Initiation of DNA Synthesis in the Prematurely Condensed Chromosomes of G_1 Cells
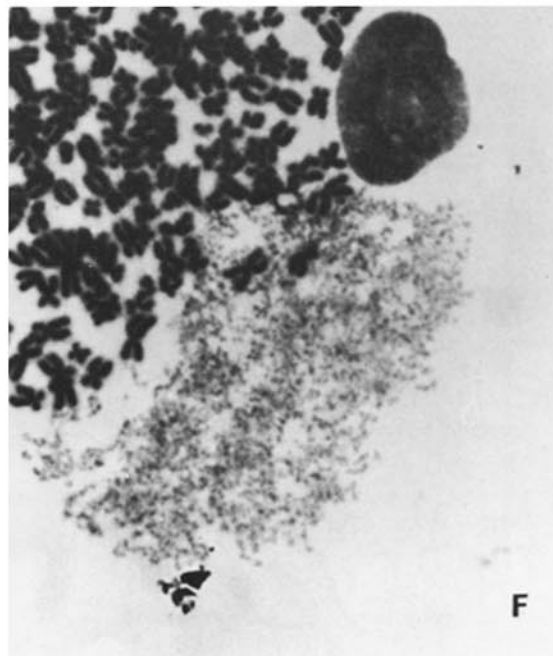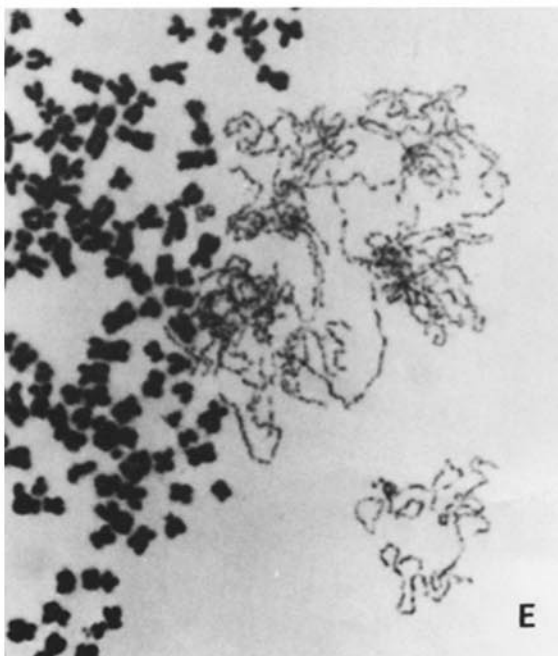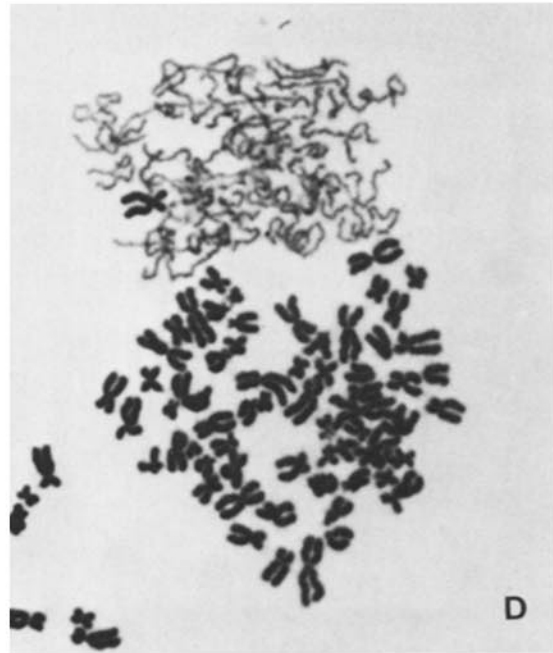
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ABSTRACT   The objective of this study was to investigate whether G_1 cells could enter S phase after premature chromosome condensation resulting from fusion with mitotic cells. HeLa cells synchronized in early G_1, mid-G_1, late G_1, and G_2 and human diploid fibroblasts synchronized in G_0 and G_1 phases were separately fused by use of UV-inactivated Sendai virus with mitotic HeLa cells. After cell fusion and premature chromosome condensation, the fused cells were incubated in culture medium containing Colcemid (0.05 μg/ml) and [^{3}H]thymidine ([^{3}H]ThdR) (0.5 μCi/ml; sp act, 6.7 Ci/mM). At 0, 2, 4, and 6 h after fusion, cell samples were taken to determine the initiation of DNA synthesis in the prematurely condensed chromosomes (PCC) on the basis of their morphology and labeling index. The results of this study indicate that PCC from G_0, G_1, and G_2 cells reach the maximum degree of compaction or condensation at 2 h after PCC induction. In addition, the G_1-PCC from normal and transformed cells initiated DNA synthesis, as indicated by their "pulverized" appearance and incorporation of [^{3}H]ThdR. Further, the initiation of DNA synthesis in G_1-PCC occurred significantly earlier than in the mononucleate G_1 cells. Neither pulverization nor incorporation of label was observed in the PCC of G_0 and G_2 cells. These findings suggest that chromosome decondensation, although not controlling the timing of a cell's entry into S phase, is an important step for the initiation of DNA synthesis. These data also suggest that the entry of a G_1 cell into S phase may be regulated by cell cycle phase-specific changes in the permeability of the nuclear envelope to the inducers of DNA synthesis present in the cytoplasm.

