Chromosomal Changes without DNA Overproduction in Hydroxyurea-treated Mammalian Cells: Implications for Gene Amplification

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ABSTRACT

It has been reported that a 6-h incubation of early S-phase Chinese hamster cells with hydroxyurea promotes DNA overproduction, i.e., replication of DNA a second time within a single cell cycle, and that this could be the basis for gene amplification in drug-treated mammalian cells. When we incubated methotrexate-resistant Chinese hamster cells that were approximately 2 h into the S phase with hydroxyurea for 6 h, DNA that had been replicated before the incubation with hydroxyurea (early S-phase DNA) was replicated again within 11 h after the hydroxyurea treatment. However, incubation with colchicine or Colcemid after hydroxyurea treatment virtually abolished this overreplication, as well as that of the amplified dihydrofolate reductase genes in these cells, indicating that the second replication had occurred in a second cell cycle. Cells collected in the first mitosis after incubation with hydroxyurea never contained overreplicated DNA but did contain abundant chromosome aberrations. Early S-phase DNA replicated again on schedule during the first few hours after mitosis. Asymmetric segregation of chromosome fragments or unequal sister chromatid exchange may be the actual basis for gene amplification in drug-treated mammalian cells.

INTRODUCTION

Gene amplification is a process whereby the number of copies per cell of a given gene increases, and consequently the amount of that gene's product also increases (1). In mammalian cells, the most frequently studied system is the development of resistance to methotrexate by amplification of the DHFR gene (2, 3). This process has been studied primarily in cultured cells; amplification events in mammalian cells have been observed in nature (4), but thus far only in aneuploid cells. Gene amplification in Drosophila (5) involves developmentally controlled processes that cause overreplication of small portions of the genome by iterative initiation at single DNA replication origins. Because intermediates are envisaged in these processes, in which multiple copies of both DNA strands lie within the same structure, the term "onion-skin model" has been coined to describe this method of gene amplification (6, 7). In applying this model to gene amplification in drug-treated mammalian cells, a seminal idea is that the two DNA growing points of a replicon diverging from its origin are stalled, i.e., completely blocked, by an inhibitor of DNA synthesis. The inhibitor may be either the selective agent itself, e.g., methotrexate, or a second DNA synthesis inhibitor, e.g., hydroxyurea. This stalling is thought to lead to derangement of a DNA replication control system, so that abnormal reinitiation at the origin(s) occurs, leading to a 2-fold or more amplification of that region of the genome.

Experimental approaches to elucidate the mechanism of gene amplification in mammalian cells have generally fallen into two categories: (a) structural analysis of the DNA or chromosomes of cells that have been selected (8–11) or (b) search for agents that will increase the spontaneous levels of gene amplification and analysis of the mode of action of these agents to arrive at a conclusion about the underlying process (12–16). Mariani and Schimke (16) presented evidence that treatment of Chinese hamster cells during early S phase with hydroxyurea, a potent inhibitor of ribonucleotide reductase, induces all DNA replicated before the addition of hydroxyurea to be replicated again within that same cell cycle. This would result in a 2-fold amplification of that DNA replicated before the block.

A problem with the concept of stalled growing points is that inhibitors of DNA synthesis such as hydroxyurea do not block DNA growing points; they only slow them in a dose-dependent manner (17, 18). Therefore, we sought another explanation for gene amplification in mammalian cells. We repeated and extended the experiments of Mariani and Schimke (16), and our results led not only to an alternative explanation for their data, but also to alternative explanations for the mechanism of gene amplification in drug-treated mammalian cells.

MATERIALS AND METHODS

Cell Culture. CHO-B11 cells (from A. Hill, Stanford University) are the same cells used by Mariani and Schimke (16) and are resistant to high concentrations of methotrexate because they contain an amplified array of approximately 50 copies of stably integrated DHFR genes. These cells were maintained as monolayers in Ham's F-12 medium lacking glycine, hypoxanthine, and thymidine and supplemented with 10% dialyzed fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and 0.5 μM methotrexate.

Cell Synchrony. Mitotic cell populations were selected by means of an automated synchrony apparatus (Talandic Research Corp., Pasadena, CA) (19). CHO-B11 cells were plated into 750-cm² roller bottles and maintained at 1 rpm. For the experiment described in Fig. 1, the synchronized cells were selected in Ham's medium lacking glycine, hypoxanthine, and thymidine. For the experiments described in Fig. 3, to ensure uniform, nonconfluent late log phase monolayers that would yield a maximum number of mitotic cells, we subcultured the cells in each roller bottle 2:1 into new roller bottles containing complete Ham's F-12 supplemented with whole serum 24 h before the beginning of synchrony.

