Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2869–2873, July 1977
Cell Biology

Regulation of DNA synthesis: Age-dependent cooperation among G1 cells upon fusion

(cell cycle/multinucleate cells/cell fusion/inducers of DNA synthesis)

POTU N. RAO, PRASAD S. SUNKARA, AND BARBARA A. WILSON

Department of Developmental Therapeutics, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

Communicated by Theodore T. Puck, April 4, 1977

ABSTRACT **The object of this study was to determine whether the inducer(s) of DNA synthesis in mammalian cells accumulates gradually throughout the G1 period or becomes available suddenly at the G1–S transition. HeLa cells, synchronized at various points in the G1 period, were fused by using UV-inactivated Sendai virus. Early G1 cells were fused with mid-G1 or late G1 cells and late G1 cells were fused with mid-G1 cells. The G1 traverse of mono-, bi-, and trinucleated cells was studied. The bi- and trinucleated cells of mid-G1 and late G1 parents traversed the G1 period significantly faster than did their mononucleated counterparts. The reduction in the duration of the G1 period was proportional to the number and age of nuclei at the time of fusion. There was no significant difference between the mono- and binucleated cells of the early G1 parent in their rates of entry into S period. In light of these findings, a model is proposed in which the inducer(s) of DNA synthesis accumulates gradually throughout the G1 period, reaching a critical level at the G1–S boundary when DNA replication is initiated; after reaching a peak during early or mid-S period, it declines to below the critical level when DNA synthesis ceases.**

The presence of factors for the initiation of DNA synthesis in S phase cells and their ability to induce this event in G1 nuclei have been amply demonstrated by cell fusion (1, 2) and nuclear transplantation (3, 4) studies in various experimental systems. However, the nature of these factors and the molecular mechanisms of their action remain to be elucidated. It is commonly understood that, in mammalian cells, the preparations for the replication of DNA are generally made during the G1 period. Inhibition of protein synthesis in G1 blocks the progression of cells into S phase, suggesting that these initiators are probably proteins. However, it is not clear whether these initiators of DNA synthesis accumulate gradually throughout G1 or become available suddenly at the G1–S transition.

The present study was aimed at clarifying this point by fusing HeLa cells, synchronized at various points in G1, and by studying the rates of progression of mono-, bi-, and trinucleated cells into S phase. We have observed that bi- and trinucleated cells entered S phase significantly earlier than the mononucleated cells, depending upon their position in G1 at the time of fusion. These results indicate that the initiators of DNA synthesis accumulate during G1 and can induce DNA replication only after reaching a critical level which is not concentration dependent.

MATERIALS AND METHODS

Cells and Cell Synchrony. HeLa cells were grown as suspension cultures at 37° in Eagle's minimal essential medium supplemented with nonessential amino acids, sodium pyruvate, glutamine, and 10% (vol/vol) heat-inactivated fetal calf serum. The spinner flasks were gassed with a mixture of 5% CO_2/95% air. Cells were maintained in exponential growth by diluting the cell suspension to a concentration of 2×10^5 cells per ml every day with fresh prewarmed medium. This cell line has a generation time of about 22 hr, with a pre-DNA-synthetic period (G1) of 10.4 hr, a DNA synthetic period (S) of 7.0 hr, a post-DNA-synthetic period (G2) of 3.5 hr, and a mitotic period of 0.9 hr (5).

For the purpose of this study, a spinner culture in exponential growth was partially synchronized in S phase by the application of an excess thymidine (dThd) (2.5 mM) block for 20 hr. Immediately after the reversal of the dThd block, the cells were plated in 100-mm Falcon plastic dishes at the rate of 4×10^6 cells per dish, incubated for 4 hr in a humidified CO_2 incubator, and then transferred to a pressure chamber containing N_2O at 80 lb/in^2 (5.5×10^5 N/m^2) with an inside temperature of 37° (6). At the end of the N_2O treatment (of not less than 9 hr), the rounded and floating mitotic cells were harvested by gentle pipetting. The mitotic index of these samples was >98%.

