DETECTION BY MEANS OF CELL FUSION
OF MACROMOLECULAR SYNTHESIS INVOLVED
IN THE RECONSTRUCTION OF THE
NUCLEAR ENVELOPE IN MITOSIS

YOSHITAKA OBARA, HERBERT WEINFELD, and AVERY A. SANDBERG
From the Roswell Park Memorial Institute, Buffalo, New York 14203

ABSTRACT
Using the cultured Chinese hamster cell line Don, G_1 or S or a mixture of
late-S/G_2 cells were prepared by release from metaphase arrest. Metaphase (M)
cells were also obtained by mitotic arrest of log-phase cultures with Colcemid
and held in metaphase; such M cells remained untreated with any other
compound and were termed standard M cells.

When interphase (I) cells were fused at pH 8.0 and 37°C with standard M
cells in the presence of Colcemid by means of UV-inactivated Sendai virus,
binucleate interphase-metaphase (I-M) cells were obtained. In a given I-M cell
there occurred within 30 min after fusion either prophasning of the I nucleus or
formation of a nuclear envelope (NE) around the chromosomes. About 20% of
early G_1 cells, 35% of cells at the G_1/S boundary, 50% of S cells, and 70% of
late-S/G_2 cells could induce NE formation.

If, before fusion, cycloheximide (CHE), an inhibitor of protein synthesis,
was present during release from M arrest, the cells entered G_1 but not S. About
20% of such early G_1 cells, like the untreated early G_1 cells, had the capacity to
induce NE formation during subsequent fusion. If the cells were blocked in S
with 5 mM thymidine (TdR), at least 80% of these cells could induce NE
formation during subsequent fusion, but in the presence of both TdR and CHE
only 35% could do so. It appeared, therefore, that protein synthesis in
interphase was required for NE formation. Experiments with actinomycin D
indicated that RNA synthesis was also necessary for acquisition of NE-induc-
ing capacity.

About 35% of G_1 cells from confluent monolayers had the NE-inducing
capacity, but prolonged exposure to CHE reduced their number to 8%.
Removal of CHE restored the ability while the cells still remained in G_1. This
result indicated that continuing protein synthesis in the G_1 cell was needed for
NE formation subsequent to fusion.

The fact that macromolecular synthesis must occur in the I cell before
fusion if NE formation was to occur in the fused I-M cell lends further support
to evidence adduced earlier that this phenomenon is a normal mitotic event.
Prophasing of the I nucleus in I-M cells did not appear to be dependent on macromolecular synthesis in the I cell; earlier results from this laboratory showed, however, that protein synthesis in the prior G\textsubscript{2} period of the M cell of the I-M pair was required for prophasing.

The fusion of a mammalian metaphase (M) cell with an interphase (I) cell by means of UV-inactivated Sendai virus results in one of two phenomena within 30 min after fusion: (a) the I nucleus of the resulting binucleate cell undergoes a series of changes, termed prophasing (9) or premature chromosome condensation (4, 19), which resemble those seen in normal mononucleate mitosis without any visible change in the chromosomes, or, alternatively, (b) the I nucleus of the binucleate cell remains unchanged, whereas the chromosomes enter into a telophase-like nucleus (TLN), becoming enclosed in an envelope which closely resembles a normal nuclear envelope (NE) (3, 13, 15). In a given interphase-metaphase (I-M) cell, when TLN formation occurs, prophasing is absent and vice versa (3, 13, 15).

The evidence that prophasing and TLN formation (i.e., NE formation) in Chinese hamster I-M cells reflect normal mitotic events of the mononucleate cell cycle has been summarized (14, 15). Prophasing is probably due to mitotic factors that are the contribution to the I-M cell by the M cell, in which they are resident at the time of fusion (7, 15). Formation of the NE of the TLN is probably under control of factors resident in the I cell at the time of fusion (3, 13–15). The same is probably true for both events in fused HeLa I-M cells (4, 5, 13, 19).

In the case of prophasing, we have presented evidence (7) that occurrence of a factor(s) in the M cell is dependent on protein synthesis during its prior G\textsubscript{2} period. We have now examined the possibility that inhibition of macromolecular synthesis in the I cell before fusing it with an untreated M cell can affect the efficiency of NE formation.