Before beginning the experiments with mitotically selected cells, we rotated the monolayers in the roller bottles at 75 rpm for 1 min each hour to remove loosely attached cells and debris and then refed the cells. This was repeated hourly for 24 h. Under these conditions, it was possible to obtain 4 to 5 x 10⁶ mitotic cells in a single selection with a mitotic index of >95%. Therefore, a single selection was sufficient to perform each of the experiments described in Figs. 3 and 4. The cell cycle was determined in each experiment with each population of B11 cells. The length of the cycle, G1 and S phase, was observed each time that the cells were used for hydroxyurea-BrdUrd experiments. To measure S phase, we pulse labeled synchronized cells for 30 min with [³H]thymidine (2 μCi/ml; specific activity, 60 Ci/mmol). Cells were then washed with SSC and trypsinized. One-half of the cells were counted in a Coulter Counter, and the remainder of the sample was counted.
used to determine $^{3}H$ cpn incorporation in the acid-precipitable cellular fraction (19).

For experiments, cells were treated essentially as described by Mariani and Schimke (16). Starting immediately after selection, the cells were incubated in medium containing BrdUrd $(10^{-3}$ M) and $[^{3}H]$thymidine (3 $\mu$Ci/ml; 50 Ci/mmol) for 8 h. At the end of this time the medium was removed, and medium containing BrdUrd $(10^{-3}$ M) and hydroxyurea (0.3 mM) was added. After 6 h this medium was removed, the cells were washed with fresh medium, and medium containing BrdUrd $(10^{-3}$ M) was added. At this point, variations in the protocol were introduced. The details of each protocol are given in "Results."

Cesium Chloride Equilibrium Density Centrifugation. Cellular DNA was isolated as previously described (20). Cell monolayers were washed with SSC and lysed with 0.1% sodium dodecyl sulfate. The lysate was incubated for 1 h with RNase (100 ng/ml, 37°C) and for 1 h with Pronase (100 ng/ml, 37°C). The preparation was extracted with chloroform:isoamyl alcohol (24:1) 2 or 3 times. The aqueous layer was then removed and dialyzed against SSC. After dialysis, the DNA samples were sheared with a Virtis homogenizer. Exactly 4.5 ml of sample were added to 5.9 g of CsCl and centrifuged at 37,000 rpm for 48 h or more in a Beckman TI-50 rotor. After centrifugation, a hole was made in the bottom of each tube, samples were collected, and the DNA in the solution was precipitated with 4% perchloric acid and passed through glass fiber filters. The $^{3}H$ on the filters was measured by liquid scintillation spectrometry.

Dot Blot Analysis. For detection of DHFR genes, fractions from CsCl gradients containing the heavy-light or heavy-heavy DNA were pooled, dialyzed, ethanol precipitated, and subjected to the dot blot procedure of Kafatos et al. (21). The probe was a $^{32}$P-labeled mouse complementary DNA (22).

Cytogenetic Analysis. Synchronized CHO-B11 cells (mitotic index, $\geq 95\%$) were treated as described for the CsCl centrifugation experiment, except that no radioisotope was added to the cultures. After the 6-h incubation with hydroxyurea (0.3 or 1.0 mM), fresh medium containing BrdUrd $(10^{-3}$ M) was added to the cultures, and 2 h later Colcemid $(2 \times 10^{-7}$ M) was added. Separate cultures were maintained in medium containing BrdUrd for 12 h before addition of Colcemid. Mitotic cells were collected at 3-h intervals after Colcemid treatment. Metaphase preparations were processed for cytogenetic analysis by routine methods and stained by the fluorescein-plus-Giemsa technique (23). This procedure permitted us to determine cytogenetically if any parts of the chromosomes had replicated more than once before arriving at metaphase. The detailed results of this analysis have been reported by Morgan et al. (24). In some experiments, the cells were allowed to go through another cell cycle in the presence of BrdUrd before collection of mitotic cells, so that we could measure the frequency of SCE.