Cells synchronized in early, middle, and late G1 phases were obtained by harvesting the cells at 3, 5, and 7 hr, respectively, after the reversal of the N_2O block. A pulse labeling of these G1 cells with [^{3}H]dThd (1.0 μCi/ml; 6.7 Ci/mmol) for 20 min did not show any incorporation of label. Prelabeled populations of early, middle, and late G1 cells were obtained by incubating a culture in exponential growth with [^{3}H]dThd (0.1 μCi/ml; 6.7 Ci/mmol) for about 24 hr before they were subjected to synchronization procedures.

Cell Fusion. The procedures for Sendai virus-induced cell fusion have been described (1). About 3×10^6 cells of each of the two synchronized populations, one prelabeled and the other unlabeled, were placed in a total volume of 1 ml of Hanks' basal salt solution, without glucose, containing about 1000 hemagglutinating units of UV-inactivated Sendai virus. The virus/cell mixture was kept at 4° for 15 min and at 37° for 45 min. After the completion of cell fusion, the fusion mixture was diluted with fresh culture medium and plated in a number of 35-mm Falcon plastic dishes. After the first sample was taken, immediately after fusion (0 time), [^{3}H]dThd (1.0 μCi/ml; 6.7 Ci/mmol) was added to all the dishes and incubation was continued. Samples were taken at regular intervals by trypsinizing one of the dishes for each fusion and depositing the cells directly on a clean slide by the use of a cytocentrifuge. The cells were fixed in absolute methanol/glacial acetic acid, 3:1 (vol/vol), extracted with cold 5% (wt/vol) trichloroacetic acid three times, and then processed for radioautography. The slides were then stained with Giemsa and scored for the frequency of labeled and unlabeled mono-, bi-, and trinucleated cells. About 500 cells of each class were scored for each data point.

For this study, six different fusions were made as follows

Abbreviations: dThd, thymidine; L, labeled cells; U, unlabeled cells; NSA-binucleates, binucleated cells containing nuclei of the same age; NDA-binucleates, binucleated cells containing nuclei of different ages.

Table 1. Calculation of rate of induction of DNA synthesis in mono- and binucleated cells of unlabeled parent in fusion A (EG1/LG1*)[a]

Time after fusion, hr	Mononucleate class U		Binucleate classes			
			L/U		U/U	
	Observed frequency	Calculated increase in LI	Observed frequency	Calculated increase in LI	Observed frequency	Calculated increase in LI
0	70.6	0	31.6	0	44.8	0
2	71.0	0	30.8	0.2	43.0	0.4
4	69.5	0.1	32.0	0	42.0	0.6
6	67.0	0.5	23.7	25.0	43.7	0.2
8	65.7	0.7	18.2	42.5	42.1	0.6
10	52.9	25.1	11.2	64.7	33.2	26.0
12	42.5	39.8	7.8	75.2	25.4	43.3
14	27.3	61.3	5.3	83.2	14.1	68.5
16	18.7	73.5	3.2	90.0	11.7	73.9

[a] LI = labeling index.

(asterisk indicates prelabeled population): (A) early G1/late G1*; (B) early G1*/late G1; (C) early G1/mid-G1*; (D) early G1*/mid-G1; (E) mid-G1/late G1*; and (F) mid-G1*/late G1. For study of the cell cycle progression of both parents and comparison of them with that of the hybrids, it was essential to prelabel one parent at a time. Hence, fusions A, C, and E are the reciprocals of fusions B, D, and F, respectively, with regard to prelabeling.

Collection and Presentation of Data. The procedures for the collection and analysis of the data have been described in detail (1). Before fusion, the cells of each population were mononucleated and either labeled or unlabeled (L or U). In the HeLa cell line used in this study, the frequency of multinucleated cells before fusion was less than 5%. After fusion, 30% of the cells in the fusion mixture were multinucleated, with binucleates more numerous than trinucleates or tetranucleates. Among the binucleated cells, there were three classes: L/L, L/U, and U/U. The L/L and U/U binucleated cells were formed by the fusion between cells of the same parental type (labeled or unlabeled), and in this study we designated them as binucleates with *nuclei* of the *same age* (NSA-binucleates). In contrast, the L/U class of binucleates were the products of fusion between early and late G1 cells and were designated as binucleates with *nuclei* of *different ages* (NDA-binucleates). The trinucleated cells comprised four groups: LLL, LLU, LUU, and UUU. Cells with four or more nuclei were not taken into consideration because they were too few to score.

