Mammalian Cell Fusion: Studies on the Regulation of DNA Synthesis and Mitosis

by

POTU N. RAO
ROBERT T. JOHNSON

Eleanor Roosevelt Institute for Cancer Research and Department of Biophysics, University of Colorado Medical Center, Denver, Colorado 80220

DNA synthesis and mitosis are inducible in multinucleate HeLa cells formed by fusion between cells in different phases of the life cycle.

FUSION of two cells in different phases of the mitotic cycle is a means by which the regulatory mechanisms for the initiation of DNA synthesis and mitosis can be examined and related to specific stages of the cell cycle. One can then ask the question: to what extent can a cell's progress in the life cycle be altered by fusion with other cells in the same or different stages ?

Synchronization and Cell Fusion

HeLa cells were grown as suspension cultures at 37° C in Eagle's minimal essential medium supplemented with sodium pyruvate, glutamine and 5 per cent foetal calf serum. The spinner flasks were gassed with a mixture of 5 per cent CO_2 in air. Cells were maintained in exponential growth by diluting the suspension to a concentration of 2×10^5 cells/ml. every day with fresh, pre-warmed medium. At 37° C, these cells have a generation time of 21·8 h with a DNA synthetic (S) period of 7·0 h; a pre-DNA-synthetic ($G1$) period of 10·4 h; a post-DNA-synthetic ($G2$) period of 3·5 h and a mitotic duration of 0·9 h (ref. 1). For the purpose of fusion, cells were synchronized by the excess thymidine double block technique as described earlier[1]. A synchronized population of cells in S period was obtained by collecting cells 1 h after the reversal of the second thymidine block, whereas the $G2$ cells were collected 6 h after the reversal of a similar block. An early $G1$ population was obtained by collecting the cells 2 h after the release of an N_2O block following the reversal of a single excess thymidine block[2].

We carried out three types of experiments involving fusion of $G1$ with S cells, $G1$ with $G2$ cells, and S with $G2$ cells (Table 1). One member of each cell pair was labelled with ^{3}H-thymidine (0·05 μCi/ml.; 6·7 Ci/mmole) previous to fusion, to permit its identification in the resulting hybrid. About 5×10^6 cells of each of the two synchronized populations were placed in a total volume of 1 ml. of Hanks basal salt solution, without glucose, containing 1,000 haemagglutinating units of ultraviolet inactivated Sendai virus. The virus–cell mixture was kept at 4° C for 15 min and at 37° C for 20 min according to the procedures described by Harris *et al.*[3]. After the completion of cell fusion, the suspension was diluted 1 : 50 with fresh medium, dispensed into forty-eight sterile plastic culture dishes (30 mm in diameter) and incubated at 37° C. For each experiment, the dishes were divided into three sets of sixteen. To one set ^{3}H-thymidine (0·2 μCi/ml.; 6·7 Ci/mmole) and colcemide ($6·7 \times 10^{-7}$ M) were added to study the pattern of DNA synthesis in the fused cells while mitosis was blocked. To the second set of dishes colcemide alone was added to study the rate of mitotic accumulation.

Table 1. PROTOCOL FOR OBTAINING SYNCHRONIZED POPULATIONS OF HeLa CELLS FOR THE THREE TYPES OF FUSION EXPERIMENTS

Time (h)	Spinner-A for prelabelled S and $G2$ populations	Spinner-B for $G2$ population	Spinner-C for $G1$ population	Spinner-D for $G1$ population
0	Start spinner-A; add ^{3}H-thymidine (0·05 μCi/ml.)	—	—	—
24	Place excess thymidine block	Start spinner-B; place excess thymidine block	—	—
30	—	—	Start spinner-C; place excess thymidine block	—
41	Release first thymidine block; add ^{3}H-thymidine (0·1 μCi/ml.)	Release first thymidine block	—	Start spinner-D; place excess thymidine
50·5	Place second thymidine block	Place second thymidine block	—	—
51	—	—	Release thymidine block; place cells in plastic culture dishes; incubate at 37° C	—
55	—	—	Transfer these dishes into N_2O chamber	—
62	—	—	—	Release thymidine block; place cells in plastic culture dishes; incubate at 37° C
64	—	—	Release N_2O block; place cells in a spinner	—
65	Divide the cell suspension into three spinners-A1, A2 and A3; release second thymidine block in A1	Release second thymidine block	—	—
66	—	—	Fuse cells from spinner-A1 with those from spinner-C to give $G1/S^*$ fusion (Expt. II)	Transfer these dishes into N_2O chamber
70	Release second thymidine block in spinners A2 and A3	—	—	—
71	—	Fuse cells from spinner-A2 with those from spinner-B to give a $S^*/G2$ fusion (Expt. I)	—	—
75	—	—	—	Release N_2O block; place cells in a spinner.
77	—	—	—	Fuse cells from spinner-A3 with those from spinner-D to give $G1/G2^*$ fusion (Expt. III)

* Prelabelled population.

Nothing was added to the third set, which served as the control and permitted study of mitotic synchrony among the nuclei in the multinucleate cells.

At regular intervals the dishes were trypsinized. From each sample $0{\cdot}75 \times 10^5$ to 1×10^5 cells were taken in a volume of about 0·5 ml. and centrifuged at 600 r.p.m. for 10 min directly on a microscope slide by means of a cytocentrifuge (Shandon–Elliot, England). The cells were fixed for 5 min in absolute ethanol : glacial acetic acid (3:1) and were given three 10 min extractions with cold 5 per cent trichloroacetic acid. The slides were then processed for autoradiography according to the procedures previously described[4].