When a mitotic cell is fused with an interphase cell, factors present in the mitotic cytoplasm induce breakdown of the nucleus and reorganization of the interphase chromatin into prematurely condensed chromosomes (PCC) (9). The morphology of PCC indicates the position of a cell in the cell cycle at the time of fusion (9, 26). PCC from G_2 cells resemble extended prophase chromosomes. S-phase PCC (S-PCC) exhibit a characteristic "pulverized" appearance attributable to the presence of both condensed and diffused regions. Active sites of replication in S-PCC appear as gaps under the light microscope (25). G_1-phase PCC consist of single chromatids that vary considerably in length and thickness, depending on the degree of advancement of the cell toward S phase before fusion. The closer a G_1 cell gets to S phase, the more extended are its PCC (6, 7, 23). Immediately before entry into S phase, individual chromosomes are no longer easily discernible, as the PCC take on a highly diffused appearance.

The increasing amount of decondensation in PCC, as cells progress through G_1, reflects intranuclear changes in chromatin organization and provides direct visual evidence for the "chro-mosome condensation cycle" originally proposed by Mazia (11). According to this model, chromosomes begin a decondensation process during telophase that continues throughout G_1. Maximum decondensation levels are associated with DNA replication during S phase. After replication, chromosomes begin to recondense, and this process continues until the maximum level of compaction is again achieved during metaphase. Many other investigators, using a variety of experimental methods, have provided evidence to support the idea of a chromatin condensation cycle (1, 3–5, 12–17, 25). At present, it is unclear to what extent chromatin decondensation during G_1 is involved in the regulation of the initiation of DNA replication. Is the total dispersion of the interphase chromosomes observed at the G_1/S boundary a necessary prerequisite, or can DNA synthesis be initiated in condensed chromosomes? We have used the PCC phenomenon to investigate this question. The results of our study show that DNA synthesis is initiated in G_1 cells after nuclear dissolution and reorganization of chromatin into prematurely condensed chromosomes. The degree to which DNA synthesis is initiated is dependent on the extent of G_1 phase

traversed by the cell before PCC induction.

essed for autoradiography, and restained with Giemsa's. PCC spreads were then relocated and scored for labeling.

MATERIALS AND METHODS

Cells and Cell Culture

A normal human diploid fibroblast line, Detroit-550 (American Type Culture Collection, Rockville, Md.), and HeLa cells were used in these experiments. Both lines were maintained as monolayer cultures in modified McCoy's 5A medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 16% fetal calf serum, 1% glutamine and penicillin/streptomycin mixture at 37°C in a humidified, 5% CO_2 atmosphere. Detroit-550 cells were in their 20th to 23rd passages during the period of these experiments.

Cell Synchrony

Mitotic HeLa cells for fusion experiments were obtained in the following manner. Exponentially growing cells were partially synchronized into S phase by a 24-h exposure to 2.5 mM thymidine. After reversal of the excess ThdR block, Colcemid (0.05 μg/ml, CIBA Pharmaceutical Co., Summit, N. J.) was added to block cells in mitosis, and incubation continued for an additional 18 h. The loosely attached mitotic cells were then collected by selective detachment. Mitotic indices of cells collected by this procedure were routinely greater than 98%.