Because the cells were incubated with [^{3}H]dThd after fusion, the frequency of the unlabeled classes decreased with time as they entered into S phase. For example, the frequency of class L/U would decrease if the unlabeled nucleus of the NDA-binucleated cells incorporated [^{3}H]dThd. The decrease in the frequency of L/U would result in a corresponding increase in the frequency of class L/L. Our present studies, and previous studies (1, 2, 7), indicate that there is a high degree of synchrony in DNA synthesis among the nuclei of binucleated cells. Hence, the decrease in the frequency of class U/U would result in a proportional increase of class L/L but not of class L/U.

On the basis of the initial class frequencies observed, the relative rate of induction of DNA synthesis was calculated as a percentage of the increase in the labeling index by the following formula (1):

$$\% \text{ increase in labeling index} = \frac{Nt_o - Nt_n}{Nt_o} \times 100$$

in which Nt_o is the number of cells in a given class at 0 time (immediately after fusion) and Nt_n is the number of cells in that class at n hr after fusion. The calculation of the rate of increase in labeling index from the observed frequency of mono- and binucleates in fusion A is shown in Table 1. Similar tables were prepared for other fusions but were not presented here. In each fusion, the rates of G1 traverse of the unlabeled G1 nuclei residing in mononucleates, NSA-binucleates, and NDA-binucleates were measured.

RESULTS

Early G1/Late G1 Fusions. In fusion A, involving prelabeled late G1 and unlabeled early G1 cells, the kinetics of the initia-

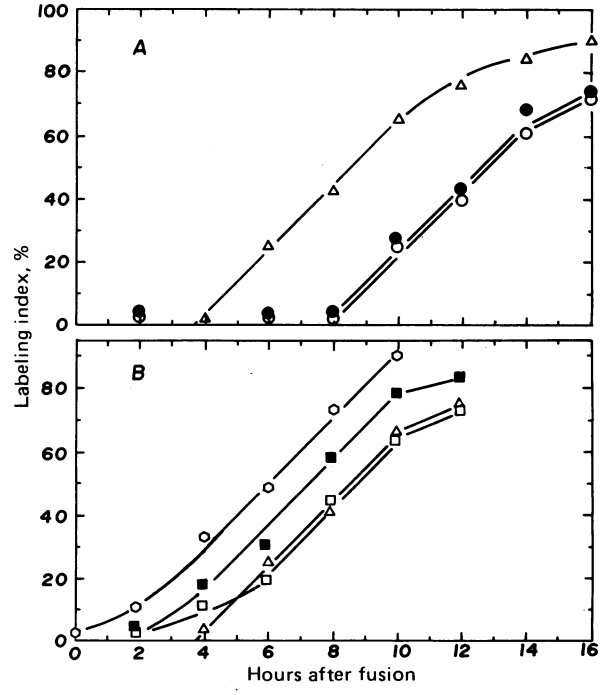

FIG. 1. Kinetics of initiation of DNA synthesis in fusions between early G1 (EG1) and late G1 (LG1) cells. (*A*) Fusion A (EG1/LG1*). The G1 traverse is shown as increase in labeling index, as a function of time, of the early G1 nuclei. ○, In mononucleates (EG1); ●, in NSA-binucleates (EG1/EG1); △, in NDA-binucleates (EG1/LG1). (*B*) Fusion B (EG1*/LG1). The increase in labeling index of the late G1 nuclei. □, In mononucleates (LG1); ■, in NSA-binucleates (LG1/LG1); △, in NDA-binucleates (EG1/LG1); and ○, in NSA-trinucleates (3LG1).