Heterophasic and Homophasic Multinucleate Cells

We use the term heterophasic multinucleate cell to describe the result of fusion between similar cells in different phases of the life cycle (Fig. 1*A*, *B*, for example). Homophasic multinucleate cells would be formed by the fusion of cells in the same phase of the cell cycle (Fig. 1*C*). The following effects of fusion were studied. (*a*) Initiation of DNA synthesis in *G*1 and *G*2 nuclei after fusion with cells in *G*1, *S* or *G*2. (*b*) Suppression of DNA synthesis in *S* and *G*1 nuclei after fusion with cells in *G*1, *S* or *G*2. (*c*) Acceleration in the time of entry into mitosis of *G*1 and *S* nuclei by the *G*2 and, conversely, the delay of entry into mitosis of *G*2 after fusion with cells in *G*1, *S* or *G*2.

In every case, a lightly prelabelled phased cell population was fused with an unlabelled one. Then a large dose (0·2 μCi/ml.) of ^{3}H-thymidine and colcemide was added immediately to one set of dishes and samples were taken at various periods of incubation to study the induction of DNA synthesis in the fused cells. The density of labelling after fusion was sufficiently greater than that of the prelabelled cells before fusion to leave little or no ambiguity about whether a given labelling had occurred before or after fusion.

The following procedure was used in scoring the effect of fusion on DNA synthesis. Before fusion the cells of each population were mononucleate and either labelled or unlabelled (L or U). The frequency of multinucleate cells in the HeLa cell suspension before fusion ranged from 3–5 per cent. After fusion about 30 per cent of the mixed population consisted of multinucleate cells of various classes (Table 2). As expected, among the fused cells binucleates were most numerous followed by tri, tetra and higher multinucleate cells in that order of decreasing frequency. For example, the binucleate cells can be either