RESULTS

When mitotically synchronized CHO-B11 cells, the cell line used by Mariani and Schimke, were incubated with BrdUrd and $[^{3}H]$thymidine for 8 h, BrdUrd and hydroxyurea for 6 h, and BrdUrd for 11 h, the results were almost identical to those reported by Mariani and Schimke (Ref. 16; Fig. 1). More than half of the DNA that had replicated in the presence of $[^{3}H]$-BrdUrd during the first 8 h after mitotic collection (early S-phase DNA) was found in the "heavy-heavy" region of the CsCl gradient, corresponding to the density of DNA labeled in both strands with BrdUrd. Therefore, the early S-phase DNA had entered another round of replication. However, when parallel cultured cells were simultaneously treated in exactly the same way except for the addition of $2 \times 10^{-5}$ M colchicine to the medium for 11 h after removal of hydroxyurea, only a very small amount of the early S-phase DNA had completed a second round of replication (Fig. 1). It should be noted that, although colchicine and Colcemid inhibit Chinese hamster cell division, they delay entry into a second cell cycle by only 2 to 3 h (25). For this reason, these spindle inhibitors are often used to induce tetraploidy (26). The small amount of heavy-heavy DNA visible in the colchicine-treated culture is very likely due to reentry into S phase of the second cell cycle after a failed attempt to undergo cell division. The experiment described in Fig. 3 was designed to address this point further.

The timing of the appearance of the heavy-heavy DNA varied from one experiment to another; occasionally there was no heavy-heavy DNA in cells incubated with or without colchicine at the 11-h posthydroxyurea time point. We attribute this variability to differences in cell cycle time brought about by the restrictive nature of the selective medium (Ham's F-12 lacking glycin, thymidine, and 5-hyoxanthine and supplemented with dialyzed fetal calf serum plus 0.5 $\mu$M methotrexate). The growth rate of cells in this medium is dependent on feeding schedules and cell density. Therefore, for the next experiment we used synchronized cells grown in complete F-12 medium supplemented with whole fetal calf serum, and we checked the cell cycle (Fig. 2) immediately before the experiment (16-h cycle time, with S phase commencing at 4 to 6 h). The synchronized cells collected at the zero time point of the cell cycle determination in Fig. 2 were used for the following experiment.

The cells were plated into one large flask and incubated with BrdUrd and $[^{3}H]$thymidine for 8 h and then with BrdUrd and hydroxyurea for 6 h. After the hydroxyurea treatment, the cells were incubated with medium containing both BrdUrd and Colcemid (a more reversible microtubule inhibitor than colchicine), so that every 2 h we could shake off the cells that had accumulated in mitosis. Again, Colcemid inhibited cell division and allowed the cells to accumulate in mitosis for a few hours, but it only postponed, rather than blocked, entry into the following
cell cycle, even in the continued presence of Colcemid. Half of the cells collected at each shake-off were lysed immediately; the other half were transferred to another flask to continue incubation for a total of 20 h after hydroxyurea treatment before being lysed. In this way, we were able to distinguish between events occurring before and after the first mitosis after hydroxyurea treatment. A diagram and explanation of the protocol for this experiment are given in Fig. 3.

The results of CsCl equilibrium density gradient analysis of the various cell samples showed the following. (a) None of the mitotic cells that accumulated with Colcemid contained measurable quantities of overreplicated (heavy-heavy) DNA at the time that they were collected (4, 6, 8, or 10 h after removal of hydroxyurea) (Fig. 4). (b) All cell populations that had been collected in mitosis and reincubated with Colcemid in a second flask (for a total of 20 h after removal of hydroxyurea) contained replicated DNA, the percentage of which increased with time of incubation after collection (Table 1). The apparent linear increase in heavy-heavy DNA with time probably reflects partial decay of synchrony induced by the hydroxyurea treatment.

Because none of the cells contained $^3$H in heavy-heavy DNA at the time of mitotic harvest, no early S-phase DNA had replicated a second time in the first cell cycle. The highest yield of mitotic cells was obtained at the collection made 6 h after removal of hydroxyurea, but there were already many cells in the mitotic harvest at 4 h. This shows that the most frequent time for cells to progress from the end of hydroxyurea treatment to mitosis was only 4 to 6 h, and many cells took less than 4 h.