**MATERIALS AND METHODS**

**Cells and Virus**

A Chinese hamster embryonic lung cell line (Don), a cell stock of the American Type Culture Collection, Rockville, Md., was used throughout the experiments as in the earlier work on TLN (3, 13, 15). This cell line was grown at 37°C as a monolayer culture in RPMI 1640 medium (12) at pH 7.4 supplemented with 10% fetal calf serum, containing 100 μg/ml each of penicillin and streptomycin. Cells growing in log phase were obtained about 15–16 h after subculture.

UV-inactivated Sendai virus, concentrated to 20,000 hemagglutinating units (HAU)/ml of glucose-free Hanks' solution, was used for cell fusion experiments. Procedures for proliferation and inactivation of the virus and preparation of virus stock were described in a previous paper (6).

**Preparations of M- and Synchronized I-Cell Populations**

M cells were obtained by mitotic arrest with Colcemid (23), as described in a previous paper (15). Log-phase monolayer cultures were exposed to 0.08 μg/ml of the mitotic inhibitor for 5 h at 37°C. After the cultures were shaken gently to detach the M cells from the culture flasks, the freed cells which were centrifuged at 1,000 rpm for 3 min were resuspended in prewarmed fresh Colcemid medium at pH 8.0 before fusion. The M-cell population had a metaphase index exceeding 95% in most cases. Such cells will be referred to as standard M cells.

I-cell populations were derived from Colcemid-arrested M cells, and in some cases they originated from confluent monolayer cultures. In the former case M cells were obtained by shaking log-phase monolayer cultures which had been treated with Colcemid, 0.02–0.04 μg/ml, for 3–4 h. After being washed free of Colcemid, the cells were placed in culture flasks in fresh medium pH 7.4 at 37°C. More than 95% of the cells were in the G\textsubscript{1} phase 1.5 h later, more than 80% in the S phase by 6 h, and at least 80% in late-S/G\textsubscript{2} phase by 10.5 h (see Fig. 1 below). In general, this synchrony was essentially the same as that observed previously (8).

Cells in these stages or in confluent monolayers were freed by trypsinization (Grand Island Biological Co., Grand Island, N. Y., 0.25%) for 3 min at 37°C with gentle shaking. The treatment of I cells with cycloheximide (CHE) or actinomycin D (AMD) appears in the individual protocols. Standard M cells were exposed only to Colcemid and no other drug.

**Cell Fusion and Observations**

The freed I cells were washed once with prewarmed fresh Colcemid medium at pH 8.0. In all fusion experiments about 2.5 × 10\textsuperscript{6} each of standard M cells and of I cells were mixed and suspended in a total of 0.5 ml of medium, pH 8.0, containing 1,000 HAU of
inactivated Sendai virus and 0.08 μg/ml of Colcemid. The cell-virus mixture was allowed to stand for 10 min at about 1°C. The suspension was then transferred to an incubator maintained at 37°C and shaken gently for 10 min. The suspension was then diluted sixfold in prewarmed medium of the same pH containing 0.08 μg/ml of Colcemid, and the diluted sample was incubated with intermittent shaking at 37°C for an additional 20 min. The cells were harvested at room temperature by centrifugation, treated with hypotonic 15 mM sodium citrate (0.5 ml) for 5 min at room temperature, and fixed by addition of the same volume of Carnoy's fixative (acetic acid:methanol, 1:3). After removing the fixative, the cells were resuspended in fresh fixative and spread on glass slides without flaming. The air-dried cells were stained with Giemsa's.

This procedure from fusion through staining was essentially the same as that described in previous papers from this laboratory (3, 13, 15).

The criteria for scoring TLN and prophasing were described earlier (3, 13, 15). At least 100 I-M binucleate or trinucleate cells were examined at random in each sample, and the frequency of TLN or prophasing was recorded.

Autoradiography

The cells were exposed to either 1 μCi/ml of [3H]thymidine (TdR) (6.7 Ci/mmol), 5 μCi/ml of [3H]uridine (24.9 Ci/mmol), or 5 μCi/ml of [3H]lysine (7.0 Ci/mmol) for periods of 10-20 min at 37°C.