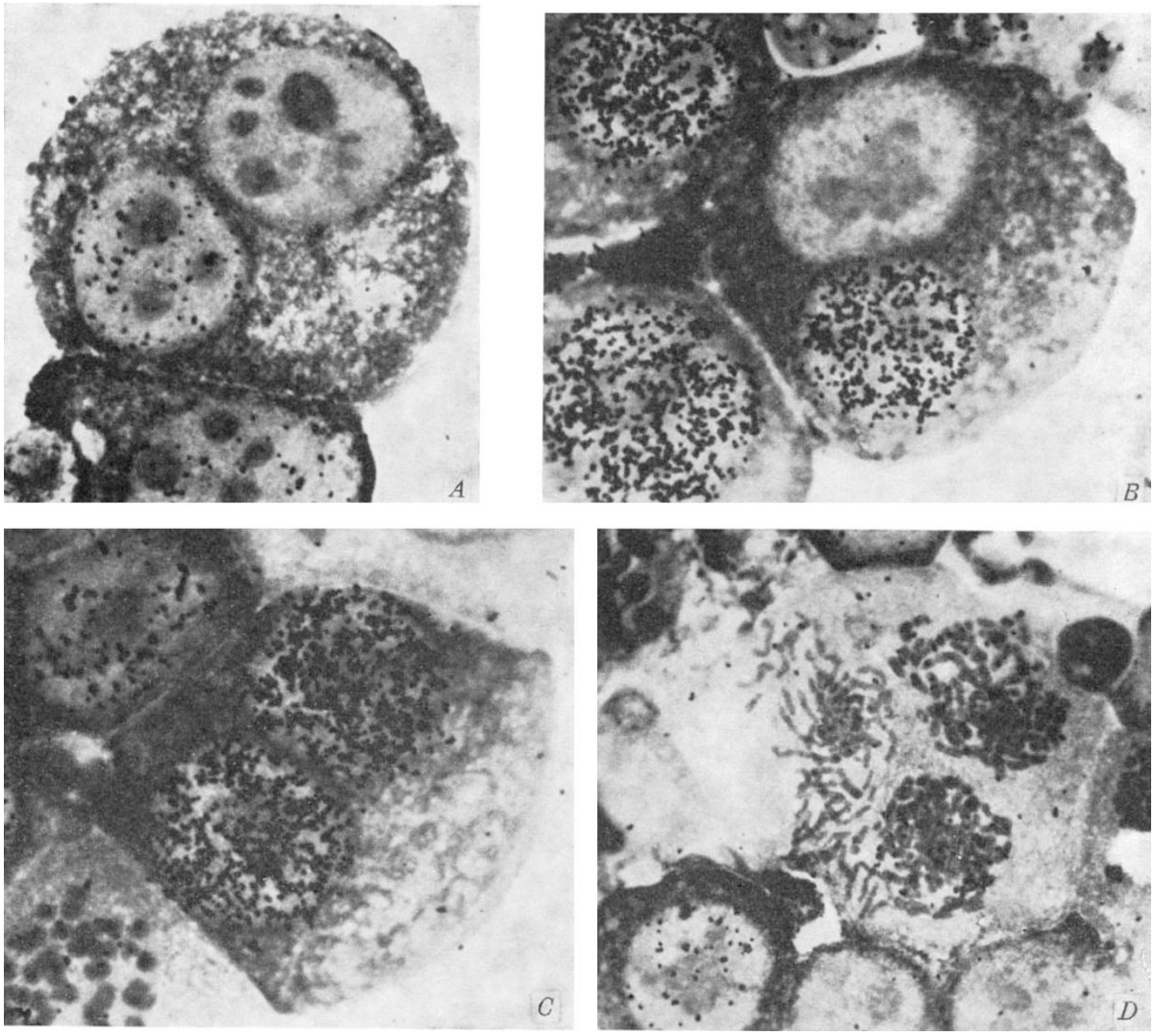

Fig. 1. *A*, Heterophasic *S*/*G*2 binucleate cell at $t = 0$ after fusion. The *S* nucleus was prelabelled with ^{3}H-thymidine. *B*, Heterophasic *S*/*G*2 binucleate cell at $t = 6$ h after fusion and incubation with ^{3}H-thymidine. The increased intensity of labelling of the *S* nucleus as compared with that in *A* arises from continued DNA synthesis after fusion. There was no uptake of ^{3}H-thymidine by the *G*2 nucleus. *C*, Homophasic *S*/*S* binucleate cell at $t = 6$ h after fusion and incubation with ^{3}H-thymidine. The intensity of labelling in each of the nuclei is comparable with that in the *S* nucleus in *B*. *D*, Heterophasic *G*1/2*G*2 trinucleate cell in synchronous mitosis (no colcemide treatment was given). *G*2 nuclei were prelabelled. Note a slightly less condensed state of the chromosomes of the unlabelled (*G*1) nucleus.

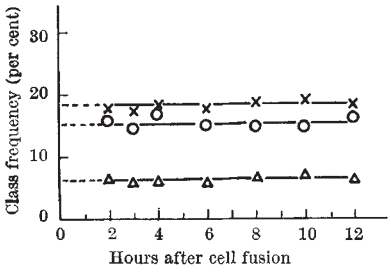

Fig. 2. The frequency of homo and heterophasic binucleate cells in the $S/G2$ fusion as a function of time after continuous labelling with ^{3}H-thymidine. The S nucleus was prelabelled. The mononucleate $G2$ class (○) is presented for comparison. ×, $S/G2$; △, $G2/G2$.

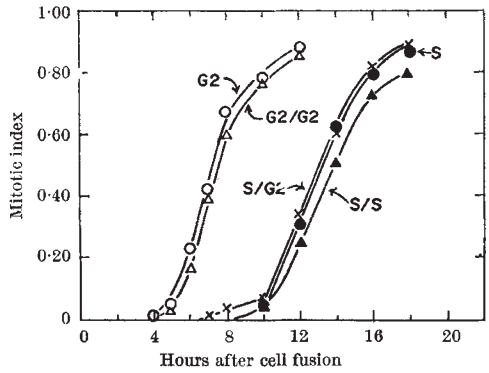

Fig. 3. Mitotic accumulation functions for the mono and binucleate S and $G2$ classes are compared with the heterophasic $S/G2$ binucleate class. Note that the pattern of mitotic accumulation in $S/G2$ is similar to that of the S parent.

U/U, L/L or L/U if two unlabelled, two labelled or one labelled and one unlabelled cells, respectively, were fused together. When the fused cells are incubated with ^{3}H-thymidine, if the unlabelled nuclei do not incorporate ^{3}H-thymidine the frequencies of the classes U/U, L/L and L/U remain constant. Otherwise their frequencies would undergo a change. The class frequencies plotted in Figs. 2, 4*A* and 6*A* were obtained in the following manner.

$$\text{Class frequency} = \frac{N_c \times 100}{N_{sp}}$$

where N_c is the number of cells in a given class at a given time and N_{sp} is the total number of cells in that sub-population at that time (Table 2).

Table 2. VARIOUS CLASSES OF CELLS THAT ARE FORMED BY A RANDOM CELL FUSION BETWEEN LABELLED AND UNLABELLED POPULATIONS

Sub-population	Fusion classes: Homophasic	Heterophasic
1. Mononucleate cells (parental types only)	L; U	
2. Binucleate cells	LL; UU	LU
3. Trinucleate cells	3L; 3U	2L/U; L/2U
4. Tetranucleate cells	4L; 4U	3L/U; 2L/2U; L/3U
5. Pentanucleate*		

L = Prelabelled nucleus. U = Unlabelled nucleus.
* The cells with five or more nuclei were too few to be included in this study.

A change in the frequency of any class was brought about by the initiation of DNA synthesis as monitored by the incorporation of ^{3}H-thymidine in one or more nuclei of a multinucleate cell. For example, the frequency of the class L/U would decrease if the unlabelled nucleus of the heterophasic binucleate cells incorporated ^{3}H-thymidine. The decrease in the frequency of the L/U would result in the proportional increase of the class L/L. In this case there is a possibility that uptake of labelled thymidine in one of the nuclei of a binucleate cell of the homophasic U/U class would result in a cell of the L/U class and this cannot be distinguished from the original L/U class formed by the fusion of a labelled cell with an unlabelled one. By studying the changes in the frequency of each class, however, one could detect DNA synthesis in one of the nuclei of the U/U class. Our findings indicate that there is a high degree of synchrony in DNA synthesis among the nuclei of the homophasic binucleate cells. Either both or none of the nuclei would synthesize DNA in a cell of the U/U class. The most important feature of this type of analysis is the built-in controls in the system. The kinetics of the heterophasic classes can readily be compared with the unfused mononucleate parents and fused homophasic classes.

From the class frequency curves the relative rate of induction of DNA synthesis was calculated and expressed as the percentage of increase in labelling as shown here.

$$\text{Increase in labelling} = \frac{Nt_0 - Nt_n}{Nt_0} \times 100$$

where Nt_0 is the number of cells in a given class at $t = 0$ h (soon after cell fusion) and Nt_n is the number of cells in that class at $t = n$ h after fusion. For the determination of mitotic index each class was treated as a separate entity.