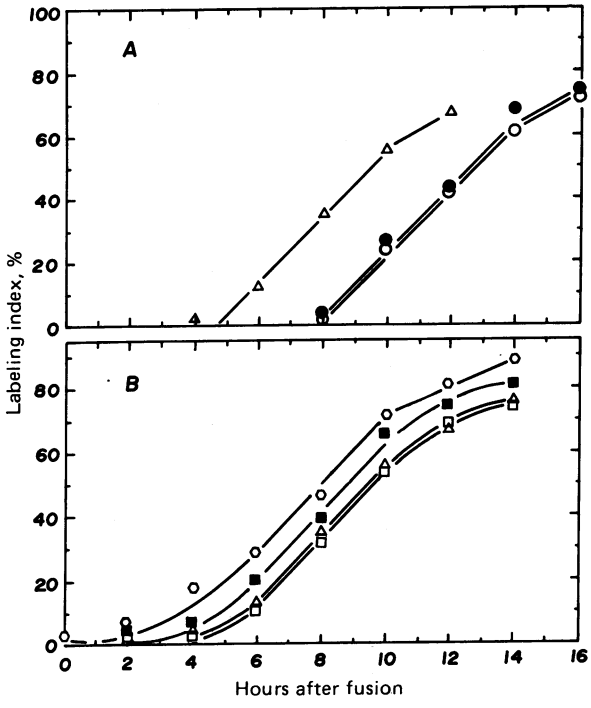

FIG. 2. Kinetics of initiation of DNA synthesis in fusions between early G1 (EG1) and mid-G1 (MG1) cells. (*A*) Fusion C (EG1/MG1*). The rate of entry of the unlabeled early G1 nuclei into S phase is plotted as a function of time. ○, In mononucleates (EG1); ●, in NSA-binucleates (EG1/EG1); △, in NDA-binucleates (EG1/MG1). (*B*) Fusion D (EG1*/MG1). The increase in the labeling index of the mid-G1 nuclei was plotted as a function of time. □, In mononucleates (MG1); ■, in NSA-binucleates (MG1/MG1); △, in NDA-binucleates (EG1/MG1); ○, in NSA-trinucleates (3MG1).

tion of DNA synthesis in the mononucleates and the NSA- and NDA-binucleates of the early G1 parent were studied. The mononucleates and NSA-binucleates entered S phase at about the same rate (Fig. 1*A*). However, the early G1 nuclei residing in the NDA-binucleates entered S phase much earlier in synchrony with late G1 nuclei. In fusion B (early G1*/late G1), the binucleates of late G1 parents reached a labeling index of 50% about 1.5 hr earlier than did the mononucleates (Fig. 1*B*). The difference between the mononucleates and trinucleates was even greater (2.75 hr). However, the NDA-binucleates were only slightly ahead (0.25 hr) of the mononucleated late G1 cells in their rate of entry into S.

In these experiments the duration of G1 appeared to be greater than the normal 10.4 hr seen in a spinner culture. This is due to the procedures of cell fusion which include cooling to 4° for 15 min and exposure to Sendai virus. Because both the fused and unfused cells were delayed to the same extent, the quality of these data is not affected. This system has the unique advantage of having the unfused mononucleated cells serve as built-in controls for comparison with the fusion products (the bi- and trinucleated cells).

Early G1/Mid-G1 Fusions. In fusion C (early G1/mid-G1*), the NDA-binucleates reached a labeling index of 50% at 9.5 hr, compared to 12.75 hr for the mononucleates and NSA-binucleates (Fig. 2*A*). The NSA-binucleates of the mid-G1 parent reached the 50% mark about an hour earlier than did their mononucleated counterparts (Fig. 2*B*). The trinucleates were even faster than the binucleates by 0.75 hr. The rate of G1 traverse of the NDA-binucleates was slightly faster than the mononucleates, but much slower than the NSA-binucleates.

Mid-G1/Late G1 Fusions. The NDA-binucleates in fusion E, in which late G1 cells were fused with mid-G1 cells, entered S phase earlier than the mononucleates or NSA-binucleates (Fig. 3*A*). This again is due to the premature induction of DNA synthesis in mid-G1 nuclei which was influenced by the entry of the late G1 component into S phase. In fusion F (MG1*/LG1), the NDA-binucleates (mid-G1/late G1) reached the 50% mark significantly earlier than did the late G1 mononucleates but were only slightly behind the late G1/late G1 binucleates (Fig. 3*B*).