To show that the presence of Colcemid did not interfere with the ability of the cells to replicate their DNA a second time within a single cell cycle, we performed a similar experiment without Colcemid. Cells from four roller bottles were used for a single mitotic selection, and all the detached mitotic cells were plated into a single roller bottle. As in the other experiments, $[\text{H}]$thymidine and BrdUrd were present for the first 8 h, followed by 0.3 mM hydroxyurea and BrdUrd for 6 h, followed by BrdUrd only (no Colcemid). After hydroxyurea was removed from the medium, the roller bottle was rotated at 75 rpm for 1 min every h, and the detached mitotic cells were aspirated from the bottle. Medium was replaced with fresh medium, and the process was repeated at hourly intervals. At alternate hours, the aspirated mitotic cells were either lysed immediately and prepared for CsCl gradient centrifugation or plated into separate flasks and incubated for a total of 12 h after hydroxyurea removal, then lysed, and prepared for CsCl gradient analysis. Fewer cells were collected at each time point than in experiments in which Colcemid accumulated cells in mitosis. Nevertheless, heavy-heavy DNA was never observed in the mitotic cells (Fig. 5A) but was observed in the cells that were allowed to continue past mitosis (Fig. 5B), indicating that, even in the absence of Colcemid, the second replication of DNA occurred only after mitosis in a second cell cycle.

In a similar experiment, cells were detached by mitotic shake-off in the absence of Colcemid at 4, 6, 8, 10, 12, 14, and 16 h after the removal of hydroxyurea and lysed immediately. The samples were pooled (to increase the sensitivity of the experiment) and analyzed by CsCl density gradient analysis (Fig. 6A), as was the DNA from the cells remaining on the monolayer (Fig. 6B). (The cells on the monolayer are cells that had escaped mitotic harvest, some of which had entered S phase of the next cell cycle.) Heavy-heavy DNA was not observed in the cells harvested at mitosis, but was observed in the cells remaining on the monolayer.

We also measured the effects of the 6-h incubation with hydroxyurea on DNA synthesis; the incorporation of $[\text{H}]$thymidine into DNA was reduced to 20% of control rates by this treatment. Therefore, the cells had progressed for about an hour's equivalent of S phase during the 6-h incubation with hydroxyurea and were not completely blocked, as reported by Mariani and Schimke (16). Our results are consistent with other reports on inhibition of DNA synthesis by hydroxyurea (17, 18).

Even though the major part of early S-phase DNA was not replicated twice in the same S phase after the hydroxyurea block, it was possible that the DHFR gene itself had been overreplicated. To determine if this was so, the heavy-light

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**Table 1 Replication of early S-phase DNA after mitosis**

<table>
<thead>
<tr>
<th>Time in second flask (h)</th>
<th>DNA replicated (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td>14</td>
<td>77</td>
</tr>
<tr>
<td>16</td>
<td>91</td>
</tr>
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</table>

*Percentage of $^3$H-labeled early S-phase DNA found in the heavy-heavy fraction of equilibrium density gradients after mitotic shake-off, transfer to a separate flask, and continued incubation (see Fig. 3).*
HYDROXYUREA AND GENE AMPLIFICATION

Fig. 5. CsCl density gradient profiles of [3H]DNA from CHO-B11 cells incubated with [3H]thymidine and BrdUrd for 8 h, then with hydroxyurea for 6 h, and then with BrdUrd only (no Colcemid) until shake-off in mitosis. A, DNA from cells lysed immediately after collection in mitosis at 12 h after hydroxyurea; B, DNA from mitotic cells transferred to a second flask at 7 h after hydroxyurea and incubated for 5 h in medium with BrdUrd. HH, heavy-heavy; HL, heavy-light.

Fig. 6. CsCl density gradient profiles of [3H]DNA from CHO-B11 cells incubated with [3H]thymidine and BrdUrd for 8 h, then with hydroxyurea and BrdUrd for 6 h, and then with BrdUrd only (no Colcemid). A, profile of DNA from mitotic shake-offs at 4, 6, 8, 10, 12, 14, and 16 h after removal of hydroxyurea. Cells were lysed immediately after shake-off, and cell samples were pooled for analysis. B, profile of DNA from the cells remaining on the monolayer. HH, heavy-heavy; HL, heavy-light.

Fig. 7. Dot blot analysis of 32P-labeled DHFR-hybridizable sequences in heavy-heavy (HH) and heavy-light (HL) DNA from CHO-B11 cells incubated with or without colchicine. Cells were treated as described in Fig. 1 but yielded a CsCl density gradient profile indicating that only about one-fifth of the DNA in cells not incubated with colchicine had rereplicated. The 32P in the heavy-heavy DNA spots was autoradiographed with (HH(i)) or without (HH) an intensifying screen. The spots represent one-fourth of the sample and were excised after autoradiography and counted in a liquid scintillation spectrometer for the presence of 3H used in the initial labeling. There were 78 cpm in heavy-heavy DNA from cells treated with colchicine and 319 cpm in heavy-heavy DNA from cells treated without colchicine. There were 10 cpm in a random, unlabeled nitrocellulose fragment.