S/G2 Fusion (Experiment I)

A prelabelled S population was fused with an unlabelled $G2$ population and the patterns of DNA synthesis and mitotic accumulation were studied in the fused and unfused cells. Because the $G2$ population had completed DNA synthesis before cell fusion, no change in the frequency of $G2$ or $G2/G2$ classes was expected nor observed (Fig. 2). The absence of any decrease in the frequency of the class $S/G2$ was an indication that there was no induction of DNA synthesis in the $G2$ nucleus. On the other hand, the $G2$ component of this heterophasic cell did not inhibit the DNA synthesis in the S nucleus as shown by the increased intensity of labelling (compare Fig. 1*A* with *B* and *C*).

The mitotic accumulation function[1,5] was studied in the set of dishes to which colcemide alone was added. The mononucleate $G2$ population was the first to enter mitosis, closely followed by the homophasic binucleate ($G2/G2$) cells (Fig. 3). Similarly, the S and S/S classes arrived at mitosis about 6 h later in that sequence. The heterophasic

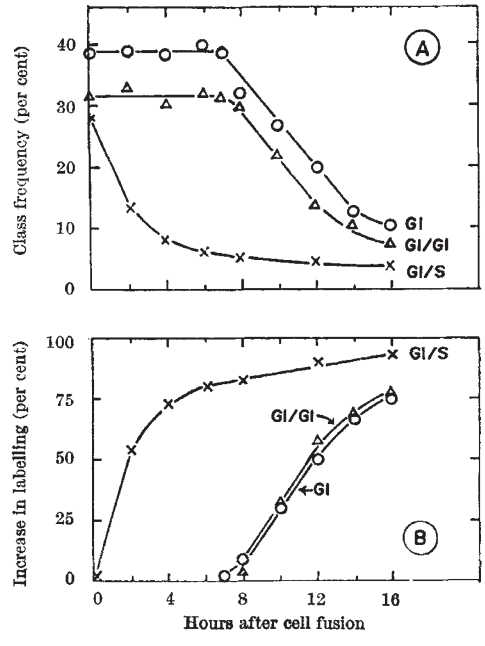

Fig. 4. Induction of DNA synthesis in the $G1$ nucleus of the $G1/S$ fusion. *A*, Class frequencies of $G1$, $G1/G1$ and $G1/S$ as a function of time after fusion and continuous incubation with ^{3}H-thymidine. The S nuclei were prelabelled. *B*, Rate of induction of DNA synthesis in the $G1$ nucleus of the $G1/S$ fused cells.

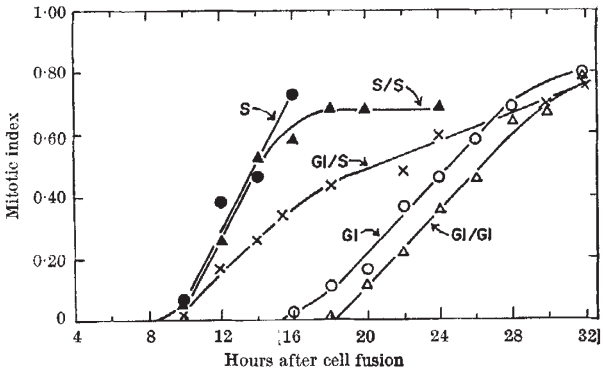

Fig. 5. Mitotic accumulations from the *G1/S* fusion, showing the time interval between the parental *S* and *G1* classes and the intermediate nature of the heterophasic *G1/S* binucleate cells.

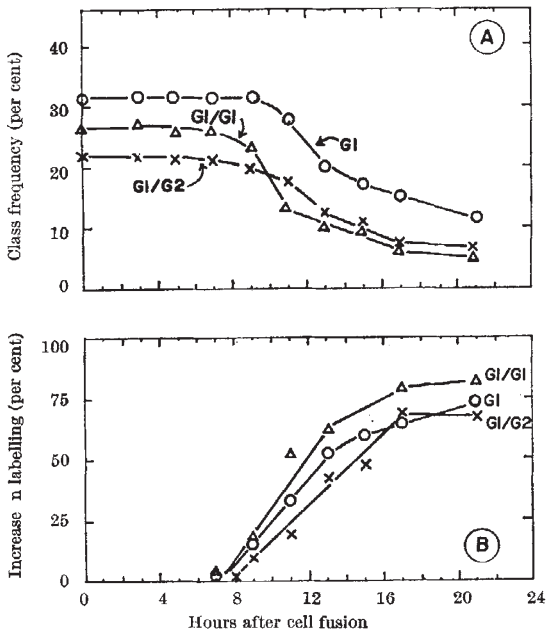

Fig. 6. *A*, The frequencies of homo and heterophasic binucleate cells in the *G1/G2* fusion as a function of time after continuous labelling with ^{3}H-thymidine. The *G2* nuclei were prelabelled. The mononucleate *G1* class is shown for comparison. *B*, Rate of induction of DNA synthesis in the heterophasic *G1/G2* binucleate cells is compared with those of *G1* parent and *G1/G1* homophasic binucleate cells.

cells (*S/G2*) arrived at mitosis about the same time as mononucleate *S* cells. The *S/G2* cells, however, were definitely ahead of the *S/S* class in their entry into mitosis, indicating a slight pull of *S* nucleus by the *G2* component towards mitosis.