The rates of G1 traverse of the three different NDA-binucleates resulting from the six fusions were compared with each other (Fig. 4). As expected, the mid-G1/late G1 binucleates had a shorter G1 period than the early G1/late G1 and early G1/mid-G1 cells. For convenience, the times required to reach a labeling index of 50% for the various classes of cells are presented in Table 2. In essence, Table 2 is a summary of Figs. 1 through 4. In addition, Table 2 makes it possible to determine the contribution of the early, middle, and late G1 cells to the advancement of cells toward S phase.

Table 2. Rates of entry of mononucleates and NSA- and NDA-binucleates into S phase, measured as time from fusion to labeling index of 50%

Cell type[a]	Time, hr	Relative contribution (hours of advancement) for each additional cell in
(*a*) LG1	8.75	
(*b*) LG /LG1	7.25	
(*a* − *b*)	1.5	LG1, 1.5
(*c*) 3LG1	6.0	
(*a* − *c*)	2.75	LG1, 1.4
(*d*) MG1	9.75	
(*e*) MG1/MG1	8.75	
(*d* − *e*)	1.0	MG1, 1.0
(*f*) 3MG1	8.0	
(*d* − *f*)	1.75	MG1, 0.9
(*g*) LG1/MG1	7.75	
(*a* − *g*)	1.0	MG1, 1.0
(*h*) EG1	12.75	
(*i*) EG1/EG1	12.7	
(*h* − *i*)	0.05	EG1, 0.05
(*j*) LG1/EG1	8.5	
(*a* − *j*)	0.25	EG1, 0.25
(*k*) MG1/EG1	9.5	
(*d* − *k*)	0.25	EG1, 0.25

[a] LG1, late G1; MG1, mid-G1; EG1, early G1; 3LG1 and 3MG1, trinucleates.

DISCUSSION

In this study we have found that there was no significant difference between the mono- and binucleates of the early G1 cells in their rate of entry into S phase (Fig. 1*A*). This is in agreement with the observations by Rao and Johnson (1) and Graves (2). In contrast, Fournier and Pardee (7) found that the binucleated cells produced by a 90-min cytochalasin-B treatment of mitotic cells (cell line BHK 21/C13) traversed the G1 period significantly faster than did mononucleated cells. A non-concentration-dependent cooperation among the two G1 components was thought to be responsible for the differences in their rates of progression into S phase (7). Working with HeLa cells, we were able not only to reproduce the above results but also to find that the timing of cytochalasin-B treatment after the reversal of the