Cells were monitored for G1, S progression or failure of such progression by grain counting. After a 10-min exposure to 1 μCi/ml of [3H]TdR, the medium was removed and after subsequent trypsinization for 3 min at 37°C the cells were treated with hypotonic 15 mM sodium citrate at room temperature for 5 min, fixed with Carnoy's solution as described above, and slides were prepared. The slides were coated with Kodak nuclear track emulsion, type NTB 2, at 45°C, and exposed for 7 days at 4°C. They were then developed in Kodak D19 for 3 min at 20°C, fixed with Kodak rapid fixer for 2 min, stained with Giemsa's and grain counts were recorded.

RESULTS

TLN Formation Using I Cells in Different Stages of the Cell Cycle

It was first necessary to establish the G1, S, and G2 periods of the cell cycle after release from Colcemid inhibition. Fig. 1 shows that the peak of the S period occurred about 6 h after the release. The G1 period lasted about 3 h when 50% of the cells had entered S (Fig. 1), and in subsequent experiments this time was taken as the point of arrival at the G1/S boundary.

Cells in different stages of the cell cycle were found to have different capacities to induce the TLN when fused with standard M cells. As shown in Table I, only about 20% of G1 cells, 50% of S cells, and about 70% of the cells in a mixed population of late-S and G2 had the capacity, respectively.

It is known that when treatment with CHE, an inhibitor of protein synthesis (21), is initiated during late prophase or metaphase, completion of cell division and nuclear reconstruction are observed even though protein synthesis is inhibited (2). However, as reported by many workers, inhibition of protein synthesis in G1 prevents progression to S (11). The same results were obtained in the present work. When CHE...
was added at 20 μg/ml to M cells at the beginning of incubation in the absence of Colcemid under the usual conditions (see Materials and Methods), the metaphase index fell from 95% to almost 0 within 90 min, but the cells were prevented from entering S and so remained in G1. Protein synthesis was completely blocked, RNA synthesis was markedly depressed, and cells did not enter S, as evidenced by failure to incorporate [3H]TdR, i.e., less than 1% of them exhibited more than 10 nuclear grains after the standard pulse (Fig. 1), whereas in the absence of CHE about 7% of the cells showed more than 40 grains. When the inhibitor was removed, the cells moved into S, with some delay compared with the progression of the M cells that had not been treated with CHE, as shown in Fig. 1.

A block of the G1 to S progression by CHE prevented the increase in TLN-forming ability as shown in Table II. It should be noted in Table II that, concomitantly with failure of CHE to prevent the M to G1 progression, CHE did not inhibit the ability of such early G1 cells to induce TLN formation. (In separate experiments it was found that 20 μg/ml of CHE added only during fusion had no effect on TLN formation or prophasing.) There is a suggestion in the results of exp. 4 in Table II that cells at the G1/S boundary have a greater capacity than cells in early G1 to induce TLN formation.

It should be noted in Table II and in additional results shown below in this paper that in all cases when frequency of TLN formation was high, the frequency of prophasing was low and vice versa. This inverse relationship has been documented previously (3, 13, 15).

The inhibitory effect of CHE, present for 5.5 h commencing with the release of the metaphase block, on subsequent TLN formation could be reversed by changing to fresh CHE-free medium for an additional 5.5 h before fusion. These results appear in Table III.

**Table I**

<table>
<thead>
<tr>
<th>Time after release from Colcemid</th>
<th>Stage</th>
<th>TLN</th>
<th>P</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 G1</td>
<td></td>
<td>22.0</td>
<td>72.7</td>
<td>5.3</td>
</tr>
<tr>
<td>5.5 S</td>
<td></td>
<td>53.7</td>
<td>36.3</td>
<td>10.0</td>
</tr>
<tr>
<td>10.5 Late-S/G1</td>
<td></td>
<td>67.7</td>
<td>26.0</td>
<td>6.3</td>
</tr>
</tbody>
</table>

A synchronized 1-cell population originated from Colcemid-arrested M cells which were collected by shaking log-phase monolayer cultures that had been exposed to Colcemid for 4 h at a concentration of 0.04 μg/ml. After Colcemid release, M cells were resuspended in prewarmed fresh medium, and incubated for 1.5, 5.5, and 10.5 h to obtain G1, S, and late-S/G1 cells, respectively, as shown in Fig. 1. Cells at each stage were fused separately with standard M cells.