G1/S Fusion (Experiment II)

In this cross the *S* population was prelabelled. Results on the induction of DNA synthesis in the *G1* nucleus of the *G1/S* fused cell are presented in Fig. 4. The frequency of *G1* and *G1/G1* classes did not change until about 8 h after fusion, when they started entering into the *S* period as indicated by the decrease in the frequency. By contrast there was a sharp decrease in the frequency of the *G1/S* class beginning at $t = 0$ h, indicating the induction of DNA synthesis in the *G1* nucleus (Fig. 4*A*). DNA synthesis was induced in more than 50 per cent of the *G1* nuclei of the heterophasic *G1/S* cells within 2 h after cell fusion (Fig. 4*B*). The 50 per cent labelling point was reached in *G1* and *G1/G1* classes about 12 h after fusion. Clearly there was a rapid induction of DNA synthesis in the nucleus of a *G1* cell soon after fusion with a cell that was synthesizing DNA. This was further confirmed by the mitotic accumulation curve for the *G1/S* class of cells which lies between those for the *S/S* and *G1/G1* classes (Fig. 5). The earlier induction of DNA synthesis in the *G1* nucleus of the *G1/S* cells resulted in an earlier initiation of mitosis in the heterophasic cells than in the homophasic *G1/G1* cells.

G1/G2 Fusion (Experiment III)

A prelabelled *G2* population was fused with a *G1* population. The frequencies of the various classes (Fig. 6*A*) studied in order to measure the rate of initiation of DNA synthesis (Fig. 6*B*) indicated that there was no significant difference between the heterophasic *G1/G2* and homophasic *G1/G1* in the pattern of labelling. In other words, the *G2* component of the heterophasic binucleate (*G1/G2*) cell had no effect on the normal course of DNA synthesis in the *G1* nucleus. The mitotic accumulation function for *G1/G2* class was quite similar to that of *G1/G1* (Fig. 7). About 20–35 per cent of the *G1*, *G1/G1* and *G1/G2* cells entered mitosis much earlier than expected because of contamination of the *G1* population of this experiment with some *G2* cells as a result of technical difficulties (compare curves *G1* and *G1/G1* of Fig. 7 with those in Fig. 5). This asynchrony in the *G1* population was responsible for the initial mitotic accumulation as shown in Fig. 7. This did not, however, prevent us from observing that in general the *G2* component did not affect the *G1* nucleus in its progression through the mitotic cycle. It was the *G2* nucleus of *G1/G2* cell that was delayed in entering into mitosis by its *G1* component.

Dosage Effect

The metabolic activity of a multinucleate cell formed by fusion between cells in different phases of the cell cycle was

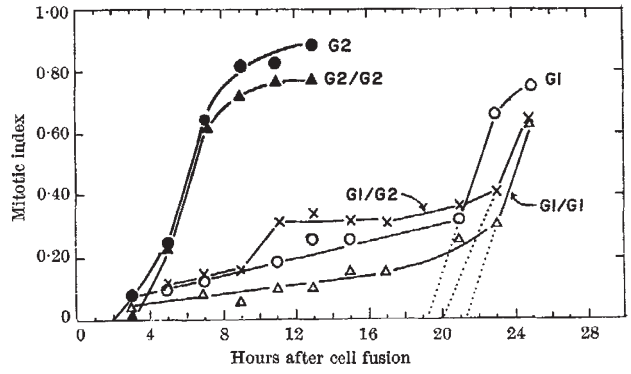

Fig. 7. Mitotic accumulations for the *G1/G2* fusion, showing the interval between the parental *G1* and *G2* types. The mitotic accumulation function for the heterophasic *G1/G2* is similar to the *G1* parent.

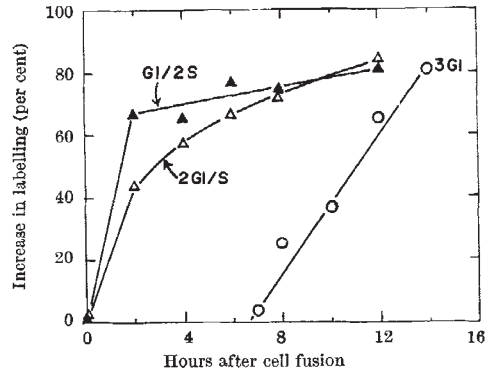

Fig. 8. Dosage effect on the induction of DNA synthesis in the *G1* nuclei of the heterophasic trinucleate cells of the *G1/S* fusion. The *S* nuclei were prelabelled.

greatly influenced by the ratio of cells in advanced to early states. The dose response of the induction of DNA synthesis and the initiation of mitosis was studied in tri and tetranucleate heterophasic cells. In the *G1/S* fusion the rate of induction of DNA synthesis in *G1* nuclei was faster in the *G1/2S* class than in *2G1/S* (Fig. 8). The dosage effect of the *S* component on the *G1* is illustrated by the time taken by a given class of cells to reach a labelling index of 50 per cent. This time was 1·5 h for *G1/2S*, 1·75 h for *G1/S* and 3·0 h for the *2G1/S* class. About 75 per cent of the *G1* nuclei in the heterophasic cells were synthesizing DNA by the time the homophasic (*3G1*) cells started entering *S* period.

In the *S/G2* and *G1/G2* fusions, no induction of DNA synthesis in the *G2* nucleus was observed even when the ratio of *S* or *G1* to *G2* was high. Similarly, an increase in the proportion of *G2* to *S* or *G1* component did not result in the inhibition of DNA synthesis in the *S* nucleus nor in the progression of the *G1* nucleus into the *S* period.