HeLa cells synchronized in G_1 were obtained through reversal of a mitotic block induced by exposure to nitrous oxide at 90 psi (18). 90% of the mitotic cells, harvested by selective detachment, had divided and entered the G_1 period within 1.5 h after reversal of the N_2O block. Unattached mitotic cells were first removed, then the attached G_1 cells were collected by trypsinization. Cells collected at 3, 5, and 7 h after reversal of the N_2O block were designated as early-, mid-, and late-G_1 cells, respectively (22).

To obtain HeLa cells in G_2, we first synchronized exponentially growing cells into S phase by the excess thymidine double-block method (2, 19). After reversal of the second ThdR block, Colcemid (0.05 μg/ml) was added to the medium, and the cells were incubated for 9 h more. After the incubation period, floating and loosely attached mitotic cells were removed by vigorous pipetting. The interphase cells that remained attached to the dish represented a population greatly enriched in G_2 cells. Contaminating S-phase cells were identified by a 30-min exposure to 2.0 μCi/ml [³H]ThdR (sp act, 6.7 Ci/mM, New England Nuclear, Boston, Mass.) before collection of the interphase cells.

Detroit-550 cells in G_0 were obtained by harvesting at 7 d after cells had reached confluence. To obtain Detroit-550 cells in G_1, we held the cells in confluence for 7 d, then trypsinized and replated them at 25% confluence. At 18 h after plating, the cells were harvested for cell fusion.

Cell Fusion, Slide Preparation, and Scoring

PCC were induced by fusing mitotic cells with synchronized interphase cell populations by use of UV-inactivated Sendai virus, as described previously (23). After PCC induction, the cells were resuspended in modified McCoy's 5A medium containing 0.5 μCi/ml [³H]ThdR (sp act, 6.7 Ci/mM) and 0.05 μg/ml Colcemid, then plated into four dishes, and returned to the incubator to await sample collection. Separate interphase populations, fused among themselves, were used as a control to measure the rate of entry of the synchronized G_1 populations into S phase. A separate control was necessary because, after the fusion process, some of the mitotic cells began to move out of mitosis and became indistinguishable from the interphase population.

At 0, 2, 4, and 6 h after fusion, cells were collected by trypsinization and deposited directly onto slides with the aid of a cytocentrifuge. In addition, chromosome spreads were made from the mitotic-interphase fusion mixture for the visualization of PCC, as described earlier (23). All slides were stained with Giemsa's.

For each time-point, ~100 PCC spreads were located under a light microscope and scored as to their degree of condensation. Condensation levels of G_1-PCC were determined, using the classification scheme developed by Hittelman and Rao (6). Under this system, G_1-PCC are separated into six categories based on their level of condensation (Fig. 1). G_1-PCC showing the maximum amount of condensation are designated as belonging to category 1. Increasing degrees of decondensation are seen in categories 2 through 5, with the maximum decondensation level represented by category 6.

After localization and classification of PCC, the slides were destained, proc-

RESULTS AND DISCUSSION

Initiation of DNA Synthesis in G_1-PCC after the Breakdown of the Nuclear Envelope

Initially, we sought to determine whether DNA synthesis, as measured by [³H]ThdR incorporation, would be initiated in the prematurely condensed chromosomes of HeLa cells in early G_1 phase. Immediately after PCC induction, some variability with regard to their degree of condensation was observed among the G_1-PCC. Most of them could be grouped into categories 2–4 of the classification scheme (Fig. 2A). However, by 2 h after fusion, PCC had achieved the maximum level of condensation (Figure 2B). We also noted that some of the condensed PCC exhibited a pulverized appearance that is indicative of DNA synthesis in progress. Further, the frequency of pulverized PCC increased as a function of time (Fig. 2B–D). The degree of pulverization varied, ranging from a banded or slightly broken appearance to a highly fragmented morphology (Fig. 3B–D).