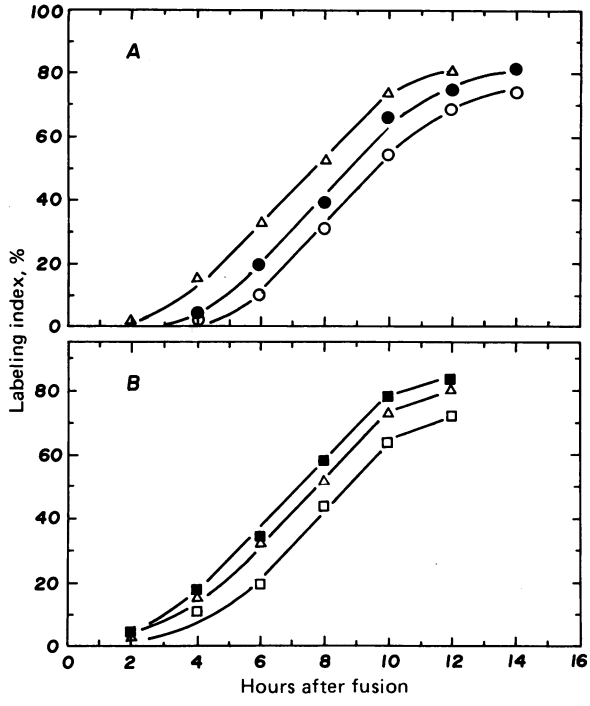

FIG. 3. Kinetics of initiation of DNA synthesis in fusions between mid-G1 and late G1 populations. (*A*) Fusion E (MG1/LG1*). The progress of mid-G1 nuclei into S phase is plotted. ○, In mononucleates (MG1); ●, in NSA-binucleates (MG1/MG1); △, in NDA-binucleates (MG1/LG1). (*B*) Fusion F (MG1*/LG1). The G1 traverse of late G1 nuclei is shown. □, In mononucleates (LG1); ■, in NSA-binucleates (LG1/LG1); △, in NDA-binucleates (MG1/LG1).

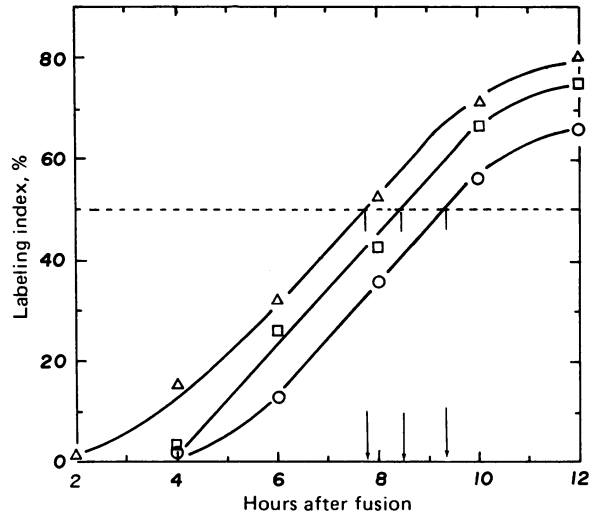

FIG. 4. Comparison of rates of G1 traverse of three "hybrids" or NDA-binucleates resulting from the six different fusions under investigation. NDA-binucleates are the products of the following fusions: □, EG1/LG1; ○, EG1/MG1; and △, MG1/LG1.

mitotic block affects the kinetics of the initiation of DNA synthesis in the mono- and binucleated cells (8). If there were a true cooperation between the different G1 components in the cytochalasin-B-induced multinucleates, one would expect a proportionate decrease in the duration of G1 associated with an increase in the number of nuclei. No such relationship was found in HeLa multinucleated cells produced by cytochalasin-B treatment (8). However, the model for non-concentration-dependent cooperative initiation of DNA synthesis proposed by Fournier and Pardee (7) is supported by the results of the present study.

This study shows that when G1 cells are fused with one another, they cooperate by pooling their resources for the purpose of DNA replication. These resources could be proteins that can induce DNA synthesis. The amount of inducer present within a G1 cell and, consequently, its contribution to the induction of DNA synthesis depend on its position in the G1 period. The farther a cell has advanced in G1, the greater would be its effect on DNA synthesis. According to Table 2, the fusion of one late G1 cell with another would reduce the duration of G1 by about 1.5 hr. On the other hand, the addition of one mid-G1 cell to either a late G1 or mid-G1 cell would shorten the G1 period by only 1 hr. When an early G1 cell is fused with a mid-G1 or late G1, the reduction in the length of G1 period is about 0.25 hr. These results clearly indicate that the levels of the inducer of DNA synthesis are significantly higher in the late G1 cells than in the mid-or early G1 cells.

The fact that the degree (hours) of advancement of a late G1 cell toward S phase was proportional to the number and the age of G1 cells added to it by fusion indicates that the inducer of DNA synthesis operates in a non-concentration-dependent manner. If the effect were concentration-dependent, there should not be any difference between the tri-, bi-, and mononucleates in their rates of entry into S phase. From this study, it appears that the total number of inducer molecules present within a cell is important for the initiation of DNA synthesis, rather than their concentration in the cytoplasm or the nucleus. When more cells are fused together, the total number of the inducer molecules increases without any significant change in their concentration. When a critical level of inducer is reached, DNA synthesis is initiated in one or more nuclei at the same time. The number of nuclei present within a cell does not appear to affect the rate of initiation. The molecular mechanisms involved in the synchronous initiation of DNA synthesis in multinucleated cells remains to be elucidated.