**Table II**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Time after release from Colcemid block</th>
<th>TNL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-CHE</td>
<td>+CHE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-CHE</td>
<td>+CHE</td>
</tr>
<tr>
<td>1–3</td>
<td>1.5</td>
<td>21.3 (17.6–24.3)</td>
<td>23.6 (19.3–27.6)</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>53.1 (44.5–61.0)</td>
<td>19.1 (15.4–22.5)</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>68.3 (69.7–71.0)</td>
<td>16.2 (14.7–17.7)</td>
</tr>
<tr>
<td>4</td>
<td>3.0*</td>
<td>37.0</td>
<td>27.0</td>
</tr>
</tbody>
</table>

The experiments were conducted in the same way as in Table I except that CHE, 20 μg/ml, was supplemented to replicate cultures at the time fresh medium was added to the M cells. For exps. 1–3, the data are presented as averages with ranges in parentheses; 300 cells were counted in each experiment. For exp. 4, 100 cells were counted. *G1/S boundary.
After Colcemid-arrested M cells were washed three times with ice-cold fresh Colcemid-free medium, the M cells were resuspended in prewarmed fresh CHE-supplemented (20 μg/ml) medium, in three T60 culture flasks, and incubated for 5.5 h. At this time, one of the CHE-treated cultures was fused with standard M cells, one of them was released from CHE inhibition by replacing with prewarmed fresh medium for 5.5 h, and simultaneously one of them was replenished with prewarmed fresh CHE medium with the same concentration of inhibitor for 5.5 h. The CHE-treated and CHE-released I cells were then fused with nontreated standard M cells.

### Table III

<table>
<thead>
<tr>
<th>Time after release from metaphase arrest</th>
<th>Treatment</th>
<th>Binucleate (1M/1I)</th>
<th>Trinucleate (1M/2I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLN  P  No change</td>
<td>TLN  P  No change</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5 CHE</td>
<td>19.1 74.2 6.7</td>
<td>—  —  —</td>
<td></td>
</tr>
<tr>
<td>11.0 CHE</td>
<td>18.6 75.2 6.2</td>
<td>25.0 64.0 11.0</td>
<td></td>
</tr>
<tr>
<td>5.5 + CHE</td>
<td>71.5 19.0 9.5</td>
<td>89.0 7.0 4.0</td>
<td></td>
</tr>
</tbody>
</table>

After Colcemid-arrested M cells were washed three times with ice-cold fresh Colcemid-free medium, the M cells were resuspended in prewarmed fresh CHE-supplemented (20 μg/ml) medium, in three T60 culture flasks, and incubated for 5.5 h. At this time, one of the CHE-treated cultures was fused with standard M cells, one of them was released from CHE inhibition by replacing with prewarmed fresh medium for 5.5 h, and simultaneously one of them was replenished with prewarmed fresh CHE medium with the same concentration of inhibitor for 5.5 h. The CHE-treated and CHE-released I cells were then fused with nontreated standard M cells.

**Effect of CHE on Cells in G1 in Confluent Monolayer**

The effect of CHE on cells in confluent monolayers was examined. Cells were allowed to grow to confluence (see Materials and Methods) and examined for their ability to incorporate [3H]TdR in 10 min supplemented at 1 μCi/ml. About 20% could do so, indicating that about 80% of the cells were in G1 (25). If such cells were exposed to CHE for 15 h only about 8% of them could induce TLN formation, whereas 36% of the untreated cells had this capacity. When the inhibitor was removed for 5 h about 28% of the cells now had the capacity. The results appear in Table V. It should be noted that after release from the CHE block no cells incorporated isotope in a 10-min pulse with [3H]TdR, indicating that all were still in the G1 period, as given in the protocol to Table V. It could also be ascertained that 5 h after release from the CHE block the cells were still in G1, independently of scoring [3H]TdR incorporation. It is known (4, 5, 19, 20, 22, 27) that prophased G1 chromatin can be distinguished from prophased G2 and S chromatin. In one of the three experiments of Table V that involved the 5-h release from the CHE block followed by fusion, of 100 I-M cells showing prophasing 75% of the prophased chromatin was of the G1 type, 20% were of the S type, and not more than 5% were of the G2 type. In the case of exposure to CHE for 20 h, the percentages were 73, 22, and about 5, respectively. The discrepancy between 100% (TdR data) and 75% (prophasing data) is probably due to some ambiguity in subjective evaluation of prophased chromatin. It should also be noted that progression to G2 after the release of the CHE block is unlikely in 5 h, because the S period is at least 6 h in duration. In Fig. 2 the appearance of prophased chromatin of I nuclei of cells from a confluent monolayer that had been exposed to CHE for 15 h...
TABLE IV