Almost all PCC exhibiting a pulverized morphology had incorporated [³H]ThdR, as observed by autoradiography (Fig. 2B–D). In general, the grain densities over the PCC appeared to be proportional to the degree of pulverization (Fig. 3F–H). Very light labeling was also seen over many of the highly condensed PCC in which pulverization was not obvious (Fig. 3E). These findings demonstrate that initiation of DNA synthesis can take place after transformation of the G_1 nucleus into PCC. No labeling was seen over the mitotic chromosomes of the cells used to induce PCC, in spite of their sharing a common cytoplasmic environment with PCC which were actively engaged in DNA synthesis. This observation suggests that structural or conformational features of the chromatin play a role in determining whether DNA synthesis can be initiated.

We next investigated whether DNA synthesis could likewise be initiated in G_1-PCC of a nontransformed cell line. For this experiment, cells from a human diploid fibroblast line, Detroit-550, were used. Initially, the kinetics of labeling with [³H]ThdR were determined after the subculturing of a confluent plateau phase culture of Detroit-550 cells (data not shown). On the basis of these data, it was determined that at ~18 h after subculturing, a majority of cells in the population had exited from the G_0 state but had not yet entered S phase. Therefore, Detroit-550 cells harvested at 18 h after subculturing were fused to mitotic HeLa cells, and the ability of the resulting G_1-PCC to incorporate [³H]ThdR was examined as before. As with HeLa G_1-PCC, we observed progressive increases in both the degree of condensation and the amount of pulverization of the Detroit-550 G_1-PCC after fusion (Fig. 2E–H). Also, autoradiography again revealed that a correlation existed between the presence of a pulverized morphology and the incorporation of [³H]ThdR. Thus, we have demonstrated that DNA synthesis can be initiated in G_1 cells of both normal and transformed cell lines following nuclear dissolution and reorganization of chromatin into prematurely condensed chromosomes.

FIGURE 1 Human diploid fibroblasts (Detroit-550) in G_1 period were fused with mitotic HeLa cells. The resulting PCC of Detroit-550 cells were classified into A, category 1; B, 2; C, 3; D, 4; E, 5; and F, 6. The darkly stained chromosomes are of the mitotic HeLa cells used to induce PCC.
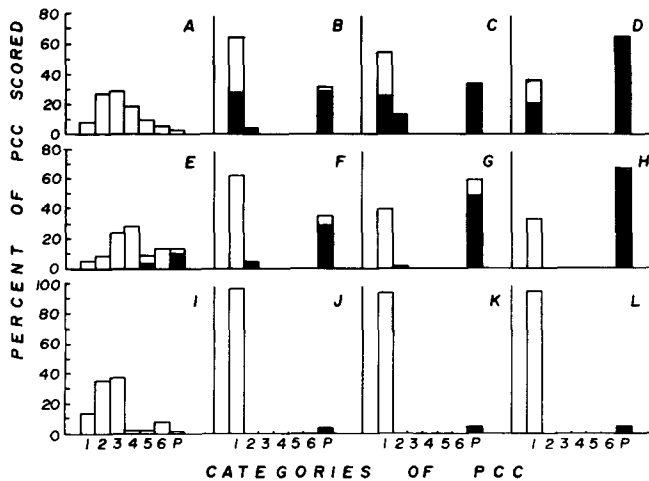
FIGURE 2 HeLa cells in early G_1 and Detroit-550 cells in G_0 and G_1 were separately fused with mitotic HeLa cells and plated in a number of dishes in medium containing [^{3}H]ThdR. Cell samples were taken at various times after fusion and processed for chromosome spreads. G_1-PCC were categorized from 1 to 6 according to their degree of condensation, as shown in Fig. 1. Category P represents PCC showing a pulverized appearance. The solid portion of each histogram bar indicates the fraction of PCC in that category scored as labeled after autoradiography. The fate of early G_1-PCC of HeLa cells is given in the top series of figures. Data are shown for A, 0 h; B, 2 h; C, 4 h; and D, 6 h after fusion. The center panel gives results for G_1-PCC from Detroit-550 cells (a normal human diploid fibroblast line) at E, 0 h; F, 2 h; G, 4 h; and H, 6 h after fusion. The bottom series of figures show the fate of G_0-PCC of Detroit-550 cells at I, 0 h; J, 2 h; K, 4 h; and L, 6 h after fusion. Note that none of the PCC of Detroit-550 cells belonging to category 1 were labeled, whereas ~50% of this category in HeLa cells was labeled. These differences could be a result of the fact that Detroit-550 cells were stimulated from a plateau phase growth while HeLa cells were cycling continuously.