The initiation of mitosis in the multinucleate HeLa cells was also dose dependent as observed in *S/G2* and *G1/G2* fusions. The data on the mitotic accumulation of the two mononucleate parents and the homophasic multinucleate cells of the *S/G2* fusion are presented in Fig. 9*A*; for the corresponding heterophasic multinucleate cells see Fig. 9*B*. The greater the ratio of *G2* : *S* nuclei in a fused cell, the earlier was the initiation of mitosis in all the nuclei of that cell. For example, a mitotic index of 0·50 was reached by the *4S* class at 14·2 h after cell fusion, whereas it was 13·3 h for *G2/S*, 11·6 h for *2G2/S*, 10·7 h for *3G2/S* and 8·5 h for the *4G2* class. Even though the pulling effect—with respect to mitosis—of the *G2* over the *G1* component in cells with a 1 : 1 ratio was negligible, an increase in the ratio of *G2* : *G1* did bring about earlier onset of mitosis in heterophasic trinucleate cells (Fig. 10). The *2G2/G1* class of cells entered mitosis quite early, however, partly because of the contamination of the *G1* population with *G2* cells.

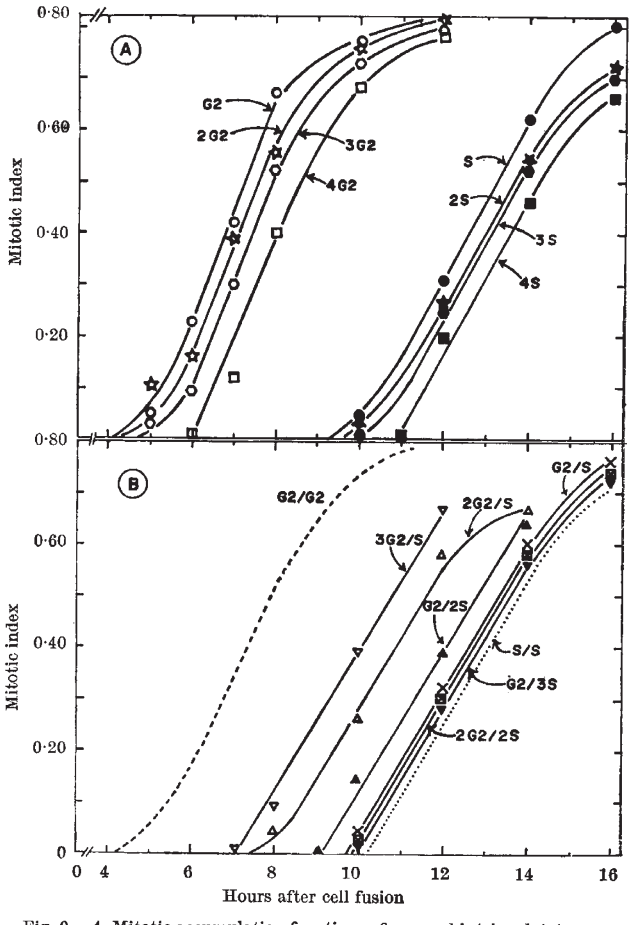

Fig. 9. *A*, Mitotic accumulation functions of mono, bi, tri and tetranucleate homophasic cells of the *S/G2* fusion. The *S* nuclei were prelabelled. The greater the number of nuclei in a cell the slower was the onset of mitosis. *B*, Dosage effect of the *G2* or *S* component on the rate of mitotic accumulation in the heterophasic multinucleate cells of the *S/G2* fusion. The homophasic binucleate *G2/G2* and *S/S* are represented by dashed and dotted lines respectively.

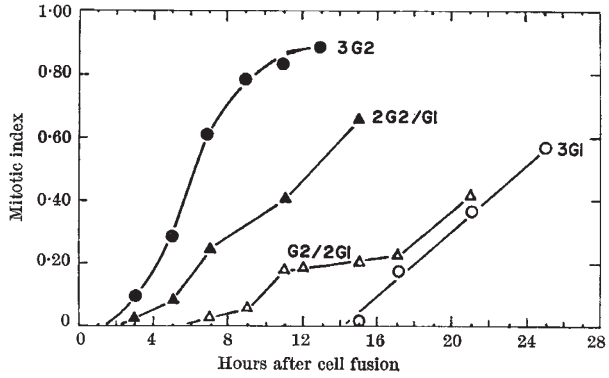

Fig. 10. Dosage effect of the *G2* component on the rate of mitotic accumulation of the heterophasic trinucleate cells of the *G2/G1* fusion. The *G2* nuclei were prelabelled.

Mitotic Synchrony

A very high degree of mitotic synchrony was observed among the nuclei in fused cells in the control set of dishes to which no colcemide or ^{3}H-thymidine was added. In the *G1/S* and *G1/G2* fusions the mitotic synchrony was almost perfect (Fig. 1*D*). Asynchronous mitoses were observed in only five out of 1,000 fused cells in mitosis. In these two fusions there was no significant difference between homophasic and heterophasic cells with regard to mitotic synchrony, but in the *S/G2* fusion some degree of mitotic asynchrony (about 11 per cent) was observed in heterophasic cells but not in the homophasic cells. Further analysis of this asynchrony revealed that in the fused cells the *G2* nuclei were always in a more advanced phase of mitosis than the *S* nuclei.

The phenomenon of anomalous chromosome condensation, or the "pulverization" of chromosomes as it was first termed by Sandberg *et al.*[6], was predominant in the *S/G2* fusion but rarely seen in *G1/S* or *G1/G2* fusions.