Table 2 Effect of hydroxyurea, methotrexate, and hydroxyurea plus methotrexate on survival of CHO-B11 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies/plate</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX (0.5 (\mu M))</td>
<td>136 ± 20</td>
<td>34</td>
</tr>
<tr>
<td>MTX (0.3 mM) + MTX (0.5 (\mu M))</td>
<td>136 ± 20</td>
<td>34</td>
</tr>
<tr>
<td>HU only (50 (\mu M))</td>
<td>279 ± 5</td>
<td>70</td>
</tr>
<tr>
<td>HU (0.3 mM) + MTX (50 (\mu M))</td>
<td>59 ± 4</td>
<td>15</td>
</tr>
</tbody>
</table>

* MTX, methotrexate; HU, hydroxyurea.
* CHO-B11 cells are resistant to 0.5 \(\mu M\) methotrexate.
* Mean ± range.

detectable signal for DHFR sequences was found in the heavy-heavy DNA of colchicine-treated cells. Had the DHFR gene in these cells replicated to the same extent as the total DNA of cells incubated without colchicine, there would have been the same number of copies of the DHFR gene in the heavy-heavy region of the gradients whether or not the cells had been incubated with colchicine.

We were unable to repeat the abrupt gene amplification results that Mariani and Schimke (16; their Fig. 3) reported for early S-phase cells that had been incubated with hydroxyurea for 6 h. Their results indicated that this treatment, followed by incubation in 100-fold excess methotrexate, virtually eliminated the cytotoxic effects of both drugs. We incubated synchronized cells with hydroxyurea for 6 h (beginning 2 h into S phase) and then transferred them into medium containing 50 \(\mu M\) methotrexate or, as one control, 0.5 \(\mu M\) methotrexate. (CHO-B11 cells are resistant to 0.5 \(\mu M\) methotrexate.) For additional controls, synchronized cells were plated directly into 0.5 \(\mu M\) or 50 \(\mu M\) methotrexate alone. The results show that both hydroxyurea plus 0.5 \(\mu M\) methotrexate and 50 \(\mu M\) methotrexate alone killed cells, and that the effects of the two treatments were additive (Table 2), not mutually protective, as reported by Mariani and Schimke (16). We cannot explain the difference between our results and those of Mariani and Schimke (16).

The predominant cytogenetic effects of the 6-h incubation
with hydroxyurea on S-phase cells were chromosome aberrations, increased polyploidy, and increased SCEs (Table 3; Fig. 8); no radioisotope was used in these experiments. We followed the fate of aberrations into the second generation and found that they were still elevated over controls but were reduced compared to the first generation.

**DISCUSSION**

Our equilibrium density gradient results without colchicine or Colcemid treatment are essentially identical to those of Mariani and Schimke (16). However, the addition of colchicine or Colcemid delayed the appearance of a second replication of early S-phase DNA. The experiments with removal of mitotic cells show that early S-phase DNA was replicated a second time only after the cells had completed mitosis and entered the next S phase, and not, as Mariani and Schimke proposed, during a single cell cycle as aberrant rereplication. Our determination of the frequency of DHFR sequences in the heavy region of density gradients shows that the DHFR gene was also not replicated twice within a single cell cycle in hydroxyurea-treated cells. These observations are consistent with reports from other studies in which overreplication of DNA within a single cell cycle was sought but not detected (27–29).

The possibility that DNA was replicated twice in a single cell cycle, but that this process was inhibited in some way by colchicine or Colcemid, is refuted by our experiments. In non-Colcemid-treated cells, there was no overreplicated DNA in cells harvested at mitosis, but early S-phase DNA replicated again after mitosis (Figs. 5 and 6).

