei after fusion between quiescent and S-phase 3T3 cells. These results are in complete agreement with those of ours.

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