Induction of DNA Synthesis

There was obviously a rapid induction of DNA synthesis in the *G1* nuclei of the *G1/S* fused cells (Fig. 4*B*). The rate of induction was dependent on the ratio of *S* to *G1* nuclei (Fig. 8). The greater the number of *S* components in the fused cell the faster was the induction of DNA synthesis in the *G1* nuclei. The *G1* component of the heterophasic *G1/S* cell did not inhibit DNA synthesis that was in progress in the *S* nucleus. These findings are generally in accord with those in other systems[3,7–12]. The rapid induction of DNA synthesis in the *G1* nuclei in a dose-dependent manner suggests that certain substances which are present in the *S* component probably migrate into *G1* nucleus and cause the initiation of DNA synthesis. There is some evidence of such factors in the studies of Thompson and McCarthy[13].

In the virus-fused HeLa cells we have found that the *G2* nucleus and cytoplasm had no effect on the normal course of DNA synthesis of an *S* nucleus (Fig. 1*A* and *B*). These heterophasic cells then complete the cycle and reach mitosis. No matter what the ratio of *G2* : *S* nuclei in a fused cell, the *S* nuclei continued DNA synthesis. In these cells there was no re-induction of DNA synthesis in the *G2* nucleus. Studies on *Physarum polycephalum*[14], *Amoeba proteus*[15] and *Stentor coeruleus*[9] resulted in similar findings except for those studies by Prescott and Gold-

stein[16,17]. We show that the presence of a *G2* component did not inhibit a *G1* nucleus from entering the *S* phase (Fig. 6*A* and *B*). Even cells with high ratios of *G2* : *G1* nuclei show normal progression of the *G1* nuclei into the *S* period. The nuclei of these *G1*/*G2* multinucleate cells subsequently entered mitosis with almost perfect synchrony (Fig. 1*D*).

With respect to DNA synthesis, therefore, we show that *G1* nuclei in the presence of an *S* component can rapidly move into S phase, thus eliminating most of the *G1* period in a dose-dependent manner. The inducers for DNA synthesis are present in cells synthesizing DNA and are probably transmitted through the cytoplasm to the *G1* nucleus following *G1*/*S* cell fusion. These inducers cannot initiate a second round of DNA synthesis in *G2* nuclei of the HeLa cells. The fact that DNA synthesis continued in *S* nuclei in the presence of a *G1* or *G2* component suggests the absence of any inhibitors of DNA synthesis during these phases. We conclude that there is a positive control of DNA synthesis in HeLa cells with the appearance of inducer substances at the start of the *S* phase. These substances are not produced in either *G1* or *G2* phase cells, and their absence during these periods—rather than the presence of specific repressor materials—is reflected in the absence of DNA replication during these phases of the cell cycle.

Initiation of Mitosis

Mitotic accumulation functions revealed that a *G2* cell, when fused with a *G1* or *S* cell in a 1 : 1 ratio, was always delayed until the latter was ready to enter into mitosis (Figs. 3 and 7). In the fused cells the *G2* component imparted some advantage with regard to an early onset of mitosis to the *G1* or *S* nucleus. In the sub-population of binucleate cells, the heterophasic cells (*G2*/*S* and *G2*/*G1*) always reached a mid point (0·50) in the mitotic accumulation a little earlier than the homophasic cells of the late parent-*G1*/*G1* or *S*/*S* (Figs. 3 and 7). The mitotic inducing effect of the *G2* cells was even more obvious with an increase in the dosage of the *G2* component in the fused cells (Figs. 9*B* and 10). In these cases the *G2* phase of the *S* or *G1* nucleus was essentially shortened in much the same way as the *G1* phase was shortened by the *S* component in the *G1*/*S* fusion. In the *G1*/*S* fusion, unlike the *S*/*G2* or *G1*/*G2* fusions, the mode of mitotic accumulation for the heterophasic *G1*/*S* cells was intermediate between the two parents because of the rapid induction of DNA synthesis in the *G1* nucleus (Fig. 5). In other words, the pre-DNA synthetic period of the *G1* nucleus was greatly reduced under the influence of the *S* component.

In the heterophasic *S*/*G2* cells the *G2* nuclei were delayed in entering mitosis by the presence of the *S* component and the greater the initial dose of the *S* component the greater was the delay (Fig. 9*B*). This may be interpreted as an equilibration of mitotic inducer substances between all the nuclei of a fused cell, so that the time of entry into mitosis depends on the critical concentration of the inducer substances and hence the ratio of *G2* to *S* components present initially. These conclusions are also applicable to the *G1*/*G2* cells (Fig. 10).

The equilibration of materials in multinucleate cells leading to dosage effects on DNA synthesis and mitosis has been described by many workers using many different systems, for example, *Amoeba*[17]; *Stentor*[18–20]; *Pelomyxa*[21]; *Physarum*[22–25]; HeLa–Ehrlich ascites heterokaryons[26]. In a series of experiments with *Physarum*, Rusch *et al.*[24] found that substances which induce mitosis were synthesized during the interphase, and reached critical levels just before mitosis. Coalescence of early and late plasmodia led to a speeding up of the lagging nuclei and a retardation of the advanced nuclei, so that complete synchrony of nuclear division was always produced. When the sizes of the late and early plasmodia were varied, the speeding up of the lagging nuclei was proportional to the size of the advanced plasmodium used. In addition, mitotic gradients were observed between partially overlapping late and early plasmodia, indicating that a gradient of mitotic inducers was indeed present. Such dosage effects in *Physarum* strongly resemble our findings, and suggest that the mechanisms responsible for the initiation of mitosis in *Physarum* have much in common with those in mammalian cells.