The apparent rapid traverse of the cell cycle after removal of hydroxyurea was probably due primarily to the fact that 0.3 mM hydroxyurea inhibits DNA synthesis to only about 20% of control. Therefore, at the end of hydroxyurea treatment, the cells had actually progressed much further through S phase than would be expected if hydroxyurea formed an absolute block to DNA synthesis. Mariani and Schimke (16) found that mitotic cells plated immediately into hydroxyurea and BrdUrd showed an appreciable amount of DHFR-hybridizing activity in heavy-light DNA. This indicates that, even in their hands, hydroxyurea was not an absolute block to replication. Thus, the presumptive overreplication they observed can be explained by this incomplete hydroxyurea block. Furthermore, several reports have indicated that other events associated with cell cycle traverse continue in the presence of a strong inhibition of DNA synthesis (30–32). Therefore, cell cycle progression may be more rapid than normal after release from an inhibition of DNA synthesis. Finally, it should be noted that Mariani and Schimke previously reported the CHO-B11 cell line to have an average cell cycle time of only 12 h (33), indicating that the 12 h during which these cells were allowed to recover from the hydroxyurea treatment would be sufficient for them to enter a second S phase, even if hydroxyurea acted as an absolute block and no other cell cycle perturbations were induced.

The “onion-skin” model has been applied to integrated SV40 sequences; in this case there seems to be no doubt that DNA replication is reinitiated within a single cell cycle (7). However, the SV40 origin of replication is absolutely required (34), and one can envisage this process as one in which the SV40 origin acts autonomously after DNA damage, i.e., escapes control by the host system, as during initiation of the lytic cycle of temperate bacteriophage. The experiments reported here show that overreplication of early S-phase DNA following a 6-h hydroxyurea block does not occur, at least within the limits of detection of this technique (~3%) in CHO cells. Although overreplication of small parts of the genome might occur at levels below this, there is still no direct evidence that overreplication occurs at any level in drug-treated mammalian cells. Therefore, alternatives to the onion-skin model should be considered.

Our results suggest two alternative mechanisms for gene amplification. (a) We have observed that hydroxyurea, even at 0.3 mM, induces large amounts and many kinds of chromosome aberrations (Table 3; Fig. 8). We speculate that the first step in gene amplification occurs during the first step of selection, when the drug, e.g., methotrexate, induces chromosome aberrations. After mitosis, a chromosome fragment containing the DHFR gene may occasionally segregate with the intact chromosome containing the gene, resulting in a cell with three instead of two DHFR genes. Fragments of various sizes are

Table 3 Aberrant and polyploid cells and SCE per chromosome induced by hydroxyurea

<table>
<thead>
<tr>
<th>Hydroxyurea (mm)</th>
<th>First division</th>
<th></th>
<th></th>
<th>Second division</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aberrant (%)</td>
<td>Polyploid (%)</td>
<td></td>
<td>Aberrant (%)</td>
<td>Polyploid (%)</td>
<td>SCE/chromosome</td>
</tr>
<tr>
<td>0</td>
<td>4.6</td>
<td>3.0</td>
<td>3.6</td>
<td>2.2</td>
<td>0.54 ± 0.023*</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>17.6</td>
<td>7.6</td>
<td>12.6</td>
<td>10.0</td>
<td>0.78 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>21.0</td>
<td>8.6</td>
<td>14.2</td>
<td>9.0</td>
<td>1.30 ± 0.086</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SE.

Fig. 8. Examples of chromosome aberrations seen in CHO-B11 cells after a 6-h hydroxyurea treatment. A, an acentric fragment (arrow) resembling a double minute; B, multiple chromosome fragments and rearrangements.
seen in the first metaphase after hydroxyurea treatment (Fig. 8). We further suggest that once these fragments are generated by methotrexate or hydroxyurea, they behave in a manner analogous to chromosome-mediated gene transfer, in which selected genes are either maintained unstably and frequently amplified (similar to double minutes) or are later stably integrated into a chromosome (35). We also found that the frequency of polyploidy increased after incubation with hydroxyurea (Table 3). We have shown that these extra chromosomes are not due to overreplication in a single cell cycle (24). It is probable that polyploid cells were selected for in the mitotic harvests because they are more resistant to the effects of the hydroxyurea treatment on cell cycle progression than are near-diploid cells.

(b) A second mechanism is suggested by the observation of increased SCE in hydroxyurea-treated cells (Table 3). Unequal SCE has often been suggested as a mechanism for initiation of gene amplification (for review, see Ref. 36). If only a very small fraction of hydroxyurea-induced SCE involves unequal exchange near the DHFR locus, it could explain the observed hydroxyurea-induced increase in frequency of gene amplification (13).

The two possible mechanisms discussed here are not mutually exclusive; together they could accommodate most of the data that describe gene amplification events in drug-treated mammalian cells.

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