The above results confirm the earlier findings by Frazier (9) on *Stentor*. It has been shown that starvation of these animals results in the inhibition of DNA synthesis. However, DNA synthesis could be reinitiated in starved *Stentor* after grafting of cytoplasm from another starved animal. The increase in the cytoplasmic volume due to grafting would result only in an increase of the number of inducer molecules but not in their concentration. These results also suggest that the inducer of DNA synthesis operates in a non-concentration-dependent manner.

From a review of the literature, one would get the impression

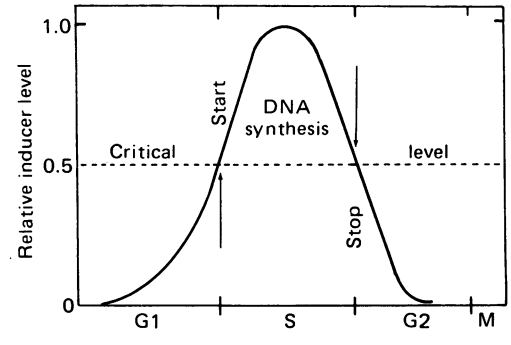

FIG. 5. Model to explain the appearance of the inducer(s) of DNA synthesis during the HeLa cell cycle. The relative level (arbitrary units) of the inducer is shown to vary according to the position of a cell in the cell cycle. G1, pre-DNA-synthetic period; S, DNA synthetic period; G2, post-DNA-synthetic period; M, mitosis. The durations of the various phases are not drawn to scale.

that the inducers of DNA synthesis become available suddenly at the G1–S transition, although no definite experimental evidence is available (10, 11). The present study, on the other hand, provides clear evidence that the inducer(s) of DNA synthesis accumulates gradually during the G1 period. In view of these observations, we propose the following model for the appearance of the inducer(s) of DNA synthesis during the cell cycle. According to this model, the inducer(s) accumulates gradually throughout the G1 period, reaching a critical level at the G1–S transition when DNA replication is initiated (Fig. 5). The level of the inducer(s) is expected to reach a peak, probably by the middle of S phase, and then gradually decline below the critical level at the S–G2 transition when DNA synthesis ceases. The studies by Johnson and Mullinger (12) showed that the late S phase cells were not as effective as early or mid-S phase cells in inducing DNA synthesis in chick erythrocytes upon fusion. The suggestion that the late S phase cells are relatively poor in the amount of the inducer fits well with our model.

This investigation was supported by U.S. Public Health Service Grants CA-16480, CA-11520, CA-19856, and CA-14528 from the National Cancer Institute and GM-23252 from the Institute of General Medical Sciences and Grant VC-163 from the American Cancer Society.

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Rao, P. N. & Johnson, R. T. (1970) *Nature* **225**, 159–164.
2. Graves, J. A. M. (1972) *Exp. Cell Res.* **72**, 393–403.
3. Gurdon, J. B. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 545–552.
4. de Terra, N. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 607–614.
5. Rao, P. N. & Engelberg, J. (1966) in *Cell Synchrony: Studies in Biosynthetic Regulation*, eds. Cameron, I. L. & Padilla, G. M. (Academic Press, New York and London), pp. 332–352.
6. Rao, P. N. (1968) *Science* **160**, 774–776.
7. Fournier, R. E. & Pardee, A. B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 869–873.
8. Rao, P. N. & Smith, M. L. (1976) *Exp. Cell Res.* **103**, 213–218.
9. Frazier, E. A. J. (1973) *Dev. Biol.* **34**, 77–92.
10. Johnson, R. T. & Rao, P. N. (1971) *Biol. Rev.* **46**, 97–155.
11. Prescott, D. M. (1976) *Adv. Genet.* **18**, 99–177.
12. Johnson, R. T. & Mullinger, A. M. (1975) *J. Cell Sci.* **18**, 455–490.