Lack of Initiation of DNA Synthesis in the PCC of G_0 and G_2 Cells

To determine whether the DNA synthesis we observed in the PCC of G_1 cells is cell cycle phase–specific, these studies were repeated with both G_0- and G_2-phase cells. PCC was induced in a confluent culture of Detroit-550 cells resting in G_0 phase. The ability of the PCC to incorporate [^{3}H]ThdR was again examined, along with their morphological appearance. Initially, most of the G_0-PCC fell into categories 1–3 of the classification scheme. ~8%, however, had a highly extended morphology (see Fig. 2I). Subsequently, almost all of the PCC reached the maximum level of compaction and retained their condensed morphology throughout the course of the experiment (Fig. 2J–L). Neither pulverization nor incorporation of label into the highly condensed PCC was seen (see Fig. 4C). Similar studies performed with HeLa cells synchronized in G_2 phase likewise failed to exhibit pulverized appearance or incorporation of [^{3}H]ThdR in G_2-PCC (data not shown).

Because PCC from G_0 and G_2 cells failed to initiate DNA synthesis, we feel that DNA repair processes are not responsible for the incorporation of [^{3}H]ThdR observed in the G_1-PCC. In addition, the high density of labeling observed over the more highly pulverized G_1-PCC is indicative of semiconservative DNA replication rather than repair. The likelihood of repair is further diminished when one considers the lack of exposure, during the course of the experiments, to agents known to induce DNA damage.

Assuming that the incorporation of [^{3}H]ThdR in G_1-PCC is attributable to semiconservative replication, we can conclude that the maximum degree of chromatin dispersion normally attained at the G_1/S boundary does not, in itself, control the timing of a cell's entry into S phase. These findings show, for the first time, that DNA synthesis can be initiated in the absence of an intact nuclear structure within a cell. A previous report had shown that S-phase PCC could continue to synthesize DNA in the absence of a nuclear structure after PCC induction (9). It could not be resolved at that time, however, whether new origins of replication were being initiated in the S-phase PCC or whether the DNA synthetic activity was limited to the continuation of chain elongation in replicons that had been initiated before the breakdown of the nucleus. Our observation of the initiation of DNA synthesis in G_1-PCC opens the possibility that new origins of replication may be initiated in the S-phase PCC as well as continued chain elongation.

The failure of PCC from G_0 and G_2 cells to initiate DNA synthesis demonstrates the cell cycle specificity of this phenomenon. A prerequisite for the initiation of DNA synthesis in PCC appears to be an entry into G_1 phase and the initiation of the cell's preparations for S phase before cell fusion. These preparations must involve both the production of the enzymes and substrates involved in the actual replication process as well as a reorganization of chromatin structure to a state that allows the replication process to occur. This activation of replication origins is probably mediated through the chromatin decondensation process. PCC from both G_0 and G_2 cells show high levels of condensation from the very early stages of PCC formation. Thus, their failure to initiate DNA synthesis supports the conclusion that the extent to which DNA synthesis is initiated in PCC is dependent upon the level of decondensation.

Our observation that PCC can reach a maximum degree of condensation within 2 h after fusion may have some practical application in the field of cytogenetics. Studies requiring the visualization and identification of chromosomes from nonproliferating cells can be carried out by inducing PCC and allowing an additional 2 h of incubation time before chromosome preparations are made. The high level of condensation of PCC makes it rather easy to obtain accurate chromosome counts. Moreover, banding techniques could be used to identify individual chromosomes.