In our fusion experiments, we have found extremely low levels of asynchronous mitoses among the multinucleate cells, in agreement with the findings of others[26–29]. Giménez-Martin *et al.*[30] examined the asynchrony of nuclear entry into mitosis in caffeine induced multinucleate onion root tip cells: as the number of nuclei per cell increased the extent of asynchronous passage into prophase among the nuclei also increased. The subsequent passage into metaphase was, however, always highly synchronous.

The nuclei in most of the naturally occurring and artificially created multinucleate cells and syncytia show a high order of synchrony of DNA synthesis and of entry into mitosis[31–35]. We have shown that even though the cells were widely separated in their metabolic states, when fused together they achieve a rapid coordination and reach mitosis synchronously during the first mitotic cycle itself. The fact that most of the multinucleate systems achieve synchrony rather quickly suggests that there are some factors operating in this direction. The induction of DNA synthesis and the initiation of mitosis are probably the two main forces operating in order to bring about synchrony in multinucleate systems.

We thank Dr Theodore T. Puck for critical reading of the manuscript; Professor Henry Harris, of the University of Oxford, for kindly supplying us with Sendai virus; and George Barella and David Peakman for technical assistance. R. T. J. is a recipient of a Damon Runyon postdoctoral fellowship. The investigation was aided by a US Public Health Service grant from the National Institute of Child Health and Human Development.

Received September 22, 1969.

1 Rao, P. N., and Engelberg, J., in *Cell Synchrony Studies in Biosynthesis Regulation* (edit. by Cameron, I. L., and Padilla, G. M.), 332 (Academic Press, New York and London, 1966).
2 Rao, P. N., *Science*, **160**, 774 (1968).
3 Harris, H., Watkins, J. F., Ford, C. E., and Schoefl, G. I., *J. Cell Sci.*, **1**, 1 (1966).
4 Rao, P. N., and Engelberg, J., *Science*, **148**, 1092 (1965).
5 Puck, T. T., and Steffen, J., *Biophys. J.*, **3**, 379 (1963).
6 Sandberg, A. A., Sofuni, T., Takagi, N., and Moore, G. E., *Proc. US Nat. Acad. Sci.*, **56**, 105 (1966).
7 Graham, C. F., Arms, K., and Gurdon, J. B., *Devel. Biol.*, **14**, 349 (1966).
8 Gurdon, J. B., *Proc. US Nat. Acad. Sci.*, **58**, 545 (1967).
9 De Terra, N., *Proc. US Nat. Acad. Sci.*, **57**, 607 (1967).
10 Graham, C. F., *J. Cell Sci.*, **1**, 363 (1966).
11 Jacobson, C. E., *Exp. Cell Res.*, **53**, 316 (1969).
12 Johnson, R. T., and Harris, H., *J. Cell Sci.* (in the press).
13 Thompson, L. R., and McCarthy, B. J., *Biochem. Biophys. Res. Commun.*, **30**, 166 (1968).
14 Guttes, S., and Guttes, E., *J. Cell Biol.*, **37**, 761 (1968).
15 Ord, M. J., *Nature*, **221**, 964 (1969).
16 Prescott, D. M., and Goldstein, L., *Science*, **155**, 469 (1967).
17 Goldstein, L., and Prescott, D. M., in *The Control of Nuclear Activity* (edit. by Goldstein, L.), 3 (Prentice-Hall, New Jersey, 1967).
18 Weisz, P. B., *J. Exp. Zool.*, **131**, 137 (1956).
19 De Terra, N., *Exp. Cell Res.*, **21**, 41 (1960).
20 Tartar, V., *J. Exp. Zool.*, **163**, 297 (1966).
21 Daniels, E. W., *J. Exp. Zool.*, **117**, 189 (1951).
22 Guttes, E., Guttes, S., and Rusch, H. P., *Fed. Proc.*, **18**, 479 (1959).
23 Guttes, E., and Guttes, S., *Experientia*, **19**, 13 (1963).
24 Rusch, H. P., Sachsenmaier, W., Behrens, K., and Gruter, V., *J. Cell Biol.*, **31**, 204 (1966).
25 Guttes, E., Dvi, V. R., and Guttes, S., *Experientia*, **25**, 615 (1969).
26 Johnson, R. T., and Harris, H., *J. Cell Sci.* (in the press).
27 Johnson, R. T., and Harris, H., *J. Cell Sci.* (in the press).
28 Oftebro, R., and Wolf, I., *Exp. Cell Res.*, **48**, 39 (1967).
29 Oftebro, R., *Scand. J. Clin. Lab. Invest.*, **22**, 79 (1968).
30 Giménez-Martín, G., Lopez-Saéz, J. F., Moreno, P., and González-Fernández, A., *Chromosoma*, **25**, 282 (1968).
31 Mazia, D., in *The Cell* (edit. by Brachet, J., and Mirsky, A. E.), 77 (Academic Press, New York and London, 1961).
32 González, M. A., *Genet. Ibérica*, **19**, 1 (1967).
33 Cone, C. D., *J. Theoret. Biol.*, **22**, 365 (1969).
34 Braun, R., Mittermayer, C., and Rusch, H. P., *Proc. US Nat. Acad. Sci.*, **53**, 924 (1965).
35 Fawcett, D. W., Ito, S., and Slautterback, D., *Biophys. Biochem. Cytol.*, **5**, 453 (1959).