Effect of CHE on the Ability of Cells at the G1/S Boundary to Induce TLN Formation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment no.</th>
<th>TLN</th>
<th>P</th>
<th>No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdR</td>
<td>1</td>
<td>84.3</td>
<td>10.3</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>83.0</td>
<td>11.3</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>83.6</td>
<td>10.8</td>
<td>5.6</td>
</tr>
<tr>
<td>TdR + CHE</td>
<td>1</td>
<td>34.6</td>
<td>57.7</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33.0</td>
<td>59.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.3</td>
<td>56.0</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Colcemid-arrested (0.04 µg/ml, 4 h) M cells were released from the mitotic arrest by washing three times with cold, fresh medium and resuspended in prewarmed fresh medium containing TdR (5 mM). After 3 h of incubation, when the cells progressed from metaphase to the G1/S boundary, the medium of the cultures was replaced by prewarmed fresh medium containing 5 mM TdR only or 5 mM TdR plus CHE (20 µg/ml), and the cultures were incubated further for 15 h. Fusion was then performed with standard M cells. At least 100 binucleate cells with TLN, P, or no change were examined in each experiment.

In a separate experiment, commencing 3 h after release from Colcemid, four cultures were exposed to 5 mM TdR for 15 h. After the 15-h period, the monolayers were washed three times with prewarmed fresh medium (no TdR) and fresh medium was added. Then (a) two cultures were immediately pulsed for 10 min at 37°C with 1 µCi/ml of [3H]TdR and (b) two were allowed to incubate for 30 min at 37°C and an identical pulse was applied. Although in case (a) only about 7% of the cells exhibited more than 10 nuclear grains, in case (b) about 85% of the cells exhibited at least 50 nuclear grains each, indicating that the 5 mM TdR had held the cells in S or near the G1/S boundary.

and then freed of the antibiotic for 5 h is compared with that produced by fusion of standard M cells with G1 cells and with S cells. These results indicate that before fusion of G1 cells with M cells, continued synthesis of cellular protein in the G1 cells is needed for efficient formation of the TLN.

Inhibition of TLN Formation by AMD

Cycloheximide may cause a reduction in the rate of translation of messenger RNA (mRNA) information into protein (16). Continued synthesis of protein in late G1 appeared to be necessary for TLN formation. It was possible that mRNA(s) needed for the synthesis of the essential protein(s) is formed in G1. If this were the case, inhibition of RNA synthesis should prevent the cells in G1 from attaining their capacity to induce TLN formation. AMD at 2 µg/ml presumably blocks mRNA synthesis in Chinese hamster cells (26); at this concentration in either nonfused I cells or fused I-M binucleate cells, RNA synthesis in the I nuclei was extensively inhibited, as shown by the autoradiographic data in Table VI. Accordingly, M cells after release from Colcemid block were exposed to CHE for 5.5 h, the time in which they would progress into G1 but not into S. They were then washed free of CHE and placed in fresh medium with or without 2 µg/ml of AMD for an additional 5.5 h. They were then fused with standard M cells and TLN formation was scored.

The results of this treatment with AMD on TLN formation are given in Table VII. When the cells were released from the CHE inhibition in the presence of AMD, the results were almost the same as those found when CHE was not removed (Table III), i.e., only about 20% of the cells could induce TLN formation in binucleate cells. In contrast, the capacity to induce TLN by the sample untreated with AMD was about three times that of the treated sample (Table VII). In the case of trinucleate cells (one M/two I), treatment with AMD of the I cells that were subsequently used to form these fused cells resulted in 50% inhibition of TLN formation (Table VII).