Initiation of DNA Synthesis in Relation to the Stages of Chromosome Decondensation during G_1

To determine whether the extent of a cell's progression through G_1 before fusion has an effect on the intensity of labeling observed over the PCC spreads, we twice repeated the experiment as described for early G_1-HeLa cells, except that the HeLa cells were synchronized into either mid- or late-G_1 phase before PCC induction. The extent of DNA synthesis, measured as the median number of silver grains counted over the labeled PCC spreads, was then determined for each time-point in the three experiments involving fusion of mitotic HeLa cells with those in early G_1, mid-G_1, and late G_1. Comparison of the median values for the different time-points failed to reveal a significant increase in the number of grains per PCC as a function of time in each of the three experiments (Table I). However, we noted a highly significant increase in labeling
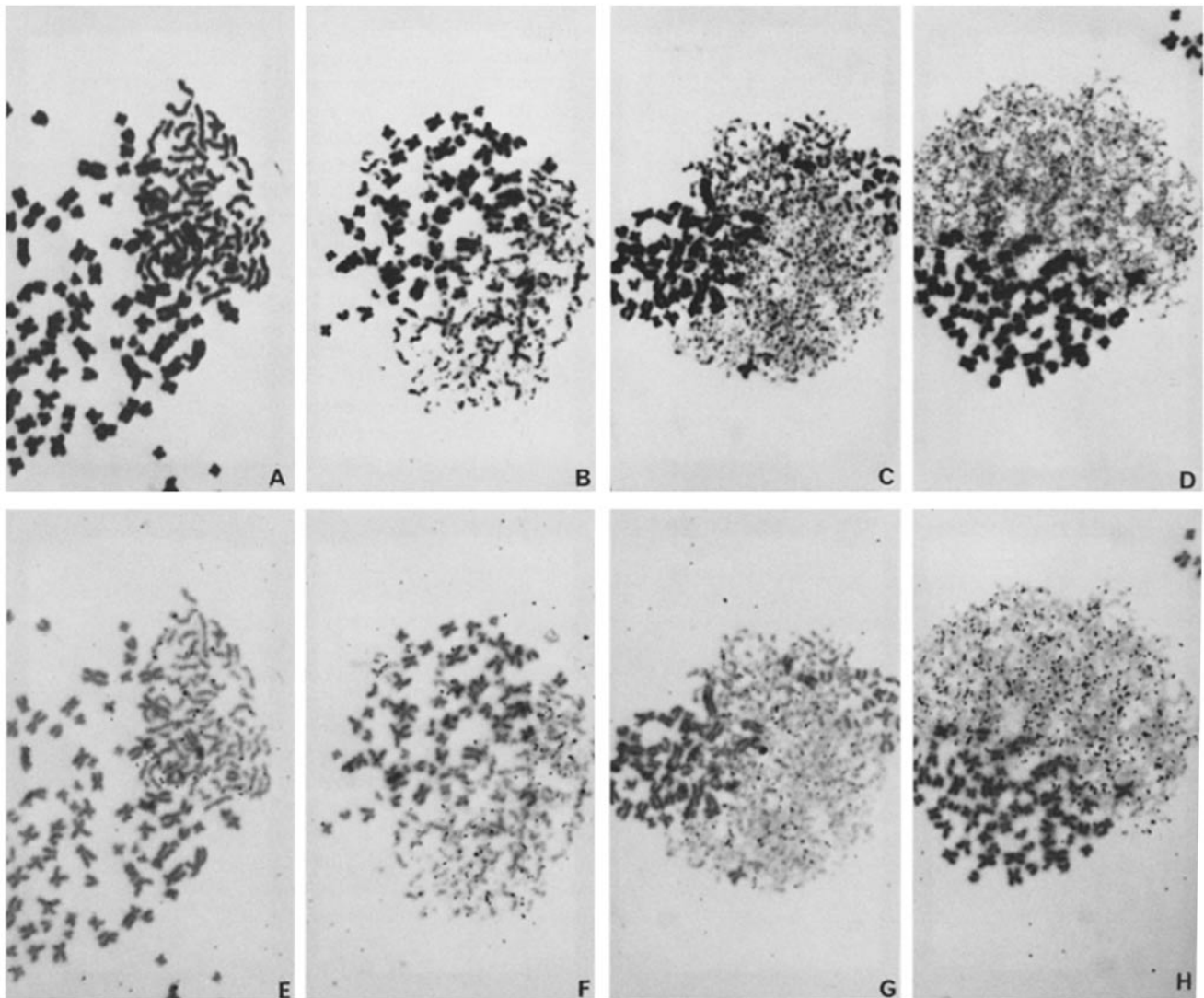
FIGURE 3 This figure shows evidence for DNA synthesis in the PCC of HeLa G₁ cells after fusion with mitotic HeLa cells. These G₁-PCC exhibit various degrees of "pulverization" and intensity of labeling. The pulverized appearance of the PCC was not detectable in *A*, was slight in *B*, and was high in *C* and *D*. Figures *E–H* are the respective autoradiographs for *A–D* showing various levels of labeling. The darkly stained metaphase chromosomes from the cells used to induce PCC show no incorporation of label.
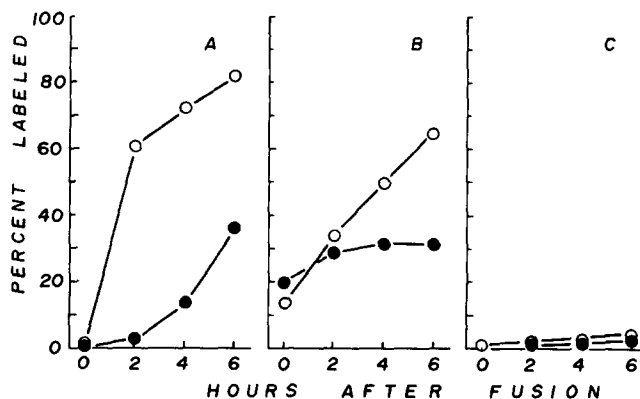