DISCUSSION

The major observable structural event, possibly the only one, that is related to TLN formation in the 30-min period when I cells are fused with M cells by UV-inactivated Sendai virus in the presence of Colcemid is the formation of NE around the metaphase chromosomes in the I-M cell (3, 13, 15). The formation of this NE is believed to be a normal mitotic event representative of NE formation in the normal mononucleate cell cycle, but in the I-M cell it has been isolated temporally from events antecedent to it in the mononucleate metaphase to telophase progression (15).

Up to now, four reasons could be marshalled for considering the NE of the TLN as a normal cellular structure: (a) Ultrastructurally, it is...
TABLE V

Reversible Inhibition by CHE of the Ability of Cells in Confluent Monolayers to Induce TLN Formation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Treatment</th>
<th>TLN (%)</th>
<th>P (%)</th>
<th>No change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>None</td>
<td>36.0 (30.0-44.0)</td>
<td>57.0 (52.0-61.0)</td>
<td>7.0 (4.0-9.0)</td>
</tr>
<tr>
<td>15</td>
<td>CHE</td>
<td>8.0 (6.0-11.0)</td>
<td>87.0 (86.0-88.0)</td>
<td>5.0 (3.0-7.0)</td>
</tr>
<tr>
<td>15</td>
<td>+CHE</td>
<td>28.3 (25.0-31.0)</td>
<td>61.7 (60.0-65.0)</td>
<td>10.0 (9.0-11.0)</td>
</tr>
<tr>
<td>20</td>
<td>-CHE</td>
<td>8.3 (7.0-9.0)</td>
<td>85.0 (82.0-89.0)</td>
<td>6.7 (4.0-9.0)</td>
</tr>
</tbody>
</table>

Three experiments were performed. In each, after seeding each Falcon plastic culture flask with about 10^6 cells, growth to confluence was obtained about 50 h later. At this time, all flasks received fresh medium. One of the flasks was incubated for an additional 15 h, and the cells were fused with standard M cells. Three of them were supplemented with CHE, 20 μg/ml, and incubated for 15 h. One of the CHE-treated flasks was fused with standard M cells, and the remaining two flasks were rinsed three times with fresh medium; one of the flasks was supplemented with 20 μg/ml of CHE, the other received no supplement. After an additional 5 h of incubation the cells were fused with standard M cells. The data are averaged for three experiments with the ranges in parentheses. 100 cells were counted in each experiment.

In each of the three experiments a monolayer after release of the CHE block was pulse-labeled with [3H]TdR (1 μCi/ml) for 10 min, and the cells were recovered by trypsinization and subjected to autoradiography. In 100 cells examined at random, no grains were detected, in contrast to frankly S-phase cells as in Fig. 1.

difficult to distinguish it from the NE of the normal I nucleus (3, 13, 15); (b) The pH dependence of its formation resembles that of the normal M to G1 progression (15); (c) The TLN after its initial formation can progress to a G1-like nucleus, including formation of nucleoli (14); and (d) The probability of formation of the TLN in a fused I-M population is directly dependent on the ratio of I nuclei to the chromosome sets within the fused cells, i.e., the formation of the NE of the TLN is dependent on a contribution from the I cell rather than from the fusion virus (3, 13, 15).

The current results constitute additional evidence that formation of the NE of the TLN is a normal mitotic event. A block of protein synthesis by CHE in the G1 period of the I cells, before their exposure to virus and fusion with standard

FIGURE 2a A binucleate cell with prophasing showing G1-type chromatin. The picture was taken from the 1.5-h sample of Table I.

FIGURE 2b A binucleate cell with prophasing showing S-type chromatin. The picture was taken from the 5.5-h sample of Table I.

FIGURE 2c A binucleate cell with prophasing showing G1-type chromatin. The picture was taken from a sample treated with CHE (20 μg/ml) for 10.5 h (see Table III).

FIGURE 2d A trinucleate cell with two prophased nuclei showing G1-type chromatin. The picture was taken from a sample in which confluent monolayer cells were exposed to CHE (20 μg/ml) for 20 h (see Table VI).

FIGURE 2e A binucleate cell showing G1-type prophasing. The picture was taken from a sample in which confluent monolayer cells were released from CHE block for 5 h after a CHE block for 15 h (see Table VI).

FIGURE 2f A binucleate cell containing TLN and an I nucleus. The picture was taken from the same sample as that in Fig. 2c.

384 THE JOURNAL OF CELL BIOLOGY · VOLUME 64, 1975
M cells, markedly reduced the number of I cells that could induce TLN formation. Similar results were obtained using cells held in S by 5 mM TdR. Additionally, inhibition of RNA synthesis in G₁ by means of AMD before fusion drastically reduced TLN formation when such treated cells were subsequently fused with the standard M cells. Thus, synthetic events in the I cell, unrelated to exposure to fusion virus, govern its capacity to induce NE formation in the I-M cell.

In sharp contrast macromolecular synthesis in the I cell is, very probably, not needed for prophasing of the I nucleus by the M cell contribution. This probability stems from the current finding that reduction of efficiency of TLN-forming capacity by treatment of I cells with CHE and AMD enhanced prophasing in such treated I-cell populations; macromolecular synthesis required for prophasing probably takes place in the prior G₀ period of the M cell before it is fused with the I cell (7). The results offer additional evidence that balances between I-cell factors and M-cell factors are crucial to NE formation or degradation (3, 13, 15).

The current findings strengthen our previous proposal (15) that the fused I-M Don cell at pH 8.0 (and probably the fused I-M HeLa cell at pH 8.5 [13]) provides a tool for studying the parameters that regulate formation of the NE as an isolated mitotic event.

With regard to such parameters the present results raise questions about the nature of the macromolecules which are needed for NE formation of the TLN. Are the proteins that are synthesized in the I cell specific for this phenomenon? Alternatively, is it due to the totality of new protein known to accumulate as the cells enlarge in the G₁ to G₂ progression or in TdR-blocked cells (1, 24)?

We are prejudiced in favor of specific macromolecules which may either be catalytic in nature or become structural components of the NE of the TLN. The pH specificity (13, 15) of TLN formation tends to support this hypothesis. Additionally, there is a precedent for such specificity in that synthesis in G₁ of HeLa cells of at least one protein needed for attachment of DNA to the nuclear membrane has been observed by Yamada and Hanakoa (28).

At first glance a surprising result is that G₂ cells probably have the highest concentration of macromolecules needed for NE formation, that is, at a stage when the cell is also synthesizing those entities needed for entry into mitosis (26) which involves disruption of the NE. In light of the probability of a balance between formative

### Table VI

<table>
<thead>
<tr>
<th>Grain counts in nuclei of 300 cells</th>
<th>AMD-treated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-TLN</td>
<td>Single I</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>21-30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31-40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>41</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Log-phase monolayer cells were exposed to Colcemid for 5 h at a concentration of 0.08 μg/ml. AMD was added to the cultures at a final concentration of 2 μg/ml for the last hour of the Colcemid treatment. The trypsin-freed, AMD-treated cells were then fused together and exposed to [³H]uridine (4.0 μCi/ml) for the final 20 min of incubation after fusion in the presence of AMD.

### Table VII

<table>
<thead>
<tr>
<th>Treatment after release from CHE block*</th>
<th>Binucleate (1M/1I)</th>
<th>Trinucleate (1M/2I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLN</td>
<td>P</td>
</tr>
<tr>
<td>+ AMD, 2 μg/ml</td>
<td>22.7</td>
<td>72.3</td>
</tr>
<tr>
<td>– AMD</td>
<td>66.8</td>
<td>24.3</td>
</tr>
</tbody>
</table>

* Treatment of M cells with CHE for 5.5 h and the subsequent release from the inhibitor was the same as described in Tables III and IV. Treatment with AMD for a subsequent period of 5.5 h is described in the text. The data are the averages of three experiments.
and disruptive agents (3, 13, 15), the cell must have some way of achieving the proper balance in prophase. One way may be by partial degradation to avoid an excess of the formative agents; we have presented evidence in the present paper that in G₁, continuing protein synthesis is necessary for efficient TLN formation which implies that the formative agents can be degraded. Since prophasing and TLN formation have different pH optima (15) another way may be by a fine adjustment of intracellular pH to allow prophase to occur, keeping the formative agents quiescent.

This work was supported in part by grant CA-16935 from the National Cancer Institute. 

Received for publication 20 May 1974, and in revised form 26 September 1974.

REFERENCES


23. STUBBLEFIELD, E., and R. KLEVECZ. 1965. Synchro-