FIGURE 4 Rates of increase in the labeling indices for PCC, ○; and unfused mononucleate G₁ cells, ●, as a function of time after fusion. Mitotic HeLa cells were fused with *A*, HeLa cells in early G₁ phase; *B*, Detroit-550 cells in G₁; and *C*, Detroit-550 cells in G₀.

intensity associated with the extent of G₁ traversed before fusion when overall median grain counts from the early-, mid-, and late-G₁ experiments were compared (Table I).

Two conclusions can be drawn from these data. First, the lack of increase in grain density as a function of time in all three experiments indicates that the amount of DNA replicated is rather limited. These cells are not able to complete the replication of the whole genome. Second, our observation of an increased labeling intensity over the PCC from the later stages of G₁ lends support to the notion that factors contributing to the initiation of DNA synthesis accumulate gradually during the G₁ period (22). One likely function for these proposed factors is in the decondensation of chromatin observed during G₁, leading to an activation of replication origins. Thus, the degree of decondensation achieved before fusion would determine the number of sites at which DNA synthesis is initiated in the G₁-PCC.

Earlier Onset of DNA Synthesis in G₁-PCC than in G₁ Nuclei

Comparison of labeling kinetics of early G₁-PCC of HeLa cells and G₁-PCC of Detroit-550 cells, with the mononucleate G₁ cells in the controls, indicated that fusion to mitotic HeLa cells resulted not only in the premature condensation of chro-

TABLE I

Relationship between the Median Number of Silver Grains per PCC and the Position of a Cell in G_1 Phase at the Time of Fusion *

Type of PCC scored		Hours after fusion	Number of PCC scored	Median grain count	95% confidence limits	χ^2 (P)
Early G_1		2	62	15	12–22	
		4	74	19	16–23	0.989
		6	27	20	13–28	$(0.50 < P < 0.75)$
	TOTAL		163	18	15–22	
Mid-G_1		2	87	27	22–34	
		4	53	24	17–28	1.58
		6	67	25	16–30	$(0.25 < P < 0.75)$
	TOTAL		207	26	22–28	
Late G_1		2	70	75.5	60–112	
		4	94	83.5	64–126	0.099
		6	90	76	60–109	$(P \cong 0.95)$
	TOTAL		254	78	67–102	
GRAND TOTAL of all time points			624	31	27–35	103.04 $(P < .005)$

* The median number of grains was determined from PCC spreads made at 2, 4, and 6 h following the addition of [^{3}H]ThdR. Chi-square analysis using the median test (28) showed no significant variation in the medians from time-point to time-point within each of the three HeLa experiments (early, mid-, and late G_1). A comparison of combined medians between the three experiments, however, revealed a highly significant increase in grain counts as the cells progress through G_1.

matin but also an earlier initiation of DNA synthesis in the G_1-PCC (Fig. 4 A and B). There is a striking similarity between these data and the labeling kinetics of G_1 nuclei in the S/G_1 fusions (20). In other words, the G_1-PCC seem to enter S phase almost as fast as G_1 nuclei after fusion between S- and G_1-phase cells.

Two alternative explanations exist to explain this observation: (a) the disorganization of the nucleus during the induction of premature chromosome condensation may lead to an earlier access of the inducers of DNA synthesis present in the cell to replication origins in the chromatin, and (b) the mitotic cells used to induce PCC provide additional factors that promote the early onset of DNA synthesis. According to this latter view, mitotic cells arrested by the prolonged (18-h) exposure to Colcemid may functionally be in G_1 phase and thus be accumulating the factors necessary for the initiation of DNA synthesis.

To test these two alternatives, we again induced premature chromosome condensation in early-G_1 HeLa cells and measured their ability to incorporate [^{3}H]ThdR after PCC formation. In this experiment, however, the HeLa mitotic cells used to induce PCC had been exposed to the Colcemid block for 24 h. Because the ability of the mitotic cells to induce PCC decreases after a prolonged (24-h) mitotic arrest (21), many of the M/G_1 fusions did not result in the breakdown of the nucleus and the formation of PCC. Therefore, by comparing labeling kinetics of PCC and intact nuclei in the M/G_1 fusion products, we were able to determine the contribution of the mitotic component toward the early onset of DNA synthesis in G_1-PCC or intact nuclei.

Our data indicate that the labeling kinetics of the intact nuclei in the M/G_1 fused cells resemble those of the mononucleate G_1 cells rather than those of G_1-PCC (Fig. 5). If the mitotic cells were contributing additional factors for the initi-
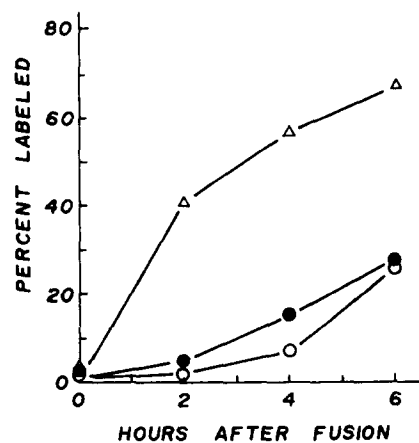

FIGURE 5 A comparison of the rates of increase in the labeling indices for unfused mononucleate early G_1 HeLa cells, O; intact early G_1 nuclei located in M/G_1-fused cells, ●; and early G_1-PCC, △.

ation of DNA synthesis, then the labeling kinetics of the M/I (mitosis/interphase) cells would be similar to those of M/PCC. Because they are not, we conclude that the mitotic cells contribute little or nothing directly to the pools of factors responsible for initiation of DNA synthesis in G_1-PCC.

These results suggest that the breakdown of the nuclear structure facilitates the early initiation of DNA synthesis in the G_1-PCC. Because nuclear envelope breakdown occurs during PCC induction, this might allow cytoplasmic factors to interact more freely with chromatin and cause early entry into S phase. Therefore, we suggest the permeability of the nuclear envelope might normally play a role in the initiation of DNA synthesis by controlling the rate of flow of such factors into the nucleus. An involvement of the nuclear envelope in the control of the

initiation of DNA synthesis has been previously postulated by others (8, 10, 24, 27).

CONCLUSION

In summary, the phenomenon of premature chromosome condensation has provided evidence toward a greater understanding of the process involved in the regulation of a cell's entry into S phase. Our observation that G_1-PCC can initiate DNA synthesis demonstrates that the maximum level of decondensation achieved by the chromosomes immediately before entry into S phase does not, in itself, control the initiation of replication. However, the level of decondensation achieved by the G_1 cell before PCC induction does appear to determine the extent to which DNA synthesis is initiated. Because the breakdown of the nuclear envelope during PCC formation would result in earlier access of the cytoplasmic inducers of DNA synthesis to the G_1-PCC leading to an earlier onset of DNA synthesis, we suggest that the permeability of the nuclear membrane to the cytoplasmic factors might normally play a role in the initiation of DNA synthesis by regulating the flow of these factors into the nucleus.

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